

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

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Abstract The *nir* and *nor* genes, which encode nitrite and nitric oxide reductase, lie close together on the DNA of *Paracoccus denitrificans*. We here identify an adjacent gene, *nnr*, which is involved in the expression of *nir* and *nor* under anaerobic conditions. The corresponding protein of 224 amino acids is homologous with the family of FNR proteins, although it lacks the N-terminal cysteines. A mutation in the *nnr* gene had a negative effect on the expression of nitrite and nitric oxide reductase. Synthesis of membrane bound nitrate reductase, of nitrous oxide reductase, and of the *cbb₃*-type cytochrome *c* oxidase were not affected by mutation of this gene. These results suggest that denitrification in *P. denitrificans* may be governed by a signal transduction network that is similar to that involved in oxygen regulation of nitrogen metabolism in other organisms.

Key words: Denitrification; FNR; Gene regulation; *nir*; *nor*; *Paracoccus denitrificans*

1. Introduction

The Gram-negative aerobic bacterium *Paracoccus denitrificans* is found in soil, sewage and sludge, environments, in which the oxygen availability can change drastically. The bacterium has a great potential to adapt its metabolism to the prevailing growth condition. Examples are found in its adaptation to low oxygen tensions. At high oxygen concentrations, the *aa₃*-type oxidase is predominant [1], whereas at decreasing oxygen tensions, a *cbb₃*-type oxidase appears to be the most abundant terminal oxido reductase [2]. Recent studies revealed that the gene cluster encoding the latter oxidase ([3]; J. Van der Oost, unpublished observation) is homologous with the *fixNOQP* cluster found in nitrogen fixing species like *Rhizobium meliloti* [4], *Azorhizobium caulinodans* [5], and *Bradyrhizobium japonicum* [6], and with the *ccoNOQP* cluster found in *Rhodobacter capsulatus* [7]. In these species, this high affinity oxidase is expressed at low oxygen tensions under control of FixK, which is an FNR homologue [8]. The transcription factor responsible for the oxygen regulation of the *cbb₃*-type terminal oxidase in *P. denitrificans* has not yet been identified. FNR-related pro-

teins have been found in a number of different organisms in which they regulate the expression of a variety of genes involved in anaerobic respiration [9].

If nitrate is available under conditions of oxygen limitation, *P. denitrificans* synthesizes oxido reductases that enable it to use nitrate, nitrite, nitric oxide, and nitrous oxide as terminal electron acceptors [10]. Because the operons encoding these anaerobic reductases are expressed in response to oxygen limitation, it has been suggested that also here an FNR homologue is involved in their transcriptional activation [11]. Two observations support this view. First, the promoter regions of the *cco* (J. van der Oost et al., unpublished observation), *nir* [12], *nor* (De Boer et al., unpublished observation), and *nos* [13] operons of *P. denitrificans*, which encode the *cbb₃*-type oxidase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, respectively, contain DNA sequences that resemble the consensus sequence of the FNR binding site (FNR box) [14]. Second, an FNR-dependent promoter from *Escherichia coli* is inducible by anaerobiosis in *P. denitrificans* [11].

This is the first report on the finding of an FNR homologue that specifically controls the expression of two closely linked gene clusters which encode the nitrite and nitric oxide reductases, the key enzymes in the process of denitrification.

2. Experimental

2.1. Organisms and growth conditions

P. denitrificans Pd1222, a DSM413 derivative, was cultivated at 30°C in flasks fully filled with either brain heart infusion broth (precultures) or mineral salts medium containing 25 mM succinate and 50 mM potassium nitrate as carbon source and electron acceptor, respectively [15]. Chlorate susceptibility was studied on plates with 25 mM chlorate as electron acceptor. When necessary, antibiotics were added to final concentrations of 40 µg of rifampin, 25 µg of streptomycin, and 100 µg of ampicillin per ml.

