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Genetics

Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*

(plasmid RK2/plasmid vehicle/conjugal transfer/*nif* genes)

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ABSTRACT **A broad host range cloning vehicle that can be mobilized at high frequency into Gram-negative bacteria has been constructed from the naturally occurring antibiotic resistance plasmid RK2. The vehicle is 20 kilobase pairs in size, encodes tetracycline resistance, and contains two single restriction enzyme sites suitable for cloning. Mobilization is effected by a helper plasmid consisting of the RK2 transfer genes linked to a ColE1 replicon. By use of this plasmid vehicle, a gene bank of the DNA from a wild-type strain of *Rhizobium meliloti* has been constructed and established in *Escherichia coli*. One of the hybrid plasmids in the bank contains a DNA insert of approximately 26 kilobase pairs which has homology to the nitrogenase structural gene region of *Klebsiella pneumoniae*.**

RK2 is a bacterial plasmid of incompatibility group P-1 that is very similar, if not identical, to plasmids having the designation RP1, RP4, and R68 (1). It confers resistance to the antibiotics ampicillin, tetracycline, and kanamycin and exists at approximately five to eight copies per chromosomal equivalent in *Escherichia coli* (2). A general feature of P-1 plasmids is their extensive host range. Such plasmids are capable of conjugal self-transfer to a wide variety of Gram-negative bacteria (3, 4). This unique property has been used as the basis for development of a plasmid cloning system in *E. coli* with widespread applicability. Although native RK2 DNA can be used directly as a recombinant DNA cloning vector, its large size [56 kilobase pairs (kb)] is a serious drawback to routine use. In order to reduce the size and still retain overall broad host range transfer capability, a cloning system has been devised that separates RK2 transfer and replication functions onto separate plasmids. The tetracycline-resistant plasmid component of this system, pRK290, contains a functional RK2 replicon and can be mobilized at high frequency by using a helper plasmid, but is non-self-transmissible. pRK290 contains single *Eco*RI and *Bgl* II sites where DNA can be inserted without loss of essential functions. The kanamycin-resistant helper plasmid, pRK2013, consists of the RK2 transfer genes cloned onto a ColE1 replicon (5). Its sole function in this system is to *trans*-complement the vector for mobilization.

This paper describes the construction of pRK290, its properties as a cloning vector, and its use in constructing a gene bank of the agriculturally important bacterium *Rhizobium meliloti*. As an initial test of the gene bank, DNA containing the nitrogenase structural gene region of *Klebsiella pneumoniae* (6, 7) was used as a hybridization probe to identify clones carrying the nitrogenase region of *R. meliloti*. One of the members of the bank was found to contain a 26-kb insert with homology to this *K. pneumoniae* probe.

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MATERIALS AND METHODS

Bacterial Strains. *E. coli* was strain HB101 *pro leu thi lacy* Str^r *endoI*$^-$ *recA*$^-$*r*$^-$*m*$^-$; *R. meliloti* 102F34 and 104B5 were kindly provided by Nitragin (Milwaukee, WI); *Serratia marcescens* MW1 is a clinical isolate obtained from D. Guiney; *Pseudomonas aeruginosa* PAO was obtained from D. Guiney; *K. pneumoniae* M5A1 was obtained from W. Brill; *Acinetobacter calcoaceticus* is a laboratory strain, originally obtained from John Ingraham.

Enzymes. Restriction endonuclease *Eco*RI was purified in our laboratory; *Bgl* II was a gift from C. Yanofsky; all other restriction enzymes were obtained from New England BioLabs. T4 DNA ligase was obtained from Bethesda Research Laboratories (Rockville, MD), and was used at a concentration of 1 unit/ml for ligations. Bacterial alkaline phosphatase was obtained from Miles and was dialyzed into 10 mM glycine, pH 9.5/0.1 mM $ZnCl_2$ for storage. DNA was treated with this enzyme at 65°C for 90 min in 10 mM Tris·HCl (pH 9.5). The reaction was terminated by phenol extraction.

Bacterial Matings. Matings were performed by mixing 10^9 cells each of the donor and recipient and filtering the suspension onto 0.45-μm Millipore filters. The filters were incubated at 30°C on nonselective agar plates for 3–6 hr before the cells were resuspended and plated.

