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Molecular Characterization of *nosA*, a *Pseudomonas stutzeri* Gene Encoding an Outer Membrane Protein Required To Make Copper-Containing N_2O Reductase

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A *Pseudomonas stutzeri* gene (*nosA*) encoding an outer membrane protein was cloned into the broad-host-range vector pRK290 and expressed in a mutant lacking the protein. Deletion analysis identified the approximate extent of the *nosA* region which was sequenced, and it was found to contain an open reading frame encoding 683 amino acids including a presumed signal sequence of 44 amino acids. The putative processed form had a molecular weight of 70,218, characteristics typical of outer membrane proteins, and considerable amino acid sequence homology with *Escherichia coli* BtuB. A short stretch of amino acids was homologous with the *E. coli* TonB-dependent outer membrane proteins, BtuB, IutA, FepA, and FhuA, suggesting a homologous function: interaction with a periplasmic protein or uptake of a specific substrate.

Denitrification involves a series of anaerobic respirations using nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), or nitrous oxide (N_2O) as the terminal electron acceptor (32). Each step is catalyzed by a distinct enzyme, the final one being a copper-containing N_2O reductase (9). Genetic analysis identified a channel-forming outer membrane protein (NosA) which contains copper, is repressible by copper (20), and is required to transfer copper from the external environment to N_2O reductase (27).

In this paper, we describe the cloning and sequencing of *nosA* and show that the predicted amino acid sequence is homologous to the C-terminal region of the *Escherichia coli* outer membrane protein, BtuB.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. LT medium was modified Luria-Bertani (LB) broth (3) supplemented with trace mineral solution (1 ml/liter) lacking added copper (27). *Pseudomonas stutzeri* and *E. coli* strains were routinely grown in LB broth (24). To test N_2O reductase function, *P. stutzeri* strains were grown for 48 h on LT plates supplemented to 10 μM $CuSO_4$ in an N_2O atmosphere.

To ensure the retention of vectors or recombinant plasmids, the following appropriate antibiotics were added in the following amounts (micrograms per milliliter): chloramphenicol, 30; tetracycline, 20; ampicillin, 50; kanamycin, 50; and spectinomycin, 100. When needed, M9 minimal medium (23) was supplemented with tetracycline to a final concentration of 10 μg/ml. All incubations were done at 37°C unless stated otherwise.

Conjugation. Conjugational matings using *E. coli* HB101 (pRK2013) as a helper were performed as described previously (10). Transconjugants were selected on M9 plates containing tetracycline.

Preparation of DNA. *E. coli* and *P. stutzeri* cultures were screened for plasmid content by the alkaline lysis procedure (2). Larger amounts of plasmid DNA were prepared by alkaline lysis, followed by purification on CsCl-ethidium bromide gradients (23). Spectinomycin (300 μg/ml) was used for the amplification of plasmid pBR329. Chromosomal DNA from *P. stutzeri* was prepared by the method of Clewell and Helinski (6) with modifications. One liter of LT medium, which contains 1.3 μM $CuSO_4$, was inoculated with an overnight culture of *P. stutzeri* JM604 and grown at 37°C with vigorous shaking. Cells were harvested at an optical density at 650 nm of 0.7, washed with 10 mM Tris buffer (pH 8.0), and suspended in 30 ml of SET buffer (10 mM Tris-HCl, 50 mM EDTA, 20% sucrose [pH 7.6]). Lysozyme was added to 1 mg/ml, and the suspension was incubated on ice. After 15 min, 0.6 ml of a 10% *N*-lauroyl sarcosine solution was added and the suspension was incubated at 65°C for 30 min and then at 37°C for 15 min. RNase was added to a final concentration of 5 μg/ml. After 30 min of incubation at 37°C, 15 mg of proteinase K was added and the incubation was continued for 2 h. The suspension was extracted with hexadecyl trimethyl ammonium bromide (CTAB), phenol, and chloroform (1). The DNA was precipitated with ethanol.

DNA manipulations. Standard molecular cloning, transformation, and electrophoresis procedures (1, 23) were used. Restriction endonucleases and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals. DNA-modifying enzymes were purchased from Bethesda Research Laboratories (BRL) and used as recommended by the manufacturer. Linkers were purchased from New England BioLabs.