2.2. DNA manipulations

General cloning techniques were carried out essentially as described in Current Protocols in Molecular Biology [16]. Plasmid DNA was isolated and purified by using the Qiagen plasmid kit, DNA fragments were purified from agarose gels by using Qiaex (Diagen GmbH, Dusseldorf, Germany). Partial *Sau3A* digested fragments, cloned in M13, were sequenced using the dye-primer and dye-terminator cycle kits from ABI and loaded onto a ABI 373A fluorescent sequencer (Applied Biosystems, Perkin and Elmer). The Macintosh computer programs used were DNA Strider 1.0 and GeneWorks 2.2.1. The E-mail servers of NCBI running the BLAST program, the FASTA and BLITZ server and the BLOCKS server were used for comparison of sequences with the sequence databases.

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The novel nucleotide sequence data published here have been submitted to the GenBank sequence data banks under accession number U17435.

2.3. Protein assays and gel electrophoresis

Spectra and oxidation and reduction kinetics of cytochromes were recorded by using an advanced version of an Aminco/SLM DW-2 UV/vis spectrophotometer. In vivo and in vitro nitrite, nitric oxide and nitrous oxide reductase activities of cell suspensions, and in cell free extracts were determined as described earlier [12]. The in vitro nitrate reductase activity was assayed with or without 20 μ M azide. SDS-PAGE was carried out using the Bio-Rad mini-protein II gel system with 13% slab gels. Sample preparation and staining of cytochromes *c* was carried out as described earlier [12]. Protein concentration was determined with the BCA kit (Pierce), using BSA as a standard.

3. Results and discussion

3.1. Isolation and sequence analysis of the *nnr* gene

The isolation, sequencing, and mutational analysis of the *nir* gene cluster involved in nitrite reduction in *P. denitrificans* has been described recently [12]. In that study, a chromosomal locus of about 20-kb was isolated that contained the *nir* gene cluster. Additional sequence analysis of this locus revealed that the gene cluster encoding nitric oxide reductase (*nor*) was located adjacent to the *nir* gene cluster (T. de Boer, unpublished data). Further downstream, at the distal part of the isolated locus, the 3'-part of an open reading frame (ORF) was found. The deduced amino acid sequence of this part of the ORF shared a high degree of homology with proteins belonging to the FNR family of transcriptional activators. The ORF was tentatively designated *nnr* (nitrite and nitric oxide reductases regulator). In order to isolate the remaining part of this gene by plasmid rescue, an internal 0.2-kb *EcoRI*–*HindIII* restriction fragment of *nnr* was isolated and cloned into the suicide vector pGRPd1 [15]. The construct was transferred to *P. denitrificans*, and integrant strains were selected for streptomycin resistance. One of these strains was isolated and designated Pd77.71. Chromosomal DNA of this strain was isolated, and restricted with *EcoRV*, after which the fragments were self-ligated and used to transform competent *E. coli*. Transformants harbouring pGRPd1 derivatives were isolated on plates with ampicillin and streptomycin. Plasmid DNA of these strains was isolated, and the *nnr* linked chromosomal *EcoRV* fragment was sequenced.

A physical map of the locus with the position of *nnr* relative to the structural *nir* and *nor* genes along with the sequencing strategy is presented in Fig. 1A. The DNA sequence of the *nnr*

gene and flanking regions was determined (accession number U17435). The NNR protein, deduced from the *nnr* coding strand, consists of 224 amino acids, and has a molecular mass of 24,581 Da. As in the case of the *anr* gene promoter region [17], but in contrast to many other *fnr* genes, the *nnr* promoter region does not contain an FNR box.

