

oriT-Directed Cloning of Defined Large Regions from Bacterial Genomes: Identification of the *Sinorhizobium meliloti* pExo Megaplasmid Replicator Region

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We have developed a procedure to directly clone large fragments from the genome of the soil bacterium *Sinorhizobium meliloti*. Specific regions to be cloned are first flanked by parallel copies of an origin of transfer (*oriT*) together with a plasmid replication origin capable of replicating large clones in *Escherichia coli* but not in the target organism. Supplying transfer genes in *trans* specifically transfers the *oriT*-flanked region, and in this process, site-specific recombination at the *oriT* sites results in a plasmid carrying the flanked region of interest that can replicate in *E. coli* from the inserted origin of replication (in this case, the F origin carried on a BAC cloning vector). We have used this procedure with the *oriT* of the plasmid RK2 to clone contiguous fragments of 50, 60, 115, 140, 240, and 200 kb from the *S. meliloti* pExo megaplasmid. Analysis of the 60-kb fragment allowed us to identify a 9-kb region capable of autonomous replication in the bacterium *Agrobacterium tumefaciens*. The nucleotide sequence of this fragment revealed a replicator region including homologs of the *repA*, *repB*, and *repC* genes from other *Rhizobiaceae*, which encode proteins involved in replication and segregation of plasmids in many organisms.

With the rapid increase in the number of completed microbial genome sequences, we are entering an era in which interests in the manipulation and functional characterization of whole genomes are flourishing. Methods and techniques involved in the manipulation of large regions of genomes will increasingly become valuable tools (for example, in the generation of mosaic organisms with various catabolic and biosynthetic capabilities). Here we describe a new procedure to clone large (>100-kb) defined regions from the genome of the nitrogen-fixing bacterium *Sinorhizobium meliloti*.

S. meliloti is a free-living gram-negative soil bacterium whose symbiotic interaction with alfalfa results in the formation of nitrogen-fixing root nodules. The genome of *S. meliloti* strain SU47 consists of three large replicons, the largest of which is 3,500 kb in size and appears to be similar to a conventional bacterial chromosome (4, 28, 36). The two other replicons are referred to as megaplasmids (47). One is 1,350 kb in size and, because it carries nodulation and nitrogen fixation genes required for symbiosis, is referred to as pSym (5, 33, 46); the other is 1,700 kb and is designated pExo since it carries two large gene clusters required for the synthesis of exopolysaccharides (alternate designations include pRmeSU47b and pSymb [4, 13, 22, 30, 33]).

In previous work, we constructed a genetic map of the pExo megaplasmid which consists of sequential Tn5-derivative transposon insertions linked to each other in transduction (16). Strains carrying pExo megaplasmid deletions between defined insertions were isolated, and a phenotypic analysis of these strains allowed us to identify several loci involved in utilization of the carbon sources dulcitol, β -hydroxybutyrate, lactose, rhamnose, and protocatechuate (17). Other known genes located on the plasmid include those involved in thiamine bio-

synthesis, purine and glycerol metabolism, dicarboxylate transport, and phosphate transport and *fix* genes (*sbmA*) (3, 6, 23, 27, 58). We estimate that the genes for all of the known phenotypes associated with pExo could be accommodated within a 70-kb region; hence, the biological role of over 95% of the megaplasmid remains to be established.

To gain further insight into the biology of pExo, we wished to identify genes on this megaplasmid using a nucleotide sequencing approach, and as part of that work, we developed a procedure to clone defined regions from microbial genomes. With this approach, we identified and partially characterized a region which appears to be the pExo megaplasmid origin of replication. The procedure is based on site-specific recombination which occurs at the origin of transfer (*oriT*) of conjugative plasmids (7, 11, 26, 38, 42, 57, 60). While such recombination is well documented, to our knowledge, it has not previously been applied for the manipulation of microbial or other genomes. Here we describe the development of the directed cloning procedure and its application, and we briefly discuss its advantages and limitations.

MATERIALS AND METHODS

Bacterial strains, plasmids, transposons, and genetic manipulations. The *S. meliloti* strains, transposons, growth media, antibiotic concentrations, and general methods for conjugation and transduction used in this work were as previously described (17, 21). *S. meliloti* and *Agrobacterium tumefaciens* GMI9023 (48) cells were incubated at 30°C, while *Escherichia coli* cells were incubated at 30 or 37°C. The integration plasmids were mobilized via the *oriT* from *E. coli* into *S. meliloti* by supplying the RK2 transfer genes in *trans* on the mobilizing plasmid pRK2013 (20) or pRK600 (22).

The *S. meliloti* *oriT*-flanked regions were captured in *E. coli* JW192 (DH5 α with *trfA278D* integrated in the chromosome, Ap^r) (59) following overnight triparental matings (with donor, helper, and recipient bacterial strains) made up of the *S. meliloti* double-integrand donor strain, *E. coli* DH5 α carrying the mobilizing plasmid pRK2013 (Km^r), and the *E. coli* DH5 α (*trfA*) recipient. Transconjugants were selected at 37°C on Luria-Bertani agar containing ampicillin (100 μ g/ml) and spectinomycin (20 μ g/ml) or ampicillin and chloramphenicol (10 μ g/ml). Following purification, transconjugants were screened for loss of pRK2013-encoded Km^r prior to the preparation of plasmid DNA.

