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Vector Insertion Mutagenesis of *Rhizobium* sp. Strain ORS571: Direct Cloning of Mutagenized DNA Sequences

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When the limited-host-range plasmid pVP2021 carrying Tn*5* was mobilized into *Rhizobium* sp. strain ORS571 and stable acquisition of Tn*5* was selected, ORS571 plasmid-genome cointegrates were exclusively obtained; direct Tn*5* transposition was never observed. In every case, genomic cointegrates exhibited an additional (third) IS*50* element that bordered VP2021 DNA sequences but maintained a single Tn*5* element. Genomic cointegrates containing IS*50* triplications were stable; neither phenotypic reversion nor resolution was detectable. Auxotrophic mutant strains (vector insertion mutants) were identified at expected frequencies among derivatives carrying ostensibly random genomic pVP2021 insertions; N_2 fixation (Nif)-defective vector insertion mutants were observed among these derivatives at a frequency of 10^{-3}. The presence of integrated pVP2021 in ORS571 *nif*::VP2021 mutant genomes enabled VP2021 to constitute an endogenous cloning vector. After *Eco*RI or *Kpn*I digestions, genomic *nif*::pVP2021 DNA sequences contiguous with integrated pVP2021 were directly cloned as new replicons without addition of an exogenous vector. Recombinant plasmids derived from two such *nif*::pVP2021 mutants hybridized to previously analyzed ORS571 Nif DNA sequences. Recombinant plasmid DNA and ORS571 Nif region DNA were found to be colinear; pVP2021 insertions could be accurately mapped. pVP2021 insertion-mutagenesis thus allows the direct cloning of ORS571 gene sequences for which mutant phenotypes can be selected or screened.

Both random and site-directed transposon Tn*5* mutagenesis have been used extensively in *Escherichia coli* as well as in a variety of other bacteria (2). Tn*5* transpositions yield operon-polar insertions that confer resistance to kanamycin/neomycin in enteric bacteria as well as resistance to streptomycin in many nonenteric bacteria (18). Tn*5* has been used with varying degrees of success as a random mutagen for bacterial genomes other than *Enterobacteriaceae*; both the frequency and the site specificity of Tn*5* transposition fluctuate considerably from strain to strain (2, 25).

We constructed Tn*5*-containing derivatives of the mobilizable, limited-host-range plasmid pSUP202 (24) for use as putative plasmid suicide (4, 11), Tn*5* mutagenesis vectors in *Rhizobium* sp. strain ORS571. After DNA transfer of pVP2021 to ORS571 during heterogeneric bacterial conjugations, stable Tn*5*-containing ORS571 strains were obtained by selecting for kanamycin (Km) and streptomycin (Sm) resistances. As we shall document, all Km^r Sm^r ORS571 strains obtained were found to contain IS*50*R-mediated complete plasmid-genome cointegrates and not direct Tn*5* transpositions. To assess the site specificity of cointegrate formation, such strains were then screened for auxotrophic and other mutant phenotypes; these analyses suggested that such genomic insertions were random.

In putative vector insertion (Vi) mutant strains, we decided to exploit the presence of VP2021 DNA for use as an endogenous cloning vector to (i) confirm the hypothesized cointegrate structures and (ii) obtain recombinant DNA clones of contiguous ORS571 genomic DNA sequences. Analyses of recombinant DNA plasmids obtained from such Vi mutant strains have allowed us to conclude that pVP2021 cointegrates result exclusively from IS*50*R-mediated transposition to the ORS571 genome, that, to the limits of detection, this insertion process is random, that such cointegrates formed are stable, and that genomic DNA sequences contiguous with insertion points obtained as recombinant plasmids are colinear with those in the ORS571 wild-type genome library.

MATERIALS AND METHODS

Bacterial strains and plasmids. Plasmid pSUP202 (24), a pBR325 derivative, contains the IncP1-specific, replicative origin of DNA transfer (*oriT* or *mob*). pSUP202 derivatives carrying random Tn*5* insertions were recovered from *E. coli* strain ECG1 carrying a single genomic Tn*5* insertion (7) (Table 1) that had been transformed with the plasmid. Approximately 10^8 ECG1(pSUP202) cells were spread on LB plates to which had been added a centered drop of kanamycin solution (25 mg ml^{-1}); plates were incubated at 37°C. Colonies growing closest to the center, and thus expressing highest-level Km^r, were recovered en masse. Plasmids were isolated by the "mini-boil" (13) technique, used to transform *E. coli* strain HB101, and high-level Km^r (0.25 mg ml^{-1}) transformants were again selected. The positions of Tn*5* insertions in resulting pVP plasmids were determined by restriction endonuclease mapping. *E. coli* strain SM10 (Table 1), which is DNA transfer proficient (Tra$^+$) for IncP1 plasmids (24), was used to mobilize plasmid pVP2021 or pVP2023 to *Rhizobium* sp. strain ORS571 during heterogeneric conjugations. *E. coli* strains MM294 and HB101 (Table 1) were used as hosts for transformations and plasmid maintenance.

