JOURNAL OF BACTERIOLOGY, Oct. 1988, p. 4658–4668
0021-9193/88/104658-11$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 170, No. 10

Molecular Cloning, Heterologous Expression, and Primary Structure of the Structural Gene for the Copper Enzyme Nitrous Oxide Reductase from Denitrifying *Pseudomonas stutzeri*

ADELHEID VIEBROCK AND WALTER G. ZUMFT*

Lehrstuhl für Mikrobiologie, Universität Karlsruhe, Kaiserstrasse 12, D-7500 Karlsruhe 1, Federal Republic of Germany

Received 4 April 1988/Accepted 21 June 1988

The *nos* genes of *Pseudomonas stutzeri* are required for the anaerobic respiration of nitrous oxide, which is part of the overall denitrification process. A *nos*-coding region of ca. 8 kilobases was cloned by plasmid integration and excision. It comprised *nosZ*, the structural gene for the copper-containing enzyme nitrous oxide reductase, genes for copper chromophore biosynthesis, and a supposed regulatory region. The location of the *nosZ* gene and its transcriptional direction were identified by using a series of constructs to transform *Escherichia coli* and express nitrous oxide reductase in the heterologous background. Plasmid pAV5021 led to a nearly 12-fold overexpression of the NosZ protein compared with that in the *P. stutzeri* wild type. The complete sequence of the *nosZ* gene, comprising 1,914 nucleotides, together with 282 nucleotides of 5′-flanking sequences and 238 nucleotides of 3′-flanking sequences was determined. An open reading frame coded for a protein of 638 residues (M_r, 70,822) including a presumed signal sequence of 35 residues for protein export. The presequence is in conformity with the periplasmic location of the enzyme. Another open reading frame of 2,097 nucleotides, in the opposite transcriptional direction to that of *nosZ*, was excluded by several criteria from representing the coding region for nitrous oxide reductase. Codon usage for *nosZ* of *P. stutzeri* showed a high G+C content in the degenerate codon position (83.9% versus an average of 60.2%) and relaxed codon usage for the Glu codon, characteristic features of *Pseudomonas* genes from other species. *E. coli* nitrous oxide reductase was purified to homogeneity. It had the M_r of the *P. stutzeri* enzyme but lacked the copper chromophore.

Denitrifying bacteria utilize nitrous oxide (N_2O) as part of a respiratory pathway of energy conservation. N_2O, being reduced to molecular nitrogen, functions as facultative terminal acceptor of an electron transfer chain in the absence of molecular oxygen. Establishment of the trace-metal requirement for N_2O respiration by *Pseudomonas stutzeri* ZoBell (34) (formerly *Pseudomonas perfectomarina*) led to the identification of N_2O reductase as a homodimeric copper protein, containing ca. four copper atoms per monomer of 62 to 74 kilodaltons (8, 70). Mutational loss of this protein causes the loss of N_2O respiration (68).

Related copper enzymes were found in the nondenitrifying but N_2O-utilizing nonsulfur purple bacterium *Rhodobacter capsulatus* (36) and in the denitrifying bacteria *Achromobacter denitrificans* (formerly *Alcaligenes* sp. strain NCIB 11015) (35) and *Rhodobacter sphaeroides* f. sp. *denitrificans* (40). A comparative biochemical study with N_2O reductase of *Paracoccus denitrificans* established a considerable similarity with the *P. stutzeri* enzyme and rectified earlier work with the former organism (56).

N_2O reductase has a novel type of copper chromophore which is not readily accommodated within the current classification of the three principal types of copper proteins (8, 13, 72; J. Riester, W. G. Zumft, and P. M. H. Kroneck, submitted for publication; H. Jin, H. Thomann, C. L. Coyle, and W. G. Zumft, submitted for publication). Several gene products are required for the assembly of the copper prosthetic group in an as yet unknown way (44, 62, 68, 72). By mapping transposon Tn*5*-induced mutations, we identified a *nos*-coding region of ca. 8 kilobases (kb), which comprises functions related to the biosynthesis of N_2O reductase (62). Mutagenesis of a region of ca. 3.5 kb resulted in chromophoreless, apoenzyme-synthesizing strains (62, 68). The same phenotype was observed in a class of frameshift mutants that had lost a 61-kilodalton protein from the outer membrane (44).

This work describes the cloning of the entire *nos* cluster, previously identified by insertional mutagenesis (62), and the determination of the primary structure of the structural gene, *nosZ*, of N_2O reductase. We have used plasmid integration and excision (38) to clone the *nos* cluster of *Pseudomonas* species. *nosZ* was identified by deletion analysis and heterologous expression of *nosZ*-carrying constructs in *Escherichia coli*.

(Preliminary accounts of this work were presented previously [72; W. G. Zumft and A. Viebrock, Eur. Bioenerg. Conf. Rep. **4**:108, 1986; A. Viebrock and W. G. Zumft, Biol. Chem. Hoppe Seyler **368**:561–562, 1987].)

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used or constructed in this work are listed in Table 1.

Media, antibiotics, and growth conditions. *E. coli* and *P. stutzeri* were grown at 37°C in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl). For solid medium, 1.5% agar (Difco Laboratories, Detroit, Mich.) was added. Unless indicated otherwise, antibiotics were used in the following concentrations (in micrograms per milliliter): ampicillin, 50; chloramphenicol, 30; kanamycin, 50; and tetracycline, 10.

Transformation and conjugation. *E. coli* was transformed by a standard method (9). Conditions for conjugation were described previously (68). Transconjugants were selected on LB plates containing ampicillin (10 μg/ml) and chloramphen-

* Corresponding author.

TABLE 1. Bacteria and plasmids used in this work

Strain or plasmid	Genotype or phenotype	Source or reference
P. stutzeri		
ZoBell	Formerly *P. perfectomarina* ATCC 14405	12
MK21	Sm^r	68
MK416	Sm^r Km^r *nos*::Tn*5*	62
E. coli		
HB101	*hsdS20 recA13 proA2 endA leu-6 thi-1 rpsL20*(Sm^r) *ara-14 galK2 lacY1 xyl-5 mtl-1 supE44*	6
S17-1	[RP4-2(Tc::Mu)(Km::Tn*7*) Tra(IncP)] *pro hsdR recA* Tp^r Sm^r	55
S605	*thr leu thi tonA lacY supE met*::Tn*5* Km^r	26
JM105	*thi rpsL*(Sm^r) *endA sbcB15 hspR4*Δ (*lac-proAB*) [F′*traD36 proAB lacI*^{q}ZΔM15]	67
Plasmids		
pBR325	Ap^r Tc^r Cm^r	5
pSUP203	pBR325-Mob; Ap^r Tc^r Cm^r	55
pJA1	pBR322-derived cosmid vector; Ap^r Tc^r	31
pNS600	pBR325 derivative	This work (Fig. 1)
pMK1	pBR325-Tc::Tn*5*; Ap^r Cm^r Km^r	This work
pAV21	pSUP203 derivative	This work (Fig. 1)
pMNS400	pSUP203 derivative	This work (Fig. 1)
pAV39, pAV42, pAV5018, pAV5021, pAV5022, pAV5023, pAV5024, pNS800	pBR325 derivatives	This work (Fig. 2)
pAV49, pAV50, pAV502, pAV5019, pAV5020	pMK1 derivatives	This work (Fig. 2)

icol (10 μg/ml). The maximum number of cells plated on selection medium did not exceed 10^7 cells per 85-mm petri dish, to prevent excessive background growth.

Recombinant DNA techniques. Plasmid DNA was prepared by alkaline lysis (4). Standard procedures were used for endonuclease digestions, ligation, filling-in of 5′ protruding ends with the Klenow fragment of DNA polymerase I, and agarose gel electrophoresis (33). Genomic DNA from *P. stutzeri* was isolated as described previously (37). For Southern blot analysis, DNA was digested to completion with different restriction enzymes, electrophoresed in 0.7% agarose gels, and transferred to nitrocellulose sheets (33). The hybridization probe was a 1.2-kb *Pst*I fragment of the *nosZ* gene. The probe was labeled with biotinylated 11-dUTP for a nonradioactive detection system (29, 69).

Construction of plasmid pMK1. *E. coli* S605, carrying Tn*5* integrated into the chromosome, was transformed with plasmid pBR325. A 0.1-ml sample of an overnight culture was plated evenly on chloramphenicol-containing medium, upon which a radial gradient of kanamycin was superimposed (highest concentration, 50 mg/ml) (26). The plates were incubated overnight at 37°C. Five large colonies, each one from a separate gradient plate and growing near the center, were analyzed for Tn*5* insertions. Four clones had integrated Tn*5* into pBR325. The insertions mapped between the *Hin*dIII and *Bam*HI sites; two of each in the opposite orientation of Tn*5*. One of the plasmids, designated pMK1, was chosen for further experiments. Plasmid pMK1 allowed the cloning of DNA (downstream from the promoter of the kanamycin-neomycin resistance gene of Tn*5*; see reference 50) into the *Bgl*III site of IS*50L* and the *Bam*HI site of pBR325.

