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10 000 copies of plasmid DNA described in this paper. Indeed, the accessibility of the target is higher on plasmid DNA than on genomic DNA that has a more complex conformational organization. In addition, replicate intermediates of the plasmid are single-stranded and so are a template for NASBA amplification. Therefore, NASBA is not a suitable method to detect transcription of plasmid DNA.

From these results, we determined that for the highly sensitive detection of eukaryotic gene transcription with NASBA, massive contamination of the RNA sample with genomic DNA may be a problem if the template is multiple copy-number genes (e.g., HERV) and if the sample is derived from a tissue with high mitotic activity. In general, DNA molecules are not static replication and transcription events that lead to the formation of single-stranded regions or permit specific primer hybridization or amplification by NASBA.

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New Genetic Tools for Use in the *Rhizobiaceae* and Other Bacteria

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During the past decade there has been considerable interest in the isolation and characterization of microbes from diverse environments. Unfortunately, genetic experiments in prokaryotes other than *E. coli* often are limited by the availability of stable, compatible vectors with various drug resistance markers. For example, we found that in our organism of interest—the symbiotic soil bacterium *Sinorhizobium meliloti* strain 1021 (derivative of SU47)—plasmids of the incompatibility groups IncQ and IncW are not stably maintained under some culture conditions (7,12,16).

Previously, our group reported the characterization of an autonomously replicating sequence (ARS) from the *S. meliloti* pSymB replicon (14). Although we constructed vectors using this ARS, we found that they were unstable in the absence of antibiotic selection (M. Barnett, unpublished results). Recently, we have made extensive use of derivatives of the broad-host-range plasmid pBBR1 from *Bordetella bronchiseptica*, which we describe here. With the use of new plasmid replicons, we needed additional resistance markers to use multiple vectors effectively, especially in the presence of marked genomic mutations. Here, we report the construction of a new Tn5 derivative carrying hygromycin resistance (Hy^r); construction of two new pBBR1MCS derivatives containing spectinomycin resistance (Sp^r) or Hy^r; and construction of pBluescript®-derived (Stratagene, La Jolla, CA, USA) suicide vectors, carrying neomycin resistance (Nm^r), Sp^r or Hy^r.

The most commonly used resistance markers in *S. meliloti* are gentamicin, neomycin, spectinomycin, streptomycin and tetracycline. Since other antibiotics such as chloramphenicol (Cm) and β -lactam compounds are not usable in our strain, we sought candidates for an additional resistance marker. Hygromycin resistance has not previously been exploited in the *Rhizobiaceae* but had

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proved useful in *E. coli*, *Saccharomyces cerevisiae*, plants and mammals (8). Therefore, we tested the IncP vector, pCIT20 (13), which carries Sp^r and Hy^r , for the ability to confer Hy^r in *S. meliloti*. The plasmid conferred resis-

tance to 25, 50 and 100 $\mu\text{g/mL}$ hygromycin, while *S. meliloti* strains lacking the plasmid failed to grow even after extended periods of time. Further work with the vectors described below demonstrated that *S. meliloti* is sensitive

to levels as low as 10 $\mu\text{g/mL}$ in solid-rich medium and 5 $\mu\text{g/mL}$ in liquid-rich medium. Resistant *S. meliloti* strains grow well at levels of 50 $\mu\text{g/mL}$ in solid-rich medium and 5 $\mu\text{g/mL}$ in liquid-rich medium. Therefore, Hy^r can be used successfully as a marker in *S. meliloti*.

To make use of the Hy^r marker, we constructed a new Tn5 derivative containing the hygromycin resistance gene, *hph*. The gene was amplified from pCIT20 and used to replace the central drug marker-containing region of Tn5 as described in Figure 1A. We have demonstrated that Tn5- Hy effectively mutagenizes *S. meliloti* and can be exchanged for Tn5 or other Tn5 derivatives by the method described by De Vos et al. (4)

Previously, the *B. bronchiseptica* pBBR1 plasmid was reported to be stably maintained in *S. meliloti* strain 2011 (2). The earliest cloning vector based on this replicon, pBBR1MCS, was not usable in *S. meliloti* because it carried Cm^r (11). We constructed two pBBR1MCS derivatives to use in our strain: one (pMB393) that carries Sp^r plus the original Cm^r marker, and another (pMB403) that contains Hy^r (Figure 2). These vectors complement the other available pBBR1 derivatives that carry kanamycin, tetracycline, ampicillin, gentamycin (10) and trimethoprim resistance markers (3). It has been previously reported that pBBR1 can coexist with IncP plasmids (2), and we have used our Sp^r and Hy^r derivatives to confirm this compatibility in *S. meliloti*. We have used green fluorescent protein (GFP) gene fusions in these vectors to visualize bacteria during all stages of symbiosis (6). The retention frequency of these vectors in the absence of selection is 60%–70% (6).