3.2. NNR of *P. denitrificans* is an FNR homologue

NNR shares a high degree of homology with proteins that belong to the Crp and FNR family of transcriptional activators. Comparison of the NNR amino acid sequence to that of ANR from *Pseudomonas aeruginosa* [18], FnrA from *Pseudomonas stutzeri* [19], and that of FixK from *R. meliloti* [20] revealed 40%, 37%, and 30% identity, and 60%, 60%, and 53% similarity, respectively. An alignment of NNR with FNR and its homologues is shown in Fig. 2A. Just like FixK of *R. meliloti*, NNR lacks the N-terminal cysteines that are typical for FNR and related proteins like ANR and FnrA. These residues are thought to be the ligands for iron, which metal has been implicated to sense the redox state in the cell. The similarity of FNR-like proteins is high in the C-terminal region that contains the helix turn helix (HTH) motif. This DNA binding region contains the highly conserved glutamic acid and serine residues that are essential for binding of the protein to the FNR box [14]. In the alignment of Fig. 2A the region that has been proposed to form a surface exposed loop is also highlighted. This patch is supposed to be the site that interacts with RNA polymerase for transcription activation [21]. The residues that are involved in this contact correspond with residues 52–58 of CRP, and 81–87 of FNR. The putative contact site of NNR contains a conserved glycine residue, but no other apparent consensus was found among the members of the FNR family. A phylogenetic tree of the FNR family of proteins, including NNR, is shown in Fig. 2B.

3.3. Mutational analysis of *nnr*

The integrative recombination between the cloned *EcoRI*–*HindIII* fragment of *nnr* and its chromosomal copy resulted in integration of the pGRPd1 derivative and the creation of two truncated versions of the *nnr* gene, one of which lacked the 3'-part including part of the HTH motif, the other of which lacked the 5'-part including the translational start. As a result,

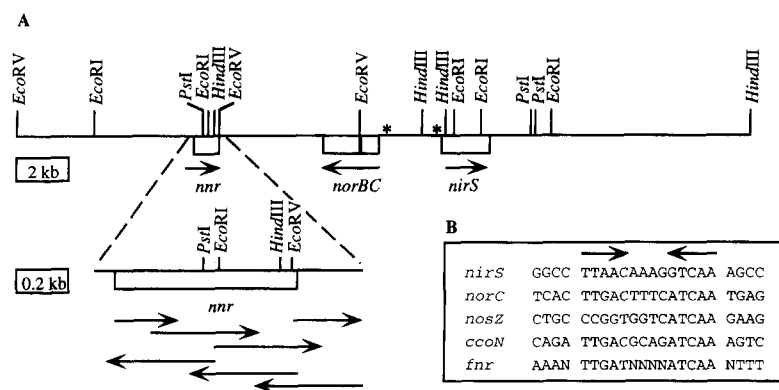


Fig. 1. (A) Physical map of the *P. denitrificans* locus encompassing the *nir* and *nor* gene clusters. The position of the *nnr* gene is indicated by the box marked *nnr*. This region is shown at higher resolution in order to show the restriction map and the *Sau3A* fragments used for sequencing. Positions of putative FNR-boxes are denoted by asterisks. (B) Alignment of the *E. coli* FNR box (lower sequence) [14] with putative FNR boxes in the promoter regions of the *nir* [12], *nor* (A. De Boer, unpublished observation), *nos* [13], and *ccoNOQP* (J. Van der Oost, unpublished observation) gene clusters of *P. denitrificans*.

