ELSEVIER

Biochimica et Biophysica Acta 1517 (2001) 436–440

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Short sequence-paper

Characterization and transcriptional analysis of *Pseudomonas fluorescens* denitrifying clusters containing the *nar*, *nir*, *nor* and *nos* genes

L. Philippot *, P. Mirleau, S. Mazurier, S. Siblot, A. Hartmann, P. Lemanceau, J.C. Germon

Institut National de la Recherche Agronomique – Laboratoire de Microbiologie des Sols et de Recherche sur la Flore Pathogène, 17, rue Sully, B.V. 1540, 21034 Dijon Cedex, France

Received 19 July 2000; received in revised form 8 November 2000; accepted 9 November 2000

Abstract

In this study, we report the cloning and characterization of denitrifying gene clusters of *Pseudomonas fluorescens* C7R12 containing the *narXLDKGHJI*, *nirPOQSM*, *norCB* and *nosRZDFYL* genes. While consensus sequences for Fnr-like protein binding sites were identified in the promoter regions of the *nar*, *nir*, *nor* and *nos* genes, consensus sequences corresponding to the NarL binding sites were identified only upstream the *nar* genes. Monitoring by mRNA analysis the expression of the *narG*, *nirS*, *norB* and *nosZ* structural genes suggests a sequential induction of the denitrification system in *P. fluorescens*. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Denitrification; mRNA; Nitrate reductase; Nitrite reductase; Nitric oxide reductase; Nitrous oxide reductase; (*Pseudomonas fluorescens*)

Denitrification is a dissimilatory process in which bacteria utilize oxidized nitrogen as electron acceptors when oxygen concentration is limited. This process consists of four reaction steps by which nitrate is reduced into dinitrogen gas by the metalloenzymes nitrate reductase, nitrite reductases, nitric oxide reductase and nitrous oxide reductase. Despite the increasing number of fully sequenced bacterial genomes, the structural genes encoding for the four denitrifying reductases have never been characterized in a single bacterium. Moreover, the sequence of structural genes encoding the membrane bound nitrate reductase still remains to be completed for a denitrifier [1–4].

In this article we report the cloning and the characterization of the *narXLDKGHJI*, *nirPOQSM*, *norBC* and *nosRZDFYL* gene clusters from *Pseudomonas fluorescens* C7R12. The kinetics of expression of the structural *narG*, *nirS*, *norB* and *nosZ* genes with various electron acceptors were then studied using dot blot hybridization on extracted mRNA.

To analyze the denitrifying genes of *P. fluorescens* C7R12, a genomic library was screened using digoxigenin-labeled PCR fragments of the *narG*, *nirS*, *norB* and *nosZ* genes as probes. Primers to amplify these genes in *P. fluorescens* C7R12 were designed according to the known sequences of these genes. Sequencing cosmid DNA containing the denitrifying genes was performed by primer walking using the dideoxy-nucleotide chain termination method. The sequences coding for the *nar*, *nir*, *nor* and *nos* genes have been deposited in the GenBank database under accession number AF197465, AF197466, AF197467 and AF197468, respectively. To study the expression of the structural genes, total RNA from *P. fluorescens* was extracted using RNeasy kit (Qiagen) from samples taken at set times directly from aerobic and anaerobic cultures containing 50 ml of LB medium or 50 ml of LB medium supplemented with 3 mM KNO_3. The RNA samples were then treated with RNase free Dnase I in the presence of RNase inhibitor. Gene expression was quantified by dot blot hybridization with *narG*, *nirS*, *norB*, and *nosZ* probes. Detection was performed using the ECF substrate (Amersham) for quantification of the transcript levels in a STORM 860 PhosphoImager (Molecular Dynamics). Reverse transcriptase–polymerase chain reaction (RT–PCR) using 200 ng of RNA as templates was carried out as recommended by the manufacturer (Promega).

