Antonie van Leeuwenhoek **66:** 111–127, 1994.

Isolation, sequencing and mutational analysis of a gene cluster involved in nitrite reduction in *Paracoccus denitrificans*

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Key words: denitrification, cd_1-type nitrite reductase, cytochrome *c*, d_1 heme biosynthesis, uroporphyrinogen III methylase, *Paracoccus denitrificans*

Abstract

By using the gene encoding the C-terminal part of the cd_1-type nitrite reductase of *Pseudomonas stutzeri* JM300 as a heterologous probe, the corresponding gene from *Paracoccus denitrificans* was isolated. This gene, *nirS*, codes for a mature protein of 63144 Da having high homology with cd_1-type nitrite reductases from other bacteria. Directly downstream from *nirS*, three other *nir* genes were found in the order *nirECF*. The organization of the *nir* gene cluster in *Pa. denitrificans* is different from the organization of *nir* clusters in some Pseudomonads. *nirE* has high homology with a S-adenosyl-L-methionine:uroporphyrinogen III methyltransferase (uro'gen III methylase). This methylase is most likely involved in the heme d_1 biosynthesis in *Pa. denitrificans*. The third gene, *nirC*, codes for a small cytochrome *c* of 9.3 kDa having high homology with cytochrome c_{55X} of *Ps. stutzeri* ZoBell. The 4th gene, *nirF*, has no homology with other genes in the sequence databases and has no relevant motifs. Inactivation of either of these 4 genes resulted in the loss of nitrite and nitric oxide reductase activities but not of nitrous oxide reductase activity. *nirS* mutants lack the cd_1-type nitrite reductase while *nirE*, *nirC* and *nirF* mutants produce a small amount of cd_1-type nitrite reductase, inactive due to the absence of heme d_1. Upstream from the *nirS* gene the start of a gene was identified which has limited homology with *nosR*, a putative regulatory gene involved in nitrous oxide reduction. A potential FNR box was identified between this gene and *nirS*.

Abbreviations: SDS – sodium dodecyl sulfate, NBT – nitroblue tetrazolium, PAGE – polyacrylamide gel electrophoresis

Introduction

Denitrification is the stepwise reduction of nitrate to diatomic nitrogen. Nitrate is reduced via nitrite, nitric oxide, nitrous oxide to nitrogen. The reduction of nitrite to gaseous nitric oxide is catalyzed by a dissimilative nitrite reductase and is the defining step for denitrification, which separates it from assimilative nitrite reduction in which nitrite is reduced to ammonium. Two types of dissimilative nitrite reductases have been isolated, a copper containing and a cytochrome *c* and d_1 containing reductase. Both are located in the periplasm in gram negative bacteria (Hochstein 1988; Stouthamer 1991).

Paracoccus denitrificans is a gram-negative bacterium capable of growing under various growth conditions. It can grow heterotrophically with a great variety of carbon compounds under aerobic conditions. In the absence of oxygen it can use nitrate, nitrite, with nitric oxide and nitrous oxide as terminal electron acceptors, but can not grow with nitric oxide as the only terminal electron acceptor (Voßwinkel et al.

Table 1. Strains and plasmids.

Strains	Relevant genotype	References
E. coli		
TOP10F'	F'tet^r (*mrr-hsdRMS-mcrBC*) lacZΔM15 *rpsL*Sm^r *endA1*	Stratagene
S17-I	Sm^r pro r$^-$ m$^+$ RP4-2 integrated (Tc::Mu)(Km^r::Tn7)	(Simon et al. 1983)
P. denitrificans		
Pd1222	Rif^r	(de Vries et al. 1989)
Pd7121	Pd1222 der., nirS ::Km^r	This study
Pd7221	Pd1222 der., *nirE*::Km^r	This study
Pd7321	Pd1222 der., *nirC*::Km^r	This study
Pd7421	Pd1222 der., *nirF*::Km^r	This study
Plasmids		
pGEM-7Zf(+)		Promega
M13mp18/mp19		(Sanger et al. 1980)
pPNIR1	11 kb HindIII Chrom. fr. Pd1222 in pGEM7-Zf(+)	This study
pPNIR3	13 kb PstI Chrom. fr. Pd1222 in pGEM7-Zf(+)	This study
pUC4K	Km^r (Tn903)	Pharmacia
pGRPD1	oriV (ColE1) Amp^r oriT Sm^r (Tn1831)	(van Spanning et al. 1990)
pRVS1	oriV (ColE1) Amp^r oriT Sm^r (Tn1831) Tn5p lacZ	(van Spanning et al. 1991)

1991). The composition of the electron transport chain varies with the growth condition and includes at least 8 *c* type cytochromes (Bosma 1989; Stouthamer 1991). Under anaerobic conditions the expression of nitrate-, nitrite-, nitric oxide- and nitrous oxide reductase is induced. The nitrite reductase of *Pa. denitrificans* is of the cd_1 type (Timkovich et al. 1982). It consists of two identical subunits of about 63 kDa, each subunit harboring one heme *c* and one heme d_1 (Hochstein 1988; Moir et al. 1993). The heme d_1 cofactor of the cd_1-type nitrite reductase is different from the *b* and *c* type hemes. It has two oxo-groups attached to the carbon tetrapyrrole skeleton and two extra methyl groups at the C2 and C7 carbon. Little is known about the biosynthetic pathway of heme d_1 but the carbon skeleton has the same origin as protoporphyrinogen IX, which is the precursor of the *b* and *c* type hemes (Matthews & Timkovich 1993). The heme d_1 biosynthesis therefore probably proceeds via the precursor of all tetrapyrrole cofactors, uroporphyrinogen III (Warren & Scott 1990). The electron donors for the cd_1-type nitrite reductase *in vivo* are not known, but cytochrome c_{551} and pseudoazurin (Ambler & Tobari 1985; Martinkus et al. 1980) can act as electron donors *in vitro* in *Pa. denitrificans* (Moir et al. 1993). Genes for the cd_1-type nitrite reductase have been isolated from three species of Pseudomonads and recently also from *Pa. denitrificans* strain IFO12442 (Ohshima et al. 1993). Here we report on the isolation of a gene cluster from *Pa. denitrificans* Pd1222 containing a gene coding for the cd_1-type nitrite reductase and 3 other *nir* genes. The construction and analysis of strains mutated in either of these genes is described.

Materials and methods

Bacterial strains, plasmids and growth conditions

The strains and plasmids used are listed in Table 1. *E. coli* was routinely grown in 2YT medium (10 g/l yeast extract, 16 g/l trypton and 5 g/l NaCl) at 37° C. *Pa. denitrificans* was grown in batch in either 2YT or mineral salts medium (Chang & Morris 1962) supplemented with Lawford trace solution (Lawford et al. 1976) and 25 mM succinate at 30° C. *Pa. denitrificans* was grown anaerobically in the same medium supplemented with 100 mM KNO_3 in completely filled stationary bottles at 30° C. Conjugations were performed as described before (van Spanning et al. 1990). Antibiotics were used at the following concentration; 100 μg/ml ampicillin, 25 μg/ml kanamycin, 40 μg/ml rifampicin and 25 μg/ml streptomycin.

DNA manipulations

General cloning techniques and DNA manipulations were essentially as described in Current Protocols in Molecular Biology (Ausubel et al. 1993). Plasmid DNA was routinely isolated using the alkaline lysis method (Birnboim 1983) from *E. coli* strain TOP10F'. Genomic DNA from *Pa. denitrificans* was isolated using the CTAB procedure (Current Protocols) from stationary growth phase cultures. In southern blot experiments, DNA was transferred from 1% agarose gels to positively charged nylon membranes (Boehringer Mannheim) via capillary transfer using 10 × SSC (1.5 M NaCl, 0.15 M Na-citrate pH 7.0). Colonies were transferred to the membrane from 2YT plates and replica plated on fresh plates. The DNA was released from the colonies by placing the blots on filter paper soaked with 0.5 M NaOH, 1.5 M NaCl, 15 min; 0.5 M Tris/HCl pH 7.5, 1.5 M NaCl, 5 min; 2 × SSC, 0.1% SDS, 5 min and washed in 3 × SSC, 0.1% SDS at 68° C, 60 min. Probes were made by labeling DNA fragments with Dig-dUTP (DIG Nucleic acid detection kit, Boehringer Mannheim) and hybridized in 5 × SSC, 1% Boehringer Blocking Reagent, 0.1% N-lauroylsarcosine, 0.02% SDS at 68° C for 18 h. When heterologous probes were used (probe # 1 and # 3) the blots were washed at lowered stringency (2 ×SSC, 0.1% SDS) at room temperature otherwise the blots were washed in 0.1 × SSC, 0.1% SDS at 68° C. The bands were visualized using alkaline phosphatase labeled anti-dig antibodies and using either the chromogenic substrate NBT/X-phosphate or the chemiluminescent substrate AMPPD (Lumigen-PPD, Boehringer Mannheim). Sequence reactions were performed on single stranded M13mp18 and mp19 clones using the dye-primer and dye-terminator cycle kits from ABI and loaded on a ABI 373A fluorescent sequencer (Applied Biosystems, Perkin and Elmer). Random clones were made by partial Sau3A digestion of clone pPNIRI and ligation in the BamHI site of M13mp19. The Macintosh^{TM} computer programs used were DNA Strider 1.0 and GeneWorks^{TM} 2.2.1. The eMail servers of NCBI running the BLAST program, the FASTA and BLITZ server at Heidelberg and the BLOCKS server were used for comparison of sequences with the sequence databases. For evolutionary relationship the Unweighted Pair Group Method with Arithmic Mean (UPGMA) method of the GeneWorks^{TM} program was used (Nei 1987). To construct the UPGMA tree, GeneWorks^{TM} does a pairwise position by position comparison of all of the sequences to determine whether or not the sequences are identical at that position. It then divides the number of mismatches by the length of the shorter sequence. The number of mismatches may be slightly higher than expected, since the algorithm corrects for the possibility that several mutations could have occurred at one site. This results in scores that reflect the relationship of one sequence to another.

