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# Close genetic relationship between *Nitrobacter hamburgensis* nitrite oxidoreductase and *Escherichia coli* nitrate reductases

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**Abstract.** The nitrite oxidoreductase (NOR) from the facultative nitrite-oxidizing bacterium *Nitrobacter hamburgensis* X14 was investigated genetically. In order to develop a probe for the gene *norB*, the N-terminal amino acid sequence of the NOR β-subunit (NorB) was determined. Based on that amino acid sequence, an oligonucleotide was derived that was used for the identification and cloning of gene *norB*. Sequence analysis of DNA fragments revealed three adjacent open reading frames in the order *norA*, *norX*, *norB*. The DNA sequences of *norX* and *norB* represented complete genes while the open reading frame of *norA* was truncated by the cloning site. The deduced amino acid sequence of protein NorB contained four cysteine clusters with striking homology to those of iron-sulfur centers of bacterial ferredoxins. NorB shares significant sequence similarity to the β-subunits (NarH, NarY) of the two dissimilatory nitrate reductases (NRA, NRZ) of *Escherichia coli*. Additionally, the derived amino acid sequence of the truncated open reading frame of *norA* showed striking resemblance to the α-subunits (NarG, NarZ) of the *E. coli* nitrate reductases.

**Key words:** *Nitrobacter hamburgensis* – Nitrite oxidoreductase genes – Nitrite oxidoreductase β-subunit – Iron-sulfur protein – *Escherichia coli* nitrate reductase

The facultative lithotroph *Nitrobacter hamburgensis* derives energy for $CO_2$ fixation via the Calvin cycle from the oxidation of nitrite to nitrate. In the absence of oxygen the cells are able to grow by nitrate reduction using organic material (Freitag and Bock 1990). Both, nitrite oxidation and nitrate reduction are catalysed by the membrane-bound nitrite oxidoreductase (NOR) (Tanaka et al. 1983; Sundermeyer-Klinger et al. 1984). The catalytically active enzyme consisted of two subunits with molecular weights of 115000 and 65000 when purified by heat treatment (Meincke et al. 1992). In the presence of detergents cytochromes $a_1$ and $c_1$ were attributed to the NOR (Tanaka et al. 1983; Sundermeyer-Klinger et al. 1984). Cytochrome $c_1$ was shown to be an integral membrane protein of a molecular weight of 32000 and considered to be the third subunit of the NOR (Sundermeyer-Klinger et al. 1984). Cofactors of NOR are molybdopterin (Krüger et al. 1987) and iron-sulfur centers (Meincke et al. 1992). The subunit composition of NOR and the ability to reduce nitrate are typical for dissimilatory nitrate reductases. As reviewed by Hochstein and Tomlinson (1988), dissimilatory nitrate reductases are membrane-bound, molybdenum-containing iron-sulfur proteins.

*Escherichia coli* possesses two dissimilatory nitrate reductases, designated NRA and NRZ. Chaudhry and MacGregor (1983) showed that the molybdenum cofactor is part of the α-subunit while the β-subunit is supposed to be an electron-channeling Fe-S protein (Blasco et al. 1989, 1990). The cytochrome *b* containing γ-subunit is a membrane-embedded protein thought to be a link between the nitrate reductase and the respiratory chain.

Here we report on the identification, cloning and sequencing of the *norAXB* genes. The genes *norA* and *norB* encode the α- and β-subunit, respectively, of NOR from *N. hamburgensis* X14.

## Material and methods

### *Bacterial strains, plasmids and growth conditions*

All bacterial strains and plasmids used in this work are listed in Table 1. *Nitrobacter hamburgensis* X14 was grown mixotrophically as previously described (Bock et al. 1983). *Escherichia coli* K12 was used as a host strain for cloning of DNA fragments and propagation of plasmids. The DNA vectors for cloning and sequencing were pIBI30 and pIBI31 (International Biotechnology Inc., New Haven, Conn., USA), and the lambda promoter vector pCE30 (Elvin et al. 1990) was used for expression of *norB* in *E. coli*.

### *Amino-terminal analysis of NorB*

Membranes of nitrifying cells of *N. hamburgensis* were isolated according to Milde and Bock (1984). The membrane proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electro-