Construction of *nos* subfragments. Plasmid pNS800 was obtained by cloning the 2.5-kb *Eco*RI-*Hin*dIII fragment of pAV21 into the corresponding sites of plasmid pBR325. Plasmids pAV39 and pAV42 were generated by digesting pAV21 partially with *Sau*3A and then ligating the fragments into the *Bam*HI site of pBR325. Ligation of the partial *Sau*3A digest of pAV21 into the *Bam*HI-*Bgl*II sites of pMK1 resulted in plasmids pAV49 and pAV50. Plasmid pAV502 was obtained from pAV50 by first digesting the restored *Bam*HI site to completion, then partially digesting pAV50 with *Bgl*II, and finally ligating the *Bam*HI and *Bgl*II sites. Plasmid pAV5018 was obtained by digesting pAV50 with *Eco*RI and allowing self-ligation of the fragments.

Plasmid pAV5018 was digested with the endonucleases *Eco*RI and *Bam*HI. The overhanging 5′ ends were filled with the Klenow enzyme, and the two fragments were ligated. From *E. coli* transformants, plasmid pAV5022 was isolated which had the inserted *Pseudomonas* DNA in the inverted orientation compared with pAV5018. Plasmids pAV5023 and pAV5024 were generated by digesting pAV5018 to completion with *Bam*HI and then partially digesting it with *Cla*I. The overhanging 5′ ends were filled in with the Klenow fragment and ligated.

Plasmid pAV50 was digested with *Eco*RI or *Sal*I. Overhanging 5′ ends were made blunt with the Klenow fragment; digestion with *Bam*HI was then carried to completion. The resulting fragments were ligated into the *Bam*HI site and the blunt-ended *Bgl*II site of plasmid pMK1. The *Bgl*II site of pMK1 was made blunt ended with the Klenow fragment before the plasmid was cut with *Bam*HI. This resulted in plasmids pAV5019 and pAV5020. Plasmid pAV5021 was derived from pAV5018 by digesting the latter completely with *Eco*RI and partially with *Bgl*II. Overhanging 5′ ends were made blunt with Klenow fragment and ligated. After ligation of the DNA fragments and selection on appropriate antibiotic-containing plates, the plasmid size of recombinant clones was screened by an electrophoretic method (14) adopted for horizontal gels (54). All plasmids were introduced into *E. coli* HB101 by transformation (9).

Preparation of cell extracts. Cells of recombinant *E. coli*

strains were grown in ampicillin-containing LB medium and collected in the cold (4°C) by centrifugation. They were washed once with 20 mM phosphate buffer (pH 7.2) and broken by two passes at 110 MPa through a French press. The homogenate was centrifuged at 42,000 × *g* for 2 h. The supernatant was used for immunochemical assays.

Immunochemical techniques. N_2O reductase was detected and quantitated in crude extracts of recombinant *E. coli* strains by Laurell electroimmunoassay. The preparation of antibodies against *P. stutzeri* N_2O reductase and the conditions for electroimmunoassay were as described (68). Cell extracts were subjected for immunoblotting to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 12% separating gel and a discontinuous buffer system (28). Proteins were transferred electrophoretically to nitrocellulose sheets (59). N_2O reductase was detected by staining the immunoconjugate with protein A-gold (7).

Purification of N_2O reductase. The enzyme from *P. stutzeri* was obtained by the standard purification procedure (8). Alternatively, the enzyme was isolated by a modified procedure in which the following chromatographic steps were substituted for preparative isoelectric focusing. After Sephadex G-100 gel filtration (8), the enzyme was dialyzed and bound to a small column of hydroxyapatite. The column was eluted with a linear gradient (1 to 10 mM) of phosphate buffer (pH 7.2). Copper-containing, colored fractions were concentrated by ultrafiltration (PM10 membrane; Amicon Corp., Lexington, Mass.) and passed, in 50 mM Tris hydrochloride buffer (pH 7.5) containing 0.1 M KCl, through a Sephacryl S-200 column (2.6 by 90 cm). The enzyme was concentrated again and subjected to nondenaturing preparative gradient polyacrylamide gel electrophoresis in a 3-mm slab gel (8), followed by electroelution and dialysis against 1 mM Tris hydrochloride buffer (pH 7.5). Samples from either method were used for the analysis of the NH_2 terminus.

E. coli-derived N_2O reductase was purified from transformants harboring plasmids pAV5018 or pAV5021 by the standard procedure developed for the *Pseudomonas* enzyme (8). Cells were grown aerobically in LB medium supplemented with 25 μg of ampicillin per ml in a 9-liter carboy. The copper concentration of the medium was either 5 or 800 μM. The protein was monitored by immunoelectrophoresis during purification. Conditions for incorporation of copper into the purified *E. coli* protein were those used for *Pseudomonas* apoenzyme reconstitution (8).

DNA sequence determination. The region of plasmid pAV5018 identified as *nosZ* was cloned into M13mp11 (33). Transfection of *E. coli* JM105, identification of clones, and isolation of M13 replicative-form and single-stranded DNA were done by previously published techniques (39).

The ca. 1.5-kb *Cla*I fragment (NS810), the ca. 1.8-kb *Bgl*II fragment (NS820), and the 0.3-kb *Pst*I fragment (NS280) of pAV5018 were cloned into the *Acc*I, *Bam*HI, and *Pst*I restriction sites of M13mp11, respectively. For each fragment, two clones were identified by complementary tests to carry the insert in opposite directions. The fragments NS810 and NS820 were progressively shortened by exonuclease III treatment (22). Double-stranded replicative-form of DNA was cut at the unique *Sma*I and *Sac*I sites in the polylinker region of M13mp11. Treatment of the DNA with exonuclease III led to unidirectional digestion of the insert from the *Sma*I site. In a 50-μl assay, 5 μg of DNA was incubated with 500 U of exonuclease III. During a period of 4 min, a 5-μl sample was removed every 60 s, starting 30 s after addition of the enzyme. DNA was treated with S1 nuclease, Klenow fragment, and T4 DNA ligase (22). *E. coli* JM105 was transfected with religated DNA. From each time point, 20 clones were isolated. The sizes of the inserts were analyzed by *Eco*RI-*Hin*dIII digestion.

DNA was sequenced by the dideoxynucleotide chain termination method (51) with deoxyadenosine 5′-[α-^{35}S]thiotriphosphate for labeling (specific activity, ca. 22 TBq/mmol) (3). DNA fragments were separated on wedge-shaped (0.2- to 0.6-mm) gradient gels (1) of 5% (wt/vol) polyacrylamide cast by the sliding-plate technique and containing 7 M urea. The complete sequence was also obtained with deoxy-7-deazaguanosine triphosphate to resolve ambiguities in G+C-rich regions (42). Sequences were evaluated with the PC/GENE software (Genofit, Geneva, Switzerland).

Analytical methods. Protein was determined by the procedure of Lowry et al. (32). Copper was measured by atomic absorption spectroscopy with background compensation by a deuterium lamp. For internal sequences, N_2O reductase was digested with trypsin and the resulting peptides were fractionated by high-pressure liquid chromatography. NH_2-terminal amino acids were determined by automated Edman degradation (18).

Chemicals. Radionuclides and the M13 sequencing kit were from Amersham-Buchler, Brunswick, Federal Republic of Germany. Restriction enzymes were purchased from GIBCO/BRL-Bethesda Research Laboratories GmbH, Eggenstein, Federal Republic of Germany; Pharmacia, Freiburg, Federal Republic of Germany; or Boehringer GmbH, Mannheim, Federal Republic of Germany. Calf intestine alkaline phosphatase was obtained from Boehringer. T4 DNA ligase, Klenow fragment, exonuclease III, S1 nuclease, and the reagents for nonradioactive DNA labeling and detection were from GIBCO/BRL. Electrophoresis chemicals were from Serva, Heidelberg, Federal Republic of Germany and protein A-gold was from Bio-Rad Laboratories, Munich, Federal Republic of Germany.

RESULTS

Cloning of the *nos* region. The cloning strategy for the *nos* region of *P. stutzeri* was based on the plasmid integration-excision system outlined in Fig. 1. A partial *Mbo*I digest of genomic DNA from mutant strain MK416 was first cloned into the cosmid vector pJA1, and subsequently a *Hin*dIII digest thereof was subcloned into pBR325. One of several isolated subclones, pNS600, contained an insert of about 12 kb (62). The larger one (ca. 9 kb) of two *Eco*RI-*Hin*dIII fragments was isolated from this plasmid. This fragment included 1.5 kb of the known *nos* region, the major part comprising adjacent DNA. Insertion of the *Eco*RI-*Hin*dIII fragment into the mobilizable vector pSUP203 resulted in plasmid pMNS400. The plasmid was introduced into *E. coli* S17-1, which carried a chromosomally integrated RP4-derived helper plasmid to provide transfer functions (55). Plasmid pMNS400 was transferred from *E. coli* to *P. stutzeri* by conjugation; transconjugants were selected for vector-mediated ampicillin resistance. Constitutive resistance of *P. stutzeri* against low concentrations of chloramphenicol (10 μg/ml) was used to select against the donor. Since plasmid pMNS400 cannot replicate in *P. stutzeri*, ampicillin resistance was rescued by insertion of the plasmid into *Pseudomonas* DNA by homologous recombination (Fig. 1).

