Nitrous oxide production and consumption: regulation of gene expression by gas-sensitive transcription factors

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Several biochemical mechanisms contribute to the biological generation of nitrous oxide (N$_2$O). N$_2$O generating enzymes include the respiratory nitric oxide (NO) reductase, an enzyme from the flavo-diiron family, and flavohaemoglobin. On the other hand, there is only one enzyme that is known to use N$_2$O as a substrate, which is the respiratory N$_2$O reductase typically found in bacteria capable of denitrification (the respiratory reduction of nitrate and nitrite to dinitrogen). This article will briefly review the properties of the enzymes that make and consume N$_2$O, together with the accessory proteins that have roles in the assembly and maturation of those enzymes. The expression of the genes encoding the enzymes that produce and consume N$_2$O is regulated by environmental signals (typically oxygen and NO) acting through regulatory proteins, which, either directly or indirectly, control the frequency of transcription initiation. The roles and mechanisms of these proteins, and the structures of the regulatory networks in which they participate will also be reviewed.

Keywords: denitrification; nitrous oxide; nitric oxide; nitrous oxide reductase; nitric oxide reductase

1. INTRODUCTION

Nitrous oxide (N$_2$O) is a water-soluble gas that attracts current interest because of its contribution to the atmospheric greenhouse effect. N$_2$O has well known and useful anaesthetic and analgesic properties, and has also found applications as an oxidant in fuels. N$_2$O is relatively inert at ambient temperature, and (unlike nitric oxide, NO) has a very low affinity for metal centres in proteins. Thus, N$_2$O is not toxic, and micro-organisms can tolerate relatively high (millimolar) concentrations. The reduction potential of the N$_2$O/N$_2$ couple is high ($E^0 = +1.35$ V at pH 7), and some bacteria exploit this property by using N$_2$O as the terminal electron acceptor in energy conserving respiratory metabolism.

N$_2$O is an intermediate (or, in some cases, end-product) of the respiratory pathway denitrification, so denitrification is a major contributor to global N$_2$O emissions [1]. The ammonia-oxidizing bacteria are another significant source of N$_2$O, although those that have been studied are also capable of partial denitrification (the respiratory reduction of nitrite to N$_2$O), so the enzymes responsible for N$_2$O production are similar to those of the denitrifying bacteria [2]. The respiratory reduction of nitrate to nitrite and ammonia (sometimes called respiratory ammonification) can also be a source of N$_2$O, since some nitric oxide (NO) is made as a by-product of this pathway and is subsequently reduced to N$_2$O. In contrast to the multiplicity of mechanisms by which N$_2$O can be generated, only a single sink for N$_2$O is known, which is the respiratory N$_2$O reductase typically found in denitrifying bacteria.

The factors that contribute to the emission of N$_2$O from bacterial populations are numerous and complex, clearly one important determinant being the cellular abundance and activities of the enzymes that produce and consume N$_2$O. A key contributor to enzyme abundance is the regulation of expression of the corresponding genes by regulatory systems and signal transduction pathways that respond to intracellular or extra-cellular signals. The goal of this review is to present a brief overview of the enzymatic mechanisms of N$_2$O production and consumption, and then to focus on the regulatory mechanisms that control the expression of the genes that encode these enzymes. The structures of the regulatory networks that integrate environmental signals and connect regulatory proteins with their target genes will also be reviewed. There is a surprising degree of diversity in the organization of regulatory networks, even among organisms that are phylogenetically quite closely related. This diversity presents a challenge when it comes to extrapolating from studies done on one organism to another, and from pure culture experiments to studies of complex microbial populations.

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2. ENZYMES OF NITROUS OXIDE SYNTHESIS

(a) Respiratory nitric oxide reductase

The major contributor to the biological production of N₂O is almost certainly the respiratory NO reductase (NOR) found in denitrifying bacteria and in some ammonia-oxidizing organisms. Three types of NOR have been described [3], as will be discussed below. All three catalyse the two-electron reduction of NO to N₂O:

\[ 2\text{NO} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O} \]

with the electrons derived from a small soluble c-type cytochrome (cytochrome \(c_{551}\) in \(P.\ aeruginosa\)) or from pseudoazurin. The qNOR is a single-subunit enzyme that accepts electrons from the quinone pool. The qCuₐNOR accepts electrons either from a membrane-associated c-type cytochrome (not shown) or from menaquinol. (b) The soluble enzymes have roles in NO detoxification. NO is reduced by a flavo-diiron protein (NorV, FprA or ROO), which accepts electrons from an NADH-dependent flavo-enzyme: NorW, high molecular weight rubredoxin (Hrb) or NADH : rubredoxin oxidoreductase (NRO). P and N denote periplasmic and cytoplasmic compartments, respectively.

