

Transcriptional and Environmental Control of Bacterial Denitrification and N₂O Emissions

Invited mini-review for upcoming 'New insights in the Nitrogen Cycle' issue

Keywords: denitrification, transcription, nitrous oxide, *Paracoccus denitrificans*, *nosZ*, sRNA

Hannah Gaimster*, Mark Alston, David Richardson, Andrew Gates and Gary Rowley.

*Corresponding author:

Email: h.gaimster@uea.ac.uk

Address: School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ

Telephone: 01603 597269

Denitrification is an important biogeochemical process, which when disrupted, leads to production of a greenhouse gas, nitrous oxide. Here we describe the factors which are important in regulating this process.

Abstract

In oxygen-limited environments, denitrifying bacteria can switch from oxygen-dependent respiration to nitrate (NO₃⁻) respiration in which the NO₃⁻ is sequentially reduced via nitrite (NO₂⁻), nitric oxide (NO) and nitrous oxide (N₂O) to dinitrogen (N₂). However, atmospheric N₂O continues to rise, a significant proportion of which is

microbial in origin. This implies that the enzyme responsible for N₂O reduction, nitrous oxide reductase (NosZ), does not always carry out the final step of denitrification either efficiently, or in synchrony with the rest of the pathway. Despite a solid understanding of the biochemistry underpinning denitrification, there is a relatively poor understanding of how environmental signals and respective transcriptional regulators control expression of the denitrification apparatus. This mini-review will describe the current picture for transcriptional regulation of denitrification in the model bacterium, *Paracoccus denitrificans*, highlighting differences in other denitrifying bacteria where appropriate, as well as gaps in our understanding. Alongside this, the emerging role of small regulatory RNAs (sRNAs) in regulation of denitrification will be discussed. We will conclude by speculating how this information, aside from providing a better understanding of the denitrification process, can be translated into development of novel greenhouse gas mitigation strategies.

Introduction

Bacteria are able to survive in a wide range of constantly fluctuating environments. The ability to thrive in such dynamic surroundings will depend on the ability of the bacteria to adapt its metabolic apparatus to utilise changes in both carbon source and electron acceptor availability. In particular, a high degree of flexibility is required to deal with temporal fluctuations in oxygen availability (Richardson *et al.*, 2009). One way some bacteria are able to cope with low oxygen levels is by using a process termed denitrification. In oxygen-limited environments, such as those encountered in a water logged soil, denitrifying bacteria can switch from O₂-respiration to nitrate (NO₃⁻) respiration in which NO₃⁻ is converted via nitrite (NO₂⁻), nitric oxide (NO) and N₂O to dinitrogen (N₂). Four essential reductases which sequentially reduce nitrate (*nar*), nitrite (*nir*), nitric oxide (*nor*) and nitrous oxide (*nos*) to dinitrogen are required for complete denitrification (Zumft, 1997). Many years' worth of work by many different groups across the globe has helped develop a comprehensive understanding of the biochemistry of the enzymes which catalyse the reactions of denitrification (Nar, Nir, Nor and Nos). Many Gram-negative bacteria catalyse the reduction of nitrate using a membrane-bound nitrate reductase (Nar),

however, it is important to note that in some other bacteria, this reaction is instead performed by a periplasmic nitrate reductase (Nap) (Bedmar *et al.*, 2005).

Both ATP and growth yield from oxygen respiration is significantly higher than from denitrification (Strohm *et al.*, 2007). Therefore, it is energetically efficient to downregulate the denitrification enzymes in the presence of oxygen. A strict regulation of the downstream NO_x reductases is also likely to be essential to avoid accumulation of the toxic denitrification intermediates NO and, to a lesser extent, NO₂⁻, depending on the pH (Baumann, 1997). Another important intermediate produced during denitrification is N₂O. Under certain conditions, the final step in denitrification is not performed efficiently and as a result N₂O is released into the atmosphere (Felgate *et al.*, 2012). Crucially, N₂O is a potent greenhouse gas with, molecule-for-molecule, a ~300-fold greater global warming potential than CO₂. It has also been described as the biggest single cause of ozone depletion over the Arctic (Ravishankara *et al.*, 2009). The atmospheric loading of N₂O is increasing by 0.25% each year, it has a long atmospheric lifetime of ~150 years and contributes to approximately 9% of the global radiative forcing of total greenhouse gas emissions (Richardson *et al.*, 2009, Skiba *et al.*, 2012, Smith *et al.*, 2012). The only known biological sink for N₂O is through its reduction to N₂ by N₂O reductase, NosZ (Richardson *et al.*, 2009).

Despite the global importance of denitrification and ever increasing N₂O emissions, comparatively little work has been carried out to map the regulatory networks that modulate the assembly and activity of the denitrification apparatus at the level of gene expression. As such, there is relatively poor understanding of how environmental signals and corresponding transcriptional regulators control synthesis of the denitrification apparatus. This mini-review will bring together what is currently known about transcriptional regulators in denitrifying bacteria and will focus on 3 main questions.

- Which transcriptional regulators sense key environmental variables?
- What processes does each sensory element regulate and how much functional overlap is there between regulators in a species and between species?

- What new techniques are emerging to help us study this and how can we use this information to help develop novel N₂O mitigation strategies?

The Gram negative α - proteobacteria *Paracoccus denitrificans* is arguably the best studied bacterial denitrifier so this mini-review will focus mainly on the study on transcriptional regulators in this organism as a paradigm, but will also highlight key differences in other organisms where relevant. *P. denitrificans* is often described as a soil-dwelling bacteria, but is found in a wide range of terrestrial and aquatic environments. *P. denitrificans* has become a model organism for two key reasons, firstly it is biochemically and genetically tractable and secondly, it grows well under denitrifying conditions in the laboratory (Spiro, 2017).

1. The role of FnrP, NNR and NarR in regulating denitrification in *P. denitrificans*

In *P. denitrificans* the expression of the Nar, Nir, Nor and Nos enzymes is regulated, at the level of gene expression, by environmental signals that include (but probably are not limited to) nitrate, nitrite, NO, oxygen and copper. This transcriptional regulation is controlled, at least in part, by regulators FnrP (fumarate and nitrate reduction protein), NNR (nitrite reductase and nitric oxide reductase regulator) and NarR (nitrate reductase regulator) (summarised in figure 1) which are all members of the FNR (fumarate and nitrate reduction) /CRP (cyclic-AMP receptor protein) super family of transcriptional regulators.