Isolation of *R. meliloti* DNA. Total DNA from *R. meliloti* was obtained from 500 ml of stationary-phase cells grown in yeast/mannitol broth (8). Washed cells were resuspended in 50 mM Tris·HCl/20 mM EDTA, pH 8.0, and lysed with predigested Pronase (500 μg/ml) and Sarkosyl (1%) for 60 min at 37°C. DNA was purified by equilibrium centrifugation first in neutral CsCl (ρ = 1.70 g/cm^3) and then in CsCl/ethidium bromide (ρ = 1.55 g/cm^3).

Size Fractionation of *R. meliloti* DNA. Total *R. meliloti* DNA was partially digested with *Bgl* II to give fragments in the range 10–30 kb; 140 μg of such DNA was heated briefly at 65°C and layered directly onto a 36-ml, 10–40% sucrose gradient in 20 mM Tris·HCl, pH 8.0/10 mM EDTA/50 mM NaCl. Centrifugation was for 18 hr at 23,000 rpm in an SW 27 rotor at 25°C. Fractions were monitored for DNA size on a 0.5% agarose gel. Those containing DNA molecules of predominantly 12–25 kb were pooled and used for construction of the gene bank.

Construction of a *R. meliloti* Gene Bank. pRK290 DNA was digested exhaustively with *Bgl* II and was then treated with bacterial alkaline phosphatase. A small background of transformants was obtained from this DNA, with or without ligation, which probably represented residual uncleaved molecules. Size-fractionated *R. meliloti* DNA, ligated to this vector, was used to transform HB101 to tetracycline resistance. The ex-

Abbreviation: kb, kilobase pair(s).

pression time after heat shock was kept short (≈40 min) to avoid the generation of siblings.

RESULTS

Three widely separated regions of RK2, designated *oriV*, *trfA*, and *trfB*, are necessary for DNA replication (Fig. 1). *oriV* is the origin of replication as determined by electron microscopy (9); *trfA* and *trfB* are regions encoding *trans*-acting replication functions (10). There is a fourth physically distinct region which encodes a *cis*-acting function necessary for conjugal mobilization to occur, termed *rlx* (11), which is thought to represent the origin of conjugal transfer. This region also contains the site of the nick for the RK2 relaxation complex (11). Because it is essential that a plasmid vector derived from RK2 contain all of these regions in addition to a drug resistance gene and appropriate cloning sites, the remaining RK2 DNA was deleted by the ordered sequence of steps shown in Fig. 2.

First, approximately 14 kb of DNA was removed by using *Kpn* I, which cleaves RK2 at four positions. Because all of the genetic information necessary for autonomous replication and tetracycline resistance is located on only two of the four fragments, transformation of *E. coli* with a total *Kpn* digest of RK2 DNA and selection for tetracycline resistance yielded transformants containing these two fragments in either of the two possible orientations. Such transformants exhibited both kanamycin sensitivity and a lack of conjugal self-transmissibility. DNA with the fragments aligned as in RK2 was then treated to remove as much DNA as possible from either side of the single *Hin*dIII site without removing *rlx* or *trfA*. This was accomplished by digesting the DNA to completion with *Hin*dIII followed by treatment with bacterial alkaline phosphatase to render it incapable of being covalently recircularized by DNA ligase. A partial digestion with *Hae* II, which cleaves RK2 at many sites, was then used to generate pseudo-random cuts on either side of the phosphatase-treated *Hin*dIII site. Whenever at least one cleavage occurred on both sides of the *Hin*dIII site, a molecule was generated that could again be circularized by DNA ligase. Provided that the cleavages did not extend into essential replication regions, such molecules could be detected by transformation. In this manner, a number of deletion derivatives were obtained. These were screened for retention of the *rlx* site by monitoring mobilization with pRK2013. The smallest such derivative was selected. It represented a deletion of approximately 11.5 kb of DNA. The last step in the construction involved removing most of the 12.1 kb of DNA between the single *Eco*RI site and the single *Bgl* II site of RK2. To do this, a previously constructed RK2 deletion derivative, pRK2501, was used (14). The distance between the single *Eco*RI and *Bgl* II sites in pRK2501 is only 1.1 kb. The two DNAs were digested jointly with *Eco*RI and *Bgl* II, ligated, and used to transform *E. coli* for tetracycline resistance. Substitution of the appropriate fragment was monitored by screening transformants for sensitivity to ampicillin and kanamycin.

