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NosR, a Membrane-Bound Regulatory Component Necessary for Expression of Nitrous Oxide Reductase in Denitrifying *Pseudomonas stutzeri*

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The regulatory element NosR was identified within the *nos* region of the denitrification gene cluster of *Pseudomonas stutzeri* ZoBell (ATCC 14405) and characterized. It is essential for expression of the N_2O reductase encoded by *nosZ* immediately downstream of *nosR*. The *nosR* region was initially identified by Tn*5* mutagenesis (W. G. Zumft, K. Döhler, and H. Körner, J. Bacteriol. 163:918–924, 1985). It consists of a single open reading frame of 2,172 nucleotides and has the coding capacity for an 81.9-kDa protein. The codon usage for *nosR*, with its high G+C content of 62.4 mol% and a preference for G or C at the third position, is characteristic for a *Pseudomonas* gene. Hydropathy analysis classified NosR as an integral membrane protein with at least seven membrane-spanning segments. No similarity to known bacterial regulator proteins was found in a data bank search. However, the C terminus of NosR shows sequence similarity to the cysteine clusters of several 2[4Fe-4S] bacterial ferredoxins. A monocistronic mRNA for *nosZ* which allowed us to monitor NosR function was identified. Complementation of Nos^- mutant MK418 (*nosR*::Tn*5*) with the *nosR* gene supplied in *trans* restored *nosZ* transcription and expression of a catalytically active N_2O reductase. In addition to evidence of the requirement for NosR, indirect evidence for involvement of the transcriptional regulator Fnr is presented.

N_2O respiration is a discrete way of energy conservation which allows a wide variety of bacteria to grow under anaerobic conditions (58, 62). N_2O reduction is usually found in bacteria as the terminal step of denitrification. The multicopper protein N_2O reductase (EC 1.7.99.6) catalyzes this reaction and constitutes an intriguing example of metal coordination, since the electron paramagnetic resonance-detectable copper in the resting enzyme shares properties with the Cu_A center of cytochrome *c* oxidase (2, 61).

As a facultative trait, expression of bacterial denitrification is anticipated to be tightly regulated (13). Regulatory elements that enable bacterial cells to respond to changes in the environment have been identified (9, 49). Evidence is accumulating that certain of those elements also control expression of denitrification. The synthesis of many anaerobically induced enzymes of *Escherichia coli* depends on the *fnr* gene product, a redox-active transcriptional activator (16). Its homolog was recently found in *Pseudomonas aeruginosa*, where it is required for nitrate respiration, arginine catabolism, and cyanogenesis (45, 57). A further control element is represented by the alternative sigma factor σ^{54}, which is encoded by the *rpoN* gene (17, 18). Mutagenesis of *rpoN* from *Alcaligenes eutrophus* abolishes anaerobic growth on nitrate (42), and putative RpoN-dependent promoters have been found for the *nosZ* genes of *A. eutrophus* and *P. aeruginosa* (61). Circumstantial evidence of a nitrite sensor for *P. stutzeri* (59) may argue for a contribution by elements of the two-component systems, perhaps analogous to the *E. coli* sensor-regulator system for nitrate, *narXL* (34, 48).

The *nos* locus of *P. stutzeri* encodes *nosZ*, the structural gene for N_2O reductase, and auxiliary components. This locus was initially identified by mapping a cluster of randomly induced transposon Tn*5* mutants (54). Immediately downstream, *nosZ* is followed by three open reading frames (ORF), *nosDFY*, which are essential for assembly of the copper chromophore of N_2O reductase (63).

Seven Tn*5*-induced mutants were identified upstream of *nosZ* within a 2.3-kb region (54). The associated phenotypes ranged from marginal expression of N_2O reductase to apparent lack of enzyme synthesis (60). Nitrite respiration and nitric oxide respiration were not grossly affected. The objective of this work was to establish the nature of the presumed regulatory phenotype of these mutants. We describe here regulatory gene *nosR* of the *nos* region within the 30-kb denitrification gene cluster of *P. stutzeri*. Complementation and transcription analysis indicate that *nosR* is a *trans*-acting component with *nosZ* as the regulatory target. The deduced NosR protein shows no sequence homology to regulatory components identified thus far.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. stutzeri* ZoBell (ATCC 14405), Tn*5*-induced *nosZ* mutant MK408, and *nosR* mutants MK403, MK406, MK409, MK410, MK413, MK414, and MK418 were described previously (54). They are derived from streptomycin-resistant *P. stutzeri* MK21, which otherwise represents wild-type traits (60). Plasmid pNS600 was used to generate the subclones for sequencing (55). Plasmids pNS10, pNS14, and pNS18 carry Tn*5* and are subclones of mutants MK410, MK414, and MK418, respectively (54). Plasmid pPF028 is a pUC18 derivative carrying the *anr* gene of *P. aeruginosa* (45). For recombinant DNA techniques and conjugative plasmid transfer, *E. coli* HB101 (7), BMH 71-18 (30), and SM10 (46) were used. Cloning and sequencing

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vectors were pBR322 (6), pBR325 (5), pSUP104 (39), M13mp11 (29), and M13mp18 (35).

