Protein Interaction Networks for Nitrate Reduction in Soil Bacteria

Alexander Rohan Jarvis

PhD

University of East Anglia

School of Biological Sciences

September 2020

This thesis is submitted in partial fulfilment of the requirements of the degree of Doctor of Philosophy at the University of East Anglia

Statement

The work submitted in this thesis is my own work, except where due reference is made to other authors, and has not been submitted to this or any other University for any degree.

Abstract

Paracoccus denitrificans is a Gram-negative model Alphaproteobacterium capable of nitrate assimilation and complete denitrification; two central pathways in the global biogeochemical nitrogen cycle. It encodes three distinct nitrate reductases, cytoplasmic Nas, respiratory Nar and periplasmic Nap. Nas supports growth through conversion of nitrate to ammonium which can be assimilated into organic molecules. Nar supports growth using nitrate as an alternative electron acceptor under anoxic conditions. Nap supports growth by reducing nitrate to remove excess electrons from the quinol-pool, produced when growth depends upon highly reduced carbon substrates. Whilst Nar and Nap have received extensive individual research the assimilatory Nas system has been comparatively under-studied.

The periplasmic nitrate reductase Nap has been shown to express strongly under growth regimes using reduced carbon substrates and thus acts as a sink for excess electrons generated by carbon metabolism. In this thesis the role of the assimilatory nitrate reductase is analysed under mixed nitrogen source conditions using continuous culture to analyse expression of nitrate reductase genes and use of carbon and nitrogen sources. The role of Nas as an alternative redox-balancing nitrate reductase with a dual assimilation function is discussed.

Previous studies have suggested a protein interaction network between three Nas proteins in *P. denitrificans*. These would form a total nitrate to ammonium reduction complex including the assimilatory nitrate and nitrite reductases as well as a small Rieske-type ferredoxin that facilitates electron transfer between the nitrite and nitrate reductase. This project identified novel methods for protein extraction from *P. denitrificans* to determine interacting partner proteins. The model for protein interactions and proposed complex forming proteins has been evolved significantly, showing a tight interaction between the nitrate reductase and a truncated form of the nitrite reductase and another tight interaction between the nitrite reductase and the Rieske-type ferredoxin. This changes the proposed role of the Rieske-type ferredoxin significantly and changes the way electron transfer in this system is understood.

Acknowledgements

Firstly, I would like to thank my supervisors Prof. David Richardson, Dr. Andrew Gates and Dr. Richard Bowater for their continued guidance and support during the last 4 years. Your support and help have been vital to my development throughout my PhD and your advice on all aspects of experimental work, writing and future career guidance has been invaluable and very gratefully received. I would also like to thank Dr. Ben Miller for providing me the opportunity to work with him as a research associate while I wrote this thesis, his project allowed me to develop new skills and his understanding of the requirements of thesis writing were gratefully received.

Further thanks must go to everyone in BIO who has helped me enormously over the last 4 years. From guidance on the use of equipment to listening to my talks and encouraging me when times were tough, I am eternally grateful. A special mention must go to Dr. Andrea Hall without whom the entirety of Chapter 3 of this thesis would not have been possible, thank you for your patience and attentiveness to the needs of this project and above all thank you for all the biscuits and chats at coffee, without those who knows if I would have ever made it! Another special mention must go to Dr. Marcus Edwards, a once fantastic post-doctoral researcher and now an even better PI. Thank you for all your hard work helping me on all levels of science and life outside as well. I am grateful you managed to stay at UEA for the duration of my project and as we have left at the same time, I wish you all the best in your future. Finally, I would like to thank the past members of the Gates group, Manuel, Ben and Nanakow who set up many of the protocols I needed and who took the time to teach me even in their final year of study/work.

The friends I have made along the way on floor 2 have been a constant support and motivation for me. Thank you, Joseph, for getting me out running and back to fitness! Thank you, Lauren, for lunches and steadying conversations through my final year, you helped me hold it together in the end! Thanks also to all the past and present members of Lab 2.30 who have been outstanding colleagues and great friends, I wish you all the luck in the world on your future endeavours.

To my brother Harry, thank you for your support over the years, you are the perfect little brother in that you are occasionally frustrating but always fantastic and it is great we both

finish our times at university together. To my Masi Ruma and Rita thank you both for being there for me my whole life and continuing to share your wisdom and recipes with me! To my mother I would like to say the biggest thank you. We both know I would not be here now without your love, guidance, support and occasional shove in the right direction. Thank you for everything you do for me, ILU. To Sam, I couldn't have asked for a more understanding and supportive girlfriend and your love and support over the past 4 years has been amazing. Thank you for everything and most of all for coming with me on this journey, I look forward to our future adventures together. Finally, I would like to thank my Nanaji and Naniji. Whilst you both are not here to see it, I know I will have made you proud and I can finally fulfil the promise that one day I would be a doctor!

Contents

Chapter 1: Introduction

1.1 The biogeochemical nitrogen cycle

Nitrogen is a vital component of all life as we understand it as well as the principal constituent of the atmosphere on Earth. It is found within all living tissues in DNA, RNA, proteins and various other vital compounds synthesised by the plethora of life. The vast majority of the nitrogen found on our planet is in the atmosphere. Approximately 80% of the gas we breathe is in the inert form of dinitrogen (N_2). This gas is inherently stable due to the triple bond between the two nitrogen atoms (Canfield, Glazer and Falkowski, 2010). This stability of N_2 presents a problem for life, principally it means that obtaining nitrogen in a reactive form to support life is difficult.

As a cycle, the biogeochemical nitrogen cycle has no start or end point and the nitrogen is reduced and oxidised according to the needs of the organism undertaking the catalysis. Possibly the most important step however, is the initial fixation of N_2 from the atmosphere into the typical precursor for organic nitrogen, ammonia (NH_3)(Fani, Gallo and Liò, 2000). This process allows for the transformation of the inert atmospheric form into the more reactive ammonia which is readily assimilated into biomass. This process is vital for life and as such many species of plants have co-evolved association with *Rhizobia* bacteria which are able to fix this atmospheric nitrogen. There are many suggestions as to the driver for this symbiotic relationship but it appears beneficial to both the microbes and the higher plants (Udvardi1t *et al.*, 1992).

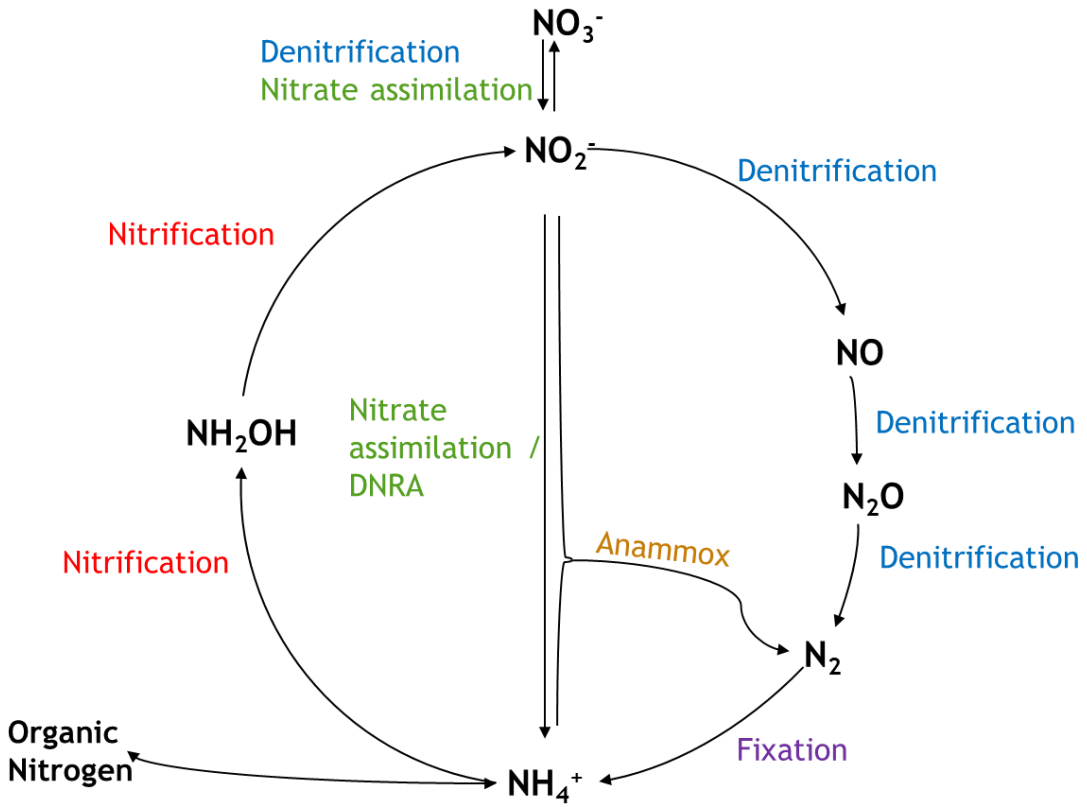


Figure 1. 1 A simplified overview of the biogeochemical nitrogen cycle with the key transformations displayed.

Ammonia generated by the fixation of atmospheric nitrogen is usually assimilated into the organic compounds found in living things. It can, however, be transformed in a respiratory process called nitrification. This oxidative process converts ammonia via hydroxylamine, to nitrite and finally to nitrate (Figure 1. 1). This process is commonly carried out by micro-organisms in the soil environment and is very important for the generation of nitrate and nitrite, which are both vital for supporting the anoxic lifestyles of a wide variety of microbes. Another effect of this process is to change the charge state of molecule in question. Whilst ammonia usually exists as ammonium (NH_4^+) in neutral pH soil (pKa 9.4), and as a product of its positive charge, it is stable within many soils. The same is not true for the negatively charged nitrite (NO_2^-) and nitrate (NO_3^-). Both of these ions are then able to leach from soils and provide nitrogen sources to a wider variety of environments, particularly organisms in aquatic ecosystems (Barnard, Leadley and Hungate, 2005).

Both nitrate and nitrite are used by microbes as alternative electron acceptors to oxygen in cellular respiration and as sources of nitrogen that are assimilated into biomass. The process of assimilation, (section 1.4), allows the conversion of inorganic nitrogen to organic nitrogen, supporting the biosphere and underpinning ecosystems. The respiratory use of nitrate and nitrite is the major method for the return of nitrogen to the atmosphere in the form of dinitrogen. This process also generates intermediate gases such as nitric oxide and nitrous oxide. Nitric oxide is cytotoxic and can have negative environmental consequences. Nitrous oxide is an increasingly problematic greenhouse gas with a higher radiative potential than the most abundant greenhouse gas, carbon dioxide (Canfield, Glazer and Falkowski, 2010). Previous work has identified an increase in denitrification in fertilised agricultural soils compared to natural forests. As much of the United Kingdom has been transformed from forested land to agricultural soils, and, as many other parts of the world continue to convert land for high intensity agriculture the increase in denitrification is set to rise with implications for the entire cycle and the bioavailability of nitrogen in soils.

1.2 The Haber-Bosch process and anthropogenic nitrogen

The Haber-Bosch process was developed over a century ago to allow for artificial fixation of atmospheric dinitrogen to ammonia. This was only the second time in the history of the Earth that organisms had discovered a way of obtaining nitrogen from the atmosphere. The original effort was undertaken by a variety of nitrogen fixing microbes, some in association with plants, and the nitrogenase enzyme was evolved to facilitate nitrogen fixation. This natural process meant that the biogeochemical nitrogen cycle was able to change slowly, and the balance of available nitrogen was maintained over time.

The advent of the Haber-Bosch process has fundamentally disrupted the flow of nitrogen in our environment. For the first time it afforded humans control over nitrogen fixation and, as Fritz Haber himself recognised in his Nobel Prize lecture, it allowed farmers to

choose the nitrogen supplied to their fields to turn poor quality land into the bread-baskets of the world (Haber, 1920). The population expansion over the last century cannot be attributed solely to the advent of this ground-breaking invention but it is commonly accepted that half of the nitrogen consumed by humans comes from Haber-Bosch processes. This means that without the use of this nitrogen source half of the worlds population would not exist (Erisman *et al.*, 2008).

Haber-Bosch nitrogen is not without its problems. Whilst the process has undoubtedly fed an increasing global population it is extremely energy intensive. Attempts have been made in more recent time to make a more environmentally friendly method with new catalysts and cooler temperatures which may go some way towards reducing the impact (Vojvodic *et al.*, 2014). Despite these attempts to make the process more efficient it is the use of the ammonia produced that is the most impactful on our environment. Comparatively little of the nitrogen fixed ends up in organic nitrogen compounds. Approximately 17% of the nitrogen input provided by Haber-Bosch makes it to humans. This relatively low efficiency is one of the major problems with the process (Erisman *et al.*, 2008).

The rapid change in the global nitrogen cycle balance brought on by the advent of the Haber-Bosch process is as yet not fully understood. The flow of nitrogen from the atmosphere to the biosphere has historically been limited by the timescale of evolution and the action of biological systems. Now that humans are able to direct the biological processes, the organisms that have evolved to utilise the conversion of inorganic nitrogenous compounds will experience an increasingly different environment. Microbes in soils are presented with far more inorganic nitrogen than they have ever been before and the adjustment to this new reality could have serious consequences for our climate and our agriculture.

1.3 Environmental and Economic consequences of nitrogen loss

Nitrogen is essential to life as it is an essential component of nucleotides and amino acids. Consequently, it is one of the most important considerations farmers must make when deciding the requirements of their crops. With Haber-Bosch nitrogen flooding the agricultural markets many farmers have opted to supplement their fields with this inexpensive, artificial fertiliser. As a result, the nitrogen lost to the environment as nitrate is increased with increasing application of nitrogenous fertiliser. The ratio of the input of available nitrogen to growth yield of crops plateaus at a point at which increasing the nitrogen does not give a further yield benefit. Furthermore, the effects of different irrigation regimes in the agricultural soils result in differences in the efficiency of the nitrogen use, dependent upon the existing level of nitrogen fertilisation (Gheysari *et al.*, 2009).

There are serious implications for the loss of nitrogen, particularly as nitrate, from agricultural soils. As previously mentioned, the negative charge state of nitrogen as nitrate is a key factor in loss of this nitrogen source. Whilst plants are able to utilise nitrate as a nitrogen source they are limited by the length and dispersal of their root systems. Nitrate and nitrite are not electrostatically attracted to many of the particles that make up common agricultural soil types. Movement of these ions away from their original application zone is therefore possible. This is extremely important in loss due to depth, whereby the nitrate and nitrite leaches deeper into the soil, past the root systems of the crops. At the increased soil depth oxygen is less readily available and so microbes that carry out denitrification linked to cellular respiration dominate. This results in loss of applied nitrogen back to the atmosphere (Burns, 1977).

The environmental consequences of this loss of nitrogen can be severe. Eutrophication is a serious problem caused by agricultural run-off entering aquatic environments and causing blooms of microbes that can feed on the nutrients. In experimental run-off scenarios where nitrate was added to a water course the results showed a several-fold

increase in phytoplankton within the short time of 17 weeks. The increase of these organisms changed the population dynamics as the dominant genera was replaced. Furthermore, the pH was changed dramatically increasing to over 9 and the oxygen level was decreased (Schindler *et al.*, 1971). A fundamental effect of oxygen depletion is the loss of aquatic life. In many instances of eutrophication there is an associated fish kill resulting in a sometimes permanent change to the ecology of the local landscape (Prepas and Charette, 2003).

Whilst the environmental impacts of eutrophication and nitrogen loss have been characterised for many decades the economic impact of this loss of vital nutrients has been given less attention. It was estimated in 2011 to cost the European Union at least €13 billion in lost revenue for farmers. Furthermore, the environmental consequences were predicted to cost up to €320 billion in clean-up and lost revenue costs. These are major economic costs associated with nitrogen loss from agriculture and it is therefore vital to study the regulation and action of the microbial communities involved in nitrogen cycling (Sutton *et al.*, 2011).

1.4 Nitrate assimilation

1.4.1 Fundamental aspects of nitrate assimilation

Nitrate assimilation is a two-step enzymatic process, typically catalysed by two oxidoreductase enzymes. Bacterial nitrate assimilation takes place in the cytoplasm of capable bacteria and provides ammonium for assimilation into biomass. The first reaction is catalysed by a molybdoenzyme of the DMSO reductase family of proteins. This enzyme is a nitrate reductase and catalyses the two-electron reduction of nitrate to nitrite (Equation 1). This reductive step is common to all nitrate reductases irrespective of end function, this will be discussed in more detail later (Lin and Stewart, 1997).

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O$$

Equation 1. The reduction of nitrate to nitrite catalysed by nitrate reductase.

The reduction of nitrate to nitrite is performed by a molybdenum centre of the enzyme. Whilst *E. coli* does not encode an assimilatory nitrate reductase, useful conclusions can be reached from the study of the respiratory nitrate reductase. This enzyme has been shown to be reduced by preparations of dithionite and subsequently re-oxidised by addition of nitrate *in vitro* by electron paramagnetic resonance spectroscopy. The authors in this study investigated the valence state of the molybdenum species present in preparations of nitrate reductase from *E. coli* and were able to conclude nitrate-dependent re-oxidation of the protein under experimental conditions (Bray *et al.*, 1976). This has been well established since the 1970's as the method utilised by nitrate reductases of both the assimilatory and respiratory types for their action.

The second stage of assimilatory nitrate reduction is the conversion of nitrite to ammonium. This is performed by the assimilatory nitrite reductase. In nature biological redox reactions frequently utilise transfers of 1 or 2 electrons in a single step. In the denitrification pathway nitrate is reduced to nitrite (2 electrons), nitrite to nitric oxide (1 electron), 2 nitric oxide molecules to 1 nitrous oxide (1 electron per NO) and finally nitrous oxide to dinitrogen (2 electrons). This process releases a variety of intermediates between nitrite and the end product of molecular dinitrogen (Figure 1. 1). This process is not repeated in assimilatory nitrite reduction. Instead there is a single step reaction that completes a 6 electron reduction of nitrite to ammonium, without the detectable release of free intermediates (Vega, Garrett and Siegel, 1975)(Equation 2).

$$NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O$$

Equation 2 The reduction of nitrite to ammonium catalysed by the siroheme-dependent nitrite reductase.

The enzymes that carry out this process all utilise a siroheme catalytic centre. This unusual prosthetic group was discovered initially in sulfite reductases which also perform a six-electron reduction from sulfite to hydrogen sulfide. Siroheme is an unusual prosthetic group for most proteins, whilst many other groups found in nitrite reductases, such as iron-sulfur clusters and FAD binding domains, are used for alternative processes in other enzymes, siroheme is only known to perform the six-electron reductions carried out by sulfite and nitrite reductases (Murphy *et al.*, 1974). It is known to be synthesised by a variety of organisms from the precursor uroporphyrinogen III, which can also be used for biosynthesis of vitamin B_{12} amongst other biologically useful compounds (Tripathy, Sherameti and Oelmüller, 2010a). Assimilatory nitrite reductases therefore utilise a niche in biochemistry, allowing for a significant chemical reduction from a novel prosthetic group. This process completes a two-step enzymatic reduction to convert inorganic nitrate to ammonium which can be directly assimilated into organic nitrogen by a wide variety of organisms.

There is an alternative to the siroheme-dependent enzyme that catalyses the reduction of nitrite to ammonium. NrfA is found in many enteric bacteria and is well characterised in *E. coli*. It is large deca-heme protein that is capable of catalysing this large reductive step. In contrast to the siroheme systems however, it is found in the periplasm and is linked to dissimilatory nitrate reduction to ammonium (DNRA). This gives the organisms that encode this system an advantage in the anaerobic conditions often encountered in the gut. It also shows that there is significant diversity in the systems that can reduce nitrite to ammonium (Clarke *et al.*, 2008).

1.4.2 Nitrate assimilation systems and diversity

Nitrate assimilation systems must contain enzymes capable of carrying out the two-step reduction of nitrate to ammonium. This comprises the core functionality of the system, however, beyond this there is a wide diversity of encoded genes for support systesms. For example, assimilation systems are typically located within the cytosol of bacteria and archaea, transporters are usually encoded that allow for uptake of nitrate and nitrite. Other genes usually encoded include some regulatory genes, these can act in different ways to promote or supress the expression of the other nitrate assimilation genes. In addition,

there are also co-localised biosynthesis genes, these can be involved in the generation of the nitrate and nitrite reductase active centres. This demonstrates the conservation and the variety of genes associated with nitrate assimilation clusters (Figure 1. 2)(Luque-Almagro *et al.*, 2011).

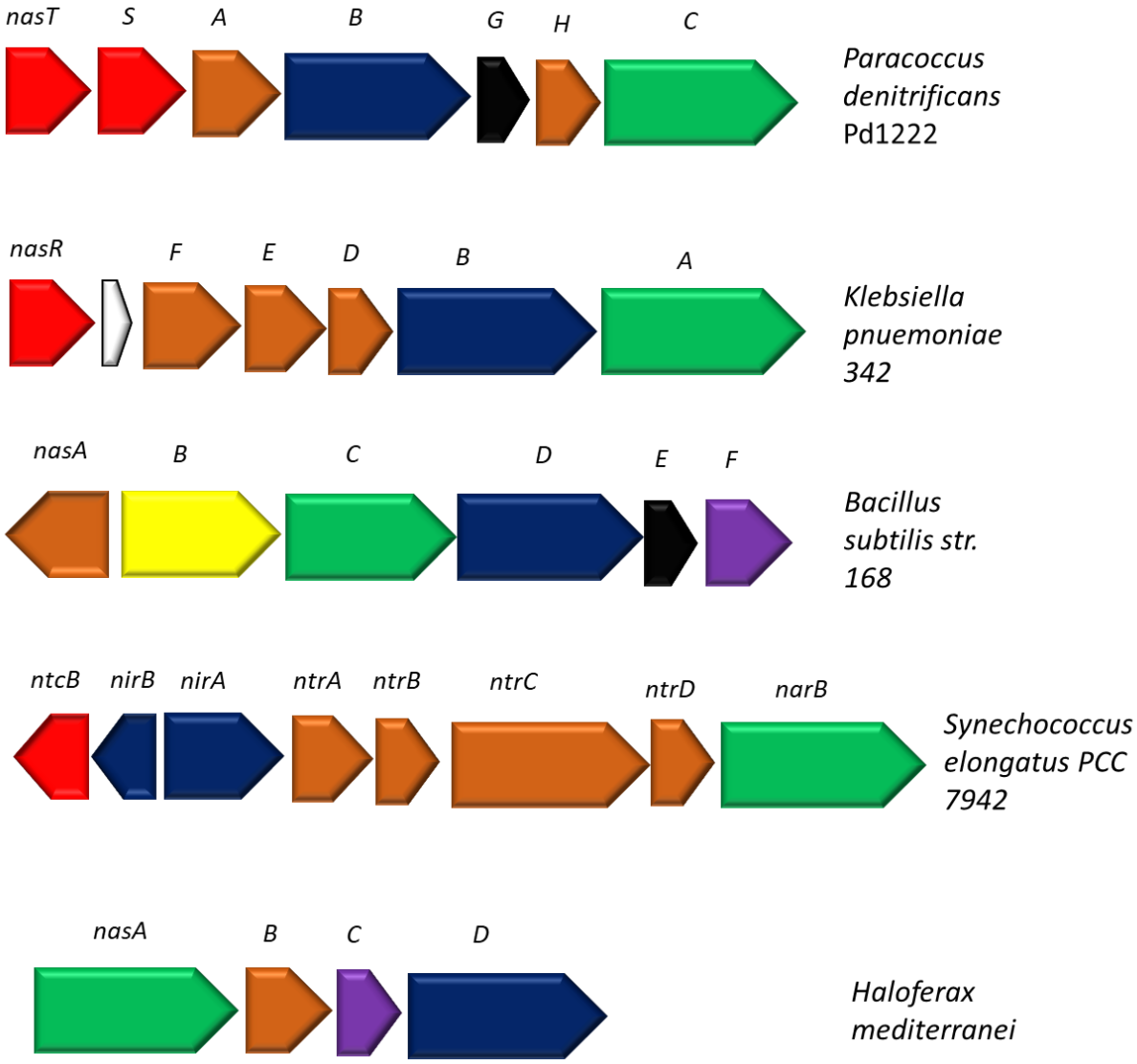


Figure 1. 2 Representative nitrate assimilation gene clusters showing the conservation of core enzyme-encoding genes and the diversity of accessory, associated genes. Selected organisms represent Gram-negative and Gram-positive bacteria, cyanobacteria and archaea. Colours represent; red, regulatory genes; green, nitrate reductase; blue, nitrite reductase; black, Rieske-type ferredoxin; brown, nitrate/nitrite transporters; yellow, FAD-containing proteins; purple, biosynthesis proteins; white, other proteins. (Luque-Almagro *et al.*, 2011).

1.4.2.1 System-specific regulation

Throughout different *nas* gene clusters there are a variety of different system specific regulatory genes. These are different to the global nitrogen regulators that interpret the nitrogen status of the whole cell and act to regulate a wide variety of genes. These regulators typically respond to direct signals such as nitrate or nitrite availability to induce expression of nitrate assimilation genes. Whilst the effects are consistent, the activation of *nas* gene clusters, the proteins encoded can be very different.

In cyanobacteria nitrate assimilation genes are commonly found. Like most bacteria these organisms have regulators that detect the whole cell nitrogen status and as such activate or repress certain genes. As nitrate assimilation is an energetically costly process, requiring 8 electrons per nitrate molecule assimilated, these genes are typically repressed in response to available nitrogen such as ammonium. Cyanobacteria sense their nitrogen status indirectly through the ratio of carbon to nitrogen by the global regulator NtcA, this will be discussed in more detail later. NtcA is important to mention as it activates a range of genes, one specific example being *ntcB*. Loss of nitrate assimilation has been shown in an *Anabaena* sp. PCC 7120 *ΔntcB* mutant indicating a role for this protein in regulation. NtcB is a member of the LysR family of bacterial transcriptional regulators and is thought to interact with the promoter region of nitrate assimilation gene clusters in cyanobacteria. In *Anabaena* sp. PCC 7120 *ntcB* has been shown to itself be regulated by an NtcA dependent promoter (Frías, Flores and Herrero, 2000). This process is therefore a system specific control of nitrate assimilation gene expression. This global regulator induces expression of a system-specific regulator which in turn activates gene expression for the structural components (Figure 1. 3). In this way cyanobacteria are able to control the expression of their nitrate assimilation genes requiring both a global regulator and a system specific regulator for function (Flores *et al.*, 2005; Ohashi *et al.*, 2011).

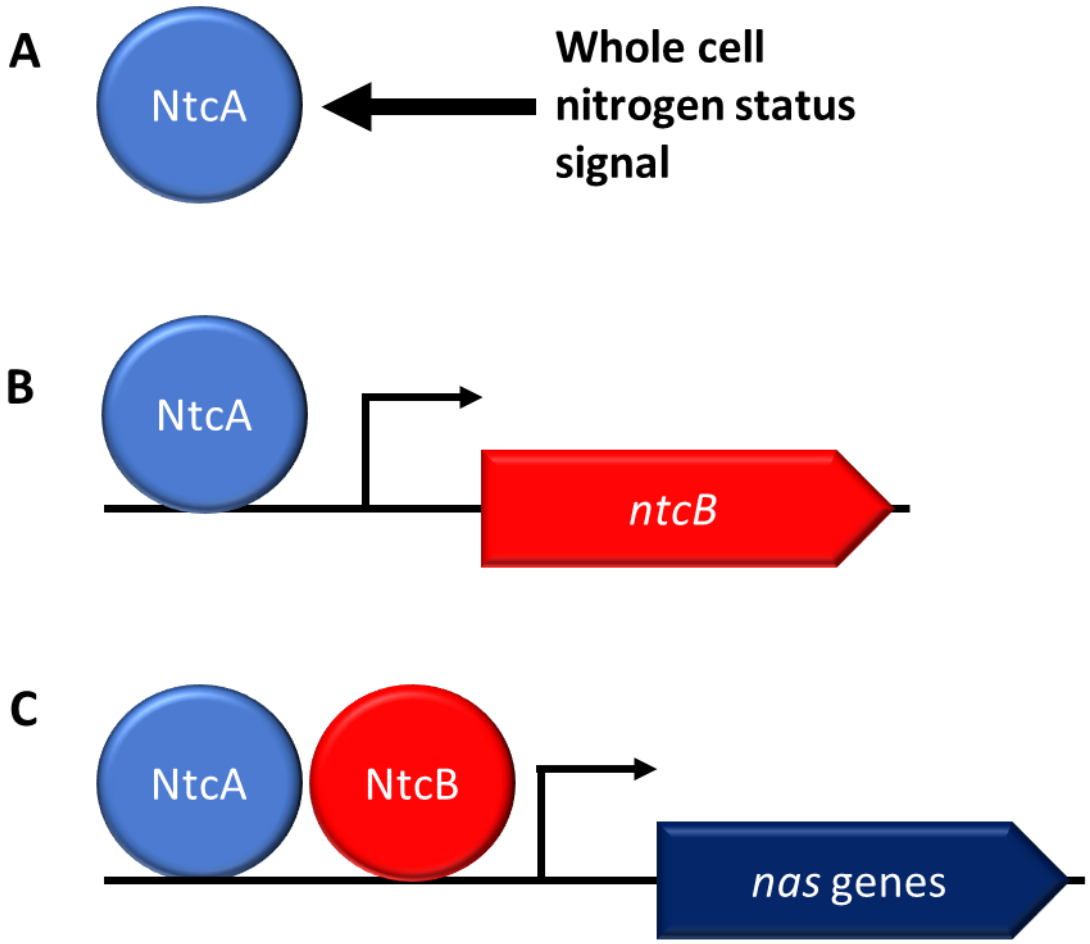


Figure 1. 3 Simplified overview of the system specific regulation of nitrate assimilation genes in cyanobacteria. NtcA detects a change in the C:N ratio of the cell activating its function (A), NtcA then activates expression of *ntcB* (B) and finally NtcA and NtcB act together to allow expression of the nitrate assimilation genes (C).

In a similar manner to cyanobacteria, many gram-positive organisms have their nitrate assimilation genes regulated at the whole cell level rather than the system specific regulation that will be discussed. The gram-positive model organism *Bacillus subtilis* encodes a nitrate assimilation system capable of supporting growth using nitrate as the sole nitrogen source. However, the organism lacks an apparent regulatory gene in its *nas* gene cluster (Figure 1. 2). This indicates that the regulation is not the same as other organisms. In *B. subtilis* the control of nitrate assimilation gene expression has been shown to be less specific than in other organisms (Nakano *et al.*, 1995). In gram-negative bacteria, such as *Paracoccus denitrificans*, a specific nitrate/nitrite sensor complex is encoded that allows for *nas* structural gene expression only in the presence of nitrate or nitrite (Luque-Almagro *et al.*, 2013). In contrast *B. subtilis* senses 'poor' nitrogen sources,

such as glutamine and nitrate, through a global regulator and induces expression of nitrate assimilation genes without the use of a specific nitrate/nitrite sensing protein (Nakano *et al.,* 1995). The regulator TnrA, which induces expression of a range of genes related to nitrogen metabolism, has been implicated in the regulation of expression from the *nasB* promoter in *B. subtilis*, as has the two-component system ResDE. Both of these protein systems appear to be involved in expression of *nas* genes, however, the process is different to that observed in both cyanobacteria and gram-negative organisms as the genes are general regulators and not associated with the *nas* gene clusters (Wray *et al.*, 1996; Nakano *et al.*, 1998).

Different systems also exist in many Gram-negative bacteria that act at a post-transcriptional level. In *Klebsiella pneumoniae* there is a single, system specific regulator involved in transcription of the other *nas* genes. This is encoded by the *nasR* gene and produces a dual function protein. The protein was identified initially in a mutagenesis screen where it was noticed the bacteria could not carry out nitrate assimilation despite the structural *nas* genes being unaffected. The region disrupted was the *nasR* gene which meant the other genes must have been under the transcriptional control of its gene product (Goldman, Lin and Stewart, 1994). The NasR protein acts as a dual functional protein. In the first instance it binds to either nitrate or nitrite and this binding allows it to act as a transcriptional anti-terminator. There is a region of the *nasF* leader mRNA sequence that forms a secondary structure that would ordinarily prevent gene expression. This is the region bound by NasR and thus stabilised for the expression of downstream genes. This binding is dependent upon NasR activation by nitrate or nitrite binding and as such it acts as a specific regulator of the system (Chai and Stewart, 1998).

In *Paracoccus denitrificans* the encoded regulators are *nasTS* whose gene products perform a similar role to NasR to control expression of the structural *nas* genes. NasS is a nitrate/nitrite binding protein with significant sequence similarity to the ABC-type transporter binding proteins of other Nas systems and NasT is a transcriptional anti-terminator protein similar to NasR. These two proteins form a complex that dissociates in the presence of nitrate or nitrite. This system works similarly to the NasR system of *K. pneumoniae* whereby nitrate or nitrite is bound by NasS in complex with NasT, the

complex dissociates releasing NasT protein which is then able to bind the RNA target and allow the full expression of the downstream genes (Luque-Almagro *et al.*, 2013).

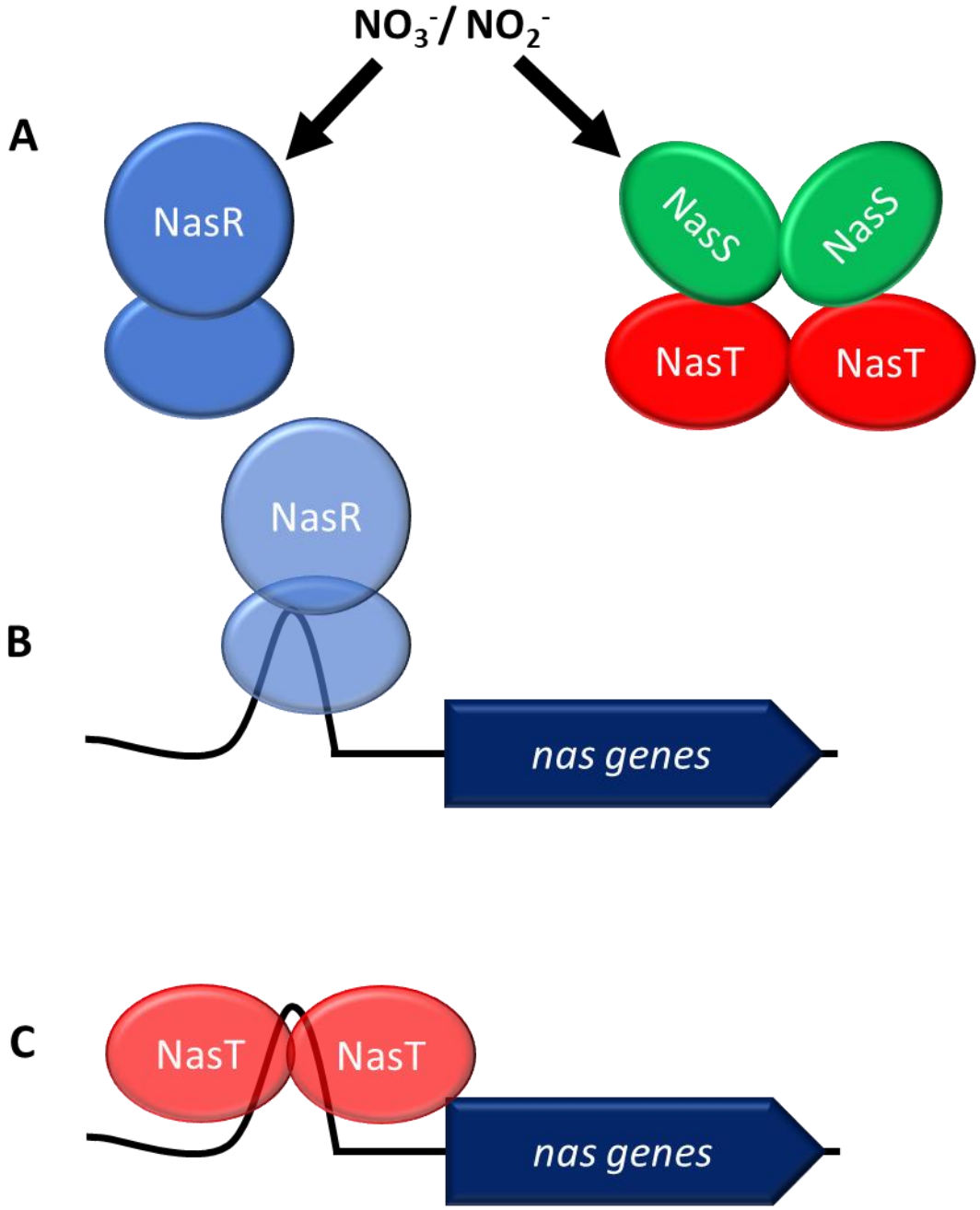


Figure 1. 4 Simplified overview of the system specific regulation of nitrate assimilation in *K. pneumoniae* and *P. denitrificans*. Nitrate or nitrite is bound by NasR or NasS (A), NasR is then able to directly interact with the *nasF* leader sequence stabilising it and preventing termination (B), NasS dissociates from NasT and NasT is able to interact with the leader sequence of the remaining *nas* genes preventing termination (C). NasR is specific to *K. pneumoniae* and NasTS is specific to *P. denitrificans*.

In both the system specific regulatory networks described for the Gram-negative organisms there is a close control of nitrate assimilation gene expression encoded directly in the *nas* gene clusters. This contrasts with the global nitrogen regulation found in Gram-positive bacteria and the intermediate system encoded by many cyanobacteria. This exemplifies the diversity of the genes and functions found in nitrate assimilation gene clusters despite the ultimate fact that each of the organisms described use these regulators to allow them to survive using nitrate as the sole nitrogen source.

1.4.2.2 Nitrate/nitrite transport

Nitrate assimilation systems are found within the cytosol where they provide ammonium to the organic nitrogen synthesis machinery. As nitrate and nitrite are charged molecules at physiologically relevant pH, they are unable to cross the bacterial membrane(s) and require specialised transport systems. Some of these systems are encoded within the nitrate assimilation gene clusters. There are a variety of different methods used by bacteria to this end. Some utilise the membrane proton motive force to symport nitrate, others use active uptake by binding the nitrate and transporting into the cell and others encode permeases that allow nitrate and nitrite into the cell according to diffusion gradients. This is another example of the variety of different systems encoded by nitrate assimilation gene clusters.

In the Gram-positive organism *B. subtilis* the gene *nasA* is used as a nitrate importer. It operates by linking the nitrate import to the proton motive force generated by the cell to import nitrate for assimilation. This process only requires a single acting transporter resulting in a very different appearance of the nitrate assimilation gene cluster. This system appears to support the soil dwelling lifestyle of *B. subtilis* and allows it to utilise the nitrate sources found in soil (Glaser *et al.*, 1995; Moir and Wood, 2001).

Many organisms commonly associated with nitrate assimilation have very different environments to the soil dwelling *B. subtilis*. Cyanobacteria have been studied as nitrate assimilating organisms for a long time, however, their aquatic, usually nutrient poor environment contrasts with the soil ecosystem. Consequently, the nitrate assimilation gene clusters appear rather different. Whilst the core functional proteins remain similar the nitrate and nitrite transport systems are very different.

Cyanobacteria such as *Synechococcus elongatus* PCC 7942 (Figure 1. 2) encode the *nrtABCD* genes within the nitrate assimilation gene cluster. These encode the components of an ATP-binding cassette (ABC) type transporter. These transporters require energy input in the form of ATP to facilitate the translocation of a substrate from outside the cell to the inside against the diffusion gradient. In cyanobacteria the system comprises of NrtA which is a lipoprotein facing out from the cell surface and is involved in the binding nitrate, the transmembrane protein NrtB which will form the necessary gap for nitrate transport and finally NrtC and NrtD are involved in the ATP-binding, generating the energy required for transport. This system is rather different to the one described for *B. subtilis* as it uses more proteins which are not linked to the proton motive force in the same way. Furthermore, the outer binding proteins of ABC-type transporters generally have a high affinity and specificity for their substrates allowing for uptake of key nutrients in the nutrient poor environment that these cyanobacteria are commonly found in. In the case of *Synechococcus elongatus*, its NrtA protein has a K_d of 0.3 µM for nitrate meaning it is able to take up nitrate at very low concentrations found in its environment (Maeda and Omata, 1997; Flores *et al.*, 2005).

In *P. denitrificans* there are two encoded transporters in the *nas* gene cluster. These are *nasA* and *nasH*. These two transporters are thought to satisfy the demand for nitrate and nitrite under assimilation conditions. In the case of NasH the protein is thought to act as nitrite importing protein. There is similarity to the bi-directional nitrite transporter NirC of *E. coli* and however deletion of this gene from *P. denitrificans* did not result in a significant loss of growth, even when grown with nitrite as a sole nitrogen source. The explanation for this may be the protonation of nitrite in solution to form free nitrous acid (HNO_2). This is an uncharged, membrane permeable molecule and dissociation in the cytoplasm (at higher pH) would yield nitrite for incorporation into biomass by Nas (Gates *et al.*, 2011).

There is another assimilatory N-oxyanion import system in *P. denitrificans*. The NasA protein shows sequence similarity to the respiratory NarK1-type nitrate/proton symporter, themselves a subset of the major facilitator superfamily (MFS) of bacterial membrane transporters. NarK is encoded by the *P. denitrificans* respiratory nitrate reduction system and it has two distinct domains. NarK1 is a nitrate/proton symporter and NarK2 is a nitrate/nitrite antiporter. In a study investigating these two distinct domains it was found that NarK1 would complement the loss of nitrate assimilation experienced by a Δ*nasA* mutant, however, NarK2 would not. This was consistent with the initially proposed idea that NasA was a nitrate/proton symporter. NarK2 would remove the nitrite generated to the periplasm and as such not allow growth on nitrate as the sole nitrogen source. As only NarK1 could complement the loss of NasA it confirms that NasA is involved in this form of uptake (Gates *et al.*, 2011; Goddard *et al.*, 2017).

Once again it can be seen that despite the core reductases and functionality encoded by nitrate assimilation gene clusters there is also a wide variety of methods used by bacteria to take up the substrates nitrate and nitrite from the environment. These methods are usually consistent with the lifestyle of the organism. As shown in this section the soil dwelling organisms, *P. denitrificans* and *B. subtilis*, tend to favour nitrate/proton symport mechanisms whereas the aquatic organisms favour the high affinity ABC-type transporter systems. This exemplifies the variability of nitrate assimilation gene clusters.

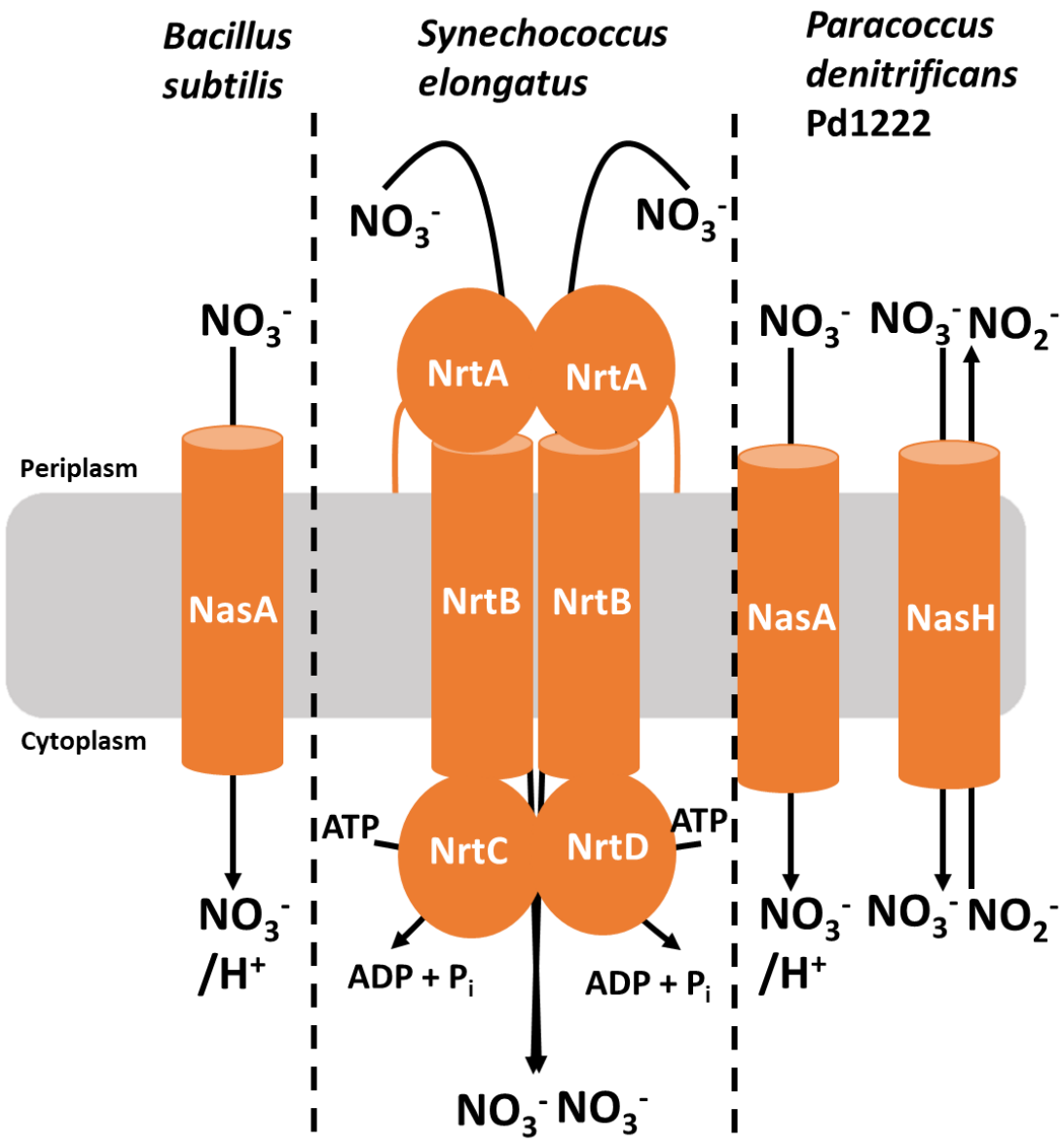


Figure 1. 5 Simplified diagram of the different nitrate/nitrite import mechanisms encoded in a variety of different *nas* gene clusters. Representative examples include the gram-positive *B. subtilis*, the cyanobacteria *S. elongatus* and the gram-negative *P. denitrificans*. Different systems are segregated by dashed lines.

1.4.2.3 Nitrate assimilation associated biosynthesis genes

In some gene clusters annotated in Figure 1. 2 there are additional genes encoding biosynthesis associated proteins. Nitrate assimilation requires complex prosthetic groups for the reduction of both nitrate and nitrite. As the co-factors required are so complicated and may be required at higher levels during *nas* expression, there are occasionally biosynthesis genes associated with the nitrate assimilation gene clusters.

In the archaeon *Haloferax mediterranei* there is a gene that is unusual for such halophilic organisms. These organisms usually require a high acidic amino acid composition for protein stability, however, the NasC protein lacks this feature in this organism. This gene has a high level of similarity to the *Halobacterium* sp. MobA protein which is involved in biosynthesis of the Mo-MGD co-factor of the nitrate reductase (Lledó *et al.*, 2005). In *H. mediterranei* the *nas* system encodes a biosynthesis protein for the specific maturation of the nitrate reductase, in other organisms these genes are not associated specifically with nitrate assimilation again showing the versatility of the *nas* systems.

In other organisms there is a gene named *cysG* that encodes a uroporphyrinogen III c-methyltransferase. Uroporphyrinogen III is a complex precursor of vitamin B_{12} and importantly of the nitrite reductase catalytic co-factor siroheme. In *E. coli* it has been shown to catalyse steps critical for the production of siroheme. In this organism it has been suggested to act as a siroheme synthase (Spencer *et al.*, 1993) (Figure 1. 6). This again shows the variety of genes encoded by the *nas* gene clusters in different organisms. Some encode accessory proteins responsible for the incorporation of complex co-factors and others appear to be completely lacking these. Other organisms appear to have shared maturation proteins with other systems such as the NarJ protein from *P. denitrificans* which is involved in both respiratory and assimilatory nitrate reductase maturation (Pinchbeck *et al.*, 2019).

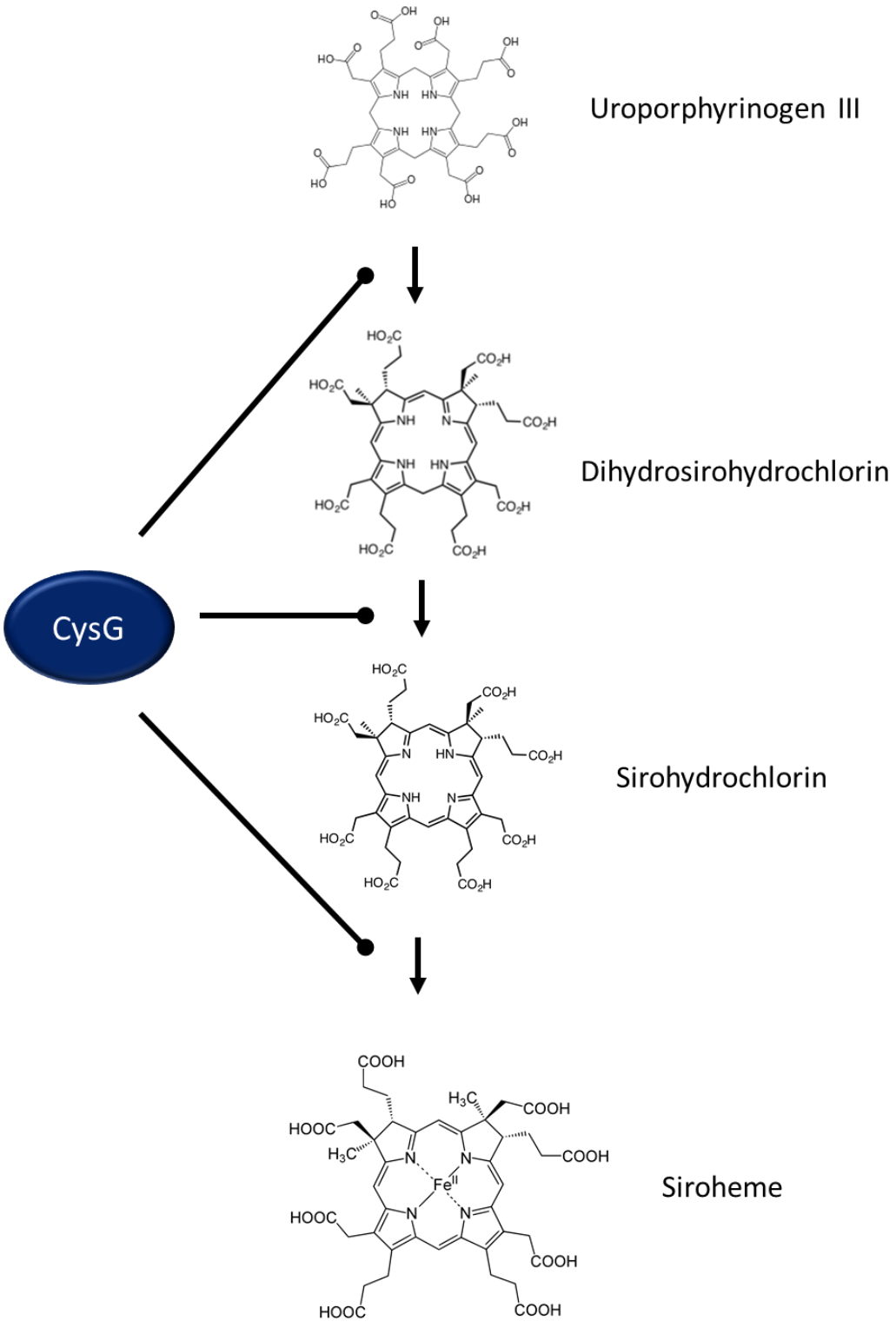


Figure 1. 6 The CysG mediated biosynthesis pathway for production of siroheme in bacteria (adapted from (Tripathy, Sherameti and Oelmüller, 2010b)).

1.5 Respiratory nitrate reduction/ denitrification

Respiratory nitrate reduction is often termed denitrification as it removes nitrate and nitrite from the water or soil and releases gases such as nitric oxide, nitrous oxide and dinitrogen. This is a major process that returns the nitrogen fixed by bacteria back to the atmosphere (Knowles, 1982). Denitrification is used by many bacteria to facilitate an anaerobic lifestyle. By coupling the reduction of inorganic nitrogen compounds to the respiratory electron transport chain and proton translocation these, mainly chemoheterotrophic, bacteria are able to respire without oxygen (Stevenson and Firestone, 1982). Denitrification removes nitrate or nitrite to a range of intermediate gases or all the way to molecular dinitrogen, usually termed 'complete denitrification'. This process requires four reactions, each catalysed by a specific subset of complex metalloenzymes (Equation 3 & Figure 1. 7).

Nitrate reductase

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \quad (E^o = +420\ mV)$$

Nitrite reductase

$$NO_2^- + e^- + 2H^+ \rightarrow NO + H_2O \quad (E^o = +375\ mV)$$

Nitric oxide reductase

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \quad (E^o = +1175\ mV)$$

Nitrous oxide reductase

$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \quad (E^o = +1355\ mV)$$

Equation 3 The equations required for complete denitrification catalysed by the various metalloenzymes named in each stage. Standard reduction potentials are shown for the nitrogen species in each equation in brackets.

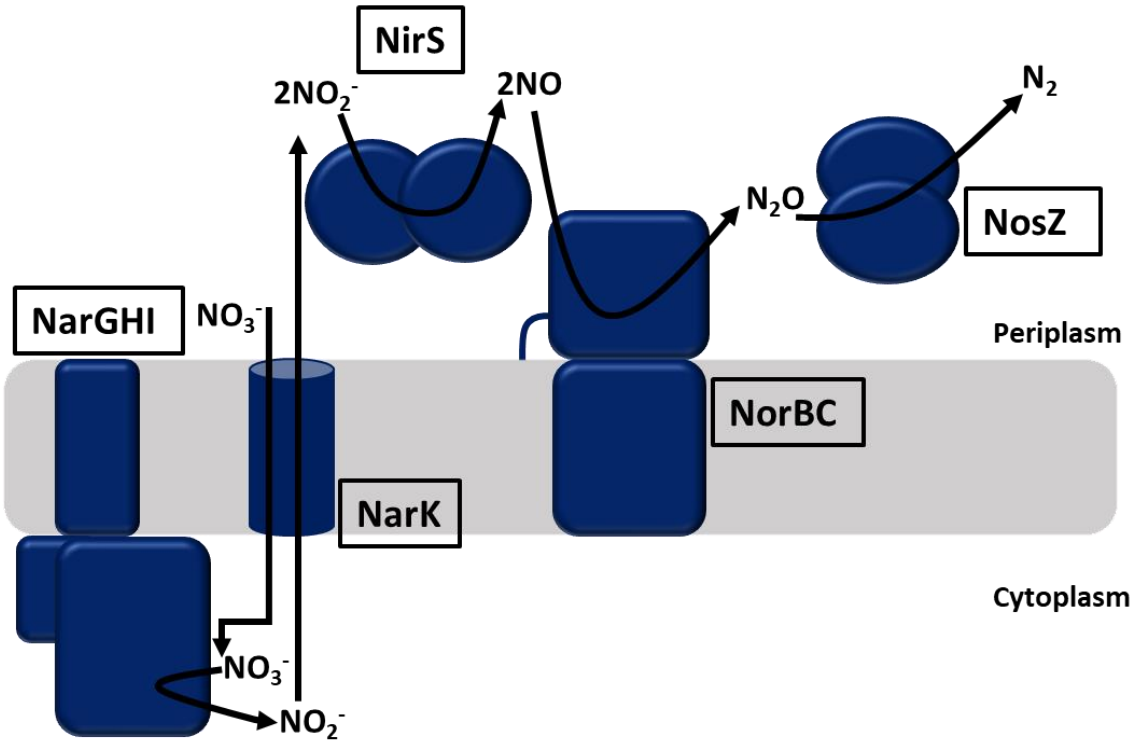


Figure 1. 7 The respiratory pathway for nitrate metabolism and complete denitrification in *P. denitrificans*. Nitrate is transported across the inner energy conserving membrane by NarK; it is reduced by NarG at the NarGHI complex to nitrite in the cytosol; the nitrite is exported to the periplasm by NarK and reduced to nitric oxide by NirS; nitric oxide is reduced to nitrous oxide by the membrane associated NorBC complex; finally, nitrous oxide is reduced to dinitrogen by NosZ.

1.5.1 Nitrate reduction by NarG

Respiratory nitrate reduction begins with the respiratory nitrate reductase complex of NarGHI. This three-protein complex links the bacterial membrane quinol pool to the reduction of nitrate. Respiratory nitrate reductase was discovered in *E. coli* in the 1960's where it was found to allow bacteria to grow using nitrate as an alternative electron acceptor to oxygen. It was also found to form a multi-protein complex allowing for *in vitro* nitrate reduction using quinol as an electron donor, only in the presence of one of the core components (Taniguchi and Itagaki, 1960).

The structure of the NarGHI complex has been solved and reveals a multi-protein complex, with integral membrane subunits. NarI is embedded in the membrane and is the first part of the electron harvesting/transfer chain and is a ubiquinol dehydrogenase. This protein oxidises quinol in the membrane releasing protons to the periplasm and taking electrons via the two *b*-type hemes it coordinates. It subsequently passes the electrons to NarH through a series of iron-sulfur clusters. Finally, the electrons pass to NarG through a further iron-sulfur cluster and end at the molybdopterin guanine dinucleotide, which is the active site of nitrate reduction (Kristjansson, Walter and Hollocher, 1978; Bertero *et al.*, 2003).

NarG is located on the cytoplasmic side of the inner bacterial membrane (Bertero *et al.*, 2003). It is the only component of the denitrification apparatus that is within the cytosol and consequently the nitrate must be transported into the cell. This is done by the NarK nitrate/nitrite antiporter (Noji *et al.*, 1989). NarK is then able to remove nitrite, generated from the reduction of nitrate by NarG, back out to the periplasm where the nitrite reductase can continue the process (Rowe *et al.*, 1994).

1.5.2 Nitrite reduction by NirS and NirK

Nitrate reductases contain a core catalytic centre based on a complex molybdenum containing co-factor. This allows them to reduce nitrate to nitrite, irrespective of the purpose or location. Nitrate can be reduced in the cytoplasm for assimilation, it can be reduced at the membrane for respiration and it can be reduced in the periplasm for the purpose of redox balancing. In all of these cases, the reduction is from nitrate to nitrite and does not require substantially different active sites. For nitrite reductases there is a different story. Assimilatory nitrite reductase contains a siroheme co-factor allowing for the single step reduction of nitrite to ammonium (section 1.4.1). In denitrification a completely different nitrite reductase is required to catalyse the single electron reduction of nitrite to nitric oxide (Equation 3). Bacteria therefore encode a wider variety of genes for this reduction. These are broken down into two particular categories, the first is the cytochrome nitrite reductases which contain a cd_1 heme arrangement and the second is

the copper containing nitrite reductases which contain two distinct copper sites that are involved in nitrite reduction (Godden *et al.*, 1991; Van Spanning *et al.*, 1995).

For the cytochrome nitrite reductases there are two distinct heme components. The first is the *c*-type heme which is covalently attached to the protein backbone and the second is the d_1 heme which is the active site of nitrite reduction (Stouthamer, 1991). Whereas nitrate reductase gained electrons from the membrane quinol pool via a complex involving a ubiquinol dehydrogenase, nitrite reductase is not membrane bound and exists in the periplasm (Alefounder and Ferguson, 1980). As such, there are conserved regions which allow interaction with dedicated soluble redox partners, cytochrome *c*-550 and pseudoazurin. These proteins shuttle electrons from the bc_1 complex to nitrite reductase (Moir and Ferguson, 1994; Williams *et al.*, 1995). Electrons enter the nitrite reductase via the *c*-type heme and are passed to the d_1 heme for nitrite reduction. There is a layer of regulation within this heme to heme transfer that maintains a steady rate of nitrite reduction (Stouthamer, 1991; Farver *et al.*, 2003).

The copper containing nitrite reductases use a different system. For these proteins the electron source is usually azurin or pseudoazurin. The electron donor reduces a single copper site of the reductase, in turn this alters the chemistry of the other site allowing for favourable electron transfer from the first copper site and resulting in nitrite reduction to nitric oxide (Abraham *et al.*, 1997; Murphy, Turley and Adman, 1997; Strange *et al.*, 1999). This demonstrates the distinct differences between the respiratory nitrate reduction apparatus and the assimilatory proteins. There is a greater diversity of mechanistic processes available for the single electron reduction of nitrite compared to the six-electron reduction to ammonium.

1.5.3 Nitric oxide reduction by NorBC

The third step of the denitrification pathway reduces two molecules of nitric oxide to generate a single molecule of nitrous oxide. This step is catalysed by the nitric oxide reductase complex of NorBC. These proteins are associated with the bacterial membrane

and face into the periplasm where they can access the nitric oxide generated by the nitrite reductase. They are heme proteins that also coordinate a non-heme iron. They are members of the heme-copper oxidase family of proteins and coordinating a *c*-type heme in NorC, two *b*-type hemes in NorB and a non-heme iron also in NorB. The proposed mechanism involves transfer of electrons from pseudoazurin to the NorC heme, this then transfers electrons to the NorB hemes where the reduction takes place. This mechanism allows for the third step of denitrification to take place, again using a complex of metalloenzymes to catalyse the reduction (Van Spanning *et al.*, 1995; Field *et al.*, 2008; Hino *et al.*, 2010).

Whilst the NorBC complex is found in denitrifying bacteria it is also important to note alternative systems. qNOR is an alternative nitric oxide reductase found mainly in non-denitrifying bacteria. It lacks the *c*-type heme present in NorC and derives its electrons from the quinol pool directly. It is thought to contain a non-heme iron and a high-spin heme combining to form the active site. This enzyme is thought to protect intracellular pathogens against NO-toxicity from host defences however, it can be combined with other denitrification systems to allow full denitrification potentially giving the bacteria that encode it an advantage in oxygen deprived environments (Hendriks *et al.*, 2000).

1.5.4 Nitrous oxide reduction by NosZ

Nitrous oxide is the final compound produced prior to the termination of complete denitrification. It is a potent greenhouse gas and ozone-depleting agent and its release is a major issue for the climate. The enzyme that reduces this molecule to molecular nitrogen is one of the most important in the entire cycle. It allows for nitrogen to return to the atmosphere and so continues the biogeochemical cycle (Stüeken *et al.*, 2016). The reduction of nitrous oxide uses a copper containing reductase. This protein interacts with pseudoazurin to gain electrons from the bc_1 complex. To reduce nitrous oxide it encodes a CuA and a CuZ centre, these act to receive electrons and reduce the nitrous oxide that binds. This final step releases dinitrogen gas back to the environment resulting in the completion of the denitrification process, with the coupling to the respiratory network of the bacteria this process allows them to survive the anoxic environment using nitrogenous

compounds as the terminal electron acceptors (Snyder, S.W. and Hollocher, 1987; Van Spanning *et al.*, 1995; Haltia *et al.*, 2003).

1.6 Redox-balancing nitrate reduction

Nitrate can be used as a source of nitrogen for biomass production and an alternative terminal electron acceptor to facilitate anoxic respiration, however, it has another important use. Some bacteria utilise the reduction of nitrate for the dissimilation of excess reducing equivalents generated under particular types of metabolism. This process of nitrate reduction to nitrite occurs in the presence of oxygen and was originally termed 'aerobic denitrification' (Richardson *et al.*, 1988a).

The enzyme that catalyses this reduction is a typical nitrate reductase with a molybdenum catalytic centre, however, it differs from Nar and Nas as it faces into the periplasm. This means that it does not require nitrate transport across the membrane to function and instead reduces nitrate to nitrite outside of the cytoplasm (Bell, Richardson and Ferguson, 1990). This enzyme is therefore the periplasmic nitrate reductase and is named Nap. Nap proteins are associated with the membrane and usually form an interaction with a membrane bound cytochrome that passes electrons from the quinol pool to the molybdenum centre of Nap. Crucially this process does not involve proton translocation as found in the NarGHI complex and so does not support energy generation in these organisms (Arnoux *et al.*, 2003; Jepson *et al.*, 2006).

The evident question is therefore, why do organisms encode a nitrate reductase that does not facilitate respiration, energy generation or nitrogen assimilation? Nap was originally found to occur in the phototrophic bacteria *Rhodobacter capsulatus* that encodes a cyclic photosynthesis system which involves a variety of redox reactions (Tichi and Tabita, 2001). When the organism was grown using reduced fatty acids, such as butyrate, it was unable to grow without auxiliary oxidants with which it could vent electrons. The photosystem would become fully reduced by the excess reducing equivalents generated by metabolism of reduced fatty acids and would therefore not function and the organism would die. To overcome this, excess electrons are used to reduce nitrate without the

necessity to generate further energy or biomass (Richardson *et al.*, 1988a). This is used by many more organisms than just the phototropic to facilitate metabolism of highly reduced compounds, others include *Paracoccus denitrificans* (Sears *et al.*, 1993; Sears, Spiro and Richardson, 1997).

Interestingly there are organisms that encode a *nap* gene cluster and also encode genes for the downstream processes of denitrification. In many of these organisms Nap has been shown as the nitrate reductase responsible for the first step of denitrification and not Nar (Bedmar, Robles and Delgado, 2005). Further studies have also indicated a greater presence of Nap encoding bacteria than Nar encoding bacteria indicating that this process is environmentally important (Carter *et al.*, 1995). As this type of nitrate reduction is wide-spread in the environment its importance to agriculture and nitrogen loss is vital to understand and take into account. In a changing climate and with changing agricultural practice care should be taken to understand the regulation and the function of redox-balancing nitrate reduction, particularly as it appears to occur in response to externally applied carbon substrates.

1.7 The carbon-nitrogen metabolism interface

1.7.1 The Tricarboxylic Acid Cycle

The Tricarboxylic Acid (TCA) cycle is a central metabolic pathway for the catabolism and anabolism of carbon compounds during aerobic growth of a wide range of organisms. It is carried out in the mitochondria of eukaryotes and the cytoplasm of many prokaryotes. It is a cyclical process that incorporates carbon and then removes it to generate reducing equivalents such as NADH and $FADH_2$ which are used to generate ATP or to provide electrons to other reductive processes. It is also linked to nitrogen metabolism as the TCA cycle intermediate 2-oxoglutarate (α-ketoglutarate) is also used to assimilate inorganic ammonium into organic nitrogen as glutamate (Nagatani, Shimizu and Valentine, 1971; Akram, 2014). It is therefore important to understand the stages involved in the TCA cycle to understand the metabolism and potentially the regulation of nitrate assimilation.