Preparation of DNA probe. *P. stutzeri* chromosomal DNA was partially digested with *Hae*III, *Alu*I, and *Rsa*I; ligated with *Eco*RI linkers; and in vitro-packaged into λgt11 following the manufacturer's instructions (Protoclone λgt11 System; Promega). Screening the in vitro-packaged genomic library with anti-NosA antibody identified several clones that produced β-galactosidase–NosA hybrid proteins. On the basis of the restriction maps of these clones and the sizes of the hybrid proteins they encoded, the *nosA*-coding region was tentatively identified. A 775-bp *Eco*RI-*Sal*I fragment

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TABLE 1. Bacterial strains and plasmids

Strains or plasmid	Genotype or phenotype	Source or reference
E. coli		
RR1	F^- *pro leu thi lacY rpsL20 hsdR hsdM ara-14 galK2 xyl-15 mtlA supE44*	R. L. Rodríguez
HB101	F^- *recA13 proA2 ara-14 xyl-5 mtl-1 lacY1 galK2 rpsL20 supE44 hsdS20 leu mcrB mrr* λ^-	BRL
DH5α	F^- ϕ80d*lacZDM15* Δ(*lacZYA-argF*)*U169 deoR endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* λ^-	BRL
P. stutzeri		
JM604	Wild type	27
JM778	*nos*	26
Plasmids		
pBR329	Ap^r Tet^r Cm^r	8
pUC18	Ap^r	42
pRK290	IncP Tet^r	10
pRK2013	*rep* (ColE1) Mob^+ Tra^+ Km^r	11
pHL1	pBR329 with 10.8-kb *Eco*RI fragment carrying *nosA*; Ap^r Tet^r	This work
pHL3	pRK290 with 10.8-kb *Eco*RI fragment from pHL1 carrying *nosA*; Tet^r	This work
pHL35	pRK290 with 7.25-kb *Eco*RI fragment carrying *nosA*; Tet^r	This work
pHL36	pRK290 with 6.0-kb *Eco*RI fragment carrying *nosA*; Tet^r	This work
pHL33	pRK290 with 4.0-kb *Eco*RI fragment partially carrying *nosA*; Tet^r	This work
pHL48	pRK290 with 3.6-kb *Eco*RI fragment partially carrying *nosA*; Tet^r	This work
pHL66	pUC18 with 3.6-kb *Eco*RI-*Xho*I fragment from pHL33; Ap^r	This work
pHL72	pUC18 with 2.7-kb *Eco*RI fragment partially carrying *nosA*; Ap^r	This work
pHL76	Same as pHL72 with insert in the opposite orientation	This work

from clone A1 (Fig. 1), which produced the longest fragment of NosA (approximately 27.3 kDa or 40% of NosA), was labeled with dioxigenin-dUTP by using a commercial kit (Genius; Boehringer Mannheim) and used as a *nosA* probe.

Cloning of *nosA*. A *P. stutzeri* genomic library was made of 10- to 15-kb *Eco*RI fragments cloned into plasmid pBR329. Chromosomal DNA partially digested with *Eco*RI was size-fractionated by 10 to 40% sucrose gradient centrifugation, ligated with the vector, and transformed into *E. coli* RR1. Screening recombinants by colony hybridization by using dioxigenin-dUTP-labeled DNA as a probe identified six positive clones, and the plasmid in one of these was designated pHL1.

Subcloning. Plasmid pHL3 was made by ligating the 10.8-kb *Eco*RI fragment of pHL1 into pRK290. Plasmids pHL33, pHL35, and pHL36 were made by self-ligating fragments of pHL3 made by partial digestion with *Xho*I. Plasmid pHL48 was made by self-ligating *Pst*I-generated fragments of plasmid pHL35. Plasmid pHL66 was made by ligating a 3.6-kb *Eco*RI-*Xho*I fragment of plasmid pHL33 and an *Eco*RI linker into pUC18. Plasmids pHL72 and pHL76 were prepared as follows: plasmid pHL66 was cut with

