A new nos gene downstream from nosDFY is essential for dissimilatory reduction of nitrous oxide by Rhizobium (Sinorhizobium) meliloti

Yiu-Kwok Chan, Wayne A. McCormick and Robert J. Watson

Author for correspondence: Yiu-Kwok Chan. Tel: +1 613 759 1663. Fax: +1 613 759 1701.
e-mail: chanyk@em.agr.ca

Eastern Cereal and Oilseed Research Centre, Agriculture & Agri-Food Canada, K. W. Neatby Bldg, Central Experimental Farm, 960 Carling Ave, Ottawa, Ontario, Canada K1A 0C6

Rhizobium (Sinorhizobium) meliloti strains capable of dissimilatory nitrous oxide reduction (Nos+) carry a nosRZDFY gene cluster on a 10-1 kb EcoRI fragment of the nod megaplasmid near the fixGHIS genes. These nos genes are arranged in three complementation groups and the 10-1 kb EcoRI fragment is sufficient to confer Nos activity to R. meliloti strains lacking such activity. An overlapping HindIII fragment containing the nosRZDFY genes but missing a 0-6 kb HindIII–EcoRI downstream segment was found incapable of imparting Nos activity to strains unable to reduce nitrous oxide, suggesting the presence of other nos gene(s) in this region. Tn5 introduced near the HindIII site resulted in mutants with a Nos− phenotype. Complete sequence analysis of nosY showed that it was well-conserved with respect to that of Pseudomonas stutzeri. Two previously unreported genes downstream of nosY in R. meliloti were also revealed. Contiguous with nosY was a sequence showing 63% identity with the ORFL protein of P. stutzeri. It appeared to be in the same operon as nosDFY and was predicted to encode a membrane lipoprotein similar to the putative NosL of P. stutzeri. Unlike the latter protein, however, amino acid sequences typical of metal-binding sites and cysteine residues indicative of the active site of protein disulphide isomerase were absent in the predicted NosL of R. meliloti. The Tn5 mutations resulting in a Nos− phenotype were localized within a 966 nucleotide gene 31 nucleotides downstream of nosDFYL with the same orientation. The new gene, nosX, was determined to be in a separate complementation group. It encoded a periplasmic protein with homology in the C-terminal domain with RnfF of Rhodobacter capsulatus and with a hypothetical Escherichia coli protein, YOJK. It was concluded that there are seven genes constituting the nos cluster in R. meliloti. They are organized in four complementation groups and in the same orientation, spanning a distance of about 9 kb on the nod megaplasmid.

Keywords: Rhizobium (Sinorhizobium) meliloti, denitrification, nitrous oxide reduction, nosY, nosX, lipoprotein

INTRODUCTION

The complete denitrification of nitrate by bacteria to dinitrogen (N₂) is generally an anaerobic respiratory process. The last step involves the dissimilatory reduction of nitrous oxide (N₂O), the free energy change of which can be coupled to phosphorylation (Zumft, 1992; Zumft & Kroneck, 1990). Denitrifying bacteria are ubiquitous in nature. Several species capable of partial or complete denitrification are N₂-fixing bacteria, including those of the Rhizobiaceae family (Chan et al., 1994). One of these is Rhizobium meliloti (Chan et al., 1989), a bacterium that enters into symbiotic partnership with alfalfa by inducing the formation of root nodules on the plant. [R. meliloti was recently renamed Sinorhizobium meliloti (De Lajudie et al., 1994) but it is referred to as a Rhizobium species in this paper for consistency with our previous work.] Although an-

