Localization of denitrification genes on the chromosomal map of *Pseudomonas aeruginosa*

Kai-Uwe Vollack,¹ Jun Xie,¹ Elisabeth Härtig,¹ Ute Römeling^{2†} and Walter G. Zumft¹

Author for correspondence: Walter G. Zumft. Fax: +49 721 608 4290.

¹ Lehrstuhl für Mikrobiologie, Universität Fridericiana, D-76128 Karlsruhe, Germany

² Karolinska Institutet, MTC, Department of Bacteriology, Box 280, Stockholm, Sweden

Cleavage of chromosomal DNA from *Pseudomonas aeruginosa* PAO by *SpeI* and *DpnI* has been used together with PFGE and Southern hybridization to establish the map location of the following principal denitrification genes: *narGH* (encoding the large and small subunits of respiratory nitrate reductase), *nirS* (cytochrome-*cd*₁ nitrite reductase), *nirE* (uroporphyrinogen-III methyltransferase for haem *d*₁ biosynthesis), *norCB* (nitric-oxide reductase complex), *nosZ* (nitrous-oxide reductase) and *nosA* (an outer-membrane protein and *OprC* homologue). The study also included several genes related to anaerobic or microaerophilic metabolism: *napA* (encoding the catalytic subunit of the periplasmic nitrate reductase), *ccoN* (catalytic subunit of the cytochrome-*cbb*₃ oxidase), *hemN* (oxygen-independent coproporphyrinogen-III oxidase), an *fnr*-like regulatory gene, and *azu* and *fdxA* (electron carriers azurin and ferredoxin, respectively). Genes necessary for denitrification are concentrated at 20 to 36 min on the *P. aeruginosa* chromosome, where they form three separate loci, the *nir*–*nor*, *nar* and *nos* gene clusters. Genomic DNA of *Pseudomonas stutzeri* ZoBell was also subjected to *SpeI* restriction and Southern analysis to assign denitrification genes to individual fragments. A homologue of *nosA* encoding a putative component of the Cu-processing apparatus for nitrous-oxide reductase was identified. In both *P. aeruginosa* and *P. stutzeri* there is evidence for the linkage of *anr* (*fnrA*) with *hemN* and *ccoN*; and for the presence of a *napA* gene.

Keywords: *Pseudomonas aeruginosa* chromosome, denitrification, *Pseudomonas stutzeri*

INTRODUCTION

The ability to denitrify nitrate to dinitrogen under anaerobic conditions is widespread among the pseudomonads. The most detailed chromosomal map of a bacterium capable of complete denitrification is currently that of *Pseudomonas aeruginosa* PAO, which comprises close to 200 identified markers (Holloway *et al.*, 1994; Liao *et al.*, 1996). Among them are about a dozen related to anaerobic nitrate respiration and denitrification. Information on the location of denitrification genes was first sought for *P. aeruginosa* by conjugational and transductional mapping (van Hartingsveldt & Stouthamer, 1973; Jeter *et al.*, 1984). An important outcome of the early genetic analysis was the finding that *P. aeruginosa* possesses distinct gene sets

encoding the respiratory (*nar*) and the assimilatory (*nas*) nitrate reductase systems (Sias *et al.*, 1980), which is now assumed to be a rule for nitrate-assimilating denitrifiers. The distinct genetic basis for the respiratory and assimilatory process is manifest in different regulatory responses to oxygen and ammonia.

A total of about 50 genes have been identified to date in various denitrifying bacteria, about 30 of them in the genus *Pseudomonas*. The genes identified comprise the structural information for the nitrogen oxide reductases, functions for metal processing, cofactor synthesis, electron donation, protein maturation and regulation. Most of the gene positions on the chromosome and locations relative to each other are not known. Only the genes encoding functions for nitrite respiration (*nir*) and nitric oxide (NO) respiration (*nor*) of denitrifiers depending on the cytochrome-*cd*₁ nitrite reductase seem to be preferentially organized in a mixed cluster comprising both the *nir* and *nor* genes (Jüngst *et al.*, 1991a; Braun &

† **Present address:** Klinische Forschergruppe, OE 4350, Medizinische Hochschule, D-30623 Hannover, Germany.

Zumft, 1992; de Boer *et al.*, 1994, 1996; Arai *et al.*, 1995). In *Pseudomonas stutzeri* the *nos* genes for nitrous-oxide (N_2O) respiration are within 14 kb of the *nir* genes, forming a single denitrification cluster of about 30 kb (Braun & Zumft, 1992); it is an open question whether this gene organization is representative of other denitrifiers. Here, we report the determination of the chromosomal map position of the principal denitrification genes of *P. aeruginosa*. A preliminary account of this work was given elsewhere (Vollack *et al.*, 1996). The same set of probes was used for assignment of *P. stutzeri* genes to *SpeI* macrorestriction fragments.

METHODS

Bacterial strains and plasmids. *P. aeruginosa* PAO (DSM 1707) and the type strain (DSM 50071^T) and *P. stutzeri* ZoBell (ATCC 14405) were grown at 37 and 30 °C, respectively. Luria–Bertani medium was used, with the addition of the following antibiotics ($\mu\text{g ml}^{-1}$) where required: ampicillin (100), chloramphenicol (30), streptomycin (200), tetracycline (10). Cells destined for agarose embedding and macrorestriction analysis were grown in 1% tryptone, 0.5% NaCl (pH 7.5) to the end of the exponential phase.

