JOURNAL OF BACTERIOLOGY, July 1985, p. 324–330
0021-9193/85/070324-07$02.00/0

Vol. 163, No. 1

Extension of the Host Range of *Escherichia coli* Vectors by Incorporation of RSF1010 Replication and Mobilization Functions

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Received 6 August 1984/Accepted 3 April 1985

The broad-host-range vectors pSUP104, pSUP106, pSUP204, pSUP304, and pSUP404 are based on conventional *Escherichia coli* vectors (such as pBR325 and pACYC184) which have been modified to include the mobilization and broad-host-range replication functions of the IncQ plasmid RSF1010. These vector plasmids now can be maintained in a wide range of bacterial genera including *Rhizobium*, *Agrobacterium*, and *Pseudomonas*. They are efficiently mobilized by RP4 and thus are of particular interest for bacteria refractory to transformation. They offer the selection markers and cloning sites characteristic of the basic *E. coli* vectors. Therefore, they can be applied and adapted to a variety of cloning strategies. However, the cloning of very large fragments (e.g., in cosmid hybrids of pSUP106) was found to affect the stability of the recombinant molecules in a Rec$^+$ background. This instability was not observed with smaller inserts of about 5 kilobases.

An important factor for the genetic manipulation of microorganisms is the availability of vector plasmids. A powerful technology based on an extensive array of phage and plasmid vectors has been developed for the enteric bacterium *Escherichia coli*. However, there are many gram-negative strains of practical and theoretical importance (for example, soil bacteria such as *Rhizobium*, *Agrobacterium*, and *Pseudomonas* spp.) for which these cloning vehicles are of very limited use. *E. coli* cloning vectors such as pBR325 (9) or pACYC184 (11) replicate within a very narrow host range. Moreover, they cannot be efficiently transferred by conjugation, and transformation systems are poorly developed for many gram-negative species. Therefore, cloning vehicles useful in a wide variety of gram-negative bacteria require not only promiscuous replication functions but also functions which allow their efficient conjugative transfer.

There are several transmissible broad-host-range plasmids that have been modified to improve their suitability as cloning vehicles in gram-negative bacteria. Examples include cloning vectors based on the IncP plasmids RK2 and RP4 (12, 19, 21, 28) as well as a series of cloning vehicles derived from the IncW plasmid pSa (22, 35). Another significant broad-host-range plasmid is RSF1010 (6, 17). It is a small multicopy plasmid which is not self-transferable but which can be mobilized by coexisting conjugative plasmids (2, 6, 37). It has been used as a basic replicon for the construction of a variety of cloning vehicles for gram-negative bacteria (1, 2, 15, 27, 32, 38). Although there is no doubt about the usefulness of these cloning vectors, many of them were not completely satisfactory for our purposes, especially in terms of selection markers and number of conventional cloning sites.

We report here the construction and properties of vector plasmids (pSUP vectors) which combine the mobilization and broad-host-range replication functions of RSF1010 with the well-known advantages of conventional cloning vectors commonly used in *E. coli*. We also describe the use of the pSUP vectors in the construction of a cosmid gene bank and in interspecific complementation tests. The pSUP plasmids presented here are referred to as replicative vectors in contrast to the nonreplicative ones described previously (34).

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MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used in this work are described in Table 1.

Media. *E. coli* and *Pseudomonas* strains were grown in Penassay broth (Difco Laboratories, Detroit, Mich.) at 37°C, and *Rhizobium* and *Agrobacterium* strains were grown in tryptone-yeast extract (7) at 30°C. Agar (1.3%) was added for solid medium. Antibiotics were used at the following concentrations unless otherwise indicated: tetracycline, 5 μg/ml; streptomycin, 200 μg/ml; rifampin, 100 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 50 μg/ml; kanamycin, *E. coli*, 250 μg/ml; and neomycin, *Rhizobium*, 100 μg/ml.

Transformation. Competent cells of *E. coli* were prepared and transformed as described by Morrison (26).

Bacterial conjugation. Matings were carried out on filter membranes as described previously (34).

Estimation of plasmid stability. Vector stability was determined by inoculating a single colony harboring the plasmid to be tested into liquid medium in the absence of any antibiotic. Immediately after inoculation, the number of bacteria was determined by plating onto nonselective medium. The cultures were diluted after they reached the end of the log phase, and samples were either transferred to fresh medium to continue the nonselective growth or plated onto nonselective agar plates. Colonies were counted to calculate the number of doublings that had occurred during cultivation. In each case, 100 colonies were tested for retention of plasmid-encoded antibiotic resistance markers. In some cases the plasmid content was additionally tested on agarose gels.

Analysis of plasmid contents. Plasmids were visualized by a rapid screening technique originally described by Eckhardt (13). The method has been adapted for horizontal gels (33).