DNA methodology. Plasmid DNA was purified with the alkaline lysis method as described by Birnboim and Doly (10). Plasmid DNA from 60- to 240-kb BAC

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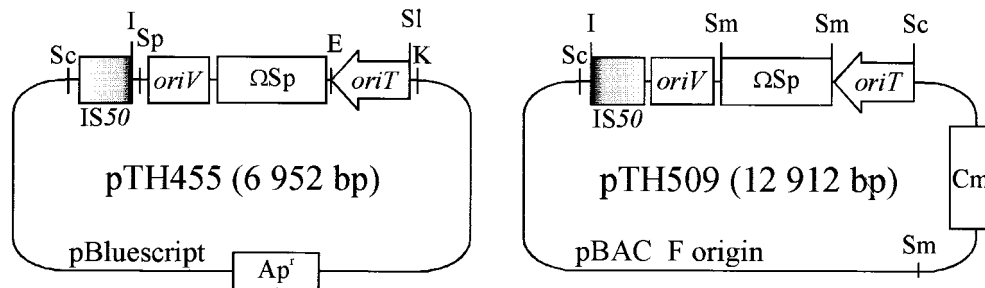


FIG. 1. Illustration of integration plasmids pTH455 and pTH509. The IS50-oriV-ΩSp-oriT integration cassettes in the two plasmid backbones (pBluescript and pBACe3.6) differ in the orientation of the IS50 fragment. The IS50 is shaded white (outside end) to black (inside end). All indicated restriction sites are unique except for the SmaI and SacI sites indicated in pTH509. E, EcoRI; I, I-SceI; K, KpnI; Sc, SacI; SI, SalI; Sm, SmaI; Sp, SpeI.

clones was purified from 2-liter cultures by alkaline lysis followed by CsCl density gradient centrifugation, yielding 50 to 250 μg, depending on the plasmid. DNA manipulations and PCRs were done according to manufacturer recommendations. Following preparation and digestion-modification, DNA samples were electrophoresed through 0.8 to 2% agarose gels in TAE (18) buffer.

Construction of integration plasmids. The various DNA fragments used in the construction of pTH455 (Fig. 1) were obtained as follows: a 760-bp EcoRI/SalI fragment containing the oriT site of RK2 was isolated from pTJS82 (50); the oriV_(RK2) was a 900-bp BamHI fragment from plasmid pMS107-GENO14 (59); a 334-bp IS50 PCR product, which included bp 20 to 314 starting from the outside end of the IS50, as well as sites for I-SceI (underlined), SpeI, and XbaI (italicized) restriction endonucleases, was synthesized by PCR using synthetic primers AB10170 (IS50 nucleotides 20 to 42; 5'-GCTCTAGAAGCGTCCTGACGGAACCTTTCC-3') and AB10171 (IS50 nucleotides 292 to 314; 5'-GGACTAGTTACGCTAGGGATAACAGGGTAATTGATCGCCTCGGCAGAAACGTTG-3') (the orientation of the PCR fragment was confirmed by DNA sequencing); and the ΩSp cassette was a 2.0-kb XmaI fragment from pHP45Ω

(44). First an oriV-ΩSp cassette (2.9 kb) was made and cloned as an EcoRI/SpeI fragment next to the oriT fragment in pBluescriptII (1) to give pTH444. The resulting oriV-ΩSp-oriT cassette was then excised as a SalI/SpeI fragment and joined to both orientations of the PCR-amplified IS50 fragment, producing the plasmids pTH455 (Fig. 1) and pTH456. These differ from one another only in the directionality of the PCR fragment.

Plasmids pTH509 (single cassette) (Fig. 1) and pTH504 (duplicated cassette) (Fig. 2) were made by cloning the IS50-oriV-ΩSp-oriT cassette from pTH455 as a 4-kb SacI fragment into SacI-digested pBACe3.6 (25).

Determination of cointegrate orientation. The 296 bp of IS50 directed the integration plasmids via single-crossover homologous recombination to one of the two IS50 elements (either the left or right IS50 element) of the target Tn5 derivatives. To distinguish the two types of cointegrates, we employed Southern blotting and PCR (Fig. 3) procedures. In the case of Southern blotting, genomic DNA from pTH509 cointegrates was digested with SmaI and probed with the mini-F plasmid pMF21 (34). Different-sized border fragments are observed for the two cointegrates. For the PCR procedure, primers specific to the integration

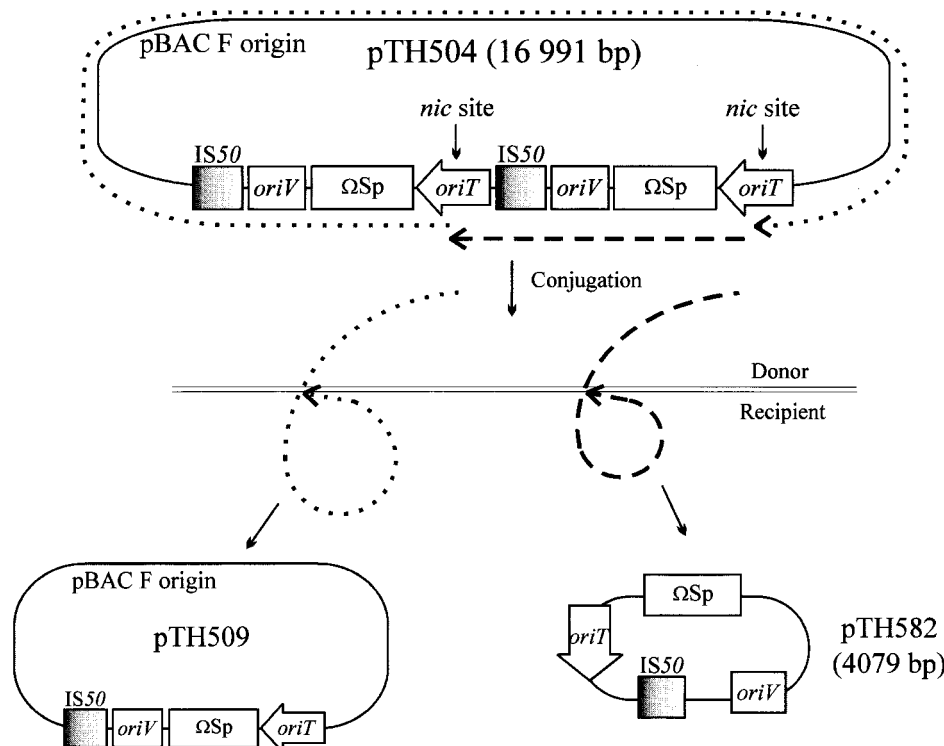


FIG. 2. Schematic representation of predicted oriT-directed transfer events during conjugation from *E. coli*(pTH504) to either *E. coli* (*trfA*) or *S. meliloti*. Transfer is initiated by nicking at the oriT *nic* site and occurs 5' to 3'. The 5' end is thought to remain covalently attached at the cell membrane and is then ligated to the 3' end of an identical *nic* site (31, 41, 51). Two such nicking-ligation events can occur in pTH504, resulting in the transfer of two distinct regions (the two types of dashed lines) originating from either oriT site. Rescue of the IS50-oriV-ΩSp-oriT cassette plasmid (pTH582) via IS50-directed recombination in *S. meliloti* at Ω5069::Tn5-132 is also shown.