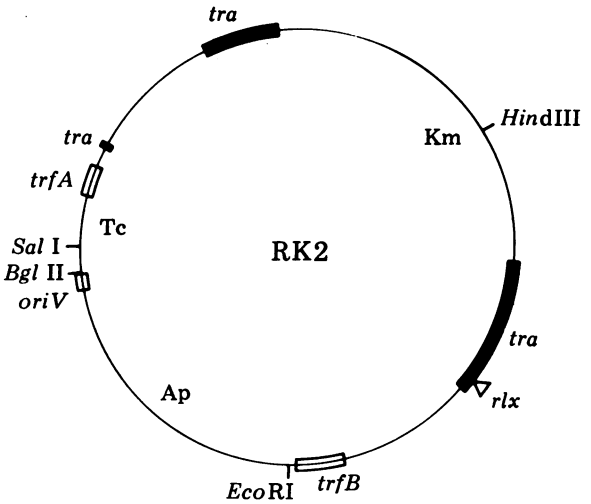

FIG. 1. Map of RK2. Ap, Tc, and Km refer to genes conferring resistance to ampicillin, tetracycline, and kanamycin, respectively. *oriV* is the origin of replication as determined by electron microscopy (9). *trfA* and *trfB* refer to *trans*-acting replication functions (10). *rlx* refers to the relaxation complex site (11). *tra* refers to regions containing genes required for conjugal transfer (12, 13).

The resulting plasmid, pRK290, is shown in Fig. 3. It is 20 kb large and has two single restriction enzyme sites into which a variety of *Eco*RI- and *Bgl* II-generated DNA fragments have been cloned successfully. Other enzyme sites within the tetracycline gene include *Sma* I and *Sal* I. The molecule specifically lacks sites for *Bam*HI, *Hin*dIII, *Pst* I, *Kpn* I, *Hpa* I, and *Xho* I. The copy number of pRK290 in *E. coli* has been found to be similar to that of RK2 (data not shown). Because interruption of either cloning site does not lead to a detectable change in colony phenotype (e.g., insertional inactivation), it is necessary to treat the restriction enzyme-cleaved vehicle with alkaline phosphatase prior to ligation. This prevents covalent recircularization of the plasmid vehicle during ligation and eliminates what would otherwise be an overwhelming background of molecules without inserts.

Transfer Properties. Table 1 shows the frequency with which pRK290 was transferred into a variety of Gram-negative bacteria as part of the binary plasmid system. Matings were performed on membrane filters as described in *Materials and Methods*. *E. coli* strain HB101 was specifically chosen as the plasmid host for two reasons. First, it is recombination deficient (*recA*$^-$), which is desirable because pRK2013 and pRK290 share regions of homology. Second, HB101 lacks the normal K-12 restriction system which might otherwise inactivate unmodified foreign DNA carried as inserts. In Table 1, the first line shows the high frequency of self-transmissibility displayed by pRK2013 in *E. coli*. In line 2 it can be seen that such self-transmissibility is completely lacking for the vector pRK290. When the complete binary plasmid system is constituted (line 3), high-frequency transfer of pRK290 occurs. Although the majority of exconjugants selected on tetracycline were found to carry both pRK2013 and pRK290, a sizeable proportion (≈15%) carried only pRK290. This is apparently due to incompatibility between the two plasmids; restreaking of cells carrying both plasmids on tetracycline leads to a rapid segregational loss of pRK2013, whereas restreaking on kanamycin leads to a loss of pRK290. Although pRK2013 uses and requires the ColE1 replicon for replication (6), the RK2-specific replication functions *trfA* and *trfB* are still present in this plasmid. These may be capable of interacting with and expressing incompatibility against other RK2 replicons such as pRK290.

The pattern observed for *R. meliloti* in Table 1 is quite different from that observed for *E. coli*. In contrast to the high rate of self-transmissibility previously seen for pRK2013, this plasmid shows a very low rate of transfer into *Rhizobium* (line 4). This presumably reflects the relatively narrow host range of the ColE1 replicon and the inability of pRK2013 to become established stably in an organism distantly related to *E. coli*. pRK290, as a component of the binary plasmid system (line 6), shows a high rate of transfer into *Rhizobium*. Although the majority of tetracycline-resistant conjugants in this situation are neomycin sensitive, reflecting the absence of pRK2013, tetracycline-resistant conjugants displaying the neomycin-resistant phenotype of pRK2013 can be detected at a frequency

