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Nitric Oxide Signaling and NO Dependent Transcriptional Control in Bacterial Denitrification by Members of the FNR-CRP Regulator Family

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Abstract

Bacterial denitrification transforms nitrate to dinitrogen. The process is expressed facultatively in response to environmental conditions. Around 50 components make up the denitrification apparatus and its assembly pathways. We are beginning to understand how exogenous signals provided by oxygen and N oxides are processed for activating the underlying gene programs. Key signals are provided by nitrate, nitric oxide, and a low oxygen tension. In the genus *Pseudomonas* the nitrate signal is processed by a two component regulatory system which activates the *nar* operon encoding respiratory nitrate reductase. Nitric oxide is not only an essential respiratory substrate of the denitrifying cell but constitutes in nanomolar concentrations also a key signal for the expression of nitrite reductase and NO reductase which control cellular NO homeostasis. The signal pathway in the genera *Pseudomonas, Paracoccus* and *Rhodobacter* involves regulators of the FNR family of transcription factors, which cluster phylogenetically in a separate subgroup. In contrast, *Ralstonia eutropha* requires a sigma-54 dependent regulator of the NtrC family for the expression of its quinol-dependent NO reductase. Important questions are directed currently at the mechanism(s) of activation of these transcription factors by NO, and avoidance of crosstalk with FNR factors at target promoters operating with identical recognition motifs.

Introduction

Nitric oxide (NO) is generated and reduced by bacterial denitrification. ''Monoxide d'azote'' (nitrogen monoxide) was described to originate from a biological process in one of the first studies on denitrification, more than one hundred years ago (Gayon and Dupetit, 1886). Cell-free NO formation by bacteria was shown by Mori and coworkers (Iwasaki *et al.*, 1956) and several lines of evidence secured NO the biological function of an obligate intermediate in nitrite denitrification. Denitrification is a respiratory mode of energy conservation based on the reduction of N oxides. The complete denitrification process consists thus in the consecutive action of four respiratory oxidoreductases acting on nitrate, nitrite, nitric oxide, and nitrous oxide (N_2O) to yield dinitrogen. The overall reaction is essential for biogeochemical N-cycling since dinitrogen fixation needs to be balanced by a reverse reaction to prevent nitrate accumulation at detrimental levels in the biosphere.

Regulatory studies of how the facultatively functioning denitrification system is expressed have been directed at a select group of species representing distinct metabolic themes. The best-studied organotrophic denitrifiers harbor the cytochrome cd_1 nitrite reductase; in contrast, *Rhodobacter sphaeroides*, a photodenitrifier, depends on the Cu-containing nitrite reductase. Extensive biochemical and genetic characterizations of the denitrification apparatus of the heterotrophic denitrifiers *Pseudomonas stutzeri* and *Pseudomonas aeruginosa* have revealed the modifications of the system within a single genus. The intensively studied aerobic modes of respiration of *Paracoccus denitrificans* (Baker *et al.*, 1998) provide the background with which to compare the anaerobic system of denitrification. *Ralstonia eutropha*, a hydrogen bacterium and cytochrome cd_1-dependent denitrifier, harbors a novel quinol-dependent NO reductase and shows unprecedented organizational and regulatory modifications of a in part megaplasmid-encoded denitrification system.

Reviews covering the multiple facets of the denitrification process have been published elsewhere, such as a description of the participating enzymes (Berks *et al.*, 1995), the biochemistry of nitrite reductase and NO generation (Silvestrini *et al.*, 1994; Cutruzzolà, 1999), NO metabolism and properties of NO reductases (Zumft, 1993; Hendriks *et al.*, 2000), and the systematics of denitrifying bacteria (Zumft, 1992). For a coverage of the cell biology, molecular genetics and biochemistry of the denitrification process the reader is referred to a comprehensive review (Zumft, 1997). Here, I describe the current status of our understanding of how the expression of the denitrification apparatus is regulated with emphasis on NO as the signal molecule and signal transduction pathways involving regulators of the FNR family. Nitrate and oxygen signaling which are necessary for establishing the overall process and are interlaced with NO dependent regulation will also be summarized.

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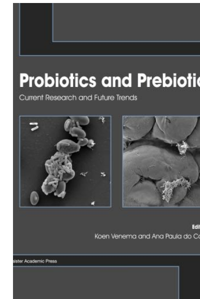

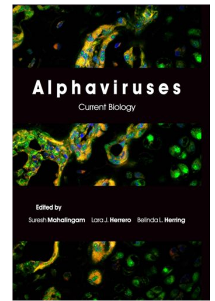

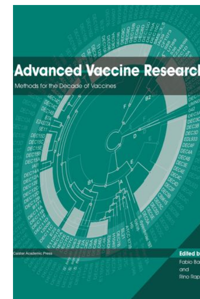

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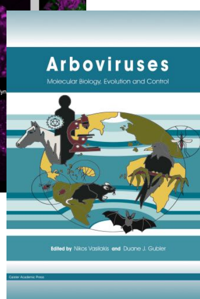

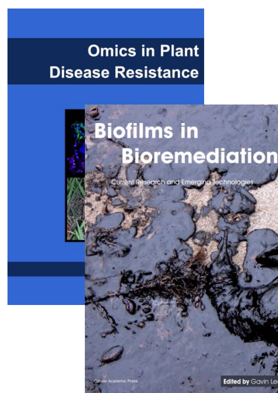

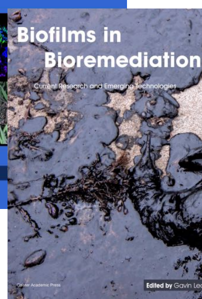

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Variations to the theme will be described and the mechanism of NO sensing discussed in light of the available experimental evidence.