Figure 1. Organization of membrane-bound and soluble NO reductase complexes. (a) The cNOR has two membrane subunits and accepts electrons from a small soluble c-type cytochrome (cytochrome \(c_{551}\) in \(P.\ aeruginosa\)) or from pseudoazurin. The qNOR is a single-subunit enzyme that accepts electrons from the quinone pool. The qCuₐNOR accepts electrons either from a membrane-associated c-type cytochrome (not shown) or from menaquinol. (b) The soluble enzymes have roles in NO detoxification. NO is reduced by a flavo-diiron protein (NorV, FprA or ROO), which accepts electrons from an NADH-dependent flavo-enzyme: NorW, high molecular weight rubredoxin (Hrb) or NADH : rubredoxin oxidoreductase (NRO). P and N denote periplasmic and cytoplasmic compartments, respectively.

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with the electrons derived from a small c-type cytochrome, the copper protein pseudoazurin or from the quinone pool. In each case, the catalytic subunit contains a binuclear centre comprising a high-spin haem \(b\) and a non-haem iron (FeB). A low spin haem \(b\) participates in electron transfer to the binuclear centre, which is the site of NO reduction. The three types of NOR differ in their subunit architectures and in the entry routes for electrons (figure 1a).

In the c-type NOR (also called scNOR, for short chain), the catalytic NorB subunit is in a complex with NorC, a small c-type cytochrome with a single membrane-spanning helix (figure 1a). Electrons from a periplasmic c-type cytochrome or from pseudoazurin pass through the haem of NorC, to the low spin haem of NorB, and thence to the binuclear centre. The best-characterized cNORs are those from \(P.\ aeruginosa\), \(P.\ denitrificans\) and \(P.\ stutzeri\). The structure of the NorBC complex from \(P.\ aeruginosa\) [4] confirmed the predicted presence of 12 membrane-spanning \(\alpha\)-helices in NorB. Biochemical experiments indicated that the protons required for NO reduction are taken from the periplasmic side of the membrane [5], and that NorB does not function as a proton pump [6]. The latter is confirmed in the structure by the absence of trans-membrane proton channels in NorB analogous to those found in the proton-translocating haem-copper oxidases, which are otherwise structurally related to NorB [4].

The \(\text{norCB}\) genes are usually co-transcribed with accessory genes designated \(\text{norD}\), \(\text{norE}\), \(\text{norF}\) and \(\text{norQ}\); the gene order \(\text{norEFBCQD}\) is typical though not universal [3]. The \(\text{norQ}\) and \(\text{norD}\) genes are always linked to \(\text{norCB}\); the other accessory genes may be distantly located or absent altogether in some genomes [3]. The functions of the accessory genes and their protein products are not well understood. NorE is a predicted membrane protein with some sequence similarity to part of subunit 3 of the cytochrome \(c\) oxidase. This led to speculation that NorE might be a component of the NOR complex [7], although purified and active preparations of the cNOR only contain NorB and NorC. Mutation of the accessory genes tends to lead to variable phenotypes in different organisms [3], but in no case is the biochemical function of the accessory proteins understood; this is an area worthy of further investigation.

The q-type NOR is a single-subunit enzyme that is similar to but larger than the B subunit of the c-type NOR, hence this enzyme has also been called the long chain NOR. The single subunit of the qNOR
has two additional trans-membrane helices (compared with NorB) that flank a periplasmic domain of approximately 200 amino acids (figure 1a). The qNOR receives electrons from the quinone pool, and the additional N-terminal domain is the presumed location of the quinol oxidase activity [8]. A qNOR has been characterized in the hyperthermophilic Archaea Pyrobaculum aerophilum [9]. The archaeal enzyme is similar to the bacterial qNORs, with the exception of covalent modifications to its haem groups. Interestingly, the genes encoding qNORs are not typically co-expressed with accessory genes, implying that the accessory proteins have functions that are specific to the activity and/or assembly of the cNORs. In Ralstonia eutropha, the norB gene that encodes the qNOR is transcribed with a gene designated norA (also called ytfE, dnrN and sccA in other organisms). The NorA/YtfE protein contains a diiron centre, and has been implicated in the repair of damaged iron sulphur clusters [10] and/or in the buffering of cytoplasmic NO [11].

An unusual hybrid NOR has been described in the Gram-positive organism Bacillus azotoformans [12, 13]. This is a two subunit enzyme that functions as a menaquinol: NO oxidoreductase, but can also accept electrons from small membrane-bound c-type cytochromes [13]. The B. azotoformans NOR contains a CuA centre that is similar to the CuA of cytochrome oxidases, and is proposed to be bound to the small subunit [12]. Hence, there appear to be two routes of electron entry into this enzyme (designated qCuA, NOR), either from the quinone pool, or from small c-type cytochromes (figure 1a).

