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Gene Fusion Vehicles for the Analysis of Gene Expression in *Rhizobium meliloti*

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A set of plasmid cloning vehicles was developed to facilitate the construction of gene or operon fusions in *Rhizobium meliloti*. The vehicles also contain a broad-host-range replicon and could be introduced into bacteria either by transformation or by transduction, using bacteriophage P2. Insertion of foreign DNA into a unique restriction endonuclease cleavage site promotes the synthesis of either the *Escherichia coli* lactose operon or the kanamycin phosphotransferase gene from transposon Tn*5*. Expression of the lactose operon could be detected by observing the color of *Rhizobium* colonies on medium that contained a chromogenic indicator. We also determined the growth conditions that make it possible to select either for or against the expression of the *E. coli* lactose operon in *R. meliloti*. Recombinant plasmids were constructed by inserting *Mbo*I restriction fragments of *R. meliloti* DNA into one of the vehicles, pMK353. Expression of β-galactosidase by a number of these recombinants was measured in both *R. meliloti* and *E. coli*.

Rhizobium bacteria form nodules on the roots of leguminous plants and, in these nodules, they reduce atmospheric nitrogen to ammonia. The ammonia is then exported to the host plant where it can be used in biosynthesis. The morphological development of *Rhizobium* organisms during the formation of productive nodules has been described previously (6, 21). However, changes in gene expression that lead to nodule formation and nitrogen fixation are not well characterized. Because the plant and bacteria are in such intimate contact within the nodule, it is difficult to get pure preparations of either for biochemical analysis. That the initial events of nodulation involve very few cells also limits the quantity of material available for study.

A potential solution to these and other problems is to adapt to *Rhizobium* bacteria the gene fusion techniques that have been developed to analyze gene expression in *Escherichia coli* (1, 4, 13). In general, these techniques involve joining DNA sequences that regulate the gene that is being studied to another gene for which more sensitive assays are available. As an example of this approach, fusion of the nitrogenase promoters to the β-galactosidase gene has been useful in determining the structure of the nitrogen fixation gene cluster in *Klebsiella* spp. (7, 15) and in studying the regulation of nitrogenase synthesis (9, 19). It would have been difficult to show that the *Klebsiella nifL* gene is important in oxygen-activated repression of nitrogenase by a direct assay for nitrogenase because nitrogenase is both repressed by oxygen and oxygen labile.

To use gene fusion techniques effectively in *Rhizobium meliloti*, it was necessary to develop a means of propagating gene fusions in *R. meliloti* and to study the properties that these fusions would have in an unfamiliar genetic background. We constructed various gene fusion vehicles that can be used in the analysis of gene expression in *R. meliloti*. These vehicles all contained a single restriction endonuclease cleavage site for insertion of DNA fragments. Some contained a complete coding sequence for an enzyme such as kanamycin phosphotransferase or *E. coli* β-galactosidase, which could be activated by the insertion of DNA that contained a promoter sequence. Others contained a truncated version of the β-galactosidase gene that lacked its normal promoter sequence, ribosome binding site, and start codon. When the DNA that is inserted into the vehicle contains these regulatory sequences and is joined in the same reading frame as the *lacZ* gene, a hybrid protein is produced (4). These hybrid proteins usually retain β-galactosidase activity. By studying the properties of *R. meliloti* strains that actively express the *lac* operon, we established conditions for detecting expression of these genes and for manipulating them genetically.

MATERIALS AND METHODS

Bacterial strains. Bacteria, bacteriophage, and plasmids are listed in Table 1. HK113 is a P2-related bacteriophage that was isolated by T. Dhillon and obtained from G. Bertani. A clear-plaque-forming mutant, HK113 *c*, was isolated by V. Camara.

Media. Minimal medium and LB complex medium used for *E. coli* (17) and yeast mannitol broth (YMB), yeast sucrose broth and minimal mannitol medium used for *Rhizobium meliloti* (24) have been described previously. Carbohydrates were added at 1% unless otherwise indicated. Lactobionic acid was filter sterilized, and care was taken to make sure the pH of lactobionic acid medium was 6.5 or lower. Agar was added at 1.5% for plates and 0.7% for overlays. Media were supplemented with tetracycline at 5 μg/ml for *R. meliloti* and 25 μg/ml for *E. coli*. Ampicillin was used at 100 μg/ml for *E. coli*, and kanamycin sulfate was used at 200 μg/ml for *R. meliloti* and 75 μg/ml for *E. coli*. The chromogenic indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was dissolved in 0.2 ml of dimethyl sulfoxide and added to media to obtain a final concentration of 40 mg/liter. Yeast extract, Casamino acids, tryptone, MacConkey agar, and agar for minimal plates were from Difco Laboratories. Mannitol, lactobionic acid, antibiotics, and

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TABLE 1. Bacterial strains, plasmids, and phage

Strain, plasmid, or phage	Relevant features	Reference or source
Strains		
R. meliloti		
104A14	Wild-type	24
E. coli		
MC1061	Δ*lacX74 rpsL*	5
RR1	*hsr hsd*	3
C-520	*supD*	25
Plasmids		
pRK248	Tet^r replicon RK2	27
pJS5	Kan^r, Chl^r, P4 *cos*	20
pMK317	Tet^r Kan^r P4 *cos*, replicon RK2	24
pMK318	Tet^r Kan^r P4 *cos*, replicon RK2	Kahn et al. manuscript in preparation
pMC275	Kan^r *lacZ*$^+$ *lacY*$^+$	4
pMC871	Kan^r *lacP*	4
pMC874	Kan^r *lacP lacZ'*	4
pMC903	Kan^r *lacP*	4
pMC931	Kan^r *lacP lacZ'*	4
Phage		
P2 *vir*1	Clear plaques, immunity sensitive	2
HK113 *c*	Clear plaques	This work

agar were from Sigma Chemical Co. X-gal was from Bachem.