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**Table 1.** Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics | Source/Reference |
|---|---|---|
| *Nitrobacter hamburgensis* X14 | | Bock et al. 1983 |
| *Escherichia coli* K12 JM109 | *recA1 endA1 gyrA96 thi hsdR17 sup*E44 *relA1* Δ *(lac-pro-AB)/F' [traD36 proA⁺B⁺ lacI^q lacZ ΔM15]* | Yanisch-Perron et al. 1985; Ausubel et al. 1989 |
| Plasmids | | |
| pIBI30 | Ap^r, *lacPOZ'*, 2.93 kb, mcsII (*Eco*RI ≫ *Hin*dIII) | Internat. Biotech. New Haven, Conn. USA |
| pIBI31 | Ap^r, *lacPOZ'*, 2.93 kb, mcsII (*Hin*dIII ≫ *Eco*RI) | Internat. Biotech. |
| pCE30 | Ap^r, Lambda *cI857*, lambda-$p_R$ lambda-$p_L$ | Elvin et al. 1990 |
| pKK1 | pIBI30; 1.9-kb *Eco*RI/*Bam*HI fragment from *N. hamburgensis* X14 carrying part of *norB* | This study |
| pKK2 | pIBI30; 7-kb *Eco*RI fragment from *N. hamburgensis* X14, carrying *norXB* genes | This study |
| pKK2d | pKK2; deletion of 1.28-kb *Xho*I fragment | This study |
| pKK3 | pCE30; 2.7-kb *Sma*I fragment from pKK2 | This study |

Ap^r, resistant to ampicillin; mcs, multiple cloning site

phoresis (SDS-PAGE) according to Laemmli (1970). After electrophoresis, proteins were electroblotted onto polyvinyliden difluoride (PVDF) membrane as described by Matsudaira (1987). Then the PVDF membrane was stained with Coomassie blue and the membrane area carrying the NOR β-subunit was cut out and directly loaded into a gas-liquid phase sequenator (Model 470 A, Applied Biosystems; Funkstadt, Germany). The amino-terminal sequence of the NOR β-subunit was determined by automated Edman degradation (Edman 1956).

### *Recombinant DNA work*

For routine work with recombinant DNA, established protocols were used (Sambrook et al. 1989). Chromosomal DNA of *Nitrobacter* was isolated using the method of Marmur (1961) modified by Koops and Harms (1985). The isolation of plasmids of *E. coli* was performed as described by Birnboim and Doly (1979).

### *DNA hybridization with an oligonucleotide probe*

Chromosomal *N. hamburgensis* DNA or recombinant plasmids from *E. coli* were separated on an agarose gel and transferred to a Gene Screen Plus membrane (NEN Du Pont, Dreieich, Germany) by Southern blotting. Prehybridization was done for 30 min at 60 °C in a solution of 1 M NaCl, 1% (w/v) SDS, and 10% (w/v) dextran sulfate (50 µl/cm² filter area). Oligonucleotide synthesis was performed by means of a DNA/RNA synthesizer (Model 394, Applied Biosystems). The oligonucleotide probe was labeled with [γ-$^{32}$P]ATP using T4 polynucleotide kinase and then added to the prehybridized filter at a concentration of $10^6$ cpm per ml of prehybridization solution. After an incubation for 18 h at 40 °C, the filter was washed twice for 5 min at room temperature in 0.3 M NaCl plus 30 mM sodium citrate (2 × SSC), twice for 30 min at 35 °C in 2 × SSC, 1% (w/v) SDS, and twice for 5 min at room temperature in 2 × SSC without SDS. The filter was wrapped in plastic foil and exposed to X-ray film.

### *DNA sequence analysis*

The DNA sequence was determined by the dideoxynucleotide chain termination method of Sanger et al. (1977) according to the sequencing strategy shown in Fig. 2.

Nested deletions were generated by exonuclease III (Erase-a-Base Kit; Promega, distributed by Serva, Heidelberg, Germany). Sequencing kits (Pharmacia Sequencing Kit; Pharmacia, Freiburg, Germany; USB Taquence Version 2.0 DNA Sequencing Kit; United States Biochemical Corporation, Dannstadt-Schauernheim, Germany) were employed using [α-$^{35}$S]ATP from Amersham-Buchler (Braunschweig, Germany).

### *Expression of norB in Escherichia coli*

The expression of *norB* in *E. coli* was performed according to Elvin et al. (1990). *E. coli* JM109 containing the recombinant expression vector was grown at 30 °C in Luria-Bertani medium (Sambrook et al. 1989) containing ampicillin (50 µg/ml) to an $OD_{595}$ = 0.5, then treated at 42 °C for 2 h. Cells in 1-ml samples were harvested before and after heating, resuspended in an SDS-PAGE loading mixture at an $OD_{595}$ of 5. The samples were treated at 100 °C for 2 min prior to application of 20-µl samples to lanes of a 0.1% (w/v) SDS/10% (w/v) acrylamide slab gel. Following electrophoresis, proteins were stained with Coomassie brilliant blue.

### *Computer analysis*

DNA and protein sequences were analysed by using the PC GENE (Genofit, Geneva, Switzerland) software package. Data search for sequence homology was performed by the PATMAT program (Henikoff et al. 1990) and sequence information was received from the EMBL Data Library (Heidelberg, Germany). Alignment of amino acid sequences was done by the MACAW program (Schuler et al. 1991).

