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), 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-cbb₃ 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-cd₃ 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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genes, forming a single denitrification cluster of about 14 kb of the nir genes of denitrifiers. Here, we report the determination of the chromosomal map positions 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_ PA0 (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 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 _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_ PA0 (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 (S 5’ AAGGARTACCSGARC CGGARTGG 3’) and a stretch from helix XI (TFEGPMMP) (5’ CATCATC CGRCCYTCGAA GGT 3’) of several CcoN proteins (Preisig et al., 1993; de Gier et al., 1996; EMBL accession number U80902). The annealing temperature was 55 °C. The complete _fdxA_ gene was amplified from genomic DNA with the primers 5’ ACCTCGGATGATTA CCTCTGTSGTSACC CGAC 3’ and 5’ TCGAATTCCTACGCGTTCCAGGTACTGCAGCTTG 3’ (annealing temperature 50 °C) derived from the amino acid sequence of _P. stutzeri_ ferredoxin (Saeiki 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_ (Sodergren & 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_ harbours a 1942 bp _Smal_ 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

<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><em>ann</em></td>
<td><em>P. aeruginosa</em> PA0</td>
<td>pPF028</td>
<td>940</td>
<td>60</td>
<td>Sawers (1991)</td>
</tr>
<tr>
<td><em>azu</em></td>
<td><em>P. aeruginosa</em> PA0</td>
<td>Genomic DNA</td>
<td>306</td>
<td>65, 50</td>
<td>Hoitink et al. (1990)</td>
</tr>
<tr>
<td><em>ccoN</em></td>
<td><em>P. stutzeri</em> ZoBell</td>
<td>Genomic DNA</td>
<td>627</td>
<td>68, 60</td>
<td>Unpublished</td>
</tr>
<tr>
<td><em>fdxA</em></td>
<td><em>P. stutzeri</em> ZoBell</td>
<td>Genomic DNA</td>
<td>318</td>
<td>65</td>
<td>Unpublished</td>
</tr>
<tr>
<td><em>napA</em></td>
<td><em>R. eutropha</em> H16</td>
<td>pANAP1</td>
<td>998</td>
<td>42</td>
<td>Siddiqui et al. (1993)</td>
</tr>
<tr>
<td><em>narG</em></td>
<td><em>E. coli</em> K-12</td>
<td>pSL962</td>
<td>881</td>
<td>55</td>
<td>Sodergren &amp; DeMoss (1988)</td>
</tr>
<tr>
<td><em>narH</em></td>
<td><em>E. coli</em> K-12</td>
<td>pSL962</td>
<td>871</td>
<td>55</td>
<td>Sodergren &amp; DeMoss (1988)</td>
</tr>
<tr>
<td><em>nirE</em></td>
<td><em>P. stutzeri</em> ZoBell</td>
<td>pUCS20</td>
<td>497</td>
<td>65, 60</td>
<td>Glockner &amp; Zumft (1996)</td>
</tr>
<tr>
<td><em>nirR</em></td>
<td><em>P. stutzeri</em> ZoBell</td>
<td>pAJ206</td>
<td>268</td>
<td>65, 50</td>
<td>Jüngst et al. (1991a); unpublished</td>
</tr>
<tr>
<td><em>nirS</em></td>
<td><em>P. stutzeri</em> ZoBell</td>
<td>pNIR9</td>
<td>1200</td>
<td>60</td>
<td>Jüngst et al. (1991b)</td>
</tr>
<tr>
<td><em>norCB</em></td>
<td><em>P. stutzeri</em> ZoBell</td>
<td>pNORCB1</td>
<td>1900</td>
<td>60</td>
<td>Zumft et al. (1994)</td>
</tr>
<tr>
<td><em>nosD</em></td>
<td><em>P. aeruginosa</em></td>
<td>pBS-X</td>
<td>285</td>
<td>65</td>
<td>Zumft et al. (1992)</td>
</tr>
<tr>
<td><em>nosR</em></td>
<td><em>P. aeruginosa</em></td>
<td>pBS-E</td>
<td>660</td>
<td>65</td>
<td>Zumft et al. (1992)</td>
</tr>
<tr>
<td><em>oprC</em></td>
<td><em>P. aeruginosa</em></td>
<td>Genomic DNA</td>
<td>775</td>
<td>68, 55</td>
<td>Yoneyama &amp; Nakae (1996)</td>
</tr>
<tr>
<td><em>rpoN</em></td>
<td><em>P. stutzeri</em> 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.
Cleavage of pUC280 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., 1991a) by EcoRI and HindIII digestion together with 64 bp of flanking vector DNA. A 1.9 kb HindIII-BglII fragment of plasmid pNOR161 carrying the norCB operon of P. stutzeri (Braun & Zumft, 1991) was initially cloned into M13mp18. norCB was subcloned as a HindIII–EcoRI fragment into pHBR325 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 2395–2417 and 2680–2660. A probe for nosR was amplified from the plasmid pBS-E with the forward and reverse primers 5' CTTCATCGGCTGATGATCAGTAG 3' and 5' GTCATCTGGCAG-TCGAGGCCCAGTAG 3', respectively (unpublished sequence data). The annealing temperature for both nosZ 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 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 SalI fragment carrying pseudomonad DNA. A 2518 bp Smal fragment was cloned into pUC18, from which an internal 999 bp XhoI fragment was isolated.