The GenBank accession number for the nucleotide sequence reported in this paper is U94899.
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Reference or source</th>
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<tr>
<td><strong>Strains</strong> R. meliloti</td>
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<tr>
<td>J1c10</td>
<td>Wild-type strain; Nos+ Rif'</td>
<td>Selvaraj et al. (1987)</td>
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<td>RmYC2160</td>
<td>Spontaneous Nal' Rif' derivative of BALSAC; Nos+ Nal' Rif'</td>
<td>Chan &amp; Wheatcroft (1993)</td>
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<td>Chan &amp; Wheatcroft (1993)</td>
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<td>RmWM1181</td>
<td>nosX::Tn5-703, nosX::Tn5-A43, nosX::Tn5-B93, nosX::Tn5-7100, J1c10 derivatives with genomic Tn5 inserts introduced by recombination; Nos- Rif' Km'</td>
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<td>Boyer &amp; Roulland-Dussoix (1969); Hanahan (1983)</td>
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<td>pYC7</td>
<td>Cosmid pVK100 carrying contiguous 8 and 14.5 kb HindIII fragments containing the nos region of R. meliloti J1c10; Te'</td>
<td>Holloway et al. (1996)</td>
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<td>pYC8</td>
<td>Cosmid pVK100 carrying contiguous 14.5 and 10.5 kb HindIII fragments containing a major nos region of R. meliloti J1c10; Te'</td>
<td>This report</td>
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<td>pWM4</td>
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<td>pWM5</td>
<td>Broad-host-range plasmid pRK310 carrying the 14.5 kb HindIII fragment of pYC7; Te'</td>
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<td>pWM1249</td>
<td>3.0-kb PstI fragment carrying the 3'-end of nosL, nosX and its downstream region in pGEM-SZf(+) (Promega); Ap'</td>
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<td>10-kb EcoRI fragment of pYC7 carrying the entire nos region in pUC19; Ap'</td>
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<td>pRK2013</td>
<td>ColEI replicon carrying RK2 transfer genes, helper plasmid for triparental matings; Km'</td>
<td>Figurski &amp; Helinski (1979)</td>
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<td>pRK600</td>
<td>pRK2013::Tn9 derivative, helper plasmid for triparental matings; Cm'</td>
<td>Finn et al. (1986)</td>
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<td>pVK100</td>
<td>Broad-host-range cosmID cloning vehicle; Km' Te'</td>
<td>Knauf &amp; Nester (1982)</td>
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*Ap', ampicillin resistance (50 µg ml⁻¹); Cm', chloramphenicol resistance (15 µg ml⁻¹); Km', kanamycin resistance (20 or 40 µg ml⁻¹); Nal', nalidixic acid resistance (10 µg ml⁻¹); Te', tetracycline resistance (5 or 10 µg ml⁻¹); Rif', rifampin resistance (100 µg ml⁻¹).

Aerobic nitrate respiration has been suggested to be indirectly controlled by fixLJ genes in rhizobia (Hennecke et al., 1993) and the nitrite reductase gene of an unclassified Rhizobium sp. has recently been characterized (Toffanin et al., 1996), the detailed investigation of denitrification genetics in this group of bacteria has largely been limited to N₂O respiration.

Strains of R. meliloti capable of dissimilatory N₂O reduction carry a gene cluster, nosRZDFY, located on the nod megaplasmid downstream from the fixGHIS genes (Chan & Wheatcroft, 1993; Holloway et al., 1996). To date, the plasmid location of denitrification genes has been reported in only one other free-living denitrifier, Alcaligenes eutrophus (Friedrich et al., 1990).
The nosRZDFY genes are known to be required for N₂O reduction (Nos) activity in the well-studied denitrifier Pseudomonas stutzeri (Cuypers & Zumft, 1992) and other free-living denitrifiers (Zumft et al., 1992): nosR is regulatory in function, nosZ is the structural gene for the periplasmic N₂O reductase, and nosDFY are genes for processing its copper cofactor. These plasmid-borne nos genes of R. meliloti have been shown by nucleotide sequence determination of nosZ and its flanking regions to show a similar genetic sequence and organization to the chromosomal nosZDFY genes of P. stutzeri (Holloway et al., 1996). They are arranged in three adjacent complementation groups located on a 10.1 kb EcoRI fragment which is sufficient to confer Nos activity to R. meliloti strains completely lacking denitrification ability or specifically lacking Nos activity (Holloway et al., 1996). In the same study, plant tests with some nos mutants constructed by Tn5 insertions in the nos region did not show any effect on symbiotic N₂ fixation activity. Hence, the nos genes concerned are probably not associated with the fix genes in their vicinity.