DNA purification. Plasmid DNA was isolated from *E. coli* by a Triton lysis method (12). Plasmid DNA from *R. meliloti* was isolated by a modification of the boiling technique of Holmes and Quigley (10). Cultures (5 ml) were grown to stationary phase in YMB. Sodium lauryl sarcosinate was added to a final concentration of 0.1%, and after 2 to 5 min, the cells were pelleted by centrifugation and the supernatant was discarded. The pellet was resuspended in residual medium, and 350 μl of STET buffer (10) and 25 μl of lysozyme (10 mg/ml) were added. The cultures were heated to boiling and incubated at 100°C for 30 s, and the viscous lysate was centrifuged at 23,300 × *g* for 10 min. The supernatant was removed and either extracted with phenol and precipitated with an equal volume of isopropanol or immediately precipitated with isopropanol. The DNA was dried and resuspended in 10 mM Tris–1 mM EDTA (pH 8). High-molecular-weight *R. meliloti* chromosomal DNA was purified by using CsCl gradients (24).

Plasmid constructions. Restriction, ligation, and transformation were by the method of Kahn et al. (12). Restriction enzymes were purchased from New England Biolabs or purified in this laboratory or the laboratory of M. Thomashow. To insert DNA into pMK353, the plasmid was completely digested with restriction endonuclease *Bam*HI and treated with calf intestinal alkaline phosphatase (New England Nuclear Corp.) (16). *R. meliloti* genomic DNA was partially digested with endonuclease *Mbo*I to produce two sets of fragments with average fragment lengths of 2 and 6 kilobases. Each set of fragments was ligated separately to pMK353, and the ligated DNA was transformed into RR1 or MC1061. After a 1-h incubation to allow tetracycline resistance (Tet^r) to be expressed, samples of transformed MC1061 were plated on LB–X-gal agar to determine the proportion of colonies that were blue and that probably contained inserts. The remaining transformed cells from each ligation were pooled, brought to a volume of 150 ml with LB-tetracycline broth, and grown overnight at 37°C. Sterile glycerol (100 ml) was added to each culture, and the gene banks were stored at −20°C.

Infection of *R. meliloti*. Bacteriophages P2 *vir*1 and HK113 *c* were grown with *E. coli* C-520 as the host and concentrated by using polyethylene glycol (8). Transducing lysates were prepared from plasmid-containing *E. coli* cells by the same method, and the number of encapsidated plasmids was determined (M. Kahn, T. Kerppola and S. Stanfield, manuscript in preparation). For unknown reasons, high transducing titers of pMK320 and pMK321 could not be obtained with P2 *vir*1, so HK113 *c* was used to encapsidate these plasmids. Late-logarithmic-phase cultures of *R. meliloti* grown at 30°C were washed twice with yeast sucrose broth and suspended in 0.1 volume of yeast sucrose broth. To 0.1 ml of concentrated cells, an equal volume of transducing lysate was added, and the mixture was incubated at 37°C for 30 min to promote infection. YMB (5 ml) was added, and the cultures were grown for 3 to 4 h at 30°C to permit the expression of antibiotic resistance. Cells were pelleted and resuspended in 1 ml of YMB broth. A 2.5-ml amount of 0.7% agar was then added, and the mixture was poured over selective media.

β-Galactosidase assays. β-Galactosidase activity was determined by measuring the rate of hydrolysis of *o*-nitrophenyl-β-D-galactoside, as described by Miller (17). For the assay of the *R. meliloti* enzyme, cultures were grown in YMB media. Various methods were used to permeabilize the cells, including sonication with and without lysozyme treatment, freezing and thawing with and without lysozyme treatment, or the addition of any of 0.1% Triton X-100, toluene, 0.1% sodium lauryl sarcosinate, or chloroform–0.1% sodium dodecyl sulfate. No difference in activity was seen in these treatments, except with the chloroform-sodium dodecyl sulfate treatment which reduced activity of the *Rhizobium* enzyme. Toluene treatment was the simplest method of permeabilizing the cells for the assay of *E. coli* β-galactosidase activity in *R. meliloti* and β-galactosidase activity of the hybrid proteins in both *E. coli* and *R. meliloti*. The activity of many of the hybrid proteins was sensitive to sodium dodecyl sulfate.

