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 RnF 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, *nosYXL*, 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

Strain or plasmid	Relevant characteristics*	Reference or source
Strains		
<i>R. meliloti</i>		
JJ1c10	Wild-type strain; Nos ⁺ Rif ^r	Selvaraj <i>et al.</i> (1987)
RmYC2160	Spontaneous Nal ^r Rif ^r derivative of BALSAC; Nos ⁻ Nal ^r Rif ^r	Chan & Wheatcroft (1993)
RmYC2164	Spontaneous Nal ^r derivative of ATCC 9930; Nos ⁻ Nal ^r Rif ^r	Chan & Wheatcroft (1993)
RmWM1181	<i>nosX</i> ::Tn5-703, <i>nosX</i> ::Tn5-A43, <i>nosX</i> ::Tn5-B93, <i>nosX</i> ::Tn5-7100, JJ1c10 derivatives with genomic Tn5 inserts introduced by recombination; Nos ⁻ Rif ^r Km ^r	This report
RmWM1182		
RmWM1183		
RmWM1195		
RmWM1192	<i>nosD</i> ::Tn5-B90, JJ1c10 derivative with genomic Tn5 inserts introduced by recombination; Nos ⁻ Rif ^r Km ^r	Holloway <i>et al.</i> (1996)
RmWM1186	<i>nosF</i> ::Tn5-A28, <i>nosF</i> ::Tn5-B13, JJ1c10 derivatives with genomic Tn5 inserts introduced by recombination; Nos ⁻ Rif ^r Km ^r	Holloway <i>et al.</i> (1996)
RmWM1190		
RmWM1184	<i>nosR</i> ::Tn5-878, <i>nosR</i> ::Tn5-B42, JJ1c10 derivatives with genomic Tn5 inserts introduced by recombination; Nos ⁻ Rif ^r Km ^r	Holloway <i>et al.</i> (1996)
RmWM1191		
RmWM1185	<i>nosZ</i> ::Tn5-893, <i>nosZ</i> ::Tn5-C11, JJ1c10 derivatives with genomic Tn5 inserts introduced by recombination; Nos ⁻ Rif ^r Km ^r	Holloway <i>et al.</i> (1996)
RmWM1194		
RmWM1179	JJ1c10::Tn5-7C35, JJ1c10::Tn5-A52, JJ1c10::Tn5-B4, genomic Tn5 inserts introduced by recombination; Nos ⁺ Rif ^r Km ^r	Holloway <i>et al.</i> (1996)
RmWM1188		
RmWM1189		
<i>E. coli</i>		
DH5 α , HB101	Host strains	Boyer & Roulland-Dussoix (1969); Hanahan (1983)
Plasmids		
pYC7	Cosmid pVK100 carrying contiguous 8 and 14.5 kb <i>Hind</i> III fragments containing the <i>nos</i> region of <i>R. meliloti</i> JJ1c10; Tc ^r	Holloway <i>et al.</i> (1996)
pYC8	Cosmid pVK100 carrying contiguous 14.5 and 10.5 kb <i>Hind</i> III fragments containing a major <i>nos</i> region of <i>R. meliloti</i> JJ1c10; Tc ^r	This report
pWM4	Broad-host-range plasmid pPW11 carrying the 10.1 kb <i>Eco</i> RI fragment of pYC7 including the entire <i>nos</i> region; Tc ^r	Holloway <i>et al.</i> (1996)
pWM5	Broad-host-range plasmid pRK310 carrying the 14.5 kb <i>Hind</i> III fragment of pYC7; Tc ^r	Holloway <i>et al.</i> (1996)
pWM1249	3.0 kb <i>Pst</i> I fragment carrying the 3'-end of <i>nosL</i> , <i>nosX</i> and its downstream region in pGEM-5Zf(+) (Promega); Ap ^r	This report
pBB155	10.1 kb <i>Eco</i> RI fragment of pYC7 carrying the entire <i>nos</i> region in pUC19; Ap ^r	Holloway <i>et al.</i> (1996)
pBB149	2.4 kb <i>Eco</i> R1- <i>Sph</i> I fragment from pBB155 including the 3'-end of <i>nosY</i> and <i>nosLX</i> in pUC19; Ap ^r	Holloway <i>et al.</i> (1996)
pBB154	1.5 kb <i>Eco</i> R1- <i>Pst</i> I fragment from pBB155 including the 3'-end of <i>nosL</i> and <i>nosX</i> in pUC19; Ap ^r	This report
pRK2013	ColE1 replicon carrying RK2 transfer genes, helper plasmid for triparental matings; Km ^r	Figurski & Helinski (1979)
pRK600	pRK2013::Tn9 derivative, helper plasmid for triparental matings; Cm ^r	Finan <i>et al.</i> (1986)
pVK100	Broad-host-range cosmid cloning vehicle; Km ^r Tc ^r	Knauf & Nester (1982)