By using a mixture of *narG*, *norB*, *nirS* and *nosZ*

* Corresponding author. Fax: +33-3-80-69-32-24;
E-mail: philippo@dijon.inra.fr

0167-4781/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0167-4781(00)00286-4

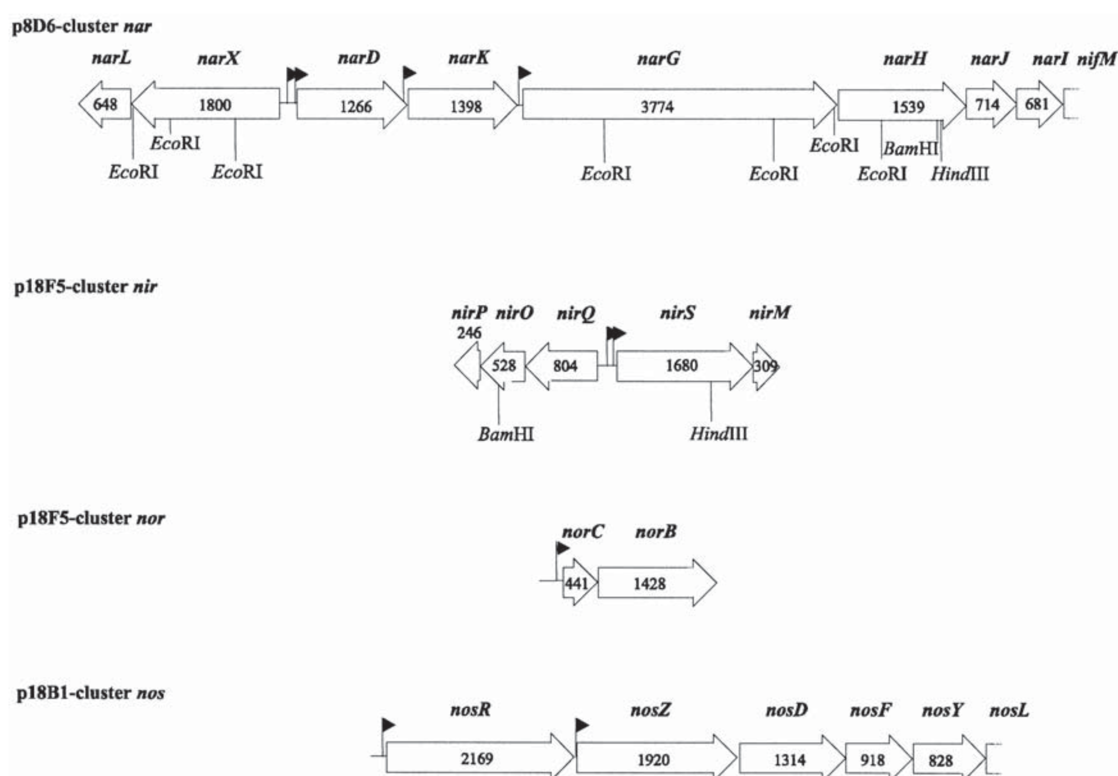

Fig. 1. Organization of the denitrification gene clusters of *P. fluorescens* C7R12. Number of nucleotides is given for each gene. Vertical flags indicate locations and directions of putative recognition motifs for an Fnr homologue protein.

fragments as probe, four clones were isolated out of a genomic library of 2300. Subsequent characterization of these clones by PCR showed that the clones 8D6 and 10F4 contained the *narG* gene, clone 18F5 contained the *norB* and *nirS* genes, and clone 18B1 contained the *nosZ* gene. A 12.6 kb DNA region of the cosmid extracted from clone 8D6 was sequenced. As a result, an open reading frame (ORF) of 3774 bp starting with a GTG codon was assigned to the *narG* gene encoding the catalytic α subunit of the membrane-bound nitrate reductase (Fig. 1). The deduced amino acid sequence shows 93%, 73%, 67%, 45% and 43% identity to the corresponding α subunit from *P. fluorescens* YT101, *P. aeruginosa*, *Escherichia coli*, *Thermus thermophilus* and *Mycobacterium tuberculosis*s, respectively. The N-terminus of the α subunit of *P. fluorescens* C7R12 shares three of the four cysteine residues conserved in molybdopterin guanine dinucleotide-binding proteins [4,5]. The first cysteine residue from this motif is substituted by a histidine residue, which replaces the cysteine as a ligand to the [4Fe–4S] cluster [3,5]. Similar to nitrate reducers, *narGHJI* is organized in an operon. In *P. fluorescens* C7R12, this *narGHJI* operon is clustered with *narXL* and *narDK* encoding a two component nitrate regulatory system [6] and putative nitrate or nitrite translocases, respectively. The derived NarX peptide consists of 599 amino acids with a molecular mass of about 67 kDa. The deducted topology of this protein is similar to those of other sensor-kinases with two transmembrane regions, separated by a periplasmic region of 120 amino acids located near the N-terminus, and a cytoplasmic histidine kinase domain. The NarL response-regulator, which consists of 215 amino acids, contains residues conserved in the histidine-receiving module in the N-terminal region and in the helix–turn–helix motif in the C-terminal region. Identification of NarXL in *P. fluorescens* C7R12 confirm the presence of this two-component nitrate regulatory system in denitrifiers [2]. The derived NarD and NarK peptides of *P. fluorescens* C7R12 showed 51% and 71% identity with the putative nitrate or nitrite transporters of *P. aeruginosa* and 31% and 49% identity with those of *P. stutzeri* (W.G. Zumft, personal communication). Transmembrane helix prediction [7] of these proteins indicates the presence of 12 helices. The identification of two genes encoding putative nitrate or nitrite translocases in *P. fluorescens* C7R12 as well as in *P. stutzeri* and *P. aeruginosa*, while only one has been sequenced in *E. coli* and in *B. subtilis*, raises the question of the presence of a redundant system of nitrate/nitrite translocation in bacteria belonging to this group.