### *Accession number*

The sequence of the gene *norB* has been deposited in the EMBL Data Library under the accession number X66067 NHNORB.

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   Met[1] Asp[2] Ile[3] Arg[4] Ala[5] Gln[6] Val[7] Ser[8] Met[9] Val[10]Phe[11]His[12]Leu[13]Asp[14]Lys[15]

5'-ATG  GAT  ATC  CGT  GCG  CAG  GTG  TCC  ATG  GTG  TTC  CAC  CTG  GAT  AA -3'
         C         C    C              G                             C
                        T              AG
```

**Fig. 1.** N-terminal amino acid sequence of the NOR β-subunit (NorB) from *Nitrobacter hamburgensis* X14 and the deduced sequences of the *norB*-specific 44mer oligodeoxynucleotides

## Results

### *The norB gene probe*

The β-subunit (NorB) of the *Nitrobacter hamburgensis* NOR was isolated from purified membranes (Milde and Bock 1984) by using SDS-PAGE. The separated NorB was electroblotted onto PVDF membrane as described by Matsudaira (1987) and then directly loaded into an amino acid sequenator (Model 470 A, Applied Biosystems). The N-terminal amino acid sequence of NorB is shown in Fig. 1. Based on the codon usage of the *N. vulgaris* T3 genes for ribulose-1,5-bisphosphate carboxylase/oxygenase (E. Sickinger, personal communication), degenerated 44mer oligonucleotides were synthesized. The oligonucleotides used are listed below the amino acids in Fig. 1. After 5'-end labeling with [$\gamma$-$^{32}$P]ATP, the degenerated oligonucleotides served as a *norB* gene probe.

### *Isolation and sequencing of norB*

The *norB* gene probe hybridized to a 1.9-kb *Eco*RI/*Bam*HI fragment and to a 7-kb *Eco*RI fragment of chromosomal *N. hamburgensis* DNA. The 1.9-kb fragment was isolated and inserted into the plasmid pIBI30, resulting in plasmid pKK1. Using the same techniques, the 7-kb fragment was also inserted into pIBI30 to generate plasmid pKK2. The latter plasmid contained the complete *norB* together with adjacent genes named *norX* and *norA* (Fig. 2) as revealed by sequencing.

The sequences of *norB* and adjacent DNA regions were determined by the dideoxy chain termination method (Sanger et al. 1977). The nucleotide sequence and the derived amino acid sequences are shown in Fig. 3. Upstream of *norB* the complete open reading frame (ORF) of *norX* and the incomplete ORF of *norA* were detected. The ORF of *norB* was identified by comparing the deduced amino acid sequences of all possible reading frames with the known N-terminal amino acid sequence of the NorB protein. ORF *norB* has a length of 1539 bp (positions 967–2505) and a coding capacity of 513 amino acids. The predicted molecular weight of NorB is 57995. The ORF is preceded by a putative ribosome-binding site at an appropriate distance (positions 952–957). At a distance of 32 nucleotides downstream of the stop codon TAG (positions 2506–2508) of *norB*, a region marked by arrows was found where dyad symmetry could form a