Vi mutagenesis of ORS571. *Rhizobium* sp. strain ORS571 was grown in SYPC (8) medium and *E. coli* SM10(pVP2021) was grown in LB, both to late-exponential growth phase. Rhizobia were 10× concentrated and then mixed with an equal volume of the *E. coli* culture. Bacterial mixtures were plated on LB plus 0.4% D-glucose and 0.2% L-glutamine; plates were incubated for 12 to 15 h at 37°C. After mating, bacteria were suspended in 50 mM phosphate buffer, pH 6.3, and replated on rhizobial minimal medium (8) containing streptomycin (200 μg ml^{-1}) and kanamycin (100 μg ml^{-1}) to select ORS571 Tn*5*-containing derivatives. ORS571 Km^r

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TABLE 1. Bacterial strains and plasmids

Strain	Phenotype/genotype[a]	Reference
E. coli		
HB101	Pro Thi Leu, *recA hsdS supE44*	
ECG1	HB101::Tn*5* *malT*, λ^r	7
MM294	Pro Thi Thr Leu, *endA hsdS*	
SM10	MM294 *recA*[::pRP4ΔTn*1*, Tc^s] Tra(IncPl)$^+$ Km^r	24
Rhizobium sp.		
ORS571	Wild type	
57801	ORS571 Rf^r, spontaneous mutant	
Vector insertion strains (::pVP2021), Km^r, Sm^r Tc^r		
60016	Nif^-	
60070	Nif^-	
57651	Asm^-	
plasmids		
pR68.45 Km^s	Tc Tra$^+$ Cma$^+$	20
pSUP202	Ap Cm Tc, pBR325 *mob*	24
pVP2021	Ap Cm Km Tc, pSUP202::Tn*5*	
pVP2022	Ap Cm Km Tc, pVP2021, Tn*5* inverted in situ	
pVP2023	Ap Cm Km *tet*::Tn*5*, pSUP202::Tn*5*	
pVP2024	Ap Cm Km, pVP2021, IS*50*R deleted by *Bam*HI	
pVP2025	Ap Cm Km, pVP2021, IS*50*R deleted by *Sal*I	
pDRN161	Ap Cm Km Tc, *Kpn*I digestion of 60016, no insert	
pDRN162	Ap Tc, *Eco*RI digestion of 60016	
pDRN163	Ap Tc, *Eco*RI digestion of 60016	
pDRN701	Ap Tc, *Eco*RI digetion of 60070	
pDRN702	Ap Cm Km Tc, *Kpn*I digestion of 60070	
pDRA51	Ap Cm Km Tc, *Kpn*I digestion of 57651, no insert	

[a] Abbreviations: Tc, tetracycline; Km, kanamycin; Sm, streptomycin; Ap, ampicillin; Cm, chloramphenicol; Rf, rifampin; Asm, ammonium assimilation; Cma, chromosome-mobilizing ability; Nif, N_2 fixation. For plasmids, resistance phenotypes are given.

Sm^r strains so obtained were screened by replica plating directly onto nutritive selection media. Ammonium assimilation (Asm)-defective mutants were identified by differential growth on minimal medium containing either L-glutamate or ammonium as sole N source (8). N_2 fixation (Nif)-defective mutants were identified by differential growth on minimal medium containing either ammonium or no added N source under 1% O_2. Plates were placed in an air-tight jar and were sparged continuously for 4 days at 30°C with a gas mixture containing 98% N_2, 1% O_2, and 1% CO_2. The N-free variation of minimal medium (8) contained 0.6% agarose (Sigma Chemical Co.) as solidifying agent and, also, 0.3 μg of D-biotin, 1.0 μg of Na_2MoO_4, and 10 μg of $FeCl_3 \cdot 6H_2O$ ml^{-1}. Other auxotrophic strains were identified by conventional auxanography. Antibiotics were used to select *E. coli* strains at the following concentrations (micrograms per milliliter): ampicillin, 100; tetracycline, 5; chloramphenicol, 50; kanamycin, 50.

DNA isolations and hybridizations. ORS571 genomic DNA was obtained by lysozyme-Sarcosyl cell lysis (17) and purified by standard isopycnic CsCl-ethidium bromide centrifugation and ethanol precipitation techniques (5). Before isopycnic centrifugations, lysates were thrice extracted with phenol and once extracted with diethyl ether. *E. coli* plasmid DNAs were prepared and purified by standard procedures (5). DNA hybridizations were conducted for 48 h on nitrocellulose (0.45 μm; Schleicher & Schuell B6) filters in 5× SSPE–0.3% sodium dodecyl sulfate–50% formamide–100 μg of carrier salmon sperm DNA ml^{-1} at 42°C (6). [α-^{32}P]dCTP-labeled, nick-translated probe DNAs were included at 5 μCi per hybridization. Hybridized filters were washed for 3 h at 50°C in 0.3% SSPE–0.1% sodium dodecyl sulfate and subjected to autoradiography.

Recombinant DNA cloning. Genomic DNA samples (1 μg) containing endogenous pVP2021 sequences were digested to completion with either *Eco*RI or *Kpn*I endonuclease (4 U). DNA samples (0.3 μg) were ligated with 0.1 U of T4 DNA ligase (Bethesda Research Laboratories) in 1 ml of ligation buffer (0.1 mg of bovine serum albumin ml^{-1}, 10 mM dithiothreitol, 0.4 mM ATP, 6 mM $MgCl_2$, 70 mM Tris-chloride, pH 7.6). These conditions kinetically favored intramolecular ligation events (9).