The sequencing of cosmid from clone 18F5 yields to two DNA fragments of 3.89 and 2.29 kb belonging to the *nirPOQSM* and *norBC* clusters, respectively. Unfortunately, since further sequencing to determine the distance between the *nir* and *nor* genes and their genetic organization failed due to the high G+C mol%, the *nir* and *nor* clusters are not presented together in Fig. 1. In the

deduced sequence of the NirS protein of *P. fluorescens* C7R12, a predicted signal sequence of 27 amino acids (aa) and a cytochrome *c* family heme-binding signature (AKEIYFQRCAGCH) are present. The predicted molecular mass of the mature protein is around 58 kDa. The *nirS* gene is followed by a gene encoding a protein showing 64% identity with the cytochrome c_{551} NirM of *P. stutzeri* [8] (Fig. 1). Upstream from the *nirS* gene, three ORF showing identity with *nirQ* and the recently characterized genes *nirO* and *nirP* [9] have been sequenced (Fig. 1). Transmembrane helix prediction [7] of NirP and NirO indicated the presence of six and three transmembrane spanning helices, respectively. In *P. fluorescens* C7R12, *norB* encodes a product of 475 aa with a calculated molecular mass of about 53 kDa. The deduced amino acid sequence shows 83% and 76% identity with NorB of *P. stutzeri* and *P. aeruginosa*, respectively. NorB of *P. fluorescens* C7R12 is predicted to contain 12 transmembrane helices. Six invariant histidine residues have been identified after alignment of NorB to the haem copper oxidase [5]. These histidine residues are ligands to the prosthetic groups associated with subunit I of cytochrome *c* oxidases. NorB of *P. fluorescens* C7R12 shares all these residues, located in helices II and X (His60, His207, His301, His302, His347 and His349), suggesting a similar function in providing ligands to the prosthetic groups. Hydropathy analysis of the deduced amino acid sequence of *norC* gene suggested the presence of one transmembrane helix near its N-terminus.

Computer analysis of the 7.68 kb DNA region from cosmid p18B1 has shown that it encodes the *nosRZDFYL* genes (Fig. 1). The identity of NosZ protein of *P. fluorescens* C7R12 with those of *P. stutzeri*, *P. aeruginosa* [10], *Achromobacter cycloclastes* [11] and *Rhizobium meliloti* [12] are 80%, 79%, 59% and 50%, respectively. Prediction of the cleavage site [13] indicated that the precursor form of NosZ of *P. fluorescens* is probably processed between positions 54 and 55. Apart from this unusually long signal peptide, sequence analysis also reveals the presence of a conserved 'twin arginine' leader motif (Arg20–Arg21) at

