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

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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, nitrite reductase), *nire* (uroporphyrinogen-III methyltransferase for haem d, biosynthesis), *norCB* (nitric-oxide reductase complex), *nosp* (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, 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-cbb3 nitrite reductase seem to be preferentially organized in a mixed cluster comprising both the *nir* and *nor* genes (Jüngst *et al.*, 1991a; Braun &
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Zumft, 1992; de Boer et al., 1994, 1996; Arai et al., 1995). In Pseudomonas stutzeri the nos genes for nitrous oxide (N,O) respiration are within 14 kb of the nir genes, forming a single denitrification cluster of about 30 kb (Braun oxide (N,O) respiration are within 14 kb of the cation genes of denitrifiers. Here, we report the determination of the whether this gene organization is representative of other stutzeri 1995). In following antibiotics genes to Pseudomonas stutzeri otherwise, standard procedures were used (Sambrook plasmids). Bacterial strains and plasmids. Luria-Bertani medium was used, with the addition of the source organisms or plasmids providing them. If not stated hybridization according to Engler-Blum (10). Cells destined for agarose embedding and macro¬ restriction fragments. Gene probes. Table 1 lists the genes used in this study and the following manipulations were done to obtain the various genes to P. stutzeri and P. aeruginosa. The following following antibiotics (µg ml⁻¹) where required: ampicillin (100), chloramphenicol (30), streptomycin (200), tetracycline (10). Cells destined for agarose embedding and macro¬ restriction analysis were grown in 1% tryptone, 0.5% NaCl (pH 7.5) to the end of the exponential phase.

The following manipulations were done to obtain the various gene probes. Plasmid pPF028 with the ann gene region from P. aeruginosa PA01 (Sawers, 1991) was cleaved by BamHI and PstI double digestion and subsequently with DdeI. The BamHI–DdeI fragment carried the complete ann 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¬ GARTACGCCGARCGGGTGG 3’) and a stretch from helix XI (TFEGPMMM) (5’ CATCATCGGRCCYTCGAA¬ GGT 3’) of several CcoN proteins (Preisig et al., 1993; de Gier et al., 1996; EMBL accession number U85092). The annealing temperature was 55 °C. The complete fdxA gene was amplified from genomic DNA with the primers 5’ ACCTCGGATGTA¬ CTTTCGTSGTSACCGAC 3’ and 5’ TCGAATTTACTAG¬ GYTCCAGGTACTGCAGCTTG 3’ (annealing temperature 50 °C) derived from the amino acid sequence of P. stutzeri ferredoxin (Saeki et al., 1988). The added 5’ extensions introduced XhoI and EcoRI 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 EcoRI and XbaI. Plasmid pSL962 carries the nar operon encoding respiratory nitrate reductase A from Escherichia coli (Hoitink & DeMoss, 1988). Internal regions of narG and narH were isolated as 881 bp and 871 bp PstI fragments, respectively, and subcloned as pUC18 derivatives. The nar probes were cleaved by PstI. The intergenic region between the nos and nir clusters of P. stutzeri harbour a 1942 bp Smal fragment carrying the nirE gene (Glockner & Zumft, 1996). The fragment was cloned into pUC18 to give plasmid pUCS20.

**METHODS**

**Bacterial strains and plasmids.** P. aeruginosa PAO (DSM 1707), and the type strain (DSM 50071T) 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 (µg ml⁻¹) where required: ampicillin (100), chloramphenicol (30), streptomycin (200), tetracycline (10). Cells destined for agarose embedding and macro¬ restriction 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

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

*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.

Table 1. Characteristics of gene probes used for mapping
Cleavage of pUC20 by Smal and BglII 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 Smal fragment was cloned into pUC18 to give pAJ610. By double digestion with PstI and Smal an internal probe for nirR was prepared from pAJ610. The nirS probe was derived from plasmid pNIR9 (Jüngst et al., 1991b) by EcoRI and HindIII digestion together with 64 bp of flanking vector DNA. A 19 kb HindIII–BglII fragment of plasmid pNOR161 carrying the norCB operon of P. stutzeri (Braun & Zumft, 1991) was initially cloned into M13 mp18. norCB was subcloned as a HindIII–EcoRI fragment into pBR325 to give plasmid pNORCB1. The HindIII–EcoRI fragment served as the norCB probe. Plasmid pBS-X carries part of nosZ from P. aeruginosa 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 1242–1261 and 2016–1997 of published 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' CGGCTGGTATGCATTGG 3' and 5' GTCATCTGGCAGTCGAGGCGATCG 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 PstI 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 together with the 5' region of oprC and rpoN were separated by PFGE in a 1% agarose gel at 10 °C and 6 V cm⁻¹. Three linearly increasing ramps of pulse intervals were applied for the Spel digest: 8–50 s for 24 h, 12–25 s for 48 h and 1–14 s for 14 h. For the separation of DpnI 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 Spel fragments (Römling & Tümmler, 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. The results of a Spel digest with that of DpnI 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 Spel 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.,
Table 2. Localization of denitrification genes in *P. aeruginosa* PAO and *P. stutzeri* ZoBell