The TCA cycle links a variety of metabolic processes into a single cycle. It allows for the catabolic oxidation of distinct carbon compounds such as glucose, short chain fatty acids and dicarboxylic acids such as succinate. In the case of the two former examples there is a metabolic pathway prior to the TCA cycle that generates the starting compound of acetyl-CoA, for the latter, succinate is an intermediate of the pathway and as such can fit into it directly. The cycle begins with acetyl-CoA (2 carbons) being linked to oxaloacetate (4 carbons) in a condensation reaction catalysed by the citrate synthase enzyme. This process generates citrate (6 carbons). The next reaction involves the isomerisation of citrate to isocitrate (6 carbons) carried out by an aconitase enzyme. Isocitrate is then converted to 2-oxoglutarate (5 carbons) by the isocitrate dehydrogenase. This step is NAD^+ linked and results in the formation of NADH and release of CO_2. 2-oxoglutarate undergoes a further decarboxylation by a 2-oxoglutarate dehydrogenase enzyme yielding succinyl-CoA (4 carbons) and further NADH and CO_2. Succinyl-CoA undergoes a further reaction with succinic thiokinase that generates GTP from GDP and succinate (4 carbons). Succinate is dehydrogenated to fumarate (4 carbons) by succinate dehydrogenase yielding $FADH_2$ in the process. Fumarate is then converted to malate (4 carbons) by the fumarase enzyme. Malate is then dehydrogenated by malate dehydrogenase to form oxaloacetate and NADH. The oxaloacetate goes on to combine with fresh acetyl-CoA and restarts the process (Figure 1. 8)(Akram, 2014).

During the TCA cycle a variety of intermediates are produced. 2-oxoglutarate is vital for the assimilation of inorganic nitrogen and acts as an important signal molecule for the control of nitrate assimilation genes (Section 1.8). Furthermore, the cycle produces a variety of cytoplasmic reducing equivalents in the form of NADH and $FADH_2$. NADH is important for nitrate assimilation in heterotrophic bacteria as it is the electron donor to the nitrate assimilation oxidoreductases (Gates *et al.*, 2011). It is therefore vital to consider the carbon source used by the organism when studying its nitrogen assimilation regulation and nitrogen source preference.

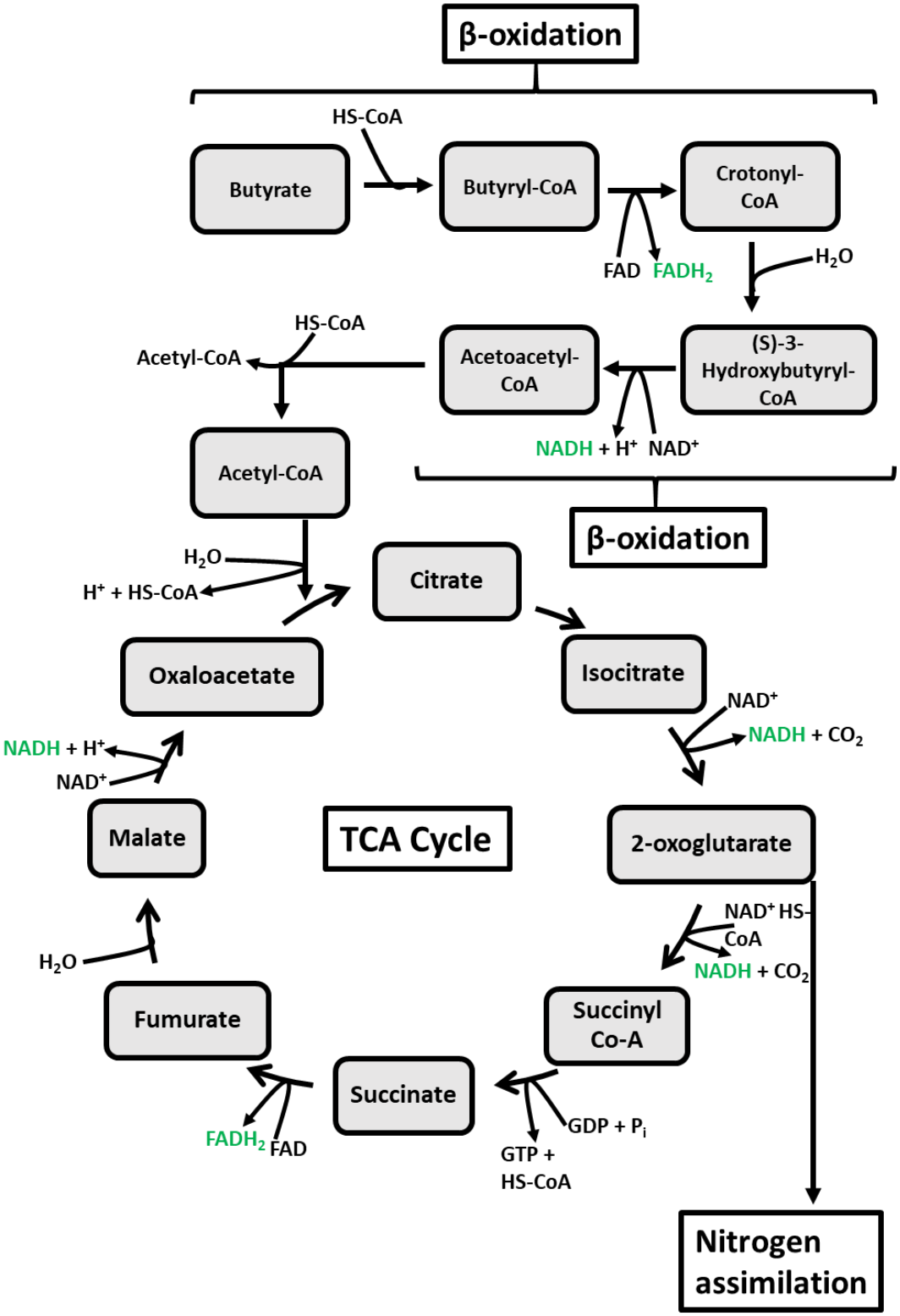


Figure 1. 8 The metabolic pathways for the breakdown of carbon substrates by *P. denitrificans*. The process of β-oxidation for the metabolism of butyrate and other fatty acids, the process of the TCA cycle for generation of energy and for the production of 2-oxoglutarate for nitrogen assimilation are highlighted. Succinate and butyrate require different metabolic pathways for their metabolism, the key differences for this thesis involve the reducing equivalents generated hence these are coloured in green.

1.7.2 β-oxidation of fatty acids

Some carbon substrates, such as succinate and malate, can fit directly into the TCA cycle. Others, such as glucose, require the process of glycolysis to generate the acetyl-CoA required. Fatty acids, however, require another process termed β-oxidation. The name is derived from the sequential removal of two carbons from a fatty acid molecule to yield acetyl-CoA each time. For short, four carbon fatty acids such butyrate the process yields two molecules of acetyl-CoA from a single round, but for longer chain lengths such as the six carbon caproate there will be another round to breakdown the product fully. Once acetyl-CoA is formed it can enter the remainder of the TCA cycle.

The first reaction of β-oxidation is the addition of an acyl-CoA to the fatty acid catalysed by an acyl-CoA dehydrogenase. This further dehydrogenates the fatty acid forming a double bond between carbons 2 and 3 and uses FAD to generate $FADH_2$. The next stage involves the hydration of the double bond generated previously resulting in a stereospecific 3-hydroxyacyl-CoA product. An oxidation step then occurs converting the hydroxyl group into a keto group, this generates NADH from NAD^+ in the process. Finally, a thiolysis reaction occurs whereby the thiol group of a coenzyme A is added between carbons 2 and 3 and results in the release of a single molecule of acetyl-CoA and a second molecule of acyl-CoA. The acyl-CoA can be released to undergo the process again until the substrate has been exhausted and broken down into acetyl-CoA entirely (Figure 1. 8)(Schulz, 1991).

This process is extremely important for bacteria as it allows for the utilisation of alternate carbon substrates. Furthermore, it generates a number of reducing equivalents in the form of NADH and $FADH_2$ which can be used for energy generation or as a fuel for energetically expensive processes such as nitrate assimilation. It is therefore worth considering the implications for nitrogen metabolism when cells are using fatty acid carbon substrates instead of TCA cycle intermediates.

1.7.3 The glutamate dehydrogenase pathway of nitrogen assimilation

Glutamate dehydrogenase (GDH) catalyses the reversible reaction of glutamate to 2-oxoglutarate with ammonia (Equation 4). Despite being discovered as a glutamate dehydrogenase it has been reported to function in many bacteria in the reverse direction, catalysing the formation of glutamate from the TCA cycle intermediate 2-oxoglutarate (Harper *et al.*, 2010).

$$\mathbf{Glutamate + NAD^{+} \leftrightarrow NH_3 + 2 - oxoglutarate + NADH}$$

Equation 4 The reversible reaction catalysed by the glutamate dehydrogenase enzyme.

Glutamate dehydrogenase is associated with the assimilation of inorganic nitrogen in the form of ammonia. This enzyme produces organic nitrogen in the form of glutamate by linking 2-oxoglutarate to ammonia. Under ammonium-dependent growth the bacteria have sufficient nitrogen for this reaction to take place, however, under nitrate assimilation conditions the flow of ammonium into the system requires the reduction of nitrate first. This requirement for nitrate reduction may slow down the supply of ammonium to the GDH pathway resulting in insufficient ammonium concentrations for the reaction to occur. Enzyme isolated from *E. coli* had a K_M in of 1.1 mM and from *P. denitrificans* it had a K_M of 14 mM for ammonia (Sakamoto, Kotre and Savageau, 1975; Kremeckova, Svrcula and Mikes, 1992). The affinity for ammonia is therefore very low in many organisms. This means under nitrogen limitation an alternative pathway must be used for high affinity ammonia assimilation (Pengpeng and Tan, 2013).

1.7.4 The glutamine synthase – glutamine oxoglutarate aminotransferase system of nitrogen assimilation

The glutamate dehydrogenase system is a low affinity ammonium assimilation pathway in many bacteria. This is useful under plentiful supply of ammonium, however, under limiting conditions where nitrate is the sole, available nitrogen source the amount of ammonium is limited by the rate of nitrate reduction. Consequently, the concentration of ammonium falls, and the bacteria have to utilise an alternate system. The Glutamine Synthase – Glutamine Oxoglutarate AminoTransferase or GS-GOGAT system encodes two pathways for the high affinity assimilation of ammonium into organic nitrogen (van Heeswijk, Westerhoff and Boogerd, 2013).

The system incorporates two distinct reactions. In the first ammonium is incorporated, in an ATP dependent reaction, into glutamate to generate glutamine. This is done by the glutamine synthase enzyme. The second stage transfers nitrogen from the glutamine to a 2-oxoglutarate, from the TCA cycle, to produce two molecules of glutamate. This can then cycle around again to produce a single glutamate from each round of the GS-GOGAT cycle. The reaction scheme uses one ATP and one NAD(P)H per ammonium incorporated (Figure 1. 9). There is a higher energy requirement for this system compared to the GDH system, possibly explaining the preference for GDH under higher ammonium conditions (Harper *et al.*, 2010; van Heeswijk, Westerhoff and Boogerd, 2013).

The GS-GOGAT system has a higher affinity for ammonium compared to the GDH pathway. In *Paracoccus denitrificans* the calculated K_M of the GDH enzyme for ammonia was 14 mM, however, in the same organism the calculated K_M of GS for ammonia was 0.03 mM (Kremeckova, Svrcula and Mikes, 1992). This demonstrates a significant increase in affinity. Under nitrate-dependent growth, microarray data has recently shown a large increase in expression of the genes for the GS-GOGAT system in *P. denitrificans*, alongside a variety of nitrogen scavenging genes such as high affinity ammonium transporters (Luque-Almagro *et al.*, 2017). The GS-GOGAT system is therefore thought to be the principal system of nitrogen assimilation when cells are cultured using nitrate as the sole nitrogen source.

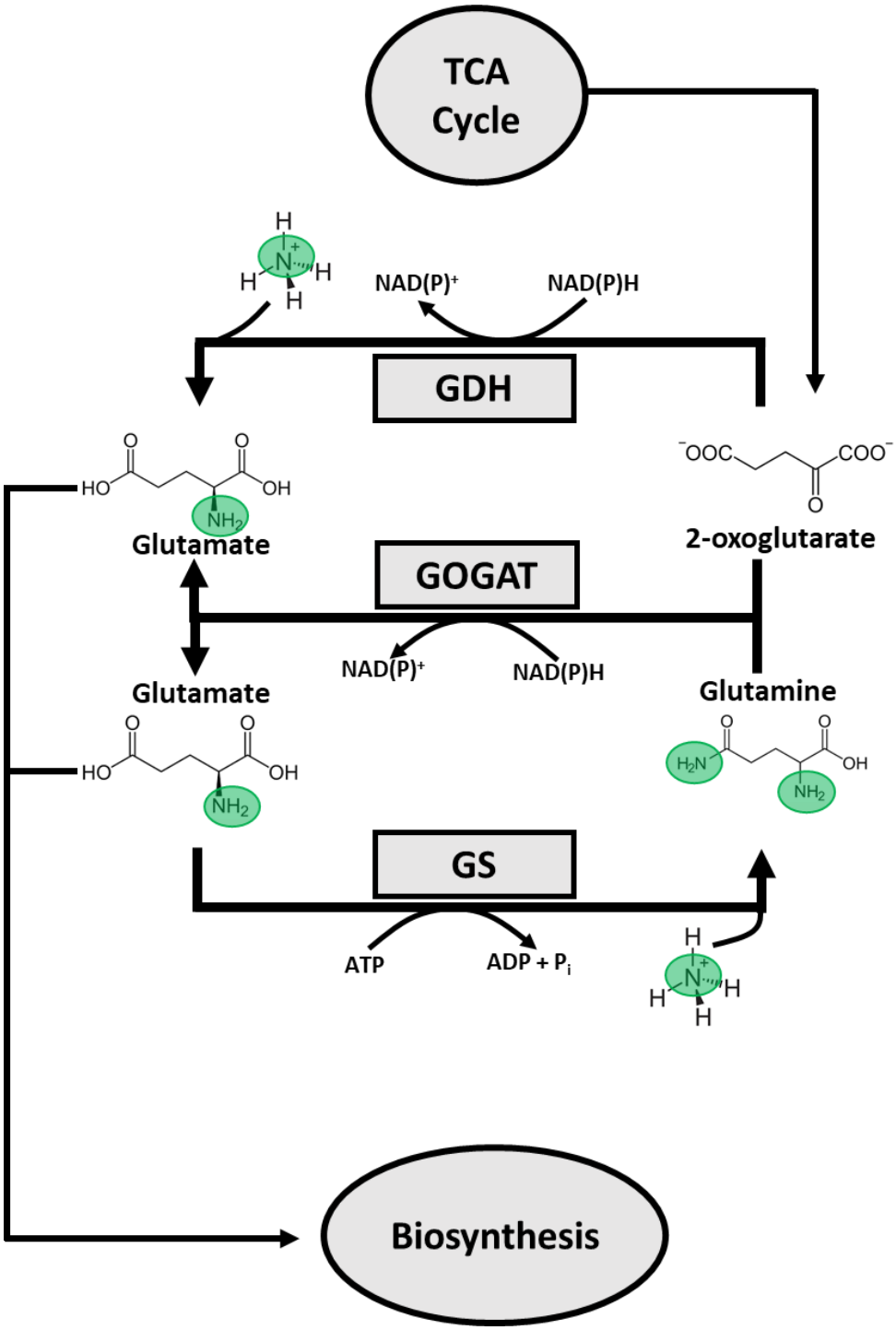


Figure 1. 9 Schematic showing the two principal systems for the assimilation of inorganic ammonium into organic glutamate in *P. denitrificans*. Glutamate dehydrogenase (GDH) assimilates 2-oxoglutarate and ammonium to produce glutamate and Glutamine synthase – oxoglutarate aminotransferase (GS-GOGAT) produces glutamine from ammonium and glutamate before the second stage generating two molecules of glutamate from glutamine and 2-oxoglutarate. The nitrogen atoms incorporated are shaded green.

1.7.5 The impact of carbon source on nitrogen metabolism

In section 1.6 'redox-balancing' nitrate reduction was discussed. The nitrate reductase Nap dissipates excess electrons by reducing nitrate. This was evident in the context of cyclic photosynthesis in *Rhodobacter* sp. for example (Richardson *et al.*, 1988a). This enzyme has also been found in other organisms, notably for this thesis *P. denitrificans*, and many of these chemoheterotrophic bacteria do not have the reaction centre and so do not perform the same phototrophic growth as *Rhodobacter* (Sears *et al.*, 1993). While the necessity of a dissipation system is clear in *Rhodobacter* during growth by cyclic photosynthesis, it is less clear in other heterotrophic bacteria. When the carbon source metabolism is considered the reason becomes clearer and poses questions regarding microbial biochemistry, nutrient competition and environmentally relevant carbon substrates.

The discovery of a periplasmic nitrate reductase in *P. denitrificans* was similar to the equivalent discovery in *Rhodobacter*. *P. denitrificans* encodes a respiratory nitrate reductase that is not expressed under aerobic growth conditions and it is also highly sensitive to azide which is a potent inhibitor. Cells were cultured on butyrate or succinate as the sole carbon source and subjected to nitrate reductase activity assays. An enzyme was found in the periplasm of cells that was insensitive to azide and was expressed under aerobic growth. Importantly, this protein was seemingly also expressed in response to growth on butyrate and not expressed as highly during succinate dependent growth (Sears *et al.*, 1993). There is a link between the carbon source used for growth and the expression of a nitrate reductase in this organism.

Further study of the aeration and carbon source oxidation state effects upon Nap in *P. denitrificans* found that aerobic metabolism of malate and butyrate, the two tested carbon sources, yielded different quantities of electrons. In the case of malate, assimilation required the consumption of reducing equivalents, whereas, butyrate anabolism generated them. Full catabolism of either carbon substrate would yield eight more electrons per molecule of butyrate than malate, as both contain four carbons this is a large difference.

This difference between consumption or generation of reducing equivalents explains the necessity of Nap in this organism (Sears, Spiro and Richardson, 1997).

In another study the preference of particular carbon substrates was analysed. The representative substrates were the four carbon, common laboratory carbon substrate succinate, the two-carbon compound acetate and the four carbon, short-chain fatty acid butyrate. When cultured on these carbon substrates the cells showed a clear hierarchical preference for consumption of succinate then acetate then butyrate. This appears to track with the oxidation state of the carbon source from least to most reduced. It also follows the expression of Nap, whereby, growth on succinate yields low expression, more activity is seen with acetate and the highest is seen with butyrate. The authors noted that it may be strange to want to dissipate electrons when growing aerobically as a higher yield would allow for higher production of ATP. The explanation could well be the over-production of ATP, thus depleting ADP and creating an imbalance in the organism. The expression of Nap was also found to occur dependent upon the carbon substrate used and not the availability of nitrate in the media. Furthermore, it was proposed that availability of carbon substrate was not the regulatory signal, instead it appears to be from the metabolism of the carbon substrate. Both acetate and butyrate have the same metabolic pathway from the formation of acetyl-CoA and an as yet unknown regulator was thought to detect the production of intermediates dependent upon the carbon source metabolism (Ellington *et al.*, 2002).

This interplay between carbon source oxidation state and nitrogen metabolism is well established for the periplasmic reductase Nap. However, nitrate assimilation has received comparatively little attention. In the studies presented in this section the media contained ammonium in sufficient concentration for growth (Harms *et al.*, 1985). The introduction of nitrate was just to see the effect of Nap in these studies. *P. denitrificans* also encodes a cytoplasmic nitrate reductase that uses NADH as an electron donor (Sears *et al.*, 1997; Gates *et al.*, 2011). As the production of excess cytoplasmic reducing equivalents is linked to the metabolism of nitrogen it is logical to question whether Nas is involved. Under a mixed nitrogen regime where neither ammonium or nitrate concentration is sufficient for maximum growth yield could the carbon source allow Nas to be used alongside ammonium assimilation?

1.8 Global regulation of nitrate assimilation by bacteria

Many bacteria live in a complex environment that can change rapidly. Those that occupy niches in soil and aquatic environments are subject to variable supplies of many vital nutrients. As a result, bacteria encode regulatory proteins and sensor networks to understand their nutrient status and respond to the changes that occur in the environment. System specific control of nitrate assimilation genes has already been discussed in this chapter (1.4.2.1), however, prior to the action of these specific regulators there is a whole cell pathway that governs global cellular metabolism.

The GlnB protein is a P_{II} protein that is involved in the initial signal transduction for nitrate assimilation gene regulation. This is the first protein that responds to a signal from the whole cell metabolic state (Bueno, Pahel and Magasanik, 1985). Under nitrogen sufficient conditions the P_{II} protein either phosphorylated in cyanobacteria to prevent it interacting with downstream effector proteins. Under nitrogen limited conditions, interpreted either by the presence of ammonium or the relative ratio of key carbon compounds to overall nitrogen status, the protein will be dephosphorylated and can go on to influence the expression and activation of the Ntc system. In heterotrophic bacteria the system uses a uridylation of the P_{II} protein under nitrogen limitation which allows activation of the downstream Ntr global regulatory system. Without this addition the protein signals nitrogen excess and the Ntr system is not activated (Weiss and Magasanik, 1988; Forchhammer and De Marsac, 1995; Merrick and Edwards, 1995). The signals for the nitrogen status of the organism are not completely understood, in cyanobacteria the signal is usually thought to be levels of 2-oxoglutarate which would build up in the cell without ammonium to link to nitrogen assimilation (Forchhammer and De Marsac, 1995).

The next level of regulation in cyanobacteria is the Ntc system, mediated by NtcA. As previously mentioned this protein can upregulate the expression of the system specific NtcB protein which is found in some *nas* gene clusters (Frías, Flores and Herrero, 2000; Flores *et al.*, 2005). This protein is a global regulator, however, and it also activates

transcription of a range of other nitrogen responsive genes such as urea transporters which is thought to be a response to low nitrogen availability and the development of a nitrogen scavenging phenotype (Vega-Palas *et al.*, 1990).

In heterotrophic bacteria the regulatory system is the Ntr system of global nitrogen regulators. This system is a typical two-component system involving an autophosphorylating kinase, NtrB, and a DNA binding transcriptional regulator, NtrC. NtrB is a histidine kinase that autophosphorylates and then transfers a phosphate to NtrC. Phosphorylated NtrC can then bind to DNA binding sites and regulate gene expression by ATPase activity and in some cases interaction with sigma transcription factors (Weiss and Magasanik, 1988; Weiss, Claverie-Martin and Magasanik, 1992; Chen and Reitzer, 1995; Wu *et al.*, 1999). This system regulates a wide variety of nitrogen metabolism genes, it has been shown to regulate nitrogenase genes in *Rhodobacter sphaeroides* and nitrate assimilation by *Paracoccus denitrificans* (Zinchenko *et al.*, 2006; Luque-Almagro *et al.*, 2017).

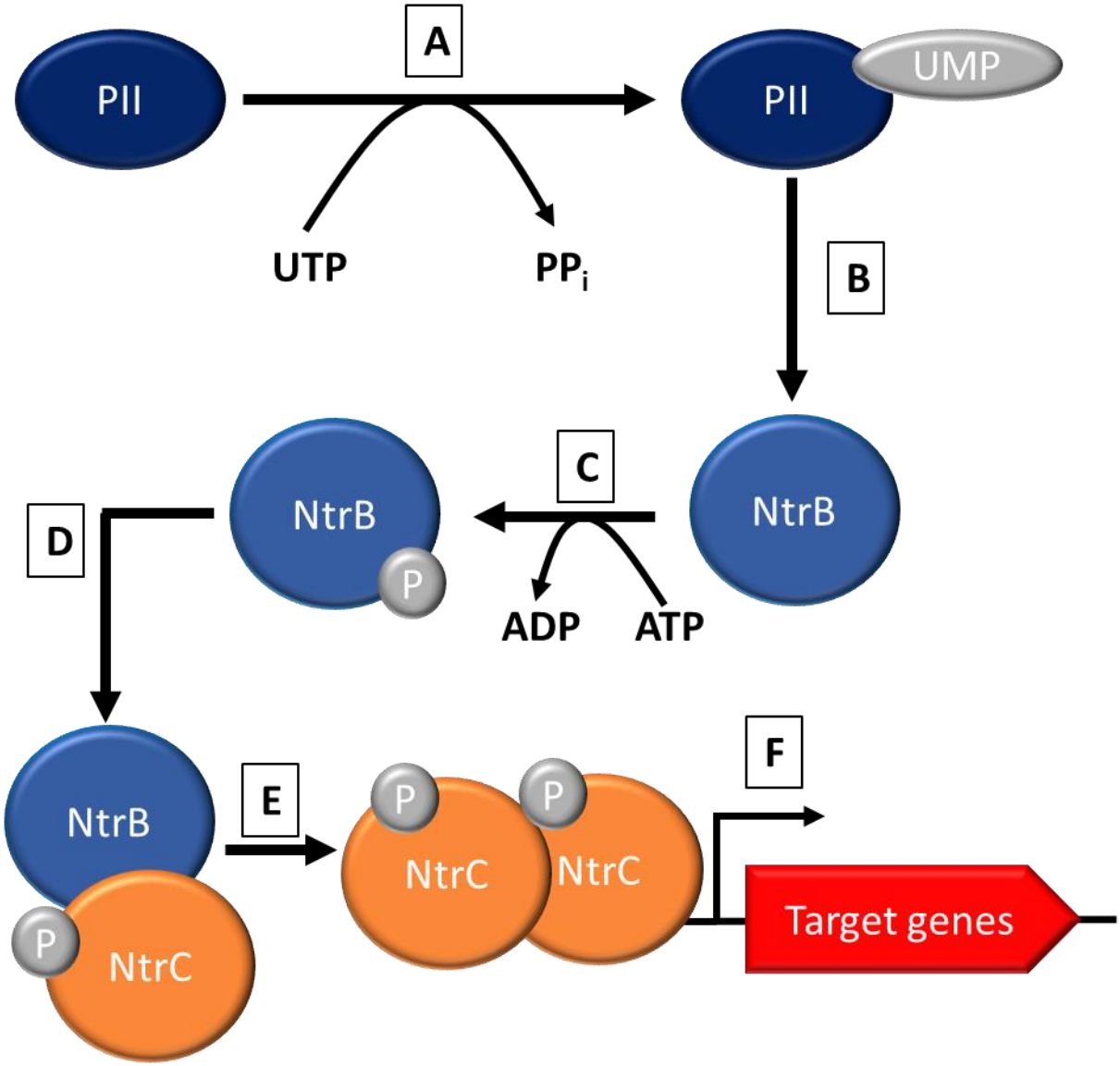


Figure 1. 10 The P_{II}/NtrBC mediated general nitrogen metabolism regulatory cascade. The PII protein is uridylated by a uridyltransferase in response to cellular nitrogen status signalling (A), the P_{II}-UMP protein is activated and activated the NtrB protein (B), the NtrB protein autophosphorylates (C), the phosphorylated NtrB transfers a phosphoryl group to NtrC activating it (D), activated NtrC dimerises at binding sites on DNA (E), finally, genes are expressed in response to NtrC binding (F).

1.9 Chaperones for maturation of nitrate reductases

Nitrate reductases use the complex molybdenum containing co-factor molybdopterin guanine dinucleotide in their active site. This co-factor is comprised of a modified pyranopterin structure linked to a molybdenum and a dinucleotide, in this case it is a guanine dinucleotide (Iobbi-Nivol and Leimkühler, 2013). In many nitrate reductases such as NasC from *P. denitrificans* this co-factor exists as a Mo-*bis*$(MGD)_2$ which contains two units of the pterin guanine dinucleotide (MGD) coordinated to the single central metal ion, Mo (Figure 1. 11) (Gates *et al.*, 2011). This co-factor is large and complex, it requires maturation by a series of proteins prior to the incorporation into nitrate reductases to form the catalytically competent holoenzymes.

The first stage of the generation of the MGD co-factor involves the synthesis of the molybdopterin (MPT) compound, this reaction has been characterised extensively in *E. coli*. MPT is generated from a precursor molecule known as precursor Z. The MPT synthase enzyme of *E. coli* uses two proteins to add sulfur to the precursor Z and thus create the dithiolene moiety that is the site of molybdenum coordination typically by bidentate ligation. The two proteins encoded are MoaE and MoaD. It is MoaD that has a thiocarboxylate group added at the C-terminus by another protein, this sulfur is derived from cysteine and will be added to the precursor Z to produce MPT. The stoichiometry of the reaction requires two MoaD proteins to generate a single MPT as addition of a single sulfur from each monomer achieves the two sulfur moiety that coordinates the molybdenum in MPT (Wuebbens and Rajagopalan, 2003). Whilst the addition of the crucial sulfurs is performed by the MPT synthase yet more proteins are required for addition of the molybdenum. In *E. coli* these are the MoeA and MogA proteins. MoeA is thought to incorporate the molybdenum into MPT and the MogA protein is thought to stabilise the MPT while this maturation takes place.

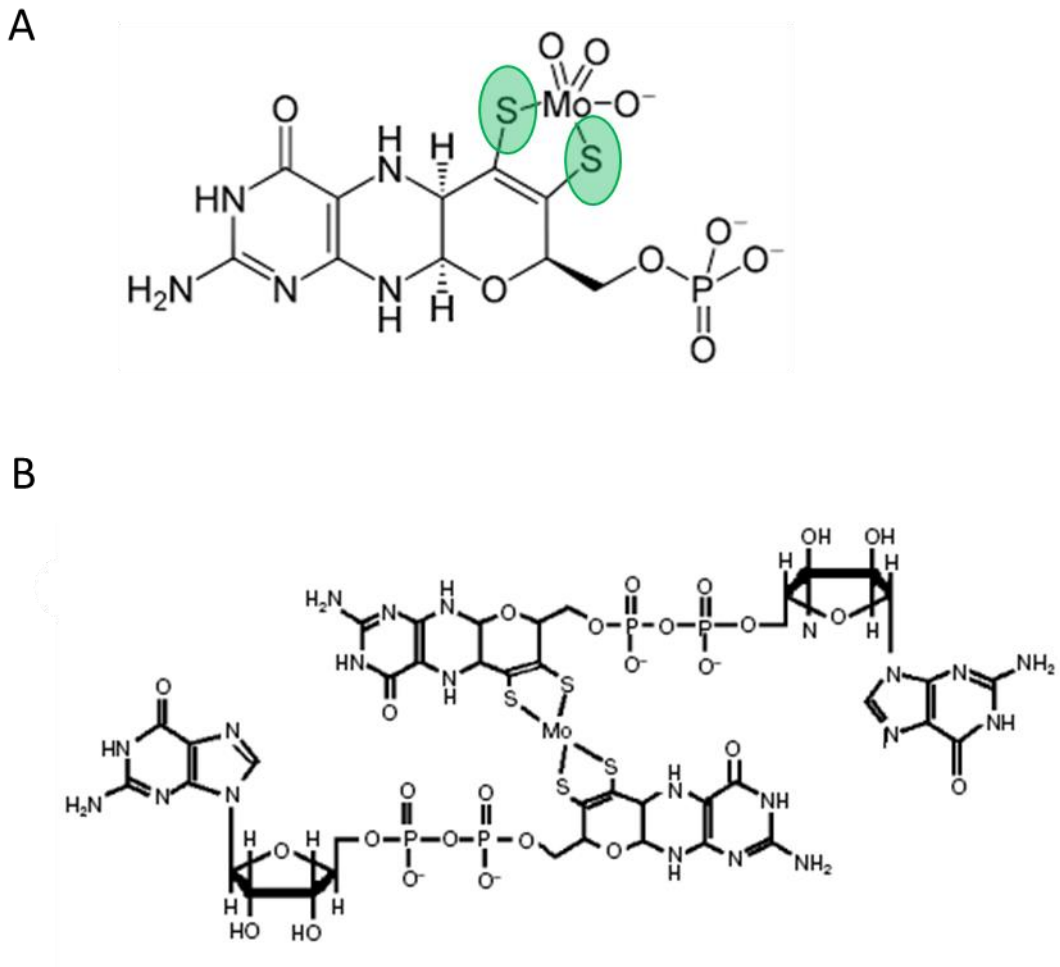


Figure 1. 11 The chemical structure of the molybdopterin (MPT) co-factor made by MPT synthase, the sulfur atoms added by MoaD are highlighted in green (A), and the Mo-*bis*(MGD)$_2$ final structure incorporated into microbial nitrate reductases and made by MobAB (in *E. coli*) (B) adapted from (Morozkina and Zvyagilskaya, 2007).

The second stage of co-factor maturation involves the linkage of the nucleotide. Nitrate reductases use a guanine derived nucleotide co-factor, however, cytosine can also be used in other systems yielding an MCD rather than an MGD co-factor (Johnson, Rajagopalan and Meyer, 1990). In *E. coli* the production of the MGD co-factor from MPT requires two proteins, MobA and MobB. MobB is not essential for molybdopterin guanine nucleotide biosynthesis, in mutation studies a *mobB* knockout mutant can still produce functional MGD but overexpression of the gene resulted in increased levels indicating that it is still functionally important (Palmer *et al.*, 1996). Further mutational studies in *Rhodobacter sphaeroides* showed MobA to be essential for the function of the DMSO reductase. This protein is in the same family as the assimilatory nitrate reductase and also

requires an MGD co-factor. Furthermore, inactive DMSO reductase isolated from *mobA* deficient cells could be reactivated with addition of isolated MobA, MPT, GTP and $MgCl_2$. This proved that MobA could formulate the MGD co-factor and this was sufficient to reactivate the DMSO reductase (Temple and Rajagopalan, 2000). The crystal structure of the *E. coli* MobA protein revealed an α/β architecture that splits two domains. The N-terminal domain was found to bind GTP in a co-crystallisation and the C-terminal domain was found to bind the MPT co-factor. This would bring these two structures together and allow for the reaction to generate MGD (Lake *et al.*, 2000).

For the DMSO reductase mentioned previously there was no additional protein required for the insertion and maturation of the MGD co-factor. This is not always the case. For the *E. coli* TMAO reductase TorD protein a *bis*$(MGD)_2$ co-factor is required. There is another Tor protein required for the correct insertion of the co-factor into TorD. This protein is TorA and it has been shown to interact with MobA and TorD, linking the final step of MGD biosynthesis to the reductase that requires the co-factor (Genest *et al.*, 2008).

The establishment of a system-specific link between MGD synthesis and co-factor insertion is exemplified by the MobA-TorD-TorA interaction chain reviewed above. Nitrate reductases have also been known to encode these system specific chaperones. For maturation of *E. coli* NarG the NarJ protein has long been known to be essential. Without the presence of NarJ, NarG is unable to function despite synthesis of MGD being uninterrupted. It was therefore concluded that NarJ inserts the MGD co-factor into the active site of NarG and activates the enzyme. This was demonstrated *in vitro* where isolated proteins from mutant backgrounds could carry out this function (Palmer *et al.*, 1996; Blasco *et al.*, 1998; Vergnes *et al.*, 2006).

The examples listed for TorA and NarJ are specific to a single system, however, recent work using the model denitrifier *P. denitrificans* has shown the NarJ protein also plays a role in maturation of the respiratory nitrate reductase and the assimilatory nitrate reductase. This is despite *narJ* being clustered with respiratory nitrate reductase genes on the genome. NarJ was shown to directly interact with both NarG and NasC in pull-down assays and a deletion of NarJ was sufficient to remove respiratory and assimilatory nitrate

reduction. This effect was similar to the removal of molybdenum from the media and confirmed the role of NarJ in maturation of NasC.

This section has shown the complexity associated with the production of complex co-factor containing metalloenzymes that bind the molybdopterin guanine dinucleotide cofactor. The assembly of a large, complex and multi-co-factor containing enzymes requires the involvement of multiple proteins and system specific chaperones for correct folding. This is important to understand and take account of when designing strategies for overexpression and purification of nitrate reductases.

1.10 *Paracoccus denitrificans*

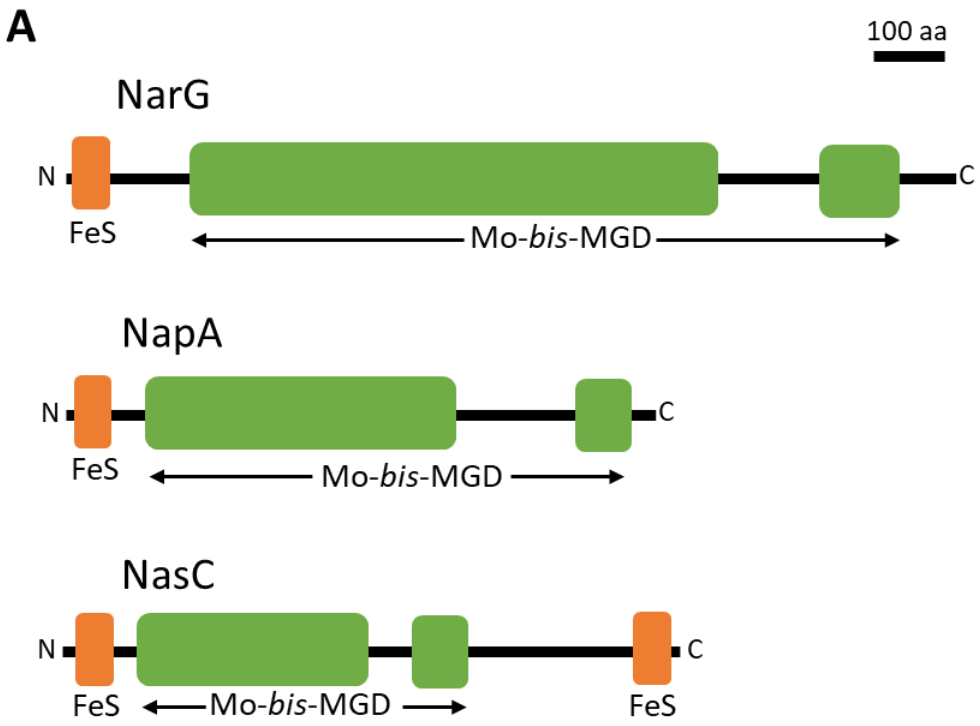
1.10.1 Organism overview

Paracoccus denitrificans was originally isolated by the Dutch microbiologist Martinus Beijerinck in 1910. Then named *Micrococcus denitrificans* it was found to reduce nitrate when grown anaerobically (Beijerinck, M.W. and Minkman, 1910). It is a non-motile Gram-negative alphaproteobacterium and has been a model organism for a number of years. As the name suggests *P. denitrificans* is able to complete respiratory nitrate reduction from nitrate to the release of dinitrogen gas. Furthermore, it has a varied central metabolism which can utilise a wide variety of carbon compounds for growth. It is able to grow heterotrophically using typical sugars such as glucose and it can use chemolithoautotrophic metabolism to grow using one-carbon compounds such as carbon disulfide. This model denitrifier has been used to study central metabolism of the eukaryotic mitochondria. Given the remarkable similarity between mitochondrial metabolism and that encoded by *P. denitrificans* it has been suggested as a descendant of the bacterial species that was engulfed and became the mitochondrion (John and F.R. Whatley, 1977). Furthermore, recent molecular biology advances have allowed for

mutations to be made in the genome and for overexpression in *P. denitrificans* Pd1222 (John and Whatley, 1975, 1977; Steinrãcke and Ludwig, 1993; Gates *et al.*, 2011).

1.10.2 Nitrate reduction by *Paracoccus denitrificans*

Paracoccus denitrificans is a paradigm denitrifier and has been extensively used as a model organism for the study of respiratory denitrification, redox-balancing nitrate reduction and, more recently, nitrate assimilation (Sears *et al.*, 1993; Van Spanning *et al.*, 1995; Gates *et al.*, 2011). This organism encodes three distinct nitrate reductases, the respiratory Nar, the redox-balancing Nap and the assimilatory Nas. Whilst there are distinct differences between these reductases there are certain core similarities. In all cases a molybdenum bis-molybdopterin guanine dinucleotide co-factor is coordinated and in all cases iron-sulfur clusters are provided for internal electron transfer.



B

```
NarG/1-1254       1 MSHLLDRLNFLKPTRKDVFSEGHGQTT-TEN---RDWEDTYRSRWRHDKIVRSTHGVNCTGSCSWKIYVKSGIVTWETQQTDYPRT   82
NapA/1-831        1 --MTISRRDLLKAQAAGIAAMAANIPLSADAQPVPGGVESLQITWSK------APGRFCGTGCGVMVGVKEGRVV-------ATHG   71
NasC/1-870        1 ------------------------------------------MQTIR------TTGPYCGVGCGVLATPDGKGGL-------GIAG   31

NarG/1-1254      83 RPDLPNHEPRGCAPGASYSWYLYSANRVKTPLIRGRLMKLWREKRKTMTPIQAWTAIQNDPQARESYTRIRGKGGFVRATWDEATE  168
NapA/1-831       72 DLLAEVNRGLNCVKGYFLSKIMYGQDRLTQPLLRKKDGV-----------------------------YAKDGEFTPVSWEEAFD  127
NasC/1-870       32 DPDHPANRGRLCVKGSALGETVGHGGRLLAPRIHG----------------------------------------REAGWDEALD   76

NarG/1-1254     169 ITAAANAYTARTYGPDRVFGFSPIPAMSMISYAAGTRY--------LSLLGGTCMSFYDWYCDLPPASPQTWGEQTDVPESADWYN  246
NapA/1-831      128 TMAAQAKRVLKDKGPTAVGMFGSGQWTIFEGYAATKLMRAGFRSNNLDPNARHCMASA------AYAFMRTFGMDEPMGCYDCFEA  207
NasC/1-870       77 LVAHRFRDTIAEHGPDSVGFYVSGQLLTEDYYVANKLMKGFIGSANIDTNSRLCMASA------VAGHRRAFGTDTVPGLYEDLEL  156

NarG/1-1254     247 AGYLLLWGSNVPQTRTPDAHFYTEARY--RGTKSAVICPDYSEAAKFGDIWLNAKQGTDAALGMAFGHVILREFHLDRQAEYFEDY  330
NapA/1-831      208 ADAFVLWGSNMAEMHPILWTRVADRRLGHPHVKVAVLSTFTHRSSDLADIPIVFKPGTDLAILNYIANHIIQTGRVNRD------F  287
NasC/1-870      157 ADTVVLVGSNLAWCHPVLYQRLAAARREARG-TRVVVVDPRRAASCDIADLHLALAPGADAALFNLLLAELIARRGLAGGD------A  235
```

Figure 1. 12 The co-factor environments of the three nitrate reductases encoded by *Paracoccus denitrificans* Pd1222 (A) and a multiple sequence alignment of the N-terminal region of the three reductases with conserved coordinating residues highlighted in black (B).

In all three proteins there is an N-terminal iron-sulfur cluster coordinated by covalent linkages to conserved cysteine residues (Figure 1. 12). Studies of the structure of *E. coli* NapA show that there is a conserved hydrogen bonding network that allows for electron transfer from the iron-sulfur cluster through to the molybdenum site (Jepson *et al.*, 2006). Whilst there is a high degree of similarity between the three reductases encoded there is a distinguishing feature of NasC. NasC forms an additional iron-sulfur cluster at the extreme C-terminus of the protein. This is a key differentiating factor compared to the other proteins. In both NapA and NarG there is a proven association with proteins involved in electron transfer from the membrane quinol pool. For NasC there is no such interaction to membrane associated proteins, instead there is an association proposed between NasC and NasB (Gates *et al.*, 2011). It is therefore important to question the use and necessity of this additional iron-sulfur cluster in interactions with potential partner proteins.

1.10.2.1 Denitrification by *P. denitrificans*

Paracoccus denitrificans is a model denitrifying organism, capable of complete nitrate reduction to dinitrogen and it encodes NarGHI, NirS, NorBC and NosZ to carry out each stage of the process (reviewed in section 1.5). By encoding respiratory Nar, the reduction of nitrate is used to generate a proton motive force and is a key part of anaerobic respiration carried out by *P. denitrificans*. This organism has been used extensively as a paradigm denitrifier and much of the bioenergetics of denitrification have already been reviewed (Carlson and Ingraham, 1983; Van Spanning *et al.*, 1995; Baumann *et al.*, 1996). More current study using *P. denitrificans* has focused on the flexibility to different environmental changes. The presence and absence of oxygen is a crucial factor in respiratory denitrification and recently *P. denitrificans* has been shown to utilise a bet-hedging approach to allow for survival in a constantly changing environment. This study also has major implications for mitigating release of nitrous oxide from agricultural land and demonstrates the new ways in which this organism can be a model for environmental effects (Lycus *et al.*, 2018).

1.10.2.2 Periplasmic nitrate reduction by *P. denitrificans*

Periplasmic nitrate reduction has been reviewed in section 1.7.5. *Paracoccus denitrificans* encodes this redox-balancing system despite lacking the cyclical photosystem encoded by the *Rhodobacter* sp. that first described it (Richardson *et al.*, 1988; Sears *et al.*, 1993). It has subsequently been used to investigate the regulation of carbon source preference and as an important part of the denitrification apparatus of other organisms (Ellington *et al.*, 2002; Bedmar, Robles and Delgado, 2005). In *P. denitrificans* the role of Nap appears to be clearly related to dissimilatory nitrate reduction as a means of removing excess electrons from the quinol pool and thus ensuring respiration and ATP synthesis homeostasis (Sears, Spiro and Richardson, 1997). This work has not focused on the alternative pathways for carbon source metabolism or indeed the effects seen with the other nitrate reduction systems. It has noted that denitrification by Nar was unchanged under aerobic conditions however as much of the study related to Nap in a microbiology context was conducted prior to the discovery of Nas in *P. denitrificans* the interplay between the two remains unclear (Sears *et al.*, 1993, 1997; Sears, Spiro and Richardson, 1997).

1.10.2.3 Nitrate assimilation by *P. denitrificans*

The discovery of an assimilatory nitrate reductase came significantly later than the discovery of both Nar and Nap. Observations of both nitrate reductase activity and of the growth and nitrate utilisation confirmed that growth of *P. denitrificans* could be sustained under aerobic and anaerobic conditions when using nitrate as a sole nitrogen source. It was noted in this first paper that there was a problem when detecting nitrate reductase activity. The physiological electron donor NAD(P)H is commonly associated with anabolic processes such as assimilation of nitrate. In this study reductase activity was measured using artificial electron donors (Sears *et al.*, 1997). This posed a question crucial to understanding assimilatory nitrate reduction, how does the nitrate reductase get electrons?

This question is yet to be fully answered, however, there have been major improvements in the understanding. In some species the answer lies in reduced ferredoxin or flavodoxin from photosynthesis, however this is not the case for *P. denitrificans* (Flores *et al.*, 2005). The nitrate assimilation system in *P. denitrificans* has been proposed to contain a core functional set of proteins. These are the nitrate reductase NasC, the nitrite reductase NasB and the small Rieske-type iron-sulfur protein NasG. This system has been studied extensively with gene deletion mutants shedding particular light on the roles played by each member. A genomic deletion of *nasB* results in a total loss of nitrate assimilation, a deletion of *nasC* results in a complete loss of nitrate assimilation but not of nitrite assimilation. This would be expected for these crucial reductases, however, a deletion of *nasG* results in loss of both nitrate and nitrite assimilation. This suggests a vital role for NasG in nitrate assimilation. As NasG is a Rieske-type iron-sulfur containing protein the apparent role may be electron transfer. This could be the link that allows electrons to move from the FAD site of NADH oxidation contained in NasB to the active site of NasC. This cannot, however, be the only role for NasG, if it were then nitrite assimilation could proceed unimpeded by the loss of NasG. It is therefore proposed that NasG may act to shuttle electrons to both of the catalytic cores in the reductases. This is proposed to act as a core nitrate reduction complex whereby nitrate is reduced to nitrite by NasC, and then this is swiftly reduced to ammonium by the nearby NasB. This system would work as a complex, the nature of which is unknown. In previous work the nitrate and nitrite reductases have been shown to separate by ion-exchange chromatography and thus the interaction is not proposed to be very tight, however beyond this it is not known (Gates *et al.*, 2011).

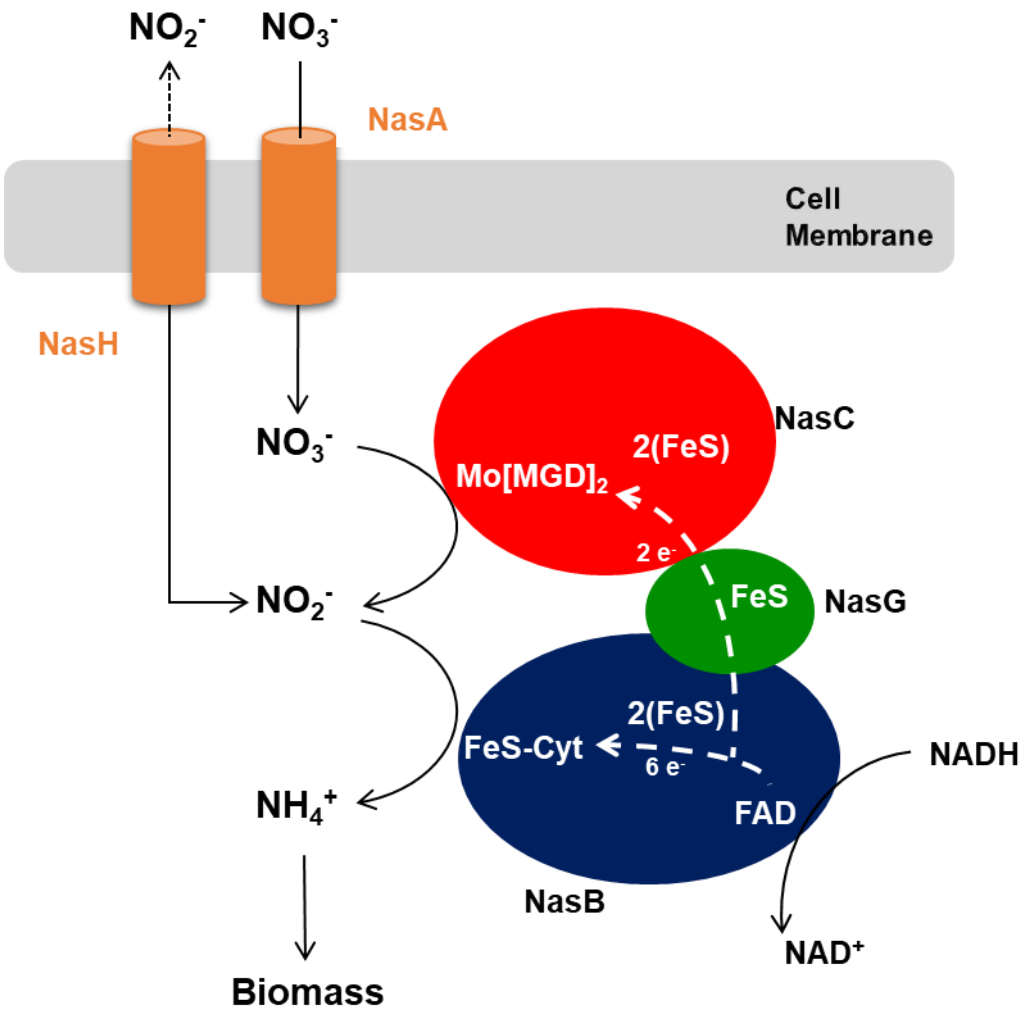


Figure 1. 13 The proposed nitrate/nitrite reductase complex for nitrate assimilation in *P. denitrificans*. NADH is oxidised by the FAD centre of NasB, electrons are then passed to the $Mo(MGD)_2$ centre of NasC for nitrate reduction and the siroheme centre of NasB for nitrite reduction, this is facilitated by NasG. Possible routes for electron transfer are denoted by white dashed lines and the relevant co-factors are annotated.

There remain many questions regarding the nitrate assimilation process in *P. denitrificans*. The nature of the proposed complex is not yet fully understood. Analysis of genomic deletion mutants has given some insights but *in vitro* work is still required to decipher the particular interactions required. Furthermore, more biochemical data is required to understand the implications of any potential complex discovered. The kinetic parameters of the two reductases must be balanced if they are to function as a complete unit and to avoid the release of free intermediates. It is also unclear what role the additional iron-sulfur cluster encoded by NasC plays in its function. Nitrate assimilation

remains the least well characterised nitrate reduction process carried out by *P. denitrificans* despite its potentially large contribution to the biogeochemical nitrogen cycle.

1.11 Aims and Objectives

At the outset of this study there were three principal aims. Firstly, previous work has focussed on the role of carbon substrates in promoting activity of the periplasmic nitrate reductase Nap. This study sought to investigate whether Nas could perform as a dual functional redox-balancing nitrate reductase and an assimilatory nitrate reductase. This question was approached using a combination of microbiological techniques, mainly continuous culture, and molecular biology (Chapter 3). Secondly, a system for the production of NasC has long been sought to generate purified protein for biochemical analysis. This thesis aimed to produce purified nitrate reductase for kinetic study and further biochemical assessment (Chapter 4). Thirdly, using a combination of molecular biology and biochemistry the protein-protein interactions required for *in vitro* nitrate reduction by NasC were assessed. This aim sought to further evolve the model of the nitrate assimilation protein interactions and to answer the question as to the role of NasG (Chapter 5).

Chapter 2: Materials and Methods

2.1 Bacterial growth media and conditions

Media was prepared using analytical grade water (Fisher scientific) (minimal media) or distilled water (rich media) and sterilised by heating to 121°C for 15 minutes using an autoclave (*LTE Scientific*). All media additives that were unable to be heated were sterilised by passage through a 0.22 µm syringe filter (*Sartorius Stedim*).

Eschericia coli and *Paracoccus denitrificans* strains were cultured at 37°C and 30°C, respectively, (unless otherwise stated). For aerobic growth bacteria were cultured in 50 mL of media in a 250 mL conical flask sealed with an air-permeable foam bung and aluminium foil. Shaking of cultures was maintained at 180 rpm by a rotary shaker to maintain aeration of cultures. For anaerobic growth of bacteria cultures were maintained in stationary, gas-tight bottles, sealed with rubber septa and degassed with oxygen-free nitrogen. Samples of headspace gas and liquid samples were withdrawn through the rubber septa as required.

For bacterial growth analysis 1 mL of culture volume was withdrawn and measured in 1 cm polystyrene cuvettes (*Sarstedt*) by the optical density at 600 nm (OD_{600}) using a spectrophotometer. Cultures were set up by inoculation with 1% (v/v) of appropriate initial culture. All *P. denitrificans* strains were washed prior to the next culture by pelleting cells, resuspending in basal media, pelleting again, and resuspending in media appropriate for the further culture of the bacteria. This was done to ensure no transfer of previous carbon or nitrogen sources.

Solid media contained 1.5% (w/v) agar (*Formedium Ltd.*). *E. coli* and *P. denitrificans* strains were grown on solid media at 37°C and 30°C, respectively, inverted and stationary. Appropriate antibiotics were added to media that was cooled to below 50°C prior to setting the plates.

2.1.1 Luria-Bertani medium

Luria-Bertani (LB) medium was prepared using distilled water (dH_2O) with 10 g tryptone, 10 g sodium chloride and 5 g yeast extract dissolved per litre (*Melford biolaboratories Ltd.*). Appropriate antibiotics were added if required (Table 2. 1).

2.1.2 Minimal salt medium

For culture of *P. denitrificans* a defined minimal medium was used. This contained 29 mM Na_2HPO_4, 11 mM KH_2PO_4, 0.4 mM $MgSO_4.7H_2O$ at pH 7.5 as detailed by Gates *et al*, 2011 (Gates *et al.*, 2011). As a variety of carbon and nitrogen sources were used, addition of nitrogen and carbon sources was performed by dilution of sterile 1 M stock solutions. The nitrate assimilation media typically contained 30 mM succinate and 10 mM nitrate as the sole carbon and nitrogen sources respectively. In addition, trace metals were added from a 500× stock solution of Vishniac-Santer trace metal solution (Vishniac and Santer, 1957). The Vishniac-Santer solution was modified to facilitate nitrate assimilation, ammonium molybdate was replaced by sodium molybdate to prevent the addition of contaminating ammonium (Gates *et al.*, 2011).

2.1.3 Media additives used

Table 2. 1 Antibiotics and additives used

Antibiotic	**[Stock] (mg/mL)**	**[Final] (µg/mL)**
Spectinomycin	25	25
Kanamycin	50	50
Gentamycin	15	15
Additive	**[Stock] (M)**	**[Final] (mM)**
IPTG	1	1
X-gal	0.1	0.1

Table 2. 2 Vishniac-Santer trace element solution

Compound	[Stock] (mM)	[Final] (µM)
EDTA	130	260
$Na_2MoO_4.2H_2O$	0.89	1.78
$ZnSO_4.7H_2O$	7.64	15.3
$FeSO_4.7H_2O$	18.5	37.0
$CaCl_2.2H_2O$	37.4	74.8
$MnCl_2.4H_2O$	25.0	50.0
$CuSO_4.5H_2O$	6.40	12.8
$CoCl_2.6H_2O$	6.72	13.4

2.2 Continuous culture

Continuous culture experiments were conducted using a BioFlo 310 benchtop fermentation vessel (*New Brunswick Scientific*). The total internal vessel volume was 1.5 L when all internal components were in place. The conditions inside the vessel were maintained as outlined in Table 2. 3. Nitrogen and carbon sources were varied according to the experimental program.

Table 2. 3 Continuous culture conditions

Condition	Setting
Temperature	30°C
Agitation	100 rpm
Dissolved oxygen	>90%
pH	7.5 ±0.2

pH probes were calibrated prior to sterilisation of the entire vessel by setting the range band between pH 7 and pH 10 using standard solutions provided by the manufacturer. The vessel was filled with minimal basal media and sealed. Sterilisation was achieved using an autoclave (*LTE Scientific*), heating to 121°C for 15 minutes. The vessel was

removed from the autoclave and connected to the monitoring station. Air was applied continuously through the vessel and agitation was initiated for a minimum of 6 hours to repolarise the dissolved oxygen probe prior to calibration. The dissolved oxygen probe was calibrated at 100% dissolved oxygen with air provided at maximum flow, 0% dissolved oxygen was calibrated after addition of oxygen-free nitrogen until the raw measurement values had stabilised. Media additives were injected through a sealed port into the basal minimal media and allowed to settle for 5 minutes. The vessel was inoculated with 1% (v/v) cells cultured in the relevant media. The setup is shown in Figure 2. 1.

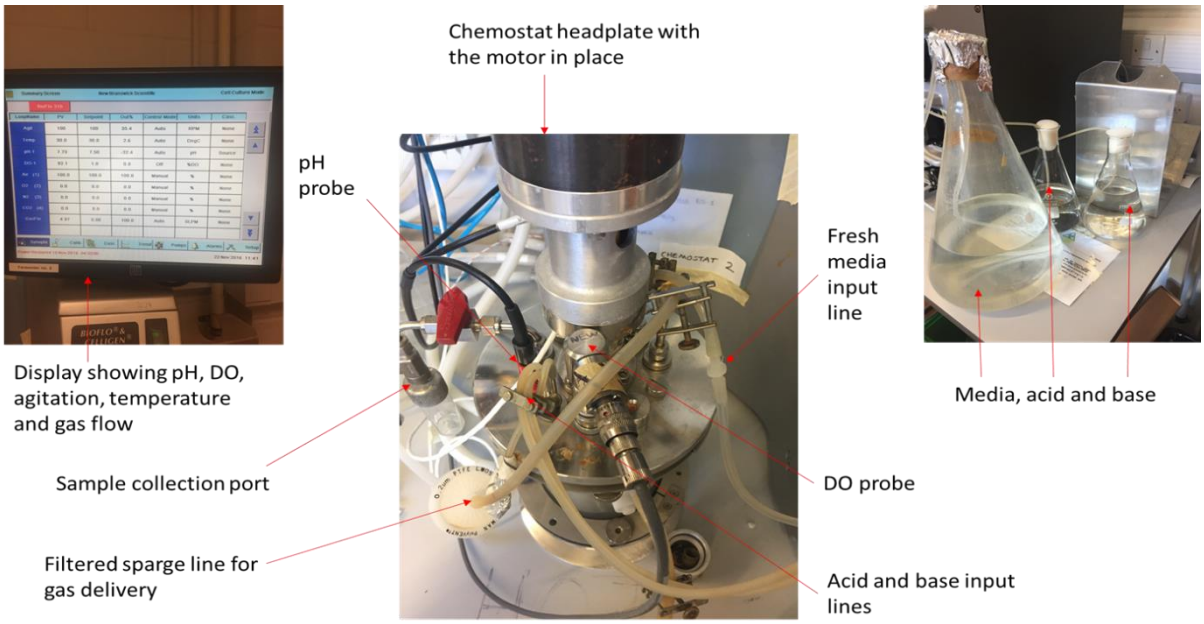


Figure 2. 1 The set-up of the continuous culture vessels.

2.3 Nitrate and nitrite concentration measurement

Nitrate and nitrite concentrations were determined by a Sievers 280i nitric oxide analyser (*Zysense*). The assay reduces nitrate or nitrite to nitric oxide gas which was subsequently detected by the analyser.

The reaction of nitrite to nitric oxide is shown by the equation: $I^- + NO_2^- + 2H^+ \rightarrow {}^1/_2I_2 + H_2O + NO$. The nitric oxide generated is gaseous and is carried by an oxygen-free nitrogen flow to the detection cell. The reaction conditions were a temperature of 40°C with a reagent of 1% (w/v) sodium iodide in 500 µL analytical grade water, made up to 5

mL with glacial acetic acid. The reaction mixture was maintained in a reaction chamber kept at the correct temperature by water circulation.

Nitrate was determined using a similar set-up however a different reaction was used shown by the equation: $2NO_3^- + 3V^{3+} + 2H_2O \rightarrow 2NO + 3VO_2^+ + 4H^+$. The reagent was made using 80 mg of vanadium (III) chloride dissolved in 10 mL 1 M hydrochloric acid and filtered through a 0.22 µm syringe filter (*Sarstedt*). The reaction chamber was maintained at 95°C for the reaction to proceed efficiently. As the above nitrate reaction can also take place with nitrite, to quantify the nitrate concentration, the concentration of nitrite needs to be subtracted from the concentration given by this reaction.

A calibration curve was created each time fresh reagent was used. All chemicals used in this protocol were HPLC grade reagents to avoid contaminating nitrite or nitrate.

2.4 Ammonium concentration determination

Ammonium concentrations were determined by an ammonia assay kit (*Sigma*) according to the manufacturers instructions. The ammonia assay reagent was reconstituted with analytical grade water according to the instructions. The absorbance at 340 nm was measured, using a spectrophotometer, on addition of cell free media, then glutamate dehydrogenase was added and after 5 minutes at 30°C the A_{340} was then measured. The ΔA_{340} was then used to determine the ammonia concentration of the sample.

2.5 Succinate and Butyrate concentration determination

Succinate and butyrate concentrations from minimal media were determined using a Bruker 500 MHz NMR spectrometer (*Bruker*). Aliquots of 600 µL of cell free media were added to 60 µL D_2O and samples were analysed in 500 MHz Wilmad 528 pp NMR tubes (*Sigma-Aldrich*). Data were acquired using a Bruker 500 MHz Avance-III NMR spectrometer fitted with a broad band BBFO z-axis gradient probe and were processed using Bruker's TopSpin 3.1.6 software. The pulse pattern included selective signal suppression of the water solvent peak to accurately resolve the succinate or butyrate

proton peaks. At the beginning of the experiment the nuclei are aligned along the Z-axis, a 90° non-selective pulse is applied along the X-axis and the magnetisation is rotated onto the Y-axis. A solvent selective 180° pulse along the X-axis inverts only the water nuclei in the opposite direction to the bulk magnetisation. A further non-selective 180° pulse inverts both the water and the bulk magnetisation such that they are still pointing in opposite directions. A further gradient is applied along the Z-axis to bring the bulk resonance back into alignment which simultaneously spreads out the signal from the water nuclei. This is repeated twice to minimise the solvent signal (Figure 2. 2).

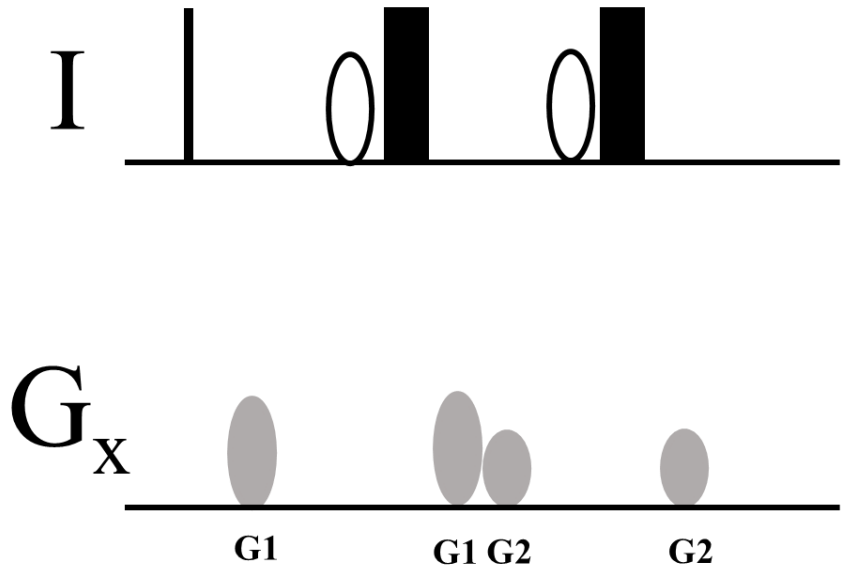


Figure 2. 2 Pulse pattern diagram for succinate and butyrate detection. Thin and thick squared bars represent non-selective 90° and 180° RF pulses respectively. Open curved bars represent water selective 180° RF pulses. Grey curved bars represent magnetic field gradients applied along the Z-axis.

All spectra were acquired automatically using macros to initially optimise the transmitter offset for the water frequency, the ^{1}H 90° pulse width and the magnetic field homogeneity. Further automated post acquisition processing performed exponential line broadening, Fourier transformation, phasing, chemical shift referencing and baseline correction. Finally the signal to noise ratio was measured for the relevant chemical shift positions relating to the specific signals produced by succinate or butyrate protons. As succinate is highly symmetrical only a single peak is produced in the region of 2 ppm and

is subsequently compared with a consistently empty area of the spectrum. Butyrate produces 3 signals and each of these were compared to a blank area of the spectrum, this allowed an internal check for the concentration as each peak compared to the standard curve should give the same butyrate concentration. The resultant peaks were analysed and compared to a standard curve, an example standard curve is shown in **Figure 2. 3**.

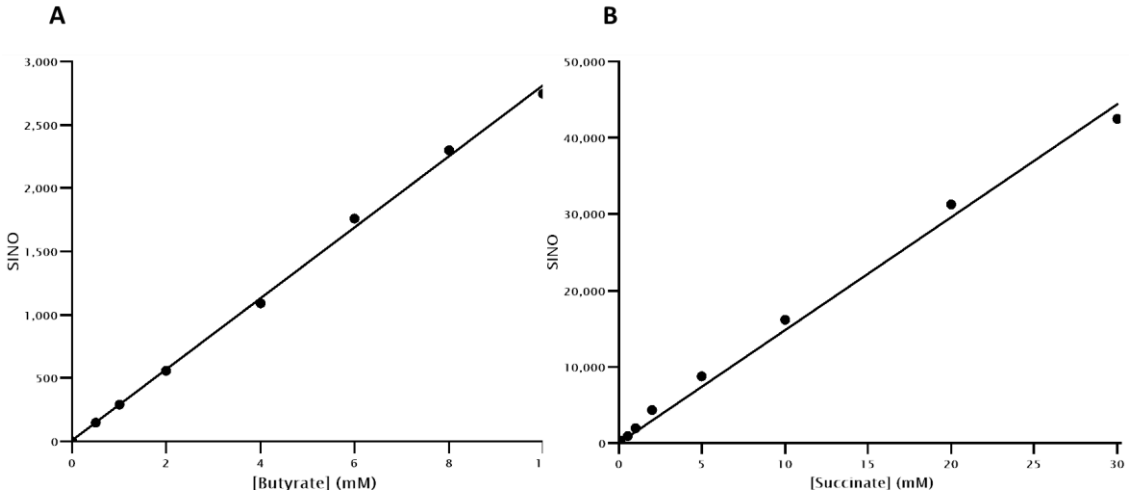


Figure 2. 3 Standard curve used to calculate butyrate concentration at the chemical shift 2.06 ppm (A) and succinate concentration at the chemical shift 2.01 ppm (B).

