

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

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Received 19 October 1992; revised version received 2 November 1992

An open reading frame, designated *nirQ*, was identified upstream of *nirS*, 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 *nirQ* 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: $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$. 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 *nirQ*, 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 *nirQ* gene product, and support a concept for denitrification with the reaction sequence $\text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O}$ forming a functional unit.

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 *nirQ* 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 $\mu\text{g}\cdot\text{ml}^{-1}$): 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'-[α -³²S]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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<u>SmaI</u>	
CCCGGGGACTTCTTCTTTTTCGCGACTTGGATGCTATGAGAGCGGCAATCCGAAAGCGCTT	60
← Fnr →	**
GACGGCAATCAAGAGAGCCACCGGCACCCCGCAACGTCAGCGCTTGTCCGGGGCGCAAC	120
** *****	Fnr
ACGCCGAAACGCTCTGGAATCGGCTTCGATTGGCAGGCATCCGGCGCGGGACTTGATCGCA	180
→ HindIII	***** RBS
ATCAAGCTTTGCCGGCGGATGGCTTGAGGGGGCAAAGCGGGGCGCGTAGCTTGGCGATGG	240

AAATAACCAACTGCTGCCGTGAGGTACTTGCCTGTGAATGCCATTGAAATCCCGACTACC	300
nirQ → M R Y L P V N A I E I P T T	14
GCCGGCACGCCTGACCGGCCCTTCTACCAACCGTTGGGCAATGAAGAGCAGCTGTTCAG	360
A G T P D A P F Y Q P L G N E E Q L F Q	34
CAGGCCTGGCAGCACGGCATGCCCGTGCTTATCAAGGGCGGACCGGCTGCCGCAAGACC	420
Q A W Q H G M P V L I K <u>G P T G C G K T</u>	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
GACGACCTGTCCGGCGGCGACCTGGTCCGGCCGACACCTGATCGGGCGACAGGGCACCTGG	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 <u>G G I C Y L D E</u>	114
	B
GTGGTGAAGCACGGCAGGACACCGCCGTGGTACTGCACCCGCTGGCCGATGATCGCCGC	660
V V E A R Q D T A V V L H P L A D D R R	134
GAAGTGTTCATCGAGCGCACCGGGCGAGGCGCTCAAGGCGCCGCCGGGCTTCATGCTGGTG	720
E L F I E R T G E A L K A P P G F M L V	154
GTGTCTACAACCCCGTTACCAAAACCTGCTCAAGGGCATGAAGCCCAGCACCCGCCAG	780
V S Y N P G Y Q N L L K G M K P S T R Q	174
CGCTTCGTGGCGATGCGCTTCGACTATCCGCCGACCGCCGAGGAAGAGCGCATCGTCGCC	840
R F V A M R F D Y P P T A E E E R I V A	194
	HindIII
AACGAGGCGCAGGTGATGCCCGCTCGCCGCCAGGTGGTCAAGCTTGGCCAGGCACTG	900
N E A Q V D A A L A A Q V V K L G Q A L	214
CGTCGGCTGGAACAGCACGATCTGGAGGAAGTCGCCTCGACCCGCTGCTGATCTTCACC	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
CCGCTGTCCGATGATCCGCAGACCGTTGCCGCGCTGATGGATGTGGTCTATGTCCACTTC	1080
P L S D D P Q T V A A L M D V V Y V H F	274
CGCTGAACGGCTCGCCCGAGCCGTCCCGGCGCCTGCGGGTGCCTGCGGATGTGCTTTTT	1140
G end	275
CATCCTCGCCGAG	1153

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 *EcoRI* fragment which carries *nirQ*, *nirS*, and about 75% of *nirT*. The *EcoRI* restriction sites result from the construction of the lambda gt11 expression library from which this fragment was originally obtained [7]. The 3.4 kb *EcoRI* fragment, a 0.7 kb *HindIII* fragment, and a 1.2 kb *SmaI-EcoRI* 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 *SmaI-EcoRI* fragment were sequenced; ambiguous regions were resolved with