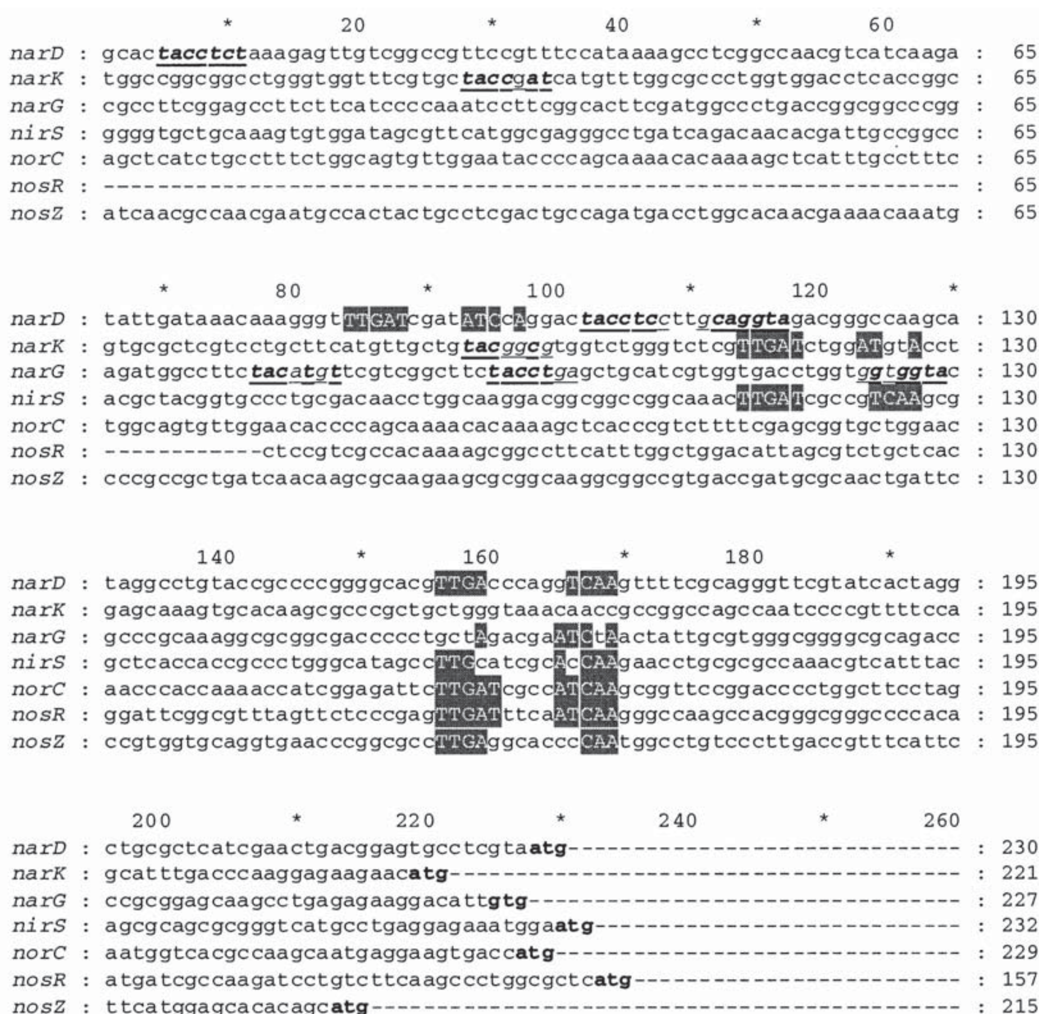

Fig. 2. Comparison of promoter regions of the *narD*, *narK*, *narG*, *nirS*, *norB*, *nosR* and *nosZ* genes. The shaded boxes indicate putative Fnr binding sites (TTGATNNNNATCAA). Potential NarL binding sites are underlined and nucleotides conserved with NarL consensus (TACYYMT) are boldfaced. Nucleotides corresponding to the start codon are boldfaced.

the beginning of the signal sequence. This twin arginine motif is conserved in other periplasmic proteins containing complex redox cofactors. Recently, Berks [14] and Weiner et al. [15] reported the identification of a novel Sec-independent system for membrane targeting and a translocation system of cofactor-containing proteins. Proteins following this pathway have a long signal peptide and a twin arginine leader motif serving as the addressing sequence which indicates that NosZ is probably translocated by this Sec-independent system. Upstream from the *nosZ* gene of *P. fluorescens* C7R12 is the *nosR* gene encoding a protein of about 80 kDa (Fig. 1). The predicted amino acid sequence of NosR from *P. fluorescens* C7R12 contains six putative transmembrane helices with a large periplasmic region of 390 amino acids between the first and the second helix. Cysteine motifs similar to those found in 4Fe–4S bacterial ferredoxins are present after helix 6 near the C-terminus of NosR. The *nosDFY* genes are located 25 nucleotides downstream from the termination codon of *nosZ*. NosD of *P. fluorescens* C7R12 is predicted to contain a signal sequence with a cleavage site between amino acids 28 and 29 and a transmembrane helix at position 13–31. Overlapping the termination codon of *nosD* is the *nosF* gene encoding a cytoplasmic protein of 33 kDa showing homology to the family of ATP/GTP-binding proteins. NosY is an integral membrane protein of 30 kDa composed of six transmembrane helices. The *nosY* ORF is followed by the 5′ end of an ORF coding for a protein with homology to the N-terminus of NosL.

