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Alfalfa Yield Response to Inoculation with Recombinant Strains of *Rhizobium meliloti* with an Extra Copy of *dctABD* and/or Modified *nifA* Expression

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The construction of rhizobial strains which increase plant biomass under controlled conditions has been previously reported. However, there is no evidence that these newly constructed strains increase legume yield under agricultural conditions. This work tested the hypothesis that carefully manipulating expression of additional copies of *nifA* and *dctABD* in strains of *Rhizobium meliloti* would increase alfalfa yield in the field. The rationale for this hypothesis is based on the positive regulatory role that *nifA* plays in the expression of the *nif* regulon and the fact that a supply of dicarboxylic acids from the plant is required as a carbon and energy source for nitrogen fixation by the *Rhizobium* bacteroids in the nodule. These recombinant strains, as well as the wild-type strains from which they were derived, are ideal tools to examine the effects of modifying or increasing the expression of these genes on alfalfa biomass. The experimental design comprised seven recombinant strains, two wild-type strains, and an uninoculated control. Each treatment was replicated eight times and was conducted at four field sites in Wisconsin. Recombinant strain RMBPC-2, which has an additional copy of both *nifA* and *dctABD*, increased alfalfa biomass by 12.9% compared with the yield with the wild-type strain RMBPC and 17.9% over that in the uninoculated control plot at the site where soil nitrogen and organic matter content was lowest. These increases were statistically significant at the 5% confidence interval for each of the three harvests made during the growing season. Strain RMBPC-2 did increase alfalfa biomass at the Hancock site; however, no other significant increases or decreases in alfalfa biomass were observed with the seven other recombinant strains at that site. At three sites where this experiment was conducted, either native rhizobial populations or soil nitrogen concentrations were high. At these sites, none of the recombinant strains affected yield. We conclude that RMBPC-2 can increase alfalfa yields under field conditions of nitrogen limitation, low endogenous rhizobial competitors, and sufficient moisture.

Rhizobium and *Bradyrhizobium* are two bacterial genera whose members induce the formation of nitrogen-fixing root nodules on legumes. Mutants of *Bradyrhizobium japonicum* which express increased nitrogenase activity compared with that of the wild-type strains have been isolated previously (22, 26, 27, 36). Soybean yield increased after inoculation with one of these mutants in a California soil that lacked indigenous bradyrhizobia. There are no reports confirming these results in soils outside California (40).

A few genotypes and phenotypes have been described as improving symbiotic nitrogen fixation. These include uptake hydrogenase (16, 17, 20), acid tolerance (12), and a hybrid regulatory nodulation gene (38). Cannon, Ronson, and colleagues (10, 32) have constructed the appropriate strains to test whether modified or increased expression of a regulatory nitrogen fixation gene (*nifA*) and C_4-dicarboxylic acid transport (*dct*) should enhance the productivity of leguminous crops under nitrogen-limiting conditions. The objective of this work was to test this hypothesis in alfalfa under agricultural conditions.

The rationale for these genes providing increased productivity is based on the possibility that symbiotic nitrogen fixation is enhanced with carefully controlled increased expression of *nifA* and *dct*. This is based on the positive regulatory role that *nifA* plays in the expression of all other *nif* genes necessary for nitrogen fixation and on the fact that a supply of dicarboxylic acids from the plant is required as a carbon and energy source for nitrogen fixation by the *Rhizobium* bacteroids in the nodule.

Strains of *Rhizobium meliloti* which contain an extra copy of *nifA* and/or *dct* genes inserted into the chromosome of two wild-type strains of *R. meliloti* were used in this study. The

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TABLE 1. Relevant characteristics of the strains used in the 1992 field trials in Wisconsin

Strain	Parent strain	Genotype of added genes	Integration vector used in construction	Integration site
PC	None (wild type)	None	None	None
RMB7201	PC	Ω	pMW193	*ino*
RMB7203	PC	*nifA*/Ω	pMW191	*ino*
RMB7240	PC	*nifA*/Km^r	pMW242	P3
RMBPC-2	PC	*dctABD*/Ω/*nifA*	pMW300	*ino*
RCR2011	None (wild type)	None	None	None
RMB7101	RCR2011	Ω	pMW193	*ino*
RMB7103	RCR2011	*nifA*/Ω	pMW191	*ino*
RMB138Ω710A	RCR2011	*dctABD*/Ω	p138Ω710A	P3
RMB139Ω710B	RCR2011	2(*dctA*/Ω)	p139Ω710B	P3

Rhizobium transport system for C_4-dicarboxylates is a single protein permease encoded by the *dctA* gene. The expression of *dctA* is regulated by the *dctB* and *dctD* genes. The genetics of dicarboxylic acid transport in *Rhizobium leguminosarum* have been extensively studied (for a review, see reference 30). The *dctB* and *dctD* genes are regulatory genes that are required for C_4-dicarboxylic acid transport in free-living but not symbiotic rhizobia. The *dctA*-encoded permease transports succinate, fumarate, and malate. The nucleotide sequences of the *dctB* and *dctD* genes have been published elsewhere (29). Immediately downstream of the coding sequence is a structure characteristic of a *rho*-independent transcriptional terminator which has shown to be functional experimentally.

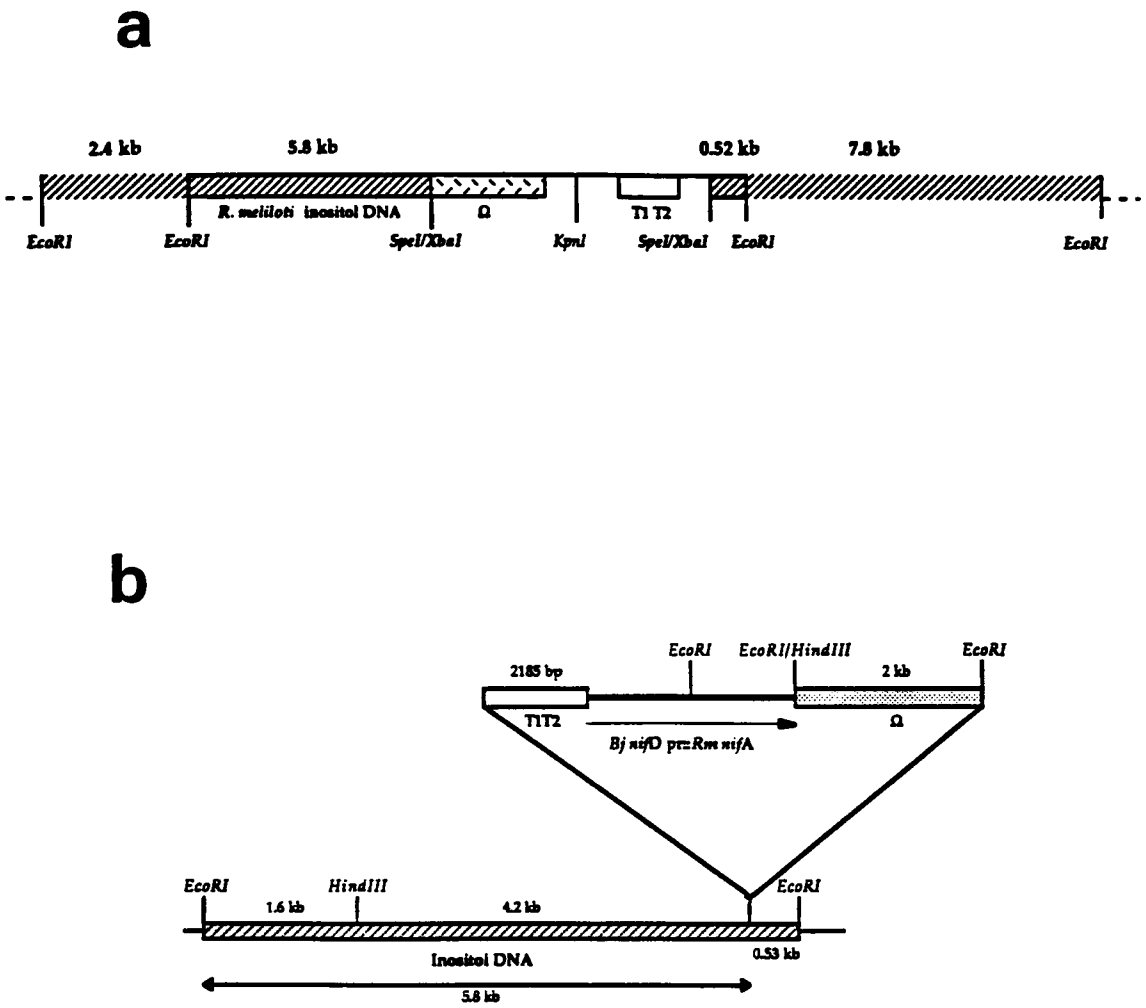

FIG. 1. Organization of the insert at the inositol locus in the chromosomes of RMB7101 and RMB7201 (a) and of RMB7103 and RMB7203 (b).

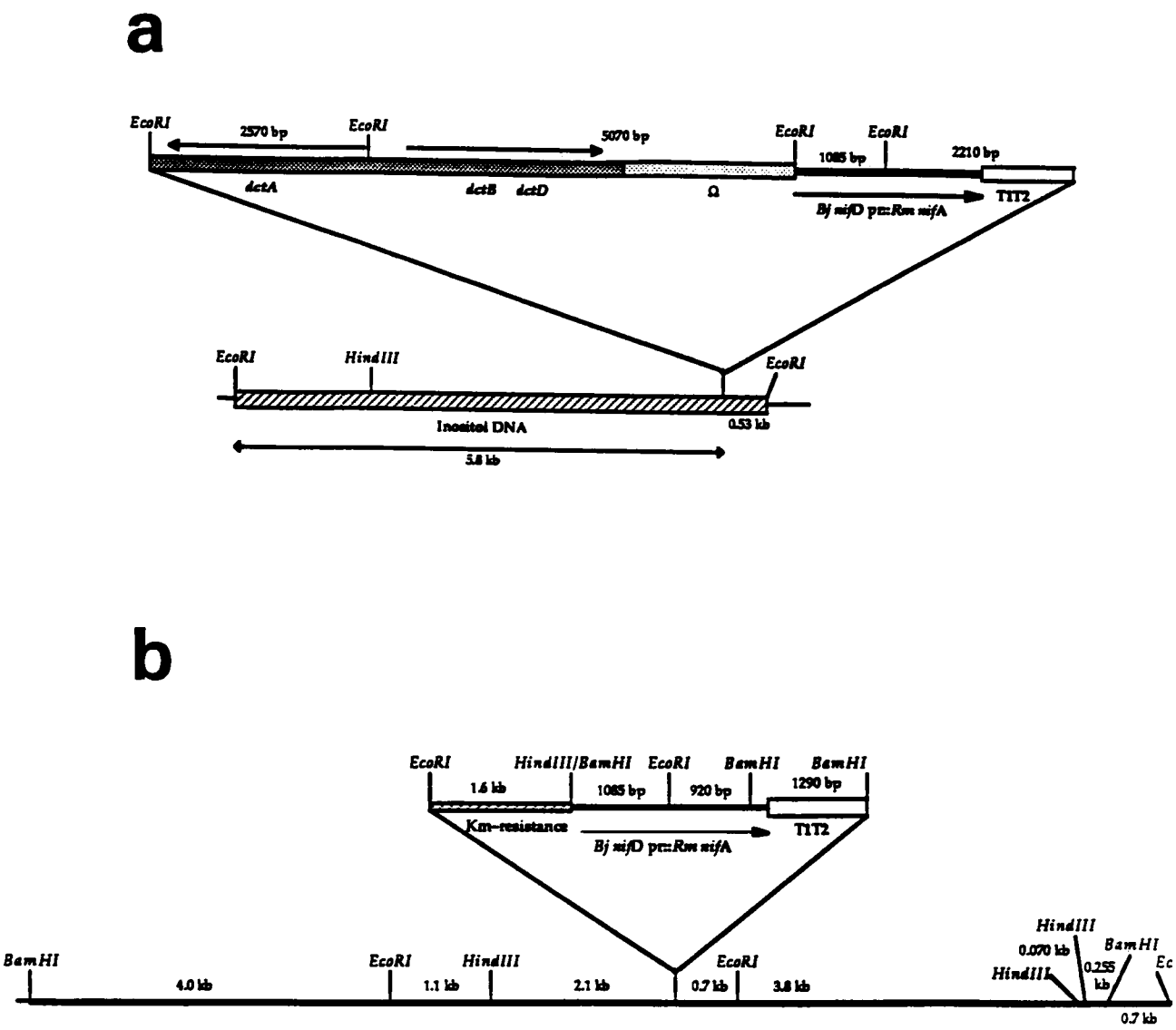

FIG. 2. Organization of the insert at the inositol locus in the chromosomes of RMBPC-2 (a) and RMB7240 (b).