Genomic DNA of ampicillin-resistant colonies was isolated and partially digested with *Kpn*I. This enzyme was initially chosen because physical mapping had indicated the absence of *Kpn*I restriction sites within the *nos* region (62).

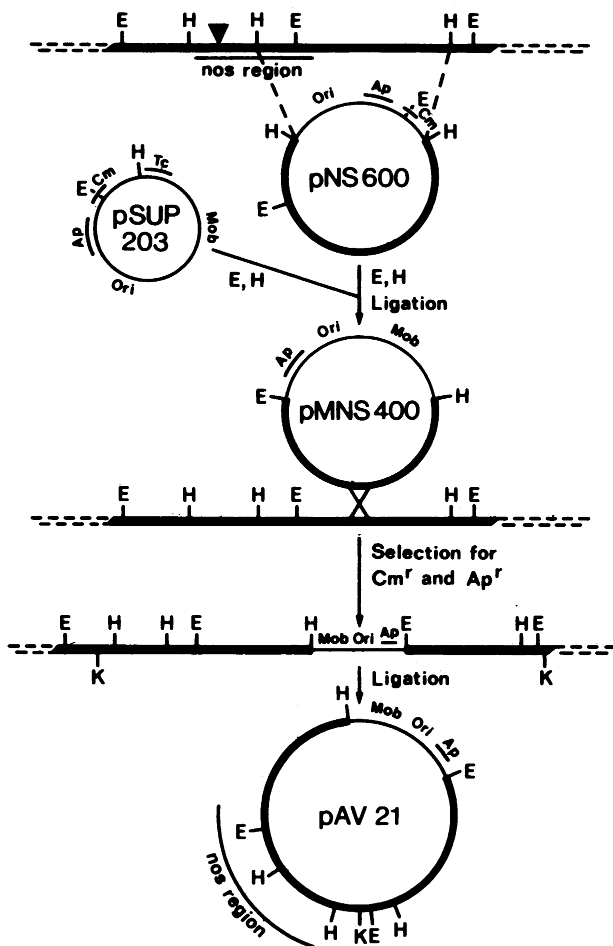

FIG. 1. Cloning by plasmid integration and excision of the *nos* gene cluster from *P. stutzeri*. The top bar represents *Pseudomonas* DNA from mutant strain MK416, carrying a transposon Tn*5* insertion (▼), which provided the *Eco*RI-*Hin*dIII fragment for construction of plasmid pMNS400. An additional *Kpn*I site within the *nos* cluster (see Fig. 2) is not shown, for the sake of clarity. For further description, see the text. Abbreviations for restriction sites: E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I.

A single *Kpn*I site was subsequently recognized, however, and only partial digestion was practicable to preserve the integrity of the *nos*-coding region. *Kpn*I-restricted DNA was self-ligated and used to transform *E. coli* HB101. Plasmid pAV21 (size, ca. 35 kb) was isolated from ampicillin-resistant transformants (Fig. 1). It carried the complete *nos* region. This was shown by comparing the physical map of the plasmid with that of the *nos* region previously identified by mapping Tn*5* insertions (62). Integration of plasmid pMNS400 into *Pseudomonas* DNA gave rise to a gene duplication interrupted by the vector moiety derived from pSUP203. Instability of plasmid pAV21 because of this duplication was not observed in the host, *E. coli* HB101.

Cloning and expression of *nosZ* in *E. coli*. The size of *Pseudomonas* DNA of plasmid pAV21 was reduced by partial digestion with *Sau*3A. Fragments were ligated to vector pBR325 or pMK1, and plasmids with inserts of more than 2 kb were defined by restriction analysis (Fig. 2). Plasmids pAV39 and pAV42 and plasmids pAV49 and pAV50 were obtained from vectors pBR325 and pMK1, respectively. All four plasmids included the DNA region in

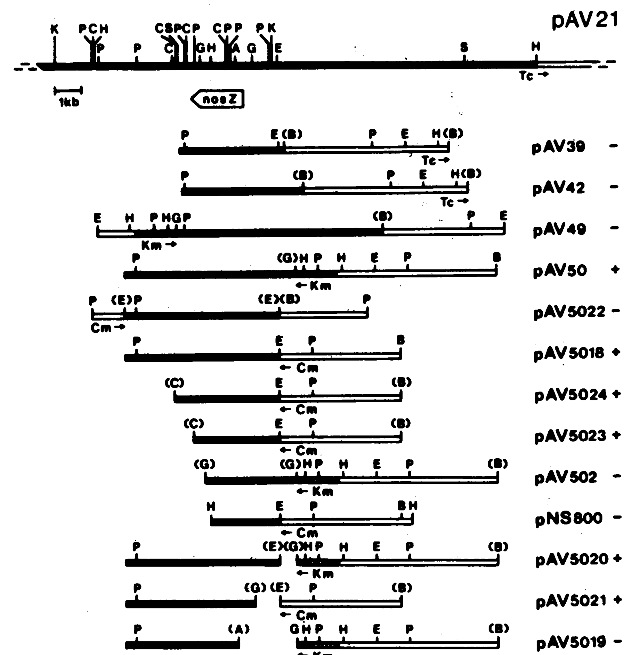

FIG. 2. Physical map of the *P. stutzeri nos* region and restriction maps of *nosZ*-carrying constructs. The position and direction of transcription of *nosZ* are shown by the open arrow below pAV21. Symbols: ▬, *Pseudomonas* DNA; ▭, vector DNA; ▥, IS*50*L element of Tn*5*. The small arrows define the promoter regions of the antibiotic resistance genes for chloramphenicol, kanamycin, or tetracycline. Restriction sites were completely mapped only for the *Kpn*I fragment. Endonuclease designations in parentheses indicate loss of the corresponding recognition sites in the construction process. The + and − signs refer to the results of *nosZ* expression in *E. coli* HB101 (see Fig. 3). Abbreviations for restriction sites not listed in Fig. 1: A, *Sal*I; B, *Bam*HI; C, *Cla*I; G, *Bgl*II; P, *Pst*I; S, *Sma*I.

which Tn*5* insertion into genomic DNA had led to the loss of the structural protein of N_2O reductase (62). *E. coli* HB101 was transformed with these plasmids, and cell extracts were immunochemically assayed for the expression of the enzyme (Fig. 3). Only *E. coli* HB101(pAV50) produced a small amount of material which cross-reacted with a monospecific antiserum against *P. stutzeri* N_2O reductase.

Plasmid pAV50 carried an insert of *Pseudomonas* DNA of about 6 kb. Subfragments with deletions from both ends of the presumed *nosZ* gene were made for more exact gene localization, and the effect of the deletions on *nosZ* expres-

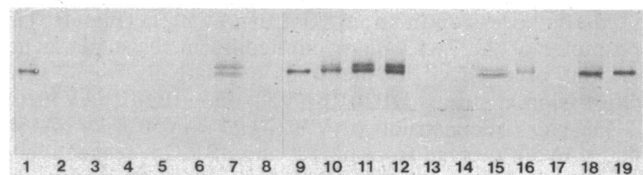

FIG. 3. Western blot analysis of crude extracts of *E. coli* HB101 transformed with the constructs shown in Fig. 2. Lanes: 1, 9, and 19, purified N_2O reductase from *P. stutzeri*; 2, pBR325; 3, pAV21; 4, pAV39; 5, pAV42; 6, pAV49; 7, pAV50; 8, pAV5022; 10, pAV5018; 11, pAV5024; 12, pAV5023; 13, pAV502; 14, pNS800; 15, pAV5020; 16, pAV5021; 17, pAV5019; 18, crude extract from *P. stutzeri*. For electrophoresis and blotting conditions, see the text. The immunoconjugate was stained with protein A-gold.

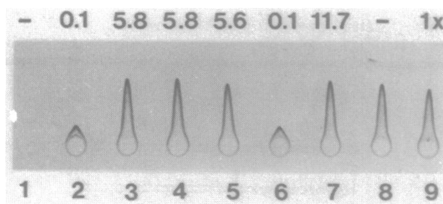

FIG. 4. Electroimmunoassay of NosZ from *E. coli*. Each well was charged with 3 μl of crude extract of *E. coli* HB101 transformed with the indicated plasmids (protein concentrations are given in parentheses). Lanes: 1, pBR325 (30 mg/ml); 2, pAV50 (30 mg/ml); 3, pAV5018 (3 mg/ml); 4, pAV5024 (3 mg/ml); 5, pAV5023 (3 mg/ml); 6, pAV5020 (30 mg/ml); 7, pAV5021 (1.5 mg/ml); 8, purified N_2O reductase (0.2 mg/ml); 9, crude extract from *P. stutzeri* (15 mg/ml). Numbers above the figure indicate the expression factor related to the wild-type *Pseudomonas* strain. Immunoelectrophoresis was run in calcium lactate-containing barbital buffer (pH 8.6) for 1 h at 3 V/cm and then for 3 h at 10 V/cm.

sion in *E. coli* was studied. Thus, a series of constructs was generated by deleting terminal segments of pAV50 with the appropriate restriction enzymes (see Materials and Methods for experimental details). Figure 2 shows the physical maps of the respective plasmids. The religated constructs were used to transform *E. coli* HB101, and isolated clones were again assayed immunochemically (Fig. 3). It became apparent that heterologous *nosZ* expression depended on the presence in the clones of a fragment of 2.2 kb with terminal *Cla*I and *Bgl*II recognition sites. The size of this DNA fragment was close to the minimal length required to code for the N_2O reductase subunit.