(b) Flavo-diiron proteins

In several non-denitrifying bacteria, an enzyme from the flavo-diiron family reduces NO to N2O. The physiological role of this enzyme seems to be NO detoxification, a reaction which may be particularly important in organisms (such as Escherichia coli) which make low concentrations of NO as a by-product of the respiratory reduction of nitrite to ammonia. Although N2O is (probably) the end-product of this pathway, the contribution that it makes to the global N2O budget is likely to be very small. In this enzyme system, NO is reduced by a flavo-diiron protein, which receives electrons from a rubredoxin domain or protein. The rubredoxin is itself reduced by an NADH-dependent flavoenzyme (figure 1b). The flavo-diiron protein of E. coli has a fused rubredoxin domain, and so is called flavrubredoxin, Fird (also called NorV). In complex with the NADH-dependent Fird oxidoreductase (NorW), this enzyme functions as an NO reductase in vitro [14] and in vivo [15]. In Moorella thermoacetica, a similar complex (figure 1b) functions as an NO reductase, though in this case the flavo-diiron protein (FprA) is reduced by an NADH-dependent high molecular weight rubredoxin, Hrb [16]. In Desulfovibrio gigas, an equivalent enzyme complex was initially characterized as an oxidase, comprising the flavo-diiron protein (ROO, for rubredoxin oxygen oxidoreductase), a standalone rubredoxin and an NADH-rubredoxin oxidoreductase. The recombinant D. gigas ROO and reduced rubredoxin were found also to reduce NO to N2O, and physiological data suggested that the enzyme functions as an NO reductase in vivo [17]. Similarly, the D. vulgaris FprA functions as an NO reductase both in vitro and in vivo [18]. The oxidase activities associated with these flavo-diiron proteins are probably not physiologically relevant, since oxygen turnover irreversibly inactivates the enzyme [16, 18]. Thus, a consensus has emerged in which the physiological role of these enzymes is to scavenge low concentrations of NO in cells growing under micro-oxic conditions.

(c) Flavohaemoglobin

The flavohaemoglobin Hmp is another NO detoxification enzyme, which is phylogenetically widespread, being found in denitrifying bacteria (such as R. eutropha) and non-denitrifiers, including E. coli. Hmp has a globin like domain, and an FAD-containing domain that binds NAD(P)H [19]. In the presence of oxygen, Hmp oxidizes NO to nitrate, an activity that has been described as an NO dioxygenase or NO denitrosylase. In the absence of oxygen, Hmp reduces NO to N2O [20]. However, the consumption of NO by Hmp under anaerobic conditions is rather slow [21], so Hmp may not be a significant source of N2O.

3. ENZYMES OF NITROUS OXIDE CONSUMPTION

(a) Respiratory nitrous oxide reductase

While N2O is generated by a diversity of enzymes, N2O consumption appears to be exclusively due to the respiratory N2O reductase Nos (also called NosZ). Genetic, biochemical and molecular aspects of N2O reduction have been reviewed comprehensively [22] and will be summarized only briefly here. Nos enzymes are soluble periplasmic copper proteins that catalyse the two-electron reduction of N2O to dinitrogen:

\[ \text{N}_2\text{O} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{N}_2 + \text{H}_2\text{O} \]

The electrons required for N2O reduction originate in the quinone pool and are transferred to Nos either via the cytochrome bc1 complex and small soluble periplasmic proteins, or via Nos-specific membrane-associated electron transfer proteins (figure 2).

The well-characterized Nos (referred to as the Z-type Nos) is a homodimeric protein in which each monomer contains two copper centres designated CuA and CuZ. Three-dimensional structures are available for the enzymes from Marinobacter hydrocarbonoclasticus (Pseudomonas nautica) and Pa. denitrificans [24, 25]. The electron transfer route to this enzyme is typically depicted as involving the cytochrome bc1 complex and small soluble periplasmic electron carriers, such as cytochrome c550 and pseudoaazurin [1]. However, there are observations that are consistent with Nos-specific proteins participating in electron transfer to the Z-type Nos. Specifically, NosR is a membrane-bound iron–sulphur flavoprotein, which has been suggested to mediate electron transfer between the quinone pool and Nos [26].