The promoter inserted upstream of the additional copy of *R. meliloti nifA* (*RmnifAp*) was the *B. japonicum nifD* promoter (*BjnifDp*), which was carefully chosen to avoid deleterious excess expression of *nifA*. The recombinant and wild-type strains described here are ideal tools to examine the effects of these genes on alfalfa biomass. This paper reports the construction of the recombinant strains and the results from the field trials begun in 1992.

MATERIALS AND METHODS

Construction and description of genetically engineered strains of *R. meliloti*. The strains used in these experiments along with genotypes of the inserts in the recombinant strains are described in Table 1. The construction of the recombinant strains is described in the Appendix. Wild-type strain PC was isolated from the nodule of an alfalfa plant in Pepin County, Wis., in 1986. It is the parent of strains RMB7201, RMB7203, RMB7240, and RMBPC-2. In Wisconsin soils, PC competes well for nodulation with indigenous strains (data not shown). Strain RCR2011 is a natural isolate, obtained from the Rothamsted Experimental Station Collection. It is the parent strain of strains RMB7101, RMB7103, RMB138Ω710A, and RMB139Ω710B. Strain RCR2011 and its derivatives have a nonmucoid colony morphology that contrasts with the mucoid colony morphology of the wild-type PC strain and its derivatives.

In each recombinant strain the added genes were inserted into one of two symbiotically silent sites. One of these sites is involved in inositol utilization and is referred to as the *ino* region. This site was described by Williams et al. (41). A second integration site was chosen because it is within a well-characterized region of the genome and because transposon insertions within this region have no effects on the symbiotic properties of *R. meliloti*. This locus is referred to as the P3 region and has been extensively characterized by Better et al. (3, 4). Earlier studies have shown by transposon Tn*5*

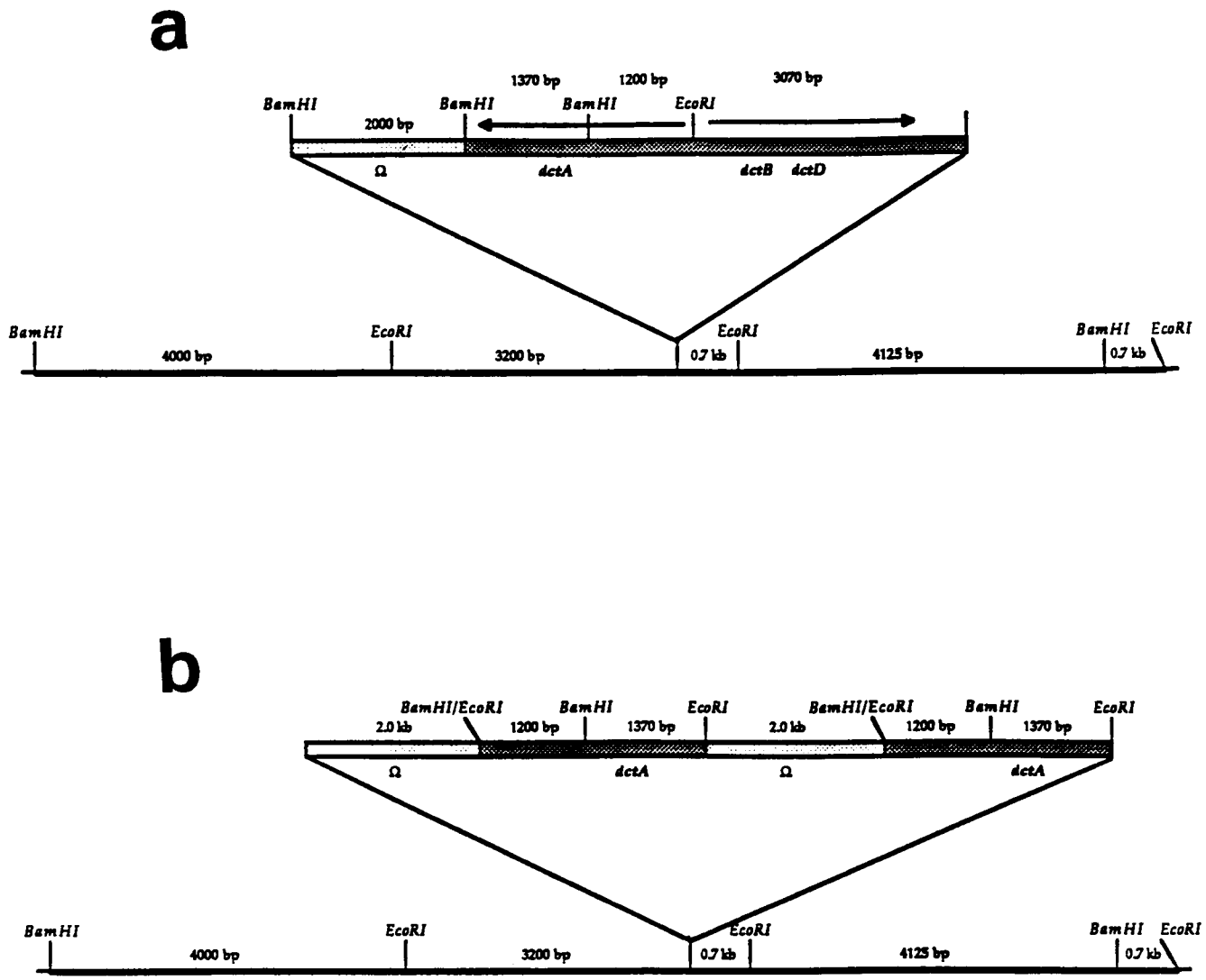

FIG. 3. Organization of the insert at the P3 site in the chromosomes of RMB138Ω710A (a) and RMB139Ω710B (b).

mutagenesis that the region between the *nifHDK* and *fixABCX* operons was symbiotically silent (13, 14, 33). Better et al. (4) showed by hybridization studies that the region contained sequences similar to the *nifHDK* and *fixABCX* promoters and termed the region P3.

Strains RMB7101 and RMB7201 were constructed in the same manner and differ only in their wild-type backgrounds, RCR2011 and PC, respectively. The structure of the Ω insert in the inositol locus of PC and RCR2011 is shown in Fig. 1a. RMB7103 and RMB7203 differ only in their wild-type backgrounds (Table 1). Both of these strains contain the *nifA*/Ω cassette integrated into the inositol locus of strains RCR2011 and PC, respectively (Fig. 1b). RMBPC-2 contains the *dctABD*/Ω/*nifA* cassette integrated into the inositol locus of strain PC (Fig. 2a). To construct RMB7240, an *nptII*/*BjnifDp*::*RmnifAp* cassette was inserted into the P3 site of PC (Fig. 2b) by marker exchange as described below, except that kanamycin resistance was used as the selectable marker for the insert. RMB138Ω 710A contains the *dctABD*/Ω cassette inserted into the P3 site (Fig. 3a). The added genes in RCR2011 to create RMB139Ω 710B include a *dctA*/Ω cassette inserted into the P3 site (Fig. 3b).

Measurement of *nifA* activity. The enhancement of *nifH* expression in *R. meliloti* with the addition of a second copy of *nifA* under the control of *BjnifDp* was demonstrated by transferring pMB210 containing an *R. meliloti nifH*::*lacZ* fusion (3) into RCR2011 and RMB7103 (Fig. 1b; Table 1) by conjugation. Between 1 and 20 nodules were collected at four time points after planting. The nodules were crushed, and bacteroids were isolated. β-Galactosidase assays were performed according to the method of Miller (25). The data were expressed as fold enhancement of *nifH* expression over that obtained with wild-type strain RCR2011.

Locations of the field trials. The field trials were conducted at the Arlington, Hancock, Lancaster, and Marshfield Agricultural Research Stations of the University of Wisconsin. The specific sites of these trials were chosen on the basis of previous cropping histories (such as lack of alfalfa on these sites during the previous 5 years), low organic matter and nitrogen content, and a low indigenous population of *R.*

TABLE 2. Soil chemical, physical, and microbiological characteristics at the field sites

Characteristic	Result at:			
	Hancock	Lancaster	Arlington	Marshfield
Distance (km) from Madison (direction[a])	130 (N)	136 (WSW)	32 (N)	240 (N)
Field site	C17	300A	372	W4
Soil type	Plainfield loamy sand	Fayette silt loam	Plano silt loam	Withee silt loam
Subsoil	Sandy outwash	Loess over sandstone	Loess over sandy loam	Clayey
Drainage	Excessive	Moderately good	High	Moderate to high
Slope (%)	<1.5	2–4	3	<1.5
Texture (%)				
Sand	87	19	17	26
Silt	96	96	77	0
Clay	4	12	16	4
pH	6.9	6.2	6.0	7.0
Organic matter (%)	0.9	1.9	3.2	3.2
Indigenous *R. meliloti* population (cells/g of dry soil)	<50	63	<50	10,000
Total N (%)	0.08	0.13	0.19	0.20
Total extractable ppm of:				
NH_4^+	7.3	13.3	15.5	22.5
NO_3^-	10.3	22.0	15.3	22.0
P	148	88	50	90
K^+	105	220	258	370
Total digestible ppm of:				
P	334	473	578	679
K^+	382	1,388	1,614	1,278
Ca^{2+}	957	1,714	2,350	2,422
Mg^{2+}	734	2,064	2,389	2,633
S	106	211	291	330
Zn^{2+}	38.1	57.9	75.2	71.2
B	4.59	4.96	4.16	5.33
Mn^{2+}	456	888	909	1,544
Fe^{2+} or Fe^{3+}	5,863	12,600	14,802	14,943
Cu^+ or Cu^{2+}	8.00	10.48	11.65	10.89
Al^{3+}	4,985	10,037	12,241	12,704
Na^+	<61.2	<61.2	<61.2	<61.2

[a] N, north; WSW, west-southwest.

meliloti. Specific characteristics of the four sites are outlined in Table 2.

Design of the field trials. A monoculture field trial was conducted at all four locations using Legend alfalfa (*Medicago sativa* L.), a multifoliate variety developed at Forage Genetics, Inc., West Salem, Wis. A fifth experiment, in which alfalfa was established with oats as a companion crop, was conducted at Arlington. The trials included two wild-type strains (PC and RCR2011), seven genetically engineered strains, and an uninoculated control. The treatments were arranged in a randomized complete-block design with eight replicates. Each trial occupied approximately 0.1 ha, including uninoculated border areas.

To minimize variability due to uneven seeding density at the beginning and end of each plot, 45-cm swaths along both sides of the replicate blocks were eliminated from the yield measurement and reserved for microbiological monitoring. On the day of each harvest, alfalfa from the swaths and from the guard plots was harvested and discarded. The remaining 3.4 m of each treatment plot was then harvested for yield. Plant sampling for nodule occupancy and rhizosphere establishment was restricted to the 45-cm ends of each plot.