Sequence motifs corresponding to binding sites of Fnr protein which activate the transcription of the genes involved in anaerobic metabolism [16] were found in the promoter regions of the *narD*, *narK nirS*, *norC*, *nosR* and *nosZ* genes (Fig. 2). However, van Spanning et al. [17] have shown in *P. denitrificans* that *nos* expression is not affected in Fnr mutants while putative Fnr binding sites have been identified upstream *nos* genes. Sequence analysis of the *narG* promoter of *P. fluorescens* C7R12 reveals one putative degenerated Fnr half binding site as observed for *P. aeruginosa* and *P. stutzeri* [2,4]. Interestingly, two putative Fnr-binding sites are present upstream from *narD and nirS*, suggesting a particular regulation of the expression of these genes during anaerobiosis. Sequence motif corresponding to the binding sites of the NarL response regulator in the presence of nitrate or nitrite has been identified only in the promoter region of the *nar* genes (Fig. 2). Accordingly, expression of the *narG* and *narK* genes but not that of the *nir*, *nor* and *nos* genes has been recently shown to be affected in a mutant of *P. stutzeri* deleted in the NarL gene [2].

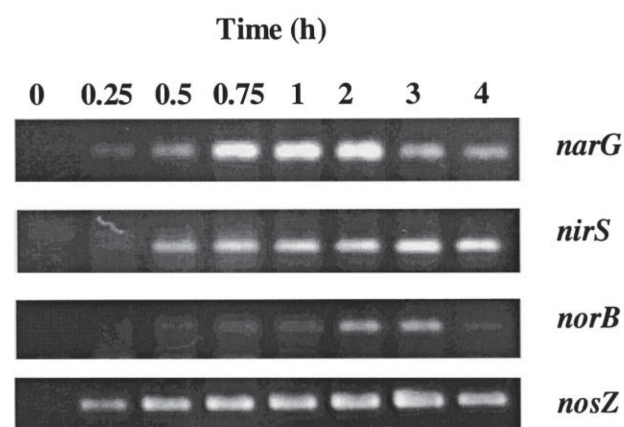

Fig. 4. RT–PCR detection of *narG*, *nirS*, *norB* and *nosZ* mRNA from *P. fluorescens* C7R12 cells 0, 0.25, 0.5, 0.75, 1, 2, 3 and 4 h after switch from aerobic conditions to anaerobic plus nitrate conditions. RT–PCR products were electrophoresed on a 1% (w/v) agarose gel.

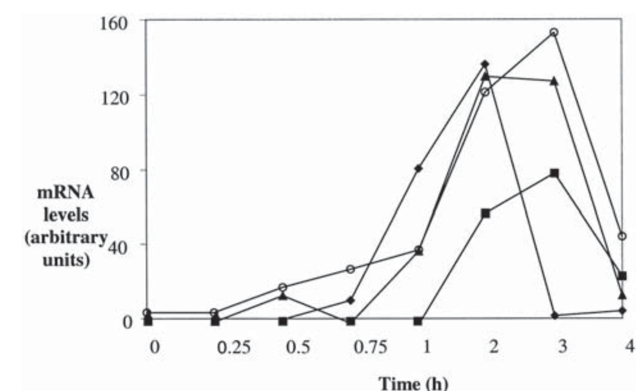

Fig. 3. Kinetics of relative mRNA levels of *P. fluorescens* C7R12 for nitrate reductase (*narG*) (◆), nitrite reductase (*nirS*) (■), nitric oxide reductase (*norB*) (▲) and nitrous oxide reductase (*nosZ*) (○) after switch from aerobic conditions to anaerobic plus nitrate conditions. For hybridization, 5 μg of total RNA was blotted onto nitrocellulose membrane (Schleicher and Schuell) in a 96-well dot blot manifold and hybridized with the *narG*, *nirS*, *norB* and *nosZ* specific probes.