Preparation of cell free extracts and isolation of periplasmic proteins

Cells were washed two times in 100 mM Tris/HCl pH 8.0 and suspended in the same buffer at an optical density of 100 cm^{-1} at 660 nm. 50 μM $MgCl_2$ and 2 μg/ml DNase I was added and the cell free extract was prepared using the French Pressure Cell. After centrifugation at 100.000 g for 60 min the supernatant was poured off and stored at - 30° C. Periplasmic proteins were isolated by preparing spheroplasts essentially as described before (Witholt et al. 1976). Protein concentration was determined using the BCA kit (Pierce) with BSA as a standard.

Protein assays and gel electrophoresis

In vitro nitrite reductase activity was determined at 30° C in a buffer containing 100 μg sample in 100 mM Tris/HCl pH 8.0, 30 μM PMS, 10 mM ascorbic acid and the reaction was started by adding KNO_2 to a final concentration of 1 mM (Matchová et al. 1993). Samples were taken every 5 min and NaOH (final concentration 10 mM) and H_2O_2 (10 mM) was added and boiled for 10 min. The nitrite concentration was determined on diluted samples by adding 1 vol. 1% sulfanilamide in 2.5 M HCl, 1 vol. 0.02% naphtylethyleen diamine (15 min at room temperature) and 4 vol. H_2O and measuring the absorbance at 540 nm using KNO_2 as a standard (0.2 mM, 0.15 mM, 0.1 mM and 0.05 mM) (Lam & Nicholas 1969). *In vivo* nitrate-, nitrite- and nitrous oxide reductase activities at 30° C were determined by following the oxidation and reduction of cytochromes after addition of the substrates. This was done by following the absorbance changes at 552 nm minus 578 nm of whole cells (OD_{660} = 100 cm^{-1}) under anaerobic conditions in an Amin-co DW2 UV/Vis spectrophotometer (Boogerd et al. 1980). *In vivo* nitric oxide reductase activity was determined by following the nitric oxide concentration using a Clark-type electrode with a polarizing voltage of 1 volt (Zimmer et al. 1985), in a buffer

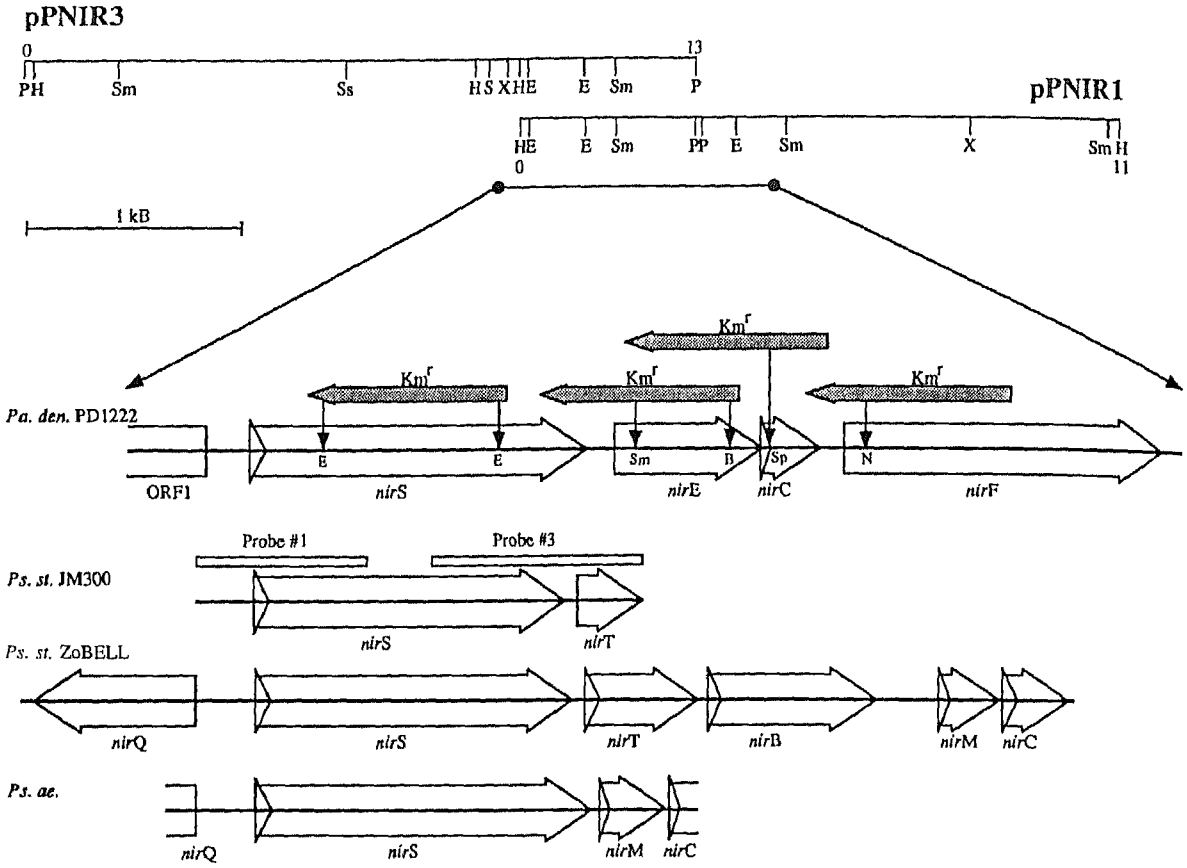


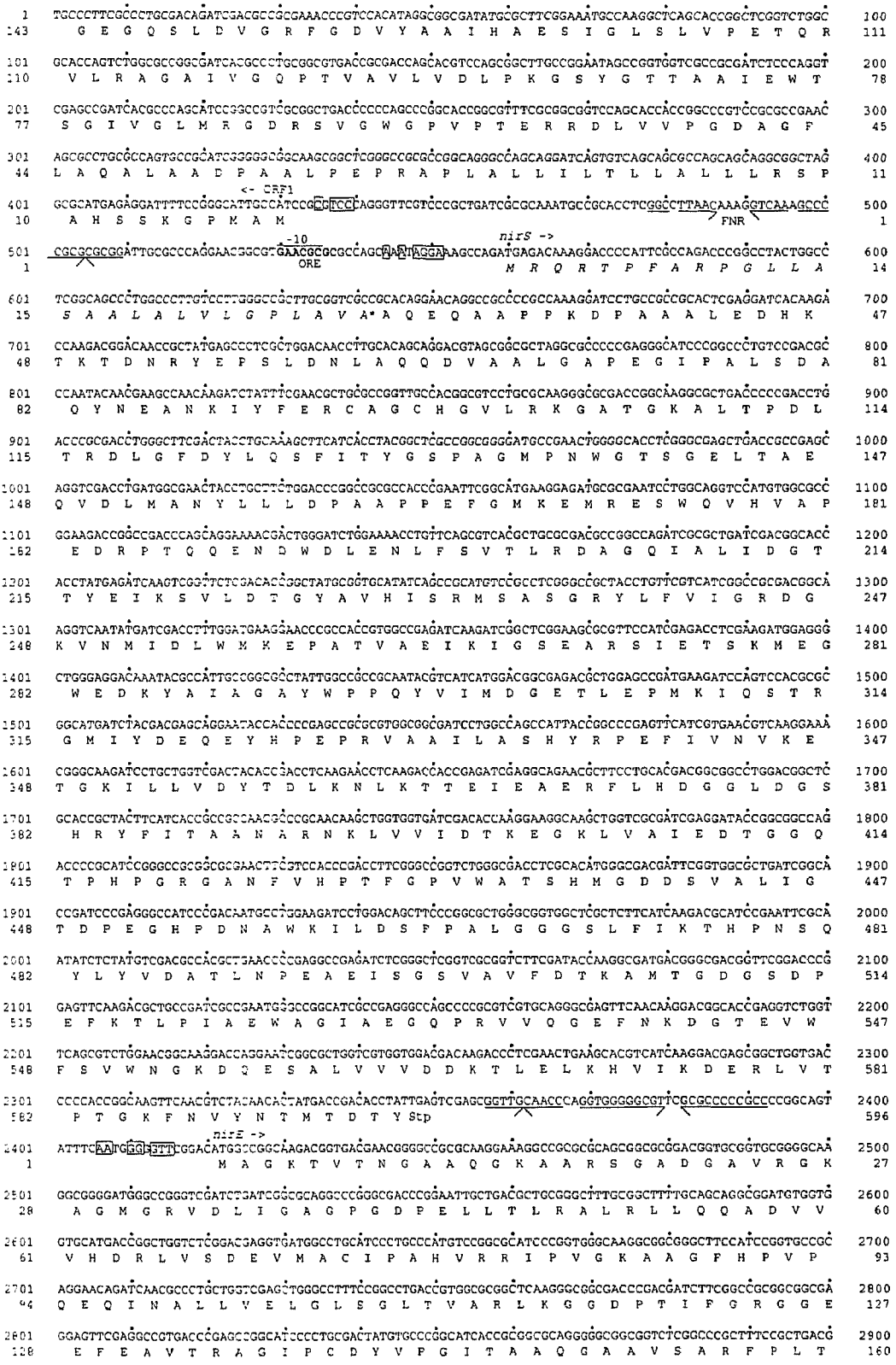
Fig. 1. Genomic fragments of *Paracoccus denitrificans* containing the *nir* locus and comparison with the organization in other bacteria. Genes are indicated by large arrows. Putative signal sequences are indicated by triangles inside the genes. Probe # 1 and # 3 designate the location of the gene fragments from *Pseudomonas stutzeri* JM300 used as heterologous probes. Sites of kanamycin insertions are indicated by grey arrows above the *Pa. denitrificans* organization. B = *Ball*, E = *Eco*RI, N = *Nrul*, P = *Pstl*, Sm = *Sma*I, Sp = *Sph*I, Ss = *Sst*I and X = *Xho*I. *Pa. den.* Pd1222 = *Pa. denitrificans* Pd1222, *Ps. st.* JM300 = *Ps. stutzeri* JM300 (Smith & Tiedje 1992), *Ps. st.* ZoBell = *Ps. stutzeri* ZoBell and *Ps. ae.* = *Ps. aeruginosa* (Zumft 1993).