FnrP acts as a transcriptional activator of the *nar* and *nos* operons in *P. denitrificans* in response to oxygen and NO, as indicated in figure 1 (Van Spanning, 1997, Bergaust *et al.*, 2012). A FnrP mutant strain of *P. denitrificans* shows a five-fold reduction in its growth rate under anaerobic, denitrifying conditions, compared to wild type *P. denitrificans*. The activity of Nar in this strain is reduced by three-fold, whilst Nir activity remained at a comparable level to that of the wild type strain (Van Spanning, 1997). In *P. denitrificans*, FnrP has four conserved cysteine residues (Cys14, 17, 25 and 113) which coordinate a 4Fe–4S cluster. It was recently demonstrated that FnrP undergoes O₂-driven [4Fe–4S] to [2Fe–2S] cluster

conversion and that this conversion leads to separation of the transcriptionally active FnrP dimer into monomers. In addition to this, this work showed that the FnrP [4Fe–4S] cluster reacts, *in vitro*, with multiple NO molecules which also leads to dissociation into monomers (Crack *et al.*, 2016). However, there has been some discussion as to whether this accurately reflects the physiologically relevant response of potentially much lower levels of NO found in denitrifying cultures (Spiro, 2017). Despite this, this *in vitro* work agrees well with work from other groups that provided *in vivo* evidence that, as well as its function as an O₂ sensor, FnrP regulates gene expression of target genes in response to NO (Bergaust *et al.*, 2012). *P. denitrificans* encodes a second transcriptional regulator, NNR, which is homologous to the FnrP protein, although it lacks the N-terminal cysteines. NNR activates the expression of the genes encoding the nitrite (*nir*) and nitric oxide reductases (*nor*) (Van Spanning *et al.*, 1995). A NNR mutant strain of *P. denitrificans* shows a two-fold reduction in its growth rate under anaerobic, denitrifying conditions, compared to wild type *P. denitrificans*. This mutant strain accumulates nitrite and was completely deficient in Nir activity. This initial characterisation of a NNR mutant showed no effect on Nos activity, but more recent work suggests NNR does regulate transcription of the *nos* operon (Bergaust *et al.*, 2012). Like FnrP, NNR is sensitive to oxygen and NO. NNR has been shown to be rapidly inactivated following a shift to aerobic growth conditions, indicating oxygen directly inactivates the protein. Genetic manipulation of NNR showed that mutant NNR alleles that encode NNR proteins that have significant activity in anaerobic cultures in the absence of NO, but remain inactive in aerobic cultures. Therefore, NNR is a dual sensor of both oxygen and NO, and this work suggested a haem-based mechanism would provide a way for NNR to sense both these entities (Lee *et al.*, 2006).

The third member of the FNR/CRP encoded by *P. denitrificans* is the nitrite/nitrate-sensitive NarR protein. This is the least studied of the three FNR/CRP family members, but has been shown to respond to nitrate and/or nitrite (indicated in figure 1). NarR was shown to be needed for maximal expression of Nar but had no other regulatory function related to denitrification. NarR has also been shown to require nitrate and/or nitrite in order to activate gene expression. However, it is also possible that NarR responds in an indirect way to nitrate and/or nitrite (Wood *et al.*, 2001, Bergaust *et al.*, 2012).

It is interesting to speculate why, in *P. denitrificans*, and in many other species of denitrifiers as will become clear throughout the review, regulation of denitrification is controlled by multiple FNR/CRP type proteins. For example, in *P. denitrificans* FnrP, NNR and NarR all have DNA binding motifs, which are, at the sequence level, indistinguishable (Spiro, 2017). Indeed, in one of the first descriptions of the role of FnrP and NNR, it was postulated that currently unidentified factors are likely to be important in the control mechanisms of these regulators. This remains an important and interesting question.

1.1 The role of FnrP and NNR homologues in regulating denitrification in other denitrifiers

Homologues of FNR proteins also exist in many other bacterial denitrifiers, including *Pseudomonas* species. In *Pseudomonas* species, control of transcriptional regulation of genes involved in denitrification is, much like in *P. denitrificans*, dominated by members of the FNR/CRP super family of transcriptional regulators. In *P. aeruginosa*, a FnrP equivalent known as ANR (anaerobic regulator of arginine deiminase and nitrate reductase) activates transcription of a gene cluster which encodes a nitrate transporter and nitrate reductase in response to limited oxygen viability (Schreiber *et al.*, 2007). ANR works in much the same way as *P. denitrificans* FnrP, in that it has a [4Fe-4S] cluster, which upon exposure to oxygen or NO is partly destroyed resulting in the transcription factor losing its ability to bind to DNA and regulate gene expression (Yoon *et al.*, 2007). In addition to this, ANR acts to promote transcription of a second regulator, DNR (dissimilatory nitrate respiration regulator), and in turn, DNR activates transcription of *nir* and *nor* in response to NO (Arai *et al.*, 1997). DNR acts as a heme-based NO sensor, the heme is 6-coordinate; external NO and CO can replace an internal ligand and specific DNA binding occurs only when the heme is nitrosylated (Lobato *et al.*, 2014).

P. stutzeri encodes 4 FNR-type proteins. Three of the FNR homologues, known as *dnrD*, *dnrE* and *dnrS*, encode regulators of a separate subgroup within the FNR family. The main difference between these proteins and other members of the family is the lack of cysteine residues for complexing the [4Fe-4S] centre typical of redox-

active FNR-type regulators. Interestingly, they also form a phylogenetic lineage separate from the FixK branch of FNR proteins (covered later in the review), which also do not have the characteristic cysteine motif. DnrD is a key regulator of denitrification and works by activation of expression of the genes for nitrite reductase and NO reductase (Vollack *et al.*, 1999). The final FNR homologue, known as FnrA, perhaps surprisingly, has no role in denitrification (Cuypers & Zumft, 1993).

