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Requirements for Cu_A and Cu-S Center Assembly of Nitrous Oxide Reductase Deduced from Complete Periplasmic Enzyme Maturation in the Nondenitrifier *Pseudomonas putida*

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Bacterial nitrous oxide (N_2O) reductase is the terminal oxidoreductase of a respiratory process that generates dinitrogen from N_2O. To attain its functional state, the enzyme is subjected to a maturation process which involves the protein-driven synthesis of a unique copper-sulfur cluster and metallation of the binuclear Cu_A site in the periplasm. There are seven putative maturation factors, encoded by *nosA*, *nosD*, *nosF*, *nosY*, *nosL*, *nosX*, and *sco*. We wanted to determine the indispensable proteins by expressing *nos* genes from *Pseudomonas stutzeri* in the nondenitrifying organism *Pseudomonas putida*. An in silico study of denitrifying bacteria revealed that *nosL*, *nosX* (or a homologous gene, *apbE*), and *sco*, but not *nosA*, coexist consistently with the N_2O reductase structural gene and other maturation genes. Nevertheless, we found that expression of only three maturation factors (periplasmic protein NosD, cytoplasmic NosF ATPase, and the six-helix integral membrane protein NosY) together with *nosRZ* in *trans* was sufficient to produce catalytically active holo-N_2O reductase in the nondenitrifying background. We suggest that these obligatory factors are required for Cu-S center assembly. Using a mutational approach with *P. stutzeri*, we also studied NosA, the Cu-containing outer membrane protein previously thought to have Cu insertase function, and ScoP, a putative membrane-anchored chaperone for Cu_A metallation. Both of these were found to be dispensable elements for N_2O reductase biosynthesis. Our experimental and in silico data were integrated in a model of N_2O reductase maturation.

Nitrous oxide (N_2O) reductase, the *nosZ* gene product, transforms nitrous oxide to N_2 as part of a respiratory mode of bacterial energy conservation. The biosynthesis of the two Cu centers of this enzyme depends on accessory proteins whose exact functions and positions in the NosZ maturation pathway are not known. This is at least in part attributable to the fact that the structure of the catalytic site, Cu_Z, remained unknown until very recently. This site turned out to be the first example of a biologically active Cu-S cluster in which four solely histidine-liganded Cu atoms are bridged by a sulfide ion (4, 44). How this conjugated metal center is assembled has become one of the most intriguing questions associated with N_2O reductase. Cu_A, the other metal center, serves as the site for electron transfer to Cu_Z and is a dicysteinyl-bridged binuclear Cu site (5, 28). N_2O reductase was among the early examples for which metal processing was recognized as part of overall enzyme biosynthesis. We conceptualized that insertion of Cu into N_2O reductase requires the bacterium to handle the toxic metal along a discrete route to prevent the occurrence of free Cu inside the cell (58). This principle is now condensed in the term metallochaperone and has been studied best in eukaryotic Cu delivery systems (for a review see reference 39).

By using random Tn*5* mutagenesis, several *nos* loci for Cu center biosynthesis were detected in *Pseudomonas stutzeri* (49, 57). Analysis of these loci led to the finding that there is a three-component assembly complex, NosDFY, whose putative ATP/GTPase, NosF, and general arrangement at and on both sides of the cytoplasmic membrane are similar to those of bacterial ABC transporters (12, 58, 59). Inactivation of any protein of this complex leads to an N_2O reductase with a low Cu content and no Cu-S site. In particular, NosZ from the *nosD* promoter mutant MK402 has been studied in detail and has been shown to exhibit only the properties of a Cu_A protein (44, 45, 56, 58).

An additional maturation component was identified in NosA, a Cu-containing outer membrane protein of *P. stutzeri* JM300 (30, 31, 37). NosA was thought to be necessary to insert Cu into N_2O reductase. The insertase or metallochaperone function was also attributed to the Cu-containing protein NosL, which has the features of a lipoprotein of the outer membrane (18, 34).

In certain denitrifying bacteria the *nos* gene cluster harbors the *nosX* gene. The precise role of this gene in NosZ biosynthesis has not been clarified, although it is necessary for N_2O utilization by *Sinorhizobium meliloti* (6) and *Paracoccus denitrificans* (46). The latter bacterium carries in addition to *nosX* a homologous gene, *nirX*, and mutagenesis of both genes is required to generate a Nos^- phenotype. Electron paramagnetic resonance hyperfine-coupling characteristics of cell extract from a *nosX nirX* double mutant indicated that the Cu_A site is altered or lacking, but the exact molecular defect is unknown. Finally, a putative assembly factor is represented by ScoP, which is a homologue of Sco1 from yeast (14, 51). The yeast factor is necessary for Cu_A assembly of cytochrome *c* oxidase (21, 38, 48). Evidence is accumulating that bacterial Sco1 homologues also function as Cu-processing factors (8, 32, 33).

Thus, we currently have a total of seven factors or candidate proteins for NosZ maturation. Given this situation, we wanted

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
E. coli DH10B	Cloning host	Gibco BRL
P. putida	Former type strain	DSM50906
P. aeruginosa PAO		25
P. stutzeri		ATCC 14405
MK21	Spontaneous Sm^r mutant of *P. stutzeri*	57
MK404	MK21 *nosD*::Tn*5*	59
MK413	MK21 *nosR*::Tn*5*	13
MK417	MK21 *nosY*::Tn*5*	59
MK498P	MK21 Δ*scoP*::Km^r	This study
MK499A	MK21 *nosA*::Km^r	This study
MK4211(pSZ)	MK21 Δ*nosZ*::Km^r complemented with *nosZ* expression vector pSZ	7
Plasmids		
cDEN1	Cosmid clone of a *Sau*3A genomic library in pJA1 carrying *P. stutzeri nos* genes	3
c14	Cosmid clone of a *Sau*3A genomic library in pJA1 carrying *scoP* of *P. stutzeri*	14
c61	Cosmid clone carrying *nosA* of *P. stutzeri*	This study
pBSL15	Source of Km^r cassette	1
pNS200	pBR325 derivative with a 4.2-kb *Hin*dIII fragment carrying *nosDFY*	59
pUC18	Cloning vector; Ap^r	54
pUC18nosAH	6-kb *Hin*dIII fragment from cosmid c61 carrying *nosA* cloned into pUCP18	This study
pUCP22	Cloning vector; Ap^r Gm^r	53
pUCP22RE	8.8-kb *Eco*47III-*Xba*I fragment with *nosRZDFYL* and *tatE* cloned into pUCP22	24
pUCP22RL	8.6-kb *Eco*47III-*Xba*I fragment with *nosRZDFYL* cloned into pUCP22	24
pUCP22RY	8.1-kb *Eco*47III-*Xba*I fragment with *nosRZDFY* cloned into pUCP22	This study
pUCP22RZ	5.3-kb *Eco*47III-*Sma*I fragment with *nosRZ* cloned into pUCP22	This study
pWM20	Template for PCR amplification of the *nosX* probe from *S. meliloti*	26

