Expression of the *nir* and *nor* genes for denitrification of *Pseudomonas aeruginosa* requires a novel CRP/FNR-related transcriptional regulator, DNR, in addition to ANR

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Abstract **A gene, designated *dnr*, was identified in the vicinity of the structural genes for nitrite reductase (*nirS*) and nitric oxide reductase (*norCB*), and the gene for activation of the reductases (*nirQ*) from *Pseudomonas aeruginosa*. It encodes a protein of 227 amino acids homologous with the CRP/FNR-family transcriptional regulators. Promoter activities for *nirS*, *nirQ* and *norCB* were considerably reduced in the *dnr* mutant as well as in the mutant of *anr*, the other *fnr*-like regulatory gene from *P. aeruginosa*. This is the first finding that two CRP/FNR-related regulators are involved in denitrification in one strain.**

Key words: Denitrification; Nitrite reductase; Nitric oxide reductase; FNR; ANR; *Pseudomonas aeruginosa*

1. Introduction

Bacterial denitrification is an anaerobic respiration to use nitrogenous oxides as terminal electron acceptors. Four enzymes, i.e. nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase, are required for complete reduction of nitrate ion to dinitrogen gas. These enzymes are induced under the low oxygen environment. Expression of the denitrification genes under the anaerobic condition is thought to be controlled by a regulatory protein similar to FNR of *Escherichia coli*. FNR is a transcriptional regulator for anaerobiosis of *E. coli* which belongs to the same family with CRP, the regulator for catabolite gene repression [1]. There is a specific recognition sequence (FNR box), TTGATATCAA, in the promoter region of the anaerobically expressed genes regulated by FNR. Many of the denitrification genes so far sequenced have motifs resemble to the FNR box in their promoter region [2–9]. The gene, *anr*, which is homologous with *fnr* of *E. coli* was found from *Pseudomonas aeruginosa* and shown to be necessary for anaerobic arginine catabolism and nitrate reduction [10,11]. The genes for dissimilatory nitrite reductase (*nirS*) and nitric oxide reductase (*norCB*) had ANR (FNR)-binding motifs in their promoter region [4,5] and the transcription of *nirS* was defective in the *anr* mutant [12]. A gene homologous with *fnr* was also isolated from *Pseudomonas stutzeri* by using the *anr* gene from *P. aeruginosa* as a hybridization probe and designated *fnrA*. Although the derived amino acid sequence of the *fnrA* gene was very similar to that of the *anr* gene, the *fnrA* mutant of *P. stutzeri* was able to grow on nitrate under the anaerobic condition. The activity of denitrification enzymes was not affected by the *fnrA* mutation whereas the activity of arginine deiminase was defective. From these results, Cuypers and Zumft predicted that FnrA is a specific regulator for arginine catabolism and that a second hypothetical FNR homolog, designated FnrD, controls the expression of the denitrification enzymes in *P. stutzeri* [13].

In this work, we found a second *crp/fnr*-related gene from *P. aeruginosa*. We propose to call the gene as *dnr* for *d*issimilatory *n*itrate *r*espiration *r*egulator. It is uncertain that *dnr* corresponds to the hypothetical regulatory gene, *fnrD*, from *P. stutzeri* at present, but the anaerobic growth by nitrate respiration and transcription of the *nir* and *nor* genes of *P. aeruginosa* completely depended on the gene.

2. Materials and methods

2.1. Bacterial strains and plasmids

P. aeruginosa PAO1 [14] was used as a source for the denitrification genes and to represent a wild-type trait. PAO6261, the *anr* mutant of *P. aeruginosa* PAO1 was provided by D. Haas [15]. A plasmid, pHAE1, which carries a 6.5 kb *Eco*RI fragment containing ORF7 and a part of the *dnr* gene was cloned in the previous work [5]. Charomid 9–36 (Nippon gene, Toyama, Japan) was used for cloning a fragment containing the complete *dnr* gene. pUC119 [16] was used for subcloning, sequencing and mutagenesis of *dnr*. For the assay of promoter activity, the *lacZ* promoter probe vector pQF50 [17] was used. *Escherichia coli* hosts were strain DH5 for Charomid and strain JM109 for other plasmids [16].