More recently, the work of some groups has turned to focus on the role of regulators during denitrification in the marine environment. One marine species where the role of FNR like proteins has been addressed is the heterotrophic α -proteobacterium *Dinoroseobacter shibae*. *D. shibae* encodes 7 FNR/CRP-like regulators (Wagner-Dobler *et al.*, 2009). Four of these regulators, FnrL, DnrD, DnrE, and DnrF, have been shown to work in concert in a regulatory network during denitrification. Only FnrL contains cysteine residues potentially involved in iron-sulfur cluster formation and so plays the crucial role as an oxygen-sensitive activator of the denitrification genes in this bacteria (Ebert *et al.*, 2017).

There are also numerous proteins homologous to NNR in a wide range of denitrifiers. *Rhodobacter sphaeroides* encodes a homologue called NnrR. Mutation of *nnrR* prevents growth on nitrite, as well as the reduction of nitrite and NO. NnrR was shown to be a transcriptional activator of nitrite reductase and nitric oxide reductase (Tosques *et al.*, 1996). In addition to this, *R. sphaeroides* encodes an additional regulator known as *nnrS* and expression of *nnrS* is controlled by NnrR (Bartnikas *et al.*, 2002).

Bradyrhizobium diazoefficiens (previously known as *Bradyrhizobium japonicum*) is a soybean symbiont which can fix nitrogen as well as perform denitrification. *B. diazoefficiens* encodes a regulator homologous to NNR. Mutation of NNR left *B. diazoefficiens* unable to grow anaerobically on nitrate or nitrite. NnrR controls expression of *nir* and *nor* genes in response to NO (Mesa *et al.*, 2003, Bueno *et al.*, 2017). *B. diazoefficiens* NnrR works to regulate expression of denitrification genes alongside FixLJ-FixK₂. In this system, *B. diazoefficiens* utilises a cytoplasmic histidine kinase, known as FixL as a sensor for oxygen. Low oxygen levels cause FixL to undergo auto-phosphorylation, and in turn, transfer the phosphate group to

FixJ. In turn, FixJ activates FixK2 that induces expression of the *napEDABC*, *nirK*, *norCBQD* and *nosRZDYFLX* denitrification genes (Bueno *et al.*, 2017; Torres *et al.*, 2017) as well as other regulatory genes [e.g., *rpoN1*, *fixK1*, and *nnrR*; (Mesa *et al.*, 2003, Mesa *et al.*, 2008)]. Thus, NnrR adds an additional control level to the FixLJ-FixK2 cascade integrating the NO signal necessary for induction of *norCBQD* genes expression in *B. diazoefficiens* (Mesa *et al.*, 2003; Bueno *et al.*, 2017). Most recently, it has been shown that FixK₂ regulates expression of the *nos* operon in response to low oxygen availabilities in *B. diazoefficiens* (Torres *et al.*, 2017).

There are other additional proteins which play roles in regulating denitrification in *B. diazoefficiens* which are homologous to RegAB, a histidine kinase/response regulator pair which is involved in modulating gene expression in response to redox changes (Elsen *et al.*, 2004). In *B. diazoefficiens* they are known as RegSR. RegR plays a key role in activation of expression of *B. diazoefficiens* *nor* and *nos* operons, in response to nitrate and low oxygen levels (Torres *et al.*, 2014). *P. denitrificans* encodes homologues of RegAB, but the role of these, if any, in regulation of denitrification is yet to be determined in this organism. Further to this, a third oxygen-responsive regulatory cascade mediated by the nitrogen fixation regulatory protein, NifA is also used in regulation of denitrification in *B. diazoefficiens*. Mutation of *nifA* caused slower growth of *B. diazoefficiens* when grown under denitrifying conditions and reduced expression of *nir* and *nor*. NifA is part of the σ^{54} enhancer binding protein family but perhaps surprisingly, the activity of NifA in this context is independent of σ^{54} (Bueno *et al.*, 2010). It seems increasingly clear that regulation of denitrification in *B. diazoefficiens* is much more complicated than in other denitrifying bacteria. It has been suggested that this is likely due to the lifestyle of this organism as a soybean symbiont, where denitrification may be playing a key role (Meakin *et al.*, 2007, Spiro, 2017).

The vast majority of work on transcriptional regulators of denitrification has been in denitrifying bacteria. However recent work has, for the first time, described a transcriptional regulator of denitrification in the Archaea (Hattori *et al.*, 2016). NarO is encoded upstream of the respiratory nitrate reductase gene of *Haloferax volcanii*. Disruption of the *narO* gene resulted in inhibited growth under denitrifying conditions,

with the expression of the recombinant NarO allowing growth to be restored. A novel CX_nCXCX₇C motif in the N terminus of NarO was identified and showed to be well conserved showing in homologous NarO proteins found in other haloarchaea (Hattori *et al.*, 2016).

2.3 Other environmental factors important in regulation of denitrification

FNR, NNR and NarR are crucial regulators in term of sensing and responding to oxygen and denitrification intermediates. However, it is also important for denitrifiers to sense and respond to other external factors. For example, it has been appreciated for many years that copper is an important factor in NosZ activity but recent transcriptomic analyses demonstrated the importance of copper in regulating expression of the *nos* operon at the level of gene expression. This work also demonstrated the importance of the accessory proteins NosC and NosR in copper dependent expression of the *nos* operon (as indicated in figure 1), with their exact role in the process still to be determined (Sullivan *et al.*, 2013). This paper also showed that the downregulation of expression of *nosZ* in copper-limited environments, leads to net emission of N₂O, which in turn fine tunes expression of genes controlled by vitamin B₁₂ riboswitches, because N₂O inactivates vitamin B₁₂ (Sullivan *et al.*, 2013).

A similar recent study investigated the effect of zinc depletion on the *P. denitrificans* transcriptome and showed that genes encoding nitric oxide reductase (*norCB*) and nitrite reductase (*nirS*) were upregulated. Interestingly, *nosC* was also shown to be upregulated nearly 10-fold. Additionally, the data also suggested that the *norCB* genes are directly regulated by the zinc uptake regulator (Zur). This is an interesting and currently unexplained result, with the authors acknowledging that more work is required to understand the physiological function of regulatory crosstalk between zinc limitation and denitrification (Neupane *et al.*, 2017).