Further analysis of cosmids covering the known nos region in R. meliloti has led us to identify a novel gene, nosX, which has not been reported in any other denitrifier. It was detected on a previously unmapped region bearing a 0.6 kb HindIII–EcoRI fragment downstream from nosDFY. Analysis of new nos mutations generated by Tn5 insertions in this region indicated that they were in a complementation group distinct from the three groups consisting of nosZDFY (Holloway et al., 1996). In addition, we have determined between nosY and nosX a nosL sequence resembling the recently sequenced ORFL in P. stutzeri (Dreusch et al., 1996). Here, we describe these new findings and compare the sequences of the nosYL genes and their translated amino acid sequences with those of P. stutzeri.

METHODS

Bacterial strains, media and culture conditions. Bacterial strains and plasmids used in this work are listed in Table 1. R. meliloti strains were grown on TYC (tryptone-yeast extract-calcium) medium (Fallik et al., 1991) at 30 °C. Escherichia coli strains were grown in Luria–Bertani medium (Sambrook et al., 1989) at 37 °C. Antibiotics used were ampicillin (50 μg ml⁻¹), chloramphenicol (15 μg ml⁻¹), kanamycin (20 or 40 μg ml⁻¹), nalidixic acid (10 μg ml⁻¹), tetracycline (5 or 10 μg ml⁻¹) and rifampin (100 μg ml⁻¹). R. meliloti strains RmYC2160 and RmYC2164 are Na⁺ derivatives of the Nos+ strains BALSAC and ATCC 9930, respectively, used as conjugation recipients.

Bacterial conjugation and molecular techniques. Tripartite matings involving helper plasmids (pRK2013 or pRK600) were used to introduce plasmids from E. coli to R. meliloti according to Ditta et al. (1980). DNA manipulations, including restriction digests, analysis by agarose gel electrophoresis, ligation and transformation, were carried out by standard methods (Sambrook et al., 1989). Tn5 mutagenesis of cosmids pYC7 and subsequent complementation analysis of the constructed R. meliloti JJ1c10 mutants were done as previously described (Holloway et al., 1996). Southern hybridization was performed with DNA probes labelled with digoxigenin-11-dUTP (Boehringer Mannheim) by random priming. Hybrid DNA was detected by using chemiluminescent digoxigenin antibodies from the same manufacturer according to Holloway et al. (1996). Other genetic manipulations have been described previously (Chan & Wheatcroft, 1993; Wu et al., 1995).

Gene sequencing. DNA sequence determination by the dideoxynucleotide chain-termination method of Sanger et al. (1977) was performed with a T7 sequencing kit (Pharmacia Biotech) or a Sequenase T7 polymerase kit (US Biochemical), and with ³²P- or ³³S-labelled dATP (Amersham). It was carried out on nested deletions generated from pWM1249 (Erase-a-Base System, Promega), pBB149 and pBB154 (Exo-Size deletion kit, New England Biolabs). Both complementary strands of the DNA were sequenced. SP6 and T7 promoter primers (Promega) were used on the pWM1249 deletions; M13 universal and reverse primers (US Biochemical) were used on the pBB149 and pBB154 deletions. Gaps in the sequence were filled by using custom synthetic primers (Bio/Can Scientific or Canadian Life Technologies). Additional sequencing was done with a Sequitherm Cycle sequencing kit (Epigencentre Technologies), following the manufacturer’s instructions. Sequence data were assembled and analysed with PC/Gene software (IntelliGenetics).

Activity assay. N₂O reduction was assayed in anaerobic R. meliloti cultures and its specific activity determined as described previously (Holloway et al., 1996).