to define the minimal set of essential components. To do this, we pursued a strategy to express *nosZ* of *P. stutzeri* and its regulatory gene, *nosR*, together with genes encoding assembly factors in the nondenitrifying organism *Pseudomonas putida*. By using a heterologous background we intended to differentiate specific *nos* functions from potential organismal adaptations and to identify those functions for which a rescue pathway may exist. Our experimental data, together with the results of an in silico study, allowed us to formulate an integrated model for NosZ maturation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids used in this study are shown in Table 1. *Escherichia coli* DH10B was grown in Luria-Bertani (LB) medium at 37°C and 240 rpm on a gyratory shaker. Pseudomonads were grown in LB medium at 30°C and 240 rpm for use in recombinant DNA work. For other purposes an asparagine- and citrate-containing medium, AC medium, was used, and this medium was supplemented with 5 μM Cu (11). *P. putida* was grown in a modified M9 medium containing 20 mM sodium citrate as the carbon source (42). This medium was supplemented with 7.5 μM Fe, 0.1 μM Mo, and 5 μM Cu. When necessary for strain maintenance, antibiotics were added at the following final concentrations: streptomycin, 200 μg/ml of medium; kanamycin, 50 μg/ml; ampicillin, 100 μg/ml (*E. coli*) or 50 μg/ml (*P. putida*); and gentamicin, 10 μg/ml (*E. coli*) or 15 μg/ml (*P. putida*).

DNA techniques. Standard protocols described previously (24) were used for genomic DNA extraction, plasmid DNA preparation and purification, agarose gel electrophoresis, dephosphorylation, and ligation of DNA. Restriction enzymes were used as recommended by the manufacturers. Transformation was done by electroporation. For Southern blot analysis the alkaline capillary transfer method was used (9). DNA hybridization was done overnight with the gene probes listed in Table 2. Detection was done by using chemiluminescence with the CDP-Star reagent (Roche Biochemicals) combined with the EasyHyb system from the same manufacturer or as described previously (20). Gene probes were prepared in a two-step PCR. The first amplification was done with the templates listed in Table 2. Purified PCR fragments were labeled in a second PCR by using a digoxigenin-labeled nucleotide mixture (Roche Biochemicals).

RNA analysis. Cells were grown under denitrifying conditions with 5 μM Cu or without added Cu (the concentration of adventitious Cu in the medium was ≤0.7 μM). RNA was isolated from fresh cells with a total RNA extraction kit (Roche Diagnostics) or was extracted by the hot phenol method from cells frozen in liquid nitrogen (52). Northern blotting was performed as described previously (52). Hybridization and detection of digoxigenin-labeled probes were carried out according to the instructions of the EasyHyb system (Roche Diagnostics).

Cloning and sequencing of *nosA* and *scoP*. A *P. stutzeri* cosmid library (3) was screened for an *oprC* homologue by Southern hybridization with probe OC at 55°C. An approximately 6-kb *Hin*dIII fragment was cloned into pUC18 with *E. coli* DH10B as the host, generating pUC18nosAH. A stretch of ca. 2.9 kb was sequenced on both strands by primer walking by using a dye terminator kit (Amersham Pharmacia Biotech) with an ALFexpress sequencer according to instructions of the manufacturer. The previously described partial sequence of *scoP* was completed. The gene was designated *orf193*; it is located on cosmid c14 downstream of *fnrA* (14).

Mutagenesis of *nosA* and *scoP*. *nosA* of MK21 was inactivated by insertion of a kanamycin resistance cassette, Km^r, into the single *Xho*I restriction site in the orientation opposite that of *nosA*, generating strain MK499A. The mutation was verified by Southern hybridization with the *nosA* probe NA, as well as by transcriptional analysis. *scoP* was inactivated by replacing an internal 160-bp *Xcm*I-*Bsa*AI fragment with a Km^r cassette in the same orientation, generating strain MK498P. The mutation was verified by Southern blot analysis.

Construction of *nosZ* expression vectors. Vector pUCP22RZ was constructed by cloning the *nosRZD′*-carrying *Eco*47III-*Sma*I fragment into *Ecl*136II- and *Sma*I-digested pUCP22. The pUCP replicon is functional in pseudomonads. pUCP22RY was assembled as described previously for pUCP22RL and pUCP22RE (24). The forward primer, 5′-ACGTGCGCAGATCAGCAATAAC C-3′, was located 34 bp upstream of the *Sma*I site in *nosD* that was used for construction of pUCP22RZ. The reverse primer, 5′-GTACTATCTAGACCGC ACACGTGACACTCG-3′ (nucleotides 129 to 146 of *nosL*), was designed to add an *Xba*I site (underlined) 176 bp downstream of the *nosY* stop codon. The *Sma*I- and *Xba*I-digested PCR product was ligated into pUCP22RZ that was digested with the same restriction enzymes, resulting in plasmid pUCP22RY(*nosRZDFY*). Due to primer design this vector also encoded the 49 N-terminal amino acids of the 191 amino acids of NosL. This fragment did not include the cysteine and histidine residues for Cu binding (34). The *P. putida* expression strains harboring plasmids pUCP22RZ, pUCP22RY, pUCP22RL, and pUCP22RE were designated RZ, RY, RL, and RE, respectively.

TABLE 2. Primers used for PCR amplification and Southern or Northern hybridization

Probe or gene	Primer	Sequence	Template	Annealing temp (°C)	Reference
apbE	Forward	5′-CCGACCATGGGCAGCAGCTN-3′	*P. putida*	40	This study
	Reverse	5′-ATCGAAGGTGTGCGAATAGN-3′			
NA	Forward	5′-GGCGCCGATGGTCGTTA-3′	c61	56	This study
	Reverse	5′-CCGGCCTTGAGTTCGACA-3′			
ND	Forward	5′-GTATCAAGGCCAGTTCACCA-3′	pNS200	50	This study
	Reverse	5′-TCATCAGGATGCCGTAGTTC-3′			
NL	Forward	5′-ACTGGCCGTGTTGCTCGCTT-3′	cDEN1	45	This study
	Reverse	5′-GCAGCAGCGCCTGATCGATT-3′			
NR	Forward	5′-TTCGAGATGGCGATCTTCACTGC-3′	cDEN1	56	This study
	Reverse	5′-TCAGGGTTCCACCACTTG-3′			
NX	Forward	5′-CGATCGCGTCGTATTCG-3′	pWM20	56	26
	Reverse	5′-ATGCGGACAGCCGAACT-3′			
NZ	Forward	5′-GTTGCTGCCACGGCTCTC-3′	cDEN1	55	This study
	Reverse	5′-GTCGGCGTCGGTGTTGTC-3′			
OC	Forward	5′-CGCGCCGACCTCCTACATCT-3′	*P. aeruginosa*	60	This study
	Reverse	5′-GAACACCGCGTCCTTGCTCC-3′			
scoP	Forward	5′-GGACATCTGCCCGACCA-3′	*P. putida*	50	This study
	Reverse	5′-CAGGTTACCGCTGTGATCCA-3′			
TatC1	Forward	5′-GGTCTGGGGCTTCATCGC-3′	*P. aeruginosa*	57	24
	Reverse	5′-CCATGCCGACCACGAAAC-3′			

