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Detection of a Nitrous Oxide Reductase Structural Gene in *Rhizobium meliloti* Strains and Its Location on the *nod* Megaplasmid of JJ1c10 and SU47†

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The gene encoding a denitrification enzyme, nitrous oxide reductase (EC 1.7.99.6), in *Rhizobium meliloti* and other gram-negative bacteria was detected by hybridization to an internal 1.2-kb *Pst*I fragment of the structural gene (*nosZ*) cloned from *Pseudomonas stutzeri* Zobell (W. G. Zumft, A. Viebrock-Sambale, and C. Braun, Eur. J. Biochem. 192:591–599, 1990). Homology to the probe was detected in the DNAs of two N_2-fixing strains of *P. stutzeri*, two denitrifying *Pseudomonas* species, one *Alcaligenes eutrophus* strain, and 36 of 56 *R. meliloti* isolates tested. Except for two isolates of *R. meliloti*, all showed nitrous oxide reduction activity (Nos^+). Therefore, at least part of the *nosZ* sequence appears to be conserved and widely distributed among denitrifiers, which include free-living and symbiotic diazotrophs. By using *Agrobacterium tumefaciens* transconjugants harboring different megaplasmids of *R. meliloti* JJ1c10 and SU47, sequence homology with the *nosZ* probe was unequivocally located on the *nod* megaplasmid. A cosmid clone of JJ1c10 in which *nosZ* homology was mapped on a 4.2-kb *Bam*HI fragment was selected. This cosmid, which conferred Nos^+ activity to the *R. meliloti* wild-type strains ATCC 9930 and Balsac (Nos^- and nondenitrifying, respectively) also restored Nos^+ activity in the mutants of JJ1c10 and SU47 in which the 4.2-kb *Bam*HI segment was deleted. Therefore, this segment contains sequences essential for *nos* gene expression in JJ1c10 and SU47 and thus confirms that the *nod* megaplasmid in JJ1c10 and SU47 which carries genes essential for symbiotic dinitrogen fixation also carries genes involved in the antagonistic process of denitrification.

Bradyrhizobium and *Rhizobium* species are bacteria that fix dinitrogen (N_2) in symbiosis with legumes (see reference 2 for a review). They are among the seven N_2-fixing genera that contain denitrifying strains (53). Denitrifiers are facultative anaerobes capable of growth and energy conservation under O_2 limitation. This is achieved by the stepwise dissimilatory reduction of nitrate or nitrite as the terminal electron acceptor coupled to proton translocation with the formation of gaseous N products (40): $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. Although most denitrifiers possess the four reductases necessary to carry out the complete denitrification pathway, some carry out a truncated one because of their lack of NO_3^- or N_2O reductase (26a). In the latter case, N_2O is accumulated as the end product. The basic biochemistry and genetics of denitrification have been well studied with the model organism *Pseudomonas stutzeri* Zobell (ATCC 14405 [formerly *Pseudomonas perfectomarina*]; 15, 50), of marine origin, and the results have recently been summarized by Cuypers and Zumft (13a). Its denitrification enzymes and corresponding genes (*nar*, *nir*, *nor*, and *nos*) have been characterized. With the exception of the *nar* complex, the structural genes of the dissimilatory reductases are closely linked and transcribed in the same direction. They are arranged in the order *nos-nir-nor*, spanning a distance of less than 30 kb, with the *nos* and *nir* structural genes separated by about 14 kb and the *nos* operon measuring about 8 kb (13a, 25a, 50).

With the exception of *Rhizobium meliloti*, apparently few *Rhizobium* species strains can denitrify, compared with *Bradyrhizobium* species (34). In a collection of distinct *R. meliloti* isolates from alfalfa nodules, 48% (57 of 120) showed significant denitrification activity (10). Recently, denitrification by detached alfalfa nodules and isolated bacteroids (3, 3a) was suggested to be inducible by nitrate, which, when present at relatively low concentrations (≤5 mM), was largely metabolized by *R. meliloti* bacteroids in nodulated alfalfa plants (3). In the same study, the level of nitrite reductase activity of the isolated bacteroids was estimated to be half that of nitrate reductase; however, nitrous oxide (N_2O) reductase activity was not studied.

There is little information on the distribution of N_2O reductase (EC 1.7.99.6) in *R. meliloti* strains. All five denitrifying strains tested by Daniel et al. (14) were reported to produce N_2 as the end product, implying the presence of N_2O reductase and the complete denitrification pathway. N_2O is reduced not only by denitrifiers but also by nitrogenase (33) and nondenitrifying N_2O utilizers, e.g., *Wollinella succinogenes* (formerly *Vibrio succinogenes*; 49). With the exception of nitrogenase, all N_2O reductases are periplasmic multicopper enzymes (see reference 51 for a review). The first denitrifying enzyme that was isolated and purified was from *P. stutzeri* Zobell. Since then, detailed biochemical and genetic studies have greatly extended the fundamental understanding of N_2O respiration in this organism (51). The structural gene for N_2O reductase, *nosZ*, has been cloned and sequenced previously (41, 54). In an unpublished investigation (30), *nosZ* was found to hybridize with the DNAs of several denitrifying *Pseudomonas* species, *Alcaligenes eutrophus*, and *Paracoccus denitrificans*, indicating that this conserved *nosZ* would be a useful probe for screening bacteria with similar N_2O reductases. Recently, von Berg and Bothe (43a) reported the successful use of NO_2^- and N_2O reductase DNA probes from *A. eutrophus* and *P. stutzeri* to screen for the distribution of denitrifying bacteria in soils. To determine the distribution and significance of

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denitrification in the symbiotic N_2-fixing species *R. meliloti*, we have used an internal 1.2-kb *Pst*I segment of the N_2O reductase structural gene (*nosZ*) from *P. stutzeri* Zobell (41, 54) to probe the DNAs of a number of *R. meliloti* isolates from various geographical locations.