Growth of cultures and seed coating. Cultures were grown in 1 liter of Luria broth supplemented with 1 g of $CaCl_2$ per liter to a density of approximately 10^9 cells per ml. The medium for all genetically engineered strains was amended with 200 μg of streptomycin per ml and 500 μg of spectinomycin per ml, except for strain RMB7240, for which 50 μg of kanamycin per ml was used rather than streptomycin and spectinomycin. The cultures were incubated in shake flasks at 28°C to early stationary phase and then washed twice with sterile phosphate-buffered saline. After the second wash, pelleted cells were suspended in 34 ml of sterile water, providing a final density of 2.95×10^{10} cells per ml. The entire washed cell suspension was inoculated directly into 66 g of Research Seeds nonsterile humus carrier, achieving an inoculant density of 10^{10} cells per g of wetted humus. Uninoculated control humus was prepared with sterile water. The inoculant strains were enumerated with appropriate antibiotic selective media on the day of coating. The uninoculated control humus was plated onto antibiotic selective medium.

The target inoculation rate was 10^6 cells per seed. This rate is higher than commercial inoculation rates in order to guarantee the highest possible nodule occupancy by the inoculant strains. Inoculated humus was applied to the seed at a rate of 18.5 g/kg.

The inoculated humus was used to coat the seed on the afternoon of the day before planting by using the adhesive vitalive furnished by Research Seeds. The field trials were planted during the first week of May 1992 except at Marshfield, which was planted during the first week of June 1992.

Protocol of the field trials. (i) Site preparation. Approximately 10 to 14 days before planting, each site was plowed to a depth of 15 cm and tilled to prepare the seed bed. Phospho-

TABLE 3. Yield results of alfalfa plants inoculated with the wild-type strains and the genetically engineered derivatives

Inoculating strain	Yield (kg of dry forage/ha)[a] at:				
	Hancock	Lancaster	Arlington		Marshfield
			Solo seeded	Combined seeding	
RMBPC-2	10.08 A	7.14 A	6.72 A	6.59 A	3.41 A
RMB7240	9.22 AB	7.05 AB	6.93 A	6.71 A	3.37 A
RMB7201	9.20 AB	7.00 AB	6.84 A	6.66 A	3.53 A
RMB7203	9.12 B	6.80 AB	6.75 A	7.03 A	3.52 A
PC	8.93 B	6.87 AB	6.80 A	6.74 A	3.41 A
RMB138Ω710A	8.93 B	7.21 A	6.80 A	6.76 A	3.10 AB
RMB139Ω710B	8.98 B	6.38 B	6.87 A	6.74 A	3.49 A
RMB7103	8.98 B	6.94 AB	6.97 A	6.99 A	2.79 B
RCR2011	9.40 AB	6.87 AB	6.89 A	6.59 A	3.36 A
None	8.55 B	7.07 AB	6.96 A	6.71 A	3.24 AB

[a] There were eight replicates per treatment arranged in a randomized complete-block design. Data represent three harvests at Hancock and two harvests at each of the other sites during the 1992 growing season. Yield results followed by the same letter within a site are not significantly different at the 5% level of confidence as determined by Tukey's least-significant-difference test. The coefficients of variance for each yield trial were 6.88% (Arlington combined), 5.12% (Arlington solo seeded), 9.52% (Hancock), 10.85% (Lancaster), and 16.0% (Marshfield).

rous, potassium, and calcium fertilizers were added as needed as suggested by soil fertility tests as a measure to ensure that nitrogen was the limiting factor at each site. The preplanting herbicide Eptam 7-E (4.3 liters/ha) as required for each field site was applied and incorporated into the soil.

(ii) Planting protocol. Seeds were coated and divided into 11-g subsamples, and the subsamples were placed in individual coin envelopes prelabelled according to plot number. The seeding rate was 20 kg/ha. A subsample of coated seeds from each treatment was reserved in the laboratory and used for viable counts on the day of planting.

A six-row self-propelled belt cone planter, adjusted to 15-cm row spacing and 1.3-cm planting depth, was used to seed all experiments. Plots were 4.3 m long. Seeding began in the uninoculated control treatment plot, which was followed by the inoculated treatment plots. Those components of the planter which came in direct contact with the seed (specifically, the cone, belt, and tubes) were disinfected with ethanol or isopropanol between rhizobium treatments. This sterilization method was adequate to prevent cross-contamination of the strains between plots.

TABLE 4. Average nodule occupancy of the inoculant strains[a]

Inoculating strain	Avg % nodule occupancy at:				
	Hancock	Lancaster	Arlington		Marshfield
			Solo seeded	Combined seeding	
RMBPC-2	96.7	94.1	94.1	97.4	18.2
RMB7240	88.6	96.9	96.7	98.7	7.8
RMB7201	95.6	92.8	98.0	97.4	8.2
RMB7203	92.8	96.5	96.0	97.8	10.3
RMB138Ω710A	90.5	84.0	94.3	95.0	3.9
RMB139Ω710B	88.2	79.6	93.2	94.0	3.4
RMB7103	87.8	90.6	92.8	89.1	0.0
RMB7101	79.3	64.6	93.6	89.7	1.3
RCR2011	93.1	97.4	93.3	94.7	0.0
None					
Sm^r Sp^r	2.6	9.5	20.7	29.8	0.0
Km^r	1.9	1.9	0.7	17.1	0.0

[a] As strain PC could not be distinguished from the indigenous rhizobia, the nodule occupancy of the PC-inoculated plants was not determined.

TABLE 5. Average rhizosphere colonization of the inoculant strains[a]

Inoculating strain	Avg colonization (log_{10} CFU/g [fresh wt] of root) at:			
	Hancock	Lancaster	Arlington	
			Solo seeded	Combined seeding
RMBPC-2	6.79	7.81	7.02	6.92
RMB7240	6.76	5.62	<3.26	6.17
RMB7201	6.49	7.10	7.14	6.64
RMB7203	6.56	7.45	7.16	6.80
RMB138Ω710A	6.74	6.70	6.87	6.90
RMB139Ω710B	6.68	7.29	6.57	6.86
RMB7103	6.67	7.58	6.83	6.90
RMB7101	6.77	6.92	6.73	6.79
None	<3.79	5.01	<3.11	<3.04

[a] Results for strains PC and RCR2011 were not determined.

Once all the plots were seeded, the border areas were seeded with a mixture of bluegrass, ryegrass, and tall fescue. The Hancock site was irrigated twice each week. Other sites were irrigated as needed. Plots were weeded by hand as needed. The Arlington companion-seeded trial was treated with the herbicide Poast after oat harvest to control volunteer oats. The insecticide dimethoate was sprayed on plots to control leafhoppers as needed.

(iii) Harvesting protocol. Harvests were scheduled according to the stage of plant maturity at each site. Whenever possible, the forage was harvested when the plants reached 1 to 10% flowering (24, 24a, 37). Companion plots at Arlington were harvested when the oats were at early anthesis. All harvested alfalfa hay from the trials was donated to the respective field stations, to be composted or used as fodder for the station herd. The forage was harvested with a 100-cm-swath sickle bar mower adjusted to cut 5 cm above the ground. A 45-cm swath was harvested from both ends of each plot to remove border effects. The remaining forage (1 by 3.4 m) in the plots was harvested for yield determination. After the forage in each plot was cut, it was collected by raking and weighed on-site with a portable balance. A 200-g subsample was collected for dry weight determination. Subsamples for dry weight determination were dried at 60°C to a constant weight at the individual stations (for at least 48 h). Yield was calculated on a dry weight basis.

Statistical analysis of data. Yield data from each harvest at each site were analyzed by the GLM procedure in SAS (34). Methods of statistical analysis, including Tukey's least-significant-difference test, which were appropriate for the experimental design of the field trials and consistent with the experimental objectives were chosen (34).

Nodule occupancy. Thirty days after planting, the roots of 10 plants were collected from each plot and transported to the laboratory in Madison, Wis. Roots were frozen at −70°C until analysis. Nodules were harvested from the plants, surface sterilized with 50% bleach for 30 s, and crushed in a microtiter dish. The cells were replica plated onto RDM medium and RDM medium with the appropriate antibiotics added as necessary for each treatment to determine whether the inoculant strain occupied the nodule. RDM medium is a modified Bergersen's synthetic medium (2) which includes 5 g of gluconic acid per liter and lacks mannitol. The two wild-type

TABLE A1. Plasmids used in the construction of the recombinant strains of *R. meliloti*

Plasmid	Genotype or purpose in cloning strategies	Reference or source
pACYC184	Cloning vector	11
pBG1A	*dctABD*	This work
pEA300	Source of T1 T2 terminator	6
pHP45Ω	Source of Ω fragment	28
pIC20H	Cloning vector	23
pIC20HΩ	Source of Ω fragment	This work
pIC20R	Cloning vector	23
pIC20RΩ	Source of Ω	This work
pJB251	Incompatible plasmid for marker exchange	41
pMB210	*nifH::lacZ*; fusion used in promoter strength assays	3
pMW113	*BjnifDp*; promoter construction	This work
pMW114	*BjnifDp*; promoter construction	This work
pMW153	*BjnifDp::RmnifAp* fusion construction	This work
pMW157	Source of T1 T2/Ω	This work
pMW180	*BjnifDp::RmnifAp* fusion construction	This work
pMW184	*ino* integration site	41
pMW186	Source of T1 T2/*BjnifDp::RmnifAp*/Ω	This work
pMW186K	Source of T1 T2/*BjnifDp::RmnifAp/nptII*	This work
pMW188	Source of *BjnifDp::RmnifAp* fusion	This work
pMW191	*ino*/T1 T2/*BjnifDp::RmnifAp*/Ω/*ino* integration vector	This work
pMW193	*ino*/T1 T2/Ω/*ino* integration vector	This work
pMW211	Source of T1 T2/*nptII*	This work
pMW212	Source of T1 T2/*BjnifDp::RmnifAp/nptII*	This work
pMW229	P3 integration site vector	This work
pMW242	P3/T1 T2/*BjnifDp::RmnifAp/nptII*/P3 integration vector	This work
pMW300	*ino*/T1 T2/*BjnifDp::RmnifAp*/Ω/*dctABD/ino* integration vector	This work
p138Ω710A	P3/Ω/*dctABD*/P3 integration vector	This work
p139Ω710B	P3/Ω/*dctA*/Ω/*dctA*/P3 integration vector	This work
pPH1JI	Incompatible plasmid used to make pJB251	19
pRj676Δ1	Source of *BjnifDp*	1
pRK138Ω	Source of Ω/*dctABD*	This work
pRK139Ω	Source of Ω/*dctA*	This work
pRK150	Source of T1 T2/*BjnifDp::RmnifAp*/Ω/*dctABD*	This work
pRK290	Broad-host-range vector	15
pRK2013	Helper plasmid for conjugation	18
pRMB3.8H	Source of *RmnifAp*	9
pUC4-K1XX	Source of *nptII* gene	Pharmacia
pWB716	Source of P3 site	8

strains, RCR2011 and PC, have no selectable markers. Strain PC could not be distinguished from the indigenous strains. As a result, we have no nodule occupancy data for this strain. Strain RCR2011 was distinguished from the indigenous rhizobia by its rough colony morphology.

Rhizosphere colonization. Three to four weeks after planting, four plant root systems were collected from one end of each replicate plot at every trial site except Marshfield. Approximately half of the plant roots collected from the plots in blocks I, IV, V, and VII were separated and used to determine inoculant population densities in the rhizosphere. The remainder of the plant samples was stored at 4°C for nodule occupancy analysis. Parent strains RCR2011 and PC were not evaluated because they do not possess selectable markers. Root samples were immersed in 40 ml of sterile water containing 0.1% Tween 80. Samples were shaken on a rotary shaker for 20 min, and the rinse was diluted in 10-fold intervals to 10^{-3} in sterile water. Aliquots (100 μl) of each of the three dilutions were spread in duplicate on RDM medium with and without the appropriate antibiotics for each treatment, and the plates incubated for up to 10 days at 25°C. Plates containing 20 to 300 colonies were counted.