RESULTS

Anomalous Tn*5* transposition occurs during *Rhizobium* sp. strain ORS571 random Tn*5* mutagenesis. The limited-host-range plasmid pSUP202 (Table 1) was loaded with Tn*5* (see Materials and Methods) for use as a Tn*5* suicide mutagenesis vector in rhizobia (4, 11). The position of Tn*5* in one such derivative plasmid, pVP2021, was determined by restriction endonuclease mapping (Fig. 1). Although Tn*5* had inserted in the proximal portion of the tetracycline resistance gene, pVP2021 still conferred detectable tetracycline resistance in *E. coli* (data not presented).

After heterogeneric matings between *E. coli* SM10(pVP2021) and *Rhizobium* sp. strain ORS571, Tn*5*-containing ORS571 derivatives were obtained at frequencies of 10^{-6} to 10^{-7} per recipient by selective plating on minimal medium containing kanamycin and streptomycin (see Materials and Methods). When *E. coli* SM10(pSUP202) (containing the parental plasmid) was used as donor in heterogeneric matings, no ORS571 Tc^r transconjugants were found (Table 2). Therefore, it was concluded that the limited-host-range pSUP202 plasmid was not stably maintained as such in ORS571. Nevertheless, all ORS571 Tn*5*-containing derivatives obtained from matings with pVP2021 remained Tc^r when so scored (Table 2). Moreover, the Tc^r phenotype of such ORS571 Tn*5*-containing derivatives failed to detectably segregate even after many generations of nonselective growth. Several such ORS571 Tn*5*-containing strains were grown in liquid culture with rich (SYPC; see Materials and Methods) medium to stationary phase and plated on such medium, and colonies were transferred by replica plating onto SYPC containing kanamycin, streptomycin, or tetracycline. No Tc^s colonies were found among 10^4 Km^r Sm^r colonies. Therefore, it was concluded that Tc^r was stably coinherited (100%) with Tn*5*. ORS571 Tn*5*-containing derivatives also exhibited low-level chloramphenicol resistance (5 μg ml^{-1}) but did not exhibit ampicillin resistance; both are pVP2021 phenotypes that are efficiently expressed in *E. coli*.

To test whether or not pVP2021 was stably maintained as a plasmid in ORS571, Tn*5*-containing derivatives were backcrossed with *E. coli*. The broad-host-range Tra$^+$ plasmid pR68.45 Km^s (Table 1) was transferred to one such ORS571 Tn*5*-containing strain by selecting for derivatives with a high-level Tc^r (30 μg ml^{-1}) phenotype. pR68.45 efficiently mobilizes both plasmid and chromosomal DNAs (12, 20); because pR68.45 Km^s and pVP2021 belong to different incompatibility groups, resulting ORS571 Tn*5*-containing

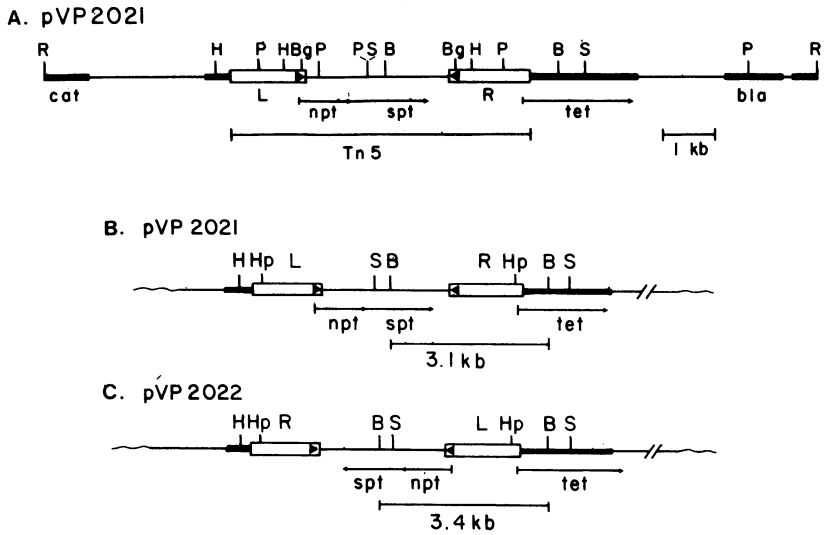

FIG. 1. Linearized restriction map of Vi mutagenesis plasmid pVP2021 (A) and inversion of Tn*5* by *Hpa*I digestion and ligation of pVP2021 in vitro (B, C). (A) Antibiotic resistance genes are shown as heavy lines: β-lactamase (bla), Ap^r; chloramphenicol acetyltransferase (cat), Cm^r; neomycin phosphotransferase (npt), Km^r Nm^r; streptomycin phosphotransferase (spt), Sm^r. Arrows designate transcriptional polarity. (B, C) A larger (3.4 versus 3.1 kb) *Bam*HI fragment was characteristic of the Tn*5* inversion construct (pVP2022).

pR68.45 Km^s strains are stable. Whereas pR68.45 Km^s could be backcrossed from ORS571 derivatives to *E. coli* MM294 at high frequencies (10^{-1} per recipient), putative pVP2021-carrying strains could be backcrossed to *E. coli* only at low frequencies (10^{-4} to 10^{-5} per recipient). It was concluded that ORS571 Tn*5*-containing strains carried, at most, 1 free pVP2021 plasmid per 10^3 clonal cells.