Genes encoding the denitrification enzymes are plasmid borne in *A. eutrophus* (21, 38a) but not in *P. stutzeri* (50). To date, there is no fully published investigation of the genetics of rhizobial denitrification. We report here that the N_2O reductase structural gene sequence is located on the *nod* megaplasmid in at least two strains of *R. meliloti*. In this species, the *nod* megaplasmid is known to carry multiple genes (*nod*, *nif*, and *fix*) essential for symbiotic N_2 fixation (4, 6, 37). This finding was unexpected, in that genes controlling antagonistic pathways of nitrogen metabolism are thus linked in *R. meliloti*.

(A preliminary account of this work has been presented previously [12].)

MATERIALS AND METHODS

Plasmids, strains, and media. The principal strains of *R. meliloti* used are ATCC 9930 (American Type Culture Collection, Rockville, Md.), Balsac (L. M. Bordeleau, Sainte-Foy Research Station, Sainte-Foy, Québec, Canada), JJ1c10 (see Table 1), and SU47 (= RCR2011 or 2011; J. Brockwell, Commonwealth Scientific and Industrial Research Organization, Canberra, Australia). The bacterial strains tested for homology to *nosZ* of *P. stutzeri* Zobell have been included and referenced in previous publications (5, 8, 11, 45, 46). Additional strains tested were *R. meliloti* Rm41 (E. Kondorosi, Hungarian Academy of Sciences, Szeged, Hungary), *Bradyrhizobium japonicum* 61A76 (R. Griffin, Department of Agriculture, Beltsville, Md.), *A. eutrophus* ATCC 17699 (=H16), and *Escherichia coli* JM83 (42) and NS220 [=HB101(pNS220)] (41, 54). Other laboratory strains and plasmids used in this work are described in Table 1. *Rhizobium*, *Azorhizobium*, and *Agrobacterium* species strains were grown aerobically on TYC (tryptone-yeast extract-calcium) medium (17) at 28°C; *Bradyrhizobium* species strains were grown in YEM at 28°C (17); and *E. coli* was grown in Luria-Bertani medium (38) at 37°C. Nutrient broth or tryptic soy broth was used to maintain and grow *Pseudomonas* species strains and other bacteria. Agar at 1.6% (wt/vol) was added for solid medium. The antibiotics used were ampicillin (50 μg/ml), chloramphenicol (15 μg/ml), kanamycin (20 or 40 μg/ml), nalidixic acid (10 μg/ml), streptomycin (25 μg/ml), tetracycline (5 or 10 μg/ml), and rifampin (100 μg/ml).

Bacterial conjugation. Tripartite matings in patches on TYC plates were used to transfer the pBR322-based plasmid pRWRm74 from *E. coli* to *R. meliloti* for site-directed DNA recombination (13). Tc^r Km^r exconjugant *R. meliloti* colonies were repeatedly subcultured in liquid medium to allow for recombination and were monitored for the appearance of Tc^s Km^r colonies by plating. Replacement of the 4.2-kb *Bam*HI fragment and loss of the cloning vehicle were verified by Southern hybridization. Transfers of cosmids from *E. coli* to *R. meliloti* in tripartite matings were carried out with the helper plasmid pRK2013 (18) or pRK600 (19).

DNA manipulations. Recombinant plasmid DNA was extracted from *E. coli* as previously described (46). DNA digests, ligations, and transformations were performed according to the method described by Sambrook et al. (38). Genomic DNAs of *R. meliloti* strains were isolated from 5-ml cultures, restricted with endonucleases, and probed in Southern blots as previously described (47). DNA probes were labeled with [^{32}P]dCTP (Dupont Canada) by nick translation and used for colony hybridization or for Southern blots according to standard procedures (38). Kodak XAR-2 film was used for autoradiography.