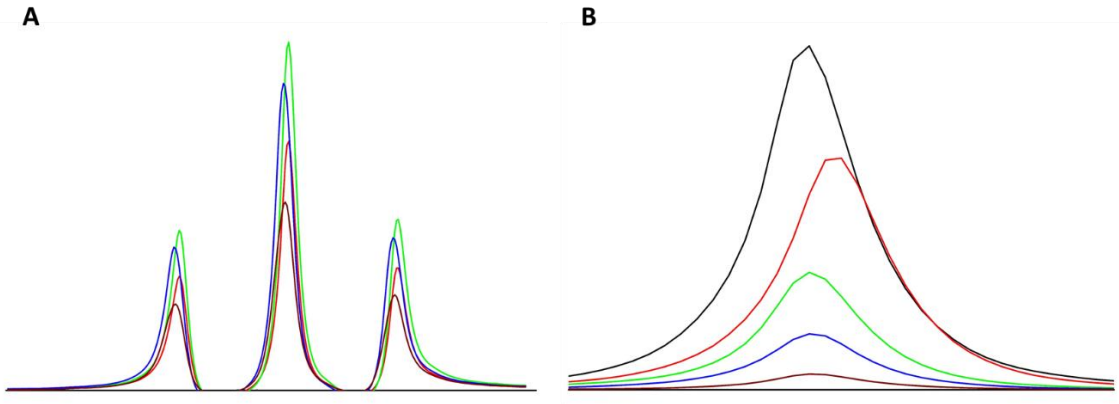


Figure 2. 4 Reduction in peak height and area for diminishing butyrate, chemical shift 2.06 ppm (A) and succinate, chemical shift 2.01 ppm (B) concentration obtained by ^{1}H NMR.

Table 2. 4 Typical acquisition and data processing parameters for all ^{1}H NMR experiments.

Parameter	Setting
No. data points	64k
No. scans	32
No. dummy scans	4
Spectral width	8000Hz
Acquisition time	4.089s
Relaxation delay	5.91s
Selective ^{1}H pulse	2ms square pulse
Temperature	298K
Gradient pulses	1ms
Gradient recovery	200μs
Gradient ratio (gp1:gp2)	31:11
Exponential line broadening	0.3Hz

2.6 Polymerase Chain Reaction

Polymerase chain reaction (PCR) reactions were carried out in a TC-512 Thermo Cycler (*Techne*) and the reagent mixture was made according to the polymerase manufacturers criteria. Reactions were performed in 0.2 mL thin walled PCR tubes.

2.6.1 MyTaq PCR

MyTaq 2× mastermix (*Bioline*) was used to perform checking PCR to quickly confirm the presence of sequences from bacterial colonies. The standard reaction set up is shown in Table 2. 5, and the standard protocol conditions in Table 2. 6.

Table 2. 5 Standard set up of MyTaq PCR mixture

Component	Volume added (µL)
MyTaq 2X Mastermix	10
ddH_2O	8.2
Forward primer (10 µM)	0.4
Reverse primer (10 µM)	0.4
Template	1.0

Table 2. 6 Standard PCR protocol of MyTaq PCR

Stage	Temperature (°C)	Time (s)	Cycles
Initial denaturation	95	300	1
Denaturation	95	30	30
Annealing	Primer dependent	30	30
Extension	72	10 s/kb	30
Final extension	72	600	1
Final hold	4	∞	1

2.6.2 Phusion PCR

For high-fidelity processes Phusion Polymerase (*ThermoFisher scientific*) was used. This was used when the resultant product was to be used in cloning or sequencing procedures. The standard reaction mixture is shown in Table 2. 7 and the PCR program in Table 2. 8.

Table 2. 7 Standard set up of Phusion PCR mixture

Component	Volume added (µL)
Phusion 5X reaction buffer	4
ddH_2O	13.4
Forward primer (10 µM)	0.5
Reverse primer (10 µM)	0.5
Template	1.0
Phusion Polymerase	0.2
dNTP's	0.4
DMSO	Up to 1 µL (optional)

Table 2. 8 Standard protocol of Phusion PCR

Stage	Temperature (°C)	Time (s)	Cycles
Initial denaturation	95	300	1
Denaturation	95	30	30
Annealing	Primer dependent	30	30
Extension	72	10 s/kb	30
Final extension	72	600	1
Final hold	4	∞	1

2.7 Reverse Transcription Quantitative Polymerase Chain Reaction

2.7.1 Cell harvesting

Cells were cultured until mid-exponential phase of growth (OD_{600} ~0.5) or were taken directly from operational continuous cultures. Unless otherwise stated 30 mL of culture was incubated with 12 mL of ice cold phenol:ethanol (5:95%) for 1 hour at 4°C. The cells were pelleted by centrifugation and excess phenol:ethanol was removed, leaving 1-2 mL

for resuspension. The cell pellet was then resuspended in residual phenol:ethanol and transferred to RNase free, sterile microcentrifuge tubes (*Sartorius stedim*). Cells were again pelleted by centrifugation and phenol:ethanol was removed entirely. Cell pellets were snap frozen in liquid nitrogen and stored at -80°C for up to 1 month prior to RNA isolation.

2.7.2 RNA purification

Total cellular RNA was isolated from harvested cells using a SV total RNA purification kit (*Promega*) according to the manufacturers instructions. To ensure complete removal of contaminating DNA, once RNA was obtained a further DNA removal step was performed by the addition of TurboDNase (*Invitrogen*) according to the manufacturers instructions. Isolated RNA was checked for integrity by absorbance ratios of $A_{260/280}$ and $A_{260/230}$, the absence of PCR products using DNA primers and positive identification of ribosomal RNA bands from an Experion automated electrophoresis system (*BioRad*).

2.7.3 Reverse transcription

To obtain cDNA for use in quantitative PCR reactions isolated RNA was reverse transcribed using a RevertAid premium first strand cDNA synthesis kit (*ThermoFisher*) according to the supplied manual. The presence of cDNA was confirmed by PCR of a gene known to be expressed in all conditions.

2.7.4 Quantitative PCR

Quantitative PCR was carried out using an AriaMx real time PCR system (*Ailgent*). Genomic DNA was used in known concentrations from 100 ng/µL to 0.001 ng/µL to create a standard curve from which cDNA could be quantified. Samples were analysed as 3 technical replicates for each of 3 biological replicates and relative expression was calculated against the control gene *rpoB*.

2.8 Preparation of nucleic acid

2.8.1 Plasmid DNA isolation

Plasmid DNA was obtained from bacterial cultures by culturing the relevant strain in LB media with any relevant antibiotics overnight at the appropriate temperature for *E. coli* or *P. denitrificans* respectively. The purification was carried out using a Qiagen miniprep kit (*Qiagen*) according to the manufacturers instructions, except from the following alterations; centrifugation steps were at 17,900 × g using a benchtop 5424 centrifuge (*Eppendorf*); a total of 3 mL of bacterial culture was used to purify plasmid DNA; 'Buffer EB' was replaced by ddH_2O as to prevent chemical contamination of downstream processes.

2.8.2 Genomic DNA isolation

Genomic DNA isolation was performed using a Wizard Genomic DNA Purification kit (*Promega*). A total of 10 mL of bacterial culture was used to purify genomic DNA and all buffers and columns were supplied with the kit. DNA was rehydrated in 100 µL ddH_2O rather than the supplied buffer to avoid chemical contamination of downstream processes. All other steps followed the manufacturers instruction.

2.8.3 Agarose gel electrophoresis

DNA was separated and visualised by agarose gel electrophoresis. Gels were cast with 1% (w/v) agarose dissolved in an appropriate volume of TAE buffer (45 mM Tris-acetate, 1 mM EDTA at pH 8). To visualise DNA, ethidium bromide (*Sigma-aldrich*) was added to the gel mixture to a 2 µM final concentration. To load DNA samples in gel wells, 10× DNA loading buffer (40% sucrose (w/v), 20% Orange G (w/v)) was added to the DNA sample. The gels were submerged in TAE buffer and 100 V was applied for 1 hour to

separate the DNA. DNA bands were imaged in a Gel Doc XR UV-transilluminator (*BioRad*).

2.8.4 Extraction of nucleic acids from agarose gel

Nucleic acids, including PCR products and linearised plasmids, were routinely purified from agarose gels for sequencing of products or cloning purposes. Gels were run according to standard procedures for an appropriate length of time according to the size of the fragment for purification. Gel slices were cut under UV light for a minimal length of time to minimise the damage to the DNA. Gel slices were dissolved and subsequent DNA was purified using a Gel Extraction kit (*Qiagen*) according to the manufacturers protocol, apart from the use of ddH_2O to elute purified DNA.

2.8.5 Restriction enzyme digestion of DNA

Restriction enzymes were sourced from New England Biolabs (*NEB*). Wherever possible NEB high fidelity enzymes were used as this allows multiple enzyme digestions to occur in a single 'CutSmart' buffer. Typically, reactions used 1 µg of DNA and were conducted at 37°C for at least 30 minutes. Reactions were stopped by heat inactivation or agarose gel electrophoresis.

2.8.6 Ligation of DNA fragments

Ligation of DNA inserts to their corresponding vectors was performed using a commercial T4 DNA Ligase (*NEB*). Ligation reactions were performed according to the manufacturers instruction with 50 ng of vector DNA and a typical molar ratio of vector to product of 1:3. Reactions were performed at 16°C overnight.

2.8.7 DNA sequencing

DNA sequencing was performed on purified PCR products and plasmids routinely to ensure there were no unintended mutations. Sequencing reactions were performed by Eurofins Genomics MWG and all DNA was provided according to the sample submission guidelines.

2.9 Transformation of plasmid DNA in *E. coli*

2.9.1 Chemically competent cells

Chemical competency of cells was achieved by the following steps. Firstly, the desired *E. coli* strain was cultured in 10 mL of LB media overnight. Once the culture had reached stationary phase, 2 mL was taken and used to inoculate a fresh 50 mL of LB media. This new culture was incubated at 37°C for 2-3 hours until an OD_{600} of ~0.5 was achieved. Cells were then pelleted by centrifugation at 5100 rpm in an Allegra 25R benchtop centrifuge (Beckman Coulter) for 10 minutes at 4°C. The media was then removed from the cell pellet and cells were resuspended in 15 mL of 0.1 M sterile $CaCl_2$ and kept at 4°C for 1 hour. Cells were then pelleted in the same way as previously. The pellet was then resuspended in 2 mL of 0.1 M sterile $CaCl_2$ and stored at 4°C for a minimum of 2 hours before use.

2.9.2 Transformation

Transformation of chemically competent cells used a standard heat-shock protocol. All temperatures were maintained by heated water baths and all reagents used were kept sterile. Between 50 and 200 µL of pre-prepared chemically competent cells were mixed with up to 100 ng of plasmid DNA. This mixture was kept on ice for 30 minutes. The cell and DNA mixture were then heated to 42°C for 45 seconds before being cooled rapidly on ice for 2 minutes. Finally, LB media was added to make a final volume of 1 mL and the cells were allowed to recover at 37°C for 1 hour. The transformed cell mixture was spread on to appropriate selective LB agar plates to screen for transformants.

2.10 Conjugation with *Paracoccus denitrificans*

Plasmid DNA was introduced into *P. denitrificans* strains using an established conjugation protocol. The relevant *P. denitrificans* strain was grown to stationary phase in 50 mL of LB media. The donor *E. coli* strain and the helper strain, containing the plasmid pRK2013 (Gates *et al.*, 2011), were initially cultured in 10 mL of LB media with appropriate antibiotics before being sub-cultured in 50 mL of LB media without antibiotics until an OD_{600} of ~ 0.5 was achieved. All strains were harvested by centrifugation at 5100 rpm in an Allegra 25R benchtop centrifuge (*Beckman Coulter*) for 10 minutes at 4°C and the supernatant was removed. Cell pellets were then resuspended in a 50% (v/v) glycerol solution and mixed. The subsequent mixture was placed on a cellulose-nitrate filter (*Sartorius stedim*) on a LB agar plate with no antibiotics and incubated for 4 days at 30°C. The post-incubation cell mixture was resuspended in a minimal volume of LB media and a dilution series was set up to 10^{-6}. From each dilution 100 µL was spread on to selective plates and incubated at 30°C until colonies were visible. Selected colonies were then tested by relevant PCR and/or sequencing to confirm successful transformation.

2.11 Production of mutant strains of *P. denitrificans*

For the generation of either gene knockout mutants or the introduction of tag sequences to the genome a strategy was used that had been previously developed for creating unmarked gene-knockout mutants (Sullivan *et al.*, 2013). To generate the 'knock-in' construct the regions ~500 bp up and downstream the stop codon of the gene were synthesised with the addition of the code for a Strep-II-tag (TGGAGCCACCCCCAATTTGAAAAA) inserted immediately adjacent to the stop codon. This region was then cloned into the suicide vector pK18*mobsacB* which is a mobilizable vector that carries genes conferring kanamycin resistance and sucrose sensitivity. This vector was introduced to wild-type *P. denitrificans* Pd1222 by tri-parental conjugation with *E. coli* carrying pRK2013 and *E. coli* DH5α containing pK18*mobsacB* + cloned sequences. Conjugations were set as described in section 2.10. The plasmid integrated into the genome via homologous recombination and produced

primary recombinants. Primary recombinants were selected for by kanamycin resistance, sucrose sensitivity and a positive amplification using M13 primers for PCR, these primers are specific to the plasmid backbone either side of the multiple cloning site. Once a primary recombinant had been identified it was grown in LB with no antibiotics for up to 4 days. The resultant mixture was serially diluted and spread on sucrose containing plates to select for colonies that are sucrose resistant, kanamycin sensitive and had a positive result using PCR that was specific to the Strep-II-tag sequence to differentiate between a mutant and a wild-type revertant. This process is shown in Figure 2. 5.

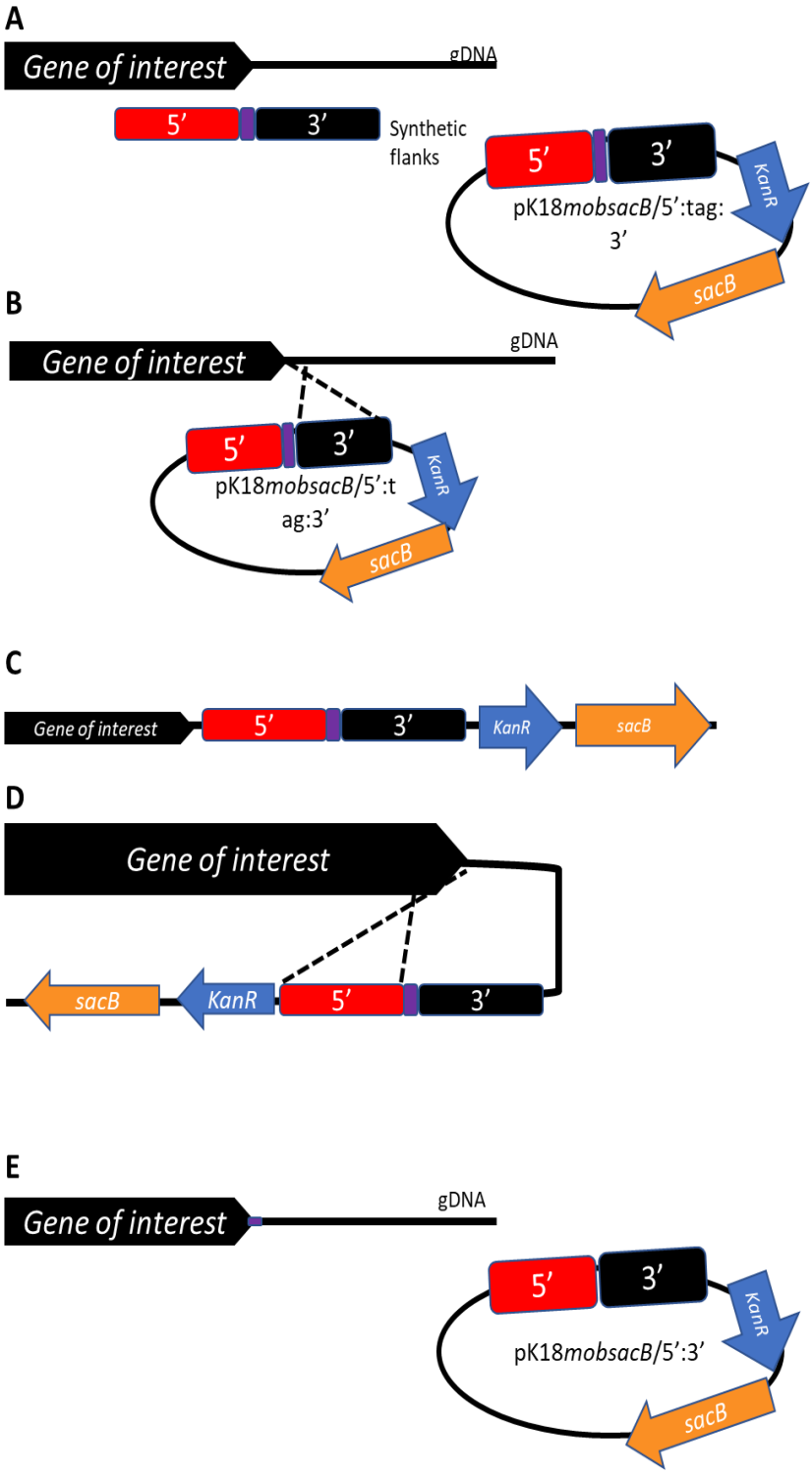


Figure 2. 5 The generation of a mutant containing the sequence for a Strep-II-tag on the 3' end of the gene. The region 5' to the stop codon, the sequence for the tag and the region 3' to the stop codon were assembled in the suicide vector pK18*mobsacB* (A), introduction into *P. denitrificans* resulted in homologous recombination by one flank (B), resulting in genomic integration of the plasmid (C). A second recombination event with the second flanking region then occurs (D) and results in the removal of the plasmid from the genome leaving behind the sequence for the Strep-II-tag (E).

2.12 Bradford assay

Protein concentration was determined using a Bradford assay reagent (*BioRad*). A standard curve of known quantities of Bovine Serum Albumin (*Sigma*) was established and used to calculate quantities for all protein samples. Appropriate volumes of protein sample were added to 800 µL ddH_2O in a 1 mL polystyrene cuvette (*Sartorius Stedim*) and the reaction was initiated by the addition of 200 µL of Bradford assay reagent (*BioRad*). Reactions were left for 5 minutes to develop a blue colour and the final absorbance at 595 nm was recorded and related back to the standard curve to determine protein concentration.

2.13 SDS-PAGE

For the identification of proteins by molecular weight, the separation technique sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used. Proteins were denatured using a loading buffer composed of 250 mM Tris-HCl pH 6.8, 10 % SDS (w/v), 50 % glycerol (v/v), 5 % β-mercaptoethanol (v/v), 0.1 % bromophenol blue (w/v). The denaturing buffer was diluted into the sample and the mixture was boiled at 95°C for 10 minutes, cooled on ice for 2 minutes and then centrifuged at 17,900 × g before being loaded on to a gel. For this work a range of acrylamide concentrations were used to ensure optimum resolution of the target proteins. For proteins below 30 kDa a 15% acrylamide gel was used, for whole cell extracts a 12% acrylamide gel was used and for proteins above 70 kDa molecular weight a 10% acrylamide gel was used. The standard components can be seen in Table 2. 9. Gels were run partially submerged in running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1 % SDS (w/v)). Precision Plus Prestained Dual Colour protein markers (*BioRad*) were used to estimate the size of sample proteins. For visualisation of proteins, InstantBlue Coomassie stain (*Expedeon*) was applied and incubated for 15 minutes.

Table 2. 9 SDS-PAGE gel components

Component	Resolving gel	Stacking gel
Polyacrylamide	X% (v/v)	4% (v/v)
1 M Tris-HCl pH 6.8	-	125 mM
1 M Tris-HCl pH 8.8	375 mM	-
Sodium Dodecyl sulfate	0.1% (w/v)	0.1% (w/v)
Tetramethylethylenediamine	0.03% (v/v)	0.03% (v/v)
Ammonium persulfate	0.05% (v/v)	0.05% (v/v)

2.14 Western blot analysis

Western blots were performed to identify specific proteins of interest, usually by the presence of an attached affinity tag. SDS-PAGE gels were run according to the protocol in section 2.13 and proteins were transferred to an iBlot mini dry transfer stack membrane (*Thermo Scientific*) using an iBlot transfer unit (*Thermo Scientific*) according to the manufacturers instructions. Addition of antibody and development of the membrane was as follows. The membrane was incubated in PBST (phosphate buffered saline + 0.1% Tween20) with 5% (w/v) dried milk powder dissolved for 1 hour at room temperature. The milk solution was then washed off with more PBST. The appropriate antibody was diluted in PBST according to the recommended dilution from the manufacturer. The antibody mixture was then applied to the membrane and kept in the dark, at room temperature, for 2 hours. The antibody was washed off with a 15 minute wash in PBST followed by 3 5 minute washes with more PBST. The development of the signal used West Pico development kit (*Thermo Scientific*) by mixing 4 mL of each buffer together in the dark and applying to this to the membrane for a further 15 minutes in the dark. The membrane was then developed in a G:Box transilluminator (*Syngene*) and imaged according to the automated system parameters.

2.15 Protein identification by MALDI-TOF/TOF

Gel slices were cut from SDS-PAGE gels, washed, treated with trypsin, and extracted according to standard procedures (Shevchenko *et al.*, 2006). The peptide solution resulting from the digest was mixed with α-cyano-4-hydroxycinnamic acid (*Sigma*) as matrix and analysed on an Autoflex™ Speed MALDI-TOF/TOF mass spectrometer **(***Bruker Daltonics™ GmbH, Coventry, UK*). The instrument was controlled by a flexControl™ (version 3.4, *Bruker*) method optimised for peptide detection and calibrated using SpheriCal™ polymer standards (*Polymer Factory, Stockholm, Sweden*). Data were processed in FlexAnalysis (*Bruker*) and the peak lists were submitted for a database search using an in-house Mascot Server (2.4.1, *Matrixscience, London, UK*). The search was performed against a *Paracoccus denitrificans* protein database downloaded from Uniprot (March 2017, 5242 sequences) using trypsin/P as enzyme with maximum 1 missed cleavage, 100 ppm mass tolerance, carbamidomethylation (C) as fixed and oxidation (M) and acetylation (protein N-terminus) as variable modifications.

2.16 Strep-II-tag affinity purification

For purifications of proteins with Strep-II-tag affinity tags, cells were lysed by 4 passages through a French Pressure Cell (*Thermo Scientific*) at 1000 psi. Cell debris was removed by ultracentrifugation using an Optima X100-K ultracentrifuge (*Beckman Coulter*) at ~280,000 × g. The resultant supernatant was filtered through a 0.45 µm syringe filter (*Sartorius Stedim*) to remove remaining large particles. The total volume was applied to a StrepTactin-XT column (*IBA scienctific*) that had been pre-equilibrated with appropriate binding buffer of pH 8 or above, to ensure efficient binding. After application of the sample the column was washed with binding buffer for 10 column volumes (CV) and then elution proceeded with addition of 20 mM biotin by 1 CV elution buffer, paused for 15 minutes in elution buffer and then continued with 3 more column volumes to elute all bound proteins. Purifications were carried out using a peristaltic pump at room temperature in an aerobic environment.

2.17 Anion-exchange chromatography

Protein purified from affinity purification was diluted up to 100× in binding buffer containing no NaCl to prevent excess salt affecting protein-column interactions. The purifications used a DEAE anion-exchange column (*GE Healthcare*) and was conducted with an AKTA Pure purification system. The column was pre-equilibrated with 5 CV of binding buffer, application of the protein sample followed until all protein had been applied, the column was then washed with 10 CV of binding buffer and elution took place over a linear gradient of increasing NaCl concentration over 20 CV.

2.18 Size-exclusion chromatography

For further purification of protein samples size-exclusion chromatography was used. Samples were concentrated to a final volume of 100 µL and injected on to a Superdex 200 10/300 GL analytical size-exclusion column. The column was pre-equilibrated in appropriate buffer and after the sample was applied the protein was eluted with 1.5 CV of buffer. Samples were collected in 1 mL fractions and protein elution was monitored by A_{280}.

2.19 UV-Visible Spectroscopy

To determine the presence of prosthetic groups and protein features UV-Visible spectroscopy was used. A 3310 Hitachi UV-Visible spectrophotometer and reduced volume quartz cuvettes with a 1 cm pathlength were used. The spectrophotometer was routinely set to measure at wavelengths from 200 to 800 nm, however for some applications it was extended to 1000 nm.

2.20 Nitrate and nitrite reductase activity assay (methyl viologen)

To determine the nitrate or nitrite reductase activity of protein samples, methyl viologen was used as an artificial electron donor to conduct activity assays. Methyl viologen can act as an electron donor to both the assimilatory nitrite and nitrate reductases of *P. denitrificans* (Gates *et al.,* 2011). Cells were cultured to mid-exponential phase (OD_{600} ~0.5) and cytoplasmic extracts were prepared as outlined by Gates *et al.,* 2011, or activity was measured using isolated proteins. Activity was measured in an anaerobic environment in a buffer containing 20 mM HEPES, 150 mM NaCl at pH 7.5. Methyl viologen was added to a final concentration of 1 mM and microlitre volumes of 10 mM sodium dithionite were added to generate the MV^{1+} electron donor. Absorbance was monitored at 600 nm over 300 seconds. The sample was added to the cuvette and a steady baseline was monitored for any background reduction. Reactions were initiated by addition of NO_3^- or NO_2^- at the stated concentrations.

2.21 Nitrate and nitrite reductase activity assay (NADH)

To determine nitrate and nitrite reductase activity with a physiological electron donor assays were conducted using NADH. Cells were cultured and harvested as described in 2.20. Activity was measured in a buffer containing 20 mM HEPES, 150 mM NaCl at pH 7.5. NADH was added to a final concentration of 0.1 mM and absorbance was measured at 340 nm over a time course of 300 seconds. Samples were diluted into the reaction cuvette and a baseline of background activity was established. Reactions were initiated by the addition of NO_3^- or NO_2^- at the stated concentrations.

2.22 Strains, primers and plasmids used

Table 2. 10 Bacterial strains used in this study.

Name	Relevant characteristics	Source
Paracoccus denitrificans		
Paracoccus denitrificans Pd1222	Wild-type strain, Rif^R, $Spec^R$	(de Vries *et al.*, 1989)
Pd∆*nasC*	*nasC* deletion variant of Pd1222, Rif^R, $Spec^R$	Donated by Dr. Nanakow Baiden
Pd∆*narG*	*narG* deletion variant of Pd1222, Rif^R, $Spec^R$	Donated by Dr. Nanakow Baiden
Pd∆*napA*	*napA* deletion variant of Pd1222, Rif^R, $Spec^R$	Donated by Dr. Nanakow Baiden
Pd∆*nasC*:∆*narG*	*nasC* and *narG* deletion variant of Pd1222, Rif^R, $Spec^R$	Donated by Dr. Nanakow Baiden
Paracoccus denitrificans Pd4449	*nasC*-Strep-II-tag variant of Pd1222, Rif^R, $Spec^R$	Present work
Paracoccus denitrificans Pd4452	*nasB*-Strep-II-tag variant of Pd1222, Rif^R, $Spec^R$	Present work
Escherichia coli		
E. coli 803	Host strain for large plasmids	(Wood, 1966)
E. coli DH5α	Host strain for pK18*mobsacB* derived plasmids	(Hanahan, 1983)

Table 2. 11 Plasmid Vectors used in this study.

Plasmid	Relevant Characteristics	Source
pRK2013	Used as a mobilisation plasmid in tri-parental conjugations, Km^R	(Figurski and Helinski, 1979)
pK18*mobsacB*	Mobilisable allelic exchange suicide vector, sucrose sensitivity, Km^R	(Schäfer *et al.*, 1994)
pLMB511	pLMB509 derivate allowing for addition of a Strep-II-tag, $Gent^R$	Donated by Dr. Manuel Soriano-Laguna
pLMB511*nasJnasC*	pLMB511 derived vector encoding an N-terminal Strep-II-tag on NasC and no tag on NarJ, $Gent^R$	Donated by Dr. Juan Cabrera Rodriguez
pARJ001	pK18*mobsacB*-derivative, construct for addition of Strep-II-tag sequence to *nasC*, Km^R	Present work
pARJ002	pK18*mobsacB*-derivative, construct for addition of Strep-II-tag sequence to *nasB*, Km^R	Present work

Table 2. 12 Oligonucleotide primers used in this study.

Name	Sequence (5'→3')	Use
Sequencing primers		
P1 (Rev.)	CATGTGGATGCCGATCCTGA	Sequencing for *nasC* knockout and Strep-II-tag integration

P2 (Rev.)	ATTTTTCAAATTGGGGGTGGCTCC	Primer for identifying Strep-II-tag integrations
P3 (For.)	GGTGCGGCTGGCCTTTTTG	Sequencing for *nasC* knockout and Strep-II-tag integration
P4 (For.)	ACCGGAAAGCTGAAACAGGA	Sequencing to confirm *napA* knockout
P5 (Rev.)	GGACATGGGTTCGGTCCAG	Sequencing to confirm *napA* knockout
P6 (For.)	TGCTTGGGTTGCAAATGCC	Sequencing to confirm *narG* knockout
P7 (Rev.)	GATCATGCGCAAGGAAGTCG	Sequencing to confirm *narG* knockout
M13 (For.)	TGTAAAACGACGGCCAGT	Sequencing primer for checking pK18*mobsacB* constructs
M13 (Rev.)	CAGGAAACAGCTATGAC	Sequencing primer for checking pK18*mobsacB* constructs
RT-qPCR primers		
RpoB_F	GCGACGTTCATCCGACCCATTACG	For use in quantitative PCR reactions
RpoB_R	TGCCCTCGACCACCTTGCGGTAGG	For use in quantitative PCR reactions

NasC_F	CTATGTCGCCAACAAGCTGA	For use in quantitative PCR reactions
NasC_R	CCAGCTCCAGATCCTCGTAG	For use in quantitative PCR reactions
NarG_F	GTATGCCCATACCGACCAGT	For use in quantitative PCR reactions
NarG_R	CCGGATGTTGTAGTCGATCA	For use in quantitative PCR reactions
NapA_F	ATGGATCCAGGTGAACAA	For use in quantitative PCR reactions
NapA_R	GTCCGAGACGACGATGAAAT	For use in quantitative PCR reactions

Chapter 3: Investigating the carbon dependent; nitrogen source preference of *Paracoccus denitrificans*

3.1 Introduction

3.1.1 The link between carbon and nitrogen metabolism

When bacteria are presented with inorganic nitrogen substrates they must find a way to incorporate the nitrogen into organic matter for growth. Across many species of plants and bacteria two distinct systems for the assimilation of ammonium to glutamate are utilised. These are the glutamate dehydrogenase (GDH) and the glutamine synthetase – glutamine oxoglutarate aminotransferase (GS-GOGAT) pathways, discussed in detail in section 1.7. These systems are intrinsically linked to the carbon metabolism of the cells as they require 2-oxoglutarate, directly produced from the TCA cycle. While both pathways use this TCA cycle intermediate, they differ in their energy requirement. The GDH pathway does not require energy input, in the form of ATP, and instead mediates the assimilation of ammonium to glutamate under high concentrations of ammonium, due to the high K_M of these enzymes for ammonium (~1 mM or higher) (Botman, Tigchelaar and Van Noorden, 2014).

The GS-GOGAT cycle requires a different mechanism to incorporate ammonium into biomass. In the first reaction glutamine is generated from ammonium, glutamate and ATP. In the second stage the glutamate is combined with ammonium, 2-oxoglutarate and NAD(P)H to generate 2 molecules of glutamate (Meers, Tempest and Brown, 1970). This clearly constitutes an increased energy demand on cells which is important in this work as carbon supply is limited.

In this chapter data are presented that link nitrogen metabolism and carbon source oxidation state. The oxidation state of the carbon source is important when the two different ammonium assimilation pathways are considered. The higher energy demand of the higher affinity system must be balanced with the lower energy demand of the low affinity system. Under different carbon source, and concentration, conditions it is feasible to predict nitrogen source preference changes.

3.1.2 The choice of carbon substrates

In this study two carbon substrates were used as sole-carbon sources for bacterial growth. *Paracoccus denitrificans* is a metabolically versatile organism capable of using a wide variety of carbon substrates including pyruvate, succinate, butyrate, malate and acetate (Gates *et al.*, 2011; Felgate *et al.*, 2012; Hahnke *et al.*, 2014; Olaya-Abril, Hidalgo-Carrillo, *et al.*, 2018). Whilst the carbon substrates used by *P. denitrificans* are numerous, few have been studied in *Paracoccus* species in relation to nitrogen metabolism.

Succinate is a typical laboratory carbon source for the culture of *Paracoccus* species. It has been used as the sole carbon source in numerous investigations of nitrate assimilation as well as anaerobic nitrate respiration (Gates *et al.*, 2011; Giannopoulos *et al.*, 2017). Succinate is a highly symmetrical dicarboxylic acid comprising of two distinct carbon environments. There are two R-COOH groups located either side of two R^1-CH_2-R^2 groups. This results in a total oxidation number of carbon of +2 across the molecule and an average oxidation number of +1/2 per carbon. This means the molecule is considered a relatively oxidised carbon source. Succinate is also a key intermediate of the TCA cycle. It is readily oxidised by bacteria to fumarate by succinate dehydrogenase, which also plays a role in the respiratory chain. Succinate is key to the TCA cycle and therefore drives production of NADH (Hederstedt and Rutberg, 1981). Whilst metabolised by the TCA cycle, which also drives production of NADH, there is no additional metabolic process carried out that drives excess production of reducing equivalents. In effect the only substrate that could provide fewer molecules of NADH in the TCA would be the highly oxidised carbon compound malate (see 1.7.1).

Butyrate contrasts with succinate as it is a short chain fatty acid comprising of an R-CH_3 group, two R^1-CH_2-R^2 groups and a R-COOH group. As with other short chain fatty acids the metabolism starts at the membrane with β-oxidation. This process breaks down short chain fatty acids in the prokaryotic cytoplasm and the cytosol of eukaryotic mitochondria to yield acetyl-CoA which flows into the TCA cycle. In this process two carbons are removed sequentially to break down the short chain fatty acid. The process is cyclical, and each cycle generates $FADH_2$ and NADH. In this way extra reducing equivalents are generated prior to those produced in the TCA cycle (Houten and Wanders, 2010). Butyrate has an overall carbon oxidation number of -4, and therefore an average oxidation number of -1 for each carbon. Compared to succinate it is a more reduced carbon source. Contrasting the metabolism of butyrate and succinate there will be a significant difference in the production of cytoplasmic reducing equivalents between metabolism of the two carbon substrates.

The difference between both the oxidation state of carbon and the difference in the metabolic pathways used by bacteria means these carbon substrates can be used to investigate carbon-nitrogen metabolic interplay. Previous studies have considered these carbon sources in relation to carbon source preference, and expression of periplasmic nitrate reductase Nap (Sears, Spiro and Richardson, 1997; Ellington *et al.*, 2002). In this study they will be used as an oxidised carbon substrate (succinate) and a reduced carbon substrate (butyrate) to investigate nitrogen assimilation by bacteria. The important interplay between the production of excess reducing equivalents, the availability of energy for ammonium assimilation and the control of bacterial nitrate assimilation will be investigated in this chapter.

3.1.3 Theory of continuous culture

Continuous culture has been used extensively to investigate bacterial responses to different respiratory substrates as well as more general bacterial metabolism. It has been used to investigate the reliance on copper for nitrous oxide reductase in *P. denitrificans* (Felgate *et al.*, 2012). It has also been used to study the ways in which *P. denitrificans* adapts from aerobic to anaerobic conditions (Giannopoulos *et al.*, 2017). In these studies bacteria were cultured initially in a batch growth phase to provide biomass for study. The

continuous culture phase was then initiated by the addition of fresh media at a steady flow rate. This addition washes away a portion of the culture resulting in a constant in and out flow. This provides nutrients to the bacteria and removes cells at a user determined rate. It is therefore important to understand the core concepts of continuous culture to understand the experiments presented in this chapter.

In the continuous culture experiments presented in this work the aim was to maintain a constant chemical environment for bacteria in order to maintain their growth rate, and therefore the culture, in the mid-exponential phase of growth. This would then allow for the determination of measurable carbon and nitrogen input and usage by growth yields and RT-qPCR. In order to achieve this the key feature to understand was the growth rate of the bacteria. Growth rate varies across the growth profile of any organism and can be significantly different depending upon the media provided. A continuous culture set up can flow fresh media into a sealed vessel and wash out excess volume, this presents a constant chemical environment and is typically called a chemostat. If the relative inflow of fresh media, and therefore outflow of culture volume, is equal to the growth rate the culture is maintained in exponential growth phase. Under steady state conditions the dilution rate (D) required is proportional to the growth rate of the bacteria (μ). Where D = μ then there will be no net change in growth. D must therefore be related to the culture vessel volume. This is done by using the equation $D = \frac{F}{V}$ where D is dilution rate in h^{-1}, V is the internal maximum vessel volume in L and F is flow rate in $L.h^{-1}$. This key metric then determines the volumetric flow rate (F) required to maintain the culture in steady state (Gilbert, 1985).

Once the biomass of the culture reaches an equilibrium with no significant loss or gain in biomass over time it can be considered in steady state growth. A culture that is in a steady growth phase can then be studied for its response to fresh nutrient addition. When bacteria are grown in batch growth experiments the continuous change of chemical environment alters their metabolic processes. The bacteria present in the culture when the fresh media is initially supplied are therefore adapted to a stationary phase culture. It is important to provide a continuous flow for a period of time to allow a change of media conditions from batch to continuous phases, and to allow division of cells sufficient to have only continuous culture adapted cells present. Data can then be analysed after this crucial time

point. Data analysed prior to this point would have batch culture influences. These factors are important considerations for the setup and maintenance of continuous culture experiments.

3.2 Results

3.2.1 Batch culture of *Paracoccus denitrificans*

In this work continuous culture techniques are used to investigate the effect carbon source oxidation state has on both the utilisation of nitrate during growth and the expression of the distinct nitrate reductase genes. Critically prior to conducting continuous culture experiments the nature of the bacterial growth must be assessed. To determine appropriate dilution rates (D) (section 3.1.3) batch culture experiments were undertaken. Bacteria were cultured in a plate reader format using either succinate or butyrate as the sole carbon substrate at the specified concentration and utilising either nitrate, ammonium or equimolar nitrate and ammonium as the sole nitrogen source(s). To further characterise the impact of the chosen carbon substrate two different concentrations were chosen to represent a 'high' carbon content and a 'low' carbon content.

The first condition investigated was the common laboratory culture condition comprising of minimal salts media with 30 mM succinate and 10 mM nitrogen source. This is a standard medium composition used to investigate nitrate assimilation (Gates *et al.*, 2011; Luque-Almagro *et al.*, 2017; Olaya-Abril, Hidalgo-Carrillo, *et al.*, 2018). *P. denitrificans* was cultured using either 10 mM NO_3^-, 10 mM NH_4^+, or 5 mM NO_3^- and 5 mM NH_4^+ (Figure 3. 1).

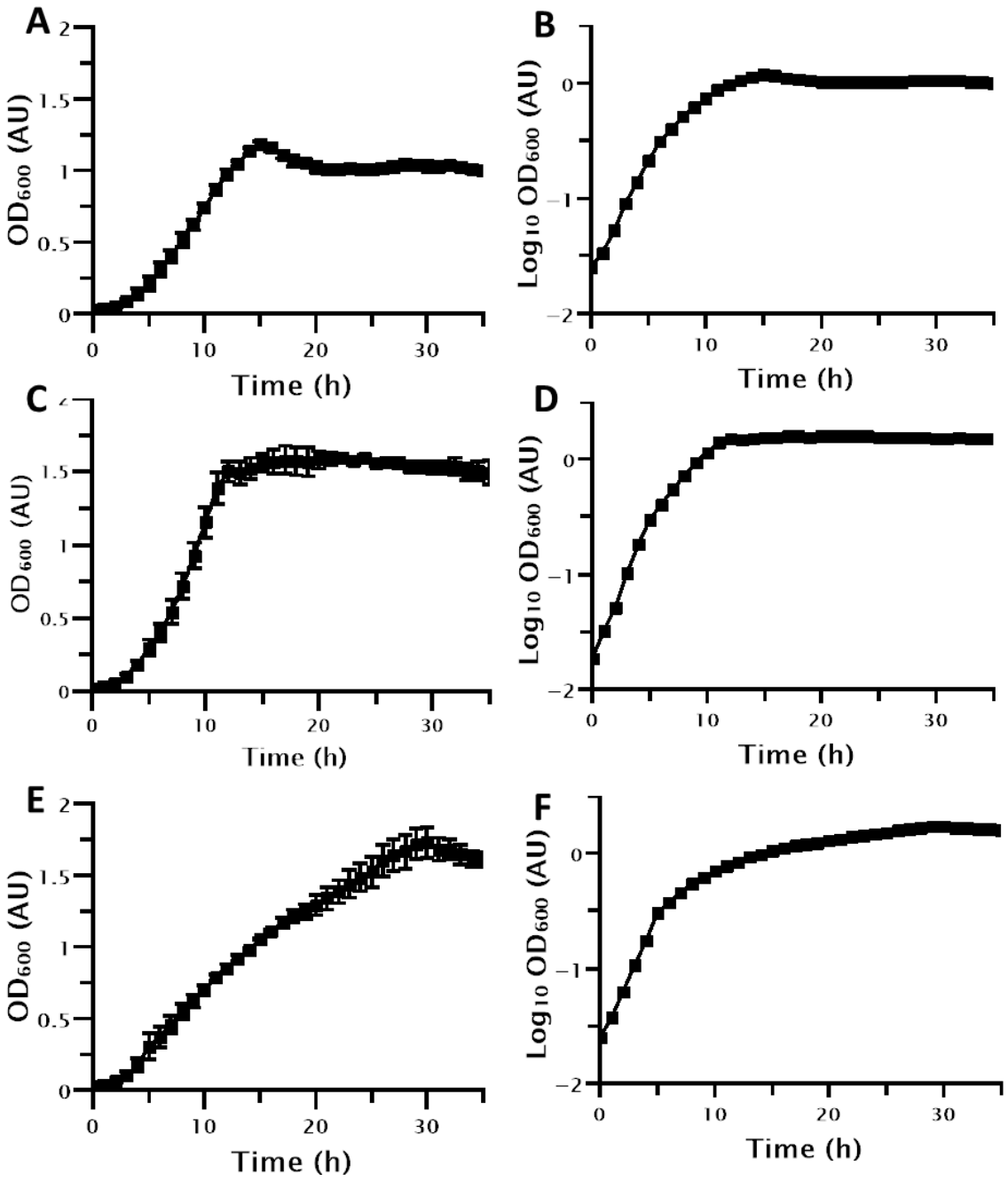


Figure 3. 1 Growth profiles of *P. denitrificans* Pd1222 cultured using 30 mM succinate in all conditions. Nitrogen sources were 10 mM NO_3^- (A&B), 10 mM NH_4^+ (C&D) and 5 mM NO_3^- and 5 mM NH_4^+ (E&F). Data presented are the average of three independent biological replicates and is shown as linear plots (A,C&E) and as semi-log plots (B, D&F).

The data presented demonstrates a preference for ammonium under this carbon source regime. This can be determined initially by the maximum OD_{600} achieved. In both culture conditions where ammonium is present (C&E) the OD_{600} reached was >1.5 whereas it was significantly lower when the bacteria were solely cultured on nitrate (A). Furthermore, the growth rates differed significantly between ammonium and nitrate grown cells. In the case of nitrate grown cells the apparent maximum growth rate (μ_{max} app.) was 0.06 h^{-1} ±0.002 however for ammonium cultured cells it was 0.07 h^{-1} ±0.001. the mixed nitrogen source experiment shows a diauxic growth pattern with a small initial growth rate of 0.08 h^{-1} ±0.004, which is comparable to the ammonium grown cells, and a second slower growth rate of 0.04 h^{-1} ±0.004, which is more comparable to nitrate grown cells. This batch growth data therefore indicates that nitrate assimilation and ammonium assimilation are not simultaneously carried out under conditions with 30 mM succinate. Whilst this was a minor difference regarding the batch culture experiments this difference is key when designing continuous culture experiments. A difference of 0.01 in dilution rate is equivalent to a reduction of media input of 66.67 $mL.h^{-1}$. This result highlights the importance of conducting these batch experiments prior to the commencement of continuous culture growth.

As discussed in section 3.1.1 it was important to analyse the nitrogen source preference under low carbon substrate availability. Under low carbon availability conditions the bacteria would need to respond with strict metabolic control in order to maintain maximum growth. *P. denitrificans* was cultured in minimal salts medium supplemented with 5 mM succinate and 10 mM nitrogen source (Figure 3. 2). This presents a significant challenge to the bacteria and as such it was important to characterise their response in batch culture. The major difference between 5 mM and 30 mM succinate is the reduction in maximum OD_{600}. This is likely due to the reduction in total carbon available to the cells. It is also worth noting the maximum apparent growth yield was highest in the ammonium cultured cells, this is likely because the bacteria did not need to generate the level of reductant required to assimilate nitrate and could therefore use the succinate for biomass generation instead.

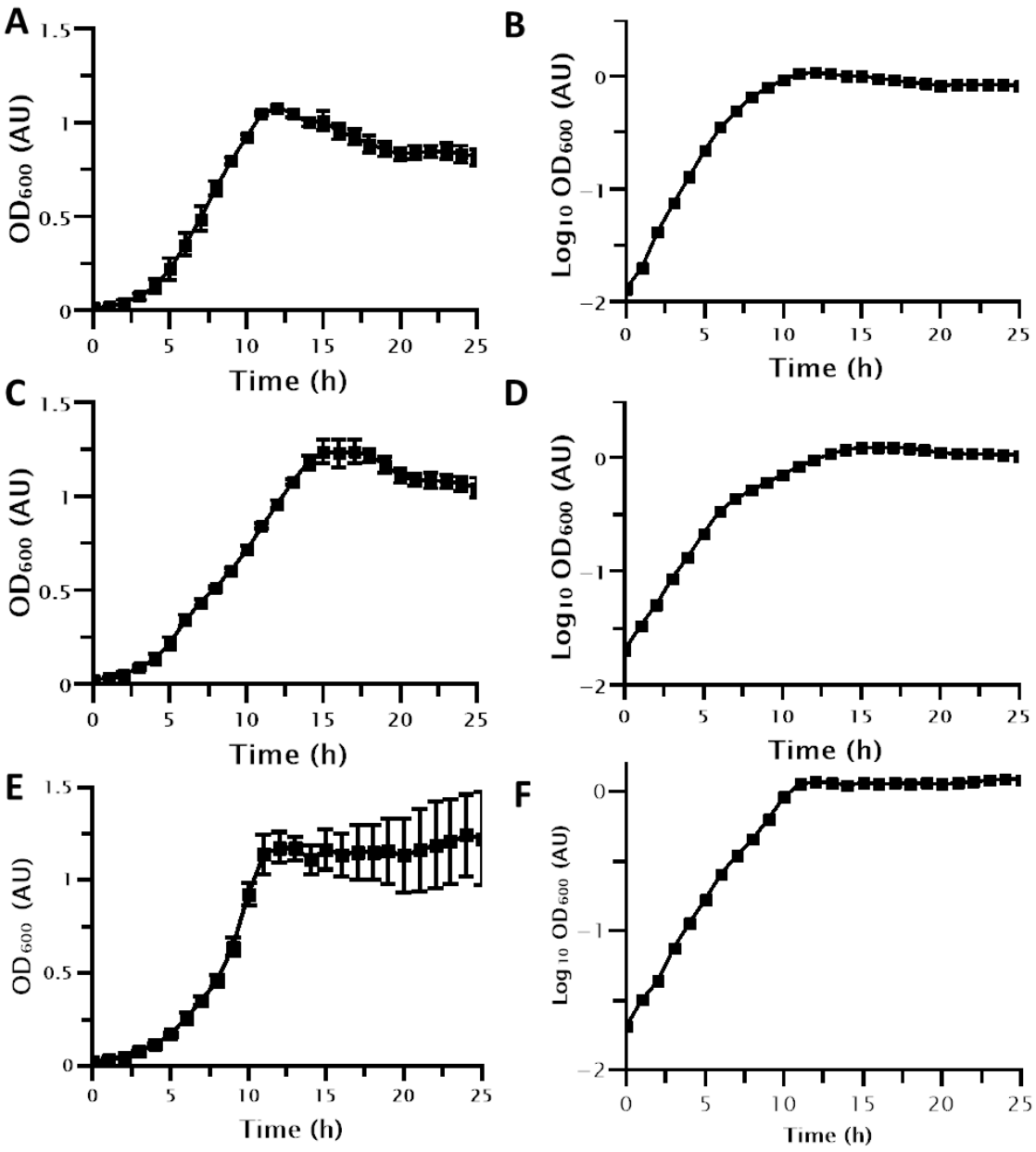


Figure 3. 2 Growth profiles of *P. denitrificans* Pd1222 cultured using 5 mM succinate in all conditions. Nitrogen sources were 10 mM NO_3^- (A&B), 10 mM NH_4^+ (C&D) and 5 mM NO_3^- and 5 mM NH_4^+ (E&F). Data presented are the average of three independent biological replicates and is shown as linear plots (A,C&E) and as semi-log plots (B, D&F).

In contrast to the 30 mM succinate condition the μ_{max} app. of the ammonium grown cells was lower (0.07 h^{-1} ±0.005) than that of the nitrate grown cells (0.09 h^{-1} ±0.01). This may be due to the truncation of maximum growth under nitrate assimilation conditions. Cells undertaking nitrate assimilation are required to use their energy source to generate higher levels of cellular reductant and consequently may suffer with lower overall growth despite having an unchanged growth rate. Whilst the 30 mM succinate, mixed nitrogen condition demonstrated a diauxic growth pattern the equivalent 5 mM succinate condition did not. It is likely only a single nitrogen source was utilised, possibly as a consequence of carbon limitation. It is notable however that the growth rate in this condition was 0.05 h^{-1} ±0.007 indicating that ammonium was likely preferential to nitrate, in agreement with the data presented for 30 mM succinate.

Butyrate constitutes a different metabolic challenge to succinate. In a previous study using the closely related organism *Paracoccus pantotrophus* a strict regulatory mechanism was proposed to govern the preference of succinate over butyrate. In this study ammonium was supplied as the nitrogen source and nitrate as an auxiliary oxidant so this conclusion does not necessarily apply to nitrate assimilation conditions (Ellington *et al.*, 2002). Nevertheless, this result indicates that butyrate would constitute a less preferential carbon source versus other more oxidised substrates. To investigate this further *P. denitrificans* was cultured using 10 mM butyrate and 10 mM nitrogen source (Figure 3. 3). Firstly, there is a significant difference, compared to succinate cultured cells, in the lag phase, which is extended. It is also worth noting the difference in maximum OD_{600} between nitrate and ammonium cultured cells is not present when cultured on 10 mM butyrate. This could indicate a difference in the way the nitrogen sources are used, or it could indicate a difference in the metabolism of the carbon substrate.

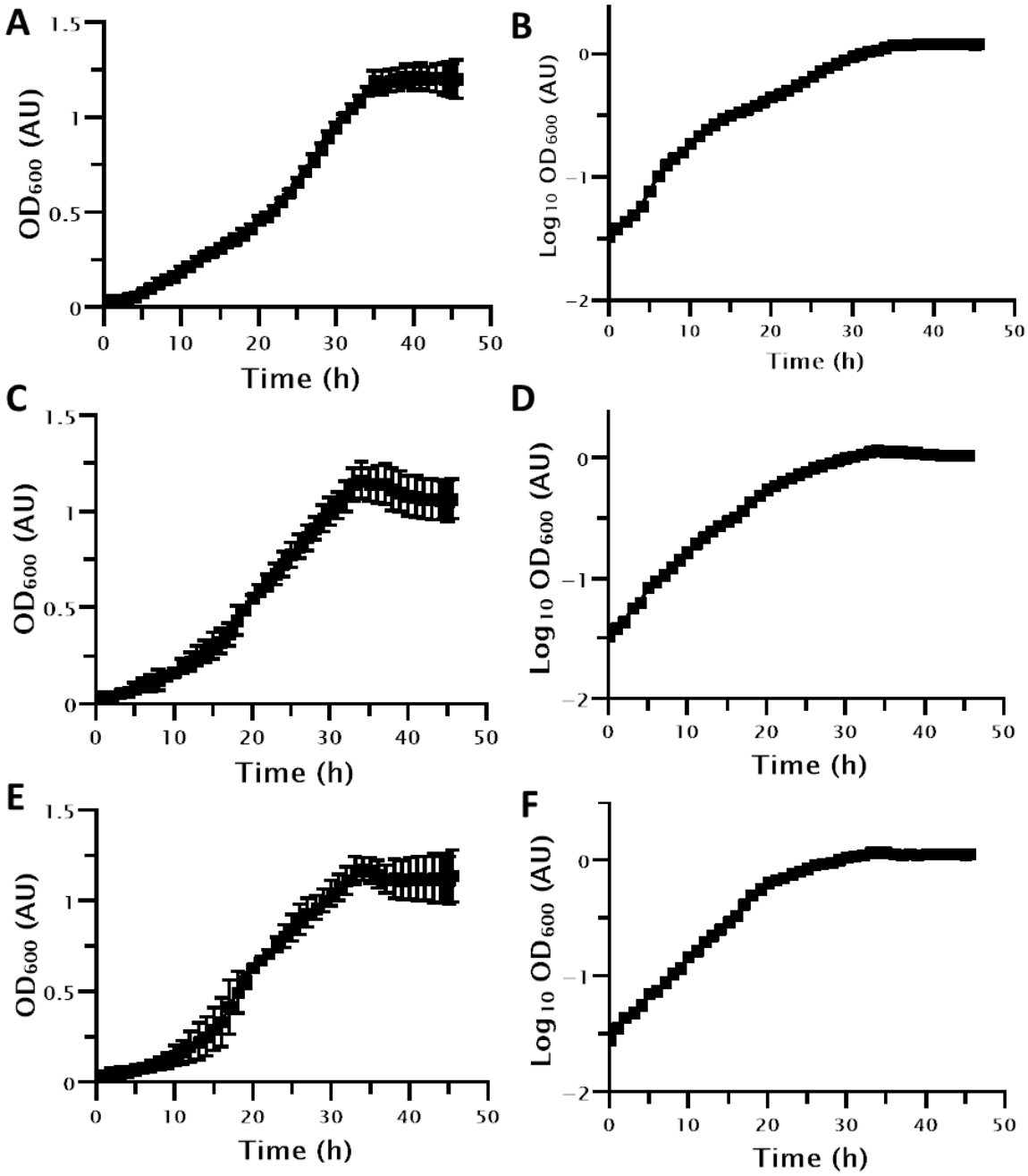


Figure 3. 3 Growth profiles of *P. denitrificans* Pd1222 cultured using 10 mM butyrate in all conditions. Nitrogen sources were 10 mM NO_3^- (A&B), 10 mM NH_4^+ (C&D) and 5 mM NO_3^- and 5 mM NH_4^+ (E&F). Data presented are the average of three independent biological replicates and is shown as linear plots (A,C&E) and as semi-log plots (B, D&F).

Under butyrate culture conditions μ_{max} app. remained unchanged between nitrate-dependent (0.02 h^{-1} ± 0.004) and ammonium-dependent (0.02 h^{-1} ± 0.007) growth. Interestingly when ammonium and nitrate are present the growth rate is slightly increased at 0.03 h^{-1} ±0.01. This is consistent with previous observations indicating the necessity of a redox vent when cells are grown utilising butyrate (Sears, Spiro and Richardson, 1997; Ellington *et al.*, 2002). It is also in contrast to the conditions tested using succinate as these demonstrated, in the high succinate condition, a diauxic growth not present in this condition.

The final condition to test was 4 mM butyrate with 10 mM nitrogen source. This represented a reduction in total carbon substrate availability similar to the 5 mM succinate condition. As previously observed in this work, a reduction in the total biomass accumulated was observed from cells cultured under the low carbon regime. This was more pronounced in the butyrate conditions compared to succinate. This is possibly due to the increased metabolic strain placed on the bacteria by the requirement of β-oxidation prior to incorporation in the TCA cycle (section 3.1.2).

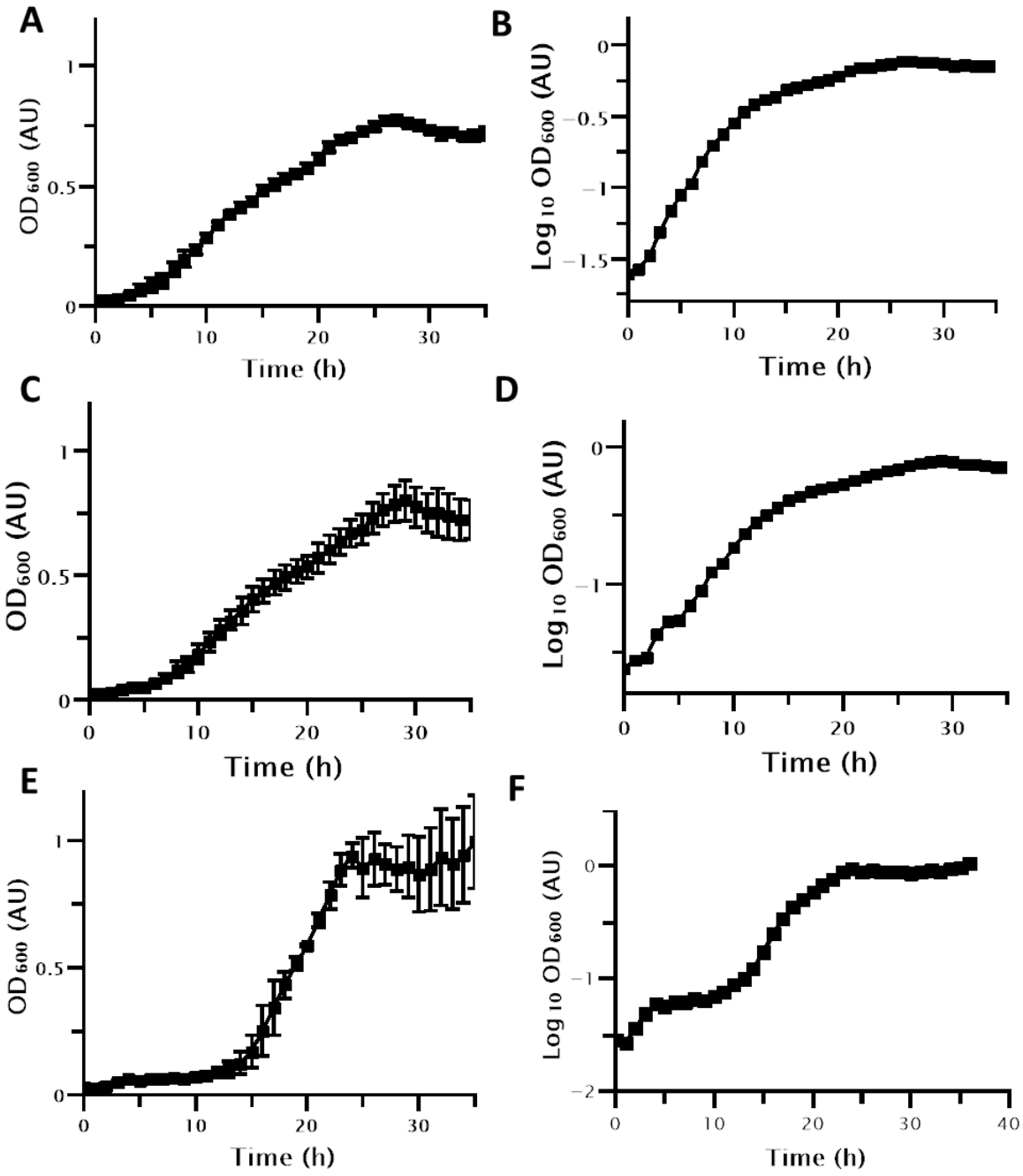


Figure 3. 4 Growth profiles of *P. denitrificans* Pd1222 cultured using 4 mM butyrate in all conditions. Nitrogen sources were 10 mM NO_3^- (A&B), 10 mM NH_4^+ (C&D) and 5 mM NO_3^- and 5 mM NH_4^+ (E&F). Data presented are the average of three independent biological replicates and is shown as linear plots (A,C&E) and as semi-log plots (B, D&F).

There are a number of differences between the 4 mM butyrate condition and the other carbon substrate concentrations tested (Figure 3. 4). Firstly, the apparent growth yield on mixed nitrogen sources was higher than on either nitrate or ammonium individually. Secondly, the μ_{max} app. of each condition differed significantly from the pattern observed thus far. There was a preference for nitrate assimilation under this condition, with μ_{max} app. of 0.05 h^{-1} ±0.007, compared to ammonium dependent growth at 0.04 h^{-1} ±0.001. More interestingly however the mixed nitrogen substrate condition gave an increased growth rate at 0.06 h^{-1} ±0.008 further indicating a preference for nitrate as the nitrogen source in agreement with the results obtained when cells were grown with 10 mM butyrate.

The batch growth data presented in this section yielded some interesting conclusions. Firstly, it was useful in providing growth rates that could be used to determine dilution rates in continuous culture experiments. Secondly, it provided useful insights into the potential for the carbon source oxidation state to alter the nitrogen source preference of the bacteria. Finally, the data shows that batch growth experiments are insufficient as a means to determine the nitrogen source preference, as comparisons of growth rate and maximum OD_{600} are inferences of nitrogen use. As a result, *P. denitrificans* was cultured in continuous culture to further examine these initial results.

3.2.2 Continuous culture with nitrate

Section 3.2.1 shows how nitrate is, potentially, a preferential nitrogen source under certain carbon substrate conditions. These data, however, do not provide a complete view of nitrate assimilation. Under batch growth conditions the availability of nitrogen substrate is constantly changing, and the bacteria are presented with a continuously changing chemical environment. Under continuous culture a steady state is maintained whereby bacteria are presented with a controlled chemical environment with constant addition of carbon and nitrogen at fixed concentrations. This is a powerful technique allowing for the assessment of the uptake and use of different nitrogen compounds.

To ensure nitrate assimilation was the sole process occurring, only nitrate was supplied at a 10 mM concentration from a reservoir of media. Cells were cultured in a batch phase with no media flow until they had reached a maximum growth yield and were in stationary phase. Media was then supplied to the vessel at a rate proportional to the μ_{max} app. of the bacteria at the mid exponential phase of their growth, this was adjusted to suit the fermentation vessel growth profiles. Cells were therefore maintained in the mid-exponential growth phase with replenishing nutrients from the constant inflow of fresh media. In the following experiments nitrate, nitrite, ammonium and carbon substrate were all measured to determine growth yields per nitrogen and carbon source. During all experiments the dissolved oxygen content was maintained above 90% to ensure changes in nitrate levels were not due to respiratory nitrate reduction. The pH was maintained by addition of small quantities of acid or base automatically by the culture control system.

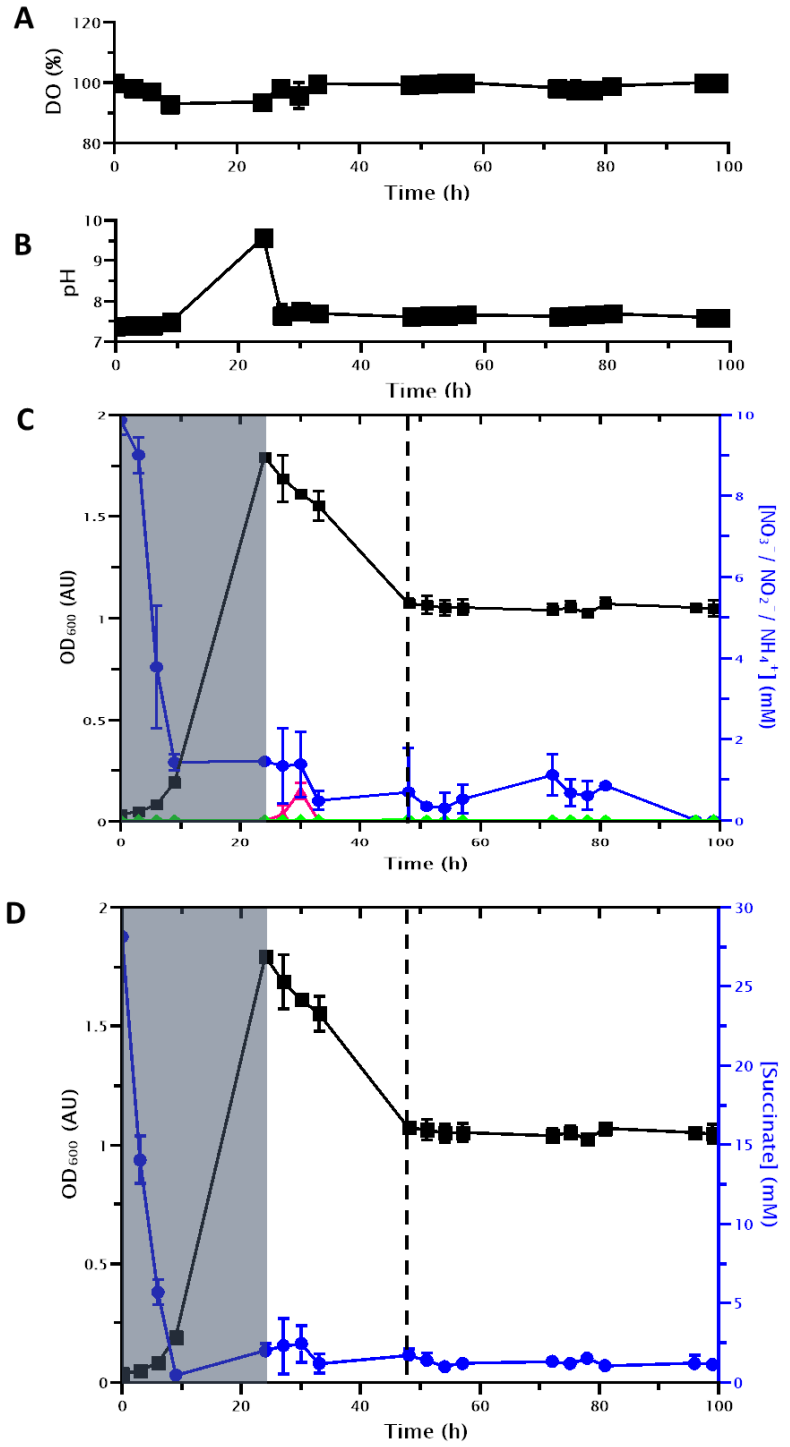


Figure 3. 5 The changes in dissolved oxygen (A), pH (B), nitrate (blue circles), nitrite (pink triangles) and ammonium (green diamonds) (C) and succinate (blue circles) (D) in the media during the continuous culture experiment with bacterial growth being shown as OD_{600} (black squares). The sole carbon source was 30 mM succinate and the sole nitrogen source was 10 mM nitrate. The grey area indicates the batch phase of the growth and the dotted line indicates the time at which the cells are in steady state. The dilution rate was 0.08 h^{-1}.