containing 100 mM Tris/HCl pH 8.0, 10 mM Succinate and about 3 mM nitric oxide. The reaction was started by adding the cells to a cell density at 660 nm at about 5 cm^{-1}. SDS PAGE was carried out using the BIO-RAD mini-protean II gel system with 10 or 15% slab gels (Laemmli 1970). The samples were diluted in sample buffer and were not boiled but left at room temperature for 10 min to prevent the loss of heme *c*. The gels were stained for covalently bound heme using 60 mg 3,3′,5,5′-tetramethylbenzidine (Janssen Chimica) dissolved in 45 ml methanol and after dilution 105 ml 0.25 M Na-acetate pH 5.2 was added. The gels were soaked for at least one hour and after addition of 250 μl 30% H_2O_2 bands were visible after about 5 min (Thomas et al. 1976).

Results

Isolation of the nitrite reductase gene cluster

Genomic DNA of *Paracoccus denitrificans* Pd1222 was digested with either *Hin*DIII or *Pst*I and, after gel electrophoresis, probed with DNA harboring part of the nitrite reductase locus of *Pseudomonas stutzeri* JM300 (Smith & Tiedje 1992). One probe, a 1.2 kb *Sal*I-*Cla*I fragment encoding the C-terminal part of the cd_1-type nitrite reductase (*nirS*) and N-terminal part of a tetra-heme cytochrome *c* (probe # 3, Fig. 1), hybridized with a single 11 kb *Hin*DIII fragment and with 2 *Pst*I fragments of 13 and 7.5 kb. A second probe was a 0.9 kb *Hin*DIII-*Acc*I fragment coding for the N-terminal part of the cd_1-type nitrite reductase of *Ps.*


-10
ORE
FNR
nirS ->
nirE ->
Stp

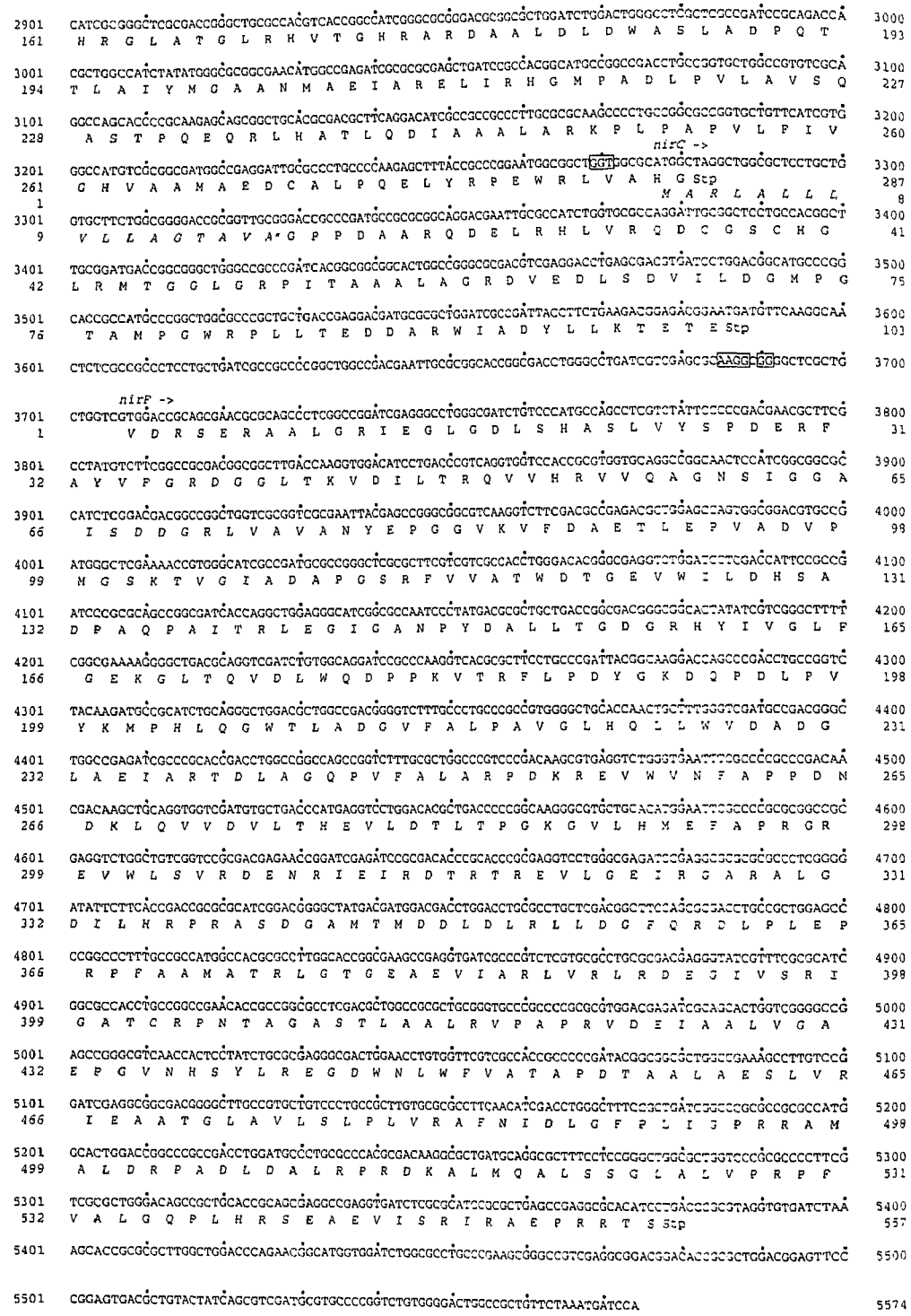
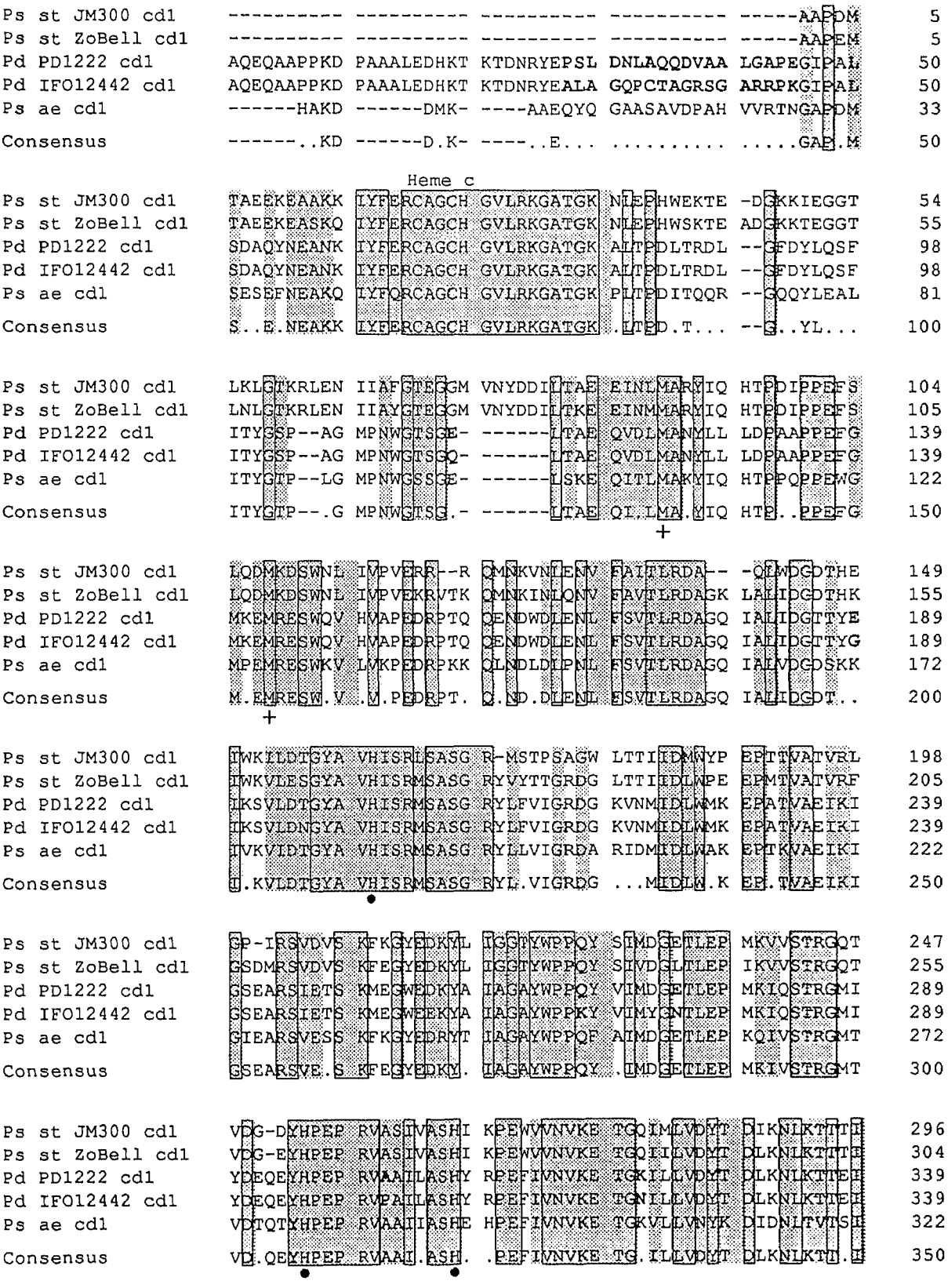


