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Identification and Analysis of the Dissimilatory Nitrous Oxide Reduction Genes, *nosRZDFY*, of *Rhizobium meliloti*†

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The complete *nos* region essential for dissimilatory nitrous oxide reduction by the endosymbiotic diazotroph *Rhizobium meliloti* was identified in a cosmid (pYC7) carrying a 10.1-kb *Eco*RI fragment of the *nod* megaplasmid. This gene region was localized by Southern hybridization and Tn*5* mutagenesis to within 8 kb downstream from the *fixGHIS* cluster. Nucleotide sequence determination of a 4.6-kb DNA segment including the structural gene *nosZ* and its flanking regions showed sequence homology and similarity in genetic organization with the *nosRZDFY* genes of *Pseudomonas stutzeri* Zobell. The genes were arranged in three complementation groups, comprising the *nosZ* structural gene, the *nosR* regulatory gene, and the *nosDFY* copper-processing genes. The derived amino acid sequence of the *R. meliloti nosZ* product (a multi-copper nitrous oxide reductase) was more similar to those of the analogous gene products of *Paracoccus* and *Pseudomonas* species than to that of *Alcaligenes eutrophus*. The *nosZ* gene was preceded by *nosR*, which encodes a regulatory protein containing C-terminal cysteine clusters similar to those present in the 4Fe-4S binding region of bacterial ferredoxins. The *nosDFY* genes, located downstream from *nosZ*, were identified as copper-processing genes encoding a periplasmic protein, an ATP/GTP-binding protein, and a membrane protein, presumably forming a copper-processing system. A consensus sequence for an Anr- or Fnr-binding site similar to that in the upstream sequence of *nosZ* in *Paracoccus denitrificans* or *P. stutzeri* was absent in *R. meliloti*. No *rpoN*-binding site preceding the *nos* genes was detected, and none of the Tn*5* insertions in the *nos* gene region affected symbiotic N_2-fixing ability.

Nitrous oxide (N_2O) respiration or its dissimilatory reduction is one of the three optional reactions that conveniently characterize denitrification (52). Only when it is coupled with nitrate and nitrite respiration (including nitric oxide reduction), resulting in gas production, is denitrification sensu stricto constituted. It is then considered the ultimate step, catalyzed by N_2O reductase (EC 1.7.99.6), in the complete denitrification reaction sequence. The reductase invariably contains copper and is periplasmically located (54), with the exception of the molybdo-iron nitrogenase complex that also catalyzes N_2O reduction. Interestingly, the respiration of N_2O is not carried out by certain denitrifiers, and not every denitrifier can grow on N_2O. However, a nondenitrifying species such as *Wollinella* can respire N_2O (51).

N_2O reductase is the first denitrification enzyme that was purified from *Pseudomonas stutzeri* Zobell (30), and its structural gene, designated *nosZ*, is also the first denitrification gene whose nucleotide sequence was determined (46). The genes required for N_2O reduction by this organism are located on the chromosome and are the best studied among the common denitrifiers. It has been identified in an 8-kb region by mutational analysis and subsequently found to consist of five genes, *nosRZDFY* (9, 45, 46, 55). The *nosDFY* genes downstream from *nosZ* have been proposed to possess copper-processing functions, while *nosR*, upstream of *nosZ*, is regulatory in function. The *nosZ* gene of *Pseudomonas aeruginosa* is highly homologous to that of *P. stutzeri* (53). Less closely related denitrifiers from which the *nos* genes have been analyzed are *Paracoccus denitrificans* (25) and *Alcaligenes eutrophus* (53). The *nos* and other denitrification genes (*nir* and *nor*, respectively, for nitrite and nitric oxide reductases) of *A. eutrophus* H16 are borne on a 450-kb plasmid (pHG1) that also carries multiple genes involved in H_2 metabolism and autotrophic CO_2 fixation (20). The extrachromosomal location of denitrification genes suggests that nondenitrifiers may acquire these genes by plasmid transfer.

Several dinitrogen (N_2)-fixing genera are known to contain denitrifiers (6). One of these is *Rhizobium meliloti* (5), which forms a well-known endosymbiosis with alfalfa in which its differentiated bacteroids fix atmospheric N_2 inside the plant root nodules. Denitrification can be readily observed in many species of the genus *Rhizobium*, in the free-living form, in legume root nodules, or as isolated bacteroids (31). Although it has not been established in alfalfa nodules elicited by denitrifying strains of *R. meliloti* whether N_2O is primarily reduced by N_2O reductase or nitrogenase, the former has been shown to be the major pathway of N_2O reduction in detached cowpea nodules formed by a denitrifying rhizobial strain (8). The *nosZ* structural gene of *R. meliloti* has been located on one of its two symbiotic plasmids (7), the 1.4-Mb *nod* megaplasmid (or pNOD) which carries *nod*, *nif*, and *fix* genes essential for nodulation and symbiotic N_2 fixation with alfalfa (34). The other symbiotic plasmid, the 1.7-Mb *exo* megaplasmid (or pEXO), carries genes required for effective nodulation and endosymbiotic metabolism. The two megaplasmids constitute almost half of the *R. meliloti* genome compared with its 3.4-Mb chromosome (43).

Although the *nosZ* gene of *R. meliloti* possesses detectable DNA homology to that of *P. stutzeri* (7), its unique location on a symbiotic megaplasmid may distinguish its *nos* locus structurally or organizationally from those reported for the common free-living denitrifiers. The objectives of this work were to identify and characterize the *nos* locus in *R. meliloti*, including

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TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Characteristics[a]	Reference(s) or source
R. meliloti		
JJ1c10	Wild-type strain; Nos$^+$ Fix$^+$ Rif^r	39
RmYC2160	Spontaneous Nal^r Rif^r derivative of BALSAC; Nos$^-$ Fix$^+$ Nal^r Rif^r	7
RmYC2164	Spontaneous Nal^r derivative of ATCC 9930; Nos$^-$ Fix$^+$ Nal^r Rif^s	7
RmWM1179	JJ1c10::Tn*5-7C35*, genomic Tn*5* insert introduced by recombination; Nos$^+$ Fix$^+$ Rif^r Km^r	This work
RmWM1184, RmWM1191	*nosR*::Tn*5-878*, *nosR*::Tn*5-B42*, JJ1c10 derivatives with genomic Tn*5* inserts introduced by recombination; Nos$^-$ Fix$^+$ Rif^r Km^r	This work
RmWM1185, RmWM1194	*nosZ*::Tn*5-893*, *nosZ*::Tn*5-C11*, JJ1c10 derivatives with genomic Tn*5* inserts introduced by recombination; Nos$^-$ Fix$^+$ Rif^r Km^r	This work
RmWM1186, RmWM1190	*nosF*::Tn*5-A28*, *nosF*::Tn*5-B13*, JJ1c10 derivatives with genomic Tn*5* inserts introduced by recombination; Nos$^-$ Fix$^+$ Rif^r Km^r	This work
RmWM1192	*nosD*::Tn*5-B90*, JJ1c10 derivative with genomic Tn*5* inserts introduced by recombination; Nos$^-$ Fix$^+$ Rif^r Km^r	This work
4R21	*rpoN* mutant of JJ1c10 derived by Tn*5* mutagenesis; Nos$^+$ Fix$^-$ Rif^r Km^r	48
GMI360 (=RmΔHG3.1)	Deletion mutant of SU47 lacking two contiguous *Hin*dIII (8 and 14 kb) fragments downstream from *fixGHIS* on pNOD; Nos$^-$ Fix$^+$ Rif^r	T. Huguet, INRA,[b] Toulouse, France
E. coli DH5α and HB101	Host strains	3, 24
Plasmids		
pRK2013	ColE1 replicon carrying RK2 transfer genes; Km^r	16
pRK600	pRK2013::Tn*9* derivative; Cm^r	17
pRK310	Broad-host-range cloning vehicle; Tc^r	12
pLAFRI	Broad-host-range cosmid cloning vehicle, IncP-1; Tc^r	19
pVK100	Broad-host-range cosmid cloning vehicle; Km^r Tc^r	29
pPW11	Derivative of pLAFRI containing *Bam*HI, *Eco*RI, and *Pst*I cloning sites; Tc^r	R. L. Robson, University of Georgia, Athens
pNS220	1.2-kb *Pst*I-internal fragment of *nosZ* from *P. stutzeri* Zobell (ATCC 14405); Ap^r Cm^r Tc^r (pBR325)	W. G. Zumft, Universität Karlsruhe, Karlsruhe, Germany; 46
pRWRm67	Cosmid clone selected from JJ1c10 *Eco*RI-digested genomic library by hybridization to pNS220; Tc^r (pLAFRI)	7
pYC7	Cosmid clone selected from JJ1c10 *Hin*dIII-digested genomic library by hybridization to pNS220; Tc^r (pVK100)	This work
pWM2	3.6-kb *Bam*HI fragment of pYC7; Tc^r (pPW11)	This work
pWM3	6.1-kb *Pst*I fragment of pYC7; Tc^r (pPW11)	This work
pWM4	10.1-kb *Eco*RI fragment of pYC7, carries entire *nos* region; Tc^r (pPW11)	This work
pWM5	14.5-kb *Hin*dIII fragment of pYC7; Tc^r (pRK310)	This work
pWM20	0.6-kb *Eco*RI-*Hin*dIII fragment of pYC7; Ap^r (pUC19)	This work
pBB144	Cosmid clone containing *Eco*RI fragments of JJ1c10 pNOD, carries *fixLJ* and adjacent *fix* genes; Tc^r (pLAFRI)	This work
pBB155	10.1-kb *Eco*RI fragment from pWM4, carries entire *nos* region; Ap^r (pUC19)	This work
pBB149	2.4-kb *Eco*RI-*Sph*I fragment of pBB155; Ap^r (pUC19)	This work
pBB150	0.6-kb *Bam*HI fragment of pBB155; Ap^r (pUC19)	This work
pBB151	3.6-kb *Bam*HI fragment of pBB155; Ap^r (pUC19)	This work
pBB152	2.1-kb *Sph*I fragment of pBB155; Ap^r (pUC19)	This work
pBB153	0.53-kb *Sph*I fragment of pBB155; Ap^r (pUC19)	This work