Nitrous oxide reduction assay. Anaerobic rhizobial cultures (8 ml in tubes [18 by 150 mm] closed with Suba-Seals) were aseptically prepared in modified Vincent's defined medium (pH 7 [43]) on the basis of a procedure previously described (10). Medium modifications (per liter) were $FeCl_3 \cdot 6H_2O$ (5 mg), $CuSO_4 \cdot 5H_2O$ (2.5 mg), $Na_2MoO_4 \cdot 2H_2O$ (25 mg), monosodium glutamate (1.1 g) as N source, mannitol (3.64 g) as C source, and vitamins (biotin, pantothenic acid, and thiamine at 20 μg each). Glucose (20 mM) was used as a carbon source for other cultures. Each culture was evacuated and backfilled three times with N_2 (zero-gas grade; Matheson Gas Products, Ottawa, Ontario, Canada) to atmospheric pressure (101.3 kPa) through sterile 0.2-μm-pore-size Gelman Teflon syringe filters. One-tenth of the headspace volume was then replaced with sterile 1% N_2O in N_2. The headspace N_2O in the cultures and uninoculated controls was monitored for up to 48 h of incubation by electron capture-gas chromatography as previously described (9). The amount of cell protein was determined by the Lowry method according to the procedure described by Chan and Marshall (11). The mean specific activity for duplicate cultures was computed and expressed in nanomoles of N_2O reduced per milligram of protein per hour.

RESULTS

Detection of *nosZ* homology in nonrhizobial gram-negative bacteria. To ascertain whether the *nosZ* probe (1.2-kb *Pst*I fragment of pNS220) from *P. stutzeri* Zobell (41, 54) would be generally useful for the detection of *nosZ*-like genes, Southern blots of restricted total cellular DNAs of pseudomonads and other nonrhizobial gram-negative bacteria were probed (Table 2).

Two N_2-fixing strains of *P. stutzeri*, CMT.9.A (29) and JM300 (5), were shown to have homology in *Pst*I fragments of the same size as the probe, suggesting that the *nosZ* gene is conserved in this species. However, no homology was detected in *P. stutzeri* ATCC 17832, which lacked N_2O reductase activity (Nos^-) but was still capable of denitrifying nitrate. The probe also hybridized to *Pst*I restriction fragments of different sizes in another two of seven *Pseudomonas* species, one of which (*Pseudomonas diazotrophicus* H8) is known to fix N_2 (44). Homology in *A. eutrophus* ATCC 17699 but not in ATCC 17706 was detected. No homology in any of the enteric species tested was detected.

All of the strains that showed DNA homology to the *nosZ* probe were shown to be Nos^+ denitrifers (Table 2). Conversely, no homology to *nosZ* was detected in any Nos^- strain, regardless of denitrification capability. Denitrifiers such as *Pseudomonas aureofaciens* and *Pseudomonas chlororaphis* are known to be Nos^- (22) and to lack N_2O reductase (28, 31). Such observations were corroborated by the failure of these species to hybridize with the *nosZ* structural gene probe. Thus, hybridization with the *nosZ* probe and N_2O reduction activity were positively correlated in the nonrhizobial strains tested and indicate the presence of functional *nos* genes.

***R. meliloti* strains and other members of the *Rhizobiaceae* family.** By hybridization, it was shown that the *P. stutzeri* *nosZ* probe possessed homology to the DNAs of 64% (36 of 56) of distinct *R. meliloti* isolates from various geographic

TABLE 1. Bacterial strains and plasmids[a]

Bacterial strain or plasmid	Description	Source or reference
Laboratory strains		
Derivatives of *R. meliloti* IZ450		
JJ1c10 (= KN1001)	Spontaneous Rif^r derivative of IZ450	1
RmRW2255	JJ1c10::pRWRm74 exconjugant; Rif^r Tc^r	This work
RmRW2267	*nos* deletion derivative of RmRW2255; Rif^r Km^r	This work
RmYC2286	RmRW2267 (pRWRm67) exconjugant; Rif^r Km^r Tc^r	This work
Derivatives of *R. meliloti* SU47		
RmRW1035	Spontaneous Rif^r derivative of SU47	This work
RmRW2262	RmRW1035::pRWRm74 exconjugant; Rif^r Tc^r	This work
RmRW2273	*nos* deletion derivative of RmRW2262; Rif^r Km^r	This work
RmYC2287	RmRW2273(pRWRm67) exconjugant; Rif^r Km^r Tc^r	This work
Derivatives of *R. meliloti* ATCC 9930		
RmYC2164	Spontaneous Nal^r derivative of ATCC 9930	This work
RmYC2165	RmYC2164(pGMI42) exconjugant; Nal^r Tc^r	This work
RmYC2171	RmYC2164(pRWRm67) exconjugant; Nal^r Tc^r	This work
Derivatives of *R. meliloti* Balsac		
RmYC2160	Spontaneous Nal^r Rif^r derivative of Balsac	This work
RmYC2161	RmYC2160(pGMI42) exconjugant; Nal^r Rif^r Tc^r	This work
RmYC2169	RmYC2160(pRWRm67) exconjugant; Nal^r Rif^r Tc^r	This work
A. tumefaciens		
GMI9050	Rif^r Str^r derivative of C58	19
At117	GMI9050(pRmeSU47a::Tn*5*-11)	19
At104	GMI9050(pRmeSU47b::Tn*5*-*oriT*)	19
PD2001	A348(pRmeJJ1c10a::Tn*5*-A1)	V. N. Iyer and P. A. Donaldson, Carleton University, Ottawa, Ontario, Canada; 24
PD91	A348(pTA2::Tn*5*-A1)	
E. coli		
GMI3420	= PK1046(pGMI42)	25
Plasmids		
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; 41, 54
pGMI42	285-kb fragment of the *nod* megaplasmid (pRmeSU47a) of SU47 with Tn*5* inserted in the *nif* region; Ap^r Bleo^r Km^r Tc^r (RP4 prime)	25, 36
pRWRm67	Cosmid clone selected from JJ1c10 *Eco*RI-digested genomic library by hybridization to pNS220; Tc^r (pLAFRI)	This work; 39
pRWRm71	10.1-kb *Eco*RI fragment of pRWRm67 selected by hybridization to pNS220; Ap^r (pRWRm70)	This work
pWMRm1	3.6-kb *Bam*HI fragment of pRWRm71 selected by hybridization to pNS220; Ap^r (pUC19)	This work
pRWRm72	5.9-kb *Eco*RI fragment, deletion derivative of pRWRm71 lacking two contiguous *Bam*HI segments; Ap^r (pRWRm70)	This work
pRWRm73	1.7-kb *Bgl*II fragment containing Km^r gene from pRWRm18 replacing DNA deleted in pRWRm72; Ap^r Km^r (pRWRm70)	This work
pRWRm74	7.6-kb *Eco*RI insert as in pRWRm73; Ap^r Km^r Tc^r (pBR322)	This work
pRWRm18	Source of 1.7-kb *Bgl*II fragment containing Km^r gene from Tn*903* (23); Ap^r Km^r Tc^r (pBR322)	46
pUC19	*E. coli* cloning vehicle; Ap^r	48
pRWRm70	Derivative of pUC19 in which *Bam*HI and *Sac*I sites are deleted; Ap^r	This work
pBR322	*E. coli* cloning vehicle; Ap^r Tc^r	7
pLAFRI	Broad-host-range cosmid cloning vehicle; IncP1 Tc^r	20
pRK2013	ColE1 replicon containing RK2 transfer genes; Km^r	18
pRK600	pRK2013::Tn*9* derivative; Cm^r	19