Fig. 2. DNA and predicted amino acid sequence of the *Pa. denitrificans nir* locus. Predicted signal sequences and their cleavage sites are indicated in italics and by asterisks, respectively. Putative Shine-Dalgarno ribosome binding sites are boxed. Palindromic sequences and inverted repeats are underlined. The putative FNR box is underlined and indicated by FNR. The putative oxygen responsive element (ORE) binding site is in boldface, the putative - 10 promoter sequence in lowercase. The sequence has been submitted to the GenBank database under accession number U05002.


Heme c

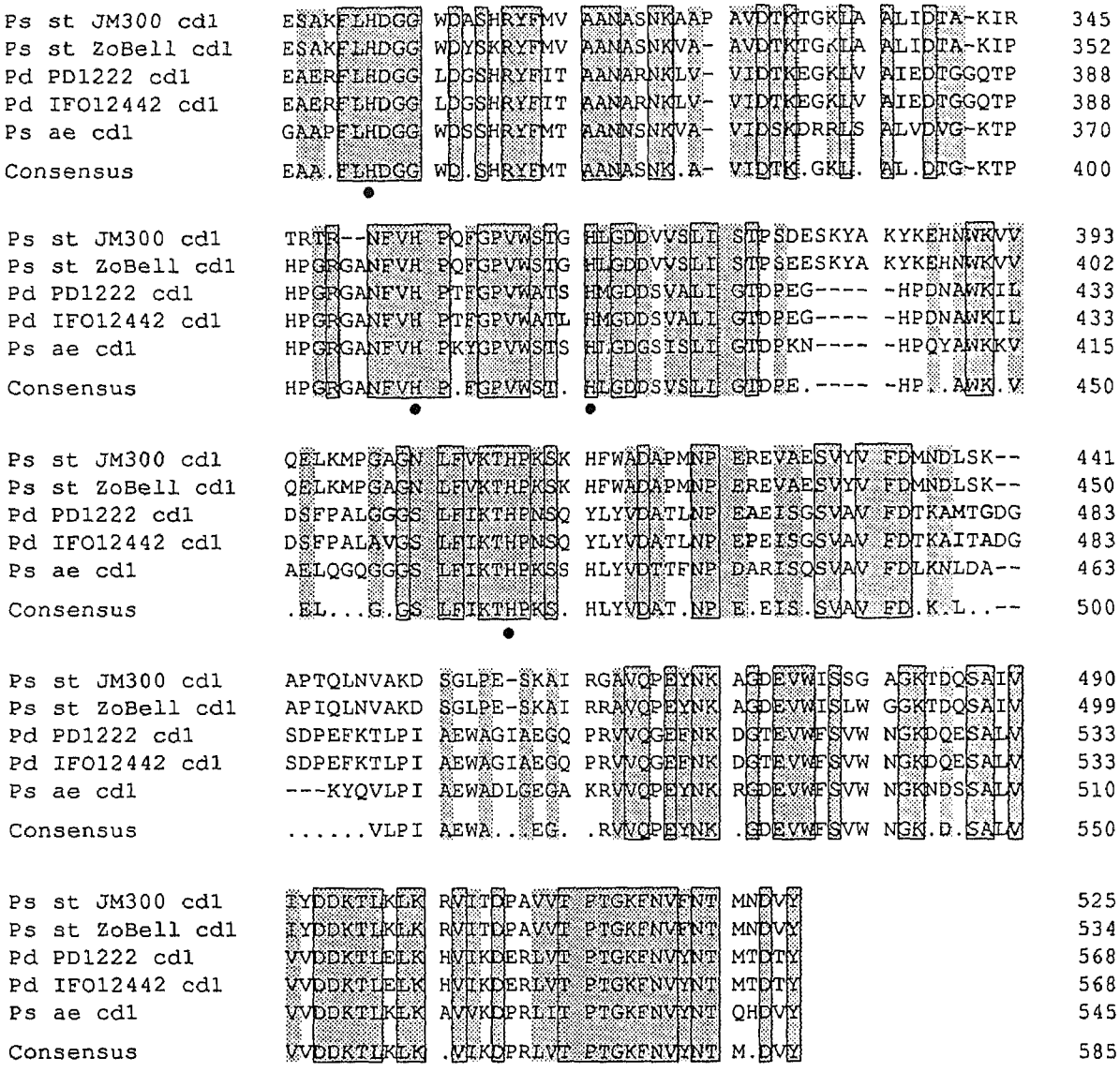


Fig. 3. Multiple sequence alignment of cd_1-type nitrite reductases. Ps st = *Pseudomonas stutzeri*, Ps ae = *Pseudomonas aeruginosa* and Pd = *Paracoccus denitrificans*. Residues conserved in all sequences are in gray boxes, conservative substitutions are only gray. Differences between the two *Pa. denitrificans* strains (Pd1222 and IFO12442) are indicated in boldface. Conserved histidine residues are indicated by a dot, conserved methionine residues by a plus sign. Two of the histidine residues could be ligands for heme d_1. One of the methionine residues could be a ligand for heme *c*.

stutzeri JM300 (probe # 1). This probe hybridized with a single 11 kb *Hin*DIII fragment and a single 13 kb PstI fragment. Genomic DNA of *Pa. denitrificans* Pd1222 was digested with either *Hin*DIII or *Pst*I and run on an agarose gel. DNA ranging in size from 5 to 15 kb was cut from the gel, extracted and ligated into the pGEM-7Zf(+) vector. Colonies from this enriched bank were screened with probe # 3 and two clones, pPNIR1 and pPNIR3 that reacted positively were isolated (Fig. 1). The observation that 2 fragments hybridized with heterologous probe # 3 on genomic DNA of Pd1222 cut with *Pst*I may be an indication that the ORF with the coding potential for a tetraheme *c* containing protein which is found in *Ps. stutzeri* JM300 is also present in *Pa. denitrificans*. The first *Pst*I site in pPNIR1 is located about 2 kb downstream from the end of the *nirS* gene. This means that the second *Pst*I fragment of 7.5 kb cannot hybridize with the *nirS* part of probe

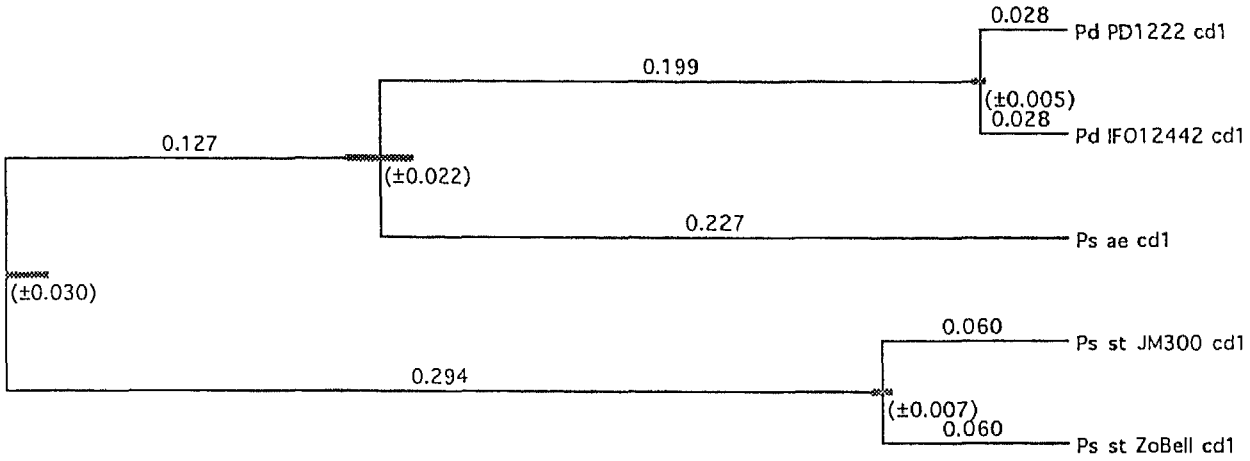


Fig. 4. UPGMA tree of aligned cd_1-type nitrite reductases from *Pa. denitrificans* and some Pseudomonads. Ps st = *Pseudomonas stutzeri*, Ps ae = *Pseudomonas aeruginosa* and Pd = *Paracoccus denitrificans*.