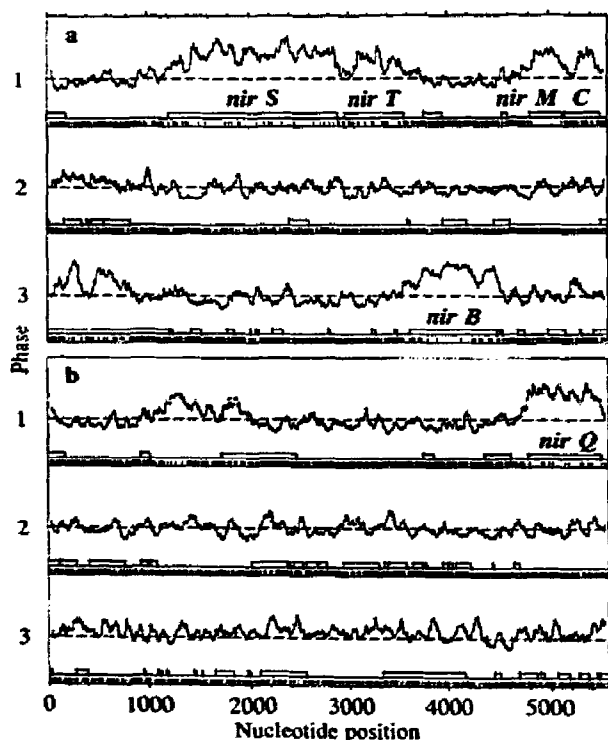


Fig. 2. Codon preference plot for the *nir* gene subcluster from *P. stutzeri*. The window size is 25 codons. The analysis is shown for the six possible phases in (a) the complementary *nirQ* sequence of Fig. 1 and the published *nirSTBMC* sequence; *nirC* was formerly ORF5 [25]; (b) the complementary strand of the *nirSTBMC* sequence and *nirQ*. 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).

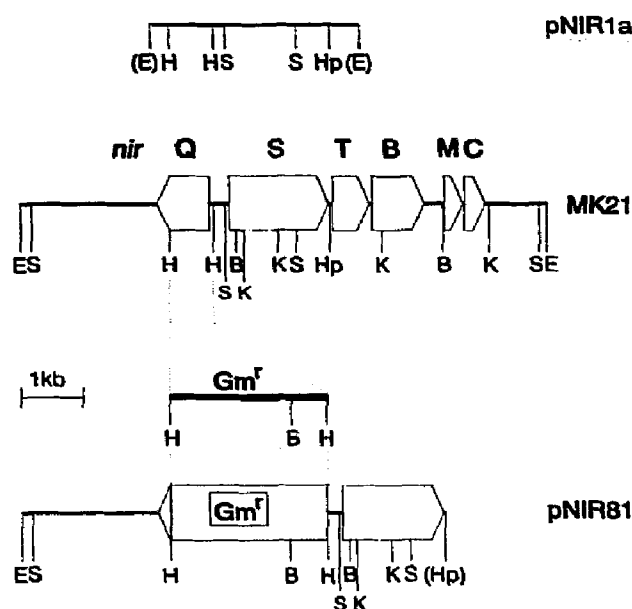


Fig. 3. Physical map of the *nir* region and strategy of deletion-replacement mutagenesis for the *nirQ* gene. The organization of denitrification genes of *P. stutzeri* wild-type (MK21) is shown by open arrows denoting gene size and direction of transcription. The bar (Gm^r) indicates the gentamicin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

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 *nirS*, *nirB* (cytochrome *c*₅₅₂), and *nirM* (cytochrome *c*₅₅₁) for which confirmatory protein sequence data are available [25]. An open reading frame, designated *nirQ* (formerly ORF8 in [6] and [25]), extends from position 259 to 1,086. It is divergently transcribed from *nirS*. 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 *nirM* somewhat stronger.

3.2. Construction of the *nirQ* deletion mutant, MK2181

Most of the *nir* gene subcluster is located on an 8.6 kb *EcoRI* fragment which was obtained from cosmid cDEN1 [6]. This fragment was first cloned into the plasmid, pBR325. A 5 kb *EcoRI-HpaI* fragment thereof, carrying the *nirS* and *nirQ* genes, was subcloned into the *EcoRI-EcoRV* restriction sites of pBR325 to yield plasmid pNIR8. For replacement of the *nirQ* gene a 0.7 kb *HindIII* fragment was deleted that begins in the pro-