Media, antibiotics, and growth conditions. *E. coli* was grown in Luria-Bertani medium at 37°C. For solid medium, 1.5% agar (Difco Laboratories, Detroit, Mich.) was added. To induce denitrifying activity, *P. stutzeri* strains were grown under O_2-limited conditions as 20-ml cultures in 100-ml flasks at 120 rpm and 30°C in a synthetic medium supplemented with 0.1% $NaNO_3$ (11). Antibiotics were used at the following concentrations (in micrograms per milliliter): ampicillin, 100; chloramphenicol, 60; tetracycline, 10; kanamycin, 50; streptomycin, 200.

Recombinant DNA techniques. Plasmid pNS675 was constructed by cloning 5.8-kb *Eco*RV fragment NS670 of pNS600 into the *Eco*RV restriction site of pBR322. This fragment contained *nosR* and 836 bp of the 5′ coding region of *nosZ*. Plasmid pNS670 was cleaved with restriction enzymes *Bam*HI and *Hin*dIII, and the fragment carrying NS670 was transferred into the identical sites of plasmid pSUP104. This yielded plasmid pENS675. Plasmid pENS 6702 was generated by ligating the 3.7-kb *Eco*47III-*Eco*RV fragment of NS670 into the *Eco*RV site of pBR322 and inserting it as a *Hin*dIII-*Bam*HI cassette into pSUP104. The orientations of the respective fragments were determined by restriction analysis. Both orientations of pENS6702 were used for complementation without affecting the results; in pENS675, the fragment was inserted in sense with respect to the promoter of the tetracycline resistance gene, *tet*, of pSUP104. Standard techniques were used for plasmid isolation, transformation, and cloning procedures (44). Conjugative plasmid transfer was described previously (60).

DNA sequence analysis. The following fragments of pNS600 were subcloned into the corresponding sites of M13mp11: the 1.23-kb *Pst*I fragment (NS610), the 1.19-kb *Pst*I fragment (NS620), the 1.8-kb *Sal*I fragment (NS650), and the 0.75-kb *Sal*I fragment (NS660) (see Fig. 1). Progressively shortened fragments of these clones were generated by treatment with exonuclease III as previously described (55). The DNA sequence was determined for both strands with the dideoxy-chain termination method using a kit based on modified T7 DNA polymerase (United States Biochemical, Cleveland, Ohio). Gaps in the sequence were filled in by using sequence-derived primers prepared with an Applied Biosystems automatic DNA synthesizer. DNA and protein sequences were analyzed with PC/GENE software (IntelliGenetics, Mountain View, Calif.).

The insertion point of transposon Tn*5* in mutant MK414 was determined by directly sequencing a 3.8-kb fragment of pNS14. It contained one end of the transposon with flanking sequences of the mutant. The synthetic primer 5′-AAGAT CAGATCCTGGAAA-3′ was used, which corresponds to bases 63 to 46 of the terminal sequence of Tn*5* (23). The point of integration of the transposon in mutants MK410 and MK418 was determined by cloning *Hpa*I-*Pst*I fragments of pNS10 and pNS18 into *Pst*I- and *Sma*I-restricted M13mp11, followed by sequencing with the universal primer.