The amount of N_2O reductase protein produced in the heterologous host was quantitated by Laurell electroimmunoassay and related to the level of enzyme in the anaerobically derepressed wild-type *Pseudomonas* strain (Fig. 4). A considerable difference in *nosZ* expression was found in strains with plasmids derived either from pMK1 or from pBR325. Expression was also dependent on the orientation of the insert. A ca. sixfold *nosZ* overexpression was found in *E. coli* HB101(pAV5018), whereas the strain with the inverted insert, HB101(pAV5022), completely lacked expression. This argued for the expression of *nosZ* from a vector promoter by transcriptional readthrough. The *Pseudomonas* promoter apparently was inactive, as indicated also by lack of enzyme expression from plasmids pAV39 and pAV42. The promoter of the chloramphenicol resistance gene of pBR325 led to strong gene expression in strains carrying plasmid pAV5018, pAV5023, or pAV5024. Deletion of an *Eco*RI-*Bgl*II fragment of 900 base pairs upstream of the presumptive translational start site led to a further doubling in *nosZ* expression in strain HB101(pAV5021) (Fig. 4). The promoter of the Tn*5* kanamycin-neomycin resistance gene, on the other hand, resulted in a relatively weak gene expression in strains HB101(pAV50) and HB101(pAV5020).

The pairs of constructs pAV5022 and pAV5018, or pAV49 and pAV50, derived from pMK1 and pBR325, respectively, carried identical or similar DNA fragments, yet in opposite directions. Expression of *nosZ* was observed only with pAV50 and pAV5018, defining the direction of transcription as shown by the arrows in Fig. 2 and 5.

DNA sequencing. The nucleotide sequence of *nosZ* was determined by the dideoxy chain termination method of ordered, overlapping fragments obtained by exonuclease III digestion. The sequencing strategy is shown in Fig. 5. Both

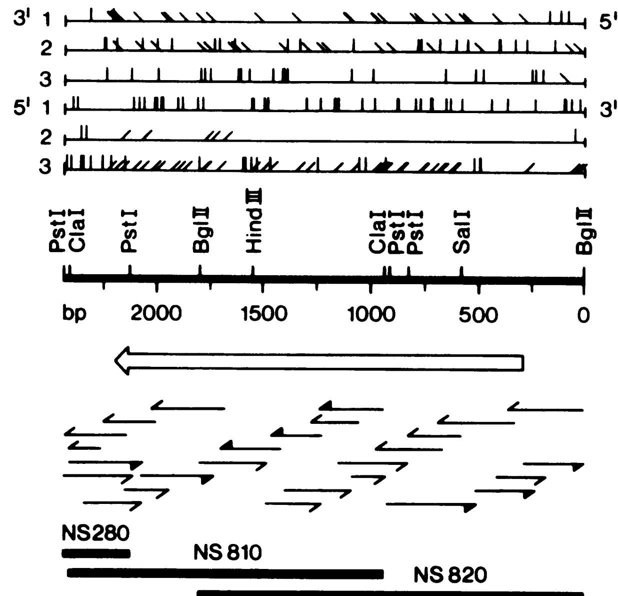

FIG. 5. Restriction map of and sequencing strategy for the *nosZ* gene of *P. stutzeri*. The open arrow shows the position and transcriptional direction of *nosZ*. Small arrows denote the extent of sequencing and the positions of subclones which were derived from the DNA fragments NS280, NS810, and NS820. Filled-in small arrows indicate clones that were sequenced with deoxy-7-deazaguanosine triphosphate in addition to dGTP. The top part shows the distribution of stop codons (|) and start codons (╲, ╱) for the six possible reading frames.

strands were sequenced. The complete nucleotide sequence is shown in Fig. 6. A single open reading frame (ORF) was found extending through 1,914 nucleotides in the transcriptional direction deduced from the *nosZ*-carrying constructs (Fig. 2 and 5). The frame was predicted as coding (17, 53); its deduced amino acid sequence is shown in Fig. 6. A potential ribosome-binding site (52) was located 10 base pairs upstream from the assumed translational start. Inverted repeats, possibly acting as transcription terminators, were found downstream of the stop codon.

The upstream, noncoding region of *nosZ* was scanned (49) for known *Pseudomonas* promoters (11). A decanucleotide, GCACCGCAGT, was found in *nosZ* at positions 171 to 180, which matches a sequence immediately upstream of the promoter of the carboxypeptidase G2 gene (41). The promoter regions of other *Pseudomonas* genes, *algD*, *kilB*, *trfA*, and *xylR*-P1, show six matches, and *toxA* and *xylCAB* show seven matches with this sequence. The CATCC pentanucleotide of the chromosomal *algD* and *toxA* genes (11) is present in *nosZ* at positions 36 to 40. A short distance downstream of the decanucleotide, *nosZ* has a σ^{60} recognition sequence, (200)GG-10-GC(213). However, *Pseudomonas* promoters do not show a highly conserved consensus sequence (11, 24), and evaluation of the significance of the decanucleotide, if any, as well as experimental identification of the *nosZ* promoter, must await further studies.