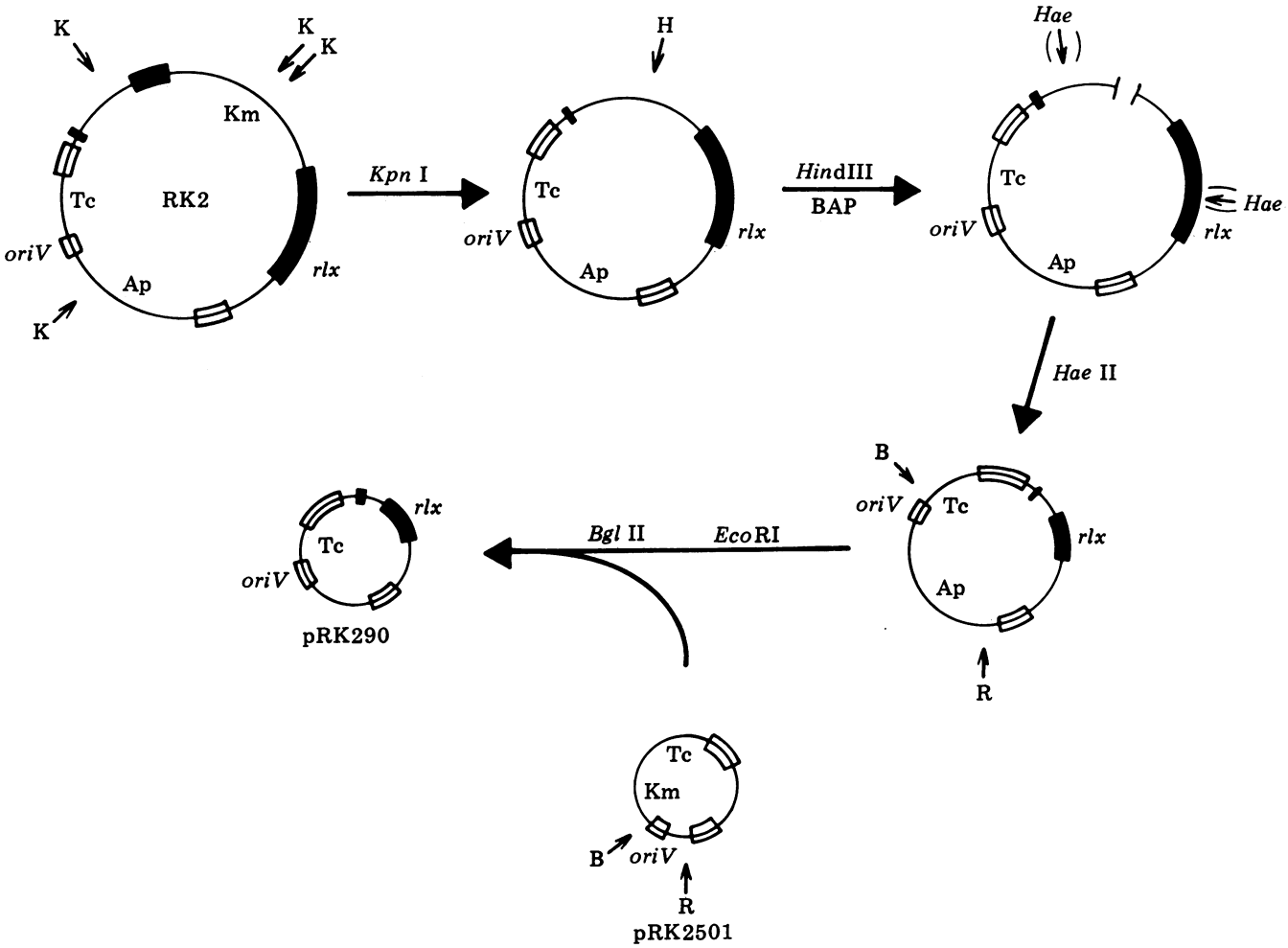

FIG. 2. Construction of pRK290. Small arrows indicate cleavage sites for restriction enzymes used at each step; for *Hae* II, the approximate position of cleavages leading ultimately to pRK290 are indicated. BAP refers to treatment with bacterial alkaline phosphatase. Solid bars represent conjugal transfer genes; open bars are essential replication regions. pRK2501 is a previously constructed RK2 deletion derivative (14) containing a *Hae* II kanamycin fragment that had been inserted *in vitro*. K, *Kpn* I; H, *Hin*dIII; *Hae*, *Hae* II; B, *Bgl* II; R, *Eco*RI.

that is considerably higher than that observed for pRK2013 alone. This suggests that rescue of pRK2013 may be occurring via homologous recombination with pRK290 in the recipient during binary system matings.