* Ap^r, ampicillin resistance (50 μ g ml⁻¹); Cm^r, chloramphenicol resistance (15 μ g ml⁻¹); Km^r, kanamycin resistance (20 or 40 μ g ml⁻¹); Nal^r, nalidixic acid resistance (10 μ g ml⁻¹); Tc^r, tetracycline resistance (5 or 10 μ g ml⁻¹); Rif^r, rifampin resistance (100 μ g 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 N_2O 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 N_2O 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 *nosRZDFY* 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 N_2 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 *nosRZDFY* (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 Nal^r 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 cosmid 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 ³³P- or ³⁵S-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 (Epicentre Technologies), following the manufacturer's instructions. Sequence data were assembled and analysed with PC/Gene software (IntelliGenetics).

Activity assay. N_2O 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 *nosZ* 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 *fixLJKNOQPGHIS* 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*, *fixNOQP* and *fixGHIS* are included in the *fix* cluster (Fischer, 1994). The range of *R. meliloti* DNA carried in cosmids (pYC7, pYC8) and broad-host-range plasmids (pWM4, pWM5) are shown with their ability to confer Nos activity to Nos⁻ backgrounds. E, *EcoRI*; H, *HindIII*.

Fig. 2. Physical map of the complete *nos* gene region required for N_2O reduction by *R. meliloti*. Tn5 insertions resulting in a Nos^- phenotype are marked by open circles; insertions not affecting the *Nos* phenotype are marked by filled circles. Labelled arrows indicate the transcriptional direction of the individual genes. The thick line indicates the extent of nucleotide sequence determination. Clones used for the sequencing are shown at the bottom. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; Sp, *Sph*I; X, *Xho*I.

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 *Eco*RI fragment. The requirement for the 0.6 kb *Eco*RI–*Hind*III fragment was confirmed by testing the *Nos* phenotypes conferred by the broad-host-range plasmids pWM4 (carrying the complete 10.1 kb *Eco*RI fragment only) and pWM5 (carrying the 14.5 kb *Hind*III 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 *Eco*RI–*Hind*III fragment downstream from *nosRZDFY*.

Tn5 mutagenesis and nucleotide sequence of *nosYLX*

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 *Eco*RI–*Hind*III segment were analysed. Four mutants with Tn5 insertions mapping near the *Hind*III 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 *Eco*RI–*Hind*III fragment while the fourth (703) was located within the adjacent 0.2 kb *Hind*III–*Xho*I 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 *Eco*RI 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 ORF of *P. stutzeri* (Dreusch *et al.*, 1996), while the other was new and unidentified. Since the region in which additional Tn5 insertions near the *Hind*III 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 *nosYL* 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

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 & Doolittle, 1982). Horizontal bars indicate the positions of transmembrane segments (Klein *et al.*, 1985).