RESULTS AND DISCUSSION

Evidence for an extended nos region downstream from nosRZDFY

We have previously described pYC7 as a cosmid clone of R. meliloti JJ1c10 DNA carrying the complete nos region near the centre of the inserted DNA on a 10.1 kb EcoRI fragment (Holloway et al., 1996). An overlapping cosmid from the same genomic library, pYC8, was also identified using a nos probe as carrying the nos region on a common 14.5 kb HindIII fragment. pYC8 carried 94% of the 10.1 kb EcoRI fragment in the overlapping region, lacking only a 0.6 kb EcoRI–HindIII fragment at the end proximal to the fixLJKNOQPHGIS cluster downstream from the nosRZDFY genes (Fig. 1). How-

![Fig. 1. Partial restriction map of the R. meliloti nod megaplasmid in the region of the nos and fix gene clusters. The boxed area indicates the newly extended nos region. Genes fixLJ, fixK, fixNOQ and fixGHIS are included in the fix cluster (Fischer, 1994). The range of R. meliloti DNA carried in cosmids (pYCY7, pYCY8) and broad-host-range plasmids (pWM4, pWM5) are shown with their ability to confer Nos activity to Nos- backgrounds. E, EcoRI; H, HindIII.](image-url)
ever, while pYC7 was able to confer the Nos+ phenotype to R. meliloti Rm2160 and Rm2164 (Table 1), strains naturally lacking the entire nos region (Chan & Wheatcroft, 1993), pYC8 was unable to do so, apparently because it did not carry the complete 10-1 kb EcoRI fragment. The requirement for the 0-6 kb EcoRI-HindIII fragment was confirmed by testing the Nos phenotypes conferred by the broad-host-range plasmids pWM4 (carrying the complete 10.1 kb EcoRI fragment only) and pWM5 (carrying the 14.5 kb HindIII fragment common to both pYC7 and pYC8). Only pWM4 was able to confer Nos activity, indicating the presence of other nos gene(s) in the vicinity of the 0.6 kb EcoRI-HindIII fragment downstream from nosRZDFY.

**Tn5 mutagenesis and nucleotide sequence of nosY**

To examine the region downstream of nosDFY, Tn5 insertions in cosmid pYC7 were obtained and those mapping in the vicinity of the 0-6 kb EcoRI-HindIII segment were analysed. Four mutants with Tn5 insertions mapping near the HindIII site were derived. Consistent with the analysis of pYC8 described above, three of these insertions (A43, B93 and 7100) were localized within the 0-6 kb EcoRI-HindIII fragment while the fourth (703) was located within the adjacent 0-2 kb HindIII-XhoI fragment (Fig. 2). Despite repeated efforts, we were unable to obtain mutants with insertions in the 2 kb segment including the nosY gene and the region immediately downstream. When cosmids containing the four Tn5 insertions were introduced into a Nos- background (RmYC2160 or RmYC2164), they were unable to confer Nos activity, although the parental cosmid, pYC7, was able to do so. Consequently, these insertions confirm the extension of the known nos region from 74 to 90% of the 10-1 kb EcoRI fragment.

To identify potential genes within the extended nos region, three overlapping fragments were cloned in pBB149, pBB154 and pWM1249 (Table 1; Fig. 2) for sequencing. In this terminal nos region two ORFs were found orientated in the same direction as nosY (Fig. 2). An ORF contiguous with nosY was homologous to the recently reported ORFL of P. stutzeri (Dreusch et al., 1996), while the other was new and unidentified. Since the region in which additional Tn5 insertions near the HindIII site (resulting in the loss of Nos phenotype) is covered by the new ORF, the latter is a functional gene which we named nosX.