Nodule isolation for β-galactosidase assay. Alfalfa (Ladak variety; Hinrichs Seed, Pullman, Wash.) was grown by using a tube method (M. Kahn and J. Somerville, manuscript in preparation). Four-day-old seedlings were inoculated with bacteria. If the strain contained a Tet^r plasmid, 3 μg of tetracycline per ml was added to the tubes to ensure that nodulation was by plasmid-containing cells (18). After 24 days, nodules and roots were removed, and surface was sterilized by immersion in 5% H_2O_2 for 30 s. They were then rinsed three times in sterile distilled water and crushed in 0.2 ml of sterile 0.5 M mannitol–20 mM Tris (pH 7.0) (26) by using a 0.2-ml Wheaton tissue grinder that had been soaked in 5% H_2O_2 and rinsed thoroughly with sterile distilled water. The homogenate was removed from the grinder, and the walls of the grinder were rinsed with 0.1 ml of grinding solution. Bacteria and bacteroids were counted by direct observation with a Neubauer hemacytometer. To estimate the fraction of bacterial forms that contained a recombinant plasmid, a 0.1-ml sample of the homogenate was serially diluted and plated on YMB and YMB-tetracycline agar. The remaining 0.2 ml was added to 0.6-ml Z buffer (17) and assayed for β-galactosidase activity by the toluene method described above.

R. meliloti mutant isolation. Log-phase cultures of *R. meliloti* 104A14 were pelleted and suspended in 0.05 M Tris-malate buffer (pH 6.5). *N'*-methyl-*N'*-nitro-*N*-nitrosoguanidine (50 μg/ml) was added, and the cultures were incubated at 37°C for 30 min. Cells were washed once with buffer, suspended in YMB broth, and grown overnight at 30°C to allow recessive mutations to segregate. In the morning, the cultures were harvested by centrifugation, suspended in minimal lactose broth, and incubated at 30°C. After 2 h, penicillin (200 μg/ml) was added, and the cultures were incubated for 2 to 4 h at 30°C until the cells lysed. The lysed cells were centrifuged at 5,860 × *g*, resuspended in YMB, and incubated overnight at 30°C. A second round of penicillin enrichment followed. After lysis, the cultures were plated on minimal medium that contained 1% lactose, 0.1% mannitol, and X-gal. We reasoned that lactose-negative (Lac^-) bacteria could only use the mannitol as a carbon source and would produce smaller colonies than the lactose-positive (Lac^+) bacteria. To identify Lac^- mutants, the small, pale colonies were then picked onto minimal medium that contained either 1% mannitol or 1% lactose.

RESULTS

Plasmid construction strategy. In designing cloning vehicles to facilitate the construction of gene fusions in *R. meliloti*, it was desirable to include other traits in addition to the assay gene. Plasmids derived from ColE1 are often used as cloning vehicles in *E. coli*, but they do not replicate in *R. meliloti* (23). Plasmid pRK248 (27), a small derivative of the broad-host-range plasmid RK2 that is able to replicate in *R. meliloti*, was used as the replicon for the vehicles. To introduce the vehicles into *R. meliloti*, we included the P4 *cos* site. This site is a DNA sequence that contains the cohesive ends of bacteriophage P4. We have shown that plasmids that contain this site are efficiently packaged into bacteriophage P2 capsids and can be transduced into *R. meliloti* 104A14 (Kahn et. al., manuscript in preparation). *R. meliloti* does not support P2 lytic growth. In addition to the Tet^r gene carried by pRK248, other antibiotic resistance genes have been included to aid in the selection of cells that contain recombinant plasmids. All plasmids contain a unique *Bam*HI or *Bgl*II site adjacent to the assay gene. Fragments generated by *Bam*HI, *Bgl*II, *Bcl*I, *Xho*II, *Mbo*I, and *Sau*3A digestion can be inserted into this site since these enzymes share the same cohesive ends (16).

Transcriptional fusion to the kanamycin phosphotransferase gene. We constructed vehicles that use the kanamycin phosphotransferase gene as the assay gene. One of these vehicles, pMK341, was constructed as shown in Fig. 1. A *Bam*HI-*Bgl*II fragment from pJS5 (L. S. Pierson III and M. Kahn, Mol. Gen. Genet., in press) that contains the kanamycin phosphotransferase gene from Tn*5* (Tn*5 kan*), the P4 *cos* site, a gene for chloramphenicol resistance (Chl^r), and a P15 replicon was inserted into the unique *Bgl*II site in pRK248 of both orientations to yield pMK341 and pMK342. The *Bgl*II cut in pJS5 removed the Tn*5 kan* promoter from its coding sequence (11). Since a *Bam*HI-*Bgl*II junction could not be cut with either enzyme, both plasmids now had a unique *Bgl*II site adjacent to the beginning of the Tn*5 kan* gene. Both plasmids were transduced into 104A14 by using P2 *vir*1, and the level of Kanamycin resistance (Kan^r) of plasmid-containing cells was determined. 104A14 carrying a single copy of the Tn*5* transposon grew on kanamycin at 1 mg/ml. Strain 104A14 was inhibited by kanamycin at 8 μg/ml, 104A14(pMK341) grew on medium that contained kanamycin at 40 μg/ml but not at 60 μg/ml, and