[a] Resistance to antibiotics abbreviated as follows: Ap^r, ampicillin; Bleo^r, bleomycin; Cm^r, chloramphenicol; Km^r, kanamycin; Nal^r, nalidixic acid; Str^r, streptomycin; Tc^r, tetracycline; Rif^r, rifampin.

TABLE 2. Hybridization of the *P. stutzeri* Zobell (ATCC 14405) *nosZ* probe to genomic DNAs from nonrhizobial gram-negative bacteria and their N_2O reduction phenotype (Nos)

Strain(s)	Size (kb) of hybridizing restriction fragment		Nos phenotype[a]
	*Pst*I	*Eco*RI	
Pseudomonas species			
P. stutzeri			
ATCC 17832	ND[b]	NT[c]	−
CMT.9.A[d]	1.2	4.8	+
JM300[d]	1.2	15	+
P. aeruginosa PAO	3.8	23	+
P. aeruginosa PAC5	1.2	23	+
P. aureofaciens	ND	NT	−
P. chlororaphis	ND	NT	−
P. diazotrophicus H8[d]	18, 14.5, 8.8, and 1.8	7.0	+
P. putida[e] mt-2b (Paw1)	ND	ND	−
Pseudomonas sp. 4B[d,e] and DC[d]	ND	NT	−
Other gram-negative bacteria			
A. eutrophus			
ATCC 17706	ND	ND	−
ATCC 17699	3.2	17	+
E. coli[e] HB101 and JM83	ND	ND	−
Klebsiella pneumoniae[e] M5aL	ND	ND	−

[a] A positive phenotype indicates an N_2O reduction activity of at least 100 nmol · mg^{-1} of protein · h^{-1}; a negative phenotype has less than 10% of this activity. See Materials and Methods for a description of the assay method.
[b] ND, none detected.
[c] NT, not tested.
[d] Diazotrophic species or strain (5).
[e] Nondenitrifying species or strain.

locations (Table 3). However, in one subgroup of these isolates originating in the Ottawa vicinity, 82% (23 of 28) showed homology while in another subgroup, isolated from West Asia (i.e., Jordan, Syria, and Turkey [16]), only 12% (1 of 8) showed homology to the probe. The size uniformity of hybridizing *Pst*I and *Eco*RI restriction fragments indicated that the *nos* structural gene region is highly conserved in *R. meliloti*.

Homology to the *nosZ* probe was not detected in three biovars of *Rhizobium leguminosarum*, three *Bradyrhizobium* species, or two other species of the *Rhizobiaceae* family tested (Table 3). Since they were also shown to be Nos^-, it

TABLE 3. Hybridization of the *P. stutzeri* Zobell (ATCC 14405) *nosZ* probe to genomic DNAs from *R. meliloti* strains and other members of the family *Rhizobiaceae* and their N_2O reduction phenotype (Nos)