2.2. Media and growth conditions

Bacterial strains were cultivated at 37°C in LB medium or on LB plate [16]. Cultivation for promoter assay was done in the synthetic medium described by Wood [18] supplemented with 5 mM sodium nitrite instead of 40 mM sodium nitrate. When necessary, antibiotics were added to the medium at the following concentrations (μg/ml): ampicillin (Ap) 100 and tetracycline (Tc) 12.5 for *E. coli*; and carbenicillin (Cb) 150 and Tc 200 for *P. aeruginosa*. Tc was not added for the cultivation to measure the β-galactosidase activity.

For oxygen-limiting condition, a 50-ml vial (70-ml total volume) containing 20 ml of the synthetic medium was used. After inoculating 200 μl of overnight culture aerobically grown in LB medium, the vial was fitted with a butyl rubber septum and an aluminum seal. The air in the vial was replaced with argon by flushing the gas through a needle for 5 min and then oxygen gas was added into the vial to 2% (1 ml) by a gastight syringe. Cultivation was done with gentle shaking.

2.3. Transformation and mutation of P. aeruginosa

Introduction of DNA into *P. aeruginosa* strains was carried out as follows: 0.2-ml overnight culture of *P. aeruginosa* was inoculated into 20 ml of LB medium. After about 2 h cultivation with vigorous shaking, cells were harvested by centrifugation, suspended in 10 ml of ice-cold buffer (100 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5) and stored on ice

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for 30 min. Then cells were recovered by centrifugation and resuspended in 1 ml of the same buffer. To 100 μl of the cell suspension, appropriate amount of DNA solution was added and stored on ice for 3 h. The suspension was heated at 45°C for 2 min, cooled on ice for 2 min, and then transferred into 1 ml of medium. After incubating at 37°C for 1 h for transformation by the broad-host-range plasmids or overnight for marker exchange mutagenesis, cells were plated onto LB plates containing antibiotics and the objective clones were selected.

2.4. DNA manipulations and β-galactosidase assay

Recombinant gene techniques were carried out by standard methods [16] or that described previously [5]. The cells to measure the enzyme activity of β-galactosidase, the gene product of *lacZ*, were incubated under the oxygen-limiting condition for 16–18 h. After washing with Z buffer [16], the cells were resuspended in Z buffer and the β-galactosidase activity was determined by the standard protocol [16].

3. Results and discussion

3.1. Cloning and sequencing of the downstream region of the nor *genes*

In our previous investigation, we identified the genes for nitric oxide reductase, *norCB*-ORF6, nearby the *nir* genes for dissimilatory nitrite reduction from *P. aeruginosa* [5]. To determine the downstream structure of the *nor* genes, we cloned the region by using a 0.8-kb *Sph*I-*Eco*RI fragment from pHA-E1 (Fig. 1) as a hybridization probe. A *Bam*HI digest of chromosomal DNA from *P. aeruginosa* PAO1 was shown to hybridize with the probe at about 8 kb in size (data not shown). *Bam*HI fragments around 8 kb were recovered from agarose gel and ligated to Charomid 9–36. Gene library was constructed in *E. coli* DH5. Three positive clones out of 650 colonies were obtained by colony hybridization. Two of the three clones carried the objective fragment (pHA-B1).

The nucleotide sequence of the downstream region of ORF6 was determined (Fig. 2). Two open reading frames, ORF7 and *dnr*, were identified in the opposite direction of the *nor* genes. ORF7 encode a protein of 64 amino acids. Molecular weight of the ORF7 gene product calculated from the translated sequence was 7.3 kDa. No protein with an overall similarity to the deduced sequence of ORF7 was found in the protein data banks (SwissPlot release 30 and PIR release 43) by DDBJ FASTA e-mail server. There is a sequence resemble to the consensus binding motif of FNR (ANR) in the 5′ region of ORF7, suggesting that the gene is expressed under the anaerobic conditions as a single transcriptional unit. The *dnr* gene

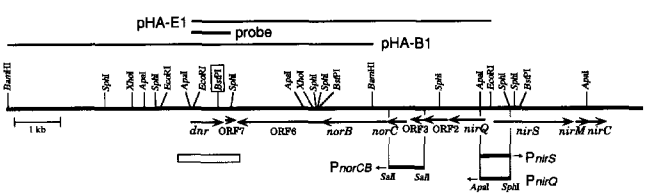

Fig. 1. Physical map of the *nir* and *nor* gene cluster from *P. aeruginosa*. Upper bars indicate the fragments cloned in the previous work (pHA-E1) [5] and this study (pHA-B1), and a fragment used as a probe for Southern and colony hybridization analyses (probe). The arrows shows the sizes and transcriptional directions of the denitrification genes. Open box indicate the region where the nucleotide sequence was determined in this work. The lower bars indicate the fragment used for the *lacZ* assay. These fragments were ligated into the *Hin*cII or *Sal*I site of pUC119, removed by *Bam*HI and *Hin*dIII digestion, and inserted into the respective sites of pQF50. The small arrows show the directions of the *lacZ* gene. Boxed *Bst*PI site is where the tetracycline resistance gene is inserted to construct the *dnr* mutant, RM536.