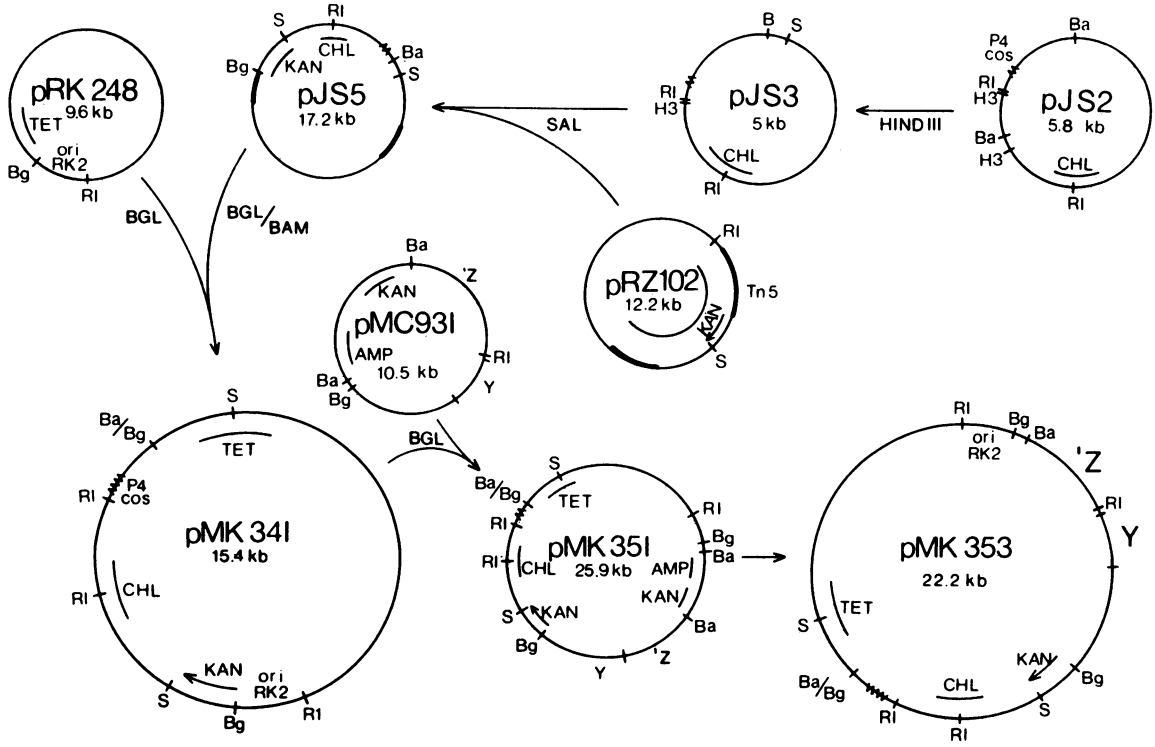

FIG. 1. Construction of pMK341 and pMK353. The arrow indicates the direction of transcription of Tn*5 kan*. Restriction enzyme sites are: Ba, *Bam*HI; Bg, *Bgl*II; X, *Xhol*; S, *Sal*I; H3, *Hin*dIII; and R1, *Eco*RI. Ba/Bg represents a *Bam*HI-*Bgl*II fusion site.

104A14(pMK342) grew in the presence of kanamycin at 100 μg/ml. Insertion of DNA into the *Bgl*II site in pMK341 therefore will detect weaker promoter sequences than insertion of DNA into pMK342.

Cloning vehicles that carry the *E. coli* lactose operon. Plasmid cloning vehicles that use the expression of *E. coli* lactose operon to indicate the activity of cloned regulatory sequences have been constructed (4). One of these, pMC275, had all of the protein-coding regions of the *lac* operon and expressed these regions under the control of a β-lactamase promoter. This promoter was active in *R. meliloti*; strains that contained a plasmid carrying the entire β-lactamase gene were ampicillin resistant (Amp^r). A second plasmid, pMC871, also carried the protein-coding regions of the *lac* operon but had no promoter sequence adjacent to them. Insertion of a DNA fragment with promoter activity next to these sequences led to high levels of *lac* operon expression. A third plasmid in this series, pMC874, had a deletion which removed the first eight amino acids of the β-galactosidase protein and required the insertion of a promoter, ribosome binding site, and start codon in the correct reading frame to express the *lac* operon. These plasmids all had ColE1-type replicons and could not replicate in *R. meliloti*.

Regions of these three plasmids that contain the lactose operon were joined to pMK317 (24) (Fig. 2). Digestion of pMK317 with *Hin*dIII and *Bam*HI produced three fragments and disrupted a kanamycin phosphotransferase gene derived from transposon Tn*903* (Tn*903 kan*). The fragment that contained the 5′ end of Tn*903 kan* also contained P4 *cos* and pRK248. Digestion of pMC275, pMC871, or pMC874 with *Bam*HI and *Hin*dIII produced fragments carrying the modified forms of the *lac* operon described above. At one end of each of these fragments was the 3′ end of the Tn*903 kan* gene. In three separate ligation reactions, *Bam*HI-*Hin*dIII digests of pMC275, pMC871, and pMC874 were joined to similarly digested pMK317. Tet^r Kan^r transformants of pMC1061 were analyzed, and three plasmids, designated pMK320, pMK321, and pMK323, were isolated. MC1061(pMK320) and MC1061(pMK321) were white, and MC1061(pMK323) was red on lactose MacConkey agar. pMK320 produced significant amounts of β-galactosidase in *R. meliloti* (Table 2) and was used to determine the characteristics of *R. meliloti* containing an actively expressed *lac* operon (see below).