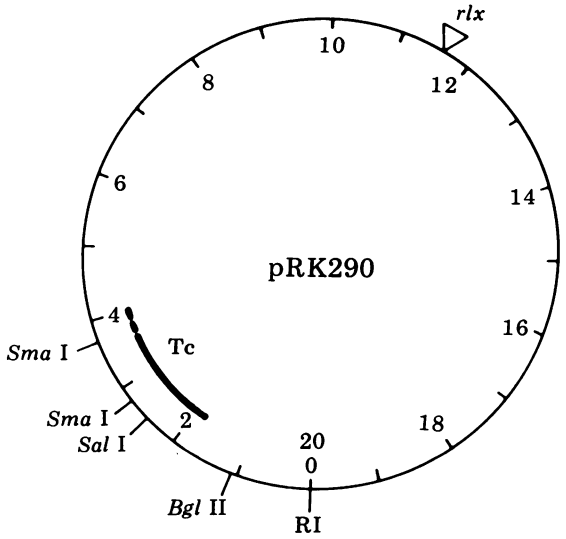

FIG. 3. Map of pRK290 (20 kb large). Coordinates are in kb. RI, *Eco*RI.

As shown in line 7 of Table 1, an important feature of the binary plasmid system is the finding that it is not necessary to have pRK2013 and pRK290 together in the same cell at the start of the mating for efficient mobilization. Triparental matings [e.g., HB101 (pRK2013) × HB101 (pRK290) × recipient] are equally efficient in promoting transfer. Cloned DNA can thus be "stored" in suitable *E. coli* strains such as HB101 until the time for transfer without necessitating the prior introduction of pRK2013.

Except for *P. aeruginosa*, the transfer patterns observed for the rest of the Gram-negative organisms surveyed in Table 1 are basically similar to that of *E. coli*. All were carried out as triparental matings. As was found for *E. coli*, both vehicle and helper plasmids exerted mutual incompatibility, leading to a rapid segregational loss of the nonselected plasmid.

Gene Bank of *R. meliloti*. One of our major objectives in constructing the pRK2013/pRK290 binary plasmid system was to provide an effective means for studying symbiotic nitrogen fixation by *Rhizobium* by using recombinant DNA technology. The molecular mechanisms whereby rhizobia are able to infect and nodulate legumes are poorly understood. We have constructed a plasmid gene bank representing the entire cellular DNA of a strain of *R. meliloti*, the species capable of nodulating alfalfa. This strain, 102F34, is reported to effectively nodulate a wide range of alfalfa varieties (15). The total cellular DNA of 102F34 was cloned into pRK290 as a collection of *Bgl* II re-

Table 1. Conjugal transfer frequencies of pRK2013/pRK290 binary plasmid system for various Gram-negative bacteria

Donor	Recipient	Tc^R conjugants/ recipients	Km^R and Nm^R conjugants/ recipients
E.c. HB101 (pRK2013)	E.c. HB101 *rif*	—	8.5×10^{-1}
E.c. HB101 (pRK290)	E.c. HB101 *rif*	0	—
E.c. HB101 (pRK2013, pRK290)	E.c. HB101 *rif*	4.0×10^{-1}	8.2×10^{-1}
E.c. HB101 (pRK2013)	R.m. 104B5 *nal*	—	1.7×10^{-7}
E.c. HB101 (pRK290)	R.m. 104B5 *nal*	0	—
E.c. HB101 (pRK2013, pRK290)	R.m. 104B5 *nal*	4.6×10^{-2}	8.4×10^{-4}
E.c. HB101 (pRK2013) + E.c. HB101 (pRK290)	R.m. 104B5 *nal*	8.3×10^{-2}	5.6×10^{-4}
E.c. HB101 (pRK2013) + E.c. HB101 (pRK290)	S.m. *nal*	6.6×10^{-2}	2.2×10^{-1}
E.c. HB101 (pRK2013) + E.c. HB101 (pRK290)	K.p. M5A1	1.4×10^{-1}	8.8×10^{-1}
E.c. HB101 (pRK2013) + E.c. HB101 (pRK290)	P.a. PAO *nal*	2.6×10^{-1}	8.4×10^{-7}
E.c. HB101 (pRK2013) + E.c. HB101 (pRK290)	A.c. *rif*	8.3×10^{-4}	3.0×10^{-4}