Putative *E. coli* MM294(pR68.45 Km^s)(pVP2021) transconjugants were tested for reacquisition of pVP2021. One such putative MM294(pR68.45) Km^s(pVP2021) strain was grown under selective (Km) conditions, plasmid DNA was isolated (see Materials and Methods), isolated plasmids were used to transform strain MM294, and Km^r transformants were selected. Restriction endonuclease mapping analyses of plasmids from several independent Km^r transformants showed the presence of a plasmid indistinguishable from the original pVP2021 (data not presented). In a second test of pVP2021 presence as free plasmid in ORS571 Km^r Sm^r Tc^r derivatives, these strains were used as potential plasmid donors in matings with strain 57801 (Table 1). Transfer of antibiotic resistance phenotypes between strains (at frequencies of $\geq 10^{-7}$) could not be detected. Moreover, plasmid DNAs, obtained from ORS571 Km^r Sm^r Tc^r strains by gentle lysis techniques, could not be detected by gel electrophoresis (data not presented). It was concluded that, although complete copies of pVP2021 could be rescued from ORS571 Tn*5*-containing derivatives, such strains rarely, if at all, maintained pVP2021 as a plasmid.

TABLE 2. Vector insertion mutagenesis of ORS571

E. coli donor strain	Selected			Scored; (Tc^r)
	Km^r	Tc^c	Km^r Sm^r	
SM10(pVP2021)			8×10^{-7}	50/50
SM10(pVP2022)			7×10^{-7}	100/100
SM10(pVP2021)[a]			8×10^{-7}	100/100
SM10(pVP2024)	1×10^{-9}			
SM10(pVP2025)	5×10^{-10}			
SM10(pVP2023)			3×10^{-6}	50/50
SM10		0		
SM10(pSUP202)		0		

[a] Reconstructed in vitro after *Hpa*I digestion (see text).

pVP2021 was tested as an insertion mutagen in ORS571. Among ORS571 Tn*5*-containg derivatives, Asm mutants, Nif mutants, and other auxotrophs were isolated at frequencies of 10^{-4}, 10^{-3}, and 10^{-2}, respectively. None of the ORS571 Asm^- or Nif^- mutants tested reverted to prototrophy at a detectable frequency. It was concluded that Tn*5* anomalously transposes in ORS571 and that ORS571 Tn*5*-containing derivatives contain Tn*5* that is somehow genomically integrated.

IS*50*R-mediated cointegrate formation is independent of its context. The hypothesis that the Tn*5* position in pVP2021 might have contributed to anomalous Tn*5* transposition in ORS571 was investigated. The Tn*5* *npt* and *spt* genes are flanked by two ~1.5-kilobase (kb) IS*50* elements, IS*50*R and IS*50*L, that differ by only a single nucleotide (22); IS*50*R and IS*50*L can transpose independently if transposition functions are supplied in *cis* (3). IS*50*R encodes two proteins, one of which (p58) mediates and the other of which (p54) regulates transposition (14, 15). DNA sequence analysis indicates that the IS*50*L sequence change has generated an in-frame ochre codon that simultaneously produces truncated, defective p58 (p53) and defective p54 (p49) as well as creates a new *npt* and *spt* gene promoter (18, 21). Therefore, the ratio of IS*50*R to IS*50*L gene products may in theory influence both the frequency and mode of transposition. If vector promoters of pVP2021 alter the p58,p54/p53,p49 ratio, e.g., producing additional proteins of one group, then this might alter transposition.

To investigate an effect of its context, the Tn*5* element in pVP2021 was inverted in vitro. Purified plasmid DNA was digested with *Hpa*I, the two resulting DNA fragments were ligated, and *E. coli* MM294 Km^r transformants were selected. Plasmids able to confer all four (excluding Sm) antibiotic resistance phenotypes were obtained from transformants and were mapped by restriction endonuclease analysis. Both reconstituted pVP2021 plasmids and plasmids exhibiting the larger *Bam*HI fragment of inverted Tn*5* were

obtained (Fig. 1B and C). In a final construction, pVP2021 IS*50*R was excised. *Bam*HI or *Sal*I was used to digest purified plasmid, DNA fragments were ligated, transformants were selected, and the desired plasmids, pVP2024 and pVP2025 (Table 1), were obtained.

Effects of these alterations to pVP2021 on anomalous Tn*5* transposition in ORS571 were assessed. *E. coli* SM10 strains carrying each of the pVP plasmids were used in heterogeneric matings with ORS571. An additional Vi mutagenesis plasmid, pVP2023, that is Tc^s by virtue of the position of its resident Tn*5* insertion (Table 1) was included in this experiment. ORS571 Km^r or Km^r Sm^r derivatives were selected and subsequently scored for Tc^r (Table 2). Frequencies of ORS571 Km^r Sm^r derivatives yielded by pVP2021, pVP2022, and pVP2023 were found to be similar and thus independent of the context of Tn*5* in the delivery plasmid (vector). One hundred independent ORS571 Km^r Sm^r derivatives from matings with both pVP2021 and pVP2022 were scored; all were Tc^r. However, plasmids pVP2024 and pVP2025 failed to yield ORS571 Km^r strains. Three conclusions were drawn from these results: (i) pVP2021 vector promoters do not interfere with direct Tn*5* transposition in ORS571, (ii) frequencies of pVP2021-mediated transposition events in ORS571 are independent of the Tn*5* position in the vector, and (iii) IS*50*R is required to establish cryptic pVP2021 in ORS571. These conclusions are consistent with a mechanism of IS*50*R-mediated pVP2021 cointegrate formation in ORS571.