according to the $-1 -3$ rule (von Heijne, 1986). The single histidine (His-108) of the *P. stutzeri* NosY is conserved in that of *R. meliloti*, but the latter has an additional histidine (His-243). All of these histidines are located outside the transmembrane helices. However, while the two cysteine residues (Cys-122 and Cys-255) of the *P. stutzeri* protein reside within separate membrane helices (Zumft *et al.*, 1990), the only cysteine residue in the *R. meliloti* counterpart (Cys-102) is located outside such helices. Because of their location, these residues are probably not metal ligands that would allow NosY to serve as a copper carrier in either organism. Nevertheless, NosY has been speculated to mediate between an energy-dependent copper-insertion process involving NosD in the periplasm and the energy-providing NosF at the cytoplasmic side of the inner membrane, as modelled after bacterial transport systems for small molecules (Zumft *et al.*, 1990).

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 ORF 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

RmNosL	M---KLTVTAILAATLF--LAG/C-QKEEDTTPSPSYLTADAMGRYCGM	43
PsNosL	MNALHRIGAGTLLAVLLAFGLTG/CGEKEEVQQSLEPVAFHDSDCHVCGM	50
	thiol/disulphide pair	
RmNosL	NVLEHPGPKGQIIQDIPEPIWFSSARDTVAFTMLPEEPRDVAIIYVSDM	93
PsNosL	IIITDFPGPKGQAV--EKRGVKKFCSTAEMLGWLQPENRLDLAKLYVHDM	98
RmNosL	GAAPSWQEPGAENWIDAKKAFYVIGSKVRGNGAAEAVPFSSERAARDFA	143
PsNosL	GRSV-WEKPDDGHLIDATSAYVVVGTSLKGAMGAS-LASFAEEQDAKALA	146
RmNosL	AKNGGRVTGFAEIPKGYVLGTGTAEQGAIIETESHGEAQING	185
PsNosL	GMHGGVLRFFEEIDQALLQEAASMQHGGMHDPNGAHNAHAGH	190
	metal-binding site	

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/Gen. 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.

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 ORF 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 (2 KLTVTAILAATLFLAGC 18) at the N terminus (Fig. 4) with a predicted lipid attachment site located at the cysteine residue (Cys-18), qualifying NosL as an outer-membrane lipoprotein (Hayashi & Wu, 1990). In addition to the conserved location of the cysteine for lipid attachment and cleavage, the presequence in both signal peptides also conforms to the consensus pattern with one lysine or arginine residue in the first seven positions. However, both the C-terminal signature sequence (170 MXHXXMHXHX $_5$ HXXHXXH 190) of a potential metal-binding site and the N-terminal active-site thiol/disulphide pair (45 CHVC 50) of a protein

Table 2. Complementation analysis of the *R. meliloti* nos region

Cosmid pYC7 carries the wild-type *nos* region. Each pYC7 derivative containing a Tn5 insertion was introduced into the *R. meliloti* nos mutant used as recipient and the transconjugants were tested for N₂O reduction activity.

Recipient	Genotype	N ₂ O reduction activity of pYC7::Tn5 transconjugants*					
		Tn5-A43	Tn5-B93	Tn5-A28	Tn5-C11	Tn5-B42	Tn5-878
RmWM1182	<i>nosX</i> ::Tn5-A43	—	(+)	+	+	+	—
RmWM1183	<i>nosX</i> ::Tn5-B93	(+)	—	+	+	+	—
RmWM1195	<i>nosX</i> ::Tn5-7100	—	—	+	+	+	—
RmWM1181	<i>nosX</i> ::Tn5-703	—	—	+	+	+	—
RmWM1186	<i>nosF</i> ::Tn5-A28	+	+	—	+	+	+
RmWM1190	<i>nosF</i> ::Tn5-B13	+	+	—	+	+	+
RmWM1192	<i>nosD</i> ::Tn5-B90	+	+	—	+	+	+
RmWM1194	<i>nosZ</i> ::Tn5-C11	+	+	+	—	+	+
RmWM1185	<i>nosZ</i> ::Tn5-893	+	+	+	—	+	+
RmWM1191	<i>nosR</i> ::Tn5-B42	+	(+)	+	+	—	—
RmWM1184	<i>nosR</i> ::Tn5-878	+	+	+	+	—	—
RmWM1179†	Wild-type	+	+	+	+	+	+

* +, Wild-type activity [approx. 750 nmol N₂O (mg protein)⁻¹ h⁻¹]; —, negative activity (<10% of wild-type activity); (+), delayed partial activity (<50% wild-type activity after >6 h incubation).