To generate disruptions or markers in the chromosome, we make use of suicide vectors that cannot replicate in *S. meliloti* and therefore integrate into the chromosome by single reciprocal recombination. To provide additional suicide vectors with different drug markers and be able to introduce them into cells by triparental conjugation using RK2 transfer functions (15), we constructed pBluescript derivatives containing either Sp^r , Nm^r or Hy^r markers and a minimal *oriT* sequence to allow conjugal transfer, as shown in Figure 2.

Figure 1. Vectors containing Tn5- Hy and the hygromycin and spectinomycin resistance genes as cassettes flanked by multiple restriction enzyme sites. (A) Construction of a Tn5 derivative carrying Hy^r . The *hph* gene (8) was amplified from pCIT20 DNA (11) using the following primers: 5'-GCTGCA-GAAAGGAATTACCAC-3' and 5'-CTAGTAACATAGATGACACCGCGC-3'. The 1.8-kb amplified product includes the cauliflower mosaic virus (CaMV) 19S promoter and nopaline synthase (NOS) 3' sequences (17). *hph* is expressed from the CaMV promoter. The native promoter and co-transcribed apramycin resistance gene are not present (9). The product was used to replace the central, drug marker-containing *Bgl*III fragment in Tn5 by digesting pTJ7.1 (provided by T. Jacobs; Tn5 located downstream from the tetracycline resistance gene in pBR322) with *Bgl*III, blunting with Klenow and inserting the *hph* gene. Tn5- Hy was then transferred to the self-mobilizable, *ColE1* origin-containing pRK600 (5), resulting in pMB443. (B) Construction of a hygromycin resistance cassette. The *hph* gene was amplified as in (A) using *Taq* DNA polymerase (Stratagene), which adds an A as a 3' overhang. pBluescript SK+ was digested with *EcoRV* and then treated with *Taq* DNA polymerase in the presence of TTP to add a T as a 3' overhang. The *hph* fragment was then ligated into pBluescript SK+ to create pVO205. All sites shown can be used to remove the *hph* cassette and are unique within the vector except for *Bss*HIII, which cuts twice within 24 bp, and *Bam*HI, which cuts flanking the *hph* gene. The sequence of *hph* within the polylinker (8,17) was corrected by DNA sequencing and has been assigned GenBank® accession no. AF190131. (C) Construction of a spectinomycin resistance cassette. A 1428-bp minimum fragment containing the *aadA* gene was removed from pM37 (J. Mulligan, unpublished results) by digesting with *Bsa*I, blunting with Klenow and then digesting with *Hind*III. To create pVO121, the *aadA* fragment was ligated into pBluescript SK+ that had been digested with *Cla*I, blunted with Klenow and then digested with *Hind*III. To create pVO122, the *aadA* fragment was ligated into pBluescript SK+ that had been digested with *EcoRV* and *Hind*III. All polylinker sites remain unique and can be used to remove the *aadA* cassette. The sequence of the *aadA* gene is contained within GenBank accession no. X12870 from bases 915–2343.

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Figure 2. Construction of pBBR1MCS- and pBluescript-derived vectors. pMB403 was constructed by amplifying DNA containing the Hy^r cassette from pCIT20 as in Figure 1 and ligating into *Dra*I-digested pBBR1MCS. pMB393 was constructed by isolating a 2.3-kb *Bam*HI-*Hind*III fragment from pM37 (J. Mulligan, unpublished results) containing the *aadA* gene (18), blunting with Klenow and ligating into the *Eco*RI site (overhangs removed) of pBBR1MCS (11). pMB419 was created by cloning the amplified Hy^r cassette into *Ssp*I-digested pBluescript SK⁻. The 300-bp *oriT*-containing *Eco*RI fragment from pBSL327 (1) was blunted with Klenow and cloned into the Klenow-blunted *Sap*I site. pMB438 was constructed by cloning the *npt* gene that encodes neomycin resistance from pMB321 (M. Barnett, unpublished results) into *Ssp*I-digested pBluescript SK⁻. The *oriT* DNA was cloned into the Klenow-blunted *Xmn*I site, and the resulting vector was partially digested with *Eco*RI, filled in with Klenow and religated. This removed the *Eco*RI sites present at the end of the *npt* and *oriT* sequence. pMB439 was constructed by cloning the *Sp*I cassette from pM37 (J. Mulligan, unpublished results) into pBluescript SK⁻ that was partially digested with *Ssp*I. This recreates an *Eco*RI site that was removed by partially digesting the vector with *Eco*RI, filling in the ends with Klenow and religating. The *oriT* DNA was cloned into the *Sap*I site as described for pMB419. In these vectors, the multiple cloning site (MCS) contains *lacZα*. Therefore, blue/white screening can be used to identify colonies that contain plasmids with inserts.

Finally, to facilitate the use of Hy^r and Sp^r genes in other plasmid constructions, we generated plasmids containing either the *hph* gene or the *aadA* gene as cassettes flanked by multiple restriction enzyme cleavage sites (Figure 1, B and C). The *aadA* gene cassette has the added advantage that it is a minimum 1.4-kb fragment that removes an extraneous E2 integration-like gene from Tn21 present in the 2.3-kb fragment used in pMB393 shown in Figure 2. These markers and vectors provide added versatility for functional genomic studies.

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