Interdependence of respiratory NO reduction and nitrite reduction revealed by mutagenesis of *nir*Q, a novel gene in the denitrification gene cluster of *Pseudomonas stutzeri*

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An open reading frame, designated *nir*Q, was identified upstream of *nir*S, the structural gene for the respiratory nitrite reductase of *Pseudomonas stutzeri* ZoBell. Its derived gene product (275 amino acids, $M_r = 30,544$) shows similarity to the NtrC protein family of transcriptional activators. Deletion-replacement mutagenesis of the *nir*Q gene resulted in the simultaneous loss of nitrite reduction and NO reduction in vivo. However, both reductases were still synthesized, with only nitrite reductase being active in vitro. NO reductase was overproduced by a factor of about 2. Our results indicate that the systems for nitrite reduction and NO reduction are functionally coupled.

Nitrite respiration; Cytochrome cd_1; Nitric oxide reductase; Denitrification; NtrC family; Nucleotide sequence; *Pseudomonas stutzeri*; *Pseudomonas aeruginosa*

1. INTRODUCTION

The stoichiometric denitrification pathway established from genetic and biochemical evidence comprises four sequential reactions: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. Nitrite reductase (cytochrome cd_1, EC 1.9.3.2) is the enzyme in the sequence that in *Pseudomonas stutzeri* forms nitric oxide (NO) as the first gaseous species. Mutational evidence [1], NO trapping [2], and isotope studies [3,4] convincingly argue for NO as the intermediate that is reduced to nitrous oxide (N_2O) by NO reductase, a cytochrome *bc* complex (EC 1.7.99.7) [5]. In spite of its role as an intermediate, NO is toxic to denitrifiers and the loss of NO reductase constitutes a conditionally lethal mutation under denitrifying conditions [1]. Genes for nitrite respiration (*nir*) and NO respiration (*nor*) are located in two adjacent clusters in *P. stutzeri* [6]. Here we characterize *nir*Q, a novel gene of the *nir* gene subcluster. Its deletion resulted in the simultaneous loss of nitrite reduction and NO reduction. Our results reveal an interdependence of both processes controlled by the *nir*Q gene product, and support a concept for denitrification with the reaction sequence $NO_2^- \rightarrow NO \rightarrow N_2O$ forming a functional unit.

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2. EXPERIMENTAL

2.1. *Bacterial strains and plasmids*

The spontaneously streptomycin resistant strain, MK21, of *P. stutzeri* ZoBell (ATCC 14405) was used to represent wild-type traits. Plasmid pNIR44 was used to generate the subclones for sequencing and for construction of plasmid pNIR1a [7]. The DNA fragment for mutagenesis of *nir*Q was obtained from the cosmid, cDEN1, which carries the entire denitrification gene cluster of ca. 30 kb [6]. The cassette with the gentamicin resistance gene of the transposon Tn21 family was derived from plasmid pR209CII [8]. For recombinant DNA techniques and conjugative plasmid transfer the *Escherichia coli* strains, HB101 [9], BMH71-18 [10], and SM10 [11], were used. Cloning and sequencing vectors were pBR325 [12], pSUP104 [13], pSUP203 [11], M13mp10,11 [14], and M13mp18 [15].

2.2. *Media, antibiotics, and growth conditions*

P. stutzeri and *E. coli* were grown for recombinant DNA work in Luria-Bertani medium at 37°C. For physiological studies *P. stutzeri* was grown in a synthetic asparagine- and citrate-containing medium and induced for denitrification by O_2 limitation in the presence of nitrate [16]. Antibiotics were used in the following concentrations (in $\mu g \cdot ml^{-1}$): ampicillin (Ap) 50; chloramphenicol (Cm) 30; gentamicin (Gm) 10; tetracycline (Tc) 10; kanamycin 50; and streptomycin 200. Cells were harvested by centrifugation and washed in 100 mM potassium phosphate buffer, pH 7. For in vitro assays the cells were broken in a French pressure cell by two passages at 136 MPa.

2.3. *DNA sequencing and Southern blotting*

DNA was sequenced by the Sanger method with deoxyadenosine $5'-[\alpha^{35}S]$thio-phosphate and Sequenase 2.0. Details of recombinant DNA work are described elsewhere [7,17]. Genomic DNA of strains MK21 and MK2181 was digested with *Eco*RI for Southern blot analysis. About 10 μg of DNA was separated on a 0.7% agarose gel, and transferred to nylon membranes (Hybond-N, Amersham, Braunschweig, Germany). The appropriate gene probes were purified from agarose by squeezing [18], extraction with phenol, and ethanol precipitation. They were denatured and labeled with digoxigenin by Klenow polymerase using random oligonucleotides as primers [19]. Hybridiza-

