JOURNAL OF BACTERIOLOGY, Nov. 1990, p. 6557–6567
0021-9193/90/116557-11$02.00/0

Vol. 172, No. 11

Transposon Vectors Containing Non-Antibiotic Resistance Selection Markers for Cloning and Stable Chromosomal Insertion of Foreign Genes in Gram-Negative Bacteria

MARTA HERRERO, VICTOR DE LORENZO, AND KENNETH N. TIMMIS*

GBF-National Research Centre for Biotechnology, Braunschweig, Federal Republic of Germany

Received 16 April 1990/Accepted 25 July 1990

A simple procedure for cloning and stable insertion of foreign genes into the chromosomes of gram-negative eubacteria was developed by combining in two sets of plasmids (i) the transposition features of Tn*10* and Tn*5*; (ii) the resistances to the herbicide bialaphos, to mercuric salts and organomercurial compounds, and to arsenite, and (iii) the suicide delivery properties of the R6K-based plasmid pGP704. The resulting constructions contained unique *Not*I or *Sfi*I sites internal to either the Tn*10* or the Tn*5* inverted repeats. These sites were readily used for cloning DNA fragments with the help of two additional specialized cloning plasmids, pUC18Not and pUC18Sfi. The newly derived constructions could be maintained only in donor host strains that produce the R6K-specified π protein, which is an essential replication protein for R6K and plasmids derived therefrom. Donor plasmids containing hybrid transposons were transformed into a specialized λ*pir* lysogenic *Escherichia coli* strain with a chromosomally integrated RP4 that provided broad-host-range conjugal transfer functions. Delivery of the donor plasmids into selected host bacteria was accomplished through mating with the target strain. Transposition of the hybrid transposon from the delivered suicide plasmid to a replicon in the target cell was mediated by the cognate transposase encoded on the plasmid at a site external to the transposon. Since the transposase function was not maintained in target cells, such cells were not immune to further transposition rounds. Multiple insertions in the same strain are therefore only limited by the availability of distinct selection markers. The utility of the system was demonstrated with a kanamycin resistance gene as a model foreign insert into *Pseudomonas putida* and a melanin gene from *Streptomyces antibioticus* into *Klebsiella pneumoniae*. Because of the modular nature of the functional parts of the cloning vectors, they can be easily modified and further selection markers can be incorporated. The cloning system described here will be particularly useful for the construction of hybrid bacteria that stably maintain inserted genes, perhaps in competitive situations (e.g., in open systems and natural environments), and that do not carry antibiotic resistance markers characteristic of most available cloning vectors (as is currently required of live bacterial vaccines).

The growing number of biotechnological applications involving the introduction into the environment of genetically modified microorganisms has highlighted the need for new cloning vectors. Such vectors need to satisfy several criteria that are less important where the organisms are contained and grown under controlled conditions. The most important of these are (i) stable inheritance of the engineered functions in the absence of selective pressure for a vector marker, (ii) a level of expression of the engineered functions that is not so high that it constitutes a selective disadvantage to the organism, particularly when competing with indigenous organisms in a target habitat, and (iii) lack of resistance to antibiotics.

Most currently available gene cloning vectors for bacteria are based on multicopy plasmids carrying antibiotic resistance selection markers. Although hybrid plasmids may be readily maintained in bacteria cultivated as monocultures under controlled environmental conditions, they are frequently unstable when host organisms are grown in the absence of selection pressure for plasmid maintenance, i.e., in the presence of antibiotics. This instability may be exacerbated when host bacteria have to compete with other organisms in natural niches. Furthermore, antibiotic resistance markers are generally perceived to be undesirable in organisms designed for release in large quantities, such as live vaccines or bacteria developed to degrade chemical pollutants. It is of course possible to increase the stability of plasmids in the absence of antibiotic pressure by using autoselection cloning vectors (30) or plasmids carrying the stabilizing *parB* (*hok/sok*) locus of R1 (13). However, strains that carry multicopy plasmids and express at a high level genes present on the insert and/or the vector often compete poorly with other bacteria already present in their environment. It is frequently preferable in some circumstances to insert cloned genes into the chromosome, where they are maintained at a low, often natural copy number and thus are not overexpressed and where, at least theoretically, they should be as stable as chromosomal genes. When transposons are used for cloning and insertion, the stability of the inserts is improved if the cognate transposase gene is located on the transposon donor replicon but outside of the inverted repeats that are its substrate, such that it is not carried with the transposon during transposition (4). Very stable insertions can in this way be obtained with elements derived, for instance, from Tn*7* (3, 12), Tn*9* (17), and Tn*10* (44) and potentially from Tn*5* as well (4, 5).

In *Escherichia coli*, the classical method for generating chromosomal inserts of foreign genes involves the use of specialized λ phage cloning vectors that can exist stably in the lysogenic state (36). Alternatively, genes can be inserted through homologous recombination when bracketed with *E. coli* chromosomal sequences (33) or by transposition if the genes can be cloned in the permissive sites of a transposon

* Corresponding author.

(15). For gram-negative bacteria other than *E. coli*, analogous procedures have been developed. The use of ColE1-derived vectors carrying a conjugal transfer (Mob) sequence is a standard way to introduce transposons into a variety of soil bacteria (38), where such vectors cannot replicate. Suicide transposon donors can also be based on unstable (thermosensitive or other) replicons or the use of specialized phage-host schemes (27). In general, most available vectors for insertion of DNA fragments in the chromosomes of soil bacteria have limited versatility with regard to their useful cloning sites, consist of the full transposon and are therefore large and unwieldy and have the numerous disadvantages of the presence of the cognate transposase, and carry selection markers based on resistances to antibiotics of clinical importance.

In this paper we report a general system for the generation of stable chromosomal inserts of foreign genes in a variety of gram-negative bacteria without the use of such antibiotics. This system is based on (i) the use of resistances to the herbicide bialaphos, to mercuric salts and organomercuric compounds, and to arsenite as selection markers; (ii) the transposition features of Tn*5* and Tn*10*, and (iii) the utilization of the general suicide delivery properties of plasmid pGP704 (29). With the system described here, DNA fragments can be stably inserted into the chromosome of target gram-negative bacteria, provided that the strain is sensitive to such markers, is able to act as recipient of RP4-mediated conjugal transfer, and can support Tn*5* and/or Tn*10* transposition.

MATERIALS AND METHODS

Strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. Phage λ1105 containing the mini-Tn*10* kanamycin transposon (44) was a gift of N. Kleckner. Phage λ*pir* (20) was obtained after spontaneous induction of *E. coli* SY327(λ*pir*) and subsequent lytic growth on *E. coli* LE392 as described previously (36). λ*pir* lysogens of *E. coli* CC118 and MV1190 were generated by infection with a phage lysate (36), and the resulting λ-insensitive colonies were tested for their ability to support *pir*-dependent replication of pGP704. The resulting strains, *E. coli* CC118(λ*pir*) and *E. coli* MV1190(λ*pir*), showed high transformation efficiencies and were used as the routine strains for maintenance of the *pir*-dependent recombinant plasmids. Solid and liquid LB media (28) were supplemented, when required, with 150 μg of ampicillin per ml, 50 to 75 μg of kanamycin per ml, 50 μg of rifamycin per ml, or 22 μg of piperacillin per ml (16). M63 minimal medium (28) was supplemented with 0.2% glucose or citrate as the carbon source and, when indicated, with 0.1% Casamino Acids. Low-phosphate, 121-salts minimal medium was prepared as described previously (22). The medium for the detection of melanin production (1) contained (per liter) 10 g of Bacto-Tryptone, 10 g of NaCl, 400 mg of tyrosine, 50 mg of $CuSO_4$, and 35 mg of isopropyl-β-D-thiogalactopyranoside. Purified bialaphos (phosphinotricin tripeptide [Ptt]) was the kind gift of T. Nagaoka (Meiji Seika Kaisha, Ltd., Yokohama, Japan).

Recombinant DNA techniques. Phage and plasmid DNA isolation, restriction endonuclease digestion, ligation, transformation, agarose electrophoresis, and other standard recombinant DNA techniques were carried out by published protocols (24). Restriction digestions followed directly by ligation were achieved with KGB buffer (26). Filling in of protruding ends was made with T4 DNA polymerase in the presence of the required deoxynucleoside triphosphates. Alternatively, fragments were made blunt ended by treatment with S1 nuclease (24). Synthetic adapters and other oligonucleotides were prepared on an Applied Biosystems automatic synthesizer. Site-directed mutagenesis to remove the single *Not*I site present in the IS*50* sequence of Tn*5* (2, 21) was made as described by Kunkel et al. (23) after a uracil-containing single-stranded DNA template was obtained from the corresponding sequence cloned in pUC119 (43). Chromosomal DNA from exconjugant *Pseudomonas putida* strains was prepared by a simplified lysis procedure with proteinase K-sodium dodecyl sulfate (34). Southern blots of digested chromosomal DNA on Biodyne nylon membranes (Pall, Glen Cove) were carried out (24) and were probed with restriction fragments labeled with [α-^{32}P]dCTP by using the nick translation kit of Bethesda Research Laboratories, Inc. After hybridization, blots were washed at high stringency (68°C, 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate).