Cell extract, cell fractionation, gel electrophoresis, and enzyme detection. The size of the inoculant in aerobic LB medium was increased from 3 to 20 ml. Cells were grown by using a two-phase growth mode in 100 ml of minimal medium in a 300-ml flask. The initial optical density at 660 nm was 0.03. The aerobic growth phase (shaking speed, 240 rpm) was changed to O_2-limited conditions when the optical density reached approximately 0.3. The shaking speed was reduced to 120 rpm, and sodium nitrate was added to a final concentration of 0.1%. After incubation overnight cells were harvested under cold conditions at 5,000 × *g*. The cell pellet was washed once with 25 mM Tris-HCl (pH 7.5) and suspended in the same buffer. The cells were disrupted by two rounds of pulsed sonication (2 min each). Insoluble material was separated by centrifugation at 15,000 × *g* for 20 min. The supernatant was used for gel electrophoresis and Western blot analysis of NosZ.

Fractionation of cells into periplasm and cytoplasm fractions was done by adapting the partial lysozyme digestion method (40). Freshly harvested cells were washed twice with 200 mM Tris-HCl (pH 7.5) and suspended on ice at a 1:1.5 (wt/vol) ratio in 200 mM Tris–1 M sucrose (pH 7.5). For each 1 g of biomass 150 μl of 0.1 mM EDTA (pH 7.6) and 3 ml of a lysozyme solution (2 mg/ml) were added. Spheroblast formation was monitored with a microscope and was stopped by adding 150 μl of 1 M $MgCl_2$ per g of cell mass. The periplasm was separated from spheroblasts by centrifugation at 4,000 × *g*, and outer membrane components were removed by ultracentrifugation at 80,000 × *g* for 1 h. The spheroblasts were washed once in 200 mM Tris–0.5 M sucrose–50 mM $MgCl_2$ and lysed by addition of distilled water and sonication as described above for whole cells. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 5% polyacrylamide stacking gel and a 10% polyacrylamide separating gel was used for protein separation. Immunochemical detection of NosZ was done with polyclonal antiserum and protein A-horseradish peroxidase conjugate (17) either colorimetrically with 4-chloro-1-naphthol or by chemiluminescence with Luminol reagent (Pierce).

Purification of NosZ and activity measurements. For purification of NosZ from *P. putida*, we started with 100 to 150 g of cell mass obtained as follows. Three 2-liter flasks containing 1 liter of M9 medium each were inoculated from agar slants. The flasks were incubated aerobically overnight and used to inoculate a 50-liter batch culture. The carboy was sparged with air at a flow rate of 0.5 liter min^{-1} through grade D2 sinter glass disks. Sodium nitrate (5.3 g) was added once the optical density reached approximately 0.3. Cells were harvested after 24 h by continuous-flow centrifugation. A typical yield was about 60 to 80 g per batch. *P. putida* RZ did not grow well in a 50-liter culture and was therefore cultured in 2-liter flasks. Cells were frozen in liquid nitrogen and stored at −20°C until they were used. NosZ was isolated under a protective argon atmosphere by using the protocol established for *P. stutzeri* (11). The enzyme was monitored in the chromatographic fractions immunochemically. The yield of NosZ from the initial cell mass ranged from 4 to 20 mg; a high yield was not associated with a particular expression strain. The NosZ activity of whole cells was measured with 50 mM citrate as the electron donor by gas chromatography (7); the purified enzyme was measured spectrophotometrically with photoreduced benzyl viologen (11). One unit of enzyme activity was defined as 1 μmol of N_2O reduced per min. The Cu contents of NosZ, culture media, and chromatographic fractions were determined by flame atomic absorption spectroscopy. UV-visible spectra were recorded with an HP-8453 diode array photometer (Hewlett-Packard).

Nucleotide sequence accession numbers. The nucleotide sequence data reported here have been deposited in the EMBL nucleotide sequence databank under accession numbers AJ507426 (*nosA*) and Z26044 (*scoP*).

RESULTS

***P. putida* as the nondenitrifying host for *nos* genes from *P. stutzeri*.** Our first objective was to find a suitable organism for heterologous NosZ biosynthesis. We focused on the genus *Pseudomonas* to avoid problems with codon usage and to work in a Nos^- background with an otherwise high level of similarity to *P. stutzeri*. *P. putida* DSM50906, the former type strain, was selected as the host from 10 pseudomonads tested for their inventory of *nos* genes by Southern hybridization. The probes used for hybridization and their sources are listed in Table 2; their locations in the corresponding genes of source organisms are shown in Fig. 1. *P. putida* tested negative for all *P. stutzeri nos* genes except *nosA*, which showed that this nondenitrifying organism did not provide specific functions for N_2O respiration. On the other hand, *P. putida* gave a strong hybridization signal for *tatC* (data not shown). Since NosZ is translocated to the periplasm via the Tat translocon (24), a *tat* strain would not be suitable for *nosZ* expression. Furthermore, we also obtained experimental evidence for the presence in this strain of *sco* and *apbE* genes by PCR amplification and/or Southern hybridization. Our work was initiated before data for the genome of *P. putida* KT2440 became available. The sequence of KT2440 confirmed our experimental findings for strain DSM50906 and showed that valid information for the former type strain may be deduced from information for the apparently very similar organism KT2440.

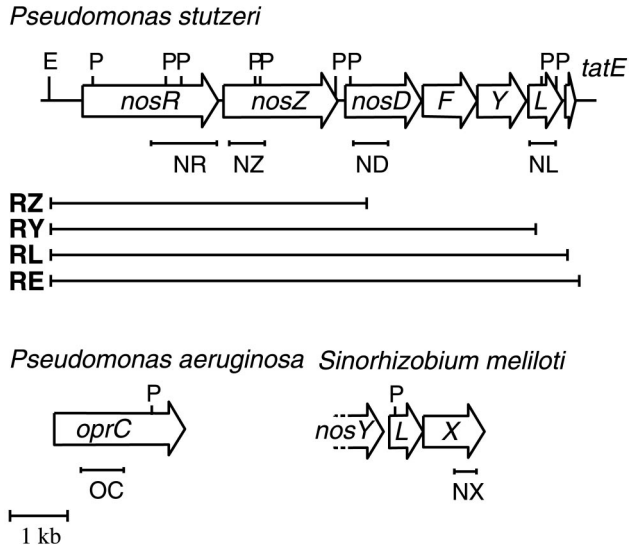

FIG. 1. *nos* gene cluster of *P. stutzeri* with maturation genes and the gene combinations used for expression in *P. putida*. RZ, RY, RL, and RE are the fragments cloned into pUCP22 that were used for heterologous expression. The locations of the DNA probes (NR, NZ, ND, NL, OC, and NX) and the corresponding source genes and host bacteria are indicated. Restriction sites: P, *Pst*I; E, *Eco*47III.