	R	Y	E	P	S	L	D	N	L	A	Q	Q	D	V	A	A	L	G	A	P	E	G	I
Pd1222	CGC	TAT	GAG	CCC	TCG	CTG	GAC	AAC	CTT	GCA	CAG	CAG	GAC	GTA	GCG	GCG	CTA	GGC	GCC	CCC	GAG	GGC	ATC
IFO12442	CGC	TAT	GAA	GCC	CTC	GCT	GGA	CAA	CCT	TGC	ACA	GCA	CGA	CGT	AGC	GGC	GCT	AGG	CGC	CCC	AAG	GGC	ATC
	R	Y	E	A	L	A	G	Q	P	C	T	A	G	R	S	G	A	R	R	P	K	G	I

Frameshift Frameshift

Fig. 5. Comparison of the DNA and the derived amino acid sequence of the two *Pa. denitrificans* strains. Frameshifts are indicated and codons which have a codon preference of less than 10% are termed rare codons and are underlined. In IFO12442 10 of 17 codons are rare codons, while in Pd1222 4 of 17 codons are rare codons.

3 because the *nirS* gene of *Pa.denitrificans* is located on a 13 kb *Pst*I fragment (= pPNIR3) and that the 7.5 kb PstI fragment could hybridize with the tetraheme part of probe # 3. The sequence presented here does not show the presence of a tetraheme cytochrome *c* as the 7.5 kb *Pst*I fragment has not been completely sequenced yet.

Sequence analysis of the nitrite reductase gene cluster

The exact location of the cd_1-type nitrite reductase gene was determined after southern blot analysis of restriction fragments of the two clones using probe # 1 and # 3. Subsequent sequence analysis of the fragments confirmed the presence of the gene for the cd_1-type nitrite reductase on these two clones, which are overlapping. By using codon preference information (Steinrücke & Ludwig 1993) 4 open reading frames (ORF's) and a possible 5th, could be identified (Fig. 2). Since the ORF's were found to be involved in nitrite reduction (see further) they were tentatively designated *nir* genes. An overview of the gene organization in *Pa. denitrificans* as compared to some Pseudomonads is given in Fig. 1.

The first gene of 1788 bp (596 aa) is the structural gene for the cd_1-type nitrite reductase, *nirS*. It contains one heme *c* binding motif (**CAGCH**) and a predicted signal sequence (Heijne 1983) of 28 aa. The predicted molecular weight of the mature protein (568 aa), including one heme *c*, is 63144 Da. The homology with other known cd_1-type nitrite reductases is given in Fig. 3. The homology of the five published cd_1-type nitrite reductases is very high around the heme *c* binding site and around some of the histidine residues. Two of these histidine residues could be the 5th and 6th ligand of the iron in the d_1 heme (Sutherland et al. 1986). Evolutionary relationship (Fig. 4) indicates that the *Pa. denitrificans* cd_1-type nitrite reductase is more related to the *Pseudomonas aeruginosa* counterpart than to the cytochrome cd_1 of either *Pseudomonas stutzeri* strains. The structural gene for the cd_1-type nitrite reductase

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CYSG_ECOLI      MDHLPIFCQL RDRDCLIVGG GDVAEPKARL LLDAGARLTV NALAFIPQFT     50
CYSG_SALTY      MDHLPIFCQL RDRDCLIVGG GDVAERKARL LLEAGARLTV NALTFIPQFT     50

CYSG_ECOLI      AWADAGMLTL VEGPFDESLL DTCWLAIAAT DDDALNQRVR QAAEARRIFC    100
CYSG_SALTY      VWANEGMLTL VEGPFDETLL DSCWLAIAAT DDDTVNQRVS DAAESRRIFC    100

CYSG_ECOLI      NVVDAPKAAS FIMPSIIDRS PLMVAVSSGG TSPVLARLLR EKLESLLPLH    150
CYSG_SALTY      NVVDAPKAAS FIMPSIIDRS PLMVAVSSGG TSPVLARLLR EKLESLLPQH    150

CYSG_ECOLI      LGQVAKYAGQ LRGRVKQQFA TMGERRRFWE KLFVNDRLAQ SLANNDQKAI    200
CYSG_SALTY      LGQVARYAGQ LRARVKKQFA TMGERRRFWE KFFVNDRLAQ SLANADEKAV    200
nirE            ---------- ---------- ---------- -----MAGKT VTNGAAQGKA     15

SUMT_PSEDE      -MIDDLFAGL PALEKGSVWL VGAGPGDPGL LTLHAANALR QADVIVHDAL     49
CYSG_ECOLI      TETTEQLINE PLDHRGEVVL VGAGPGDAGL LTLKGLQQIQ QADVVVYDRL    250
CYSG_SALTY      NATTERLFSE PLDHRGEVVL VGAGPGDAGL LTLKGLQQIQ QADIVVYDRL    250
SUMT_BACME      ---------- ----MGKVYL VGAGPGDPDL ITLKGLKAIQ QADVILYDRL     36
nirE            ARSGADGAVR GKAGMGRVDL IGAGPGDPEL LTLRALRLLQ QADVVVHDRL     65
SUMT_METIV      ---------- ------VVYL VGAGPGDPEL ITLKAVNVLK KADVVLYDKP     34

SUMT_PSEDE      VNEDCLKLAR PGAVLEFAGK RGGKPSPKQR DISLRLVELA RAGNRVLRLK     99
CYSG_ECOLI      VSDDIMNLVR RDADRVFVGK RAGYHCVPQE EINQILLREA QKGKRVVRLK    300
CYSG_SALTY      VSDDIMNLVR RDADRVFVGK RAGYHCVPQE EINQILLREA QKGKRVVRLK    300
SUMT_BACME      VNKDLLEYAK SDADIIYCGK LPNYHTLKQE TINNFLVKFA KKGKIVTRLK     86
nirE            VSDEVMACIP AHVRRIPVGK AAGFHPVPQE QINALLVELG LSGLTVARLK    115
SUMT_METIV      ANEEILKYA- EGAKLIYVGK QAGHHYKSQN EINTLLVEEA KENDLVVRLK     83

SUMT_PSEDE      GGDPFVFGRG GEELALLVEH QVPFRIVPGI TAGIGGLAYA GIPVTHREVN    149
CYSG_ECOLI      GGDPFIFGRG GEELETLCNA GIPFSVVPGI TAASGCSAYS GIPLTHRDYA    350
CYSG_SALTY      GGDPFIFGRG GEELETLCHA GIPFSVVPGI TAASGCSAYS GIPLTHRDYA    350
SUMT_BACME      GGDPFVFGRG GEELAELVQQ GISFEIVPGI TSGIAAAAYA GIPVTHREYS    136
nirE            GGDPTIFGRG GEEFEAVTRA GIPCDYVPGI TAAQGAAVSA RFPLTHRGLA    165
SUMT_METIV      GGDPFVFGRG GEELLALVEE GIDFELVPGV TSAIGVPTTI GLPVTHRGVA    133

SUMT_PSEDE      HAVTFLTGHD SSGLVPDRIN WQGIASGSPV IVMYMAMKHI GAITANLIAG    199
CYSG_ECOLI      QSVRLITGHL KTG---GELD WENLAAEKQT LVFYMGLNQA ATIQQKLIEH    397
CYSG_SALTY      QSVRLVTGHL KTG---GELD WENLAAEKQT LVFYMGLNQA ATIQEKLIAF    397
SUMT_BACME      ASFAFVAGHR KDS-KHDAIK WDSLAKGVDT LAIYMGVRNL PYICQQLMKH    185
nirE            TGLRHVTGHR ARD-AALDLD WASLADPQTT LAIYMGAANM AEIARELIRH    214
SUMT_METIV      TSFTVVTGHE DPT---KCKK QVGWDFKADT IVILMGIGNL AENTAEIMKH    180

SUMT_PSEDE      GRSPDEPVAF VCNAATPQQA VLETTLARAE ADVAAAGLEP PAIVVVGEVV    249
CYSG_ECOLI      GMPGEMPVAI VENGTAVTQR VIDGTLTQL- GELA-QQMNS PSLIIIGRVV    445
CYSG_SALTY      GMQADMPVAL VENGTSVKQR VVHGVLTQL- GELA-QQVES PALIIVGRVV    445
SUMT_BACME      GKTSATPIAL IHWGTCADQR TVTGTLGTIV DIVKEEQIEN PSMIIVGEVV    235
nirE            GMPADLPVLA VSQASTPQEQ RLHATLQDIA AALARKPLPA PVLFIVGHVA    264
SUMT_METIV      -KDPETPVCV IENGTMEGQR IITGTLENI- ---AGKDIKP PALVVL-EML    224

SUMT_PSEDE      RLRAALDWIG ALDGRKLAAD PFANRILRNP A                        280
CYSG_ECOLI      GLRDKLNW-- ---------- -FSNH----- -                        457
CYSG_SALTY      ALRDKLNW-- ---------- -FSNH----- -                        457
SUMT_BACME      N--------- ---------- -FS------- -                        238
nirE            AMAEDCALPQ ELYRPEWRLV AHG------- -                        287
SUMT_METIV      SM-------- ---------- -FLKK----- -                        230
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Fig. 6. Multiple sequence alignment of the derived amino acid sequence of *nirE* from *Pa. denitrificans* compared to the corresponding sequences from other bacteria. CYSG_ECOLI and CYSG_SALTY are the siroheme synthases encoded by the gene *cysG* from *E. coli* and *S. typhymurium* respectively. SUMT_PSEDE, SUMT_BACME and SUMT_MATIV are the uro'gen III methylases of *Pseudomonas denitrificans, Bacillus megaterium* and *Methanobacterivanovii* respectively. Residues conserved in all sequences are in gray boxes, conservative substitutions are only in gray.

of *Pa. denitrificans* strain IFO12442 has recently been published (Ohshima et al. 1993). The main difference with the one published here is located in the IFO12442 DNA between base 405 and 456, coding for the N-terminal region. This difference is the consequence of two frameshifts and the codon usage in this part of the sequence differs very much from codon preferences published for *Pa. denitrificans* (Steinrücke & Ludwig 1993) as can be seen by the dramatic increase in the number of rare codons (Fig. 5). Other differences in the DNA sequence are also found, sometimes leading to differences in the amino acid sequence (Fig. 3). These differences may be due to strain differences. Downstream from the *nirS* gene, two inverted repeats were identified. The first, a palindromic sequence, is also present in the sequence published by Ohshima et al. (1993). A second, larger inverted repeat could be identified just downstream from the palindromic sequence. This inverted repeat is not present in the sequence published by Ohshima et al. (1993).