Mobilization and transposition. Plasmids were transferred from donor strain *E. coli* SM10(λ*pir*) into the different target strains by mobilization with a filter mating technique. Filters with the mixture of donor and recipient strains in a 1:4 ratio were incubated for 8 h at 37°C on the surface of LB plates (with 50 μM isopropyl-β-D-thiogalactopyranoside in the case of Tn*10* derivatives). Cells were then suspended in 10 mM $MgSO_4$, and the appropriate dilutions were plated on selective medium (see below). Since the frequency of transfer cannot be measured with the delivery system used, the rate of transposition occurrence cannot be accurately calculated. Therefore, operational frequencies were estimated as the ratio of recipient cells that had received transposon markers versus the total number of recipients.

Selection of non-antibiotic markers. Conditions for the selection of *P. putida* KT2442 exconjugants acquiring the non-antibiotic markers used were as follows.

(i) Resistance to bialaphos. The mating mixture was suspended and diluted in 10 mM $MgSO_4$ as described above and directly plated out and grown at 37°C on M63 minimal medium containing either 100 μg of bialaphos per ml, 100 μM iron chelator 2,2′-bipyridyl and 0.2% sodium citrate [for nutritional counterselection of the donor *E. coli* SM10(λ*pir*) strain], or 0.2% glucose–0.1% Casamino Acids–100 μg of Ptt per ml–100 μM 2,2′-bipyridyl–50 μg/ml of rifamycin for antibiotic counterselection.

(ii) Resistance to mercury and organomercurial compounds. Hg^{2+}-resistant (Hg^r) exconjugants were selected also by direct plating of suspensions of the mating mixture and growth at 37°C on M63 medium with 0.2% glucose, 0.1% Casamino Acids, 1.5 μg of $HgCl_2$ per ml, and 50 μg of rifamycin per ml. Colonies grown on this selective medium were further checked for resistance to organomercurial compounds by patching on LB plates containing 8 to 10 μg of phenyl mercuric acetate per ml.

(iii) Resistance to arsenite. Before plating on selective medium for arsenite resistance (Ars^r), the mating mixture was subjected to phosphate starvation by overnight growth at 37°C in a liquid culture of minimal 121-salts, 0.2% sodium citrate, and 100 μM phosphate. Ars^r colonies were then selected at 37°C on plates with 121-salts, 0.2% sodium citrate, and 100 μM phosphate with 12 mM $NaAsO_2$ and 100 μM 2,2′-bipyridyl. Insufficient phosphate starvation before selection on arsenite selective plates resulted in the growth of numerous artifactual small colonies that did not maintain the resistance phenotype upon reisolation. These were easily

TABLE 1. Bacteria, phage, and plasmids

Species or plasmid	Description	Reference or source
E. coli		
SY327(λ*pir*)	Δ(*lac-pro*) *argE*(Am) *recA56 nalA* Rf^r(λ*pir*)	29
CC118	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*	25
CC118(λ*pir*)	As CC118, lysogenized with λ*pir* phage	This study
MV1190	Δ(*lac-proAB*) *thi supE* Δ(*srl-recA*)*306*::Tn*10*(F′:*traD36 proAB lacI*^{q}*Z* ΔM15)	43
MV1190(λ*pir*)	As MV1190, lysogenized with λ*pir* phage	This study
LE392	*supF supE hsdR galK trpR metB lacY tonA*	36
CJ236	*dut-1 ung-1 thi-1 relA1* [pCJ105/Cm^r]	23
SM10(λ*pir*)	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu, Km^r, λ*pir*	29
P. putida KT2442	*hsdR*, Rif^r	27
K. pneumoniae 492	Prototrophic, mccE492$^+$	9
λ1105	*Eco*RI fragment with mini-Tn*10*-Km/p*tac* IS*10*$_R$ in λRP167	44
Plasmids		
pUC9, pUC18, pUC19	Ap^r; α-*lac*/MCS[a]	46
pUC119	Ap^r; α-*lac*/MCS pUC19, M13 *ori*	43
p18Sfi	Ap^r; identical to pUC18 but with *Sfi*I-*Eco*RI-*Sal*I-*Hin*dIII-*Sfi*I as MCS (*Sfi*I site sequence of Fig. 6)	This study
p18Not	Ap^r; identical to pUC18 but with *Not*I-*Eco*RI-*Sal*I-*Hin*dIII-*Not*I as MCS	This study
pUC18Sfi	Ap^r; identical to pUC18 but with *Sfi*I-polylinker of pUC18-*Sfi*I as MCS (*Sfi*I site sequence of Fig. 6)	This study
pUC18Not	Ap^r; identical to pUC18 but with *Not*I-polylinker of pUC18-*Not*I as MCS	This study
pDB37	Ap^r; p*iucA′-′lacZ*, aerobactin promoter as a 78-bp *Eco*RI-*Bam*HI restriction fragment	10
pMJR1560	Ap^r; *lacI*^{q} inserted between *Kpn*I and *Pst*I sites of pUC18	39
pBG195	Ap^r; *bar* gene inserted in pUC19 as a 2-kb *Bam*HI fragment	42
pHG106	Ap^r; broad-spectrum *mer* genes of pDU1358 inserted as a 4.2-kb *Hin*dIII fragment in pBR322	14
pUM3	Ap^r; *ars* genes of R773 inserted as a 4.3-kb *Hin*dIII fragment in pBR322	7
pGP704	Ap^r; *ori* R6K, *mob* RP4, MCS of M13tg131	29
pTet	Ap^r Tet^r; *tnp* gene of Tn*5*-IS*50*$_R$ inserted as a 1.5-kb *Sal*I fragment in a pBR322-derived replicon	11
pJOE810	Ap^r; *mel* genes inserted in a pBR322-derived replicon	1
pUT	Ap^r; *tnp** gene of Tn*5*-IS*50*$_R$ inserted in *Sal*I site of pGP704	This study
pBOR7	Ap^r; *Bam*HI-to-*Pst*I sites of pUC19 replaced by a single *Sfi*I site (*Sfi*I sequence of Fig. 6)	This study
pBOR8	Ap^r; *lacI*^{q} gene of pMJR1560 inserted as a 1.2-kb *Pst*I-*Sac*I fragment in pGP704	This study
pBOR10	Ap^r; Ptt^r cassette of Fig. 1 as an *Sfi*I insert in pBOR7	This study
pBOR11	Ap^r; Hg^r cassette of Fig. 1 as an *Sfi*I insert in p18Sfi	This study
pBOR12	Ap^r; Ars^r cassette of Fig. 1 as an *Sfi*I insert in pUC18Sfi	This study
pLOFKm	Ap^r; Tn*10*-based delivery plasmid with Km^r	This study
pLOFPtt	Ap^r; Tn*10*-based delivery plasmid with Ptt^r	This study
pLOFHg	Ap^r; Tn*10*-based delivery plasmid with Hg^r	This study
pLOFArs	Ap^r; Tn*10*-based delivery plasmid with Ars^r	This study
pLOFPttKm	Ap^r; same as pLOFPtt with a Km^r gene at the *Not*I site	This study
pLOFHgKm	Ap^r; same as pLOFHg with a Km^r gene at the *Not*I site	This study
pLOFArsKm	Ap^r; same as pLOFArs with a Km^r gene at the *Not*I site	This study
pUTKm	Ap^r; Tn*5*-based delivery plasmid with Km^r	This study
pUTPtt	Ap^r; Tn*5*-based delivery plasmid with Ptt^r	This study
pUTHg	Ap^r; Tn*5*-based delivery plasmid with Hg^r	This study
pUTArs	Ap^r; Tn*5*-based delivery plasmid with Ars^r	This study
pUTPttKm	Ap^r; same as pUTPtt with a Km^r gene at the *Not*I site	This study
pUTHgKm	Ap^r; same as pUTHg with a Km^r gene at the *Not*I site	This study
pUTArsKm	Ap^r; same as pUTArs with a Km^r gene at the *Not*I site	This study
pABOR70	Ap^r; Ptt^r/Tn*10*-based *mel* delivery vector	This study

[a] MCS, Multiple cloning site.

differentiated from authentic resistant colonies (either spontaneous or those carrying the *ars* genes), which were larger.