Other environmental factors including pH have also been linked to the transcriptional regulation of the enzymes involved in denitrification. In particular, it has been well documented that the ratio N₂O:N₂ produced is increased when the pH of soils is lower, reviewed by (Šimek & Cooper, 2002). There has been some suggestion that

pH can impact expression of *nosZ*. In a mixed culture experiment using soils from field experiments, the *nosZ* : *nirS* ratio was higher in pH 6.1 than in pH 8.0 soil. Furthermore, the level of *nosZ* transcripts were maintained at higher levels in pH 6.1 soil throughout the period of active denitrification, whereas in pH 8.0 soil, a rapid decline was observed (Liu *et al.*, 2010). However, more recent work on pure cultures of *P. denitrificans* suggests that relative transcription rates of *nosZ* versus *nirS* and *norB* were unaffected by pH, leading to the conclusion that the higher levels of N₂O emitted from low pH cultures was due to lower levels of NosZ protein synthesis/assembly rather than transcription (Bergaust *et al.*, 2010).

2. The role of sigma factors in regulating denitrification

Sigma factors are used by many bacterial species as a mechanism through which the expression of a group of genes, which are usually functionally linked, can be controlled. Sigma factors are capable of controlling the expression of genes under their influence through their ability to enable transcriptional activation of specific gene promoters through recognition of the promoter region. This promoter recognition function requires the formation of a σ -bound RNA polymerase holoenzyme complex. The primary sigma factor of the bacterial cell, σ^{70} , is responsible for the transcription of most essential and growth-related genes (reviewed in (Feklístov *et al.*, 2014).

Regarding denitrification, sigma factor σ^{54} (encoded by the *rpoN* gene) was originally described as it is involved in the expression of nitrogen-regulated genes; since then many other physiological functions are related to this factor (Kustu *et al.*, 1989). One organism which requires σ^{54} for anaerobic growth on nitrate is *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*), suggesting that σ^{54} is essential to regulate the expression of genes involved in denitrification in this organism (Warellmann *et al.*, 1989). The sigma-54 dependence of denitrification in this organism can be at least partly explained by the fact that *R. eutropha* uses a sigma-54 dependent regulator, NorR, to activate transcription of the *nor* operon ((Pohlmann *et al.*, 2000).

In contrast to this, a *Pseudomonas aeruginosa rpoN* mutant grows anaerobically on nitrate. However, σ^{54} was shown to control diverse sets of genes, including those encoding glutamine synthetase, urease, and flagellin (Totten *et al.*, 1990). In *Pseudomonas stutzeri* analysis of the levels of Nar, Nir, Nor and Nos proteins in *RpoN*-null mutants showed that σ^{54} affected both nitrite reductase and nitric oxide reductase activity, but not the transcription of their structural genes (*nir* and *nor*) – suggesting σ^{54} plays a role in post translational processes rather than at the level of gene expression (Härtig & Zumft, 1998). Interestingly, *P. denitrificans* encodes a σ^{54} homologue (amongst other alternative sigma factors) and the potential role of this sigma factor in regulation of the denitrification apparatus is currently under investigation in the authors' laboratory.

3. The emerging role of sRNAs in regulation of denitrification

Another mechanism by which transcription of genes can be regulated which has been rather neglected in *P. denitrificans* and indeed, denitrifying bacteria more generally, is by using small RNAs (sRNAs). Bacterial sRNAs are an emerging class of regulatory RNAs which are ~40-500 nucleotides in length. sRNAs are either able to repress the expression of target mRNA transcripts with which they share similar complementarity by preventing translation or decreasing mRNA stability. sRNAs are also able to activate expression of gene targets via mRNA pairing. sRNAs often act to regulate multiple targets; this means that a single sRNA can globally modulate a particular physiological response to an environmental change, in a way analogous to a transcription factor reviewed in (Papenfort & Vanderpool, 2015). sRNAs have been particularly well studied in enteric pathogenic bacteria such as *E. coli* and *Salmonella* species. Indeed, a sRNA was recently shown to play a role in nitrogen metabolism in *Salmonella*, SdsN regulated the levels of the nitrate- and nitrite-responsive NarP transcription factor (Hao *et al.*, 2016). Some work has been done on describing the sRNA landscape in denitrifiers, perhaps most notably on *P. aeruginosa*, but mainly in the context of virulence (Gómez-Lozano *et al.*, 2012). A description of the sRNA complement expressed by in the marine denitrifier *Ruegeria pomeroyi* was described, however, again not specifically under denitrifying conditions (Rivers *et al.*, 2016).

Recently, our laboratory has identified 167 putative sRNAs across the *P. denitrificans* genome specifically under denitrifying conditions. Figure 2 shows the putative sRNAs encoded on chromosome 2 on *P. denitrificans*. Importantly, 35% of these sRNAs were differentially expressed under high N₂O and low N₂O emitting conditions respectively, suggesting they may play a role in production or reduction of N₂O. Use of target prediction software for the sRNAs suggested that the most commonly predicted targets were transcriptional regulators such as the Xre, Fis and TetR families, as indicated in figure 2 (Gaimster *et al.*, 2016). This finding was consistent with the work of other groups where global regulators are subject to regulation by multiple sRNAs (Lee & Gottesman, 2016). Additionally, many predicted targets of the sRNAs included proteins implicated in transport. Interestingly, this was the most commonly predicted target of sRNAs in *R. pomeroyi*. It is therefore possible that regulation of transporters may be a conserved role for sRNA across these related species. We therefore suggest that sRNAs might play a crucial role, via targeting transcriptional regulators or transporter proteins, in modulating the denitrification pathway of *P. denitrificans* and related denitrifying bacteria.

In addition to this, we have also recently sought to define the transcriptional landscape in *P. denitrificans* also under high N₂O and low N₂O emitting conditions using differential-RNAseq (dRNAseq). dRNAseq differentiates between processed and unprocessed transcripts through treatment of one RNA library with a terminator exonuclease. This results in enrichment of primary transcripts, allowing us to identify individual -10 and -35 promoter motifs by characterizing the first nucleotide of a transcript, known as the Transcription Start Site (TSS) (Sharma *et al.*, 2010). From this dataset we have identified a number of N₂O responsive TSS along the *nos* operon, and an additional N₂O responsive promoter upstream of *nosC*, the first gene in the *nos* operon, which explains the differential expression of this gene in comparison to the rest of the operon as reported in (Sullivan *et al.*, 2013). This points to a novel transcriptional regulator controlling this cluster, which we are currently characterizing as part of our other investigations.