Partial digestions. Partial digestions with restriction enzymes were carried out according to the procedure of Maniatis et al. (23). To linearize plasmid DNA with *Sau*3A, we determined the enzyme concentration which would produce the largest amount of molecules in the range of linear, full-sized plasmid DNA. To enrich the proportion of

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
E. coli		
SM10	Rec⁻ derivative of C600 with RP4-2Tc::Mu integrated in the chromosome	34
S17-1	Rec⁻ derivative of 294 with RP4-2Tc::Mu Km::Tn7 in the chromosome	34
294	*hsdR* Pro	
K802	*hsdR* Gal Met *supE*	39
HB101	Pro Leu Thi *lacY* Sm^r *endA recA hsdR hsdM*	10
R. meliloti 2011	Sm^r	J. Dénarié
R. leguminosarum 897	Phe Trp Sm^r	18
A. tumefaciens		
LBA275	pTi pAt Rf^r	P. Hooykaas
LBA4301	pAt Rf^r Rec⁻	20
P. aeruginosa PAO5	Rf^r Trp	V. Krishnapillai
Plasmids		
pKT210	RSF1010 derivative, Cm^r Sm^r	M. Bagdasarian
pACYC177	Ap^r Km/Nm	11
pACYC184	Cm Tc	11
pBR325	Ap Cm Tc	9
RP4Tc::Mu	Ap Km Nm Tc::Mu	R. Simon
RP4Tc::Tn7	Ap Km Nm Tc::Tn7	R. Simon

full-length molecules in relation to uncut DNA and smaller digestion products, the digest was loaded onto a sucrose gradient from 5 to 20% (wt/vol) and centrifuged in a swinging bucket rotor (SW40; Beckman Instruments, Inc., Fullerton, Calif.) for 4 h at 35,000 rpm. Fractions were collected and tested on agarose gels. Those fractions containing linearized molecules were pooled, dialyzed, and used for cloning. DNA isolation, restriction analysis, ligation, and cosmid cloning have been described elsewhere (29, 30, 31, 34).

RESULTS

Construction of replicative pSUP vectors. The source of mobilization and broad-host-range replication functions was the RSF1010-derived pKT210 vector (Fig. 1). Random fragments of pKT210 were cloned into linearized *E. coli* vectors, and the composite plasmids were tested for their ability to be mobilized and replicated in the soil bacterium *Rhizobium meliloti*.

The construction of pSUP104 based on the *E. coli* vector pACYC184 (11) is shown in Fig. 2. pKT210 DNA was partially digested with the enzyme *Sau*3A to yield randomly generated restriction fragments. To conserve the unique *Bam*HI cloning site as well as the Tc^r gene, pACYC184 was linearized with *Sau*3A. After dephosphorylation, the linearized pACYC184 vector DNA was ligated to the partial *Sau*3A digest of pKT210, and samples of the ligation mix were used to transform *E. coli* SM10 (34). This is a mobilizing strain, characterized by a chromosomally integrated RP4 derivative which provides all RP4-specific transfer functions for mobilization of RSF1010 and its derivatives. SM10 transformants selected for the Tc^r specified by pACYC184 were pooled and used as donor cultures in subsequent en masse matings with *R. meliloti* 2011. All resulting transconjugants must have obtained a pACYC derivative carrying both the mobilization and replication functions of pKT210.

A number of *R. meliloti* transconjugants were further analyzed for coexisting Cm^r and for molecular size of the transferred hybrid plasmids. Appropriate plasmids were reintroduced into *E. coli* and further characterized for mobilization frequency, host range, and restriction pattern. The plasmid finally selected was called pSUP104.

Mobilizable broad-host-range derivatives of pACYC177 (11) and pBR325 (9) were obtained with the same strategy. One derivative of pACYC177, pSUP304.2, was modified to encode Cm^r instead of Ap^r (which is not very useful in many gram-negative bacteria) by replacing the *Pst*I fragment with a DNA region of about 1.9 kilobases (kb) carrying a Cm^r gene including an *Eco*RI restriction site originating from phage P1Cmts (W. Klipp, unpublished data). The plasmid thus constructed is pSUP404.2. A cosmid derivative, called pSUP106, was produced by inserting a 0.4-kb fragment containing the λ *cos* site (25) into the unique *Pst*I site of pSUP104.

Properties of replicative pSUP vector plasmids. (i) Physical and cloning properties. The cloning vectors finally selected were pSUP104 and pSUP106 originating from pACYC184, pSUP204 based on pBR325, and pSUP304.1, pSUP304.2, and pSUP404.2 derived from pACYC177 (Fig. 3). They all contained a pKT210 insert of about 6 kb including recognition sites for *Pst*I and *Bst*EII. In heteroduplex experiments with pSUP104, this fragment was homologous to a unique stretch of DNA in pKT210 (from position 2.0 to position 8.0 on the map of pKT210 [Fig. 1]). The *Pst*I site could be used for cloning (as shown by the construction of the cosmid pSUP106), whereas removal of the *Bst*EII site resulted in loss of broad-host-range replication.