To discriminate between authentic transposition and cointegration events, exconjugants were examined in all cases for loss of the *bla* marker of the delivery vector by checking sensitivity to piperacillin (16).

RESULTS AND DISCUSSION

Use of non-antibiotic selection markers as cloning tools. Gram-negative strains engineered for a variety of in situ applications must meet a number of practical requirements for their safe and efficacious performance. These include not only the absence of traits that may give them an advantage over nonengineered strains but also the capability to retain the acquired genotype and phenotype in the absence of selection. Moreover, despite the widespread occurrence of multiple antibiotic resistances, the release of antibiotic-resistant organisms into the environment is often perceived as undesirable. To address this concern, we surveyed potential dominant markers that would allow the genetic engineering of species of genera like *Pseudomonas* without the use of clinical antibiotics or nutritional selection. One feature we

required of such markers is that the rate of spontaneous tolerance to the selective agent should be relatively high so that the marker would not confer a significant selective advantage to the engineered organism if introduced into the environment. Resistances to some heavy metals (37) and herbicides (18) were considered likely to meet our requirements. Three such resistances were adapted into selection markers of cassettes for two novel transposon-based systems that afford stable chromosomal insertions. We used the universal suicide donor properties of plasmid pGP704 (29; J. J. Mekalanos, personal communication) to construct delivery plasmids for Tn*10*- and Tn*5*-based transposon vectors as explained below. The construction of the cassettes and of the cloning and delivery systems involved multiple intermediate steps, which, for the sake of clarity, will be only outlined (a more detailed description is available upon request).

Construction of a bialaphos resistance cassette. Bialaphos is a tripeptide nonselective herbicide consisting of phosphinotricin (an antimetabolite of L-glutamic acid) and two L-alanine residues. After removal of the alanine moieties in vivo by peptidases, phosphinotricin is released intracellularly, where it inhibits the enzyme glutamine synthetase of species ranging from bacteria to plants (8, 40, 42, 45). The *bar* gene from bialaphos-producing *Streptomyces hygroscopicus* encodes a phosphinotricin acetylase that confers immunity to the herbicide and that can be expressed in *E. coli* if transcribed efficiently (42). The *bar* structural gene was initially excised as a 770-bp *Mlu*I-*Stu*I restriction fragment of pBG195 (42). To achieve expression of the *bar* gene in a variety of gram-negative bacteria, we placed its transcription under the iron-regulated aerobactin promoter of pColV-K30 (10). The resulting unit (approximately 900 bp) was modified by the addition of *Not*I and *Xba*I sites downstream of the *bar* gene and finally flanked by *Sfi*I linkers (Fig. 1).

Construction of a mercury-organomercurial compound resistance cassette. The mercury-organomercurial compound resistance determinant forms part of the broad-spectrum *mer* operon of plasmid pDU1358 originally isolated from *Serratia marcescens* (14). A 4.3-kb *Hin*dIII restriction fragment from pDU1358 cloned into pBR322 (pHG106) affords constitutive resistance to mercuric salts and to a variety of organomercurial compounds like phenyl mercuric acetate (14). A 3-kb *Hin*dIII-*Eco*RV fragment of pHG106 containing the intact *mer* TPAB genes and their native promoter (31) was cloned into *Hin*dIII-*Hin*cII-digested p18Sfi vector (Table 1). This resulted in the cloned fragment becoming flanked by *Sfi*I sites (Fig. 1).

Construction of an arsenite resistance cassette. The origin of the arsenite resistance marker is the enterobacterial plasmid R773. A 4.3-kb *Hin*dIII fragment cloned in pBR322 (pUM3) confers arsenite and arsenate resistance when expressed through the *tet* promoter of the vector (7). This fragment, containing the *arsABC* genes, was first placed downstream of the aerobactin promoter. A smaller fragment containing the *arsAB* structural genes (which determine only resistance to arsenite [6, 7, 35]) was subsequently cloned between the *Eco*RI and *Kpn*I sites of pUC18Sfi, resulting in a resistance cassette bracketed by *Sfi*I sites (Fig. 1).

Tn*10*-based transposon vector delivery systems. Phage λ1105, a derivative of λRP167, carries a 5.1-kb *Eco*RI insert containing the mini-Tn*10*Km element and the transposase gene of $IS10_R$ located outside the inverted repeats of the mobile element and downstream of the p*tac* promoter (44). To obtain a transposon delivery plasmid with a host-independent regulation of its transposition, the *Eco*RI fragment

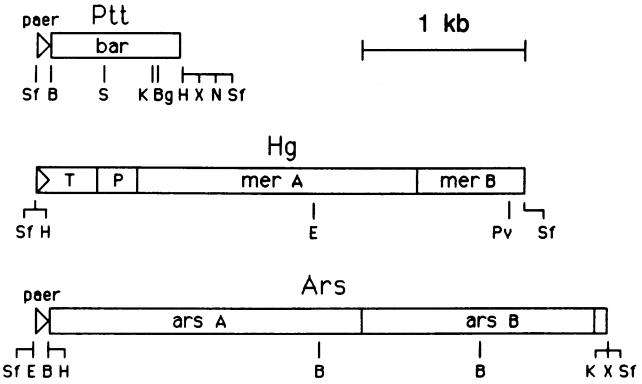

FIG. 1. Structure of the three selection marker cassettes. The *bar* gene of *S. hygroscopicus* conferring resistance to bialaphos (Ptt), part of the broad-spectrum *mer* cluster of the *S. marcescens* plasmid pDU1358, and two of the four *ars* genes of the enterobacterial plasmid R773 were used as the basis for construction of the *Sfi*I restriction fragments shown. The triangles indicate the directions of transcription of the corresponding genes, which are driven by either a native promoter (triangle internal to the box) or by a heterologous aerobactin promoter (Paer, external triangle). Important restriction endonuclease sites are indicated: Sf, *Sfi*I; B, *Bam*HI; S, *Sal*I; K, *Kpn*I; Bg, *Bgl*II; H, *Hin*dIII; X, *Xba*I; N, *Not*I; E, *Eco*RI; Pv, *Pvu*I. The Ptt^r *Sfi*I restriction fragment was inserted into the single *Sfi*I site of pBOR7 to produce pBOR10, the Hg^r *Sfi*I cassette was cloned in vector p18Sfi to produce pBOR11, and the Ars^r *Sfi*I element was cloned in pUC18Sfi to produce pBOR12 (Table 1). The *Eco*RI site originally present in the p18Sfi vector was lost during the construction of pBOR11.

was ligated to pBOR8, a derivative of pGP704 containing the *lacI*^{q} gene from plasmid pMJR1560 (39) (Fig. 2). This plasmid is unable to replicate in host strains devoid of the R6K-specified π protein product of the *pir* gene. pGP704 contains the conjugal transfer origin (*oriT* sequence) of the RP4 plasmid and can therefore be transferred to a variety of gram-negative bacteria when provided in *trans* with mobilization (Mob) functions (29). The *Mlu*I fragment internal to the inverted repeats containing the original kanamycin resistance gene of the mini-Tn*10* was replaced by a fragment containing the *Sfi*I-Ptt cassette shown in Fig. 1, appropriately modified by the addition of the *Not*I site and *Mlu*I adaptors, which produced pLOFPtt (Fig. 3). This construction has unique *Sfi*I, *Not*I, and *Xba*I cloning sites between the mini-Tn*10* inverted repeats. The Ptt resistance marker (Ptt^r) of pLOFPtt was subsequently exchanged by kanamycin, Hg^{2+}-phenyl mercuric acetate, and arsenite resistances to produce plasmids pLOFKm, pLOFHg, and pLOFArs, respectively (Fig. 3).

A common structural feature of these vectors is the presence of a sequence of *Mlu*I-*Sfi*I-*Not*I-*Mlu*I sites between the inverted repeats of the transposable element. This sequence, originally generated by the insertion of a synthetic adapter, was devised to provide two infrequent restriction sites (*Sfi*I and *Not*I), which could be used for the cloning of foreign DNA fragments and introduction of a selection determinant, respectively. Constructions devoid of an insert between the inverted repeats were inviable in our hands. Therefore, except for in pLOFPtt, which has the two sites available for cloning, we inserted a selection marker into one of the two sites while leaving the other free for cloning purposes, a task facilitated by the intermediate cloning plasmids described below. The interchange of selection markers was easily effected because of these design features.