Tc, tetracycline; Km, kanamycin. pRK290 is Tc resistant; pRK2013 is Km resistant. E.c., *E. coli*; R.m., *R. meliloti*; S.m., *S. marcescens*; K.p., *K. pneumoniae*; P.a., *P. aeruginosa*; A.c., *A. calcoaceticus*.

striction enzyme fragments. These fragments were derived from a partial enzyme digestion of 102F34 DNA followed by size fractionation on a 10–40% sucrose gradient. DNA, approximately 15–20 kb in size, was ligated to *Bgl* II-digested pRK290 DNA that had been treated with bacterial alkaline phosphatase; it was then used to transform *E. coli*. A representative sample of the total transformants (≈300) was then analyzed for plasmid DNA content by published procedures (14). A *Bgl* II restriction digest pattern of 15 such DNAs is shown in Fig. 4. pRK290 is the uppermost band in each lane except lane 5. The size of each insert is estimated from the sum of the *Bgl* II fragments released during digestion. Lane 3, which contains no plasmid DNA, is an infrequent occurrence; 929 of 1285 transformants, or approximately 72%, were estimated to carry DNA insertions. Overall, the average size of the inserts was 19 kb. If the molecular weight of the *R. meliloti* genome is assumed to be about the same as that of the *E. coli* genome, 4200 kb (16), then one can calculate that there is a 98% chance that a given unique sequence of DNA will be represented in this bank (17).

We have experienced no instability of cloned *Rhizobium* DNAs in HB101 maintained under selective pressure. Reproducible plasmid restriction patterns are obtained from cloned DNAs even after prolonged culture on solid medium. In the absence of selection, the rate of plasmid loss is generally low (<1% per generation) although higher rates of loss have been seen for particular clones. After reintroduction into *Rhizobium* there is no apparent decrease in stability.

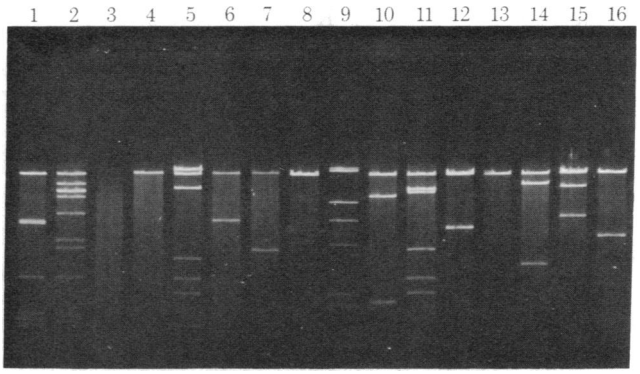

FIG. 4. *Bgl* II-digested plasmid DNAs from the *R. meliloti* gene bank. Plasmid DNA was isolated from HB101 transformants by published procedures (14) and digested with *Bgl* II. The uppermost band in each lane except lane 5 is pRK290. Lane 9 is a set of DNA size standards derived from a *Hin*dIII total digest of bacteriophage λ DNA (top to bottom): 28.0 kb, 23.7 kb, 9.5 kb, 6.7 kb. 4.3 kb, 2.2 kb, and 2.0 kb.

As an initial test of the gene bank, we have used colony hybridization (18) to probe clones for DNA homologous to the nitrogenase structural genes of *K. pneumoniae*. These genes have been cloned as a 6.9-kb fragment of DNA onto PACYC184 (6, 7). It has been shown that the resulting plasmid, pSA30, has a region of approximately 1–2 kb that hybridizes specifically to the DNA of nitrogen-fixing organisms but not to the DNA of nonfixers (19). A single clone was identified which carries, as part of a 26-kb insert, a 3.6-kb *Bgl* II fragment with strong homology to pSA30 (Fig. 5). In a complete *Bgl* II digest of total *Rhizobium* DNA, a DNA fragment of identical size was identified as having the highest homology to pSA30.