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  SmaI
CCCGGGACTTCTTCTTTTTGCGACTTGGATGCTATGAGAGCGGCAATCCGAAAGCGCTT    60
  ← Fnr →                                                **
GACGGCAATCAAGAGAGCCACCGGCACCCCCCGAACGTGACGCCTTGTCGCGGGCCGCAAC  120
**     *****                                          ← Fnr
ACGCCGAAACGTCTGGAATCGGCTTCCATTGGCAGGCATCCGGCGCGGGACTTGATCGCA   180
→  HindIII                                        ***** RBS
ATCAAGCTTTGCCGGCGGATGGCTTGAGGGGGCAAAGCGGGGCCGGTAGCTTGGCGATGG   240
*****
AAATAACCAACTGCTGCCGTGAGGTACTTGCCTGTGAATGCGATTGAAATCCCGACTACC   300
              nirQ → M  R  Y  L  P  V  N  A  I  E  I  P  T  T    14

GCCGGCACGCCTGACGCGCCCTTCTACCAACCGTTGGGCAATGAAGAGCAGCTGTTCCAG   360
A  G  T  P  D  A  P  F  Y  Q  P  L  G  N  E  E  Q  L  F  Q       34

CAGGCCTGGCAGCACGGCATGCCCGTGCTTATCAAGGGCCCGACCGGCTGCGGCAAGACC   420
Q  A  W  Q  H  G  M  P  V  L  I  K |G  P  T  G  C  G  K  T|      54
                                                A
CGTTTCGTACAGCACATGGCGCATCGCCTGAATCTGCCGCTGTACACCGTGGCCTGCCAT   480
R  F  V  Q  H  M  A  H  R  L  N  L  P  L  Y  T  V  A  C  H       74

GACGACCTGTCGGCGGCCGACCTGGTCGGCCGACACCTGATCGGCGCACAGGGCACCTGG   540
D  D  L  S  A  A  D  L  V  G  R  H  L  I  G  A  Q  G  T  W       94

TGGCAGGACGGTCCGCTGACCCGCGCGGTCCGCGAAGGAGGCATCTGCTACCTGGACGAA   600
W  Q  D  G  P  L  T  R  A  V  R  E |G  G  I  C  Y  L  D  E|     114
                                              B
GTGGTGGAAGCACGGCAGGACACCGCCGTGGTACTGCACCCGCTGGCCGATGATCGCCGC   660
V  V  E  A  R  Q  D  T  A  V  V  L  H  P  L  A  D  D  R  R      134

GAACTGTTCATCGAGCGCACCGGCGAGGCGCTCAAGGCGCCGCCGGGCTTCATGCTGGTG   720
E  L  F  I  E  R  T  G  E  A  L  K  A  P  P  G  F  M  L  V      154

GTGTCCTACAACCCCGGTTACCAAAACCTGCTCAAGGGCATGAAGCCCAGCACCCGCCAG   780
V  S  Y  N  P  G  Y  Q  N  L  L  K  G  M  K  P  S  T  R  Q      174

CGCTTCGTCGGCGATGCGCTTCGACTATCCGCCGACCGCCGAGGAAGAGCGCATCGTCGCC   840
R  F  V  A  M  R  F  D  Y  P  P  T  A  E  E  E  R  I  V  A      194
                                    HindIII
AACGAGGCGCAGGTCGATGCCGCCGCTCGCCGCCCAGGTGGTCAAGCTTGGCCAGGCACTG   900
N  E  A  Q  V  D  A  A  L  A  A  Q  V  V  K  L  G  Q  A  L      214

CGTCGGCTGGAACAGCACGATCTGGAGGAAGTCGCCTCGACCCGCCTGCTGATCTTCACC   960
R  R  L  E  Q  H  D  L  E  E  V  A  S  T  R  L  L  I  F  T      234

GCACGCATGATCCGCTCCGGCATGACGCCGCGGCAGGCCTGCCTGGCCTGCCTCGCCGAA  1020
A  R  M  I  R  S  G  M  T  P  R  Q  A  C  L  A  C  L  A  E      254

CCGCTGTCGGATGATCCGCAGACCGTTGCCGCGCTGATGGATGTGGTCTATGTCCACTTC  1080
P  L  S  D  D  P  Q  T  V  A  A  L  M  D  V  V  Y  V  H  F      274

GGCTGAACGCGTCGCCCAGCCCGTCCCGGCGCCCTGCCCGGGTGACCTCGCGATGTGGTTTTT  1140
G  end                                                         275

CATCCTCGCCGAG                                                 1153
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Fig. 1. Nucleotide and derived amino acid sequences of the nirQ gene from P. stutzeri ZoBell. RBS, putative ribosome-binding site; Fnr, potential binding site for the transcriptional activator of anaerobic gene expression [36]; the consensus motif, TTGATN₄ATCAA, is labeled by asterisks; A, B, presumed nucleotide-binding motifs [37]. Potential stem-and-loop structures are underlined by opposing arrows. The first 189 nucleotides overlap with the nirS gene previously described [25]. The nirQ sequence has been submitted to the EMBL data bank and assigned the accession number Z17423.

tion was done at 42°C in 50% formamide [17]. Detection was with antidigoxigenin antibodies following the protocol of the manufacturer (Boehringer GmbH, Mannheim, Germany).