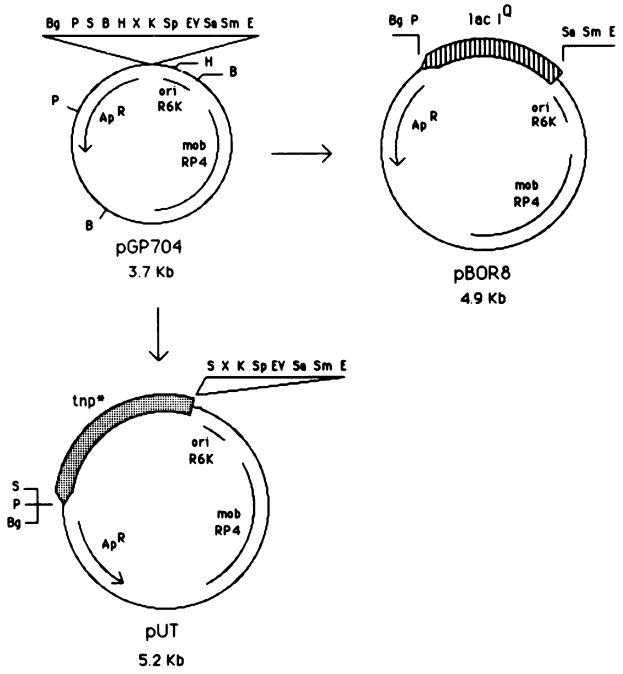

FIG. 2. Construction of delivery plasmids for Tn*10*- and Tn*5*-derived transposon vectors. The two plasmids used as suicide donors for the transposon vectors described in this work are derived from pGP704 (29). A 1.2-kb *Pst*I-*Sac*I restriction fragment from pMJR1560 containing the *lacI*^{q} gene was cloned between corresponding sites in pGP704 to yield pBOR8, the delivery system used for the Tn*10*-based vectors (Fig. 3). Similarly, a 1.5-kb *Sal*I fragment from pTet (9) containing a Tn*5* *tnp* gene devoid of *Not*I sites (*tpn**) was inserted into the single *Sal*I site of pGP704. The resulting construction (pUT) was the delivery system used for the Tn*5*-derived series of vectors (see Fig. 7 and the text for explanations). The approximate locations of the *bla* gene (Ap^r), which confers resistance to a variety of β-lactam antibiotics, including piperacillin (14), the R6K origin of replication (oriR6K), and the RP4 *oriT* region (mobRP4) are indicated. The points on the filled inserts indicate the transcription directions of the corresponding genes. Restriction sites: B, *Bam*HI; S, *Sal*I; K, *Kpn*I; Bg, *Bgl*II; H, *Hin*dIII; X, *Xba*I; N, *Not*I; E, *Eco*RI; P, *Pst*I; Sp, *Sph*I; EV, *Eco*RV; Sa, *Sac*I; Sm, *Sma*I.

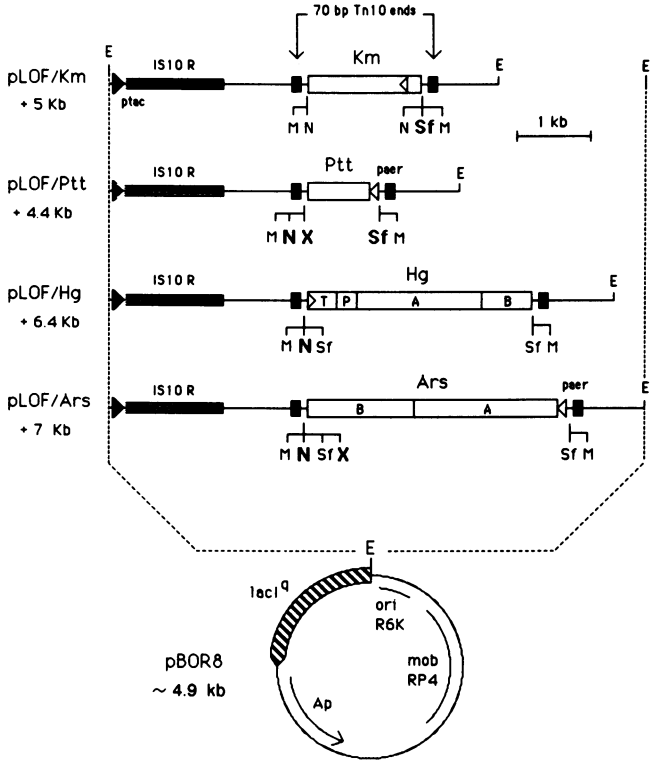

FIG. 3. Structure of the Tn*10*-based transposon vector delivery plasmids. The common portion of the constructions corresponding to the delivery plasmid pBOR8 (Fig. 2) is shown at the bottom. The elements of the transposition system (IS*10*$_R$ transposase in *cis* driven by the *ptac* promoter and the Tn*10* inverted repeats that are its substrate [44]) are drawn to scale with respect to the restriction map of the *Eco*RI inserts. The resistance genes located in each of the transposons and the size of such *Eco*RI inserts are indicated with their names to the left. The first plasmid, pLOFKm, was used throughout this work as a control mini-transposon donor with a standard antibiotic resistance (kanamycin). Restriction sites important for cloning or for generating other transposable derivatives are indicated: M, *Mlu*I; X, *Xba*I; N, *Not*I; E, *Eco*RI; Sf, *Sfi*I. Unique sites available for the insertion of foreign DNA fragments to be transposed to the chromosome are indicated in boldface type.

To investigate the utility of the plasmids shown in Fig. 3 as vehicles for inserting DNA fragments into bacterial chromosomes, we used the constructions indicated in Fig. 4, which carry a Km^r indicator gene inserted at the single *Not*I site of the pLOF vectors. pLOFKm, pLOFPttKm, pLOFHgKm, and pLOFArsKm were then transformed into the donor strain *E. coli* SM10(λ*pir*) and mated with *P. putida* KT2442. Exconjugants that had acquired the non-antibiotic selection markers were then scored for the Km^r phenotype and loss of the delivery plasmid marker (piperacillin resistance [Pip^r]). The frequencies of these events are described in Table 2. In all cases, the selection markers were correlated with the Km^r Pip^s phenotype. In our hands, resistance to mercuric salts in minimal-citrate medium gave the cleanest selection (provided that the concentration of $HgCl_2$ had been optimized), whereas resistance to Ptt selected in a minimal-glucose medium gave insertions at frequencies close to the spontaneous appearance of Ptt-tolerant colonies. The frequency of Ptt tolerance could, however, be decreased when selection was made on minimal-citrate medium. Resistance to arsenite was also an efficient selective marker when the conditions indicated in Materials and Methods were rigorously followed.

To ascertain whether transpositions were to different locations of the chromosome, whether they were single or multiple events, and whether the transposase gene was indeed lost after transposition, we carried out Southern blot analyses of chromosomal DNA from eight independent *P. putida* Ptt^r Km^r Pip^s exconjugants arising from the *E. coli* SM10(λ*pir*)(pLOFPttKm) × *P. putida* KT2442 mating (Fig. 5). In each case, chromosomal DNA was digested with either *Mlu*I, which excised the whole region bracketed by the Tn*10* inverted repeats as a 2.4-kb fragment, or *Xho*I, which cuts at a unique site in the hybrid transposon. The blots were then hybridized to either an insert probe or a transposase probe. The autoradiographs of Fig. 5 clearly show that (i) the phenotype acquired by the exconjugants is due to the insert originally present on the donor plasmid pLOFPttKm, (ii) only single insertions occurred in each of the exconjugants, (iii) all insertions analyzed were at distinct locations in the chromosome (the possible existence of hot spots was thus not further investigated), and (iv) the *tnp*

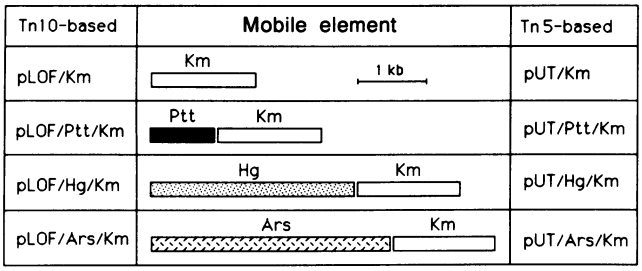

FIG. 4. Model delivery plasmids for the selection of chromosomal insertions. The drawing shows the organization of the inserts bracketed by either the Tn*10* or Tn*5* inverted repeats present in the pLOF (Fig. 3) and pUT (Fig. 7) series to which an indicator kanamycin resistance gene was added at their single *Not*I sites (pLOFKm and pUTKm were used as controls). The names of the resulting plasmids are indicated. Each construction was transformed into the donor strain *E. coli* SM10(λ*pir*) and subsequently mated with *P. putida* KT2442 as described in Materials and Methods. Transconjugants were selected on the basis of the non-antibiotic resistance marker and then scored for the Km^r phenotype and for loss of delivery system marker (Table 2).

transposase gene was not present in exconjugants. Similar analyses with the other vectors were not made, although they are assumed to behave identically.