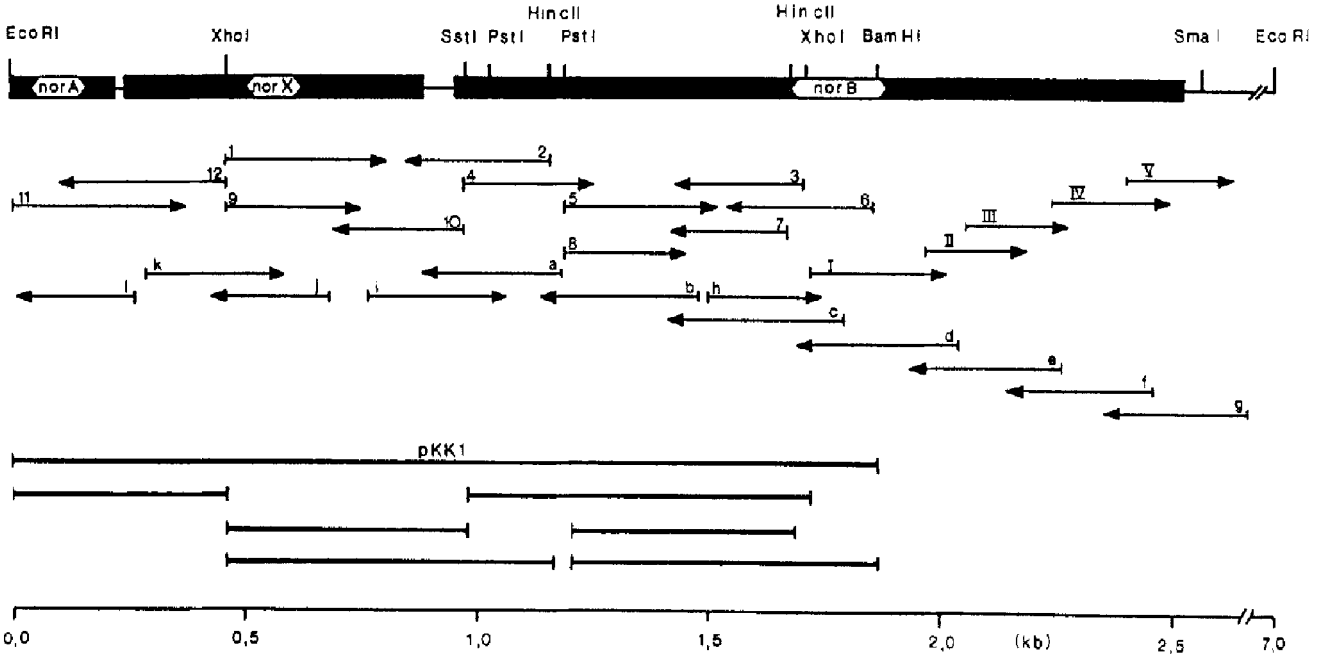


**Fig. 2.** Physical map of the partially sequenced 7-kb *Eco*RI fragment from *N. hamburgensis* X14 and sequencing strategy. The *arrows* show the positions, directions and the extent of sequences obtained. Subclones were produced by digestion of the plasmid pKK1 with different restriction enzymes. The subcloned fragments of pKK1 are depicted by *black bars*. The sequences obtained with these subclones are indicated by the arrows 1–12. For sequencing the second half of *norB*, deletion plasmids of a derivative of plasmid pKK2 (plasmid pKK 2d) were constructed using exonuclease III. These regions are marked by *arrows I–V*. Based on the obtained sequence information from subclones synthetic oligonucleotides were designed and used as sequencing primers. These regions are indicated by the *arrows a-l*

10-bp stemmed hairpin loop. This region may represent a potential transcription termination structure whose free energy was calculated to be −97,1 kJ/mol.

The incomplete ORF of *norA*, ending with a TAG stop codon at positions 232–234, encloses 231 bp corresponding to a peptide of 77 amino acids. ORF *norX* extends for 648 bp (positions 250–898) and terminates with a TAA stop codon at positions 899–901. The gene *norX* may code for a polypeptide of 216 amino acids with a molecular weight of 23645. A putative ribosome-binding site (positions 233–238) is preceding the *norX* start codon at a distance of 11 bp.

No putative promoter sequence could be detected. The absence of a conspicuous promoter sequence upstream of *norX* or *norB* and the existence of a single putative termination structure downstream of *norB* indicate that the genes *norA*, *norX*, and *norB* are probably organized in an operon.

The hydrophobicity profiles of NorB and NorX (not shown) suggested that both proteins are not membrane-bound.

### *Expression of norB in E. coli*

The calculated molecular weight of NorB is 57995. This value differs from that determined by SDS-PAGE (Sundermeyer-Klinger et al. 1984; Meincke et al. 1992). To prove that there have been deletions the gene *norB* was overexpressed in *Escherichia coli*. The gene product was compared with NorB from *N. hamburgensis* membranes.

For expression of *norB* the 2.7-kb *Sma*I fragment from pKK2 was inserted into the *Sma*I site of the lambda