As observed in section 3.2.1 (Figure 3. 1) nitrate assimilation gave a growth rate of 0.06 h^{-1}. The experiment presented in Figure 3. 5 highlights a critical issue with batch growth observations as the dilution rate required to maintain steady state was higher than previously calculated. Overall maximum OD_{600} was also significantly higher in the continuous culture condition. This was likely due to increased aeration from internal gas delivery instead of orbital shaking, and from efficient substrate mixing by internal propellers.

The metabolite data shows the utilisation of nitrate in this condition. This is unsurprising as it is the sole nitrogen source and is therefore being used for assimilation into biomass. The respiration of nitrate can be discounted due to the high levels of dissolved oxygen present. It can also be seen that there is no extracellular ammonium present indicating that the nitrate consumed is being efficiently incorporated into biomass. While there is a small amount of nitrite detected at the end of the batch phase this is lost during the steady state phase. The observation of this small amount of nitrite is consistent with previous studies in *P. denitrificans* where nitrite is seen in the media at the beginning of stationary phase (Gates *et al.*, 2011). This could be explained by cell death releasing partially metabolised nitrogen or a disconnect between the nitrate and nitrite reductases resulting in excess nitrite production which is removed from cells. It is unlikely to be the result of denitrification by the respiratory nitrate reductase Nar as the oxygen level is high.

The dramatic fall in the concentration of succinate exemplifies the energy demand made upon cells undertaking nitrate assimilation (Figure 3. 5 part D). Approximately 29 mM succinate is required to assimilate ~10 mM nitrate. As the carbon substrate is required for carbon assimilation as well as energy generation the 8-electron reduction of nitrate to ammonium presents a significant burden on the cells. However, at no point does the succinate concentration fall to 0 suggesting the availability of carbon substrate is not limiting in this condition. A low carbon substrate concentration can therefore be used to analyse the changes in nitrate assimilation with a limited carbon substrate.

There is a significant difference between the growth and metabolite data from the low succinate condition compared to the high succinate condition (Figure 3. 6). Most striking is the severely reduced maximum OD_{600}. This is most likely due to the lack of available carbon source for generation of biomass. While nitrate was consumed, the amount remaining in the media was significant. There was no loss of nitrogen from the cells as nitrite or ammonium indicating efficient assimilation of nitrate. In contrast to the high succinate condition, all succinate was consumed and remained below the detection limit of the assay during the steady state phase. This indicates that succinate was limiting in this condition.

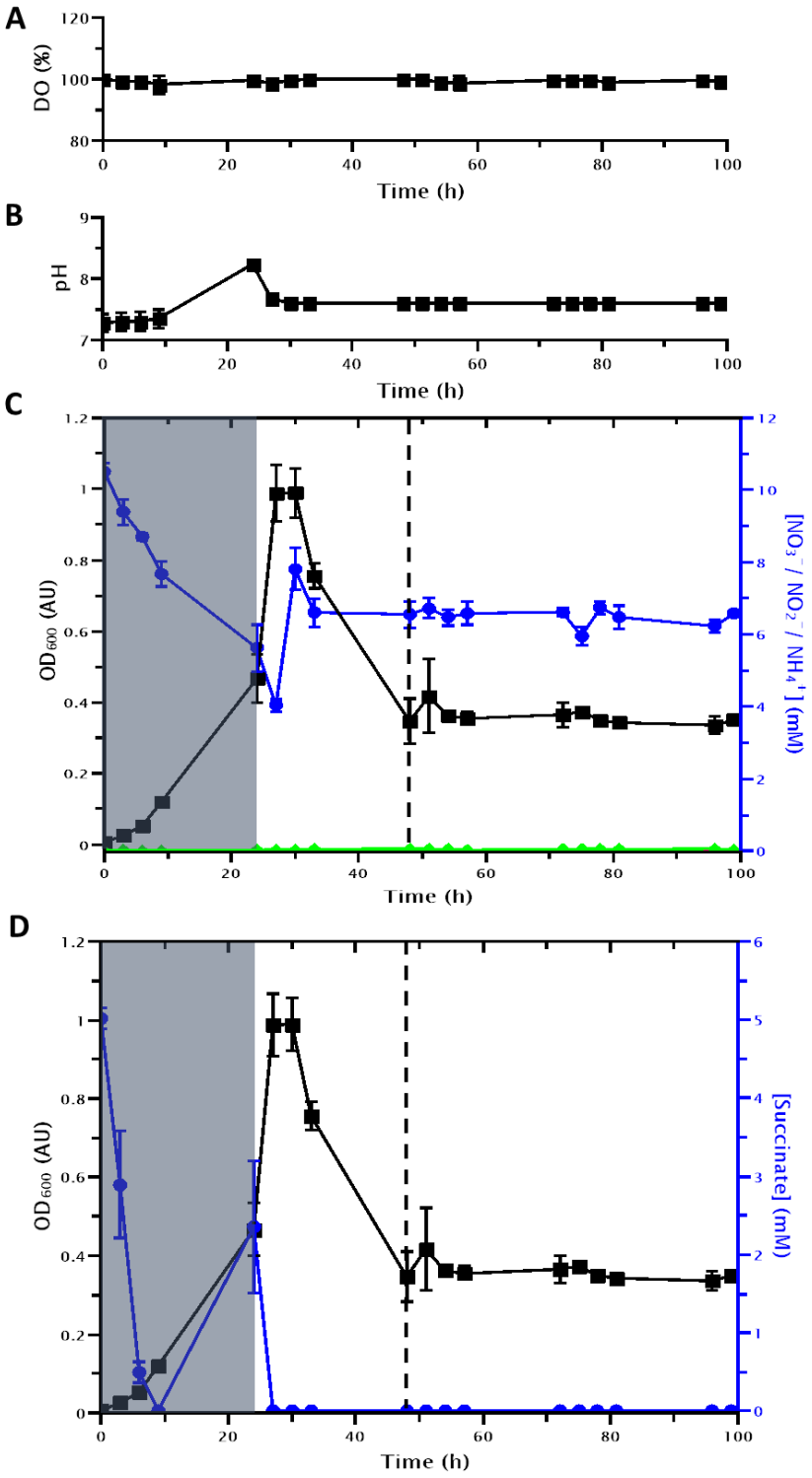


Figure 3. 6 The changes in dissolved oxygen (A), pH (B), nitrate (blue circles), nitrite (pink triangles) and ammonium (green diamonds) (C) and succinate (blue circles) (D) in the media during the continuous culture experiment with bacterial growth being shown as OD_{600} (black squares). The sole carbon source was 5 mM succinate and the sole nitrogen source was 10 mM nitrate. The grey area indicates the batch phase of the growth and the dotted line indicates the time at which the cells are in steady state. The dilution rate was 0.08 h^{-1}.

Table 3. 1 Growth yields for continuous culture experiments with 30 or 5 mM succinate and 10 mM nitrate.

Condition	$OD_{600}.mM^{-1}$ Succinate	$OD_{600}.mM^{-1}$ Nitrate
30 mM Succinate	0.039 ±0.0008	0.112 ±0.004
5 mM Succinate	0.07 ±0.002	0.086 ±0.004

When growth yields are analysed for the continuous culture experiments the 30 mM succinate condition represents a low growth per mM succinate utilised but a relatively high growth per mM nitrate utilised (Table 3. 1). This result could be expected in a condition where carbon is sufficient, as not all the succinate is needed for biomass generation and as such more can be catabolised to provide reductant for assimilation of nitrate. The reciprocal relationship is seen in the 5 mM succinate condition. Where carbon is in limited supply the bacteria appear to make a more efficient anabolic use of it and as such growth per mM succinate is almost double that of the 30 mM succinate condition. This increased yield from succinate, however, appears to come at a cost. The growth yield per mM nitrate is significantly lower in the 5 mM succinate experiment which would be expected as the bacteria cannot generate enough reducing equivalents to drive effective nitrate assimilation. Furthermore, there could be an inefficient link between the carbon and ammonium interface for biomass generation.

The results presented for the succinate experiments so far show a conventional story. When carbon is plentiful, nitrate is assimilated readily and the use of the carbon substrate can be varied. When the amount of carbon is limited then energetically expensive processes such as nitrate assimilation suffer, and the bacteria respond by tighter regulation over carbon usage. As the hypothesis was nitrate assimilation is regulated, in part, by the oxidation state of the carbon substrate, it was pertinent to examine nitrate assimilation continuous cultures using butyrate as the sole carbon source.

In the first experiment 10 mM butyrate was used to represent a high carbon substrate condition. Whilst this is significantly less overall carbon than the 30 mM succinate condition it is the highest concentration *P. denitrificans* will readily tolerate. Previous

studies investigating carbon source preference in the related organism *P. pantotrophus* also used a 10 mM butyrate concentration sufficient to induce changes (Ellington *et al.*, 2002). There are several evident differences when butyrate is the sole carbon source (Figure 3. 7). Firstly, the lag phase is significantly longer which is expected from the batch growth experiments in Figure 3. 3 and Figure 3. 4. Secondly the dilution rate required was lower than the succinate experiments which was predicted from the previous batch growth experiments. This demonstrates the requirement of batch experiments to understand the bacterial growth prior to the commencement of continuous culture experiments.

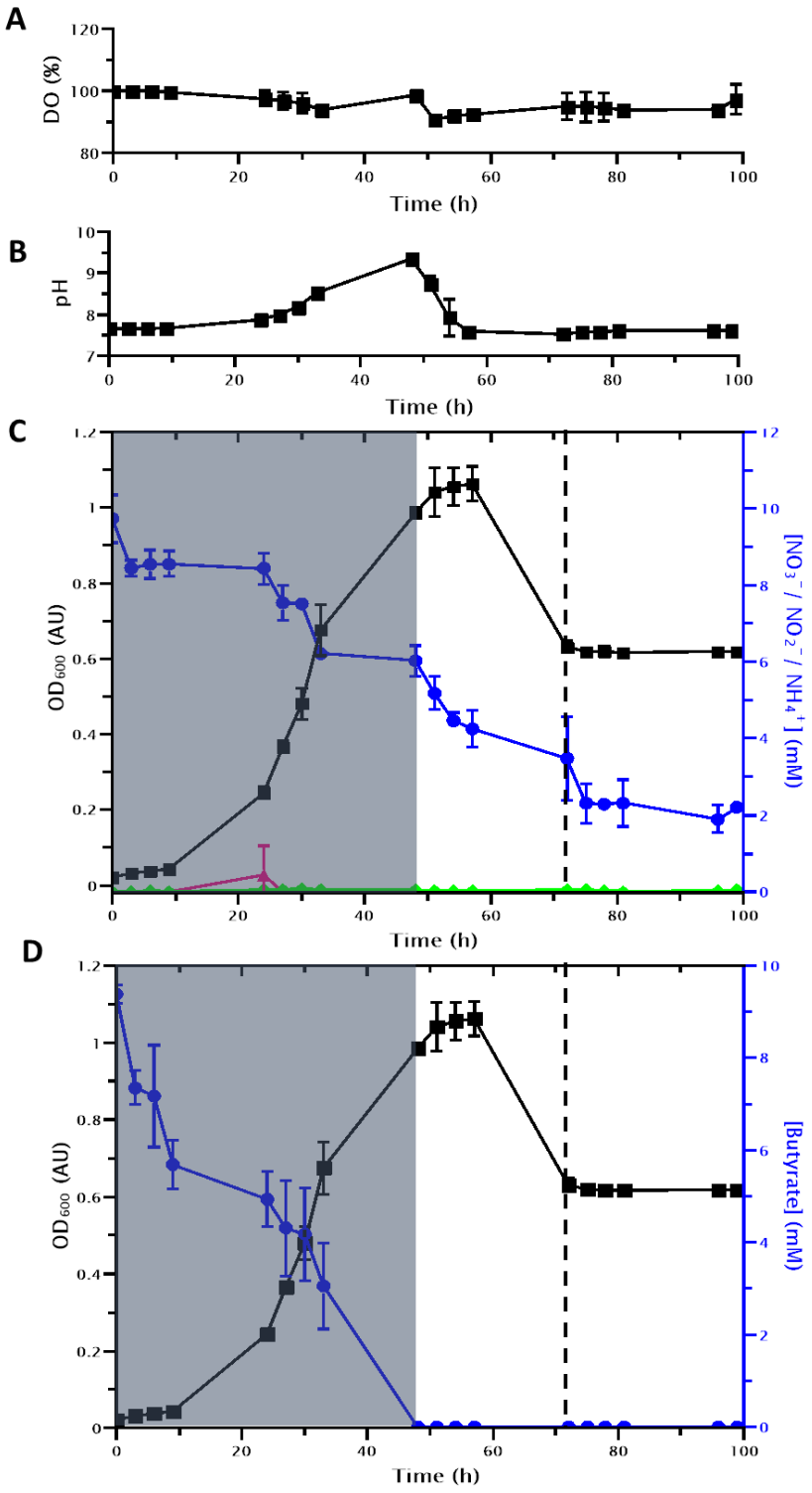


Figure 3. 7 The changes in dissolved oxygen (A), pH (B), nitrate (blue circles), nitrite (pink triangles) and ammonium (green diamonds) (C) and butyrate (blue circles) (D) in the media during the continuous culture experiment with bacterial growth being shown as OD_{600} (black squares). The sole carbon source was 10 mM butyrate and the sole nitrogen source was 10 mM nitrate. The grey area indicates the batch phase of the growth and the dotted line indicates the time at which the cells are in steady state. The dilution rate was 0.05 h^{-1}.

Comparing the 10 mM butyrate experiment to the 30 mM succinate experiment, there are some key differences. Firstly, the level of butyrate remains below the detection limit for the duration of the steady state. This shows that the carbon substrate is not completely sufficient for growth. This could explain the difference in the total use of nitrate. Comparing these two conditions there is an additional ~1 mM nitrate left in the 10 mM butyrate culture. This is notable however, as there is far less total carbon. Another key difference is the change in dilution rate. In section 3.2.1 batch growth experiments showed very low (~0.02 h^{-1}) maximum growth rates when *P. denitrificans* was cultured on 10 mM butyrate. In the continuous culture experiment, however, the dilution rate was increased to 0.05 h^{-1}. This shows again the difference between batch and continuous culture and the limitations of conducting batch growth experiments only.

To assess the effects of further carbon limitation, on butyrate grown cultures, a low condition of 4 mM butyrate was used. This represents a similar overall lack of total carbon as the 5 mM succinate experiment however, it provides a different metabolic pathway requirement for the bacteria. Under this condition we would expect the bacteria to suffer with a reduced ability for nitrate assimilation, however, as butyrate is a more reduced carbon source its metabolism will yield a greater number of reducing equivalents and therefore nitrate assimilation can still be maintained.

The maximum growth yield was maintained at a high level in the low butyrate condition in contrast to the equivalent experiments using succinate (Figure 3. 8). It is, however, obvious that nitrate utilisation was impacted when the concentration of butyrate was lowered. The nitrate concentration remains stable at ~5 mM. This result could be explained by a difference in the use of nitrate. In the 10 mM butyrate culture there is an evident nitrite peak during the exponential growth phase, this would be consistent with the expression of the periplasmic energy dissipating nitrate reductase Nap. If Nap is being used to dissipate excess electrons during growth then the reduction of nitrate to nitrite may release nitrite into the media from the periplasm. In the 4 mM butyrate culture the nitrite peak comes at the beginning of the stationary phase which is consistent with nitrate-dependent growth on succinate as discussed previously.

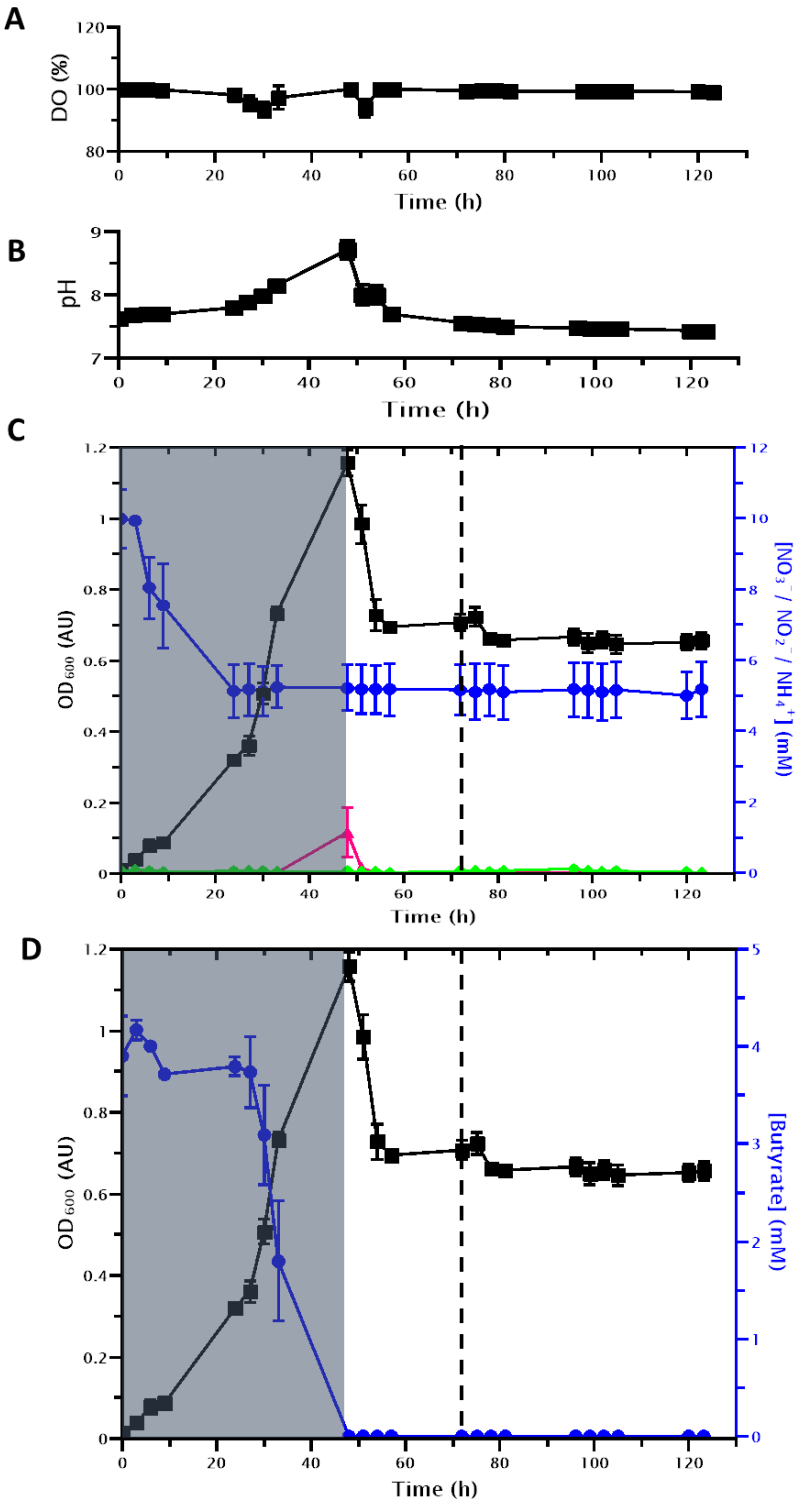


Figure 3. 8 The changes in dissolved oxygen (A), pH (B), nitrate (blue circles), nitrite (pink triangles) and ammonium (green diamonds) (C) and butyrate (blue circles) (D) in the media during the continuous culture experiment with bacterial growth being shown as OD_{600} (black squares). The sole carbon source was 4 mM butyrate and the sole nitrogen source was 10 mM nitrate. The grey area indicates the batch phase of the growth and the dotted line indicates the time at which the cells are in steady state. The dilution rate was 0.05 h^{-1}.

Table 3. 2 Growth yields for continuous culture experiments with 10 or 4 mM butyrate and 10 mM nitrate.

Condition	$OD_{600}.mM^{-1}$ Butyrate	$OD_{600}.mM^{-1}$ Nitrate
10 mM Butyrate	0.066 ±0.002	0.082 ±0.005
4 mM Butyrate	0.105 ±0.01	0.145 ±0.05

When the growth per mM butyrate is considered there is an increased efficiency in the growth yield when the carbon source is limited (Table 3. 2). This is not a surprising result as there is likely a metabolic switch allowing greater assimilation of carbon under these limiting conditions. In the case of nitrate, the results are unexpected. As nitrate assimilation is an energetically unfavourable process, reducing the carbon source availability should decrease the growth yield per mM nitrate used. Instead, there is an increase in the amount of growth from each mM of nitrate used. This indicates a more efficient use of nitrate favouring nitrate assimilation over the energy dissipating mechanisms.

When the data presented in this section are brought together some stark differences appear in the use of nitrate depending on the oxidation state of the carbon source provided to the bacteria. Firstly, the utilisation of succinate and butyrate appears to follow the same pattern. When the either carbon source is in its higher concentration the growth yield per unit carbon source supplied is lowered compared to the low carbon condition. This indicates that as there is more carbon available to catabolise for energy. Excess carbon can then be used to generate reducing equivalents for the assimilation of nitrate while still having enough to sustain biomass generation. When the carbon substrates are lowered the growth yields per mM carbon used increase. This is likely due to auxiliary, non-essential processes being limited and the generation of biomass being prioritised.

The decrease in growth yield per mM nitrate for the 5 mM succinate condition was expected, as there was less carbon, there were fewer reducing equivalents generated to assimilate nitrate and less overall carbon to link the generated ammonium to. In the case of 4 mM butyrate however, there is a different story. Nitrate assimilation appears to be

increased in this condition. The increase could be explained by a change in the use of nitrate. It has been reported previously that high concentrations of butyrate induce the expression of the energy dissipating nitrate reductase Nap (Sears *et al.*, 1993). This explains the increase in growth yield when cells are cultured with a lower concentration of butyrate. If butyrate is in a higher concentration then excess electrons enter the quinol pool in the membrane, this makes them less accessible to Nas, however it induces the expression of Nap leading to dissimilatory nitrate reduction and therefore less growth per mM nitrate used. With less butyrate available fewer electrons enter the quinol pool and Nap is not highly expressed. This then allows Nas to assimilate the nitrate rather than having to 'share' the supply with Nap.

This section has provided a more comprehensive view of nitrate assimilation. The results presented have highlighted the necessity of investigating metabolic preferences in continuous culture rather than simply in batch. While it is useful to define growth rates in batch culture prior to continuous culture it cannot exactly predict the dilution rate required. Investigation of growth yields under continuous culture conditions leads to novel conclusions about nitrate assimilation, mainly that low butyrate conditions increase the yield of growth on nitrate and not, as we have previously predicted, high butyrate conditions. Finally, this work has established a potential role for Nas as an alternative 'electron sink' for bacteria when they are unable to pass electrons to other electron sinks.

3.2.3 Continuous culture with mixed nitrogen sources

In section 3.2.2 the date presented suggest a link between the lower butyrate concentration and an increase in growth yield when nitrate is the sole nitrogen source. In this section results are presented from continuous culture experiments where nitrogen was supplied as nitrate and ammonium in equimolar concentrations. The current model would suggest ammonium is the preferred nitrogen source and as such would be used before any nitrate is used for assimilation, when presented with a mixture of the two sources the nitrate assimilation gene expression should be altered by the presence of ammonium. Ammonium does not require any reduction or oxidation to pair with organic molecules

to make organic nitrogen compounds, as such it is logical to assume it is preferential. Most previous work has focussed on using either ammonium or nitrate to investigate nitrate assimilation however, the application of both in continuous culture has yet to be investigated.

In the first set of experiments succinate was used as the sole carbon substrate. Nitrate and ammonium were supplied at a concentration of 5 mM each resulting in 10 mM total nitrogen available. This represents the same total nitrogen as the previous nitrate assimilation conditions, but in this case, it provides a dilemma to the bacteria. While ammonium is conventionally preferable to nitrate in these conditions there is not enough nitrogen in the form of ammonium to sustain the same usage as the nitrate assimilation condition. This means the ability of the bacteria to modulate their metabolism to co-assimilate nitrate and ammonium will be tested.

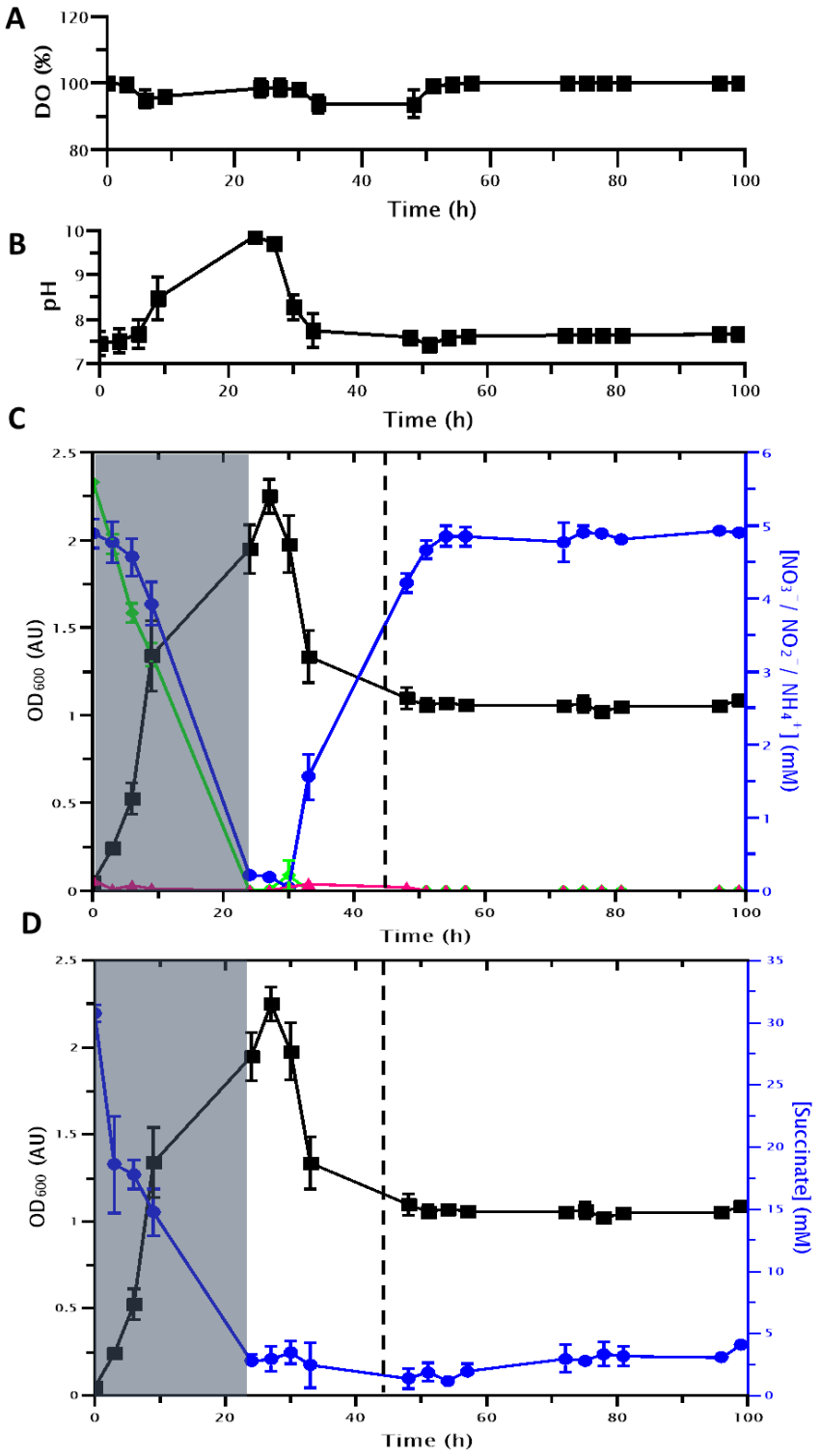


Figure 3. 9 The changes in dissolved oxygen (A), pH (B), nitrate (blue circles), nitrite (pink triangles) and ammonium (green diamonds) (C) and succinate (blue circles) (D) in the media during the continuous culture experiment with bacterial growth being shown as OD_{600} (black squares). The sole carbon source was 30 mM succinate and the nitrogen sources were 5 mM nitrate and 5 mM ammonium. The grey area indicates the batch phase of the growth and the dotted line indicates the time at which the cells are in steady state. The dilution rate was 0.1 h^{-1}.

The maximum growth of the bacteria in this mixed condition is considerably higher than when cultured on nitrate only (Figure 3. 9). This result is consistent with the initial observations in batch culture. The dilution rate was also increased in this condition. This is consistent with the idea that the bacteria grow more efficiently when cultured on ammonium. The succinate utilisation is also different when the bacteria are presented with a mixture of both nitrogen sources. In this case there is ~2 mM more succinate remaining in the culture media. This is, again, consistent with the hypothesis that under succinate growth conditions the bacteria would assimilate ammonium before nitrate. Under these conditions they do not need to generate the extra reducing equivalents to reduce their nitrogen source into a useful form, so they do not require as much carbon.

The key data presented here is the nitrogen metabolite data. Nitrate is consumed after ammonium starts to be consumed. Nitrate would be expected to be the second-choice nitrogen source, so this fits well with the current theory. The bacteria do appear to use both nitrogen sources in the batch growth phase which makes sense as they are able to generate extra reducing equivalents from the excess succinate to drive nitrate assimilation. Critically however, they do not do this until they have consumed a significant amount of available ammonium. Once fresh media is supplied to the culture after the initial batch phase the bacteria appear to revert to a strict preference for ammonium. This results in nitrate levels maintaining at the starting concentration and ammonium levels remaining undetectable as the bacteria are consuming it. This experiment is an important control as a preference for ammonium is expected with this relatively oxidised carbon substrate and this experiment confirms the current theory.

Next, the consequence of limiting carbon was investigated. In the equivalent experiment to section 3.2.2 the succinate concentration was reduced to 5 mM. Previously this resulted in a drop in nitrate reduction, explained by a lack of reducing capability and carbon linked assimilation, and a more efficient use of succinate for biomass generation.

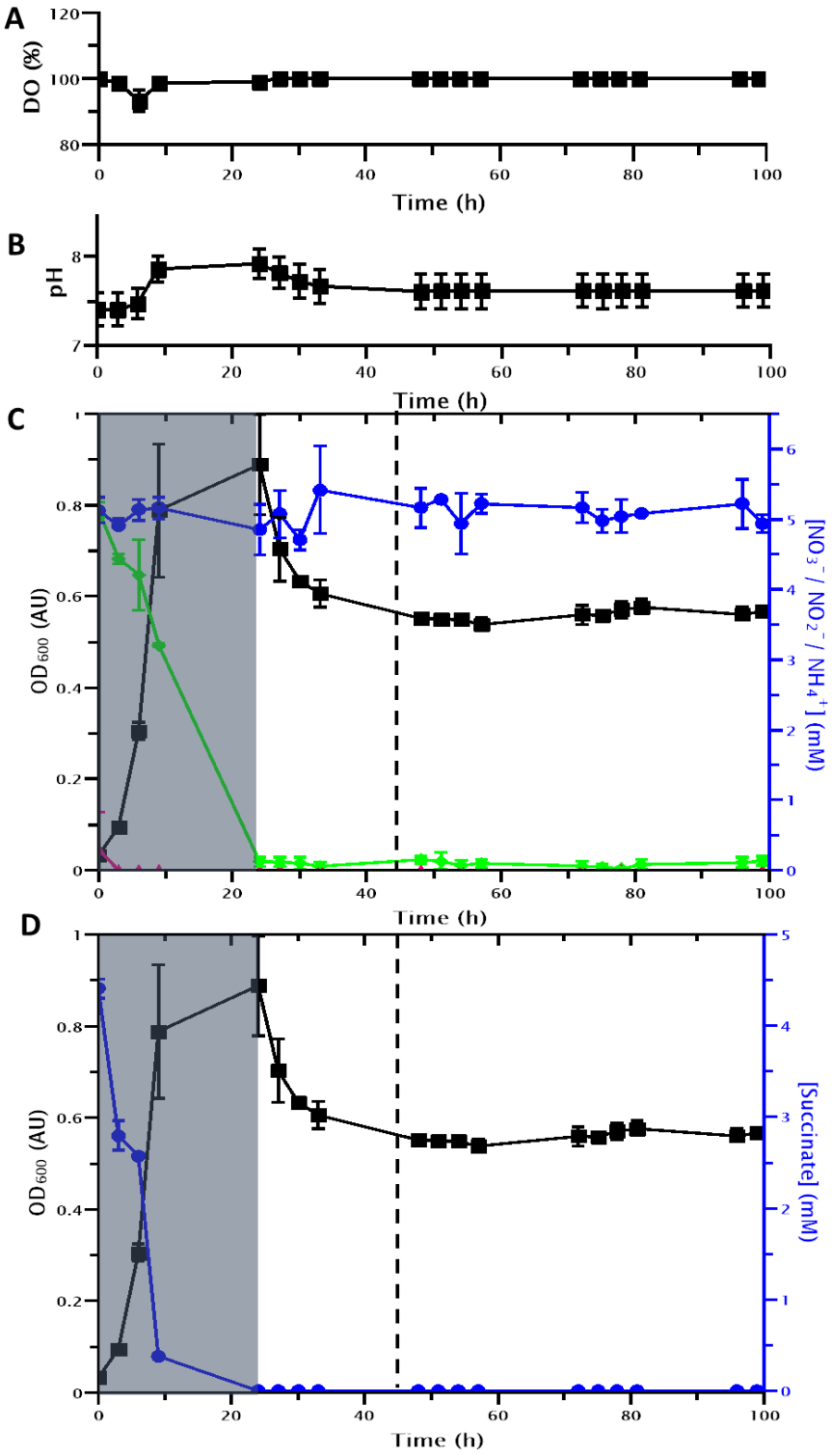


Figure 3. 10 The changes in dissolved oxygen (A), pH (B), nitrate (blue circles), nitrite (pink triangles) and ammonium (green diamonds) (C) and butyrate (blue circles) (D) in the media during the continuous culture experiment with bacterial growth being shown as OD_{600} (black squares). The sole carbon source was 5 mM succinate and the nitrogen sources were 5 mM nitrate and 5 mM ammonium. The grey area indicates the batch phase of the growth and the dotted line indicates the time at which the cells are in steady state. The dilution rate was 0.1 h^{-1}.

Firstly, the growth yield from the low carbon condition is severely compromised compared to both the batch growth and the nitrate assimilation growth (Figure 3. 2, Figure 3. 6, Figure 3. 10). This appears to be as a result of the limiting concentration of succinate. Whilst a reasonable concentration of succinate is left in the culture media of the equivalent high succinate condition this was not repeated with low succinate (Figure 3. 9, Figure 3. 10). The succinate concentration falls to an undetectable level very rapidly and remains unchanged during the continuous culture phase. This is consistent with a condition severely limited in total available carbon.

Most importantly the low succinate concentration gives a clear view of the nitrogen source preference under this carbon source. While ammonium concentration falls rapidly (Figure 3. 10 C), as it did in the high succinate condition (Figure 3. 9), there is noticeably no reduction in nitrate concentration. When the nitrate-dependent growth condition is considered (Figure 3. 6) no reduction of nitrate would be expected as less than 5 mM total nitrogen was required to sustain growth. Nitrite was also measured and remained undetectable suggesting there was no additional nitrate reduction. These results therefore indicate a clear preference for ammonium over nitrate in *P. denitrificans,* when succinate it the sole carbon source.

Table 3. 3 Growth yields for continuous culture experiments with 30 or 5 mM succinate, 5 mM nitrate and 5 mM ammonium.

Condition	**$OD_{600}.mM^{-1}$ Succinate**	**$OD_{600}.mM^{-1}$ Nitrogen**
30 mM Succinate	0.039 ±0.0004	0.188 ±0.004
5 mM Succinate	0.129 ±0.004	0.112 ±0.009

Growth yields explain the preference for ammonium under succinate-dependent growth (Table 3. 3). In the high succinate condition the growth yield per mM succinate used remains the same as it was in nitrate-dependent growth. This indicates there is sufficient carbon for creation of biomass and that the residual succinate in the culture media explains the difference in requirement when bacteria need to generate reductant for nitrate assimilation. The growth yield per mM succinate increases in the lower succinate

condition. The bacteria did not reduce any nitrate in this condition and therefore the succinate utilised did not need to generate the higher level of reductant required in the nitrate assimilation experiment. This means the bacteria can assimilate more carbon and hence growth yield is increased per mM utilised.

The critical results from Table 3. 3 are the yields per mM nitrogen. In both conditions there is a considerable increase in growth yield. This is explained by the nitrogen source they are assimilating. In both conditions the growth is ammonium-dependent and nitrate is not used. Ammonium is already able to enter the nitrogen assimilation pathways and hence requires no additional reductant input to generate biomass. This all combines to allow the bacteria to efficiently assimilate nitrogen and produce a higher growth per mM used.

In the previous nitrate-dependent growth experiments an apparent increase in utilisation of nitrate assimilation was seen when cells were cultured on butyrate. It is important to analyse these results in the context of competing nitrogen sources. In this section the data show ammonium is the preferred nitrogen source when cells are cultured on succinate. Therefore, there is a need to investigate if this hierarchy is maintained when cells are using butyrate.

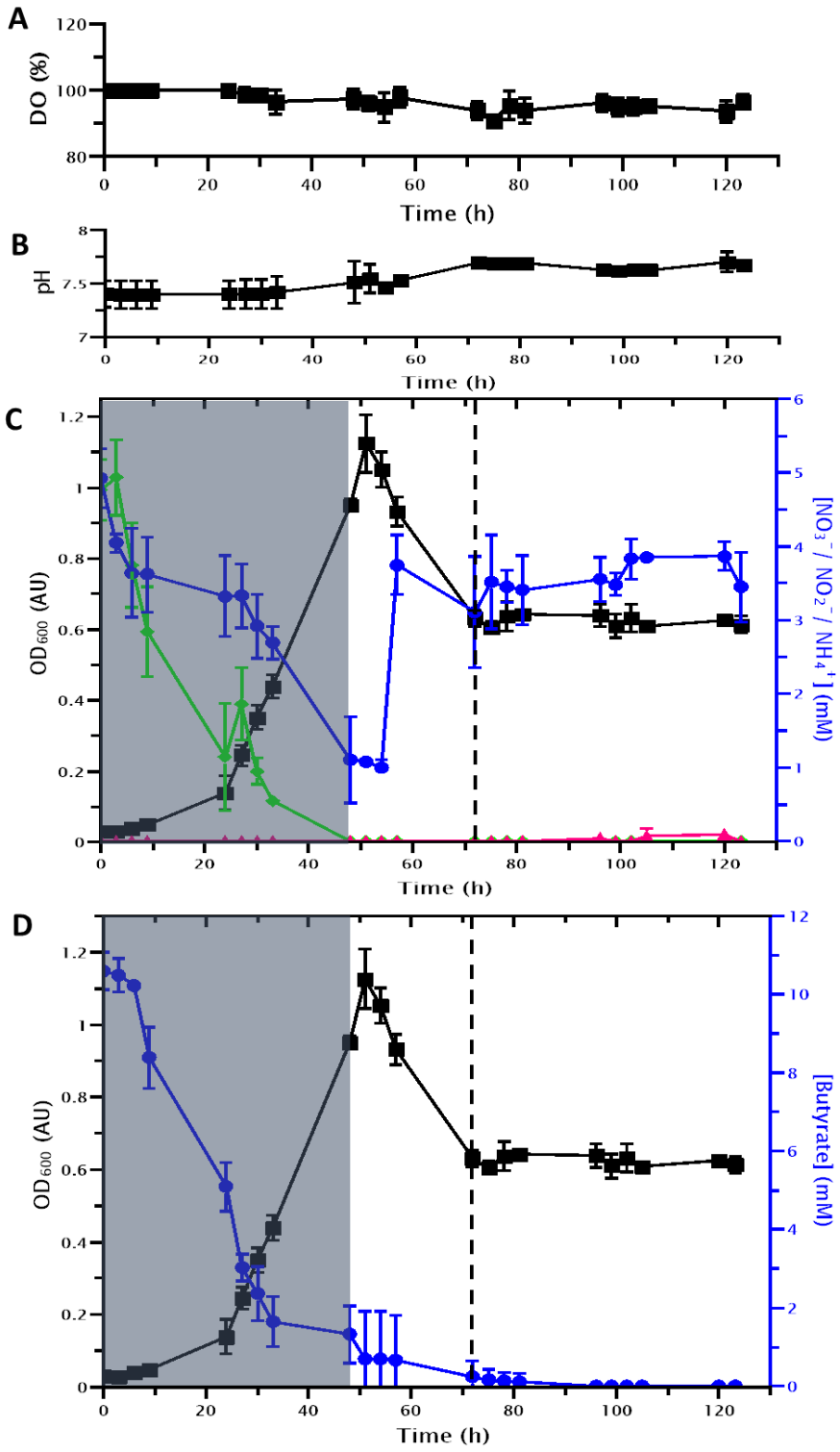


Figure 3. 11 The changes in dissolved oxygen (A), pH (B), nitrate (blue circles), nitrite (pink triangles) and ammonium (green diamonds) (C) and butyrate (blue circles) (D) in the media during the continuous culture experiment with bacterial growth being shown as OD_{600} (black squares). The sole carbon source was 10 mM butyrate and the nitrogen sources were 5 mM nitrate and 5 mM ammonium. The grey area indicates the batch phase of the growth and the dotted line indicates the time at which the cells are in steady state. The dilution rate was 0.08 h^{-1}.

In the high butyrate with mixed nitrogen source experiment the butyrate is consumed past the detection limit, as it was in the nitrate-dependent growth experiment (Figure 3. 11). This is consistent with the data presented thus far which indicates *P. denitrificans* will optimally consume >25 mM total carbon. Butyrate can be considered limiting at this concentration, irrespective of the nitrogen source. The reduction in butyrate concentration is consistent with the nitrate-dependent growth and there is nothing to suggest that butyrate metabolism is affected by the change in nitrogen sources, unlike the succinate condition where less succinate was used.

The nitrogen changes are the most interesting results from Figure 3. 11. Comparing the high succinate and high butyrate conditions, with nitrate and ammonium present, there are some key differences. Nitrate and ammonium are consumed at the same time in the butyrate growth, however under a succinate regime there is a delay until ammonium reaches lower levels. This could be put down to nitrate assimilation or dissimilatory nitrate reduction due to Nap. Continuous culture then allows us to investigate this further. If nitrate assimilation was a dominant process, nitrate levels would be expected to fall in the continuous culture phase but instead they recover to ~1 mM less than the supply concentration. This indicates that nitrate is being utilised, but it is more consistent with reduction via Nap rather than Nas. The current dogma that ammonium is preferred appears held up by this data.

Nitrate assimilation appeared to increase growth efficiency under the lower butyrate concentration in both batch culture and in nitrate-dependent continuous culture. It is therefore necessary to investigate the nitrogen source preference under this carbon regime. In theory ammonium may be a preferential nitrogen source due to the depleted carbon supply. There would be less energy source available for the generation of reducing equivalents required for nitrate assimilation so ammonium would be preferred as it does not require the redox state changes that nitrate does in order to be assimilated.

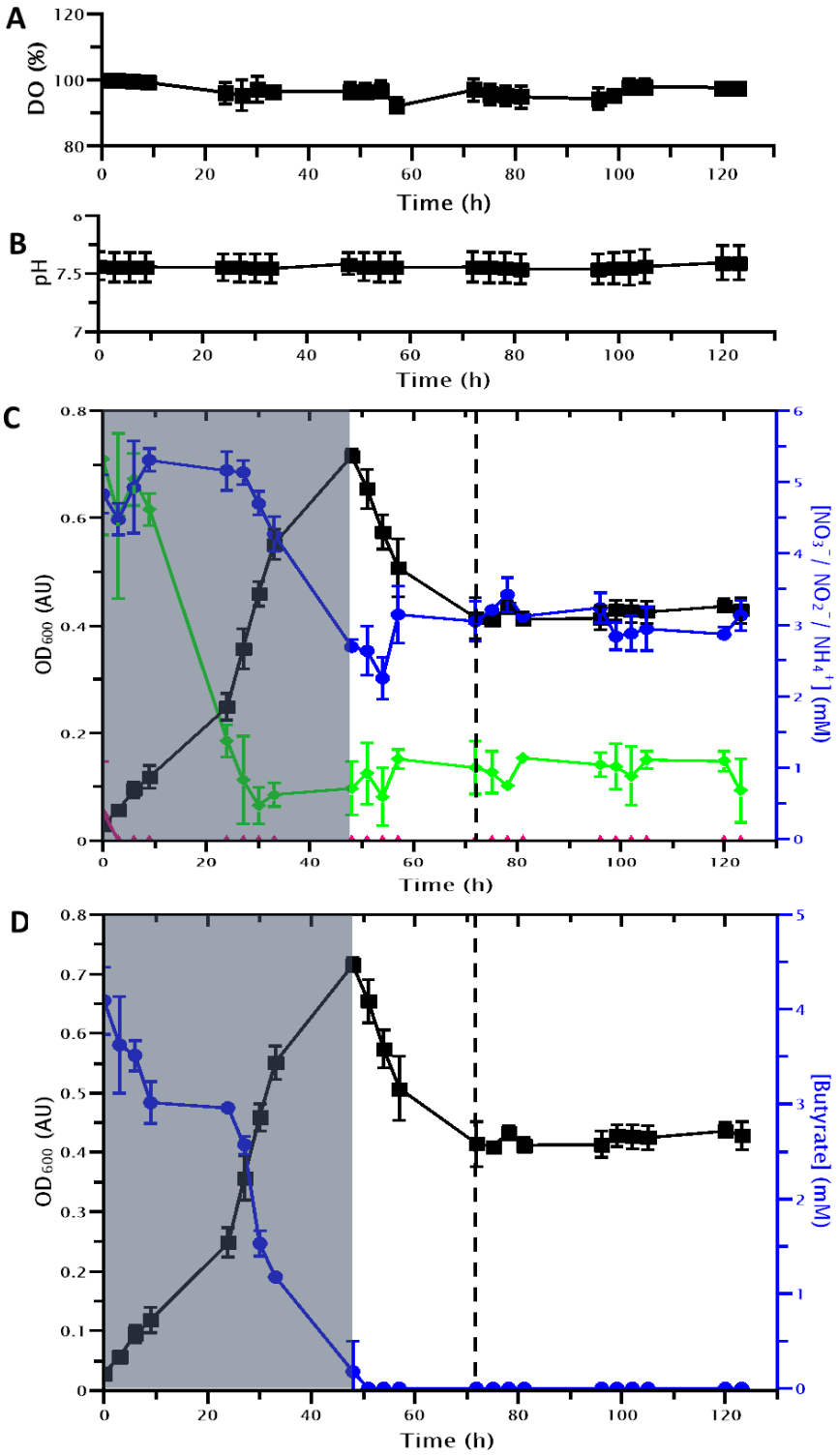


Figure 3. 12 The changes in dissolved oxygen (A), pH (B), nitrate (blue circles), nitrite (pink triangles) and ammonium (green diamonds) (C) and butyrate (blue circles) (D) in the media during the continuous culture experiment with bacterial growth being shown as OD_{600} (black squares). The sole carbon source was 4 mM butyrate and the nitrogen sources were 5 mM nitrate and 5 mM ammonium. The grey area indicates the batch phase of the growth and the dotted line indicates the time at which the cells are in steady state. The dilution rate was 0.08 h^{-1}.

Figure 3. 12 shows a very clear difference between high and low butyrate. When *P. denitrificans* was cultured using succinate the only difference between high and low concentration was a lack of nitrate assimilation in the batch culture phase of growth in the low condition. When cultured on butyrate there are several notable changes. Firstly, nitrate and ammonium are not initially co-consumed. Some ammonium is used first before nitrate starts to be utilised. Secondly, nitrate is still being used while ammonium is detectable in the culture media. Thirdly, nitrate levels are ~1 mM lower and, ammonium levels are ~1 mM higher than when cells are grown with 10 mM butyrate. This indicates that the cells are using nitrate assimilation at the same time as assimilating ammonium.

Table 3. 4 Growth yields for continuous culture experiments with 10 or 4 mM butyrate, 5 mM nitrate and 5 mM ammonium.

Condition	**$OD_{600}.mM^{-1}$ Butyrate**	**$OD_{600}.mM^{-1}$ Nitrogen**
10 mM Butyrate	0.059 ±0.002	0.104 ±0.009
4 mM Butyrate	0.105 ±0.01	0.069 ±0.008

For butyrate growth yield there is very little difference between the mixed nitrogen sources here and the nitrate-dependent data (Table 3. 4). This suggests that the cells find the generation of adequate reducing equivalents less difficult when growing with butyrate. There are however significant differences with the nitrogen metabolism. In high butyrate conditions the growth yield is higher per mM nitrogen consumed. This is likely as a result of the comparative ease of assimilating ammonium rather than nitrate. The consumption of nitrate for redox balancing was at low levels and so does not affect the yield data significantly. For low butyrate the yield is negatively impacted. This is probably as a result of inefficient use of nitrogen. When cells are using nitrate and ammonium simultaneously, they are required to expend energy assimilating the nitrate and as such they must use some carbon substrate to do this, resulting in a sacrifice in overall growth. This may result in lower growth yield but could be significantly preferable to the build-up of excess reducing equivalents in the cytoplasm.

The work presented in this section illustrates the differences in nitrogen source preference dependent upon the oxidation state of the carbon source. Furthermore, it highlights the importance of considering different concentrations of available resources. Intuitively, it may seem logical to assume higher concentrations of reduced carbon substrates should drive nitrate assimilation in cells, however, this work shows that it is instead when cells are cultured on lower concentrations of this carbon substrate that nitrate assimilation appears to be preferred.

3.2.4 Continuous culture with ammonium

Section 3.2.3 presented some interesting data. It showed that nitrate assimilation can be carried out concurrently with ammonium assimilation under certain carbon source conditions. This result however raised an interesting question. Nitrate assimilation occurs in low butyrate conditions alongside ammonium assimilation, however, there was a lower growth yield than when cells are cultured solely with nitrate. It is therefore important to investigate the two butyrate regimes presented in this chapter, when the cells are undertaking ammonium-dependent growth.

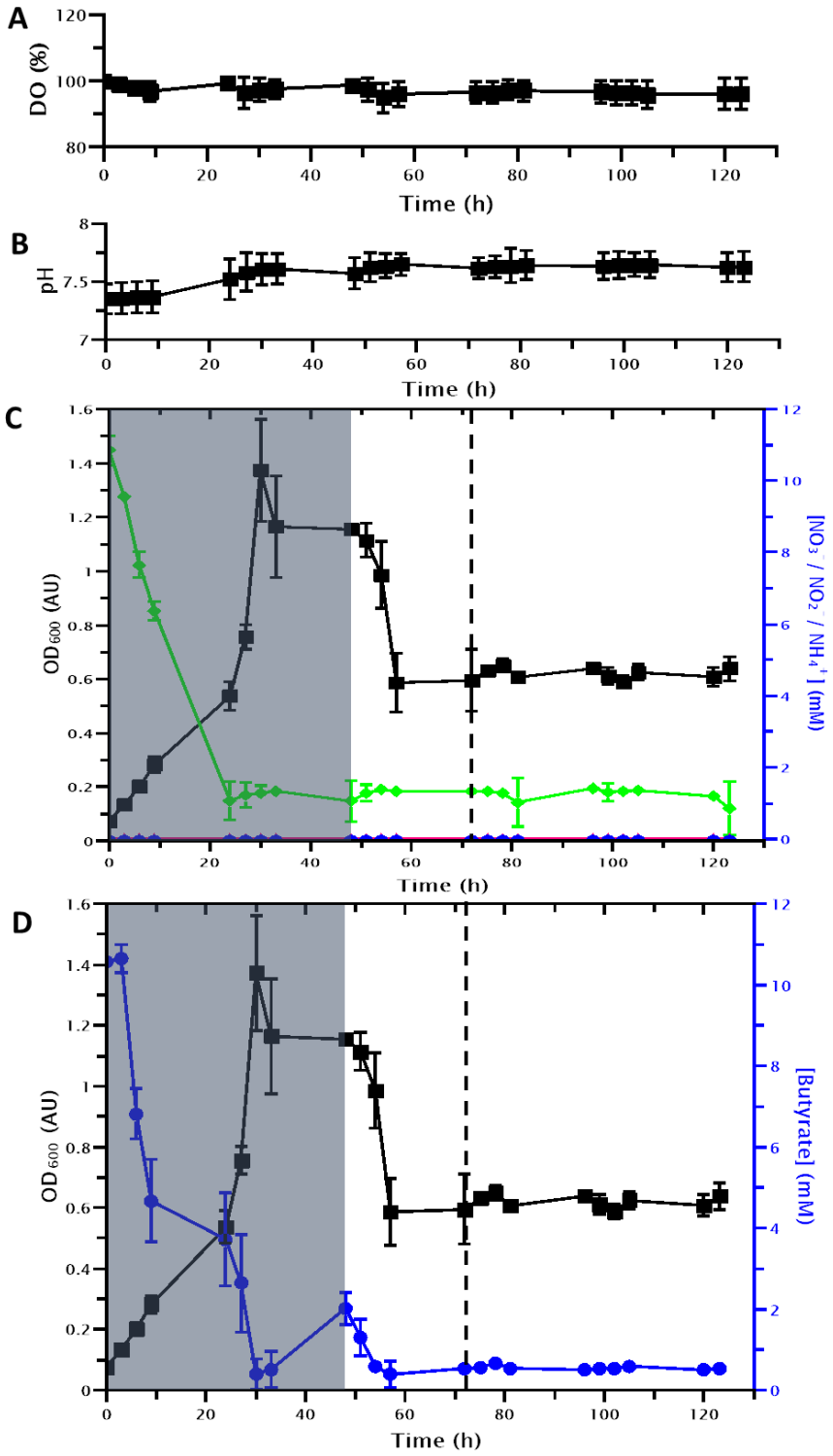


Figure 3. 13 The changes in dissolved oxygen (A), pH (B), nitrate (blue circles), nitrite (pink triangles) and ammonium (green diamonds) (C) and butyrate (blue circles) (D) in the media during the continuous culture experiment with bacterial growth being shown as OD_{600} (black squares). The sole carbon source was 10 mM butyrate and the nitrogen source was 10 mM ammonium. The grey area indicates the batch phase of the growth and the dotted line indicates the time at which the cells are in steady state. The dilution rate was 0.05 h^{-1}.

Butyrate is a challenging substrate for *P. denitrificans* to assimilate. The bacteria grow to a high maximum OD_{600}, however they appear to struggle to assimilate all of the available nitrogen despite having sufficient levels of remaining butyrate in the media (Figure 3. 13). This is indicative of cells struggling elsewhere in their metabolism. This is likely to be a result of excess electrons in the quinol pool. This will result in difficulty in respiration and without nitrate to act as an electron acceptor in a redox vent the cells are unable to re-balance their redox state. This is consistent with the previous data that showed a small amount of nitrate usage when nitrogen sources were mixed. This experiment shows that the nitrate usage in the mixed nitrogen source continuous cultures was due to Nap acting to balance the redox state of the cells. Therefore, when no nitrate is available the cells struggle to assimilate all carbon and nitrogen.

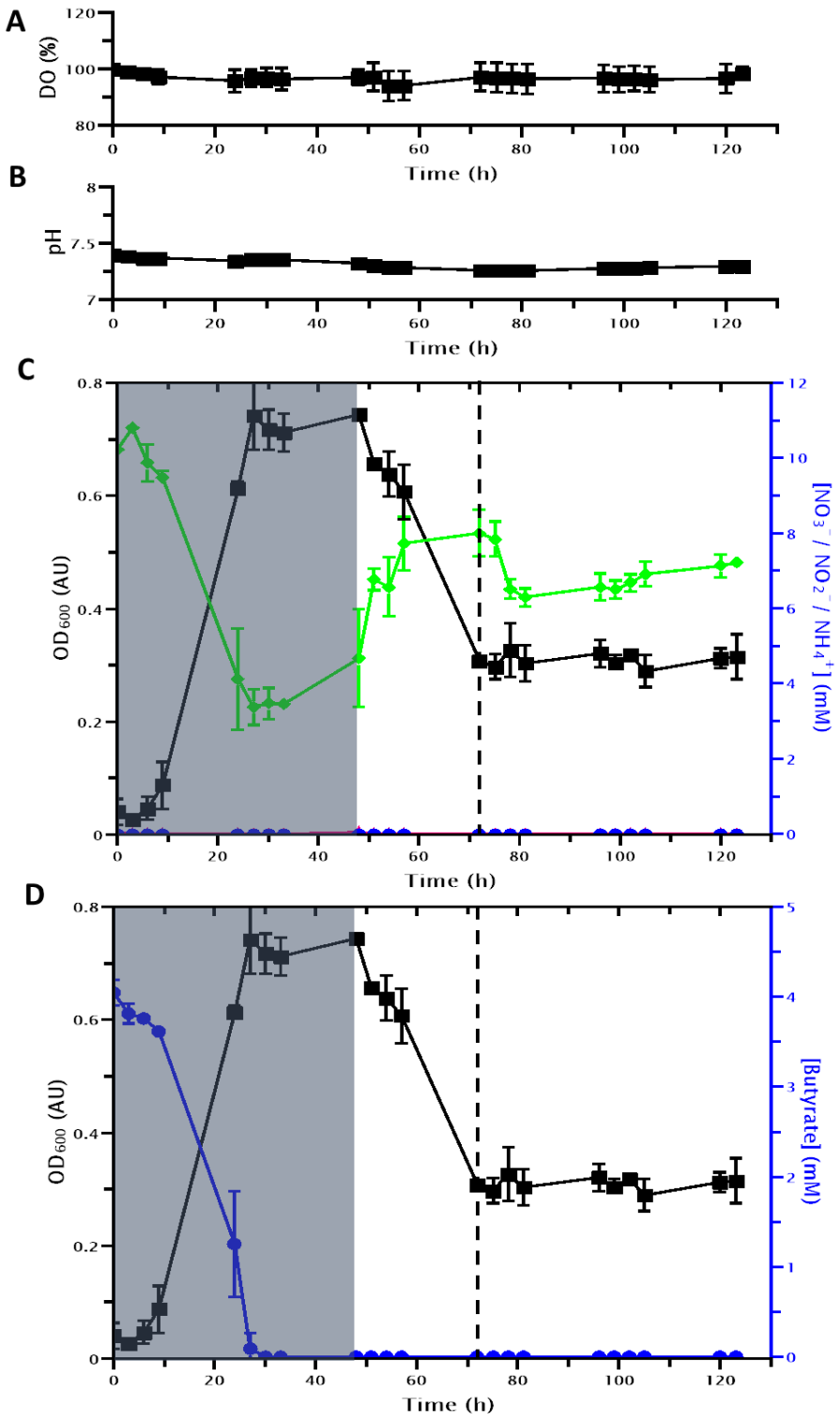


Figure 3. 14 The changes in dissolved oxygen (A), pH (B), nitrate (blue circles), nitrite (pink triangles) and ammonium (green diamonds) (C) and butyrate (blue circles) (D) in the media during the continuous culture experiment with bacterial growth being shown as OD_{600} (black squares). The sole carbon source was 4 mM butyrate and the nitrogen source was 10 mM ammonium. The grey area indicates the batch phase of the growth and the dotted line indicates the time at which the cells are in steady state. The dilution rate was 0.03 h^{-1}.

Figure 3. 14 shows the extremely detrimental effect an absence of nitrate has on cells under this condition. The butyrate is still completely used suggesting this is a truly limiting condition. The total amount of nitrogen used in the batch phase is similar to that of co-nitrate and ammonium assimilation however, in the continuous culture there is significantly lower nitrogen supporting growth. Cell growth is also reduced compared to cultures with nitrate present by ~0.1 OD_{600}. This suggests that the cells are struggling to grow. It is also important to note that the dilution rate was revised significantly lower indicating a lower growth rate consistent with the batch growth observations in section 3.2.1.

Table 3. 5 Growth yields for continuous culture experiments with 10 or 4 mM butyrate and 5 mM ammonium.

Condition	**$OD_{600}.mM^{-1}$ Butyrate**	**$OD_{600}.mM^{-1}$ Nitrogen**
10 mM Butyrate	0.062 ±0.0002	0.064 ±0.002
4 mM Butyrate	0.076 ±0.004	0.092 ±0.004

Table 3. 5 displays the growth yield data for these two continuous culture experiments. Ammonium-dependent growth had no effect on the yield from butyrate for the 10 mM condition. It did however result in a lower yield for the 4 mM condition consistent with the idea that the cells are struggling to generate biomass under this condition. For the high butyrate condition, the yield from nitrogen was lower than all previously tested nitrogen source conditions. This is likely due to the cells struggling to function without the redox vent to nitrate being available. For the low condition the yield per mM nitrogen was higher than with mixed nitrogen sources however it was lower than when cultured on nitrate only. This result means cells can assimilate the nitrogen, however in the context of lower overall growth and a lower growth rate it is still consistent that cells prefer to be able to assimilate nitrate rather than simply assimilate ammonium. This is most probably a result of balancing of the cytoplasmic reductant pool which preferences overall growth and growth rate, even if the growth yield with only ammonium present is higher.

The data presented in this section confirms that nitrate is important for *P. denitrificans* when cultured using reduced carbon sources such as butyrate. When cells are presented with ammonium only, they struggle to assimilate carbon and nitrogen in a carbon concentration dependent manner. In high carbon conditions cells struggle to utilise all the available carbon, likely as a result of damaging the delicate redox balance state of the cell. In the low condition cells are able to utilise all the carbon, however they struggle to assimilate as much nitrogen, and they grow more slowly. This is likely a result of build-up of cytoplasmic reductant with nowhere to vent it.

3.2.5 RT-qPCR

Throughout this chapter metabolite data collected from operational continuous cultures has provided insights into the utilisation of nitrate and ammonium. It is important to investigate these conclusions further using gene expression data. In this section data is presented from reverse-transcription quantitative PCR to quantify the expression of the three independent nitrate reductases encoded in the genome of *P. denitrificans*. Data are presented as expression relative to the housekeeping gene *rpoB*, which does not change expression in the conditions tested. Cells were harvested from continuous culture vessels after the cells had been in a steady state phase for more than at least 24 hours, to ensure gene expression data was relevant to this stage, not the batch phase.

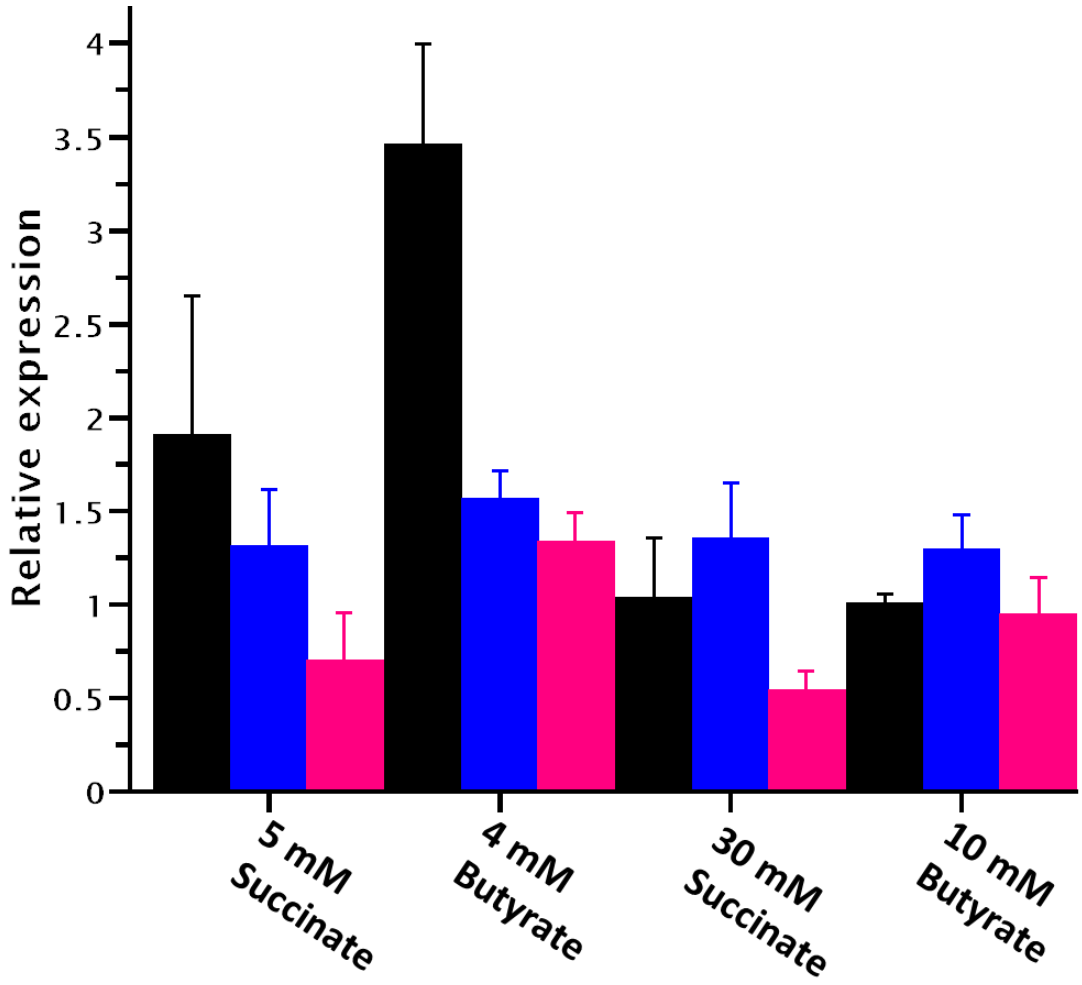


Figure 3. 15 Relative expression of the nitrate reductases *nasC* (black), *narG* (blue) and *napA* (pink). Expression is relative to the control gene *rpoB*. The data presented is from nitrate-dependent growth in continuous culture and the carbon source and concentration is stated for each experiment.

Figure 3. 15 shows the relative expression of nitrate reductase genes under nitrate-dependent growth conditions (presented in 3.2.2). Whilst there is expression of *narG* in all cultures this can be discounted as the dissolved oxygen content was kept >90% at all times and there was no change in expression in any condition. The second key result relates to *napA* expression. There was always a significant increase in expression of this gene when the culture was dependent on the reduced carbon substrate ($P<0.05$). This agrees with the theory discussed in 3.1.2. Finally, there was no significant difference in expression of the assimilatory nitrate reductase *nasC* between high butyrate and high succinate conditions. In the low carbon conditions, however, there were significant differences. Under low butyrate continuous culture *nasC* was expressed significantly

higher than under low succinate (P<0.01). This is likely a result of the increased reductant generated by the butyrate metabolism. The RT-qPCR data displayed in Figure 3. 15 agrees with the metabolite data that shows that low butyrate conditions result in a high growth yield from nitrate. This is due to the increased expression of *nasC* resulting in an efficient use of nitrate for biomass generation.

