Localization of denitrification genes on the chromosomal map of Pseudomonas aeruginosa

Kai-Uwe Vollack,1 Jun Xie,1 Elisabeth Härtig,1 Ute Römling2† and Walter G. Zumft1

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

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 conjugal and transducental 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-cd1 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.
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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 & 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 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 *anr* gene region from *P. aeruginosa* PAO1 (Sawers, 1991) was cleaved by BamHI and PstI double digestion and subsequently with DdeI. The BamHI–DdeI 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′ AAGGARTACCGSGARCGGGARTGG 3′) and a stretch from helix XI (TFEGPMM) (5′ CATCATCGRCCCTGGAA-GGT 3′) of several CcoN proteins (Preisig et al., 1993; de Gier et al., 1996; EMBL accession number US8092). The annealing temperature was 55 °C. The complete fdxA gene was amplified from genomic DNA with the primers 5′ ACCTCGAGATGACCTTCGTSGCACCACGAC 3′ and 3′ TCGAAATTCTACTGGTGCAATGGCTGC 5′ (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 pANAPI carries a *napA* fragment from *Ralstonia eutrophus* (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 Xbal. 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 *SmaI* 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>anr</td>
<td><em>P. aeruginosa</em> PAO</td>
<td>pPF028</td>
<td>940</td>
<td>60</td>
<td>Sawers (1991)</td>
</tr>
<tr>
<td>azu</td>
<td><em>P. aeruginosa</em> PAO</td>
<td>Genomic DNA</td>
<td>306</td>
<td>65, 30</td>
<td>Hoitink et al. (1990)</td>
</tr>
<tr>
<td>ccoN</td>
<td><em>P. stutzeri</em> ZoBell</td>
<td>Genomic DNA</td>
<td>318</td>
<td>65</td>
<td>Unpublished</td>
</tr>
<tr>
<td>fdxA</td>
<td><em>P. stutzeri</em> ZoBell</td>
<td>Genomic DNA</td>
<td>998</td>
<td>42</td>
<td>Siddiqui et al. (1993)</td>
</tr>
<tr>
<td>narG</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>narH</td>
<td><em>E. coli</em> K-12</td>
<td>pSL962</td>
<td>497</td>
<td>65, 60</td>
<td>Glockner &amp; Zumft (1996)</td>
</tr>
<tr>
<td>nirE</td>
<td><em>P. stutzeri</em> ZoBell</td>
<td>pUC20</td>
<td>268</td>
<td>65, 50</td>
<td>Jüngst et al. (1991a);</td>
</tr>
<tr>
<td>nirR</td>
<td><em>P. stutzeri</em> ZoBell</td>
<td>pAJ206</td>
<td>1200</td>
<td>60</td>
<td>Jüngst et al. (1991b)</td>
</tr>
<tr>
<td>nirS</td>
<td><em>P. stutzeri</em> ZoBell</td>
<td>pNIR9</td>
<td>1900</td>
<td>60</td>
<td>Unpublished</td>
</tr>
<tr>
<td>norCB</td>
<td><em>P. stutzeri</em> ZoBell</td>
<td>pNORCB1</td>
<td>285</td>
<td>65</td>
<td>Zubert et al. (1994)</td>
</tr>
<tr>
<td>nosD</td>
<td><em>P. aeruginosa</em></td>
<td>pBS-X</td>
<td>360</td>
<td>60</td>
<td>Zumft et al. (1992)</td>
</tr>
<tr>
<td>nosR</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>nosZ</td>
<td><em>P. stutzeri</em> ZoBell</td>
<td>pSN220</td>
<td>1220</td>
<td>65</td>
<td>Viebrock &amp; Zumft (1988)</td>
</tr>
<tr>
<td>oprC</td>
<td><em>P. aeruginosa</em></td>
<td>pM220</td>
<td>775</td>
<td>68, 55</td>
<td>Yoneyama &amp; Nakae (1996)</td>
</tr>
<tr>
<td>rpoN</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 pUCS20 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 19 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 pBR325 to give plasmid pNORCB1. The HindIII–EcoRI fragment served as the norCB probe. Plasmid pBS-X carries part of nosZ from P. aeruginosa a 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' GTCATCTGGCAGTGCCGCA 3', respectively (unpublished sequence data). The annealing temperature for both nosR and nosD 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 a 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 Sall fragment carrying rpoN from Pseudomonas putida (Köhler et al., 1989). The rpoN gene was cloned into pUC18, from which an internal 999 bp Xhol 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 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 pm) nylon membrane (Amersham) by the alkaline capillary method (Chomczynski, 1992). Detection of DNA was by chemiluminescence (Engler-Blum et al., 1993) and an anti-digoxi-genin 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 ecosN, 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 chromosomes map. 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.,