All vector plasmids retained the antibiotic resistance genes and restriction sites characteristic of the basic *E. coli*

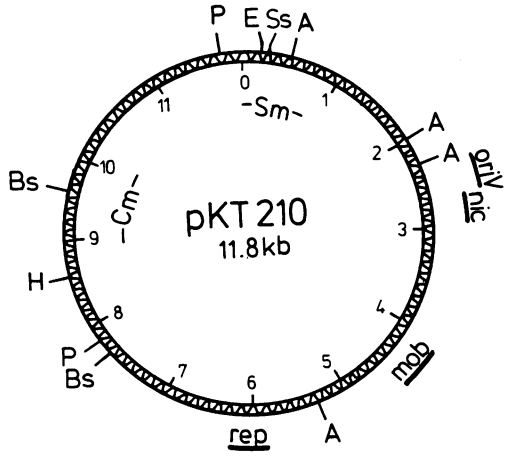

FIG. 1. Map of the RSF1010 derivative pKT210 (1, 4). pKT210 is a vector of 11.8 kb that encodes Cm^r and Sm^r. It was constructed by inserting a *Pst*I fragment carrying Cm^r into the single *Pst*I site of RSF1010 and allows cloning into *Eco*RI and *Sst*I (inactivation of streptomycin) and *Hin*dIII. The positions of *oriV* (origin of vegetative replication), *mob* (mobilization functions), *nic* (relaxation nick site), and *rep* (*trans*-acting replication functions) are indicated according to Bagdasarian et al. (4). Restriction sites: P, *Pst*I; E, *Eco*RI; Ss, *Sst*I; A, *Ava*I; Bs, *Bst*EII; H, *Hin*dIII.

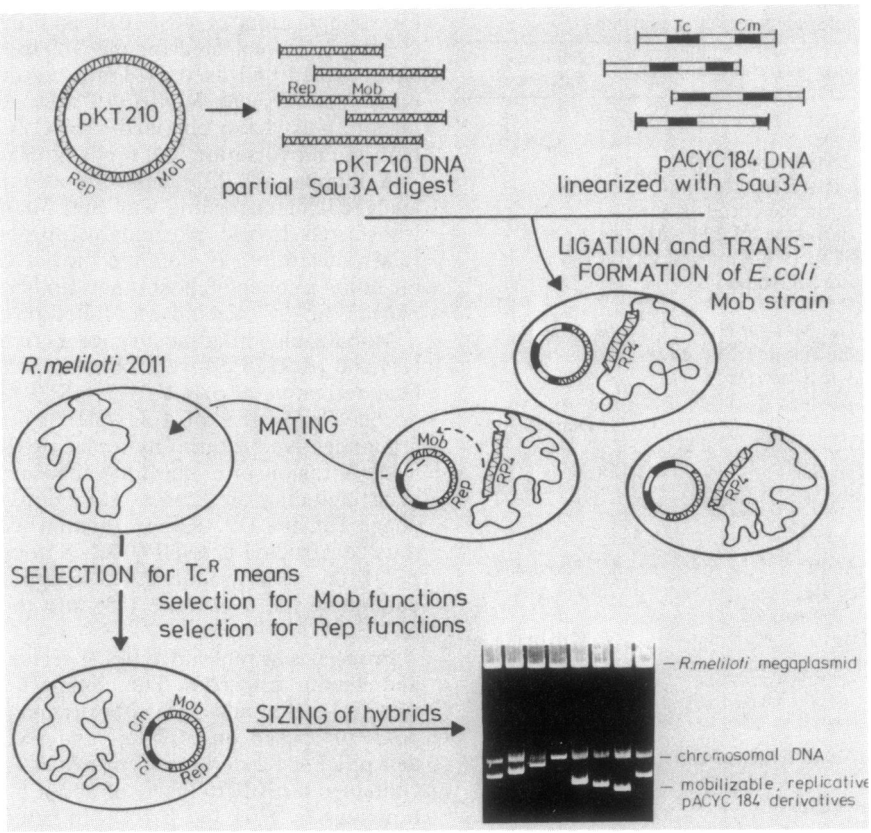

FIG. 2. Strategy for construction of pSUP104. A partial *Sau*3A digest of pKT210 was ligated to *Sau*3A-linearized DNA of pACYC184. A mobilizing strain, carrying an integrated RP4 derivative, was transformed with the ligation mix. Tc^r transformants were mated with *R. meliloti* 2011, selecting for Tc^r transconjugants. The mobilizable and replicative pACYC184 hybrids were further characterized in terms of coexisting Cm^r. Eckhardt gels revealed the presence of hybrid molecules of different sizes, in addition to the *R. meliloti* megaplasmid (5). More detailed analysis of appropriate molecules resulted in the selection of pSUP104.

vector. In pSUP204, an additional *Bam*HI site was generated in the course of the *Sau*3A cloning procedure, so that *Bam*HI (as well as *Pst*I) may be used for replacement cloning. The same event occurred in pSUP304.1, which carries a single *Bam*HI site available for cloning. This site was not created in pSUP304.2. pSUP404.2 offers a single *Eco*RI site located within the Cm^r gene.