Conventionally nitrate reductases would be expected to be down regulated in response to ammonium, under aerobic conditions. Gene expression was explored from the experiments shown in 3.2.3. Again, the three nitrate reductase genes were tested for their expression as shown previously.

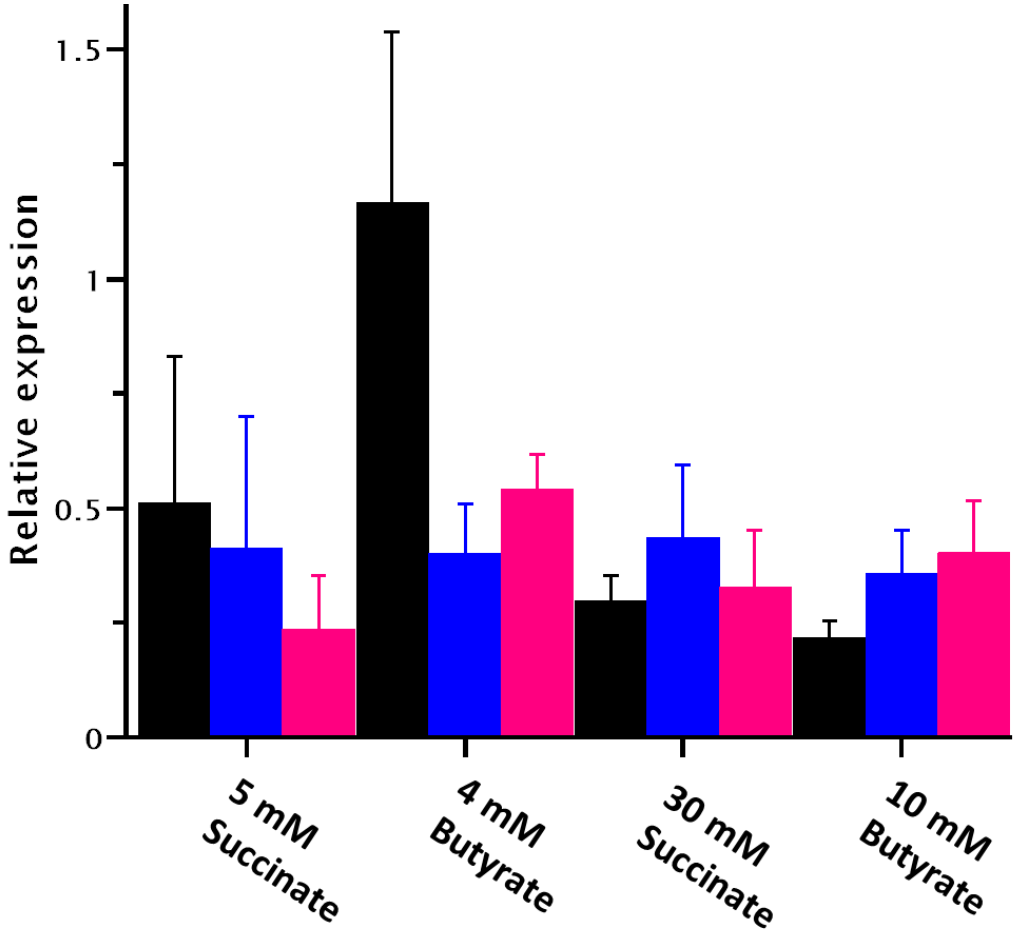


Figure 3. 16 Relative expression of the nitrate reductases *nasC* (black), *narG* (blue) and *napA* (pink). Expression is relative to the control gene *rpoB*. The data presented is from mixed nitrogen source growth in continuous culture and the carbon source and concentration is stated for each experiment.

Figure 3. 16 shows an overall reduction in the expression of all tested genes. This is unsurprising from the succinate conditions as no utilisation of nitrate was observed in these cultures. The most significant result indicates a higher expression of *nasC* under low butyrate conditions compared to the low succinate condition ($P<0.05$). This is significant as it confirms that the reduction in nitrate concentration was due to nitrate assimilation rather than dissimilation by Nap. The expression of *napA* was increased in the low butyrate concentration, however *nasC* expression is at a level sufficient to facilitate nitrate assimilation.

In a final control the expression of nitrate reductases under the ammonium-dependent growth conditions, presented in 3.2.4, was explored. This experiment determined whether the expression of the nitrate reductases is related to the presence of nitrate and the carbon source or simply the carbon source alone.

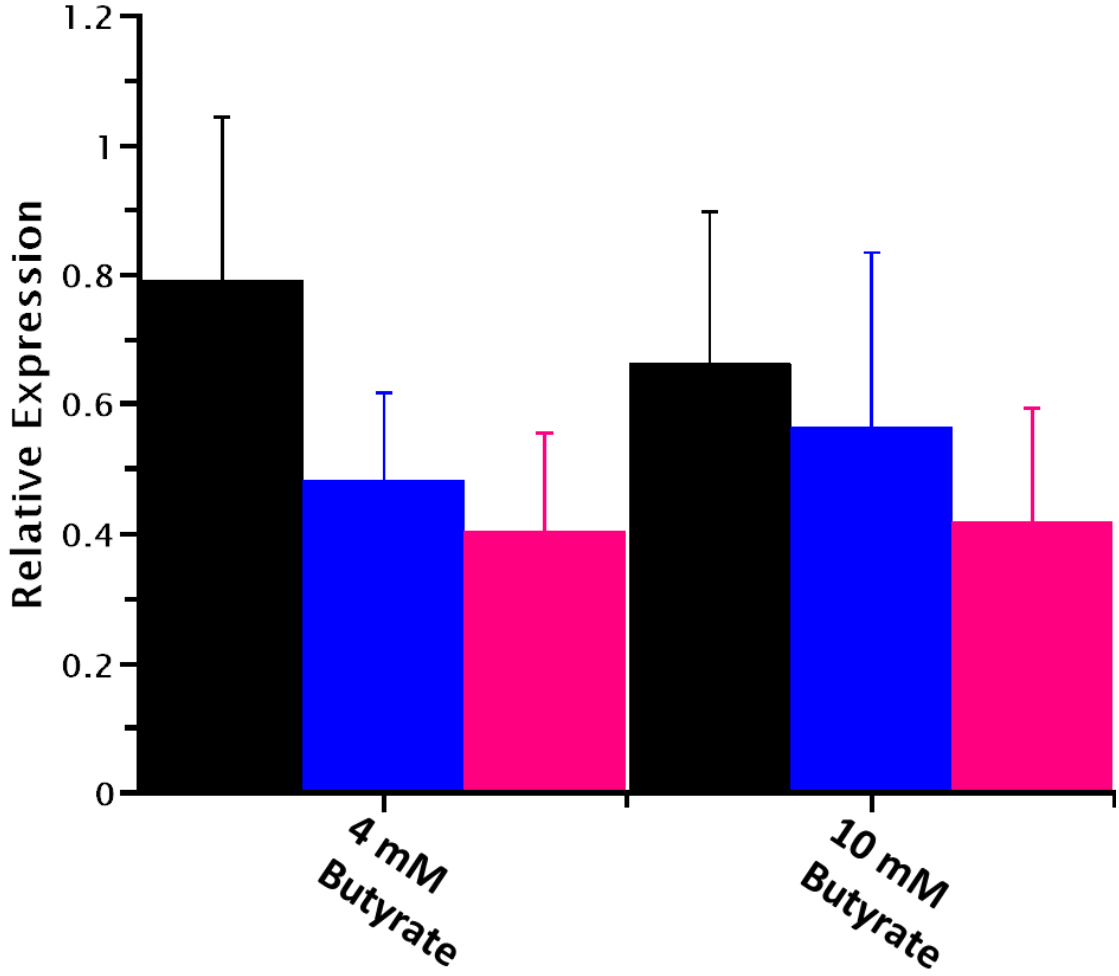


Figure 3. 17 Relative expression of the nitrate reductases *nasC* (black), *narG* (blue) and *napA* (pink). Expression is relative to the control gene *rpoB*. The data presented is from ammonium-dependent growth in continuous culture and the carbon source and concentration is stated for each experiment.

The data presented in Figure 3. 17 shows that although some expression of these reductases was detected it was at low levels and is unlikely to be supporting functional nitrate reduction. Most importantly the data presented shows that *nasC* expression is increased in the presence of nitrate, even when ammonium is also present, only when the cells are cultured on low concentrations of butyrate. It is this key result that combines with the metabolite data to confirm nitrate assimilation is possible when ammonium is present, and it is dependent upon the oxidation state and concentration of the carbon source.

3.3 Discussion

3.3.1 Continuous culture is superior to batch culture for investigating bacterial metabolism

In the first set of experiments in this chapter the batch growth profiles of *P. denitrificans* were investigated when the bacteria were cultured on different carbon and nitrogen sources. From this data it was suggested nitrate assimilation may be possible under mixed ammonium and nitrate conditions, when the cells were cultured with butyrate. This assertion was based on the lack of diauxic growth profiles in the butyrate condition compared to the succinate condition. In fact, as seen in the remainder of the chapter there is nitrate assimilation occurring in the 4 mM butyrate, mixed nitrogen source condition but not in the 10 mM condition. From the batch growth data this result would not have been possible.

In batch growth conditions data are taken from a constantly changing chemical environment. This reduces the trust in the conclusions drawn, as the concentrations of substrates change constantly. When 30 mM succinate, mixed nitrogen sources (Figure 3. 9) and 10 mM butyrate, mixed nitrogen sources (Figure 3. 11) are compared, the changes in nitrate concentration occur differently. In the high succinate condition the levels of ammonium fall prior to the nitrate levels, but this pattern is not seen in the high butyrate condition. Instead the levels fall simultaneously. In this batch growth it could then be argued that nitrate assimilation or dissimilation was taking place. This would most likely depend upon the part of the growth analysed and as such drastically different conclusions would be reached. Thanks to the continuous culture phase of the experiment nitrate is observed increasing in concentration and remaining at the feed concentration. This means it is not readily assimilated in either of these conditions when ammonium is present. This contrasts the conclusions that would have been made had batch growth curves been studied in isolation. It demonstrates the importance of continuous culture in investigating metabolic preferences.

3.3.2 Nitrate assimilation is possible even in the presence of ammonium

In this study the nitrogen metabolism of *P. denitrificans* has been investigated when cells were exposed to two different carbon substrates, under high or low carbon source availability. The results presented demonstrate an upregulation of functional assimilatory nitrate reductase under low level butyrate conditions. Furthermore, the growth yield per nitrate utilised is increased in nitrate-dependent growth, when cells are grown on low concentrations of butyrate. Both, key results indicate low butyrate concentrations favour nitrate assimilation, a result that is not consistent across the succinate conditions tested. Ultimately, the most interesting result of this chapter is the significantly increased expression of *nasC* under mixed nitrogen source growth with a low concentration of butyrate, combining this with the metabolite data it is consistent with the utilisation of nitrate. The conclusion must be nitrate assimilation is occurring under conditions where ammonium is also present.

In previous studies investigating regulation of nitrate assimilation in *P. denitrificans*, carbon substrates have been the typical laboratory source succinate. In one study it was suggested nitrate assimilation proteins were not present under ammonium growth, a result that makes perfect sense when no nitrate was present. Despite higher concentrations being used (50 mM) the data presented in this chapter suggests no utilisation phenotype would have been seen, as on both high and low succinate, ammonium is consistently preferred (Luque-Almagro *et al.*, 2017). Furthermore, to produce nitrate assimilation proteins the NasTS nitrate/nitrite regulatory sensor has been shown to be vital in allowing peptide production (Luque-Almagro *et al.*, 2013). This perhaps further highlights the reason nitrate assimilation has not been seen in ammonium grown cells. Prior to the identification of the assimilatory nitrate reductase in *P. denitrificans* the periplasmic nitrate reductase Nap was found. In this study the researchers used high concentrations of butyrate and found periplasmic nitrate reductase activity. The data presented in this chapter agrees that butyrate is important in the up-regulation of expression of *napA*, however as the previous work used high concentrations of butyrate, nitrate assimilation would not necessarily be detected here (Sears *et al.*, 1993). Furthermore, multiple studies have found that NarG is not found under aerobic conditions, so nitrate respiration can be discounted in any of the

culture conditions presented in this chapter (Sears *et al.*, 1993; Sears, Spiro and Richardson, 1997).

It is interesting that higher butyrate concentrations did not yield nitrate assimilation phenotypes. A possible explanation for this unexpected result could be due to the formation of polyhydroxyalkanoates. *P. denitrificans* is known to produce these structures in cells partially in response to increased levels of acetyl-CoA (Olaya-Abril, Luque-Almagro, *et al.*, 2018). This higher level of acetyl-CoA would certainly be present under high butyrate conditions as this is where β-oxidation meets the TCA cycle. Acetyl-CoA is converted to acetoacetyl-CoA in one reaction and is then further converted to (R)-3-hydroxybutyryl-CoA in an NADPH consuming pathway. Finally, this is polymerised to form poly(3-hydroxybutyrate). This pathway consumes cellular reductant and stores carbon substrates under high carbon conditions (Prieto *et al.*, 2007; Rehm, 2007). Under a high butyrate concentration this may be preferential to nitrate assimilation as cells can store carbon while it is plentiful, however when it is limited then they must use it for energy generation and biomass production. Combining this process with redox balancing nitrate reduction could be sufficient for cells grown on high butyrate. Conversely with little carbon source available the cells may need to consume all the carbon for energy generation and biomass and, as such require an alternative redox vent such as Nas. Alternatively, the metabolism of high concentrations of butyrate may result in a toxic effect on the cells. In this scenario the cells may expend energy maintaining their physiology and as such do not produce alternative redox vents such as Nas. If this is the case a lower concentration of butyrate may simply offer a lower toxic effect to cells and they are therefore able to assimilate nitrogen readily rather than triggering a variety of stress responses.

In conclusion the data presented here demonstrates nitrate assimilation is possible under butyrate-dependent growth conditions and not under succinate-dependent conditions. It is also clear that this is partially in response to the concentration of the aforementioned carbon sources. This demonstrates that nitrate assimilation is an alternative electron sink in cells struggling to deal with their increased levels of cytoplasmic reductant, when growing on reduced carbon substrates.

3.3.3 There is an alternative regulator of nitrate assimilation in bacteria that is responsive to the oxidation state of carbon sources

This thesis shows nitrate assimilation is possible under the conditions discussed in the previous section. This result is a contradiction of the current regulatory network that has been proposed. The conclusion from this work is that there must be an alternative regulator controlling nitrate assimilation that is responsive to carbon source oxidation state.

To make sense of this result the known regulatory levels in *P. denitrificans* must be considered. The critical step in production of Nas proteins is at the level of RNA, controlled by the nitrate/nitrite sensory complex of NasTS. This two-component complex acts as a control mechanism for production of Nas proteins. It operates as a complex that dissociates upon binding of nitrate or nitrite to the NasS protein. This leads to dissociation of the NasT protein which in turn acts as a transcriptional anti-terminator. NasT binds to *nasA* leader RNA stabilising the transcript and allowing for further production. This process has been shown to be vital to *P. denitrificans* and a mutant lacking *nasT* was unable to grow on nitrate (Luque-Almagro *et al.*, 2013). This control mechanism presumably prevents the energetically costly production of nitrate assimilation proteins under conditions where nitrate or nitrite are not present. In the work presented in this chapter there is always a supply of nitrate in the media and as such we the NasTS system would still be operational in these cultures. Therefore the control of expression must be at a higher level.

The global nitrogen regulatory system NtrBC has been well characterised in enteric bacteria and the phosphorylation mechanisms are well understood (Swanson, Alex and Simon, 1994). In bacteria this system acts to promote or repress expression of a wide variety of genes. It works by the autophosphorylation of NtrB, this in turn phosphorylates NtrC which can then act to initiate expression of various genes (Sanders *et al.*, 1992; Luque-Almagro *et al.*, 2017). In the literature to date it has been stated that this system

responds to a lack of nitrogen, explained as a lack of ammonium, and in turn activates genes for nitrate assimilation, ammonium scavenging and increased uptake of carbon substrates for production of reducing equivalents for nitrate assimilation (Luque-Almagro *et al.*, 2017). It is therefore likely that it is this level of global nitrogen control that is involved in dual nitrate and ammonium assimilation.

In cyanobacteria a global nitrogen metabolism control system has been shown to control expression of nitrate assimilation genes. This system relies on NtcA to sense the change in nitrogen availability and then instigate the expression of nitrate assimilation genes. This is very similar to the NtrBC system which responds to a lack of ammonium. Interestingly the cyanobacterial system responds to the levels of 2-oxoglutarate rather than directly to the level of ammonium present. The theory implies that 2-oxoglutarate levels in the cell rise when ammonium is limited as there is no available ammonium to generate organic nitrogen. The cell therefore interprets the increase in 2-oxoglutarate as a nitrogen starvation and induces expression of nitrate assimilation genes (Ohashi *et al.*, 2011).

In *P. denitrificans* the production of poly(3-hydroxybutyrate) (PHB) has been studied for its potential use in the production of bioplastics. Interestingly the study found that a mutant lacking the global nitrogen regulator *ntrB* hyper-accumulates PHB (Olaya-Abril, Luque-Almagro, *et al.*, 2018). This is interesting as the results from this chapter could be explained by PHB production being used by butyrate cultured cells as a storage/reducing equivalent consuming pathway under carbon sufficient conditions. It is therefore interesting that a mutant unable to use nitrate assimilation carries out this PHB over-production. This provides a link between carbon and nitrogen metabolism regulatory control and implies that there is an as yet unknown regulatory level that responds to cellular carbon conditions. This would likely be acting in a similar way to the Ntc system, directly involved in intermediates produced during assimilation of butyrate and nitrogen.

To carry this work forward it would be pertinent to investigate the NtrBC system further in continuous culture conditions shown in this chapter. It may be beneficial to look at global transcriptomic data from these experiments to try to identify up-regulated

regulatory genes. Finally, there is always a possibility that novel regulatory systems may be in play. While G-quadruplex DNA structures have be known for a time in eukaryotes, it has recently been shown that nitrate assimilation by *P. denitrificans* is affected by stabilisation of one such quadruplex (Waller *et al.*, 2016). It would be a significant surprise if there was only one example of regulation by these quadruplexes in *P. denitrificans* and it is therefore important to further study these systems for their potential regulatory effects in this system.

Chapter 4: Construction of a *nasC* expression strain

4.1 Introduction

4.1.1 Nitrate reductase co-factors

Nitrate reductases require a number of co-factors to perform the reductive process of converting nitrate to nitrite. Amongst the variety of different nitrate reductases, found in both eukaryotic and prokaryotic systems, molybdenum is critical to the active centre of the enzymes (Rajagopalan, K.V. and Johnson, 1992). In *P. denitrificans* the molybdenum centre is comprised of the molybdopterin guanine dinucleotide co-factor which performs the catalytic reduction. This is conserved amongst the three distinct nitrate reductases in *P. denitrificans* (Gates, Richardson and Butt, 2008; Gates *et al.*, 2011). It therefore stands to reason that limitation in molybdenum co-factor is a key issue for overexpression and purification of the assimilatory nitrate reductase NasC.

Whilst it is important to provide the active centre of the enzyme, nitrate reductases require a supply of electrons to facilitate substrate reduction. In both the respiratory nitrate reductase (NarG) and the periplasmic, energy dissipating nitrate reductase (NapA) the electron source is the membrane quinol pool. In both systems a membrane anchored heme-containing protein is involved in electron acquisition. The electrons are then passed through to the active sites of the nitrate reductases by a variety of different iron-sulfur clusters. There is evidence that the potential of these clusters is, in part, determined by the correct molybdenum co-factor being present (Berks *et al.*, 1995; Magalon *et al.*, 1997; Rothery *et al.*, 1998). In assimilatory systems the proteins are generally not associated with the membrane and are found in the cytoplasm. They must gain their electrons from an alternative source. In cyanobacteria the system is linked by ferredoxin to photosynthesis (Flores *et al.*, 2005). In heterotrophic bacteria, such as *P. denitrificans*,

the electron source comes from the NAD(P)H pool (Gates *et al.*, 2011). Irrespective of the source of the electrons or indeed the type of nitrate reductase being investigated iron-sulfur clusters are another critical component of consideration.

4.1.2 NarJ as a chaperone for NasC in *P. denitrificans*

Correct folding of proteins is important for their ultimate function. In the case of complex metalloenzymes such as nitrate reductases the correct protein fold also includes the molybdenum co-factor and biogenesis of iron-sulfur clusters. The chaperone NarJ has been identified in respiratory nitrate reductase systems for a number of years. In *E. coli* it was shown to be important for insertion of molybdenum co-factor into the NarGH complex. This work was extended to show apo-NarGH could be reactivated by the addition of recombinant NarJ. The work on *E. coli* assumed that the interaction between NarJ and NarG was specific to the respiratory system only, a conclusion that makes sense given the location in the same gene cluster and the data provided (Blasco *et al.*, 1998). Recently, however, NarJ from *P. denitrificans* has been shown to be a dual functional maturation protein, important for NasC molybdenum maturation not just NarG (Pinchbeck *et al.*, 2019).

Work by Pinchbeck and colleagues used a variety of different techniques to establish NarJ as a chaperone in *P. denitrificans* specifically relating to molybdenum insertion into both NarG and NasC. Growth experiments highlighted the importance of molybdenum for nitrate assimilation, without this key metal there is no activity detected and growth is severely limited. With a deletion of the *narJ* gene the results are akin to molybdenum deficiency. This was combined with gene expression data to confirm *narJ* is transcribed under aerobic conditions, in contrast to *narG*. Finally, recombinant NarJ was shown to interact with both NasC and NarG. This established NarJ as a crucial chaperone for assimilatory nitrate reduction in *P. denitrificans* (Pinchbeck *et al.*, 2019). If a successful strain is produced for the expression and purification of active NasC, NarJ will presumably be important as a critical co-factor assembly protein.

4.1.3 The aims of this chapter

In this chapter the aim was to create a strain capable of production of active NasC that could subsequently be isolated. This has yet to be done with *P. denitrificans* NasC. In previous studies investigating different organisms, purification of assimilatory nitrate reductases involved a variety of ion-exchange chromatography techniques alongside size exclusion (Martinez-Espinosa, Marhuenda-Egea and Bonete, 2001). In analysis of nitrate assimilation in *P. denitrificans* the nitrate reductase NasC was removed from the nitrite reductase NasB by anion-exchange chromatography, however, the proteins themselves were never isolated (Gates *et al.*, 2011). In the two methodologies employed the proteins analysed were natively produced and had no modifications. In this work a Strep-II-tag was added to aid affinity purification. This tag was chosen ahead of more conventional poly-histidine tags as it does not involve metal binding and as such should not disrupt the metal co-ordination required in NasC. As nitrate reductases are complex proteins requiring a variety of cofactor assemblies this work used a range of techniques to attempt to maximise the availability of chaperones that may be required. Ultimately, this work attempted to analyse the kinetic properties of NasC in order to further understand its biochemistry and to establish more detail about the role of nitrate assimilation in a range of environments.

4.2 Results

4.2.1 Nitrate reductase mutants

As highlighted in section 4, nitrate reductases are large, complex proteins that require assembly of multiple elements to function correctly. Nitrate reductases share common elements across the Nas, Nar and Nap systems, as *P. denitrificans* encodes all three of these systems recent work has shown a dual function for NarJ in the maturation of NasC and NarG (Pinchbeck *et al.*, 2019). A shared chaperone may be positive for the bacteria as it is more energetically favourable to produce a single protein, however it presents a challenge for the over-expression of recombinant protein. If wild-type *P. denitrificans* cells are cultured for over-expression they have the capacity to produce three distinct nitrate reductases that use the same or similar critical co-factors, most significantly molybdenum. This study therefore began with analysis of pre-established unmarked gene deletion mutants of the three nitrate reductases to establish an over-expression strain optimal for generation of functional NasC.

4.2.1.1 Characterisation of gene deletion mutants

To verify single gene deletions primers were designed to provide a PCR product encompassing the up and downstream regions of each nitrate reductase gene, allowing for simple size differentiation by agarose gel electrophoresis and providing a sample of DNA for sequencing to verify the deletion.

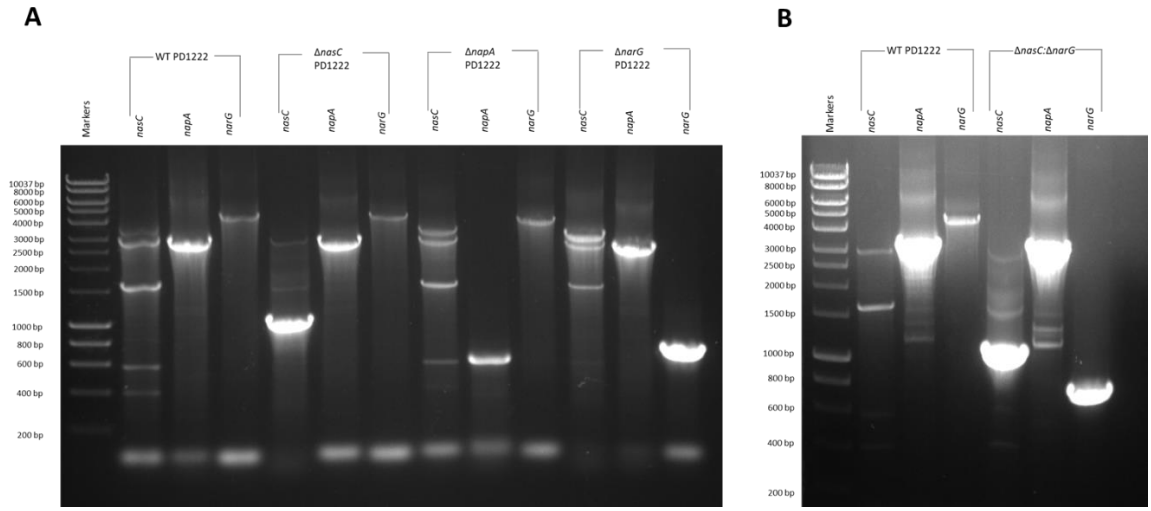


Figure 4. 1 Agarose gel electrophoresis of PCR products generated from each single gene deletion mutant (A) and the double nitrate reductase mutant (B) and wild-type *P. denitrificans* as a control (A&B).

The large products originate from the three nitrate reductase genes in the wild-type lanes. In each mutant the results show a product size reduction, corresponding to the deletion of the specific coding sequence for each deleted reductase (Figure 4. 1). Furthermore, the results verify that only the intended nitrate reductase had been deleted in each case, for example in the Δ*narG* strain *narG* is removed and *nasC* and *napA* are unaffected. As it was possible to purify the PCR products from gel extractions, DNA sequencing confirmed the correct deletions and confirmed that none had polar effects on downstream genes.

Growth analyses were carried out to determine the phenotype of each mutant grown aerobically with nitrate as the sole nitrogen source, to test the nitrate assimilation system. Mutants lacking *nasC* do not grow at all and the others are able to successfully grow (Figure 4. 2).

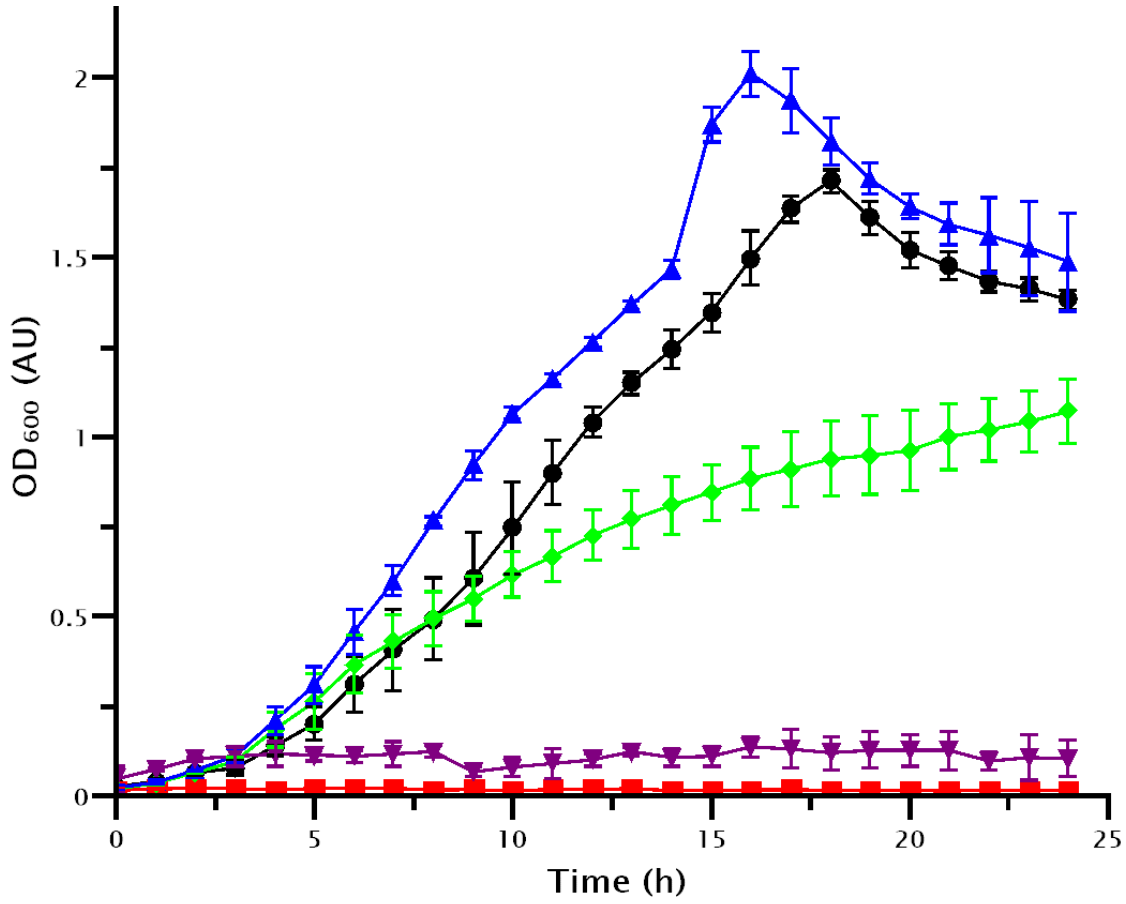


Figure 4. 2 Growth of *P. denitrificans* wild-type (black circles), Δ*napA* (blue triangles), Δ*narG* (green diamonds), Δ*nasC* (red squares) and Δ*nasC:narG* (purple inverted triangles). Cells were cultured using 30 mM succinate and 10 mM NO_3^- as the sole carbon and nitrogen sources respectively, at 30°C and shaking at 180 rpm to maximise aerobicity.

Aerobic phenotypes followed expectations; next anaerobic growth analyses were carried out to determine the correct phenotype for strains lacking *narG*. Both the Δ*narG* and Δ*nasC:narG* strains were not able to grow in the presence of nitrate as the sole nitrogen and respiratory substrate (Figure 4. 3). Interestingly anaerobic, nitrate co-respiration/assimilation resulted in recovery of the Δ*nasC* mutant, probably due to a functional crossover between NarG and NasC.

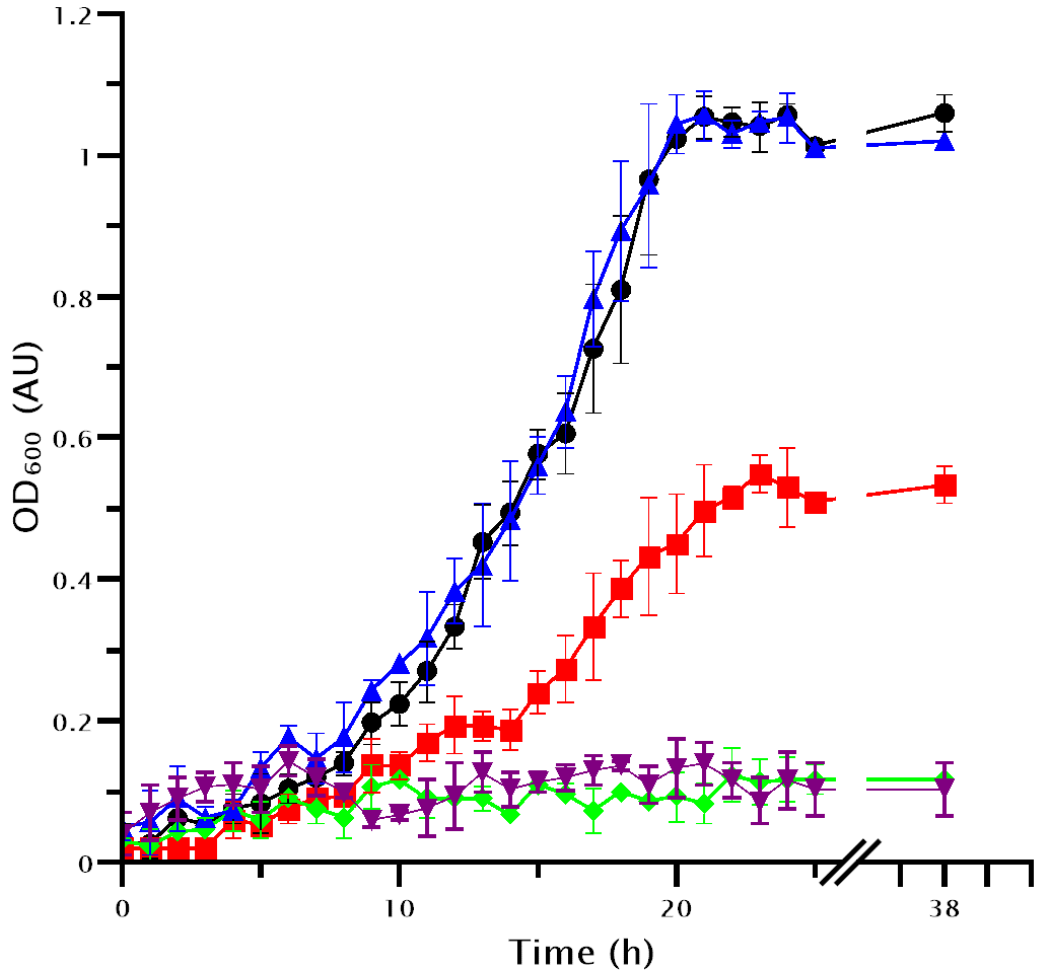


Figure 4. 3 Growth of *P. denitrificans* wild-type (black circles), Δ*napA* (blue triangles), Δ*narG* (green diamonds), Δ*nasC* (red squares) and Δ*nasC:narG* (purple inverted triangles). Cells were cultured using 30 mM succinate and 30 mM NO_3^- as the sole carbon and nitrogen sources respectively, at 30°C and stationary in sealed, oxygen free nitrogen sparged bottles to maximise anaerobicity.

The growth curves confirmed that the nitrate reductase mutants conformed to the expected phenotypes. This meant these strains could be explored for overexpression of a recombinant, plasmid borne copy of *nasC* with the addition of a Strep-II-tag to aid affinity purification.

4.2.2 Transformation of mutant strains with the overexpression plasmid

Previous work has highlighted the importance of the dual functional maturation chaperone NarJ. This protein functions as a molybdenum chaperone for the correct assembly of the catalytic centres of both NarG and NasC in *Paracoccus* (Pinchbeck *et al.*, 2019). For this reason, it was hypothesised that previous attempts to generate successful *nasC* overexpression constructs was due to the absence of functional NarJ. Therefore, *narJ* was included in the overexpression construct so that *nasC* and *narJ* would be co-overexpressed and the chaperone would be able to maintain the required loading of co-factor. The vector shown has the two genes arranged to generate a NarJ protein without an affinity tag and a NasC protein with a Strep-II-tag (Figure 4. 4). The plasmid produced was under the control of a taurine inducible promoter.

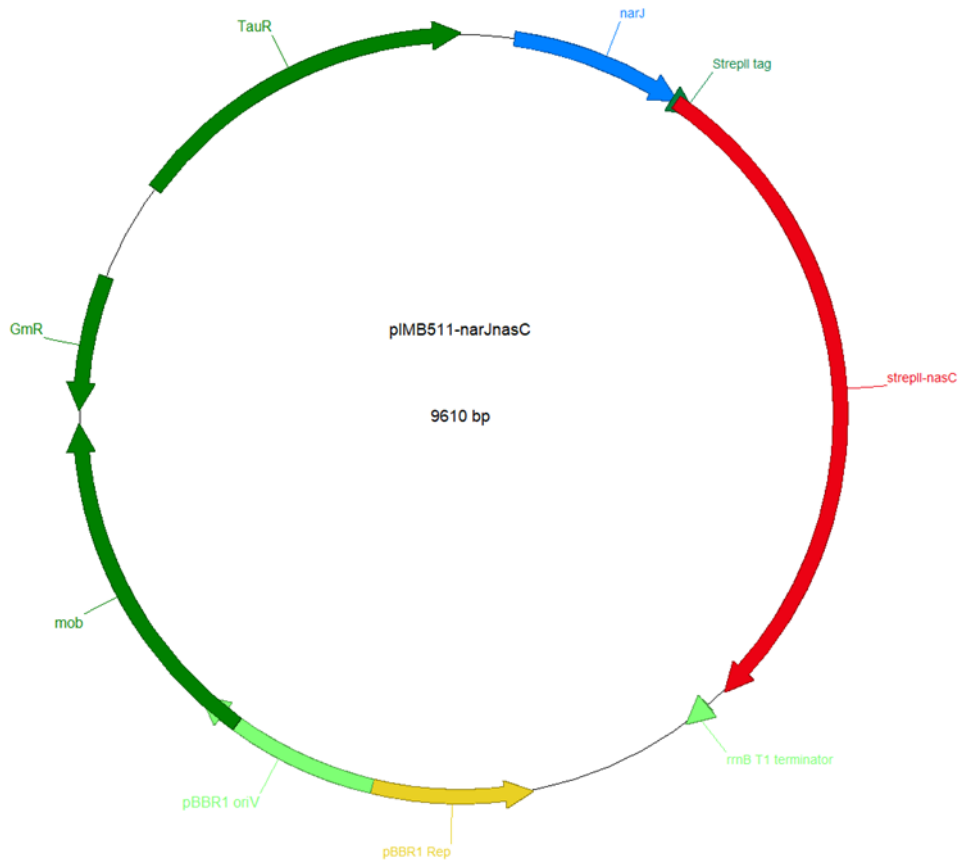


Figure 4. 4 Vector map of pLMB511*narJnasC* showing the relevant characteristics of the vector.

The expression vector was used to transform *P. denitrificans* wild-type and mutant strains by a tri-parental conjugation from *E. coli* DH5α and a helper strain containing the pRK2013 plasmid. Gentamycin resistant colonies were tested using PCR for the production of a *narJ-nasC* fragment only possible from the construct. Positive colonies were then grown, and plasmid DNA was isolated and sequenced to confirm the correct sequence was present.

4.2.3 Overexpression trials in mutant backgrounds

To establish the correct expression time, inducer concentration and mutant background to use in large scale growths various expression trials were carried out. As taurine is a carbon and nitrogen containing molecule a variety of concentrations were tested to find a point at which it could act as a strong inducer of expression without being a preferred nitrogen source.

4.2.3.1 Wild-type P. denitrificans

In the first experiment the wild-type strain was used to establish the data expected when no genetic modifications were made. Cells were grown on 5 mM nitrite, 5 mM nitrate until an OD_{600} of ~0.4 and expression was induced by the addition of either 1, 5 or 10 mM taurine. Cells were grown aerobically as previously described, using 30 mM succinate as the sole carbon source in minimal salts media. The gel image shown in Figure 4. 5 shows that there is no obviously enlarged band relating to the 92 kDa NasC present under any condition tested. A subsequent Western blot analysis using a commercially available Strep-II-tag HRP conjugate antibody (*Merck*) did not reveal a band relating to the correct size for NasC (data not shown).

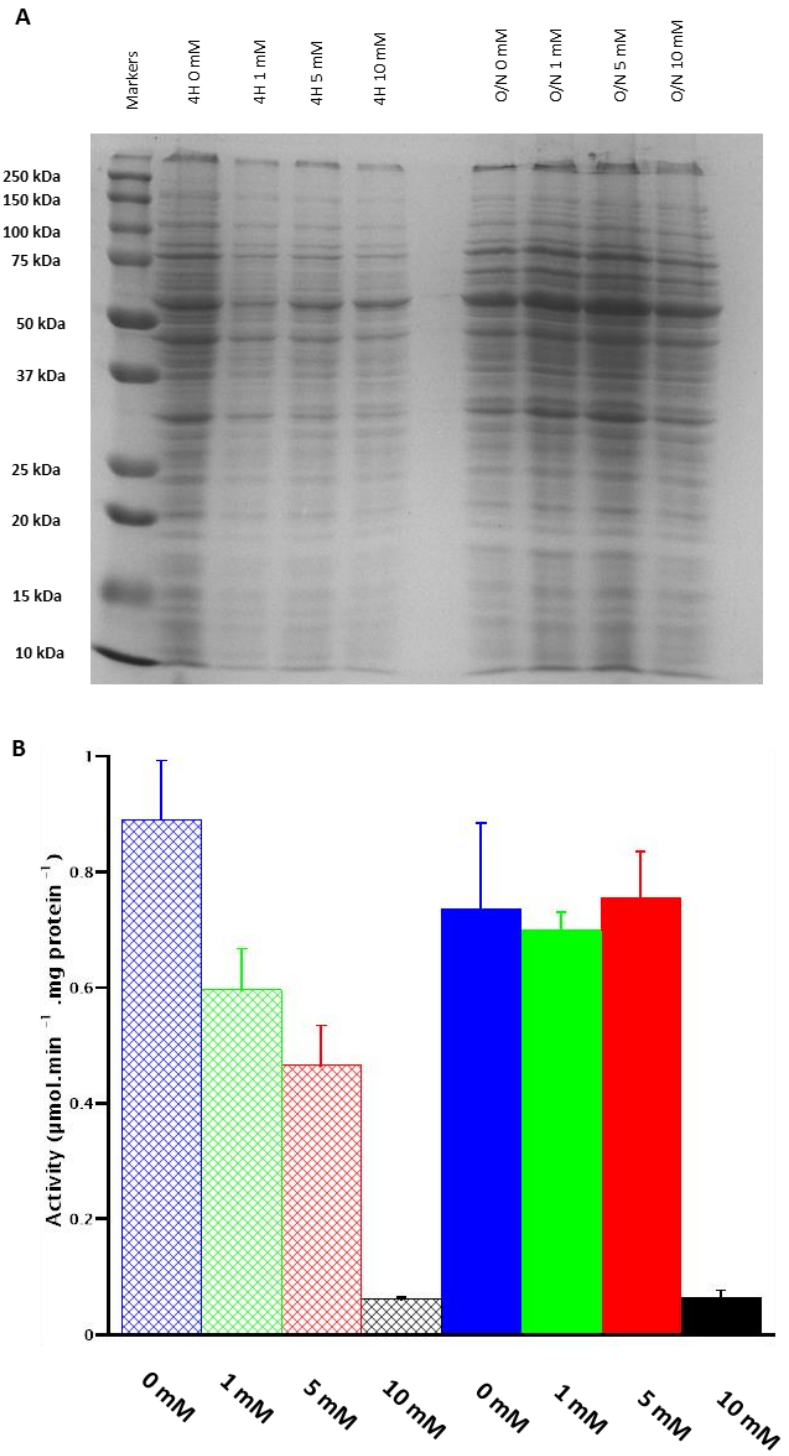


Figure 4. 5 **(A)** SDS-PAGE analysis of overexpression trials under a wild-type background. Lanes are labelled by length of expression where 4H means 4 hours expression length and O/N means overnight. The final concentration of taurine added to induce expression is shown for each lane. **(B)** Methyl viologen dependent nitrate reductase activity from cell lysates obtained from each overexpression condition. Hashed bars represent data from 4 hours of expression and solid bars represent data from overnight expression. The final concentration of taurine is shown underneath each bar.

Nitrate reductase activity was determined by a methyl viologen assay. The results show that the activity was highest when no taurine was added to the cultures (Figure 4. 5). Irrespective of the expression time 10 mM taurine appeared to down-regulate the amount of nitrate reductase present, possibly due to taurine being a preferred nitrogen source at this concentration. This result showed no clear overexpression condition and as such it was important to analyse the overexpression conditions for the gene deletion backgrounds.

4.2.3.2 ΔnasC P. denitrificans

The Δ*nasC* strain should provide a background that has the appropriate maturation proteins but, does not have a nitrate reductase to mature. In this case the chaperones should be freely available to the recombinant version of NasC. It was important to culture the bacteria in media containing both nitrate and nitrite as this strain was unable to grow solely utilising nitrate. Nitrate was included in the media to stimulate expression of any required nitrate reductase maturation genes. There was no evident overexpressed protein at the expected size present on the SDS-PAGE gel (Figure 4. 6). In this gel strains were included that contain a single copy of either *nasC* or *narJ* as controls. In both cases no clear overexpression band could be seen, and a western blot failed to detect the Strep-II-tag NasC from either construct. This antibody is not effective in the detection of NasC-Strep-II-tag proteins, as confirmed in future experiments (Section 4.2.6).

In almost all cases no nitrate reductase activity was observed (Figure 4. 6). This confirmed that there were no other nitrate reductases produced under the chosen growth condition. The only condition that produced any activity was 5 mM taurine overnight. This activity was significantly lower than that seen for the wild-type lysates, indicating that the level of expression was lower than native expression. Overall it was concluded that this strain was not useful for overexpression of *nasC*.

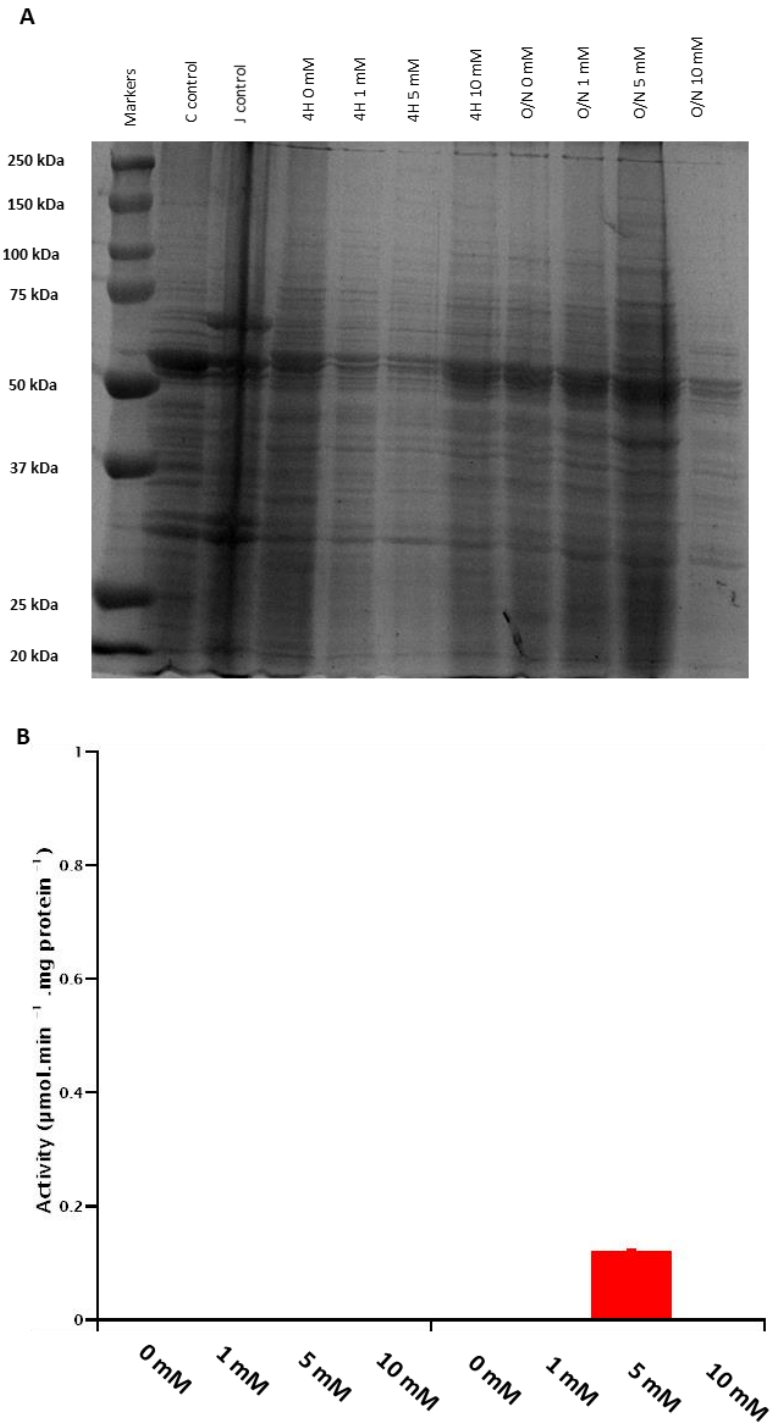


Figure 4. 6 (A) SDS-PAGE analysis of overexpression trials under a Δ*nasC* background. Lanes are labelled by length of expression where 4H means 4 hours expression length and O/N means overnight. The final concentration of taurine added to induce expression is shown for each lane. 'C control' and 'J control' refer to single gene copy overexpression strains to act as controls. **(B)** Methyl viologen dependent nitrate reductase activity from cell lysates obtained from each overexpression condition. Hashed bars represent data from 4 hours of expression and solid bars represent data from overnight expression. The final concentration of taurine is shown underneath each bar.

4.2.3.3 ΔnarG P. denitrificans

As there is a shared chaperone for the maturation of both NasC and NarG, a strain lacking *narG* should allow the available maturation proteins to mature only NasC. Again, there was no evident overexpressed protein under any of the tested conditions (Figure 4. 7). Cells were cultured using nitrate and nitrite to be consistent with previous expression tests. When activities were measured for each of the conditions tested there was a pattern of descending activity with increasing taurine concentration. This has been seen before in these activity trials and is likely due to *P. denitrificans* preferentially metabolising the taurine as a nitrogen source. It is interesting that there appears to be an increase in the activity under the 5 mM taurine conditions, when compared to the 1 and 10 mM conditions. This could be as a result of *nasC* expression compensating with some activity. Ultimately no condition restored total activity to that seen in wild-type lysates without induction of expression so this would be unlikely to yield significant amounts of active protein.

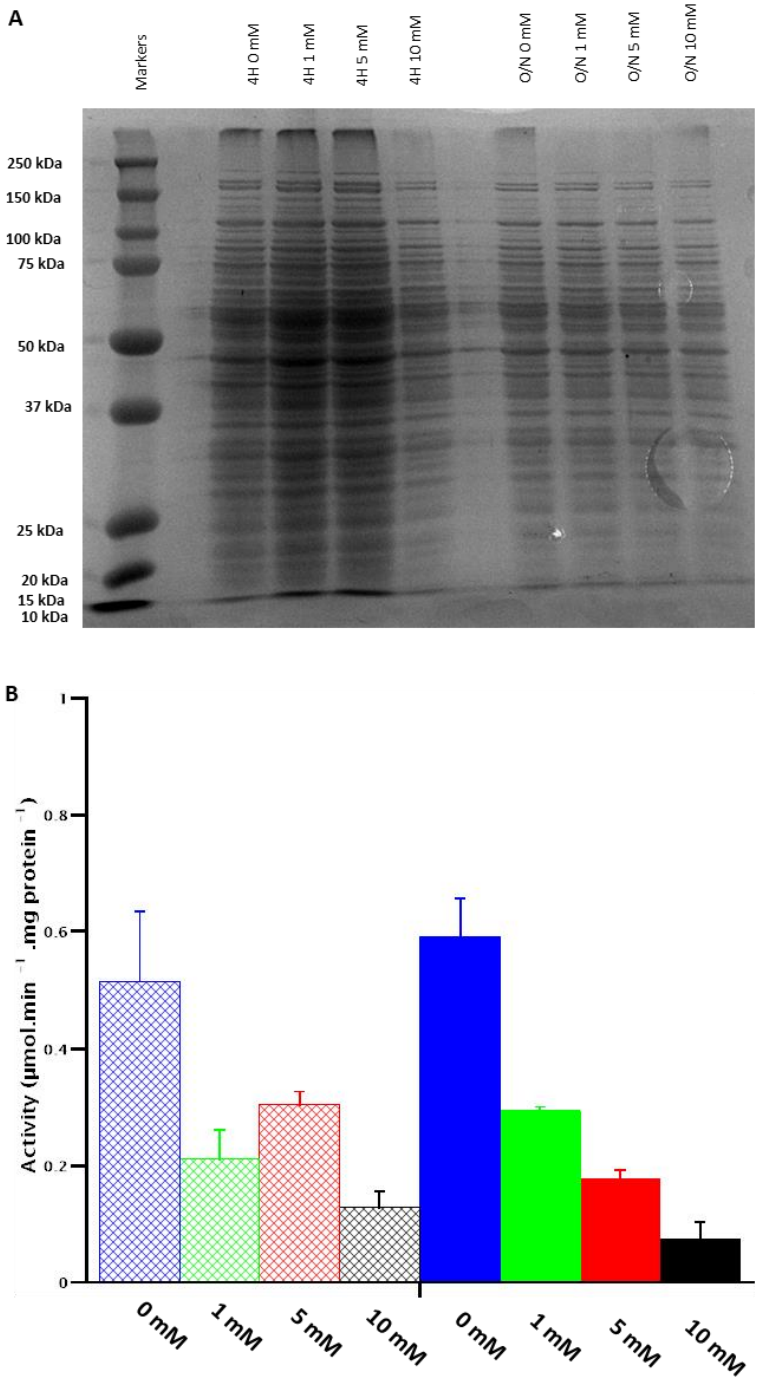


Figure 4. 7 (A) SDS-PAGE analysis of overexpression trials under a Δ*narG* background. Lanes are labelled by length of expression where 4H means 4 hours expression length and O/N means overnight. The final concentration of taurine added to induce expression is shown for each lane. **(B)** Methyl viologen dependent nitrate reductase activity from cell lysates obtained from each overexpression condition. Hashed bars represent data from 4 hours of expression and solid bars represent data from overnight expression. The final concentration of taurine is shown underneath each bar.

4.2.3.4 ΔnapA P. denitrificans

The link between the respiratory and assimilatory nitrate reductases in maturation of the active site has been considered however, the redox-balancing reductase NapA has not been shown to have a shared chaperone with NasC. This does not discount the possibility that such a link exists. Therefore, perhaps deletion of this gene may result in an increased overexpression of recombinant *nasC*. There was no overexpression of any particular protein, a recurring theme in these experiments. Activity assays show a similar story to the Δ*narG* background. At 5 mM taurine there appears to be a modest increase in activity and a high taurine concentrations nitrate reduction appears to be redundant (Figure 4. 8). These results again show that no condition is able to increase activity from the wild-type with no induction and, as such this strain does not give the required overexpression profile.

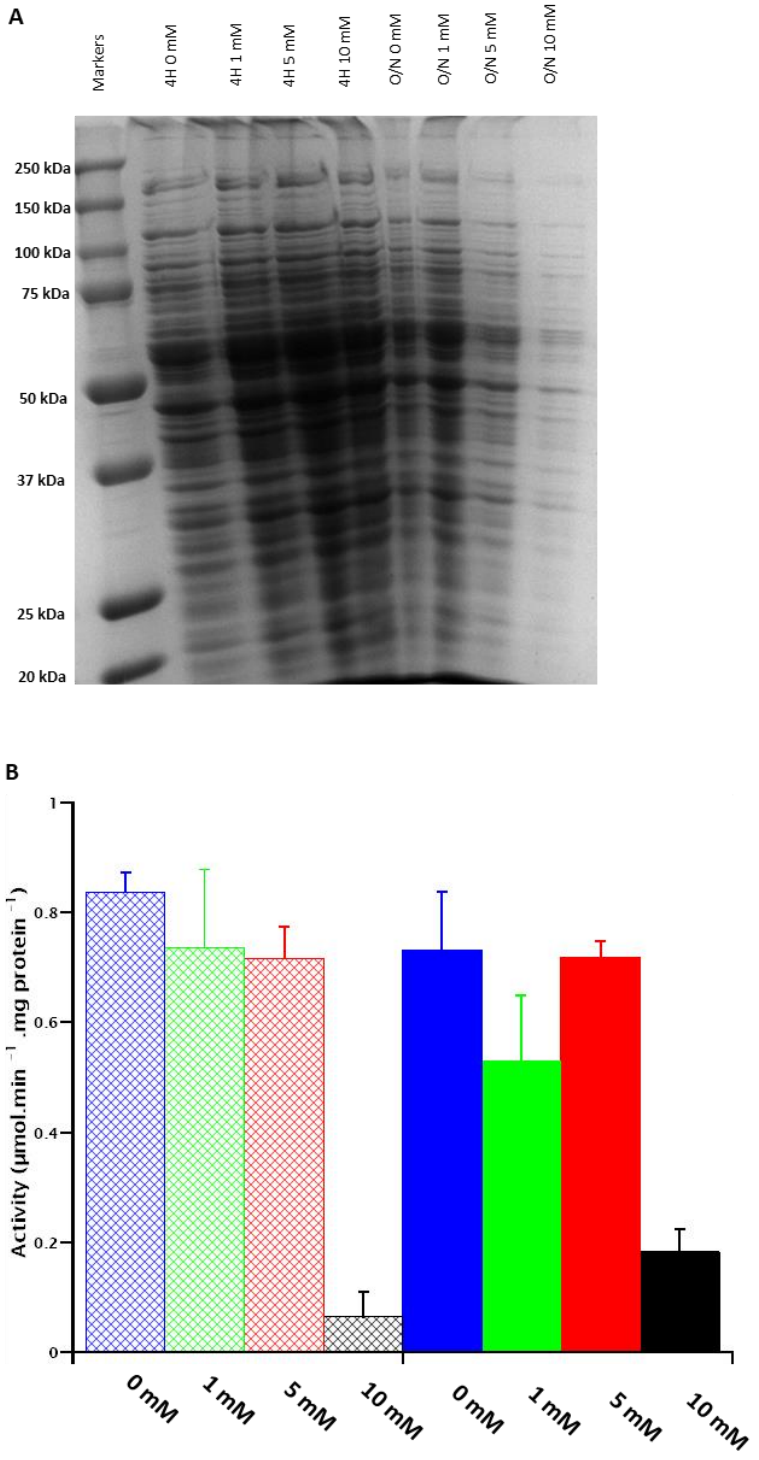


Figure 4. 8 (A) SDS-PAGE analysis of overexpression trials under a Δ*napA* background. Lanes are labelled by length of expression where 4H means 4 hours expression length and O/N means overnight. The final concentration of taurine added to induce expression is shown for each lane. **(B)** Methyl viologen dependent nitrate reductase activity from cell lysates obtained from each overexpression condition. Hashed bars represent data from 4 hours of expression and solid bars represent data from overnight expression. The final concentration of taurine is shown underneath each bar.

4.2.3.5 ΔnasC:narG P. denitrificans

Finally, it was appropriate to test the expression in a double mutant background where both the nitrate reductases using a shared maturation protein were deleted (Δ*nasC:narG*). Under this condition the effect of limited co-factor loading could be eliminated as no native nitrate reductases should be produced. Unsurprisingly there were no clearly overexpressed proteins in the SDS-PAGE analysis (). Activity assays were also completed with samples from each condition and the results essentially repeat those for the Δ*nasC* background. The overnight expression with 5 mM taurine was the only condition that provided any nitrate reductase activity, which was again significantly less than the wild-type native expression.

These results confirm firstly, that deletion of nitrate reductases to make maturation proteins available to recombinantly expressed proteins does not increase levels of expression or activity. Secondly, the results presented show a critical problem with the overexpression vector system used in this research, as higher taurine concentrations reduce expression of nitrate reductases and possibly other unknown maturation proteins required leading to inefficient overexpression.

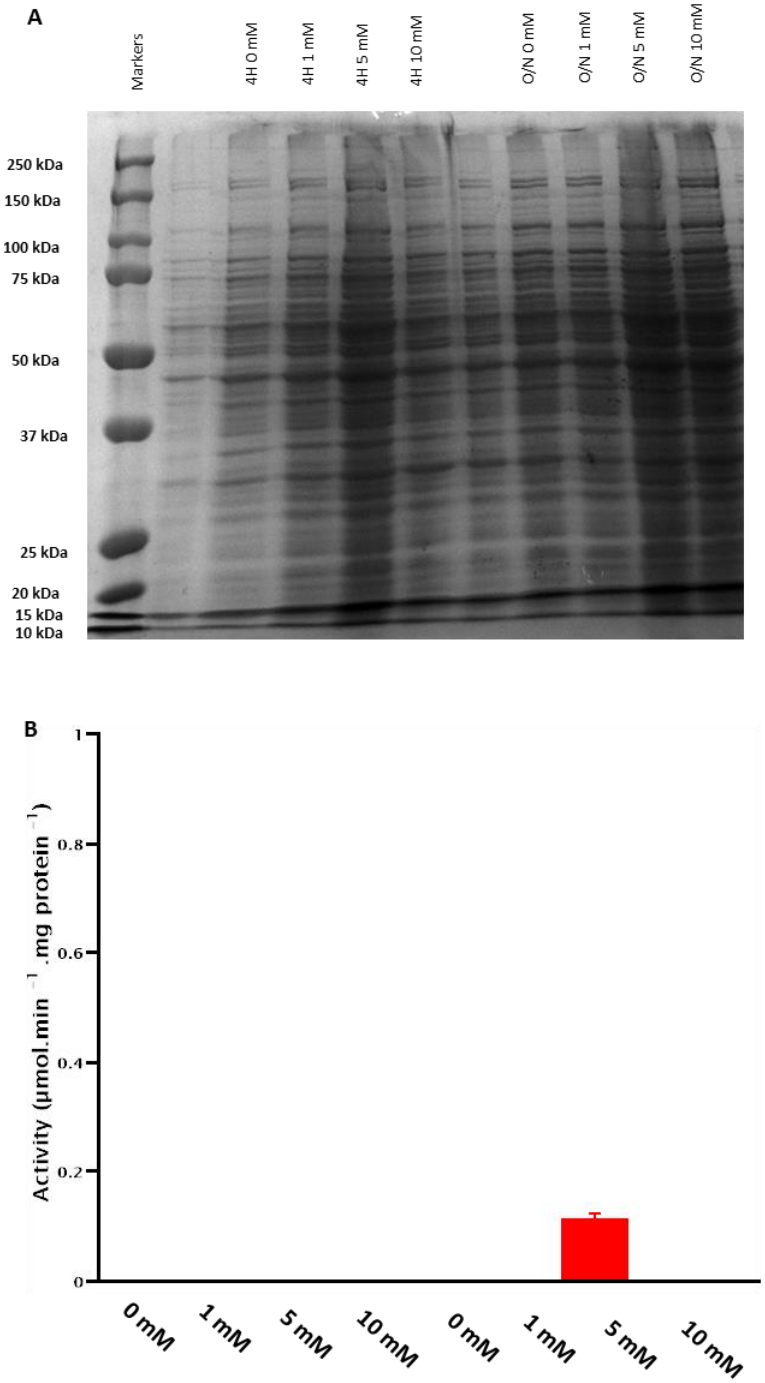


Figure 4. 9 (A) SDS-PAGE analysis of overexpression trials under a Δ*nasC:narG* background. Lanes are labelled by length of expression where 4H means 4 hours expression length and O/N means overnight. The final concentration of taurine added to induce expression is shown for each lane. **(B)** Methyl viologen dependent nitrate reductase activity from cell lysates obtained from each overexpression condition. Hashed bars represent data from 4 hours of expression and solid bars represent data from overnight expression. The final concentration of taurine is shown underneath each bar.

4.2.4 Construction of a native NasC-Strep-II-tag strain

As the data presented in section 4.2.3 demonstrated, overexpression of *nasC* is extremely difficult in *P. denitrificans*. At no point did the activity in the trial expression conditions exceed that of native *nasC* expression in uninduced cultures. As the highest apparent expression of active NasC was under the native promoter control in response to nitrate dependent growth it would be logical to isolate the protein from this source.

To isolate NasC protein the sequence for a Strep-II-tag was introduced to the 3' end of the *nasC* coding sequence immediately prior to the stop codon. This was done using a method of homologous recombination explained in section 2.11. On addition of the tag sequence any NasC produced would include the tag sequence to aid affinity purification. The plasmid pARJ001 was constructed to include the recombining flanks either side of the Strep-II-tag sequence that was introduced (Figure 4. 10).

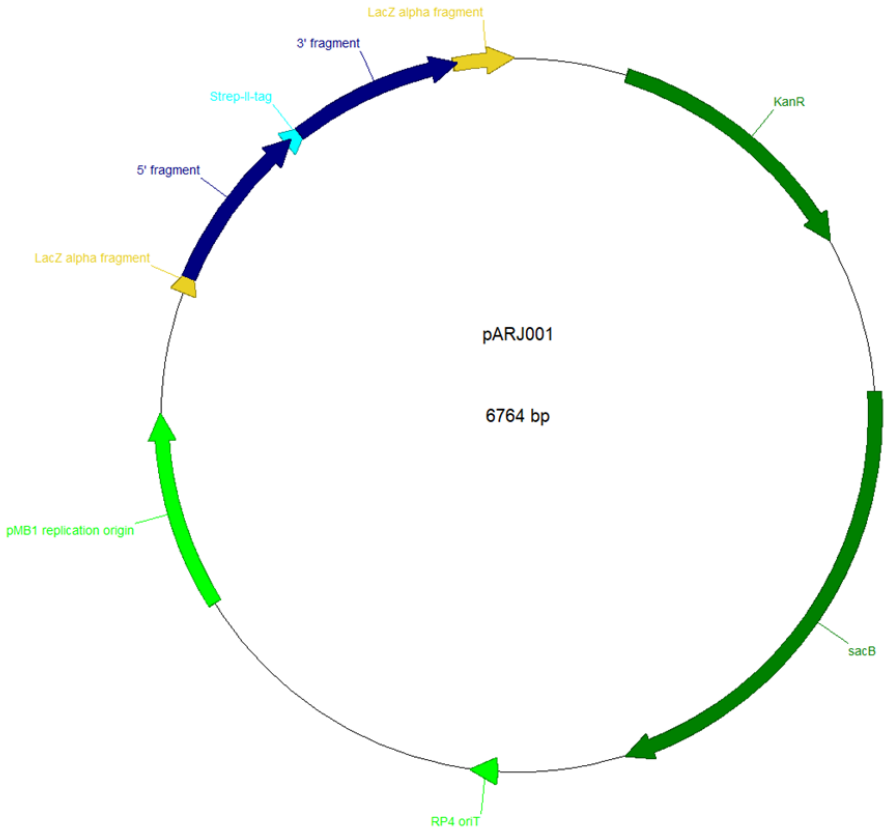


Figure 4. 10 Plasmid map of the *pK18mobsacB* derivative plasmid pARJ001 encoding the 5' and 3' fragments either side of the Strep-II-tag sequence to be added to the genome. Other highlighted genes include the kanamycin resistance gene (*kanR*) and the levan sucrase gene (*sacB*).