RNA and DNA extraction. RNA and genomic DNA were extracted by adapting a published method (8). Cells induced for denitrification from the early exponential growth phase (optical density at 660 nm, ≈0.2 to 0.4), equivalent to a total optical density at 660 nm of ≈20, were pelleted, washed, and suspended on ice in 1 ml of NTE (0.1 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA). The cells were poured into a preheated (80°C) mixture of 5 ml of phenol-chloroform-isoamyl alcohol (25:24:1), 4 ml of NTE, and 0.5 ml of 10% sodium dodecyl sulfate (SDS). After 5 min of incubation at 80°C and shaking, the phases were separated at 15°C by centrifugation for 5 min at 8,000 × *g*. The aqueous phase was collected and extracted twice at room temperature with the phenol-chloroform-isoamyl alcohol mixture specified above. After a final extraction with chloroform-isoamyl alcohol (24:1), the nucleic acids were precipitated with ethanol. The pellet was dissolved in Tris-EDTA buffer and made 2 M in LiCl with a 4 M solution to separate RNA from DNA. The RNA was collected by centrifugation and purified by a second LiCl precipitation. The RNA pellet was dissolved in sample buffer, subjected to electrophoresis on a 1.2% formaldehyde gel, and directly blotted onto a nylon membrane (Hybond-N; Amersham-Buchler GmbH, Braunschweig, Germany) as described elsewhere (38). The membrane was hybridized and washed in 50% formamide at 45°C (44). The DNA was recovered by ethanol precipitation from the supernatant of the first LiCl treatment.

Protein analysis. The protein pattern of bacterial cells was analyzed by discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) with a 10% separating gel (25). The N_2O reductase protein was detected after blotting on nitrocellulose filters by a monospecific polyclonal antiserum (60). The chromogenic substrate used to stain immunoblots was 4-chloro-1-naphthol (32). N_2O reductase activity of whole cells was measured by gas chromatography (11).

Nucleotide sequence accession number. The nucleotide and amino acid sequences for the *P. stutzeri nosR* gene have been submitted to GenBank and assigned accession number Z13988.

RESULTS

Sequence of the *nosR* gene. *nosR* was cloned and sequenced from subfragments of plasmid pNS600. This plasmid also carries the major part of *nosZ* (55). Figure 1 shows the genetic organization of the *nos* region and the physical map of *nosR*. Sequence analysis of the region defined by Tn*5* mutants MK418 and MK410 revealed a single ORF (Fig. 2). Preliminary mapping of the transcriptional start site indicated that *nosR* transcription is initiated downstream of the first possible start codon for translation (GTG), at positions 262 to 264. A plausible initiation site is at position 472 (see the discussion of the Fnr motif, below). The corresponding, potential ORF for translation is preceded by a ribosome-binding site, AGG, and has the capacity to encode a polypeptide of 724 amino acids with an M_r of 81,872 (Fig. 2). The codon usage for *nosR* shows the characteristics of a typical *Pseudomonas* gene, with a high overall G+C content (62.4 mol%) and a preference for G or C at the third codon position, as shown previously for the *nosZ* gene (55). Inverted repeats at the end of *nosR* were not prominent.

nosR is preceded by a canonical Fnr-binding motif (TT GAT-N_4-ATCAA) (16) at positions 397 to 410, which may be associated with a somewhat poor −10 region, TACCAT, at positions 434 to 439 (Fig. 2). This, as well as the lack of an apparent −35 region, is typical for positively regulated genes (40). If the TACCAT motif were the actual −10 region, transcription would initiate with A at position 446 and the Fnr-binding motif would be centered at −42.5, as in *E. coli* (47, 56). A less well-conserved Fnr-binding motif, with the second half of the palindromic motif degenerated, was found in front of *nosZ*, at positions 2617 to 2631 (Fig. 2). Sequence analysis of the *nosZ* genes from two other denitrifying bacteria revealed a gene homologous to *nosR* in *P. aeruginosa* but not in *A. eutrophus*. The *nosR* gene of *P. aeruginosa* is also located immediately upstream of *nosZ* (61).