Strains	Size (kb) of hybridizing restriction fragment		Nos phenotype[a]
	*Pst*I	*Eco*RI	
R. meliloti			
IZ450, JJ1c10, NGR185, R304, R423, Rm41, SU47, U45, 102F34, 102F51, 102F70, M275,[b] BT5,[c] MB1,[c] MB3,[c] MB7,[c] MB10,[c] MB18,[c] MBA9,[c] MBA18,[c] MBA34,[c] 143,[c] 247,[c] 408,[c] 472,[c] 982,[c] 1094,[c] 1132,[c] 1187,[c] 1218,[c] 1232,[c] 1398,[c] 1426,[c] and 1648-A[c]	6.6	10.1	+
ATCC 9930, Balsac,[d] DMG117, NRG43, 2012, 2035, CC2003, CC2013, M1,[b] M3,[b] M102,[b] M161,[b] M205,[b] 15A6,[b] 74B12,[b] I-6-5,[b] MBA38,[c] 268,[c] 1227,[c] and 1642-B[c]	ND[e]	ND	−
DMG118 and MBA25[c]	6.6	10.1	−
R. leguminosarum			
bv. phaseoli (127K12b and CFN-Rph215), trifolii (3D1k19a), and *viciae* (DMG112)	ND	ND	−
Bradyrhizobium spp.			
B. japonicum 61A76, *Bradyrhizobium* sp. 520 (*Lupinus*), and RCR3824 (*Vigna*)	ND	ND	−
Other species of the family *Rhizobiaceae*			
Azorhizobium caulinodans ORS571 and *A. tumefaciens* C58	ND	ND	−

[a] See footnote *a* in Table 2.
[b] Isolate from Ottawa vicinity (45).
[c] Isolate from West Asia (16).
[d] Nondenitrifying strain.
[e] ND, none detected.

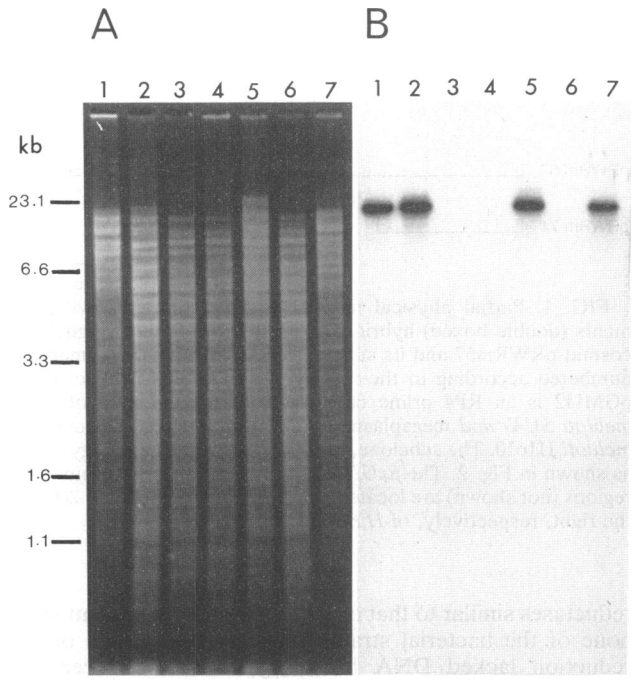

FIG. 1. Hybridization of the *nosZ* probe to genomic DNAs from *R. meliloti* strains and *A. tumefaciens* transconjugants. (A) Ethidium bromide-stained 0.8% agarose gel showing genomic DNA digested with *Eco*RV; (B) autoradiograph of the subsequent Southern blot hybridized (at 42°C; 50% formamide) with a purified and ^{32}P-labeled probe, the 1.2-kb *Pst*I internal fragment of the N_2O reductase structural gene (*nosZ*) from *P. stutzeri* Zobell carried in pNS220. Lanes: 1, *R. meliloti* SU47; 2, *A. tumefaciens* At117, carrying the *nod* megaplasmid (pRmeSU47a) of SU47; 3, *A. tumefaciens* At104, carrying the *exo* megaplasmid (pRmeSU47b) of SU47; 4, *A. tumefaciens* GMI9050, not carrying any *R. meliloti* megaplasmids; 5, *A. tumefaciens* PD2001 carrying the *nod* megaplasmid (pRmeJJ1c10a) of JJ1c10; 6, *A. tumefaciens* PD91, carrying the cryptic plasmid (pTA2 [pRmeIZ450c]) of IZ450; 7, *R. meliloti* JJ1c10.

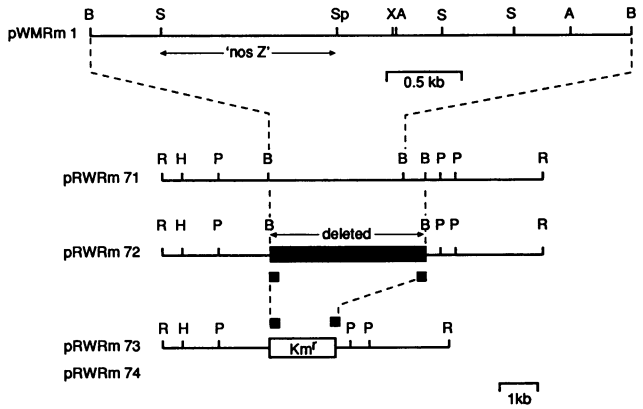

FIG. 2. Restriction map of pRWRm71, which contains the 10.1-kb *Eco*RI fragment of JJ1c10 having homology to *nosZ* of *P. stutzeri* Zobell. Maps of pRWRm72, pRWRm73, and pRWRm74 show the contiguous 3.6- and 0.6-kb *Bam*HI segments deleted from pRWRm71 and their replacement by a 1.7-kb *Bgl*II fragment containing a Km^r gene; the insert of pRWRm73 was switched to the vector pBR322 in pRWRm74 for mobilization into *R. meliloti* hosts. pWMRm1 is a subclone containing the 3.6-kb *Bam*HI segment in which the *nosZ* homology is more precisely located. Restriction sites are abbreviated as follows: A, *Ava*I; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sal*I; Sp, *Sph*I; X, *Xho*I.

is unlikely that they possess an N_2O reductase different from that of *R. meliloti*. Not every strain listed in Table 3 was tested for nitrate or nitrite respiration; hence, the percentage of denitrifying strains in this collection is unknown.