The method allowed for primary integration of the plasmid into the genome conferring resistance to the antibiotic kanamycin and sensitivity to the sugar sucrose. This also allowed for diagnostic PCR detection by the vector borne M13 primer binding sites (Figure 4. 11 A). Once this was confirmed secondary recombination events were selected for by the reversal of these vector-borne traits. Colonies were screened for resistance to sucrose, sensitivity to kanamycin and a negative result in M13 primer PCR. Finally, a check was completed screening for the presence of the Strep-II-tag sequence (Figure 4. 11 B). This confirmed that the colonies had not reverted to the wild-type genotype and instead encoded the tag in the appropriate location. Successful colonies were isolated, genomic DNA was prepared and the entire gene was amplified and sequenced to confirm that only the intended mutation had occurred. This strain was then named *Paracoccus denitrificans* Pd4449.

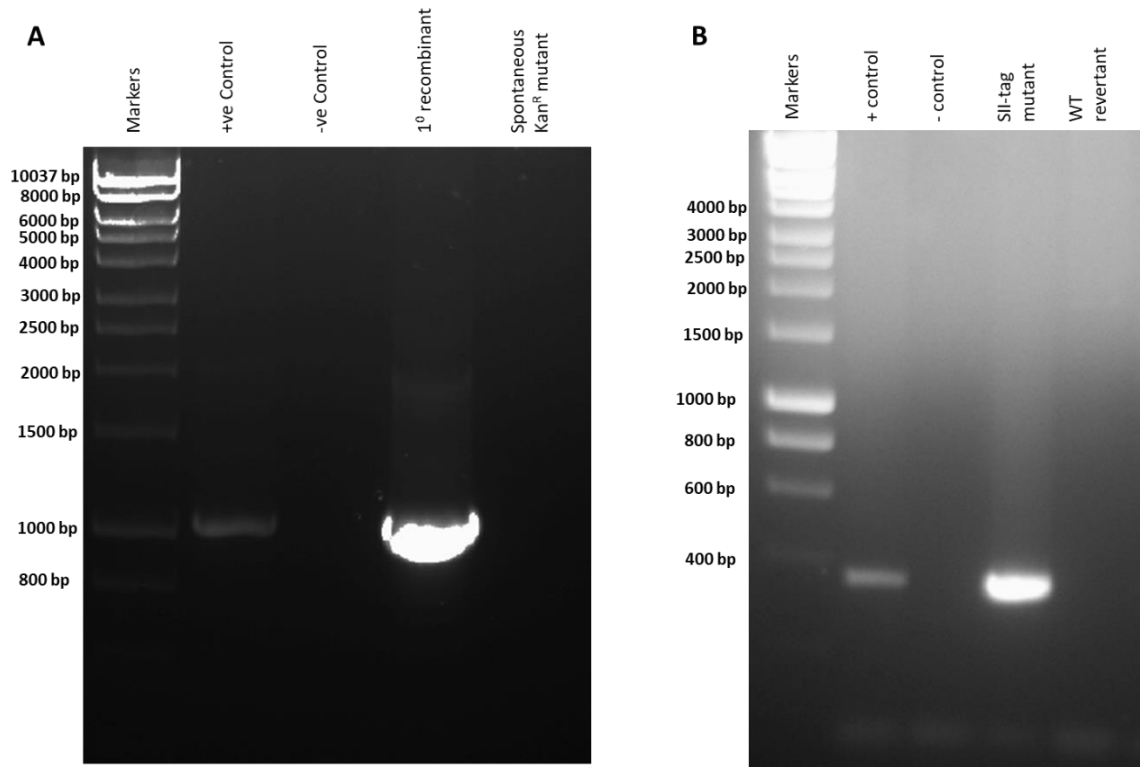


Figure 4. 11 PCR products amplified from M13 PCR (A) or Strep-II-tag specific PCR (B). Positive controls were isolated pARJ001 and negative controls were wild-type *P. denitrificans* Pd1222 genomic DNA (A&B). The expected size of the M13 PCR product was ~1 kb and the expected size of the Strep-II-tag specific PCR product was ~380 bp.

Once the strain was constructed it was important to test the growth phenotype to ensure that NasC was still functional. Cells were grown in minimal salts media using 30 mM succinate and, either, 10 mM ammonium or 10 mM nitrate as the sole carbon and nitrogen source. The results show that there is a minor phenotype from the Pd4449 strain in both conditions (Figure 4. 12). The fact that Pd4449 was still able to grow effectively under nitrate assimilation conditions confirmed that NasC was functional, as a *nasC* deletion results in no growth under these media conditions. Therefore, the addition of a Strep-II-tag to the C-terminus of NasC is not detrimental to its function.

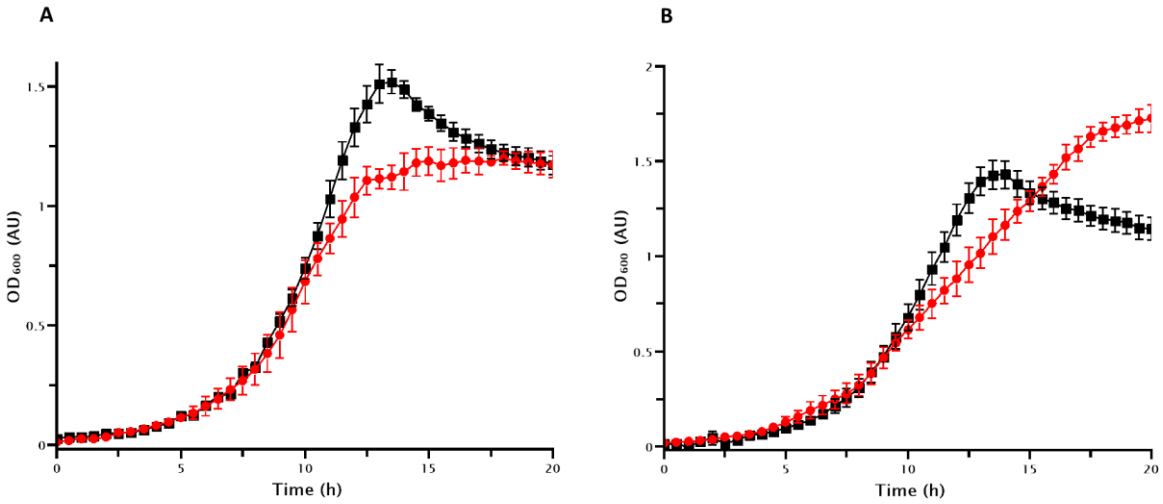


Figure 4. 12 Growth analysis of wild-type *P. denitrificans* Pd1222 (black squares) and the new strain *P. denitrificans* Pd4449 (red circles). Cells were cultured using 30 mM succinate and either 10 mM nitrate (A) or 10 mM ammonium (B).

4.2.5 Nitrate reductase activity in Pd4449

Assimilatory nitrate reductase activity can be identified in the Pd4449 strain by analysis of growth on nitrate. This, however, does not give any detail on the specific activity in these cells. To investigate this further, cells were grown in a nitrate-dependent growth condition and whole cell lysates were analysed by methyl viologen activity assays. These results confirmed that, in both the wild-type Pd1222 and the Pd4449 strain the nitrate reductase conforms to Michealis-Menten kinetics (Figure 4. 13). For both samples the calculated K_M values were within error of each other, 130 ±13 µM and 116 ±16 µM for Pd1222 and Pd4449 respectively, confirming that the enzyme being analysed was the same and not a mixture of an alternative nitrate reductase. Interestingly there was a higher V_{max} in the Pd4449 strain compared to the Pd1222 wild-type strain, 1.80 ±0.076 µmol.min^{-1}.mg prot.$^{-1}$ and 0.89 ±0.027 µmol.min^{-1}.mg prot.$^{-1}$ respectively. These experiments were conducted using whole cell lysates and therefore it cannot be stated with certainty that there were equal concentrations of nitrate reductase present in each sample, however, this result suggests that the addition of the affinity tag has interrupted some part of the normal function of the enzyme. It is possible that this is why there is a small growth phenotype compared to wild-type cells.

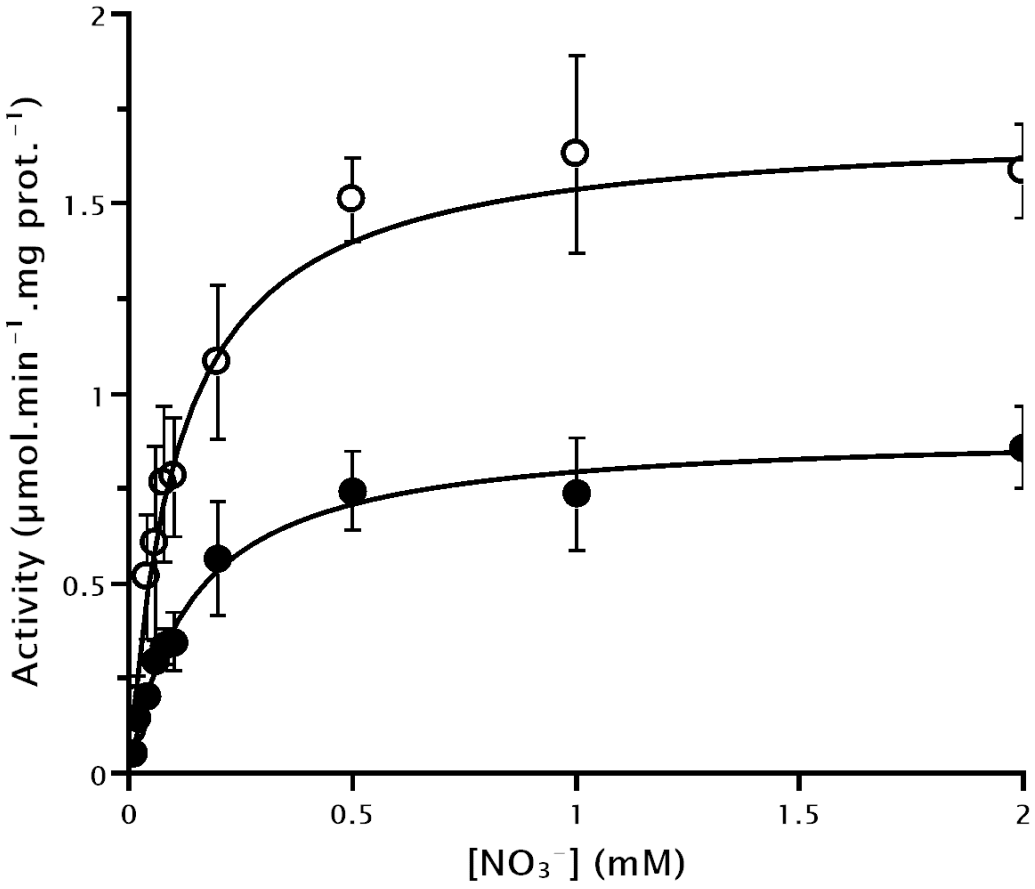


Figure 4. 13 Methyl viologen dependent nitrate reductase activity with changing nitrate concentration displaying Michaelis-Menten kinetic parameters. Samples were whole cell lysates from either Pd1222 (filled symbols) and Pd4449 (open symbols). Error bars are standard deviation from three biological replicates. Values of K_M and V_{max} were calculated as 130 ±13 µM, 0.89 ±0.027 µmol.min^{-1}.mg prot.$^{-1}$ and 116 ±16 µM, 1.80 ±0.076 µmol.min^{-1}.mg prot.$^{-1}$ for Pd1222 and Pd4449 respectively.

Methyl viologen dependent activity assays confirm the presence of active enzyme in the Pd4449 strain. To further investigate the difference between the mutant and the wild-type strains the physiological electron donor NADH was used in similar assays. In this experiment the K_M values obtained of 68 ±12 µM and 57 ±12 µM for Pd1222 and Pd4449 respectively were within error of each other. There was however a significant difference in the V_{max} values obtained. This result was seen in the methyl viologen based assays however the reverse was seen with NADH activity. The V_{max} values were 190 ± 13 nmol.min^{-1}.mg prot.$^{-1}$ and 129 ± 9 nmol.min^{-1}.mg prot.$^{-1}$ for Pd1222 and Pd4449

respectively (Figure 4. 14). The addition of the affinity tag sequence to NasC could interfere with the ability of NasC to interact with its respective partner proteins, most significantly the source of electrons NasB. This disturbance may allow for greater access to NasC from methyl viologen, which donates electrons directly. Conversely it may reduce the ability of NasB to pass electrons to NasC and as such the highest activity would be lowered.

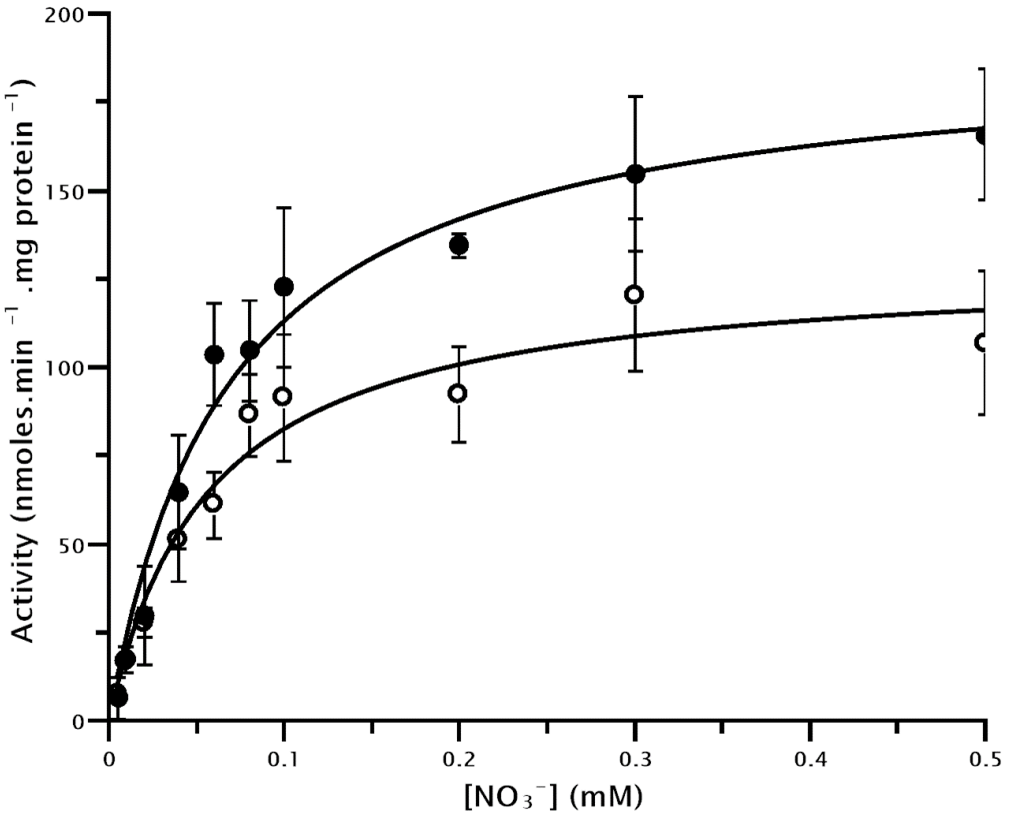


Figure 4. 14 NADH dependent nitrate reductase activity with changing nitrate concentration displaying Michaelis-Menten kinetic parameters. Samples were whole cell lysates from either Pd1222 (filled symbols) and Pd4449 (open symbols). Error bars are standard deviation from three biological replicates. Values of K_M and V_{max} were calculated as 68 ±12 µM, 190 ± 13 nmol.min^{-1}.mg prot.$^{-1}$ and 57 ±12 µM, 129 ± 9 nmol.min^{-1}.mg prot.$^{-1}$ for Pd1222 and Pd4449 respectively.

This section confirms that the assimilatory nitrate reduction apparatus is functional in the new strain Pd4449. It confirms that the protein is not significantly inhibited in nitrate reduction, shown by the consistent K_M values calculated for both strains. As there was a

strain encoding a functional NasC with an added Strep-II tag it a variety of protein purification experiments were attempted.

4.2.6 Purification of NasC

4.2.6.1 Attempted purification from a wild-type background

In the following experiments attempts were made to isolate NasC by affinity chromatography from the Pd4449 strain. It was important to first attempt the same strategy of purification using the wild-type strain. This was done as a control experiment to determine whether a nitrate reductase could bind to the column used. This was important as there are multiple different nitrate reductases in *P. denitrificans* of similar sizes and ensuring only NasC activity was analysed was important.

Cells were cultured in minimal salts media containing 30 mM succinate and 10 mM nitrate under aerobic conditions. This was to encourage maximum expression of *nasC* and to try to prevent the expression of the other two nitrate reductase genes. Harvested cells were lysed by a French Pressure Cell as outlined in the methods section and the buffer system used for all purifications was 50 mM Tris, 5 mM EDTA and 5 mM ascorbate to provide a reducing environment in the buffer system to maintain stability of iron-sulfur containing proteins. Insoluble material was removed by ultracentrifugation and the soluble lysate was applied to a StrepTactin-XT matrix. The column was washed in buffer for 10 column volumes and protein was eluted by the addition of 20 mM biotin. Eluted protein was concentrated to 1 mL final volume at a protein concentration of 0.32 mg/mL. A total of 10 µg of protein was analysed by SDS-PAGE to visualise proteins isolated and the remainder was used to analyse the nitrate reductase activity by a methyl viologen dependent assay.

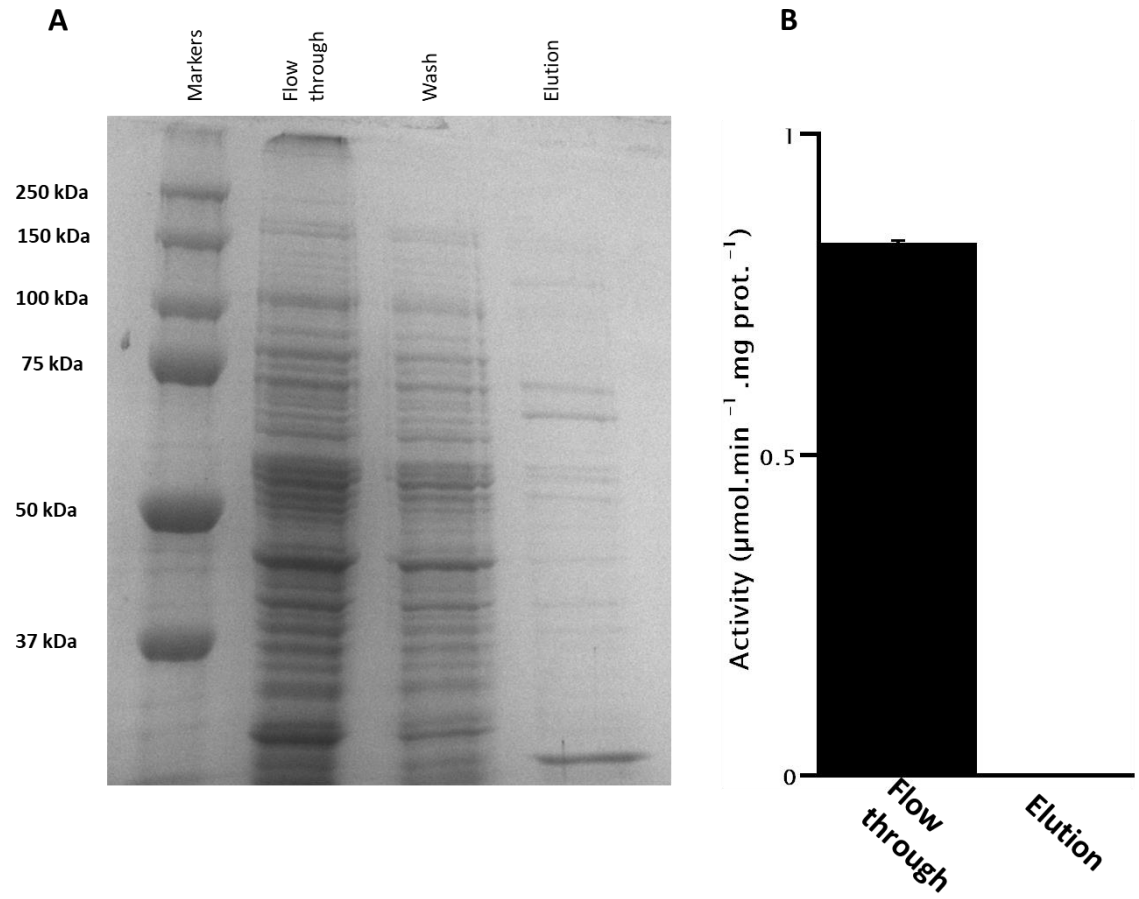


Figure 4. 15 SDS-PAGE analysis of protein isolated from wild-type Pd1222 cells grown on nitrate as the sole nitrogen source (A), and the nitrate reductase activity from methyl viologen dependent assays using isolated proteins from the elution and the protein that did not bind to the column (B).

There was no obvious band at the correct size for NasC (~92 kDa) (Figure 4. 15 A). There was no nitrate reductase activity found in the elution fraction and the activity present in the flow through is comparable to whole cell lysate data presented for wild-type cells previously in this chapter (Figure 4. 15 B). This experiment therefore confirms that a nitrate reductase from Pd1222 cells grown on nitrate as the sole nitrogen source is not purified by the StrepTactin-XT affinity chromatography method.

4.2.6.2 Affinity purification of NasC

As the previous section has shown it was not possible to recover an active nitrate reductase from Pd1222 cells. The logical next step was, therefore, to culture Pd4449 with nitrate as the sole nitrogen source and repeat the previous attempted purification. The SDS-PAGE analysis of this purification contrasts to the analysis from Pd1222 cells (Figure 4. 16). A clear band can be seen in the correct size region between 75 and 100 kDa. This appears to be the most enriched protein despite obvious contamination present on the gel. Furthermore, the eluted protein was extremely active in methyl viologen dependent nitrate reductase activity assays.

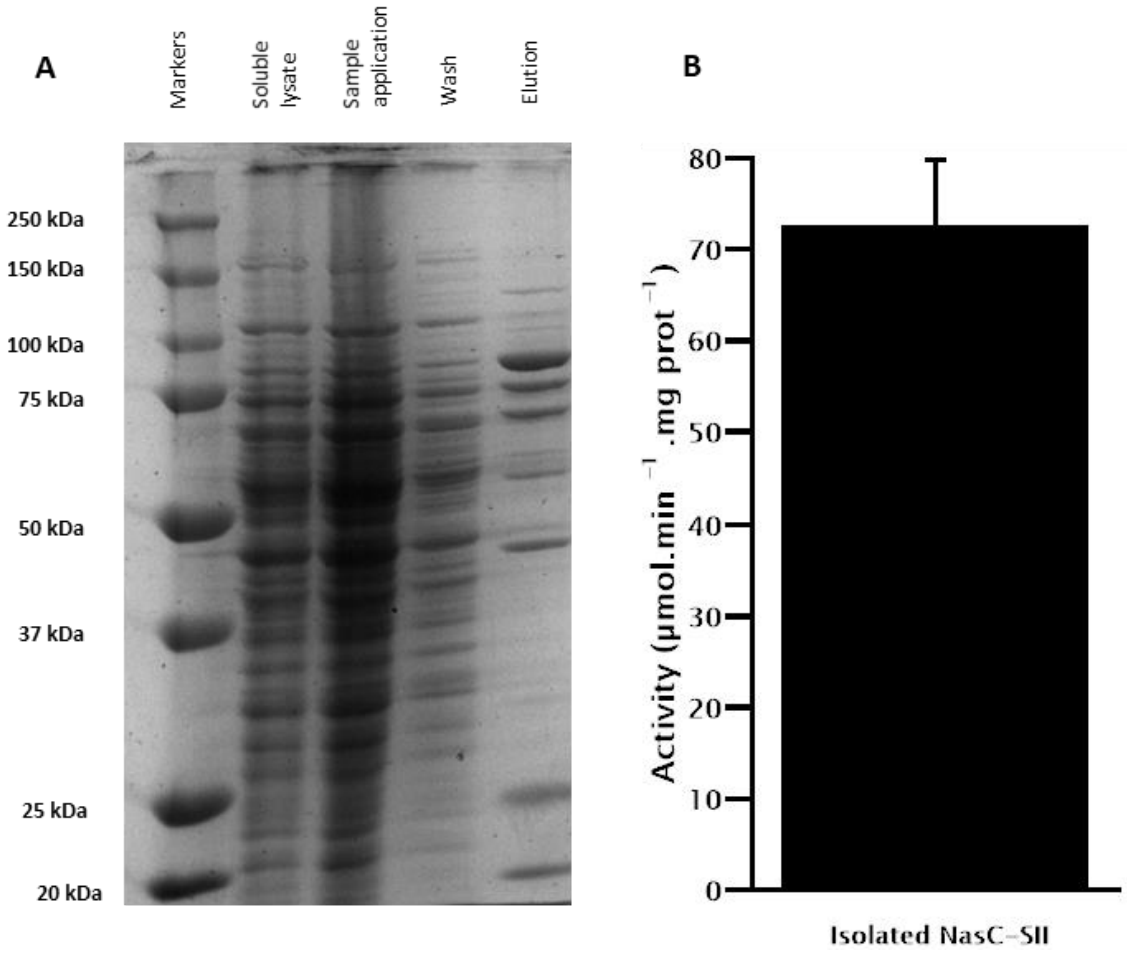


Figure 4. 16 SDS-PAGE analysis of protein isolated from Pd4449 cells grown on nitrate as the sole nitrogen source (A) and the associated nitrate reductase activity from isolated protein (B). Typical yields were 2-3 mg of eluted protein from 12 L of bacterial growth.

Whilst this protocol resulted in a reasonable yield of assimilatory nitrate reductase, and with activity being maintained, there is significant contamination present on the gel. Therefore, the following sections will detail attempts made to further purify the protein.

4.2.6.3 Anion exchange chromatography

The first attempt to further purify NasC used anion exchange chromatography. Eluted protein was diluted into a low salt buffer and applied to a DEAE FF column (*GE Healthcare*). Protein was eluted by a linear gradient of increasing NaCl. SDS-PAGE analysis demonstrates how NasC was purified away from the contaminants seen in the previous section however the protein obtained was inactive in reductase assays (Figure 4. 17). This was most likely due to the long time the protein spent bound to the column in an aerobic environment potentially leading to degradation. Due to the loss of activity anion exchange chromatography was judged ineffective for further purification of NasC.

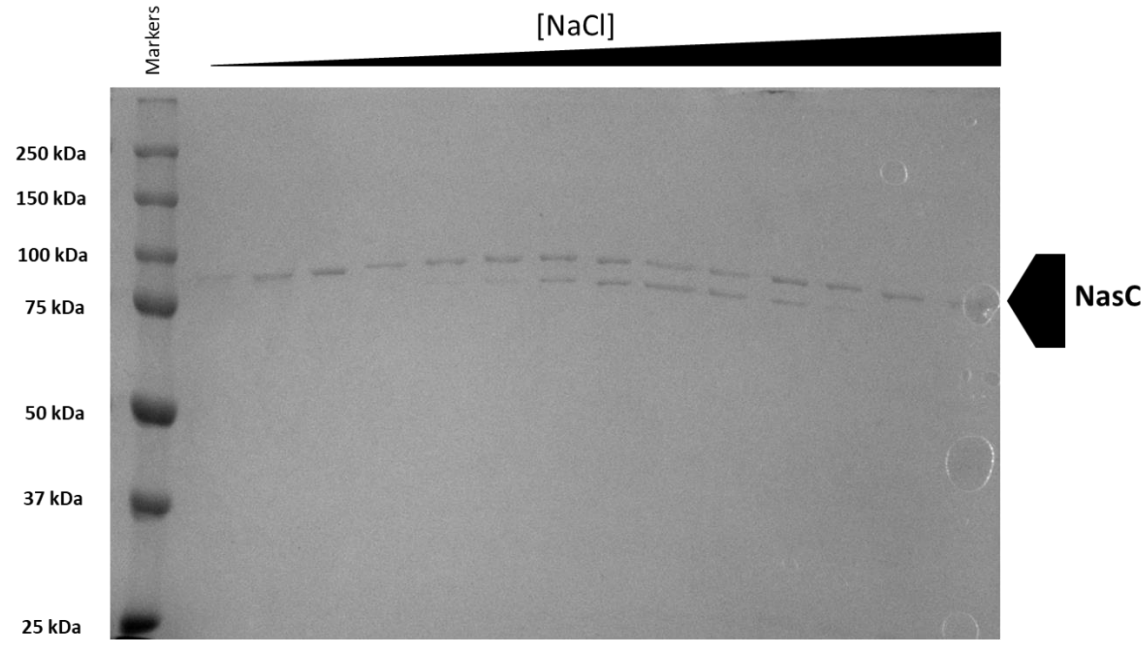


Figure 4. 17 SDS-PAGE analysis of anion exchange chromatography of a NasC sample prepared from affinity chromatography.

4.2.6.4 Size exclusion chromatography

The alternative approach of size exclusion chromatography was used next to try to maintain active NasC while removing the contaminating proteins. To minimise the time proteins took to elute from the column an analytical size exclusion column was selected. The total sample was concentrated to a volume of 100 µL and applied directly to the column. Elution used the same TEA buffer used in affinity purifications. This method resulted in significant loss of protein there was still evident contamination in the fraction containing NasC (Figure 4. 18). There was also no activity detected in the sample containing NasC and this method was judged ineffective in purifying the protein.

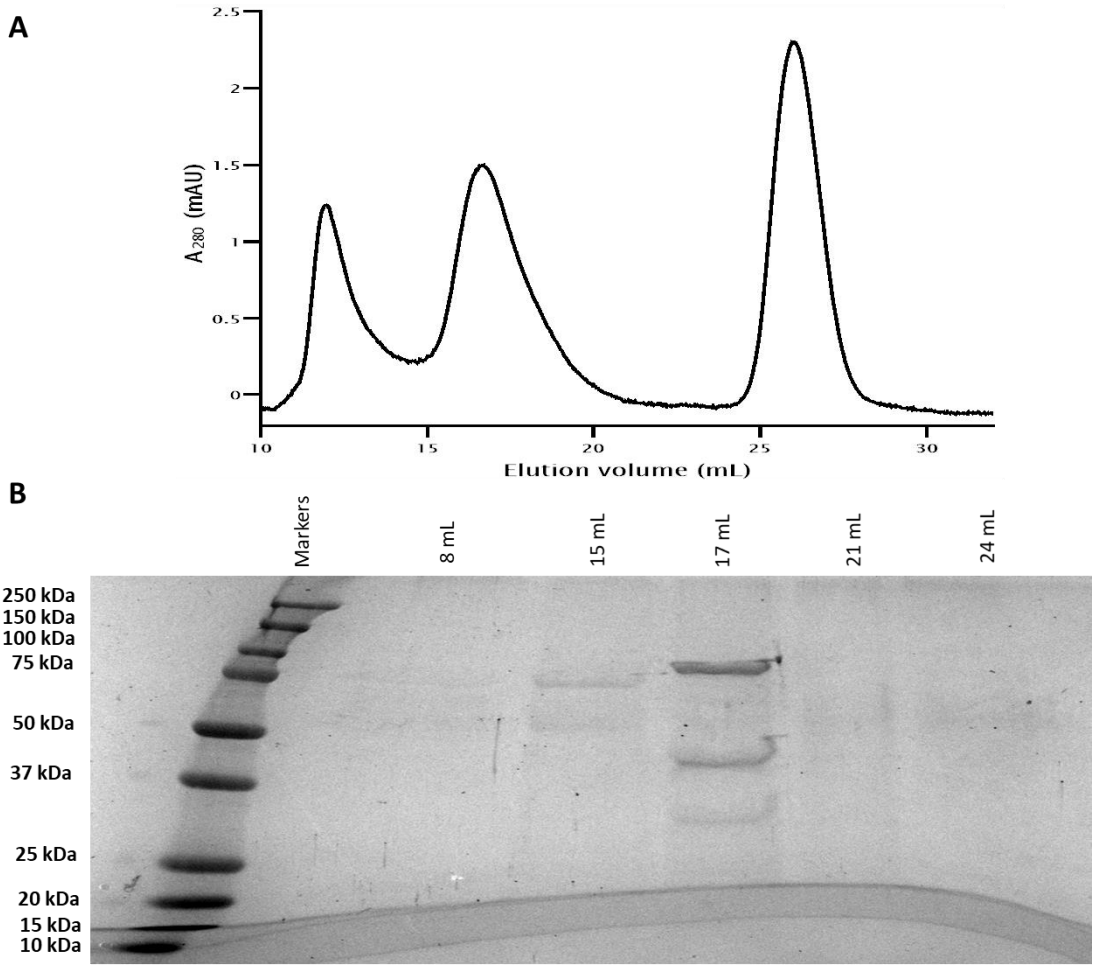


Figure 4. 18 Chromatogram showing the absorbance at 280 nm over the elution profile for the size exclusion run (A) and the subsequent SDS-PAGE analysis performed highlighting particular fractions that cover the three peaks observed.

4.2.7 Kinetics of the enriched NasC protein

As it proved challenging to fully purify active NasC, activity assays were completed on the available protein. This constituted an enrichment of protein allowing for measurement of key kinetic parameters. The protein sample was taken immediately from affinity purifications and used in methyl viologen activity assays to investigate the affinity for nitrate and the maximum rate of reaction. Methyl viologen can act as an artificial electron donor to a wide variety of different redox enzymes and as such can be used to provide electrons to NasC for the reduction of nitrate. In this context methyl viologen dependent assays were used as NasC has been previously reported to lack a functional domain for oxidation of NADH. Separation from the partner protein NasB appears to prevent NasC from functioning (Gates *et al.*, 2011).

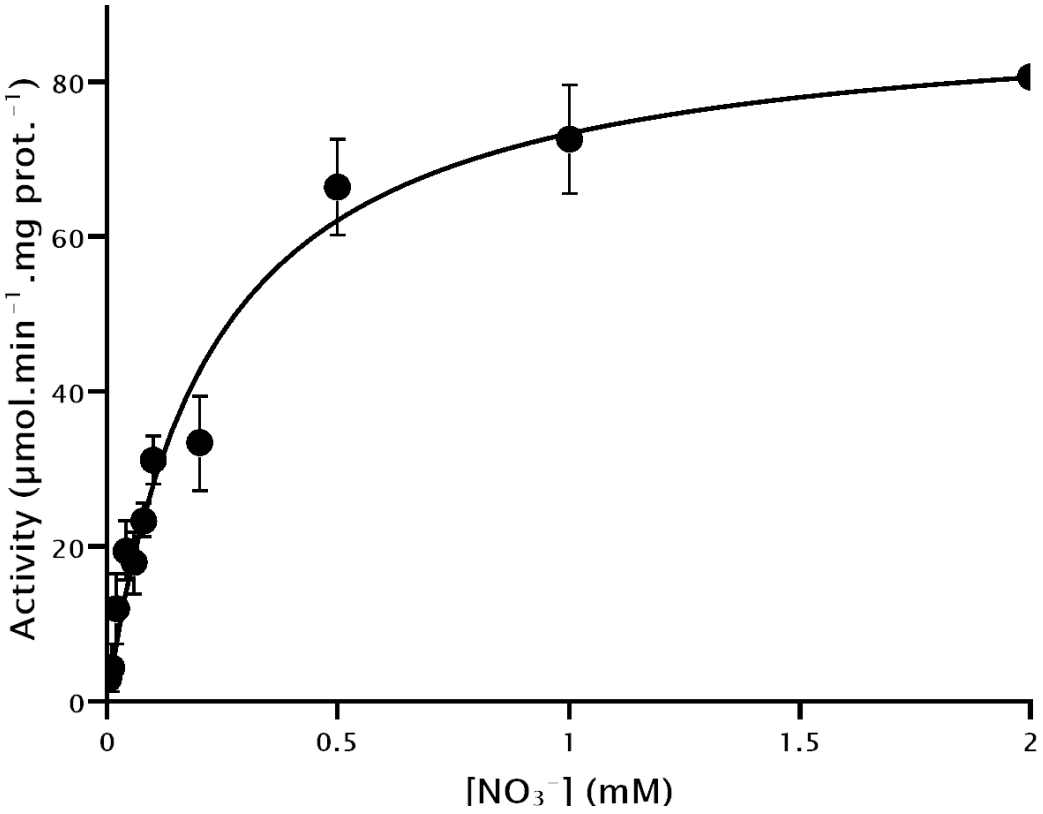


Figure 4. 19 Methyl viologen dependent nitrate reduction activity of enriched NasC with changing nitrate concentration. Data are the average of 3 biological replicates with error bars representing the standard deviation. The kinetic fit was generated by a non-linear regression analysis according to the Michaelis-Menten expression. Values for K_M and V_{max} were calculated as 220 ±34 µM and 89 ±4.6 µmol.min^{-1}.mg prot^{-1} respectively.

Initial observations show a marked increase in the observed nitrate reductase activity of enriched protein compared to the whole cell lysate data presented earlier in this chapter. This is due to the increased purity of the sample, with the removal of the majority of contaminant proteins the activity per mg of protein is logically increased. The next key feature is the calculated K_M. This constant is a measure of the affinity of the protein for its substrate. A low K_M is indicative of a high affinity for the substrate and our value of 220 ±34 μM is similar to observations of other similar nitrate reductases (Gates, Richardson and Butt, 2008). This value for K_M may appear higher than expected, *P. denitrificans* can consume nitrate at levels undetectable in the media, however it is consistent with previous studies using methyl viologen as the electron donor. In one such study the K_M calculated using an alternative approach gave a value of 45 μM, while in the same study the methyl viologen dependent assay gave a K_M of 168 μM. This highlights the overestimation made when using methyl viologen as the electron donor (Gates, Richardson and Butt, 2008). Whilst it is not ideal to use an electron donor that may overestimate these values it was necessary due to the previously determined inability of isolated NasC to use physiological electron donors.

The maximum velocity of reaction (V_{max}) is an important measure of the efficacy of an enzyme to turnover its substrate. A high V_{max} indicates an enzyme is highly capable of turning over the substrate and driving a fast reaction. An enzyme that can turnover a lot of substrate may be vital to cellular growth to have a high maximum rate to generate maximum reduced nitrogen for assimilation. Previous studies regarding NasC from *P. denitrificans* have shown maximum reaction rates in the order of nmol.min^{-1}.mg prot^{-1}. In this work, when the protein is enriched to this extent, the maximum velocity of the reaction is extremely high, an order of magnitude above previous values (Gates *et al.*, 2011). This is likely due to other proteins being in the sample reducing the specific activity values observed.

This work has identified reliable values for maximum velocity of the nitrate to nitrite reaction of the assimilatory nitrate reduction system in *P. denitrificans* and has shown the affinity of this protein for nitrate. This confirms that the nitrate reductase of *P. denitrificans* is highly adapted with a high affinity for nitrate and a high velocity. This combination of traits fits with the high growth rate of the bacteria when grown using

nitrate as the sole nitrogen source. It also explains how nitrate can be so effectively assimilated and removed rapidly from the culture media in the continuous culture experiments in this thesis (3.2).

4.2.8 Purification from NasC overexpression

In the previous section an expected methyl viologen dependent nitrate reductase activity profile for NasC was established. This allows for comparisons between purifications of NasC from overexpression conditions to investigate the reason low levels of activity were observed in the expression trials. In these experiments two nitrate reductase knockout backgrounds were used. The first was the Δ*narG* strain and the second was the Δ*nasC:narG* strain. These strains were selected as they do not encode the respiratory nitrate reductase. There is a single shared chaperone between the respiratory and assimilatory nitrate reductases in *P. denitrificans*, however other similar proteins are not known. Therefore, the use of alternative chaperones, that provide co-factor to the recombinant NasC, can be limited in strains deficient in the other reductases.

4.2.8.1 Overexpression in Δ*narG*

In the first experiment the Δ*narG* was used. Cells were cultured in minimal media containing 30 mM succinate, 5 mM nitrate and 5 mM nitrite until and OD_{600} of ~0.5. Expression was induced by addition of 5 mM taurine as this gave the most positive expression result in the previous expression trials. The cultures were then incubated at 30°C overnight to allow expression of NasC. Cells were harvested by centrifugation and the affinity purification protocol used for the recovery of NasC from Pd4449 was carried out.

The protein yield from this experiment was lower than from the Pd4449 strain. This is consistent with the lower activity observed in the overexpression trials conducted

previously. In total, the yield was typically 0.8 mg/mL in a volume of approximately 1.5 mL from 10 L of cultured bacteria. This was significantly less than the 2-3 mg recovered from Pd4449 and suggests that the overexpression is lower than the native expression of NasC. The eluted protein was not completely pure and there were other contaminating proteins in the sample (Figure 4. 20).

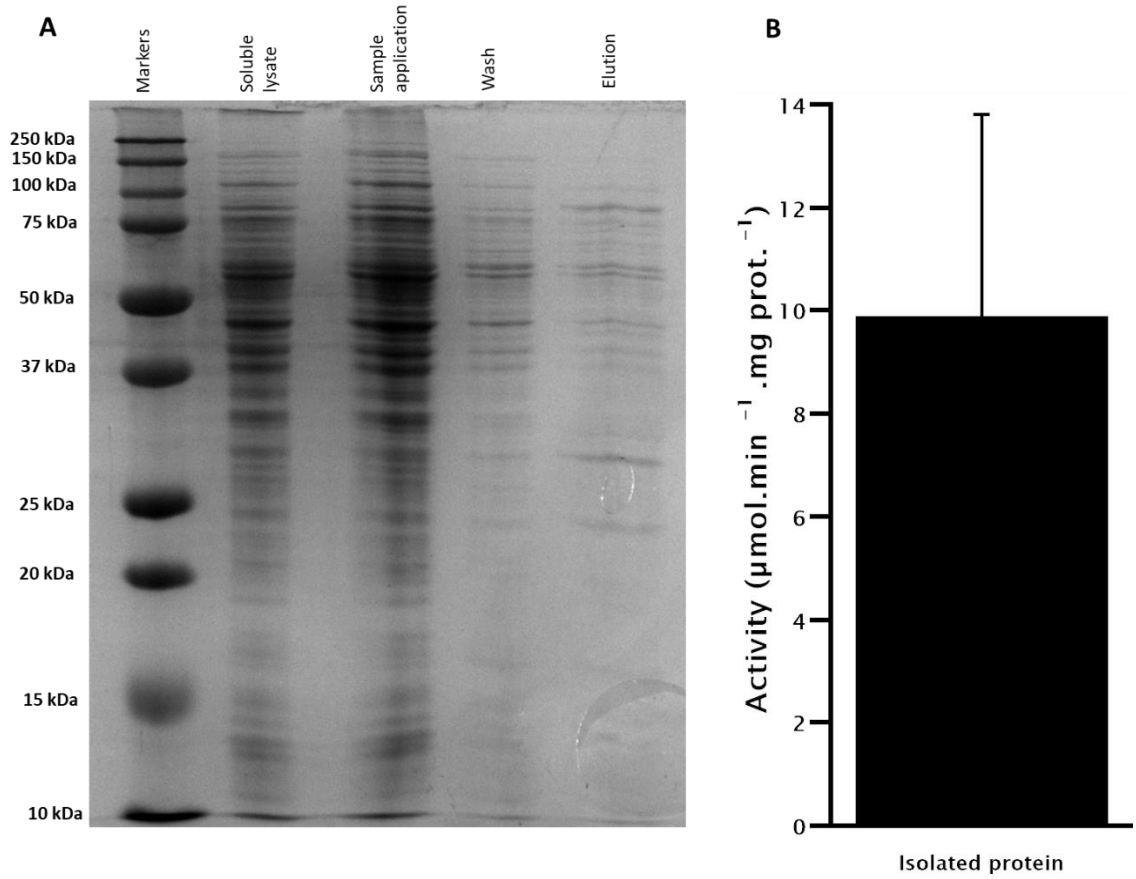


Figure 4. 20 SDS-PAGE analysis of protein prepared from the Δ*narG* strain (A) and the subsequent methyl viologen nitrate reductase activity of that eluted protein using 1 mM nitrate (B).

The key result from this experiment was the activity measured from the eluted protein. This confirmed that NasC had been isolated, however it shows that it is significantly less active than the protein isolated from Pd4449. The activity of ~10 µmol.min^{-1}.mg prot^{-1} is approximately 7 times lower than that measured from Pd4449. This could be due to inefficient loading of recombinant NasC with relevant co-factor. Using the Δ*narG* background did mean there was still active NasC being produced from the genome and as such it would require appropriate maturation. It is therefore plausible that a NasC specific maturation protein is required and as a result this recombinant version was unable to function optimally.

4.2.8.2 Overexpression in Δ*nasC:narG*

Cells were cultured as previously described using 30 mM succinate, 5 mM nitrate and 5 mM nitrite. This was more important when using this background as there was no native assimilatory nitrate reductase available. Cells were grown to an OD_{600} of ~0.5 and expression was induced by the addition of 5 mM taurine. The taurine concentration was chosen as it was the only condition that gave a nitrate reductase activity in the overexpression trials. Expression took place overnight at 30°C after which cells were harvested by centrifugation. Cells were lysed by French pressure cell and the purification was carried out as previously detailed.

Total protein yield was very low from this overexpression resulting in 0.6 mg from 10 L of bacterial growth. This seems to confirm that overexpressed NasC is lower than native expression. There were a lot of contaminating bands present in the SDS-PAGE analysis (Figure 4. 21). This is likely due to the low concentration of NasC present, leaving space for the contaminants to effectively bind to the column. When the activity of the sample is analysed there is a lower activity than the isolated protein from Pd4449. This is in agreement with the purification from the Δ*narG* background. It appears that there is another important factor present in the production of active NasC in addition to NarJ.

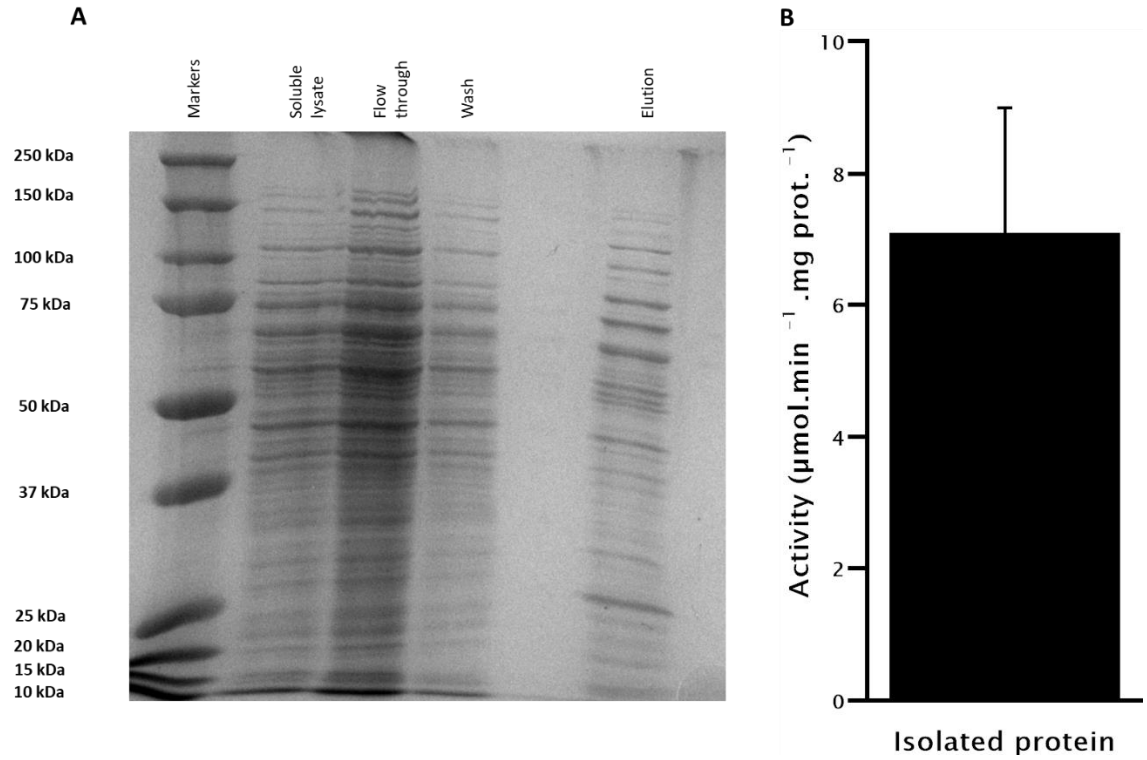


Figure 4. 21 SDS-PAGE analysis of protein prepared from the Δ*nasC:narG* strain (A) and the subsequent methyl viologen nitrate reductase activity of that eluted protein using 1 mM nitrate (B).

4.3 Discussion

4.3.1 Overexpression of NasC cannot be achieved in the current vector system

In this work attempts were initially made to develop an overexpression system for the rapid production of NasC. To achieve this, a construct encoding equal expression of the chaperone NarJ alongside a tagged variant of NasC was designed and constructed. In theory this would allow for production of a NasC protein that was fully loaded with co-factor. In fact, the data in section 4.2.3 shows that overexpression did not produce more nitrate reductase activity than was expected from the native background. Furthermore, there was no clear band present in SDS-PAGE analysis that would relate to NasC. It was not possible to detect the Strep-II-tag by western blot analysis, this result is likely due to an effect of NasC obscuring the recognition site as isolated NasC with a Strep-II-tag did not produce a signal either.

In the experiments shown in section 4.2.3 the original idea was a reduction in the potential nitrate reductases present would free up chaperones that may be dual functional between the different systems. This expected result was not observed in any condition. In a background lacking NasC and NarG the highest measured nitrate reductase activity was not sufficient to restore activity to a wild-type, non-induced level. Furthermore, this work revealed a flaw in the overexpression system used in *P. denitrificans*. Conventionally pLMB509 (10 or 11 also) are used for overexpression systems. These vectors require taurine to promote expression of the cloned genes. This study shows that taurine is shutting down the native nitrate assimilation system as the nitrate reductase activity measured declines significantly when concentrations above 5 mM are used. This indicates it may be a preferential nitrogen source to nitrate.

In previous studies using *P. denitrificans* the metabolic pathway for the use of taurine has been proposed and studied (Brüggemann *et al.*, 2004). Furthermore, the precursor for

taurine, hypotaurine, has been shown to be broken down to release its nitrogen as ammonium by *P. denitrificans* (Felux *et al.*, 2013). This means high taurine concentrations used to overexpress proteins in *P. denitrificans* may be contributing to a down-regulation of genes involved in nitrate assimilation. This result explains the lack of success of overexpression of NasC in *P. denitrificans* and confirms that the addition of a Strep-II-tag sequence to the genome was the correct approach for isolation of active NasC.

4.3.2 Multiple maturation proteins are required for NasC expression

Whilst NarJ was originally selected as the most important chaperone for maturation of NasC, the overexpression data presented here indicates that it is not the only one required. Despite *narJ* being expressed at the same level as *nasC*, the data shows the activity of the isolated NasC is lower than natively produced NasC. In section 4.2.8 the isolated proteins from this overexpressed *nasC* had approximately 10% of the activity of the native protein. As the units of activity are per mg protein then these are specific activities quoted in this chapter. Whilst there was evident contamination in both overexpression purifications trialled it was largely comparable to the native purification gels. This means the drop in specific activity cannot simply be explained by a reduction in relative amount of NasC in the sample. This leaves the conclusion that the NasC isolated from overexpression was impaired in nitrate reduction. This may well be a result of inefficient co-factor loading and as such the conclusion can be drawn that NarJ is not sufficient to produce correctly folded and loaded NasC.

NarJ has been shown to directly interact with both NasC and NarG in *P. denitrificans*. Furthermore, the mutant lacking *narJ* has been shown to grow in the same way wild-type cells do when limited in molybdenum. This makes the clear link between NarJ and a proposed molybdenum chaperone system (Pinchbeck *et al.*, 2019). In *E. coli* NarJ has been proposed to hold the conformation of NarG in a state receptive to insertion of the molybdenum co-factor (Vergnes *et al.*, 2006). Vergnes and colleagues did suggest that NarJ in *E. coli* was not involved in the insertion of the molybdenum co-factor directly,

instead that it held the apo-enzyme in a state receptive to insertion from the biosynthetic machinery. This suggests that inclusion of NarJ in this construct could aid in the incorporation of molybdenum co-factor but as it is not capable of delivering this itself other proteins are required.

Whilst the biosynthetic pathway for the production of molybdenum co-factor in *E. coli* has been well studied and reviewed the same cannot be said for *P. denitrificans*. Production of co-factor has been broken down into a series of critical steps starting with the production of cyclic pyranopterin, the conversion of this to molybdopterin, the insertion of the molybdenum and then finally the reaction to link the nucleotide. This requires multiple biosynthetic gene clusters and a specific molybdate uptake protein in *E. coli* (Iobbi-Nivol and Leimkühler, 2013). In *P. denitrificans* it is unclear which step, if any, is limiting this co-factor production. In future this process should be further understood and used to generate better overexpression constructs with upregulated molybdenum co-factor production.

4.3.3 The kinetics of nitrate reduction by NasC

The first experiments analysing the kinetics of nitrate reduction focussed on whole cell lysate samples. In these it was impossible to characterise the concentration of NasC in each lysate and as a result the activities are not specific. Comparisons of nitrate reduction in lysates from Pd1222 and Pd4449 were used to determine any effect of the addition of the affinity tag. The results indicate a difference in the maximum rate of reaction between the two strains. In the methyl viologen studies, presented in Figure 4. 13, there was an approximate doubling in the reaction rate at substrate saturation by lysate from Pd449 compared to that from Pd1222. This was initially dismissed as the rates were not specific and consequently a greater concentration of NasC in the Pd4449 sample could not be discounted. The K_M calculated for each sample was within error and as such the same protein in each experiment was being analysed.

When the NADH-dependent nitrate reductase activity of the same cell lysates discussed was tested the converse relationship was observed with respect to maximal reaction rate. In this experiment the Pd4449 lysate showed reduced maximum rate compared to that from Pd1222. This result could be explained by the same logic used to dismiss the previous relationship, however, it did introduce an interesting hypothesis. As discussed previously in this thesis, NasC is unable to oxidise NADH to gain electrons for its function and it requires the partner protein NasB to do this (Gates *et al.*, 2011). Therefore, the addition of the affinity tag to NasC could disrupt the association with NasB and result in two different assay results. In the first, methyl viologen dependent assay, the disruption in association provides greater access to NasC for the methyl viologen and results in a higher measured activity. In the second, NADH-dependent assay, the disruption of association between NasC and NasB results in a reduced rate of electron transfer and consequently lowers the measured activity. In this case the affinity for nitrate is not changed and no significant difference between the measured K_M is seen. Irrespective of these proposed differences the ability of the strain to grow and the important kinetic parameter K_M being unaffected confirmed that a native NasC was being analysed, largely unaffected by the addition of the tag.

When attempts were made to isolate NasC by various forms of protein chromatography it was not possible to remove the contaminating proteins from NasC, while maintaining nitrate reductase activity. Consequently, the kinetics were analysed in an 'enriched' sample. An expected rise in the activity was observed, largely due to the removal of the non-specific proteins, and the K_M was similar to that measured in lysates. The artificial electron donor methyl viologen was used to analyse the activity of NasC in this case as a complete complex of NasBGC was not observed by SDS-PAGE and as such it was concluded this would be the most accurate way of gaining kinetic insights. Interestingly the activity of NasC measured in this sample was comparable to a range of different nitrate reductases. In some of the first measurements of the *E. coli* NarGH complex the maximum reaction rate was quoted as 75 $\mu mol.min^{-1}.mg\ prot^{-1}$ which is very similar to the calculated value of 89 ±4.6 $\mu mol.min^{-1}.mg\ prot^{-1}$ for *P. denitrificans* (Enoch, H.G. and Lester, 1975). Furthermore, the evolutionarily related nitrate reductase of *P. pantotrophus* was isolated and analysed using similar methods and was found to have highly comparable activity as well as a similarly high K_M (Gates, Richardson and Butt, 2008).

The kinetic data observed for enriched samples of NasC shows that this protein is a highly active and efficient nitrate reductase. The maximum activity puts in a field of respiratory enzymes that have a high turnover rate. It is shown to be distinct from the lower activity measured from the archaeal protein from *Haloferax mediterranei* which was significantly lower (Martinez-Espinosa, Marhuenda-Egea and Bonete, 2001). This is unsurprising as the two organisms occupy very different ecological niches. Whilst the K_M values calculated from methyl viologen dependent assays may be overestimates they do indicate a high affinity for the substrate in the micromolar range. This would be more than sufficient to maintain growth and competition, particularly in agricultural soil environments which typically have high nitrate concentrations (Milham *et al.*, 1970; Willems *et al.*, 1997).

Chapter 5: Investigating the protein-protein interactions between Nas proteins

5.1 Introduction

5.1.1 The proteins involved in nitrate assimilation systems

In *P. denitrificans* the nitrate assimilation system comprises of the nitrate/nitrite transporters (NasA and NasH), the two-component regulatory complex (NasT-NasS), the functional nitrate reductase (NasC), Rieske-type protein (NasG) and the nitrite reductase (NasB). This is a typical core set of genes found across a variety of heterotrophic bacteria. The transport of nitrate or nitrite into the cell is the first stage of nitrate assimilation. Following uptake, the nitrate is reduced to nitrite by the nitrate reductase, the subsequent nitrite is then further reduced to ammonium by the nitrite reductase. This process is energetically expensive as it requires eight electrons for each molecule of nitrate converted to ammonium, as a result the strict control exerted by the regulators is key. In other bacteria, notably cyanobacteria, the transport system used differs. Typically, these organisms use ABC-type transporters to take up the required nitrate or nitrite. From the uptake the differences are minimal as similar molybdoenzymes and siroheme containing reductases are required for the reduction of nitrate and nitrite respectively (Luque-Almagro *et al.*, 2011). These different protein groups must come together to facilitate the total reduction of nitrate to nitrite.

There are conserved proteins in nitrate assimilation gene clusters. Unsurprisingly, these include the nitrate and nitrite reductases, NasC and NasB respectively in *P. denitrificans*, however, there is typically another protein conserved amongst these two. This protein is called NasG in *P. denitrificans* but is also known as NirD, NasD and NasE in other

organisms. In many cases it is a Rieske-type iron-sulfur protein that is thought to facilitate electron transfer. The descriptions of this protein are varied in the literature, with NasG of *P. denitrificans* being given the status of an independent protein whereas other descriptors from other organisms describe it as a subunit of the nitrite reductase. This is indicative of the unknown nature of its role. It leads to the question of whether it is truly involved in electron transfer between Nas proteins or perhaps it is a component of the NasB nitrite reductase (Luque-Almagro *et al.*, 2011).

The proposition of a system that facilitates the total reduction of nitrate to ammonium in *P. denitrificans* is proposed to involve several protein-protein interactions. Principally the concept involves a complex between NasC, NasG and NasB (Figure 5. 1). The logic for this theory stems from the predicted co-factors associated with each of these proteins. Firstly, NasC is a molybdenum-dependent nitrate reductase, this protein is predicted to contain a molybdenum active site and a series of iron-sulfur clusters. What is notable, for a reductase, is an apparent lack of an electron ingress site. This could have been in the form of an NADH oxidising domain or an additional protein that is bound to NasC and facilitates electron transfer from another source. In both Nap and Nar systems the nitrate reductases are connected to quinol dehydrogenases that provide electrons from the membrane quinol pool. As NasC is a cytoplasmic protein, not known to be associated with a membrane, this is not the case. As a result, NasC does not have an obvious way of obtaining electrons for its function. NasB however, has both the functional reduction site with a siroheme co-factor and a flavin adenine dinucleotide (FAD) binding domain thought to allow oxidation of NADH. Therefore, it appears that NasB is the source of all electrons that flow into nitrate assimilation in *P. denitrificans.* This leaves the small Rieske-type protein NasG. With an iron-sulfur cluster bound, this protein is thought to act as an electron shuttle between NasB and NasC and completes the three protein interaction complex (Gates *et al.*, 2011).

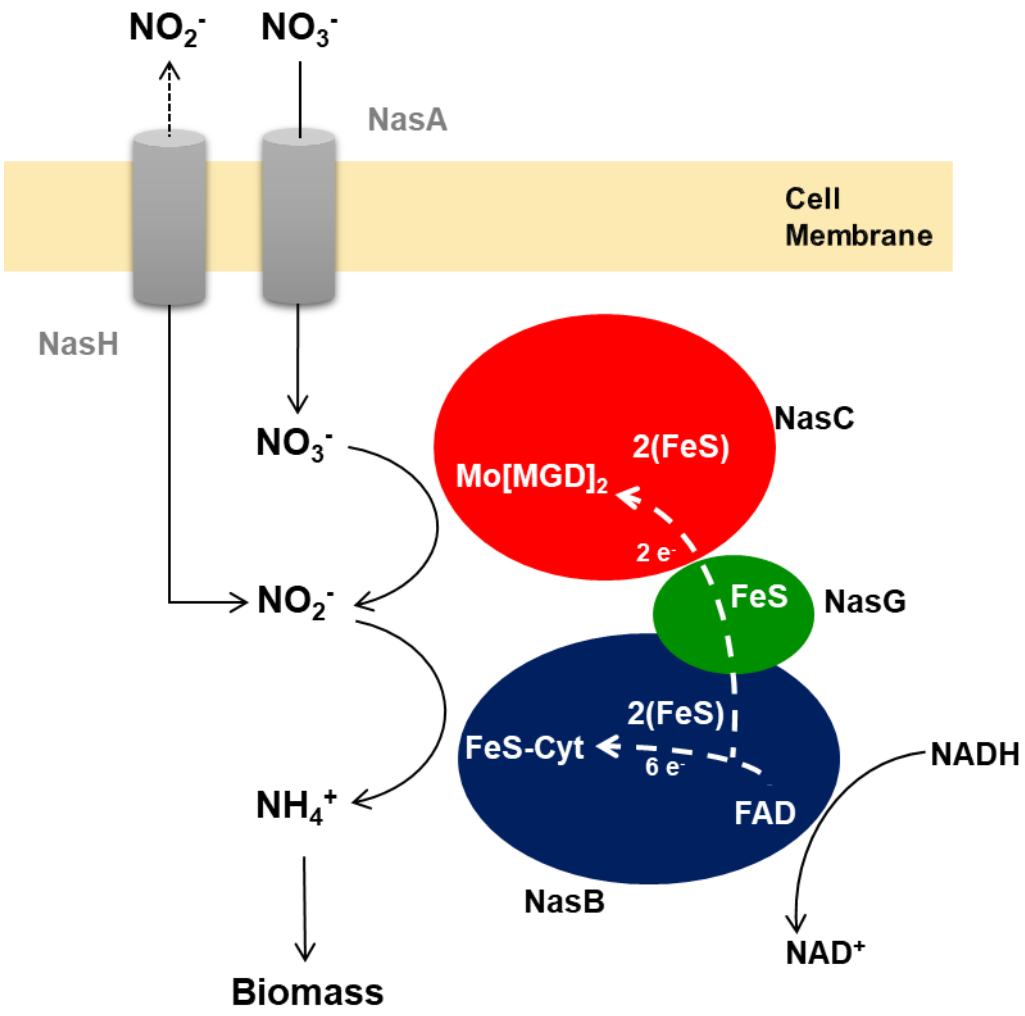


Figure 5. 1 A model representation of the proposed system for nitrate assimilation in *P. denitrificans*. Key features of the catalytic proteins are shown, and the proposed route of electron flow is denoted by a dashed line.

5.1.2 The aims of this chapter

In this chapter the aim was to further the understanding of protein-protein interactions involved in nitrate assimilation in *P. denitrificans*. The principal interest was the interactions between the catalytic subunits of NasC and NasB, with the further aim to clarify a role for NasG in facilitating electron transfer. By using a combination of Strep-II-tag variants of both NasC and NasB to attempt to isolate interacting partners using affinity chromatography and protein identification by MALDI-TOF/TOF mass spectrometry. Furthermore, the electron transfer route from NADH oxidation to nitrate

and nitrite reduction was investigated by *in vitro* assays of isolated proteins. The overarching aim of this chapter, therefore, was to further evolve the protein interaction model developed by Gates *et al.* in 2011, leading to a better understanding of the roles and requirements necessary for nitrate assimilation in *P. denitrificans* and by association the organisms that use a similar core set of genes.

5.2 Results

5.2.1 Construction of a NasB-Strep-II-tag strain

5.2.1.1 Construction of Paracoccus denitrificans Pd4452

To investigate the possibility of a NasBGC protein complex a new *Paracoccus* strain that encoded a C-terminal Strep-II-tag variant of NasB was required. Combining this with the equivalent NasC strain from Chapter 4 insights could be gained into the interactions between Nas proteins. The construction of this strain was conducted as described in section 2.11. In the first stage the plasmid pARJ002 was constructed to encode the up and downstream flanking regions either side of the Strep-II-tag sequence that was being added (Figure 5. 2).

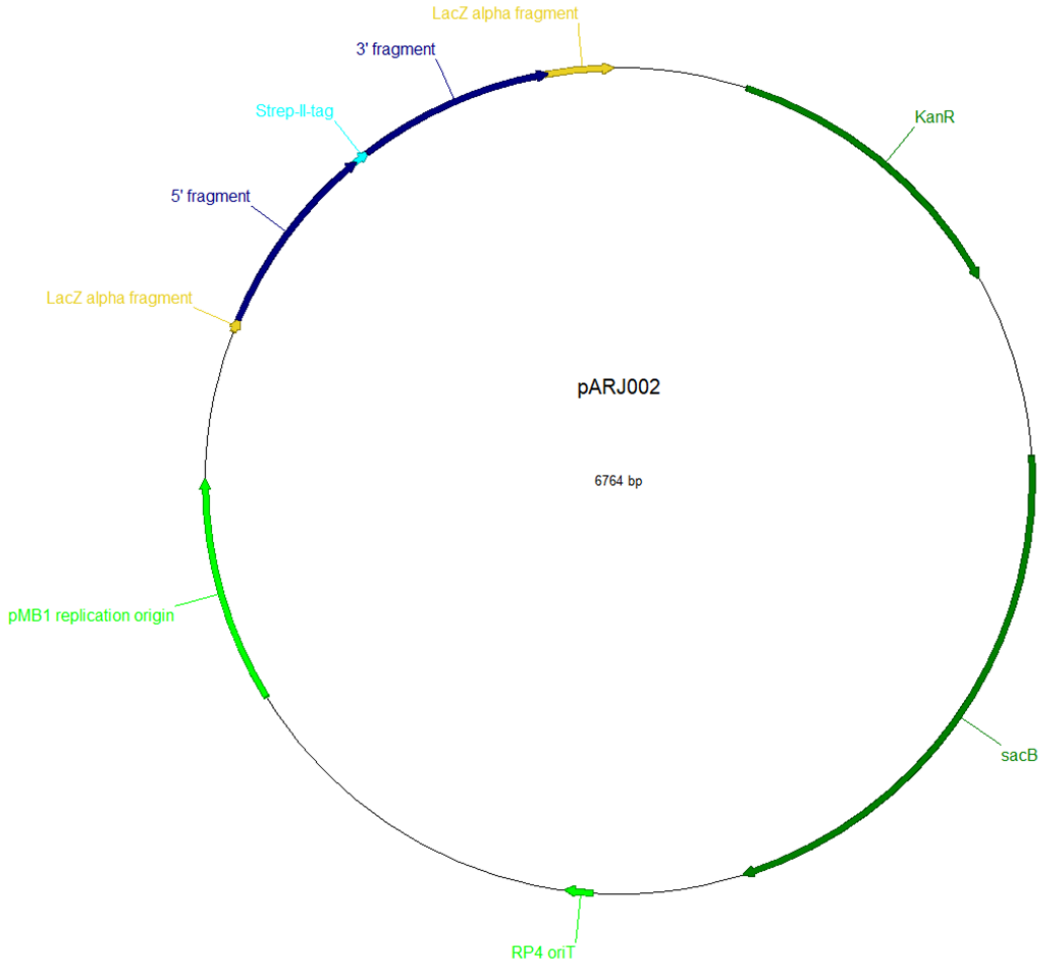


Figure 5. 2 Plasmid map of the *pK18mobsacB* derivative plasmid pARJ002 encoding the 5' and 3' fragments either side of the Strep-II-tag sequence to be added to the genome. Other highlighted genes include the kanamycin resistance gene (*kanR*) and the levan sucrase gene (*sacB*).