The Z-type Nos is encoded by a gene called nosZ that is typically linked to other nos genes, whose products have roles in the maturation of the active enzyme. The functions of the accessory Nos proteins...
are not well understood, but they include an ABC transporter (NosFYD) that may export a sulphur compound to the periplasm, an outer membrane copper porin (NosA), an outer membrane anchored copper protein (NosL), a periplasmic flavoprotein (NosX) and NosR [22]. The catalytic Nos subunit is exported as an apo protein to the periplasm via the Tat transport system, which is unusual, since Tat typically secretes fully assembled and folded proteins. Thus, the copper centres are inserted into Nos in the periplasm, and it seems likely that some or all of the periplasmic accessory Nos proteins have roles in assembly of the copper clusters. As is also the case for the c-type NOR, the maturation of Nos is an area that demands further investigation.

An unusual variant of Nos is found in Wolinella succinogenes and some of its relatives. This enzyme (called the c-type Nos) is characterized by an additional C-terminal mono-haem cytochrome c domain. Export to the periplasm is by the Sec secretory system rather than the Tat pathway used by the Z-type Nos. Electron transfer to the c-type Nos involves membrane-associated iron–sulphur proteins (NosG and NosH) which mediate electron transfer between the quinone pool and NosZ, perhaps via small soluble periplasmic c-type cytochromes [23]. The physiological role of the Nos in W. succinogenes is not certain, given that the organism reduces nitrite to ammonia. One possibility is that the enzyme acts on the N₂O produced by detoxifying activities that remove the NO made as a by-product of nitrite respiration [23].

4. REGULATORY PROTEINS

The genes and operons encoding the enzymes of N₂O production and consumption are controlled in different organisms by a diverse array of transcriptional regulators (figure 3). The signals to which these regulatory proteins respond are also diverse, and include oxygen, NO, nitrate and the activity of the electron transport chain. In most species that have been studied, multiple environmental signals are integrated by regulatory pathways to ensure that the NO and N₂O reductases are expressed optimally according to the prevailing needs of the organism. In the following sections, the properties of the various regulatory proteins will first be reviewed briefly, with a focus on mechanistic aspects. Then, the signalling networks that connect regulatory proteins with their target genes in selected model organisms are described. The intention is to present only a thumbnail of each regulatory system. Thus, references to the literature are not exhaustive, with a focus on reviews, where available, and recent developments. Regulators of genes of the entire

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Figure 2. Electron transfer pathways to the Z-type and c-type nitrous oxide reductase, exemplified by those of Pa. denitrificans and W. succinogenes, respectively. In Pa. denitrificans, electron transfer from the cytochrome bc₁ complex is via cytochrome c₅₅₀ or pseudoazurin. In the W. succinogenes system, the small mono-haem c-type cytochromes NosC₁ and NosC₂ may be periplasmic or attached to the membrane, and may or may not be on the electron transfer route from NosGH to NosZ [23]. P and N denote periplasmic and cytoplasmic compartments, respectively.
Figure 3. Domain organizations of proteins involved in regulating expression of genes encoding the respiratory nitrite and NO reductases. Domain architectures were generated from primary structures of representative proteins with the simple modular architecture research tool, SMART [27]. The proteins shown are those discussed in the text, with the exception of NsrR, for which SMART predicts no conserved domains. Known or suspected cofactors are indicated, along with conserved sequence motifs that are discussed in the text. The P-box in NarL is in a periplasmic loop (although SMART does not predict transmembrane helices for NarL) and is the site of nitrate binding [28]. For RegB and its orthologues, SMART predicts a single trans-membrane helix in place of the experimentally verified helices 3 and 4 [29]. The GGXXNPFF motif occurs between helices 3 and 4, and is likely to be part of a quinine-binding site [30]. PAS, domain found in Per-Arnt-Sim proteins; PAC, domain occurring C-terminal to a subset of PAS domains; HisKA, dimerization and phosphoacceptor domain of histidine kinases; HATPase_c, histidine kinase-like ATPases; REC, cheY-homologous receiver domain; HTH LuxR, helix-turn-helix domain in LuxR family of response regulators, cNMP, cyclic nucleotide-monophosphate-binding domain; HTH CRP, helix-turn-helix domain in cAMP receptor protein family; GAF, domain present in phytochromes and cGMP-specific phosphodiesterases; AAA, ATPases associated with a variety of cellular activities; HAMP, domain found in histidine kinases, adenyl cyclases, methyl-binding proteins and phosphatases; FMN bind, flavin-binding site.
denitrification pathway are considered, since the flux through the early reactions of the pathway will be a contributor to the emission of $\text{N}_2\text{O}$.