2.4. Analytical methods
Protein concentration was measured by the procedure of Lowry.

Nitrite and NO reduction were assayed in vivo with lactate as the electron donor. Ascorbate-reduced phenazine methosulfate (PMS) was used as electron donor for in vitro measurements [5,20]. The reduction of NO or nitrite was followed by gas chromatography [21]. Nitrite reductase and NO reductase were detected in cell extracts by immunoblotting [22]. After SDS-gel electrophoresis [23] the proteins

were transferred to nitrocellulose membranes by semi-dry blotting and reacted with polyclonal antisera. Antibodies were raised against both electrophoretically separated cytochrome subunits of NO reductase as described for cytochrome cd, [20]. The immunoconjugate was detected with a protein A-horseradish peroxidase conjugate (Sigma Chemie GmbH, Deisenhofen, Germany) and 4-chloro-1-naphthol as chromogen [24].

3. RESULTS

3.1. Sequence of nirQ and analysis of the derived gene product

Sequence data were obtained from NIR44, a 3.4 kb *Eco*RI fragment which carries *nir*Q, *nir*S, and about 75% of *nir*T. The *Eco*RI restriction sites result from the construction of the lambda gt11 expression library from which this fragment was originally obtained [7]. The 3.4 kb *Eco*RI fragment, a 0.7 kb *Hin*dIII fragment, and a 1.2 kb *Sma*I–*Eco*RI fragment derived thereof, were cloned into M13 vectors and sequenced with the M13 universal primer or with sequence-derived primers. Both directions of the 1.2 kb *Sma*I–*Eco*RI fragment were sequenced; ambiguous regions were resolved with
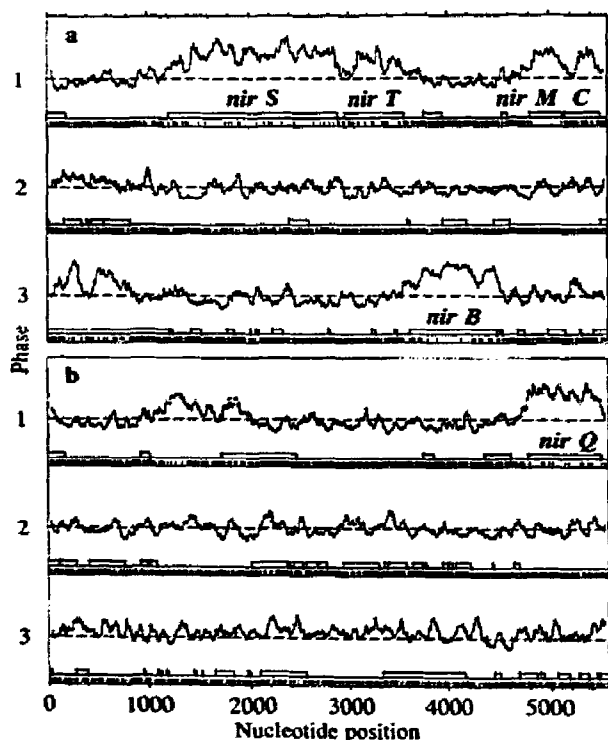

Fig. 2. Codon preference plot for the *nir* gene subcluster from *P. stutzeri*. The window size is 25 codons. The analysis is shown for the six possible phases in (a) the complementary *nir*Q sequence of Fig. 1 and the published *nir*STBMC sequence: *nir*C was formerly ORF5 [25]; (b) the complementary strand of the *nir*STBMC sequence and *nir*Q. Open bars in each panel indicate open reading frames: those defined as coding are identified by the corresponding gene acronyms. Stippled bars represent the frequency of rare codons. The algorithm of [38] was used as implemented in the HUSAR software, release 2.1 (Deutsches Krebsforschungszentrum, Heidelberg, Germany).
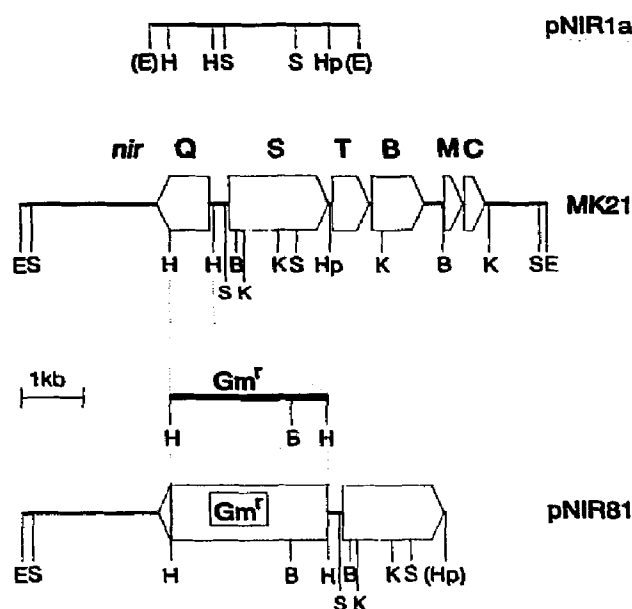