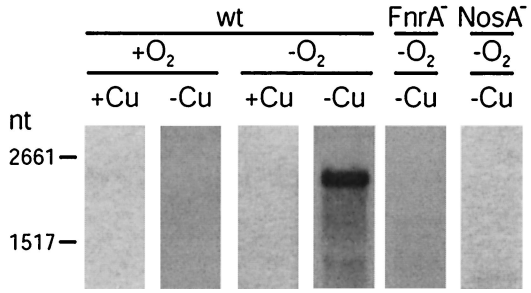

FIG. 2. Oxygen and copper repress transcription of *nosA* from *P. stutzeri*. Strains were grown in AC medium under aerobic ($+O_2$) or O_2-limiting ($-O_2$) conditions with 5 μM Cu (+Cu) or without Cu supplementation (−Cu) (residual Cu concentration, ≤0.7 μM Cu). wt, MK21 having wild-type features; FnrA⁻, mutant MKR2; NosA⁻, mutant MK499A. Total RNA was isolated and subjected to Northern blot analysis with the *nosA* probe NA (Table 2) as described in Materials and Methods. For calibration, digoxigenin-labeled RNA molecular weight marker I from Roche Diagnostics was used. nt, nucleotides.

Cloning and inactivation of *nosA*. It has been suggested that the outer membrane protein NosA is required for NosZ biosynthesis and has a Cu insertase function for NosZ of *P. stutzeri* JM300 (31, 37). A homologue of NosA, OprC, was isolated from *Pseudomonas aeruginosa* (55), but its supposed role in NosZ biosynthesis was not studied further. Because of the presence of the potential maturation gene *nosA* in *P. putida*, we set out to isolate this gene first from *P. stutzeri* ZoBell and to study whether it is necessary for NosZ maturation in an organism in which NosZ is well characterized. We screened a genomic cosmid library of *P. stutzeri* (3) with the *P. aeruginosa oprC* probe and identified a homologous locus on cosmid c61. A 6-kb *Hin*dIII fragment was cloned into pUC18, resulting in pUC18nosAH. The subclone was sequenced and analyzed for coding regions. A 2,157-bp open reading frame (ORF) had the ability to encode a 719-amino-acid protein with an M_r of 79,552, the expected mass of NosA. High levels of sequence similarity with OprC from *P. aeruginosa* and NosA from *P. stutzeri* JM300 showed that we had isolated the homologous gene. We also found that an ORF on the complementary strand, immediately downstream of *nosA*, encodes a putative transport protein with similarity to homologues in *Brucella melitensis* (SwissProt accession no. Q8YH60), *Xylella fastidiosa* (Q9PG65), *Vibrio cholerae* (Q9KV91), and *Yersinia pestis* (AAM85288).

We studied *nosA* transcription in *P. stutzeri* by Northern blot analysis. The principal regulatory signals for *nosA* expression were absence of oxygen and absence of Cu (Fig. 2). Hardly any transcriptional activity was found in aerobic cells in medium that was supplemented with Cu (5 μM) or in aerobic unsupplemented medium composed of pro analysis ingredients (Cu concentration, ≤0.7 μM). Under O_2-limiting conditions *nosA* was derepressed only in the absence of Cu. Transcription of *nosA* was not increased further by shifting cells to denitrification conditions. O_2-dependent regulation involved the Crp-Fnr-type regulator FnrA, since the *fnrA* mutant MKR2 showed no *nosA* transcript (Fig. 2).

To investigate whether NosA is necessary for NosZ biosynthesis, we constructed a *nosA* knockout mutant (see Materials and Methods). No transcript was found in mutant MK499A under conditions of maximal *nosA* transcription, which verified the mutational event (Fig. 2). MK499A exhibited no phenotype with respect to N_2O reduction. The N_2O consumption by whole cells of the mutant was comparable to that by parent strain MK21 under both Cu-deficient and Cu-supplemented conditions (data not shown). When *nosA* was repressed by Cu, no effect on NosZ synthesis was observed in the wild type. NosA was therefore excluded from the minimal set of specific factors required for NosZ biosynthesis.

Role for ScoP? Bacterial homologues of yeast Sco1 are involved in Cu processing for heme-copper oxidases. Since homologues of *sco1* are also part of *P. putida* genomes (see above), we used the same approach that was used with *nosA*. In *P. stutzeri* a *sco1* homologue, designated *scoP* (formerly *orf193*), is located downstream of *fnrA* and close to a *ccoNOQP* gene cluster encoding a cbb_3-type oxidase (14, 51). The previously described partial sequence of *orf193* was completed. Sco1 proteins have a conserved CxxxCP motif and a histidine residue, which are important for Cu binding. Sco1 homologues of *P. stutzeri* and *P. putida* both harbor these critical sequences.

scoP of *P. stutzeri* was mutagenized by replacing an internal fragment with a kanamycin resistance cassette. The deletion removed the region coding for the functionally important cysteine motif (data not shown). The resulting mutant, MK498P, was not affected in terms of growth on N_2O (in AC medium sparged with N_2O and not supplemented with Cu). When assayed by gas chromatography, it showed the same rate of N_2O reduction as the wild type. Thus, ScoP is not an obligatory factor for NosZ maturation. Overall, the studies with *P. stutzeri* allowed us to consider the presence of NosA and Sco1 homologues in *P. putida* as a supportive but not an indispensable background.

Expression of *nos* genes in *P. putida*. We constructed a set of expression vectors to examine which factors are essential for NosZ biosynthesis (Fig. 1). The pUCP22RZ vector carried *nosR* and *nosZ* and allowed us to test whether Cu incorporation occurred in the *P. putida* host in the absence of maturation factors. Two other expression vectors, pUCP22RY and pUCP22RL, carried the *nos* gene cluster of *P. stutzeri* either with or without *nosL* and allowed us to probe whether NosL has a specific role in NosZ maturation. The *nos* genes were all under the control of their native promoters. Plasmid pUCP22RE carried the transport gene *tatE* in addition to the *nos* genes (Fig. 1).