The 1.2-kb *Pst*I fragment from the *nosZ* gene was used as the hybridization probe for Southern blot analysis. It hybridized to a single fragment when genomic DNA was digested with the endonucleases *Bam*HI, *Eco*RI, *Kpn*I, *Sac*I, and *Sma*I, all of which have no recognition sites within the *nosZ* gene. A duplication of the *nosZ* gene within the genome of *P. stutzeri* is therefore considered unlikely.

```
BglII  10        20        30        40        50        60        70        80        90        100       110       120
AGATCTGTGCCAACGAATGCGAAGTGCAGGCGATTCATCCGGACGGGCATATCAACCACAACGAATGCCATTACTGCCTGGACTGCCAGATGACCTATCACAACGAGAACAAGTGTCCGC

       130       140       150       160       170       180       190       200       210       220       230       240
CGCTGATTGCGAAGAACAAGCGCGCCCGGCGTGACAAGAAGGCCCCAGCTGCACCGCAGTTGATCCCCGTGCAAGTGGTGGAACCCTGAGCGCGCCACTGCGAGCCGCTCGAAACTGGTT

       250       260       SD        280       290       300       310       320       330       340       350       360
GTTAGACCTGTGTTTGAAGACGCCCCAAAGGAGCGAAACCCCATGAGCGACAAAGATTCCAAGAACACTCCGCAAGTGCCCGAGAAACTCGGCCTGAGCCGCCGCGGCTTCCTCGGCGCC
                                            M  S  D  K  D  S  K  N  T  P  Q  V  P  E  K  L  G  L  S  R  R  G  F  L  G  A
                                                                       10                            20

       370       380       390       400       410       420       430       440       450       460       470       480
AGCGCAGTCACCGGTGCCGCCGTTGCTGCCACGGCTCTCGGCGGCGCGGTGATGACCCGCGAATCCTGGGCGCAGGCCGTCAAGGAGTCCAAGCAGAAGATCCACGTCGGCCCCGGCGAG
 S  A  V  T  G  A  A  V  A  A  T  A  L  G  G  A  V  M  T  R  E  S  W  A  Q  A  V  K  E  S  K  Q  K  I  H  V  G  P  G  E
   30                            40                            50                            60

       490       500       510       520       530       540       550       560       570       580 SalI      600
CTGGATGACTACTACGGCTTCTGGTCCGGCGGTCACCAGGGTGAAGTCCGCGTGCTGGGCGTGCCGTCGATGCGCGAGCTGATGCGTATCCCGGTGTTCAACGTCGACTCGGCCACCGGC
 L  D  D  Y  Y  G  F  W  S  G  G  H  Q  G  E  V  R  V  L  G  V  P  S  M  R  E  L  M  R  I  P  V  F  N  V  D  S  A  T  G
   70                            80                            90                           100

       610       620       630       640       650       660       670       680       690       700       710       720
TGGGGGCTGACCAACGAAAGCCGCCACATCATGGGCGACAGCGCCAAGTTCCTCAACGGTGACTGCCACCACCCGCACATCTCCATGACCGACGGCAAGTACGATGGCAAGTACCTGTTC
 W  G  L  T  N  E  S  R  H  I  M  G  D  S  A  K  F  L  N  G  D  C  H  H  P  H  I  S  M  T  D  G  K  Y  D  G  K  Y  L  F
   110                           120                         * 130                           140

       730       740       750       760       770       780       790       800       810       820    PstI         840
ATCAACGACAAGGCCAACAGCCGCGTTGCGCGTATCCGTCTGGACATCATGAAGTGCGACAAGATGATCACCGTGCCGAACGTGCAGGCGATCCACGGTCTGCGTCTGCAGAAGGTGCCG
 I  N  D  K  A  N  S  R  V  A  R  I  R  L  D  I  M  K  C  D  K  M  I  T  V  P  N  V  Q  A  I  H  G  L  R  L  Q  K  V  P
   150                           160                *          170                           180

       850       860       870       880       890       900    PstI       920       930       940    ClaI         960
CACACCAAGTACGTATTCGCCAACGCCGAGTTCATCATCCCGCACCCGAACGATGGCAAGGTCTTCGATCTGCAGGACGAGAACAGCTACACCATGTACAACGCCATCGATGCGGAAACC
 H  T  K  Y  V  F  A  N  A  E  F  I  I  P  H  P  N  D  G  K  V  F  D  L  Q  D  E  N  S  Y  T  M  Y  N  A  I  D  A  E  T
   190                           200                           210                           220

       970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
ATGGAAATGGCCTTCCAGGTCATCGTTGACGGCAACCTCGACAACACCGACGCCGACTACACTGGCCGTTTCGCTGCTGCTACCTGCTACAACTCGGAGAAGGCCTTCGATCTGGGCGGC
 M  E  M  A  F  Q  V  I  V  D  G  N  L  D  N  T  D  A  D  Y  T  G  R  F  A  A  A  T  C  Y  N  S  E  K  A  F  D  L  G  G
   230                           240                           250               *           260

      1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
ATGATGCGTAACGAGCGCGACTGGGTGGTGGTGTTCGATATCCACGCCGTCGAAGCAGCGGTCAAAGCTGGCGATTTCATCACCCTGGGCGACTCCAAGACGCCTGTGCTCGATGGTCGC
 M  M  R  N  E  R  D  W  V  V  V  F  D  I  H  A  V  E  A  A  V  K  A  G  D  F  I  T  L  G  D  S  K  T  P  V  L  D  G  R
   270                           280                           290                           300

      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
AAGAAGGATGGCAAGGACAGCAAGTTCACCCGTTACGTGCCAGTGCCGAAAAACCCGCACGGCTGCAACACCTCCTCCGATGGCAAATACTTCATCGCCGCCGGCAAGCTCTCGCCAACC
 K  K  D  G  K  D  S  K  F  T  R  Y  V  P  V  P  K  N  P  H  G  C  N  T  S  S  D  G  K  Y  F  I  A  A  G  K  L  S  P  T
   310                           320                         * 330                           340

      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440
TGCTCGATGATCGCCATCGACAAGCTGCCCGACCTGTTCGCCGGCAAGCTGGCCGATCCGCGTGATGTGATCGTGGGTGAGCCTGAGCTGGGTCTCGGCCCGCTGCACACCACCTTCGAC
 C  S  M  I  A  I  D  K  L  P  D  L  F  A  G  K  L  A  D  P  R  D  V  I  V  G  E  P  E  L  G  L  G  P  L  H  T  T  F  D
 * 350                           360                           370                           380

      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550 HindIII
GGCCGCGGTAACGCCTACACCACGCTGTTCATCGACAGCCAGGTGGTCAAGTGGAACATGGAAGAAGCTGTTCGTGCCTACAAGGGCGAGAAGGTCAACTACATCAAGCAGAAGCTTGAT
 G  R  G  N  A  Y  T  T  L  F  I  D  S  Q  V  V  K  W  N  M  E  E  A  V  R  A  Y  K  G  E  K  V  N  Y  I  K  Q  K  L  D
   390                           400                           410                           420

      1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680
GTGCACTACCAGCCGGGTCACCTGCACGCGTCGCTGTGTGAAACCAATGAAGCCGATGGCAAGTGGCTGGTAGCACTGTCCAAGTTCTCCAAGGACCGCTTCCTGCCGGTTGGCCCGCTG
 V  H  Y  Q  P  G  H  L  H  A  S  L  C  E  T  N  E  A  D  G  K  W  L  V  A  L  S  K  F  S  K  D  R  F  L  P  V  G  P  L
   430                          *440                           450                           460

      1690      1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
CATCCCGAGAACGACCAACTGATCGACATCTCCGGCGACGAGATGAAGCTGGTACATGACGGCCCGACCTTTGCCGAACCGCATGACTGCATCATGGCTCGCCGTGATCAGATCAAGACC
 H  P  E  N  D  Q  L  I  D  I  S  G  D  E  M  K  L  V  H  D  G  P  T  F  A  E  P  H  D  C  I  M  A  R  R  D  Q  I  K  T
   470                           480                           490               *           500

   BglII        1820      1830      1840      1850      1860      1870      1880      1890      1900      1910      1920
AAGAAGATCTGGGACCGCAACGATCCGTTCTTCGCCCCGACCGTGGAAATGGCGAAGAAGGACGGCATCAACCTCGATACCGACAACAAGGTCATTCGCGACGGCAACAAGGTGCGCGTG
 K  K  I  W  D  R  N  D  P  F  F  A  P  T  V  E  M  A  K  K  D  G  I  N  L  D  T  D  N  K  V  I  R  D  G  N  K  V  R  V
   510                           520                           530                           540

      1930      1940      1950      1960      1970      1980      1990      2000      2010      2020      2030      2040
TACATGACCTCGATGGCGCCGGCGTTCGGCGTGCAGGAGTTCACCGTCAAGCAGGGCGATGAAGTCACCGTGACCATCACCAACATCGACCAGATCGAAGACGTCTCCCACGGCTTCGTG
 Y  M  T  S  M  A  P  A  F  G  V  Q  E  F  T  V  K  Q  G  D  E  V  T  V  T  I  T  N  I  D  Q  I  E  D  V  S  H  G  F  V
   550                           560                           570                           580

      2050      2060      2070      2080      2090      2100      2110      2120      2130   PstI       2150      2160
GTGGTCAACCATGGCGTGAGCATGGAGATCAGCCCGCAGCAGACTTCTTCCATCACCTTTGTCGCTGACAAGCCAGGCCTGCACTGGTACTACTGCAGCTGGTTCTGCCATGCGCTGCAC
 V  V  N  H  G  V  S  M  E  I  S  P  Q  Q  T  S  S  I  T  F  V  A  D  K  P  G  L  H  W  Y  Y  C  S  W  F  C  H  A  L  H
   590                           600                           610                     *   620    *

      2170      2180      2190      2200      2210      2220      2230      2240      2250      2260      2270      2280
ATGGAAATGGTCGGCCGCATGATGGTCGAGCCGGCCTAAGCTGTTCGAGCCATCGCCAATAAGCCGGTGATAGTTCAGCGACTGCGCGAGGAGCCTCGGTCCTGCCGAGGTTGTTCGCCGC
 M  E  M  V  G  R  M  M  V  E  P  A  ---
   630

      2290      2300      2310      2320      2330      2340      2350      2360      2370      2380      2390      2400
AGTGTTTACCCGCCTGGAAGCTGAAGAAGGTCAAAACGCAGTGTTCAAAGCTCAGGCTACTTTCTCGCGGTACTCAGCGGCAGTTTCGCTGCTGCTTTTATTCAGTGGTGCTGCCCAGGC

     ClaI  2420     PstI
GGCACCGCAATCGATTACCACCTTGCCGCTGCAG
```

FIG. 6. Nucleotide sequence and deduced amino acid sequence of the *nosZ* gene from *P. stutzeri*. Mapped restriction sites (Fig. 2 and 5) and a potential ribosome-binding sequence (SD) are indicated above the nucleotide sequence. Dashes denote the stop codon; inverted repeats are underlined by opposing arrows; asterisks emphasize cysteine residues. Numbers are aligned with the last digit to the corresponding nucleotide or amino acid.

TABLE 2. Comparison of the amino acid composition of NosZ and N_2O reductase from *P. stutzeri*

Amino acid	No. of residues in:			
	NosZ[a]	NosZ[b]	N_2O reductase[c]	ORF[d]
Asp	54	52		51
Asx	82	79	72.9	60
Asn	28	27		9
Thr	35	33	32.3	23
Ser	33	29	31.4	25
Glu	31	30		35
Glx	49	47	54.0	65
Gln	18	17		30
Pro	31	29	34.2	14
Gly	52	48	52.8	81
Ala	49	44	49.8	84
Cys	9	9	4.1	8
Val	53	50	49.8	100
Met	25	24	20.3	5
Ile	33	33	32.4	22
Leu	38	35	37.0	78
Tyr	18	18	17.4	3
Phe	28	27	26.8	21
His	23	23	24.2	49
Lys	45	42	43.4	4
Arg	26	24	24.0	53
Trp	9	9	4.6	4

[a] Translation of the entire ORF of *nosZ*.
[b] Assuming processing of the enzyme at Ala-35 (M_r 67,280).
[c] Data from reference 70 recalculated for M_r 67,280.
[d] Cryptic gene product corresponding to complementary ORF of *nosZ* (M_r 74,277).

Analysis of NosZ. The gene product predicted from the ORF consists of a protein of 638 amino acids and M_r 70,822. The mature protein is likely to have a lower M_r (65,777 to 67,280), depending on the exact size of an export signal sequence (see below). M_r values of 62,000 and 74,000 were previously obtained in different sodium dodecyl sulfate-polyacrylamide gel electrophoresis systems (8, 70). The chemically determined amino acid composition (70), with the exception of unstable residues, corresponded well to the composition predicted from the nucleotide sequence (Table 2). Deviations of 10 to 18% were found in the residues Glx, Pro, Gly, and Ala; these deviations, unless inherent to the method, could reflect a microheterogeneity of the very first preparations of N_2O reductase. The protein has nine cysteine residues as opposed to the four determined chemically. Direct gas-phase sequencing of tryptic peptides isolated by reversed-phase high-pressure liquid chromatography gave the sequences IPVFNVDSA, YVFANAEFII, and GNA YTTLFI, which corresponded to the internal sequences of NosZ at amino acid positions 96 to 104, 190 to 199, and 389 to 397, respectively.