Nitric Oxide, an Essential Nutrient

Whenever a bacterium encounters a high NO concentration over a prolonged time, the toxic aspect of NO will be dominating irrespective of the organism being a denitrifier or not. NO at 0.5 mM completely prevents the transcription of nitrite reductase and NO reductase genes in *P. stutzeri* (Vollack and Zumft, 2001). Even in denitrifiers the loss of NO reductase is a lethal event due to NO accumulation, whereas a *nir-nor* double mutant, that is unable to reduce nitrite, is viable (Braun and Zumft, 1991). Thus, hardly a report on nitric oxide metabolism fails to state the toxicity of NO, giving the impression that a cell would do all to avoid this compound. However, the view manifest in such statements needs differentiation.

In bacteria, NO is reaction product and substrate of denitrification at the same time, i.e., an ordinary cell metabolite. The physiologically relevant NO concentration is in the low nanomolar range. Toward this end NO can be compared to a trace metal, whose deleterious or beneficial properties depend on the concentration to which a bacterium is exposed. Ample evidence for the metabolic role of NO stems from several complementary approaches. Central findings comprise the identification and purification of the NO reductase complex (Heiss *et al.*, 1989), isolation and mutational inactivation of the reductase structural genes, *norC* and *norB* (Braun and Zumft, 1991), demonstration of the built-up of a steady state concentration of NO during denitrification (Goretski *et al.*, 1990), proof for the occurrence of NO in the environment (Conrad, 1996), and evidence for a role of NO reductase in the evolution of aerobic respiration (Giuffrè *et al.*, 1999). As will be shown, NO has also the function of a signal molecule in activating gene programs for its own metabolism besides its role in cellular respiration.

Enzymology of NO Homeostasis

The steady state concentrations of free NO during denitrification was found in the range of 20 to 30 nM and 50 nM, by direct measurement of NO (Goretski *et al.*, 1990) or by gas stripping (Zafiriou *et al.*, 1989), respectively. Nitrate-denitrifying *P. aeruginosa* develops a steady state concentration of 1 to 2 nM NO (Kalkowski and Conrad, 1991). The apparent K_m value for NO consumption by denitrifying bacteria and soil samples ranges from about 1 to 70 nM (for review, see Conrad, 1996). Thus, the bacterial NO-metabolizing system is fully operative at an extremely low NO concentration.

Evolutionary Convergence for NO Generation by Heme- or Cu-Containing Nitrite Reductases

NO homeostasis is determined by NO production and sink reactions. The major enzymatic NO generators of denitrifiers are two types of respiratory nitrite reductases (Cutruzzolà, 1999; Watmough *et al.*, 1999). They are found on an either/or basis without recognizable taxonomic pattern in denitrifying bacteria suggestive of multiple horizontal gene transfer events. The *nirS* gene encodes the tetraheme cytochrome cd_1 nitrite reductase in *P. aeruginosa*, *P. stutzeri*, *Pa. denitrificans*, *Paracoccus pantotrophus* (formerly *Thiosphaera pantotropha*), *R. eutropha*, and several other denitrifiers. The mixed heme protein carries a heme C in the electron transfer domain and an unusual porphyrinedione, referred to as heme D_1, at the catalytic site. The second type of nitrite reductase contains Cu, and is encoded by the *nirK* gene. Representative species harboring this enzyme are *R. sphaeroides*, *Achromobacter cycloclastes*, *Alcaligenes xylosoxidans*, *Alcaligenes faecalis* S-6*, Pseudomonas* sp. G-179, *Bacillus halodenitrificans*, and also the archaeon *Haloferax denitrificans* (Inatomi and Hochstein, 1996). The crystal structure of both types of nitrite reductases has been determined. Although cytochrome cd_1 and Cu-containing nitrite reductases both exhibit some oxygen reductase activity, no evidence exists that this property would attribute them a dual physiological function in anaerobic and aerobic respiratory metabolism. Primary and secondary structures of the two types of nitrite reductase are without relationship indicating independent evolutionary trajectories. Thus, the biochemistry of NO generation provides us with a fine example of evolutionary convergence, where the cell employs in distinct proteins different metals and cofactors to achieve the same ends for catalysis and cellular bioenergetics.

Diversity of Electron Donation to a Conserved Binuclear Heme-Nonheme Iron Site in Nitric Oxide Reductases

It is NO reductase that reverses dinitrogen fixation and catalyzes N-N bond formation by reductively transforming NO to N_2O. The enzyme appears to belong to an evolutionary ancient lineage of respiratory complexes (Saraste and Castresana, 1994; van der Oost *et al.*, 1994). An X-ray structure is not yet available, but the catalytic subunit, NorB, has been modeled onto the subunit II of cytochrome *c* oxidase (Kannt *et al.*, 1998). The model is thought to come close to the real situation due to the tight correspondence in the transmembrane helices, putative histidine ligands, and limited extra membrane domains allowing little structural variability. Within the concept of an evolutionary linkage between the two types of enzymes it is striking that certain NO reductases have oxygen reductase activity (Fujiwara and Fukumori, 1996), whereas the cytochrome oxidases of *Thermus thermophilus* have significant NO reductase activity (Giuffrè *et al.*, 1999).