TABLE 2. Determination of β-galactosidase activity

Strain[a]	Activity[b]
E. coli	
C-520	0.4
C-520 (+IPTG)	550
MC1061	0.06
MC1061(pMK320)	2,050
MC1061(pMK330)	0.
MC1061(pMK353)	0.7
R. meliloti	
104A14	1.2
104A14(pMK320)	1,250
104A14(pMK330)	1.6
104A14(pMK353)	2.0
Medicago staiva (alfalfa)	
Uninfected root	0.01, ND
104A14-infected nodule	0.17, 3.5
104A14(pMK320)-infected nodule	2.0, 20

[a] *E. coli* cultures were grown in LB medium except for C520 (+IPTG) which was supplemented with 10^{-3} M isopropyl-thio-β-D-galactoside. *R. meliloti* cultures were grown in YMB.

[b] For *E. coli* and *R. meliloti*, activity units are 10^3 times the change in absorbance (420 nm) produced by the hydrolysis of ONPG per min per absorbance unit (600 nm) of culture. Activity units for *M. sativa* are 10^3 times the change in absorbance (420 nm) produced by the hydrolysis of ONPG per min per mg of wet tissue (first value) or per 10^8 bacterial cells (second value). Values are the average of four or more determinations. ND, Not done.

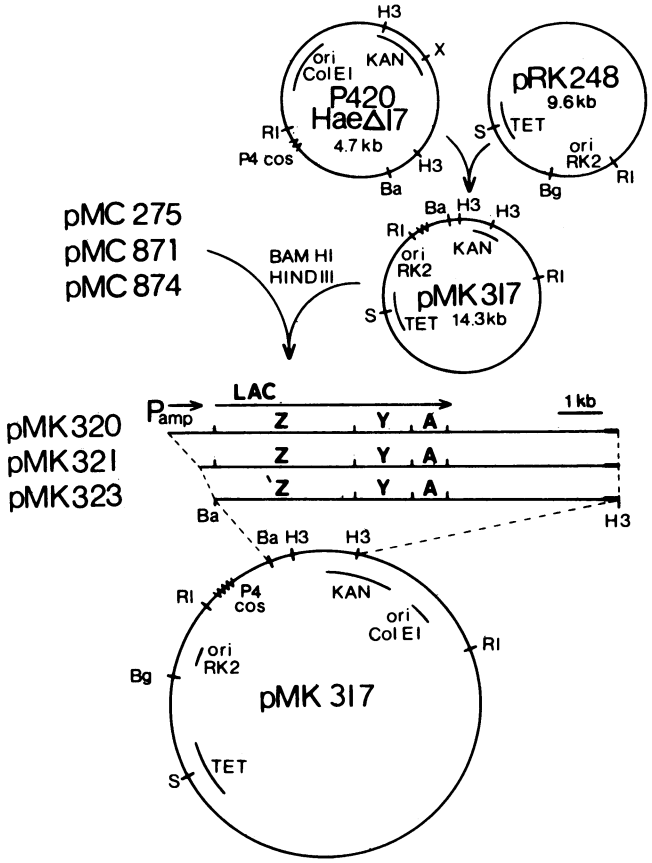

FIG. 2. Construction of pMK317 and the *lac* fusion vehicles. Only the portions of pMC275, pMC871, and pMC874 used in the constructions of pMK320, pMK321, and pMK323 are shown. The arrow represents the direction of *lac* DNA transcription.

Transcription initiated in pMK317 resulted in about 900 U of β-galactosidase activity in strains of *E. coli* and *R. meliloti* that carry pMK321. We believed that this was too high for pMK321 to be used as a transcriptional fusion vehicle and that this level of transcription might be sufficient to read through DNA fragments inserted into the translational fusion vehicle pMK323 and activate β-galactosidase synthesis. In an attempt to lower this background, we constructed another translational fusion vehicle, pMK330 (Fig. 3). pMK318 (Kahn et al., manuscript in preparation) was cut at its unique *Bam*HI site and joined to a *Bam*HI-*Bgl*II fragment derived from pMC931 (4), a plasmid that contains the version of the *lac* operon in which β-galactosidase is missing its first eight amino acids. In a similar construction, pMK327 was constructed by using a *Bam*HI-*Bgl*II fragment from pMC903 (4), a plasmid that carries a promoterless *lac* operon similar to