IS*50*R mediates stable cointegrate formation between pVP2021 and the ORS571 genome. The genomic structures of ORS571 Tn*5*-containing strains were examined by Southern hybridization experiments. Genomic DNAs were prepared from six Asm^- and two Nif^- ORS571 Tn*5*-containing mutants. Purified genomic DNAs were digested with *Eco*RI or *Bam*HI. *Eco*RI does not cut Tn*5*, *Bam*HI cuts Tn*5* once, and both enzymes cut pSUP202 once. DNA fragments were subjected to agarose gel electrophoresis and transferred to nitrocellulose filters (see Materials and Methods). Three ^{32}P-labeled DNA probes (see Materials and Methods) were prepared for use in genomic DNA hybridization experiments: (i) a 2.6-kb *Bgl*II Tn*5* internal DNA fragment, (ii) a 1.35-kb *Hpa*I-*Bgl*II IS*50* DNA fragment, and (iii) pSUP202. Restriction endonuclease-digested genomic DNAs were hybridized with each of these three DNA probes (data not presented). ORS571 wild type showed no detectable hybridization with any DNA probe; all eight mutant strains showed DNA homology with each DNA probe. For each mutant strain one *Eco*RI genomic DNA fragment hybridized with the Tn*5* internal DNA probe (data not presented). Therefore, a single copy of intact Tn*5* was present in the genomes of these eight mutants. However, for each mutant strain more than two genomic *Bam*HI DNA fragments hybridized with the IS*50* DNA probe (data not presented). For all mutant strains, results from these hybridization experiments were consistent with the interpretation that each strain contained a single pVP2021-genomic cointegrate, comprising a single Tn*5* element and an additional (third) IS*50* element.

Nif complementation tests. To determine the structures of putative pVP2021 genomic cointegrates in detail, we wished to identify mutants in a region of the genome that had otherwise been physically mapped; the region encoding the *nifHDK* genes fulfilled these criteria. Therefore, identified ORS571 *nif*::VP2021 mutant strains were subjected to Nif^+ complementation tests. Recombinant phage from a lambda EMBL3 library of ORS571 genomic DNA had been isolated that contained DNA sequences homologous to *Klebsiella pneumoniae nifHDK* genes (8, 19). These phage had been used to prepare a physical map of this region of the ORS571 genome. Plasmid clones homologous to one such phage, λNif6, that contained the ORS571 *nifHDK* genes were isolated and used to construct partial merodiploids of ORS571 *nif*::VP2021 mutant strains. (Details of the ORS571 genomic library construction, the physical mapping of the genome, and the subclonings will be described separately [C. K. Raymond, R. G. K. Donald, A. I. Laroch, D. Nees, and R. A. Ludwig, manuscript in preparation].) Among such *nif*::VP2021 mutants, two, strains 60016 and 60070 (Table 1), that were complemented to Nif^+ by such techniques were chosen as representative examples. Because the VP2021 insertions in strains 60016 and 60070 were expected to lie within the physically mapped *nif* genomic region, strains 60016 and 60070 were chosen for recombinant DNA cloning experiments.

Direct recombinant DNA cloning of ORS571 Vi mutants via endogenous VP2021 sequences. To test the proposed mechanism of IS*50*R-promoted cointegration in ORS571, and to obtain recombinant DNA clones of ORS571 *nif*::VP2021 mutant DNAs, a direct recombinant DNA cloning strategy was devised based on the presumed integration of pVP2021 contiguous with Nif DNA sequences in these mutant strains. In brief, this cloning protocol entailed restriction endonuclease digestion of genomic mutant DNA, intramolecular ligation of DNA fragments, and transformation of *E. coli* HB101. In a first protocol, *Kpn*I was chosen to digest mutant genomic DNAs because the enzyme does not attack pVP2021. If genomic cointegrates existed, then after intramolecular ligation of genomic *Kpn*I DNA fragments, recombinant plasmids would be expected to contain ORS571 DNA contiguous with pVP2021 in the *nif*::VP2021 mutant genomes. Because *Kpn*I digestion of mutant genomic cointegrates would be expected to yield recombinant plasmids containing an IS*50* triplication, the stability of such recombinant plasmids was suspect on theoretical grounds. At least two copies of IS*50* would be in a directly repeated orientation and thus be a template for IS*50*R-mediated resolution (26) or homologous recombination, both of which would result in excision and loss due to segregation of the recombinant DNA insert. Therefore, in a second protocol, *Eco*RI was substituted for *Kpn*I; *Eco*RI cuts the *cat* gene DNA sequence of pVP2021 once (Fig. 1). After *Eco*RI digestion and intramolecular ligation, recombinant DNA plasmids would contain only a stable IS*50* inverted duplication or a single copy of IS*50*, depending upon the orientation of Tn*5* in the genomic cointegrate (Fig. 2). A selectable replicon would necessarily result and would contain a replicative origin and at least one antibiotic resistance gene (*tet* or *bla*).