The *R. eutropha* NO reductase is distant in a phylogenetic comparison to the *Pseudomonas* NO reductase. This is due to an N-terminal sequence extension of 280 amino acids, which increases the mass of the catalytic subunit from about 53 to 75 kDa (Cramm *et al.*, 1997). The extension comprises two putative transmembrane helices (to raise the total

number from 12 to 14) and a periplasmic domain extending between helices one and two. Different from the situation of *P. stutzeri*, *P. aeruginosa*, *Pa. denitrificans* and other closely studied denitrifiers, the *Ralstonia norB* gene is not associated with a second gene, encoding the cytochrome *c* subunit of NO reductase. The purified enzyme has no cytochrome *c*-oxidizing activity but shows NO dependent quinol-oxidation (Cramm *et al.*, 1999). Homologues of the *Ralstonia*-type enzyme are encoded in the genomes of *Neisseria* sp., *Synechocystis* sp., *Bacillus stearothermophilus*, *Staphylococcus aureus*, *Mycobacterium avium* and *Corynebacterium diphtheriae*. It has been proposed to refer to this enzyme as 'qNOR' to differentiate it from the cytochrome dependent 'cNOR' (Hendriks *et al.*, 2000). A third variant of NO reductase has been found recently in the grampositive denitrifier *Bacillus azotoformans*. This dimeric enzyme is quinol dependent but carries also a Cu_A center (Suharti *et al.*, 2001), known otherwise only from N_2O reductase and cytochrome *c* oxidase. The electron paramagnetic resonance spectrum shows a well resolved hyperfine splitting due to the binuclear Cu_A center, whereas the optical spectrum is characteristic of the heme B content.

Chemical analysis, electron paramagnetic resonance and optical spectroscopy for all purified NO reductases indicate that the catalytic subunit of the enzyme has low-spin and high-spin hemes and nonheme iron, as established initially for the NO reductases of *P. stutzeri* (Cheesman *et al.*, 1998; Kannt *et al.*, 1998) and *Pa. denitrificans* (Hendriks *et al.*, 1998; Moënne-Loccoz and de Vries, 1998). Heme and nonheme iron form the binuclear catalytic site where NO is reduced to N_2O (a center homologous to the binuclear oxygen-reducing site of cytochrome *c* oxidase and other heme-copper oxidases). The catalytic subunit of NO reductase has conserved its architecture and is apparently interlaced during evolution with that of the active site of the heme-copper oxidases and their electron donation pathways. The later vary in both the NO reductases and terminal oxidases among cytochrome *c*, quinone, and Cu_A redox centers.

Protective Mechanisms

Within a limited concentration range bacteria are able to cope with NO, and some seem to have developed protective measures to extend the range of NO tolerance or counteract a particular NO susceptibility. The best studied examples of protective proteins, other than the NO reductases themselves, are flavohemoglobin and cytochrome c'. Both proteins cannot be presumed to belong to the obligatory inventory of a denitrifier; yet, where they occur, they act as alternative sinks and take part in NO homeostasis. Flavohemoglobins have a wide distribution among bacteria. This includes the denitrifiers *R. eutropha* (Cramm *et al.*, 1994), *P. stutzeri* (Vollack and Zumft, 2001), and *P. aeruginosa* (Stover *et al.*, 2000). Flavohemoglobin binds heme B and FAD. The flavoprotein domain is structurally similar to that of ferredoxin-NADP-reductase. Flavohemoglobin is involved in NO detoxification and depending on the availability of oxygen, it eliminates NO in an NADH dependent reaction either by its oxygenase activity, generating mainly nitrate, or by reducing NO to N_2O (for review, see Poole and Hughes, 2000). The synthesis of flavohemoglobin in *Escherichia coli* is induced by 0.2 mM sodium nitroprusside (SNP), *S*-nitrosothiols, or nitrite. Cytochrome c' is a periplasmic heme protein that has its heme C-binding motif located C-terminally. The heme iron is high-spin. It binds NO and CO and confers distinct optical properties to this protein. Cytochrome c' is found in several denitrifiers and is thought to function as an NO scavenger or buffer for NO (Yoshimura *et al.*, 1988, 1993; Moir, 1999; Cross *et al.*, 2000). Recent evidence suggests that cytochrome c' indeed functions as an NO reductase (Cross *et al.*, 2001).

Nitric Oxide as a Signal Molecule

The discovery of the function of NO as a regulatory signal in bacterial denitrification has its roots in observations with nitrite reductase mutants. Inactivation of *nirS*, the structural gene for cytochrome cd_1, not only causes loss of nitrite reduction but also decreases NO reduction and leads to a low level of NO reductase expression (Ye *et al.*, 1992; de Boer *et al.*, 1994; Palmedo *et al.*, 1995; Arai *et al.*, 1999). This effect is also observed when nitrite reduction is impeded other than by inactivating *nirS*, for instance genes required for heme D_1 biosynthesis. A crucial observation to explain these results came from interspecies exchange of *nirK*. In spite of the different biochemical nature of the two types of nitrite reductases, it is possible to express *nirK* in active form in a $NirS^-$ background (Glockner *et al.*, 1993). This relieves the low level expression of NO reductase. Since the common denominator of NirK and NirS proteins is the generation of NO, we proposed NO as inducer of its own reductase and existence of an NO-signaling mechanism (Zumft *et al.*, 1994; Palmedo *et al.*, 1995).

