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The Structural Genes of the Nitric Oxide Reductase Complex from *Pseudomonas stutzeri* Are Part of a 30-Kilobase Gene Cluster for Denitrification

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A gene cluster of 30 kilobases required for denitrification in *Pseudomonas stutzeri* ZoBell was identified and mapped. It harbors genes necessary for the respiratory reduction of nitrite (*nir* genes), nitric oxide (*nor* genes), and nitrous oxide (*nos* genes). Fifteen genes, 13 of which are transcribed in the same direction, have been located on a 56-kb *Bam*HI fragment. They are arranged in three subclusters in the order *nos-nir-nor*.

Denitrification is the branch of the N cycle that returns elementary nitrogen to the atmosphere by transforming ionic N oxides stepwise to dinitrogen with the metalloenzymes nitrate reductase (EC 1.7.99.4), nitrite reductase (EC 1.7.2.1 and EC 1.9.3.2), and nitrous oxide (N_2O) reductase (EC 1.7.99.6) (14). The N oxide-dependent electron transfer is coupled to proton translocation and energy conservation (29). Recently, we have provided genetic evidence for *Pseudomonas stutzeri* that N-N bond formation in vivo requires nitric oxide (NO) reductase (EC 1.7.99.7) and that the transformation of nitrite to nitrous oxide obligatorily involves this enzyme in addition to nitrite reductase (cytochrome cd_1) (2, 31). Catalysis of N_2O formation from nitrite by nitrite reductase alone occurs in denitrifiers with copper-containing nitrite reductase on the basis of studies with the isolated enzyme and isotope labeling (16, 30).

Nitric oxide reductase from *P. stutzeri* is a membrane-bound, heteromeric cytochrome *bc* complex, with the apparent M_r of the cytochrome *c* subunit in sodium dodecyl sulfate gel electrophoresis being 17,000 and that of the cytochrome *b* subunit being 38,000 (13). A similar enzyme was found in *Paracoccus denitrificans* (3). Nitric oxide-dependent ATP synthesis has been demonstrated with membrane vesicles from this bacterium (4). The structural genes for cytochrome cd_1, *nirS*, and nitrous oxide reductase, *nosZ*, together with functions for electron donation, regulatory components, and metal processing, are genetically closely linked in *P. stutzeri* (18, 19, 32). However, the location of the *norCB* genes, which encode the nitric oxide reductase complex, relative to the *nir* and *nos* regions remained unknown.

In the genetically well-characterized species *P. aeruginosa*, a contiguous arrangement of genes with related functions is rare; not unexpectedly, the few known *nar* and *nir* loci are dispersed randomly on the chromosome (15). For *Alcaligenes eutrophus* H16, a close linkage of *nosZ*- and *nir*-related functions was shown (28). Genes required for denitrification of this organism are located on a megaplasmid which also determines chemolithotrophy (27). *nosZ* of *Rhizobium meliloti* is part of the pSym megaplasmid, which confers plant-invasive and nitrogen-fixing properties on the bacterium (5). Although many important phenotypic properties of pseudomonads are plasmid borne (10), we have not found direct or circumstantial evidence for the existence of a plasmid in the ZoBell strain of *P. stutzeri* used in this work. Here we show that the structural genes *norC* and *norB* for the cytochrome *c* and cytochrome *b* subunits of *P. stutzeri* NO reductase form part of a sizable denitrification gene cluster. We describe the current extent of this cluster and currently defined functions.

Bacterial strains and growth conditions. *P. stutzeri* ZoBell (ATCC 14405) was originally isolated as a marine pseudomonad and was formerly known as *P. perfectomarina* (8). The *Escherichia coli* strains HB101 (1), used as the recipient for plasmid transformation, and JM105 (26), used as the recipient for transfection with M13 derivatives, were used for recombinant DNA work. *P. stutzeri* and *E. coli* were grown in Luria-Bertani medium at 30 and 37°C, respectively (25).

Isolation of the cytochrome *c* subunit of NO reductase and cloning of the *nor* region. The NO reductase complex was solubilized with Triton X-100 from membranes of anaerobically nitrate-grown cells of *P. stutzeri* and purified to electrophoretic homogeneity by ion exchange and gel chromatography (13). The purified enzyme complex was separated into its subunits by an electrophoresis system (21) adapted to a preparative purpose. The cytochrome *c* subunit, which was obtained in a higher yield at the required degree of purity than the cytochrome *b* subunit, was eluted in the electrophoresis buffer by diffusion from the polyacrylamide gel and precipitated by ammonium sulfate at 45% saturation. A stretch of 20 amino acids of the N-terminal sequence was determined by Edman degradation. The octapeptide Glu-Thr-Phe-Thr-Lys-Gly-Met-Ala with a minimal codon ambiguity served as the template for the mixed 23-mer oligonucleotide NOR1 [5′-GA(A,G)ACCTTCACCAAGGG(G,T) ATGGC-3′]. The oligonucleotide was purified by gel filtration on Sephadex G-50 and labeled with [γ-^{32}P]ATP (>5,000 Ci/mmol; Radiochemical Centre, Amersham, England) by T4 polynucleotide kinase (Boehringer GmbH, Mannheim, Germany). Recombinant DNA techniques followed procedures described elsewhere (18, 25).