(ii) Host range and mobilization frequencies. Mobilization of the vector plasmids into different bacterial species has been tested with *E. coli* S17-1, a mobilizing donor (34), as the source of RP4 transfer functions. As summarized in Table 2, the vector plasmids could be mobilized at frequencies comparable to those for normal RP4 transfer or even higher. Efficient transfer occurs also into strains of *Pseudomonas stutzeri* (V. Krishnapillai, personal communication), *Rhizobium trifolii*, and *Rhodopseudomonas capsulata* (not shown in Table 2).

Transfer of the vector plasmids can also be accomplished via triparental matings. This has been shown for the transfer of pSUP104 within *R. meliloti* strains, with *E. coli* donors harboring Tc^s RP4 derivatives to provide the transfer functions. With RP4Tc::Tn*7*, plasmid pSUP104 was mobilized from one *R. meliloti* strain to another at frequencies of 7×10^{-3} per recipient cell; however, the RP4 derivative was also transferred at very high frequencies into the recipient strain. In contrast, RP4Tc::Mu was equally efficient in promoting transfer of pSUP104 (3×10^{-3}) but could not be stably established in the majority of recipient cells (6×10^{-6}) because of the well-known Mu-induced suicide effect (7, 36).

(iii) Stability in different hosts. The vector plasmids were tested for stable maintenance in different organisms without selective pressure (Table 3). All host strains used were Rec^+. The two plasmids pSUP104 and pSUP106 (not shown in Table 3) were stably maintained in all hosts tested. Plasmid pSUP304.1 exhibited a certain instability in *R. meliloti* after prolonged nonselective cultivation, but it was stably replicated in the other hosts. Stable maintenance was also observed for pSUP304.2 and pSUP404.2 in all recipients (data not shown). In contrast, pSUP204 showed a marked instability in the non-*E. coli* hosts. In all cases tested, loss of the Tc^r marker could be clearly correlated with the absence of the plasmid on agarose gels.

Cloning with replicative pSUP vectors. (i) Use of pSUP106 for cosmid cloning. pSUP106 was used to construct a cosmid gene bank from *R. meliloti* 2011 DNA by *Eco*RI cloning (Fig. 4). After in vitro packaging, the strains K802 (Rec^+) and S17-1 (Rec^-) were both infected with the same volume of phage lysate. With K802 as a recipient a 10-fold-higher yield of Tc^r cells was obtained compared with S17-1. However, only the Rec^- S17-1 clones showed molecules in the expected size range of approximately 50 kb when analyzed on agarose gels. The majority of hybrid plasmids in K802 had obviously undergone deletions or rearrangements so that plasmids of different sizes were generated. To prove the correlation between recombination and degradation, full-sized cosmid hybrids were mobilized from S17-1 into Rec^+ strains of *E. coli* and *R. meliloti*. Analysis of the plasmid content again revealed degenerated molecules of various