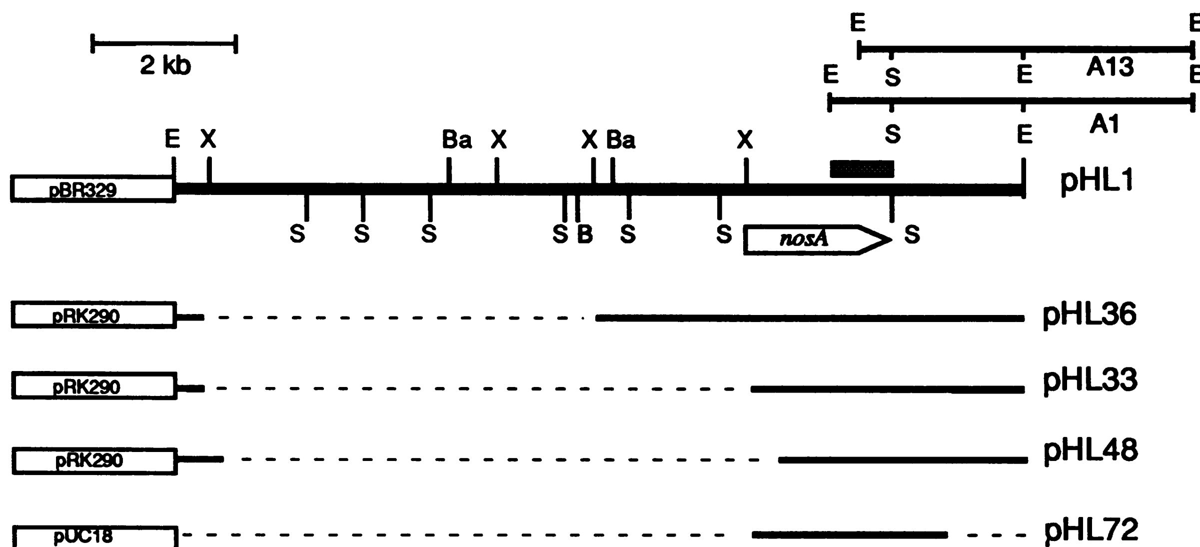

FIG. 1. Plasmids containing the *nosA* region. A1 and A13 are partial *nosA* clones from a λgt11 library. Plasmid pHL1 contains a 10.8-kb *Eco*RI fragment carrying the entire *nosA*-coding region. The shaded box represents the region of DNA used to make a probe. Dashed lines represent deleted regions. Vectors pBR329, pRK290, and pUC18 were not drawn to scale. Restriction site abbreviations: Ba, *Bam*HI; B, *Bgl*II; E, *Eco*RI; S, *Sal*I; X, *Xho*I.

*Hin*dIII, digested with exonuclease III and SI nuclease, ligated with *Eco*RI linkers, and cut with *Eco*RI; fragments of interest were ligated into pUC18.

Southern hybridization. Southern hybridization was done according to established procedures (1). DNA probes were labeled with dioxigenin-dUTP as described above. Nitrocellulose membranes (Magnagraph; Micron Separations, Inc.) were used as the transfer medium. Hybridization conditions were identical to those used to screen for the *nosA* clone.

DNA sequence determination. DNA sequences were determined by the dideoxynucleotide chain termination method (35) with recombinant pUC18 templates and synthetic oligonucleotide primers synthesized by a DNA synthesizer (model 381A; Applied Biosystems, Inc.). Sequencing reactions were performed by using a commercially available T7 polymerase sequencing kit (Pharmacia). The reaction mixtures were separated on a sequencing gel as previously described (41). Sequences were analyzed with PC/GENE (IntelliGenetics) and by GCG (University of Wisconsin-Madison).

Determining the N-terminal sequence of NosA. Samples for protein sequencing were prepared as described previously (28). Stacking gel was cast with 1.5% SeaKem agarose (FMC BioProducts). Proteins were transferred to an Immobilon-P membrane (Millipore Corp.). The band corresponding to NosA was cut out and subjected to protein sequencing by Edman degradation.

Other techniques. Minicell (15) and maxicell (41) experiments were done as previously described. Purification of NosA, preparation of antibody and cell extracts, and immunoblotting analyses were done as described previously (20). One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (19) and two-dimensional (O'Farrell) gels (29) were done as previously described. Protein content was determined as described previously (37) by using bovine serum albumin as the standard.

Nucleotide sequence accession number. The sequence reported has been assigned GenBank accession no. M60717.

RESULTS

Preparation of a *nosA*-specific DNA probe. A *P. stutzeri* genomic library constructed in λgt11 was screened with anti-NosA antibody. Among several positive clones, clone A1 (Fig. 1) expressed the largest fusion protein; it contained a 27.3-kDa NosA fragment which corresponds to about 40% of NosA (data not shown). Clone A13 (Fig. 1) expressed a smaller fragment, about 20% of NosA (data not shown). By comparing the sizes of the fusion proteins and the restriction maps of the clones, a tentative *nosA*-coding region was identified. The 775-bp *Eco*RI-*Sal*I fragment of the A1 clone was inferred to correspond to the C-terminal region of NosA, and the internal *Sal*I site was inferred to correspond roughly to the C terminus. The 775-bp *Eco*RI-*Sal*I fragment was used as a probe to screen a plasmid library in order to obtain a clone carrying all of *nosA*.

Analysis of positive clones. The *P. stutzeri* genomic library constructed in plasmid pBR329 was screened by colony hybridization. The six positive clones found among approximately 1,600 recombinants were analyzed by digestion with *Eco*RI, *Sal*I, *Xho*I, *Bam*HI, and *Bgl*II. All clones carried 10.8-kb *Eco*RI fragments which contained identical restriction fragments derived from one *Bgl*II site, two *Bam*HI sites, four *Xho*I sites, seven *Sal*I sites, and numerous *Pst*I and *Pvu*II sites (Fig. 1, pHL1). As expected, pHL1 from the plasmid library and the A1 clone from the λgt11 library shared a region with identical restriction fragments. Sequence homology was confirmed by Southern blot analysis. The 775-bp *nosA* probe hybridized with the 2.6-kb *Sal*I fragment and the 3.6-kb *Xho*I-*Eco*RI fragment of pHL1 (data not shown). As judged by the size of the insert, plasmid pHL1 appeared to contain the entire region coding for NosA.