Gene probes. Table 1 lists the genes used in this study and the source organisms or plasmids providing them. If not stated otherwise, standard procedures were used (Sambrook *et al.*, 1989). Plasmid DNA was isolated by alkaline cell lysis. Genomic DNA isolation followed a general protocol for Gram-negative bacteria (Chen & Kuo, 1993). DNA fragments were recovered from agarose using the NucleoSpin kit (Macherey & Nagel).

The following manipulations were done to obtain the various

gene probes. Plasmid pPF028 with the *anr* gene region from *P. aeruginosa* PAO1 (Sawers, 1991) was cleaved by *Bam*HI and *Pst*II double digestion and subsequently with *Dde*II. The *Bam*HI–*Dde*II fragment carried the complete *anr* gene. The *azu* gene was amplified from genomic DNA of *P. aeruginosa* PAO (Meade *et al.*, 1982) with primers derived from the sequence positions 573–593 and 858–878 (annealing temperature 53 °C) (Hoitink *et al.*, 1990). A PCR fragment amplified from *P. stutzeri* genomic DNA and cloned into plasmid pMOS (Pharmacia) was used as the probe for *ccoN*. Its identity as the *ccoN* gene was confirmed by sequencing. Degenerate primers were designed from the conserved loop region (KEYAEPEWY) connecting the transmembrane domains V and VI (5' AAG-GARTACGCSGARCCGGARTGG 3') and a stretch from helix XI (TFEGPMM) (5' CATCATCGGRCCYTCGAA-GGT 3') of several CcoN proteins (Preisig *et al.*, 1993; de Gier *et al.*, 1996; EMBL accession number U58092). The annealing temperature was 55 °C. The complete *fdxA* gene was amplified from genomic DNA with the primers 5' ACCTCGAGATGACCTTCGTSAGTSACCGAC 3' and 5' TCGAATTCTAGCGYTCCAGGTACTGCAGCTTG 3' (annealing temperature 50 °C) derived from the amino acid sequence of *P. stutzeri* ferredoxin (Saeki *et al.*, 1988). The added 5' extensions introduced *Xho*I and *Eco*RI sites for cloning of the PCR product into pBluescript SK(–). Plasmid pANAP1 carries a *napA* fragment from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) H16 (Siddiqui *et al.*, 1993). The *napA* probe was excised together with 12 bp vector DNA by a double digestion with *Eco*RI and *Xba*I. Plasmid pSL962 carries the *nar* operon encoding respiratory nitrate reductase A from *Escherichia coli* (Sodergren & DeMoss, 1988). Internal regions of *narG* and *narH* were isolated as 881 bp and 871 bp fragments, respectively, and subcloned as pUC18 derivatives. The *nar* probes were cleaved by *Pst*II. The intergenic region between the *nos* and *nir* clusters of *P. stutzeri* harbours a 1942 bp *Sma*I fragment carrying the *nirE* gene (Glockner & Zumft, 1996). The fragment was cloned into pUC18 to give plasmid pUCS20.

Table 1. Characteristics of gene probes used for mapping

Gene	Source organism	Plasmid or DNA	Probe size (bp)*	Hybridization temperature (°C)†	Reference for plasmid or gene
<i>anr</i>	<i>P. aeruginosa</i> PAO	pPF028	940	60	Sawers (1991)
<i>azu</i>	<i>P. aeruginosa</i> PAO	Genomic DNA	306	65, 50	Hoitink <i>et al.</i> (1990)
<i>ccoN</i>	<i>P. stutzeri</i> ZoBell	Genomic DNA	627	68, 60	Unpublished
<i>fdxA</i>	<i>P. stutzeri</i> ZoBell	Genomic DNA	318	65	Unpublished
<i>napA</i>	<i>R. eutropha</i> H16	pANAP1	998	42	Siddiqui <i>et al.</i> (1993)
<i>narG</i>	<i>E. coli</i> K-12	pSL962	881	55	Sodergren & DeMoss (1988)
<i>narH</i>	<i>E. coli</i> K-12	pSL962	871	55	Sodergren & DeMoss (1988)
<i>nirE</i>	<i>P. stutzeri</i> ZoBell	pUCS20	497	65, 60	Glockner & Zumft (1996)
<i>nirR</i>	<i>P. stutzeri</i> ZoBell	pAJ206	268	65, 50	Jüngst <i>et al.</i> (1991a); unpublished
<i>nirS</i>	<i>P. stutzeri</i> ZoBell	pNIR9	1200	60	Jüngst <i>et al.</i> (1991b)
<i>norCB</i>	<i>P. stutzeri</i> ZoBell	pNORCB1	1900	60	Zumft <i>et al.</i> (1994)
<i>nosD</i>	<i>P. aeruginosa</i> ^T	pBS-X	285	65	Zumft <i>et al.</i> (1992)
<i>nosR</i>	<i>P. aeruginosa</i> ^T	pBS-E	660	65	Zumft <i>et al.</i> (1992)
<i>nosZ</i>	<i>P. stutzeri</i> ZoBell	pNS220	1220	65	Viebrock & Zumft (1988)
<i>oprC</i>	<i>P. aeruginosa</i> ^T	Genomic DNA	775	68, 55	Yoneyama & Nakae (1996)
<i>rpoN</i>	<i>P. stutzeri</i> ZoBell	Cosmid c167	999	65	Unpublished

* Probes *azu*, *ccoN*, *fdxA*, *nosD*, *nosR* and *oprC* were labelled by PCR; the other probes were labelled by random priming.