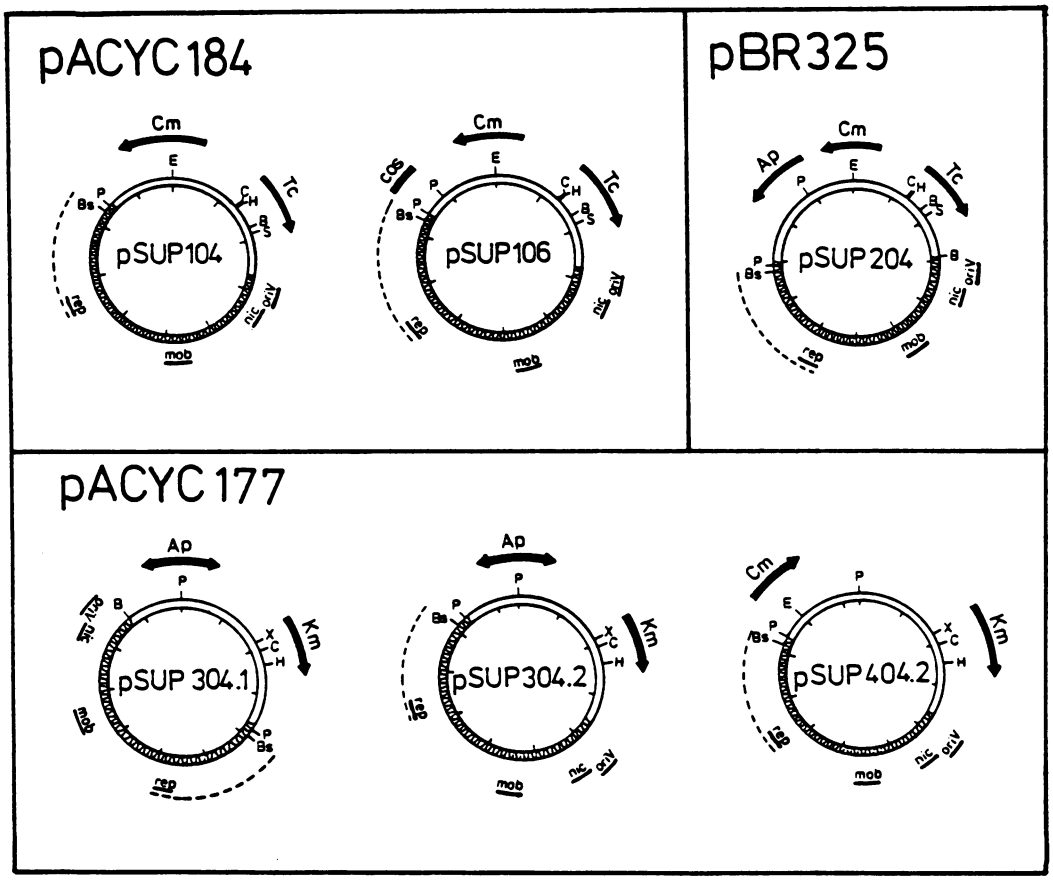

FIG. 3. Physical and genetic maps of replicative pSUP vectors. The regions encoding Ap^r, Cm^r, Km^r, and Tc^r as well as the location of mobilization (*mob nic*) and replication (*oriV rep*) functions derived from RSF1010 (~~~) are indicated. The probable extension of the *trans*-acting *rep* genes is indicated by dashed lines (M. Bagdasarian, personal communication). The length of pSUP104 (9.5 kb) is derived from electron microscope measurements; the sizes of pSUP204 (ca. 12 kb), pSUP304.1 (ca. 10.8 kb), pSUP304.2 (ca. 10.5 kb), and pSUP404.2 (ca. 11.3 kb) are estimated from restriction enzyme analysis. For pSUP304.1 a second *Bst*EII site located within the pACYC177 part (approximately at position 3.0) could be identified (not indicated in the map). Restriction sites: P, *Pst*I; E, *Eco*RI; H, *Hin*dII; B, *Bam*HI; S, *Sal*I; X, *Xho*I; C, *Cla*I; Bs, *Bst*EII.

lengths in the Rec^+ recipients (data not shown). The results indicate that pSUP106 is no longer stably replicated in a Rec^+ background when carrying a very large insert.

(ii) Use of pSUP304.1 in complementation tests. A pSUP205 cosmid bank of *R meliloti* 2011 (34) was screened for clones able to suppress the UV sensitivity of the *recA*$^-$ *E. coli* strain HB101. One hybrid selected was further characterized. A *Bam*HI fragment of approximately 5 kb sufficient to confer UV resistance to HB101 was subcloned into pSUP304.1 and introduced into a UV-sensitive Rec^- strain of *Agrobacterium tumefaciens* (LBA4301). All transconjugants were able to tolerate UV doses as high as the control *A. tumefaciens* LBA275 (Rec^+), indicating interspecific complementation of the Rec^- mutation.

DISCUSSION

The small size, high copy number, and wide host range of RSF1010 have made this plasmid particularly attractive for the construction of cloning vectors. It has been modified to

TABLE 2. Host range and mobilization frequencies of pSUP104, pSUP204, and pSUP304.1[a]

Recipient strain	Mobilization frequency (antibiotic and concn[b]) for donor strain:			
	S17-1(pSUP104)	S17-1(pSUP204)	S17-1(pSUP304.1)	C600(RP4)
E. coli CSH51	4.2×10^{-1} (Tc_5)	3.2×10^{-1} (Tc_5)	5.0×10^{-1} (Km_{25})	3.7×10^{-1} (Tc_5)
R. meliloti 2011	1.6×10^{-1} (Tc_5)	8.5×10^{-2} (Tc_5)	3.6×10^{-1} (Nm_{100})	1.4×10^{-1} (Tc_5)
R. leguminosarum 897	1.5×10^{-2} (Tc_5)	1.3×10^{-1} (Tc_5)	1.5×10^{-1} (Nm_{75})	2.5×10^{-1} (Tc_5)
A. tumefaciens LBA275	8.3×10^{-3} (Tc_3)	1.2×10^{-2} (Tc_3)	1.0×10^{-1} (Nm_{100})	3.8×10^{-1} (TC_3)
P. aeruginosa PAO5	2.7×10^{-4} (Tc_{50})	3.4×10^{-4} (Tc_{50})	1.4×10^{-5} (Nm_{100})	5.0×10^{-6} (Tc_{50})

[a] Only some of the strains which were able to support replication of the pSUP vectors are shown. Mobilization frequency into these recipients was determined with the mobilizing donor strain S17-1. All matings were performed on NC filters; transconjugants were selected for the acquisition of one of the plasmid-encoded resistance markers. Transfer of the RP4 wild-type plasmid served as a positive control.

[b] Subscripts indicate the concentration of antibiotics in micrograms per milliter; Tc, tetracycline; Km, kanamycin, *E. coli*; Nm, neomycin, *Rhizobium* spp.