Characterization of the structural *narG*, *nirS*, *norB* and *nosZ* genes within the same bacterium has enabled us to further study the expression of these genes by monitoring simultaneously their levels of mRNA with various electron acceptors. Investigation of the effect of nitrogenous oxide on mRNA levels under aerobic conditions shows that the presence of nitrate did not allow a detectable induction of expression of the denitrifying genes, including *narG*. Similar results were obtained by Baumman et al. [18] in an aerobically growing continuous culture of *P. denitrificans*. Similarly, no hybridization signal was detected in anaerobiosis without nitrogenous oxides. In contrast, a very strong hybridization signal was recorded for all the studied genes after a 1 h incubation in denitrifying conditions (Fig. 3). RT–PCR experiments confirmed that there was no expression of the *narG*, *nirS*, *norB*, and *nosZ* denitrifying genes under aerobic conditions at t0 whereas after 30 min incubation under anaerobic conditions with nitrate as electron acceptor, all the studied genes were expressed (Fig. 4). Altogether, these results indicate that both anaerobiosis and nitrogen oxides are required for significant activation of the expression of the denitrification genes (Fig. 3). However, in our experimental conditions,

discrimination between NO_3^-, NO_2^-, NO and N_2O as inducers of the expression of the *narG*, *nirS*, *norB* and *nosZ* genes was not possible. Kinetics of expression of the four structural genes *narG*, *nirS*, *norB* and *nosZ* had not been previously reported. In *P. fluorescens*, the highest expression of *narG* was recorded after 2 h of incubation, whereas *nirS*, *norB* and *nosZ* showed the highest expression after 3 h (Fig. 3). Moreover, no mRNA for nitrate reductase could be quantified after 3 h (Fig. 3). Transient expression of denitrifying mRNAs has been previously reported [2,18]. The decrease in the overall mRNA levels may be due to the presence of fewer molecules inducing mRNA synthesis or due to the stability of mRNAs, which can vary considerably. These data suggest the presence of a sequential induction of the denitrification system in *P. fluorescens* C7R12 with an induction of the nitrate reductase system followed by a coordinate induction of the nitrite-, nitric oxide- and nitrous oxide-reductase systems. It is interesting to notice that in *P. stutzeri*, the *nir*, *nor* and *nos* genes are forming a supercluster on the chromosome while the *nar* genes are never linked with the denitrification genes sensu stricto [19].

The characterization and cloning of the genes encoding all the denitrifying reductases in the same bacterium should facilitate further investigations into the interlaced regulatory processes of nitrate, nitrite, nitric oxide and nitrous oxide reduction [20,21]. How the sequential induction of the denitrification genes is accomplished in *P. fluorescens* is also of interest.

The authors are grateful to S. Nazaret for helpful advice, and to B. Maarouri and A. Morange for technical assistance. We thank Professor W.G. Zumft for sharing results prior to publication. The work was supported by the PNSE program of the Institut National des Sciences de l'Univers.

References

[1] B.C. Berks, M.D. Page, D.J. Richardson, A. Reilly, A. Cavill, F. Outen, S.J. Ferguson, Sequence analysis of subunits of the membrane-bound nitrate reductase from a denitrifying bacterium: the integral membrane subunit provides a prototype for the dihaem electron-carrying arm of a redox loop, Mol. Microbiol. 15 (1995) 319–331.

[2] E. Härtig, U. Schiek, K.-U. Vollack, W.G. Zumft, Nitrate and nitrite control of respiratory nitrate reduction in denitrifying *Pseudomonas stutzeri* by a two component regulatory system homologous to NarXL of *Escherichia coli*, J. Bacteriol. 181 (1999) 3658–3665.

[3] L. Philippot, A. Clays-Josserand, R. Lensi, I. Trinsoutrot, P. Normand, P. Potier, Purification of the dissimilative nitrate reductase of *Pseudomonas fluorescens* and the cloning and the sequencing of its corresponding genes, Biochim. Biophys. Acta 1350 (1997) 272–276.

[4] L. Philippot, O. Hojberg, Dissimilatory nitrate reductases in bacteria, Biochim. Biophys. Acta 1446 (1999) 1–23.