† Hybridization according to Engler-Blum *et al.* (1993), except for *napA* which was according to the Boehringer Mannheim protocol.

Cleavage of pUCS20 by *Sma*I and *Bgl*II gave the *nirE* probe. The *nirR* gene was obtained from plasmid pAJ206, which was isolated from the Tn5 mutant MK206 of *P. stutzeri* (Jüngst *et al.*, 1991a). A 2518 bp *Sma*I fragment was cloned into pUC18 to give pAJ610. By double digestion with *Pst*I and *Sma*I an internal probe for *nirR* was prepared from pAJ610. The *nirS* probe was derived from plasmid pNIR9 (Jüngst *et al.*, 1991a) by *Eco*RI and *Hind*III digestion together with 64 bp of flanking vector DNA. A 1.9 kb *Hind*III–*Bgl*II fragment of plasmid pNOR161 carrying the *norCB* operon of *P. stutzeri* (Braun & Zumft, 1991) was initially cloned into M13mp18. *norCB* was subcloned as a *Hind*III–*Eco*RI fragment into pBR325 to give plasmid pNORCB1. The *Hind*III–*Eco*RI fragment served as the *norCB* probe. Plasmid pBS-X carries part of *nosZ* from *P. aeruginosa*^T together with the 5' region of *nosD* (Zumft *et al.*, 1992). *nosD* was amplified and labelled by PCR with primers derived from the sequence positions 2395–2417 and 2680–2660. A probe for *nosR* was amplified from the plasmid pBS-E with the forward and reverse primers 5' CTTCATCGGCTGGTATGCATTGG 3' and 5' GTCATCTGGCAGTCGAGGCAGTAG 3', respectively (unpublished sequence data). The annealing temperature for both *nosD* and *nosR* was 60 °C. The *nosZ* probe of *P. stutzeri* was obtained as a *Pst*I digest from plasmid pNS220 (Zumft *et al.*, 1992; Viebrock & Zumft, 1988). The probe for the *oprC* gene was amplified and labelled by PCR (annealing temperature 60 °C) from genomic DNA of *P. aeruginosa*^T with primers derived from the published sequence positions 1242–1261 and 2016–1997 (Yoneyama & Nakae, 1996). To identify the *rpoN* gene on cosmid c167 in a gene bank of *P. stutzeri* we used plasmid pNTR1, which includes a 3.4 kb *Sal*I fragment carrying *rpoN* from *Pseudomonas putida* (Köhler *et al.*, 1989). The *rpoN* gene was cloned into pUC18, from which an internal 999 bp *Xho*I fragment was isolated.

Most probes were non-radioactively labelled with a random-priming reaction kit using dUTP-digoxigenin and following the protocol of the supplier (Boehringer Mannheim). Reaction mixtures were incubated at 37 °C for 24 h to increase the yield of label. Probes for *azu*, *fdxA*, *nosD*, *nosR* and *oprC* were labelled by hot-start PCR (reagents from Perkin-Elmer) including 5% formamide to allow for the high GC content of pseudomonad DNA. The temperature programme consisted of 30 cycles each of 1 min at 95 °C, 1 min at the annealing temperature specific for the probe, and 1 min at 72 °C; after the last cycle the mixture was kept for another 5 min at 72 °C. The extent of labelling was assayed by dot-blot analysis with commercially available DNA.

Physical mapping of genes. Genomic DNA of *P. aeruginosa* and *P. stutzeri* was prepared in small blocks of 1% low-gelling agarose type VII (Sigma) and cleaved *in situ* with *Spe*I or *Dpn*I (Römling *et al.*, 1994). DNA was separated by PFGE in a CHEF-DR II chamber (Bio-Rad) in 1.5% agarose (USB) at 10 °C and 6 V cm⁻¹. Three linearly increasing ramps of pulse intervals were applied for the *Spe*I digest: 8–50 s for 24 h, 12–25 s for 22 h and 1–14 s for 14 h. For the separation of *Dpn*I fragments, the 22 h ramp was modified to 12–35 s. DNA thus separated was transferred to a Hybond-N+ (0.45 µm) nylon membrane (Amersham) by the alkaline capillary method (Chomczynski, 1992). Detection of DNA was by chemiluminescence (Engler-Blum *et al.*, 1993) and an anti-digoxigenin Fab fragment–alkaline phosphatase conjugate (Boehringer Mannheim). For double and triple hybridizations, the mixture of probes was adjusted to yield in most cases signals of comparable intensity. The probes for *ccoN*, *nirE*, *nosZ* and *oprC* gave more than one band at low stringency. The signal

persisting at the increased hybridization temperature was taken as specific for the gene under consideration.