Subsequent work has provided supportive evidence for this concept. The preferred experimental approach consists in the use of an artificial NO donor (mostly the nitrosating agent SNP) for whole cells. The signal activity of NO is monitored with *lacZ* reporter gene fusions of putative target promoters provided in *trans* or introduced chromosomally. Such studies with the *nirK* promoter of *R. sphaeroides* (Kwiatkowski and Shapleigh, 1996; Tosques *et al.*, 1996a) and the *nirS and norC* promoters of *Pa. denitrificans* (van Spanning *et al.*, 1999; Hutchings and Spiro, 2000) have shown that SNP serves as an activator. The effective concentration of NO as an inducer was determined with *P. stutzeri* by adding NO gas to a cell suspension and following RNA transcripts. Five to 50 nM NO were sufficient for a maximal transcription of the *nirSTB* and *norCB* operons (Vollack and Zumft, 2001). Interestingly, the effective NO concentration is close to that building up during steady state denitrification.

The *nir* and *nor* genes of *Pseudomonas* sp. and *Pa. denitrificans* are activated by millimolar concentrations of nitrite (Arai *et al.*, 1991, 1999; Härtig and

Zumft, 1999; Murai *et al.*, 2000). Expression of *nirS* from *P. aeruginosa* shows a linear response up to 5 mM nitrite. The principal nitrite response can now be explained by the generation of NO from nitrite. However, it is open whether the nitrite effect is entirely due to NO generation or whether there are genuine regulatory responses triggered by nitrite (or also nitrate), and acting on *nir* and *nor* genes. With regard to NO as the signal molecule, the question has to be answered where this signal comes from during the transition from aerobiosis to denitrification. There may be alternative NO sources in the cell. Nitrate respiration has been brought into the context of NO formation (Ji and Hollocher, 1989; Kalkowski and Conrad, 1991), but this requires further experimental scrutiny with regard to gene regulation.

Nitrate and Oxygen Signaling

When nitrate is added to anoxic cells it induces first respiratory nitrate reductase and generates nitrite (Körner and Zumft, 1989; Körner, 1993; Baumann *et al.*, 1996). This, in turn, may trigger the NO-specific signal transduction pathway by forming NO enzymatically or nonenzymatically. The activation of the *narGHJI* operon, encoding respiratory nitrate reductase is under the control of NarXL, a nitrate (to some extent also nitrite) responsive two-component system. The genes for the nitrate sensor, *narX*, and the response regulator, *narL*, are located upstream of the *nar* operon in *P. stutzeri* (Härtig *et al.*, 1999), *P. aeruginosa* (Rompf *et al.*, 1998b), and *Pseudomonas fluorescens* (Philippot *et al.*, 2001). Knockout mutagenesis of *narXL* prevents *nar* expression in *P. stutzeri*, but the other reductases of denitrification are not affected. The *Pseudomonas* NarXL regulatory system is structurally and functionally homologous to that of *E. coli* (Darwin and Stewart, 1996). Induction of denitrification in *Pa. denitrificans* shows a well established nitrate response (Baumann *et al.*, 1996), but it is not clear whether this is mediated by a NarXL system. Full expression of the *nar* operon of *Pa. pantotrophus* requires besides nitrate also NarR, a regulator with similarity to FNR factors without the cysteine motif (see below) (Wood *et al.*, 2001).

A nitrite sensitive two-component system has been identified in *Neisseria gonorrhoeae*, which activates *aniA*, encoding the Cu-containing dissimilatory nitrite reductase (Lissenden *et al.*, 2000). Because of the effector specificity, the system is being referred to as NarPQ, akin to the *E. coli* two-component system with preference for nitrite. Nitrite denitrification is a general trait of the genus *Neisseria*. *N. gonorrhoeae* reduces low concentrations of nitrite, but not nitrate, and generates N_2O. The reaction involves a nitrite reductase, located unusually in the outer membrane, and a quinol-dependent NO reductase. The nitrite reductase is essential for establishing anaerobic growth. The qNOR of nondenitrifying bacteria is often found associated with a nitrite reductase (Hendriks *et al.*, 2000). This may confer a selective advantage to a bacterium and perhaps is important also for establishing pathogenicity. A bacterium that is exposed in its habitat to NO under microaerophilic or intermittently to oxic and anoxic conditions can eliminate NO and nitrite (the latter formed from NO oxidation) by reduction to nonreactive N_2O, provided that it disposes over both nitrite reductase and NO reductase.