To our knowledge, this is the first report of a Tn*10*-based transposition system shown to be functional in *P. putida* at low but workable frequencies. Whether its transposition mechanism is identical to that in *E. coli* (19) deserves further study.

We have used this system to introduce foreign inserts of up to 10 kb into the chromosome of *P. putida* (de Lorenzo, unpublished data). Although the upper size limit of the inserts has not been systematically studied, it is to be expected that transposition frequencies will decrease with increasing size of insert. However, since only a few insertions (perhaps only one) may be required to generate a desired hybrid, low transposition frequencies for large inserts may not be problematic for most purposes. An exhaustive survey of the performance of the transposition system with the different non-antibiotic markers in various gram-negative bacteria was not carried out, although they have behaved as expected in the examples examined so far.

Tn*5*-based transposon vector delivery systems. The concepts underlying the design of the preceding Tn*10*-based system were used and further expanded in the construction of equivalent Tn*5*-based transposon cloning vectors. The use of a Tn*5*-based system carrying non-antibiotic selection markers has at least two advantages: (i) Tn*5* is the transposon with the broadest host range known (4, 5), and (ii) the inverted repeats flanking the transposable element are as short as 19 bp (32). These features provided several possibilities for engineering that are hindered in the case of Tn*10* (see below).

For the construction of the delivery plasmids, two synthetic 64-mers containing the Tn*5* inverted repeats were hybridized to obtain the adaptor shown in Fig. 6, with the configuration *Xba*I-I end-*Sfi*I-*Not*I-O end-*Eco*RI. The adaptor was directly inserted into the corresponding sites of pGP704, and a kanamycin resistance gene was introduced as a *Not*I fragment in the resulting plasmid. Before adding the Tn*5*-specific transposase gene, we removed the single *Not*I site present in the middle of the *tnp* gene of IS*50*$_R$ (2, 21). To do this, the *tnp* gene was cloned as a 1.5-kb *Sal*I fragment obtained from pTet (11) into the *Sal*I site of pUC119 (43). Uracil-containing single-stranded DNA of this construction (23, 43) was then hybridized to a synthetic 24-mer carrying a single mismatch that changed the GCG codon specifying Ala-168 of the *tnp* gene to the Ala codon GCC. This eliminated the *Not*I site without changing the structure of the *tnp* product. The modified transposase gene, devoid of *Not*I

TABLE 2. Transposition frequencies of hybrid transposons

System base	Donor plasmid[a]	Selection	Resistance frequency		Analysis of exconjugants (no. of colonies)			
			Spontaneous[b]	Induced[c]	Sample[d]	Spontaneous[e]	Cointegrates[f]	Transposition[g]
Tn*10*	pLOFKm	Km[h]	$<10^{-8}$	3×10^{-6}	100	ND	5	95
	pLOFPttKm	Ptt/gluc[i]	3×10^{-5}	4×10^{-5}	58	53	2	3
		Ptt/cit[j]	6×10^{-6}	1×10^{-5}	80	70	1	9
	pLOFHgKm	$HgCl_2$[k]	$<10^{-8}$	7×10^{-7}	48	0	12	36
	pLOFArsKm	$NaAsO_2$[l]	$<10^{-8}$	5×10^{-7}	20	2	2	16
Tn*5*	pUTKm	Km[h]	$<10^{-8}$	2×10^{-6}	50	ND	2	48
	pUTPttKm	Ptt/gluc[i]	3×10^{-5}	6×10^{-5}	58	33	0	25
		Ptt/cit[j]	6×10^{-6}	1×10^{-5}	116	87	1	28
	pUTHgKm	$HgCl_2$[k]	$<10^{-8}$	3×10^{-6}	36	2	0	34
	pUTArsKm	$NaAsO_2$[l]	$<10^{-8}$	2×10^{-6}	41	0	7	34

[a] Plasmids indicated were transformed into the donor strain *E. coli* SM10(λ*pir*), which carries RP4 mobilization determinants in its chromosome. Each donor was then mated with *P. putida* KT2442 (Rif^r) for 8 h and plated on selective medium as described in Materials and Methods. Plasmids pLOFKm and pUTKm were used as controls.

[b] The figures indicate the frequencies of spontaneous appearance of colonies resistant to the selective agent in recipient cells not mated with the transposon donor.

[c] Frequency of acquisition of resistance marker by the recipient cells after mating with the corresponding donor.

[d] After mating, the indicated numbers of independent colonies of the recipient strain resistant to the non-antibiotic selective agent (Ptt, Hg, or Ars) were scored for the presence of the accompanying Km^r indicator gene and for the loss of delivery plasmid marker *bla* (resistance to piperacillin).

[e] Number of Km^s colonies, defining the spontaneous mutants resistant to the selective agent. ND, Not determined.

[f] Number of Km^r Pip^r colonies resulting from cointegrate formation between the delivery plasmid and the chromosome.

[g] Number of Km^r Pip^s colonies produced by transposition of the mobile element.

[h] Selection on LB plates containing 50 μg of rifamycin per ml and 75 μg of kanamycin per ml.

[i] Selection on M63 plates containing 0.2% glucose, 50 μg of rifamycin per ml, 0.1% Casamino Acids, 100 μg of Ptt per ml, and 100 μM 2,2′-bipyridyl.

[j] Selection on M63 plates containing 0.2% citrate, 100 μg of Ptt per ml, and 100 μM 2,2′-bipyridyl.

[k] Selection on M63 plates containing 0.2% glucose, 50 μg of rifamycin per ml, 0.1% Casamino Acids, and 1.5 μg of $HgCl_2$ per ml.

[l] Selection on 121-salts plates containing 0.2% citrate, 100 μM P_i, 12 mM $NaAsO_2$, and 100 μM 2,2′-bipyridyl.

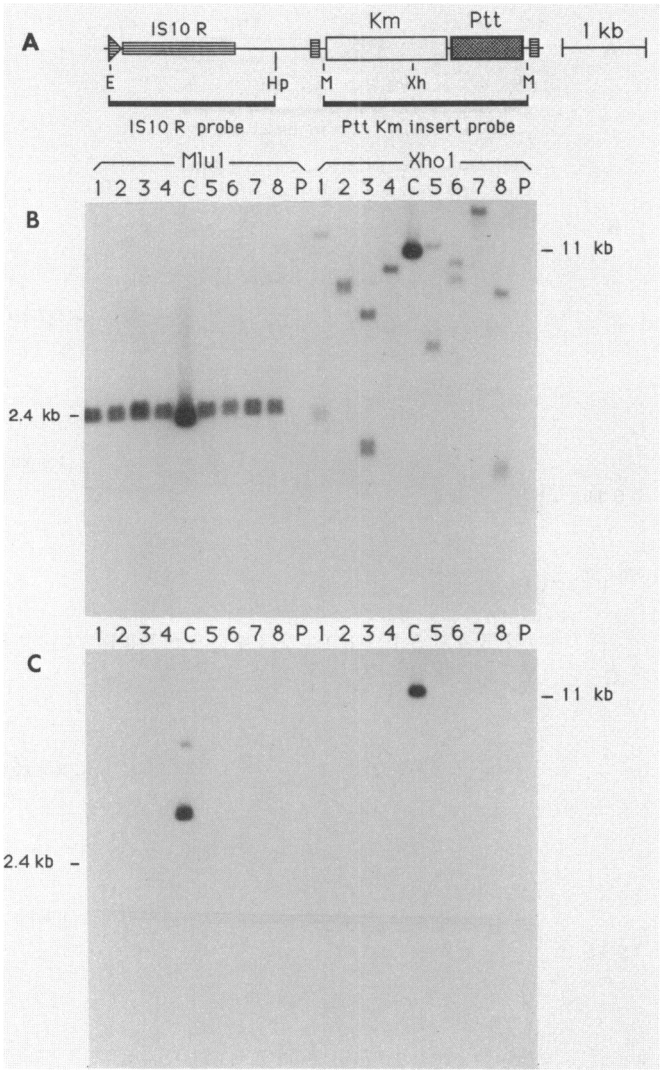

FIG. 5. Southern analysis of bialaphos-resistant *P. putida* transconjugants that acquired resistance to kanamycin after mating with *E. coli* SM10(λ*pir*)(pLOF PttKm). (A) Organization of the transposition-related elements contained in the donor plasmid pLOFPttKm and location of the restriction fragments used as probes in the hybridizations (E, *Eco*RI; Hp, *Hpa*I; M, *Mlu*I; Xh, *Xho*I). (B and C) The chromosomal DNA from eight *P. putida* transconjugants (numbered 1 to 8) with a phenotype Ptt^r Km^r Pip^s and that of the unmated strain (P) were digested to completion with *Mlu*I or *Xho*I and electrophoresed through an agarose gel; the gel was then sandwich replicated onto two nylon membranes. These were separately hybridized with either the insert probe (B) or a transposase probe (C). Lane C is a control containing donor plasmid pLOFPttKm digested with the indicated enzymes. The sizes of the fragments are indicated (bands from control digestions of CsCl-purified plasmid DNA run slightly faster than those of the chromosomal counterparts).