DISCUSSION

The binary plasmid system described here should greatly facilitate the genetic analysis of cloned DNAs in organisms in which transformation systems cannot easily be established. *R. meliloti*, for example, is extremely refractory to transformation by homologous RK2 DNA, yet pRK290 can be introduced into this organism at frequencies on the order of a few percent. Similarly, high-transfer frequencies were observed for other Gram-negative bacteria (Table 1). Although the extent to which restriction systems in these organisms may have been circumvented by the lack of appropriate sites in pRK290 is not known (except for *K. pneumoniae* because pRK290 lacks *Kpn* I sites), a reduction in transfer frequency of several orders of magnitude could clearly be tolerated. We experienced no difficulty conjugally transferring cloned DNA into a number of *Rhizobium* strains.

The extent to which the helper plasmid pRK2013 will cotransfer with pRK290 during mobilization experiments depends on the recipient. Among those bacteria that were examined, pRK2013 was found to transfer efficiently to *E. coli*, *K. pneumoniae*, *S. marcescens*, and *A. calcoaceticus*. For each of these hosts, however, a single restreaking on tetracycline was usually sufficient to segregate pRK290 clone. In contrast, pRK2013 was not maintained stably either in *R. meliloti* or in *P. aeruginosa*, and the majority of exconjugants contain only pRK290.

When present alone in a particular recipient, pRK290 is incapable of further transfer. As such, a certain degree of biological containment is inherent in the use of this system. The

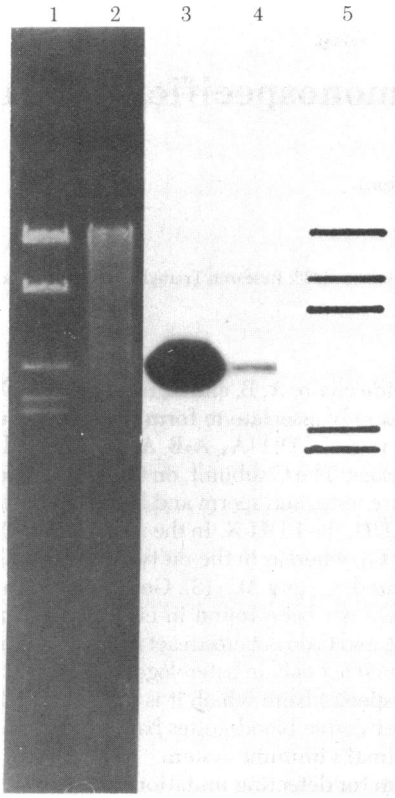

FIG. 5. 1, *Bgl* II digest of *R. meliloti* 102F34 gene bank clone 375; 2, *Bgl* II digest of total *R. meliloti* 102F34 DNA. 3, Autoradiogram of 1 after Southern transfer (20) and hybridization to pSA30; 4, autoradiogram of 2 after Southern transfer and hybridization to pSA30. 5, DNA size standards derived from a *Hin*dIII digest of bacteriophage λ (top to bottom): 23.7 kb, 9.5 kb, 6.7 kb, 2.2 kb, and 2.0 kb.

same applies to the storage of cloned fragments in *E. coli*. Because transfer can be accomplished via triparental matings, pRK290 need never come in contact with the mobilizing plasmid except during the actual mating.

The *R. meliloti* gene bank described in this work contains an average fragment size of 19 kb. A particular effort was made to clone large inserts for several reasons. Large inserts obviously increase the probability of preserving functional gene clusters intact. One of our major goals is to use these clones for the genetic analysis of nodulation and nitrogen fixation. For *K. pneumoniae*, a free-living nitrogen fixer, a cluster of at least 15 genes extending over 24 kb of DNA are required for nitrogen fixation (6). For *Rhizobium*, an even greater number of genes may be necessary for effective symbiosis. Large inserts are also desirable insofar as they reduce the absolute number of clones comprising the gene bank. This may be particularly important for studies with *Rhizobium* in which plant screening procedures may be contemplated. Lastly, cloning large fragments is necessary to eliminate nonrandom DNA representation caused by the use of a restriction enzyme to fragment the DNA.

Very little is known about the organization and control of nitrogen fixation (*nif*) genes in *Rhizobium* species. By using the gene bank, we have been able to tentatively identify a portion of the *nif* structural gene set in *R. meliloti*. A single cloned insert of 26 kb was found which has a unique region of homology to *K. pneumoniae nif* DNA. This result provides preliminary confirmation of the gene bank because homology to the *K. pneumoniae nif* structural gene region has been detected in all nitrogen-fixing organisms surveyed so far (19 organisms). Attempts are now being made to identify other clones containing genes that play a role in the symbiotic nitrogen fixation process.

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