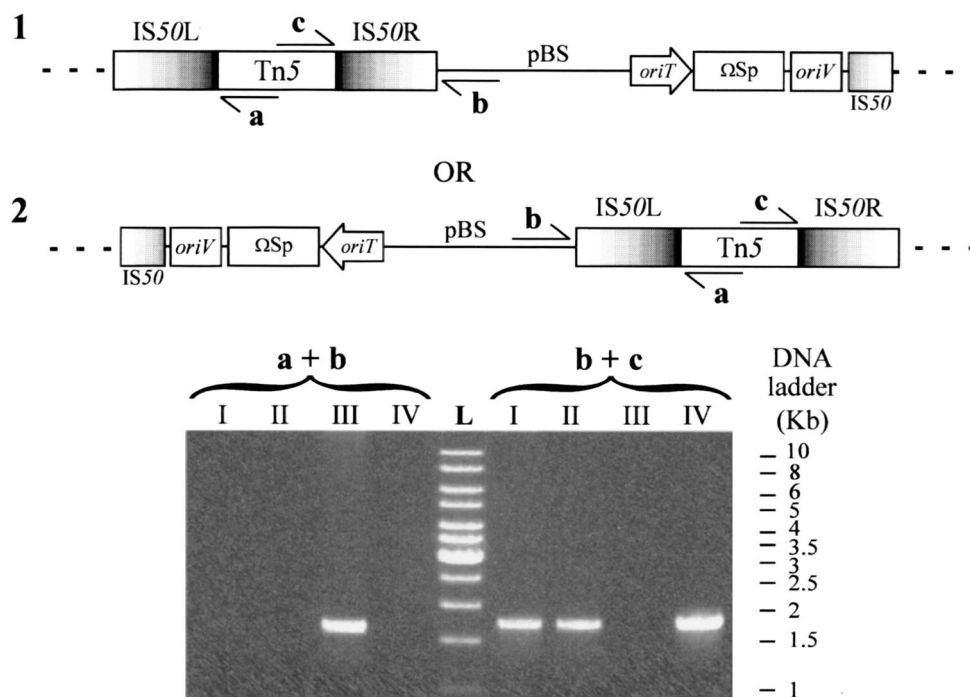


FIG. 3. Schematic representation of the two possible cointegrate structures (structures 1 and 2) between the integration plasmid pTH455 and a Tn5 transposon, and an agarose gel demonstrating both integration events as determined by PCR. Homologous recombination of pTH455 can occur at either of the two IS50 sequences, IS50R (structure 1) or IS50L (structure 2). The PCR primers (a, b, and c) are indicated by half arrows. The dashed line represents pExo DNA. Genomic DNA preparations of four integrants (I to IV) were used for PCR with primer sets a-b and b-c. Sample III carries pTH455 in IS50L, whereas the other three samples carry pTH455 in IS50R. Determination of pTH509 integration into both IS50 elements of Tn5-132 transposons is accomplished in a similar fashion with different primer sets. L, DNA ladder; pBS, pBluescript.

vector and to the Tn5 insertion derivative were used. For pTH455, the integration vector primer (5'-ATGTGCTGCAAGGCGATTAAGTTGGGTAAC-3') combined with either of two primers (5'-TGTTGTGCCAGTCATAGCCGA ATAGCC-3' or 5'-GCGTGTCTGGGAGATTGGACGACAGC-3') specific for opposite strands of the central region of Tn5 were used (Fig. 3). For pTH509, the integration vector primer (5'-TTCTCGAACCTCCCGCCGCTAACG-3') was used with either of two primers (5'-TTTCTAAGGACAGCAACCAT TTGTTAAATCAG-3' or 5'-TTCAGTGATCCATTGCTGTGACAAAGGG AATC-3') specific for opposite strands of the central region of Tn5-132. These primer pairs generated unique products depending on whether integration occurred at the right or left IS50 (Fig. 3).

Generation of plasmid pTH515. The *oriT* cassette plasmid, pTH582, was rescued in *S. meliloti* via IS50-directed cointegrate formation following the transfer of pTH504 into *S. meliloti* carrying Ω 5069::Tn5-132 (Fig. 2, lower half). *Sp*^r Cm^r transconjugants were recovered, and PCR analysis of these strains showed that they carried pTH582 integrated at either of the IS50 elements of the Tn5-132. Double-cointegrate strains carrying the four combinations of Ω 5069::Tn5-132::pTH582 and Ω 5056::Tn5::pTH455 were then constructed by transduction (strains RmK188 to RmK191). The 60-kb region flanked by Ω 5056 and Ω 5069 was rescued from two of these double-cointegrate strains (RmK189 and RmK190) by selecting for *Sp*^r transfer into *E. coli* DH5 α (*trfA*). Transconjugants from the RmK189 donor were Km^r and Tc^r, while RmK192 generated Km^r and Tc^r transconjugants. These results are consistent with *oriT*-directed recombination from the outer (RmK189) and inner (RmK190) IS50 elements, respectively, of the two transposon insertions. The predicted structure of the Km^r Tc^r RmK190 transconjugant plasmids is such that they should contain only the 60-kb Ω 5056- and Ω 5069-flanked region and the *oriV*-FRT cassette from the integration plasmids; one such plasmid was retained and designated pTH515.

Sequence analysis. The ClustalW program (55) was used to align nucleotide and amino acid sequences. All GenBank searches to compare nucleotide sequences against those in databases at the National Center for Biotechnology Information were accomplished using BLAST 2.0 programs (2).