Therefore, strain 60016 and 60070 genomic DNAs were isolated, purified, and digested to completion with either *Kpn*I or *Eco*RI (see Materials and Methods). DNA fragments were religated under dilute conditions theorized to preferentially yield intramolecular ligation products (9), these ligation mixes were used to transform *E. coli* HB101, and Km^r, Tc^r, or Ap^r transformants were selected (see Materials and Methods). Resident plasmids of several transformants derived from both 60016 and 60070 (pDRN701, pDRN702, pDRN162, and pDRN163, Table 1) were analyzed by restriction endonuclease mapping and compared with pVP2021. Common DNA fragments as well as novel DNA fragments were observed; it was therefore inferred that these plasmids were recombinant.

Recombinant plasmids pDRN162 (8.8 kb) and pDRN163 (7.8 kb) exhibited DNA fragments that hybridized with the

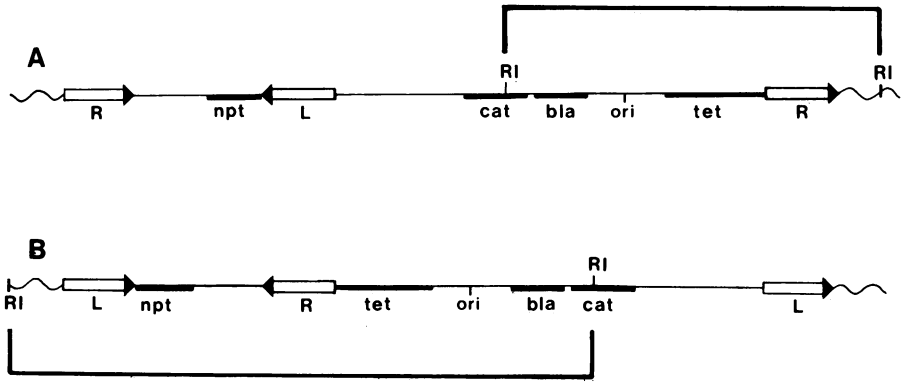

FIG. 2. Theoretical products of pVP2021 cointegration via either (A) IS*50*R- or (B) IS*50*L-mediated events. Digestion of such genomic DNAs with *Eco*RI and subsequent intramolecular ligation of DNA fragments would yield recombinant plasmids as indicated (bars above or below genomic structures). Note that an intact Tn*5* element would be contained in recombinant plasmids derived from IS*50*L-mediated cointegration; such plasmids would confer a Km^r phenotype. Recombinant plasmids derived from IS*50*R-mediated cointegration would confer a Km^s phenotype.

^{32}P-labeled SalI recombinant DNA fragment of λNif6 phage (Fig. 3A and 4). pDRN161 (Table 1), a product of *Kpn*I digestion of strain 60016 DNA, did not contain DNA homologous with ORS571 wild-type genomic DNA sequences. Detailed physical analysis of pDRN161 indicated that it was similar to pVP2021 except that Tn*5* was inverted. In contrast, pDRN162 and pDRN163 plasmids, derived from *Eco*RI digestion of strain 60016 DNA, were Ap^r and Tc^r and contained, respectively, 2.4 and 1.4 kb of insert DNA (Fig. 3A) that hybridized to the λNif6 SalI genomic DNA fragment. pDRN162 and pDRN163 are comprised of pVP2021-derived vector DNA (from the *Eco*RI site to the IS*50* adjacent to the *tet* gene); the remaining DNA is therefore the 60016 genomic DNA insert (Fig. 3A). That the additional *Eco*RI fragment of pDRN162 was contiguous in the 60016 genome was evidenced by the presence of a homologous DNA fragment in λNif6; therefore pDRN162 is the product of an incomplete *Eco*RI digest. Because the physical map of ORS571 wild-type DNA in this region has been deduced from that of λNif6, the position of the *nif*::pVP2021 insertion in strain 60016 was precisely determined (Fig. 4).

Like plasmids pDRN162 and pDRN163, recombinant plasmid pDRN701 (10.6 kb), derived from *Eco*RI digestion of strain 60070, conferred Ap^r and Tc^r, and physical mapping showed that these plasmids contained identical vector (pVP2021-derived) sequences (Fig. 3A and B). The 4.2-kb DNA insert in pDRN701 hybridized to the λNif6 genomic SalII (5.7-kb) but not the SalI (6.3-kb) DNA fragment probe (Fig. 3B, and 4; hybridization data not shown).