The first stage of the process involved integration of the plasmid into the genome of *Paracoccus denitrificans* Pd1222. Successful primary recombination events were selected for by the resistance to the antibiotic kanamycin and sensitivity to the sugar sucrose. Colonies that conformed to the expected phenotype were tested by a diagnostic PCR using the vector-borne M13 primer sites (Figure 5. 3 A). Colonies that showed the expected PCR result were then grown in LB media to facilitate secondary recombination events. Colonies were isolated and tested for the reversal of the vector derived phenotype. Colonies with the correct phenotype had genomic DNA isolated and tested with two diagnostic PCR tests. The first used the M13 primers and no product was expected, the second used primers specific to the tag sequence and the *nasB* gene (Figure 5. 3 B). In a

final test the entire gene was amplified and sequenced to ensure there were no unintended mutations. The new strain was named *Paracoccus denitrificans* Pd4452.

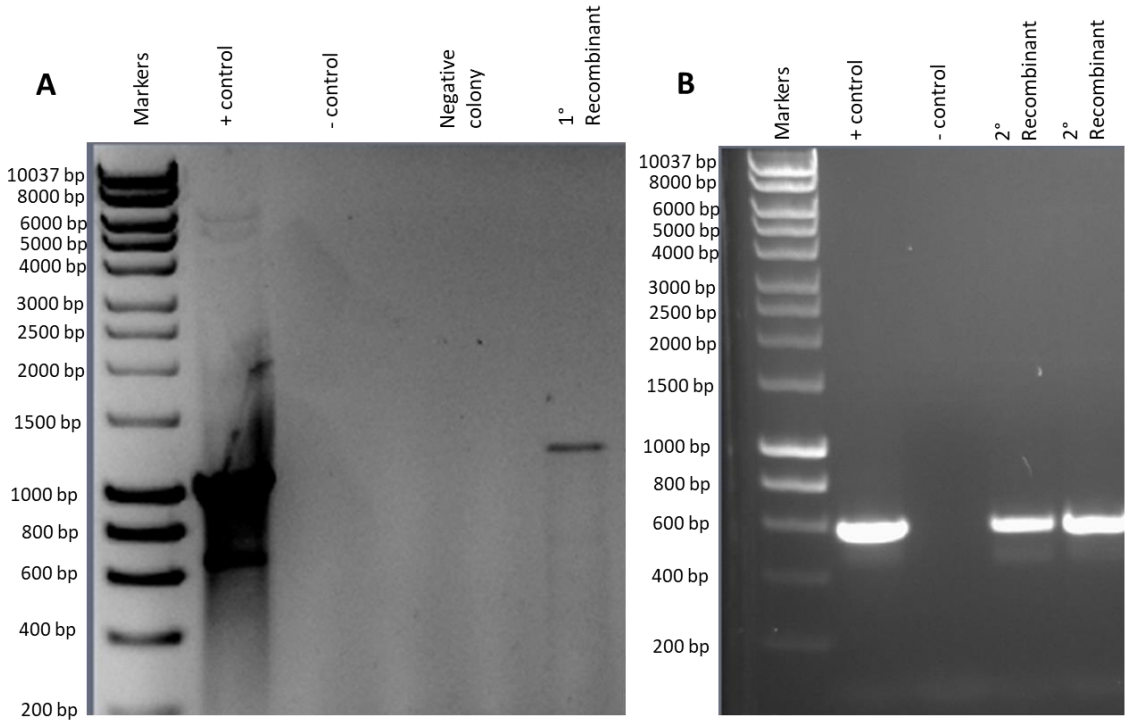


Figure 5. 3 PCR products amplified from M13 PCR (A) or Strep-II-tag specific PCR (B). Positive controls were isolated pARJ002 and negative controls were wild-type *P. denitrificans* Pd1222 genomic DNA (A&B). The expected size of the M13 PCR product was ~1 kb and the expected size of the Strep-II-tag specific PCR product was ~584 bp.

5.2.1.2 Phenotype of Pd4452

Once the new Pd4452 strain had been constructed it was important to analyse the growth phenotype under nitrate dependent and independent growth. The results show that the introduction of the tag sequence had little effect on the growth of the bacteria (Figure 5. 4). This was consistent whether the cells were dependent upon nitrate or not. This was a positive result as the previous strategy with a tagged NasC variant gave active enriched protein for kinetic analysis.

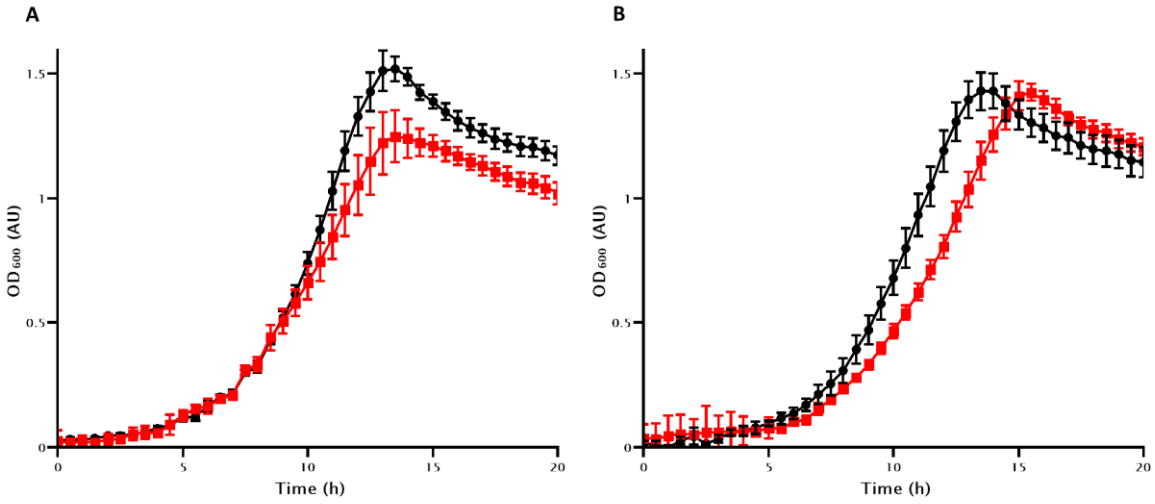


Figure 5. 4 Growth analysis of wild-type *P. denitrificans* Pd1222 (black circles) and *P. denitrificans* Pd4452 (red squares). Cells were cultured using 30 mM succinate and either 10 mM nitrate (A) or 10 mM ammonium (B).

5.2.2 Isolation of NasB and interacting partners

Next attempts were made to determine the nitrite reductase activity of soluble cell lysates using methyl viologen as the electron donor. Whilst this was possible for the wild-type cells it proved impossible with Pd4452. Attempts were made to lyse the cells anaerobically using chemical lysis as well as the traditional methods using sonication in an aerobic environment. In all cases the nitrite reductase activity of wild-type cells was maintained and Pd4452 lost activity immediately. As an activity was not determined for the NasB protein from cell lysates attempts were made to isolate the protein by affinity chromatography.

Cells were lysed by a French Pressure Cell and debris was removed by ultracentrifugation as previously described for NasC. The resultant soluble lysate was filtered and applied to a StrepTactin XT matrix. This was washed with 10 column volumes of a buffer containing 50 mM Tris, 5 mM EDTA and 5 mM ascorbate at pH 8. Bound protein was eluted in 4 column volumes of the same buffer containing 20 mM biotin. Each fraction was analysed by SDS-PAGE and a Western blot to identify the NasB protein (Figure 5. 5). The results

show a dominant band in the SDS-PAGE gel at approximately the correct size for NasB and a signal at the same point was detected in the Western blot.

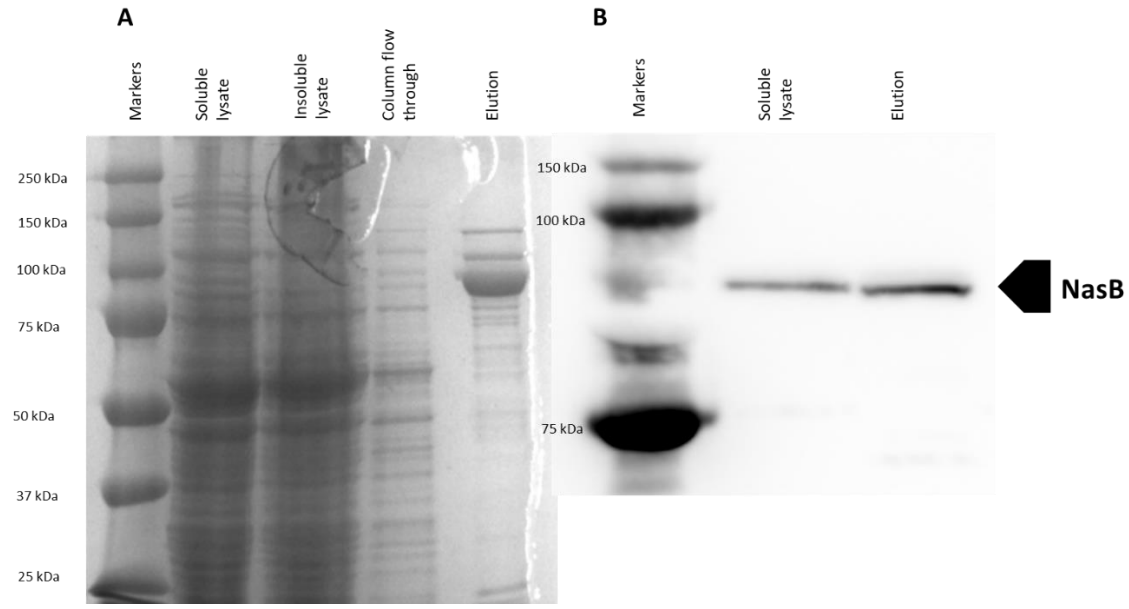


Figure 5. 5 SDS-PAGE analysis (A) of protein samples taken during affinity chromatography of soluble protein from Pd4452 cells grown using nitrate as the sole nitrogen source. Western blot analysis (B) of the same purification shown in A. An anti-Strep-II-tag antibody was used.

The enriched protein sample was tested using both methyl viologen and NADH to determine the nitrite reductase activity present. As expected, neither electron donor gave activity. To attempt to understand the reason for this the UV-Visible spectrum of the protein extract was investigated (Figure 5. 6). NasB is predicted to bind the co-factors FAD, siroheme and iron-sulfur clusters. These co-factors all have distinctive features in the UV-Vis spectrum. The data suggests that the protein has all the relevant co-factors associated. The absorbances for the siroheme co-factor present at ~400 and ~580 nm conform to that expected for the NirB type protein and the 4:1 ratio shown previously for *E. coli* NirB is maintained (Jackson, Cornish-Bowden and Cole, 1981). Furthermore, the shoulder at ~450-500 nm corresponds to FAD being present. These data suggest co-factor loading is not the problem.

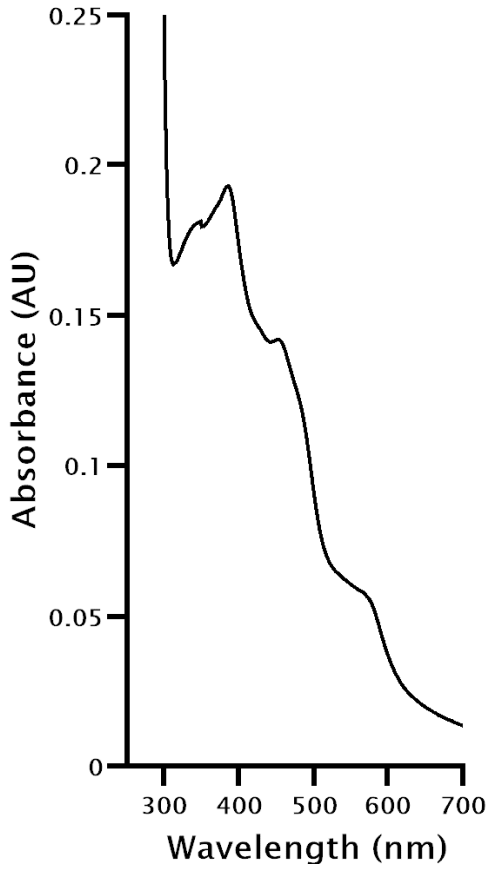


Figure 5. 6 UV-Visible spectrum of enriched NasB-containing protein sample after elution from affinity chromatography. Key features include the siroheme associated peaks at ~400 and ~580 nm and the FAD associated shoulder at ~450-500 nm.

5.2.3 Investigating the identity of co-eluting proteins

5.2.3.1 Interacting partners from Pd4449

In the SDS-PAGE analysis of proteins isolated from Pd4449 a significant amount of contamination can be seen (Figure 5. 7). The Strep-II-tag affinity tag is supposed to be able to yield highly pure protein from a single step purification however this is not the case here. Consequently, it was interesting to investigate the identities of the contaminating proteins. It was possible that interacting partner proteins from the proposed total nitrate reduction to ammonium complex may be found. Proteins may be co-eluting from the column by an interaction maintained from the *in vivo* state. This was made more

likely as the source of the protein is genomic expression rather than recombinant expression, therefore interacting partner proteins are more likely to co-elute.

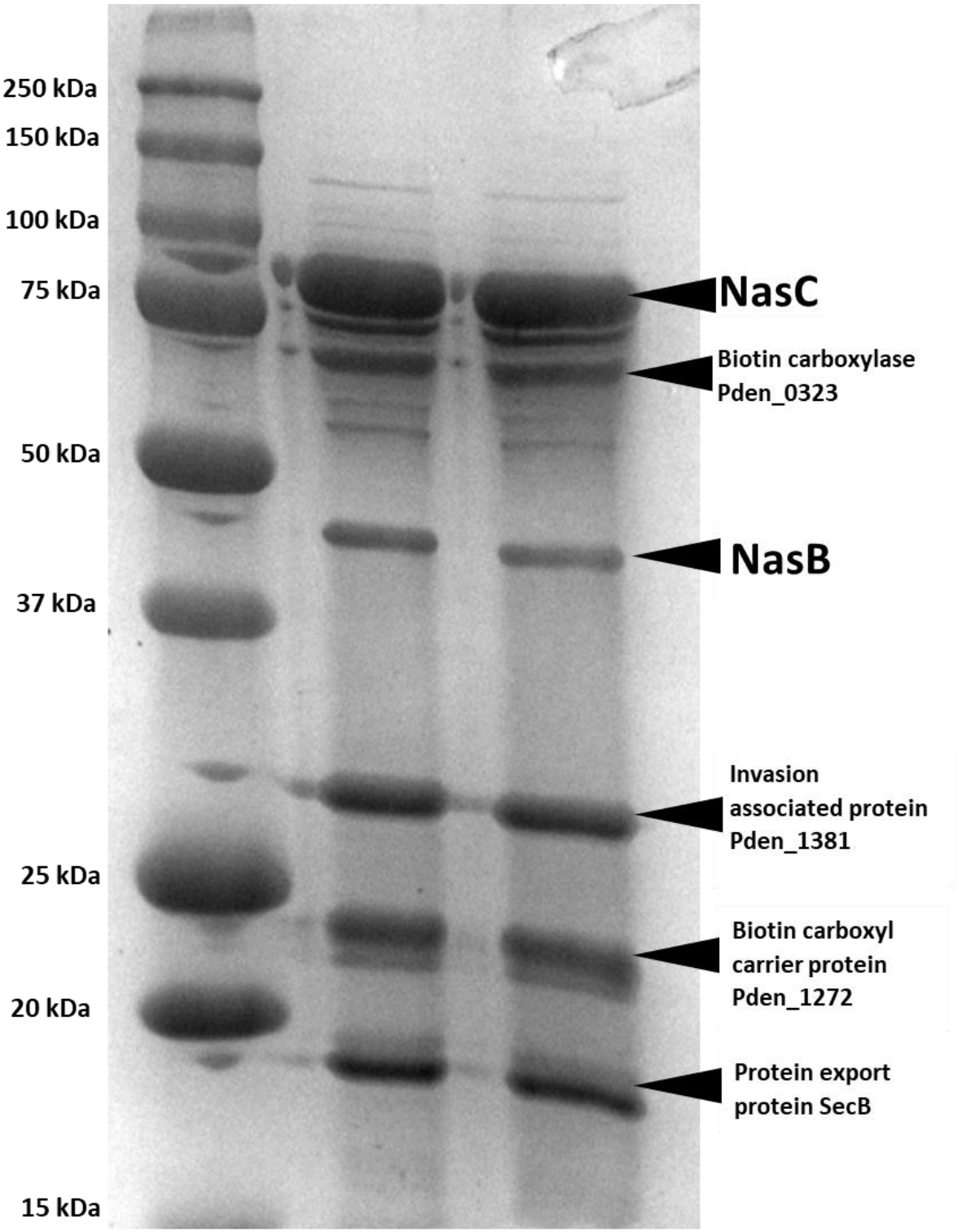


Figure 5. 7 SDS-PAGE analysis of protein isolated from Pd4449. Gel slices were taken from each of the two protein lanes displayed, prepared and used in MALDI-TOF/TOF mass spectrometry to investigate the isolated protein identity.

Table 5. 1 Protein identities obtained from Mascot database search displaying the sequence coverage and E-value for all identified proteins.

Protein	Significance score*	% coverage	E value
NasC	135	24%	1.7e-10
Pden_0323	134	27%	2.1e-10
NasB	245	36%	1.7e-21
Pden_1381	95	33%	1.6e-06
Pden_1272	79	29%	6.2e-05
Pden_2784 (SecB)	128	63%	8.3e-10

* Significance <50 were not confidently identified and have not been included.

The results show that the majority of contaminating proteins are related to biotin. These proteins are unlikely to be associated with the NasC and are instead likely to be interacting with the matrix of the column. To elute protein bound to the column biotin is used to out-compete the binding of the tag and as such biotin binding proteins may interact with the column non-specifically. The results also show that the band assumed to be NasC was indeed NasC. This confirms the protein isolation was correct and verifies the protein identity. Finally, the last protein of interest was identified as NasB. This is the assimilatory nitrite reductase that may form part of a complex between NasC and NasG. NasG was not identified as an interacting protein. It is interesting that the protein size is apparently smaller than its predicted mass and when the coverage data are analysed, only the N-terminus of the protein is identified suggesting it is not a full length NasB (Table 5. 1 & Figure 5. 8). The sequence analysis shows a separation of domain organisation in NasB. Towards the N-terminus the FAD binding domain is encoded and is used for the oxidation of NADH to gain electrons. The active site of nitrite reduction is towards the C-terminus of the amino acid sequence. The fragment recovered from NasC-SII purification attempts can oxidise NADH but is unable to reduce nitrite. Therefore, it could be a different model of protein interaction where NasC and the NasB fragment (N-NasB) interact to provide electrons to NasC and the full length NasB independently reduces nitrite.

A

```
MKKLVVIGAG  MASGRLLEQL  FEAAPGEWHV  TLFNAEPRGN  YNRLMLSPVL  SGEKTYEQIV

        70          80          90         100         110         120
THDADWYAAH  GVDCRFGEPV  VRIDRANRAV  YSNAGGAPYD  ALVIATGSAP  FIIPVPGRDL

       130         140         150         160         170         180
PGVVTYRDLE  DTNAMIEAGV  AGKDAVVIGG  GLLGLEAAAG  MAARGARVTV  VHLMGHLMER

       190         200         210         220         230         240
QLDPAAGYLL  QRDLERRGIK  VHCKGATKAI  LGHGQAEAVL  LEDGTVHPAD  LVCMAVGIRP

       250         260         270         280         290         300
EVRLANDAHL  EVERGIVVDD  ALRTSDPHIF  ALGECVEHRG  QVFGLVAPLY  DQAKVLARTL

       310         320         330         340         350         360
LGEEAAFRPV  QTATKLKVTG  CDLFSAGDFA  EGEGREDIVF  RDPGRGIYKR  LVLENDRIVG

       370         380         390         400         410         420
VVMYGDTADG  NWFYGLMKDE  TDVGEMRDTL  IFGPAFQGGA  KLDPMAAVAA  LPPEAEICGC

       430         440         450         460         470         480
NGVCKGAITD  AVLNGATTLE  AVRACTKASG  SCGTCTGLVE  QVMKLTLGDG  FVAPAPAGMC

       490         500         510         520         530         540
KCTDHSHEDV  RRLIRSMGLK  SIPAVMQELG  WKTVGGCHSC  RPALNYYLLA  EYPLDYRDDR

       550         560         570         580         590         600
QSRFVNERNH  ANIQKDGTYS  VVPRMWGGVT  TPSELRAIAD  AAEKYAVPMV  KVTGGQRIDL

       610         620         630         640         650         660
LGVKKEDLPH  MWADLNAAGL  VSGHAYSKGL  RTVKTCVGSE  FCRFGTQDST  GLGIRLEKLL

       670         680         690         700         710         720
WGSWTPHKVK  LGVSGCPRNC  AEATCKDVGV  VCVDSGYQIS  VAGAAGMEVK  ETEPLATTPS

       730         740         750         760         770         780
EDEAVEIITA  FVQLYREHAR  YLDRPYKWVA  KVGLDWVREQ  IEDVPTRRAL  VERFEISQSV

       790         800         810
YRRDPWAEHS TTTETPKWAP LADLTLEAAE
```

B

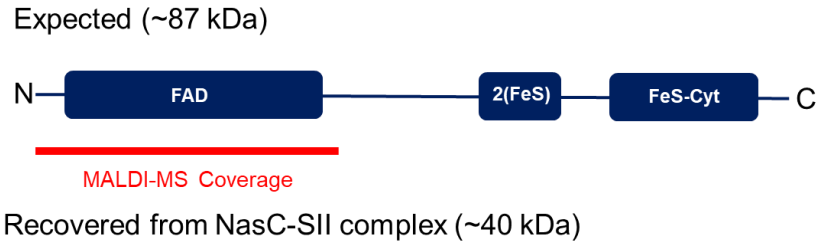


Figure 5. 8 The amino acid sequence of NasB from *P. denitrificans* Pd1222, black sequence is covered by the MALDI-MS data and grey sequence was not identified, underlined sequences are the fragments detected (A). Domain organisation of NasB

showing the pyridine nucleotide binding domain (FAD), the iron-sulfur coordination domain (2(FeS)) and the siroheme binding site (FeS-Cyt) (B).

Analysis of a size-exclusion chromatography of enriched NasC-SII sample reveals further evidence of a tight NasC, N-NasB interaction. When NasC is eluted from the column there is another protein band at the correct gel position for the N-NasB (Figure 5. 9). These two proteins appear to have maintained their interaction throughout the chromatography process, indicating a tight interaction. This implies that there may be a functional reason for an interaction of this kind.

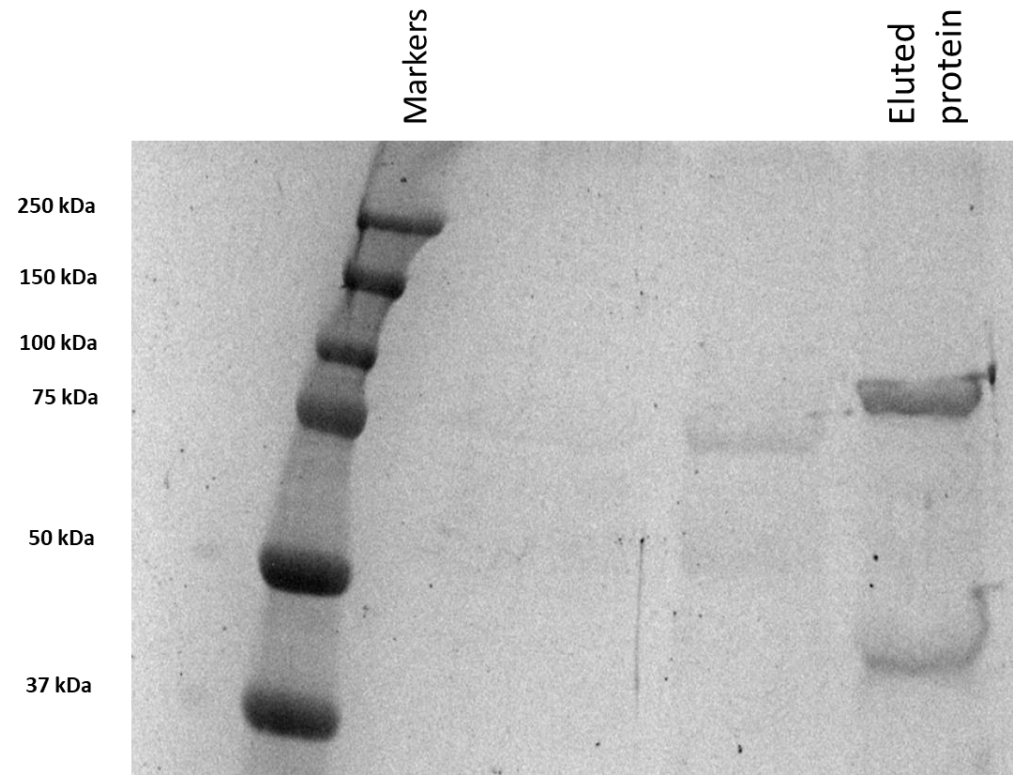


Figure 5. 9 SDS-PAGE analysis of proteins isolated from size-exclusion chromatography. The sample loaded was of enriched NasC from Pd4449 cells and the fractions run on the gel correspond to the peak fraction collected.

5.2.3.2 Interacting partners from Pd4452

In the SDS-PAGE analysis of enriched NasB, obtained from Pd4452 cells cultured using nitrate as the sole-nitrogen source, there is less contamination compared to the Pd4449 result, however there are still additional proteins that were not identified (Figure 5. 10). The same approach was taken whereby gel slices were prepared and used in MALDI-TOF/TOF to determine the identity of the protein bands. The results of this experiment confirmed that the large band assumed to be NasB was indeed a full-length copy of the protein. Furthermore, two smaller proteins were identified as the same contaminating proteins found in the Pd4449 experiment, these being 'Pden_1381' and 'SecB' (Table 5. 2). NasC is also absent in this enriched protein sample. Whilst N-NasB was found to co-elute with NasC in the reciprocal experiment the same was not observed in this one. There is, however, a key difference between the two NasB types, as the previously identified non-tagged NasB was found to be truncated in some way and in this experiment it appears to be full-length.

Table 5. 2 Protein identities obtained from Mascot database search displaying the sequence coverage and E-value for all identified proteins.

Protein	**Significance score***	**% coverage**	**E value**
NasB + NasG mixture	326	45% NasB and 66% NasG respectively	1.3e-29
NasB	365	55%	1.7e-33
Pden_1381	88	33%	8.7e-06
Pden_2784 (SecB)	107	52%	1e-07

* Significance <50 were not confidently identified and have not been included.

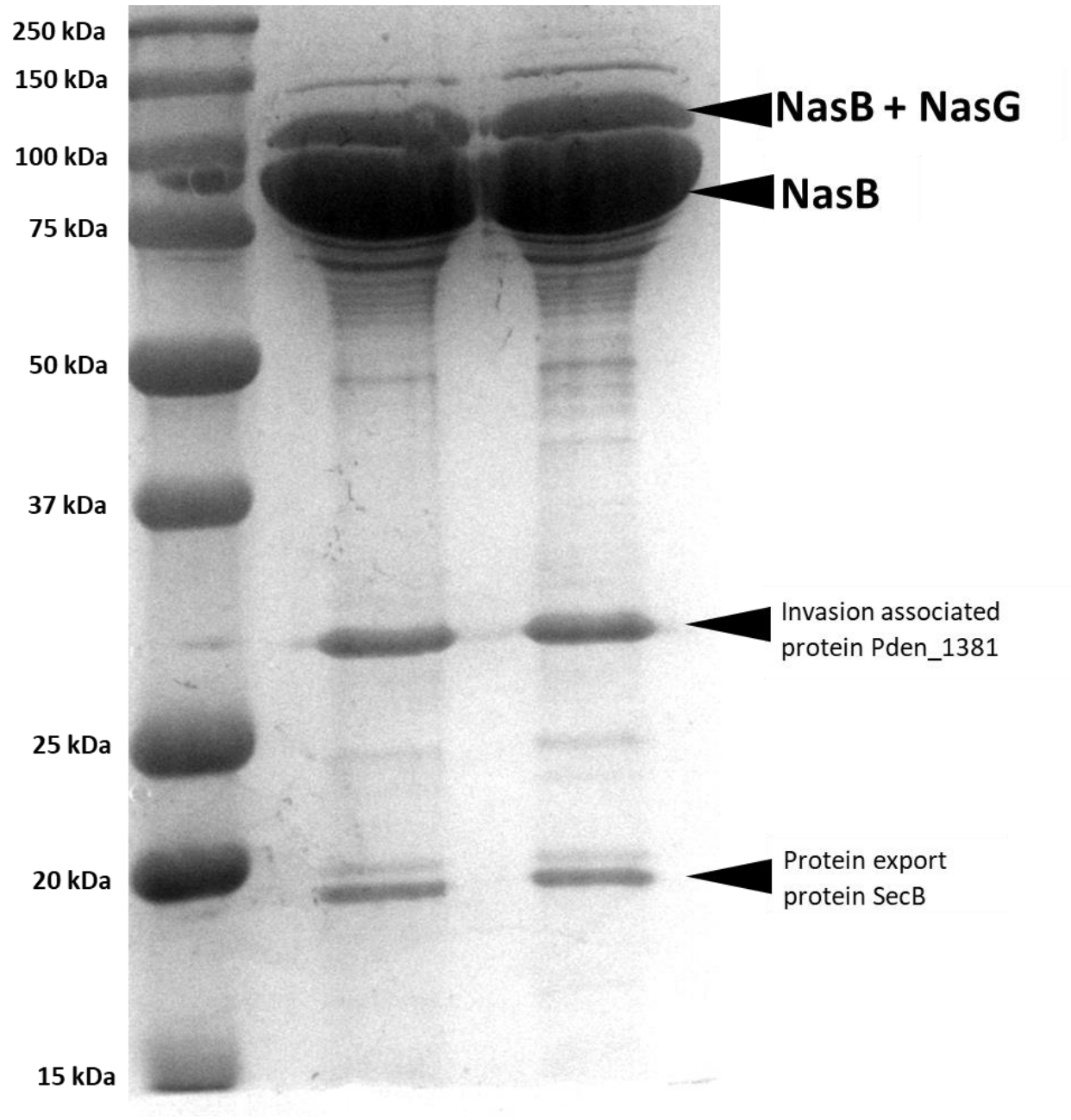


Figure 5. 10 SDS-PAGE analysis of protein isolated from Pd4452. Gel slices were taken from each of the two protein lanes displayed, prepared and used in MALDI-TOF/TOF mass spectrometry to investigate the isolated protein identity.

```
MKKLVVIGAG MASGRLLEQL FEAAPGEWHV TLFNAEPRGN YNRLMLSPVL SGEKTYEQIV

        70         80         90        100        110        120
THDADWYAAH GVDCRFGEPV VRIDRANRAV YSNAGGAPYD ALVIATGSAP FIIPVPGRDL

       130        140        150        160        170        180
PGVVTYRDLE DTNAMIEAGV AGKDAVVIGG GLLGLEAAAG MAARGARVTV VHLMGHLMER

       190        200        210        220        230        240
QLDPAAGYLL QRDLERRGIK VHCKGATKAI LGHGQAEAVL LEDGTVHPAD LVCMAVGIRP

       250        260        270        280        290        300
EVRLANDAHL EVERGIVVDD ALRTSDPHIF ALGECVEHRG QVFGLVAPLY DQAKVLARTL

       310        320        330        340        350        360
LGEEAAFRPV QTATKLKVTG CDLFSAGDFA EGEGREDIVF RDPGRGIYKR LVLENDRIVG

       370        380        390        400        410        420
VVMYGDTADG NWFYGLMKDE TDVGEMRDTL IFGPAFQGGA KLDPMAAVAA LPPEAEICGC

       430        440        450        460        470        480
NGVCKGAITD AVLNGATTLE AVRACTKASG SCGTCTGLVE QVMKLTLGDG FVAPAPAGMC

       490        500        510        520        530        540
KCTDHSHEDV RRLIRSMGLK SIPAVMQELG WKTVGGCHSC RPALNYYLLA EYPLDYRDDR

       550        560        570        580        590        600
QSRFVNERNH ANIQKDGTYS VVPRMWGGVT TPSELRAIAD AAEKYAVPMV KVTGGQRIDL

       610        620        630        640        650        660
LGVKKEDLPH MWADLNAAGL VSGHAYSKGL RTVKTCVGSE FCRFGTQDST GLGIRLEKLL

       670        680        690        700        710        720
WGSWTPHKVK LGVSGCPRNC AEATCKDVGV VCVDSGYQIS VAGAAGMEVK ETEPLATTPS

       730        740        750        760        770        780
EDEAVEIITA FVQLYREHAR YLDRPYKWVA KVGLDWVREQ IEDVPTRRAL VERFEISQSV

       790        800        810
YRRDPWAEHS TTTETPKWAP LADLTLEAAE
```

Figure 5. 11 The amino acid sequence of NasB from *P. denitrificans*. Underlined sequence shows identified fragments.

In contrast to the truncated form of NasB, the protein identified in this experiment has positively identified fragments from across the entire sequence. It is likely the N-NasB fragment is truly truncated and there was no problem with detection of ions originating from the C-terminus. The final result obtained from this experiment was the most surprising. This was the two-protein mixture identified from the distinct larger band above NasB. This showed a mixture of NasB and NasG. This is the first time NasG has been identified co-eluting with NasB. Furthermore, they maintain this interaction when

run on a gel that contains SDS indicating a very strong interaction. These results further expand the understanding of the nitrate assimilation apparatus in *P. denitrificans* as they show that the interactions between partner proteins does not conform to the model proposed earlier in this chapter.

5.2.4 Investigating the NasC-NasB interaction

As the NasB protein was isolated but not functional, the effect of the NasG interaction could not be determined. The isolated protein did, however, provide some useful biochemical information. The UV-Visible spectrum obtained showed the features that would be expected for a siroheme containing nitrite reductase, furthermore, they indicate the presence of FAD which would be critical to the protein function. This information can act as a baseline with which to test the NasC protein enrichment. By taking an equivalent UV-Visible spectrum of NasC, containing NasB identified from MALDI-TOF/TOF, the presence of siroheme could be investigated.

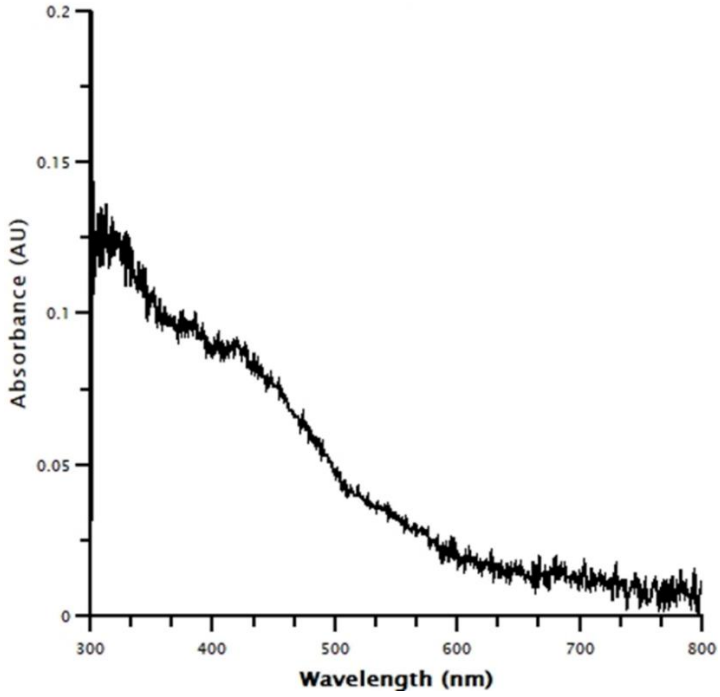


Figure 5. 12 UV-Visible spectrum of enriched NasC/NasB-containing protein sample after elution from affinity chromatography. Key features of siroheme are absent.

The result of this UV-Vis experiment shows no features of siroheme present (Figure 5. 12). This suggests that the NasB does not contain siroheme, which is not covalently bound, and as a result it is not able to reduce nitrite. When the sample was tested in nitrite reductase activity assays, with both methyl viologen and NADH as electron donors, it was unable to reduce the nitrite. This protein mixture was able to reduce nitrate using the artificial electron donor methyl viologen. Therefore, the idea that NasB could be interacting with the NasC in order to provide electrons from NADH for nitrate reduction was investigated. When the sample was tested for NADH dependent nitrate reduction activity was found (Figure 5. 13).

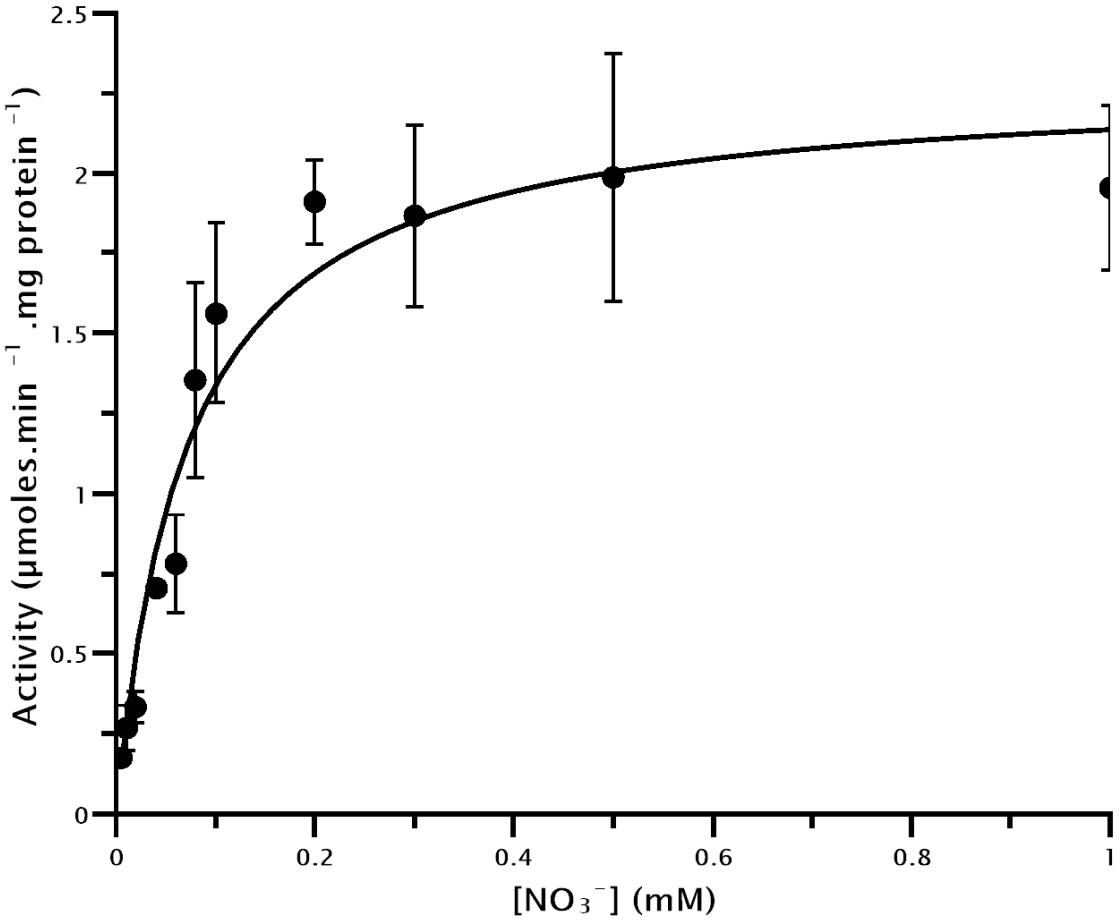


Figure 5. 13 Nitrate reductase activity with changing substrate concentration. Data is the average of 3 biological replicates with error bars representing standard deviation and the fit line is a non-linear regression using the Michealis-Menten equation. Values of K_M and V_{max} were calculated as 71 ± 15.2 µM and 2.29 ± 0.15 µmol.min^{-1}.mg prot.$^{-1}$ respectively.

These results indicate that this is the functional complex for nitrate reduction in *P. denitrificans*. The maximum rate of reaction of ~2 µmol.min^{-1}.mg prot.$^{-1}$ is a high rate of reaction indicating that the electron transfer between NasB and NasC is occurring at a high enough rate to sustain assimilation. Furthermore, a K_M of 71 ± 15.2 µM indicates a high enough affinity for the substrate to ensure a turnover of nitrate to ammonium under nitrogen limited conditions. Crucially this is the first time that nitrate reduction has been observed from a protein sample that does not contain the full-length NasB and NasG proteins. This means the model of protein interactions must be revisited, as NasG cannot be the electron shuttle from NasB to NasC. This work, therefore, re-defines the functions of the small Rieske-type protein NasG, poses interesting questions for future study regarding post or pre-translational modification of NasB and helps to shape the understanding of the protein complexes involved in this part of the global nitrogen cycle.

5.3 Discussion

5.3.1 The importance of using Strep-II-tag mutant strains

Throughout this chapter the *in vivo* protein-protein interactions of Nas proteins have been investigated. In order to achieve this aim Strep-II-tag mutant strains were used, where the addition of a sequence to the C-terminus of the protein allowed for affinity purification of protein and associated interacting partners. The results obtained in this chapter further highlight the importance of this genetic manipulation in approaching these complex questions. It was impossible, in Chapter 4, to construct an overexpression system for NasC and as such no attempt was made to generate a similar system for NasB. This meant isolation of either protein was not possible by conventional techniques and this new approach was required to investigate the protein interactions. This had unexpected, positive consequences for the results of this work. Full length NasB does not co-purify with any type of NasC but appears to with NasG. However, NasC does co-purify with a shortened version of NasB indicating that the interactions are different to the current model. This result would not have been possible by mixing recombinantly purified NasB and NasC as they would have been full length proteins which do not interact. This all highlights the importance of testing the *in vivo* interaction by this method.

5.3.2 A new model for Nas protein interactions

In the original model of protein interactions involved in nitrate assimilation a complex of NasB, NasC and NasG was suggested. The reason for a complex appeared to be two-fold. In the first case electron transfer to NasC requires NasB, and in the second case the proximity of the nitrite reductase to the nitrate reductase would be advantageous in limiting the build-up of cytotoxic nitrite. The available evidence for electron transfer comes from 2011 where Gates *et al,* found a mutant containing a *nasB* deletion was deficient in NADH-dependent nitrate reduction but the same mutant retained methyl viologen dependent activity. As MV can donate electrons directly to NasC it appeared a

functional copy of NasC was indeed present, however, the inability of this reductase to utilise the physiological electron donor NADH meant NasB was essential to provide NADH oxidation and electron transfer to NasC. Furthermore, a deletion of *nasG* resulted in a loss of both nitrate and nitrite reductase activity dependent upon NADH, in the same conditions MV-dependent activity was still observed. The authors interpreted this result as showing that NasG was the link between NasB and NasC for electron transfer and the model shown in Figure 5. 1 was developed (Gates *et al.*, 2011).

In the experiments presented in this thesis a full length NasB-SII was isolated from affinity chromatography, but it was inactive. It is possible that the lack of activity was caused by oxygen damaging iron-sulfur clusters critical for electron transfer. The NasG protein was identified in a tight association with NasB from SDS-PAGE analysis. This is very interesting as it is the first time a direct interaction between the two proteins has been observed. As NasG did not have an affinity tag it would not have bound to the column unless it was associated with the tagged NasB-SII. This result appears to validate the observations of Gates *et al*, where they found a deletion of *nasG* resulted in an inability for NADH-dependent nitrite reductase activity. It is plausible that NasG acts as an electron transfer path from the NADH dehydrogenase domain of NasB to the siroheme nitrite reductase domain and this is supported by s structural interdependence. This would be consistent with previous observations that suggest NasG is potentially highly unstable without NasB being present (Gates *et al.*, 2011).

These data can be combined with *nas* operons previously reviewed where co-localisation of the nitrite reductase *nasB* (*nirB/nasA/nasD*) and the small Rieske-type protein *nasG* (*nasD/nasE/nirD*). The nitrite reductases noted in this review were of the NirB type requiring a FAD co-factor and utilising NADH or NAD(P)H as their electron donors. In the event of an alternative NirA type nitrite reductase, which uses ferredoxin to gain electrons, there is no annotated *nasG* present. This implies a necessity in this co-localisation (Luque-Almagro *et al.*, 2011)(section 1.4.2). These results suggest NasG is associated strongly with NasB protein but not involved with NasC. This would make sense as *nasC* genes are found in operons lacking *nasG* implying there is an alternative interaction passing electrons to NasC (Luque-Almagro *et al.*, 2011).

The nature of NasB must be considered in these experiments. A full-length copy of the NasB-SII protein was found in affinity purification experiments however, the same fragment was not observed in the equivalent NasC-SII experiment. A smaller fragment of the NasB protein that corresponded to the N-terminus of the protein (N-NasB) was found. From these data the suggestion is NasB is actually split into two distinct domains, one is an NADH dehydrogenase domain co-ordinating a FAD cofactor and the other is the active enzyme centre containing the siroheme responsible for the reduction of nitrite to ammonium (Figure 5. 14). This could be a product of post-transcriptional modification resulting in differential expression of the two forms or from a post-translational cleavage of the N-terminus.

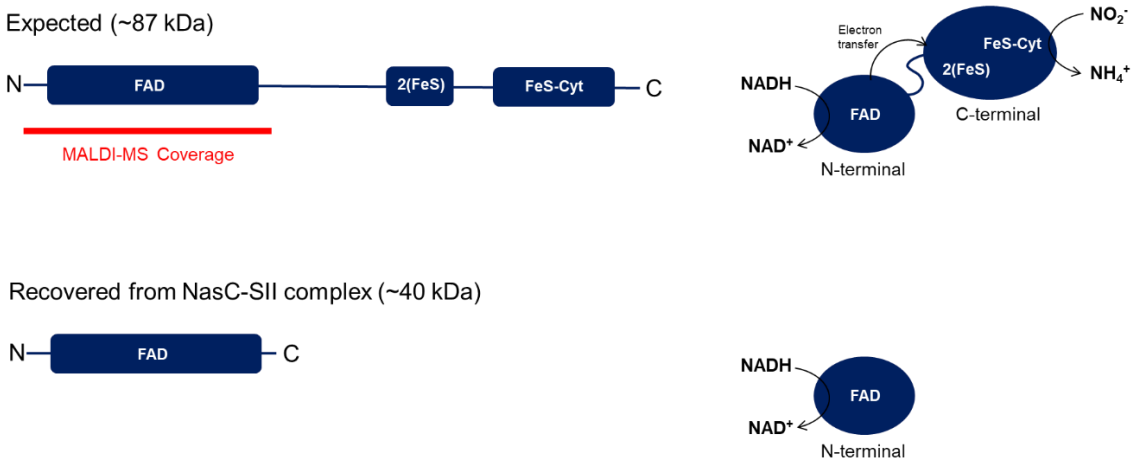


Figure 5. 14 Diagram demonstrating the expected size and domain architecture of full length NasB and the fragment detected in the MALDI-TOF/TOF experiment.

As the domains appear split in this distinct manner protein identification data from NasC-SII affinity purification can be considered in a new light. An alternative model can be suggested, whereby there are two electron transfer pathways. In the first stage the NADH dehydrogenase domain of NasB oxidises NADH to gain electrons, electrons are transferred in two ways, the first path could be directly to NasC from the interaction between NasC and N-terminus NasB, the second path would take the electrons from the N-terminus to the active centre in the C-terminus via NasG. This model therefore explains the necessity of NasG for electron transfer and explains the NasC-NasB interaction seen in the results (Figure 5. 15). Alternatively, there could be a split system whereby NasC interacts with N-NasB to reduce nitrate and NasB interacts with NasG for nitrite

reduction. This alternative model would completely alter the way this proposed complex is understood (Figure 5. 15).

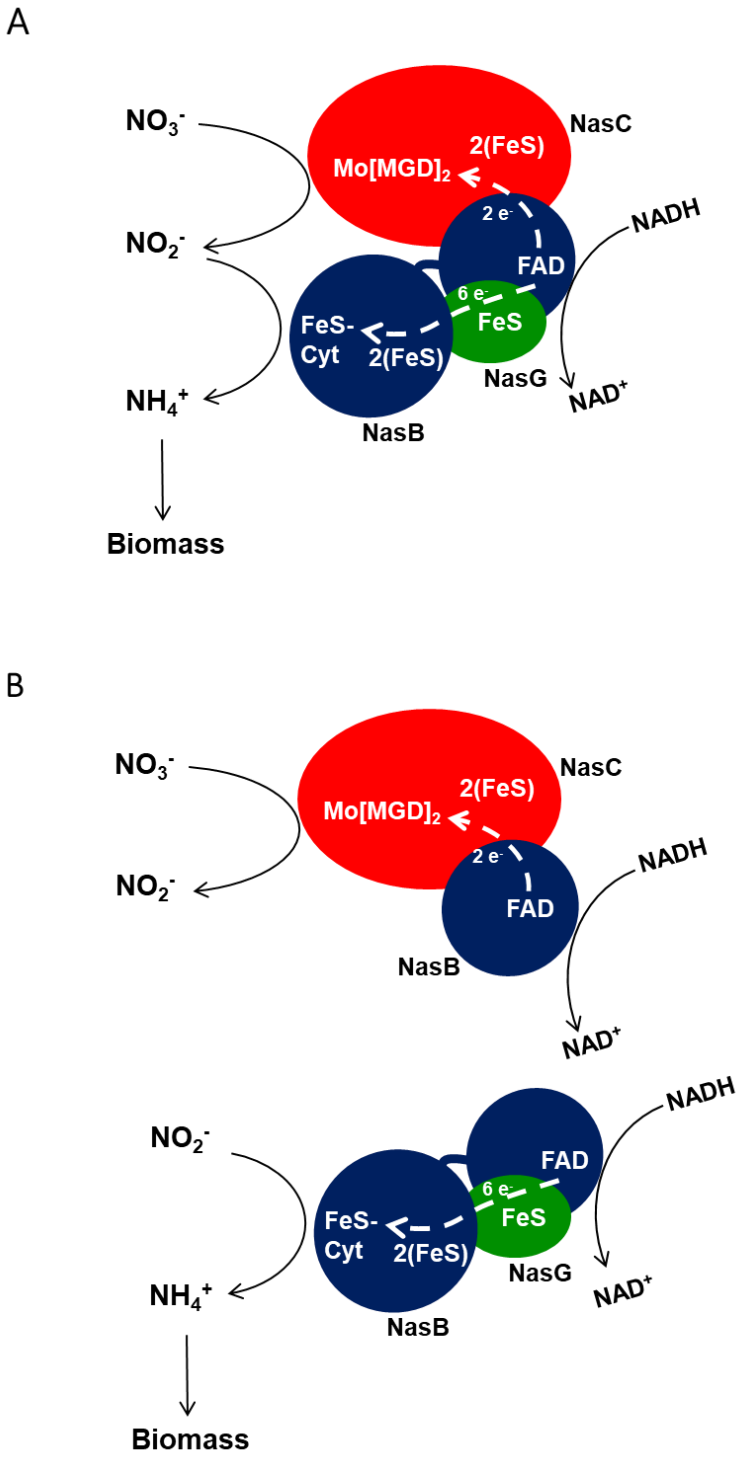


Figure 5. 15 The two proposed new models for electron transfer (white arrows) and protein-protein interactions between the catalytic Nas proteins. NasC is shown in red, NasB in blue and NasG in green. There is either a total complex with two different protein interactions (A) or a split complex with separate nitrate and nitrite reductase components (B).

Chapter 6: General discussion and future perspectives

6.1 Nitrate assimilation occurs in the presence of ammonium

6.1.1 Discussion

The first key finding of this thesis was the presence of nitrate assimilation under mixed ammonium and nitrate growth conditions (Chapter 3). At the outset of this experimental regime nitrate assimilation was proposed to act as an alternative electron vent to the characterised periplasmic nitrate reductase. Logically, this may be beneficial as the bacteria can generate useful organic nitrogen from Nas whereas Nap offers little more than a convenient ventilation system. Nitrate was used only when cells were cultured with butyrate as the sole carbon source. This was expected as previous studies have highlighted the carbon source dependent expression and use of the periplasmic nitrate reductase Nap (Sears *et al.*, 1993; Sears, Spiro and Richardson, 1997). Initially it was thought low butyrate concentrations would result in a preference for ammonium due to a decreased concentration of cytoplasmic reducing equivalents, generated by the β-oxidation of butyrate. Surprisingly the data shows the reverse, when butyrate concentration is highest there is low expression of *nasC* and when it is lowest then expression of *nasC* is increased to a level seen in cells growing with nitrate as the sole nitrogen source. This shows nitrate assimilation occurring in conditions where ammonium is still present in the media.

There are several important implications of this result. This finding evolves our understanding of nitrate assimilation as a process and opens up the possibility of it occurring in environments that had previously been discounted due to higher levels of ammonium. This opens the door to further study of nitrate assimilation under mixed

nitrogen and mixed carbon sources to discover more about the regulation of nitrogen metabolism in soil bacteria. Whilst these scientific implications are important there are other areas in which this work has relevance. In agriculture the application of a range of fertilisers results in increased concentrations of different compounds including fatty acids like butyrate and caproate. This thesis shows nitrate assimilation can occur when ammonium is present, dependent upon the dominant carbon source and the concentration of it. Where levels of fatty acids are built up in soils the dynamics of nitrate assimilation and denitrification may differ and this could change the amount of nitrogen lost or retained in a field.

Previous work investigating agricultural fields for composition of carbon substrates has largely focussed on plant health and growth. Studies have shown that fertilised fields increase the fatty acid content in the soils. This was judged in one study to constitute a benefit to plant health. The interspersion of different crops over time decreased the total fatty acid content slightly, this demonstrates the importance of considering different culture methods for food production (Jandl *et al.*, 2005; Chung *et al.*, 2017). Other studies have stated that increased composition of fatty acids in soils is a result of increased microbial stress. This may be true as a release of fatty acids due to microbial death and lysis in response to difficult conditions is possible however free carbon substrates are readily used by remaining microorganisms. The type of dominant carbon source released therefore has implications for the nitrogen metabolism by soil bacteria (Tabuchi, Kato and Nioh, 2008).

This work has highlighted a missing link in the regulation of both carbon and nitrogen metabolism. The global regulatory system NtrBC has been implicated in the accumulation of polyhydroxyalkanoates (PHAs) when a mutant strain lacking NtrB hyperaccumulated polyhydroxybutyrate (PHB). The study concerning the *ntrB* mutant suggested a possible role for the regulatory system in sensing the abundance of accumulated acetyl-CoA and/or the increase in cytoplasmic NADH concentrations. It could also be an effect of a malfunctioning nitrogen regulatory system resulting in inefficient use of reducing equivalents and therefore another regulator is involved in producing PHB as an electron sink (Olaya-Abril, Luque-Almagro, *et al.*, 2018). Another study focussed on a mutant lacking the partner protein NtrC. In this work the authors demonstrated an increase in

PHB production even under unusual conditions of low carbon to nitrogen ratios. They also demonstrated an increase in the cytoplasmic NAD(P)H to $NADP^+$ ratio in the mutant versus the wild-type. They suggested a model whereby NtrC regulates glucose metabolism, modulating the increase in acetyl-CoA and maintaining a consistent balance between carbon use and cellular oxidation state. When this was interrupted by the mutation the cells were forced to utilise an alternative pathway to balance their oxidation state and this involved synthesis of PHB, a reductive pathway. This implicates NtrBC in the regulation of both carbon and nitrogen metabolism, demonstrating the clear link between the two (Sacomboio *et al.*, 2017). There was no indication that the NtrBC system directly modulated expression of PHB synthesis genes or other related carbon metabolism pathways. There is a hole in the regulatory network that links this cellular oxidation and carbon state to regulation of genes involved in metabolism.

This work has shown a gap in the regulatory network for nitrogen assimilation in *P. denitrificans*. Previously identified systems have focussed on signals of nitrogen limitation, such as increased 2-oxoglutarate, that shows a requirement for increased ammonium supply and hence production of nitrate assimilation genes. The periplasmic nitrate reductase Nap is known to be expressed without the presence of nitrate. This indicates the underlying regulation is not associated with the presence of nitrate. One of the proposals was that the carbon substrate is the trigger for the expression of *nap*. This makes sense as acetate and butyrate result in *nap* gene expression and they have a shared metabolic pathway prior to the TCA cycle. TCA cycle intermediates such as succinate or malate do not elicit the same response. This means there must be an alternative regulator that detects the breakdown products of butyrate metabolism, possibly acetyl-CoA, and induces *nap* expression (Sears, Spiro and Richardson, 1997). A regulator that responds to the metabolic pathway use by the bacterium would explain the results obtained within this thesis. Under high butyrate concentrations there may be a preference for storage of the carbon substrate as PHB and as such the flow of carbon to the TCA cycle is diminished and any key compounds do not accrue. Under low butyrate conditions then the bacterium increases the breakdown of butyrate and this increases the concentration of the sensed intermediates which allows for the expression of the assimilatory nitrate reductase. Under high or low succinate regimes there is no such effect as the metabolic pathway being utilised is different and no intermediates accumulate. Therefore, it is likely a regulator at

this level does exist and is involved in the regulation of nitrogen metabolism. Future work should seek to address this gap in the literature.

6.1.2 Future perspectives

This work has highlighted specific conditions under which co-assimilation of nitrate and ammonium occurs in *P. denitrificans*. The primary take away is that it is possible for this co-assimilation to occur and as such it would be useful to further investigate. As described in section 6.1.1 there is a complex environment in which these organisms live with a variety of different carbon and nitrogen substrates available. The regulation of nitrate and ammonium assimilation is therefore vital for long-term sustainable use of fertiliser and intensive, high-yield agriculture.

To begin with the use of the nitrogen should be quantified. This could involve continuous culture experiments where nitrate is contains ^{15}N. Under the low butyrate condition presented in this work it would then be possible to determine the amount of nitrate assimilated into biomass. This would give a greater insight into the amount of carbon substrate that must be used to assimilate the nitrate. Further experiments of this type could then look at increasing and decreasing the concentration of butyrate in the media to find the range in which nitrate assimilation is occurring and, at each point, what the contribution is relative to the entire nitrogen budget. This would allow us to understand the influences of concentration in the soil environment more accurately and could result in specific recommendations for artificial fertiliser.

The concentration of PHB accumulated by the cells grown in these specific regimes could be determined to determine if the effect is this switch in metabolism. This would have major implications for the model and could be included with the previous suggestion to ascertain the concentration of carbon substrate that yield accumulation. Combining this work further experiments could also look at the effects of different mixed concentrations of the two chosen carbon substrates. Previous work has shown a preference for succinate over butyrate however, these experiments were not focussed on nitrate assimilation

conditions and it would be useful to see if this effect is seen when carbon sources are similarly mixed (Ellington *et al.*, 2002).

The work presented in this thesis provides specific criteria for further investigation. Firstly, nitrate assimilation is possible in the presence of ammonium. Secondly, nitrate assimilation is responsive to the oxidation state of the carbon source provided. Thirdly, carbon substrate concentration is the final critical factor to consider when understanding dual nitrate and ammonium assimilation. Future work should look to investigate the regulation involved in this process now that this work has shown it is possible.

6.2 The activity of the nitrate reductase NasC

6.2.1 Discussion

This thesis has shown the complexity involved in studying the proteins involved in nitrate reduction. The data presented in Chapter 4 shows that overexpression of nitrate reductases is difficult and requires some improvement. One of the primary issues was the use of the common overexpression vector for alphaproteobacteria pLMB509. This vector is used widely amongst alphaproteobacteria for expression of a variety of genes from a taurine inducible promoter (Tett *et al.*, 2012). The data presented in this thesis shows that there needs to be a better system for expression of nitrogen metabolism proteins in *P. denitrificans*. Taurine can be used by *P. denitrificans* for growth or for dissimilation and under the conditions used for overexpression of NasC the bacteria appears to have a preference for taurine as a nitrogen source over nitrate (Brüggemann *et al.*, 2004; Felux *et al.*, 2013). This likely down regulates the associated chaperones and biosynthesis pathways for correct co-factor incorporation into NasC. When protein was purified using this system the specific activity was significantly lower than the protein isolated from a genomic copy. This work has highlighted the need to generate new expression systems not reliant on such a key nutrient and the importance of care when using overexpression

as a means of protein study. Isolated protein appears fully formed and active however the activity may be lowered by mistakes made in the overproduction.

NasC isolated from *P. denitrificans* Pd4449 showed a markedly increased activity compared to the protein isolated from the overexpression strains. This was likely due to the maturation process. In Pd4449 the maturation was entirely subject to normal cellular controls and as such it produced functional protein as if it were wild-type. In contrast the protein produced from the overexpression strains was subject to additional maturation support from NarJ, which was co-encoded on the plasmid, however, this was not sufficient to produce increased activity. This means there is a range of biosynthetic gene clusters that need to be investigated that produce co-factor for nitrate reductase. To produce functional protein these must be working optimally. The suggestion from this work indicates a reduced expression of biosynthesis genes in response to increased taurine concentration as the inducer of plasmid gene expression.

The enriched protein samples obtained showed a high nitrate reductase activity in assays using methyl viologen as the electron donor. The V_{max} in these experiments was 89 ±4.6 μmol.min^{-1}.mg prot^{-1} which is very high. This shows that the protein is highly active and rapidly reduces nitrate to nitrite. This likely allows for the rapid growth observed when *P. denitrificans* is cultured using nitrate as the sole nitrogen source. The nitrogen fixing bacteria *Azotobacter vinelandii* also encodes an assimilatory nitrate reductase in a similar gene cluster to *P. denitrificans*. This protein (termed NasB in *A. vinelandii*) has a relatively high activity of approximately 30 μmol.min^{-1}.mg prot^{-1} which supports growth using nitrate as a sole nitrogen source. This organism has a significantly slower growth rate than *P. dentirficans*, taking around 50 hours to achieve peak biomass, and has a lower specific activity (Gangeswaran, Lowe and Eady, 1993). In slower growing organisms the specific activity calculated for assimilatory nitrate reductases is significantly lower. In the halophilic archaeon *Haloferax mediterranei* there is an assimilatory nitrate reductase. When isolated from cells cultured using nitrate as the sole nitrogen source the activity was assessed and found to be nmol.min^{-1}.mg prot^{-1} which is an order of magnitude lower than those calculated for the faster growing bacteria (Martinez-Espinosa, Marhuenda-Egea and Bonete, 2001). The slow-growing green algae *Chlorella* sp. also encode an assimilatory nitrate reductase, however, there are many structural differences between

this and the bacterial enzymes and they cannot be directly compared using physiological electron donors. The rate of electron transfer to the different types of nitrate reductase would result in incorrect comparisons. Methyl viologen is thought to donate electrons directly to the molybdenum active site that is conserved amongst nitrate reductase and as such comparisons can be made when this electron donor is used. The *Chlorella* nitrate reductase has a very similar calculated activity compared to *P. denitrificans* of approximately 76 μmol.min^{-1}.nmol heme^{-1}. Each nitrate reductase subunit contains a heme group in this type of enzyme and as such the activities were quantified using the heme concentration (Solomonson *et al.*, 1986). This high activity corresponds to a relatively slow growth rate for this organism, however, as a eukaryote the demand for cellular nitrogen is higher and growth is more complex and slower in general (Shi, Zhang and Chen, 2000).

The high rate of reaction observed for NasC from *P. denitrificans* may be explained by a proposed evolutionary link between the assimilatory and periplasmic nitrate reductases. The periplasmic redox-balancing enzyme NapA is thought to have originated from a cytoplasmic assimilatory enzyme that was fused with a twin-arginine translocation signal that moved it to the periplasm (Jepson *et al.*, 2006). When the nitrate reductase activity of Nap from *Paracoccus pantotrophus* has been assayed using methyl viologen as the electron donor the activity has been shown as 60-95 μmol.min^{-1}.mg prot^{-1}. The data presented in Chapter 4 shows that the assimilatory nitrate reductase from the closely related organism *P. denitrificans* has a V_{max} within this range (Berks *et al.*, 1994; Gates, Richardson and Butt, 2008). The assimilatory nitrate reductase, NasC, from *P. denitrificans* is able to rapidly reduce nitrate to nitrite to assist with the high growth rate this organism has when cultured using nitrate as a sole nitrogen source. Comparisons with closely related nitrate reductases show a broad agreement with the calculated values in this study and offer further evidence as to the connection between the assimilatory nitrate reductases and the periplasmic redox balancing nitrate reductases.

6.2.2 Future Perspectives

The limiting factor in this research was the stability of NasC. When the protein sample was enriched there were contaminating proteins found by mass spectrometry. Whilst NasB was also identified in this study, discussed later, there were unrelated proteins likely involved in biotin metabolism and hence interacting with the column in a non-specific manner. Attempts were made to further isolate the NasC protein, these involved anion-exchange and size-exclusion chromatography. It was possible to further isolate NasC using these two techniques, however, there was a complete loss of nitrate reductase activity. NasC is a complex protein that requires a molybdenum co-factor and iron-sulfur clusters to function effectively, when these are exposed to different conditions they may be unstable and result in the protein being unable to function. Iron-sulfur clusters are used in many regulatory proteins to detect the presence of oxygen and influence gene expression. They do this by altering the stability and structure of the cluster and the protein itself to activate or inactivate it as required (Rouault and Klausner, 1996). Iron-sulfur clusters are often thought to be oxygen sensitive and as such when NasC is isolated and exposed to the air there is a degradation of these crucial co-factors. In this case the protein would be degraded and not function correctly. This could be a critical problem that has inhibited further purification of the protein. Future work should focus on the stability of NasC. Purifications using anaerobic chambers could assist in iron-sulfur cluster stability. In this study an ascorbate based buffer was utilised to provide a reducing environment and promote stability, however, it may not be the best buffer system to use for extended purification methods.

6.3 The NasBGC protein complex

6.3.1 Discussion

The final major result obtained in this study was the evolution of the model for the proposed nitrate reductase complex. This was based on a combination of data obtained in Chapters 4 and 5. The previous model proposed, involved NasG acting as an electron transfer protein that facilitated electron transfer from the NADH oxidising domain of NasB to the molybdenum active site of NasC (Figure 6. 1). A number of key findings make this previously proposed system unfeasible.