RESULTS AND DISCUSSION

Dispersion of denitrification genes on the chromosome of *P. aeruginosa*

The 5.9 Mb chromosome of *P. aeruginosa* is composed of 38 *Spe*I fragments (Römling & Tümmeler, 1991), of which 27, ranging from 40 to 517 kb, were separated under the present conditions by one-dimensional PFGE. The probes derived from plasmids or strains specified in Table 1 were prepared as described in Methods and used for macrorestriction analysis of denitrification genes. Comparing the results of a *Spe*I digest with that of *Dpn*I allowed the unequivocal assignment of genes used in the current study, mostly with an accuracy of 2 min on the chromosomal scale. Fig. 1 shows representative results obtained for hybridizing the *Spe*I digest with various gene probes. Genes were usually detected first by single hybridization, followed by double or triple hybridizations with gene combinations that assigned the hybridization signals unambiguously to fragments on the macrorestriction ladder. The genes *anr* (Ye *et al.*,

Fig. 1. Southern blot analysis of denitrification genes of a *Spe*I digest of genomic DNA of *P. aeruginosa* PAO. Lane a shows the pattern of a *Spe*I digest with fragment labels to the left. Gene probes are indicated above the lanes. For multiple hybridizations the order of the probe labels corresponds to the order of the signals below. Conditions for hybridization were as given in Table 1.

Table 2. Localization of denitrification genes in *P. aeruginosa* PAO and *P. stutzeri* ZoBell

Gene	Gene product and/or function	<i>P. aeruginosa</i> restriction fragment		Gene position (min)	Size of <i>P. stutzeri</i> <i>SpeI</i> fragment (kb)
		<i>SpeI</i>	<i>DpnI</i>		
<i>anr</i> (<i>fnrA</i>)	FNR homologue, transcription factor	R*	C*	57.8–59.7	146
<i>azu</i>	Azurin, presumed electron donor for NirS	B	J ₂	4.1–6.7	No signal
<i>ccoN</i>	Catalytic subunit of the cytochrome- <i>cbb</i> ₃ oxidase	R	C	57.8–59.7	146
<i>fdxA</i>	Ferredoxin, homologue of <i>Azotobacter</i> Fd I	T	H	30.1–31.0	190
<i>hemN</i> †	O ₂ -independent coproporphyrinogen-III oxidase	R	C	57.8–59.7	146
<i>napA</i>	Periplasmic nitrate reductase, large subunit	M	A	61.6–64.2	109
<i>narG</i> , <i>narH</i>	Respiratory nitrate reductase, large and small subunits	A	H	27.2–28.4	40
<i>nirE</i>	Uroporphyrinogen III methyltransferase	H	F ₁	19.9–20.5	142
<i>nirR</i>	Product affects expression of <i>nirS</i>	No signal			74
<i>nirS</i>	Cytochrome <i>cd</i> ₁ nitrite reductase	H‡	F ₁	19.9–20.5	142
<i>norCB</i>	NO reductase complex	H	F ₁	19.9–20.5	142
<i>nosD</i>	Product required for copper insertion into NosZ	C	N	33.7–35.8	142
<i>nosR</i>	Putative regulator for <i>nosZ</i>	C	N	33.7–35.8	142
<i>nosZ</i>	N ₂ O reductase	C	N	33.7–35.8	142
<i>oprC</i> (<i>nosA</i>)	Copper-containing outer-membrane protein	A	F ₁	21.8–24.4	103
<i>rpoN</i>	Sigma factor σ^{54}	E§	I§	71.8–74.9	67

* Mapped by Ye *et al.* (1995).

† Positions derived from comparative sequence analysis.

‡ Mapped on fragment *SpeI*-H only (Holloway *et al.*, 1994).§ Mapped by Farinha *et al.* (1993).

1995) and *rpoN* (Farinha *et al.*, 1993) have been mapped previously and served as positive controls for our procedures. Table 2 summarizes the location of genes for denitrification for both sets of restriction fragments and the resulting positions on the chromosomal map of *P. aeruginosa* PAO.

The probes *narG* and *narH*, encoding the catalytic (α) and small (β) subunits of respiratory nitrate reductase, respectively, identified the same *SpeI* and *DpnI* fragments. Genes for respiratory nitrate reductase, *narGHJ*, form an operon in nitrate-respiring bacteria, an organization that is anticipated also for *P. aeruginosa*. The *nar* genes are not linked to the denitrification genes *sensu stricto*. Many denitrifiers possess a third nitrate reductase in the form of a periplasmic dissimilatory-type enzyme. Thus far, evidence for this reductase has not been provided for the denitrifying pseudomonads. Using a *R. eutropha napA* probe that encodes the N-terminal part of the catalytic subunit of periplasmic nitrate

reductase, we have identified a homologue of *napA* in *P. aeruginosa* (Fig. 1). The *napA* locus is not linked to the *nar* genes (Table 2).

We have previously identified the structural gene for nitrous-oxide (N₂O) reductase, *nosZ*, of *P. aeruginosa* and established a *nosRZD* sequence (Zumft *et al.*, 1992). As shown here, this locus is separate both from the *nar* and *nir-nor* loci (Fig. 1, Table 2), and organized differently from that of *P. stutzeri*, where the *nos* genes are within ≈ 14 kb of the *nir* locus. The product of a further *nos* gene, *nosA*, has been suggested to provide a Cu-processing function for N₂O reductase synthesis (Mokhele *et al.*, 1987). The *nosA* gene encodes an outer-membrane protein and was initially recognized from its property as a phage receptor of *P. stutzeri* JM300 (Clark *et al.*, 1989). Until recently *nosA* had no known homologue in any other denitrifying bacterium. This situation has changed with the description of *oprC* from *P. aeruginosa*, whose product exhibits 65% sequence

identity with NosA (Yoneyama & Nakae, 1996). We have mapped *oprC* at ≈ 23 min at a separate locus from other denitrification genes (Table 2). In particular, the *nosA* homologue is not part of the *nos* gene cluster (see also below).