RESULTS

Cloning strategy. We have developed a procedure to clone DNA fragments whose boundaries are specifically defined by two *oriT* sites in parallel orientation. In the case of the work

described below, we have inserted *oriT* sites at specific Tn5 and Tn5-132 insertions whose map locations were previously determined. These Tn5 derivative insertions contain a central antibiotic resistance gene region flanked by inverted 1.5-kb IS50 insertion elements. Tn5 encodes neomycin and kanamycin resistance (Nm^r Km^r), while Tn5-132 encodes tetracycline and oxytetracycline resistance (Tc^r Ot^r), and strains carrying two such differentially marked insertions are readily constructed by transduction (21). The *oriT*-flanked target regions are captured via *oriT*-directed recombination concomitant with their transfer to *E. coli*. Replication of the resulting closed circular DNA molecule in *E. coli* is directed from an origin of replication which was inserted along with *oriT*.

Integration vectors. To carry out the in vivo cloning procedure, we constructed integration cassettes (IS50-*oriV*- Ω Sp-*oriT*) containing (i) 296 bp of the IS50 elements of Tn5, (ii) the origin of replication (*oriV*) from plasmid RK2, (iii) a gene encoding spectinomycin resistance (in the Ω Sp) for selection in *S. meliloti*, and (iv) the origin of transfer (*oriT*) from plasmid RK2. The IS50 fragment directed the integration plasmid via single-crossover homologous recombination to one of the two IS50 elements of the target Tn5 derivatives. To obtain strains carrying two parallel IS50-*oriV*- Ω Sp-*oriT* cassettes at the inner IS50 elements flanking the region of interest, it was necessary to make two integration cassettes which differ from each other only in the orientation of the IS50 PCR fragment. One cassette (pTH455) was present in the pBluescriptII vector, while the other (pTH509) was in the chloramphenicol-resistant (Cm^r) pBACe3.6 vector, which replicates from the F plasmid origin of replication and allows recovery of large DNA fragments in *E. coli* (Fig. 1).

Demonstration of *oriT*-directed site-specific recombination.

To demonstrate that the *oriT* employed in our experiments could act as a site for specific recombination during conjugal transfer, we constructed another plasmid, pTH504, carrying a duplication of the IS50-*oriV*- Ω Sp-*oriT* cassette of pTH509 (Fig. 2). Conjugal transfer and joining at the *oriT* sites of pTH504 should generate two plasmids (as outlined in Fig. 2): one identical in structure to pTH509 (Fig. 1) and the other composed of only the IS50-*oriV*- Ω Sp-*oriT* cassette (pTH582 in Fig. 2). Unlike the Cm^r Sp^r plasmid pTH509, pTH582 encodes only Sp^r and requires TrfA for replication (at the *oriV* of RK2) in *E. coli*.

Plasmid pTH504 was conjugated from the recombination-deficient (*recA*) *E. coli* strain S17-1, which carries an RK2 derivative integrated into the chromosome and efficiently mobilizes plasmids carrying the RK2 *oriT* (52), into the recipient *E. coli* DH5 α (*recA* *trfA*⁺), which produces sufficient TrfA protein to efficiently initiate replication of plasmids carrying the RK2 *oriV* (59). Examination of the plasmid DNA from Sp^r transconjugants revealed that 90% had two plasmids, one of which was the same size as pTH509 while the other was identical to the IS50-*oriV*- Ω Sp-*oriT* circularized cassette plasmid (pTH582). Seven percent of the transconjugants carried pTH582 alone, 3% carried pTH509 alone, and none of the transconjugants examined contained a plasmid of the same size as pTH504 (data not shown). As a control, when plasmid DNA prepared from the S17-1 pTH504 donor strain was used to transform Sp^r into the DH5 α *trfA* recipient, all of the transformants carried plasmids with the same structure as the pTH504 donor plasmid (24 transformants were examined). These results are consistent with *oriT* acting as a site for specific recombination during conjugation.

***oriT*-directed recombination in *S. meliloti*.** To test for *oriT*-directed recombination in *S. meliloti* and establish that the *oriT*-flanked region can be cloned by conjugation, it was necessary to first introduce *oriT* at two different positions in pExo. In initial experiments, one *oriT* was presented in the form of Ω 5111::Tn5-*oriT*, while the second was obtained via pTH504 (described above) integration at Ω 5142::Tn5-132. Tn5-*oriT* is a transposon in which a 760-bp *oriT*-containing fragment from RK2 was cloned into the central *Bam*HI restriction site of Tn5 (62). We had previously constructed strains carrying the Tn5-*oriT* transposon in both possible orientations at the insertion site Ω 5111 (17). These two insertions were transduced (selecting for Nm^r) into each of two strains in which pTH509 was integrated at the IS50L and IS50R of the insertion Ω 5142::Tn5-132, located 140 kb clockwise from Ω 5111 on the pExo genetic map (Fig. 4). A schematic representation of the pExo region in the resulting four double-integrant strains (carrying two integration vectors) (RmK255 to RmK258) is shown in Fig. 5. Of the four strains, only RmK257 carries the necessary combination of two parallel *oriT* sites flanking the BAC F origin of replication. Therefore in this strain, *oriT*-directed recombination upon conjugal transfer should generate a large, 150-kb plasmid carrying the F origin and the Cm^r gene from the pBAC backbone of pTH509. The results obtained from triparental matings into *E. coli* showed that 10 out of 10 Cm^r and Sp^r transconjugant plasmids from the RmK257 donor were very large (ca. 150 kb) and identical as judged from their common restriction fragment patterns. Alternatively, all of the Sp^r plasmids examined from the three other matings were identical to the pTH509 targeting vector (resulting from pTH509 cointegrate resolution). As the two *oriT* sites in strain RmK256 are in direct orientation, it will transfer a plasmid similar in size to that obtained from RmK257; however, the resulting plasmid would be Nm^r Ot^r Sp^r Cm^s and would not