[a] Resistance to antibiotics: Ap^r, ampicillin; Cm^r, chloramphenicol; Km^r, kanamycin; Nal^r, nalidixic acid; Tc^r, tetracycline; Rif^r, rifampin.
[b] INRA, Institut National de la Recherche Agronomique.

the nucleotide sequencing of *nosZ* and its flanking regions. The sequence of *nosZ* and the translated amino acid sequence were also compared with those of several free-living denitrifiers. Genetic delineation by transposon mutagenesis and complementation analysis revealed three adjacent complementation groups identified as the *nosR*, *nosZ*, and *nosDFY* genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. *R. meliloti* RmYC2160 and RmYC2164 are Nal^r derivatives of the Nos$^-$ strains BALSAC and ATCC 9930, respectively, used as conjugation recipients. *R. meliloti* was grown aerobically on TYC (tryptone-yeast extract-calcium) medium (15) at 30°C. *Escherichia coli* strains were grown in

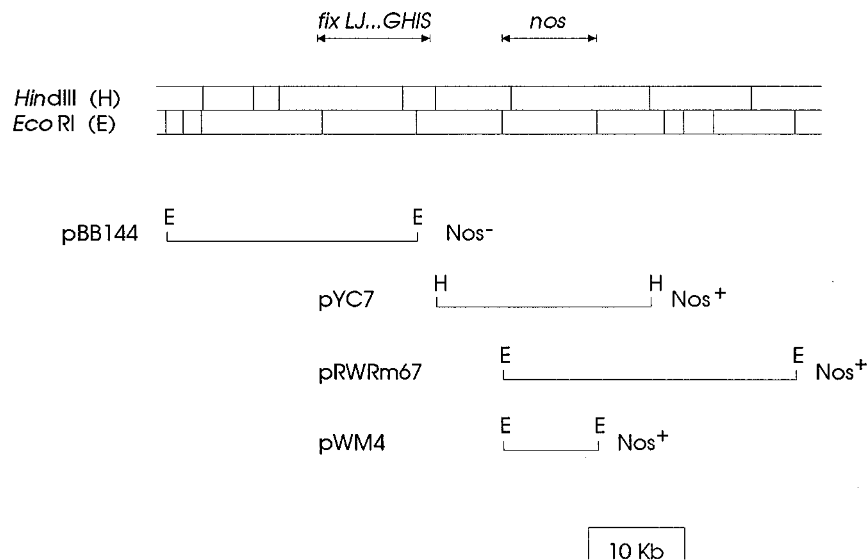

FIG. 1. Restriction map of pNOD in *R. meliloti* JJ1c10 showing the position of the *nos* region relative to the *fix* gene cluster. Cosmids and broad-host-range plasmids carrying *nos* or *fix* genes and their abilities to confer Nos activity to *R. meliloti* ATCC 9930 and BALSAC are shown at the bottom. The *fix* gene cluster includes *fixLJ*, *fixK*, *fixNOQP*, and *fixGHIS* (18).

Luria-Bertani medium (36) at 37°C. The antibiotics used were ampicillin (50 μg/ml), chloramphenicol (15 μg/ml), kanamycin (20 or 40 μg/ml), nalidixic acid (10 μg/ml), tetracycline (5 or 10 μg/ml), and rifampin (100 μg/ml).

Bacterial conjugation and DNA manipulations. Plasmids were introduced into *R. meliloti* from *E. coli* by tripartite mating using either pRK2013 or pRK600 as the helper plasmid (13). DNA restriction, agarose gel electrophoresis, ligation, and transformation were carried out by standard methods (36). Tn*5* mutagenesis of cosmid pYC7 was done as described by Ditta (11). Cosmids with Tn*5* insertions were recombined into the wild-type *R. meliloti* JJ1c10 genome by the marker-exchange technique of Ruvkun and Ausubel (35). In Southern hybridization experiments, DNA probes were labeled with digoxigenin-11-dUTP (Boehringer Mannheim Canada, Laval, Quebec, Canada) by random priming as recommended by the manufacturer. Chemiluminescent digoxigenin antibodies from the same manufacturer were used to detect the hybrids upon exposure of the DNA blots to Kodak X-Omat AR or DuPont Reflection autoradiography films. Other genetic manipulations have previously been described (7, 50).

DNA sequencing. Nucleotide sequence was determined by the dideoxynucleotide chain termination method (37). DNA sequencing was performed with a Sequenase T7 polymerase kit (U.S. Biochemical Corp., Cleveland, Ohio) and ^{33}P-labelled dATP (Amersham Canada, Oakville, Ontario, Canada). It was done by using a series of overlapping plasmids derived from pBB155 and nested deletions constructed from pBB151 (Exo-Size deletion kit; New England Biolabs, Mississauga, Ontario, Canada) and pBB153 (Table 1). The primers used were the M13 universal and reverse primers (U.S. Biochemical Corp.) on deletions, while gaps in the sequence were filled by using synthetic primers prepared with a Biosearch 8700 oligonucleotide synthesizer. Additional sequencing was done with a Sequitherm Cycle sequencing kit (Epicentre Technologies, Madison, Wis.) according to the manufacturer's instructions. Sequence data were assembled and analyzed with PC/Gene software (IntelliGenetics Inc., Mountain View, Calif.).

Activity assays. The specific activity of N_2O reductase in *R. meliloti* strains was determined in 8-ml anaerobic tube cultures as previously described (7) except that TYC medium was used instead of the modified Vincent's defined medium and the cells were washed with saline (0.85% NaCl solution) before protein analysis. Similarly grown cells were also used to assay dissimilatory nitrite (NO_2^-) and nitric oxide (NO) reductase activities. Sodium nitrite (0.1 mM) and NO (164 nmol) were added as the denitrification substrates for the NO_2^- and NO reductase assays, respectively. In the former, the NO_2^- in the medium was monitored colorimetrically by the Griess reaction. To monitor NO reduction to N_2O, which accumulated in the headspace in the presence of 10% (vol/vol) acetylene, N_2O was determined by electron capture gas chromatography (4). Both assays were carried out within 24 h of incubation.

Plant tests. *Medicago sativa* (cv. Anchor) seedlings were prepared and grown axenically in autoclaved vermiculite saturated with an N-free plant nutrient solution (41) in test tubes (25 by 150 mm) closed with translucent caps (two plants per tube). Twenty plants were inoculated with each *R. meliloti* strain to be tested and maintained in a constant-environment chamber at 22°C under the light regimen previously described (49). After 8 weeks, the plants were inspected for growth as an indicator of the Fix (N_2 fixation) phenotype by comparison with uninoculated control plants and those inoculated with the effective wild-type *R. meliloti* JJ1c10.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to GenBank/EMBL data banks and assigned accession number U47133.