A functional denitrification apparatus depends on the expression of genes for haem d_1 biosynthesis. The first such gene, *nirE*, was found in *Paracoccus denitrificans* immediately downstream of *nirS*. The derived protein shows high similarity with methyltransferases acting on uroporphyrinogen III (de Boer *et al.*, 1994). It has been an open question whether genes for haem d_1 biosynthesis are part of the *nir* region of *P. aeruginosa*. Our results show that *nirE* clusters with *nirS* and *norCB*. Previously, it had been deduced from sequencing that the vicinity of *nirS* harbours the genes encoding NO reductase and a regulator of the FNR family (Arai *et al.*, 1995). During the preparation of this paper the *P. aeruginosa* sequence was extended downstream of *nirS* and revealed a gene cluster necessary for haem d_1 biosynthesis including *nirE* (Kawasaki *et al.*, 1997). Two signals for *nirE* were detected in *P. aeruginosa*, which were associated with the *SpeI*-H fragment and the *SpeI*-D/E double band. On raising the hybridization temperature from 60 to 65 °C the *SpeI*-H signal, which is part of the *nir* gene cluster, persisted. The signal not associated with the *nir* cluster is presumed to represent a distinct methyltransferase gene, for instance *cysG* or *cobA*, required for the biosynthesis of sirohaem and cobalamin, respectively.

Electron donors to *P. aeruginosa* nitrite reductase *in vitro* are cytochrome c_{551} and the blue copper protein azurin, encoded by *nirM* and *azu*, respectively. Whereas the gene for cytochrome c_{551} is adjacent to *nirS* (Nordling *et al.*, 1990), we found that the gene encoding azurin is not linked to a denitrification gene. Whether azurin has a physiological role at all in denitrification is still a matter of controversy and continuing investigation (Vijgenboom *et al.*, 1995). Although the bacterial cell has the capability to subject unlinked genes to a common control mechanism, the absence of the *azu* gene from clusters of denitrification genes, together with the fact that it is not found consistently associated with denitrification (for instance *P. stutzeri* does not give a hybridization signal, see Table 2) casts doubt on an essential role for azurin in denitrification.

The *anr* gene encodes in *P. aeruginosa* a global regulator for anaerobic metabolism including the denitrification process (Galimand *et al.*, 1991; Ye *et al.*, 1995). *anr* maps at ≈ 59 min independently from denitrification genes. We found that the *ccoN* probe, encoding the catalytic subunit of the cytochrome-*cbb*₃ oxidase, detected the same fragment as the *anr* probe. Upstream of *anr* a sequence has been described that exhibits homology between *P. stutzeri* and *P. aeruginosa* (Cuyper & Zumft, 1993). A database search showed that the separate ORFs A and B of *P. aeruginosa* (EMBL accession number X57736) have to be joined and together represent the *hemN* gene encoding the oxygen-independent coproporphyrinogen-III oxidase for the

Fig. 2. Distribution of the loci for nitrogen oxide utilization on the chromosomal map of *P. aeruginosa* PAO. The map arrangement and map coordinates are derived from Holloway *et al.* (1994).

anaerobic biosynthesis of protohaem (EMBL accession number X97981).

Fig. 2 shows a linear representation of the *P. aeruginosa* chromosome with the results of this study. Genes for denitrification are concentrated in the 20–36 min segment of the chromosome. They are distributed in three regions harbouring the genes for nitrite and NO reduction at ≈ 20 min, separate from those for nitrate reduction at ≈ 28 min and N_2O reduction at ≈ 34 min. The periplasmic nitrate reductase system, *nap*, constitutes a further locus at ≈ 63 min. In their dispersion over a large part of the chromosome, denitrification genes form an intrinsic part of the overall genome organization of *P. aeruginosa* and not an accessory trait that is transferable as a 'denitrification island' among prokaryotes. We have previously argued that denitrification can be considered as the modular assembly of at least three respiratory processes utilizing nitrate, nitrite and nitrous oxide (Zumft, 1997).

Several other loci associated with nitrate utilization, but functionally poorly defined, have been mapped by classical techniques (see Holloway *et al.*, 1994). Given the precision of conjugational mapping, the loci *nir*-9006 (21 min) and *nar*-9001 (30 min) are close enough to

Fig. 3. Localization of denitrification genes on macrorestriction fragments of *P. stutzeri* ZoBell. (a) *SpeI* digest of *P. aeruginosa* used for size calibration; (b) *SpeI* pattern of genomic DNA from *P. stutzeri* and Southern blot results with the gene probes indicated above the lanes. Conditions for hybridization were as given in Table 1.

the *nirS* and *narGH* loci, respectively, to make identity likely. For *narD*, a role in molybdenum transport or processing for nitrate reductase is suggested since the respective gene defect is suppressed by a high dose of molybdate (van Hartingsveldt & Stouthamer, 1973).