Fig. 3. Physical map of the *nir* region and strategy of deletion–replacement mutagenesis for the *nir*Q gene. The organization of denitrification genes of *P. stutzeri* wild-type (MK21) is shown by open arrows denoting gene size and direction of transcription. The bar (Gm^r) indicates the gentamicin interposon which became part of plasmid pNIR81 (vector DNA not shown). The *Hpa*I restriction site in pNIR81 was lost during the construction. Plasmid pNIR1a was used for complementation of strain MK2181. Restriction enzymes: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; S, *Sma*I.

dITP. A sequence of 1,153 nucleotides was established (Fig. 1). The coding strand was identified from a codon preference table (Fig. 2), generated from the genes *nir*S, nirB (cytochrome c_{552}), and *nir*M (cytochrome c_{551}) for which confirmatory protein sequence data are available [25]. An open reading frame, designated *nir*Q (formerly ORF8 in [6] and [25]), extends from position 259 to 1,086. It is divergently transcribed from *nir*S. The gene has the capacity to encode a protein of 275 amino acids ($M_r = 30,544$). Since the derived product has an overall hydrophilic character and has no features of a secretory presequence, we assume a cytoplasmic location. Specific sequence motifs are indicated in Fig. 1. The incidence of rare codons (Fig. 2) suggests that most *nir* genes may be expressed at a comparable, moderately high level, with *nir*M somewhat stronger.

3.2. Construction of the nirQ deletion mutant, MK2181

Most of the *nir* gene subcluster is located on an 8.6 kb *Eco*RI fragment which was obtained from cosmid cDEN1 [6]. This fragment was first cloned into the plasmid, pBR325. A 5 kb *Eco*RI–*Hpa*I fragment thereof, carrying the *nir*S and *nir*Q genes, was subcloned into the *Eco*RI–*Eco*RV restriction sites of pBR325 to yield plasmid pNIR8. For replacement of the *nir*Q gene a 0.7 kb *Hin*dIII fragment was deleted that begins in the pro-
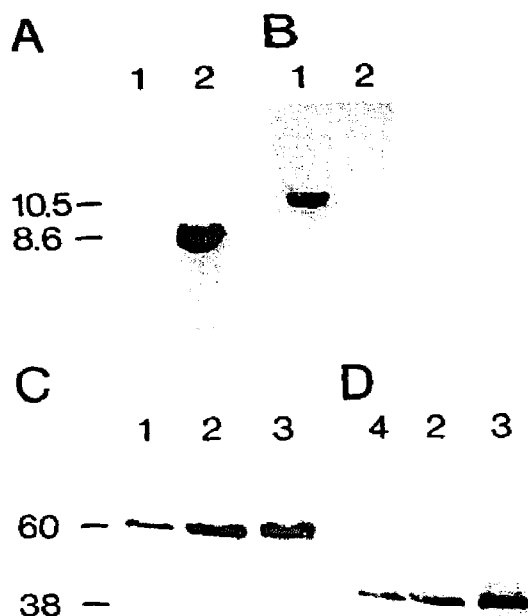
Fig. 4. Evidence for mutagenesis of MK2181 by DNA-DNA hybridization and immunoblot analysis for nitrite reductase and NO reductase. (A) Hybridization with the 0.7 kb HindIII fragment of the nirQ gene; (B) hybridization with the 2.6 kb HindIII gentamicin interposon. Lane 1, mutant strain MK2181; lane 2, parental strain MK21. Fragment size is given in kb. Immunoblot analysis for (C) cytochrome cd_1; (D) the cytochrome b subunit of NO reductase. Lane 1, purified cytochrome cd_1, 0.5 µg; lanes 2 and 3, cell extracts of strain MK21 and mutant MK2181, respectively (25 µg applied in C, 80 µg in D); lane 4, purified cytochrome b. 0.5 µg. Apparent molecular mass in kDa.

moter region of nirQ and covers 76% of the translated region (Fig. 3). Care was taken in the replacement strategy to retain the two Fnr-binding motifs of the intergenic region, in case the one proximal to nirQ would also influence nirS expression.