RESULTS

Identification and delineation of the *nos* region. Wild-type *R. meliloti* JJ1c10 expresses the complete denitrification pathway. Denitrification activities are, however, totally absent from *R. meliloti* BALSAC, while strain ATCC 9930 is a denitrifier but unable to reduce N_2O (7). We have previously described a region of the *R. meliloti* JJ1c10 pNOD megaplasmid carried by a pLAFRI cosmid, pRWRm67, capable of imparting N_2O reductase activity (Nos) to *R. meliloti* ATCC 9930 and BALSAC (7). In this cosmid, the *nos* region was near the junction of the vector and the inserted *R. meliloti* DNA. To facilitate analysis of the *nos* genes and adjacent regions by Tn*5* mutagenesis, we first isolated other cosmids with the *nos* genes located near the center of the inserted DNA. A genomic library of JJ1c10 DNA cloned in cosmid pVK100 after partial digestion with *Hin*dIII was probed with pNS220, which carries a major internal portion of the *P. stutzeri nosZ* gene. Cosmid pYC7, carrying contiguous 8-kb and 14-kb *Hin*dIII fragments (Fig. 1), was thus obtained. Cosmid pYC7 is similar to pRWRm67 in that it is able to confer the Nos$^+$ phenotype to both *R. meliloti* ATCC 9930 and BALSAC, and both cosmids contain a 10.1-kb *Eco*RI fragment in which the *nos* region has been localized. The requirement for this fragment was confirmed by demonstrating that pWM4, a derivative carrying only the 10.1-kb *Eco*RI fragment (Fig. 1), was able to confer Nos activity. Additional assays using transconjugants of BALSAC containing pWM4, pYC7, or pRWRm67 showed that the *nir* and *nor* genes were not expressed and therefore are probably not located in this region of pNOD.

Parity between *R. meliloti* JJ1c10 and SU47 in the *nos* region and its vicinity has previously been demonstrated (7) by juxtaposing the partial physical maps of pRWRm67 and pGMI42, an RP4 prime factor carrying a 285-kb fragment of pNOD

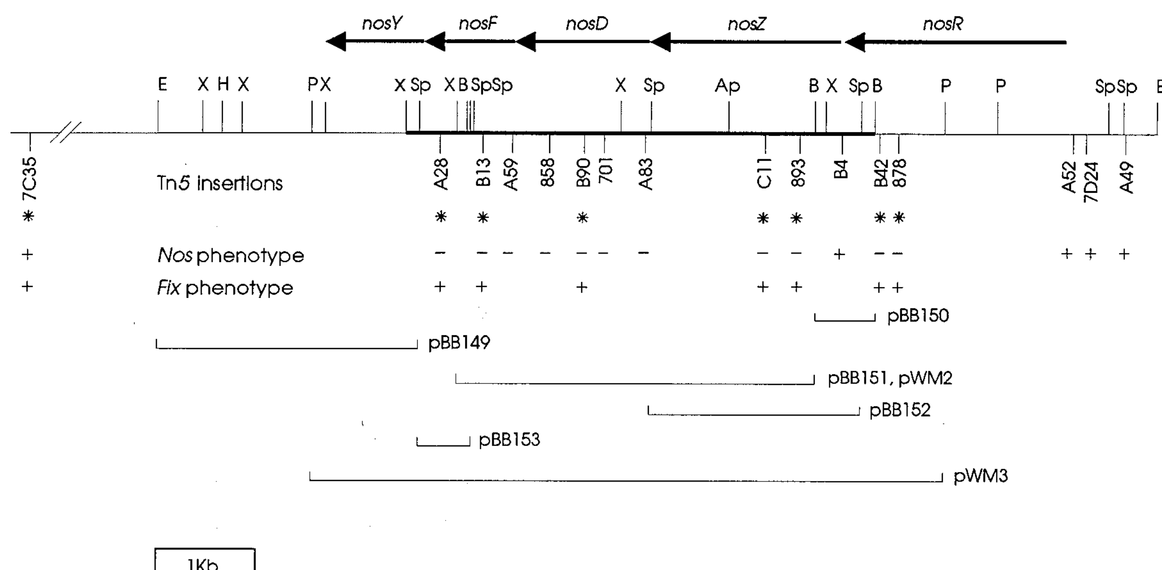

FIG. 2. Organization and restriction map of the *nos* region in *R. meliloti*. Locations and orientations of the *nosRZDFY* genes are shown above the map, while Tn*5* insertion sites derived in pYC7 and their resultant phenotypes are shown below it. Tn*5* sites with asterisks denote those which have been recombined into the *R. meliloti* JJ1c10 genome. Insertion Tn*5*::*7C35* is located 2 kb away from the 10.1-kb *Eco*RI fragment. These derivatives were assayed on plants to determine their Fix phenotypes. Nos phenotypes were determined by testing the derivatives with genomic Tn*5* insertions for N_2O reduction activity. To determine the Nos phenotype for the cosmid Tn*5* insertions, they were first conjugally transferred into *R. meliloti* BALSAC. The thickened portion of the *nos* region indicates the extent of nucleotide sequence determination. The lengths of the *nosR* and *nosY* genes were inferred from those of *P. stutzeri*. Overlapping clones prepared for this study are shown at the bottom. Restriction sites: Ap, *Asp*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sp, *Sph*I; X, *Xho*I.

(pRmeSU47a) of SU47 (28). Equivalence in this region was also shown by testing the Nos phenotype of strain GMI360, a deletion mutant of SU47 in which contiguous 8- and 14-kb *Hin*dIII fragments downstream from *fixGHIS*, comparable to those present in pYC7, were removed (1). This mutant was found to be Nos^-, and a Nos^+ phenotype was restored by conjugal transfer of pWM4. To verify that the *nos* genes are linked to the *fix* region of *R. meliloti* JJ1c10, we have analyzed Fix^- Tn*5* mutants of this strain from a collection of Tn*5* insertion derivatives (39) and identified those in which the insertions were mapped in a region of pNOD homologous to *fixLJKNOQPGHIS*. Hybridization and mapping analysis of this JJ1c10 *fix* region, cloned in cosmid pBB144, verified its structural and functional similarity to the *fix* region of *R. meliloti* SU47 (2), including the reiteration of the *fixNOQP* genes (data not shown). In both strains, the 10.1-kb *Eco*RI fragment containing the *nos* region is 8 kb downstream from *fixGHIS* (Fig. 1).

Tn*5* mutagenesis and nucleotide sequence of the *nos* genes. Plasmid pBB155 (containing the entire *nos* region) was constructed by cloning the 10.1-kb *Eco*RI fragment from pWM4 into pUC19. A restriction map of this fragment and subclones used in its analysis are shown in Fig. 2. Hybridization using the specific *nosZ* gene probe of *P. stutzeri* carried in pNS220 localized the *R. meliloti nosZ* gene to an internal 3.6-kb *Bam*HI fragment cloned in plasmid pBB151. Subsequent sequencing data showed that the *nosZ* gene extended into the adjacent 0.6-kb *Bam*HI fragment cloned in pBB150.

The *nos* region was mutagenized by Tn*5* insertions into cosmid pYC7 in order to analyze its constituent genes. Of 15 insertions mapped within the 10.1-kb *Eco*RI fragment, 11 eliminated the ability of pYC7 to impart Nos activity to *R. meliloti* ATCC 9930 or BALSAC transconjugants (Fig. 2). The insertions which resulted in loss of Nos activity were distributed throughout a 5-kb central portion of the fragment, consistent with the hybridization data described above. However, the limits of the complete *nos* region had not been closely defined since no Tn*5* insertions resulting in a Nos^+ phenotype were obtained immediately adjacent to this central region. Also, plasmid pWM3, carrying a 6.1-kb *Pst*I fragment which encompasses a region larger than that identified by mutagenesis, did not impart a Nos^+ phenotype to *R. meliloti* BALSAC (Fig. 2).

We sequenced a 4.6-kb segment of the *nos* region including a 3.6-kb *Bam*HI fragment containing *nosZ* as well as its flanking DNA. The sequence contained the complete *nosZ*, *nosD*, and *nosF* genes, the 3′ end of *nosR*, and the 5′ end of *nosY*, organized as nearly contiguous genes in the same orientation (Fig. 2 and 3). The G+C content of this 4.6-kb *nos* region was about 62 mol%, which is consistent with the overall *R. meliloti* composition of 62 to 63 mol% (27).