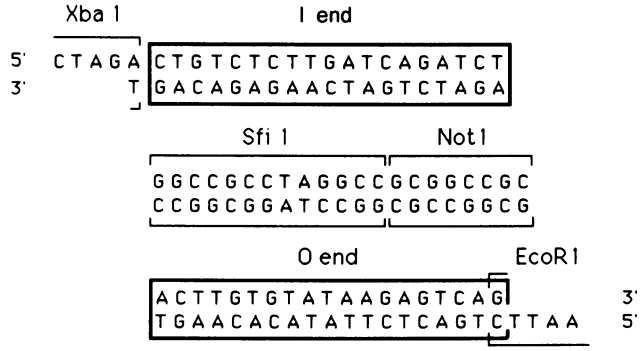

FIG. 6. Synthetic adaptor utilized for the engineering of the Tn*5*-based transposon vectors. The nucleotide sequence shown was designed to exploit the full potential of the Tn*5* transposition mechanism (5) for the introduction of foreign inserts into the chromosome of various gram-negative bacteria. The flanking restriction sites are adapted to fit the polylinker of pGP704 (Fig. 2). I and O 19-bp Tn*5* ends are devoid of transcriptional terminators and do not have translation stop codons in two of the three possible reading frames. This makes possible the utilization of the internal unique *Sfi*I and *Not*I sites for a variety of purposes, including the cloning of heterologous genes along with non-antibiotic resistance selection markers, as shown in this work, and also the utilization of the basic scheme for the in vivo construction of gene fusions (8a), a task facilitated by the auxiliary plasmids (Table 1) described in the text.

sites, is referred to below as *tnp** and was eventually cloned into the *Sal*I site of the pGP704 derivative in an orientation that was optimal for promoting transposition of the adjacent hybrid transposon (11). The corresponding construction is called pUTKm (Fig. 7).

As was the case with the Tn*10*-based system (see above), we were unable to generate stable plasmids lacking insertions between the inverted IS*50* repeats. The construction of the delivery vectors with the non-antibiotic resistance selection markers therefore involved plasmid intermediates containing inserts between these repeats. A detailed map of the final plasmids, the pUT series, is shown in Fig. 7. All have a single *Not*I site for cloning between the inverted repeats and *Sfi*I sites bracketing a selection marker, which can be exchanged for any other selection or reporter gene provided that it is inserted as an *Sfi*I fragment.

To investigate the utility of the pUT vectors for generating chromosomal insertions, we used the constructions indicated in Fig. 4, which carry a kanamycin resistance indicator gene in the single *Not*I site of the plasmids. *E. coli* SM10(λ*pir*) donor strains that had been transformed with pUTKm, pUTPttKm, pUTHgKm, and pUTArsKm were mated with *P. putida* KT2442, and transconjugants that had acquired the non-antibiotic selection markers were scored for Km^r and loss of the donor plasmid marker (Pip^r) (Table 2). As before, the selection markers were linked to Km^r Pip^s at acceptable frequencies. The use of the Tn*5*-based system improved the use of Ptt as selection marker and generally increased the frequency of authentic transposition versus that of cointegrate formation.

Figure 8 shows the results of the Southern blot analyses of chromosomal DNA from eight independent *P. putida* Ptt^r Km^r Pip^s transconjugants arising from the *E. coli* SM10 (λ*pir*)(pUTPttKm) × *P. putida* KT2442 mating. In this case the DNAs were digested with *Bam*HI-*Not*I, which excised the region bracketed by the Tn*5* inverted repeats as two fragments of 1.5 kb and 800 bp, or with *Xho*I, which cuts at the internal *Xho*I site. The blots were then hybridized with either an insert probe or a transposase probe. As observed in the Tn*10*-based counterpart, the Southern blots showed the presence of the insert delivered by the donor plasmid pUTPttKm, the loss of the transposase gene, the transposition of the element to different chromosomal locations, and the uniqueness of the insertion event.

Auxiliary plasmids. To simplify the transfer of cloned genes into the unique sites present in the pLOF and pUT plasmid series, four auxiliary plasmids were constructed

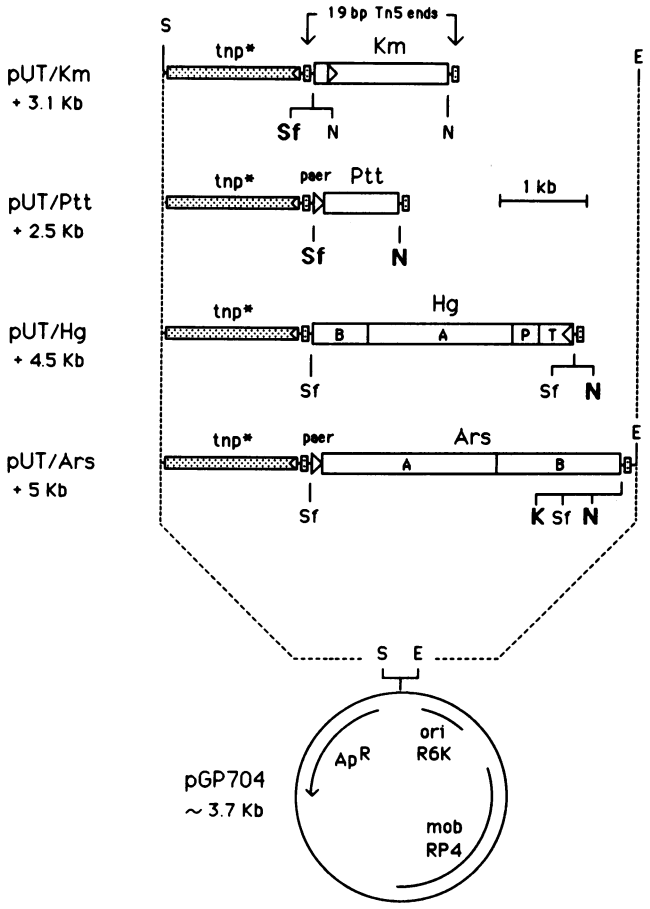

FIG. 7. Tn*5*-based insertion delivery plasmids. The drawing displays the Tn*5*-based counterparts of the vectors shown in Fig. 3. The common portion of the constructions corresponding to the delivery plasmid pGP704 (Fig. 2) is shown at the bottom. The elements of the transposition system include the Tn*5* 19-bp terminal ends and an $IS50_R$ *tnp* gene devoid of *Not*I sites (*tpn**) oriented divergently of the I end for optimal transposition efficiency (11). The resistances provided by each of the elements are also indicated with their names to the left of the figure. Plasmid pUTKm is the control minitransposon donor with a standard antibiotic resistance (kanamycin). Important restriction sites are indicated: N, *Not*I; E, *Eco*RI; Sf, *Sfi*I; S, *Sal*I; K, *Kpn*I. Unique sites for the insertion of foreign DNA fragments are indicated in boldface type.