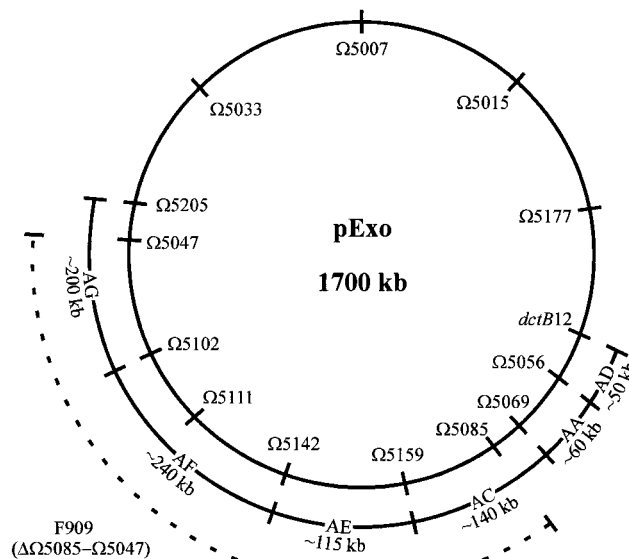


FIG. 4. Circular map of the pExo megaplasmid illustrating the relative locations of representative Tn5 derivative transposon insertions (Ω). The dashed line represents the region deleted in strain RmF909. The six regions (AA, AC, AD, AE, AF, and AG) which were flanked by the IS50-*oriV*- Ω Sp-*oriT* cassettes and subsequently transferred to *E. coli* are indicated. The approximate sizes of the flanked regions are shown.

have the BAC *oriV* (Fig. 5). In the above-described experiments, *E. coli* Sp^r transconjugants were obtained at a frequency of 10^{-6} per donor.

A second experiment was conducted employing strains carrying Ω 5079::Tn5-*oriT* together with pTH509 integrated at either Ω 5069::Tn5-132 or *dctB12*::Tn5-132. The respective intervening regions were rescued in *E. coli* only when the *oriT* sites were in parallel and the transferred region carried the F origin. Collectively these data demonstrate that *oriT*-directed site-specific recombination occurs and that pExo DNA between parallel *oriT* sites can be efficiently captured in *E. coli*.

Directed in vivo cloning of large contiguous DNA fragments.

Tn5 derivatives at insertions *dctB12*, Ω 5056, Ω 5069, Ω 5159, Ω 5142, Ω 5102, and Ω 5205 were targeted with pTH455 and pTH509 integration plasmids (see Fig. 4 for insertion locations). After the orientations of the cointegrates were determined, the desired double-integrant strains were constructed by transduction of Nm^r from the Tn5::cointegrate into the Tn5-132 (Ot^r Nm^s) cointegrate recipient strains. The megaplasmid regions were captured by selecting for Sp^r or Cm^r transconjugants from triparental matings consisting of the *S. meliloti* double-integrant donor strain, *E. coli* DH5 α carrying the mobilizing plasmid pRK2013, and the Ap^r *E. coli* DH5 α (*trfA*) as a recipient. These experiments were complicated by the fact that resolution of the pTH455 and pTH509 cointegrates via recombination at the IS50 elements also yielded Sp^r or Cm^r transconjugants. However, the latter were readily distinguished by their small size relative to plasmids carrying the megaplasmid regions.

Employing this procedure, six contiguous pExo megaplasmid regions of 50, 60, 140, 115, 240, and 200 kb were rescued as plasmids in *E. coli* (regions AD, AA, AC, AE, AF, and AG in Fig. 4). Independent transconjugant cultures from the same donor regions generated identical plasmid restriction pattern profiles (data not shown), indicating that plasmid rearrangements

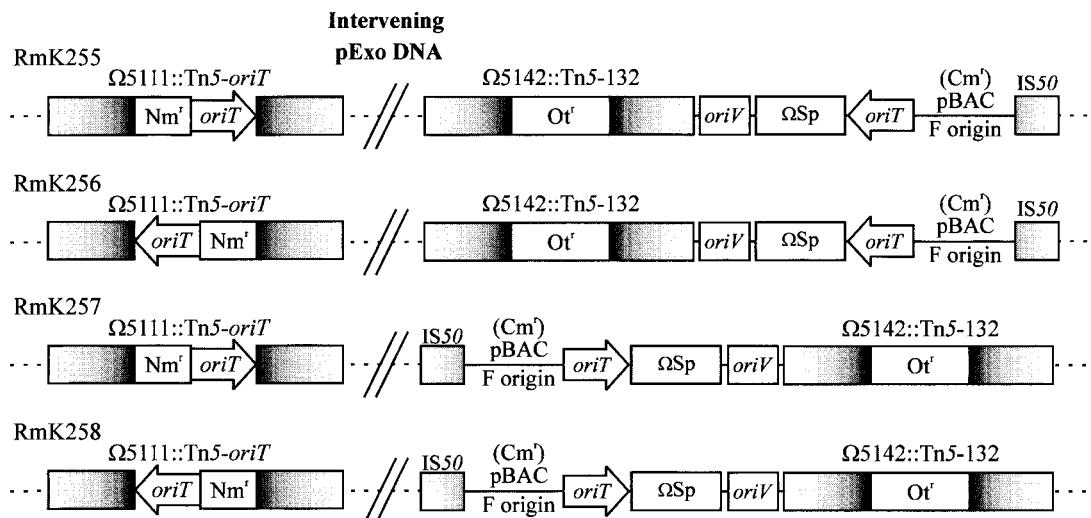


FIG. 5. Schematic representation of the four possible combinations of $\Omega 5111::Tn5-oriT$ with $\Omega 5142::Tn5-132::pTH509$ (RmK255 to RmK258). RmK255 and RmK258 carry indirect $oriT$ sites. Although RmK256 and RmK257 both carry direct (parallel) $oriT$ sites, only in RmK257 do the $oriT$ sites flank the chloramphenicol-resistant BAC vector (with the F origin of replication). A more detailed map of pTH509 is shown in Fig. 1. The dashed line represents pExo DNA. Cm^r , Nm^r , Ot^r , and Sp refer to chloramphenicol, neomycin, oxytetracycline, and spectinomycin resistance determinants, respectively.

were rare. In addition, the total sizes of the restriction fragments were consistent with those predicted for the targeted regions. When Southern blots of restricted total wild-type DNA were probed with the cloned megaplasmid regions, the pattern of the hybridizing fragments corresponded to those present in the cloned DNA (Fig. 6). In contrast, similar blots with DNA from the pExo deletion derivative RmF909

($\Delta\Omega 5085-5047$) showed few weakly hybridizing bands, presumably arising from some reiterated sequences. These data suggest that the cloned DNA was from the predicted pExo regions and that the DNA was not rearranged during the cloning process.