The *nosZ* gene and its product. Analysis of the sequence in the region which hybridized to the *nosZ* gene probe showed a 1,917-nucleotide open reading frame (ORF) with homology to the *nosZ* genes of *P. stutzeri*, *P. aeruginosa*, *Paracoccus denitrificans* and *A. eutrophus*. The ORF is preceded by a good match to a ribosome-binding site, AGGAG (40). The *R. meliloti* ORF encodes a precursor protein of 639 amino acids, compared with 638 amino acids for *P. stutzeri*, 652 for *Paracoccus denitrificans*, and 643 for *A. eutrophus* (Fig. 4).

The NosZ precursor protein of *P. stutzeri* contains a leader peptide which is cleaved during export to its periplasmic location (53). Similarly, the *R. meliloti* protein displayed N-terminal sequence characteristics indicative of an exported protein. Prokaryotic secretory signal cleavage sites in the *R. meliloti* NosZ precursor polypeptide were predicted to occur according to the −1 −3 rule (47) at positions 46 and 27, with poorer scoring possibilities at positions 25 and 32. Analysis of the sequence for potential transmembrane helices by the method of Rao and Argos (33) predicted a 16-residue membrane-spanning helix occuring in the region between residues 15 and 42. A consensus sequence of GXXRRXFLG commencing 7 to 17 residues from the N terminus has been identified in the