Conclusions and Outlook

This review sought to bring together the current knowledge regarding the role of transcriptional regulators in denitrification. Despite much diversity regarding the specific regulators and their roles across denitrifiers, important, and somewhat conserved, roles are played by members of the FNR/CRP super family of regulators. These regulators respond to a wide range of environmental signal in various ways, with the most important being oxygen and NO. Specific combinations of multiple transcriptional regulators are used by the different species of bacteria to induce a precise regulatory response. Although many of the individual characteristics of these regulators as well as other environmental factors involved in denitrification have been described, much remains to be done to fully elucidate how these regulators work in concert in the cell to create a functionally successful denitrifying phenotype.

Despite almost 20 years of intensive research on the biochemistry and modular nature of denitrification in *P. denitrificans* and other denitrifying bacteria, we still have many key questions that remain unanswered, regarding how environmental signals are integrated into the regulation of this organism, and the associated global transcriptional networks that mediate this rapid respiratory adaptation. These questions must be answered if we are serious about mitigating N₂O from microbial origins.

New approaches and recently developed technologies are likely to be crucial in helped resolve some of the remaining unanswered questions highlighted throughout the review. Taking a transcriptomics based approach has been useful in helping to define the entire regulon of a given transcription factor. For example, the regulon of the least well-studied transcriptional regulator in *P. denitrificans* NarR has been recently been defined using a microarray approach (Giannopoulos *et al.*, in press). In a $\Delta narR$ strain expression of *nar* and the *nap* nitrate reductases were down regulated, when compared to wild type *P. denitrificans*. Additionally, expression of *nir* and *nos* were upregulated compared to wild type *P. denitrificans*, as were the *cycA* and *pazS* genes encoding the electron transfer proteins cytochrome c550 and pseudoazurin, respectively. This transcriptome data suggests that NarR can serve to both induce (*nar*, *nap*) and repress (*nir*, *nos*, *pasZ*, *cycA*) components of the

denitrification respirome, whereas previously this regulator was thought to only play a part in the regulation of *narGHI* (Wood *et al.*, 2001).

The recent discovery of *nosZ* clade II organisms (Jones *et al.*, 2013) has potential to help further understand transcriptional regulation in denitrification. Understanding the environmental cues and transcriptional regulators which allow them to act as a sink for N₂O in the soil (Jones *et al.*, 2014) could potentially allow us to exploit them as a potential N₂O mitigation strategy. It is also likely that organisms with a partial complement of denitrification genes and enzymes are important players in N₂O production and consumption, reviewed recently by (Hallin *et al.*, 2017).

In addition to these new techniques, work of some groups has recently taken a computational approach to understand the dynamics of gene regulation in denitrification. In a model of *P. denitrificans*, by suggesting all cells express *nosZ*, with only a small minority expressing *nar* and *nir* and *nor*, this can explain the transient accumulation of NO₂⁻ and N₂O observed in cultures growing under denitrifying conditions (Hassan *et al.*, 2016). Furthermore, another recent modelling study reported that it is likely that delayed enzymatic reactions could be primarily controlled by transcriptional responses, highlighting the importance of regulators in denitrification (Song *et al.*, 2017).

Continued use of new technologies will help generate a robust and comprehensive picture of the transcriptional organization of the genome of *P. denitrificans* and other denitrifying bacteria under a range of physiologically and environmentally relevant conditions. This will in turn, hopefully help inform future experiments of laboratories in the search to identify novel mitigation strategies to reduce N₂O emissions. For example, it is possible to imagine a strategy whereby fertiliser components are carefully manipulated to ensure crop yields remain high whilst reducing the amount of N₂O emitted from the soil, perhaps by targeting expression of critical nodes in the regulatory cascades that controls denitrification and *nosZ* expression. An example of this based on our current studies ((Sullivan *et al.*, 2013, Gaimster *et al.*, 2016) could be copper content of fertilisers. It will also be important to move these approaches from pure culture experiments to more realistic soil

microcosm experiments, with the ultimate goal of applying this to real world environments.

Funding

This work was supported by the Biotechnology and Biological Sciences Research Council (United Kingdom) grant number BB/L022796/1.