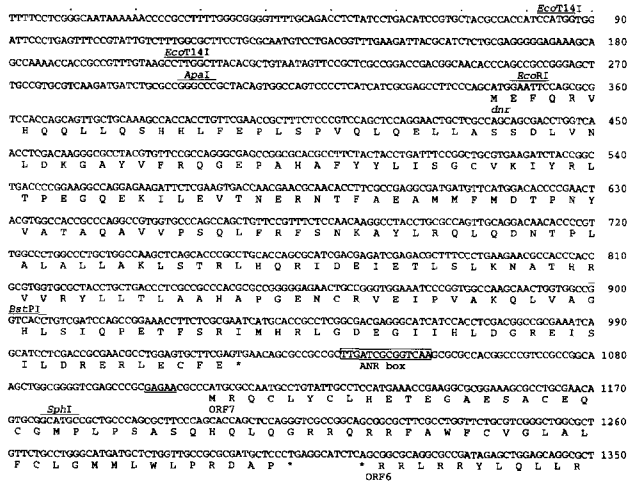

Fig. 2. Nucleotide sequence of the *dnr* gene and its flanking region. Restriction sites are overlined and a ribosome-binding site is underlined. Boxed sequence is a consensus binding motif of ANR. The nucleotide sequence data has been deposited with GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession number, D50019.

encode a protein of 227 amino acids. Calculated molecular weight of the deduced gene product is approximately 26 kDa. Homology search with the protein data banks showed that the translated sequence of *dnr* had an overall similarity with the CRP/FNR-family regulatory proteins. Those regulators had around 20% amino acid identity with the *dnr* gene product. DNR does not carry the cAMP-binding residues and the cysteine residues conserved in the CRP and FNR type proteins. An evolutionary tree of DNR and similar regulators are designated in Fig. 3. NNR from *Paracoccus denitrificans* which was recently found to be encoded in the vicinity of the *nir* and *nor* genes [19] showed the highest identity (33.3%).

3.2. Construction of a dnr *mutant of* P. aeruginosa

The 4.5-kb *Xho*I fragment containing the *dnr* gene from pHA-B1 was cloned into the *Sal*I site of pUC119. The *dnr* gene on the plasmid was disrupted by inserting the 1.4-kb *Eco*RI-*Ava*I fragment carrying the Tc-resistance gene (*tet*) from pBR322 into the unique *Bst*PI site (Fig. 1). Cohesive ends of the fragments was blunted before ligation. The constructed plasmid, pHA536, was introduced into *P. aeruginosa* PAO1 and a clone which shows Tc-resistant and Cb-sensitive phenotype was selected as described previously [20]. The obtained mutant was designated as strain RM536. Insertion of the *tet* gene on the chromosome of RM536 was confirmed by Southern hybridization analysis (data not shown). RM536 showed normal growth under the aerobic conditions. However, the mutant could not grow under the anaerobic (denitrifying) conditions. Anaerobic growth of RM536 was completely restored by introducing the 875-bp *Apa*I-*Sph*I fragment containing the *dnr* gene by a broad-host-range expression vector (data not shown).

3.3. Promoter activities for the nir *and* nor *genes in the* dnr *and* anr *mutant strains*

In our previous investigations, we found the ANR-binding motifs in the promoter regions of *nirS* and *norCB* [4,5]. *nirQ*,

which is divergently transcribed with *nirS*, was also suggested to be under the control of ANR [12]. Dependence of these denitrification genes on *dnr* or *anr* was examined by using *lacZ* as a reporter gene. The fragments containing the promoter regions for *nirS*, *nirQ* and *norCB* (Fig. 1) were ligated into a broad-host-range promoter probe vector, pQF50 [17] at the upstream of *lacZ*. The wild type (PAO1), *dnr*$^-$ (RM536) and *anr*$^-$ (PAO6261) strains were transformed by the constructed plasmids. The transformants were grown under the oxygen-limiting condition with 5 mM sodium nitrite and the activities of β-galactosidase, the gene product of *lacZ*, were measured (Table 1). As a control, the activity for the *tac* promoter (*Bam*HI fragment of pKK223-3 [21]) was also measured in the same way. Transcription from the *tac* promoter was not so affected by the *dnr* or *anr* mutation. However, the activities of the three denitrification promoters were considerably reduced by the mutation of *dnr* or *anr*. These results clearly indicated that the second *crp/fnr*-related regulatory gene, *dnr*, is necessary for the expression of the *nir* and *nor* genes. It is also the first finding that *anr* is required for the transcription of the nitric oxide reductase gene (*norCB*).