Further downstream gene *nirE* was found. *nirE* (861 bp, 287 aa) encodes a protein with a deduced molecular weight of 30 kDa. The protein is overall hydrophilic and has no apparent signal sequence. Thus this protein is probably located in the cytoplasm. It has high homology to a S-adenosyl-L-methionine:uroporphyrinogen III methyltransferase (uro'gen III methylase; EC 2.1.1.10) which is involved in siroheme synthesis in *E. coli* (Bell et al. 1989; Peakman et al. 1990) and *Salmonella typhymurium* (Wu et al. 1991) and in vitamin B_{12} (cobalamin) synthesis in *Pseudomonas denitrificans* (Crouzet et al. 1990), *Bacillus megaterium* (Robin et al. 1991), *Methanobacterium ivanovii* (Blanche et al. 1991) and *S. typhymurium* (Roth et al. 1993). A multiple alignment analysis with other uro'gen III methylases is given in Fig. 6. The uro'gen III methylase of *E. coli* encoded by the *cysG* gene, has recently been renamed siroheme synthase, because it is also involved in the oxidation of precorrin-2 and the chelation of iron to yield siroheme (Spencer et al. 1993). The *cysG* gene is part of the *nir*-operon in *E. coli* and *S. typhymurium* (Wu et al. 1991). The *nirE* gene product only shows homology to the C terminal part of the siroheme synthase of *E. coli* and *S. typhymurium* which are much larger than the *nirE* gene product (457 aa vs. 287 aa) but the *nirE* gene product has about the same size as the uro'gen III methylases involved in vitamin B_{12} (cobalamin) synthesis in *Ps. denitrificans*, *B. megaterium* and *M. ivanovii*. *nirC*, (309 bp, 103 aa) encodes a small cytochrome *c* with high homology (52% identical) with cytochrome c_{55X},

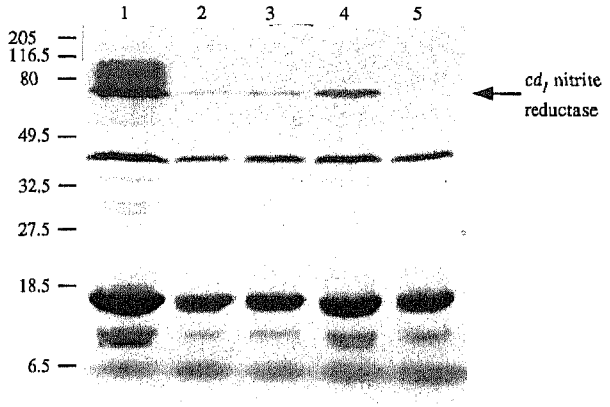

Fig. 7. SDS/PAGE of cell free extracts loaded on a 15% gel and stained for covalently bound heme *c*. Cell free extracts were prepared of *Pa. denitrificans* wild type and the 4 nir mutants grown anaerobically on 2YT. Lane 1, Pd1222, wild type; Lane 2, Pd7421, *nirF* mutant; Lane 3, Pd7321, *nirC* mutant; Lane 4, Pd7221, *nirE* mutant; Lane 5, Pd7121, *nirS* mutant. The location of the band corresponding to the cd_1-type nitrite reductase is indicated. The molecular masses of protein standards are shown (kDa).

the product of *nirC* (formerly called ORF5) of *Ps. stutzeri* ZoBell (Jüngst et al. 1991; Jüngst & Zumft 1992). The homology with other small cytochromes *c* is much lower. A signal sequence could be deduced with a length of 17 aa. The mature protein of 86 aa has a molecular weight of 9.3 kDa. Based on the high homology with *nirC* the third *nir* gene is given the same name. The translation start of *nirC* falls inside the nirE gene, which could mean that *nirE* and *nirC* are translationally coupled (Normark et al. 1983).

Codon preference information indicated that a 4th ORF was located downstream from *nirC*. This gene, *nirF*, starts with a GTG codon and a ribosome binding-site (Shine-Dalgarno or SD site, (Shine & Dalgarno 1975)) could be deduced although the spacing between the SD-site and the GTG start codon is somewhat large (16 bp), *nirF* (1671 bp, 557 aa) encodes a protein of 60.3 kDa. No signal sequence could be deduced and hydrophobicity analysis suggests that the protein is located in the cytoplasmic space. Screening of the databases revealed no significant homology with other proteins. Furthermore no motifs of any relevance could be identified.

Upstream from the *nirS* gene the start of ORF1 with an orientation opposite to that of *nirS* could be identified. The gene has some limited homology to the *nosR* regulatory gene of the nitrous oxide gene cluster

in *Pa. denitrificans* (Hoeren et al. 1993) and *Ps. stutzeri* ZoBell (Cuypers et al. 1992) (results not shown).

In the intergenic region between *nirS* and ORF1, a number of possible regulatory sites were identified.

- Located at - 71.5 bp as counted from the translation start of the *nirS* gene, a potential FNR-box could be identified. The sequence GGCCTTAACAAAGGTCAAGCC is a non-perfect inverted repeat, which does conform to the FNR- consensus (TTGAT—-ATCAA (Spiro & Guest 1990)) to a great extent.
- A region could be found which conforms to the - 10 promoter consensus sequence of *Pa. denitrificans* as proposed by Steinrücke et al. (1993).
- At the same location the binding site for a so called oxygen responsive element (ORE) (Steinrücke & Ludwig 1993) could be identified.
- A short palindromic sequence is located between the FNR box and the ORE/- 10 region. The importance of these regions in the regulation of the nitrite reduction is under further investigation.

Construction and analysis of the mutant strains

Figure 1 shows the sites of insertion and orientation of the kanamycin resistance markers. Fragments containing the mutated genes were ligated into one of the suicide vectors, pGRPD1 and pRVS1. These constructs were used to transform the mobilizing strain *E. coli* S17-I. The suicide constructs were transferred to *Pa. denitrificans* Pd1222 via conjugation. Ex-conjugants were screened for kanamycin resistance and streptomycin sensitivity. The correctness of the mutations was checked with southern blot experiments. Four mutant strains were isolated with kanamycin-box insertions in: 1) *nirS*, Pd7121; 2) *nirE*, Pd7221; 3) nirC, Pd7321 and 4) *nirF*, Pd7421. The properties of the mutant strains are summarized in Table 2. The effect of the mutations on anaerobic growth was examined by growing the mutants and wild type anaerobically in batch culture on 2YT supplemented with KNO_3 or mineral medium with succinate and KNO_3. Anaerobic growth of the mutants was severely inhibited and nitrite accumulated in the medium (30 mM on 2YT). SDS-PAGE with heme-staining of cell free extracts of anaerobically grown cells revealed that only in mutant strain Pd7121, the heme *c* signal of the cd_1-type nitrite reductase was not detectable (Fig. 7). The amount of heme *c* signal in the other 3 mutants was greatly reduced as compared to the wild type. Western blot analyses with antibodies raised against the cd_1-type nitrite reductase of *Pa. denitrificans* showed that the amount of cd_1-type nitrite reductase protein correlated with the heme *c* signal (results not shown). Serial dilutions of cell free extracts were loaded on SDS-PAGE and stained for heme *c*. This showed that the wild type Pd1222 contained about 10 times as much nitrite reductase as the mutant strains Pd7221, Pd7321 and Pd7421. No other changes in the number or relative amounts of heme *c* bands on SDS-PAGE were apparent. The small 9.3 kDa cytochrome *c* encoded by *nirC* is not detectable on SDS-PAGE with heme staining because the *nirC* mutant strain gave the same band pattern as the wild type. Spectra of dithionite reduced cell free extracts revealed the loss of the typical d_1 absorbance bands at 630 and 655 nm (Timkovich et al. 1982) indicating the loss of heme d_1 (Fig. 8). The small, broad absorbance peak at about 660 nm is not residual bound heme d_1 as this small peak is also observed in the *nirS* mutant strain in which the cd_1-type nitrite reductase is completely absent. A small peak at 595 nm was present in spectra of all 4 mutant strains but not in the wild type. All 4 mutants lacked *in vivo* nitrite reductase activity as determined by time resolved dual wavelength traces. *In vitro* nitrite reductase activity with ascorbate/PMS as artificial electron donor was also not detectable in cell free extracts prepared from the mutants. Mutants grown anaerobically had no *in vivo* nitric oxide reductase activity as measured with a Clark-type electrode. The *in vivo* nitrous oxide reductase activity of whole cells of all the mutant strains was comparable to that of the wild type.