The HindIII fragment was replaced in pNIR8 by the 2.6 kb Gm^r interposon derived from plasmid pR209CII. This resulted in plasmid pNIR81 (Fig. 3). For transfer to P. stutzeri and marker exchange, the 7.1 kb EcoRI–BamHI fragment of pNIR81 (composed of the 6.9 kb insert and a 0.2 kb EcoRV–BamHI fragment from the vector) was subcloned into the mobilizable and non-replicative plasmid pSUP203 to yield the replacement vector, pSUN81. The donor strain for conjugation was E. coli SM10, the recipient was P. stutzeri MK21. The double crossover event was screened for by Gm^r and Ap^s. Mutant MK2181 was chosen and phenotypically characterized. The gene replacement was verified by Southern analysis (Fig. 4A,B).

3.3. Properties of mutant MK2181

Cell extract of MK2181 was assayed for the presence of nitrite reductase and NO reductase by immunoblot-

ting. Both reductases were found (Fig. 4C,D). NO reductase was usually monitored with the cytochrome b-specific antiserum; identical results were obtained with the cytochrome c-specific antiserum (data not shown). Based on densitometer scans of Western blots, NO reductase in strain MK2181 was overproduced vs. the wild-type level by a factor of about 2. Table I gives the activity measurements with intact cells and cell extract, both for nitrite reduction and NO reduction. Whole cells of the nirQ mutant had lost their NO-reducing activity; nitrite reduction was weak. On anaerobic growth in the presence of nitrate, the mutant accumulated nitrite. However, nitrite reductase was active in vitro in strain MK2181, whereas NO reductase remained inactive (Table I). Nitrate reduction and N_2O reduction of mutant cells were not affected.

To complement the deleted nirQ gene, plasmid pNIR1a was used [7]. This plasmid was constructed by cloning NIR44 as the 3.4 kb EcoRI fragment into the Cm^r gene of the mobilizable and replicative vector, pSUP104, oriented in such a way that the transcriptional direction of nirQ and that of the resistance gene were identical (Fig. 3). Plasmid pNIR1a was introduced by conjugation into MK2181 and transconjugants were plated on LB agar containing gentamicin and tetracycline. Strain MK2181(pNIR1a) showed again nitrite and NO reduction (Table I). Since expression from pNIR1a complemented the defect of MK2181, the mutation in nirQ is not polar on a downstream-located, putative denitrification gene.

4. DISCUSSION

Our results reveal a functional coupling between nitrite reduction and NO reduction mediated by the nirQ product. The complementation of strain MK2181 with plasmid pNIR1a implies that the nirQ gene is an independent transcriptional unit. The nirQ and nirS genes represent an example of divergent transcriptional organization [28], the significance of which has to be ex-

Table I

Activities of nitrite reductase and NO reductase in vivo and in vitro of wild-type and the nirQ mutant of P. stutzeri ZoBell

Strain	Enzymatic activity (nmol per min per mg protein)			
	With nitrite as substrate		With NO as substrate	
	In vivo	In vitro	In vivo	In vitro
MK21(Sm^r)	21	37	17	30
MK2181(ΔnirQ)	traces	42	0	0
MK2181(pNIR1a)	11	nd^a	19	nd

^{a}nd, not determined

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NirQ QHGMP VL IK G P TG C GK T---RFVQHMAH R LNLPLYTVA C HDDLS  78
XylR RGRVS VL LL G E TG V GK EVIARSVHLRSE R AEQPFVAVN C AA--I  295
NifA RWDTT VL VR G E SG T GK ELIANAIHHNSP R AAAAFVKFN C AA--L  272
NtrC RSSIS VL IN G E SG T GK ELVAHALHRHSP R AKAPFIALN M A--I  199
DctD DTDVD VL VA G E TG S GK EVVAQILHQWSH R RKGNFVALN C GA--L  205
HrpS QLGID VL LS G E TG T GK DTIAQRIHTISG R -KGRLVAMN C AA--I   68
          *                      **  **   *        *   *        *


NirQ AAD L VGRH L I G AQGTWWQDGPLT R AV R ----E GG IC YLDE VV  116
XylR PPD L IESE L F G VDKGAYTGAVNA R AG R FERAN GG TI FLDE VI  337
NifA PDN L LESE L F G HEKGAFTGAVRQ R KG R FELAD GG TL FLDE IG  314
NtrC PKD L IESE L F G HEKGAFTGANTI R QG R FEQAD GG TL FLDE IG  241
DctD PET V IESE L F G HERGAFTGAQKR R TG R IEHAS GG TL FLDE IE  247
HrpS PES L AESE L F G VVSGAYTGADRS R VG Y IEAAQ GG TL YLDE ID  110
     * *      ***      *       * *  **                  *        *