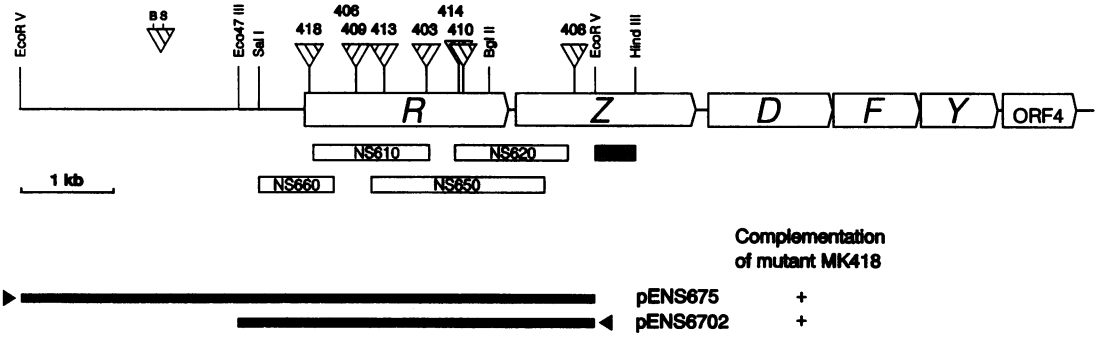

FIG. 1. Physical map of the *nos* gene cluster of *P. stutzeri*. The cluster comprises the regulatory gene *nosR*; the structural gene for N_2O reductase, *nosZ*; the genes for copper chromophore assembly, *nosDFY*; and the unassigned gene ORF4. The open arrows show the relative positions of the *nos* genes and their directions of transcription. The positions of Tn*5* insertions in the respective mutants are shown by triangles, and the hatching indicating the orientation of Tn*5* (B, *Bam*HI; S, *Sma*I). The insertions of MK403, MK408, MK413, MK406, and MK409 were mapped previously; the latter two could not be positionally differentiated (54). The shaded bar represents the location of the hybridization probe used in Northern blot analysis. Open bars show the positions of fragments NS610, NS620, NS650, and NS660, which were used for sequencing. The solid bars below show fragments NS670 and NS6702, cloned in plasmids pENS675 and pENS6702, respectively, which were used for complementation of mutant MK418. Arrowheads indicate the relative position of the vector-encoded *tet* promoter. Only restriction sites relevant to this work are shown.

An alternative ORF of 1,907 bp transcribed in the same direction as *nosR* exists in another reading frame, from positions 681 to 2588. However, the first possible initiation codon for translation (GTG) of this ORF starts at position 1,716. The codon usage of this ORF is atypical and excludes it from being a valid *Pseudomonas* gene.

Analysis of the *nosR* gene product. Prediction of the secondary structure classifies NosR as an integral membrane protein. The algorithms of Klein et al. (21) and Rao and Argos (41) predict seven transmembrane helices, whereas that of Eisenberg et al. (15) predicts nine. The central domain of the protein consists of a large hydrophilic region (Fig. 3A). We searched the MIPSX (release 17) and the SWISS-PROT (release 20) data banks with the FASTA algorithm (37) for proteins with overall sequences similar to that of NosR. No matches were found. The only remarkable sequence signatures were two cysteine clusters in the C-terminal, hydrophilic domain of NosR. They show similarity to the cysteine clusters of certain bacterial 2[4Fe-4S] ferredoxins (Fig. 3B). However, the cysteine cluster domain has no conspicuous similarity to the ferredoxin from *P. stutzeri* (43) or to *Azotobacter* ferredoxin I. The hypothesis has been advanced that the latter may act as transcriptional regulator (50). Upstream of both clusters are two further motifs of Cys-Xaa-Xaa-Xaa-Cys-Pro (positions 524 to 529 and 623 to 628). The N-terminal domain of the NosR protein harbors a single cysteine residue at position 29. At positions 215 to 234 within the central hydrophilic domain, a putative helix-turn-helix motif of prokaryotic positive regulatory factors (36) was found. The motif is just slightly below the default value of 17.0 defined as the threshold by the REGULAT program (3). Beyond that, no other sequence motifs were apparent.

Characterization of *nosR* mutants. To determine the insertion sites of transposon Tn*5* in *nosR* mutants MK410, MK414, and MK418, *Eco*RI fragments of the genomic DNAs of the mutants were subcloned into plasmid pBR325. *Hpa*I-*Pst*I fragments from mutants MK410 and MK418, carrying one end of Tn*5* and the flanking *Pseudomonas* DNA, were cloned into M13mp11 for DNA sequence analysis with the universal primer. The integration site of mutant MK414 was determined by directly sequencing a 3.8-kb *Hin*dIII fragment carrying one end of Tn*5* and flanking *nosR* sequences with a Tn*5*-derived primer (see Materials and Methods). Tn*5* in mutant MK418 was located at position 527, in MK414 it was at position 2109, and in MK410 it was at position 2153 (Fig. 2). In the latter two mutants, the insertion sites of the transposon were only 44 bp apart. They were previously not resolved by physical mapping (54).

Expression of *nosZ* in the *nosR* mutants was examined by Western blot (immunoblot) analysis and revealed a positional effect of Tn*5* (Fig. 4). The degree of *nosZ* expression increased with the proximity of Tn*5* to the structural gene to a maximal level of about 5% versus that of the wild type. Determination of the precise integration sites of Tn*5* in mutants MK410 and MK414 was essential in this respect and confirmed the positional effect: mutant MK410 expressed more NosZ protein than did MK414 (Fig. 4). This phenotype substantiated the earlier notion of a regulatory role for NosR (60). Western blot analysis of strain MK408, a *nosZ* Tn*5* insertion mutant, revealed no cross-reacting material (Fig. 1 and 4).