```
ORF norA=>
GGCACCTGCATGTACTACCACGCGGTCGAGCGTACGGTGTACATTCCGAAATCGCAGGAG   60
G T C M Y Y H A V E R T V Y I P K S Q E

CGTAAGTGGCGTGGCGGCGGACACAACAGCCTGACGCGTACTCGTATCAACCCGCTGTTC  120
R K W R G G G H N S L T R T R I N P L F

CTGGCCGGTGGCTATGCCCAGTTCACGTACGGCTGGAACTATTGGGGTCCGACTGGTATC  180
L A G G Y A Q F T Y G W N Y W G P T G I

TTAACGCGCGACACCCACGTCGTCGTGCGCAAGATGGAGAAGTTGGAATGGTAGGTGATC  240
L T R D T H V V V R K M E K L E W ***
     norX=>
TCGCGACGCATGATCATGATCACGAACATGATCATGAACCGGCTCCGGCGCATTTCCGTG  300
       M I M I T N M I M N R L R R I S V   17

CGCCCTGAGCGGGTTAGCGTCGGGAAATACGTGACCATTAACTACACGCTGCGTACCAAC  360
R P E R V S V G K Y V T I N Y T L R T N   37

GGTACGATCCGTCACATTCGATCTGCTGTGTGGGGCGACGAACCGTTCGAATATCAGCAG  420
G T I R H I R S A V W G D E P L E Y Q Q   57

GGCAGTGGGCGACTCCTGCCTGCCCTCGAGGGCAAGGTTGCGGGCGAGCGCTTCGAGGTG  480
G S G R L L P A L E G K V A G E R F E V   77

ATTTTGCCTCCCGAAGAGGCTTACGGAGAACGAGACAAGGCGTTCGCAGCAGCGAGTGCCG  540
I L P P E E A Y G E R D K A L Q Q R V P   97

GCTGAAACGTTTGGTGGTGTTGATCAAATAGAGCCGGGTATGTGTTTTCTCGCGCATAGC  600
A E T F G G V D Q I E P G M C F L A H S  117

GACGACGAGCGTCGGGTTGAGAACGTGTTGGTCACCGAGGTTGATGAGGATAACGGGTAT  660
D D E R R V E N V L V T E V D E D N G Y  137

GTGGTGGTTGATACCAATCACCCGCTCGCCGGCATGACGCTTGAGTTTGAGGGTAGCGTT  720
V V V D T N H P L A G M T L E F E G S V  157

GTGGCAGTGCGGGACACTCCAACCCCGGCGGGACATGTCGATCCGACGATGGCGAGTATG  780
V A V R D T P T P A G H V D P T M A S M  177

GGCGATGGGCTGGCGCTTGCTCCGGCCCAAGCGACAATTGACTCGCGGCGCAAGCGGACG  840
G D G L A L A A A Q A T I D S R R K R T  197

ATTGATGAGTCACCGAAACCATCGACGCCCGGCACTGTCAATGACACTGGCAACGCATAA  900
I D E S P K P S T P G T V N D T G N A ***  216

TCGCGAAGCGATAACCTAAAACGAAGCCATAAGAACGAAGCCATAGGAACGAAGGAGTAC  960
     norB=>
TACAACATGGACATCCGAGGTCAAGTTTCGATGGTCTTTCACCTGGATAAGTGCATCGGT 1020
     M D I R A Q V S M V F H L D K C I G   18