Expression of the *nar* operon depends also on a signal generated by low oxygen tension or full anaerobiosis. This response can be mediated by an FNR-type transcription factor such as ANR in *P. aeruginosa* (Ye *et al.*, 1995) or FnrP in *Pa. denitrificans* (van Spanning *et al.*, 1997). In *N. gonorrhoeae* the expression of the *aniA* gene is dependent also on an FNR-type regulator. The anaerobic regulator of *P. stutzeri* acting on the *nar* operon has not been found yet. Although this bacterium harbors with FnrA a canonical FNR-type regulator, its inactivation does not affect nitrate respiration (Cuypers and Zumft, 1993). Although, the overall picture for activating the *narGHJI* operon and *aniA* seems to involve regulators of the NarXL and the FNR families, mediating nitrate/nitrite and oxygen response, respectively, this dual signal transduction pathway has to be demonstrated yet for any one of the model denitrifiers. If we consider denitrification a rather ancient respiratory mode, several modifications may have been realized. In *Bradyrhizobium japonicum* oxygen is sensed by the heme protein FixL of the FixLJ two-component regulatory system. The response regulator FixJ activates $fixK_2$ encoding an FNR-related transcription factor activating *nar* (Nellen-Anthamatten *et al.*, 1998). It is not known whether a further signal originating from an N oxide is fed into the regulatory cascade.

The DNR Group of FNR-Related Transcription Factors

On sequencing the regions downstream of the *nor* operons of *P. aeruginosa* (Arai *et al.*, 1995), *Pa. denitrificans* (van Spanning *et al.*, 1995), *R. sphaeroides* (Tosques *et al.*, 1996b) and *P. stutzeri* (Zumft, 1997; Vollack *et al.*, 1999) open reading frames were found, whose products showed similarity to the *E. coli* transcription factor FNR. Knockout mutagenesis of these factors revealed in each case that they are necessary for the transcriptional control of nitrite reductase and NO reductase genes, i.e., *nirS*, *nirK*, and *norCB*. Their action assures the coordinate expression of the principal enzymes for NO homeostasis. The factors were termed DNR for ‘dissimilatory nitrate respiration regulator’ in *P. aeruginosa*, and NNR or NnrR for ‘nitrite, nitric oxide reductase regulator’ in *Pa. denitrificans* and *R. sphaeroides*, respectively. In *P. stutzeri*, where at least four FNR-related transcription factors coexist, the factor acting on the *nirSTB* and *norCB* operons was termed DnrD (formerly FnrD), to indicate its involvement in denitrification.

The denitrification regulators are characterized by lack of the cysteine motif necessary for binding a [4Fe-4S] cluster that mediates the oxygen response of FNR-type factors. Initially, DNR and NNR proteins were considered as FixK-like factors of the FNR family (Zumft, 1997). FixK transcription factors also lack the FeS cluster and are functionally involved in nitrogen fixation. Examination of the relationship of

the denitrification regulators with the FixK-like factors, however, show them to group in an own branch in the phylogenetic tree of the FNR family (Vollack *et al.*, 1999) and are referred to collectively here as DNR group. DNR factors, except that from *Synechocystis* sp., share the same recognition helix for DNA binding, which is a conserved ExxSR motif and makes contacts to TA and GC base pairs (Spiro, 1994).

The glutamate residue in the recognition helix is absent from NnrR of *R. sphaeroides*, *Pseudomonas* sp. G179, and *A. faecalis* S-6. A number of other putative NnrR-related factors found in genome projects share overall sequence conservation but not (providing the absence of sequence uncertainties) the conserved recognition motif. The current status shows a correlation of NnrR-related factors targeting *nirK* genes for Cu-containing nitrite reductase on one side (*R. sphaeroides* 2.4.3 and 2.4.1, *R. sphaeroides* f. sp. *denitrificans*, *Pseudomonas* sp. G-179, and *A. faecalis* S-6), and DNR-related factors acting on *nirS* genes for cytochrome cd_1 nitrite reductase on the other side (*P. aeruginosa*, *P. stutzeri*, and *Pa. denitrificans*) (unpublished data). If the structural and functional divide consolidates, it may be appropriate to consider separate subgroups for DNR and NnrR factors and adopt labels according to phylogenetic placement.

Other than the FNR-type factors, the DNR-NnrR factors are not involved in oxygen sensing. Mutation of the single cysteine of NNR does not cause loss of NNR activity (Hutchings *et al.*, 2000). This distinguishes this protein from the oxygen-sensing Flp, an FNR-like factor of *Lactobacillus* (Gostick *et al.*, 1998). Flp mediates oxygen response by the redox reaction of (–SH groups of cysteines on neighboring subunits. Several other proteins are similar to DNR factors. Of those, only the function of HbaR from *Rhodopseudomonas palustris* has been investigated. HbaR acts on *hbaA* encoding 4-hydroxybenzoate-CoA ligase of the pathway for degradation of aromatic compounds (Egland and Harwood, 2000). It is thought to become activated as a transcription factor by interaction with 4-hydroxybenzoate. Such a function is supported from genetic evidence, but demonstration of HbaR binding directly to the target promoter in response to the effector molecule is still necessary.

A Sigma Factor σ^{54} Dependent NtrC-Related Transcription Factor in *Ralstonia*

In *R. eutropha* the gene encoding a quinol-dependent NO reductase is duplicated on the chromosome and the megaplasmid. Either reductase is functional and a mutation in both structural genes is required to abolish the ability of the bacterium to reduce NO (Cramm *et al.*, 1997). The NO reductase gene, *norB,* is organized as an operon with an upstream located *norA* gene (Pohlmann *et al.*, 2000). NorA is a homologue of DnrN and not an NO reductase gene proper. Homologues of DnrD and NorA are ScdA of *Staphylococcus aureus*, YtfE of *E. coli* (accession number P39313) and *Haemophilus influenzae* (accession number P45312), and a slightly smaller protein (mol. mass 18 kDa) of *Neisseria meningitidis* (accession numbers AAF41739 and CAB84804) (Vollack and Zumft, 2001). The precise function of these proteins is unknown but their existence in different bacteria attributes them a broader significance. Mutagenesis of *dnrN* affects transcript pattern and mRNA stability of the *nirS* operon in *P. stutzeri*.