The DNA fragments were amplified by PCR prior to cloning, which may have caused accidental mutations. Therefore, we performed complementation studies with *nos* mutants to ensure the integrity of these genes. All of the expression vectors were able to restore N_2O reduction in vivo in MK413 (*nosR*::Tn*5*) and MK4211 (Δ*nosZ*::Km^r), and all of the expression vectors except pUCP22RZ complemented MK404 (*nosD*::Tn*5*) and MK417 (*nosY*::Tn*5*) (data not shown). We could not test the functionality of NosL by complementation because the *nosL* mutant MK424 exhibits a Nos^+ phenotype (18). However, as no requirement was established for NosL (see below), this did not affect our conclusions.

The growth conditions included low oxygen tension in the presence of nitrate to enable inorganic nitrate metabolism of the host strain. We used these conditions as a precautionary measure without specifically addressing the regulatory requirements in the host. Nitrate utilization has been reported previously for *P. putida* (19), and the genome of strain KT2440 contains the structural gene for assimilatory nitrate reductase. *P. putida* strain DSMZ 1088-260 was described as a heterotrophic nitrifier that reduces nitrate to nitrite with evolution of some NO under anaerobic conditions (15). A nanomolar concentration of NO was shown to be effective for inducing *nosZ* transcription (52).

Traces of NosZ were present in *P. putida* RZ grown under aerobic conditions (Fig. 3A). Under denitrifying conditions enzyme synthesis was enhanced and led to a continuous increase in the NosZ concentration in the growing culture over 18 h. There was no difference in the overall strength of *nosZ* expression between strains RZ and RE of *P. putida*. However, only one band was detected in Western blot analysis with the pUCP22RE construct, whereas the RZ derivative (Fig. 3A), as well as RY and RL, produced two protein bands accompanied by weak satellite signals.

NosZ is translocated to the periplasm of *P. putida*. Formation of two NosZ species with different masses is related in *P. stutzeri* to defects in transport and processing of the signal peptide. To probe for the location of NosZ in *P. putida*, the cytoplasmic and periplasmic cell compartments were isolated and analyzed immunochemically. With all constructs we found the largest amount of NosZ in its processed form in the periplasm (Fig. 3B). Some NosZ contamination or nonspecific cleavage of pre-NosZ was apparent in the cytoplasmic fraction. In strains RZ, RY, and RL we found unprocessed NosZ in the cytoplasm; however, we did not find unprocessed NosZ in the cytoplasm of strain RE, which contains *tatE* in addition to the *nos* genes. Thus, coexpression of *tatE* was not essential, but it ensured complete export of the enzyme to the periplasm.

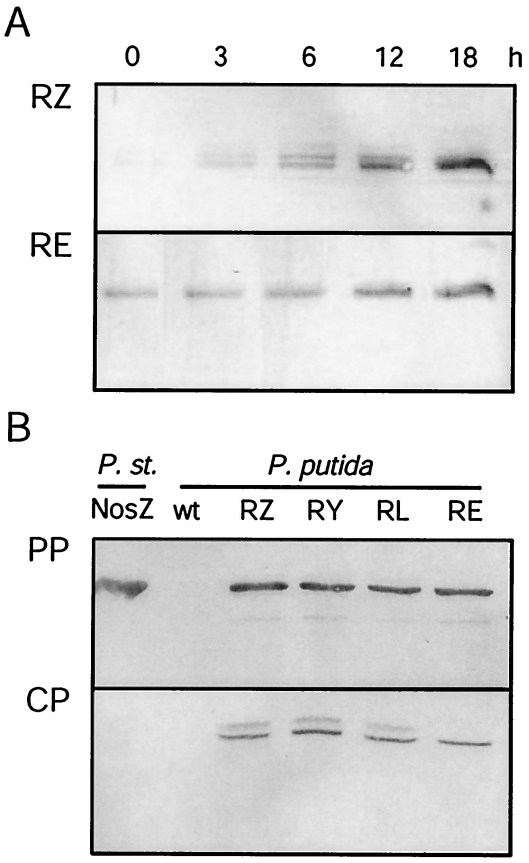

FIG. 3. NosZ is expressed in *P. putida* and translocated to the periplasm. (A) Cell extracts from growing cultures of *P. putida* strains RZ and RE were analyzed by Western blotting over an 18-h period. Coexpression of *tatE* in strain RE resulted in complete translocation of NosZ. (B) Western blot analysis of periplasm (PP) and cytoplasm (CP). RZ, RY, RL, and RE are the different *nos* gene combinations expressed in *P. putida* DSM50906 (see Fig. 1). wt, nontransformed wild type; *P. st.*, processed NosZ protein from *P. stutzeri* MK21. For the conditions used for cell growth, cell fractionation, preparation of cell extract, and immunoblotting see Materials and Methods.

***P. putida* synthesized active NosZ with three coexpressed maturation factors.** Since NosZ was located in the expression strains in its innate functional compartment, we wanted to determine whether these strains were capable of N_2O utilization. Activity measurements were obtained by gas chromatography by using whole cells and citrate as the electron donor. Data were collected over a 2-h period. No N_2O consumption was detected with the *P. putida* wild type or any of the expression strains. Although NosZ reached its functional site, whole cells were not able to respire N_2O. We isolated the periplasm of *P. putida* RL and fractionated it by gel permeation chromatography on Sephacryl S-300 to analyze NosZ for the presence of Cu as described previously (7). A Cu peak was clearly associated with NosZ (data not shown). Thus, the absence of cellular N_2O reduction was not due to a lack of prosthetic Cu. A likely explanation for the lack of in vivo activity is absence of an appropriate electron donor; i.e., endogenous electron donors of *P. putida* are not able to couple to NosZ. We attempted to complement the defect by transforming approximately 20-kb

TABLE 3. Characteristics of recombinant NosZ proteins from *P. putida*

Strain	Absorbance (nm)[a]	NosZ species	Sp act (U/mg of protein)[b]
RZ	480, 535, 785	Type V (Cu_A)	0.026 ± 0.028 (3)
RY	485sh, 539, 630sh, 782	Type I (Cu_A and Cu_Z)	1.224 ± 0.180 (4)
RL	480sh, 538, 625sh, 780	Type I	0.662 ± 0.093 (4)
RE	485sh, 535, 637, 777	Types I and II (Cu_Z*)	2.007 ± 0.340 (4)

[a] sh, shoulder.
[b] Mean ± standard deviation (number of measurements).