A cosmid library of genomic DNA from *P. stutzeri* was constructed, purified on a cesium chloride gradient, and partially digested with *Sau*3A under conditions to yield fragments of 30 to 50 kb (25). These fragments were cloned into the *Bam*HI site of cosmid vector pJA1 (24). The radiolabeled oligomer was used to screen cosmid clones of the genomic library by colony hybridization (11). Two clones of the library harboring cosmids cDEN1 and cDEN6 that

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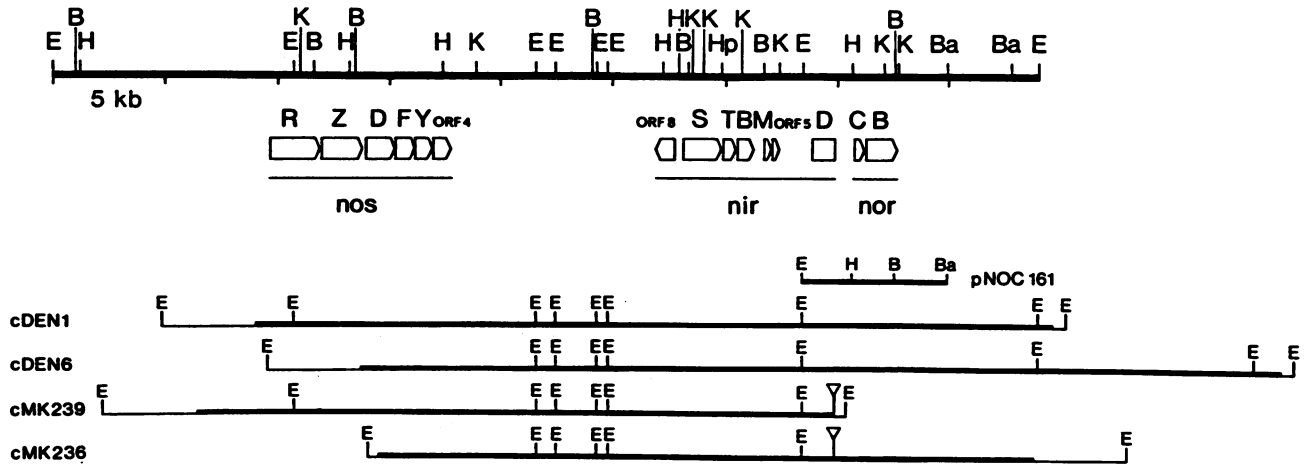

FIG. 1. Physical map of the gene cluster encoding denitrification components of *P. stutzeri*. Open arrows denote the genes for NO respiration (*nor*), nitrite respiration (*nir*), and N_2O respiration (nos) and their directions of transcription. The currently known extent of these subclusters is shown by the thin horizontal lines. Functional assignments are described in the text. Restriction analysis was done for the enzymes *Bam*HI (Ba), *Bgl*II (B), *Eco*RI (E), *Hin*dIII (H), *Hpa*I (Hp), and *Kpn*I (K). The vector DNA of plasmid pNOC161 is not shown. The lower part of the figure shows the *Eco*RI restriction sites of four cosmids which were used to establish the position of the *norCB* genes relative to the other subclusters. The Tn*5* insertion sites in the *nir* cosmids cMK239 and cMK236 are shown by open triangles. ▬, *Pseudomonas* DNA; ——, vector DNA from cosmids.

hybridized with NOR1 were characterized further by restriction fragment analysis (Fig. 1). The fragments of single and double digestions with *Eco*RI, *Hin*dIII, *Bam*HI, and *Bgl*II were separated electrophoretically and revealed on hybridization the relative position of the nor region within the cosmids.

The restriction fragments of cDEN1 and cDEN6 which were positive with NOR1 differed only in the size of the *Hin*dIII fragment, i.e., 9.8 versus 11 kb, respectively (Table 1). The hybridization with NOR1 of genomic DNA cleaved with *Hin*dIII revealed that the original *Hin*dIII fragment in the *P. stutzeri* genome is ca. 11 kb in size. Therefore, cDEN1 has a truncated *Hin*dIII fragment which must be located at one end of the 35-kb cosmid insert. The 6.7-kb *Eco*RI-*Bam*HI fragment of cDEN1, which was also positive with NOR1, was cloned into pBR325 to yield plasmid pNOC161. The smallest fragment of cosmid cDEN1 which still hybridized with the radiolabeled oligonucleotide was a 1.9-kb *Hin*dIII-*Bgl*II fragment. Restriction analysis of pNOC161 showed that this *Hin*dIII-*Bgl*II fragment was part of the plasmid (Fig. 1).