Most probes were non-radioactively labelled with a random-primer 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 SpeI or DpnI (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 SpeI digest: 8–50 s for 24 h, 12–25 s for 22 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-digoxigeninFab 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 eccN, 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 SpeI 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. Comparing the results of a SpeI 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 SpeI 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><em>P. aeruginosa</em> gene position (min)</th>
<th><em>P. stutzeri</em> Spel fragment (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>anr (fnrA)</td>
<td>FNR homologue, transcription factor</td>
<td>R*</td>
<td>57.8–59.7</td>
<td>146</td>
</tr>
<tr>
<td>azu</td>
<td>Azurin, presumed electron donor for NirS</td>
<td>B, J₂</td>
<td>4.1–6.7</td>
<td>No signal</td>
</tr>
<tr>
<td>ccoN</td>
<td>Catalytic subunit of the cytochrome-cbb₃ oxidase</td>
<td>R, C</td>
<td>57.8–59.7</td>
<td>146</td>
</tr>
<tr>
<td>fdxA</td>
<td>Ferredoxin, homologue of Azotobacter Fd I</td>
<td>T, H</td>
<td>30.1–31.0</td>
<td>190</td>
</tr>
<tr>
<td>hemN†</td>
<td>O₂-independent coproporphyrinogen-III oxidase</td>
<td>R, C</td>
<td>57.8–59.7</td>
<td>146</td>
</tr>
<tr>
<td>napA</td>
<td>Periplasmic nitrate reductase, large subunit</td>
<td>M, A</td>
<td>61.6–64.2</td>
<td>109</td>
</tr>
<tr>
<td>narG, narH</td>
<td>Respiratory nitrate reductase, large and small subunits</td>
<td>A, H</td>
<td>27.2–28.4</td>
<td>40</td>
</tr>
<tr>
<td>nirE</td>
<td>Uroporphyrinogen III methyltransferase</td>
<td>H F₁</td>
<td>19.9–20.5</td>
<td>142</td>
</tr>
<tr>
<td>nirR</td>
<td>Product affects expression of nirS</td>
<td>No signal</td>
<td></td>
<td>74</td>
</tr>
<tr>
<td>nirS</td>
<td>Cytochrome cd₁ nitrite reductase</td>
<td>H F₁</td>
<td>19.9–20.5</td>
<td>142</td>
</tr>
<tr>
<td>norCB</td>
<td>NO reductase complex</td>
<td>H F₁</td>
<td>19.9–20.5</td>
<td>142</td>
</tr>
<tr>
<td>nosD</td>
<td>Product required for copper insertion into NosZ</td>
<td>C N</td>
<td>33.7–35.8</td>
<td>142</td>
</tr>
<tr>
<td>nosR</td>
<td>Putative regulator for nosZ</td>
<td>C N</td>
<td>33.7–35.8</td>
<td>142</td>
</tr>
<tr>
<td>nosZ</td>
<td>N₂O reductase</td>
<td>C N</td>
<td>33.7–35.8</td>
<td>142</td>
</tr>
<tr>
<td>oprC (nosA)</td>
<td>Copper-containing outer-membrane protein</td>
<td>A F₁</td>
<td>21.8–24.4</td>
<td>103</td>
</tr>
<tr>
<td>rpoN</td>
<td>Sigma factor ε⁴4</td>
<td>E S I S</td>
<td>71.8–74.9</td>
<td>67</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, narGHJJI, 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 downsteam 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 \( ^\circ C \) 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 \textit{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 \textit{anr} gene encodes in \( P. \) aeruginosa a global regulator for anaerobic metabolism including the denitrification process (Galimand et al., 1991; Ye et al., 1995). \textit{anr} maps at \( \approx 59 \) min independently from denitrification genes. We found that the ccoN probe, encoding the catalytic subunit of the cytochrome-\( \textit{cbb}_3 \) oxidase, detected the same fragment as the \textit{anr} probe. Upstream of \textit{anr} a sequence has been described that exhibits homology between \( P. \) stutzeri and \( P. \) aeruginosa (Cuypers & 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 \textit{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, \textit{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 \textit{nir}-9006 (21 min) and \textit{nar}-9001 (30 min) are close enough to...
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 et al., 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 \( \approx 3.8 \text{ Mb} \) for P. stutzeri ZoBell. Restriction of DNA with CeuI, however, yields only four fragments totalling 4.3 Mb (Ginar 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 \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) 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 \( \mu \text{M} \). 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).

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. Azobacter 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 Azobacter vinelandii ferredoxin I (Sack 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.

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