**(a) FixL**

FixL is a multi-domain sensory histidine kinase which, in its active state, phosphorylates a conserved aspartic acid residue in the receiver domain of its cognate response regulator FixJ (figure 3). Sensory input into this system is via the PAS (Per-Arnt-Sim) domain of FixL, which coordinates a single molecule of haem. There is some structural diversity in FixL proteins from different organisms. For example, the *Bradyrhizobium japonicum* FixL is soluble and cytoplasmic, while the *Sinorhizobium meliloti* protein is membrane-associated, such that its PAS domain is anchored on the cytoplasmic face of the membrane (figure 3). In all cases that have been studied, FixL is in its ‘on’ state in the absence of oxygen, in which it auto-phosphorylates prior to phospho-transfer to FixJ. Phosphorylated FixJ is active for DNA binding and regulation of the transcription of target genes. Molecular oxygen binds to FixL (with an affinity in the range 50–150 μM), converting the ferrous iron of the haem from high spin to low spin. The consequent movement of the iron in the plane of the porphyrin ring triggers a series of conformational changes that inhibit the kinase activity of FixL. There is a great deal of structural and biochemical information for FixL proteins, so the mechanism by which oxygen regulates the kinase activity of FixL is quite well understood [31–33]. FixL can bind other haem ligands (such as CO and NO) but these do not inhibit the kinase activity, and so their interaction with FixL is probably not physiologically significant.

**(b) FNR**

FNR from *E. coli* is a prototypical member of the FNR/CRP superfamily of transcriptional activators. Four conserved cysteines of FNR coordinate a $[4\text{Fe}−4\text{S}]^{2+}$ cluster, which is converted to a $[2\text{Fe}−2\text{S}]^{2+}$ cluster on exposure to oxygen. This transition is accompanied by a reduced tendency of FNR to dimerize, and so a reduced affinity for its DNA targets. Details of the mechanism of the reaction of the cluster with oxygen (which may proceed via a $[3\text{Fe}−4\text{S}]^{1+}$ intermediate) are beginning to emerge [32,34]. Prolonged reaction with oxygen results in a cluster-free apo-protein, which is the form isolated from aerobically grown cells. Orthologues of FNR from other organisms (such as FnrP, ANR and FnrN) are presumed to work in similar ways, although the limited information that is available suggests that the oxygen sensitivity of the cluster can be fine-tuned by the protein environment in different FNR relatives [35,36].

Iron–sulphur proteins are potential targets for NO, and the reaction of FNR proteins with NO has been documented for proteins from several sources [35,37–39]. The product of this reaction has not been well characterized, but may include dinitrosyl iron complexes [37], and/or, perhaps, a $[2\text{Fe}−2\text{S}]$ cluster [38]. However, FNR proteins do not provide the dominant mechanism for NO sensing, and so the reaction of FNR with NO may serve only to fine-tune the expression of genes encoding the enzymes of denitrification (see below).

**(c) RegBAlPrrBA**

RegB and its orthologues are membrane-associated histidine kinases that phosphorylate their cognate response regulators (figure 3). The RegBA system was first described in the photosynthetic bacterium *Rhodobacter capsulatus*, and its close relative *Rhodobacter sphaeroides* (in which it is called PrrBA). RegBA/PrrBA two component systems have been well characterized in these organisms, and other members of the alpha proteobacteria, where they are typically involved in regulating energy generating or consuming processes, including denitrification [29]. RegB/PrrB functions as a redox sensor, such that it is active as a kinase under conditions that lead to a relatively reduced state of the cell. Thus, in the facultatively anaerobic *Rhodobacter* species, RegB/PrrB behaves as if it is inactivated by oxygen, though this is not a direct effect. Instead, the RegB/PrrB proteins appear to measure the ‘redox state’ of the cell by multiple mechanisms. In one mechanism, metal-dependent oxidation of a key cysteine residue (Cys-265 in the *R. capsulatus* RegB, figure 3) causes a dimer to tetramer transition, and inactivation of the kinase [40]. Purified RegB also contains bound ubiquinone, and oxidation of the quinone inhibits kinase activity [30]. Quinone binding requires a conserved GGXXNP motif that is located in a short periplasmic loop (figure 3, [40]). This mechanism presumably allows RegB to monitor the redox state of the electron transport chain. The PrrB protein of *R. sphaeroides* appears to be controlled by different mechanisms. In this case, it has been suggested that the cytochrome $cbb_3$ oxidase serves as a redox sensor for PrrB, in a manner that does not require oxidation activity [41]. Signal transfer is independent of the quinone pool, implying a more direct interaction between the $cbb_3$ oxidase and PrrB. Furthermore, while PrrB kinase activity is inhibited by ubiquinone, this effect does not require the GGXXNP motif or the membrane domain [41]. Thus, the current evidence points to a multitude of mechanisms in the RegB/PrrB sensor kinases.