Discussion

Here we report on the isolation and sequencing of a part of the cd_1-type nitrite reductase locus of *Paracoccus denitrificans* Pd1222. At least 4 genes involved in nitrite reduction in the order *nirSECF* were identified. *nirS* is the structural gene for the cd_1-type nitrite reductase. The deduced molecular weight of the mature protein including one covalently bound heme *c* is 63144 Da. The deduced molecular weight of the cytochrome cd_1 subunit becomes 63088 if the iron of the heme *c* is excluded from the calculation. This would be in agreement with the value of 63100 ± 14 Da as determined by electrospray mass spectrometry (Moir et al. 1993). The cd_1-type nitrite reductase does not differ significantly from other published cd_1-type nitrite reductases, although it is slightly larger. Screening of the sequence databases indicated that the

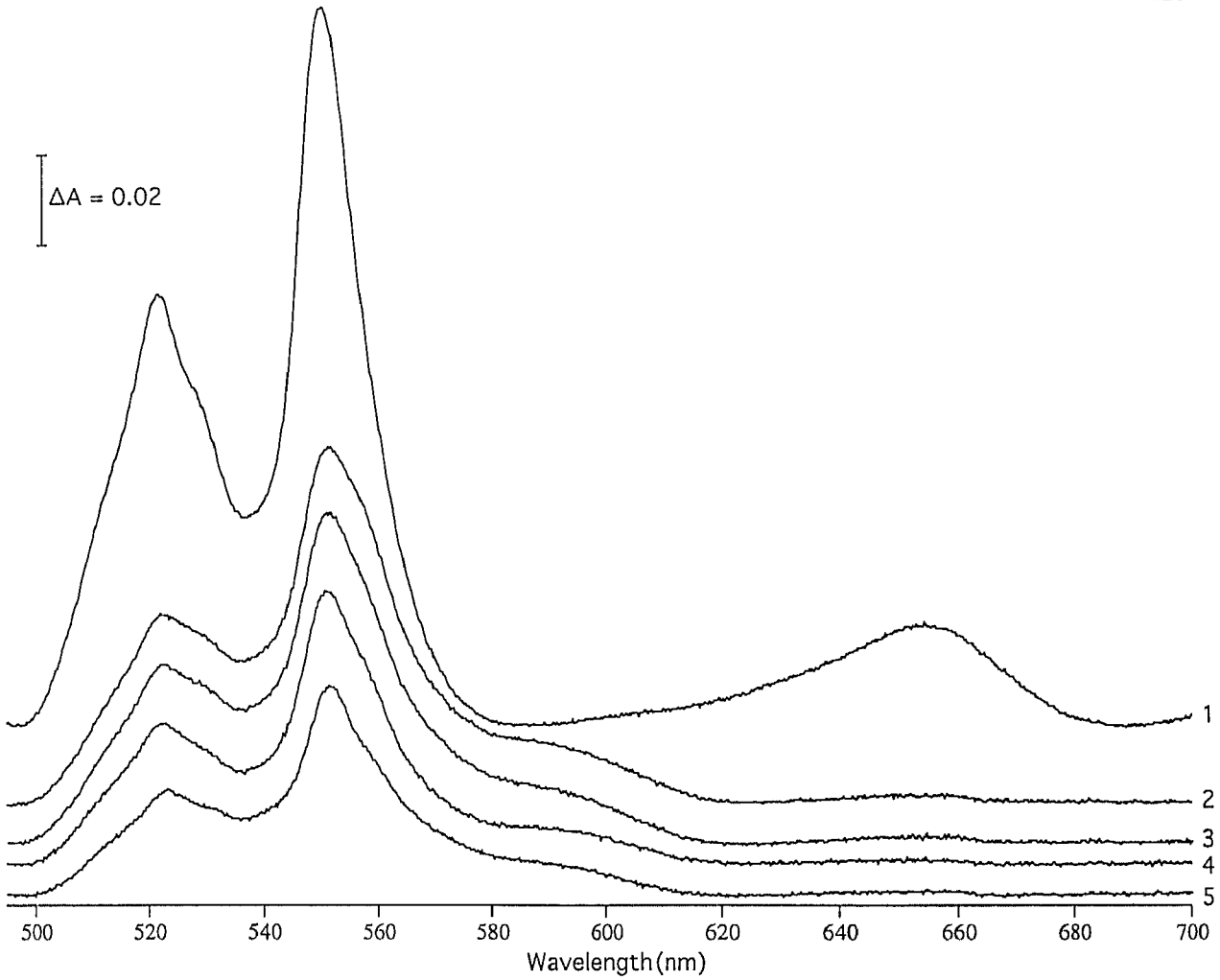

Fig. 8. Spectra of cell free extracts of *Pa. denitrificans* Pd1222 (wt) (1) and *nir* mutants Pd7121 (*nir*S::Km^r) (5), Pd7221 (*nir*E::Km^r) (3), Pd7321 (*nir*C::Km^r) (4) and (Pd7421 (nirF::Km^r) (2) grown anaerobically on 2YT. The peak at 655 with the shoulder at 630 nm are attributed to heme d_1 and are absent in all mutants, indicating the loss of heme d_1. The spectra are normalized on protein content of the cell free extract and a fixed, arbitrary baseline is subtracted.

nirE gene encodes a protein resembling S-adenosyl-L-methionine:uroporphyrinogen III methyltransferase (uro'gen III methylase). This uro'gen III methylase is probably involved in the heme d_1 biosynthesis in *Pa. denitrificans* since mutants lacking the gene produce an inactive cd_1-type nitrite reductase depleted of heme d_1. Uroporphyrinogen III is the last common intermediate of the modified tetrapyrroles biosynthetic pathways. Siroheme, factor F_{430}, chlorophyll, heme and vitamin B_{12} are all derivatives of uro'gen III (Warren & Scott 1990). Uro'gen III methylase performs the methylation of uroporphyrinogen III at the C2 and C7 carbon, yielding precorrin-2 (Blanche et al. 1989; Warren et al. 1990). Precorrin-2 is the last common intermediate of the siroheme synthesis in *E. coli* and *S. typhymurium* (Goldman & Roth 1993) and the vitamin B_{12} (cobalamin) synthesis in *Pseudomonas denitrificans* (Blanche et al. 1989; Crouzet et al. 1990), *Bacillus megaterium* (Robin et al. 1991), *Methanobacterium ivanovii* (Blanche et al. 1991) and *Salmonella typhymurium* (Goldman & Roth 1993; Roth et al. 1993). Siroheme is a cofactor of the nitrite- and sulfite reductase in *E. coli* (Jackson et al. 1981) and siroheme is structurally homologous to heme d_1, one of the cofactors of the cd_1-type nitrite reductase (Chang et al. 1986), in that they are both methylated at carbon C2 and

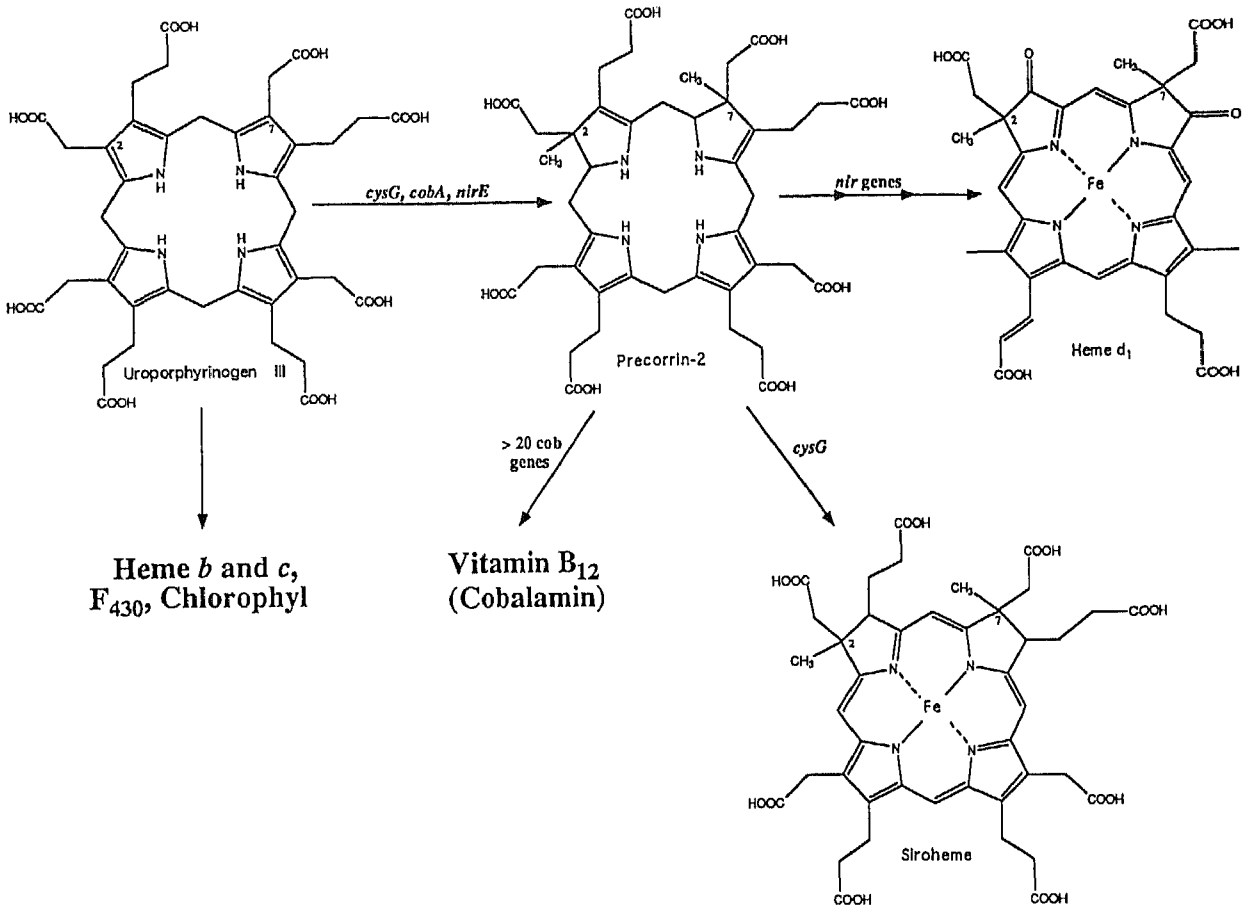

Fig. 9. Biosynthetic pathways of modified tetrapyrroles. It is suggested here that precorrin-2 is a precursor of heme d_1 and *nirE* catalyzes the first step in heme d_1 biosynthesis. *nirE* methylates uroporphyrinogen III at carbon 2 and 7 to yield precorrin-2. Other *nir* functions are needed to complete the synthesis of d_1. Carbon C2 and C7 are indicated by numbers and the synthetic pathways for vitamin B_{12} and other modified tetrapyrroles are indicated. *cysG* (*E. coli*) and *cobA* (*Ps. denitrificans*) are genes encoding uro'gen III methylases.