TGCCACACCTGCAGCATCGCGTGCAAGAACATCTGGACCGATCGCAAGGGCACCGAGTAC 1080
C H T C S I A C K N I W T D R K G T E Y   38

ATGTACTGGAACAACGTGGAGACCAAGCCGGGTACGGGATACCCGACACGTTGGGAAGAC 1140
M Y W N N V E T K P G T G Y P T R W E D   58

CAGACCAAGTATCGCGGCGGCTGGGTGGTCGACGGGACGAGGCAGAAGAGCCTGCGGCTG 1200
Q T K Y R G G W V V D G T R Q K S L R L   78

CGGCTGCAGGGCAAATCGGGAACGCTGAGCAATATCTTCTACAACCCCTATCTGCCGACG 1260
R L Q G K W G T L S N I F Y N P Y L P T   98

CTCGACGACTATTTCGAGCCATGGACCTACGACTATCAGAACCTGATCAACGCGCCCCTG 1320
L D D Y F E P W T Y D Y Q N L I N A P L  118

GCCGACGAGCAACCGACCGCCGCGCCATCTCCATGGTCACGGGCAAGTACATGGACACG 1380
A D E Q P T A R A I S M V T G K Y M D T  138

ATCGAGGCGGGCCCGAACTGGGACGACGATCTTGGCGGCTCGCAGGTCTACGCCAACAAC 1440
I E A G P N W D D D L G G S Q V Y A N N  158

GATCCGAACTTCGATGGCGCCTCCGACGAGGAAATGCGCCAGATCAACGAGATCAACAGC 1500
D P N F D G A S D E E M R Q I N E I N S  178

ACGGTCTTTTTCTACCTGCCGCCGCATCTGCAACCATTGTCTCAATCCGGGCTGCGTGGCG 1560
T V F F Y L P R I C N H C L N P G C V A  198

GCGTGCCCGCAGGGAGCGATCTACAAGCGCGGCGAGGACGGCGTGGTGCTGGTCAGCCAG 1620
A C P Q G A I Y K R G E D G V V L V S Q  218

GAGCGCTGCCGTGCCTGGCGCATGTGCGTCTCCGGCTGCCCGTACAAGAAGACCTACTTC 1680
E R C R A W R M C V S G C P Y K K T Y F  238

AACTGGTCGACCGGCAAGGCCGAAAAGTGCATCCTGTGCTATCCGCGCCTCGAGAGCGGC 1740
N W S T G K A E K C I L C Y P R L E S G  258

CACGCTCCGGCCTGCTTCCACTCGTGCGTGGGACGCATCCGGTATATCGGCCTCGTGCTC 1800
H A P A C F H S C V G R I R Y I G L V L  278

TACGATGCCGATGCGATCGAGGAAACCGCCAAGGCCCCGCAAGACCAGTTGGTGATGGCG 1860
Y D A D A I E E T A K A P Q D Q L V M A  298

CAGCGCAACATCATCAAGGATCCGTTTGATCCTGATATCATCGCGGCTGCCAGGGCCAAC 1920
Q R N I I K D P F D P D I I A A A R A N  318

GGTATCCCCGATTCGAAGATCGAGGCGGCGCAGAAGTCGCCGGTCTACCAGTTCGTCAAG 1980
G I P D S K I E A A Q K S P V Y Q F V K  338

AAGTGGGGCATTGCGCTGCCGCTGCATCCGGAGTTCCGCACGCTGCCGATGCTGTTCTAC 2040
K W G I A L P L H P E F R T L P M L F Y  358

GTACCGCCGCTGGGGCCGGTGCTCGCCAAGGTGGAAAACGGCGTGTACGACAACATTGCC 2100
V P P L G P V L A K V E N G V Y D N I A  378

TCGGAATCGCGACTGGGTCCGTTGATGAGTTCACTCGAAAGGTCGCGTATACGCCTTCGC 2160
S E S R L G P L M S S L E R S R I R L R  398

TATATGGCGAGCGCTGTTGTCGGGCGGTAATGAGGACATCATCCGCGACGTCTACAAGAAG 2220
Y M A S L L S G G N E D I I R D V Y K K  418

CTAGTCGCGGTGCGCGTCTATATGCGCTCGAAGAAGGTCAAGGACATTCCGGAGGAAGAG 2280
L V A V R V Y M R S K K V K D I P E E E  438

GTGCAAAGGGCGCTCGCCGAAGGCAAGACCACTGCGGCCGCAGGTCGAGGCGGTCTGGCGG 2340
V Q R A L A E G K T T A A E V E A V W R  458

CTCACCTCGTTGCCGACCTTCGAGGAACGCTTTGTGGTTCCGCCGATGGAGCGTGAGACA 2400
L T S L P T F E E R F V V P P M E R E T  478

GCAGTCGATTCGCTGTTCCCGCAGCTCGATCCGGTGTCACGCAACTACCCGGTTCGGAAG 2460
A V D S L F P Q L D P V S R N Y P V R K  498

GGTGAGGTCGGCGTCGGCTTCCACACCGACCCGGCGAGAGGACCATAGGCTTCTTCTCGT 2520
G E V G V G F H T D P A R G P ***        513

CACGATGCAGGCGCGAGACAGCGGTTGGGTTTCATATACCCAACCGCCGGGTCTCGCGTA 2580

ATCGTCCGGCTCGCAAGGCAGGCCGAGAGTTTATAGATCGGTCATCATAGCAGATCATGA 2640

GTCGCTTTTCGCACCGCGCAGAGGTTCTGTATTCGAATCGCTCCAAGGTTTTCCCCTTGT 2700

TCGCGGACATCCTCGATTATCCCGTCCCGGATTGGA                          2736
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**Fig. 3.** Nucleotide sequence of the *norB* gene and its flanking genomic DNA of *N. hamburgensis* X14. The proposed ribosome-binding sites are underlined and the deduced amino acid sequences of the ORFs *norA*, *norX*, and *norB* are indicated below the nucleotide sequence. *Asterisks* below the sequence identify stop codons. *Arrows* mark regions of dyad symmetry

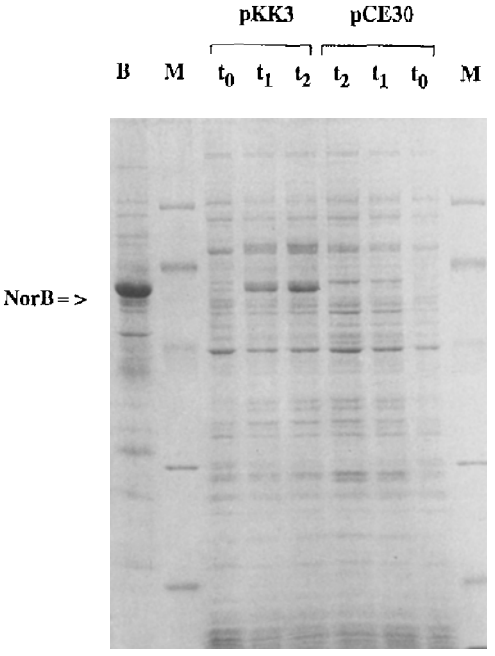


**Fig. 4.** Comparison by SDS-PAGE of the NorB protein from *Nitrobacter* membranes and that overexpressed in *Escherichia coli*. *Lanes*: *B*, NorB from *Nitrobacter* membranes (electroeluted from a gel of 2% (w/v) agarose in SDS-PAGE running buffer after electrophoresis of *Nitrobacter* membranes); **pKK3**, samples of *E. coli* containing pKK3 taken before ($t_0$) and 1 ($t_1$) or 2 h ($t_2$) after exposure of the cells to 42 °C; **pCE30**, samples of *E. coli* containing pCE30 (without insert) taken before ($t_0$) and 1 ($t_1$) and 2 h ($t_2$) after exposure to 42 °C; *M*, molecular weight markers: phosphorylase b (97400), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), soybean trypsin inhibitor (21500) and α-lactalbumin (14400)