Identification of the structural genes for nitric oxide reductase. A positive identification of the structural gene *norC* for the cytochrome *c* subunit of NO reductase was sought from DNA sequencing of pNOC161. The 1.9-kb *Hin*dIII-*Bgl*II fragment was cloned into the *Hin*dIII and *Bam*HI restriction sites of the M13 vectors mp18 and mp19 (26) and sequenced by the dideoxy method with deoxyadenosine-5′-[α-^{35}S]thiophosphate and Sequenase 2.0. The N-terminal sequence of the cytochrome *c* subunit, SETFTKGMARNIYFGGSVFF, was found in the derived amino acid sequence of this fragment close to the *Hin*dIII restriction site. The *norC* gene was immediately followed by an open reading frame (ORF) having the capacity to code for a strongly hydrophobic protein. Evidence that this ORF represents the structural gene, *norB*, of the cytochrome *b* subunit came from a deletion-replacement mutation of this region which rendered the resulting Nor$^-$ strain MK321 immunonegative with an antiserum against cytochrome *b* (2). The direction of transcription of both ORFs is the same as for 11 other genes of the cluster (Fig. 1).

TABLE 1. Restriction fragment analysis of the *nor* region of *P. stutzeri*

Endonuclease(s)	Size of hybridization signal (kb)[a]		
	cDEN1	cDEN6	gDNA
*Bgl*II	6.0	6.0	6.0
*Eco*RI	10.5	10.5	~10
*Hin*dIII	9.8	~11	~11
*Bam*HI-*Eco*RI	6.7	6.7	
*Bgl*II-*Hin*dIII	1.9	1.9	

[a] The indicated cosmid clones and genomic DNA (gDNA) were hybridized with the radiolabeled oligonucleotide NOR1.

Linkage of *nor* and *nir* genes. Linkage of the *nir* and *nos* gene subclusters had previously been shown by restriction fragment analysis and Southern hybridization of cosmids cMK236 and cMK239, which were cloned from Tn*5*-induced Nir$^-$ mutants (18). The structural genes for cytochrome cd_1 and N_2O reductase are about 14 kb apart. The question arose whether the very same region would also harbor the structural genes for NO reductase, thus establishing a genuine cluster that combines the coding potential for the essential functions of denitrification. The DNA of the *nir* cosmids was therefore hybridized with the radiolabeled NOR1 oligonucleotide. Only cosmid cMK236 gave a positive signal (Fig. 2A). This indicated that the *nor* region had to be located in the part of cMK236 that was missing from cMK239 (Fig. 1).

The nearly entire 10.5-kb *Eco*RI fragment with the *nirD* locus lies at one end of the cosmid insert of cMK236 (contiguous with the *nirS* *Eco*RI fragment). The insert of cosmid cMK239 carries no more than 1.2 kb of this *Eco*RI fragment. The part of the *Eco*RI fragment present only in cosmid cMK236 has a 1.9-kb *Hin*dIII-*Bgl*II fragment within a 6.7-kb *Eco*RI-*Bam*HI fragment. This was consistent with the hybridization pattern of cDEN1 and the restriction analysis of pNOC161. The restriction pattern of the previ-

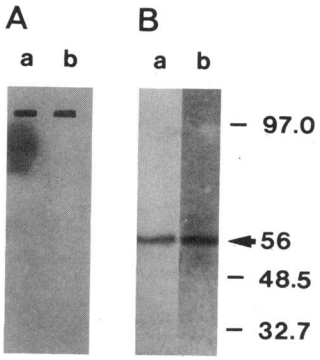

FIG. 2. Southern hybridization for localization of the *nor* region of *P. stutzeri*. (A) Autoradiography of the *nir* cosmids cMK236 and cMK239, which were hybridized with the NOR1 probe for the *norC* gene. The DNA of cMK236 (lane a) and cMK239 (lane b) was cleaved with *Eco*RI, separated in a 1% agarose gel, and hybridized with ^{32}P-labeled NOR1 after transfer to nitrocellulose. (B) Localization of *nor* and *nir* genes on the 56-kb *Bam*HI fragment. Genomic DNA of *P. stutzeri* was cleaved with *Bam*HI and separated in a 1.4% agarose gel by pulsed-field gel electrophoresis. The gel was run for 40 h in 22.5 mM Tris-borate buffer (pH 8.3)–0.5 mM EDTA with a pulse time of 3 s and a constant voltage of 6 V/cm. The buffer was cooled to 14°C and continuously circulated. The DNA was blotted onto a nylon membrane and screened with the biotin-labeled hybridization probes for *norCB* (lane a) and *nirS* (lane b). The arrowhead indicates the *Bam*HI fragment. Size standards in kilobases are shown.

ously established *nir* and *nos* gene subclusters was also found in cosmid cDEN1 (Fig. 1). This defined the location of the *nor* region downstream of *nirD* and clearly within the same 10.5-kb *Eco*RI fragment. With the cosmid cDEN1, the entire, unmutagenized region of the three denitrification subclusters identified thus far has now been cloned on a single DNA fragment.