Complementation analysis. The *nosR* gene was subcloned for complementation as 5.8-kb *Eco*RV fragment NS670 (Fig. 1) into pSUP104, a vector with a broad-host-range replicon which allows its propagation in *Pseudomonas* sp. The NS670 fragment was cloned into the *Eco*RV site of the tetracycline resistance gene to yield plasmid pENS675. A deletion derivative of pENS675, plasmid pENS6702, was also constructed to limit the complementation ability to the *nosR* gene. Protein NosZ was detected immunochemically in both complemented mutants, and in vivo N_2O reduction was restored (Fig. 5). Control strains showed no cross-reacting material. The immunochemically detectable amount of NosZ in strain MK418(pENS675) was around 30% of the wild-type level. The N_2O-reducing activity was lowered accordingly. The complemented mutant thus expressed an N_2O reductase with a specific activity comparable to that of the wild type. This demonstrates that on complementation a functional enzyme was synthesized. Transfer of the constructs into *nosZ* mutant MK408 did not lead to complementation (data not shown).

A *nosZ*-specific transcript. Total RNA was prepared from mutants MK21, MK408, and MK418 and from complemented mutant MK418(pENS675); all strains were induced for denitrification. Expression of the chromosomally encoded *nosZ* gene was detected by Northern (RNA) blot