promoter vector pCE30 (Elvin et al. 1990) to produce pKK3. The protein patterns presented in Fig. 4 show that after the temperature shift from 30 °C to 42 °C the strain containing pKK3 overproduced a protein with an electrophoretic mobility very similar to the NorB protein isolated from *N. hamburgensis* membranes.

### *Sequence comparisons*

The predicted products of *norA*, *norX*, and *norB* were investigated by using data bank searches for sequence similarities. Proteins with significant sequence similarities were found for NorB and the incomplete NorA but not for NorX. NorB exhibited 45% and 46% sequence identity to the proteins NarH and NarY (Fig. 5) which are the β-subunits of the two dissimilatory nitrate reductases NRA and NRZ of *E. coli* (Blasco et al. 1989, 1990). Taking conservative amino acid changes into account, the calculated overall resemblance increases to 61% and 63%, respectively. The N-terminal region up to Lys-362 of NorB is characterized by especially high similarity. For instance, the four marked cysteine clusters are nearly identical in its amino acid sequence. The internal region (Gly-363 to Glu-477) is less conserved while the C-terminal section (Thr-478 to the C-terminus) exhibits significant variation.

The amino acid sequence derived from the incomplete gene *norA* shows significant similarity to the C-terminal sequences of the proteins NarG and NarZ (Fig. 6) which are the α-subunits of the two dissimilatory nitrate reductases NRA and NRZ of *E. coli* (Blasco et al. 1989, 1990). Identities of 49% and 45.5% for NarG and NarZ,


**Fig. 5.** Alignment of the deduced NorB sequence to the NarH and NarY sequences of *E. coli*. The *asterisks* between the sequences indicate identical amino acids, and conservative amino acid changes (:) are defined as any within the following groups: I, L, M, V, A, G; D, E, N, Q; F, H, Y, W; K, R; S, T; C and P. *Dashes* indicate gaps introduced to optimize alignment. Cysteine clusters are *boxed*

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NarG  GMTMMYHAQERIVNLPGSEITQQRGGIHNSVTRITPKPTHMIGGYAHLAYGFNYYGTVGS-NRDEFVVVRKMKNIDWLDGEGNDQVQESVI 1238
      *  * *** ** * :* *:    ***:***:**    * :::**** **:**:* *  ** :****** ::*
NorA  GTCMYYHAVERTVYIPKSQERKWRGGGHNSLTRTRINPLFLAGGYAQFTYGWNYWGPTGILTRDTHVVVRKMEKLEW              77
      *  * *** ** : ** *:    ***:***:**    * :::***** :*:**:* *  ** :::***  ::*
NarZ  GMTMMYHAQERIMNIPGSEVTGMRGGIHNSVTRVCPKPTHMIGGYAQLAWGFNYYGTVGS-NRDEFIMIRKMKNVNWLDDEGRDQVQEAKK 1246
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**Fig. 6.** Alignment of the deduced sequence of the incomplete NorA peptide to the C-termini of NarG and NarZ of *E. coli*. For explanations see legend of Fig. 5

respectively, were calculated. Considering conservative amino acid changes, the values increase to 65% and 66%, respectively.

## Discussion

The genetic characterization of the key enzyme of *Nitrobacter hamburgensis* revealed that the $\beta$-subunit and part of the $\alpha$-subunit of NOR exhibit significant sequence similarities with the $\beta$- and $\alpha$-subunits of the two dissimilatory nitrate reductases of *Escherichia coli*. This represents the first genetic evidence for a close structural and functional relationship between NOR of *N. hamburgensis* and NRA and NRZ of *E. coli*.

The distribution of cysteines within the four NorB cysteine clusters is identical to the one in iron-sulfur centers of different bacterial ferredoxins as well as nitrate reductases and succinate dehydrogenase of *E. coli*. The amino acid composition of the cysteine clusters of NorB and of various electron transfer proteins is shown in Fig. 7. Cluster I contains a $CX_2CX_2CX_3$ arrangement of the cysteines typical for the [4 Fe–4 S] centers of the ferredoxins I and II of *Desulfovibrio desulfuricans* Norway (Bruschi and Guerlesquin 1988). Cluster II, $CX_2CX_4CX_3C$, is similar to the [3 Fe–3 S] center of *Azotobacter vinelandii* and *Pseudomonas ovalis* ferredoxins (Bruschi and Guerlesquin 1988). The arrangement of cysteines in Cluster III, $CX_5CX_3C$, is the same as described for *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum* ferredoxins (Bruschi and Guerlesquin 1988). Furthermore, patterns of cluster III exhibit the same structure like that of the Fe-S subunit (SdhB) of succinate dehydrogenase of *E. coli*. The latter is supposed to form a [4 Fe–4 S] center (Darlison and Guest 1985). Finally, cluster IV, $CX_2CX_{11}CX_3C$, resembles the [4 Fe–4 S]-type found in the ferredoxins of *Rhodopseudomonas palustris* and *Chromatium vinosum* (Bruschi and Guerlesquin 1988).