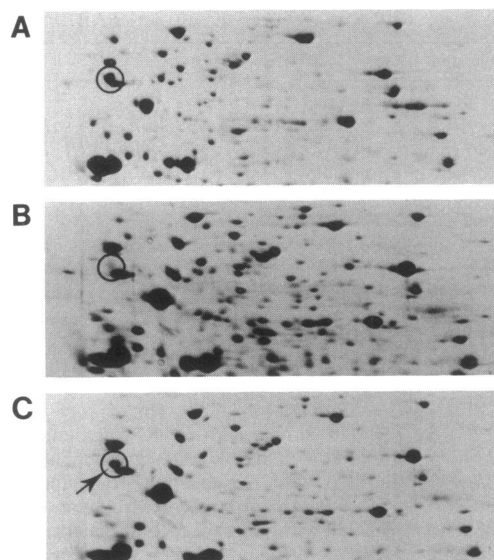

FIG. 2. Expression of NosA from plasmid pHL3. Plasmid pHL3 was prepared by subcloning a 10.8-kb insert from pHL1 into pRK290. A crude extract of *P. stutzeri* JM778(pHL3) was analyzed on two-dimensional gels. Proteins were separated by their isoelectric points (left, acidic; right, basic) and subunit molecular weights (top, large; bottom, small). (A) JM604 (wild type); (B) JM778 lacking NosA; (C) JM778(pHL3). The arrow indicates restored NosA; circles indicate location of NosA.

Expression of NosA. To test the expression of NosA from pHL1, the plasmid was introduced into mini- and maxicell-producing strains of *E. coli*. No proteins were produced in significant amounts. Attempts were made to determine whether *nosA* might be expressed anaerobically, but the minicell-producing strain did not grow well enough to make the test possible. In order to test NosA expression in *P. stutzeri*, cloned DNA from plasmid pHL1 was transferred to a broad-host-range vector, pRK290, generating plasmid pHL3 which was introduced by conjugation into a mutant strain (JM778) of *P. stutzeri* lacking NosA. Strain JM778 was generated by frameshift mutagenesis (26). The resulting transconjugant was shown by two-dimensional gels (Fig. 2) and Western blot (immunoblot) analysis (Fig. 3) to express NosA. The NosA spot was identified by comparing the neighboring protein pattern to gels on which NosA had been located (27). However, as judged by the size and intensity of the spot, a relatively small amount of NosA was expressed. This expression was repressible by exogenous copper; when grown with 20 μM copper, no cross-reacting material against anti-NosA antibody was detected in JM778(pHL3) (Fig. 3).

Plasmid pHL3 was also introduced into a series of frameshift mutants, JM753, JM761, JM774, JM778, JM779, JM780, and JM782 (27), which were unable to produce NosA or grow on N_2O as the electron acceptor. None gained the ability to grow with N_2O as an electron acceptor. It was necessary to follow *nosA* expression by Western blot (immunoblot) analysis.

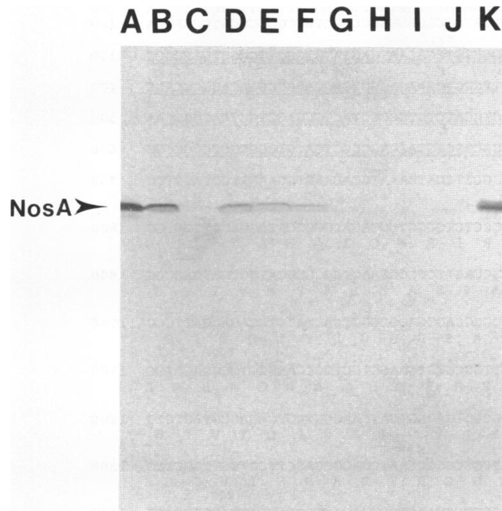

FIG. 3. Identification of subclones expressing NosA. Crude extracts of JM778 harboring *nosA* clones were prepared as described in Materials and Methods. Proteins were separated on SDS-PAGE (9%), transferred to a nitrocellulose membrane, and immunostained with anti-NosA antibody. Lanes: A and K, purified NosA; B, JM604; C, JM778; D, JM778(pHL3); E, JM778(pHL36); F, JM778 (pHL33); G, JM778(pHL48); H, JM778(pRK290); I, JM778(pHL3) grown at 20 μM copper; J, JM778(pHL36) grown at 20 μM copper.

The size of the *nosA*-coding region was identified by deletion analysis. A series of subclones generated by deleting various *Xho*I fragments (Fig. 1) were tested by Western blot analysis for their ability to express NosA. Plasmids pHL3, pHL33, and pHL36 could express NosA in a *nosA* background (Fig. 3); plasmid pHL48, a further deletion of plasmid pHL33, could not. To decrease the size of the DNA to be sequenced, the 3' end of the insert in pHL33 was deleted as described in Materials and Methods. Deletions extending beyond the internal *Sal*I site (which appeared to be the C terminus of *nosA* on the basis of the size of the expected protein) produced cross-reacting material of lower molecular weight (data not shown).