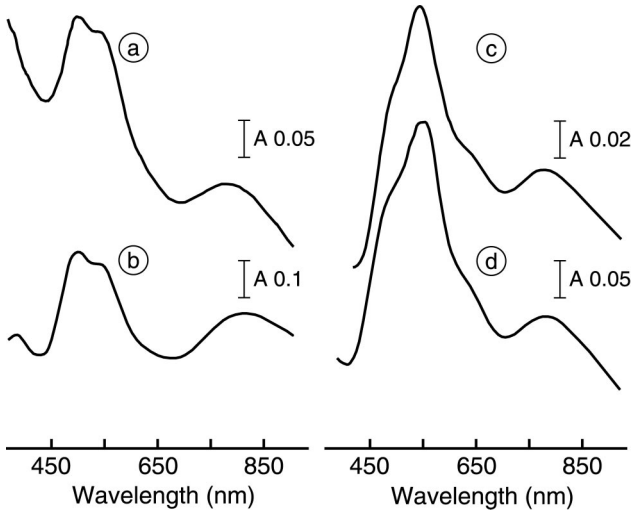

FIG. 4. Electronic absorption spectra of recombinant NosZ proteins. Spectrum a is the spectrum for NosZ isolated from *P. putida* strain RZ expressing *nosR* and *nosZ*. The isolated protein exhibits a Cu_A-type spectrum. Spectrum b is the spectrum for a Cu_A-type protein, represented by the soluble domain from *P. denitrificans* cytochrome *c* oxidase subunit II and reconstituted in vitro with Cu(II) (29). Spectrum c is the spectrum for NosZ isolated from *P. putida* strain RY expressing *nosRZDFY*. The isolated protein exhibits the spectrum of the purple species (type I) of NosZ, which represents the high-activity enzyme form. Spectrum d is the spectrum for the purple species of NosZ isolated from denitrifying *P. stutzeri* MK4211(pSZ) (7). All spectra were recorded in 50 mM Tris-HCl (pH 7.5).

fragments of genomic *P. stutzeri* DNA cloned in vector pUCP22RY into *P. putida*, but we could not convert the heterologous host into an N_2O-respiring bacterium.

As the next step we wanted to determine whether enzyme purified from the expression strains exhibited in vitro activity. All recombinant enzymes except the enzyme obtained from *P. putida* RZ reduced N_2O (Table 3). Bleaching of benzyl viologen was proportional to the amount of NosZ in the assay mixture. The specific activity ranged from 0.7 to 2 $U \cdot mg$ of $protein^{-1}$ and was in the range of values found with enzyme preparations from *P. stutzeri* (11, 44). Holo-NosZ contains a total of 12 Cu atoms in a dimeric molecule. The numbers of Cu atoms in the isolated NosZ proteins of *P. putida* strains were lower (8.9 Cu atoms for RL and 9.6 Cu atoms for RE) but were reasonably close to the theoretical value.

UV-visible absorption spectra of the purified enzyme were recorded. NosZ from *P. putida* RZ had the spectral properties of a Cu_A-only protein (Fig. 4, spectrum a, and Table 3). The same features were found with NosZ from the *nosD* mutant MK402 (45) or a reconstituted recombinant enzyme isolated from *E. coli* (50). The properties were also nearly identical to the properties of an engineered soluble Cu_A-binding domain of cytochrome *c* oxidase from *P. denitrificans* after reconstitution with $CuCl_2$ (Fig. 4, spectrum b). In this case absorption maxima were present at 363, 480, 530, and 808 nm (29). In accordance with the spectral properties, we found a metal content of 3.8 Cu atoms, as expected for a Cu_A-only NosZ.

Absorption maxima at 540 and 780 nm with a slight shoulder at 480 nm are characteristic of the purple form (type I) of NosZ isolated under anaerobic conditions (11, 45). NosZ obtained from *P. putida* RY displayed a spectrum that was nearly superimposable with the spectrum of the purple enzyme from *P. stutzeri* (Fig. 4, spectra c and d). NosZ from *P. putida* RL and NosZ from *P. putida* RE (slightly more pronounced) also exhibited a small peak around 630 nm. This peak is indicative of a spectral contribution from the pink form (type II) of NosZ. The pink form is generated during aerobic purification. It seems to be a mixture of species that have lost part of Cu_Z and have reacted with oxygen to form a Cu_Z* species having a yet-to-be-defined structural modification (43). *P. putida* had to be aerated during the entire growth cycle, which may have affected the enzyme and resulted in partial oxygen damage. Overall, NosZ from *P. putida* showed the same variability in Cu content, enzyme activity, and spectral variations as *P. stutzeri*. We attribute enzyme lability and the bulk of catalytic site heterogeneity to the Cu-S cluster.

DISCUSSION

NosZ maturation, a matter of topology. In initiating this study it was important to resolve the transport of NosZ by finding evidence for the Tat translocon in host strain DSM50906 by Southern hybridization. In all *P. putida* strains carrying a *nosZ* expression plasmid the enzyme was found in the periplasm; i.e., the Tat system of *P. putida* processed NosZ without requiring any specific recognition factor for the reductase (Fig. 3B). In *P. putida* RZ part of NosZ was found in its preform, indicating that there was saturation of the translocation system or some difficulty in the processing of the heterologous protein. This was relieved by coexpression of *tatE* from *P. stutzeri*. The findings for *P. putida* parallel those reported previously for *P. stutzeri* (24), in which *tatE* seems to have a dedicated but not obligatory role in NosZ translocation within the Tat translocon.

Formulating the sequence of assembly events requires knowledge concerning in which cellular compartment the process takes place. By subjecting cells to Cu deficiency in a metal-extracted medium it is possible to separate NosZ export and Cu insertion and show that the maturation process is periplasmic and not cytoplasmic (36). We confirmed the previous findings by fractionating *P. stutzeri* cell compartments, performing Western blotting, and measuring the activity of whole cells by gas chromatography. Cu-deficient cells of *P. stutzeri* export NosZ to the periplasm but exhibit no or little

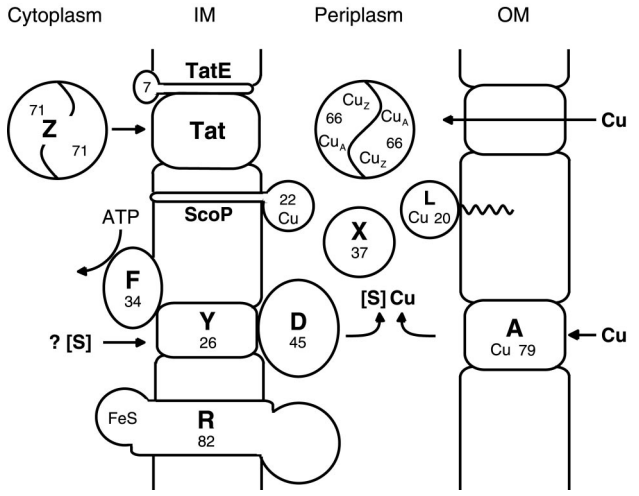

FIG. 5. Components of the maturation process for N_2O reductase and topology. Single uppercase letters indicate the products of *nos* genes. The numbers indicate the approximate protein masses (in kilodaltons). Cu-containing proteins are indicated. NosZ is shown as a dimer, but otherwise no inferences about stoichiometries of protein complexes are drawn, nor is the composition of the Tat translocon indicated other than to show the supportive role of TatE. NosF has ATPase activity (Honisch and Zumft, unpublished data). NosD belongs to a protein family with carbohydrate and sugar hydrolase signatures (10). [S] is a sulfur donor whose chemical nature is not known. NosL is shown with a lipid anchor. Cu may enter the periplasm via NosA or another porin. The membrane-bound NosR carries putative FeS groups in its cytoplasmic domain and may have other functions in addition to acting as a transcriptional regulator for *nosZ*. IM and OM, cytoplasmic and outer membranes, respectively. For further discussion see the text.