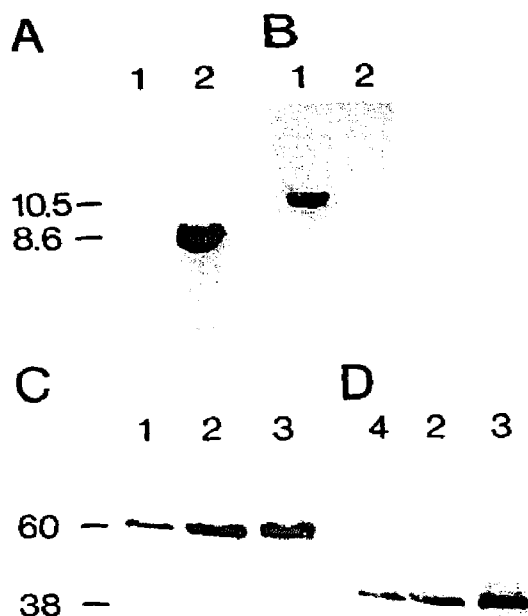


Fig. 4. Evidence for mutagenesis of MK2181 by DNA-DNA hybridization and immunoblot analysis for nitrite reductase and NO reductase. (A) Hybridization with the 0.7 kb *Hind*III fragment of the *nirQ* gene; (B) hybridization with the 2.6 kb *Hind*III gentamicin interposon. Lane 1, mutant strain MK2181; 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 μ g; lanes 2 and 3, cell extracts of strain MK21 and mutant MK2181, respectively (25 μ g applied in C, 80 μ g in D); lane 4, purified cytochrome *b*, 0.5 μ g. Apparent molecular mass in kDa.

motor 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 *Hind*III 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 *Eco*RI-*Bam*HI fragment of pNIR81 (composed of the 6.9 kb insert and a 0.2 kb *Eco*RV-*Bam*HI 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^r. 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 N₂O 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 *Eco*RI 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(Δ <i>nirQ</i>)	traces	42	0	0
MK2181(pNIR1a)	11	nd ^a	19	nd

^and, not determined

NirQ	OHGMP	VL	IK	G	P	TG	C	GK	T---	RFVQHMAH	R	LNLPLYTVA	C	HDDL	78							
XylR	RGRVS	VL	LL	G	E	TG	V	GK	EVIA	SVHLRSE	R	AEQPFVAVN	C	AA--I	295							
NifA	RWDIT	VL	VR	G	E	SG	T	GK	ELIAN	AIHHNSP	R	AAAAFVKFN	C	AA--L	272							
NtrC	RSSIS	VL	IN	G	E	SG	T	GK	ELVA	HALHRHSP	R	AKAPFI	ALN	M	A--I	199						
DctD	DTDVD	VL	VA	G	E	TG	S	GK	EVVA	QILHQWSH	R	RKGNFVALN	C	GA--L	205							
HrpS	QLGID	VL	LS	G	E	TG	T	GK	DTIA	QRIHTISG	R	-KGRLVAMN	C	AA--I	68							
NirQ	AAD	L	VGRH	L	I	G	AQGT	WQD	GPLT	R	AV	R	---	E	GG	IC	YLDE	VV	116			
XylR	PPD	L	IESE	L	F	G	VDKG	AYT	GAVNA	R	AG	R	FERAN	GG	TI	FLDE	VI	337				
NifA	PDN	L	LESE	L	F	G	HEKGA	FTG	AVRO	R	KG	R	FELAD	GG	TL	FLDE	IG	314				
NtrC	PKD	L	IESE	L	F	G	HEKGA	FTG	ANTI	R	QG	R	FEQAD	GG	TL	FLDE	IG	241				
DctD	PET	V	IESE	L	F	G	HERGA	FTG	AQKR	R	TG	R	IEHAS	GG	TL	FLDE	IE	247				
HrpS	PES	L	AESE	L	F	G	VVSG	AYT	GADRS	R	VG	Y	IEAAQ	GG	TL	YLDE	ID	119				
NirQ	EARQ	DTAVV	L	HP-	L	ADDR	E	LFI	ER	T	G	EALK	APPG	FMLV	VS	SYNPGY	161					
XylR	ELTP	RAQAT	L	LRV	L	QE--	G	E	--	L	ER	V	G	GDRT	RV	DVRL	TATNENL	379				
NifA	ESSA	SFOAK	L	LRI	L	QE--	G	E	--	M	ER	V	G	GDET	LR	VNVRI	IAATNRHL	356				
NtrC	DMPL	DVQTR	L	LRV	L	AD--	G	Q	--	F	YR	V	G	GYAP	VK	VDVRI	IAATHQNL	283				
DctD	SNPA	ATQVK	M	LRV	L	EM--	R	E	--	I	TP	L	G	TNEV	RP	VNLR	VAAAKIDL	289				
HrpS	SMPL	SLQAK	L	LRV	L	ET--	R	A	--	L	ER	L	G	STST	IK	LDVC	VIAAQS	152				
NirQ	QN	-----	LLKGMK	P	ST	R	Q	R	----	FVAMR	F	-----	D	182								
XylR	EEAV	KMG	FRAD	L	FFRL	NV	FPVHI	P	PL	R	E	R	VEDI	PLLVEH	F	LRRH	HKE	427				
NifA	EEEE	VLGH	FRED	L	YRLN	VMP	IAL	P	PL	R	E	R	QED	IAELA-H	F	LVR	KIAH	403				
NtrC	EQRV	QEGK	FRED	L	FHRL	NV	IRVHL	P	PL	R	E	R	REDI	PRLARH	F	LQV	AARE	331				
DctD	GDP	AVRG	DFRED	L	YRLN	VVT	ISI	P	PL	R	E	R	RDDI	PLLFSH	F	AAR	AAER	355				
HrpS	DDAV	EQGK	FRDL	Y	FRNL	VLT	LQL	P	PL	R	T	Q	PERI	LPLFKR	F	MAAA	AKE	200				