An origin of replication from the pExo megaplasmid. We have previously demonstrated that the *S. meliloti* pExo megaplasmid can replicate in *A. tumefaciens* (22). In the course of our experiments we rescued region AA (Fig. 4) as an Sp^r plasmid designated pTH515. This plasmid consists of the 60-kb $\Omega 5056$ - and $\Omega 5069$ flanked region, together with the $IS50-oriV-\Omega Sp-oriT-IS50$ cassette fragment (see Materials and Methods). Plasmid pTH515 DNA generated Sp^r transformant colonies only in *E. coli* strains expressing the RK2 plasmid replication initiation protein TrfA (data not shown). This was expected, since replication of pTH515 should occur from the RK2 $oriV$ present in the $ori-\Omega Sp-oriT$ cassette fragment. Plasmid pTH515 was readily transferred (frequency of $>10^{-2}$) from *E. coli* (*trfA*) into *A. tumefaciens* in triparental matings in which pTH515 was mobilized with *E. coli* carrying the plasmid pRK600 (22), which cannot replicate in *A. tumefaciens*. Plasmid DNA isolated from the resulting transconjugants was the same size and had the same restriction patterns as the pTH515(AA) donor plasmid DNA (data not shown), suggesting that this DNA was replicating autonomously in the *A. tumefaciens* cytoplasm.

We have determined the nucleotide sequence of region AA in pTH515 as part of a project to determine the complete nucleotide sequence of the pExo megaplasmid (data not shown). Analysis of the AA sequence revealed the presence of three contiguous genes whose products are homologous to known and predicted plasmid replication proteins RepA, RepB, and RepC from the related microorganisms *Agrobacterium rhizogenes* (40), *Rhizobium* sp. strain NGR234 (24), *A. tumefaciens* (53, 54), *Rhizobium etli* (45), and *Rhizobium leguminosarum* (56). Moreover, an alignment of the *repB-repC* intergenic regions from the *Agrobacterium* and *Rhizobium* plasmids revealed high levels of similarity between these sequences (Fig. 7). It has been suggested that this highly conserved inter-

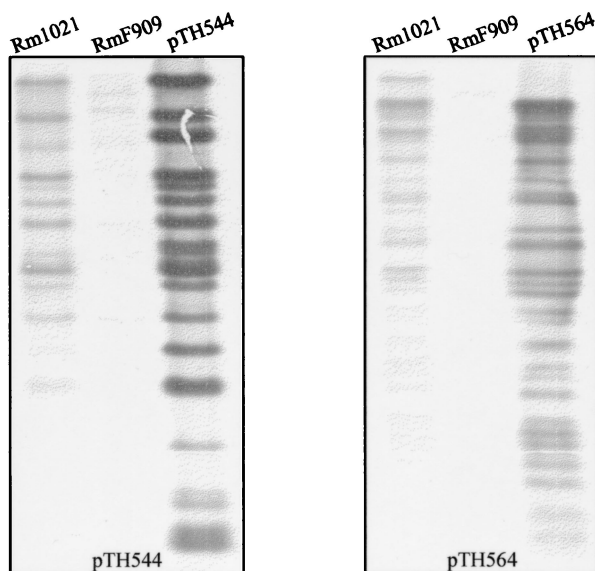


FIG. 6. Southern blots demonstrate that the recovered plasmids in *E. coli* harbor *S. meliloti* DNA. Genomic DNA was prepared from wild-type strain Rm1021 and from deletion derivative RmF909. *Bam*HI-restricted genomic DNA was hybridized with labeled pTH544 and pTH564 DNAs as indicated. The Rm1021 hybridization pattern closely resembles the probe pattern, showing that the plasmids carry pExo DNA. Moreover, DNA from RmF909, which lacks pExo DNA between $\Omega 5085$ and $\Omega 5047$ (Fig. 4), did not hybridize with either probe. BAC plasmid pTH544 carries DNA between $\Omega 5159$ and $\Omega 5142$ (Cm^r Sp^r Nm^r), and BAC plasmid pTH564 carries DNA between $\Omega 5142$ and $\Omega 5102$ (Cm^r Sp^r Nm^r).