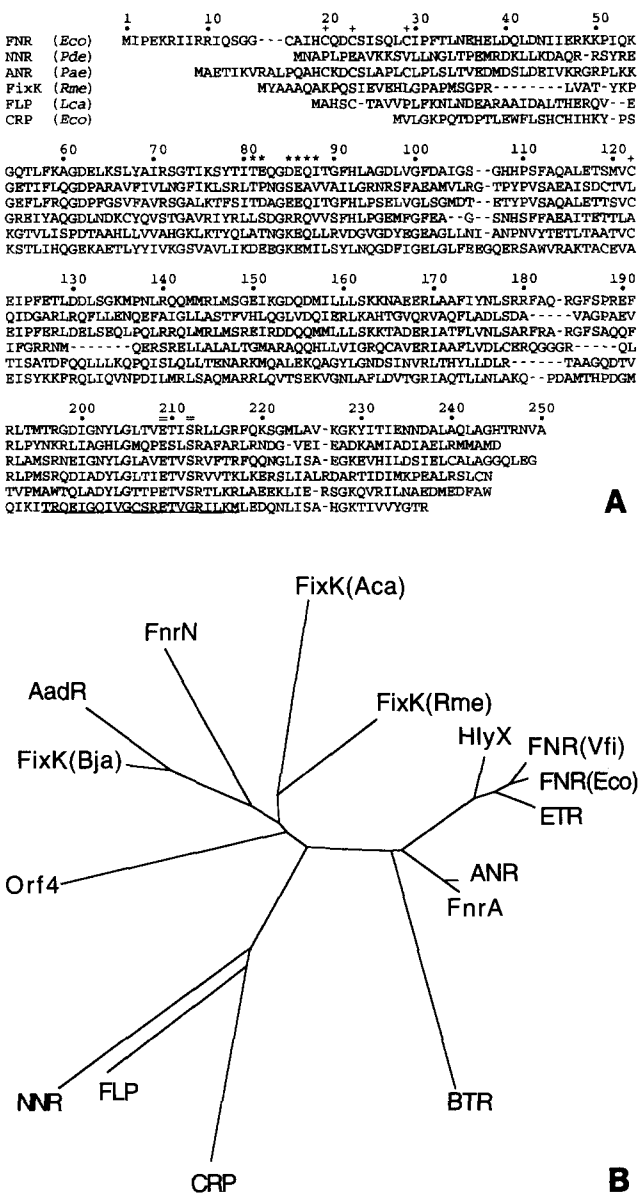


Fig. 2. (A) Alignment of NNR with FNR of *E. coli*, ANR of *Ps. aeruginosa*, FixK of *R. meliloti*, FLP of *Lactobacillus casei* and the cAMP receptor protein of *E. coli*. Cysteine residues required for activity of the *E. coli* FNR protein are indicated by crosses and asterisks indicate residues that have been implicated in interacting with RNA polymerase. The DNA binding domain is underlined, and residues thought to be involved directly in DNA binding are indicated by equals signs. Numbering is shown for the *E. coli* FNR protein. (B) Phylogenetic tree of the FNR family of proteins. All known proteins which are predicted to share the same DNA binding specificity as FNR are included (with the exception of the *Salmonella typhimurium* protein which is virtually identical to the *E. coli* protein), along with the cyclic AMP receptor protein. The tree was generated from an alignment of all 16 sequences, using the programs PROTDIST and NEIGHBOR of the PHYLIP package. The cAMP receptor protein was used as the outgroup, and as the root of the tree.

the mutant strain Pd77.71 should be unable to synthesize the full-size protein. In the event that a truncated version of the protein is synthesized, it is expected to be inactive since it lacks the DNA binding HTH motif. During anaerobic growth on succinate and nitrate, strain Pd77.71 differed from its wild-type in that it accumulated nitrite. Moreover, the mutant did not

produce gas, and reached a 5-fold lower cell density. Spectral analysis revealed that the cytochromes *b* and *c* content was dramatically decreased, while also the absorption peaks at 630 and 655 nm, characteristic for the presence of the *cd*₁-type nitrite reductase [12], were absent (results not shown). Analyses of soluble cytochromes indeed confirmed the absence of nitrite reductase in the *nnr* mutant (Fig. 3). NorC, which is the heme-*c* containing subunit of nitric oxide reductase, was much reduced in the membranes of the mutant, while all other heme-*c* containing membrane proteins were present at wild-type levels. The mutant displayed neither in vivo nor in vitro nitrite reductase activity, while the nitric oxide reductase activity was lowered to 20% of the wild-type level. These findings suggest that expression of nitrite and nitric oxide reductases was strongly compromised by the mutation in *nnr*. This result was not unexpected, since the induction profile of both nitrite and nitric oxide reductase already suggested transcription regulation by an FNR-like mechanism. In addition, FNR boxes have been found in the promoter regions of the *nir* and *nor* gene clusters (Fig. 1B).