† The Tn5 insert in RmWM1179 (JJ1c10::Tn5-7C35) is outside the *nos* gene cluster.

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.

ACKNOWLEDGEMENTS

We thank Roselyn Heys for assistance in DNA sequence determination and Teresa Martin for helping in complementation analysis. We also appreciate the useful comments on the manuscript by Thérèse Ouellet and Laurian Robert. This paper is ECORC contribution number 971117.

REFERENCES

- Boyer, H. W. & Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* **41**, 459–472.
- Chan, Y.-K. & Wheatcroft, R. (1993). Detection of a nitrous oxide reductase structural gene in *Rhizobium meliloti* strains and its location on the *nod* megaplasmid of JJ1c10 and SU47. *J Bacteriol* **175**, 19–26.
- Chan, Y.-K., Barran, L. & Bromfield, E. S. P. (1989). Denitrification activity of phage types representative of two populations of indigenous *Rhizobium meliloti*. *Can J Microbiol* **35**, 737–740.
- Chan, Y.-K., Barraquio, W. L. & Knowles, R. (1994). N₂-fixing pseudomonads and related soil bacteria. *FEMS Microbiol Rev* **13**, 95–118.
- Cuyper, H. & Zumft, W. G. (1992). Regulatory components of the denitrification gene cluster of *Pseudomonas stutzeri*. In *Pseudomonas: Molecular Biology and Biotechnology*, pp. 188–197. Edited by E. Galli, S. Silver & B. Witholt. Washington, DC: American Society for Microbiology.
- De Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestrojuan, G., Neyra, M., Collins, M. D., Dreyfus, B., Kersters, K. & Gillis, M. (1994). Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., and *Sinorhizobium teranga* sp. nov. *Int J Syst Bacteriol* **44**, 715–733.
- Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. (1980). Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci USA* **77**, 7347–7351.
- Deusch, A., Riester, J., Kroneck, P. M. H. & Zumft, W. G. (1996). Mutation of the conserved Cys165 outside of the Cu_A domain destabilizes nitrous oxide reductase but maintains its catalytic activity: evidence for disulfide bridges and a putative protein disulfide isomerase gene. *Eur J Biochem* **237**, 447–453.
- Eisenberg, D., Schwarz, E., Komaromy, M. & Wall, R. (1984). Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J Mol Biol* **179**, 125–142.
- Fallik, E., Chan, Y.-K. & Robson, R. L. (1991). Detection of alternative nitrogenases in aerobic gram-negative nitrogen-fixing bacteria. *J Bacteriol* **173**, 365–371.
- Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci USA* **76**, 1648–1652.
- Finan, T. M., Kunkel, B., Vos, G. F. D. & Signer, E. R. (1986). Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J Bacteriol* **167**, 66–72.
- Fischer, H.-M. (1994). Genetic regulation of nitrogen fixation in rhizobia. *Microbiol Rev* **58**, 352–386.