In contrast, physical mapping of recombinant plasmid pDRN702 (39 kb), derived from *Kpn*I digestion of 60070 DNA, showed that it contained a 24-kb DNA insert; the remaining DNA represented a complete copy of pVP2021 (Fig. 3C). Consistent with this, pDRN702 exhibited all four

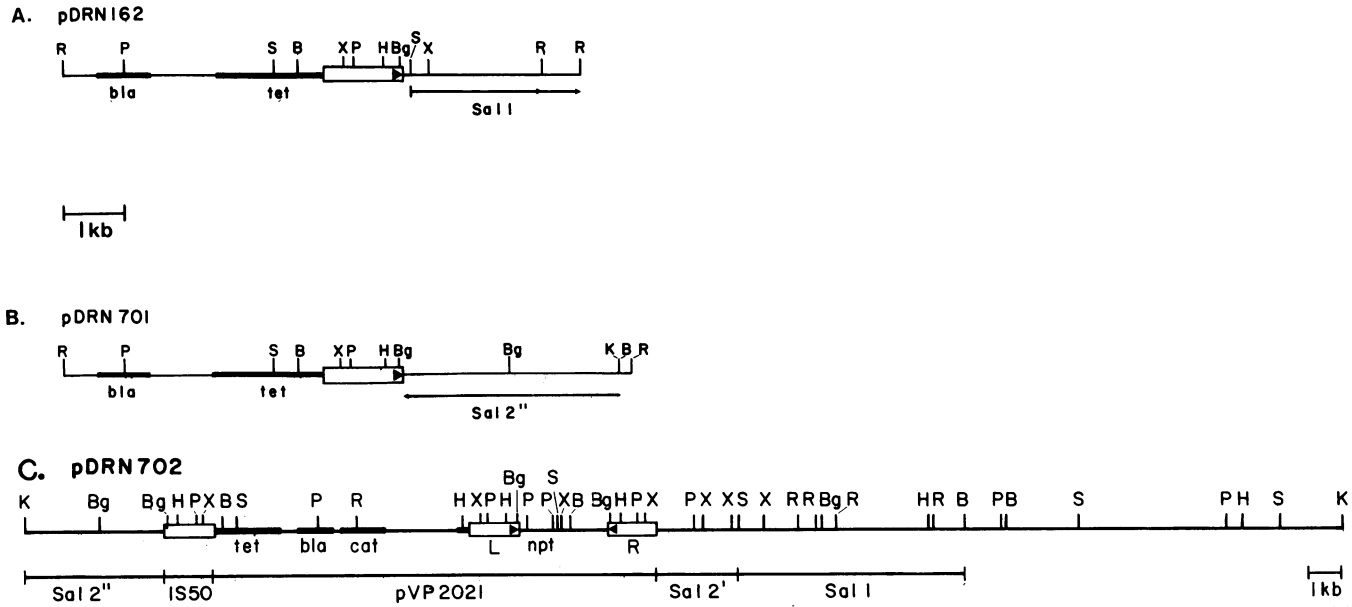

FIG. 3. Physical map of recombinant plasmids derived from: (A, B) *Eco*RI and (C) *Kpn*I genomic DNA digests of Nif^- strains (A) 60016 and (B, C) 60070. Small arrows in (A) and (B) denote the orientation of recombinant sequences relative to λNif6. pDRN163 is identical to pDRN162 except it lacks the terminal 1-kb *Eco*RI fragment. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; Hp, *Hpa*I; P, *Pst*I; R. *Eco*RI; S, *Sal*I; X, *Xho*I.

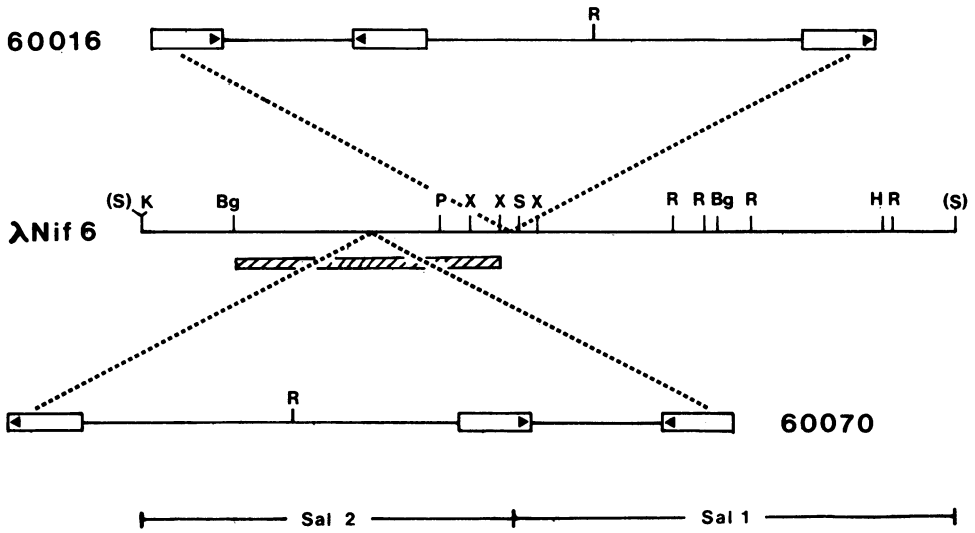

FIG. 4. Colinearity of physical maps of the recombinant phage λNif6 and DNA sequences derived from Vi mutant strains 60016 and 60070. The hatched bar indicates the region of homology between the *Rhizobium* sp. strain ORS571 genome and the *K. pneumoniae nifHDK* genes (10, 19). Map positions of pVP2021 insertions in strains 60016 and 60070 are indicated. *SalI* sites in parentheses are present in the vector λEMBL3 and not in the ORS571 genome. DNA fragments Sal 1 (6.3 kb) and Sal 2 (5.7 kb) of λNif6 were isolated and used to prepare ^{32}P-labeled hybridization probes for physical analyses of recombinant plasmids (see text; Fig. 3). Abbreviations are as given in the legend to Fig. 3.