The regulatory situation in *R. eutropha* is quite different from that of other denitrifiers studied thus far. Upstream of *norA* and transcribed in the opposite direction, the *norR* gene encodes a regulator of the NtrC family. An identical, duplicated gene arrangement is found on the chromosome and the megaplasmid (Pohlmann *et al.*, 2000). The C-terminus of the NorR proteins consists in a putative DNA-binding domain; the central domains have conserved ATP-binding motifs of response regulators interacting with RpoN. Expression of *norR* is sigma-54 dependent, explaining the previous observation of an RpoN requirement for denitrification in this bacterium. *R. eutropha* responds to nitrate with the activation of the promoters of *nirS*, *norA*, and *nosZ*. *nirS* and *nosZ* are activated in a co-ordinate manner, whereas *norA* activation is sequential in relation to the other two genes. The *norA* operon responds also to SNP. In a *nirS* mutant SNP still acts as a strong activating signal, but activation by both nitrate and nitrite is reduced to about one third of wild-type level. This indicates that nitrate and/or nitrite metabolism generate the NO signal, but might also provide an independent further signal.

Expression of qNOR of *N. gonorrhoeae* is induced by the NO donor spermine-NO in an *aniA* null background suppressing NO formation from nitrite (Householder *et al.*, 2000). Induction is possible both aerobically and anaerobically. Gene expression is not dependent on FNR or NarP but may require a DNR or NtrC factor yet to be identified. In the case of *Synechocystis* sp. PCC6803 it is remarkable that the gene for qNOR is next to an open reading frame whose gene product clearly belongs into the DNR branch of regulators. This opens the possibility that the regulation of NO reductase in this cyanobacterium is DNR dependent, a potential variation for qNOR regulation versus the situation realized in *R. eutropha*.

NO Responsive Promoters

DNR factors serve as activators of *nir* and *nor* genes but it has to be established that this is a direct dependence of the respective promoters. In as much as sequences of *nir* and *nor* genes became available promoter regions could be searched for consensus motifs with a putative regulatory function. In all *nirS* and *norC* promoters partially palindromic motifs were found with a high similarity to the binding motif for FNR, here referred to as the DNR box (Figure 1). The center of these motifs is located 40.5 to 43.5 nucleotides upstream of the transcript start, which makes the promoters to belong to class II. In this class the activator makes contact promoter-proximal to the β-subunit of RNA polymerase, overlapping the −35 promoter element, and promoter-distal with the C-terminal domain of the α-subunit (Busby and

***nirS* promoter**

TTGATtgccGTCAA	-43.5	*P. stutzeri*
TTGATtccgGTCAA		*P. aeruginosa*
TTaAcaatgGTCAA	-41.5	*Pa. pantotrophus*
TTaAcaaagGTCAA	-41.5	*Pa. denitrificans*

***norC* promoter**

TTGATtgccATCAA	-40.5	*P. stutzeri*
TTGATtgccATCAA		*P. aeruginosa*
TTGActttcATCAA	-43.5	*Pa. denitrificans*

Figure 1. Recognition sites for DNR and NNR regulators in the promoters of denitrification genes. Where known, the distance of the center of the motif from the transcript start is indicated.

Ebright, 1999). Direct binding of a DNR-NNR factor to the DNR box is suggested from indirect evidence but has not been shown experimentally yet. The DNR boxes of the *nirS* promoter (Saunders *et al.*, 1999) and the *norC* promoter (Hutchings and Spiro, 2000) of *Pa. denitrificans* have been altered in several nucleotides. Such altered promoters fused to *lacZ* reporter constructs have no β-galactosidase activity under anaerobic conditions in the wild-type background, indicating that NNR binds to the palindromic sequence in this bacterium. Thus, the loss of *nir* and *nor* expression in *dnr* or *nnr* mutants is due to DNR or NNR dependent promoters.

The *nor* genes of *Pa. denitrificans* are organized as a six-gene operon, *norCBQDEF*, yielding transcripts of 5.4 and 2.2 kb. The former covers the entire operon, whereas the short transcript corresponds to *norCB* and may be the product of mRNA processing (Murai *et al.*, 2000). A function of the additional genes of the operon has not been clearly established yet. The *norC* promoter of *Pa. denitrificans* has two transcription-start sites (Hutchings and Spiro, 2000). The major transcript initiates 43.5 nucleotides downstream of the DNR box. A minor transcript is found also in aerobically grown cells. However, it is absent in an *nnr* mutant, which suggests that NNR plays also a role in the *aerobic* expression of the *nnr* gene. Deletion of the DNA region upstream of this promoter does not cause a loss of anaerobic promoter activity, underlining that the DNR box is the major *cis*-acting element for *nor* operon expression.