Complementary-strand analysis and codon usage. Analysis of ORFs in the six absolute phases within the *nosZ* region revealed an uninterrupted reading frame of 2,097 nucleotides in the opposite direction of transcription to that of the *nosZ* ORF (Fig. 5). A similar situation, for instance, is observed within the genus *Streptomyces*, for which extensive ORFs are frequent in more than one phase and lead to difficulties in identifying the coding strand (2). The complementary ORF was indeed predicted as coding by two independent methods (17, 53). It could code for a gene product of 699 amino acids and M_r 74,277, rather close to the subunit M_r of N_2O reductase. However, the amino acid composition of this hypothetical gene product is quite different from that of N_2O reductase, especially since it has a very low content of Asn, Met, Tyr, and Lys and a high content of Val, Leu, His, and Arg (Table 2). Also, no complete correspondence was found within this ORF to the chemically determined internal amino acid sequences.

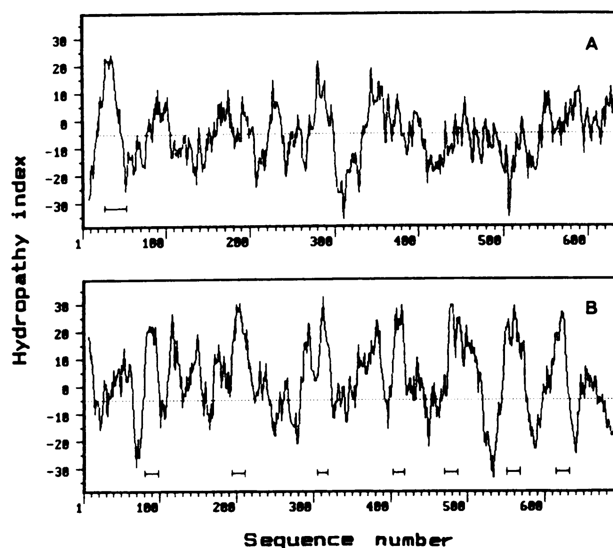

FIG. 7. Hydropathy plot of NosZ (A) and the cryptic protein of the complementary strand (B). Profiles were calculated by the method described previously (27) with a window size of 15 residues. Positions of the predicted membrane-spanning helices (25) are shown.

The profiles of the hydropathy index (27) of NosZ and the complementary, cryptic gene product were conspicuous with respect to the number of apparent membrane helices in the latter (Fig. 7). Thirteen membrane-associated helices were predicted for the complementary gene product by the algorithm of Eisenberg et al. (15); seven (25) or at least three (43) were predicted as membrane spanning, classifying the protein as integral. In contrast, no transmembrane helices, except that of the presequence, were predicted for NosZ by the same three algorithms, as expected for a globular, periplasmic enzyme (see below).

Analysis of codon usage also argues that the ORF in question may not represent a genuine *Pseudomonas* gene (Table 3). The G+C content of *nosZ* from *P. stutzeri* is 60.2%, placing it at the lower end of the G+C contents of the "stutzeri" group, which range from 60.6 to 66.3% (47). From DNA renaturation kinetics, a G+C content of 62% was determined for the genome of the ZoBell strain of *P. stutzeri* (12). Codon composition within the *nosZ* gene is biased toward a high G+C content (83.9%) in the degenerate base position (except for Glu codons), similar to previously characterized genes from other *Pseudomonas* species (10, 19, 48, 58, 60). A low G+C content in the second position (38.1%) and a lower than average content in the first position (58.6%) are thus balanced, allowing for the high overall G+C content of *Pseudomonas* DNA (2). Codon composition within the complementary ORF is opposite to that of *nosZ*, with G+C contents of 80.9, 41.8, and 57% in the first, second, and third codon positions, respectively (Table 3).

NH_2-terminal analysis. The NH_2 terminus of NosZ is likely to carry an export signal sequence extending to 35 residues (M_r of the mature protein without the copper atoms, 67,280); however, five further cleavage sites, in conformity with the −1,−3 rule (64), are present up to residue 52. These cleavage

TABLE 3. Codon usage in the *nosZ* gene of *P. stutzeri*

Codon	Amino acid	No. of times codon used[a]	Codon	Amino acid	No. of times codon used	Codon	Amino acid	No. of times codon used	Codon	Amino acid	No. of times codon used
UUU	Phe	2 (6)	UCU	Ser	1 (5)	UAU	Tyr	0 (0)	UGU	Cys	1 (5)
UUC	Phe	26 (15)	UCC	Ser	13 (0)	UAC	Tyr	18 (3)	UGC	Cys	8 (3)
UUA	Leu	0 (0)	UCA	Ser	0 (3)	UAA	---	1 (0)	UGA	---	0 (1)
UUG	Leu	0 (2)	UCG	Ser	7 (2)	UAG	---	0 (0)	UGG	Trp	9 (4)
CUU	Leu	1 (42)	CCU	Pro	2 (1)	CAU	His	5 (21)	CGU	Arg	10 (5)
CUC	Leu	9 (17)	CCC	Pro	4 (1)	CAC	His	18 (28)	CGC	Arg	16 (12)
CUA	Leu	0 (0)	CCA	Pro	3 (12)	CAA	Gln	2 (1)	CGA	Arg	0 (8)
CUG	Leu	28 (17)	CCG	Pro	22 (0)	CAG	Gln	16 (29)	CGG	Arg	0 (22)
AUU	Ile	1 (2)	ACU	Thr	3 (2)	AAU	Asn	1 (2)	AGU	Ser	0 (6)
AUC	Ile	32 (20)	ACC	Thr	29 (10)	AAC	Asn	27 (7)	AGC	Ser	12 (9)
AUA	Ile	0 (0)	ACA	Thr	0 (1)	AAA	Lys	5 (3)	AGA	Arg	0 (1)
AUG	Met	25 (5)	ACG	Thr	3 (10)	AAG	Lys	40 (1)	AGG	Arg	0 (5)
GUU	Val	5 (29)	GCU	Ala	9 (14)	GAU	Asp	20 (33)	GGU	Gly	10 (30)
GUC	Val	19 (35)	GCC	Ala	26 (41)	GAC	Asp	34 (18)	GGC	Gly	41 (30)
GUA	Val	3 (18)	GCA	Ala	3 (9)	GAA	Glu	15 (26)	GGA	Gly	0 (14)
GUG	Val	26 (18)	GCG	Ala	11 (20)	GAG	Glu	16 (9)	GGG	Gly	1 (7)

[a] Data in parentheses show the codon usage for the putative gene product corresponding to the complementary ORF of *nosZ*.

sites are carboxy terminal to Ala-32, Ala-50, Ala-52, Thr-45, or Ala-36, in this order of decreasing likeliness. The predictive accuracy of these sites is 75 to 80% (64). A presequence of 35 residues appears long, but not exceptionally so, for bacterial export signals (63, 65), including one found in *P. aeruginosa* (48). Protein processing is expected in light of the periplasmic location of N_2O reductase in denitrifying bacteria (36, 44, 61). Location of the enzyme from *P. stutzeri* in the periplasm (N. Minagawa and W. G. Zumft, Biol. Metals, in press) was indicated by cell fractionation (45) and the chloroform shock method (16). The only transmembrane helix of this enzyme is associated with the unprocessed NH_2 terminus, predicted to extend from Gly-22 through Thr-45 (GFLGASAVTGAAVAATALGGAVMT) (43) or Phe-23 through Leu-39 (25); this structure is likely to be functionally related to the export process.

Direct sequencing of *P. stutzeri* N_2O reductase purified by either method described in Materials and Methods did not produce significant yields of amino acids, indicative of NH_2-terminal blockage. The nature of the block was not determined, and it is not known when NH_2-terminal blockage occurred. Substitution of isoelectric focusing by other methods of fractionation did not render the NH_2 terminus accessible, excluding this part of the purification procedure as a cause of the problem. Until its resolution, the NH_2 terminus as well as the definitive M_r of the mature enzyme will have to remain unknown.

Characterization of *E. coli* N_2O reductase. Cell extracts of different *nosZ*-carrying clones of *E. coli* were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent immunoblotting. Results of Western immunoblots shown in Fig. 3 indicated that the plasmids of positive clones directed the synthesis of N_2O reductase proteins with the same molecular mass as the native enzyme from *P. stutzeri*. Although the occurrence of double bands is suggestive of posttranslational protein processing, blockage of the NH_2 terminus of *E. coli* N_2O reductase has, again, so far hampered progress in this area.