Sequence of NosA. Plasmid pHL72, which was the product of a deletion, contained a 2.7-kb fragment (Fig. 1) which was sequenced. Even though pHL33 expressed NosA in *P. stutzeri*, the nucleotide sequence of the insert lacked a segment at the 5' end of *nosA* corresponding to five amino acids, so further-upstream sequences were determined by using pHL36 as a template. Expression of NosA from pHL33 is probably initiated from a promoter in either the vector or the 0.4-kb *Eco*RI-*Xho*I fragment.

The complete nucleotide sequence is shown in Fig. 4. An open reading frame extends through 2,049 nucleotides in the direction of transcription deduced from the *nosA*-carrying constructs (Fig. 1). There are two GTG codons upstream of the assumed start codon, but it is unlikely that either of them serves as an initiator because of the absence of an apparent ribosome-binding site. In all six frames, this was the only open reading frame long enough to encode NosA. As predicted, the stop codon, TGA, was near the internal *Sal*I site of pHL33, only 10 bp away from it. The stop codon was followed by inverted repeats that might act as a transcriptional terminator (34). A potential ribosome-binding site (36) was located 7 bp upstream from the assumed translation initiation codon ATG. A potential promoter sequence, cCACCGCAtT, was found between positions 622 and 631. This sequence showed only two mismatches (lowercase letters) with the sequence proposed to be the promoter for N_2O reductase (NosZ) from the *P. stutzeri* ZoBell strain (39).

Analysis of encoded protein sequence. The open reading frame encoded a polypeptide of molecular weight 74,924. Because NosA is located in the outer membrane (27), potential sites for signal peptidase cleavage were searched by a previously described method (40). Sites at the positions Ala-44 and -45, Ala-45 and -46, and Ala-32 and -33 were predicted with decreasing likeliness. Since the N-terminal sequence of purified NosA was found to be Ala-Glu-Ser-Val-Asp, the cleavage site was assigned to Ala-44 and -45, which is preceded by Ala-42–Leu-43–Ala-44, a typical recognition site. This site would produce a signal peptide of 44 amino acids, which is long but not exceptionally so, and a mature protein with a molecular weight of 70,218 containing 639 amino acid residues, a prediction which is in reasonable agreement with the previously reported M_r value of 65,000 (20). Very hydrophobic proteins like NosA frequently show electrophoretic mobilities that differ significantly from that expected on the basis of molecular weight (16, 38). The predicted isoelectric point of the mature peptide was 4.57, which is in good agreement with a previously reported value (27).

The G+C content of *nosA* was 63.9%, which is typical of *P. stutzeri* (31), and the G+C content of the degenerate base position within the coding triplet was 80.7% (Table 2), similar to other genes from *Pseudomonas* species (39, 41).

NosA showed a very high content of hydrophobic amino acids (Table 3), Leu, Gly, and Ala, a composition typical of an integral membrane protein (18). These three amino acids constitute 31% of the protein and signal peptide. In total, the protein consists of 49.6% hydrophobic amino acids (Ala, Gly, Ile, Leu, Val, Phe, Trp, and Tyr). However, the method of Kyte and Doolittle (18) predicts only one transmembrane segment between amino acid positions 29 and 45 which lies within the signal peptide. The sequence contained an appreciable content of charged amino acids (26%), which were fairly evenly distributed along the sequence and, like other outer membrane proteins, lacked extensive segments of hydrophobic residues (5, 14, 25, 30).

Sequence comparison with other proteins. The translated amino acid sequence was compared with the 14,301 entries of the protein data base (NBRF-Protein) by using FASTA software, which employs the algorithm of Lipman and Pearson (21). The vitamin B_{12} receptor protein (BtuB) of *E. coli* (13) showed the highest similarity score. As shown in Fig. 5, BtuB was 24% identical to NosA in the 171-amino-acid overlap in this C-terminal region. The C terminus of NosA (from 596 to 604) has nine amino acids, seven of which are identical to the corresponding amino acids of BtuB. This region is also highly conserved among other TonB-dependent receptors, including BtuB, IutA, FepA, and FhuA (Fig. 6). The region from 437 to 444 of NosA also contained six identical amino acids. The homology between NosA and other outer membrane proteins was low.