**(d) NNR/NnrR/DNR and NarR**

The FNR/CRP superfamily includes several proteins that regulate the expression of genes encoding the respiratory NO reductase. These proteins have been variously designated NNR/NnrR and DNR, and evidence from *in vivo* experiments indicates that these proteins activate transcription in response to NO. In fact, the NNR/NnrR/DNR proteins do not form a single coherent group, rather they fall into two of the phylogenetically distinct branches of the wider FNR/CRP superfamily [42]. The mechanism(s) of NO sensing by these proteins has proved difficult to establish. NNR from *P. denitrificans* and DNR from *P. aeruginosa* require haem for their NO-dependent activity in heterologous reporter systems in *E. coli* [43,44]. The structure of the sensory domain of DNR reveals a hydrophobic pocket that might be a haem-binding site, and purified apo-DNR can bind...
one equivalent of haem [45]. The haem-reconstituted DNR binds NO and CO, and also shows some DNA-binding activity that is neither stimulated nor inhibited by NO [45]. The current model proposes that full activation of DNR requires haem and NO, though the complete details of this mechanism remain to be established [46]. Results from in vivo experiments suggest that NNR/NnrR/DNA activity is sensitive to oxygen [43,47]; this would be consistent with the physiological roles of these proteins and with a haem-based sensing mechanism.

NarR is another FNR/CRP family member, which in Pa. denitrificans regulates expression of the respiratory nitrate reductase and a nitrate transport system in response to nitrate and/or nitrite [48]. NarR can also be activated by azide, which is suggestive of a metal-based sensing mechanism [49].

(c) NorR
In the denitrifying bacterium R. eutropha, the respiratory NO reductase gene norB (of which there are two copies, one on the chromosome and one on a megaplasmid) is activated in response to NO by a protein designated NorR [50]. The NorR protein of E. coli activates transcription of the norVW genes, which encode the FixR and its redox partner [51,52]. NorR-dependent transcription requires RNA polymerase containing the alternative sigma factor, σ^54, so NorR binds to the σ^54-dependent enhancer-binding protein (EBP) family of transcriptional activators. NorR has a three-domain structure that is typical of EBPs, with a C-terminal DNA-binding domain, a central domain from the AAA^+ family that has ATPase activity and interacts with RNA polymerase, and an N-terminal signalling domain [50]. The N-terminal GAF domain of NorR contains a mono-nuclear non-haem iron, which is the binding site for NO. Formation of a mono-nitrosyl complex at this centre disrupts an intra-molecular interaction, by which the GAF domain inhibits the activity of the AAA^+ domain in the absence of NO [53,54]. The non-haem iron is believed to be coordinated by the side chains of three aspartate residues, an arginine and a cysteine [55,56].

(f) NarXL
The NarXL proteins are a two-component sensor regulator system that responds to nitrate and/or nitrite. NarXL and their orthologues NarQP have been well characterized in E. coli [57]. In the denitrifying bacteria, these proteins are not known to regulate expression of the genes encoding the enzymes of N_{2}O production and consumption. However, by regulating the expression of nitrate reductase genes, NarXL do have a role to play in some organisms in controlling the rate of flux through the denitrification pathway. The sensor kinase NarX has two trans-membrane helices (not shown in the SMART prediction in figure 3) that flank an approximately 100 residue periplasmic domain that contains the 'P-box', a region implicated in nitrate and nitrite binding by genetic and biochemical evidence [58,59]. In the three-dimensional structure of the periplasmic domain of NarX, nitrate binds to a single site at the monomer–monomer interface, where it makes contact with residues from the P-box [28]. Comparison with the structure of the apo-protein suggests that a piston-like displacement between the trans-membrane helices might be involved in coupling ligand binding to the kinase activity of NarX [28]. This signal is propagated through the cytoplasmic HAMP domain, and the signalling helix, which is a C-terminal extension of the HAMP domain [60].

(g) FixK
Rhizobial FixK proteins are FNR/CRP family members that occupy intermediate positions in the regulatory hierarchies that control the expression of genes involved in nitrogen fixation and denitrification. In B. japonicum, expression of the gene encoding FixK2 is upregulated under micro-oxic conditions by FixLJ. Interestingly, this transcriptional regulation of the fixK_2 gene appears not to be reflected in changes in the abundance of FixK_2 protein, a paradox that remains to be resolved [61]. Nevertheless, the fact that FixK_2 is regulated at the level of its expression originally led to the view that FixK_2 activity is not itself sensitive to environmental signals. Recently, however, it has been shown that the activity of the B. japonicum FixK_2 is sensitive to oxidative stress. Specifically, oxidation of a cysteine residue (either to a disulphide bridge, or to a sulphonic acid derivative) reduces the activity of FixK_2 and so causes downregulation of its target genes [61]. This regulatory mechanism may be peculiar to B. japonicum, given that the cysteine residue involved is not conserved in other FixK proteins.