TABLE 3. Stability of pSUP104, pSUP204, and pSUP304.1 in different hosts[a]

Plasmid	Host strain	Antibiotic and concn[b]	% Retention of marker at generation:		
			20	40	60
pSUP104	*R. meliloti* 2011	Tc_5	100	100	100
	R. leguminosarum 897	Tc_5	100	100	92
	A. tumefaciens LBA271	Tc_3	100	100	100
	E. coli 294	Tc_5	100	100	100
pSUP204	*R. meliloti* 2011	Tc_5	60	32	8
	R. leguminosarum 897	Tc_5	56	6	2
	A. tumefaciens LBA275	Tc_3	86	40	3
	E. coli 294	Tc_5	96	95	92
pSUP304.1	*R. meliloti* 2011	Nm_{100}	100	68	10
	R. leguminosarum 897	Nm_{75}	100	100	100
	A. tumefaciens LBA275	Nm_{100}	100	100	100
	E. coli 294	Km_{25}	100	100	100

[a] Stability of the vector plasmids was tested as described in the text. All host strains used in this experiment were Rec^+.

[b] Subscripts indicate the concentration of antibiotics in micrograms per milliliter; Tc, tetracycline; Nm, neomycin, *Rhizobium* spp.; Km, kanamycin, *E. coli*.

include a number of selection markers and single restriction sites (1, 2, 3, 32, 38). For the construction of replicative pSUP vector plasmids we chose another approach; instead of modifying the RSF1010 plasmid by inserting suitable resistance genes, we incorporated part of RSF1010 into well-characterized cloning vehicles, thus combining the mobilization and broad-host-range replication functions of RSF1010 with the advantageous features of conventional *E. coli* vectors (i.e., pACYC177, pACYC184, and pBR325).

Each of the pSUP vectors carries an identical DNA fragment of RSF1010 of about 6 kb, randomly inserted by *Sau*3A cloning, which is homologous to a unique stretch of pKT210, known to carry determinants essential for mobilization and replication (4). It would appear that this fragment is the minimum region necessary to encode both functions. Attempts to reduce the size of pSUP304.1 by removing the *Bst*EII fragment resulted in loss of the broad-host-range replication properties. This is in agreement with findings indicating the presence of three *rep* genes in RSF1010, one of which overspans the *Bst*EII site (M. Bagdasarian, personal communication).

Because of this cloned fragment, the pSUP vectors are readily mobilized by RP4 or its derivatives (e.g., pRK2013 [14]) into all gram-negative bacteria tested so far. We observed frequencies of 10^{-2} to 10^{-1} for *R. meliloti*. Similar high rates were experienced with other gram-negative recipients, including a number of *Rhizobium* and *Pseudomonas* species, *A. tumefaciens*, and *R. capsulata*.

The vector plasmids pSUP104 and pSUP106 were stably replicated in a variety of Rec^+ organisms under nonselective conditions, whereas frequent plasmid loss was observed for the pBR325 derivative pSUP204. Since the RSF1010 fragment encoding broad-host-range replication is identical in all the vector plasmids, it is likely that the pBR325 replicon causes the unstable inheritance of pSUP204. Similar observations have been reported for pBR322-RSF1010 hybrids in Rec^+ strains (1, 38). Since pSUP204 was only tested in Rec^+ and Rec^- strains of *E. coli* (without any significant difference in stability) we have no data on its segregation rate in Rec^- derivatives of *Rhizobium* or *Pseudomonas* spp.

The stability of pSUP106 cosmid hybrids is also not satisfactory; we experienced a high degree of plasmid degradation in a Rec^+ background. Introduction of the recombinant molecules into a Rec^- *E. coli* strain stabilized the