Localization of denitrification genes on *SpeI* macrorestriction fragments of *P. stutzeri*

We have also used *SpeI* to restrict genomic DNA of *P. stutzeri* ZoBell and assigned the gene probes of Table 1 to individual fragments. The *P. stutzeri* fragments were calibrated using the *SpeI* ladder of *P. aeruginosa*. We found 25 fragments, ranging in size from 40 to 319 kb, at least six of which consisted of overlapping multiple fragments (Fig. 3). The sum of the *SpeI* fragments, accounting for putative double bands, extrapolated to a genome size of ≈ 3.8 Mb for *P. stutzeri* ZoBell. Restriction of DNA with *CeuI*, however, yields only four fragments totalling 4.3 Mb (Ginard *et al.*, 1997) and implies a somewhat higher number of *SpeI* fragments.

FnrA is the homologue in *P. stutzeri* of the *P. aeruginosa* regulator ANR. However, unlike ANR, FnrA does not affect denitrification directly. The *fnrA* gene is located in *P. stutzeri* on the 146 kb *SpeI* fragment which also reacts

positively with the *ccoN* probe (Table 2). Sequencing of this region showed that *hemN*, *fnrA* and *ccoN* are linked (unpublished data). The denitrification gene cluster in *P. stutzeri* was located, using *nirS* and *nosZ* as indicators, on a 142 kb *SpeI* fragment. The *narG* operon was found on the smallest *SpeI* fragment of 40 kb. *napA* was located on the 109 kb *SpeI* fragment. Given the evidence from *P. aeruginosa*, it is likely that these loci are not linked with the 30 kb denitrification cluster of *P. stutzeri* (Fig. 3, Table 2).

The *oprC* gene (*nosA* homologue) was detected in *P. stutzeri* ZoBell on the 103 kb *SpeI* fragment (hybridization temperature 55 °C). The role of the NosA/OprC proteins in Cu homeostasis and/or N₂O reductase biosynthesis of the pseudomonads is still insufficiently established (Lee *et al.*, 1991; Yoneyama & Nakae, 1996). The mutational absence of NosA in *P. stutzeri* JM300 is associated with the formation of an enzymically inactive N₂O reductase that lacks copper (Mokhele *et al.*, 1987). NosA of *P. stutzeri* and OprC of *P. aeruginosa* both form voltage-gated outer-membrane channels with a slight preference for Cu; the binding of one to three Cu atoms has been reported. The synthesis of NosA/OprC is repressed if the Cu concentration in the medium is above 10 μ M. A limited sequence similarity has been observed between NosA/OprC and TonB-dependent outer-membrane receptors for siderophores or vitamin B₁₂. NosA, it was hypothesized, may be part of a Cu ion or Cu chelate uptake system (Lee *et al.*, 1991).

nirR represents a novel gene whose identification is thus far limited to *P. stutzeri*. It affects the expression level of *nirS* and nitrite reduction activity (Jüngst *et al.*, 1991a). The gene is located outside the denitrification gene cluster and encodes a protein of 25.6 kDa that has no noteworthy similarity with known proteins in databases (unpublished results). The gene probe did not give a signal with *P. aeruginosa* DNA.

The expression of denitrification genes may require an alternative sigma factor for which σ^{54} is a candidate. The *rpoN* gene is located on a 67 kb *SpeI* fragment in *P. stutzeri*. *Azotobacter* ferredoxin I has been proposed to be involved in the oxidative stress response mediated by the *sox* regulon (Yannone & Burgess, 1997), and a similar role is feasible for the pseudomonads. *fdxA* of *P. stutzeri* encodes a 7-Fe low-potential ferredoxin that is homologous to *Azotobacter vinelandii* ferredoxin I (Saeki *et al.*, 1988). The *fdxA* locus maps separately from other denitrification loci (Table 2). The *fdxA* gene is of importance to denitrification since the *sox* regulon is also activated by nitric oxide (Nunoshiba *et al.*, 1993) and the *sox* system may be part of a defence mechanism against NO in denitrifiers.

ACKNOWLEDGEMENTS

We are indebted to J. A. DeMoss, B. Friedrich, S. Harayama, G. Sawers and R. A. Siddiqui for providing plasmids. We thank K. Schmidt for helpful advice. The work was supported