N_2O reductase produced by *E. coli* HB101(pAV5018) was purified to homogeneity by the method used for *P. stutzeri*. N_2O reductases of *E. coli* and *P. stutzeri* were immunochemically indistinguishable. The absorption spectrum of the *E. coli* protein purified from cells grown in a low-copper medium (5 μM Cu^{2+}) is shown in Fig. 8. The characteristic absorbance of the copper chromophore around 530 nm and in the near-ultraviolet range was absent. Analysis for copper by atomic absorption spectroscopy was negative. The enzyme could be partially reconstituted in vitro by adding copper (Fig. 8). Assembly of the copper chromophore was incomplete, however, resulting in a protein with an electronic spectrum similar to that of the in vitro-reconstituted apoenzyme of *P. stutzeri* (8). *E. coli* N_2O reductase purified from cells grown in the high-copper medium (0.8 mM Cu^{2+}) contained approximately one atom of copper per dimer (versus ca. eight atoms for the *Pseudomonas* enzyme). The optical spectrum showed weak features in the visible range, suggestive of some spontaneous copper incorporation.

N_2O reductase was also purified from the most highly overproducing strain, *E. coli* HB101(pAV5021). The protein from this strain was remarkably insoluble and tended to precipitate. This is a frequent effect in highly overproducing strains and is attributed to altered protein folding (46). Since no attempts were made to solubilize the aggregating material, much N_2O reductase protein was lost, and the final yield was lower than that from the less highly overproducing strain.

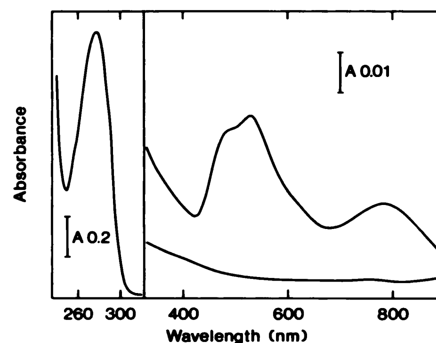

FIG. 8. Absorption spectrum of purified nitrous oxide reductase from *E. coli* HB101(pAV5018). The left-hand panel and the lower trace in the right-hand panel show the spectrum of the protein as isolated; the upper trace shows the spectrum after protein reconstitution with copper as described in Materials and Methods.

DISCUSSION

The method of plasmid integration and excision has proven to be an efficient cloning approach for the *nos* genes of *P. stutzeri*. The general applicability of this method, together with the availability of transposon Tn*5* mutants, should also provide access to other denitrification genes, such as the *nir* (69) and *nar* (S. Blümle, H. Körner, and W. G. Zumft, unpublished results) systems. In preliminary accounts of this work, we referred to the structural gene for N_2O reductase as *nosA* (71; Viebrock and Zumft, Biol. Chem. Hoppe Seyler **368**:561–562, 1987), which has since been assigned to an outer membrane protein involved in copper processing for this enzyme (44). To prevent confusion in terminology, we have modified our previous nomenclature.

Expression of the *nosZ* gene in *E. coli* did not lead to the formation of N_2O reductase holoenzyme. Apparently, *E. coli* does not provide the necessary functions for copper chromophore assembly. Even in the presence of a high (0.8 mM) copper concentration in the medium, no functional holoenzyme was found. This supports the view that formation of the copper prosthetic group of N_2O reductase is catalyzed and requires additional gene products besides the structural protein (44, 62, 68, 72). This might not be the case for all other copper proteins, since, e.g., superoxide dismutase, having only a single copper atom per monomer (besides Zn), was shown to be functional when expressed in *E. coli* (20). Enzyme activity was found to depend on the copper concentration in the medium and required at least 0.39 mM Cu^{2+} (21). The gene for *Alcaligenes faecalis* S6 azurin also led to a functional product when expressed in *E. coli* (66).

The amino acid sequence of NosZ was used to screen the 4,721 entries of the Protein Identification Resource data bank of the National Institutes of Health, Bethesda, Md., (release 14.0) but did not show significant similarities. A more extensive comparison with the copper proteins azurin, plastocyanin, tyrosinase, ceruloplasmin, hemocyanin, and superoxide dismutase showed insufficient amino acid identities to clearly establish a relation with N_2O reductase. Two regions, because of their clustering of Cys, His, and Met residues, however, are conspicuous for copper binding. Cys-128 is found within a homologous sequence, GDCHHP (and four further matching residues close by), of *Desulfovibrio vulgaris* cytochrome c_3. The carboxy-terminal positioned residues Cys-618, Cys-622, and His-626 show a spacing which matches that of the proposed Cu_A site of cytochrome *c* oxidase subunit II (CoxII) (23, 57):

```
244–255 CS E LCG I NHA Y M CoxII (Paracoccus spp.)
        : :   :     :   :
618–629 CSWFCHALHMEM NosZ (Pseudomonas spp.)
```

A serine residue adjacent to Cys-618 is conserved in NosZ and the cytochrome oxidase sequences known so far (57).

The Cu_A site of cytochrome oxidase consists of two sulfur ligands and two N,O-ligands (30). A $Cu^{II}S_2(Cys)_2N(His)$ site was recently proposed for at least one type of copper center of N_2O reductase (13). Data from extended X-ray absorption fine structure of copper (R. A. Scott, personal communication), electron spin echo (Jin et al., submitted), and electron paramagnetic resonance measurements (Riester et al., submitted) are consistent with the presence of an equivalent of a Cu_A site in N_2O reductase.

ACKNOWLEDGMENTS

We are indebted to E. Mendez and R. Frank for NH_2-terminal analyses and to R.F. for providing the internal amino acid sequences. We thank A. Schwickerath and S. Mümmler for excellent technical assistance and S. Löchelt for Southern hybridizations. A. Messerschmidt did the data bank search and drew our attention to the cytochrome c_3 similarity. R. Simon, A. Pühler, and W. Lindenmaier kindly provided strains. We also thank R. A. Scott for communicating unpublished results.

The work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

LITERATURE CITED

1. **Ansorge, W., and S. Labeit.** 1984. Field gradients improve resolution on DNA sequencing gels. J. Biochem. Biophys. Methods **10**:237–243.
2. **Bibb, M. J., P. R. Findlay, and M. W. Johnson.** 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. Gene **30**:157–166.
3. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80**:3963–3965.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
5. **Bolivar, F.** 1978. Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant molecules. Gene **4**:121–136.
6. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
7. **Brada, D., and J. Roth.** 1984. "Golden blot"—detection of polyclonal and monoclonal antibodies bound to antigens on nitrocellulose by protein A-gold complexes. Anal. Biochem. **142**:79–83.
8. **Coyle, C. L., W. G. Zumft, P. M. H. Kroneck, H. Körner, and W. Jakob.** 1985. Nitrous oxide reductase from denitrifying *Pseudomonas perfectomarina*. Purification and properties of a novel multicopper enzyme. Eur. J. Biochem. **153**:459–467.
9. **Dagert, M., and S. D. Ehrlich.** 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene **6**:23–28.
10. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. *Pseudomonas aeruginosa* infection in cystic fibrosis: nucleotide sequence and transcriptional regulation of the *algD* gene. Nucleic Acids Res. **15**:4567–4581.
11. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. Alginate biosynthesis: a model system for gene regulation and function in *Pseudomonas*. Bio/Technology **5**:469–477.
12. **Döhler, K., V. A. R. Huss, and W. G. Zumft.** 1987. Transfer of *Pseudomonas perfectomarina* Baumann, Bowditch, Baumann, and Beaman 1983 to *Pseudomonas stutzeri* (Lehmann and Neumann 1896) Sijderius 1946. Int. J. Syst. Bacteriol. **37**:1–3.
13. **Dooley, D. M., R. S. Moog, and W. G. Zumft.** 1987. Characterization of the copper sites in *Pseudomonas perfectomarina* nitrous oxide reductase by resonance Raman spectroscopy. J. Am. Chem. Soc. **109**:6730–6735.
14. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. Plasmid **1**:584–588.
15. **Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall.** 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J. Mol. Biol. **179**:125–142.
16. **Ferro-Luzzi Ames, G., C. Prody, and S. Kustu.** 1984. Simple, rapid, and quantitative release of periplasmic proteins by chloroform. J. Bacteriol. **160**:1181–1183.
17. **Fickett, J. W.** 1982. Recognition of protein coding regions in DNA sequences. Nucleic Acids Res. **10**:5303–5318.
18. **Gausepohl, H., M. Trosin, and R. Frank.** 1986. An improved gas-phase sequenator including on-line identification of PTH amino acids, p. 149–160. *In* B. Wittmann-Liebold, J. Salnikov, and V. A. Erdman (ed.), Advanced methods in protein micro-

sequence analysis. Springer-Verlag KG, Berlin.