The result of the comparison presented clearly shows that the four cysteine clusters of NorB share striking similarities with those of the $\beta$-subunits NarH and NarY of the two dissimilatory nitrate reductases NRA and NRZ of *E. coli* (Blasco et al. 1989, 1990). For NRA and NRZ three [4 Fe–4 S] clusters and one [3 Fe–4 S] cluster were demonstrated to be present using EPR spectroscopy (Johnson et al. 1985; Guigliarelli et al. 1992). Blasco et al. (1989) provided evidence for the existence of an Fe-S subunit that contained four iron-sulfur centers. Because of these findings, it seems reasonable to assume that NorB

| | | C C C C |
|---|---|---|
| *N. hamburgensis* | NorB (Cluster I) | C I G C H T C S I A C K |
| *E.coli* | NarH (Cluster I) | C I G C H T C S V T C K |
| *E.coli* | NarY (Cluster I) | C I G C H T C S V T C K |
| *Desulfovibrio desulfuricans* Norway | FdI | C I G C E S C V E L C P |
| *D.desulfuricans* Norway | FdII | C I G C G E C V D V C P |

| | | C C C C |
|---|---|---|
| *N. hamburgensis* | NorB (Cluster II) | C N H C L N P G C V A A C P |
| *E. coli* | NarH (Cluster II) | C E H C L N P A C V A T C P |
| *E. coli* | NarY (Cluster II) | C E H C L N P S C V A T C P |
| *Azotobacter vinelandii* | Fd | C I K C K Y T D C V E V C P |
| *Pseudomonas ovalis* | Fd | C I K C K Y T D C V E V C P |

| | | C C C |
|---|---|---|
| *N. hamburgensis* | NorB (Cluster III) | C R A W R M C V S G C P |
| *E. coli* | NarH (Cluster III) | C R G W R M C I T G C P |
| *E. coli* | NarY (Cluster III) | C R G W R L C I S G C P |
| *E. coli* | SdhB (Cluster III) | C T F V G Y C S E V C P |
| *Sulfolobus acidocaldarius* | Fd | C I A D G S C I T A C P |
| *Thermoplasma acidophilum* | Fd | C I A D G A C M D V C P |

| | | C C C C |
|---|---|---|
| *N. hamburgensis* | NorB (Cluster IV) | C I L C Y P R L E S G H A P A C F H S C V |
| *E. coli* | NarH (Cluster IV) | C I F C Y P R I E A G Q P T V C S E T C V |
| *E. coli* | NarY (Cluster IV) | C I F C Y P R I E S G Q P T V C S E T C V |
| *Rhodopseudomonas palustris* | Fd | C T E C .....8 aa.....C V A V C P |
| *Chromatium vinosum* | Fd | C T E C .....8 aa.....C V E T C P |

**Fig. 7.** Comparison of the cysteine clusters I–IV of NorB with those of various bacterial ferredoxins (Fd) as well as NarH, NarY and SdhB of *E. coli*. The Fd amino acid sequences are presented according to Bruschi and Guerlesquin (1988). The NarH and NarY amino acid sequences are from Blasco et al. (1989, 1990), and the SdhB amino acid sequence is from Darlison and Guest (1984)

may also contain three [4 Fe–4 S] centers and one [3 Fe–4 S] center. Therefore, NorB as a part of the membrane-associated protein complex of the NOR may function as an electron-channeling protein between the nitrite-oxidizing NorA, not yet characterized completely, and the membrane-integrated electron transport chain.

Even though the genetic analysis is not completed yet, the present results provide sufficient evidence for a close evolutionary relationship of the nitrate reductases from *E. coli* and the NOR from *N. hamburgensis*. This is consistent with the biochemical data which suggested close functional similarity between both enzyme complexes (Hochstein and Tomlinson 1988; Sundermeyer-Klinger et al. 1984). If this assumption is correct it might be speculated that NOR and nitrate reductases have a common ancestor. It is unknown whether the "ancient" nitrate reductase was able to catalyze the reverse reaction oxidizing nitrite to nitrate. To address this question further investigations on nitrite oxidoreductases and nitrate reductases should be performed.

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