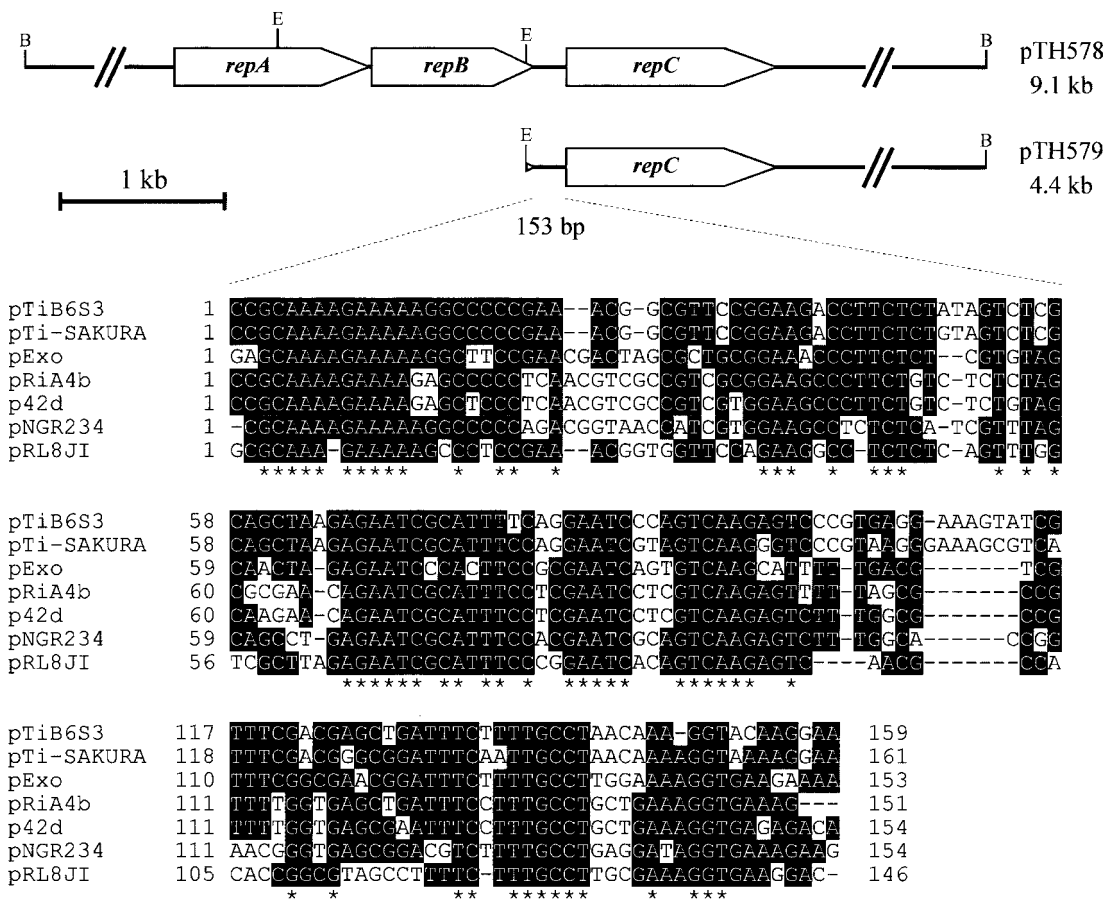


FIG. 7. Subclones of the replicator region and a multiple alignment of the *repB*-*repC* intergenic region nucleotide sequences from large plasmids. Two subclones from the AA region, a 9.1-kb *Bam*HI fragment (with the *repABC* genes) and a 4.4-kb *Bam*HI/*Eco*RI fragment (with only the *repC* gene and *repBC* intergenic region), were transferred into *A. tumefaciens* (see text). An alignment of the *repBC* intergenic regions from several *Agrobacterium* and *Rhizobium* plasmid replicator regions is shown: the Ti plasmid (pTiB6S3) of *A. tumefaciens* B6S3 (51), the Ti plasmid (pTi-SAKURA) of *A. tumefaciens* MAFF301001 (50), the pExo megaplasmid of *S. meliloti* SU47 (this study), the Ri plasmid (pRiA4b) of *A. rhizogenes* (37), the symbiotic plasmid (p42d) of *R. etli* CFN42 (42), the symbiotic plasmid (pNGR234) of *S. meliloti* NGR234 (21), and the cryptic plasmid (pRL8JI) of *R. leguminosarum* (53). Hyphens indicate gaps introduced to give the best sequence alignments. Conserved nucleotide sequences are shaded; nucleotides identical for all seven sequences are indicated with asterisks.

genic sequence may constitute an incompatibility determinant or may harbor the origin of replication (7, 45, 54).

To delineate regions required for replication of the AA region in *Agrobacterium*, we employed a Gm^r pUC19 plasmid derivative, pUC30T, which cannot replicate in this organism (50a). Plasmid pUC30T subclones which carried the *repA*, *repB*, and *repC* genes or *repC* and part of *repB* were found to replicate in *A. tumefaciens* (Fig. 7). A comparison of the stability of the latter subclone versus that of a subclone carrying all three *rep* genes revealed that following growth for seven generations, 90% of the cells retained the plasmid carrying all three *rep* genes. Only 20% of the cells retained the partial-*repB*-*repC* plasmid, suggesting that the *repA* and/or *repB* gene products contribute to plasmid stability.

DISCUSSION

We report a procedure whereby large defined fragments from the *S. meliloti* genome have been cloned via intramolecular site-specific recombination, directed by the origin of transfer (*oriT*) of the IncP group plasmid RK2. In principle, the capture of *oriT*-flanked regions can be applied to any bacteria in which conjugal transfer systems are established. Conjugal plasmid DNA transfer is initiated through the generation of a

single-strand nick at the plasmid *oriT*, and in this process a protein (TraI in the case of RK2) is bound via a phosphodiester linkage to the 5' end nucleotide. DNA transfer from the nick occurs in a 5'-to-3' direction, and plasmid transfer terminates with joining of the 5' and 3' ends, thus generating a closed circle. The precise biochemical mechanism for the termination of transfer has yet to be resolved; however, genetic studies have demonstrated that *oriT* acts as a sequence-specific recombination site during the conjugative transfer process (7, 19, 31, 41, 43, 51, 57, 60).

The transfer of *oriT*-flanked regions as reported in this study has clear analogies to the transfer of T-DNA from *A. tumefaciens* into plant cells (31, 49, 63). The transferred T-DNA is flanked by 25-bp direct repeats, called the right border (RB) and left border (LB), which are similar in sequence to *oriT* nick region sequences from IncP group plasmids. As in the case of *oriT*, the RB functions to transfer the T-DNA unidirectionally (39). Upon T-DNA transfer, the bottom DNA strand at the RB and LB is cleaved by a site-specific endonuclease complex. The resulting single strand (with the VirD2 protein attached at the 5' end) is transferred into plant cells, where it integrates into the genome as a linear fragment. In the case of *oriT*-flanked regions, we envisage that the 5' end resulting from the

nick at one *oriT* is joined to the 3' end from the nick site at the other *oriT* and that the resulting closed circle replicates in *E. coli* via the introduced origin of replication. Another instance involving *oriT*-directed recombination arose from classic studies in which F-prime plasmids, made up of the F plasmid carrying chromosomal DNA, were employed in genetic analyses in *E. coli*. Some of these F-prime plasmids were found to be transfer defective, and subsequent analysis of the DNA sequences from the chromosome-F plasmid boundary regions of these plasmids indicated they were formed via recombination between the F (Hfr) *oriT* and F *oriT*-like sequences in the chromosome (29).