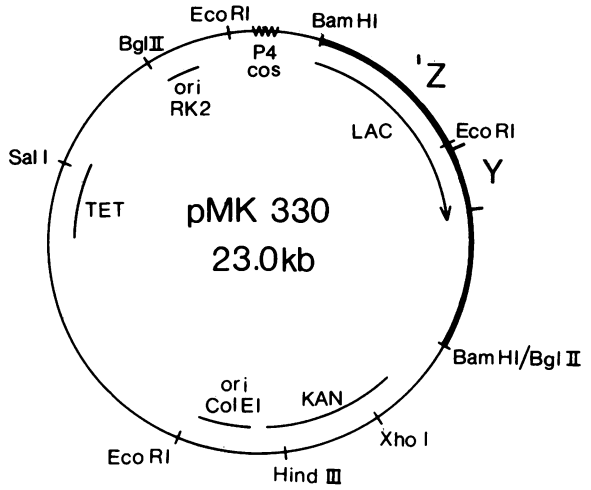

FIG. 3. Structure of translational fusion vehicle pMK330. A *Bam*HI-*Bgl*II fragment containing *lac* DNA derived from pMC931 (heavy line) was inserted into the *Bam*HI site of pMK318 (thin line). The arrow indicates the direction of *lac* DNA transcription.

that found in pMC871. pMK327 produced about 500 U of β-galactosidase in *R. meliloti* 104A14.

Plasmids pMK348 and pMK353 were constructed in a further attempt to reduce background expression. To construct the translational fusion vehicle pMK353, pMC931 (4) was digested with *Bgl*II and the entire plasmid was ligated into the *Bgl*II site of pMK341 (Fig. 1). Plasmid DNA from Amp^r Tet^r Chl^r Kan^r transformants was isolated, and those that had the structure of pMK351 (Fig. 1) were digested with *Bam*HI to remove one of the P15 replicons, Amp^r and Kan^r. This left a single *Bam*HI site adjacent to β-galactosidase. Plasmid pMC903 is a transcriptional fusion vehicle with a structure similar to that of pMC931 (4). An analogous construction that started with pMK341 and pMC903 generated the transcriptional fusion vehicle pMK348. pMK348 produced about 170 U of β-galactosidase activity in *R. meliloti*. The construction of pMK330 and pMK353 thus reduced but did not completely eliminate the possibility that transcription through DNA inserts might have activated expression of the *lac* operon.

Behavior of the lactose operon cloning vehicles in *R. meliloti*. The Lac$^+$ plasmid, pMK320, and the Lac$^-$ translational fusion vehicles, pMK330 and pMK353, were introduced into *R. meliloti* 104A14 by transduction. Tet^r transductants of 104A14 were screened on mannitol–X-gal agar to determine *lac* gene expression qualitatively. X-gal is hydrolyzed by β-galactosidase to produce a blue pigment; the intensity of the blue color is an index of the β-galactosidase activity in the cells (17; see below). 104A14(pMK320) produced very dark blue colonies on mannitol–X-gal agar. 104A14, 104A14(pMK330), and 104A14(pMK353) produced light blue colonies on this medium.

β-galactosidase activity in *E. coli* and *R. meliloti* was determined (Table 2). Strains that contained the translational fusion vehicles pMK330 and pMK353 showed the same low level of β-galactosidase activity as plasmidless strains. *E. coli* and *R. meliloti* strains that contained pMK320 had significant β-galactosidase activity.

β-galactosidase activity in alfalfa root nodules was also measured (Table 2). Nodules formed by 104A14 or by 104A14(pMK320) had substantially higher β-galactosidase activity than did uninfected root tissue. The activity of β-galactosidase in nodules formed by 104A14(pMK320) was about 10 times the activity in nodules formed by 104A14 per milligram of nodule and about six-fold higher per bacterial cell. When the β-galactosidase values for the nodules formed by 104A14(pMK320) were corrected for the proportion of viable cells (10 to 55%) that were Tet^r and thus contained pMK320, the activity per plasmid-containing cell was calculated to be about twice that found in free-living 104A14(pMK320).

Isolation of *R. meliloti* DNA sequences with promoter activity. The gene fusion vehicles described above were constructed to aid in analyzing the activity of *R. meliloti* control sequences during nodule development. As a step toward understanding the properties of these vehicles, partial *Mbo*I digests of genomic DNA from *R. meliloti* 104A14 were ligated to pMK353 as described above. Transductants of *R. meliloti* were screened on minimal mannitol–X-gal medium to identify colonies that were darker blue than normal and to determine β-galactosidase activity in these colonies. It was found that the darker blue colonies had more β-galactosidase activity. Plasmid DNA from these isolates was used to transform MC1061 to compare the levels of expression of the same fusion protein in *E. coli* and *R. meliloti*. Fusions were also isolated from *E. coli* by transforming the ligation mixtures into MC1061 and screening for blue colonies on LB–X-gal or M9-glucose-Casamino acids-X-gal agar. Plasmids from blue colonies were introduced into *R. meliloti*.

The strength of promoter sequences derived from *R. meliloti* DNA in *R. meliloti* and *E. coli* is shown in Fig. 4. None of the plasmids that were originally isolated from the darker blue *R. meliloti* colonies had a level of expression of less than 5 U. This was probably an artifact of our isolation procedure, since the faint blue color of 104A14 did not allow us to recognize cells that contain plasmids with very weak promoters. It is possible that by using the Lac$^-$ mutants described below, greater sensitivity could be achieved. In these constructs, the β-galactosidase activity was always lower in *E. coli* than in *R. meliloti*. This finding was true despite the higher copy number of the plasmids in *E. coli*.