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene product and/or function</th>
<th><em>P. aeruginosa</em> restriction fragment</th>
<th>Gene position (min)</th>
<th>Size of <em>P. stutzeri</em> Spel fragment (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Spel</td>
<td>DpnI</td>
<td></td>
</tr>
<tr>
<td>anr (fnrA)</td>
<td>FNR homologue, transcription factor</td>
<td>R*</td>
<td>C*</td>
<td>57.8–59.7</td>
</tr>
<tr>
<td>azu</td>
<td>Azurin, presumed electron donor for NirS</td>
<td>B</td>
<td>J2</td>
<td>4.1–6.7</td>
</tr>
<tr>
<td>ccoN</td>
<td>Catalytic subunit of the cytochrome-cbb3 oxidase</td>
<td>R</td>
<td>C</td>
<td>57.8–59.7</td>
</tr>
<tr>
<td>fdxA</td>
<td>Ferredoxin, homologue of Azotobacter Fd I</td>
<td>T</td>
<td>H</td>
<td>30.1–31.0</td>
</tr>
<tr>
<td>hemN†</td>
<td>O₂-independent coproporphyrinogen-III oxidase</td>
<td>R</td>
<td>C</td>
<td>57.8–59.7</td>
</tr>
<tr>
<td>napA</td>
<td>Periplasmic nitrate reductase, large subunit</td>
<td>M</td>
<td>A</td>
<td>61.6–64.2</td>
</tr>
<tr>
<td>narG, narH</td>
<td>Respiratory nitrate reductase, large and small subunits</td>
<td>A</td>
<td>H</td>
<td>27.2–28.4</td>
</tr>
<tr>
<td>nirE</td>
<td>Uroporphyrinogen III methyltransferase</td>
<td>H</td>
<td>F₁</td>
<td>19.9–20.5</td>
</tr>
<tr>
<td>nirR</td>
<td>Product affects expression of nirS</td>
<td>No signal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nirS</td>
<td>Cytochrome cd₃ nitrite reductase</td>
<td>H₃</td>
<td>F₁</td>
<td>19.9–20.5</td>
</tr>
<tr>
<td>norCB</td>
<td>NO reductase complex</td>
<td>H</td>
<td>F₁</td>
<td>19.9–20.5</td>
</tr>
<tr>
<td>nosD</td>
<td>Product required for copper insertion into NosZ</td>
<td>C</td>
<td>N</td>
<td>33.7–35.8</td>
</tr>
<tr>
<td>nosR</td>
<td>Putative regulator for nosZ</td>
<td>C</td>
<td>N</td>
<td>33.7–35.8</td>
</tr>
<tr>
<td>nosZ</td>
<td>N₂O reductase</td>
<td>C</td>
<td>N</td>
<td>33.7–35.8</td>
</tr>
<tr>
<td>oprC (nosA)</td>
<td>Copper-containing outer-membrane protein</td>
<td>A</td>
<td>F₁</td>
<td>21.8–24.4</td>
</tr>
<tr>
<td>rpoN</td>
<td>Sigma factor σ⁴⁴</td>
<td>E₉</td>
<td>I₇</td>
<td>71.8–74.9</td>
</tr>
</tbody>
</table>

* 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, *narGHJI*, 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 \( \approx 23 \) 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 \( d \), 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 \( d \), 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 \( d \), 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 \( \text{vitro} \) 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 \( \approx 59 \) min independently from denitrification genes. We found that the ccoN probe, encoding the catalytic subunit of the cytochrome-\( c_{bb} \), 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 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 \( \approx 20 \) min, separate from those for nitrate reduction at \( \approx 28 \) min and \( N_2O \) reduction at \( \approx 34 \) min. The periplasmic nitrate reductase system, \( nap \), constitutes a further locus at \( \approx 63 \) 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 conjugal mapping, the loci nir-9006 (21 min) and nar-9001 (30 min) are close enough to

![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).](image-url)
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 $\approx 3.8$ 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 $55 ^\circ C$). The role of the NosA/OprC proteins in Cu homeostasis and/or $N_2O$ 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 $N_2O$ 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 $\mu$M. A limited sequence similarity has been observed between NosA/OprC and TonB-dependent outer-membrane receptors for siderophores or vitamin B$_{12}$. 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 reductase 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 $\sigma^{44}$ 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 defense mechanism against NO in denitrifiers.

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