19. **Hadero, A., and I. P. Crawford.** 1986. Nucleotide sequence of the genes for tryptophan synthase in *Pseudomonas aeruginosa*. Mol. Biol. Evol. **3**:191–204.
20. **Hallewell, R. A., F. R. Masiarz, R. C. Najarian, J. P. Puma, M. R. Quiroga, A. Randolph, R. Sanchez-Pescador, C. J. Scandella, B. Smith, K. S. Steimer, and G. T. Mullenbach.** 1985. Human Cu/Zn superoxide dismutase cDNA: isolation of clones synthesising high levels of active or inactive enzyme from an expression library. Nucleic Acids Res. **13**:2017–2034.
21. **Hartman, J. R., T. Geller, Z. Yavin, D. Bartfeld, D. Kanner, H. Aviv, and M. Gorecki.** 1986. High-level expression of enzymatically active human Cu/Zn superoxide dismutase in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83**:7142–7146.
22. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**:351–359.
23. **Holm, L., M. Saraste, and M. Wikström.** 1987. Structural models of the redox centres in cytochrome oxidase. EMBO J. **6**: 2819–2823.
24. **Inouye, S., Y. Asai, A. Nakazawa, and T. Nakazawa.** 1986. Nucleotide sequence of a DNA segment promoting transcription in *Pseudomonas putida*. J. Bacteriol. **166**:739–745.
25. **Klein, P., M. Kanehisa, and C. DeLisi.** 1985. The detection and classification of membrane-spanning proteins. Biochim. Biophys. Acta **815**:468–476.
26. **Klipp, W., and A. Pühler.** 1984. Determination of coding regions on multicopy plasmids: analysis of the chloramphenicol acetyltransferase gene of plasmid pACYC184, p. 224–235. *In* A. Pühler and K. N. Timmis (ed.), Advanced molecular genetics, Springer-Verlag KG, Berlin.
27. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
29. **Leary, J. J., D. J. Brigati, and D. C. Ward.** 1983. Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bio-blots. Proc. Natl. Acad. Sci. USA **80**:4045–4049.
30. **Li, P. M., J. Gelles, S. I. Chan, R. J. Sullivan, and R. A. Scott.** 1987. Extended X-ray absorption fine structure of copper in Cu_A-depleted, *p*-(hydroxymercuri)benzoate-modified, and native cytochrome *c* oxidase. Biochemistry **26**:2091–2095.
31. **Lindenmaier, W.** 1985. Vektor-Wirt Systeme zur DNA-Klonierung in *E. coli*, p. 65–85. *In* N. Blin, M. F. Trendelenburg, and E. R. Schmidt (ed.), Molekular-und Zellbiologie. Springer-Verlag KG, Berlin.
32. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
33. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Matsubara, T., K. Frunzke, and W. G. Zumft.** 1982. Modulation by copper of the products of nitrite respiration in *Pseudomonas perfectomarinus*. J. Bacteriol. **149**:816–823.
35. **Matsubara, T., and M. Sano.** 1985. Isolation and some properties of a novel violet copper protein from a denitrifying bacterium, *Alcaligenes* sp. Chem. Lett. **1985**:1053–1056.
36. **McEwan, A. G., A. J. Greenfield, H. G. Wetzstein, J. B. Jackson, and S. J. Ferguson.** 1985. Nitrous oxide reduction by members of the family *Rhodospirillaceae* and the nitrous oxide reductase of *Rhodopseudomonas capsulata*. J. Bacteriol. **164**: 823–830.
37. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149**:114–122.
38. **Mejean, V., J.-P. Claverys, H. Vasseghi, and A.-M. Sicard.** 1981. Rapid cloning of specific DNA fragments of *Streptococcus pneumoniae* by vector integration into the chromosome followed by endonucleolytic excision. Gene **15**:289–293.
39. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
40. **Michalski, W. P., D. H. Hein, and D. J. D. Nicholas.** 1986. Purification and characterization of nitrous oxide reductase from *Rhodopseudomonas sphaeroides* f. sp. *denitrificans*. Biochim. Biophys. Acta **872**:50–60.
41. **Minton, N. P., and L. E. Clarke.** 1985. Identification of the promoter of the *Pseudomonas* gene coding for carboxypeptidase G2. J. Mol. Appl. Genet. **3**:26–35.
42. **Mizusawa, S., S. Nishimura, and F. Seela.** 1986. Improvement of the dideoxy chain termination method of DNA sequencing by use of deoxy-7-deazaguanosine triphosphate in place of dGTP. Nucleic Acids Res. **14**:1319–1324.
43. **Mohana Rao, J. K., and P. Argos.** 1986. A conformational preference parameter to predict helices in integral membrane proteins. Biochim. Biophys. Acta **869**:197–214.
44. **Mokhele, K., Y. J. Tang, M. A. Clark, and J. L. Ingraham.** 1987. A *Pseudomonas stutzeri* outer membrane protein inserts copper into N_2O reductase. J. Bacteriol. **169**:5721–5726.
45. **Pages, J.-M., J. Anba, A. Bernadac, H. Shinagawa, A. Nakata, and C. Lazdunski.** 1984. Normal precursors of periplasmic proteins accumulated in the cytoplasm are not exported post-translationally in *Escherichia coli*. Eur. J. Biochem. **143**:499–505.
46. **Pain, R.** 1987. Protein folding for pleasure and for profit. Trends Biochem. Sci. **12**:309–312.
47. **Palleroni, N. J.** 1984. Genus I. *Pseudomonas* Migula 1894, 237^{AL} (Nom. cons. Opin. 5, Jud. Comm. 1952, 237), p. 141–199. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
48. **Pritchard, A. E., and M. L. Vasil.** 1986. Nucleotide sequence and expression of a phosphate-regulated gene encoding a secreted hemolysin of *Pseudomonas aeruginosa*. J. Bacteriol. **167**:291–298.
49. **Pustell, J., and F. C. Kafatos.** 1982. A high speed, high capacity homology matrix: zooming through SV40 and polyoma. Nucleic Acids Res. **10**:4765–4782.
50. **Rothstein, S. J., R. A. Jorgensen, K. Postle, and W. S. Reznikoff.** 1980. The inverted repeats of Tn*5* are functionally different. Cell **19**:795–805.
51. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
52. **Shine, J., and L. Dalgarno.** 1975. Determinant of cistron specificity in bacterial ribosomes. Nature (London) **254**:34–38.
53. **Sheperd, J. C. W.** 1981. Method to determine the reading frame of a protein from the purine/pyrimidine genome sequence and its possible evolutionary justification. Proc. Natl. Acad. Sci. USA **78**:1596–1600.
54. **Simon, R.** 1984. High frequency mobilization of gram-negative bacterial replicons by the in vitro constructed Tn*5*-Mob transposon. Mol. Gen. Genet. **196**:413–420.
55. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–791.
56. **Snyder, S. W., and T. C. Hollocher.** 1987. Purification and some characteristics of nitrous oxide reductase from *Paracoccus denitrificans*. J. Biol. Chem. **262**:6515–6525.
57. **Steinrücke, P., G. C. M. Steffens, G. Panskus, G. Buse, and B. Ludwig.** 1987. Subunit II of cytochrome *c* oxidase from *Paracoccus denitrificans*. DNA sequence, gene expression and the protein. Eur. J. Biochem. **167**:431–439.
58. **Takagi, J. S., M. Tokushige, and Y. Shimura.** 1986. Cloning and nucleotide sequence of the aspartase gene of *Pseudomonas fluorescens*. J. Biochem. **100**:697–705.
59. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci.

USA **76**:4350–4354.

60. **Unger, B. P., I. C. Gunsalus, and S. G. Sligar.** 1986. Nucleotide sequence of the *Pseudomonas putida* cytochrome P-450_{cam} gene and its expression in *Escherichia coli*. J. Biol. Chem. **261**:1158–1163.
61. **Urata, K., K. Shimada, and T. Satoh.** 1982. Periplasmic location of nitrous oxide reductase in a photodenitrifier, *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*. Plant Cell Physiol. **23**:1121–1124.
62. **Viebrock, A., and W. G. Zumft.** 1987. Physical mapping of transposon Tn*5* insertions defines a gene cluster functional in nitrous oxide respiration by *Pseudomonas stutzeri*. J. Bacteriol. **169**:4577–4580.
63. **von Heijne, G.** 1985. Signal sequences. The limits of variation. J. Mol. Biol. **184**:99–105.
64. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14**:4683–4690.
65. **Watson, M. E. E.** 1984. Compilation of published signal sequences. Nucleic Acids Res. **12**:5145–5164.
66. **Yamamoto, K., T. Uozumi, and T. Beppu.** 1987. The blue copper protein gene of *Alicaligenes faecalis* S-6 directs secretion of blue copper protein from *Escherichia coli* cells. J. Bacteriol. **169**:5648–5652.
67. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
68. **Zumft, W. G., K. Döhler, and H. Körner.** 1985. Isolation and characterization of transposon Tn*5*-induced mutants of *Pseudomonas perfectomarina* defective in nitrous oxide respiration. J. Bacteriol. **163**:918–924.
69. **Zumft, W. G., K. Döhler, H. Körner, S. Löchelt, A. Viebrock, and K. Frunzke.** 1988. Defects in cytochrome cd_1-dependent nitrite respiration of transposon Tn*5*-induced mutants from *Pseudomonas stutzeri*. Arch. Microbiol. **149**:492–498.
70. **Zumft, W. G., and T. Matsubara.** 1982. A novel kind of multi-copper protein as terminal oxidoreductase of nitrous oxide respiration in *Pseudomonas perfectomarinus*. FEBS Lett. **148**:107–112.
71. **Zumft, W. G., A. Viebrock, and H. Körner.** 1988. Biochemical and physiological aspects of denitrification. *In* J. A. Cole and S. Ferguson (ed.), The nitrogen and sulphur cycles, p. 245–279. Cambridge University Press, Cambridge.
72. **Zumft, W. G., A. Viebrock, J. Riester, P. M. H. Kroneck, D. M. Dooley, and C. L. Coyle.** 1987. Nitrous oxide reductase, spectroscopy and molecular biology. J. R. Neth. Chem. Soc. **106**:352.