References

- Arai H, Kodama T & Igarashi Y (1997) Cascade regulation of the two CRP/FNR-related transcriptional regulators (ANR and DNR) and the denitrification enzymes in *Pseudomonas aeruginosa*. *Molecular Microbiology* **25**: 1141-1148.
- Bartnikas TB, Wang Y, Bobo T, Veselov A, Scholes CP & Shapleigh JP (2002) Characterization of a member of the NnrR regulon in *Rhodobacter sphaeroides* 2.4.3 encoding a haem–copper protein. *Microbiology* **148**: 825-833.
- Baumann vdMJ, Snozzi M, Zehnder AJ. (1997) Inhibition of denitrification activity but not of mRNA induction in *Paracoccus denitrificans* by nitrite at a suboptimal pH. *Antonie Van Leeuwenhoek* **72**: 183-189.
- Bedmar EJ, Robles EF & Delgado MJ (2005) The complete denitrification pathway of the symbiotic, nitrogen-fixing bacterium *Bradyrhizobium japonicum*. *Biochemical Society Transactions* **33**: 141-144.
- Bergaust L, Mao Y, Bakken LR & Frostegård Å (2010) Denitrification Response Patterns during the Transition to Anoxic Respiration and Posttranscriptional Effects of Suboptimal pH on Nitrogen Oxide Reductase in *Paracoccus denitrificans*. *Applied and Environmental Microbiology* **76**: 6387-6396.
- Bergaust L, van Spanning RJM, Frostegård Å & Bakken LR (2012) Expression of nitrous oxide reductase in *Paracoccus denitrificans* is regulated by oxygen and nitric oxide through FnrP and NNR. *Microbiology* **158**: 826-834.
- Bueno E, Mesa S, Sanchez C, Bedmar EJ & Delgado MJ (2010) NifA is required for maximal expression of denitrification genes in *Bradyrhizobium japonicum*. *Environmental Microbiology* **12**: 393-400.
- Bueno E, Robles EF, Torres MJ, Krell T, Bedmar EJ, Delgado MJ & Mesa S (2017) Disparate response to microoxia and nitrogen oxides of the *Bradyrhizobium japonicum* napEDABC, nirK and norCBQD denitrification genes. *Nitric Oxide* **68**: 137-149.
- Crack JC, Hutchings MI, Thomson AJ & Le Brun NE (2016) Biochemical properties of *Paracoccus denitrificans* FnrP: reactions with molecular oxygen and nitric oxide. *JBIC Journal of Biological Inorganic Chemistry* **21**: 71-82.
- Cuyper H & Zumft WG (1993) Anaerobic control of denitrification in *Pseudomonas stutzeri* escapes mutagenesis of an fnr-like gene. *Journal of Bacteriology* **175**: 7236-7246.
- Ebert M, Laaß S, Thürmer A, Roselius L, Eckweiler D, Daniel R, Härtig E & Jahn D (2017) FnrL and Three Dnr Regulators Are Used for the Metabolic Adaptation to Low Oxygen Tension in *Dinoroseobacter shibae*. *Frontiers in Microbiology* **8**.
- Elsen S, Swem LR, Swem DL & Bauer CE (2004) RegB/RegA, a Highly Conserved Redox-Responding Global Two-Component Regulatory System. *Microbiology and Molecular Biology Reviews* **68**: 263-279.

- Feklístov A, Sharon BD, Darst SA & Gross CA (2014) Bacterial Sigma Factors: A Historical, Structural, and Genomic Perspective. *Annual Review of Microbiology* **68**: 357-376.
- Felgate H, Giannopoulos G, Sullivan MJ, Gates AJ, Clarke TA, Baggs E, Rowley G & Richardson DJ (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**: 1788-1800.
- Gaimster H, Chalklen L, Alston M, Munnoch JT, Richardson DJ, Gates AJ & Rowley G (2016) Genome-Wide Discovery of Putative sRNAs in *Paracoccus denitrificans* Expressed under Nitrous Oxide Emitting Conditions. *Frontiers in Microbiology* **7**.
- Gómez-Lozano M, Marvig RL, Molin S & Long KS (2012) Genome-wide identification of novel small RNAs in *Pseudomonas aeruginosa*. *Environmental Microbiology* **14**: 2006-2016.
- Hallin S, Philippot L, Löffler FE, Sanford RA & Jones CM (2017) Genomics and Ecology of Novel N₂O-Reducing Microorganisms. *Trends in Microbiology*.
- Hao Y, Updegrave TB, Livingston NN & Storz G (2016) Protection against deleterious nitrogen compounds: role of σ (S)-dependent small RNAs encoded adjacent to *sdiA*. *Nucleic Acids Research* **44**: 6935-6948.
- Härtig E & Zumft WG (1998) The Requirement of RpoN (Sigma Factor ζ 54) in Denitrification by *Pseudomonas stutzeri* Is Indirect and Restricted to the Reduction of Nitrite and Nitric Oxide. *Applied and Environmental Microbiology* **64**: 3092-3095.
- Hassan J, Qu Z, Bergaust LL & Bakken LR (2016) Transient Accumulation of NO₂(-) and N₂O during Denitrification Explained by Assuming Cell Diversification by Stochastic Transcription of Denitrification Genes. *PLoS Computational Biology* **12**: e1004621.
- Hattori T, Shiba H, Ashiki K-i, Araki T, Nagashima Y-k, Yoshimatsu K & Fujiwara T (2016) Anaerobic Growth of Haloarchaeon *Haloferax volcanii* by Denitrification Is Controlled by the Transcription Regulator NarO. *Journal of Bacteriology* **198**: 1077-1086.
- Jones CM, Graf DRH, Bru D, Philippot L & Hallin S (2013) The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *ISME J* **7**: 417-426.
- Jones CM, Spor A, Brennan FP, Breuil M-C, Bru D, Lemanceau P, Griffiths B, Hallin S & Philippot L (2014) Recently identified microbial guild mediates soil N₂O sink capacity. *Nature Clim Change* **4**: 801-805.
- Kustu S, Santero E, Keener J, Popham D & Weiss D (1989) Expression of sigma 54 (*ntrA*)-dependent genes is probably united by a common mechanism. *Microbiological Reviews* **53**: 367-376.
- Lee H-J & Gottesman S (2016) sRNA roles in regulating transcriptional regulators: Lrp and SoxS regulation by sRNAs. *Nucleic Acids Research* **44**: 6907-6923.
- Lee Y-Y, Shearer N & Spiro S (2006) Transcription factor NNR from *Paracoccus denitrificans* is a sensor of both nitric oxide and oxygen: isolation of *nnr** alleles encoding effector-independent proteins and evidence for a haem-based sensing mechanism. *Microbiology* **152**: 1461-1470.
- Liu B, Mørkved PT, Frostegård Å & Bakken LR (2010) Denitrification gene pools, transcription and kinetics of NO, N₂O and N₂ production as affected by soil pH. *FEMS Microbiology Ecology* **72**: 407-417.
- Lobato L, Bouzahir-Sima L, Yamashita T, Wilson MT, Vos MH & Liebl U (2014) Dynamics of the Heme-binding Bacterial Gas-sensing Dissimilative Nitrate Respiration Regulator (DNR) and Activation Barriers for Ligand Binding and Escape. *The Journal of Biological Chemistry* **289**: 26514-26524.
- Meakin GE, Bueno E, Jepson B, Bedmar EJ, Richardson DJ & Delgado MJ (2007) The contribution of bacteroidal nitrate and nitrite reduction to the formation of nitrosylhaemoglobin complexes in soybean root nodules. *Microbiology* **153**: 411-419.
- Mesa S, Bedmar EJ, Chanfon A, Hennecke H & Fischer H-M (2003) *Bradyrhizobium japonicum* NnrR, a Denitrification Regulator, Expands the FixLJ-FixK(2) Regulatory Cascade. *Journal of Bacteriology* **185**: 3978-3982.