Total-nitrogen and crude-protein analyses. The total N concentration in the forage was determined by the micro-Kjeldahl method of Bremner and Breitenbeck (6). Crude protein was calculated as total N × 6.25.

Soil analyses. Six soil samples were collected from each site at a depth of up to 10 cm. The elements determined in these samples are listed in Table 2. These were measured by the Soil Testing Laboratory of the University of Wisconsin—Madison as described by Schulte et al. (35). The number of *R. meliloti* cells in the soil at each site was determined by most-probable-number analysis as described previously (39).

Strain and plasmid availability. Requests for strains and plasmids described in this work should be directed to Thomas J. Wacek.

RESULTS

Activity of *BjnifDp*. The expression of *R. meliloti nifH::lacZ* was 1.5, 1.3, 1.3, and 1.2 times higher in RMB7103 than in RCR2011 when nodules were collected at 12, 20, 21, and 29 days after planting.

Site characteristics. Sites chosen at all four locations had no alfalfa planted in the last 5 years and thus were likely to harbor a very low population of indigenous *R. meliloti* (38). Most-probable-number analysis of the soil samples from each site showed that the number of indigenous *R. meliloti* cells was very low at each site except Marshfield (Table 2).

Biomass production. Significant seeding-year biomass increases were obtained at the Hancock site in 1992. Inoculation with strain RMBPC-2 increased the yield of alfalfa by 17.9% over that in the uninoculated plot and 12.9% over that obtained by inoculation with the wild-type strain at Hancock (Table 3). These increases were significantly higher at the 5% level of confidence. The RMBPC-2 treatment yielded significantly ($P > 0.05$) more forage than the PC treatment and noninoculation at each harvest (data not shown). Inoculation with this strain also provided the highest mean yield of alfalfa compared with that after the other treatments, but these increases were not significantly higher (Table 3).

No significant differences between results of any treatments at either trial at Arlington were observed (Table 3). At Lancaster, none of the yields with recombinant strains were higher than those with either the wild-type strains or the uninoculated controls (Table 3). The yield with one of the recombinant strains (RMB138Ω710A) was significantly higher than that with another recombinant strain (RMB138Ω710B). However, the RMB138Ω710A treatment did not give significantly higher yields than did either the wild-type strain treatment or the uninoculated control.

At Marshfield, none of the recombinant strains gave higher yields than did either the wild-type strains or the uninoculated controls (Table 3). One of the strains, RMB7103, did give significantly lower yields than did the wild-type strain from which it was derived, RCR2011. The RMB7103 treatment did not give lower yields than did the uninoculated control.

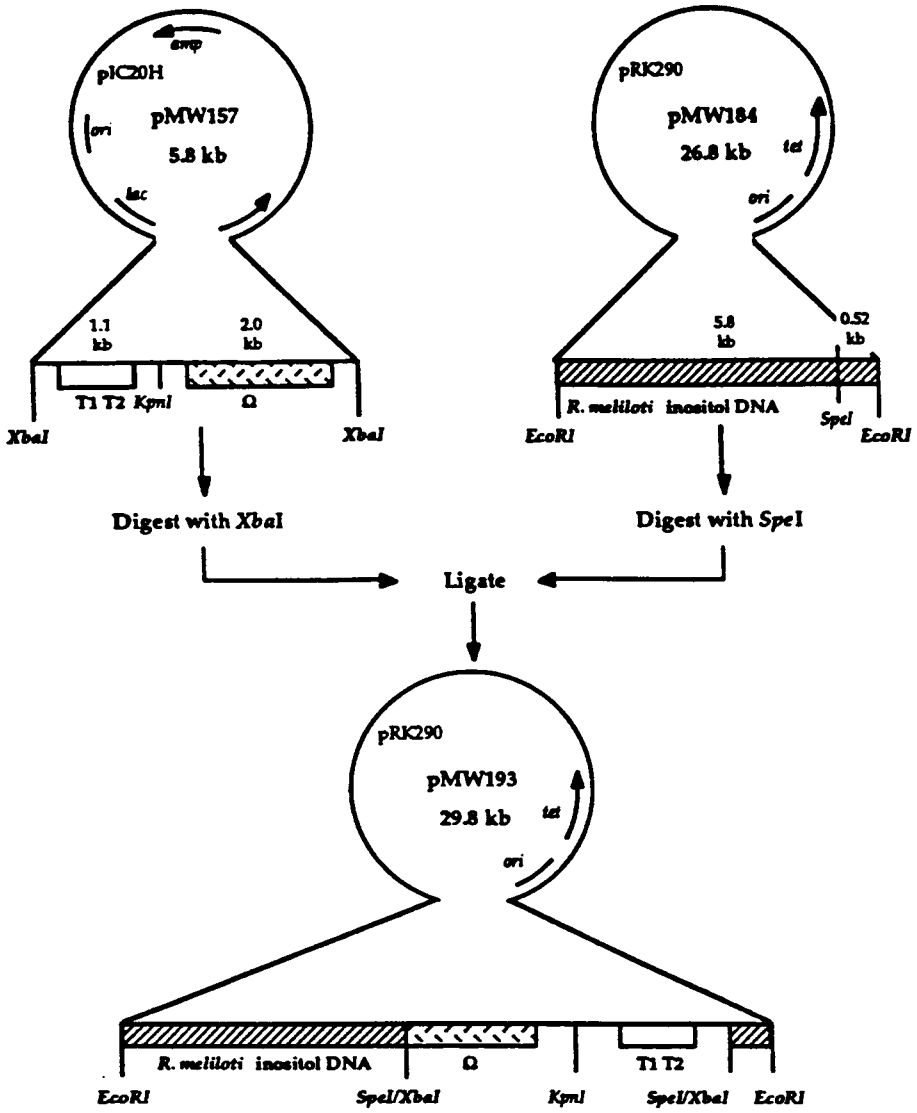

FIG. A1. Construction of the integration vector, pMW193, used to integrate the T1 T2/Ω cassette into the inositol locus in the chromosomes of RMB7101 and RMB7201.

Nodule occupancy and rhizosphere colonization. Over 85% of the nodules in the inoculated plots were occupied by the inoculum strain at the Arlington, Hancock, and Lancaster sites (Table 4). The inoculum strains also colonized the rhizosphere of the alfalfa roots in large numbers at these sites (Table 5). These results show that all inoculant strains were successfully established in similarly high population densities at these three sites for all treatments. Nodule occupancy at the Marshfield site was very low for each recombinant strain and for the RCR2011 wild-type strain (Table 4). Similarly, rhizosphere colonization at the Marshfield site was below our limit of detection (data not shown).

Nitrogen content of forage biomass. The nitrogen content of the tissue from the uninoculated, PC, and RMBPC-2 plots at Hancock was determined. As no yield increases were observed with the other strains, these parameters were not measured in the other plots at Hancock or in any plots at Arlington, Lancaster, and Marshfield. Strain RMBPC-2 did not affect total-nitrogen or crude-protein concentrations in the above-ground tissue compared with those in the plants which were inoculated with PC or those which were uninoculated. The mean nitrogen concentrations over all three harvests were 3.89, 3.81, and 3.78% for the uninoculated, PC-treated, and RMBPC-2-treated plants, respectively. The mean crude-protein levels were 24.31, 23.81, and 23.63% for the uninoculated, PC-treated, and RMBPC-2-treated plants, respectively. These levels were not significantly different between treatments at the 5% level of confidence.

Although the nitrogen concentration of the forage tissue did not increase in the RMBPC-2-treated plots compared with that in the PC-treated or uninoculated plots, there was significantly more total nitrogen per plot in the RMBPC-2-treated plots. The RMBPC-2-treated plots contained 14.4 and 12.1% more N per plot than the uninoculated and PC-treated plots, respectively. Similarly, the total amount of crude protein in the RMBPC-2-treated plot was 14.4 and 11.7% higher than in the uninoculated and PC-treated plots, respectively.

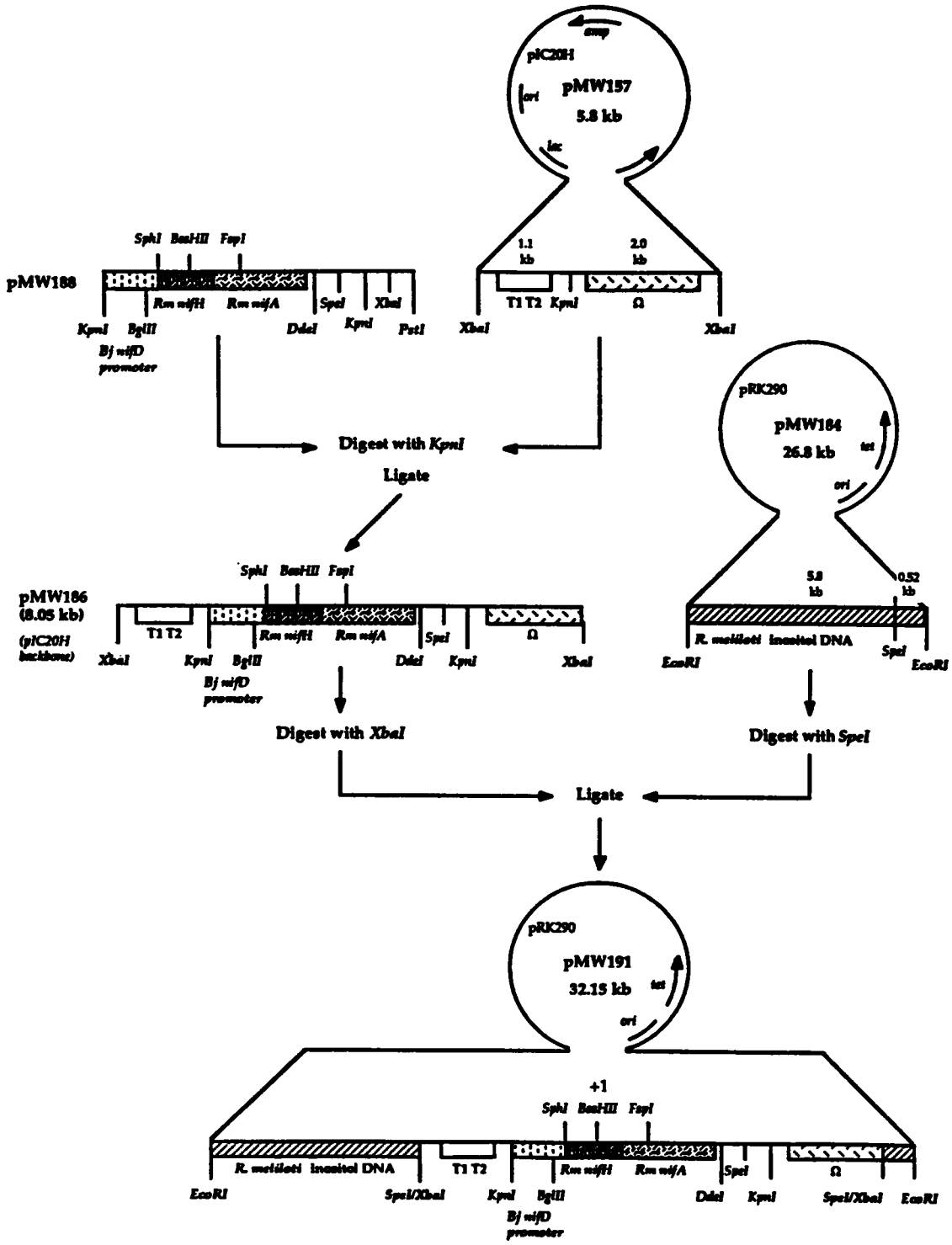

FIG. A2. Construction of the integration vector, pMW191, used to integrate the T1 T2/Ω cassette into the inositol locus in the chromosomes of RMB7103 and RMB7203.