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     nosR
     I  R  M  F  E  W  L  K  R  W  P  E  C  G  S  P  C  Q  R  C  A  K  E  C  P  V  Q  S  I  H  P  E  G  A  I  N  V  N  E  C
   1 GGATCCGCATGTTCGAGTGGCTGAAGCGGTGGCCGGAATGCGGCTCGCCCTGCCAGCGCTGCGCCAAGGAGTGTCCGGTCCAGTCCATCCACCCTGAGGGCGCCATCAATGTCAACGAGT
     I  Y  C  M  H  C  Q  E  L  Y  H  D  D  Q  R  C  P  H  M  I  Q  V  R  L  K  R  E  K  F  M  A  L  S  T  P  A  S  R  G  E
 121 GCATCTACTGCATGCACTGCCAGGAGCTTTACCACGACGACCAGCGCTGTCCGCACATGATCCAGGTGCGGCTGAAACGCGAAAAGTTCATGGCGCTTTCCACCCCGGCATCGCGGGGG
                                                                                                nosZ
     A  P  A  K  T  V  V  T  H  K  G  A  P  I  R  K  A  D  A  A  P  E  N  P  V  -                  M  S  N  E  E        5
 241 AAGCGCCGGCGAAGACCGTCGTAACCCACAAGGGCGCCCCGATCAGAAAGGCTGACGCGGCGCCGGAAAACCCCGTCTGAAGCCAACATTCAAGGAGAGGCACAAATGTCAAACGAAGAA
     T  K  M  R  L  N  R  R  Q  M  L  G  T  T  A  F  M  A  A  A  G  A  V  G  A  G  G  A  L  T  L  S  G  G  T  A  T  P  A  R   45
 361 ACCAAGATGCGTCTTAACAGACGACAGATGCTCGGCACGACCGCCTTTATGGCGGCAGCCGGCGCGGTGGGCGCAGGCGGCGCGCTGACGCTGTCGGGCGGCACGGCAACGCCGGCGAGG
     A  Q  E  T  S  G  S  S  Y  E  V  K  P  G  E  L  D  E  Y  Y  V  F  F  S  S  G  Q  S  G  E  I  R  I  V  G  A  P  S  M  R   85
 481 GCGCAGGAAACATCCGGCTCGAGCTACGAAGTGAAGCCCGGCGAGCTCGACGAGTACTATGTGTTCTTCTCATCCGGTCAGTCGGGCGAAATCCGTATCGTCGGCGCCCCCTCGATGCGC
     E  M  M  R  I  P  V  F  N  R  C  S  A  T  G  W  G  Q  T  N  E  S  R  K  V  M  T  E  G  L  L  P  E  T  V  E  F  L  K  D  125
 601 GAGATGATGCGGATCCCCGTCTTCAACCGGTGCAGTGCAACCGGCTGGGGCCAGACCAATGAAAGCCGCAAGGTCATGACCGAGGGCCTACTGCCGGAAACGGTCGAGTTCCTGAAGGAT
     Q  G  G  L  Y  L  N  G  D  L  H  H  P  H  P  S  F  T  D  G  T  Y  D  G  R  Y  L  Y  A  N  D  K  S  N  S  R  V  C  R  I  165
 721 CAGGGGGGCCTTTATCTGAACGGCGACCTTCACCATCCGCATCCCTCGTTTACAGACGGCACCTATGACGGGCGCTATCTCTACGCCAACGACAAGTCCAACTCCCGCGTCTGCCGCATT
     R  L  D  V  M  K  C  D  K  I  I  Q  L  P  N  Q  H  T  V  H  G  L  R  V  Q  K  Y  P  K  T  G  Y  V  F  C  N  G  E  D  A  205
 841 CGGCTCGACGTCATGAAGTGCGACAAGATCATCCAGCTTCCGAACCAGCACACTGTTCACGGCCTTCGGGTCCAGAAATATCCGAAGACCGGGTACGTCTTCTGCAATGGCGAGGACGCC
     V  P  V  P  N  D  G  K  T  M  G  D  K  N  S  Y  Q  A  I  F  T  A  V  D  G  E  T  M  E  V  A  W  Q  V  M  V  D  G  N  L  245
 961 GTGCCGGTTCCGAATGACGGCAAGACCATGGGTGACAAGAACTCCTACCAGGCGATATTTACCGCCGTGGACGGCGAGACGATGGAGGTCGCCTGGCAGGTGATGGTCGACGGCAACCTC
     D  N  V  D  A  D  Y  Q  G  K  Y  C  F  A  T  C  Y  N  S  E  E  G  F  T  L  A  D  M  M  A  S  E  Q  D  W  V  V  I  F  N  285
1081 GACAACGTCGACGCCGACTACCAGGGCAAGTATTGCTTCGCCACCTGCTACAACTCCGAAGAGGGCTTTACCCTGGCGGACATGATGGCGAGCGAGCAGGACTGGGTCGTCATCTTCAAC
     L  K  R  I  E  E  A  V  A  K  G  D  Y  K  E  I  G  G  V  P  V  L  D  G  R  K  G  S  P  Y  T  R  Y  V  P  V  P  N  S  P  325
1201 CTGAAGCGGATCGAGGAGGCGGTCGCAAAGGGCGACTACAAGGAAATCGGCGGCGTGCCGGTGCTGGACGGCCGCAAGGGTTCGCCCTACACGCGCTACGTACCGGTGCCGAACAGCCCG
     H  G  I  N  T  A  P  D  G  I  H  V  V  A  N  G  K  L  S  P  T  V  T  V  F  D  V  R  K  F  D  D  L  F  D  D  K  I  Q  A  365
1321 CACGGCATCAATACGGCGCCCGACGGCATCCACGTCGTTGCGAATGGCAAGCTCTCGCCGACCGTGACCGTGTTCGACGTGCGCAAGTTCGACGATCTCTTCGACGACAAGATCCAGGCC
     R  D  T  V  V  A  E  P  E  L  G  L  G  P  L  H  T  A  Y  D  G  K  G  N  A  Y  T  T  L  F  I  D  S  Q  V  C  K  W  N  I  405
1441 CGCGACACCGTTGTCGCCGAGCCGGAACTCGGTCTGGGCCCATTGCACACCGCCTACGACGGCAAAGGCAACGCCTACACGACGCTGTTCATCGACAGCCAGGTTTGCAAATGGAACATC
     E  D  A  K  R  A  Y  A  G  E  K  V  D  P  I  R  H  K  L  D  V  H  Y  Q  P  G  H  N  H  T  S  M  G  Q  T  K  E  A  D  G  445
1561 GAGGACGCCAAGCGGGCCTATGCAGGCGAGAAGGTCGATCCCATACGCCACAAGCTCGATGTCCACTACCAGCCCGGCCACAATCACACGTCCATGGGGCAGACCAAGGAAGCGGACGGC
     K  W  L  I  S  L  N  K  F  S  K  D  R  Y  L  N  V  G  P  L  K  P  E  N  D  Q  L  I  D  I  S  G  D  E  M  V  L  V  H  D  485
1681 AAATGGCTGATTTCGCTGAACAAGTTCTCCAAGGACCGCTATTTGAACGTCGGCCCGCTCAAGCCGGAAAACGACCAACTGATCGATATTTCCGGCGACGAGATGGTACTCGTGCACGAC
     N  P  T  F  A  E  P  H  D  A  T  I  V  H  A  S  K  I  N  P  V  H  V  W  N  R  D  D  P  F  F  A  D  A  V  A  Q  A  K  A  525
1801 AACCCGACCTTTGCCGAGCCCCATGACGCGACGATCGTTCATGCCTCCAAGATCAATCCAGTCCACGTCTGGAACAGGGACGATCCTTTCTTCGCCGATGCGGTAGCCCAGGCGAAGGCC
     D  N  I  D  L  M  V  D  S  E  V  I  R  D  G  N  K  V  R  V  Y  M  T  S  A  A  P  A  F  G  L  D  D  F  T  V  K  Q  G  D  565
1921 GACAACATTGACCTGATGGTCGATTCGGAAGTCATCCGTGACGGCAACAAGGTCCGCGTCTACATGACGTCCGCCGCCCCGGCCTTCGGTCTGGACGACTTCACCGTCAAGCAGGGCGAC
     E  V  T  V  Y  V  T  N  I  D  E  V  E  D  L  T  H  G  F  C  I  V  N  Y  G  I  N  M  E  V  A  P  Q  A  T  A  S  V  T  F  605
2041 GAGGTTACGGTCTATGTGACGAATATCGACGAGGTCGAGGATCTCACGCATGGATTCTGCATCGTCAACTACGGCATCAACATGGAGGTTGCACCGCAAGCGACCGCTTCCGTCACCTTC
                                                                                                         nosD
     K  A  S  R  P  G  V  Y  W  Y  Y  C  T  W  F  C  H  A  M  H  M  E  M  K  G  R  M  L  V  E  A  Q  G  A  -  M  S  R  P  N  I 639/6
2161 AAGGCGAGCAGACCGGGCGTCTACTGGTACTACTGCACCTGGTTCTGCCATGCGATGCACATGGAGATGAAGGGTCGCATGCTCGTGGAGGCGCAGGGCGCTTGATGTCTCGTCCGAACA
     S  A  F  G  M  A  A  L  A  A  V  I  L  A  C  P  V  S  A  A  T  I  R  K  S  A  D  G  L  P  L  Q  P  V  L  D  R  A  S  P   46
2281 TATCGGCCTTCGGGATGGCGGCGCTTGCCGCCGTCATCCTCGCCTGTCCCGTATCAGCGGCGACGATCCGCAAGTCCGCCGACGGCTTGCCGCTCCAGCCTGTGCTCGACCGCGCCTCGC
     G  D  V  I  V  L  Q  G  E  H  Q  G  P  V  T  I  D  K  T  L  T  L  E  G  E  P  G  A  L  V  M  G  N  G  K  G  S  V  I  T   86
2401 CCGGCGACGTCATCGTGCTGCAAGGCGAACATCAGGGACCGGTCACGATCGACAAGACGCTGACGCTCGAAGGCGAACCTGGAGCCCTTGTCATGGGCAACGGCAAAGGCAGCGTGATAA
     V  K  A  P  Q  S  I  V  R  G  L  E  V  R  G  S  G  K  D  L  Y  G  M  D  S  G  I  F  V  A  Q  T  A  S  G  A  R  V  E  K  126
2521 CAGTGAAGGCGCCGCAGTCGATCGTCCGCGGGCTCGAGGTCAGAGGGTCGGGCAAGGACCTCTATGGAATGGACTCCGGCATCTTCGTGGCGCAGACGGCGAGCGGAGCGCGCGTGGAGA
     N  T  I  I  G  N  L  V  G  I  Y  L  H  G  A  R  D  S  W  A  L  G  N  R  I  I  G  L  R  E  G  R  I  S  E  A  G  D  G  I  166
2641 AAAACACGATCATCGGCAATCTCGTCGGGATCTATCTTCACGGCGCACGGGATTCCTGGGCGCTAGGCAACCGGATCATCGGCTTGCGCGAGGGCAGGATCAGCGAGGCGGGAGACGGCA
     S  V  W  N  A  P  G  A  R  V  V  D  N  D  V  S  Y  G  R  D  G  I  F  S  K  T  S  K  R  N  V  F  R  G  N  R  F  R  E  L  206
2761 TATCGGTCTGGAACGCGCCGGGCGCTCGGGTCGTCGACAATGACGTCAGCTATGGGCGGGACGGTATATTCAGCAAGACAAGCAAGCGGAACGTCTTCCGCGGCAATCGCTTTCGTGAAT
     R  F  A  V  H  Y  M  Y  T  N  D  S  E  I  S  D  N  V  S  T  G  N  A  V  G  Y  A  I  M  Y  S  D  R  L  K  I  K  G  N  R  246
2881 TGCGGTTCGCAGTGCACTACATGTACACGAACGACAGCGAGATCAGCGACAACGTCTCGACGGGCAACGCTGTCGGCTACGCGATCATGTATTCGGACCGCCTCAAGATAAAGGGCAATC
     S  D  G  D  R  D  H  G  L  L  L  N  Y  A  N  N  S  R  I  T  G  N  I  V  V  G  R  L  Q  P  A  D  R  W  L  K  A  R  S  S  286
2941 GCTCGGACGGGGATCGCGACCACGGACTTCTGTTGAACTACGCGAACAACTCTCGGATCACCGGCAACATCGTCGTCGGCCGCCTTCAACCCGCGGACCGTTGGCTGAAGGCGCGATCCT
     G  H  G  V  P  K  T  D  E  E  N  Q  T  A  G  A  D  R  R  L  G  P  E  K  C  V  F  I  Y  N  A  N  K  N  R  F  R  D  N  V  326
3121 CCGGACATGGCGTTCCGAAGACCGACGAAGAGAACCAGACCGCAGGTGCAGACCGCCGCCTCGGGCCGGAAAAATGCGTATTCATCTACAACGCCAACAAGAACCGCTTCCGGGACAACG
     F  E  G  C  A  I  G  I  H  F  T  A  G  S  E  G  N  L  I  S  S  N  S  F  I  N  N  R  N  Q  V  K  Y  V  G  T  R  H  L  D  366
3241 TGTTCGAGGGCTGCGCGATCGGCATCCACTTCACGGCAGGCTCTGAGGGCAACCTGATCAGCAGCAACTCCTTCATCAACAATCGGAATCAGGTCAAGTATGTGGGAACGCGCCACCTCG
     W  S  S  E  G  Q  G  N  Y  W  S  D  N  P  A  F  D  L  D  G  D  G  I  G  D  N  P  Y  R  P  N  D  L  I  D  K  V  L  W  T  406
3361 ATTGGTCGTCCGAAGGACAGGGCAATTACTGGAGCGACAATCCCGCTTTCGACCTGGACGGCGACGGTATTGGCGACAACCCCTACAGGCCCAACGATCTGATCGACAAGGTGTTGTGGA
     S  P  Q  A  K  L  L  T  T  S  P  A  V  Q  V  I  R  W  A  Q  A  Q  F  P  A  I  L  P  G  G  V  V  D  S  R  P  L  M  V  P  446
3481 CCTCCCCACAGGCAAAACTGCTGACCACGAGTCCCGCGGTCCAGGTCATCCGCTGGGCGCAGGCGCAGTTTCCGGCAATCCTGCCCGGCGGCGTCGTCGACAGCCGCCCGCTGATGGTGC