NosZ activity. The activity is restored by adding Cu to cells arrested for protein synthesis (data not shown). This means that periplasmic NosZ undergoes Cu-dependent maturation posttranslocationally. On the other hand, NosZ can be retained in the cytoplasm by mutating the Tat-specific signal peptide (17) or by inactivating the principal transport gene, *tatC* (24). In either case cytoplasmic NosZ does not incorporate Cu.

NosZ synthesis by *P. putida* and an integrated model for enzyme maturation. We propose a topological model for NosZ maturation which integrates the current experimental data and in silico evidence (Fig. 5). Apo-NosZ is exported prior to and independent of cofactor insertion. A Cu_A-only NosZ protein is formed by *P. putida* RZ in the absence of *P. stutzeri*-specific *nos* maturation functions. In contrast, Cu_Z assembly depends on the coexpression of *nosDFY*, and only under such conditions was a catalytically active NosZ protein obtained with the spectral features of both the Cu_A and Cu_Z species (NosZ type I). This attributes to NosDFY a role in the assembly of the Cu_Z center, and its function is more likely to be in the provision of sulfur than that of Cu. Cu_A can be reconstituted in vitro into the apoprotein from exogenous Cu, whereas attempts to do this for Cu_Z have failed. This fact can be explained by the lack of an appropriate sulfur source. We propose that sulfur is provided from a cytoplasmic source through the action of the NosDFY ABC transporter system. Cu from the medium may pass through NosA or another cation-permeable pore and is delivered to the site of Cu cluster biosynthesis by a Cu chaperone, possibly NosL. Pathways for Cu and sulfur donation thus converge in the periplasm for Cu-S cluster formation. Beyond the minimal requirement for NosDFY in the heterologous host, proteins of *P. putida* may provide rescue functions, and considering these proteins helped us address further requirements for NosZ maturation. The nonobligatory components of our model and the arguments for why they should be considered are discussed below.

NosA, the putative component of a metal uptake system. A phylogenetic tree shows that the NosA proteins occur in a tight subcluster of a larger family of siderophore-binding or heme receptor proteins, such as HasR, FhuA, and other proteins (Fig. 6). A relationship between NosA and TonB-dependent proteins was noted previously (30, 55). A TonB-box C motif exists near the C terminus, but a corresponding TonB box near the N terminus has not been found. Other than the association with the genes encoding NosX and NosL (see below), NosA is not consistently associated with *nos* genes. Conspicuously, NosA homologues occur in the nondenitrifying bacteria *Yersinia pestis* CO92, (41), *P. putida* KT2440, and *Pseudomonas fluorescens* PFO-1. The high degrees of amino acid sequence identity of these proteins (up to 54%) with NosA from denitrifiers suggest that there is functional uniformity.

The observed regulatory responses allow the conclusion that the outer membrane protein, NosA, functions in anaerobic metabolism, and since it is repressed by Cu, its role seems to be limited to conditions in which the Cu supply is low. NosA might be involved in Cu uptake and represent the outer membrane pore for Cu ion or Cu chelator passage (30) rather than provide a Cu insertase activity for NosZ. The promoter region has a CTTCCCGAAA sequence similar to that of the binding region for the Cu-dependent regulator CopR of *Pseudomonas syringae* (35). Furthermore, the *nosA* promoter has a degenerate FNR box, TTGAC-N_1-GTCAA, and because of the spacing of the palindromes, only one half-site may act as a recognition site for the FnrA regulator for the upregulation observed under anaerobic conditions (Fig. 2).

NosA was purified as a Cu-containing protein. Its spectral properties and mode of Cu binding are not known (30). Alignment of the NosA proteins reveals a set of conserved Cys, His, and Met residues which could bind Cu as blue or type 1 Cu. *Neisseria gonorrhoeae* has in AniA an outer membrane protein that functions as a nitrite reductase and has a type 1 Cu electron transfer site (27); however, NosA has no sequence similarity to AniA. While chemically induced *nosA* mutants of *P. stutzeri* JM300 synthesize a virtually Cu-free N_2O reductase (37), the *nosA* knockout mutant MK499A of *P. stutzeri* ATCC 14405 displayed no phenotype with respect to NosZ activity and Cu content (this study). It is possible that the inability of JM300 mutants to reduce N_2O was due to the lack of protein E (30), which might be a component of the NosDFY assembly apparatus.

ScoP, a candidate protein for Cu_A assembly. *P. putida* RZ synthesizes a NosZ protein whose spectral features clearly show that only Cu_A is metallated (Fig. 4, spectrum a). It is feasible that the biosynthesis of Cu_A was dependent on a host function for synthesis of the same center of cytochrome aa_3. *P. putida* KT2440 has an aa_3-type oxidase. Studies of cytochrome aa_3 biogenesis in yeast support the hypothesis that incorpora-

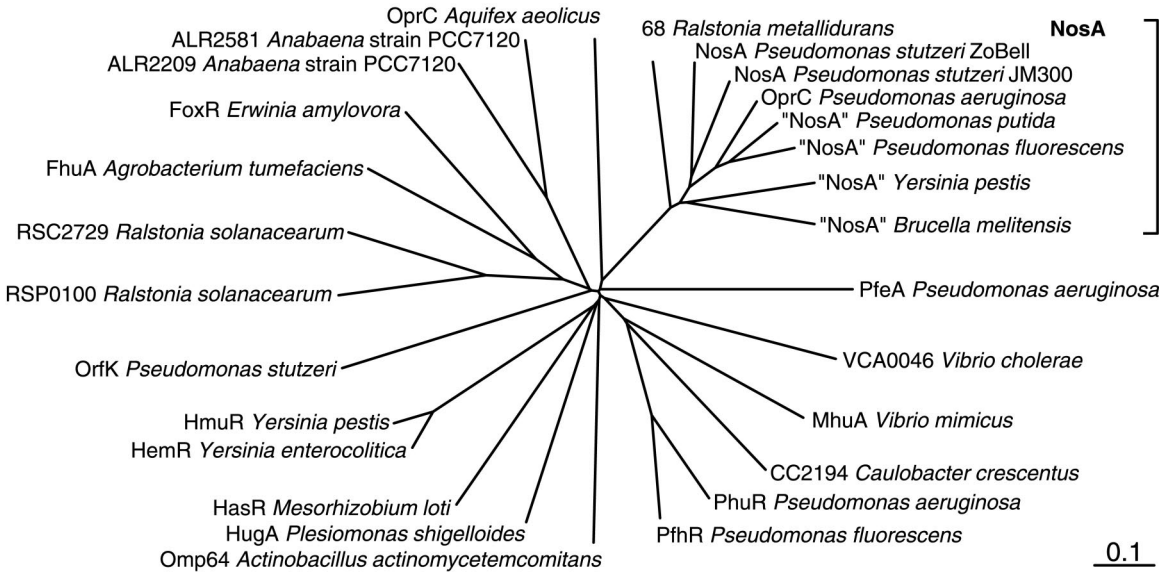

FIG. 6. Phylogenetic tree of NosA proteins and homologues. The SwissProt data bank and genome project databases were searched with *P. aeruginosa* OprC by using FASTA3. A cutoff value of 10^{-4} was used. The tree was constructed with CLUSTAL X and TreeView 1.6.6. Several bacteria contain more than one member of this family.