These higher levels in total N and crude protein were significantly higher in the RMBPC-2-treated plots relative to those in the uninoculated and PC-treated plots at the 5% level of confidence.

DISCUSSION

Chromosomal integration of an additional copy of *nifA* into *R. meliloti* under the ap propriate promoter control was proposed to enhance nitrogen fixation and, as a result, alfalfa

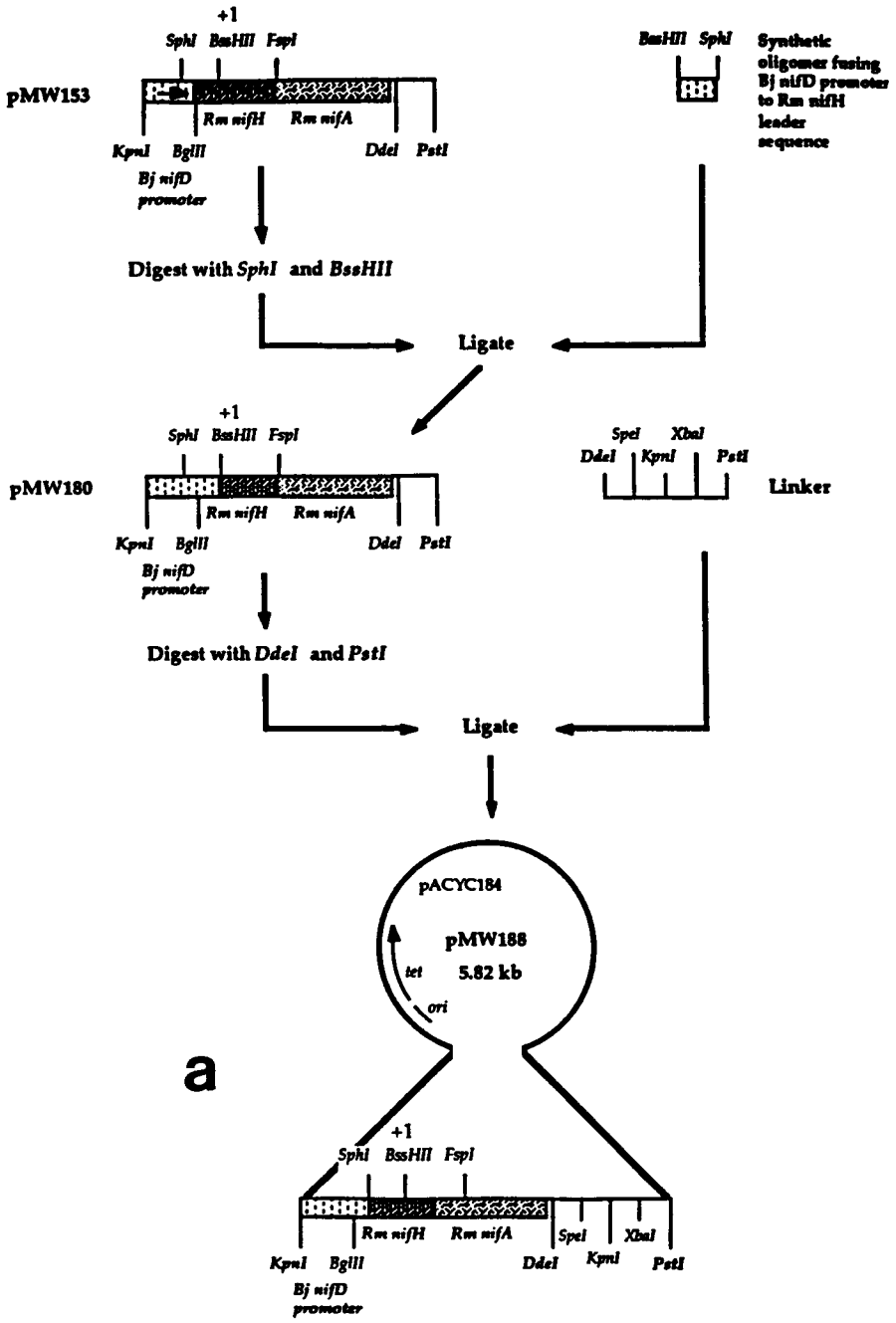

FIG. A3. Construction of the vector used as the source of *BjnifDp::RmnifAp*, pMW188 (a); the vector carrying *BjnifDp*, pMW114 (b); and the source plasmid for the *BjnifDp::RmnifAp* fusion, pMW153 (c).

biomass. For *dct* expression, the unaltered, wild-type promoter was used since its use was not detrimental to alfalfa productivity in the greenhouse (data not shown). In the case of *nifA*, preliminary experiments in the greenhouse showed that the promoter control of the second copy of *nifA* must be optimized for alfalfa biomass production (data not shown). When expression of the second *nifA* gene was too high, alfalfa biomass decreased by 15% compared with that with the wild type (data not shown). When the expression was too low, no increase in alfalfa biomass was observed. To provide optimum gene expression which enhanced alfalfa biomass in the greenhouse, we chose *BjnifDp*. This promoter provides additional *nifA* expression compared with that in the wild type but does not provide so much expression that plant productivity is harmed. The *B. japonicum nifD* promoter was chosen because it is a heterologous promoter not found in *R. meliloti*. Both of these factors contribute to its lower strength in *R. meliloti* compared with that of other promoters, such as the *cat* and *R. meliloti nifH* promoters.

Despite all of the methods to genetically engineer superior strains of rhizobia, there is no evidence that these strategies increase legume yield under agricultural conditions. Yield

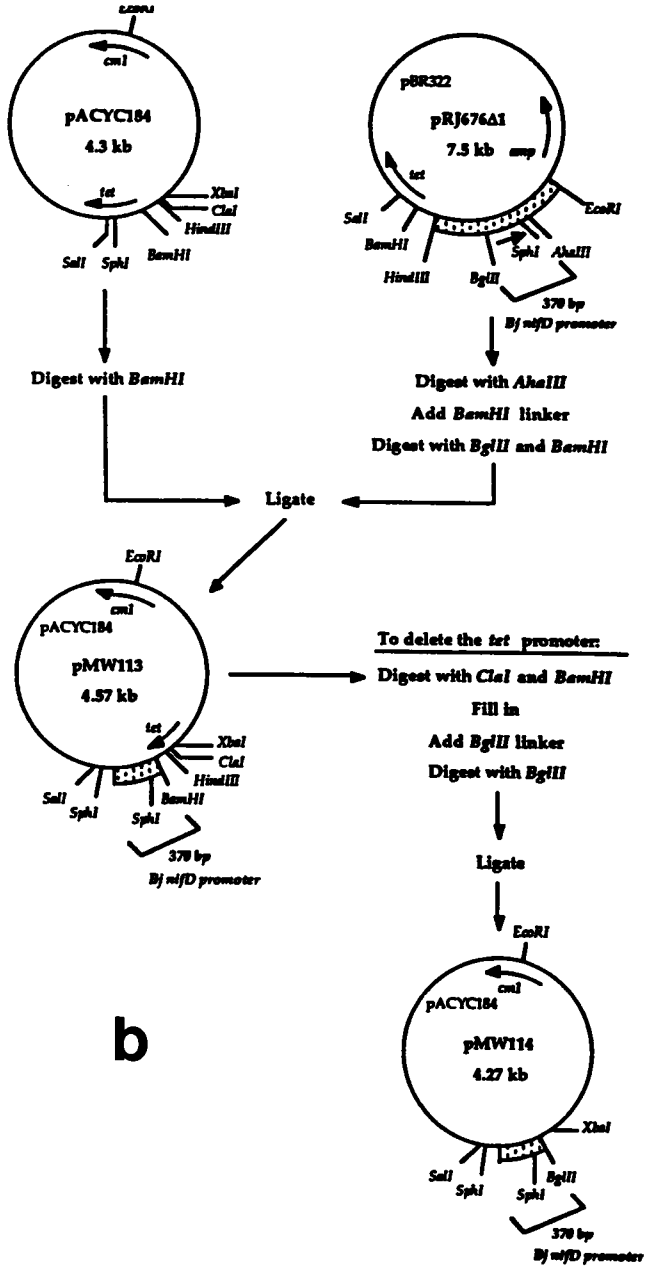

FIG. A3—*Continued.*

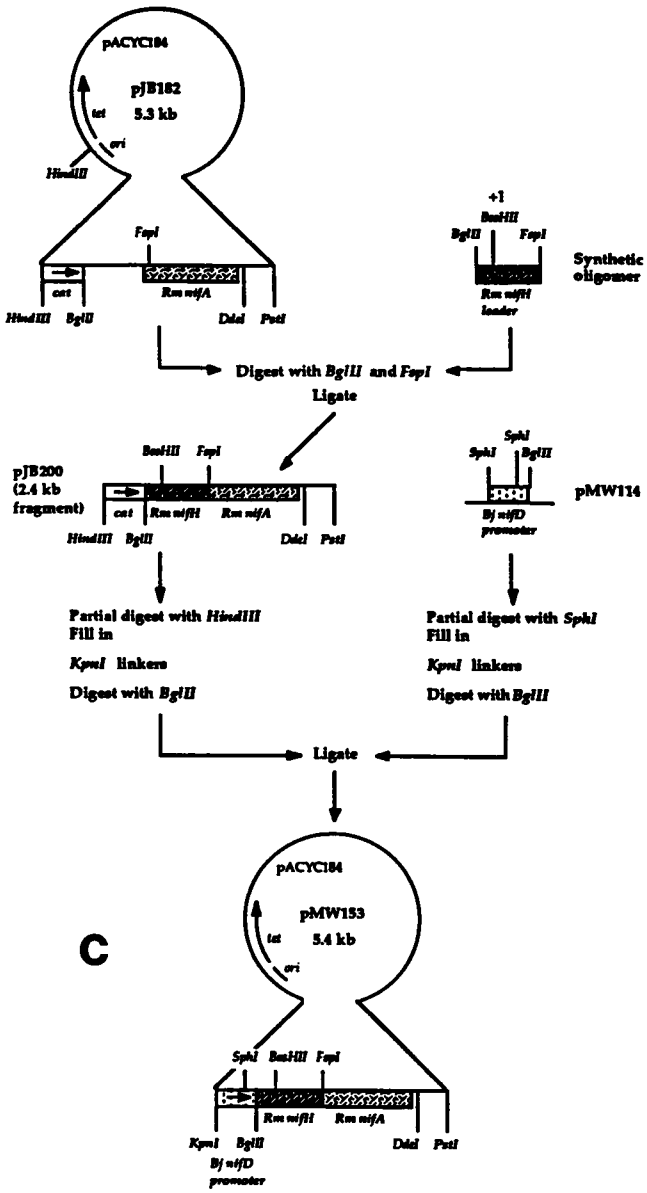

FIG. A3—*Continued.*

increases from such strains are difficult to observe for a number of reasons. First, nitrogen is often not limiting under these conditions. If nitrogen is not limiting, increased nitrogen availability to the plant will have no effect on yield. In many of these cases, either there is too much nitrogen in the soil or water is the limiting factor. In our experiments we have chosen sites with low nitrogen content and irrigated when necessary to be certain that water is not limiting.