                            nosF
     A  G  R  V  A  V  Q  M- S  G  T  V  E  I  A  G  V  S  K  C  Y  G  D  S  T  V  V  R  D  I  S  F  G  L  G  A  A  E  T  V  453/33
3601 CGGCAGGAAGGGTGGCCGTGCAATGAGCGGGACCGTCGAGATAGCCGGCGTCAGCAAGTGCTACGGAGACTCGACCGTAGTGCGGGACATTTCATTCGGCTTGGGAGCAGCCGAAACCGT
     A  L  V  G  H  N  G  A  G  K  T  T  L  I  K  L  M  L  G  L  I  R  P  T  K  G  L  V  R  V  L  G  E  N  P  A  T  G  D  F   73
3721 GGCGCTCGTCGGGCATAACGGTGCCGGCAAGACCACCCTCATCAAGCTGATGCTCGGCCTAATCCGGCCGACAAAAGGCCTGGTGCGGGTCCTGGGAGAAAATCCGGCGACCGGCGATTT
     A  V  R  Q  R  L  G  Y  L  P  E  S  V  S  F  N  M  A  L  T  G  R  E  T  L  R  F  Y  A  R  L  K  Q  V  D  G  A  A  T  G  113
3841 CGCGGTACGGCAACGCCTCGGCTATCTGCCGGAGAGCGTCTCCTTCAACATGGCGCTGACCGGGCGGGAGACTCTGCGCTTCTACGCACGGCTGAAGCAGGTGGACGGTGCAGCCACCGG
     D  L  F  E  R  V  G  L  A  Q  E  A  V  D  R  P  V  R  T  Y  S  K  G  M  R  Q  R  L  G  L  A  Q  A  L  L  G  M  P  R  I  153
3961 CGATCTTTTCGAGCGCGTCGGCCTCGCCCAGGAAGCCGTCGACCGGCCGGTCCGGACCTATTCCAAGGGCATGCGACAGCGCCTGGGGCTGGCGCAGGCCCTGCTCGGCATGCCACGGAT
     L  L  L  D  E  P  T  S  G  L  D  P  A  L  R  R  N  F  Y  E  L  I  T  E  L  R  A  K  G  T  T  V  L  L  S  S  H  A  L  T  193
4081 CCTGCTGCTCGACGAACCAACCAGCGGCCTTGACCCGGCCCTGCGGCGGAACTTCTACGAGCTGATAACCGAGCTGCGGGCGAAGGGCACCACCGTGCTGCTCTCGTCGCACGCGCTTAC
     E  L  E  G  R  A  D  R  V  I  I  V  N  K  G  V  K  I  A  D  G  T  L  E  Q  L  R  R  I  A  R  L  P  T  R  I  S  L  K  L  233
4201 CGAACTCGAGGGCAGGGCCGACCGCGTGATCATCGTCAACAAGGGCGTGAAGATCGCAGACGGAACGCTTGAGCAGCTTCGTCGCATCGCCCGGCTGCCGACACGCATCAGCCTAAAGCT
     S  Q  A  G  A  T  P  A  W  L  N  G  G  M  K  W  C  R  G  P  D  G  A  V  D  A  E  V  S  S  D  R  K  I  A  L  L  H  D  I  273
4321 CTCCCAGGCCGGCGCGACGCCTGCCTGGCTGAATGGCGGCATGAAATGGTGCCGCGGCCCGGACGGCGCCGTCGACGCGGAAGTCTCCTCGGACCGCAAGATCGCGCTGCTGCATGACAT
                                                                                                  nosY
     T  S  D  A  A  L  L  S  G  L  T  I  T  E  P  T  L  D  D  L  Y  A  H  F  L  N  G  G  V  T  K  M- S  N  I  L  T  I  A  G  304/9
4441 CACCTCGGATGCTGCGCTGCTCTCCGGCCTTACAATCACTGAACCCACGCTCGACGACCTCTATGCGCATTTTCTCAACGGCGGGGTAACGAAATGAGCAATATCCTTACCATCGCCGGC
     K  E  I  Q  E  G  M
4560 AAGGAGATCCAGGAAGGCATG
```

FIG. 3. Nucleotide and deduced amino acid sequences of the *R. meliloti* N_2O reductase structural gene (*nosZ*) and its flanking regions. Possible initiation and termination codons are underlined. The deduced amino acid sequences of the *nos* gene products are shown above the nucleotide sequence, with the residues numbered on the right; nucleotides are numbered on the left.

FIG. 4. Comparison of the *R. meliloti* (Rm) NosZ protein sequence derived from the *nosZ* sequence with NosZ proteins of other denitrifiers. The amino acids are aligned with those in the analogous proteins of *Paracoccus denitrificans* (Pd), *P. stutzeri* (Ps), and *A. eutrophus* (Ae), using the CLUSTAL program of PC/Gene. The N-terminal portions of the proteins were aligned manually. Potential cleavage sites for leader sequences were determined by using the PSIGNAL program of PC/Gene. In the N termini, the residues in reverse type are the first residues in the predicted mature proteins. In the C termini, the residues in reverse type are cysteines and histidines thought to bind Cu in the Cu_A site. Symbols: *, identical amino acids; ., related amino acids. Amino acid residues are numbered on the right.

leader sequences of *P. stutzeri* and *A. eutrophus* NosZ (53) but is missing the phenylalanine residue in the *Paracoccus denitrificans* NosZ protein (25). In the *R. meliloti* NosZ, a match was found to the internal arginine pair and the terminal Leu-Gly of this sequence: ^{9}RLN**RR**QM**LG**^{17} (Fig. 4).

Sequence homology searches revealed a region of approximately 60 amino acids in the C terminus of the NosZ protein, residues 567 to 627, with considerable homology to cytochrome *c* oxidase (subunit II), as first noted by Zumft et al. (53). When aligned to a Cu_A-binding site signature sequence of C(S/A)XXCGXXH (26) starting at residue 617 within this 60-amino-acid region, the sequence of *R. meliloti* NosZ positionally matches the two cysteine residues (C-617 and C-621) and the histidine residue (H-625) which are believed to bind the copper metal ligand (Fig. 4).

The *nosR* gene and its product. Upstream from the *R. meliloti nosZ* gene is the 3′ end of an ORF which showed significant homology to the end of the *nosR* gene of *P. stutzeri* and to partial sequences of the *nosR* of *P. aeruginosa* and *Paracoccus denitrificans*. The termination codon of *nosR* was relatively close (29 bp) to the ATG codon of *nosZ* (Fig. 3), compared with 79 bp in the sequence of *P. stutzeri*, and comparable (25 bp) to what was found for *Paracoccus denitrificans*. No inverted repeat transcription terminator was detected in the *R. meliloti* DNA sequence at the same position as was detected in *P. stutzeri.*

When translated, the *R. meliloti* NosR protein included eight cysteines in two clusters which were conserved in comparison with the C termini of the three analogous proteins that have been deduced (Fig. 5). A short segment of the sequence including the first cluster showed identity in database searches with several 4Fe-4S bacterial ferredoxins. A match was seen to the last three of the four cysteines in the ferredoxin consensus sequence, $CX_2CX_2CX_3C$(P/E/G) (32), while the first two cysteines were separated by three instead of two residues (Fig. 5). Insufficient sequence was determined to investigate whether *R. meliloti* NosR shares the predicted helix-turn-helix structure of *P. stutzeri* NosR (9). The C terminus of the *R. meliloti* NosR was 14 to 21 amino acids longer than that of the other bacterial NosR proteins and showed diminishing similarity distal to the cysteine clusters.

The *nosDFY* genes and their products. Partially overlapping the *nosZ* termination codon is an ORF of 1,359 nucleotides with homology to the *nosD* gene of *P. stutzeri*. A potential ribosome-binding site, GGAGG, was observed 12 bp upstream of the ATG initiation codon (Fig. 3). The ORF coded for a protein of 453 amino acids, 39% identical with that of *P. stutzeri* NosD (55). Little homology was present between the N termini of the *R. meliloti* and *P. stutzeri* NosD proteins or to those of *P. aeruginosa* and *Paracoccus denitrificans*, for which partial sequences are known (Fig. 6A). This portion of NosD is expected to contain a secretion signal sequence, consistent with a periplasmic location for the mature protein. In agreement with this, a membrane-spanning helix was predicted at positions 6 to 28, and cleavage of the N terminus is predicted between the A and T residues at positions 26 and 27.

Overlapping the end of *nosD* was another ORF of 912 nucleotides with homology to *P. stutzeri nosF*. The protein coded by this ORF was four amino acids shorter and 46% identical to that consisting of 308 amino acids predicted for *P. stutzeri* NosF. The encoded protein also showed homology to the family of ATP/GTP-binding proteins with transport functions, of which the most significant matches obtained were to the NodI proteins from several rhizobia. The NodI protein from *Rhizobium leguminosarum* bv. viceae (14) showed 28% identity of its 311 amino acids compared with the *R. meliloti* NosF. The identical amino acids were distributed throughout the protein

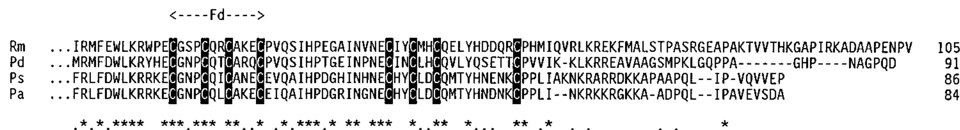

FIG. 5. Comparison of the C terminus of the *R. meliloti* (Rm) NosR protein derived from the *nosR* sequence with C termini of NosR proteins of other denitrifiers. The amino acids are aligned with those in the analogous proteins of *Paracoccus denitrificans* (Pd), *P. stutzeri* (Ps), and *P. aeruginosa* (Pa), using the CLUSTAL program of PC/Gene. Symbols: *, identical amino acids; ., related amino acids. Amino acid residues are numbered on the right. Cysteine clusters are shown in reverse type. The ferredoxin (Fd) consensus sequence for the Fe-S binding site is indicated.