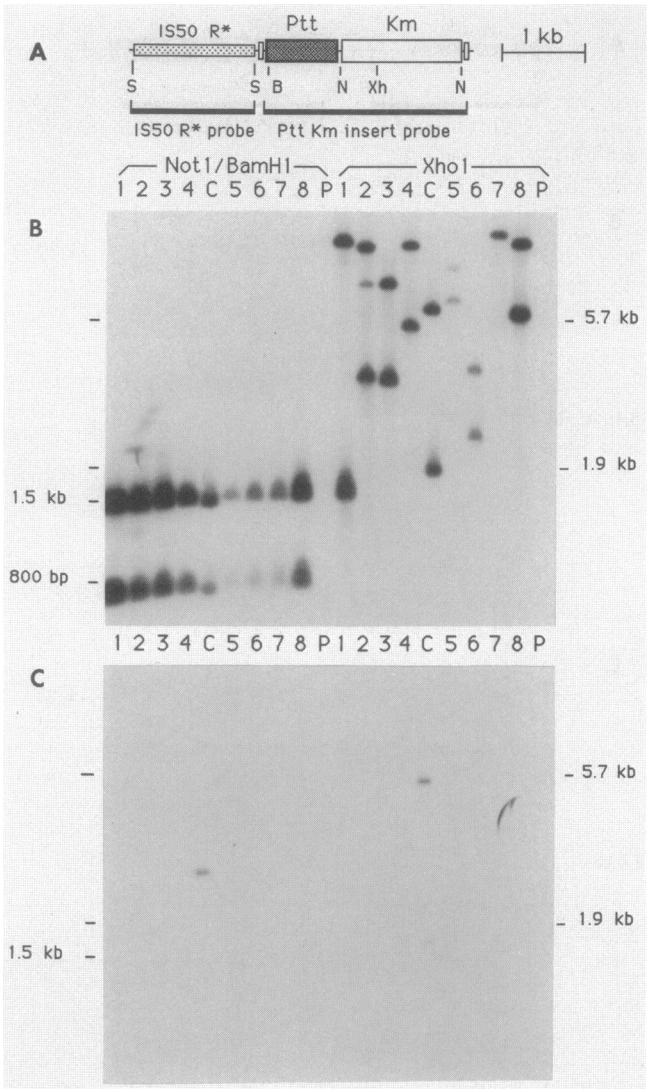

FIG. 8. Southern analysis of bialaphos-resistant *P. putida* transconjugants that acquired resistance to kanamycin after mating with *E. coli* SM10(λ*pir*)(pUTPttKm). Conditions and explanation for this figure are identical to those in the legend to Fig. 4, except the organization of the donor plasmid (A). Restriction sites: S, *Sal*I; B, *Bam*HI; N, *Not*I; Xh, *Xho*I. Digestions were made with *Not*I-*Bam*HI and *Xho*I, and the twin blots were hybridized to an insert probe (B) or to a transposase probe (C). Lanes C were loaded with the donor plasmid pUTPttKm digested with the enzymes indicated. In the upper gel, the extra band observed in the *Xho*I digestion in lane 2 might have arisen from incomplete restriction.

(Table 1). p18Not and p18Sfi are similar to pUC18 (46), but the polylinker region has been altered such that a short polylinker *Eco*RI-*Sal*I/*Hin*cII-*Hin*dIII is bracketed by two *Not*I sites and two *Sfi*I sites, respectively. On the other hand, pUC18Not and pUC18Sfi carry the complete pUC18 polylinker between, respectively, two *Not*I sites and two *Sfi*I sites. All plasmids retain the blue/white colony screening feature with 5-bromo-4-chloro-3-indolyl-β-D-galactoside, which is characteristic of the pUC plasmids (46), although the blue colonies containing p18Not and p18Sfi are paler than those of the original pUC18. With p18Not and pUC18Not, any gene cloned in the polylinker can be excised as a *Not*I fragment and transferred into the single *Not*I site of the pLOF and pUT delivery plasmids, provided that there are no additional *Not*I sites in the insert. Since *Not*I cleavage sequences are very infrequent, this will be the case in the majority of instances. Similarly, genes cloned in the polylinker of p18Sfi and pUC18Sfi can be excised as *Sfi*I fragments and transferred to the *Sfi*I sites of pLOF and pUT plasmids.

Applications of the pLOF and pUT delivery systems. The pLOF and pUT series have been developed as versatile tools for the genetic analysis and genetic engineering of a wide variety of gram-negative bacteria. The modular nature of the functional parts of the systems will permit the facile introduction of new selection markers as they become available and appropriately modified for specific purposes. Both series can be used as convenient broad-host-range mini-Tn*10* and mini-Tn*5* delivery systems to isolate stable insertion mutants of species for which procedures of transposition mutagenesis are poorly developed. For instance, we used pLOF-derived

Km^r and Ptt^r elements to mutagenize wild strains of *Klebsiella pneumoniae* (de Lorenzo, unpublished data). To our knowledge, with the exception of the specialized Tn*phoA* donor pRT733 (41), this is the first attempt to generalize a system based on the RP4-*oriT* and R6K/*pir* for the delivery of transposable elements to a wide range of bacteria. The availability of the functionally distinct Tn*10*- and Tn*5*-based vectors on the broad-transfer-range suicide delivery system should diminish problems of hot spots for insertion and transposon host range that may be encountered in some target organisms.

The principal application of the vectors is, however the insertion of foreign genes into the chromosome of target bacteria (e.g., our insertion of a melanin group of *Streptomyces antibioticus* into *K. pneumoniae* via a Tn*10* element; Fig. 9) to generate stable, non-antibiotic-resistant hybrids, particularly for environmental purposes, where selective pressure for maintenance of the cloned gene(s) is lacking, where overexpression of insert sequences may be undesirable in case it confers a selective disadvantage upon the host cell, and where predictability of behavior and function is important. The frequencies of spontaneous tolerance to the agents used for selection are not substantially below those of the transposition (Table 2). Although the non-antibiotic resistance selection markers developed are effective for laboratory manipulations, they should not endow hybrid strains with selective advantages in natural habitats under any environmental conditions that we can imagine. Mercury resistance is known to be widespread in nature and frequently encoded on transmissible plasmids. Mercury pollutants would thus act as a selective force for a variety of indigenous organisms and would not be expected to favor the engineered strains. Arsenite resistance is also widespread. Similarly, as we have shown here the frequency of spontaneous resistance to bialaphos is high (in the order of 10^{-5}; Table 2). Therefore, in soils treated with the herbicide, a significant population of indigenous tolerant organisms should assure that engineered organisms containing the Ptt marker have little selective advantage.

In the Ptt and Ars resistance cassettes (Fig. 1), the selection markers are located downstream of the iron-regulated aerobactin promoter of *E. coli* (10). As shown in this work, both markers are also effectively expressed in *P. putida*, but it is uncertain in this case whether they are transcribed from the aerobactin promoter. Addition of the iron chelator 2,2′-bipyridyl to selection plates does result in a reduction of background growth, but growth of resistant cells is unaffected by the iron content of the medium. This indicates either constitutive expression of the aerobactin promoter in *P. putida* or its overriding by readthrough transcription originating from chromosomal promoters. The absence of transcriptional terminators bracketting the inverted repeats of both types of transposons will permit the selection markers to be transcribed from appropriately oriented indigenous promoters (Fig. 9). In some cases, however, the introduction of termination signals upstream and/or downstream of the inserts could be desirable, as for example in cases where hybrid transposons are used to study transcriptional regulation in monocopy (B. Kessler, V. de Lorenzo, and K. N. Timmis, unpublished data). Introduction of appropriate terminators or other signals is of course facilitated by the availability of numerous restriction sites in the auxiliary plasmids.

An important aspect of pLOF and pUT delivery systems is that the transposase gene is not located within the transposon and thus that chromosomes carrying one hybrid transposon will tolerate further insertion events. This should afford successive insertions of foreign inserts in the same strain through the use of different selection markers. We have used this property to insert different portions of the control elements of the TOL plasmid catabolic operons into the chromosome of *P. putida* KT2442 to study their regulation in monocopy dosage (Kessler et al., unpublished data).

The availability in the auxiliary plasmids of two sets of polylinkers flanked by *Not*I or *Sfi*I sites simplifies the construction of specialized transposons for different purposes. This, combined with the lack of transcriptional-translational termination signals in the 19-bp Tn*5* inverted repeats, allows the generation of a whole variety of transposon cassettes for operon or gene fusions to reporter genes or regulatory signals (see, e.g., reference 8a).