Mesa S, Hauser F, Friberg M, Malaguti E, Fischer H-M & Hennecke H (2008) Comprehensive Assessment of the Regulons Controlled by the FixLJ-FixK(2)-FixK(1) Cascade in *Bradyrhizobium japonicum*. *Journal of Bacteriology* **190**: 6568-6579.

Neupane DP, Jacquez B, Sundararajan A, Ramaraj T, Schilkey FD & Yukl ET (2017) Zinc-Dependent Transcriptional Regulation in *Paracoccus denitrificans*. *Frontiers in Microbiology* **8**: 569.

Papenfors K & Vanderpool CK (2015) Target activation by regulatory RNAs in bacteria. *FEMS Microbiology Reviews* **39**: 362-378.

Pohlmann A, Cramm R, Schmelz K & Friedrich B (2000) A novel NO-responding regulator controls the reduction of nitric oxide in *Ralstonia eutropha*. *Molecular Microbiology* **38**: 626-638.

Ravishankara AR, Daniel JS & Portmann RW (2009) Nitrous oxide (N₂O): the dominant ozone-depleting substance emitted in the 21st century. *Science* **326**: 123-125.

Richardson D, Felgate H, Watmough N, Thomson A & Baggs E (2009) Mitigating release of the potent greenhouse gas N₂O from the nitrogen cycle – could enzymic regulation hold the key? *Trends in Biotechnology* **27**: 388-397.

Rivers AR, Burns AS, Chan L-K & Moran MA (2016) Experimental Identification of Small Non-Coding RNAs in the Model Marine Bacterium *Ruegeria pomeroyi* DSS-3. *Frontiers in Microbiology* **7**.

Schreiber K, Krieger R, Benkert B, Eschbach M, Arai H, Schobert M & Jahn D (2007) The Anaerobic Regulatory Network Required for *Pseudomonas aeruginosa* Nitrate Respiration. *Journal of Bacteriology* **189**: 4310-4314.

Sharma CM, Hoffmann S, Darfeuille F, *et al.* (2010) The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature* **464**: 250-255.

Šimek M & Cooper JE (2002) The influence of soil pH on denitrification: progress towards the understanding of this interaction over the last 50 years. *European Journal of Soil Science* **53**: 345-354.

Skiba U, Jones SK, Dragosits U, *et al.* (2012) UK emissions of the greenhouse gas nitrous oxide. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* **367**: 1175-1185.

Smith KA, Mosier AR, Crutzen PJ & Winiwarter W (2012) The role of N₂O derived from crop-based biofuels, and from agriculture in general, in Earth's climate. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* **367**: 1169-1174.

Song H-S, Thomas DG, Stegen JC, Li M, Liu C, Song X, Chen X, Fredrickson JK, Zachara JM & Scheibe TD (2017) Regulation-Structured Dynamic Metabolic Model Provides a Potential Mechanism for Delayed Enzyme Response in Denitrification Process. *Frontiers in Microbiology* **8**.

Spiro S (2017) CHAPTER 13 Regulation of Denitrification. *Metalloenzymes in Denitrification: Applications and Environmental Impacts*, p. 312-330. The Royal Society of Chemistry.

Strohm TO, Griffin B, Zumft WG & Schink B (2007) Growth Yields in Bacterial Denitrification and Nitrate Ammonification. *Applied and Environmental Microbiology* **73**: 1420-1424.

Sullivan MJ, Gates AJ, Appia-Ayme C, Rowley G & Richardson DJ (2013) Copper control of bacterial nitrous oxide emission and its impact on vitamin B(12)-dependent metabolism. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 19926-19931.

Torres MJ, Argandoña M, Vargas C, Bedmar EJ, Fischer H-M, Mesa S & Delgado MJ (2014) The Global Response Regulator RegR Controls Expression of Denitrification Genes in *Bradyrhizobium japonicum*. *PLoS ONE* **9**: e99011.

Torres MJ, Bueno E, Jiménez-Leiva A, Cabrera JJ, Bedmar EJ, Mesa S & Delgado MJ (2017) FixK(2) Is the Main Transcriptional Activator of *Bradyrhizobium diazoefficiens* nosRZDYFLX Genes in Response to Low Oxygen. *Frontiers in Microbiology* **8**: 1621.

Torres MJ AM, Vargas C, Bedmar EJ, Fischer H-M, Mesa S, *et al.* (2014) The Global Response Regulator RegR Controls Expression of Denitrification Genes in *Bradyrhizobium japonicum*. *PLoS ONE* **9**: e99011.

Tosques IE, Shi J & Shapleigh JP (1996) Cloning and characterization of *nnrR*, whose product is required for the expression of proteins involved in nitric oxide metabolism in *Rhodobacter sphaeroides* 2.4.3. *Journal of Bacteriology* **178**: 4958-4964.

Totten PA, Lara JC & Lory S (1990) The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. *Journal of Bacteriology* **172**: 389-396.

Van Spanning DBA, Reijnders WN, Westerhoff HV, Stouthamer AH, Van Der Oost J (1997) FnrP and NNR of *Paracoccus denitrificans* are both members of the FNR family of transcriptional activators but have distinct roles in respiratory adaptation in response to oxygen limitation. *Mol Microbiol* **23**: 893-907.

Van Spanning RJM, De Boer APN, Reijnders WNM, Spiro S, Westerhoff HV, Stouthamer AH & Van der Oost J (1995) Nitrite and nitric oxide reduction in *Paracoccus denitrificans* is under the control of NNR, a regulatory protein that belongs to the FNR family of transcriptional activators. *FEBS Letters* **360**: 151-154.

Vollack K-U, Härtig E, Körner H & Zumft WG (1999) Multiple transcription factors of the FNR family in denitrifying *Pseudomonas stutzeri*: characterization of four *fnr*-like genes, regulatory responses and cognate metabolic processes. *Molecular Microbiology* **31**: 1681-1694.

Wagner-Dobler I, Ballhausen B, Berger M, *et al.* (2009) The complete genome sequence of the algal symbiont *Dinoroseobacter shibae*: a hitchhiker's guide to life in the sea. *ISME J* **4**: 61-77.