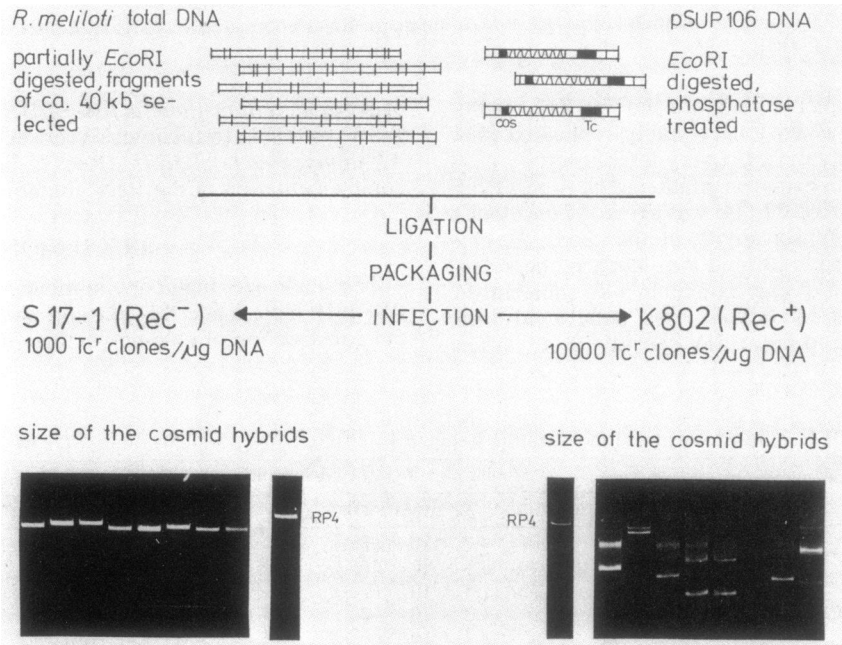

FIG. 4. Cosmid cloning with pSUP106. Partially *Eco*RI-digested total DNA of *R. meliloti* 2011 was cloned into cosmid pSUP106. After in vitro packaging, strains S17-1 (Rec^-) and K802 (Rec^+) were infected in parallel, selecting for Tc^r clones. Size determinations of the hybrids revealed full-size molecules (ca. 50 kb, compared with the length of RP4, which is ca. 60 kb) in the Rec^- host, but mainly smaller products in the Rec^+ recipient.

large plasmids, but the instability recurred upon transfer into strains of *Rhizobium* or *Agrobacterium*. Attempts to isolate cosmid derivatives that are stably maintained in Rec$^+$ strains have been unsuccessful so far. R1162, a plasmid that presumably is identical to RSF1010 (16), was observed to be unstable in *E. coli* when containing chromosomal DNA of *Pseudomonas* sp. strain AM (15). Meyer et al. (24) found that insertion of foreign DNA into nonessential regions of R1162 created plasmid instability in *Pseudomonas putida*. Whether this unstable behavior is an effect of plasmid size, of the high copy number, or of some unknown feature of RSF1010 replicon organization remains to be clarified. Further elucidation of the replication and regulation mechanisms of RSF1010 is necessary to solve these problems.

Cloning of smaller fragments seems not to affect the stability of replicative pSUP vectors. We inserted a *Bam*HI fragment of about 5 kb originating from *R. meliloti* 2011 into pSUP304.1 and could complement the UV sensitivity of *E. coli* HB101 to a Rec$^+$ phenotype. Similar interspecific complementation experiments have been reported by Better and Helinski (8). pSUP304.1 containing the *Bam*HI fragment was also able to restore UV resistance in *A. tumefaciens* Rec$^-$. It is assumed that we have cloned *rec* genes from *R. meliloti* 2011 which are able to suppress UV sensitivity in *E. coli* and *A. tumefaciens* by interspecific complementation.

ACKNOWLEDGMENTS

We thank M.-L. Wilke, P. Grönger, and S. Kautz for technical assistance and M. O'Connell and M. Hynes for their help in preparing the manuscript.

This work was supported by grant Pu 28/12-2 from Deutsche Forschungsgemeinschaft.