NirQ	YPPT	AEER	IVANE	AQ	VDAA	LAAQ	VVK	L	GQAL	RRLE	-----	218										
XylR	YGKT	TLGL	SDRAME	AC	LHYQ	WPG	NIRE	L	ENAL	ERG	VIL	TES	NES	IN	VES	---L	F	478				
NifA	SQGR	TLRIS	DGAI	RL	LLME	YSW	PGNVRE	L	ENCL	ERS	AVL	SES	G	LID	RDVI	---L	F	454				
NtrC	LGVE	AKLL	HPETE	AAL	TRLA	WPG	NVRO	L	ENTC	RWL	TVMA	AGQ	EV	LIQ	DLPG	ELF	385					
DctD	FRRD	VPPL	SPD	VRRH	LASH	TW	PGNVRE	L	SHYA	ERV	V	LG	VEG	GGA	-----	381						
HrpS	LNVA	SADV	CP	LLQ	VLLG	HEW	PGNIRE	L	KAA	AKRH	V	LG	F	PV	LG	V-----	244					
NirQ	-----	QHD	LEEV	AST	RL	LIFT	ARM	IRSG	MT	TPRQ	246											
XylR	PGLA	TATE	GD	RLS	SE	GR	LEES	CGD	SW	FRQ	I	IDQ	--	GVS	LED	LEAG	LMRT	AMDR	CG	531		
NifA	NHRD	NPPKA	--	LASS	GP	AE	----	DG	WLD	NS	LDE	--	RQ	RLI	-----	AA	LEK	AG	493			
NtrC	ESTV	AEST	SQM	QPD	SWAT	LLA	QWAD	RAL	RS	GHQ	NLL	SEA	QPE	LERT	LLT	TAL	RHTQ	441				
DctD	----	AA	VP	QPT	GAT	-----	LPER	LERY	EAE	I	IRD	TLS	AND	413								
HrpS	----	DP	QSE	H	LACG	-----	LK	SQ	LRA	IE	KAL	I	QSS	LKR	HR	276						
NirQ	ACL	ACL	A	EP	L	SDD	PQ	T	VAAL	MDV	VYV	HFG	275									
XylR	QNIS	QA	A	RL	L	GL	TRP	A	MAY	R	LK	KLD	P	S	L	S	V	K	A	MGR	566	
NifA	WVQA	KA	A	RL	L	GM	TPR	Q	VAY	R	I	Q	I	M	D	I	T	M	P	R	L	524
NtrC	GHK	QEA	A	RL	L	GW	GRN	T	LTR	K	L	KEL	G	M	E	468						
DctD	GDV	RRT	I	EA	L	GIP	RK	T	FYD	K	LQ	RH	G	I	N	R	G	G	Y	S	SRK	448
HrpS	NCID	AA	S	LE	L	DMP	RR	T	LYR	R	I	KEL	Q	I	302							

HTH

Fig. 5. Amino acid alignment of the derived *nirQ* 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].

explored. The complex phenotype observed on deletion of *nirQ* 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

nirQ 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 *moxR* 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 *nirS* also influences NO reduction. Tn5 insertions in *nirS* of *P. fluorescens* cause a low activity of NO reduction. The ^{18}O exchange pattern is drastically altered in these mutants [31]. While *nirS* 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 *nirQ* and *nirS* two Fnr-binding motifs are tentatively assigned to their vicinal genes. Cytochrome *cd₁* is synthesized anaerobically. The presence of two Fnr consensus motifs suggests that the *fnr* gene is involved both in the regulation of *nirS* and *nirQ* [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 *nirS* [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 *nirQ*.

<i>nirQ P. stutzeri</i>	17	TPDA-PFYQPLGNEEQLFQQAWQHGMFVLIKG	47
		: : :	
ORF1 <i>P. aeruginosa</i>	1	MRDATPFYEATGHEMQVFERAWRHGLPVLLKG	32

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

Acknowledgements: We are grateful to H. Cuypers for helpful advice, and to H. Körner for the data bank search. D. Erhard and B. Wohlfart provided skillful technical assistance. We thank P. Majer and A. Dreusch for photographic and computer artwork. The work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

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