antibiotic resistance phenotypes (Ap, Cm, Tc, Km) of pVP2021. DNA fragments of pDRN702 hybridized with both genomic Sal 1 and Sal 2 DNA fragment probes (Fig. 3C and 4). The physical map of the pDRN702 24-kb DNA insert was found to be colinear with that of pDRN701 (Fig. 3B). Again, because the physical map of ORS571 wild-type DNA in this region has been deduced from that of the λNif6 DNA insert, the position of the *nif*::pVP2021 insertion in strain 60070 was precisely determined (Fig. 4).

It was concluded that Vi mutant strains 60016 and 60070 contained genomic cointegrates of pVP2021 because the affected *Rhizobium* genomic DNA could be uniquely obtained by recombinant DNA cloning, after having exploited the endogenous vector. Moreover, the entire pVP2021 DNA sequence was present in each genomic cointegrate because recombinant plasmids such as pDRN702 produced by *Kpn*I digestion of Vi mutant DNAs contained within them the entire pVP2021 DNA sequence.

More than 80 independently isolated ORS571 Vi mutant strains with distinct phenotypes have directly yielded unique recombinant DNA plasmids after *Eco*RI digestion of genomic DNAs, intramolecular ligations, and transformations (data not presented). The structures of every parental genomic cointegrate have been deduced. In every case, IS*50*R was utilized as template for replicative cointegrate formation (Fig. 2A). Cointegrate formation via IS*50*L has not been observed (Fig. 2B). Correspondingly, all recombinant plasmids generated by *Eco*RI digestion of Vi mutant strains were Km^s; each contained only a single IS*50*R element and no complete Tn*5* element (Fig. 3). Moreover, ORS571::VP2021 insertion mutant strains exhibit with equal frequency the IS*50*R module oriented in both genomic IS*50*R configurations (Fig. 3 and 4) as exemplifed by strains 60016 (orientation I) and 60070 (orientation II).

DISCUSSION

Why does IS*50*R-mediated cointegration and not direct Tn*5* transposition occur in ORS571? If such cointegrates are intermediates in Tn*5* transposition, then resolution may be partly dependent on host recombination/repair functions (e.g., type RecBC RecF nucleases) that may vary between bacteria. Previous results (26) suggest that IS*50* p58 protein can catalyze not only transposition but also resolution of tandem, directly repeated IS*50* sequences that configure transient cointegrate structures in *E. coli*. However, other studies have concluded that Tn*5* transposes in *E. coli* by a conservative, post-replicative mechanism because cointegrate structures are not observed (1, 2). Our results show that IS*50*R-mediated cointegrates are formed and are stable in ORS571. Resolution of these structures by recombination across direct IS*50*R repeats would lead to a single copy of IS*50*R and an expected loss of VP2021 sequences. Several experiments indicate that ORS571 is recombination proficient and contains a gene homologous with *E. coli recA*$^+$ (unpublished data).

We cannot rigorously infer that cointegrate formation occurs by a mechanism of concerted, replicative IS*50*R-mediated transposition as opposed to a two-step process involving an IS*50*R transposition to the genome and a subsequent integrative IS*50*R-mediated resolution or homologous recombination. The latter mechanism implies that IS*50*R is able to transpose independently in ORS571; the presented results do not address this possibility. However, the available evidence suggests that a concerted cointegration mechanism is more likely. Assuming a reasonable frequency for acquisition of pVP2021 in heterogeneric matings (10^{-1}), ORS571 Vi strains are obtained at frequencies similar to those observed for Tn*5* transpositions in other bacteria ($\sim 10^{-5}$). Were the two-step mechanism occurring, Vi mutants should occur at vastly lower frequencies than those observed unless IS*50*R transposes at extremely high frequency in ORS571. The second type of evidence supporting a concerted cointegration mechanism is that the cointegrates are extremely stable. Were they to be formed at detectable rates by a second step involving IS*50*R-mediated resolution or homologous recombination, we might expect

to see resolution of the cointegrate itself, i.e., disintegration, at measurable frequencies. However, disintegration is extremely slow ($<10^{-6}$ per cell per generation).

Nevertheless, it is surprising that plasmids such as pDRN702 (and others not discussed) contain the IS*50* triplication and are stable in *E. coli* Rec$^-$ strains. In contrast, plasmids pDRA51 (not discussed) and pDRN161, recovered from *Kpn*I digestions of ORS571 genomic cointegrates, lacked *Kpn*I sites, lacked recombinant DNA sequences and were in fact identical to pVP2021 and pVP2022, respectively. These plasmids are the expected products of IS*50*R-mediated disintegrations in transformed *E. coli recA* strains. That pDRN702 maintains a *Kpn*I site and additional DNA may indicate that some ORS571 strains, such as 60070, may have been stabilized by undetected alterations, perhaps in IS*50* resolution/recombination sites (23).

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