DISCUSSION

P. stutzeri NosA participates in the pathway of copper transfer from the environment to N_2O reductase, which is located on the periplasmic side of the cytoplasmic membrane (27). Purified NosA contains copper and forms channels in artificial membranes (20). Its expression is repressible by

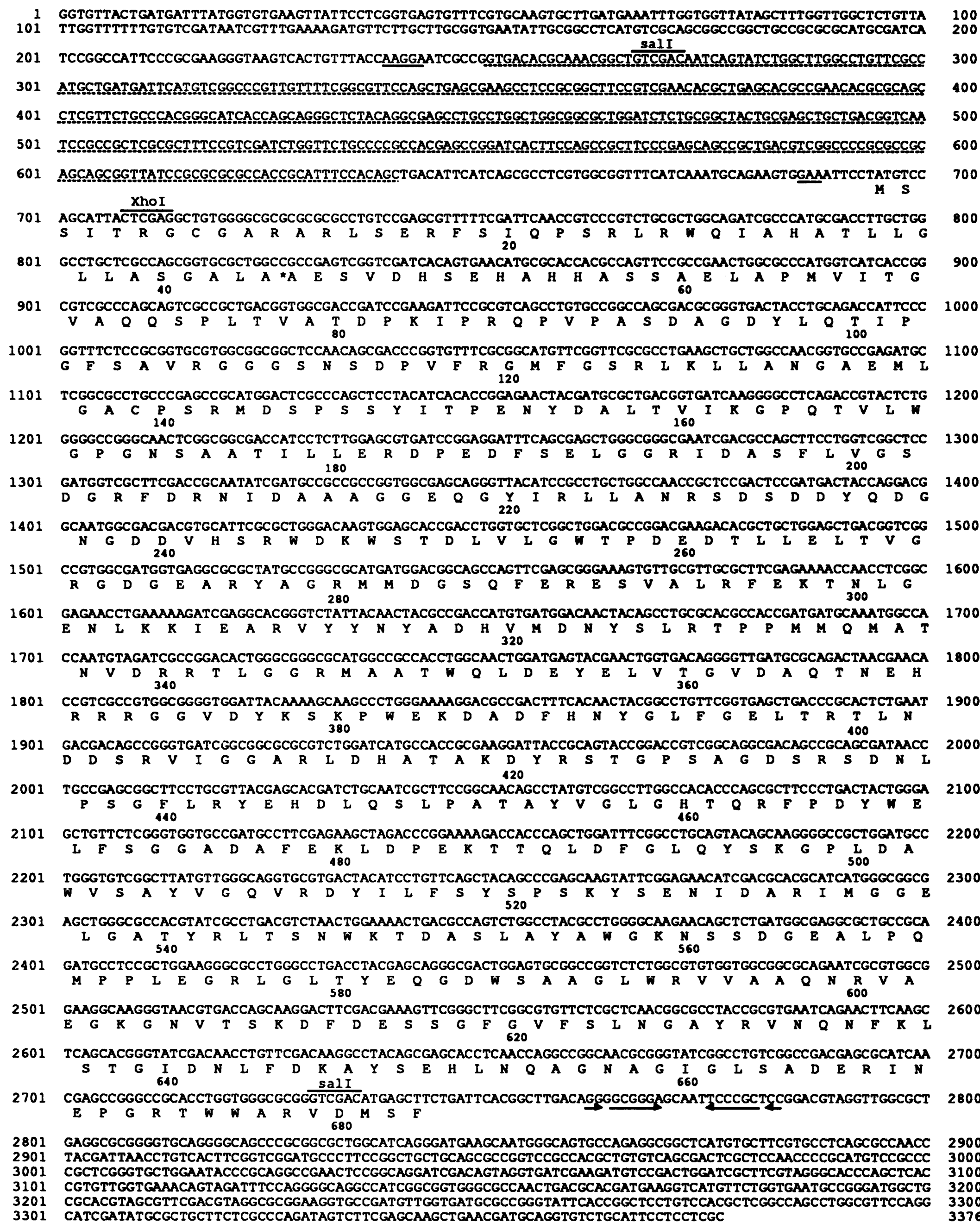

FIG. 4. Nucleotide sequence and deduced amino acid sequence of the *P. stutzeri nosA* gene. Potential ribosome-binding sequences are underlined; inverted repeats are underlined by opposing arrows; the asterisk (between amino acids 44 and 45) indicates the signal peptide cleavage site; sequences with dashed underlining represent other open reading frames; and restriction sites are indicated above the nucleotide sequence.

exogenous copper. As judged by the phenotype of the mutant strain, NosA appears to be specific for copper, but in vitro studies failed to confirm this observation (20). To answer questions about the physiological role of NosA, the *nosA*-coding region was cloned. All the positive clones found in the λgt11 library expressed partial fragments of NosA. The inability to recover the complete gene probably reflected an inadequate genomic library, even though the restriction enzymes employed cut the chromosomal DNA frequently enough to generate fragments in appropriate sizes for the cloning vector. We used a genomic library constructed in a plasmid to recover the entire *nosA*-coding region by using a partial *nosA* fragment as a probe.