The positive correlation between *nosZ* hybridization and the Nos$^+$ phenotype in the various *R. meliloti* isolates demonstrated the usefulness of the *nosZ* probe for detecting functional N_2O reductases. However, two exceptions (isolates DMG118 and MBA25) were found in which N_2O reductase activity was not detected despite clear DNA homology with the probe (Table 3).

Genomic location of sequence homology with *nosZ* in *R. meliloti* JJ1c10 and SU47. To determine the genomic location of homology with the *nosZ* probe in *R. meliloti*, Southern hybridization experiments with *Agrobacterium tumefaciens* transconjugants carrying either the *nod* or the *exo* megaplasmid of *R. meliloti* were performed. The *nosZ* probe hybridized strongly with a distinct 23-kb *Eco*RV DNA fragment of *R. meliloti* JJ1c10 and SU47 genomic DNAs (Fig. 1). It also hybridized with a restriction fragment of similar size in *A. tumefaciens* transconjugants containing the *nod* megaplasmid of JJ1c10 or SU47, but not with the genomic DNA of the *A. tumefaciens* host itself or a transconjugant containing the *exo* megaplasmid of SU47. Hence, *nosZ*-like sequences were clearly identified on the *nod* megaplasmid in two distinct strains of *R. meliloti*; however, the genomic locations of the homology in other strains have not yet been verified.

Confirmation of *nosZ*-like gene locus and function. With pNS220 as a probe, one cosmid (pRWRm67) carrying *nosZ* homology on a 10.1-kb *Eco*RI fragment was selected by colony hybridization from an *Eco*RI genomic library of JJ1c10 (Table 1). Restriction mapping and subcloning of this fragment yielded pRWRm71 with *nosZ* homology identified on a 4.2-kb *Bam*HI segment (Fig. 2). A derivative of pRWRm71, pRWRm74, was made in which the vector was switched from pUC19 to pBR322 for mobilization into *R. meliloti*, and in which the internal 4.2-kb *Bam*HI segment was replaced by a 1.7-kb *Bgl*II fragment from Tn*903* conferring kanamycin resistance (Table 1; Fig. 2). By mating HB101(pRWRm74) with JJ1c10 and SU47 in the presence of a helper plasmid to assist conjugal transfer, site-directed deletion mutants were obtained from the exconjugants by recombination, resulting in the loss of the wild-type *R. meliloti* sequence and pBR322 (Fig. 3).

The deletion mutants (RmRW2267 and RmRW2273) were found to be Nos$^-$, and the wild-type Nos$^+$ activity was restored by the introduction of pRWRm67 (Table 4). These results clearly show that the denitrifying strains JJ1c10 and SU47 were Nos$^+$ only when they contained DNA homologous to the 4.2-kb segment of JJ1c10 which was deleted in pRWRm74 (Fig. 3; Table 4). Therefore, the *Bam*HI segment contains sequences essential for N_2O reduction in these strains. Subsequently, a 1.2-kb *Sal*I-*Sph*I subfragment in which homology to the *nosZ* probe was exclusively found was mapped (Fig. 2). Cosmid pRWRm67 also conferred Nos$^+$ activity to *R. meliloti* ATCC 9930, which is capable of denitrifying nitrate to N_2O only, and to Balsac, which is a nondenitrifying strain (Tables 3 and 4) (10).

The location of the region homologous to *nosZ* of *P. stutzeri* in the *nod* megaplasmid of *R. meliloti* SU47 was determined by hybridization of the *nosZ* probe to pGMI42, which is an RP4 prime carrying 285 kb of the *nod* megaplas-