Warellmann RD, ROBERT, A., BENDER, AND, BARBEL & FRIEDRICH' (1989) An *rpoN*-Like Gene of *Alcaligenes eutrophus* and *Pseudomonas facilis* Controls Expression of Diverse Metabolic Pathways, Including Hydrogen Oxidation. *JOURNAL OF BACTERIOLOGY*, 1093-1099.

Wood NJ, Alizadeh T, Bennett S, Pearce J, Ferguson SJ, Richardson DJ & Moir JWB (2001) Maximal Expression of Membrane-Bound Nitrate Reductase in *Paracoccus* Is Induced by Nitrate via a Third FNR-Like Regulator Named NarR. *Journal of Bacteriology* **183**: 3606-3613.

Yoon SS, Karabulut AC, Lipscomb JD, *et al.* (2007) Two-pronged survival strategy for the major cystic fibrosis pathogen, *Pseudomonas aeruginosa*, lacking the capacity to degrade nitric oxide during anaerobic respiration. *The EMBO Journal* **26**: 3662-3672.

Zumft W (1997) Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61**: 533-616.

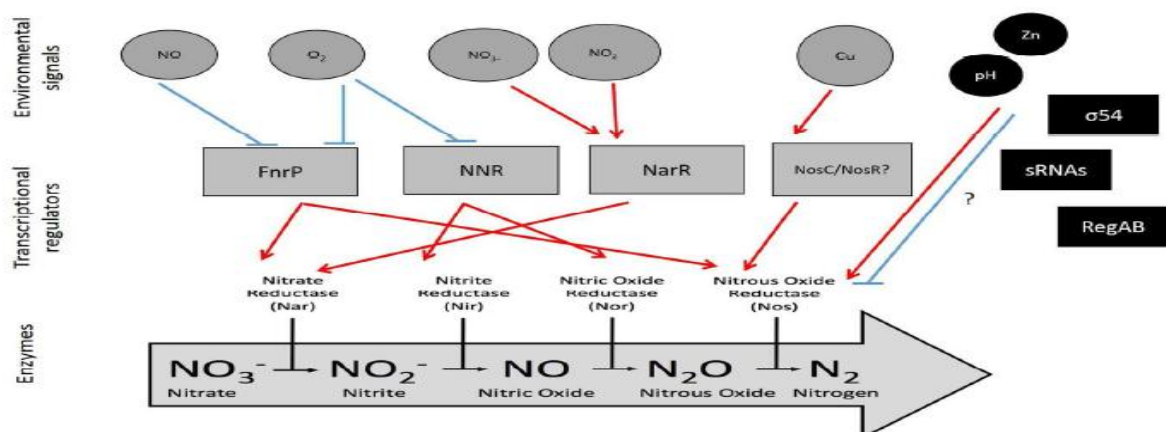


Figure 1: An overview of known (grey) and potentially novel (black) environmental and transcriptional regulators involved in denitrification in *P. denitrificans*. The three layers show the regulatory signals, regulatory proteins and the enzymes involved in each step of denitrification

respectively. Arrows between the upper and middle layers indicate signalling events, while arrows between the middle and lower layers indicate regulation at the level of gene expression. Blue arrows indicate an inhibitory effect whilst red indicate an activating effect.

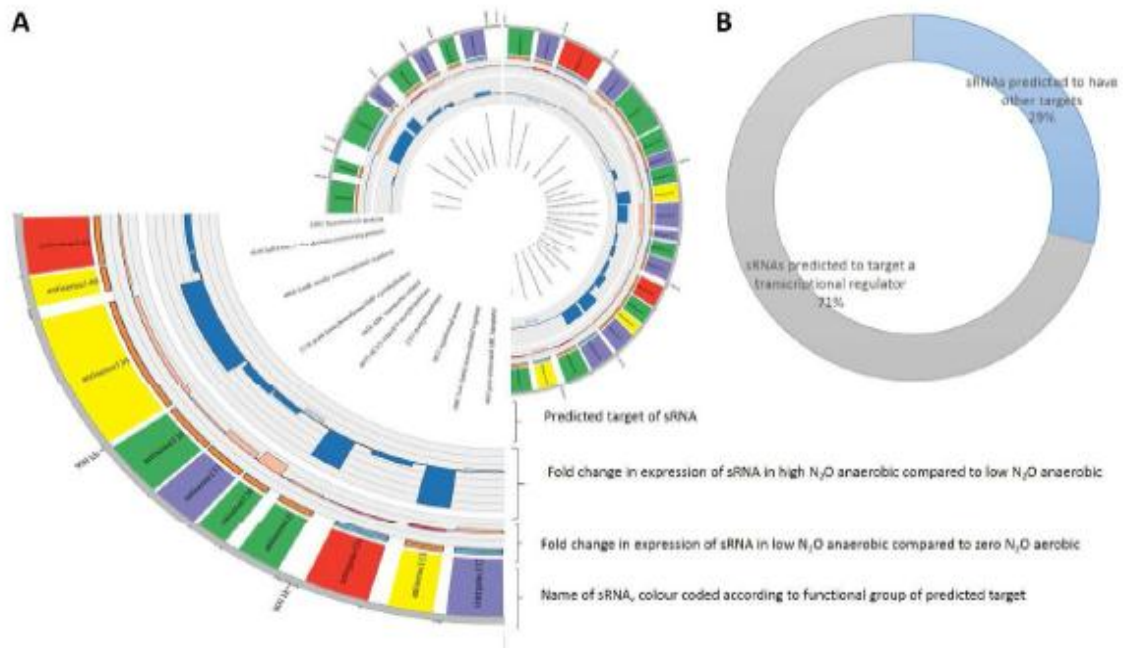


Figure 2: An overview of putative sRNAs in *P. denitrificans*. A: For clarity, only sRNAs encoded by chromosome 2 are shown. The names of the sRNAs are colour coded according to functional group of predicted target as follows metabolism – green, transport – purple, hypothetical – red and regulation – yellow. B: a pie chart to show many predicted targets of the sRNAs are annotated as transcriptional regulators. Figure produced using data from Gaimster et al., 2016.