tion of Cu_A is catalyzed by Sco1 and the Cu chaperone Cox17. Cu(I) is thought to be transferred from Cox17 to Sco1, which inserts Cu into Cu_A of subunit II (23, 38). In *Bacillus subtilis* the Sco1 homologue YmpQ affects cytochrome *c* oxidase but not menaquinol oxidase, thus favoring a role in Cu_A synthesis (32). The soluble domain of the Sco1 homologue PrrC from *Rhodobacter sphaeroides* has thiol disulfide oxidoreductase activity which can be used for Cu mobilization (33). Thus, with respect to a Sco requirement, we found a homologue in each of the available genomes of *nosZ*-harboring strains. It is interesting in this context that *Rhodobacter capsulatus* contains the homologue SenC (accession no. Q52720), and even though this bacterium has no oxidase with a Cu_A center, it disposes over NosZ. However, in spite of these multiple lines of indirect evidence, the ScoP protein was dispensable for NosZ biosynthesis in *P. stutzeri*. We presume that loss of ScoP did not result in a recognizable phenotype because metallation of Cu_A also proceeds spontaneously or a substitute protein involved in Cu processing takes over.

***nosL* is a constant partner of *nos* gene clusters.** Sixteen individually analyzed denitrifiers and entire genomes show that in each case a *nosDFYL* gene cluster is present and conserved. *nosL* is cotranscribed with *nosDFY* in *P. stutzeri* (U. Honisch and W. G. Zumft, unpublished data), which indicates that NosL has a function related to NosZ maturation. NosL was purified from *Achromobacter cycloclastes* as a Cu-containing protein. While the Cu(I) site of NosL is remarkably stable in the presence of oxygen, the Cu(II) form has little affinity for Cu and releases the metal (34). These properties support the hypothesis that NosL has a metallochaperone role and that the putative function is to guide Cu from the site of periplasmic entry to N_2O reductase. Nevertheless, the plausible role of NosL in NosZ maturation is not obligatory. Coexpression of *nosL* was not a requirement for a functional NosZ in *P. putida*.

Random Tn*5* mutagenesis and selection for loss of growth on N_2O resulted in mutants with insertions in each gene of the *nosRZDFYL* cluster except *nosL* (6, 26, 49). Since NosL is a nonselectable marker, this suggests that there is a functional substitute for NosL or NosL is dispensable. Also, a *nosL* mutant lacks a recognizable phenotype (18). No *nosL* homologue was detected in DSM50906 by Southern hybridization, and none was evident in the genome of KT2440, which suggests that *P. putida* has several ways to process Cu for its Cu proteins, which may provide a rescue function for NosZ biosynthesis.

ApbE as a functional NosX homologue in NosZ maturation. In individual studies of *nos* gene clusters, a *nosX* gene was found in *S. meliloti* and several other bacteria but not in the well-studied denitrifiers *P. stutzeri* and *P. aeruginosa*. Since the absence of *nosX* in the pseudomonads would bring into question the role of this gene in encoding an essential maturation component, we addressed the distribution of *nosX* in genomes of denitrifiers in silico and also searched for a potential rescue function.

NosX proteins exhibit high sequence similarity among themselves but not with other proteins (46), although sequence similarity to RnfF has been noted (6). RnfF is a membrane-bound periplasmic protein belonging to an *R. capsulatus* complex presumably involved in electron transport for nitrogen fixation (47). A data bank search revealed that *P. putida* KT2440 carries along with *apbE* a potential *nosX* homologue; the same gene was amplified by PCR from strain DSM50906. ApbE is a 36-kDa monotopic inner membrane protein, and most of its soluble domain is located in the periplasm (2). A periplasmic location but not membrane association is essential for the ApbE function directed at ThiH. The latter protein is a putative FeS protein involved in the last step in synthesis of the 4-methyl-5-β-hydroxyethylthiazole monophosphate moiety of thiamine monophosphate. ApbE is thought to be involved in the redox-dependent synthesis or repair of ThiH as part of a

membrane-associated complex (22). ApbE shows sequence similarity to RnfF, and most importantly, *rnfF* is able to complement a *Salmonella enterica* serovar Typhimurium *apbE* mutation (2). We argue that NosX and ApbE are functionally interchangeable members of the same protein family in order to account for the dispensability of *nosX* coexpression in the *P. putida* background. The mass of NosX is comparable to the mass of ApbE, and NosX is predicted to be periplasmic because of a signal sequence with features for Tat targeting (46). Location of NosX in the outer cell compartment is consistent with the site of Cu cluster biosynthesis. The release of Cu and/or sulfide from the corresponding donor molecules requires redox steps, and NosX may fulfill such a role.

We found that there was a consistent association of the core *nosRZDFY* cluster with the *nosX* or *apbE* genes as determined by a data bank search. *A. cycloclastes* (accession no. AF047429), *Bradyrhizobium japonicum* (AJ002531), *B. melitensis* (16), *P. denitrificans* (46), *Rhodopseudomonas palustris* (genome), and *S. meliloti* (6, 26) all have a *nosRZDFYLX* gene organization. *nosX* or *apbE* loci may also be distant from *nos* genes. In *N. gonorrhoeae* the *nosX* gene is 42 kb downstream of *nosL* and a putative *apbE* gene is 18 kb upstream of *nosR*. A single *apbE* locus, separated from the *nos* cluster, is present in the genome of *P. aeruginosa* (PA2993). Screening the genomes of *Ralstonia metallidurans*, *Ralstonia solanacearum*, *Burkholderia mallei*, and *Burkholderia pseudomallei* for an *apbE* homologue in each case resulted in an ORF approximately 1 kb upstream of *nosZ* in the opposite transcriptional orientation. These ORFs may represent functional *nosX* genes. In the latter group of bacteria *nos* clusters are organized so that *nosR* is located either immediately downstream of *nosZ* (*R. metallidurans* and *R. solanacearum*; *nosX-nosZRDFYL*) or downstream of *nosL* (*B. pseudomallei* and *B. mallei*; *nosX-nosZDFYLR*).

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