The *nirS* gene of *P. stutzeri* is transcribed as an operon to yield a *nirSTB* transcript together with a monocistronic *nirS* mRNA. *nirT* was the first member of a now sizable gene family encoding tetraheme cytochromes. Several of them are involved in electron donation to enzymes, for instance NapC to periplasmic nitrate reductase. The half-life of the *nirS* mRNA is about 13 min, which is also the case for the *norCB* and *nosZ* mRNAs (Härtig and Zumft, 1999). The *nirS* gene of *Pa. pantotrophus* is not part of an operon (Saunders *et al.*, 2000). In anaerobically grown cells a transcript of about 1800 nucleotides is found, corresponding to the size of the structural gene, which is absent from aerobically grown cells. This is important in the context whether this bacterium is indeed an aerobic denitrifier. The molecular analysis supports now what was already in doubt since aerobic cells have no detectable cytochrome cd_1 (Moir *et al.*, 1995).

The *nirS* promoter of *Pa. denitrificans* is activated by NNR, however, it requires also the *nirI* gene product, encoded just upstream of *nirS* and in opposite direction (Saunders *et al.*, 1999). The role of NirI is puzzling. It exerts a positional effect on *nirS* transcription, since it is required upstream of *nirS* and cannot be supplied in *trans*. A *nirI* strain has no detectable *nirS* promoter activity. NirI is homologous to NosR, a membrane-bound putative regulator of *P. stutzeri* required for the transcription of *nosZ*, the structural gene for nitrous oxide reductase. *nosZ* is not expressed in a *nosR* mutant, but NosR has no effect on *nirS* (Cuypers *et al.*, 1992). *nosR* is always found associated with *nosZ*, but *nirI* is not a constant component of *nir* gene clusters in the genetically analyzed denitrifiers; if essential, it must be located elsewhere. The principal topology of NosR has been determined (Cuypers *et al.*, 1995; Zumft, 1997). It consists of an N-terminal transmembrane anchor followed by a large periplasmic domain which is followed by five transmembrane helices and ends in a C-terminal cytoplasmic domain with one or two putative FeS centers. NirI shows the same topology. The precise function of these interesting membrane proteins in the transcriptional activation of denitrification genes remains to be explored in more detail.

Considerable interest is focusing now on the question of how members of the DNR group are activated by NO. Constitutive overexpression of *dnrD*, presumably leading also to a higher DnrD level in the cell, is insufficient to activate DnrD dependent target promoters unless the N oxide signal is also provided (Vollack and Zumft, 2001). This is a strong argument for the necessity of DnrD activation by NO and the existence of interconvertible active and inactive states. The pathway seems to be mechanistically not tied to denitrification to allow for the finding that NNR expressed in *E. coli* can be activated by SNP (Hutchings *et al.*, 2000). Figure 2 depicts various hypotheses of activating the regulator by NO, which are discussed below. It is feasible that there is more than one signal transduction pathways involving the NO-dependent transcription factors of denitrification. Remarkably, the *norA* promoter of *R. eutropha*, which is dependent on the NtrC-related regulator NorR, is linearly responsive toward SNP up to 2 mM (Pohlmann *et al.*, 2000).

Interaction of NO with a transcription factor may be of a direct or an indirect nature. NO may be sensed by and influence an enzymatic activity, for instance a nucleotide cyclase, and regulate DNR activity by providing a cofactor. DNR could also be modified covalently by an interacting protein or a cascade of

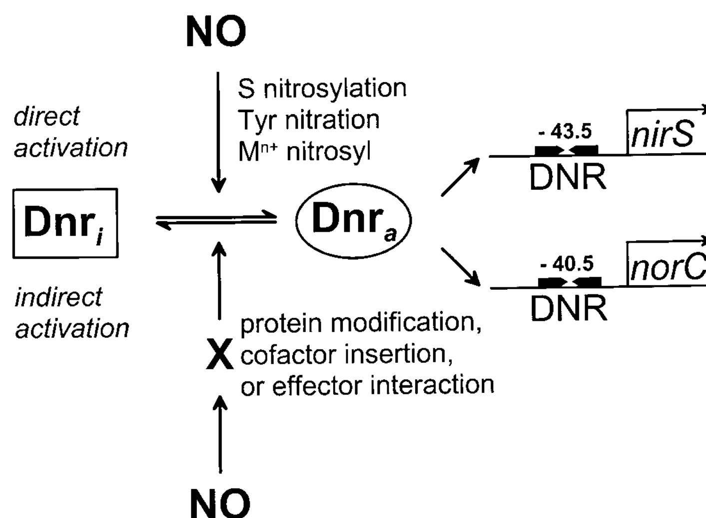

Figure 2. Model of interlacing NO and Dnr-mediated signal transduction. Dnr factors (the same holds for Nnr factors) are assumed to exist in active and inactive conformations, determining DNA binding to the recognition sequence, or DNR box, located approximately 40 bp upstream of the transcription start. Various mechanisms, direct or indirect and all of them still hypothetical, allow for the interconversion of the two Dnr forms. Details are discussed in the text. Activation has to be reversible but the requirements of the reverse reaction remain open at this moment.

proteins, one of which acts as an NO sensor. Supportive evidence for such a complex pathway is not available. More findings seem to exclude currently certain possibilities rather than they would clearly support a particular mechanism. NO is a radical and the aspect of NO toxicity has led to the identification of a number of NO-dependent reactions, which potentially can provide a mechanism for signal transduction by altering the binding activity of a DNR factor toward its target promoter. Thus, a cellular reactant with NO may also be considered a candidate effector for activation of DNR. Reaction of NO with superoxide leads to formation of peroxynitrite, which in turn could lead to nitration of a tyrosine. A conserved tyrosine found in NNR and other factors activating *nir* and *nor* genes has been mutated to phenylalanine, and although NNR activity was reduced, this tyrosine residue is not essential (Hutchings *et al.*, 2000). Under anaerobic conditions of the N_2O-grown cell, DnrD is active, which does exclude the possibility of tyrosine nitration since peroxynitrite cannot be formed (Vollack and Zumft, 2001).