![Fig. 1. Southern blot analysis of denitrification genes of a SpeI digest of genomic DNA of P. aeruginosa PAO. Lane a shows the pattern of a SpeI 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.](image-url)
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>P. aeruginosa restriction fragment</th>
<th>P. stutzeri Spel fragment (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>anr (fnrA)</td>
<td>FNR homologue, transcription factor</td>
<td>R* C* 57.8–59.7</td>
<td>146</td>
</tr>
<tr>
<td>azu</td>
<td>Azurin, presumed electron donor for NirS</td>
<td>B J2 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 57.8–59.7</td>
<td>146</td>
</tr>
<tr>
<td>fdxA</td>
<td>Ferredoxin, homologue of Azotobacter Fd I</td>
<td>T H 30.1–31.0</td>
<td>190</td>
</tr>
<tr>
<td>hemN†</td>
<td>O2-independent coproporphyrinogen-III oxidase</td>
<td>R C 57.8–59.7</td>
<td>146</td>
</tr>
<tr>
<td>napA</td>
<td>Periplasmic nitrate reductase, large subunit</td>
<td>M A 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 27.2–28.4</td>
<td>40</td>
</tr>
<tr>
<td>nirE</td>
<td>Uroporphyrinogen III methyltransferase</td>
<td>H F1 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>74</td>
</tr>
<tr>
<td>nirS</td>
<td>Cytochrome cd1 nitrite reductase</td>
<td>H F1 19.9–20.5</td>
<td>142</td>
</tr>
<tr>
<td>norCB</td>
<td>NO reductase complex</td>
<td>H F1 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 33.7–35.8</td>
<td>142</td>
</tr>
<tr>
<td>nosR</td>
<td>Putative regulator for nosZ</td>
<td>C N 33.7–35.8</td>
<td>142</td>
</tr>
<tr>
<td>nosZ</td>
<td>N2O reductase</td>
<td>C N 33.7–35.8</td>
<td>142</td>
</tr>
<tr>
<td>oprC (nosA)</td>
<td>Copper-containing outer-membrane protein</td>
<td>A F1 21.8–24.4</td>
<td>103</td>
</tr>
<tr>
<td>rpoN</td>
<td>Sigma factor σ^4</td>
<td>E§ I§ 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, narGHJL, 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 d₁ 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 d₁ 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 d₁ 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 c₅₅₃ and the blue copper protein azurin, encoded by nirM and azu, respectively. Whereas the gene for cytochrome c₅₅₃ 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-cbb₃ 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 (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 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 ≈ 20 min, separate from those for nitrate reduction at ≈ 28 min and N₂O 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 conjugal mapping, the loci nir-9006 (21 min) and nar-9001 (30 min) are close enough to
fragments of given in Table

For nitrate reductase is suggested since the likely. For

We have also used the

indicated above the lanes. Conditions for hybridization were as

respective gene defect is suppressed by a high dose of

restriction of DNA with

extrapolated to a

fragments (Fig. 3). The sum of the

found 25 fragments, ranging in size from 40 to 319 kb, at

among six of which consisted of overlapping multiple

least six of which consisted of overlapping multiple

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 \( \approx 3.8 \) Mb for P. stutzeri ZoBell. Restriction of DNA with CevI, 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 N\(_2\)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 N\(_2\)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 \( \mu \)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 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 \( \sigma^4 \) 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 (Saecki 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...
by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

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