NirQ EARQDTAVV L HP- L ADDRR E LFI ER T G EALKAPPGFMLVVSYNPGY  161
XylR ELTPRAQAT L LRV L QE--G E --L ER V G GDRTRKVDVRLITATNENL  379
NifA ESSASFQAK L LRI L QE--G E --M ER V G GDETLRVNVRIIAATNRHL  356
NtrC DMPLDVQTR L LRV L AD--G Q --F YR V G GYAPVKVDVRIIAATHQNL  283
DctD SMPAATQVK M LRV L EM--R E --I TP L G TNEVRPVNLRVVAAAKIDL  289
HrpS SMPLSLQAK L LRV L ET--R A --L ER L G STSTIKLDVCVIASAQSSL  152
         *    **    **           *         *   * ****  *


NirQ QN--------------LLKGMK P ST R Q R -----FVAMR F ------D  182
XylR EEAVKMGRFRADLFFRLNVFPVHI P PL R E R VEDIPLLVEH F LRRHHKE  427
NifA EEEVRLGHFREDLYYRLNVMPIAL P PL R E R QEDIAELA-H F LVRKIAH  403
NtrC EQRVQEGKFREDLFHRLNVIRVHL P PL R E R REDIPRLARH F LQVAARE  331
DctD GDPAVRGDFREDLYYRLNVVTISI P PL R E R RDDIPLLFSH F AARAAER  355
HrpS DDAVEQGKFRRDLYFRLNVLTLQL P PL R T Q PERILPLFKR F MAAAAKE  200
    *                      *      *              *    *


NirQ YPPTAEEERIVANEAQVDAALAAQVVK L GQALRRLE-----------------  218
XylR YGKKTLGLSDRAMEACLHYQWPGNIRE L ENALERGVILTESNESINVES---LF  478
NifA SQGRTLRISDGAIRLLMEYSWPGNVRE L ENCLERSAVLSESGLIDRDVI---LF  454
NtrC LGVEAKLLHPETEAALTRLAWPGNVRQ L ENTCRWLTVMAAGQEVLIQDLPGELF  385
DctD FRRDVPPLSPDVRRHLASHTWPGNVRE L SHYAERVVLGVEGGGA---------  381
HrpS LNVASADVCPLLQQVLLGHEWPGNIRE L KAAAKRHVLGFPVLGV---------  244
                      ****  *              *


NirQ ------------------------QHDLEEVASTRLLIFTARMIRSGMTPRQ  246
XylR PGLATATEGDRLSSE-GRLEEESGDSWFRQIIDQ--GVSLEDLEAGLMRTAMDRCG  531
NifA NHRDNPPKA--LASS-GPAE----DGWLDNSLDE--RQRLI--------AALEKAG  493
NtrC ESTVAESTSQMQPDSWATLLAQWADRALRSGHQNLLSEAQPELERTLLTTALRHTQ  441
DctD ----AAVPPQPTGAT-------------------LPERLERYEAEIIRDTLSAND  413
HrpS ----DPQSEEHLACG------------------LKSQLRAIEKALIQQSLKRHR  276
                                                        **


NirQ ACLACL A EP L SDDPQ T VAALMDVVYVHFG                     275
XylR QNISQA A RL L GLTRP A MAYRLKKLDPSLSVKAMGR                566
NifA WVQAKA A RL L GMTPR Q VAYRIQIMDITMPRL                   524
NtrC GHKQEA A RL L GWGRN T LTRKLKELGME                       468
DctD GDVRRT I EA L GIPRK T FYDKLQRHGINRGGYSSRK                448
HrpS NCIDAA S LE L DMPRR T LYRRIKELQI                        302
          *  *      **
              ----- HTH -----
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Fig. 5. Amino acid alignment of the derived *nir*Q gene product with regulatory proteins of the NtrC family. XylR from *Pseudomonas putida* [39], NifA from *Klebsiella pneumoniae* [40], NtrC from *E. coli* [41], DctD from *Rhizobium leguminosarum* [42], and HrpS from *Pseudomonas syringae* pv. *phaseolicola* [43]. Highly conserved positions are boxed, well-conserved positions are marked with asterisks. HTH, region of C-terminal helix-turn-helix motifs [44]. The N-terminal parts of the proteins are not included. The sequence alignment was made by the program CLUSTAL [45].

plored. The complex phenotype observed on deletion of *nir*Q is not amenable to a straightforward interpretation. NO reductase is overproduced in MK2181, but is inactive in vivo and in vitro. The latter observation strongly suggests regulation at the enzyme level, hitherto not described for any denitrification enzyme. We have not observed a mobility shift in the mutant vs. the wild-type protein that could give an indication for protein modification. It should be noted, however, that the electrophoretic mobility of the strongly hydrophobic cytochrome *b* subunit is much higher than would correspond to its mass (53,137 Da) derived from the primary structure (C. Braun and W. G. Zumft, unpublished data). Nitrite reduction is simultaneously affected by

*nir*Q deletion, but only in vivo. A functional enzyme was assayed in cell extract with ascorbate-reduced PMS.