Although we have not measured nitrogen fixation by the strains described here, we have shown that the total amount of N in the Hancock plots inoculated with RMBPC-2 was significantly higher than in the uninoculated or PC-inoculated plots. Also, Birkenhead et al. (5) added copies of the *dct* locus from *R. meliloti* to *B. japonicum* and observed increased free-living nitrogenase activity. Although we did not measure increased expression of *dctA* or *dctABD* from *R. leguminosarum* in the recombinant strains possessing one or two extra copies of these genes, there is sufficient evidence in the literature to support the view that these genes are expressed in *R. meliloti*. Jiang et al. (21) showed that the *dct* genes from *R. meliloti* and *R. leguminosarum* are highly homologous and that the *dct* genes from *R. meliloti* were capable of complementing *dct* mutants of *R. leguminosarum*. Ronson et al. (31) showed that *dct* mutants of *R. leguminosarum* bv. trifolii were complemented by *dct* from *R. leguminosarum* bv. viceae.

Second, often legumes are inoculated in a manner that prevents the inoculum strains from occupying the nodules. This is caused by (i) inoculation with strains that are unadapted to the soil, (ii) the presence of high populations of indigenous rhizobia which prevent nodulation by the inoculum strains, and/or (iii) insufficient numbers of viable rhizobia in the

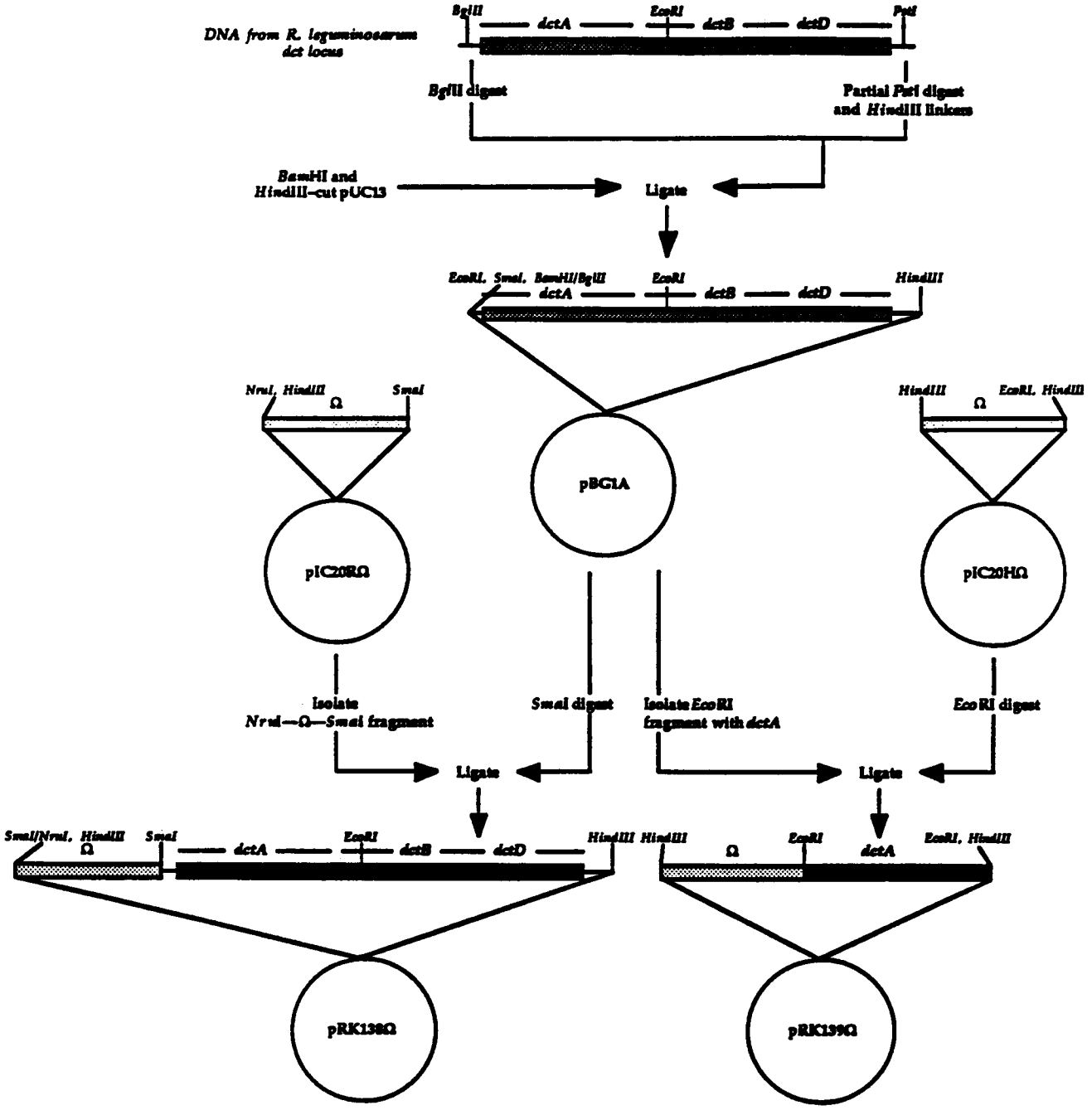

FIG. A4. Construction of the source plasmids for Ω/*dctABD* (pRK138Ω) and Ω/*dctA* (pRK139Ω), the inserts of which were used in the construction of the integration vectors p138Ω710A and p139Ω710B.

inoculum. In our experiments, we have largely prevented these problems by (i) using recombinant inoculant strains which are derived from a competitive strain native to Wisconsin, (ii) choosing sites with a low population of indigenous rhizobia, and (iii) coating seeds with numbers of rhizobia which are about 10^2 to 10^3 times higher than the commercial average.

Third, the genes chosen to enhance the rhizobia may not provide the benefit expected. In our case, we have done enough preliminary work in the greenhouse and the field to expect success under the proper nitrogen-limiting conditions (data not shown).

Fourth, often the plants are not grown to maturity or yields are not monitored over the entire growing season, preventing the experimenter from detecting small yield increases. In our experiments, alfalfa was harvested at the onset of flowering, which is optimal for biomass yield, and at least two harvests were made over the growing season.

Fifth, often the experimental design is not adequate to detect small but significant yield increases. Errors include a lack of uniformity in the plot, the use of too few replicates to be able to detect small yield increases, uneven fertilizer or water application, and weed or herbicide damage. In our

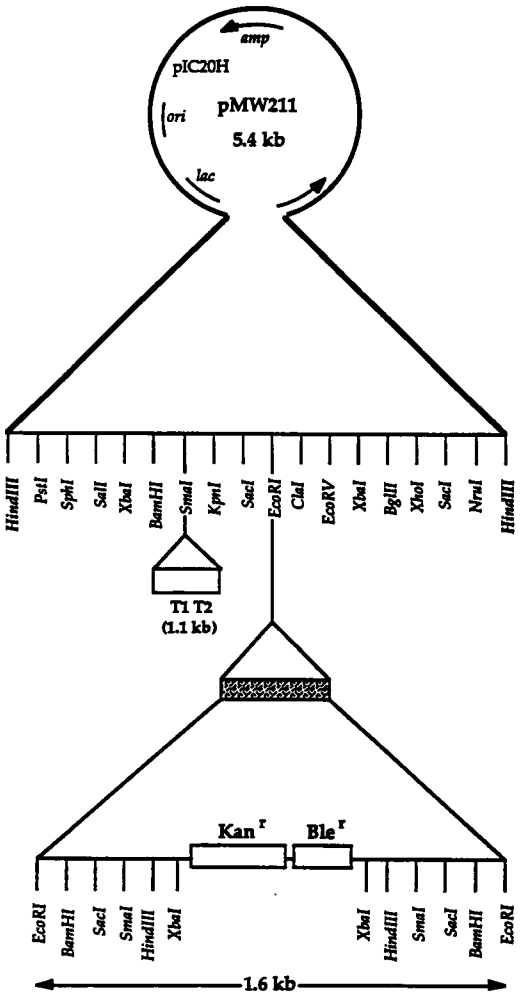

FIG. A5. Structure of the source plasmid of the *Hin*dIII fragment containing T1 T2/*nptII*, pMW211.

experiments, we carefully chose sites which would decrease plot variability, used eight replicates per treatment, and used the appropriate management practices to ensure even distribution of fertilizer and water as well as the absence of weeds.

We have addressed all of these problems in our experiments so that we can answer the question of the efficacy of our recombinant strains without ambiguity. In our first field experiments in 1992, we demonstrated a significant yield increase with one of the recombinant strains in the site where nitrogen was limiting. In other sites where soil nitrogen content was higher or where nodule occupancy by the inoculant strains was very low, no yield effects were observed.

As a result of the above precautions, we were able to limit nodulation by the indigenous strains in two ways: by inoculating with large numbers of rhizobia and by using sites with a low population of indigenous rhizobia. Nodule occupancy was very high at Arlington, Hancock, and Lancaster, where the indigenous populations of *R. meliloti* were very low. Thus, yield differences at these sites can be attributed to the inoculum strains. At Marshfield, where the indigenous population was high, very low nodule occupancy was observed. Thus, the yield difference between the RMB7103 and RCR2011 treatments at Marshfield cannot be attributed to the inoculum strains.

By far the most intriguing result of these experiments is the yield response of RMBPC-2 at Hancock. Hancock is the site where we expected to see the greatest differences between treatments because this site has the lowest soil N and organic matter content of all of the sites used in 1992. Strain RMBPC-2 was the only strain used in these trials which contains an additional copy of both *nifA* and *dctABD*. This suggests that both are necessary to provide increased biomass under nitrogen-limiting conditions.

APPENDIX

Construction and description of genetically engineered strains of *R. meliloti*. The plasmids used to construct the strains described in this paper are described in Table A1. The integration sites, *dctABD* and *nifA* genes, antibiotic resistance markers, promoters, and terminators are described in detail within the description of each recombinant strain below.

RMB7101 and RMB7201. Strains RMB7101 and RMB7201 were constructed in the same manner and differ only in their wild-type backgrounds, RCR2011 and PC, respectively. The Ω cassette was inserted in the inositol locus of PC and RCR2011. The Ω fragment confers resistance to spectinomycin and streptomycin, was obtained from plasmid pHP45Ω (28), permits selection for insertion of the integrated genes, and also improves our ability to detect these strains in nodules and in the rhizosphere. Prentki and Krisch (28) bracketed the gene with transcription and translation termination signals from bacteriophage T4 and restriction site polylinkers of known sequence. The Ω fragment was subsequently altered either by subcloning it into pIC20R or pIC20H or by adding linkers to facilitate the construction of the cassettes described below.

The construction of the integration vector, pMW193, used to insert the Ω cassette into the wild-type strains was described by Williams et al. (41) and is shown diagramatically in Fig. A1. The integration vector pMW193 includes the T1 T2 terminators to prevent transcriptional read-through of the insert DNA from genomic promoters. The T1 T2 terminator was isolated as a 1,100-bp *Pvu*II fragment from pEA300 (7) and cloned into the *Sma*I site of pIC20H (23), forming plasmid pMW157. This DNA is composed of a tandem repeat of two 500-bp fragments from the *Escherichia coli rrnB* gene (5S rRNA), each fragment containing two *rrnB* terminators. The purpose of the addition of the T1 T2 terminators was to prevent transcription from promoters located upstream of the cassette inserted into the chromosome.

The integration of genes into the inositol locus of *R. meliloti* RCR2011 by marker exchange to construct RMB7101 is described below. The same procedure was used to integrate the Ω cassette into strain PC, creating RMB7201.