by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

REFERENCES

- Arai, H., Igarashi, Y. & Kodama, T. (1995). Expression of the *nir* and *nor* genes for denitrification in *Pseudomonas aeruginosa* requires a novel CRP/FNR-related transcriptional regulator, DNR, in addition to ANR. *FEBS Lett* **371**, 73–76.
- de Boer, A. P. N., Reijnders, W. N. M., Kuenen, J. G., Stouthamer, A. H. & van Spanning, R. J. M. (1994). Isolation, sequencing and mutational analysis of a gene cluster involved in nitrite reduction in *Paracoccus denitrificans*. *Antonie Leeuwenhoek* **66**, 111–127.
- de Boer, A. P. N., van der Oost, J., Reijnders, W. N. M., Westerhoff, H. V., Stouthamer, A. H. & van Spanning, R. J. M. (1996). Mutational analysis of the *nor* gene cluster which encodes nitric oxide reductase from *Paracoccus denitrificans*. *Eur J Biochem* **242**, 592–600.
- Braun, C. & Zumft, W. G. (1991). Marker exchange of the structural genes for nitric oxide reductase blocks the denitrification pathway of *Pseudomonas stutzeri* at nitric oxide. *J Biol Chem* **266**, 22785–22788.
- Braun, C. & Zumft, W. G. (1992). The structural genes of the nitric oxide reductase complex from *Pseudomonas stutzeri* are part of a 30-kilobase gene cluster for denitrification. *J Bacteriol* **174**, 2394–2397.
- Chen, W. & Kuo, T. (1993). A simple and rapid method for the preparation of gram-negative bacterial genomic DNA. *Nucleic Acids Res* **21**, 2260.
- Chomczynski, P. (1992). One-hour downward alkaline capillary transfer for blotting of DNA and RNA. *Anal Biochem* **201**, 134–139.
- Clark, M. A., Tang, Y. J. & Ingraham, J. L. (1989). A NosA-specific bacteriophage can be used to select denitrification-defective mutants of *Pseudomonas stutzeri*. *J Gen Microbiol* **135**, 2569–2575.
- Cuypers, H. & Zumft, W. G. (1993). Anaerobic control of denitrification in *Pseudomonas stutzeri* escapes mutagenesis of an *fur*-like gene. *J Bacteriol* **175**, 7236–7246.
- Engler-Blum, G., Meier, M., Frank, J. & Müller, G. A. (1993). Reduction of background problems in non-radioactive Northern and Southern blot analyses enables higher sensitivity than ³²P-based hybridizations. *Anal Biochem* **210**, 235–244.
- Farinha, M. A., Ronald, S. L., Kropinski, A. M. & Paranchych, W. (1993). Localization of the virulence-associated genes *pilA*, *pilR*, *rpoN*, *fliA*, *fliC*, *ent*, and *fbp* on the physical map of *Pseudomonas aeruginosa* PAO1 by pulsed-field electrophoresis. *Infect Immun* **61**, 1571–1575.
- Galimand, M., Gamper, M., Zimmermann, A. & Haas, D. (1991). Positive FNR-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa*. *J Bacteriol* **173**, 1598–1606.
- de Gier, J.-W. L., Schepper, M., Reijnders, W. N. M. & 9 other authors (1996). Structural and functional analysis of *aa₃*-type and *cbb₃*-type cytochrome *c* oxidases of *Paracoccus denitrificans* reveals significant differences in proton-pump design. *Mol Microbiol* **20**, 1247–1260.
- Ginard, M., Lalucat, J., Tümmeler, B. & Römling, U. (1997). Genome organization of *Pseudomonas stutzeri* and resulting taxonomic and evolutionary considerations. *Int J Syst Bacteriol* **47**, 132–143.
- Glockner, A. B. & Zumft, W. G. (1996). Sequence analysis of an internal 9.72-kb segment from the 30-kb denitrification gene cluster of *Pseudomonas stutzeri*. *Biochim Biophys Acta* **1277**, 6–12.
- van Hartingsveldt, J. & Stouthamer, A. H. (1973). Mapping and characterization of mutants of *Pseudomonas aeruginosa* affected in nitrate respiration in aerobic or anaerobic growth. *J Gen Microbiol* **74**, 97–106.
- Hoitink, C. W. G., Woudt, L. P., Turenhout, J. C. M., van de Kamp, M. & Canters, G. W. (1990). Isolation and sequencing of the *Alcaligenes denitrificans* azurin-encoding gene: comparison with the genes encoding blue copper proteins from *Pseudomonas aeruginosa* and *Alcaligenes faecalis*. *Gene* **90**, 15–20.
- Holloway, B. W., Römling, U. & Tümmeler, B. (1994). Genomic mapping of *Pseudomonas aeruginosa* PAO. *Microbiology* **140**, 2907–2929.
- Jeter, R. M., Sias, S. R. & Ingraham, J. L. (1984). Chromosomal location and function of genes affecting *Pseudomonas aeruginosa* nitrate assimilation. *J Bacteriol* **157**, 673–677.
- Jüngst, A., Braun, C. & Zumft, W. G. (1991a). Close linkage in *Pseudomonas stutzeri* of the structural genes for respiratory nitrite reductase and nitrous oxide reductase, and other essential genes for denitrification. *Mol Gen Genet* **225**, 241–248.
- Jüngst, A., Wakabayashi, S., Matsubara, H. & Zumft, W. G. (1991b). The *nirSTBM* region coding for cytochrome *cd₁*-dependent nitrite respiration of *Pseudomonas stutzeri* consists of a cluster of mono-, di-, and tetraheme proteins. *FEBS Lett* **279**, 205–209.
- Kawasaki, S., Arai, H., Kodama, T. & Igarashi, Y. (1997). Gene cluster of dissimilatory nitrite reductase (*nir*) from *Pseudomonas aeruginosa*: sequencing and identification of a locus for heme *d₁* biosynthesis. *J Bacteriol* **179**, 235–242.
- Köhler, T., Harayama, S., Ramos, J.-L. & Timmis, K. N. (1989). Involvement of *Pseudomonas putida* RpoN sigma factor in regulation of various metabolic functions. *J Bacteriol* **171**, 4326–4333.
- Lee, H. S., Abdelal, A. H. T., Clark, M. A. & Ingraham, J. L. (1991). Molecular characterization of *nosA*, a *Pseudomonas stutzeri* gene encoding an outer membrane protein required to make copper-containing N₂O reductase. *J Bacteriol* **173**, 5406–5413.
- Liao, X., Charlebois, I., Ouellet, C. & 9 other authors (1996). Physical mapping of 32 genetic markers on the *Pseudomonas aeruginosa* PAO1 chromosome. *Microbiology* **142**, 79–86.
- Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E. & Ausubel, F. M. (1982). Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *J Bacteriol* **149**, 114–122.
- Mokhele, K., Tang, Y. J., Clark, M. A. & Ingraham, J. L. (1987). A *Pseudomonas stutzeri* outer membrane protein inserts copper into N₂O reductase. *J Bacteriol* **169**, 5721–5726.
- Nordling, M., Young, S., Karlsson, B. G. & Lundberg, L. G. (1990). The structural gene for cytochrome *c₅₅₁* from *Pseudomonas aeruginosa*: the nucleotide sequence shows a location downstream of the nitrite reductase gene. *FEBS Lett* **259**, 230–232.
- Nunoshiba, T., deRojas-Walker, T., Wishnok, J. S., Tannenbaum, S. R. & Demple, B. (1993). Activation by nitric oxide of an oxidative-stress response that defends *Escherichia coli* against activated macrophages. *Proc Natl Acad Sci USA* **90**, 9993–9997.
- Preisig, O., Anthamatten, D. & Hennecke, H. (1993). Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. *Proc Natl Acad Sci USA* **90**, 3309–3313.
- Römling, U. & Tümmeler, B. (1991). The impact of two-dimensional pulsed-field gel electrophoresis techniques for the consistent and