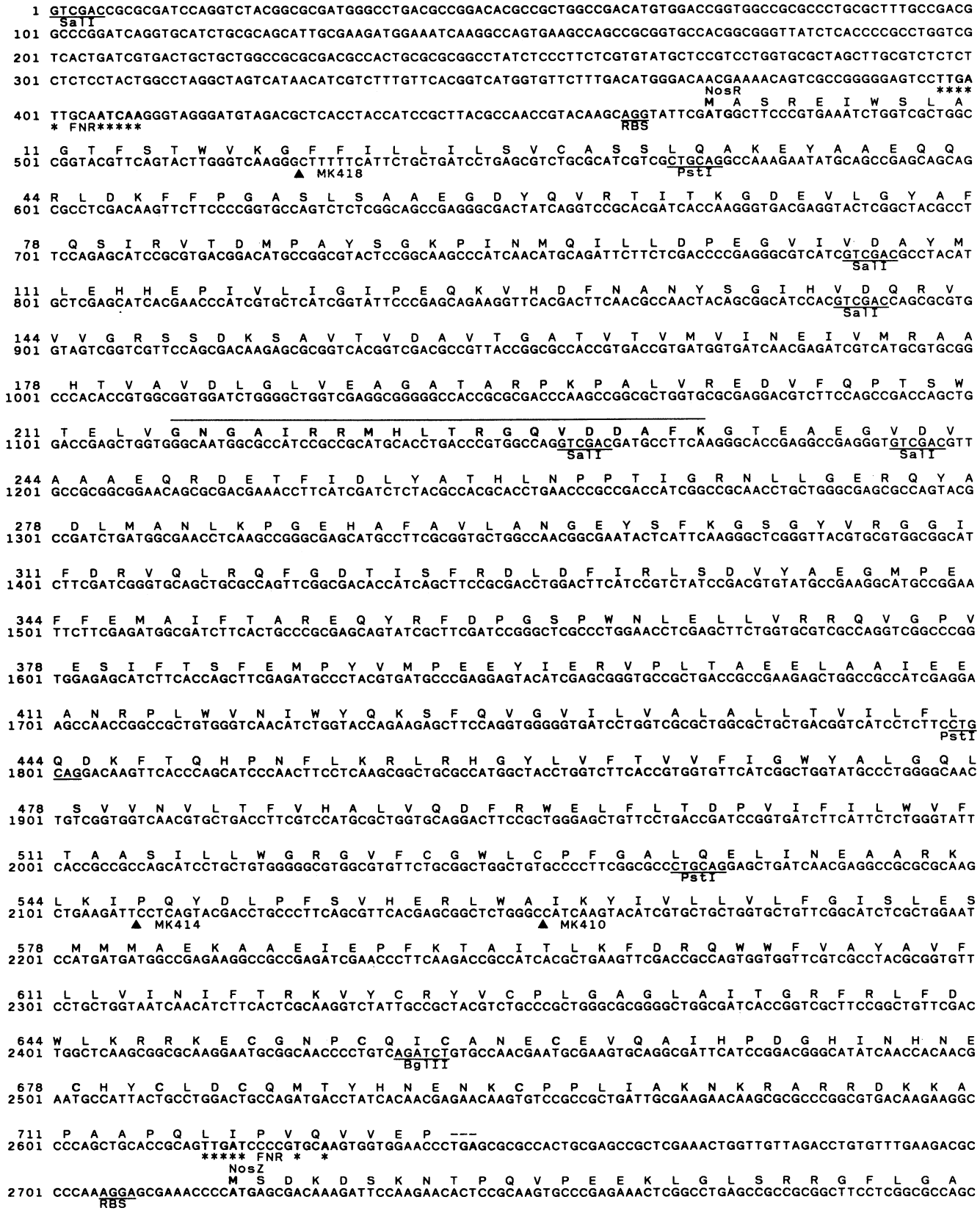

FIG. 2. Nucleotide sequence of *nosR*. Numbering of the DNA sequence starts at the *SalI* restriction site shown in Fig. 1. Restriction sites and presumed ribosome-binding sites (RBS) are underlined. Triangles denote the positions of transposon Tn*5* in mutants MK410, MK414, and MK418. Potential Fnr-binding motifs are indicated by asterisks. The deduced amino acid sequence is given in the one-letter code above the first nucleotide of the respective codon. A putative helix-turn-helix motif at amino acid positions 215 to 234 is overlined. The *Bgl*II restriction site at position 2438 gives the beginning of the published *nosZ* sequence (accession number, M22628 [55]).

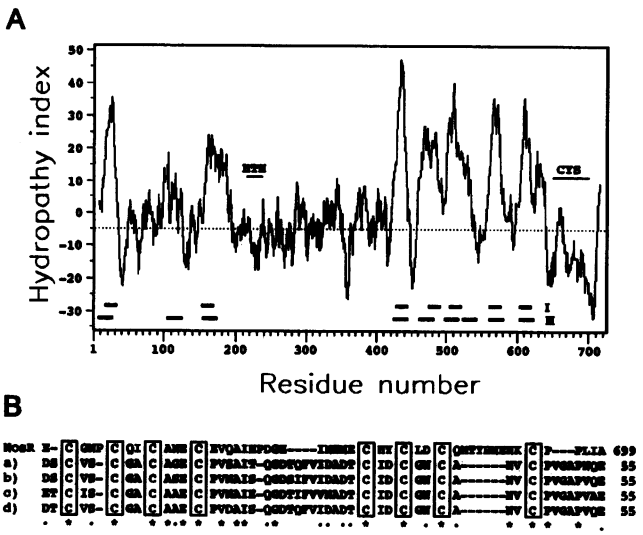

FIG. 3. Properties of the NosR protein. (A) Hydropathy profile, determined as described by Kyte and Doolittle (24), with a window size of 15 residues. The bars above the profile indicate the locations of the helix-turn-helix motif (HTH) and the ferredoxinlike cysteine clusters (CYS). The heavy bars below the profile indicate predicted membrane-spanning helices calculated by the algorithm of Klein et al. (21) (I) or Eisenberg et al. (15) (II). (B) Amino acid sequence alignment of the cysteine clusters of NosR with that of the ferredoxins from *Clostridium butyricum* (a), *C. pasteurianum* (b), *Rhodospirillum rubrum* (c), and *C. perfringens* (d). Asterisks indicate identical residues, and dots show conservative replacements. The numbers refer to the position of the last indicated residue. Conserved cysteine residues are boxed. Source of ferredoxin sequences: SWISS-PROT data bank, release 20.

analysis with a 0.5-kb *Eco*RV-*Hin*dIII fragment from the central region of *nosZ* as the hybridization probe (Fig. 1). A single 2.25-kb mRNA was identified in MK21, showing that *nosZ* is transcribed as a monocistronic RNA (Fig. 6). Its size

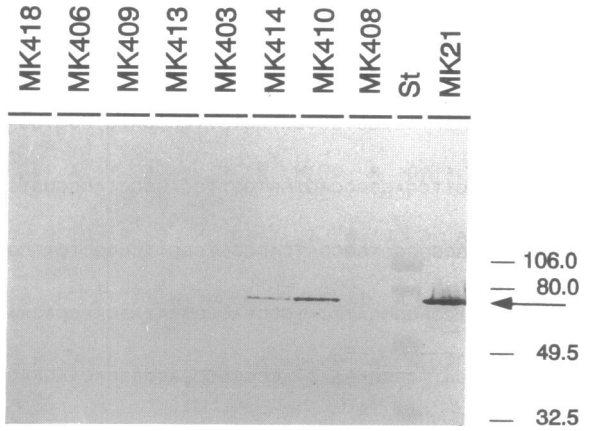

FIG. 4. Western blot analysis of *nosR* mutants of *P. stutzeri*, showing the positional effect of Tn*5* on *nosZ* expression. For Tn*5* insertion sites, see Fig. 1. In each lane, 20 μg of cell extract protein was loaded and separated by SDS-PAGE. As a control, 20 μg of extract of *nosZ* Tn*5* mutant MK408 and 5 μg of wild-type strain MK21 were loaded. Five micrograms of protein of a prestained standard mixture (St) (Bio-Rad, Munich, Germany) was loaded; molecular masses are indicated in kilodaltons. The arrow shows the position of the NosZ protein.