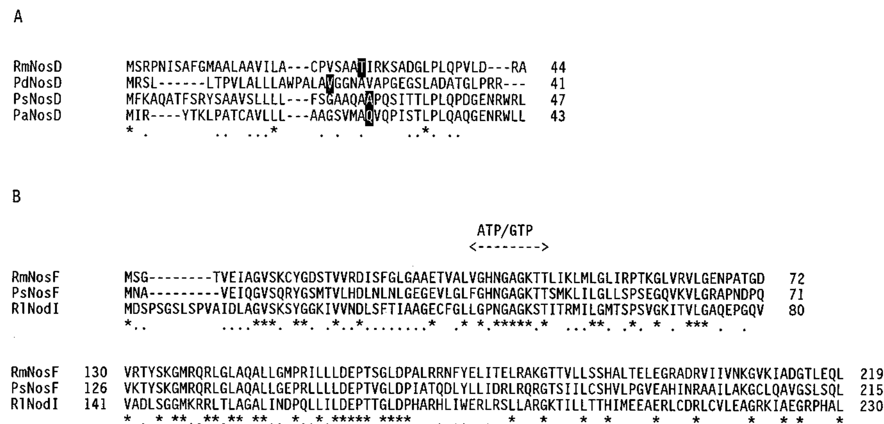

FIG. 6. (A) Alignment of the N termini of NosD proteins to compare their leader peptide sequences and potential cleavage sites. Potential cleavage sites were determined by using the PSIGNAL program of PC/Gene. In each sequence, the residue in reverse type is the first residue in the predicted mature protein. (B) Alignment of two regions of NosF proteins which are comparable to the ATP/GTP-binding NodI protein. The ATP/GTP-binding signature sequence is indicated. Alignment notations are the same as those in Fig. 5. Rm, *R. meliloti*; Pd, *Paracoccus denitrificans*; Ps, *P. stutzeri*; Pa, *P. aeruginosa*; Rl, *R. leguminosarum* bv. viceae.

sequences but were concentrated mostly in the N-terminal 65 residues and within a central region of about 82 residues between positions 133 and 214 (Fig. 6B). These two regions likely contribute to the tertiary structure required for ATP/GTP binding, since both showed sequence similarities to equivalent regions in other proteins of this type and a signature sequence for ATP/GTP binding (38) is present in the N-terminal region. Although the *R. meliloti* NosF and NodI proteins are both associated with rhizobia, this relationship is probably mainly coincidental since the NodI protein is also 29% similar to the NosF protein of *P. stutzeri.*

The *nosF* ORF was followed immediately by the 5′ end of an ORF coding for a protein with homology to the N terminus of *P. stutzeri* NosY (55). As for *nosF*, the *nosY* ATG initiation codon overlapped with the termination codon of the preceding gene within the sequence ATGA and was preceded by a purine-rich region which may include a poorly matched ribosome-binding site (Fig. 3). The portion of the *nosY* gene which was sequenced encoded 95 amino acids but included a gap on one strand (data not shown). This portion of the sequence was 49% identical to the corresponding portion of *P. stutzeri* NosY. The N terminus was very hydrophobic, with 22% leucine residues, and included two potential membrane-spanning regions. Unlike the *P. stutzeri* NosY protein, it lacked a secretory signal sequence. Its characteristics were found typical of an integral membrane protein.

Complementation analysis of the *nos* region. Table 2 summarizes complementation analysis of the *nos* gene region obtained by mating selected pYC7::Tn*5* cosmids into JJ1c10 derivatives containing genomic insertions in the *nos* region. Assays of N_2O reduction activities in these transconjugants demonstrated that the *nos* genes were organized in three complementation groups, *nosR*, *nosZ*, and *nosDF*(*Y*). Assignment of *nosR* to a separate complementation group also agreed with the observation that we obtained insertion Tn*5-B4*, between or near the junction of *nosR* and *nosZ*, which resulted in a Nos^+ phenotype (Fig. 2). Since no insertions were obtained in the *nosY* gene, it could not be conclusively assigned to a group by complementation analysis. However, given the functional relatedness of *nosDF* and *nosY* and their overlapping arrangement, it seems likely the three genes are cotranscribed.

The organization of the *nosRZDFY* genes in *R. meliloti* (Fig. 2) is comparable to that determined for *P. stutzeri*. However, no consensus sequence for the Fnr-binding site ($TTGATN_4ATCAA$) (44) or Anr-binding site ($TTGACN_4ATCAG$) (21) was detected, in contrast to the upstream sequence of *Paracoccus denitrificans nosZ* (25) and the downstream sequence of *P. stutzeri* (10).

Searches of the *nos* gene sequence of *R. meliloti* revealed no binding sites for the RNA polymerase RpoN subunit. Regulation of N_2O reduction via RpoN was tested by activity assay in *R. meliloti* 4R21, an *rpoN* mutant. The mutant was found capable of reducing N_2O as efficiently as its wild-type parent.

Recombination of selected Tn*5* insertions into the wild-type genome and testing for symbiosis on plants showed that *fix* genes were not associated with *nos* genes located in this region (Fig. 2). This result is consistent with the observation that deletion mutant GMI360, which lacks the entire *nos* gene region, is Fix^+ (1).

DISCUSSION

The *nosRZDFY* gene cluster defining the *nos* locus of *R. meliloti* JJ1c10 was exclusively identified on a 10.1-kb *Eco*RI fragment located 8 kb downstream from *fixGHIS* on the *nod* megaplasmid. The *nos* genes were localized within an 8-kb central segment of the fragment. Comparisons of this *fix-nos* region between strains JJ1c10 and SU47 by mapping, hybrid-

TABLE 2. Complementation analysis of the *R. meliloti nos* gene region by using Tn*5* insertion mutants[a]

Cosmid	Host strain with Tn*5* insertion recombined in pNOD megaplasmid: *R. meliloti* JJ1c10::Tn*5*-							
	7C35	*A28*	*B13*	*B90*	*C11*	*893*	*B42*	*878*
pYC7	+	+	+	+	+	+	+	+
pYC7::Tn*5-A28*	+	−	−	−	+	+	+	+
pYC7::Tn*5-C11*	+	+	+	+	−	−	+	+
pYC7::Tn*5-B42*	+	+	+	+	+	+	−	−
pYC7::Tn*5-878*	+	+	+	+	+	+	−	−
		←*nosDF*(*Y*)→			←*nosZ*→		←*nosR*→	

[a] Each cosmid was introduced into the *R. meliloti* mutant strains with Tn*5* insertions by conjugation and tested for N_2O reduction activity. Cosmid pYC7 carries the wild-type *nos* region. The Tn*5* insert in JJ1c10::Tn*5-7C35* is outside the *nos* gene cluster. +, wild-type activity (ca. 750 nmol of N_2O/mg of protein per h); −, negative activity (<10% of wild-type activity). The *nos* genes corresponding to the three complementation groups are indicated below the columns.

ization, and genetic complementation demonstrated that this portion of the pNOD megaplasmid is functionally and structurally very similar in the two strains. Other denitrification genes were not detected in this portion of the megaplasmid. In *P. stutzeri*, the *nir* and *nor* gene clusters are located about 8 kb downstream from the *nos* genes (10), whereas the equivalent location is occupied by the aforementioned *fix* gene cluster in *R. meliloti*. Since Nos function is not universal in *R. meliloti*, as indicated by the Nos^- phenotype of ATCC 9930 and BALSAC, the existence of genetic variants of pNOD differing in the *nos* gene region is demonstrated.

The organization of the *R. meliloti nos* locus is similar to that of *P. stutzeri*, the organism in which denitrification genetics has been most investigated. In both species, the five *nos* genes are adjacent and transcribed in the same direction. The *R. meliloti nosZ* gene is 68% homologous to that of *P. stutzeri*, while the *nosD* and *nosF* genes are 58 and 63% homologous, respectively. In *P. aeruginosa* (53) and *Paracoccus denitrificans* (25), the *nosZ* genes are also flanked upstream by *nosR* and downstream by *nosD*, but other *nos* genes have yet to be identified. *A. eutrophus* appears to be fundamentally different in *nos* gene organization since the presence of *nosR* and *nosD* was not evident upon analysis of the sequence adjacent to *nosZ* (53).

Our complementation data suggest that the *R. meliloti nos* region contains three complementation groups, and therefore probably three transcriptional units, corresponding to the *nosR*, *nosZ*, and *nosDFY* genes. Considering the regulatory role of *nosR*, separate transcriptional units are to be expected for the *nosR* and *nosZ* genes. These data are consistent with complementation and mRNA hybridization data showing that *nosR* and *nosZ* are independently transcribed in *P. stutzeri* (9). In *R. meliloti*, the *nosR* and *nosZ* genes are separated by only 29 bp; hence the *nosZ* promoter and regulatory signals must be encoded within the 3′ end of *nosR*.