Other gene clusters that are thought to be under the control of FNR-like transcriptional activators are those encoding membrane-bound nitrate reductase, nitrous oxide reductase, and the *cbh*₃-type oxidase, all of which are synthesized in response to oxygen limitation. Because the promoter regions of the *ccoN* (J. Van der Oost, unpublished observation), and *nosZ* [13] genes contain sequences similar to the FNR box (Fig. 1B), we also checked the presence and activities of the latter three redox enzymes in the *nnr* mutant strain. From gel electrophoresis of proteins and subsequent heme staining, it was deduced that the *cbh*₃-type oxidase, represented by the 30 and 45 kDa heme stained proteins in the membrane fraction (J. Van der Oost, unpublished observation), was still present in the mutant strain at wild-type levels (Fig. 3A). Therefore, although an identical FNR box TTGAC-N4-ATCAA is found in the promoter regions of the *ccoNOQP* and the *norCB* gene clusters, only the latter one appears to be a target site for NNR. Evidence that expression of membrane-bound nitrate reductase was not affected in the mutant strain was 3-fold. First, just like the wild-type strain, cells of the *nnr* mutant strain are killed on plates with chlorate, which is converted to toxic chlorite by the membrane-bound nitrate reductase. Second, membranes iso-

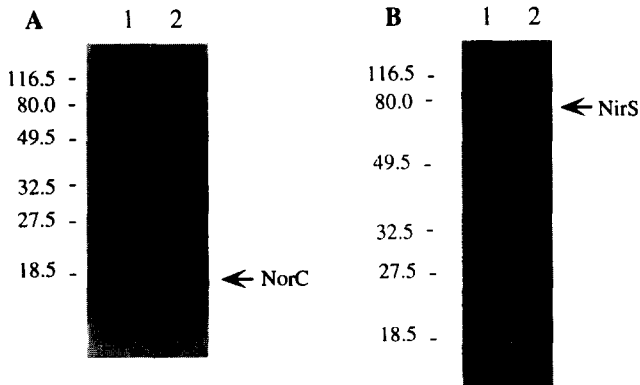


Fig. 3. SDS-PAGE of heme *c* containing proteins from membranes (A) and the soluble fraction (B) of *P. denitrificans* wild-type (lanes 1) and the *nnr* mutant strain (lanes 2). The positions of the marker proteins are indicated by their relative molecular masses. Mobilities of NorC and NirS are as indicated.

lated from the mutant displayed nitrate reductase activity just like membranes from the wild-type strain, and this activity was inhibited by azide. Both features are indicative of the presence of a fully active dissimilatory nitrate reductase [22]. Third, it was demonstrated that nitrate-induced oxidation and reduction rates of cytochromes *c* were similar in whole cell suspensions of both the wild-type strain and the *nnr* mutant strain. The *nnr* mutant strain expresses wild-type levels of nitrous oxide reductase activity, as judged from the kinetics of oxidation and reduction of cytochromes *c* upon addition of nitrous oxide.

3.4. Conclusions

In only a few cases has it been demonstrated that an FNR homologue controls the process of denitrification [18,23,24]. The data presented in this paper demonstrate that NNR of *P. denitrificans* is a protein that is involved in the anoxic regulation of nitrite and nitric oxide reductase activity. Sequence homology suggests that NNR belongs to the FNR-family of transcriptional activators. However, in contrast to the *E. coli* FNR protein, NNR from *P. denitrificans* lacks the cysteine cluster at the N-terminus. The control of NNR on denitrification appears to be exclusively on the level of expression of nitrite and nitric oxide reductases, probably acting as a fine-tuning regulator. Possibly, *P. denitrificans* synthesizes one or more other members of the FNR family, different from NNR, which control the synthesis of nitrate reductase, nitrous oxide reductase, and the *cbb₃*-type cytochrome *c* oxidase. The situation that more FNR homologues with discrete regulatory functions are found in the same species has also been suggested in *Shewanella putrefaciens* [25], *Ps. stutzeri* [19], and *B. japonicum* [24].

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