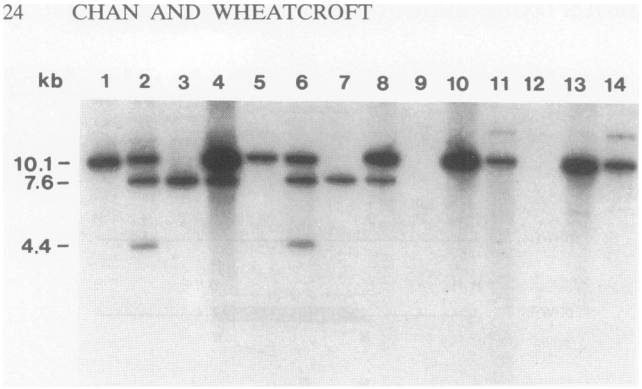

FIG. 3. Autoradiogram of *Eco*RI-digested total cellular DNAs of the strains listed in Table 4 probed with ^{32}P-labeled pRWRm71. Lanes 1 to 8 demonstrate site-specific exchanges of DNA from pRWRm74 for the wild-type regions in *R. meliloti* strains JJ1c10 and SU47 and transfer of pRWRm67 to the constructed Nos$^-$ mutants. The DNAs are from JJ1c10 (lane 1), in which there is hybridization to the 10.1-kb fragment that was cloned in pRWRm71; RmRW2255 (lane 2), an exconjugant of JJ1c10 containing pRWRm74 integrated by a single recombination such that there is hybridization to its 4.4-kb pBR322 component and its 7.6-kb insert; RmRW2267 (lane 3), a derivative of RmRW2255 in which a second recombination had occurred such that the pBR322 component and the 10.1-kb wild-type fragment were lost; RmYC2286 (lane 4), an exconjugant of RmRW2267 containing pRWRm67 which complements the mutation; RmRW1035 (lane 5), a spontaneous Rif^r derivative of SU47; RmRW2262 (lane 6), an exconjugant equivalent of pRmRW2255 containing pRWRm74; RmRW2273 (lane 7), an SU47 derivative equivalent of RmRW2267; and RmYC2287 (lane 8), an SU47 derivative equivalent of RmYC2286. Lanes 9 to 14 demonstrate the absence of hybridization in ATCC 9930 or Balsac unless pRWRm67 or pGMI42 is present. The DNAs are from RmYC2164 (lane 9), a spontaneous Nal^r derivative of ATCC 9930; RmYC2171 (lane 10), an exconjugant of RmYC2164 containing pRWRm67; RmYC2165 (lane 11), an exconjugant of RmYC2164 containing pGMI42; RmYC2160 (lane 12), a spontaneous Nal^r Rif^r derivative of Balsac; RmYC2169 (lane 13), an exconjugant of RmYC2160 containing pRWRm67; and RmYC2161 (lane 14), an exconjugant of RmYC2160 containing pGMI42. The relatively faint hybridization bands in lanes 11 and 14 are due to the homology of the Ap^r gene of the probe vector with that of pGMI42.

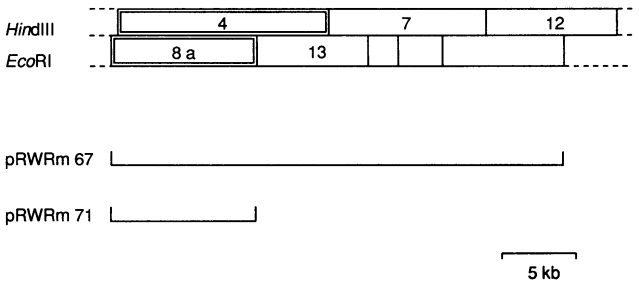

FIG. 4. Partial physical map of pGMI42 (top), showing fragments (double boxed) hybridizing to the *nosZ* probe, aligned with cosmid pRWRm67 and its subclone pRWRm71. The fragments are numbered according to the method described by Batut et al. (6). pGMI42 is an RP4 prime carrying a 285-kb fragment of the *R. meliloti* SU47 *nod* megaplasmid (25). pRWRm67 is a cosmid of *R. meliloti* JJ1c10. The subclone, pRWRm71, carries homology to *nosZ* as shown in Fig. 2. The *fixGHIS* (26) and the *nifHDK* promoter (6) regions (not shown) are located about 20 kb to the left and 200 kb to the right, respectively, of *Hin*dIII fragment 4.

mid (pRmeSU47a) of SU47 (6, 25, 36); cosmid pRWRm67, which carries the homologous region of JJ1c10; and its subclone, pRWRm71. Parity between JJ1c10 and SU47 in this DNA region was demonstrated by juxtaposing the partial physical map of pGMI42 with those of pRWRm67 and pRWRm71 (Fig. 4). In pGMI42, the *nos* region was thus mapped to be within *Eco*RI fragment 8a (10.1 kb, equivalent to the insert of pRWRm67) and *Hin*dIII fragment 4 (14.2 kb). The identification of *nos* in this previously silent region is located between the *fixGHIS* and *nifHDK* gene regions, i.e., about 20 kb upstream of *fixGHIS* and 200 kb downstream from the *nifHDK* promoter (26). As expected, Nos$^+$ activity in both ATCC 9930 and Balsac was also conferred by pGMI42 (Table 4).

DISCUSSION

We have shown by DNA probing that sequences homologous to that of *nosZ* of *P. stutzeri* are present in many gram-negative denitrifying bacteria, including those capable of fixing N_2. Excellent positive correlation was found between N_2O reduction activity and the presence of a *nosZ*-like sequence, thus demonstrating the usefulness of the *nosZ* probe for screening bacteria for copper-containing N_2O reductases similar to that of *P. stutzeri*. In the present study, none of the bacterial strains found to be capable of N_2O reduction lacked DNA homology to *nosZ*. Recently, a copper-independent N_2O reductase was implicated in *Flexibacter canadensis* (27), the DNA of which was found to lack homology with *nosZ* (32). However, two isolates of *R. meliloti* lacking N_2O reductase activity showed good homology to the probe (Table 3). It is speculated that their Nos$^-$ phenotype is due to genetic defects located outside the structural gene region related to N_2O reduction. DNA sequence not involved in N_2O reduction but with homology to *nosZ* has not been reported.