Our data show that *S. meliloti* regions flanked by RK2 *oriT* sites can be efficiently rescued in *E. coli*. However, the precise mechanism through which the excised region is generated, that is, whether *oriT*, in association with transfer proteins, catalyzes recombination-ligation in the donor strain prior to DNA transfer or in the recipient following transfer, remains unknown. In this respect, it is interesting that conjugation-independent site-specific recombination at the *oriT* of plasmid R1162 has been reported (35), and similar recombination at the *oriT* of plasmid R388 is known to be mediated by the R388 transfer protein TrwC (32 see also reference 15). In principle, the capture of *oriT*-flanked regions can be applied to any bacteria and has potential in the efficient cloning of gene clusters for subsequent use in the generation of mosaic organisms.

The application of the in vivo cloning procedure allowed us to generate six contiguous BAC clones totaling 800 kb of *S. meliloti* pExo DNA without generating any overlapping redundancy (Fig. 4). Nucleotide sequencing of the AA, AC, AE, and AF clones confirmed that the boundaries originating from the same insertion lie within the same gene (data not shown) and carry the predicted 9-bp duplication generated upon Tn5 insertion (9).

In the analysis of the 60-kb AA region, we have located a gene region that appears to constitute the replication origin (*oriV*) of the pExo megaplasmid. Within this region are three genes (*repA*, *repB*, and *repC*) whose products are homologous to other proteins involved in plasmid replication. The RepA and RepB proteins show significant sequence similarity to proteins, such as SopA and SopB of the F plasmid, which function in partitioning of various plasmids and bacterial chromosomes. On the other hand, with a single exception, all of the pExo RepC protein homologs were from plasmids present in members of the *Rhizobiaceae* (over 40% identity to *Rhizobium* sp. strain NGR234, *A. rhizogenes*, *A. tumefaciens*, *R. leguminosarum*, and *R. etli*). The exception was RepC from plasmid pTAV320, which is present in the α -proteobacterium *Paracoccus versutus* (previously *Thiobacillus versutus*). Thus, these RepC proteins likely represent a distinct class of plasmid replication proteins. Our results on the relative stabilities of clones carrying the pExo RepABC versus RepC alone in *A. tumefaciens* lend support to the inferred roles of these proteins in plasmid replication (RepC) and segregation (RepA and RepB) (8, 37, 45, 54, 56, 61).

Margolin and Long (35) identified a pExo autonomously replicating sequence (ARS) of 800 bp which required *trans*-acting factors from an unidentified region of pExo for its replication. As *S. meliloti* derivatives which lack the ARS region retain pExo, the authors suggested that pExo contains multiple origins. The ARS is located over 700 kb from the *repABC* region, and hence these two regions are clearly distinct. It will be of interest to determine whether the *repABC* genes are sufficient to support replication of the ARS, and in this respect we note that comparisons of the ARS and *repABC* region have failed to reveal any shared sequence motifs.

In an earlier study we were unable to obtain *S. meliloti* strains in which the (*repABC*)-*oriV*-containing region had been deleted (17). The simplest explanation is that this region carries the major replication genes and associated origin of replication. In the absence of these, the remainder of pExo (ca. 1,600 kb) would be lost from the cell, directly resulting in loss of cell viability. Alternatively, the 60-kb region carrying *repABC-oriV* may harbor other genes that are required for cell viability or a regulatory gene that controls a toxic gene similar to *kil-kor* systems. Further study is required to resolve this issue.

With the cloning approach described here, we have obtained clones of 140, 200, and 240 kb in size, considerably larger than previously reported BAC clones containing prokaryotic DNA. This may reflect limitations in the in vitro cloning methodologies used, although clones carrying inserts of up to 700 kb have been obtained for eukaryotic DNA. In the case of a recent BAC library prepared from the total genome of the bacterium *Mycobacterium tuberculosis* (12), the authors reported particular difficulty in obtaining BAC clones with inserts of greater than 100 kb. Moreover, in the case of a recently reported BAC library of the *S. meliloti* chromosome, the average insert size was 80 kb (14). Brosch et al. (12) suggested that the limit on insert size could be due to plasmid instability resulting from the lethal overexpression of certain *M. tuberculosis* genes in *E. coli*. In our study, we have observed that the *E. coli* colony size varied depending on the particular region of the *S. meliloti* genome present in the BAC clone; however, we have not yet identified a region that was recalcitrant to cloning in *E. coli*. This may reflect poor transcriptional activity of *S. meliloti* promoters in *E. coli*.

Our novel in vivo procedure has several advantages over conventional BAC or cosmid cloning approaches: (i) through the insertion of *oriT* at defined sites, the cloning can be directed to specific regions; (ii) very large fragment sizes can readily be obtained in a single plasmid; and (iii) in the context of genome sequencing, the fact that one can generate contiguous, nonoverlapping clones eliminates redundant sequencing of overlapping regions and precludes underrepresentation or gaps within a BAC genomic library. The possible disadvantage is the requirement of a genetic or physical map of the regions being cloned to target the integration of *oriT* sites to the genome. In addition, once *oriT* is inserted at defined locations, it is necessary to construct strains carrying pairs of *oriT* insertions in parallel orientation. In our case, the latter was readily accomplished by transduction.

We are currently determining the nucleotide sequences of the pExo regions described in this report (<http://life.biology.mcmaster.ca/brian/Rhizobium/pEXO.html>). This project is part of an international project to determine the nucleotide sequence of the complete *S. meliloti* genome (<http://sequence.toulouse.inra.fr/meliloti.html>). The sequence of the pSym megaplasmid is currently being determined from an enriched random shotgun library of pSym DNA purified by pulsed-field agarose gel electrophoresis (S. Long and colleagues, <http://cmgm.stanford.edu/~mbarnett/genome.htm>), whereas the sequence of the 3,500-kb chromosome is being determined from a recently reported minimal 49-BAC clone library (14).

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