The *nosDFY* genes are apparently not coregulated with *nosZ* since they are present in a separate transcriptional unit. This arrangement should permit the *nosDFY* genes to be expressed at a lower level than *nosZ*, as they are probably required only in catalytic amounts to mediate the incorporation of copper into the NosZ protein. As in *P. stutzeri*, the *R. meliloti nosDFY* genes overlap in their termination and initiation codons, using the sequence ATGA. In *P. stutzeri*, the presence of a separate complementation group for the *nosDFY* genes has not been established, though they are presumed on the basis of their functional relatedness and the overlapping gene arrangement to be present on a single transcriptional unit. Similarly, the inclusion of *nosY* in the same operon as *nosDF* has not been established by complementation in *R. meliloti* since we did not obtain a Tn*5* insertion in that gene.

The derived amino acid sequence of *R. meliloti* NosZ protein is 59% identical to the sequence of NosZ of *P. stutzeri*, and a further 12% of the amino acids are conservative replacements. In a multiple alignment of the known NosZ amino acid sequences, 31% of residues were identical and another 32% were similar, indicating a high degree of conservation. The *R. meliloti* NosZ was more similar to that of *Paracoccus denitrificans* (61%) and those of *P. aeruginosa* and *P. stutzeri* while less similar to the *A. eutrophus* NosZ (42%).

Little sequence conservation was found in the N termini of the NosZ proteins where their secretory signal sequences were located. Although membrane-spanning helices are a common feature, sequence homology is not usually observed preceding the cleavage sites of proteins processed for export. Nevertheless, a short conserved sequence, GXXRRXFLG, has been noted in the leader sequences of previously described NosZ proteins (53). Only four of the six amino acids specified in this sequence, the pair of arginines and the leucine-glycine, are conserved in *R. meliloti* NosZ. We observed that a nearby alanine residue is also conserved such that RRXXLGXXA remains as a common consensus sequence of unknown function in the leader sequences of all NosZ proteins which have been examined.

On the basis of amino acid sequence alignment of three NosZ proteins and their comparison with cytochrome *c* oxidases, Zumft and coworkers (53) proposed a Cu_A-binding site in the C termini of N_2O reductases between residues Asp-580 and Met-629. Alignment of *R. meliloti* and *P. stutzeri* NosZ proteins showed 30 identical and 8 conservative replacements in this region. The identical residues included the histidine (H-582) of a ^{579}D(V/L)XHX(W/F/Y)^{584} sequence which was separated by 29 amino acids from a second sequence, ^{614}(W/F/Y)$X_2CX_3CX_3HX_2M^{628}$. In the latter, the cysteines (C-617 and C-621) and histidine (H-625) were identical residues which made up part of the cytochrome *c* oxidase signature. Together with the histidine residue from the first sequence, they are believed to be the copper ion ligands.

In *P. stutzeri*, the *nosDFY* gene products are believed to be involved with copper processing and insertion into N_2O reductase (55). Transposon mutagenesis of these genes resulted in the production of catalytically inactive N_2O reductase which is recognizable by polyclonal anti-N_2O reductase antibodies. The *nosY* of *P. stutzeri* is a hydrophobic protein with six predicted membrane-spanning helices and an export signal. Its NosD appears to be an exported periplasmic protein, while its NosF is an ATP/GTP-binding protein involved with transport functions. We found that the latter characteristics were similarly implicated by the nucleotide sequence of *R. meliloti nosF*. The *nosD*, *nosF*, and *nosY* genes of *R. meliloti* are similar to those of *P. stutzeri*, which also appear to be components of a transport system. A model of these genes based on bacterial transport of sugars and phosphate was proposed (55), with NosY as one of two intrinsic membrane proteins, NosF as a cytoplasmic ATP-binding protein, and NosD as a periplasmic protein which presumably functions to insert copper into the exported reductase apoenzyme (NosZ). We identified the sequence of the gene downstream from *nosZ* in *R. meliloti* as *nosD* by its homology with the *P. stutzeri nosD* gene and with the sequences of the *P. aeruginosa* and *Paracoccus denitrificans nosD* 5′ fragments; it does not resemble any other entries in the GenBank database. No function is known for the encoded protein except for its involvement in the incorporation of copper into N_2O reductase. The *R. meliloti nosF* gene product was very similar to *P. stutzeri* NosF; at the amino acid level, 46% of the residues were identical. The sequence contained an ATP/GTP-binding site motif A (P loop), (A/G)X_4GK(S/T) (38), in the sequence ^{37}GHNGAGKT^{44}. In addition to this motif, the protein showed overall homology to the nucleotide-binding proteins of bacterial transport systems rather than to other ATP-binding proteins such as kinases and ATPases. It has been suggested that cytoplasmic *nosF* provides energy for copper uptake and insertion into N_2O reductase through the membrane-spanning NosY (55), but this has yet to be supported by direct experimental evidence.

One regulator of N_2O reduction is the NosR protein, which is encoded upstream of the *nosZ* structural gene. NosR from *R. meliloti* is similar in its C terminus to those of *P. stutzeri*, *P. aeruginosa*, and *Paracoccus denitrificans*, particularly in the presence of two cysteine clusters related to bacterial ferredoxins; the cysteines are probably necessary for Fe-S binding. Consistent with its postulated role as a membrane-bound positive regulator of *nosR* expression, the NosR protein from *P. stutzeri* includes a helix-turn-helix motif which may bind to

DNA and membrane-spanning helices. In *R. meliloti*, mutation of the *nosR* gene abolished N_2O reduction but did not affect reduction of nitrite or nitric oxide. This is consistent with NosR involvement in a regulatory mechanism which is substrate specific, possibly by redox sensing of the periplasmic NosZ protein.

Binding sites for Fnr (44) and the sigma factor σ^{54} (RpoN) (23) or their homologs, which are global regulators involved in controlling denitrification in other free-living denitrifiers such as *P. stutzeri* (10), were not detected in the sequenced *R. meliloti nos* region. Moreover, we found that *R. meliloti* did not contain sequences characteristic of the *rpoN*-binding site present in the sequenced *nos* gene region and that mutation in *rpoN* did not impair N_2O reduction.

This is the first report on the detailed characterization of a denitrification gene region in a symbiotic N_2 fixer. The location of *nos* genes on the pNOD megaplasmid, one of the two symbiotic megaplasmids of *R. meliloti*, is of special interest. In addition to carrying genes necessary for symbiosis and N_2 fixation, these megaplasmids probably also harbor genes that are essential for free-living growth (17) and therefore could account for their stability. Although none of the four denitrification reactions has been shown to be supportive of N_2 fixation or advantageous for the propagation of rhizobia, denitrification could help their survival by keeping the concentrations of undesirable toxic denitrification substrates (such as nitrite and nitric oxide) low in the microaerobic cell or bacteroid milieu. It appears that rhizobia are capable of carrying out significant denitrification for this purpose but are not highly active compared with the common free-living ones (22, 42). Hence, rhizobia are probably not responsible for the loss of significant amounts of soil nitrogen. The megaplasmid and chromosomal location of the *nos* genes of *R. meliloti* and *P. stutzeri*, respectively, as well as the similarity in organization and nucleotide sequence of these genes reinforce the view of the megaplasmids as chromosomes (43).

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