In this experiment, the proteins with β-galactosidase activity were hybrids between a *R. meliloti* protein and *E. coli* β-galactosidase. The higher activity in *R. meliloti* could be explained if these hybrid proteins were more sensitive to cellular proteases in *E. coli* than in *R. meliloti*. We examined this possibility directly. Spectinomycin was added to cultures that contained fusion proteins to inhibit protein synthesis, and β-galactosidase activity was measured for the next 4 h. The proteins were stable in *E. coli* (data not shown).

Selection in *R. meliloti* by using β-galactosidase gene fusions. To select developmentally or nutritionally regulated promoters or to alter promoters by mutation, it would be useful if *Rhizobium* growth were dependent on the level of *lac* gene expression. Since 104A14 is able to use lactose as a sole carbon source, Lac$^-$ mutants of 104A14 were isolated by nitrosoguanidine mutagenesis and penicillin enrichment. These mutants grew poorly on minimal lactose agar and produced white colonies when grown on minimal mannitol–X-gal agar. Unlike the Lac$^-$ mutant isolated by Ucker and Signer (29), these mutants did not have significantly decreased levels of β-galactosidase and might be defective in β-galactoside transport. If the *E. coli* β-galactosidase or β-galactoside permease genes could complement these mutants, they would be able to grow on lactose as sole carbon source. *R. meliloti* carrying recombinant plasmids

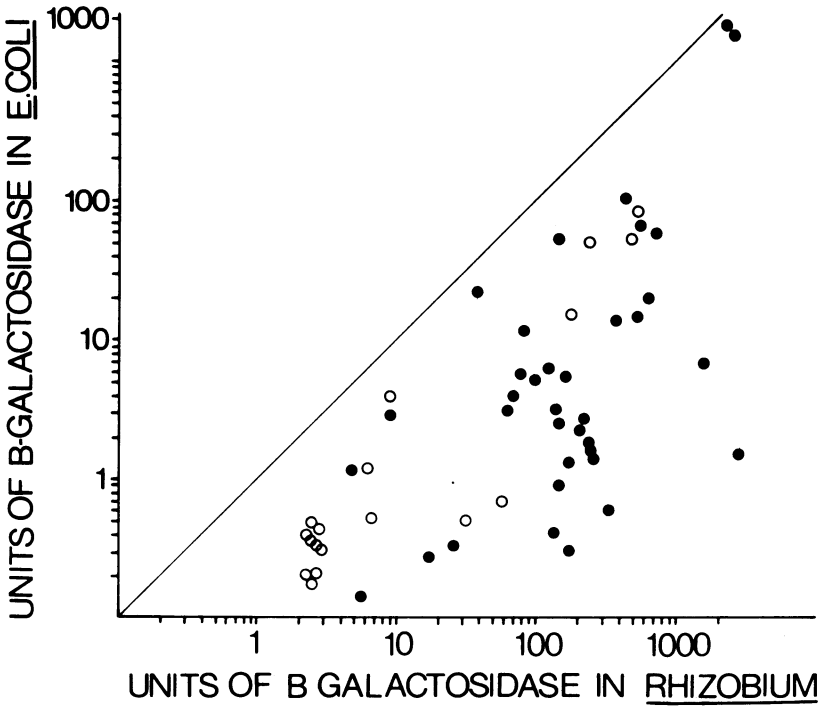

FIG. 4. Comparison of the promoter activity of *Rhizobium* DNA in *E. coli* and *R. meliloti*. Units are defined in Table 2. ○, Plasmid containing an *R. meliloti* DNA sequence that was first isolated in *E. coli*; ●, plasmid containing an *R. meliloti* DNA sequence that was first isolated in *R. meliloti*.

expressing these genes could therefore be selected even if they were very rare.

Lac^+ plasmid pMK320 was introduced into Lac^+ strain 104A14 and Lac^- strains 104A14 *lac-2* and 104A14 *lac-11* by transduction. These were screened for growth on lactose medium (Table 3). Surprisingly, none of the plasmid-containing strains grew on lactose—not even 104A14(pMK320). The presence of Lac^- plasmid pMK330 did not affect the growth of either the wild-type or Lac^- 104A14. As shown elsewhere (28), inhibition of growth requires the presence of a hydrolyzable β-galactoside in the medium and the production of both *E. coli* β-galactosidase and β-galactoside permease. It occurs even when an alternative carbon source is available. Since the toxicity of lactose to 104A14(pMK320) results from the expression of *E. coli lac* operon genes (28), this property can be used to select against promoters that activate these genes by placing cells on mannitol-lactose medium or on lactobionic acid medium (see below) and isolating resistant bacteria.