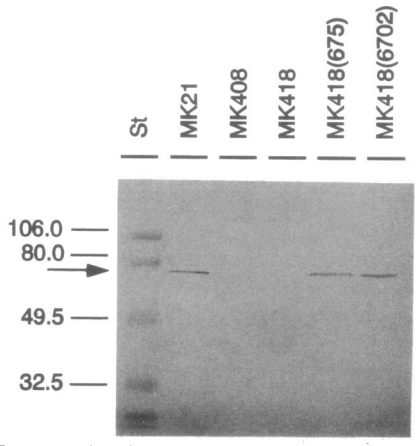

FIG. 5. Immunochemical detection of NosZ protein in complemented *nosR* mutant MK418 by Western blot analysis. Two micrograms of protein from a cell extract of *P. stutzeri* MK21 (wild type) and 20 μg of protein of strains MK408, MK418, MK418(pENS675), and MK418(pENS6702) were each separated by SDS-PAGE and immunoblotted against anti-N_2O reductase antibody. The arrow points to the NosZ protein. Molecular masses of standard proteins (St) are shown in kilodaltons.

corresponds to that of the coding region for *nosZ* of 1,914 bp plus a total of about 200 bp of noncoding sequences upstream and downstream (55). The complemented mutant synthesized a substantially increased amount of *nosZ*-specific mRNA (Fig. 6). Mutant MK408 showed no transcript at all (data not shown), whereas mutant MK418 exhibited only a marginal amount of *nosZ*-specific RNA (Fig. 6). The latter presumably corresponds to the trace

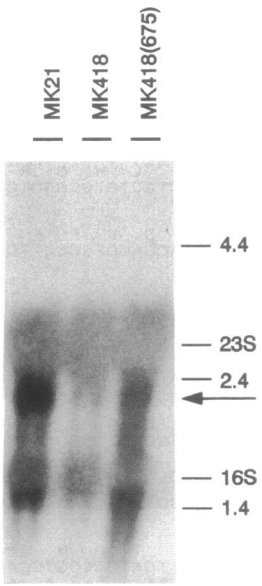

FIG. 6. Northern blot analysis of *P. stutzeri* RNA. Total RNAs from the wild type (MK21), the *nosR* mutant (MK418), and the complemented mutant MK418(pENS675) were separated on a 1.2% formaldehyde agarose gel. Hybridization was performed with the *nosZ*-specific 0.5-kb *Eco*RV-*Hin*dIII fragment (Fig. 1). The 2.25-kb transcript of *nosZ* is indicated by the arrow. The 16S and 23S rRNAs of *P. stutzeri* and an RNA ladder (Bethesda Research Laboratories Life Technologies Inc.) served as standards (sizes are in kilobases).

amount of N_2O reductase usually found in this strain. An additional signal in the Northern blot directly below the 16S rRNA is likely to represent a degraded RNA species (26). A *nosR*-specific transcript was not identified.

Evidence for the *anr* gene in *P. stutzeri* ZoBell. An earlier attempt to detect the *fnr* gene in *P. stutzeri* with an *E. coli*-derived probe was unsuccessful (27). However, the finding of the *fnr* homolog *anr* in *Pseudomonas* sp. (45, 57), and of several Fnr-binding motifs in the denitrification gene cluster (13), prompted a new search for the presence of *fnr* in *P. stutzeri* ZoBell. Genomic DNA of the ZoBell strain was isolated and subjected to Southern blot analysis. Hybridization of the filter-bound DNA with a 0.9-kb *Bam*HI-*Dde*I fragment of plasmid pPF028, which carries the *anr* gene of *P. aeruginosa*, showed signals with fragments generated by enzymes *Sma*I, *Eco*RI, and *Bam*HI (data not shown). A double band in *Eco*RI-digested DNA indicated that the internal *Eco*RI site of *anr* of *P. aeruginosa* is conserved in the *anr* gene of *P. stutzeri* ZoBell.