3.4. Conclusions

It has been reported that a regulator, ANR, is required both for denitrification and anaerobic arginine catabolism of *P. aeruginosa* [10–12,22,23]. Recent study also confirmed that the activation of the entire denitrification pathway is under the control of ANR [15]. ANR is highly homologous with FNR of *E. coli* and has conserved cysteine residues which are shown to be necessary for sensing anoxia [1]. Thus, ANR is thought to be involved in the anoxic regulation of the denitrification genes. In addition to the oxygen limitation, the presence of respiratory substrates for denitrification such as nitrate and nitrite is required for the expression of denitrification enzymes [24–27]. However, the mechanism to sense these nitrogenous oxides has not been clarified. In this report, we found the second *crp/fnr*-related regulatory gene, *dnr*, from *P. aeruginosa*. Both of the two regulatory genes, *dnr* and *anr*, were indicated to be necessary for the expression of the *nir* and *nor* genes on the stage of transcription. DNR lacks the N-terminal cysteine cluster which may participate in sensing the intracellular redox potential. There is a possibility that DNR activate the expression of the denitrification genes by the presence of nitrogenous oxide. Recently, Van Spanning et al. found the gene similar to *dnr* from *P. denitrificans* and designated *nnr* [19]. They showed by heme staining analyses that the gene is necessary for the expression of functional nitrite reductase and nitric oxide reductase enzymes. The transcription of the genes for these reductases may be regulated by the *nnr* gene product. The anoxic regulator correspond to FNR from *E. coli* or ANR from *P. aeruginosa* have not been identified from *P. denitrificans*. The ANR-like regulator, FnrA, was found from *P. stutzeri*, but FnrA was not necessary for the expression of the denitrification enzymes [13]. The CRP/FNR-related regulator which regulate the expression of the denitrification genes have not yet been found from the bacterium. The finding reported here is the first example of the involvement of two CRP/FNR-related regulators in denitrification in one strain. We are now investigating the effects of the *dnr* mutation on the expression of the other denitrification genes and the genes for anaerobic arginine catabolism.

Table 1
Both of the *dnr* and *anr* genes are required for the transcription of the denitrification genes of *P. aeruginosa*

Strain (genotype)	β-Galactosidase activity for promoters[a]			
	nirS	*nirQ*	*norCB*	*tac*
PAO1 (wild type)	2.4	0.8	7.6	4.2
RM536 (*dnr*)	0.1	–	–	3.9
PAO6261 (*anr*)	0.1	–	0.1	4.3

[a]Specific activity of β-galactosidase is expressed in Miller kilo-units. The data are mean values from three or four independent experiments. '–' indicate the mean value is lower than 50 Miller units.

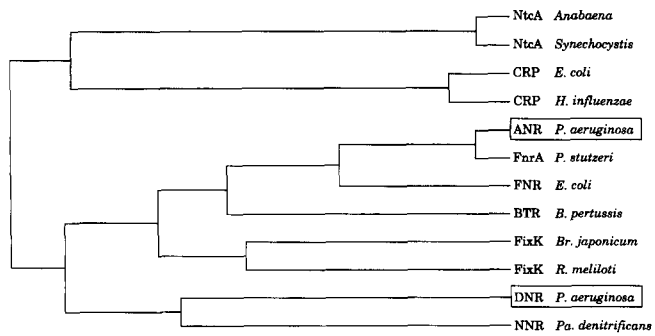

Fig. 3. Evolutionary tree of the CRP/FNR-related regulatory proteins. The abbreviations used for the genera are: E., *Escherichia*; H., *Haemophilus*; P., *Pseudomonas*; B., *Bordetella*; Br., *Bradyrhizobium*; R., *Rhizobium*; Pa., *Paracoccus*. *Anabaena* and *Synechocystis* are represent sp. PCC7120 and sp. PCC6803, respectively. Regulators from *P. aeruginosa* are boxed. The tree was generated by the program of the GENETYX package (Software Development, Tokyo, Japan) using UPGMA method.

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