To select for expression of the *lac* operon, we needed a mutant of 104A14 that was resistant to the toxic effect of lactose. Lactobionic acid, a gluconic acid β-galactoside, was used as a carbon source for this selection. Strain 104A14 grew very poorly on minimal lactobionic acid medium, and we did not observe any spontaneous mutants of 104A14 that grew rapidly on this medium. Strain 104A14(pMK320) did not grow at all on lactobionic acid medium. We believe that lactobionic acid was being metabolized by the *E. coli lac* operon proteins that were expressed in *R. meliloti* and that this inhibited cell growth. When 104A14(pMK320) was plated on tetracycline medium containing lactobionic acid as the sole carbon course, resistant mutants grew at a frequency of 10^{-6}. This frequency is lower than that observed for lactose resistance (28), since retention of the plasmid-carried genes was necessary for growth on lactobionic acid but not on lactose. A lactobionic acid-resistant strain, 104A14 *lacX28*, was isolated by curing pMK320 from one of these colonies. This was accomplished by growing 104A14 *lacX28* on nonselective medium and then isolating white colonies on mannitol–X-gal medium. The cured strain did not grow on lactobionic acid medium but when pMK320 was reintroduced into the strain, it grew well on both lactobionic acid and lactose (Table 3). This indicated that the mutation in 104A14 *lacX28* was in the *R. meliloti* genome and not on the plasmid. Strain 104A14 *lacX28*(pMK330) grew as poorly as 104A14 *lacX28* on lactobionic acid media but grew on lactose, glucose, or galactose. By using the set of recombinant plasmids derived from pMK353 as described above, we showed that plasmids that led to the production of nine or more units of β-galactosidase could support the growth of 104A14 or 104A14

TABLE 3. Effect of β-galactosides on *R. meliloti* strains

Carbon source	Strain	Colony growth with[a]:		
		No plasmid	pMK320	pMK330
Lactose	104A14	+[a]	−	+
	104A14 *lac-2*	±	−	±
	104A14 *lac-11*	±	−	±
	104A14 *lacX28*	+	+	+
Lactobionic acid	104A14	±	−	±
	104A14 *lacX28*	±	+	±

[a] Colony growth was rated as follows: +, good growth at frequency expected; ±, very small colonies at frequency expected; and −, No growth of most cells and growth by mutants at low frequency.

lacX28 on lactobionic acid agar. Strain 104A14 *lacX28* formed effective nodules on alfalfa.

DISCUSSION

To facilitate the cloning and analysis of transcriptional and translational control signals in *R. meliloti*, we developed cloning vehicles for generating operon fusions in vitro. DNA sequences that have promoter activity and, in some vehicles, a properly placed ribosome-binding site and initiation codon could be inserted into a unique restriction endonuclease cleavage site in the plasmid. This led to the expression of either kanamycin phosphotransferase (Tn*5 kan*) or β-galactosidase activity. The vehicles were designed to be introduced into bacteria by transformation (14, 22) or by transduction with bacteriophage P2 or a related bacteriophage. They contained a broad-host-range replicon which should be able to replicate in a variety of gram-negative bacteria.

Conditions have been found that make it possible to screen or select either for or against promoter activity in *R. meliloti*. *R. meliloti* strains that express the *E. coli lac* operon are sensitive to the addition of lactose or other β-galactosides (28). This effect requires that the strains produce at least 160 U of β-galactosidase (28). Since about one-half of the promoters that we studied (Fig. 4) produced less than 160 U of activity, we are attempting to find conditions to increase the sensitivity of this selection. Mutants with decreased expression of the *E. coli* genes were resistant to killing. Among these mutants should be mutants with defects in the *R. meliloti* control sequences that activated the expression of the *lac* operon.

We isolated a mutant of *R. meliloti* 104A14 that grew on lactobionic acid as sole carbon source only when it contained an expressed copy of the *lac* operon. This provided a sensitive selection for DNA sequences that activate these genes. Since a low level of expression of the operon was required for growth on lactobionic acid but a high level was required for toxicity, selection for lactobionic resistance in 104A14 should select for a high proportion of promoter mutations in plasmids that contain strong promoters. This is currently being investigated.

A set of promoter-containing plasmids was constructed by inserting *R. meliloti* DNA into the translational fusion vehicle, pMK353. Recombinant plasmids were initially found by their effect on the color of bacterial colonies on indicator medium and this color was subsequently found to be correlated with the level of β-galactosidase in *E. coli* and *R. meliloti*. The relative enzyme activity produced by each plasmid in both organisms was then measured (Fig. 4). There was considerable scatter in these data but there is a positive correlation between enzymatic activity in the two organisms. This suggests that there are features within the sequences that activate the synthesis of the hybrid proteins that are common to both bacteria. These data also suggest that selection in *E. coli* for DNA sequences that complement *E. coli* mutants has a reasonable probability of success. Activity in *R. meliloti* was consistently higher than in *E. coli*. Nevertheless, of the 45 inserts that had levels of expression in *R. meliloti* greater than background, 13 (29%) had more than 10% of this activity in *E. coli* and 34 (76%) had more than 1%.

The growth conditions used in this work to identify DNA sequences that activate β-galactosidase expression were clearly different from those that the bacteria will be exposed to in the nodules or during nodulation. We are currently attempting to approximate nodulation conditions more closely to identify genes that are expressed by *R. meliloti* during the development of root nodules.

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