The SwissProt and PIR data banks, releases 22 and 33, respectively, were screened with the FASTA program [26] for sequence similarities to NirQ. The NirQ protein has a limited sequence similarity to the NtrC family of transcriptional activators (Fig. 5). The similarity is strongest within the nucleotide-binding regions. Most members of this protein family belong to the two-component systems of regulators [27]. The presence of a potential DNA-binding motif indicates that the NirQ protein would constitute the regulatory component. Whether this is the case and whether a complementary sensing component exists requires further studies.

The similarity search based on the nucleotide-binding region also picked up the TyrR protein involved in the regulation of aromatic amino acid biosynthesis of *E. coli* [29], and the MoxR protein ($M_r = 36,900$) necessary for methylotrophic growth of *Paracoccus denitrificans* [30]. Insertional inactivation of the *mox*R gene results in the loss of methylamine dehydrogenase activity, but the enzyme subunits and cytochrome c_{551}, a component of the electron transport chain to the enzyme, are still synthesized. MoxR is believed to directly modify a subunit of methylamine dehydrogenase, or influence expression of genes required for the formation of an active enzyme. The immediate target(s) on which the NirQ protein acts need to be identified to find out whether known common denominators for the systems of nitrite and NO reduction, like protein transport or heme *c* requirement, or other possibilities of transcriptional regulation or post-transcriptional modification are involved.

An independent line of evidence indicates that mutagenesis of *nir*S also influences NO reduction. Tn5 insertions in *nir*S of *P. fluorescens* cause a low activity of NO reduction. The ^{18}O exchange pattern is drastically altered in these mutants [31]. While *nir*S mutants of *P. stutzeri* are not markedly affected in their NO-reducing activity [20], the amount of NO reductase is substantially reduced (H. Körner, personal communication). This raises the possibility that nitrite reduction and NO reduction interact at several levels. A tight cellular control of the two processes would prevent accumulation of the toxic denitrification intermediate, NO. The steady-state concentration of free NO during denitrification of *P. stutzeri* ZoBell has been estimated at about 30 nM. In other denitrifying bacteria it can be as low as 1 nM [32].

Within the intergenic, non-coding region of *nir*Q and *nir*S two Fnr-binding motifs are tentatively assigned to their vicinal genes. Cytochrome cd_1 is synthesized anaerobically. The presence of two Fnr consensus motifs suggests that the *fnr* gene is involved both in the regulation of *nir*S and *nir*Q [33]. The NirQ protein would thus be synthesized only under denitrifying conditions. Two Fnr-binding motifs have also been found in strain

JM300 of *P. stutzeri* upstream of *nir*S [34]. Very likely they are part of an identical gene arrangement. The sequence translated from a nucleotide fragment (ORF1) reported in [35] for *P. aeruginosa* indicates a gene homologous to *nir*Q.

<pre>
nirQ P. stutzeri   17 TPDA-PFYQPLGNEEQLFQQAWQHGMPVLIKG 47
                            || ||| | | |:| || ||:|||:||
ORF1 P. aeruginosa   1 MRDATPFYEATGHEMQVFERAWRHGLPVLLKG 32
</pre>

This gene is also divergently transcribed from *nir*S (= *den*A). An anaerobically induced promoter activity was found in the transcriptional direction of *nir*Q [35]. The presently available sequence data (with the reservation that they still lack overlap at an *Eco*RI site) appear to indicate for *P. aeruginosa* only one Fnr motif in the intergenic region of *nir*S and *nir*Q.

REFERENCES

[1] Braun, C. and Zumft, W.G. (1991) J. Biol. Chem. 266, 22785–22788.

[2] Goretski, J. and Hollocher, T.C. (1988) J. Biol. Chem. 263, 2316–2323.

[3] Firestone, M.K., Firestone, R.B. and Tiedje, J.M. (1979) Biochem. Biophys. Res. Commun. 91, 10–16.

[4] Goretski, J. and Hollocher, T.C. (1990) J. Biol. Chem. 265, 889–895.

[5] Heiss, B., Frunzke, K. and Zumft, W.G. (1989) J. Bacteriol. 171, 3288–3297.

[6] Braun, C. and Zumft, W.G. (1992) J. Bacteriol. 174, 2394–2397.

[7] Jüngst, A., Braun, C. and Zumft, W.G. (1991) Mol. Gen. Genet. 225, 241–248.