RMB7103 and RMB7203. Strains RMB7103 and RMB7203 differ only in their wild-type backgrounds. Both of these strains contain the *nifA*/Ω cassette integrated into the inositol locus of strains RCR2011 and PC, respectively. The construction of the integration vector used for these strains, pMW191, is shown in Fig. A2. The integration of the insert in pMW191 into the inositol locus of RCR2011 and PC was done by marker exchange as described below. To construct pMW191, the *Xba*I fragment of pMW186 containing the *nifA*/Ω cassette was inserted by cohesive-end ligation into the *Spe*I site of pMW184 (41). The construction of pMW186 was done by insertion of the *Kpn*I fragment of pMW188 containing a *BjnifDp*::*RmnifAp* fusion into the unique *Kpn*I site of pMW157. The construction of pMW188 is shown in Fig. A3a.

The construction of *BjnifDp*::*RmnifAp* is illustrated in Fig. A3. The source of the *nifD* promoter from *B. japonicum* was pRJ676Δ1 (1). Analysis of the sequence of the *B. japonicum nifD* gene reveals an *Aha*III site 9 bp upstream of the translational start site and a *Bgl*II site 365 bp further upstream. These sites were used for subcloning the *B. japonicum nifD* promoter. pRJ676Δ1 was digested with *Aha*III, *Bam*HI linkers were added, the mixture was digested with *Bam*HI, *Bgl*II linkers were inserted, and the plasmid was religated to yield pMW114. The *B. japonicum nifD* promoter and leader sequence can be isolated, together with approximately 200 bp of the tetracycline resistance locus (in reverse orientation to the promoter), from pMW114 as an *Sph*I-*Bgl*II fragment (Fig. A3b). The *BjnifDp* was isolated from pMW114 by *Sph*I digestion. The resulting fragment was filled in with the Klenow

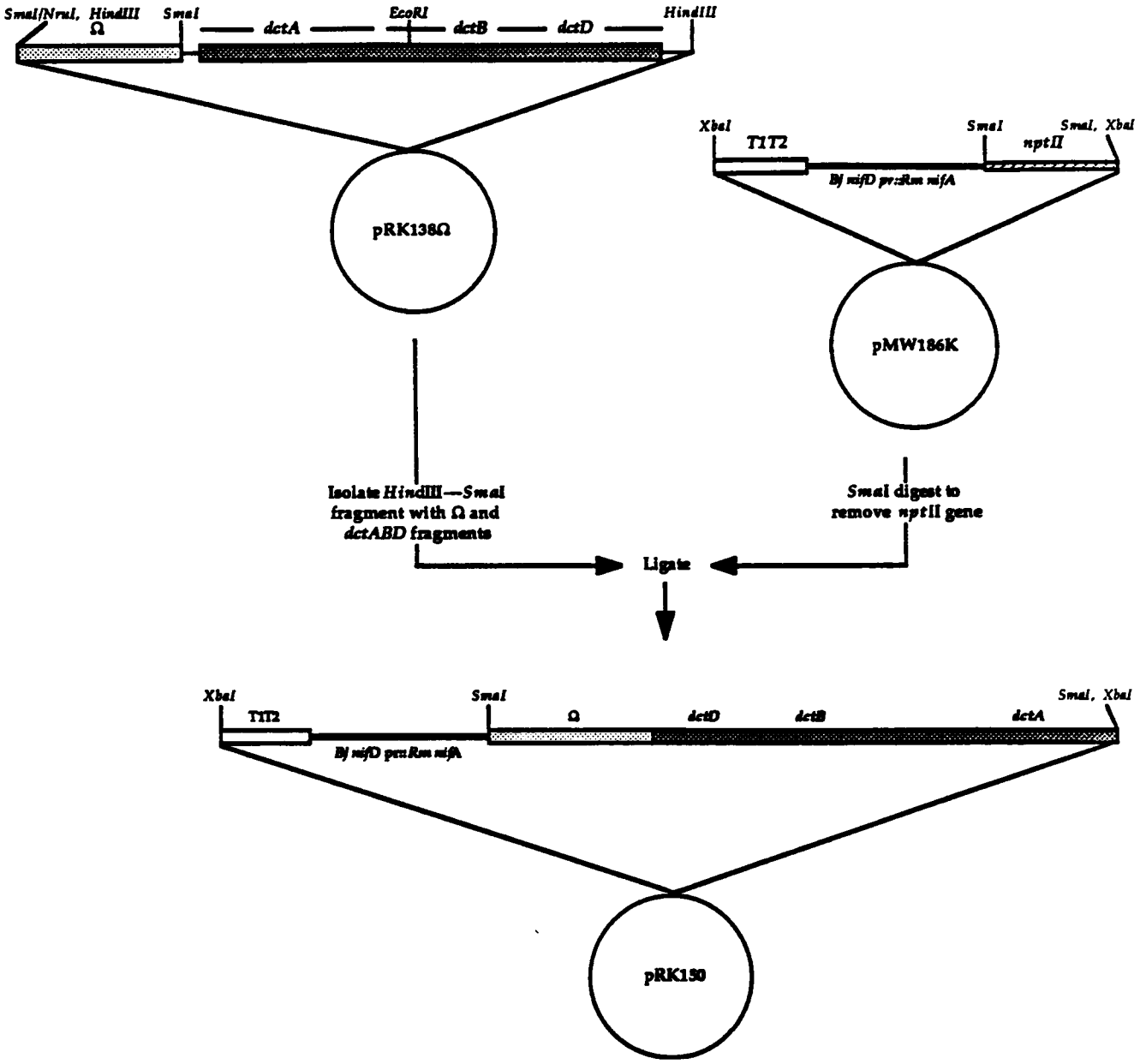

FIG. A6. Construction of pRK150, the source of the *Xba*I fragment containing T1 T2/*BjnifDp*::*RmnifAp*/Ω/*dctABD*. pRK150 contains the insert used to construct pMW300, which was then integrated into the chromosome of strain PC to create RMBPC-2.

fragment, ligated to *Kpn*I linkers, and digested with *Bgl*II. That digest was ligated to a similar digest of pJB200, the vector containing *RmnifAp* with a *RmnifHp* leader sequence upstream (Fig. A3c). The ligation product was called pMW153 (Fig. A3c).

The original source of *RmnifAp* was a 3.9-kb *Hin*dIII fragment from pRmB3.8H (9). Through a series of many ligation steps too numerous to describe here, *RmnifAp* was placed downstream of the *cat* promoter in pJB182. The *cat* promoter, cloned as a *Hin*dIII-*Pst*I fragment into pACYC184, was removed from pJB182 by digestion with *Bgl*II and *Fsp*I. A synthetic oligomer containing a *RmnifHp* leader sequence with *Bgl*II and *Fsp*I ends was ligated to the pJB182 digest, creating pJB200 (Fig. A3c). As part of the construction of pJB182, the *Xmn*I site of pACYC184 was converted with linkers to a *Hin*dIII site. *RmnifAp* contains an *Fsp*I site at the first codon of its coding sequence, while the *cat* promoter in pJB182 contains a *Bgl*II site upstream of the transcriptional start site. An oligonucleotide linker was synthesized with the sequence of the *R. meliloti nifH* leader region with *Fsp*I and *Bgl*II ends and inserted into pJB182, giving pJB200.

The *B. japonicum nifD Sph*I-*Bgl*II promoter fragment was then added to pJB200, and at the same time a *Kpn*I site was constructed at the 5′ end of the promoter. pMW114 was partially digested with *Sph*I, *Kpn*I linkers were added, the mixture was digested with *Kpn*I and *Bgl*II, and the appropriately sized fragment was isolated. pJB200 was partially digested with *Hin*dIII, *Kpn*I linkers were added, the mixture was digested with *Kpn*I and *Bgl*II, and the appropriate fragment was isolated. The fragments from pMW114 and pJB200 were ligated together, giving pMW153. pMW153 contains the *B. japonicum nifD* promoter and leader sequences and the *R. meliloti nifH* leader sequence.

To construct the exact fusion at the +1 site of transcription of the *B. japonicum nifD* promoter and the *R. meliloti nifH* leader, a 21-bp oligomer with *Sph*I-*Bss*HII ends was added to the *Sph*I-*Bss*HII-

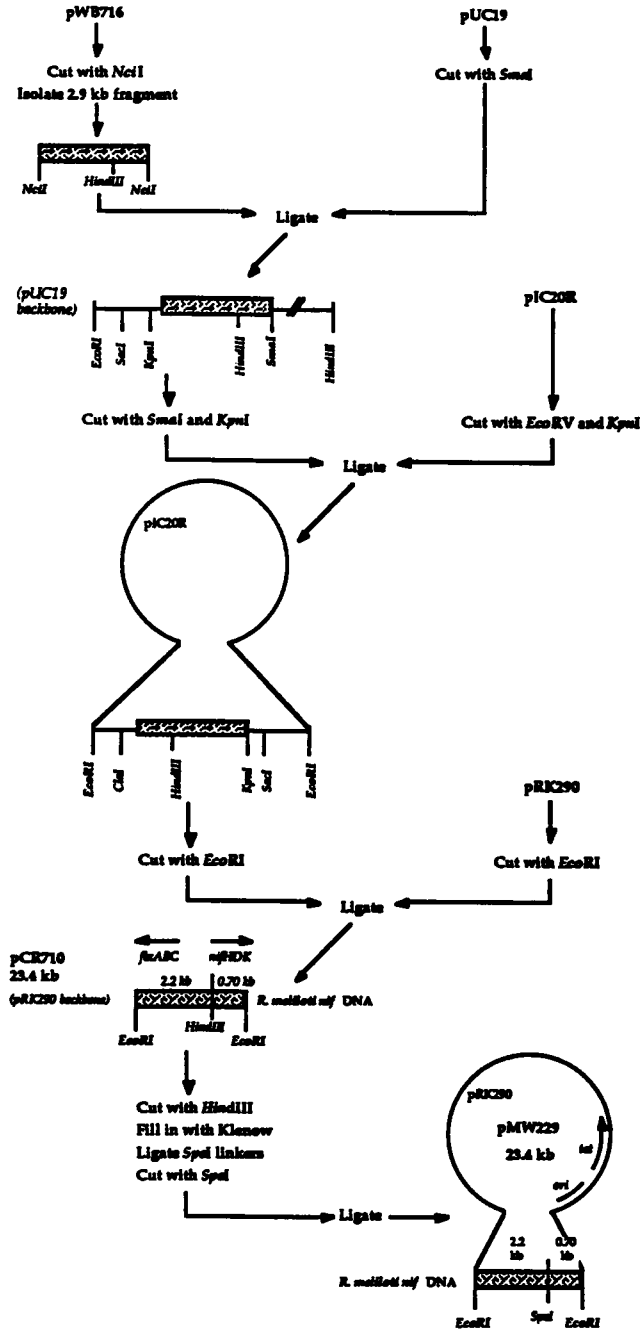

FIG. A7. Construction of the vector containing the P3 integration site, pMW229.

digested pMW153, giving pMW180. The sequence of the oligomer was 5'-CCGGTTGCAAAGTCTTGGGGG-3' and included *Sph*I and *Bss*HII overhangs on the 3' and 5' ends of the complementary strand, respectively. The 18th base of this oligomer is the 1st base of the *RmnifHp* leader. pMW180 contains the *B. japonicum nifD* promoter fused precisely at the +1 site of transcription to the *R. meliloti nifH* leader sequence, which is in turn fused at the +1 site of translation to the *R. meliloti nifA*. To enable the fusion gene to be isolated as a *Kpn*I fragment, *Kpn*I, *Spe*I, and *Xba*I sites were added between the *Dde*I and *Pst*I sites at the 3' end of *nifA*, giving pMW188.

The *Kpn*I fragment containing the fusion of the *B. japonicum nifD* promoter and *R. meliloti nifA* was cloned into the *Kpn*I site of pMW157, giving pMW186. In pMW186, the fusion is flanked by T1 T2 and Ω terminators. This region was cloned as an *Xba*I fragment into the *Spe*I site of pMW184, giving pMW191. pMW191 was then used to integrate the fusion gene with the terminator sequences into the *ino* site of RCR2011 and PC. The resulting strains were referred to as RMB7103 and RMB7203, respectively. The orientation and structure of the inserted DNA in RMB7103 and RMB7203 are exactly the same as in pMW191.