[5] B.C. Berks, S.J. Ferguson, J.W.B. Moir, D.J.D. Richardson, Enzymes and associated electron transports systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions, Biochim. Biophys. Acta 1232 (1995) 97–173.

[6] T. Nohno, S. Noji, S. Taniguchiand, T. Saito, The *narX* and *narL* genes encoding the nitrate-sensing regulators of *Escherichia coli* are homologous to a family of prokaryotic two component regulatory genes, Nucleic Acids Res. 17 (1989) 2947–2957.

[7] E.L.L. Sonnhammer, G.V. Heijne, A. Krogh, A hidden Markov model for predicting transmembrane helices in protein sequences, in: Proceedings of the Sixth International Conference on Intelligent Systems for Molecular Biology, Menlo Park, CA, AAAI Press, 1998, pp. 175–182.

[8] A. Jüngst, S. Wakabayashi, H. Matsubara, W.G. Zumft, The *nirSTBM* region for cytochrome cd_1-dependent nitrite respiration of *Pseudomonas stutzeri* consists of a cluster of mono-, di-, and tetraheme proteins, FEBS 2 (1991) 205–209.

[9] H. Arai, T. Kodama, Y. Igarashi, The role of the *nirQOP* genes in energy conservation during anaerobic growth of *Pseudomonas aeruginosa*, Biosci. Biotechnol. Biochem. 62 (1998) 1995–1998.

[10] W.G. Zumft, A. Dreusch, S. Löchelt, H. Cuypers, B. Friedrich, B. Schneider, Derived amino acids sequences of the *nosZ* gene (respiratory N_2O reductase) from *Acaligenes eutrophus*, *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* reveal potential copper-binding residues, Eur. J. Biochem. 208 (1992) 31–40.

[11] K. Inatomi, Analysis of the nitrous oxide reduction genes, *nosZDFYL*, of *Achromobacter cycloclastes*, DNA Res. 31 (1998) 365–371.

[12] P. Holloway, W. McCormick, R.J. Watson, Y.-K. Chan, Identification and analysis of the dissimilatory nitrous oxide reductase genes, *nosRZDFY*, of *Rhizobium meliloti*, J. Bacteriol. 178 (1996) 1505–1514.

[13] H. Nielsen, J. Engelbrecht, S. Brunak, G.v. Heijne, Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites, Protein Eng. 10 (1997) 1–6.

[14] B.C. Berks, A common export pathway for proteins binding complex redox cofactors?, Mol. Microbiol. 22 (1996) 393–404.

[15] J.H. Weiner, P.T. Bilous, G.M. Shaw, S.P. Lubitz, L. Frost, G.H. Thomas, J.A. Cole, R.J. Turner, A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins, Cell 93 (1998) 93–101.

[16] S. Spiro, J.R. Guest, FNR and its role in oxygen-regulated gene expression in *Escherichia coli.*, FEMS Microbiol. Rev. 75 (1990) 399–428.

[17] R.J. Van Spanning, A.P. De Boer, W.N. Reijnders, H.V. Westerhoff, A.H. Stouthamer, J. Van Der Oost, FnrP and NNR of *Paracoccus denitrificans* are both members of the FNR family of transcriptional activators but have distinct roles in respiratory adaptation in response to oxygen limitation, Mol. Microbiol. 23 (1997) 893–907.

[18] B. Baumann, M. Snozzi, A.J. Zehnder, J.R.V.D. Meer, Dynamics of denitrification activity of *Paracoccus denitrificans* in continuous culture during aerobic-anaerobic changes, J. Bacteriol. 178 (1996) 4367–4374.

[19] W.G. Zumft, H. Korner, Enzyme diversity and mosaic gene organization in denitrification, Antonie Van Leeuwenhoek 71 (1997) 43–58.

[20] A. Jüngst, W.G. Zumft, Interdependence of respiratory NO reduction and nitrite reduction revealed by mutagenesis of *nirQ*, a novel gene in the denitrification gene cluster of *Pseudomonas stutzeri*, FEBS 3 (1992) 308–314.

[21] J.F. Ghiglione, L. Philippot, P. Normand, R. Lensi, P. Potier, Disruption of *narG*, the gene encoding the catalytic subunit of respiratory nitrate reductase, also affects nitrite respiration in *Pseudomonas fluorescens* YT101, J. Bacteriol. 181 (1999) 5099–5102.