[8] Wohlleben, W., Arnold, W., Bissonnette, L., Pelletier, A., Tanguay, A., Roy, P.H., Gamboa, G.C., Barry, G.F., Aubert, E., Davies, J. and Kagan, S.A. (1989) Mol. Gen. Genet. 217, 202–208.

[9] Boyer, H.W. and Roulland-Dussoix, D. (1969) J. Mol. Biol. 41, 459–472.

[10] Messing, J., Gronenborn, B., Müller-Hill, B. and Hofschneider, P.H. (1977) Proc. Natl. Acad. Sci. USA 74, 3642–3646.

[11] Simon, R., Priefer, U. and Pühler, A. (1983) Bio/Technology 1, 784–791.

[12] Bolivar, F. (1978) Gene 4, 121–136.

[13] Priefer, U.B., Simon, R. and Pühler A. (1985) J. Bacteriol. 163, 324–330.

[14] Messing, J. (1983) Methods Enzymol. 101, 20–78.

[15] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.

[16] Coyle, C.L., Zumft, W.G., Kroneck, P.M.H., Körner, H. and Jakob, W. (1985) Eur. J. Biochem. 153, 459–467.

[17] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

[18] Heery, D.M., Gannon, F. and Powell, R. (1990) Trends Genet. 6, 173.

[19] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[20] Zumft, W.G., Döhler, K., Körner, H., Löchelt, S., Viebrock, A. and Frunzke, K. (1988) Arch. Microbiol. 149, 492–498.
[21] Frunzke, K. and Zumft, W.G. (1984) J. Chromatogr. 299, 477–483.
[22] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[23] Laemmli, U.K. (1970) Nature 227, 680–685.
[24] Nakane, P.K. (1968) J. Histochem. Cytochem. 16, 557–560.
[25] Jüngst, A., Wakabayashi, S., Matsubara, H. and Zumft, W.G. (1991) FEBS Lett. 279, 205–209.
[26] Pearson, W.J. and D.J. Lipman (1988) Proc. Natl. Acad. Sci. USA 85, 2444–2448.
[27] Gross, R., Aricò, B. and Rappuoli, R. (1989) Mol. Microbiol. 11, 1661–1667.
[28] Beck, C.F. and Warren, R.A.J. (1988) Microbiol. Rev. 52, 318–326.
[29] Cornish, E.C., Argyropoulos, V.P., Pittard, J. and Davidson, B.E. (1986) J. Biol. Chem. 261, 403–410.
[30] Van Spanning, R.J.M., Wansell, C.W., De Boer, T., Hazelaar, M.J., Anazawa, H., Harms, N., Oltman, L.F. and Stouthamer, A.H. (1991) J. Bacteriol. 173, 6948–6961.
[31] Ye, R.W., Arunakumari, A., Averill, B.A. and Tiedje, J.M. (1992) J. Bacteriol. 174, 2560–2564.
[32] Goretski, J., Zafiriou, O.C. and Hollocher, T.C. (1990) J. Biol. Chem. 264, 11535–11538.
[33] Cuypers, H. and Zumft, W.G. (1992) in: Pseudomonas, Molecular Biology and Biotechnology (Galli, E., Silver, S. and Witholt, B. eds.) pp. 188–197, American Society for Microbiology, Washington, DC.
[34] Smith, G.B. and Tiedje, J.M. (1992) Appl. Environ. Microbiol. 58, 376–384.
[35] Arai, H., Igarashi, Y. and Kodama, T. (1991) FEBS Lett. 280, 351–353.
[36] Green, J., Trageser, M., Six, S., Unden, G. and Guest, J.R. (1991) Proc. R. Soc. Lond. B 244, 137–144.
[37] Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) EMBO J. 1, 945–951.
[38] Gribskov, M., Devereux, J. and Burgess, R.R. (1984) Nucleic Acids Res. 12, 539–549.
[39] Inouye, S., Nakazawa, A. and Nakazawa, T. (1988) Gene 66, 301–306.
[40] Arnold, W., Rump, A., Klipp, W., Priefer, U.B. and Pühler, A. (1988) J. Mol. Biol. 203, 715–738.
[41] Miranda-Rios, J., Sanchez-Pescador, R., Urdea, M. and Covarrubias, A.A. (1987) Nucleic Acids Res. 15, 2757–2770.
[42] Ronson, C.W., Astwood, P.M., Nixon, P.M. and Ausubel, F.M. (1987) Nucleic Acids Res. 15, 7921–7934.
[43] Grimm, C. and Panopoulos, N.J. (1989) J. Bacteriol. 171, 5031–5038.
[44] Pabo, C.O. and Sauer, R.T. (1984) Annu. Rev. Biochem. 53, 293–321.
[45] Higgins, D.G. and Sharp, P.M. (1989) Comp. Appl. Biol. Sci. 5, 151–153.