About two-thirds of the *R. meliloti* isolates tested in the present work were shown to possess N_2O reductase activity and presumed to be denitrifying strains. The ability to reduce N_2O as a diagnostic test for denitrifying *R. meliloti* is qualified by the possible existence in this species of N_2O utilizers which are not true denitrifiers. A few denitrifying strains (e.g., ATCC 9930) are known to be capable of reducing nitrate or nitrite but not N_2O. Such strains would be excluded by the N_2O reduction test as denitrifiers, but their proportion (although not determined) is probably small.

TABLE 4. Nitrous oxide reduction activities of *R. meliloti* strains and their derivatives[a]

Strain	Description	% Activity[b]
JJ1c10	IZ450; Rif^r	100
RmRW2255	JJ1c10::pRWRm74	86
RmRW2267	*nos* deletion derivative of RmRW2255	3
RmYC2286	RmRW2267(pRWRm67)	85
RmRW1035	SU47; Rif^r	96
RmRW2262	RmRW1035::pRWRm74	94
RmRW2273	*nos* deletion derivative of RmRW2262	8
RmYC2287	RmRW2273(pRWRm67)	109
RmYC2164	ATCC 9930; Nal^r	6
RmYC2171	RmYC2164(pRWRm67)	105
RmYC2165	RmYC2164(pGMI42)	102
RmYC2160	Balsac; Nal^r Rif^r	6
RmYC2169	RmYC2160(pRWRm67)	86
RmYC2161	RmYC2160(pGMI42)	91

[a] Nal^r, nalidixic acid resistant; Rif^r, rifampin resistant.
[b] A total of 100% activity was 743 ± 15 nmol · mg^{-1} of protein · h^{-1}.

The proportions of denitrifiers in two natural populations of *R. meliloti* at adjacent sites have been shown to differ markedly (10). Differences in soil type and vegetation which affect drainage, aeration, inorganic nitrogen content, and the availability of organic matter are expected to regulate the distribution of denitrifiers. The apparently high and low percentages of denitrifiers (82 and 12%, respectively) in the Ottawa and West Asian subcollections of *R. meliloti* isolates in the present survey may well reflect the immediate environment from which they were first isolated.

The discovery that a gene which is essential for N_2O reduction is located on the *nod* megaplasmid of *R. meliloti* JJ1c10 and SU47 was unexpected, since this megaplasmid is known to carry genes which are necessary for nodulation and symbiotic nitrogen fixation (4, 6, 37). Denitrification has not previously been considered to be a symbiotic function, since it is antagonistic to the process of N_2 fixation. The *nos* deletion mutants (RmRW2267 and RmRW2273) derived from JJ1c10 and SU47 were found to be visually indistinguishable from the wild type in their symbiosis with alfalfa in preliminary laboratory experiments (35). However, denitrification may serve an ancillary function which is not required for symbiosis per se.

Both cosmid pRWRm67, which carries the JJ1c10 DNA homologous to *nosZ* of *P. stutzeri*, and pGMI42, which carries the homologous region of the *nod* megaplasmid of *R. meliloti* SU47 (25), restored the wild-type (Nos^+) phenotype by complementation of deletions in both JJ1c10 and SU47 (Table 4). In this experiment, a 4.2-kb *Bam*HI segment, which was deleted in the mutants, was shown to be required for Nos^+ expression. In addition, cosmid pRWRm67 was found to be sufficient to confer the Nos^+ phenotype to exconjugants of the unrelated strains ATCC 9930 and Balsac (which are Nos^- denitrifier and nondenitrifier, respectively), neither of which possessed homology to *nosZ*. Hence, this cosmid may carry an entire *nos* operon if the latter is similar in size (ca. 8 kb) to that of *P. stutzeri* Zobell (41), unless the Nos^- strains are defective only in the *nos* structural gene. At first, pRWRm67 was the only cosmid with *nosZ* homology obtained from the *Eco*RI genomic library of JJ1c10. Our inability to obtain a series of cosmids with overlapping regions was probably due to the large sizes of the adjacent *Eco*RI fragments in this region of pGMI42 (6). Subsequently, two overlapping cosmids with *nosZ* homology were selected from another genomic library of JJ1c10 prepared from its total DNA digested with *Hin*dIII (32).

The observation that there is a *nos* gene in the *nod* megaplasmid of some, but not all, *R. meliloti* strains demonstrates the variability of this megaplasmid with regard to both structure and function. It may be more than a coincidence that the genes (*nif* and *nos*) involved in apparently antagonistic nitrogen metabolic processes are located on the same megaplasmid. It is also striking that one-third of the isolates tested had no detectable homology to the *nosZ* probe, which nevertheless appears to be well conserved in the remainder. Since denitrification genes are known to be borne on smaller plasmids in other bacterial species, e.g., *A. eutrophus* (21, 38a), the transfer of *nos* genes to *R. meliloti* may have been mediated by such plasmids.

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