LITERATURE CITED

1. **Bagdasarian, M., M. M. Bagdasarian, S. Coleman, and K. N. Timmis.** 1979. New vector plasmids for cloning in *Pseudomonas*, p. 411–422. *In* K. N. Timmis and A. Pühler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North-Holland Publishing Co., Amsterdam.
2. **Bagdasarian, M., R. Lurz, B. Rückert, F. C. H. Franklin, M. M. Bagdasarian, J. Frey, and K. N. Timmis.** 1981. Specific purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors and a host-vector system for gene cloning in *Pseudomonas*. Gene **6**:237–247.
3. **Bagdasarian, M., and K. N. Timmis.** 1982. Host:vector systems for gene cloning in *Pseudomonas*. Curr. Top. Microbiol. Immunol. **96**:46–67.
4. **Bagdasarian, M., M. M. Bagdasarian, R. Lurz, A. Nordheim, J. Frey, and K. N. Timmis.** 1982. Molecular and functional analysis of the broad host range plasmid RSF1010 and construction of vectors for gene cloning in gram-negative bacteria, p. 183–197. *In* S. Mitshuhashi (ed.), Drug resistance in bacteria. Japan Scientific Societies Press, Tokyo.
5. **Banfalvi, Z., V. Sakanyan, C. Koncz, A. Kiss, I. Dusha, and A. Kondorosi.** 1981. Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *R. meliloti*. Mol. Gen. Genet. **184**:318–325.
6. **Barth, P. T., and N. J. Grinter.** 1974. Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. J. Bacteriol. **120**:618–630.
7. **Beringer, J. E., J. L. Beynon, A. V. Buchanan-Wollaston, and A. W. B. Johnston.** 1978. Transfer of the drug-resistance transposon Tn*5* to *Rhizobium*. Nature (London) **276**:633–634.
8. **Better, M., and D. R. Helinski.** 1983. Isolation and characterization of the *recA* gene of *Rhizobium meliloti*. J. Bacteriol. **155**:311–316.
9. **Bolivar, F.** 1978. Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant molecules. Gene **4**:121–136.
10. **Boyer, H. W., and D. Rouland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
11. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
12. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system from gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. U.S.A. **77**:7347–7351.
13. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid **1**:584–588.
14. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. U.S.A. **76**:1648–1652.
15. **Gautier, F., and R. Bonewald.** 1980. The use of plasmid R1162 and its derivatives for gene cloning in the methanol-utilizing *Pseudomonas* Am1. Mol. Gen. Genet. **178**:375–380.
16. **Grinter, N. J., and P. T. Barth.** 1976. Characterization of SmSu plasmids by restriction endonuclease cleavage and compatibility testing. J. Bacteriol. **128**:394–400.
17. **Guerry, P., J. van Embden, and S. Falkow.** 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. **117**:619–630.
18. **Johnston, A. W. B., and J. E. Beringer.** 1976. Pea root nodules containing more than one *Rhizobium* species. Nature (London) **263**:502–504.
19. **Kahn, M., R. Kolter, C. Thomas, D. Figurski, R. Meyer, E. Remaut, and D. R. Helinski.** 1979. Plasmid cloning vehicles derived from plasmids ColE1, F, R6K, and RK2. Methods Enzymol. **68**:268–280.
20. **Klapwijk, P., P. van Beelen, and R. Schilperoort.** 1979. Isolation of a recombination deficient *Agrobacterium tumefaciens* mutant. Mol. Gen. Genet. **173**:171–175.
21. **Knauf, V. C., and E. W. Nester.** 1982. Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid **8**:45–54.
22. **Leemans, J., J. Langenakens, H. DeGreve, R. Deblaere, M. Van Montagu, and J. Schell.** 1982. Broad host range cloning vectors derived from the W plasmid pSa. Gene **19**:361–364.
23. **Maniatis, R., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Meyer, R., R. Laux, G. Boch, M. Hinds, R. Bayly, and J. A. Shapiro.** 1982. Broad-host-range IncP-4 plasmid R1162: effects of deletion and insertions on plasmid maintenance and host range. J. Bacteriol. **152**:140–150.
25. **Meyerowitz, E. M., G. M. Guild, L. S. Prestidge, and D. S. Hogness.** 1980. A new cosmid vector and its use. Gene **11**:271–282.
26. **Morrison, D. A.** 1977. Transformation in *Escherichia coli*: cryogenic preservation of competent cells. J. Bacteriol. **132**:349–351.
27. **Nagahari, K., and K. Sakaguchi.** 1978. RSF1010 plasmid as a potentially useful vector in *Pseudomonas* species. J. Bacteriol. **133**:1527–1529.
28. **Olsen, R. H., G. DeBusscher, and W. R. McCombie.** 1982. Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. J. Bacteriol. **150**:60–69.
29. **Priefer, U.** 1983. Isolation of plasmid DNA, p. 14–25. *In* A. Pühler and K. N. Timmis (ed.), Advanced molecular genetics. Springer Verlag, New York.
30. **Priefer, U.** 1983. Characterization of plasmid DNA by agarose gel electrophoresis, p. 26–37. *In* A. Pühler and K. N. Timmis (ed.), Advanced molecular genetics. Springer Verlag, New York.
31. **Priefer, U., R. Simon, and A. Pühler.** 1983. Cloning with

cosmids, p. 190–202. *In* A. Pühler and K. N. Timmis (ed.), Advanced molecular genetics. Springer Verlag, New York.
32. **Sakaguchi, K.** 1982. Vectors for gene cloning in *Pseudomonas* and their application. Curr. Top. Microbiol. Immunol. **96**:31–45.
33. **Simon, R.** 1984. High frequency mobilization of gram-negative bacterial replicons by the in vitro constructed Tn*5*-Mob transposon. Mol. Gen. Genet. **196**:413–420.
34. **Simon, R., U. Priefer, and A. Pühler.** 1982. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Biotechnology **1**:784–791.
35. **Tait, R. C., T. J. Close, R. C. Lunquist, M. Hagiya, R. L. Rodriguez, and C. I. Kado.** 1983. Construction and characterization of a versatile broad host range cloning system for gram-negative bacteria. Biotechnology **1**:269–275.
36. **Van Vliet, F., B. Silva, M. Van Montagu, and J. Schell.** 1978. Transfer of RP4::Mu plasmids to *Agrobacterium tumefaciens*. Plasmid **1**:446–455.
37. **Willets, N., and C. Crowther.** 1981. Mobilization of the nonconjugative IncQ plasmid RSF1010. Genet. Res. **37**:311–316.
38. **Wood, D. O., M. F. Hollinger, and M. B. Tindol.** 1981. Versatile cloning vector for *Pseudomonas aeruginosa*. J. Bacteriol. **145**:1448–1451.
39. **Wood, W. B.** 1966. Host specificity of DNA produced by *Escherichia coli*: bacterial mutations affected the restriction and modification of DNA. J. Mol. Biol. **16**:118–133.