The *nir* gene subcluster comprises seven known gene loci (Fig. 1). Besides the *nirS* gene, it carries the *nirTBM* genes, which code for three *c*-type cytochromes that, in part, function in electron donation to cytochrome cd_1. ORF5 is likely to code for a further monoheme *c*-type cytochrome (19). ORF8 has a limited sequence similarity to the NtrC family of regulators; it is transcribed in the opposite direction to *nirS* and encodes a putative regulatory component for denitrification (17). The *nirD* locus has been established from Tn*5* mutagenesis. The function of its gene product(s) may be related to the processing of cytochrome cd_1 or to heme d_1 biosynthesis (18, 31).

The *nos* gene subcluster is located 9 kb upstream of the *nir* cluster. *nosZ* is followed in the 3′ direction by three genes, *nosDFY*, whose products have a role in the acquisition or processing of copper to form a catalytically active N_2O reductase (32). ORF4 is functionally still unassigned. Its derived gene product has a predicted presequence similar to that of lipoproteins (9). There is no sequence similarity of ORF4 to *nosA* of *P. stutzeri* JM300 (23), which encodes an outer membrane protein that is also necessary for copper processing related to N_2O reductase biosynthesis. The *nosR* gene upstream of *nosZ* codes for a *trans*-acting regulatory component required for *nosZ* expression (7).

The *nor*- and *nir*-specific subclusters are nearly contiguous, perhaps suggesting closely coupled regulation of *nor* and *nir* gene expression. Mutational loss of the NO reductase is a lethal event under anaerobic, denitrifying growth conditions because of the toxicity of accumulating NO (2). The detrimental effect of NO would be circumvented if the expression of *nir* and *nor* genes were synchronized. Immunochemical evidence indicates that the synthesis of NO reductase is anaerobically regulated and initiated at approximately the same low partial pressure of oxygen as that of cytochrome cd_1 (20).

A 56-kb *Bam*HI fragment harbors the entire denitrification cluster of *P. stutzeri*. Genomic DNA of *P. stutzeri* was cleaved with *Bam*HI, and the restriction fragments were separated in a contour-clamped homogeneous electric field (6). The electrophoresis conditions were optimized for the separation of 50- to 60-kb fragments. The best resolution was achieved with 2 to 3 μg of DNA per slot. The size standards were prepared from lambda DNA. Ligation resulted in multimers of the 48.5-kb lambda monomer; restriction with *Sal*I cleaved the monomer into 32.7- and 15.8-kb fragments. After electrophoresis the DNA was transferred to nylon membranes by diffusion, and hybridized with the following probes: NIR44, the *nirS*-carrying 3.4-kb insert of a lambda gt11 phage isolate from a genomic expression library (18), and the *norCB*-specific, 1.9-kb *Hin*dIII-*Bgl*II fragment of pNOC161. The fragments were cut from the recombinant plasmids and isolated by electrophoresis (12). The probes were labeled by oligonucleotide priming with biotin-7-dATP for use in a nonradioactive detection system (22).

The *nirS* and *norCB* probes representing their respective subclusters were localized on a 56-kb *Bam*HI fragment by hybridization (Fig. 2B). Taking into account that the NS220 probe, an internal 1.2-kb *Pst*I fragment from the structural gene of N_2O reductase, also hybridized with the 56-kb fragment (18), all three subclusters have been localized on a single *Bam*HI fragment. This agrees with the independent restriction analysis of the three regions, all of which lack *Bam*HI restriction sites (this work and reference 18).

The sizable cluster of genes required for denitrification by *P. stutzeri* covers approximately 30 kb (Fig. 1). Genetic analysis of the flanking and intergenic regions of the *nos*, *nir*, and *nor* gene subclusters is likely to result in the identification of further genes for the denitrification process, extending their number beyond the currently known 15. Open questions concern the location of genes for the nitrate-reducing system (*nar* genes) relative to the characterized denitrification gene cluster and comparative studies to reveal what kind of variation is manifest in the organization of the homologous genes of other denitrifying bacteria, particularly those with a plasmid-borne denitrification system.

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