**The nosY genes and their products**

Sequence determination of the 5' end of nosY has shown that its ATG start codon overlaps with the stop codon of the preceding nosF gene within the sequence ATGA (Holloway et al., 1996). Complete sequencing of nosY revealed that it consists of 828 nucleotides encoding 275 amino acids. The sequence was 63% identical with the 831 nucleotide nosY gene of P. stutzeri, which also overlaps with the preceding nosF gene and is believed to be involved in copper chromophore synthesis (Zumft et al., 1990). Its translated amino acid sequence showed an overall 47% identity with the 276 residue P. stutzeri NosY integral membrane protein. R. meliloti NosY was also a hydrophobic membrane protein, which contained 20% leucine residues and six transmembrane helices (at positions 21-37, 53-69, 109-125, 150-166, 176-192 and 250-266) as predicted by the methods of Eisenberg et al. (1984) and Klein et al. (1985). When juxtaposed, the remarkably similar hydropathy profiles and largely overlapping transmembrane segments of the two proteins are readily revealed (Fig. 3). However, unlike the P. stutzeri protein, a potential signal sequence cleavage site in the R. meliloti NosY was not apparent.
The partially overlapping arrangement of the start/stop codons in the consecutive nosDFY genes of R. meliloti (Holloway et al., 1996) continued in the nosY stop codon as it was also found to overlap with the start codon of the following ORF. This ORF was 558 nucleotides long and showed 63% identity with the 573 nucleotide ORFL of P. stutzeri (Dreusch et al., 1996). Since we were unsuccessful in obtaining Tn5 insertions in the region between nosF and nosX (Fig. 2), we could not definitively establish that nosDFYL belong to the same complementation group. However, the relatively close linkage of these genes in R. meliloti and their apparent lack of transcriptional terminators downstream of nosY suggest that the nosDFYL cluster in this organism represents genes belonging to one transcription unit. The organization of nosDFYL in P. stutzeri is somewhat different. Its ORFL is located 28 nucleotides downstream of nosY but still lacks a determinable transcription start site independent of the presumed nosDFY operon (Dreusch et al., 1996). Since the ORF is conserved between P. stutzeri and R. meliloti, it is likely that the sequence encodes a functional gene in both organisms.

R. meliloti JJ1c10 is the second denitrifying organism after P. stutzeri reported to possess a nosL-like gene. The derived amino acid sequence of the 185 residue R. meliloti NosL is 31% identical with the tentative 190 residue P. stutzeri NosL (Dreusch et al., 1996). Both gene products are predicted to be membrane lipoproteins. The 18 residue precursor signal peptide of R. meliloti NosL, which is six residues shorter than that of P. stutzeri, consists of one transmembrane helix at the C-terminal end of the signal peptide. According to the hydrophobicity index calculations of Kyte & Doolittle, 1982, NosL has a negative hydrophobic index of -0.15 for the first 96 amino acids, which is consistent with a single transmembrane helix orientation. NosL also contains two cysteine residues, one near the N terminus and one within the first 100 amino acids. These cysteine residues are conserved in both R. meliloti and P. stutzeri NosL, indicating that NosL is a membrane lipoprotein. The orientation of the NosL sequences is shown in Fig. 4. Alignment of the NosL sequences of R. meliloti (Rm) and P. stutzeri (Ps). A slash in each underlined consensus lipoprotein signal peptide sequence denotes a cleavage site (Hayashi & Wu, 1990) according to the PROSITE program of PC/Genome. Unique signature sequences in P. stutzeri NosL (Dreusch et al., 1996) are double-underlined and labelled. An asterisk denotes identical amino acids; a dot, related amino acids. Amino acid residues are numbered on the right.

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**Fig. 3.** Hydropathy profile of the predicted NosY protein of R. meliloti (a) juxtaposed with that of P. stutzeri (b). Computation was based on an interval of 15 amino acids (Kyte et Doolittle, 1982). Horizontal bars indicate the positions of transmembrane segments (Klein ef al., 1985).

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**Fig. 4.** Alignment of the NosL sequences of R. meliloti (Rm) and P. stutzeri (Ps). A slash in each underlined consensus lipoprotein signal peptide sequence denotes a cleavage site (Hayashi & Wu, 1990) according to the PROSITE program of PC/Genome. Unique signature sequences in P. stutzeri NosL (Dreusch et al., 1996) are double-underlined and labelled. An asterisk denotes identical amino acids; a dot, related amino acids. Amino acid residues are numbered on the right.
disulphide isomerase reported for the NosL of *P. stutzeri* (Dreusch et al., 1996) are not evident in the *R. meliloti* homologue (Fig. 4). Only two histidine residues, His-178 and His-184, were found at the C terminus of the rhizobial product, where His-184 could be a conserved residue. No potential metal-binding motifs in the sequence were identified. Of the thiol/disulphide pair 178 and His-184, were found at the C terminus of the ductase. In *P. stutzeri*, that a non-specific protein disulphide isomerase encoded function as a protein disulphide isomerase. This implies outside the organism has not been verified. Hence, the real function of the putative NosL, especially in *P. stutzeri*, remains unclear and awaits further clarification.