To verify the identity of positive clones found in the plasmid library, we attempted to express the gene in *E. coli* by using mini- and maxicells. No proteins were expressed in significant amounts. Lack of expression might reflect the unusual properties of NosA, which is produced anaerobi-

TABLE 2. Codon usage in *P. stutzeri nosA*[a]

Codon	Amino acid	No. of times used	Codon	Amino acid	No. of times used	Codon	Amino Acid	No. of times used	Codon	Amino acid	No. of times used
TTT	Phe	3	TCT	Ser	2	TAT	Tyr	6	TGT	Cys	1
TTC	Phe	20	TCC	Ser	10	TAC	Tyr	21	TGC	Cys	1
TTA	Leu	0	TCA	Ser	0	TAA		0	TGA		0
TTG	Leu	3	TCG	Ser	15	TAG		0	TGG	Trp	15
CTT	Leu	2	CCT	Pro	4	CAT	His	5	CGT	Arg	12
CTC	Leu	10	CCC	Pro	4	CAC	His	8	CGC	Arg	29
CTA	Leu	1	CCA	Pro	1	CAA	Gln	4	CGA	Arg	2
CTG	Leu	46	CCG	Pro	21	CAG	Gln	19	CGG	Arg	5
ATT	Ile	4	ACT	Thr	4	AAT	Asn	6	AGT	Ser	7
ATC	Ile	16	ACC	Thr	19	AAC	Asn	22	AGC	Ser	25
ATA	Ile	0	ACA	Thr	4	AAA	Lys	4	AGA	Arg	0
ATG	Met	15	ACG	Thr	9	AAG	Lys	17	AGG	Arg	0
GTT	Val	3	GCT	Ala	1	GAT	Asp	21	GGT	Gly	16
GTC	Val	8	GCC	Ala	36	GAC	Asp	35	GGC	Gly	43
GTA	Val	2	GCC	Ala	4	GAA	Glu	11	GGA	Gly	1
GTG	Val	19	GCG	Ala	24	GAG	Glu	26	GGG	Gly	11

[a] Data were obtained from the deduced amino acid sequence of *nosA* including the signal peptide.

cally and involved in denitrification, a process that does not occur in *E. coli*. The metabolically related protein, *P. stutzeri* N_2O reductase, was not expressed in *E. coli* either (39).

Since the 10.8-kb *nosA* clone was unable to complement *nosA* strains, the genetic defects of the frameshift mutants remain unexplained. When the mutants were analyzed on two-dimensional gels, they also lacked (in addition to NosA) a protein of unknown function, designated protein e (27). It is uncertain whether the inability to complement was caused by lack of a protein which was not supplied by the *nosA* clone. We also checked the possibility of polar effects of the frameshift. However, the cloned DNA was large enough to contain several upstream genes. On the basis of the DNA sequence, no downstream genes were found. Therefore, the nature of the frameshift mutations is not understood. We also attempted to complement *nosA* mutations caused by resistance to a NosA-specific phage (4). Since these are spontaneous mutants selected by phage resistance, they were most probably missense or point mutations. However, these mutants reverted during genetic manipulations at a high frequency to the wild type, rendering them unsuitable for this use. Despite these difficulties, the expression of NosA by cloned DNA in a mutant strain lacking the protein (as identified by two-dimensional gels, Western blot analysis, and repressibility by copper) clearly demonstrated the identity of the cloned DNA.

The nucleotide sequence determined by using the fragment that carried the *Xho*I site at its 5′ end turned out to be incomplete, lacking the first five amino acids of the signal peptide. Expression from pHL33 appeared to be caused by a fusion transcript extending from the vector or the 0.4-kb *Eco*RI-*Xho*I fragment. However, the information obtained from the sequence agreed with previous findings including molecular weight and isoelectric point data. A signal peptidase cleavage site that was typical of an outer membrane protein was identified. The predicted cleavage site was confirmed by determining the N-terminal amino acid sequence of the purified protein. The codon usage and high

TABLE 3. Amino acid composition of *P. stutzeri* NosA

Amino acid	No. of residues in NosA	
	With signal peptide[a]	Without signal peptide[b]
Ala	65	58
Arg	48	42
Asn	28	28
Asp	56	56
Cys	2	1
Gln	23	21
Glu	37	36
Gly	71	67
His	13	12
Ile	20	17
Leu	62	55
Lys	21	21
Met	15	14
Phe	23	22
Pro	30	29
Ser	59	53
Thr	36	34
Trp	15	14
Tyr	27	27
Val	32	32

[a] Translation of the entire open reading frame of *nosA*.
[b] Processed NosA without signal peptide.

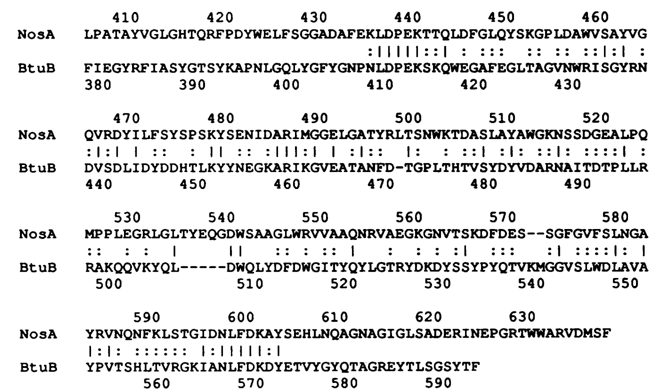

FIG. 5. Comparison of the deduced amino acid sequence of *nosA* to that of *E. coli btuB*. The sequences were aligned to yield maximum homology with respect to identical (vertical lines) and functionally similar (double dots) amino acids.