(b) NosR and NirI
NirI and NosR are related proteins that are required for transcription of the nitrite reductase and N_2O reductase genes, respectively, in some organisms. Both are polytopic membrane proteins with a periplasmic flavin-containing domain, and two cytoplasmic iron sulphur clusters (figure 3). The presence of a covalently bound flavin and two [4Fe–4S] clusters has been confirmed biochemically for the NosR protein of Ps. stutzeri [26]. Both NirI and NosR also contain cytoplasmic CXXCPXCP motifs, which may be metal ion-binding sites, or sites for thiol chemistry. In Ps. stutzeri, the nosR gene is adjacent to and upstream of the nosZ structural gene, and the nosD operon, which encodes proteins involved in Nos maturation, but the three are in separate transcription units. Transposon insertions in nosR abolished or severely reduced production of the monocistronic nosZ mRNA [62]. Similarly, transcription of the nosD operon is abolished in a nosR mutant [63]. Although nosR is upstream of nosZ and the nosD operon, these results are probably not due to polarity, since both nosR and nosZ are mono-cistronic. Thus, NosR behaves as a factor that is required for the transcription of nosZ and the nosD operon. The membrane location and domain organization of NorR, and specifically the absence of a predicted DNA-binding domain, argue against a direct role in transcriptional control.
Moreover, deletion analysis of NosR showed that only the periplasmic flavin-containing domain is required for nosZ expression [26]. Thus, the mechanism by which NosR controls expression of its target genes remains mysterious, but is likely to be indirect. Interestingly, in *P. aeruginosa*, NosR is not required for transcription of the nosZ gene [64]. NosR also has a role to play in the activity of Nos, perhaps by acting as an electron donor to the enzyme [26]. As is discussed by Wunsch & Zumft [26], *W. succinogenes* expresses a c-type Nos, and has no nosR gene. Interestingly, the cytoplasmic domain of NosH of *W. succinogenes* (figure 2) resembles NosR in that it coordinates two [4Fe–4S] clusters and has two CXXXP motifs. Perhaps the cytoplasmic domains of NorR and NosH play similar roles in *N*₂Ο reduction.

The NirI protein has been less extensively characterized, but shows some interesting similarities and differences to NosR. In *Pa. denitrificans*, nirI is divergently transcribed from *nirS*, which is the structural gene encoding nitrite reductase. Mutation of *nirI* abolished activity of the *nirS* promoter [65]. Thus, NirI has a similar structure to NosR and seems also to be required for the expression of its target gene. Careful attempts to complement a nirI mutation with nirI cloned on a plasmid were unsuccessful. Complementation could only be achieved by integrating nirI into the chromosome, into a position directly upstream of *nirS*. The basis for this unusual observation is not understood [65]. Note that nosR cloned on a plasmid is capable of complementing a *Ps. stutzeri* nosR mutant [26, 62].

In summary, NosR and NirI are related multidomain membrane proteins, with redox centres in both the periplasm and cytoplasm. Both behave as regulators of gene expression, although this is unlikely to be a direct effect requiring DNA binding, particularly in the case of NosR.

(i) *NsrR*

NsrR was originally identified as a negative regulator of the respiratory nitrite reductase gene in the ammonia oxidizer *Nitrosomonas europaea* [66], an organism that also expresses an NOR and so is capable of *N*₂Ο production. An orthologue of NsrR is a repressor of the *hmp* gene of *E. coli* that encodes the NO detoxifying flavohaemoglobin [67]. In the denitrifying pathogenic organisms *Moraxella catarrhalis*, *Neisseria meningitidis* and *Neisseria gonorrhoeae*, NsrR is a repressor of the *norB* gene encoding the respiratory NO reductase [68–70]. NsrR is a negative regulator that is inactivated by exposure to NO, the mechanism probably involving NO-mediated modification of a protein-bound iron–sulphur cluster [71].

5. REGULATORY NETWORKS

The wiring diagrams that connect the regulators discussed above to their target genes are surprisingly variable in different organisms [72, 73]. In the context of factors that directly influence *N*₂Ο production and consumption, mechanisms that regulate expression of the *nor* and *nos* genes can be considered in isolation from the remainder of the denitrification pathway.

Figure 4a displays a compendium of regulatory interactions that impinge upon the *nor* and *nos* genes in model denitrifiers (with some exceptions, see below). It should be stressed that not all of these interactions are present in all denitrifiers, and no one organism has all of the regulatory interactions shown in the figure. Rather, the diagram depicts the totality of interactions that have been described in those organisms that have been studied. Clearly, the important environmental signals that control expression of the *nor* and *nos* genes are the concentrations of oxygen and NO, and the key regulatory proteins use iron-based mechanisms to sense oxygen and NO. Notably absent is a mechanism for regulating the *nos* genes by the abundance of *N*₂Ο. So, as far as is known, the denitrifying bacteria do not induce expression of the *nos* genes in response to elevated *N*₂Ο. Figure 4b shows an equivalent diagram for the regulators of the genes encoding detoxification activities, that is Hmp and the flavo-diiron protein (FIRd, in the case of *E. coli*). Exceptions to this division between denitrification and detoxification include *R. eutropha*, *M. catarrhalis* and *Neisseria* species, in which the respiratory NO reductase gene *norB* is regulated by NorR or NsrR [68–70], rather than by the regulators that generally regulate denitrification genes, shown in figure 4a.