- Friedrich, B., Böcker, C., Eberz, G. & 8 other authors (1990). Genes for hydrogen oxidation and denitrification form two clusters on megaplasmid pHG1 of *Alcaligenes eutrophus*. In *Pseudomonas: Biotransformations, Pathogenesis, and Evolving Biotechnology*, pp. 408–419. Edited by S. Silver, A. M. Chakrabarty, B. Iglewski & S. Kaplan. Washington, DC: American Society for Microbiology.
- Glockner, A. B. & Zumft, W. G. (1996). Sequence analysis of an internal 9.72 kb segment from the 30 kb denitrification gene cluster of *Pseudomonas stutzeri*. *Biochim Biophys Acta* 1277, 6–12.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* 166, 557–580.
- Hayashi, S. & Wu, H. C. (1990). Lipoproteins in bacteria. *J Bioenerg Biomembr* 22, 451–471.
- von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res* 14, 4683–4690.
- Hennecke, H., Anthamatten, D., Babst, M. & 8 other authors (1993). Genetic and physiologic requirements for optimal bacteroid function in the *Bradyrhizobium japonicum* soybean symbiosis. In *Advances in Molecular Genetics of Plant-Microbe Interactions*, vol. 2, pp. 199–207. Edited by E. W. Nester & D. P. S. Verma. Dordrecht: Kluwer.
- Holloway, P., McCormick, W., Watson, R. J. & Chan, Y.-K. (1996). Identification and analysis of the dissimilatory nitrous oxide reduction genes, *nosRZDFY*, of *Rhizobium meliloti*. *J Bacteriol* 178, 1505–1514.
- Klein, P., Kanehisa, M. & DeLisi, C. (1985). The detection and classification of membrane-spanning proteins. *Biochim Biophys Acta* 815, 468–476.
- Knauf, V. C. & Nester, E. W. (1982). Wide host range cloning vectors: a cosmid clone bank of *Agrobacterium Ti* plasmid. *Plasmid* 8, 45–54.
- Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 157, 105–132.
- Rao, J. K. M. & Argos, P. (1986). A conformational preference parameter to predict helices in integral membrane proteins. *Biochem Biophys Acta* 869, 197–214.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74, 5463–5467.
- Schmehl, M., Jahn, A., zu Vilsendorf, A. M., Hennecke, S., Masepohl, B., Schuppler, M., Marxer, M., Oelze, J. & Klipp, W. (1993). Identification of a new class of nitrogen fixation genes in *Rhodobacter capsulatus*: a putative membrane complex involved in electron transport to nitrogenase. *Mol Gen Genet* 241, 602–615.
- Selvaraj, G., Hooper, I., Shantharam, S., Iyer, V. N., Barran, L., Wheatcroft, R. & Watson, R. J. (1987). Derivation and molecular characterization of symbiotically deficient mutants of *Rhizobium meliloti*. *Can J Microbiol* 33, 739–747.
- Toffanin, A., Wu, Q., Maskus, M., Casella, S., Abruna, H. D. & Shapleigh, J. P. (1996). Characterization of the gene encoding nitrite reductase and the physiological consequences of its expression in the nondenitrifying *Rhizobium 'bedysari'* strain HCNT1. *Appl Environ Microbiol* 62, 4019–4025.
- Wu, Q., Knowles, R. & Chan, Y.-K. (1995). Production and consumption of nitric oxide by denitrifying *Flexibacter canadensis*. *Can J Microbiol* 41, 585–591.
- Zumft, W. G. (1992). The denitrifying prokaryotes. In *The Prokaryotes*, 2nd edn, pp. 554–582. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.
- Zumft, W. G. & Kroneck, P. M. H. (1990). Metabolism of nitrous oxide. In *Denitrification in Soil and Sediment*, pp. 37–55. Edited by N. P. Revsbech & J. Sørensen. New York: Plenum Press.
- Zumft, W. G., Viebrock-Sambale, A. & Braun, C. (1990). Nitrous oxide reductase from denitrifying *Pseudomonas stutzeri*: genes for copper-processing and properties of the deduced products, including a new member of the family of ATP/GTP-binding proteins. *Eur J Biochem* 192, 591–599.
- Zumft, W. G., Dreusch, A., Löchelt, S., Cuypers, H., Friedrich, B. & Schneider, B. (1992). Derived amino acid sequences of the *nosZ* gene (respiratory N_2O reductase) from *Alcaligenes eutrophus*, *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* reveal potential copper-binding residues: implications for the Cu_A site of N_2O reductase and cytochrome-*c* oxidase. *Eur J Biochem* 208, 31–40.

Received 24 February 1997; revised 24 April 1997; accepted 25 April 1997.