NNR has a single cysteine whose mutation to serine has even a lesser effect on NNR activity than the tyrosine-phenylalanine exchange. This argues against an –SH nitrosylation mechanism. From structural considerations, we have excluded above the possibility that DNR and NNR factors are FeS proteins. A mechanism of a direct nitrosylation of an FeS cluster, as shown for SoxR of the *sox* regulon involved in the NO stress response of *E. coli* (Ding and Demple, 2000), or nitrosylation of the FeS cluster of the iron regulatory protein 1 of the eukaryotic cell (Wardrop *et al.*, 2000), is not feasible thus. This leaves us currently with the challenging and intriguing problem of how DNR, NNR and NorR are converted in the cell by NO to their active state as transcription factors.

Eluding Crosstalk with Other FNR-Type Regulators

Denitrification is a facultative process and its gene program is expressed under conditions of low oxygen tension. The [4Fe-4S] protein FNR of *E. coli* is the paradigm of an oxygen responsive transcriptional regulator (Kiley and Beinert, 1999; Green *et al.*, 2001). The first of an FNR-type regulator in denitrifying bacteria, ANR, was discovered in the context of studying arginine and pyruvate catabolism of *P. aeruginosa*. Several denitrifying bacteria are now known where FNR-type regulators, mediating oxygen response, and DNR factors, mediating NO response, coexist. Since DNR and FNR-type regulators recognize the same binding site in their respective target promoters it leads to the question of discriminatory elements (van Spanning *et al.*, 1997; Zumft, 1997). Binding of these regulators could be highly discriminatory, recognizing a single nucleotide which, however, seems not to be the case. There is no distinct modification in the DNR box that would provide a discriminatory element compared with the FNR box. DNR boxes of the *nor* promoters correspond to the TTGAT-N_4-ATCAA consensus of the FNR box (Figure 1). Intriguingly, a half site of the DNR box on *nirS* promoters is GTCAA rather than ATCAA. However, since the same regulator is assumed to bind to both the *nirS* and *norC* promoters it is clear that there is no discriminatory element in a GTCAA versus an ATCAA half site. A promoter analysis addressing the influence of neighboring sequences on a specific recognition event is not yet available. It is conceivable that further proteins have to bind at the promoter. The *nirS* promoter of *P. stutzeri* has a clear -10 sigma factor σ^{70} element (Härtig and Zumft, 1999), but such sequence is not evident in the *Pa. pantotrophus* pendant (Saunders *et al.*, 2000). It is likely that the fine tuning of

promoters with FNR and DNR boxes is not uniformly throughout the different genera.

ANR serves not only as positive anaerobic regulator, but also as an *aerobic* regulator for nitrite reductase (Ka *et al.*, 1997) and the *hemN* gene of *P. aeruginosa*. The latter encodes the oxygen independent coproporphyrinogen III oxidase (Rompf *et al.*, 1998a). Under denitrifying conditions both ANR and DNR are required for *hemN* expression. On the other hand, ANR and FnrP function also as repressors for certain genes (Ray and Williams, 1997; van Spanning *et al.*, 1997). NNR (van Spanning *et al.*, 1997) and DnrD (Vollack *et al.*, 1999) downregulate *nar*. Nitrate reductase activity of *Pa. denitrificans* is increased in an *nnr* mutant; in *P. stutzeri* inactivation of *dnrD* causes an increase in the activity and level of the enzyme as well as the amount of *nar* mRNA (unpublished data). DNR factors have been found to operate sometimes also in a hierarchical relationship to FNR-type factors to form a two-step regulatory cascade. The ANR→DNR hierarchy in *P. aeruginosa* (Arai *et al.*, 1997) can explain the integration of oxygen and substrate signals for the expression of denitrification genes. Other examples, not related to denitrification, are FnrA→DnrS of *P. stutzeri* targeting anaerobic ribonucleotide reductase (Vollack *et al.*, 1999), and AadR→HbaR of *R. palustris* regulating degradation of aromatics (Egland and Harwood, 2000).

In conclusion, during the past few years we have seen the uncovering of a remarkable versatility of sensory mechanisms integrated into the FNR scaffold. The best understood process is that of oxygen-sensing via the [4Fe-4S] cluster transformation and reassembly mechanism in the canonical FNR-type proteins (Kiley and Beinert, 1999). Then there is the alternative oxygen-sensing mechanism via (SH redox chemistry by FLP proteins (Gostick *et al.*, 1998). Further, we have NO-sensing by DNR and NnrR-related factors as principal facet of regulating denitrification, as described here, and there is the possibility of 4-hydroxybenzoate-sensing by HbaR (Egland and Harwood, 2000). Finally, CO-sensing has been integrated into the FNR-CRP family by developing a hemoprotein variant (Lanzilotta *et al.*, 2000).

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