Clearly, the flux from nitrate to NO will also influence *N*₂Ο production, and so it is appropriate to consider the regulation of all steps of the denitrification pathway. Figure 5 shows the mechanisms of regulation of denitrification genes in six model organisms. Again, it is evident that regulatory interactions can be diverse, even in closely related organisms. For example, while NosR is required for expression of the *nosZ* gene in *Ps. stutzeri*, this is not the case in *Ps. aeruginosa* [64]. In three members of the alpha proteobacteria that have been studied quite extensively, the mechanisms of oxygen regulation of denitrification genes are rather different. In *B. japonicum*, the haem protein FixL is the key sensor of oxygen, while in *Pa. denitrificans* it is the iron–sulphur protein FnrP, and in *R. sphaeroides* oxygen is sensed indirectly by PrrB (figure 5). Thus, there apparently are not universal mechanisms for the regulation of denitrification genes, and so extrapolating from studies done on one model organism to other species is unlikely to be appropriate. Given the degree of diversity that there is in the rather small number of model organisms, it seems likely that novel regulatory mechanisms will be encountered as the set of model species expands. Further, extrapolating from laboratory studies done on a small number of model systems to complex poly-species communities in the field would seem to be especially problematic.

6. CONCLUSIONS

The environmental signals that most commonly influence the expression of genes involved in denitrification are the concentrations of oxygen and NO. Since denitrification is an anaerobic respiration, it makes good physiological sense for denitrification genes to be upregulated by low oxygen concentrations. NO is an intermediate of the pathway, and is somewhat toxic.
Regulation of denitrification gene expression by NO is therefore presumed to be a mechanism to coordinate NO production and consumption so as to avoid its accumulation to toxic levels. There is no such requirement to maintain a low N$_2$O concentration in denitrifiers, and N$_2$O does not appear to be a signal that regulates the expression of any of the denitrification genes. From the point of view of mitigating N$_2$O releases from denitrification, the absence of regulation by N$_2$O is a significant observation, since denitrifying populations do not (as far as is known) respond to N$_2$O accumulation by making more of the N$_2$O

**Figure 4.** Regulatory proteins that control expression of the genes encoding enzymes that produce and consume N$_2$O. (a) Summary of regulatory interactions that control expression of the nor (NO reductase) and nos (N$_2$O reductase) genes in model denitrifying bacteria. The domain organizations of membrane proteins NosR and FixL are shown as the SMART predictions from figure 3. Filled arrow heads represent the control of protein activity, open arrow heads denote the control of gene expression (so, for example, FixL activates its partner protein FixJ, which then activates expression of the gene-encoding FixK$_2$. NosR is required for transcription of the nosZ gene (in Ps. stutzeri but not in Ps. aeruginosa) but this effect is likely to be indirect. (b) Summary of regulatory interactions that control expression of genes encoding NO detoxification activities (norVW and hmp), and the norB gene encoding a respiratory NO reductase in *R. eutropha*, *M. catarrhalis* and *Neisseria* spp. Negative regulatory interactions are denoted by lines with a “T” end.
Nevertheless, understanding those signals that do influence the expression of denitrification genes, and those that influence the abundance and activities of the corresponding enzymes, is very likely to be helpful to any effort to intervene in the activities of the denitrifying bacteria.

REFERENCES


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Figure 5. Regulatory networks controlling expression of denitrification genes in a selection of model organisms. In each case, the diagram is organized into three layers, these being the regulatory signals, regulatory proteins and the structural genes. Thus, arrows between the upper and middle layers represent signalling events, while arrows within the middle layer, and between the middle and lower layers represent gene regulation. Proteins boxed by double lines are two-component systems (histidine kinase and response regulator). Genes and operons associated with denitrification include those designated nap and nar (for periplasmic and membrane-bound nitrate reductase, respectively), nir (nitrite reductase), nor (NO reductase) and nos (N2O reductase). The nos genes are not shown for B. japonicum since their regulation is not understood, while these genes are absent from Agrobacterium tumifaciens. The ability to express N2O reductase in Rhodobacter strains is variable, and nos gene expression has not been studied in this genus.