Table 2. Summary of properties of *Pa. denitrificans* wild type and *nir* mutants.

Strain 7-10	Genotype	Nitrite reductase				Reductase activity				
						in vivo	*in vitro*	*in vivo*	*in vivo*	
		Heme *c*	Western	d_1		NO_2-	NO_2-	NO	N_2O	Final OD_{660}
Pd1222	wild type	+++	+++		+++	+++	+++	+++	+++	2.5
Pd7121	nirS::Km^r	-	-	-		-	-	-	+++	0.8
Pd7221	nirE::Km^r	+	+	-		-	-	-	+++	0.8
Pd7321	nirC::Km^r	+	+	-		-	-	-	+++	0.8
Pd7421	nirF::Km^r	+	+	-		-	-	-	+++	0.8

Heme *c* indicates the amount of a 65 kDa band on SDS/PAGE of cell free extract with heme staining.
Western indicates the amount of a 65 kDa band on western blot using antibodies raised against *Pa. denitrificans* cd_1-type nitrite reductase. d_1 denotes the presence of heme d_1 determined by the absorbance peaks at 655 nm and 630 nm. Enzyme activities are as described in Material and methods.
Final OD_{660} is the maximum optical density of the culture grown anaerobically on 2YT. +++ = as in wild type; + = decreased amount as compared to the wild type; - = not detectable.

C7 (Fig. 9). The carbon skeleton of heme d_1 originates from glutamic acid (Matthews & Timkovich 1993) and the methyl groups at C2 and C7 from methionine, presumably via S-adenosylmethionine in *Ps. aeruginosa* (Yap-Bondoc et al. 1990). Based on the high homology with uro'gen III methylases and on analysis of *nirE* mutant strains, it is suggested here that the *nirE* gene product catalyses the first step in heme d_1 biosynthesis, the methylation of uro'gen III at the C2 and C7 carbon (Fig. 9). The function of the small 9.3 kDa cytochrome c (encoded by *nirC*) is unknown. Since this heme containing protein is not detected on gels it is unlikely that it plays a role in electron transport to cd_1-type nitrite reductase. Pseudoazurin (Moir et al. 1993), c_{550} (van Spanning et al. 1990) and a 40 kDa cytochrome *c* (Matchová et al. 1993) are more likely candidates to play a role in electron transport to the cd_1-type nitrite reductase. The *nirC* gene product might play a role in maturation of cd_1-type nitrite reductase. A *nirC* mutant of *Pa. denitrificans* (Pd7321) yielded a non-functional (*in vivo* and *in vitro*) cd_1-type nitrite reductase while also the ability to reduce nitric oxide was lost. Tn5 insertion mutants of the counterpart of *nirC* in *Pseudomonas fluorescens* AK15 gave similar results (Ye et al. 1992). The function of the *nirF* gene could not be deduced from its primary sequence. It does not have a signal sequence and is probably a cytoplasmic protein as judged by its overall hydrophylic nature. It does not show any homology with other known proteins. Mutant strain Pd7421 (*nirF*::Km^r) has the same phenotype as Pd7221 and Pd7321 in that it produces a small amount of non-functional cd_1-type nitrite reductase, lacking the d_1 heme. The GTG codon has been appointed as the start of the *nirF* gene on the bases of codon usage information (Steinrücke & Ludwig 1993). The use of GTG as a start codon is rare but also found for the start of the gene encoding the Fe-S protein of the bc_1 complex of *Pa. denitrificans* (Kurowski & Ludwig 1987). Upstream from *nirS* in this study, the start of ORF1 was identified with the transcription direction opposite to that of nirS. ORF1 has limited homology to *nosR*, a regulatory gene involved in the regulation of the nitrous oxide reduction in *Ps. stutzeri* ZoBell. *nosR* is located upstream and transcribed in the same direction as the structural gene of nitrous oxide reductase, *nosZ*, of *Ps. stutzeri* ZoBell (Cuypers et al. 1992) and *Pa. denitrificans* (Hoeren et al. 1993). The *nosR* gene product is probably a membrane bound protein, the C-terminus of which shows homology with cysteine clusters of 2[4Fe-4S] containing ferrodoxins. Ohshima et al. (1993) reported an ORF located upstream of *nirS* in the same orientation as *nirS* in *Pa. denitrificans* IFO12442 (Ohshima et al. 1993). When codon preference information is taken in consideration (Steinrücke & Ludwig 1993) this is unlikely. Only in the opposite orientation can the start of an ORF be found which does not have many rare codons. Between ORF1 and *nirS* an imperfect inverted repeat is present that resembles the consensus sequence of a FNR-box. Hoeren et al. (1993) described two possible FNR boxes located upstream from the *nosZ* gene encoding the nitrous oxide reductase in *Pa. denitrificans*. It has been shown that an FNR regulated reporter gene is activated under anaerobic conditions in *Pa. denitrificans* (Spiro 1992). This indicates that the FNR regulation mechanism is also present in *Pa. denitrificans*. The organization of the *Pa. denitrificans nir* gene cluster is clearly different from the organization of the *nir* clusters of *Ps. stutzeri* and *Ps, aeruginosa* (Fig. 1). In *Ps. stutzeri* ZoBell, 4 cytochromes *c* are found downstream from *nirS*. Only cytochrome c_{55x} encoded by *nirC* is present in the sequence data presented here for *Pa. denitrificans*. However hybridizing patterns with part of the *nirT* gene from *Ps. stutzeri* JM300 as a probe suggest that the *nirT* gene is also present in *Pa. denitrificans*. *nirE, nirF* and ORF1 have not been described before in relation to nitrite reduction in other denitrifying bacteria.

Regulation of nitrite and nitric oxide reduction is coupled

All 4 *Pa. denitrificans nir* mutant strains simultaneously lost the ability to reduce nitrite and nitric oxide. Assuming a substrate induction mechanism, the expression of the nitric oxide reductase genes could be blocked when there is no nitric oxide being produced by the reduction of nitrite via the cd_1-type nitrite reductase. In *Ps. stutzeri* ZoBell, inactivation of *nirQ* also resulted in the simultaneous loss of nitrite and nitric oxide reducing capabilities *in vivo*. *nirQ* encodes a putative regulatory protein of the *ntrC* protein kinase family. In the *nirQ* mutant, the nitrite reductase was active *in vitro* while the nitric oxide reductase was not, although present at twice the amount as compared to the wild type (Jüngst & Zumft 1992). In Tn5 insertion mutants of *Ps. stutzeri* ZoBell that lost nitrite reductase activities the level of nitric oxide reductase protein was reduced to 5–12% as compared to the wild type. However, the *in vivo* nitric oxide reductase activity of the mutants was comparable to the wild type (Zumft et al. 1988). Inactivation of nitric oxide reductase in *Ps.*

stutzeri ZoBell resulted in a decrease of 90% in nitrite reductase activity *in vivo*, while the nitrite reductase protein was present at 60–80% as compared to the wild type (Zumft et al. 1994). These are indications that regulation of nitrite and nitric oxide reducing activities are coupled, but the mechanism of the regulation in *Ps. stutzeri* ZoBell seems different from that in *Paracoccus denitrificans*. In *Ps. stutzeri* ZoBell and *Ps. aeruginosa* the *nirQ* gene is found directly upstream of nirS (Jüngst & Zumft 1992; Zumft 1993). Preliminary sequence data indicate that the counter part of *nirQ* in *Pa. denitrificans* is located about 4.5 kb further upstream from *nirS* (result to be published elsewhere). The nitrous oxide reductase activity was not affected in the 4 *Pa. denitrificans nir* mutant strains described above. This indicates that the regulation of the nitrous oxide reductase operon is independent of the presence of a functional nitrite reductase.

Acknowledgements

We thank Dr. J.M. Tiedje for providing us with the cd_1-type nitrite reductase gene cluster of *Pseudomonas stutzeri* JM300. Dr. Petr Kozel is acknowledged for sending the antibodies raised against the cd_1-type nitrite reductase from *Pa. denitrificans*. This research was supported by the Netherlands Biological Research Foundation (BION) with financial aid from the Netherlands Organization for the Advancement of Science (NWO).

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