DISCUSSION

Characterization of the transposon Tn*5*-induced mutations in *nosR*, a 2.3-kb region immediately upstream of the coding region for N_2O reductase, has led to the identification of the first regulatory gene in the denitrification gene cluster. The view that *nosR* encodes a regulatory function was fostered by the finding of a positional effect of mutations in *nosR* towards a background level of *nosZ* expression in Nos^- strains. This was particularly evident from the determination of the exact position of Tn*5* in mutants MK410 and MK414. The transposable element in MK410 is proximal to *nosZ* (the next closest insertion site in mutant MK414 is 44 bp upstream) and allows a level of about 5% of NosZ expression. The positional effect was independent of the orientation of Tn*5* in the mutants.

Cloning and sequencing showed that *nosR* has the capacity to encode a polypeptide of 81.9 kDa which is in all likelihood membrane bound. Complementation analysis indicated that NosR functions as a positive regulatory element activating the transcription of *nosZ*. Its activity is supplied in *trans*. A putative helix-turn-helix motif within the central hydrophilic domain of NosR supports this view. The presence of this motif might also explain the positional effect of mutations in *nosR* on *nosZ* expression. The view of NosR as a transcriptional activator, combining sensor and activator functions, is the minimal one that is compatible with the available data. We cannot exclude the possibility that NosR interacts with a second, unidentified component.

Other membrane-bound transcriptional activators are ToxR (14, 31) and FecI (53). No sequence similarity between these components and NosR is evident, however. In terms of membrane topology, a regulatory component with high similarity to NosR appears to be the PucC protein (52). This hydrophobic protein of 461 amino acids is encoded in the *puc* operon of *Rhodobacter capsulatus* and has a minimum of eight putative transmembrane segments. It is essential for expression of the *pucAB* genes which encode light-harvesting complex LHII. Protein PucC is seen as a structural component required for assembly of the antenna complex rather than a transcriptional activator, even though Tn*5*-induced mutation NK3 in *pucC* results in a sixfold reduction of *pucAB* transcription and completely prevents LHII expression (51).

The identification of a monocistronic mRNA specific for *nosZ* gives the first indication of the units in which the *nos* genes are transcribed. Individual promoters for *nosR*, *nosZ*, and *nosDFY* have to be expected, and the latter three genes probably form an operon. Complementation of *nosR* mutant MK418 by pENS675 and pENS6702, restoring NosZ activity, confirms this independently. The failure to identify a *nosR*-specific mRNA may be due to transcript size. The expected 2.5-kb transcript may be masked by the 23S rRNA; alternatively, the lack of a pronounced secondary structure in the 3′-noncoding region of *nosR* could indicate a very unstable transcript (28, 33).

The effect of NosR on the other *nos* genes remains to be explored. *lacZ* fusions in *nosZ* and *nosDF* showed gene activation in response to anaerobic conditions (12). The analysis of such fusions in a *nosR* mutant will allow study of *nosR*-dependent *nos* gene expression, and *nosR-lacZ* fusions should help to identify factors that regulate *nosR* itself. The ferredoxinlike cysteine clusters at the C terminus of NosR suggest a metal-binding site which may be involved in redox sensing. Such a function is postulated for the Fe-cysteine cluster of the Fnr protein (16).

Synthesis of the four N oxide reductases that constitute the denitrification pathway is strongly repressed by oxygen (22). The findings of Fnr homologs in *P. aeruginosa* (45, 57), *Rhizobium meliloti* (4), *R. leguminosarum* (10), *Azorhizobium caulinodans* (20), and *Bradyrhizobium japonicum* (1), required there for nitrogen fixation, indicates a wide distribution for Fnr as a bacterial regulator for anaerobic processes. Two findings suggest that Fnr is involved in expression of the denitrification pathway of *P. stutzeri* ZoBell. The *anr* gene was identified in Southern blots of genomic DNA from this bacterium, and Fnr-binding motifs are present in *nosR* and *nosZ* and also in the promoter regions of the structural genes for nitrite reductase and nitric oxide reductase (13, 19). NosZ is synthesized in *P. stutzeri* aerobically at a low level, but full expression is observed only under anaerobiosis in the presence of nitrate (22), suggesting a synergistic action of oxygen and a nitrate-sensing system. In contrast, anaerobic conditions, together with the presence of N_2O, do not fully derepress NosZ synthesis (22, 60). Expression of N_2O reductase is thus regulated by NosR, Fnr, and N oxide-sensing components in a yet to be defined relationship.

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