**The nosX gene and its product**

A 966 nucleotide ORF in the same orientation as *nosDFYL* was found 31 nucleotides downstream from the stop codon of *nosL*. Because of the abolition of Nos activity by the Tn5 insertions, it represented a functional gene, known here as nosX. Its 3' end, which also marked the termination of the *nos* region, was located 96 nucleotides from the EcoRI site at the end of pBB149 and pBB154 used for the sequence determination (Fig. 2).

Analysis of nosX showed that it encoded a peripheral membrane protein of 321 amino acids with one possible transmembrane helix (position 270–293) predicted by the method of Rao & Argos (1986) toward its C-terminal domain, and a secretory leader sequence with a putative cleavage site detected between residue 31 and 32 that conformed to the −1−3 rule (von Heijne, 1986). NosX was found to exhibit about 27% (86 residues) sequence identity with RnfF of *Rhodobacter capsulatus*, a secretory membrane protein of 523 amino acids with a signal sequence of 44 amino acids (Schmehl et al., 1993). A majority (72%) of the identical amino acids were located away from the N-terminal domains of both NosX and RnfF. The NosX C terminus also showed similar homology (53 identical residues) with YOJK, an unpublished hypothetical 22.1 kDa protein of unknown function (SWISS-PROT database accession number P33943). There is no significant identity between NosX and any of the putative ORF proteins derived from the 9.72 kb segment that links the *nir-nor* and *nos* gene clusters for denitrification in *P. stutzeri* (Glockner & Zumft, 1996). Hence, an equivalent nosX gene in the vicinity of *nosL* is not apparent in *P. stutzeri*, probably reflecting a species difference. A comparison of the consensus portion toward the C termini of NosX, RnfF and YOJK showed that 15% of the residues are identical and another 35% are well-conserved (Fig. 5). RnfF has been determined to be an iron–sulphur protein involved in electron transport to nitrogenase which is essential for diazotrophic growth (Schmehl et al., 1993). Its N-terminal portion contains a cysteine motif (C-X,-C-X-C-X,-C) typical of [4Fe–4S] proteins. Since NosX contains only one cysteine residue (Cys-297), it obviously lacks cysteine motifs and is not an iron–sulphur protein. Although nosX was demonstrated to be essential for N₂O reduction, its function is presently unknown. It is not expected to be involved in N₂ fixation since an *R. meliloti* strain with its entire *nos* region deleted was not affected in its Fix phenotype (Holloway et al., 1996). It will be important to know if nosX-like genes exist in other denitrifiers.

**Complementation analysis of the complete nos region**

Complementation analysis of the *nos* region was carried out to include the end of the 10.1 kb EcoRI fragment downstream from *nosY* by mating cosmids pYC7::Tn5-A43, pYC7::Tn5-B93 and other previously selected pYC7::Tn5 cosmids into J111c10 derivatives containing genomic insertions in the *nos* region (Table 2). N₂O reduction assays of the transconjugants showed that nosX was in a complementation group separate from nosR, nosZ and nosDF(YL) and, therefore, represented a different operon. It was noted that some of the complementation results using mutations in *nosX* paired with mutations in *nosR* were anomalous, particularly the results obtained using pYC7::Tn5-878. One explanation is that the nosX product may interact with NosR, which is regulatory in function.

In the absence of Tn5 insertions in *nosY* and *nosL*, the present grouping of the *nosDF(YL)* genes is based on the
overlapping start/stop codon arrangement of the individual genes. From these results and our previous observations (Holloway et al., 1996), we suggest that the nos genes of R. meliloti are organized in four complementation groups, spanning a distance of about 9 kb on the nod megaplasmid. All of these adjacent groups, namely, nosR, nosZ, nosDF(YL) and nosX, are transcribed in the same direction. The present findings serve as a basis for future investigations into the coordination of nos and other denitrification genes in rhizobia.

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REFERENCES


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