- complete mapping of bacterial genomes: refined physical map of *Pseudomonas aeruginosa* PAO. *Nucleic Acids Res* **19**, 3199–3206.
- Römling, U., Heuer, T. & Tümmler, B. (1994). Bacterial genome analysis by pulsed field gel electrophoresis techniques. *Adv Electrophor* **7**, 355–405.
- Saeki, K., Wakabayashi, S., Zumft, W. G. & Matsubara, H. (1988). *Pseudomonas stutzeri* ferredoxin: close similarity to *Azotobacter vinelandii* and *Pseudomonas ovalis* ferredoxins. *J Biochem* **104**, 242–246.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sawers, R. G. (1991). Identification and molecular characterization of a transcriptional regulator from *Pseudomonas aeruginosa* PAO1 exhibiting structural and functional similarity to the FNR protein of *Escherichia coli*. *Mol Microbiol* **5**, 1469–1481.
- Sias, S. R., Stouthamer, A. H. & Ingraham, J. L. (1980). The assimilatory and dissimilatory nitrate reductases of *Pseudomonas aeruginosa* are encoded by different genes. *J Gen Microbiol* **118**, 229–234.
- Siddiqui, R. A., Warnecke-Eberz, U., Hengsberger, A., Schneider, B., Kostka, S. & Friedrich, B. (1993). Structure and function of a periplasmic nitrate reductase in *Alcaligenes eutrophus* H16. *J Bacteriol* **175**, 5867–5876.
- Sodergren, E. J. & DeMoss, J. A. (1988). *narI* region of the *Escherichia coli* nitrate reductase (*nar*) operon contains two genes. *J Bacteriol* **170**, 1721–1729.
- Viebrock, A. & Zumft, W. G. (1988). Molecular cloning, heterologous expression, and primary structure of the structural gene for the copper enzyme nitrous oxide reductase from denitrifying *Pseudomonas stutzeri*. *J Bacteriol* **170**, 4658–4668.
- Vijgenboom, E., Busch, J. E. & Canters, G. W. (1995). Physiological role and expression of the blue copper protein azurin in *Pseudomonas aeruginosa*. *J Inorg Biochem* **59**, 720.
- Vollack, K.-U., Römling, U. & Zumft, W. G. (1996). Mapping of denitrification genes from *Pseudomonas aeruginosa* by macro-restriction analysis. *Biospectrum* **1996**, 70 (abstract).
- Yannone, S. M. & Burgess, B. K. (1997). Identification of a palindromic sequence that is responsible for the up-regulation of NADPH-ferredoxin reductase in a ferredoxin I deletion strain of *Azotobacter vinelandii*. *J Biol Chem* **272**, 14454–14458.
- Ye, R. W., Haas, D., Ka, J.-O., Krishnapillai, V., Zimmermann, A., Baird, C. & Tiedje, J. M. (1995). Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. *J Bacteriol* **177**, 3606–3609.
- Yoneyama, H. & Nakae, T. (1996). Protein C (OprC) of the outer membrane of *Pseudomonas aeruginosa* is a copper-regulated channel protein. *Microbiology* **142**, 2137–2144.
- Zumft, W. G. (1997). Cell biology and the molecular basis of denitrification. *Microbiol Mol Biol Rev* **61**, 533–616.
- Zumft, W. G., Dreusch, A., Löchelt, S., Cuypers, H., Friedrich, B. & Schneider, B. (1992). Derived amino acid sequences of the *nosZ* gene (respiratory N₂O reductase) from *Alcaligenes eutrophus*, *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* reveal potential copper-binding residues: implications for the Cu_A site of N₂O reductase and cytochrome-*c* oxidase. *Eur J Biochem* **208**, 31–40.
- Zumft, W. G., Braun, C. & Cuypers, H. (1994). Nitric oxide reductase from *Pseudomonas stutzeri*: primary structure and gene organization of a novel bacterial cytochrome *bc* complex. *Eur J Biochem* **219**, 481–490.

Received 10 July 1997; revised 19 September 1997; accepted 2 October 1997.