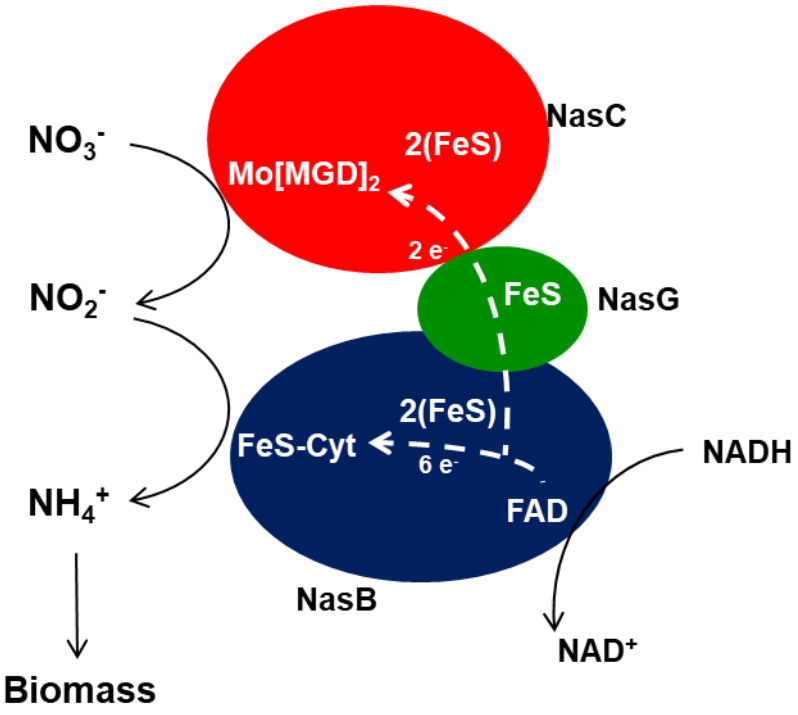


Figure 6. 1 A representation of the protein-protein interactions proposed for the NasBGC putative complex. Routes of theoretical electron transfer are designated by white dashed lines.

The major finding that precludes the possibility of this complex forming, as proposed, comes from the isolation of full length NasC and a truncated form of NasB. In this

analysis there was no band corresponding to NasG, however, the protein sample had nitrate reductase activity using both methyl viologen and NADH as electron donors. This demonstrated a previously unknown functional transfer between NasB and NasC without the presence of NasG. This means the proposed electron transfer route cannot be correct. The route of electron transfer from NasB to NasC is most likely mediated by direct interaction between the two proteins. As there was no Strep-II-tag added to NasB in these experiments there is no reasonable suggestion that it would co-elute with a tagged NasC protein, unless there was a direct protein-protein interaction. The data also show there is no NasG present in the eluted protein sample. The lack of NasG confirms the interaction between NasC and NasB.

These data shows that there is an interaction between the two proteins and the strength of that interaction is sufficient to survive a purification procedure. The Strep-II-tag variant of NasB also revealed some interesting points. Firstly, it showed that NasC does not co-elute with the full length NasB protein. Secondly, it showed that NasG co-elutes with NasB and that the interaction is strong enough to form a combined protein band under SDS-PAGE conditions. This result combines with the tagged NasC gel result to show two distinct protein-protein interactions. The first is between the N-terminus of NasB and the full length NasC. The second is between NasB and NasG, without the involvement of NasC.

This work opens up alternative possible conformations that the Nas proteins can form. It is possible that the interaction between NasB and NasC provides the electron flow to the NasC catalytic centre and the interaction between NasB and NasG is required for internal electron transfer for nitrite reduction. In this case the proteins could all come together in a complex where the association between NasB and NasC is the strongest (). Alternatively, there could be no single complex and instead two separate protein complexes. This would involve a split form of the NasB protein, the N-terminus produced and associated with full length NasC and the full length NasB produced and interacting only with NasG (). In this version there would be a complex for nitrate reduction and a separate complex for nitrite reduction. This may be more likely as there is no adequate explanation for the apparent truncation of NasB in the NasC tagged protein eluate. If NasC interacts with the N-terminus of NasB then it should also co-elute with a C-

terminally tagged NasB, which does not happen. It is likely that the split system is used by *P. denitrificans* as there is no evidence for the formation of a large complex in either NasB or NasC isolation studies.

Previous studies investigating the nature of the *P. denitrificans* proposed Nas complex have shown mutational analysis that shows NasG is required for growth on nitrate as a sole nitrogen source. This is not disputed by these findings as NasG may simply be an integral part of NasB and therefore nitrate can only be reduced to nitrite and no further, resulting in cell death. Further analysis showed nitrate and nitrite reduction could be separated in cell lysates by ion exchange chromatography (Gates *et al.*, 2011). This result could confirm either proposed system whereby the NasC, N-terminus NasB complex is separated from the NasB-NasG complex or where NasC is separated from the NasB-NasG complex.

This work has shown that the model originally envisaged for the Nas protein complex cannot be correct. The routes of electron transfer within this complex must be different. Furthermore, the work has proven that a form of NasB is required for electron transfer from NADH to NasC. NasG was previously thought of as an electron transfer protein between NasB and NasC, however, these experiments show that there must be an alternative function for this protein. Finally, the work presented in this thesis confirms that there are strong protein-protein interactions between the Nas proteins and that the nature of electron transfer between these proteins warrants further investigation as many *nas* gene clusters encode highly similar genes to *nasBGC*.

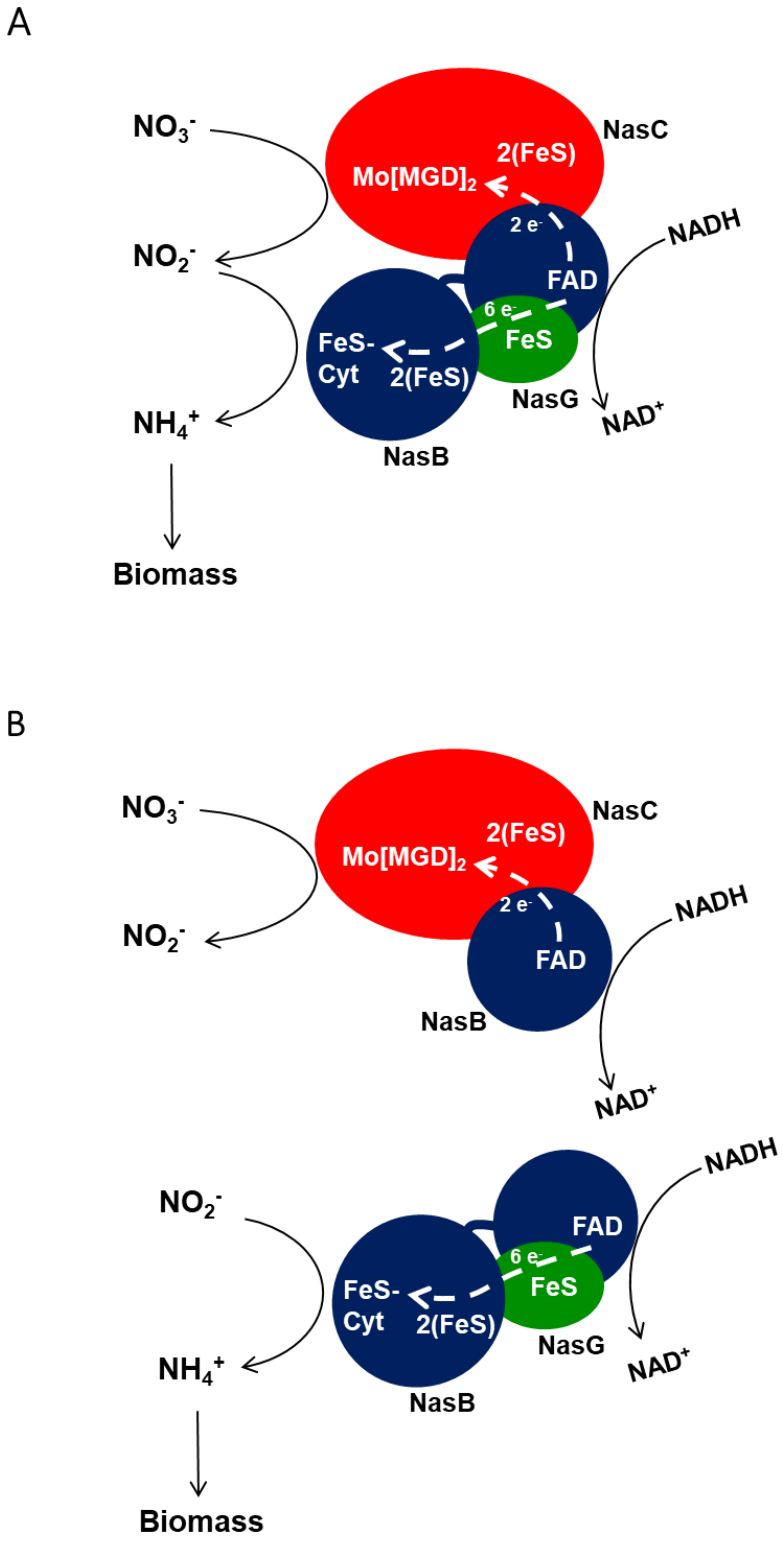


Figure 6. 2 The proposed models for protein-protein interactions between the NasBGC proteins. A three component system involving electron transfer (white dashed lines) from the N-terminus of NasB to the active site of NasC by direct interaction and internal electron transfer mediated by NasG to the siroheme centre of NasB (A). Two separate

two component systems using the N-terminus of NasB to pass electrons to NasC by direct contact and a separate NasB-NasG complex for nitrite reduction (B).

6.3.2 Future perspectives

It is important to determine the specific requirements for a modular Nas system. In the future, engineered bacterial strains capable of nitrogen removal and storage from agricultural or wastewater run-off may encode for Nas proteins that would assist in this process, storing nitrogen safely as biomass. To engineer such a strain the modularity of the Nas proteins will need to be understood fully and any required processing partners, such as specific proteases, will need to be considered.

This work has further evolved the model of protein interactions involved with the Nas reductases. It has also generated further questions that should be addressed. The most pressing remaining question is the clarification between the two models. Primary analysis of the nucleotide sequence for *nasB* did not reveal an obvious secondary structure in either the DNA or RNA that could explain a split in the domain structure of the final protein, yielding two proteins, a full length and a truncated form. This does not preclude the possibility of an unidentified site within this sequence, however, if this split is formed here it is unlikely to come from a known sequence motif. Specific peptide recognition sites are notoriously difficult to identify in primary sequences and no such clear sites are found in the NasB amino acid sequence. This does not rule out the possibility of peptide cleavage resulting in a truncated form of NasB that is associated with NasC. These unknowns are worth further investigation.

To determine which proposed model is correct there are several different approaches which could be undertaken. The first point of investigation should seek to identify whether there is a two-complex or a one-complex system. Using whole proteome analysis the reads for the N-terminus of NasB could be quantified relative to the C-terminus and if there are approximately twice as many hits for the N-terminus it would indicate a two-complex system. If the number of hits is approximately equal then it would indicate a

single form of NasB being produced which is consistent with a single complex system. From this stage it would be interesting to investigate the point at which the proteins are separated, if indeed they are at all. This could be done by RT-qPCR using primers specific for the 5' end of the *nasB* gene, and then several primer pairs that are spaced along the remaining sequence. If there is an modification in the mRNA copy number resulting in a high proportion of 5' transcripts, generating N-terminus NasB protein (the truncated form), then the result should indicate the length of this transcript and there should be a sharp decline in copy number detected after the end of this shortened transcript. Finally, it would be possible to delete the active site encoding sequence of NasB and generate an artificial truncated form. In this strain growth on nitrate as the sole nitrogen source would be impossible, however, growth combining nitrate and glutamate would allow synthesis of the NasC protein and, should the N-terminus of NasB be the only required protein interaction, nitrate reduction in cytoplasmic fractions should be detected using NADH and methyl viologen.

This experimental plan could help to decipher the correct model for Nas protein interactions from the data generated in this thesis. The increased understanding of the required interactions would allow for rational design of synthetic gene clusters dependent upon required module units of Nas proteins. This would also assist in designating these modular units for bioinformatic searches affording future researchers the ability to identify these Nas systems in sequenced genomes with a better idea of the required components. This would all give a greater understanding of the prevalence and importance of nitrate assimilation in environments where it has previously been overlooked.

6.4 Final conclusions and future perspectives

The work set out in this thesis generates a number of interesting new findings, however, it poses several future questions worth investigation. The first major finding was the expression and use of nitrate assimilation genes where ammonium was still present in the media. This was previously unheard of and it defies the current views of the literature. There is a logical case for nitrate and ammonium co-assimilation and in the complex

environment of soil and water courses it is unlikely organisms are exposed solely to either nitrate or ammonium, it is therefore likely that the co-assimilation of different nitrogen species occurs routinely in the soil environment.

Studies looking at the long-term fate of nitrogenous fertiliser applied to agricultural soils has shown that a considerable amount of it is lost to the atmosphere as nitrogenous gases such as N_2 and N_2O and that an amount is retained within the soil for long periods of time (Sebilo *et al.*, 2013). Other work has focused on the fate of ammonium added as artificial fertiliser and has found that a significant amount is turned over into nitrate by nitrifying organisms. This introduces the combined issue of promoting growth of denitrifying organisms that remove nitrogen from the soil and the mobility of nitrate to leave the soil and pollute waterways (Kraft *et al.*, 2014). This study by Kraft *et al*. suggested a range of environmental conditions that could determine where this nitrate could end up. The study mentions carbon source and ratio to carbon to nitrogen as important factors in the determination of the end product of nitrate respiration. This misses out nitrate assimilation and shows that there is a clear lack of research in this area. This thesis begins to fill in some of the space that has been unexplored and under researched. This work has demonstrated that the picture is more complex than we have previously thought and further investigation into the co-assimilation of nitrate and ammonium is required. The understanding of the regulatory mechanisms that govern the function of a co-assimilation system would allow for the development of future fertilisers that could increase the retention of biologically available nitrogen in agricultural soils by taking advantage of this natural phenomenon. This could greatly decrease the application of nitrogenous fertiliser and reduce our dependence on artificially fixed nitrogen, in turn reducing pollution from leaching and removal of nitrogen to the atmosphere.

This thesis has evolved the model of interacting Nas proteins that facilitate electron transfer and subsequent nitrate and nitrite reduction. This is important biochemical detail that shows how interactions between the system components may increase or decrease the efficiency of the system. Furthermore, it demonstrates the importance of all partner proteins when considering engineered systems. Nitrate assimilation is a fantastic candidate system for nitrate removal from wastewater as it secures the nitrate in organic matter that cannot be easily broken down and respired back to the atmosphere. Future

work may harness nitrate assimilation to remove nitrate from wastewater, however, the system will only be efficient if the correct protein components are assembled and the interactions between them are efficient. This is a crucial reason for the interest in protein-protein interactions in the Nas system. Future work should therefore focus on determining the correct model from the two proposed in this thesis.

Investigation of the Nas system found in the soil bacterium *Paracoccus denitrificans* has yielded an interesting model system for study. The future work can build from the knowledge created in this thesis and apply it to new, unexplored areas. The *Roseobacter* clade of marine microbes encompasses a group of closely related alphaproteobacteria. They are significant players in the marine environment and constitute up to 30% of bacteria in pelagic environments (Luo and Moran, 2014; Daniel, Simon and Wemheuer, 2018). Despite their abundance the role of these organisms in global biogeochemical cycling has been understudied. They are perhaps best known for their role in sulfur cycling however genomic analysis has shown a high prevalence of nitrate reduction genes in these organisms (Buchan, González and Moran, 2005; Luo and Moran, 2014). When confirmed and sequenced *Roseobacter* sp. are analysed *nas* gene clusters are found in slightly under 50% of genomes. When compared to the prevalence of the respiratory nitrate reductase gene *narG* or the periplasmic nitrate reductase gene *napA* it is clear that *nas* genes are far more abundant (Figure 6. 3). This implies nitrate assimilation may be more important in this group than nitrate respiration.

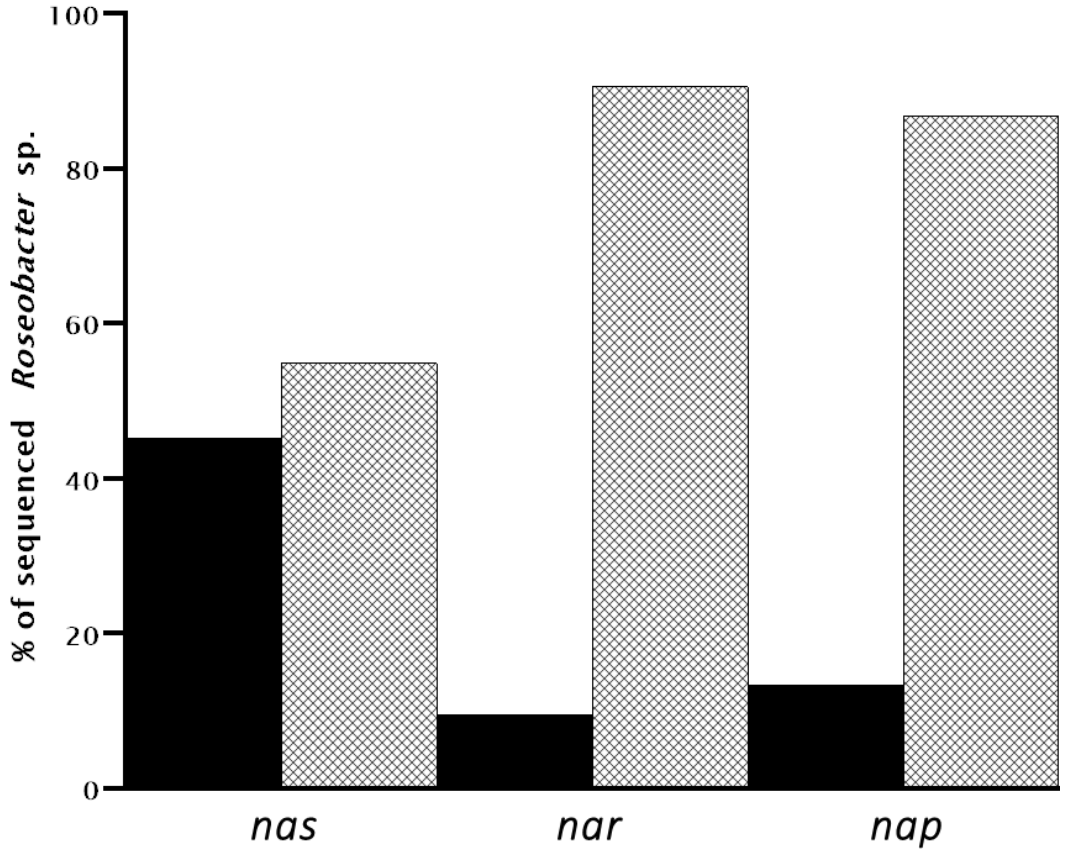


Figure 6. 3 Percentage of sequenced and confirmed *Roseobacter* sp. that contain *nas, nar* or *nap* genes in their published genomes. Solid bars represent organisms where the genes are found, and hashed bars represent organisms where genes are absent.

This under-studied group of marine organisms have been suggested in a variety of biotechnological application. Some species in this group associate with algae that are increasingly being used for renewable fuel and chemical production. These bacteria have previously been considered contaminants, however, more recent data suggests they play a positive role in algal growth and health and as such may be beneficial in these biotechnological applications (Ramanan *et al.*, 2016). Other studies have suggested a probiotic usage of *Roseobacter* species. This probiotic increases fish health by protecting against pathogenic organisms. In one study it was found to increase the survival of larval stage scallops (Ruiz-Ponte *et al.*, 1999; Bentzon-Tilia and Gram, 2017). These intensive aquaculture methods have other effects upon the environment they sit within. They increase the local concentration of both ammonium and nitrate in the water supply locally to the farm (Tovar *et al.*, 2000). Considering the increased concentration of these two key nitrogen substrates the inclusion of any probiotic bacteria needs to be thought about

carefully to ensure they will function in this environment. The results of this thesis show that ammonium does not prevent nitrate assimilation from occurring under specific carbon substrate conditions. This may help the case for inclusion of these organisms in aquaculture not simply as probiotics but as bioremediatory bacteria as well. It is unlikely that significant amounts of nitrogen would be lost by denitrification from this process as many organisms encode only the *nas* genes (Figure 6. 3)(Luo and Moran, 2014).

This thesis has also evolved the model for Nas system protein interactions. Understanding the required interactions helps in understanding the potential requirements of the Nas systems. In a typical *Roseobacter nas* gene cluster the module nature of Nas proteins is found. There are genes similar to the *nasTS* regulators found in *P. denitrificans*, there is an ABC-type transporter encoded by *nasFED*, the functional Nas protein units are encoded by an assimilatory nitrite reductase gene, *nasB*, a Rieske-type iron-sulfur protein, *nasG*, and a molybdenum containing assimilatory nitrate reductase gene *nasC* (Figure 6. 4). Taking the advanced model of interactions shown in *P. denitrificans* in this thesis the protein-protein interactions are likely highly similar. This means the organisms encoding all of these units' function in a similar way to those in *P. denitrificans*. This will help to identify functional Nas systems within prospective probiotic organisms and assess their likelihood as beneficial nitrate reducers.

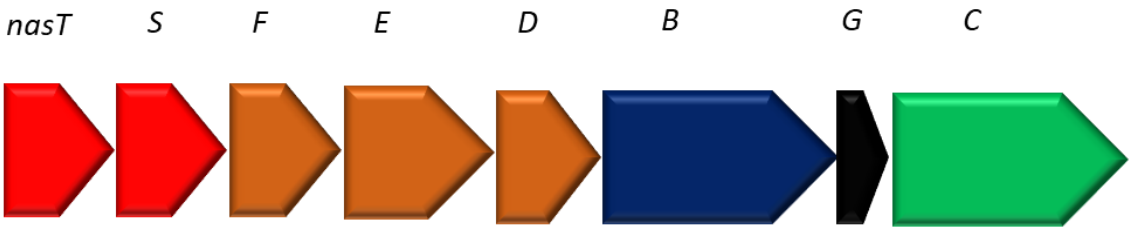


Figure 6. 4 Representative *nas* gene cluster found in *Roseobacter* sp. genomes. Red genes are regulatory, orange genes are involved in nitrate/nitrite transport, blue is the nitrite reductase, black is the Rieske-type iron-sulfur cluster protein and green is the nitrate reductase.

Ultimately this thesis has furthered the understanding of protein-protein interaction within the Nas system and has identified a new environmental circumstance under which nitrate

is assimilated. There are a number of exciting new research areas that can be explored using the knowledge generated during this project. The interaction networks identified will assist with the mining of future genomes to identify Nas systems and the proposed requirements they have for function. The newly identified area of nitrate assimilation with ammonium present changes the way we have seen nitrogen source preference by bacteria and opens the door to further research, especially using organisms that lack periplasmic nitrate reductases to further investigate the possibility of the Nas system reducing nitrate as an electron sink. This thesis has furthered our scientific knowledge of nitrate assimilation by bacteria and has improved our ability to map and understand the interactions between the different proteins and systems involved in nitrogen metabolism.

References

Abraham, Z. H. L. *et al.* (1997) 'PH-dependence for binding a single nitrite ion to each type-2 copper centre in the copper-containing nitrite reductase of Alcaligenes xylosoxidans', *Biochemical Journal*. Portland Press Ltd, 324(2), pp. 511–516. doi: 10.1042/bj3240511.

Akram, M. (2014) 'Citric Acid Cycle and Role of its Intermediates in Metabolism', *Cell Biochemistry and Biophysics*. Humana Press Inc., pp. 475–478. doi: 10.1007/s12013-013-9750-1.

Alefounder, P. R. and Ferguson, S. J. (1980) 'The location of dissimilatory nitrite reductase and the control of dissimilatory nitrate reductase by oxygen in Paracoccus denitrificans', *Biochemical Journal*. Portland Press, 192(1), pp. 231–240. doi: 10.1042/bj1920231.

Arnoux, P. *et al.* (2003) 'Structural and redox plasticity in the heterodimeric periplasmic nitrate reductase', *Nature Structural Biology*. Nature Publishing Group, 10(11), pp. 928–934. doi: 10.1038/nsb994.

Barnard, R., Leadley, P. W. and Hungate, B. A. (2005) 'Global change, nitrification, and denitrification: A review', *Global Biogeochemical Cycles*, 19(1). doi: 10.1029/2004GB002282.

Baumann, B. *et al.* (1996) 'Dynamics of denitrification activity of Paracoccus denitrificans in continuous culture during aerobic-anaerobic changes', *Journal of Bacteriology*. American Society for Microbiology, 178(15), pp. 4367–4374. doi: 10.1128/jb.178.15.4367-4374.1996.

Bedmar, E. J., Robles, E. F. and Delgado, M. J. (2005) 'The complete denitrification pathway of the symbiotic, nitrogen-fixing bacterium Bradyrhizobium japonicum', in *Biochemical Society Transactions*. Portland Press, pp. 141–144. doi: 10.1042/BST0330141.

Beijerinck, M.W. and Minkman, D. C. J. (1910) 'Bildung und verbrauch von stickoxydul durch bakterien', *Zentbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. II*, 25, pp. 30–63.

Bell, L. C., Richardson, D. J. and Ferguson, S. J. (1990) 'Periplasmic and membrane-bound respiratory nitrate reductases in *Thiosphaera pantotropha*', *FEBS Letters*. John Wiley & Sons, Ltd, 265(1–2), pp. 85–87. doi: 10.1016/0014-5793(90)80889-Q.

Bentzon-Tilia, M. and Gram, L. (2017) 'Biotechnological Applications of the Roseobacter Clade', in. Springer, Cham, pp. 137–166. doi: 10.1007/978-3-319-47935-4_7.

Berks, B. C. *et al.* (1995) 'Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions', *BBA - Bioenergetics*, pp. 97–173. doi: 10.1016/0005-2728(95)00092-5.

BERKS, B. C. *et al.* (1994) 'Purification and characterization of the periplasmic nitrate reductase from Thiosphaera pantotropha', *European Journal of Biochemistry*. John Wiley & Sons, Ltd, 220(1), pp. 117–124. doi: 10.1111/j.1432-1033.1994.tb18605.x.

Bertero, M. G. *et al.* (2003) 'Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A', *Nature Structural Biology*. Nature Publishing Group, 10(9), pp. 681–687. doi: 10.1038/nsb969.

Blasco, F. *et al.* (1998) 'NarJ is a specific chaperone required for molybdenum cofactor assembly in nitrate reductase A of *Escherichia coli*', *Molecular Microbiology*, 28(3), pp. 435–447. doi: 10.1046/j.1365-2958.1998.00795.x.

Botman, D., Tigchelaar, W. and Van Noorden, C. J. F. (2014) 'Determination of Glutamate Dehydrogenase Activity and Its Kinetics in Mouse Tissues using Metabolic Mapping (Quantitative Enzyme Histochemistry)', *Journal of Histochemistry & Cytochemistry*. SAGE PublicationsSage CA: Los Angeles, CA, 62(11), pp. 802–812. doi: 10.1369/0022155414549071.

Bray, R. C. *et al.* (1976) 'Electron paramagnetic resonance studies on the molybdenum of nitrate reductase from Escherichia coli K12', *Biochemical Journal*, 155(1), pp. 201–203. doi: 10.1042/bj1550201.

Brüggemann, C. *et al.* (2004) 'Enzymes and genes of taurine and isethionate dissimilation in Paracoccus denitrificans', *Microbiology*. Society for General Microbiology, 150(4), pp. 805–816. doi: 10.1099/mic.0.26795-0.

Buchan, A., González, J. M. and Moran, M. A. (2005) 'Overview of the marine Roseobacter lineage', *Applied and Environmental Microbiology*. American Society for

Microbiology, pp. 5665–5677. doi: 10.1128/AEM.71.10.5665-5677.2005.

Bueno, R., Pahel, G. and Magasanik, B. (1985) 'Role of glnB and glnD gene products in regulation of the glnALG operon of Escherichia coli.', *Journal of bacteriology*. American Society for Microbiology Journals, 164(2), pp. 816–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2865248 (Accessed: 13 February 2020).

Burns, I. G. (1977) 'Nitrate movement in soil and its agricultural significance', *Outlook on Agriculture*, 9(3), pp. 144–148. doi: 10.1177/003072707700900308.

Canfield, D. E., Glazer, A. N. and Falkowski, P. G. (2010) 'The evolution and future of Earth's nitrogen cycle.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 330(6001), pp. 192–6. doi: 10.1126/science.1186120.

Carlson, C. A. and Ingraham, J. L. (1983) 'Comparison of denitrification by Pseudomonas stutzeri, Pseudomonas aeruginosa, and paracoccus denitrificans', *Applied and Environmental Microbiology*. American Society for Microbiology, 45(4), pp. 1247–1253. doi: 10.1128/aem.45.4.1247-1253.1983.

Carter, J. P. *et al.* (1995) 'Soil and sediment bacteria capable of aerobic nitrate respiration', *Applied and Environmental Microbiology*. American Society for Microbiology, 61(8), pp. 2852–2858. doi: 10.1128/aem.61.8.2852-2858.1995.

Chai, W. and Stewart, V. (1998) 'NasR, a novel RNA-binding protein, mediates nitrate-responsive transcription antitermination of the Klebsiella oxytoca M5al nasF operon leader in vitro', *Journal of Molecular Biology*. Academic Press, 283(2), pp. 339–351. doi: 10.1006/jmbi.1998.2105.

Chen, P. and Reitzer, L. J. (1995) 'Active contribution of two domains to cooperative DNA binding of the enhancer-binding protein nitrogen regulator I (NtrC) of Escherichia coli: Stimulation by phosphorylation and the binding of ATP', *Journal of Bacteriology*. American Society for Microbiology, 177(9), pp. 2490–2496. doi: 10.1128/jb.177.9.2490-2496.1995.

Chung, I. M. *et al.* (2017) 'Effects of soil type and organic fertilizers on fatty acids and vitamin E in Korean ginseng (Panax ginseng Meyer)', *Food Research International*. Elsevier Ltd, 102, pp. 265–273. doi: 10.1016/j.foodres.2017.10.003.

Clarke, T. A. *et al.* (2008) 'Escherichia coli Cytochrome c Nitrite Reductase NrfA', in *Methods in Enzymology*. Academic Press Inc., pp. 63–77. doi: 10.1016/S0076-

6879(07)37004-3.

Daniel, R., Simon, M. and Wemheuer, B. (2018) ‘Editorial: Molecular Ecology and Genetic Diversity of the Roseobacter Clade’, *Frontiers in Microbiology*. Frontiers Media S.A., 9(JUN), p. 1185. doi: 10.3389/fmicb.2018.01185.

Ellington, M. J. K. *et al.* (2002) ‘Hierarchy of carbon source selection in Paracoccus pantotrophus: strict correlation between reduction state of the carbon substrate and aerobic expression of the nap operon.’, *Journal of bacteriology*. American Society for Microbiology Journals, 184(17), pp. 4767–74. doi: 10.1128/JB.184.17.4767-4774.2002.

Enoch, H.G. and Lester, R. L. (1975) ‘The purification and properties of formate dehydrogenase and nitrate reductase from Escherichia coli.’, *Journal of Biological Chemistry*, 250(17), pp. 6693–6705.

Erisman, J. W. *et al.* (2008) ‘How a century of ammonia synthesis changed the world’, *Nature Geoscience*, 1(10), pp. 636–639. doi: 10.1038/ngeo325.

Fani, R., Gallo, R. and Liò, P. (2000) ‘Molecular evolution of nitrogen fixation: The evolutionary history of the nifD, nifK, nifE, and nifN genes’, *Journal of Molecular Evolution*. Springer New York, 51(1), pp. 1–11. doi: 10.1007/s002390010061.

Farver, O. *et al.* (2003) ‘Allosteric control of internal electron transfer in cytochrome cd1 nitrite reductase’, *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 100(13), pp. 7622–7625. doi: 10.1073/pnas.0932693100.

Felgate, H. *et al.* (2012) ‘The impact of copper, nitrate and carbon status on the emission of nitrous oxide by two species of bacteria with biochemically distinct denitrification pathways’, *Environmental Microbiology*, 14(7), pp. 1788–1800. doi: 10.1111/j.1462-2920.2012.02789.x.

Felux, A. K. *et al.* (2013) ‘Paracoccus denitrificans PD1222 utilizes hypotaurine via transamination followed by spontaneous desulfination to yield acetaldehyde and, finally, acetate for growth’, *Journal of Bacteriology*, 195(12), pp. 2921–2930. doi: 10.1128/JB.00307-13.

Field, S. J. *et al.* (2008) ‘The Respiratory Nitric Oxide Reductase (NorBC) from Paracoccus denitrificans’, in *Methods in Enzymology*. Academic Press Inc., pp. 79–101. doi: 10.1016/S0076-6879(07)37005-5.

Figurski, D. H. and Helinski, D. R. (1979) ‘Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans.’, *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 76(4), pp. 1648–52. doi: 10.1073/pnas.76.4.1648.

Flores, E. *et al.* (2005) ‘Photosynthetic nitrate assimilation in cyanobacteria’, *Photosynthesis Research*. Kluwer Academic Publishers, 83(2), pp. 117–133. doi: 10.1007/s11120-004-5830-9.

Forchhammer, K. and De Marsac, N. T. (1995) ‘Functional analysis of the phosphoprotein P(II) (glnB gene product) in the cyanobacterium Synechococcus sp. strain PCC 7942’, *Journal of Bacteriology*. American Society for Microbiology, 177(8), pp. 2033–2040. doi: 10.1128/jb.177.8.2033-2040.1995.

Frías, J. E., Flores, E. and Herrero, A. (2000) ‘Activation of the Anabaena nir operon promoter requires both NtcA (CAP family) and NtcB (LysR family) transcription factors’, *Molecular Microbiology*, 38(3), pp. 613–625. doi: 10.1046/j.1365-2958.2000.02156.x.

Gangeswaran, R., Lowe, D. J. and Eady, R. R. (1993) ‘Purification and characterization of the assimilatory nitrate reductase of Azotobacter vinelandii’, *Biochemical Journal*. Portland Press, 289(2), pp. 335–342. doi: 10.1042/bj2890335.

Gates, A. J. *et al.* (2011) ‘A composite biochemical system for bacterial nitrate and nitrite assimilation as exemplified by Paracoccus denitrificans.’, *The Biochemical journal*. Portland Press Limited, 435(3), pp. 743–53. doi: 10.1042/BJ20101920.

Gates, A. J., Richardson, D. J. and Butt, J. N. (2008) ‘Voltammetric characterization of the aerobic energy-dissipating nitrate reductase of Paracoccus pantotrophus: exploring the activity of a redox-balancing enzyme as a function of electrochemical potential.’, *The Biochemical journal*. Portland Press Limited, 409(1), pp. 159–68. doi: 10.1042/BJ20071088.

Genest, O. *et al.* (2008) ‘Dedicated metallochaperone connects apoenzyme and molybdenum cofactor biosynthesis components’, *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 283(31), pp. 21433–21440. doi: 10.1074/jbc.M802954200.

Gheysari, M. *et al.* (2009) ‘Nitrate leaching in a silage maize field under different irrigation and nitrogen fertilizer rates’, *Agricultural Water Management*, 96(6), pp. 946–

954. doi: 10.1016/j.agwat.2009.01.005.

Giannopoulos, G. *et al.* (2017) 'Tuning the modular *Paracoccus denitrificans* respirome to adapt from aerobic respiration to anaerobic denitrification', *Environmental Microbiology*. Wiley/Blackwell (10.1111), 19(12), pp. 4953–4964. doi: 10.1111/1462-2920.13974.

Gilbert, P. (1985) 'The theory and relevance of continuous culture', *Journal of Antimicrobial Chemotherapy*. Oxford University Press (OUP), 15(suppl A), pp. 1–6. doi: 10.1093/jac/15.suppl_a.1.

Glaser, P. *et al.* (1995) 'Identification and isolation of a gene required for nitrate assimilation and anaerobic growth of Bacillus subtilis', *Journal of Bacteriology*. American Society for Microbiology Journals, 177(4), pp. 1112–1115. doi: 10.1128/jb.177.4.1112-1115.1995.

Goddard, A. D. *et al.* (2017) 'The Paracoccus denitrificans NarK-like nitrate and nitrite transporters-probing nitrate uptake and nitrate/nitrite exchange mechanisms', *Molecular Microbiology*. Wiley/Blackwell (10.1111), 103(1), pp. 117–133. doi: 10.1111/mmi.13546.

Godden, J. W. *et al.* (1991) 'The 2.3 angstrom x-ray structure of nitrite reductase from Achromobacter cycloclastes', *Science*. American Association for the Advancement of Science, 253(5018), pp. 438–442. doi: 10.1126/science.1862344.

Goldman, B. S., Lin, J. T. and Stewart, V. (1994) 'Identification and structure of the nasR gene encoding a nitrate- and nitrite-responsive positive regulator of nasFEDCBA (nitrate assimilation) operon expression in Klebsiella pneumoniae M5al', *Journal of Bacteriology*. American Society for Microbiology, 176(16), pp. 5077–5085. doi: 10.1128/jb.176.16.5077-5085.1994.

Hahnke, S. M. *et al.* (2014) 'An improved medium for the anaerobic growth of Paracoccus denitrificans Pd1222', *Frontiers in Microbiology*. Frontiers Research Foundation, 5(JAN). doi: 10.3389/fmicb.2014.00018.

Haltia, T. *et al.* (2003) 'Crystal structure of nitrous oxide reductase from Paracoccus denitrificans at 1.6 Å resolution', *Biochemical Journal*. Portland Press, 369(1), pp. 77–88. doi: 10.1042/BJ20020782.

Hanahan, D. (1983) 'Studies on transformation of Escherichia coli with plasmids',

Journal of Molecular Biology. Academic Press, 166(4), pp. 557–580. doi: 10.1016/S0022-2836(83)80284-8.

Harms, N. *et al.* (1985) 'Isolation and characterization of Paracoccus denitrificans mutants with defects in the metabolism of one-carbon compounds.', *Journal of bacteriology*. American Society for Microbiology Journals, 164(3), pp. 1064–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3905763 (Accessed: 18 February 2020).

Harper, C. J. *et al.* (2010) 'Glutamate dehydrogenase and glutamine synthetase are regulated in response to nitrogen availability in Myocbacterium smegmatis', *BMC Microbiology*. BioMed Central, 10(1), p. 138. doi: 10.1186/1471-2180-10-138.

Hederstedt, L. and Rutberg, L. (1981) 'Succinate dehydrogenase - a comparative review', *Microbiological Reviews*, pp. 542–555.

van Heeswijk, W. C., Westerhoff, H. V. and Boogerd, F. C. (2013) 'Nitrogen Assimilation in Escherichia coli: Putting Molecular Data into a Systems Perspective', *Microbiology and Molecular Biology Reviews*. American Society for Microbiology, 77(4), pp. 628–695. doi: 10.1128/mmbr.00025-13.

Hendriks, J. *et al.* (2000) 'Nitric oxide reductases in bacteria', *Biochimica et Biophysica Acta - Bioenergetics*. Elsevier, 1459(2–3), pp. 266–273. doi: 10.1016/S0005-2728(00)00161-4.

Hino, T. *et al.* (2010) 'Structural basis of biological N2O generation by bacterial nitric oxide reductase', *Science*. American Association for the Advancement of Science, 330(6011), pp. 1666–1670. doi: 10.1126/science.1195591.

Houten, S. M. and Wanders, R. J. A. (2010) 'A general introduction to the biochemistry of mitochondrial fatty acid β-oxidation', *Journal of Inherited Metabolic Disease*, pp. 469–477. doi: 10.1007/s10545-010-9061-2.

Iobbi-Nivol, C. and Leimkühler, S. (2013) 'Molybdenum enzymes, their maturation and molybdenum cofactor biosynthesis in Escherichia coli', *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. Elsevier B.V., 1827(8–9), pp. 1086–1101. doi: 10.1016/j.bbabio.2012.11.007.

Jackson, R. H., Cornish-Bowden, A. and Cole, J. A. (1981) 'Prosthetic groups of the NADH-dependent nitrite reductase from Escherichia coli K12.', *The Biochemical*

journal. Portland Press Ltd, 193(3), pp. 861–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7030314 (Accessed: 24 March 2018).

Jandl, G. *et al.* (2005) 'Contribution of primary organic matter to the fatty acid pool in agricultural soils', *Soil Biology and Biochemistry*. Pergamon, 37(6), pp. 1033–1041. doi: 10.1016/j.soilbio.2004.10.018.

Jepson, B. J. N. *et al.* (2006) 'Evolution of the soluble nitrate reductase: Defining the monomeric periplasmic nitrate reductase subgroup', in *Biochemical Society Transactions*. Portland Press, pp. 122–126. doi: 10.1042/BST0340122.

John, P. and Whatley, F. R. (1975) 'Paracoccus denitrificans and the evolutionary origin of the mitochondrion', *Nature*. Nature Publishing Group, 254(5500), pp. 495–498. doi: 10.1038/254495a0.

John, P. and Whatley, F.R. (1977) 'The bioenergetics of Paracoccus denitrificans', *Biochimica et Biophysica Acta (BBA) - Reviews on Bioenergetics*. Elsevier, 463(2), pp. 129–153. doi: 10.1016/0304-4173(77)90006-4.

John, P. and Whatley, F. R. (1977) 'The bioenergetics of Paracoccus denitrificans', *BBA Reviews On Bioenergetics*. Elsevier, pp. 129–153. doi: 10.1016/0304-4173(77)90006-4.

Johnson, J. L., Rajagopalan, K. V. and Meyer, O. (1990) 'Isolation and characterization of a second molybdopterin dinucleotide: Molybdopterin cytosine dinucleotide', *Archives of Biochemistry and Biophysics*. Academic Press, 283(2), pp. 542–545. doi: 10.1016/0003-9861(90)90681-N.

Knowles, R. (1982) 'Denitrification', *Microbiological Reviews*. American Society for Microbiology (ASM), pp. 43–70. doi: 10.1128/mmbr.46.1.43-70.1982.

Kraft, B. *et al.* (2014) 'Nitrogen cycling. The environmental controls that govern the end product of bacterial nitrate respiration.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 345(6197), pp. 676–9. doi: 10.1126/science.1254070.

Kremeckova, H., Svrcula, B. and Mikes, V. (1992) 'Purification and some properties of glutamate dehydrogenase and glutamine synthetase from Paracoccus denitrificans', *Journal of General Microbiology*. Microbiology Society, 138(8), pp. 1587–1591. doi: 10.1099/00221287-138-8-1587.

Kristjansson, J. K., Walter, B. and Hollocher, T. C. (1978) 'Respiration-dependent proton translocation and the transport of nitrate and nitrite in Paracoccus denitrificans and other

denitrifying bacteria', *Biochemistry*, 17(23), pp. 5014–5019. doi: 10.1021/bi00616a024.

Lake, M. W. *et al.* (2000) 'The crystal structure of the Escherichia coli MobA protein provides insight into molybdopterin guanine dinucleotide biosynthesis', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 275(51), pp. 40211–40217. doi: 10.1074/jbc.M007406200.

Lecture, N. and Haber, F. (1920) *The synthesis of ammonia from its elements*.

Lin, J. T. and Stewart, V. (1997) 'Nitrate assimilation by bacteria', *Advances in Microbial Physiology*. Academic Press, pp. 1–30. doi: 10.1016/s0065-2911(08)60014-4.

Lledó, B. *et al.* (2005) 'Identification and transcriptional analysis of nitrate assimilation genes in the halophilic archaeon Haloferax mediterranei', *Gene*. Elsevier, 361(1–2), pp. 80–88. doi: 10.1016/j.gene.2005.07.011.

Luo, H. and Moran, M. A. (2014) 'Evolutionary Ecology of the Marine Roseobacter Clade', *Microbiology and Molecular Biology Reviews*. American Society for Microbiology, 78(4), pp. 573–587. doi: 10.1128/mmbr.00020-14.

Luque-Almagro, V. M. *et al.* (2011) 'Bacterial nitrate assimilation: Gene distribution and regulation', in *Biochemical Society Transactions*, pp. 1838–1843. doi: 10.1042/BST20110688.

Luque-Almagro, V. M. *et al.* (2013) 'Nitrogen oxyanion-dependent dissociation of a two-component complex that regulates bacterial nitrate assimilation.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 288(41), pp. 29692–702. doi: 10.1074/jbc.M113.459032.

Luque-Almagro, V. M. *et al.* (2017) 'Transcriptional and translational adaptation to aerobic nitrate anabolism in the denitrifierParacoccus denitrificans.', *The Biochemical journal*. Portland Press Limited, 474(11), pp. 1769–1787. doi: 10.1042/BCJ20170115.

Lycus, P. *et al.* (2018) 'A bet-hedging strategy for denitrifying bacteria curtails their release of N2O', *Proceedings of the National Academy of Sciences of the United States of America*. NLM (Medline), 115(46), pp. 11820–11825. doi: 10.1073/pnas.1805000115.

Maeda, S. I. and Omata, T. (1997) 'Substrate-binding lipoprotein of the cyanobacterium Synechococcus sp. strain PCC 7942 involved in the transport of nitrate and nitrite', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 272(5), pp. 3036–3041. doi: 10.1074/jbc.272.5.3036.

Magalon, A. *et al.* (1997) ‘Characterization by electron paramagnetic resonance of the role of the Escherichia coli nitrate reductase (NarGHI) iron-sulfur clusters in electron transfer to nitrate and identification of a semiquinone radical intermediate’, *Journal of Bacteriology*. American Society for Microbiology, 179(16), pp. 5037–5045. doi: 10.1128/jb.179.16.5037-5045.1997.

MartÄ±Ì• nez-Espinosa, R. M., Marhuenda-Egea, F. C. and Bonete, M. J. (2001) ‘Purification and characterisation of a possible assimilatory nitrite reductase from the halophile archaeon *Haloferax mediterranei*’, *FEMS Microbiology Letters*. Oxford University Press, 196(2), pp. 113–118. doi: 10.1111/j.1574-6968.2001.tb10550.x.

MEERS, J. L., TEMPEST, D. W. and BROWN, C. M. (1970) ‘“Glutamine(amide): 2-Oxoglutarate Amino Transferase Oxido-reductase (NADP)”, an Enzyme Involved in the Synthesis of Glutamate by Some Bacteria’, *Journal of General Microbiology*. Microbiology Society, 64(2), pp. 187–194. doi: 10.1099/00221287-64-2-187.

Merrick, M. J. and Edwards, R. A. (1995) ‘Nitrogen control in bacteria.’, *Microbiology and Molecular Biology Reviews*, 59(4).

Milham, P. J. *et al.* (1970) ‘Analysis of plants, soils and waters for nitrate by using an ion-selective electrode’, *The Analyst*, 95(1133), pp. 751–757. doi: 10.1039/an9709500751.

Moir, J. W. B. and Ferguson, S. J. (1994) ‘Properties of a Paracoccus denitrificans mutant deleted in cytochrome C550 indicate that a copper protein can substitute for this cytochrome in electron transport to nitrite, nitric oxide and nitrous oxide’, *Microbiology*. Microbiology Society, 140(2), pp. 389–397. doi: 10.1099/13500872-140-2-389.

Moir, J. W. B. and Wood, N. J. (2001) ‘Nitrate and nitrite transport in bacteria’, *Cellular and Molecular Life Sciences*. Birkhauser Verlag Basel, pp. 215–224. doi: 10.1007/PL00000849.

Morozkina, E. V. and Zvyagilskaya, R. A. (2007) ‘Nitrate reductases: Structure, functions, and effect of stress factors’, *Biochemistry (Moscow)*, 72(10), pp. 1151–1160. doi: 10.1134/S0006297907100124.

Murphy, M. E. P., Turley, S. and Adman, E. T. (1997) ‘Structure of nitrite bound to copper-containing nitrite reductase from Alcaligenes faecalis: Mechanistic implications’, *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 272(45), pp. 28455–28460. doi: 10.1074/jbc.272.45.28455.

Murphy, M. J. *et al.* (1974) 'Siroheme: A new prosthetic group participating in six electron reduction reactions catalyzed by both sulfite and nitrite reductases', *Proceedings of the National Academy of Sciences of the United States of America*, 71(3), pp. 612–616. doi: 10.1073/pnas.71.3.612.

Nagatani, H., Shimizu, M. and Valentine, R. C. (1971) 'The mechanism of ammonia assimilation in nitrogen fixing bacteria', *Archiv für Mikrobiologie*. Springer-Verlag, 79(2), pp. 164–175. doi: 10.1007/BF00424923.

Nakano, M. M. *et al.* (1995) *Nitrogen Regulation of nasA and the nasB Operon, Which Encode Genes Required for Nitrate Assimilation in Bacillus subtilis †, JOURNAL OF BACTERIOLOGY*. Available at: http://jb.asm.org/ (Accessed: 7 February 2020).

Nakano, M. M. *et al.* (1998) *Nitrogen and Oxygen Regulation of Bacillus subtilis nasDEF Encoding NADH-Dependent Nitrite Reductase by TnrA and ResDE, JOURNAL OF BACTERIOLOGY*. Available at: http://jb.asm.org/ (Accessed: 7 February 2020).

Noji, S. *et al.* (1989) 'The *narK* gene product participates in nitrate transport induced in *Escherichia coli* nitrate-respiring cells', *FEBS Letters*. John Wiley & Sons, Ltd, 252(1–2), pp. 139–143. doi: 10.1016/0014-5793(89)80906-8.

Ohashi, Y. *et al.* (2011) 'Regulation of nitrate assimilation in cyanobacteria', *Journal of Experimental Botany*, pp. 1411–1424. doi: 10.1093/jxb/erq427.

Olaya-Abril, A., Hidalgo-Carrillo, J., *et al.* (2018) 'Exploring the Denitrification Proteome of Paracoccus denitrificans PD1222', *Frontiers in Microbiology*. Frontiers, 9, p. 1137. doi: 10.3389/fmicb.2018.01137.

Olaya-Abril, A., Luque-Almagro, V. M., *et al.* (2018) 'Poly(3-hydroxybutyrate) hyperproduction by a global nitrogen regulator NtrB mutant strain of Paracoccus denitrificans PD1222', *FEMS Microbiology Letters*. Oxford University Press, 365(1). doi: 10.1093/femsle/fnx251.

Palmer, T. *et al.* (1996) 'Involvement of the narJ and mob gene products in distinct steps in the biosynthesis of the molybdoenzyme nitrate reductase in Escherichia coli', *Molecular Microbiology*. John Wiley & Sons, Ltd, 20(4), pp. 875–884. doi: 10.1111/j.1365-2958.1996.tb02525.x.

Pengpeng, W. and Tan, Z. (2013) 'Ammonia Assimilation in Rumen Bacteria: A Review', *Animal Biotechnology*. Taylor & Francis Group , pp. 107–128. doi:

10.1080/10495398.2012.756402.

Pinchbeck, B. J. *et al.* (2019) 'A dual functional redox enzyme maturation protein for respiratory and assimilatory nitrate reductases in bacteria', *Molecular Microbiology*, p. mmi.14239. doi: 10.1111/mmi.14239.

Prepas, E. E. and Charette, T. (2003) 'Worldwide Eutrophication of Water Bodies: Causes, Concerns, Controls', in *Treatise on Geochemistry*. Elsevier Inc., pp. 311–331. doi: 10.1016/B0-08-043751-6/09169-6.

Prieto, M. A. *et al.* (2007) 'Synthesis and degradation of polyhydroxyalkanoates', in *Pseudomonas*. Springer Netherlands, pp. 397–428. doi: 10.1007/978-1-4020-6097-7_14.

Rajagopalan, K.V. and Johnson, J. L. (1992) 'The pterin molybdenum cofactors', *Journal of Biological Chemistry*, 267(15), pp. 10199–10202.

Ramanan, R. *et al.* (2016) 'Algae-bacteria interactions: Evolution, ecology and emerging applications', *Biotechnology Advances*. Elsevier Inc., pp. 14–29. doi: 10.1016/j.biotechadv.2015.12.003.

Rehm, B. H. A. (2007) *Biogenesis of Microbial Polyhydroxyalkanoate Granules: a Platform Technology for the Production of Tailor-made Bioparticles*. Available at: www.cimb.org (Accessed: 26 October 2019).

Richardson, D. J. *et al.* (1988a) 'The role of auxiliary oxidants in maintaining redox balance during phototrophic growth of Rhodobacter capsulatus on propionate or butyrate', *Archives of Microbiology*. Springer-Verlag, 150(2), pp. 131–137. doi: 10.1007/BF00425152.

Richardson, D. J. *et al.* (1988b) 'The role of auxiliary oxidants in maintaining redox balance during phototrophic growth of Rhodobacter capsulatus on propionate or butyrate', *Archives of Microbiology*. Springer-Verlag, 150(2), pp. 131–137. doi: 10.1007/BF00425152.

Rothery, R. A. *et al.* (1998) 'The molybdenum cofactor of Escherichia coli nitrate reductase a (NarGHI): Effect of a mobAB mutation and interactions with [Fe-S] clusters', *Journal of Biological Chemistry*, 273(13), pp. 7462–7469. doi: 10.1074/jbc.273.13.7462.

Rouault, T. A. and Klausner, R. D. (1996) 'Iron-sulfur clusters as biosensors of oxidants and iron', *Trends in Biochemical Sciences*. Elsevier Ltd, pp. 174–177. doi: 10.1016/S0968-0004(96)10024-4.

Rowe, J. J. *et al.* (1994) ‘Nark is a nitrite-extrusion system involved in anaerobic nitrate respiration by Escherichia coli’, *Molecular Microbiology*. John Wiley & Sons, Ltd, 12(4), pp. 579–586. doi: 10.1111/j.1365-2958.1994.tb01044.x.

Ruiz-Ponte, C. *et al.* (1999) ‘The benefit of a Roseobacter species on the survival of scallop larvae’, *Marine Biotechnology*. Springer New York, 1(1), pp. 52–59. doi: 10.1007/PL00011751.

Sacomboio, E. N. M. *et al.* (2017) ‘The transcriptional regulator NtrC controls glucose-6-phosphate dehydrogenase expression and polyhydroxybutyrate synthesis through NADPH availability in Herbaspirillum seropedicae’, *Scientific Reports*. Nature Publishing Group, 7(1), pp. 1–12. doi: 10.1038/s41598-017-12649-0.

Sakamoto, N., Kotre, A. M. and Savageau, M. A. (1975) ‘Glutamate dehydrogenase from Escherichia coli: purification and properties.’, *Journal of bacteriology*. American Society for Microbiology Journals, 124(2), pp. 775–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/241744 (Accessed: 16 February 2020).

Sanders, D. A. *et al.* (1992) ‘Phosphorylation site of NtrC, a protein phosphatase whose covalent intermediate activates transcription’, *Journal of Bacteriology*, 174(15), pp. 5117–5122. doi: 10.1128/jb.174.15.5117-5122.1992.

Schäfer, A. *et al.* (1994) ‘Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum’, *Gene*. Elsevier, 145(1), pp. 69–73. doi: 10.1016/0378-1119(94)90324-7.

Schindler, D. W. *et al.* (1971) ‘Eutrophication of Lake 227, Experimental Lakes Area, Northwestern Ontario, by Addition of Phosphate and Nitrate’, *Journal of the Fisheries Research Board of Canada*, 28(11), pp. 1763–1782. doi: 10.1139/f71-261.

Schulz, H. (1991) ‘Beta oxidation of fatty acids’, *Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism*. Elsevier, pp. 109–120. doi: 10.1016/0005-2760(91)90015-A.

Sears, H. J. *et al.* (1993) ‘The identification of a periplasmic nitrate reductase in *Paracoccus denitrificans*’, *FEMS Microbiology Letters*. Narnia, 113(1), pp. 107–111. doi: 10.1111/j.1574-6968.1993.tb06496.x.

Sears, H. J. *et al.* (1997) ‘Identification of an assimilatory nitrate reductase in mutants of

Paracoccus denitrificans GB17 deficient in nitrate respiration', *Archives of Microbiology*, 167(1), pp. 61–66. doi: 10.1007/s002030050417.

Sears, H. J., Spiro, S. and Richardson, D. J. (1997) 'Effect of carbon substrate and aeration on nitrate reduction and expression of the periplasmic and membrane-bound nitrate reductases in carbon-limited continuous cultures of Paracoccus denitrificans Pd1222', *Microbiology*. Microbiology Society, 143(12), pp. 3767–3774. doi: 10.1099/00221287-143-12-3767.

Sebilo, M. *et al.* (2013) 'Long-term fate of nitrate fertilizer in agricultural soils', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 110(45), pp. 18185–18189. doi: 10.1073/pnas.1305372110.

Shevchenko, A. *et al.* (2006) 'In-gel digestion for mass spectrometric characterization of proteins and proteomes', *Nature Protocols*. Nature Publishing Group, 1(6), pp. 2856–2860. doi: 10.1038/nprot.2006.468.

Shi, X. M., Zhang, X. W. and Chen, F. (2000) 'Heterotrophic production of biomass and lutein by Chlorella protothecoides on various nitrogen sources', *Enzyme and Microbial Technology*. Elsevier Science Inc, 27(3–5), pp. 312–318. doi: 10.1016/S0141-0229(00)00208-8.

Snyder, S.W. and Hollocher, T. . (1987) 'Purification and some characteristics of nitrous oxide reductase from Paracoccus denitrifican', *Journal of Biological Chemistry*, 262(14), pp. 6515–6525.

Solomonson$, L. P. *et al.* (1986) *THE JOURNAL OF BIOLOGICAL CHEMISTRY Functional Domains of Assimilatory NADH:Nitrate Reductase from Chlorella**.

Van Spanning, R. J. M. *et al.* (1995) 'Regulation of oxidative phosphorylation: The flexible respiratory network of Paracoccus denitrificans', *Journal of Bioenergetics and Biomembranes*. Kluwer Academic Publishers-Plenum Publishers, 27(5), pp. 499–512. doi: 10.1007/BF02110190.

Spencer, J. B. *et al.* (1993) 'The *Escherichia coli cysG* gene encodes the multifunctional protein, siroheme synthase', *FEBS Letters*. John Wiley & Sons, Ltd, 335(1), pp. 57–60. doi: 10.1016/0014-5793(93)80438-Z.

SteinrÃ¼cke, P. and Ludwig, B. (1993) ' Genetics of Paracoccus denitrificans ', *FEMS*

Microbiology Letters. Oxford University Press (OUP), 104(1–2), pp. 83–117. doi: 10.1111/j.1574-6968.1993.tb05865.x.

Stevenson, F. J. and Firestone, M. K. (1982) 'Biological Denitrification', in *Nitrogen in Agricultural Soils*. John Wiley & Sons, Ltd, pp. 289–326. doi: 10.2134/agronmonogr22.c8.

Stouthamer, A. H. (1991) 'Metabolic regulation including anaerobic metabolism in Paracoccus denitrificans', *Journal of Bioenergetics and Biomembranes*. Kluwer Academic Publishers-Plenum Publishers, 23(2), pp. 163–185. doi: 10.1007/BF00762216.

Strange, R. W. *et al.* (1999) 'Structural and kinetic evidence for an ordered mechanism of copper nitrite reductase', *Journal of Molecular Biology*. Academic Press, 287(5), pp. 1001–1009. doi: 10.1006/jmbi.1999.2648.

Stüeken, E. E. *et al.* (2016) 'The evolution of Earth's biogeochemical nitrogen cycle', *Earth-Science Reviews*. Elsevier, 160, pp. 220–239. doi: 10.1016/J.EARSCIREV.2016.07.007.

Sullivan, M. J. *et al.* (2013) 'Copper control of bacterial nitrous oxide emission and its impact on vitamin B12-Dependent metabolism', *Proceedings of the National Academy of Sciences of the United States of America*, 110(49), pp. 19926–19931. doi: 10.1073/pnas.1314529110.

Sutton, M. A. *et al.* (2011) 'Too much of a good thing', *Nature*, pp. 159–161. doi: 10.1038/472159a.

Swanson, R. V., Alex, L. A. and Simon, M. I. (1994) 'Histidine and aspartate phosphorylation: two-component systems and the limits of homology', *Trends in Biochemical Sciences*, 19(11), pp. 485–490. doi: 10.1016/0968-0004(94)90135-X.

Tabuchi, H., Kato, K. and Nioh, I. (2008) 'Season and soil management affect soil microbial communities estimated using phospholipid fatty acid analysis in a continuous cabbage (*Brassica oleracea* var. *capitata*) cropping system', *Soil Science and Plant Nutrition*. Taylor & Francis Group , 54(3), pp. 369–378. doi: 10.1111/j.1747-0765.2008.00242.x.

Taniguchi, S. and Itagaki, E. (1960) 'Nitrate reductase of nitrate respiration type from E. COLI. I. Solubilization and purification from the particulate system with molecular characterization as a metalloprotein', *BBA - Biochimica et Biophysica Acta*. Elsevier,

44(C), pp. 263–279. doi: 10.1016/0006-3002(60)91562-6.

Temple, C. A. and Rajagopalan, K. V. (2000) ‘Mechanism of assembly of the bis(molybdopterin guanine dinucleotide)molybdenum cofactor in Rhodobacter sphaeroides dimethyl sulfoxide reductase’, *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 275(51), pp. 40202–40210. doi: 10.1074/jbc.M007407200.

Tett, A. J. *et al.* (2012) ‘Regulatable vectors for environmental gene expression in Alphaproteobacteria’, *Applied and Environmental Microbiology*. American Society for Microbiology, 78(19), pp. 7137–7140. doi: 10.1128/AEM.01188-12.

Tichi, M. A. and Tabita, F. R. (2001) ‘Interactive Control of Rhodobacter capsulatus Redox-Balancing Systems during Phototrophic Metabolism’, *JOURNAL OF BACTERIOLOGY*, 183(21), pp. 6344–6354. doi: 10.1128/JB.183.21.6344-6354.2001.

Tovar, A. *et al.* (2000) ‘Environmental impacts of intensive aquaculture in marine waters’, *Water Research*. Pergamon, 34(1), pp. 334–342. doi: 10.1016/S0043-1354(99)00102-5.

Tripathy, B. C., Sherameti, I. and Oelmüller, R. (2010a) ‘Siroheme: An essential component for life on earth’, *Plant Signaling and Behavior*, 5(1), pp. 14–20. doi: 10.4161/psb.5.1.10173.

Tripathy, B. C., Sherameti, I. and Oelmüller, R. (2010b) ‘Siroheme: An essential component for life on earth’, *Plant Signaling and Behavior*, 5(1), pp. 14–20. doi: 10.4161/psb.5.1.10173.

Udvardi1t, M. K. *et al.* (1992) *Evolution of the (Brady)Rhizobium-Legume Symbiosis: Why Do Bacteroids Fix Nitrogen?*, *Symbiosis*.

Vega-Palas, M. A. *et al.* (1990) ‘Identification and cloning of a regulatory gene for nitrogen assimilation in the cyanobacterium Synechococcus sp. strain PCC 7942’, *Journal of Bacteriology*, 172(2), pp. 643–647. doi: 10.1128/jb.172.2.643-647.1990.

Vega, J. M., Garrett, R. H. and Siegel, L. M. (1975) *Siroheme: A Prosthetic Group of the Neurospora crassa Assimilatory Nitrite Reductase" Downloaded from, THE JOURNAL OF BIOLOGKX CHEMISTRY*. Available at: http://www.jbc.org/ (Accessed: 5 February 2020).

Vergnes, A. *et al.* (2006) ‘NarJ chaperone binds on two distinct sites of the aponitrate

reductase of Escherichia coli to coordinate molybdenum cofactor insertion and assembly', *Journal of Biological Chemistry*, 281(4), pp. 2170–2176. doi: 10.1074/jbc.M505902200.

VISHNIAC, W. and SANTER, M. (1957) 'The thiobacilli.', *Bacteriological reviews*. American Society for Microbiology (ASM), 21(3), pp. 195–213. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13471458 (Accessed: 10 April 2018).

Vojvodic, A. *et al.* (2014) 'Exploring the limits: A low-pressure, low-temperature Haber-Bosch process', *Chemical Physics Letters*. Elsevier B.V., 598, pp. 108–112. doi: 10.1016/j.cplett.2014.03.003.

de Vries, G. E. *et al.* (1989) 'Isolation and characterization of Paracoccus denitrificans mutants with increased conjugation frequencies and pleiotropic loss of a (nGATCn) DNA-modifying property', *Archives of Microbiology*. Springer-Verlag, 152(1), pp. 52–57. doi: 10.1007/BF00447011.

Waller, Z. A. E. *et al.* (2016) 'Control of bacterial nitrate assimilation by stabilization of G-quadruplex DNA', *Chemical Communications*. Royal Society of Chemistry, 52(92), pp. 13511–13514. doi: 10.1039/c6cc06057a.

Weiss, V., Claverie-Martin, F. and Magasanik, B. (1992) 'Phosphorylation of nitrogen regulator I of Escherichia coli induces strong cooperative binding to DNA essential for activation of transcription', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 89(11), pp. 5088–5092. doi: 10.1073/pnas.89.11.5088.

Weiss, V. and Magasanik, B. (1988) 'Phosphorylation of nitrogen regulator I (NR(I)) of Escherichia coli', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 85(23), pp. 8919–8923. doi: 10.1073/pnas.85.23.8919.

Willems, H. P. L. *et al.* (1997) 'Nitrate removal in riparian wetland soils: Effects of flow rate, temperature, nitrate concentration and soil depth', *Water Research*, 31(4), pp. 841–849. doi: 10.1016/S0043-1354(96)00315-6.

Williams, P. A. *et al.* (1995) 'Pseudospecific docking surfaces on electron transfer proteins as illustrated by pseudoazurin, cytochrome c550 and cytochrome cd1 nitrite reductase', *Nature Structural Biology*. Nature Publishing Group, 2(11), pp. 975–982. doi: 10.1038/nsb1195-975.

Wood, W. B. (1966) ‘Host specificity of DNA produced by Escherichia coli: Bacterial mutations affecting the restriction and modification of DNA’, *Journal of Molecular Biology*. Academic Press, 16(1), pp. 118-IN3. doi: 10.1016/S0022-2836(66)80267-X.

Wray, L. V. *et al.* (1996) ‘TnrA, a transcription factor required for global nitrogen regulation in Bacillus subtilis’, *Proceedings of the National Academy of Sciences of the United States of America*, 93(17), pp. 8841–8845. doi: 10.1073/pnas.93.17.8841.

Wu, S. Q. *et al.* (1999) ‘General nitrogen regulation of nitrate assimilation regulatory gene nasR expression in Klebsiella oxytoca M5al.’, *Journal of bacteriology*. American Society for Microbiology Journals, 181(23), pp. 7274–84. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10572131 (Accessed: 14 February 2020).

Wuebbens, M. M. and Rajagopalan, K. V. (2003) ‘Mechanistic and mutational studies of Escherichia coli molybdopterin synthase clarify the final step of molybdopterin biosynthesis’, *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 278(16), pp. 14523–14532. doi: 10.1074/jbc.M300453200.

Zinchenko, V. *et al.* (2006) ‘Mutation in ntrC gene leading to the derepression of nitrogenase synthesis in Rhodobacter sphaeroides’, *FEMS Microbiology Letters*. Oxford University Press (OUP), 147(1), pp. 57–61. doi: 10.1111/j.1574-6968.1997.tb10220.x.