The *R. meliloti nifA* gene has been fused to and is expressed from the *B. japonicum nifD* promoter. The fusion contains several segments. The 5' end of the fusion contains 193 bp from pBR322 which are not expressed. The sequence was included because of the presence of convenient restriction sites which facilitated the construction of the gene. This is followed by the *B. japonicum nifD* promoter that includes 300 bp upstream of the transcription initiation site. The untranslated leader sequence between the transcription initiation site and the translation initiation codon was derived from the *R. meliloti nifH* gene and includes a 21-bp synthetic segment of DNA that was synthesized to permit a precise fusion at the translation initiation codon. This is followed by 1,622 bp from *R. meliloti* that include the entire *nifA* gene plus 41 bp downstream of the termination codon.

RMBPC-2. Strain RMBPC-2 contains the *dctABD/*Ω*/nifA* cassette integrated into the inositol locus of strain PC. The construction of the *dctABD/*Ω cassette on pRK138Ω is shown in Fig. A4. The 5,645-bp *dct* locus from *R. leguminosarum* was cloned as a *Bgl*II-*Pst*I fragment. *Hin*dIII linkers were added to the *Pst*I overhang. The resulting fragment was cloned into pUC13, which was cut with *Bam*HI and *Hin*dIII, creating pBG1A. The Ω cassette was isolated by *Nru*I and *Sma*I digestion of pIC20RΩ followed by ligation into the *Sma*I site of pBG1A. The resulting plasmid, pRK138Ω, was digested with *Hin*dIII and *Sma*I to isolate the *dctABD/*Ω fragment, which was then ligated into the *Sma*I site of pMW186K to form pRK150.

The source of the *BjnifDp::RmnifAp/nptII* cassette was pMW186K. The plasmid pMW186K was made by cohesive end ligation of a *Kpn*I fragment of pMW188 (Fig. A3b) containing *BjnifDp::RmnifAp* into the unique *Kpn*I site of pMW211 (Fig. A5). The Ω cassette in pMW157 (Fig. A1) was replaced with *nptII* to create pMW211. The Ω cassette was removed from pMW157 by *Eco*RI digestion and replaced with a 1.6-kb *Eco*RI fragment containing *nptII*. The source of *nptII* was pUC4-K1XX.

The *Xba*I fragment of pRK150 (Fig. A6) containing the *BjnifDp::RmnifAp/*Ω*/dctABD* insert was cloned into the *Spe*I site of pMW184 by cohesive-end ligation. The resulting plasmid, pMW300, was used to integrate the cassette into PC by marker exchange as described below.

RMB138Ω710A. Strain RMB138Ω710A contains the *dctABD/*Ω cassette inserted into the P3 site. To accomplish this, an integration vector, pMW229, which included the 2.9-kb P3 site was constructed (Fig. A7). The P3 integration site used to introduce the enhancement cassettes was the *Hin*dIII site located within the P3 region between the *nifHDK* and *fixABCX* operons of the *nif* cluster on megaplasmid pRmSU47a.

The construction of the P3 site integration vectors requires pCR710 and pMW229. Plasmid pMW229 differs from pCR710 only by the conversion of the *Hin*dIII integration site in pCR710 to a *Spe*I site. The P3 region and flanking DNA were isolated as a 2.9-kb *Nci*I fragment from cosmid pWB716 (8) which covers the *nif* region. The isolated 2.9-kb *Nci*I fragment was blunt end ligated into the *Sma*I site of vector pUC19 (42). This recreated a *Sma*I site at one end of the fragment and enabled the fragment to be excised as a *Sma*I-*Kpn*I fragment and cloned into vector pIC20R (23) cut with *Eco*RV and *Kpn*I. The fragment was then excised with *Eco*RI and cloned into the unique *Eco*RI site of pRK290 (15), giving pCR710. The integration vector pCR710 contains a unique *Hin*dIII site for incorporation of genes for subsequent integration into the P3 region of the *R. meliloti* genome. To facilitate the transfer of cassettes containing *Spe*I or *Xba*I ends to this vector, plasmid pMW229 was constructed. This was accomplished by digesting pCR710 with *Hin*dIII, filling in the ends with the Klenow fragment, adding *Spe*I linkers, and religating to form pMW229.

The source of the *dctABD/*Ω cassette for RMB138Ω710A was pRK138Ω (Fig. A4). The *dctABD/*Ω cassette was isolated by *Hin*dIII-*Sma*I digestion of pRK138Ω and inserted into the *Spe*I site of pMW229 by blunt-end ligation. The resulting integration plasmid was referred to as p138Ω710A. Integration of the insert in p138Ω710A into

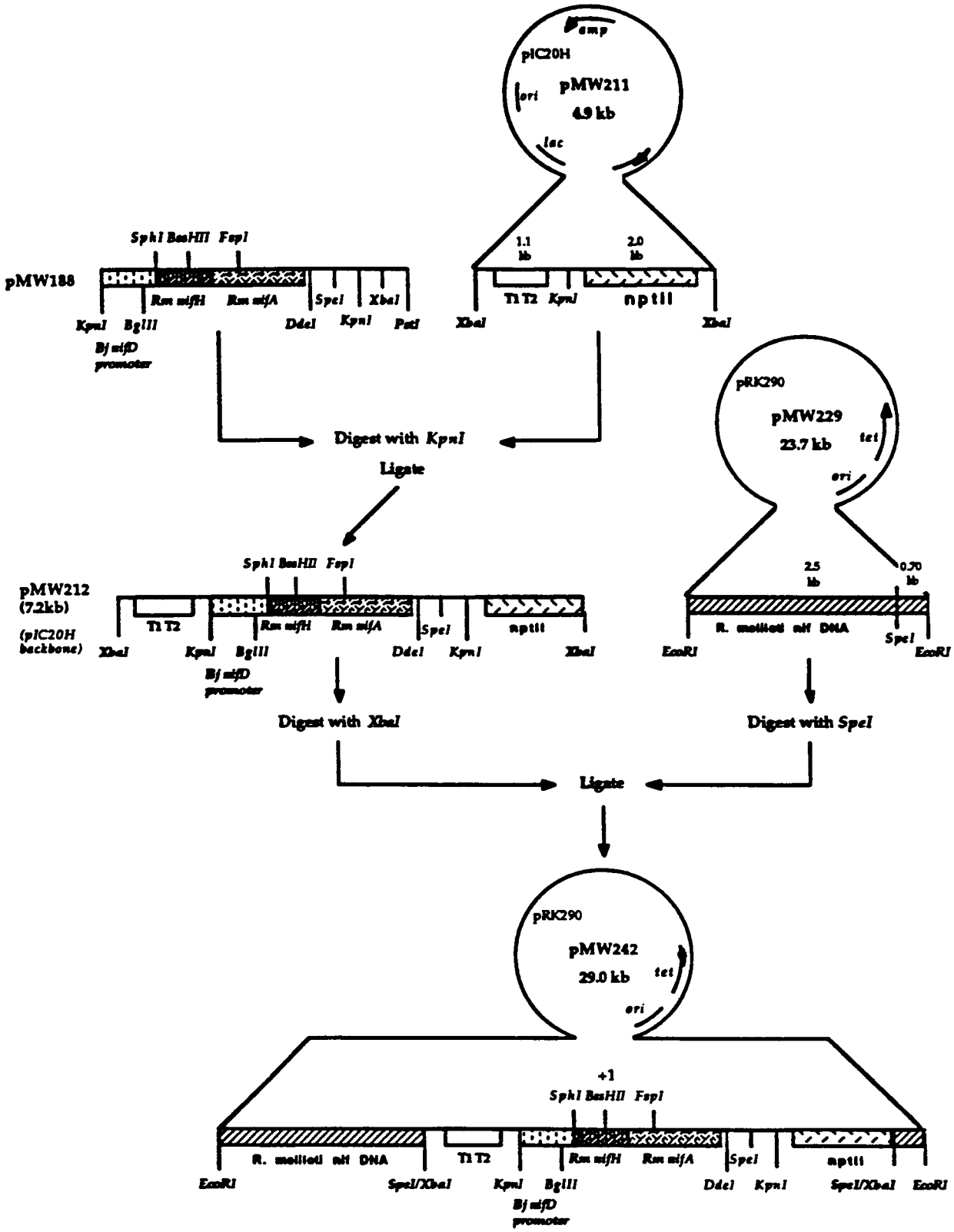

FIG. A8. Construction of the integration vector, pMW242, used to insert T1 T2/*BjnifDp*::*RmnifAp*/*nptII* into the P3 site of RMB7240.

the P3 locus of RCR2011 was done by marker exchange as described below.

RMB139Ω710B. The genes added to RCR2011 to create RMB 139Ω710B include a *dctA*/Ω cassette inserted into the P3 site. The source of the *dctA*/Ω cassette was pRK139Ω, the construction of which is shown in Fig. A4. The *Eco*RI fragment of pBG1A containing *dctA* was cloned into the *Eco*RI site of pIC20HΩ to form pRK139Ω. The *Hin*dIII fragment of pRK139Ω containing *dctA*/Ω was then cloned into the *Spe*I site of pMW229 (Fig. A7) to generate p139Ω710B. Integration of this insert into RCR2011 was done as described below.

RMB7240. To construct RMB7240, an *nptII/BjnifDp::RmnifAp* cassette was inserted into the P3 site of PC by marker exchange as described below except that kanamycin resistance was used as the selectable marker for the insert. This was done by cloning the *Kpn*I fragment of pMW188 (Fig. A3b) into the *Kpn*I site of pMW211 (Fig. A5) to create pMW212 (Fig. A8). The *Xba*I fragment of pMW212 containing *nptII/BjnifDp::RmnifAp* was inserted into the *Spe*I site of pMW229 (Fig. A7) to create the integration vector pMW242 (Fig. A8).

Integration procedure. The enhancement cassettes containing the engineered derivatives plus the antibiotic resistance marker were transferred to an integration vector (pMW191, pMW193, and pMW300 for the *ino* site and pMW242, p138Ω710A, and p139Ω710B for the P3 site) and subsequently integrated into either the P3 or *ino* site of the genome by a two-step incompatibility approach. The integration vectors were conjugated into the host strain by triparental mating using pRK2013 as the helper plasmid (18) and 20 μg of cinoxacin per ml as a counterselection. The plasmid was selected for by resistance to 5 μg of tetracycline per ml coded on the pRK290 backbone of the integration vectors and resistance to 100 μg of streptomycin per ml plus 100 μg of spectinomycin per ml due to the Ω portion of the enhancement cassette or to 50 μg of neomycin per ml if the *nptII* gene was present. Incompatible plasmid pJB251 (41), which is a spectinomycin-sensitive derivative of pPH1JI (19) and confers resistance to 40 μg of gentamicin per ml, was then crossed into the *R. meliloti* strain carrying the plasmid-borne enhancement cassette. The exconjugants from this cross were selected for resistance to gentamicin, spectinomycin, and streptomycin or to gentamicin plus neomycin. Plasmid pJB251 is incompatible with the integration vectors used here since each carries a P-group origin of replication. Therefore, selection for gentamicin, spectinomycin, and streptomycin resistance (or gentamicin plus neomycin resistance) plus sensitivity to tetracycline allowed identification of the strains which have the enhancement cassette integrated into the integration site by homologous recombination while losing the replicon from the integration vectors. The pJB251 plasmid was cured by inoculating the resultant strains onto alfalfa and screening bacteria isolated from nodules for sensitivity to gentamicin. The *ino* site integrants were further tested for the inability to grow on inositol as a sole carbon source. The structure of the recombinant strains was confirmed by Southern blot hybridization analysis (data not shown).

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