BtuB	562	G	K	I	A	N	L	F	D	K	R	Y	E	20
IutA	657	F	S	I	E	N	L	F	D	R	D	Y	T	31
FepA	671	G	G	V	D	N	L	F	D	K	R	L	W	40
FhuA	677	L	H	V	N	N	L	F	D	R	E	Y	V	25
NosA	593	T	G	I	D	N	L	F	D	K	A	Y	S	34

FIG. 6. Homologies between BtuB, IutA, FepA, FhuA, and NosA. The number on the left of the sequences is the number of residues between the mature N terminus and the beginning of the region shown; the number on the right is the number of residues from the C terminus. Sequences for *btuB* (13), *iutA* (17), *fepA* (22), and *fhuA* (7) are from published data.

G+C content at the degenerate codon position, which was typical of *Pseudomonas* spp., support the correctness of the reading frame. The amino acid composition determined from the sequence showed characteristics typical of outer membrane proteins. However, the overall homology with other outer membrane proteins was low except for its homology to *E. coli* BtuB. Since NosA contains copper, the amino acid sequence of *nosA* was compared with other copper-containing proteins. However, no substantial homology was found. The sequences characteristic of the Cu_A type found in N_2O reductase of *P. stutzeri* ZoBell was not detected. Since NosA is not involved in the pathway of electron transport, the binding of copper to NosA may involve different ligands.

On the basis of several previous observations, we suggested that a periplasmic binding protein might interact with NosA (4, 20, 27). The strongest evidence was the finding of two types of mutants isolated by resistance to a NosA-specific phage (4); copper-remedial mutants could grow anaerobically on N_2O when excess copper was added to the growth medium, whereas noncopper-remedial mutants could not. The copper-remedial type of mutants might retain a functional periplasmic protein to incorporate copper into N_2O reductase. Interestingly, a potential coding region extending from bases 250 to 639 was found upstream of the *nosA* gene. The potential gene was separated by 32 bp from the *nosA* gene and was preceded by a potential ribosome-binding site. The 390-bp open reading frame encoded a protein with a molecular weight of 14,224. The codon usage was also biased toward a high G+C content (76%) in the degenerate codon position. The preceding bases of the 390-bp open reading frame were rich in A+T (51%). Sequences of an A+T-rich region were proposed as promoter sequences for *E. coli* outer membrane protein genes, facilitating the unwinding of the duplex DNA and resulting in efficient transcriptional initiation (14, 25). However, it is uncertain whether the 390-bp open reading frame is able to encode a protein. Such a protein might participate in the copper pathway. Even though the 390-bp open reading frame was not followed by a typical transcriptional terminator, the formation of a single transcriptional unit with *nosA* requires more extensive investigation.

Recently, the *nos* region of the *P. stutzeri* ZoBell strain was sequenced, and the configuration of the genes and the protein products was found to be similar to that of a bacterial transport system for small molecules (43). This region which is located downstream of *nosZ*, the structural gene for N_2O reductase, was reported to be responsible for the acquisition and processing of copper for the enzyme. The model proposes the participation of at least three proteins, a periplasmic protein (NosD), a cytoplasmic nucleotide-binding protein (NosF), and an inner membrane protein (NosY).

The significance of the highly conserved stretch of amino acids among BtuB, IutA, FepA, FhuA, and NosA remains speculative. Besides being located in the outer membrane, these proteins share other features. They are involved in the transport of metals, with the exception of BtuB, which participates in vitamin B_{12} uptake. TonB has been proposed to transmit metabolic energy to these outer membrane receptors, allowing them to release their bound substrates into the periplasm (12, 33). They are all inducible by limitation of their substrates. The molecular weights are also similar (60,000 to 80,000). The highly conserved stretches of amino acids are localized at the C-terminal region of these proteins. The channel-forming property of NosA is distinct from the TonB-dependent receptors. The similarity between NosA and TonB-dependent outer membrane proteins may suggest similarities in the mechanism of substrate uptake; free copper in the environment could be transported into the cell in the form of a copper-chelate complex. Like others, the uptake of copper through NosA may require metabolic energy involving a TonB-like periplasmic protein as an energy transducer. As evidenced by the firm binding of copper to NosA (20), the release of copper from NosA may be an energy-dependent step.

Even though regions of high homology to BtuB were detected, the functional, topological, and evolutionary significance of the sequences needs further investigation.

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