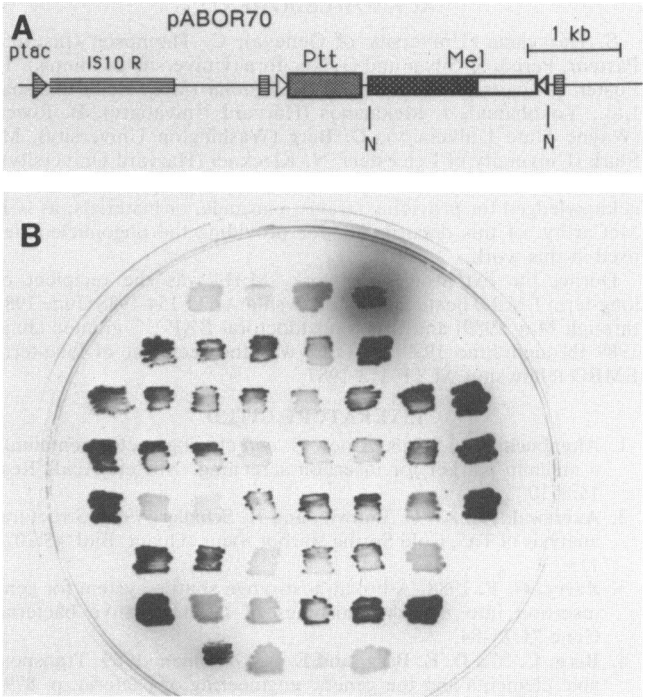

FIG. 9. Insertion and expression of melanin genes from *S. antibioticus* in *K. pneumoniae* by means of a transposon vector. The melanin genes in pJOE810 were excised as an approximately 2-kb *Ssp*I-*Pvu*II restriction fragment, converted into a *Not*I fragment, and cloned in the single *Not*I site internal to the Tn*10* inverted repeats of a bialaphos-based delivery system to yield pABOR70. (A) The relevant portion of the melanin donor plasmid is shown with its elements. (B) *E. coli* SM10(λ*pir*)(pABOR70) was mated with *K. pneumoniae* E492, and Ptt^r exconjugants were screened for melanin production. *E. coli* SM10(λ*pir*)(pABOR70), which carries the *mel* genes in multiple copies (and therefore overproduces melanin), was used as a positive control (first row, fourth patch). Unmated *K. pneumoniae* (first row, second patch) was introduced in the same plate as a negative control. Colony hybridization with the transposase probe of Fig. 5 indicated that 43 of 48 transconjugants lacked the *tpn* gene after acquisition of the Ptt^r Mel$^+$ phenotype (data not shown). Variations in melanin production are attributed to transcription from different chromosomal promoters located upstream of the newly inserted *mel* structural genes (originally read from a p*lac* promoter indicated as a triangle). Uneven pigmentation of some patches might have resulted from cross-feeding. Bialaphos resistance proved to be an excellent selection marker for insertional mutagenesis of the otherwise intractable strain *K. pneumoniae* (9).

ACKNOWLEDGMENTS

S. Harayama (University of Geneva), C. Thompson (Institute Pasteur, Paris), S. Silver and G. Nucifora (University of Illinois), T. Foster (University of Dublin), T. Nagaoka (Meiji Seika Kaisha, Ltd., Yokohama), J. Mekalanos (Harvard University), B. Rosen (Wayne State University), D. Berg (Washington University), M. Stark (University of Leicester), N. Kleckner (Harvard University), and J. Altenbuchner (University of Regensburg) are gratefully acknowledged for providing strains, plasmids, or materials, as is J. McCarthy, of this department, for providing the oligonucleotides used in this work.

During the extent of this work, M.H. was the recipient of long-term EMBO postdoctoral fellowship ALTF154-1988 (June 1988 through May 1989) and was a postdoctoral BAP-EC grantee (June 1989 through June 1990). V.D.L. was the recipient of long-term EMBO fellowship ALTF 113-1987.

LITERATURE CITED

1. **Altenbuchner, J.** 1988. A new *E. coli* cloning vector containing a melanin marker for insertion screening. Nucleic Acids Res. **16**:8710.
2. **Auerswald, E. A., G. Ludwig, and H. Schaller.** 1980. Structural analysis of Tn*5*. Cold Spring Harbor Symp. Quant. Biol. **45**:107–113.
3. **Barry, G. F.** 1988. A broad-host-range shuttle system for gene insertion into the chromosomes of Gram-negative bacteria. Gene **71**:75–84.
4. **Berg, C. M., D. E. Berg, and E. A. Groisman.** 1989. Transposable elements and the genetic engineering of bacteria, p. 879–926. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
5. **Berg, D. E.** 1989. Transposon Tn*5*, p. 185–210. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
6. **Chen, C., T. K. Misra, S. Silver, and B. P. Rosen.** 1986. Nucleotide sequence of the structural genes for an anion pump. The plasmid encoded arsenical resistance operon. J. Biol. Chem. **261**:15030–15038.
7. **Chen, C., H. L. T. Mobley, and B. P. Rosen.** 1985. Separate resistances to arsenate and arsenite (antimonate) encoded by the arsenical resistance operon of R factor R773. J. Bacteriol. **161**:758–763.
8. **De Block, M., J. Botterman, M. Vandewiele, J. Dockx, C. Thoen, V. Gossele, N. Rao Movva, C. Thompson, M. Van Montagu, and J. Leemans.** 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. **6**:2513–2518.

8a. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in grma-negative eubacteria. J. Bacteriol. **172**:6568–6572.

9. **de Lorenzo, V., J. L. Martínez, and C. Asensio.** 1984. Microcin mediated interactions between *Klebsiella pneumoniae* and *Escherichia coli* strains. J. Gen. Microbiol. **130**:391–400.
10. **de Lorenzo, V., S. Wee, M. Herrero, and J. B. Neilands.** 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. J. Bacteriol. **169**:2624–2630.
11. **Dodson, K. W., and D. E. Berg.** 1989. Factors affecting transposition activity of IS*50* and Tn*5* ends. Gene **76**:207–213.
12. **Drahos, D. J., B. C. Hemming, and S. McPherson.** 1986. Tracking recombinant organisms in the environment: β-galactosidase as a selectable non-antibiotic marker for fluorescent pseudomonads. Bio/Technology **4**:439–444.
13. **Gerdes, K.** 1988. The *parB* (*hok-sok*) locus of the plasmid R1: a general purpose plasmid stabilization system. Bio/Technology **6**:1402–1405.
14. **Griffin, H. G., T. J. Foster, S. Silver, and T. K. Misra.** 1987. Cloning and DNA sequence of the mercuric- and organomercurial-resistance determinants of plasmid pDU1358. Proc. Natl. Acad. Sci. USA **84**:3112–3116.
15. **Grinter, N. J.** 1983. A broad-host-range cloning vector transposable to various replicons. Gene **21**:133–143.
16. **Harayama, S., and M. Rekik.** 1989. A simple procedure for transferring genes cloned in *Escherichia coli* vectors into other Gram-negative bacteria: phenotypic analysis and mapping of TOL plasmid gene *xyl*K. Gene **78**:19–27.
17. **Joseph-Liauzun, E., R. Fellay, and M. Chandler.** 1989. Transposable elements for efficient manipulation of a wide range of Gram-negative bacteria: promoter probes and vectors for foreign genes. Gene **85**:83–89.
18. **Kishore, G. M., and D. M. Shah.** 1988. Amino acid biosynthesis inhibitors as herbicides. Annu. Rev. Biochem. **57**:627–663.
19. **Kleckner, N.** 1989. Transposon Tn*10*, p. 227–268. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
20. **Kolter, R., M. Inuzuka, and D. R. Helinski.** 1978. Transcomplementation-dependent replication of a low molecular weight origin fragment from plasmid R6K. Cell **15**:1199–1208.
21. **Krebs, M. P., and W. S. Reznikoff.** 1986. Transcriptional and translational initiation sites of IS*50*. Control of transposase and inhibitor expression. J. Mol. Biol. **192**:781–791.
22. **Kreuzer, K., C. Pratt, and A. Torriani.** 1975. Genetic analysis of regulatory mutants of alkaline phosphatase of *E. coli*. Genetics **81**:459–468.
23. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Manoil, C., and J. Beckwith.** 1985. Tn*pho* A: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**:8129–8133.
26. **McClelland, M., J. Hanish, M. Nelson, and Y. Patel.** 1988. KGB: a single buffer for all restriction endonucleases. Nucleic Acids Res. **16**:364.
27. **Mermod, N., P. R. Lehrbach, R. H. Don, and K. N. Timmis.** 1986. Gene cloning and manipulation in *Pseudomonas*, p. 325–355. *In* J. R. Sokatch (ed.), The bacteria, vol. 10. Academic Press, Inc., New York.
28. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170**:2575–2583.
30. **Nakayama, K., S. M. Kelly, and R. Curtiss III.** 1988. Construction of an ASD$^+$ expression-cloning vector: stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. Bio/Technology **6**:693–697.
31. **Nucifora, G., L. Chu, S. Silver, and T. K. Misra.** 1989. Mercury operon regulation by the *merR* gene of the organomercurial resistance system of plasmid pDU1358. J. Bacteriol. **171**:4241–4247.
32. **Phadnis, S. H., and D. E. Berg.** 1987. Identification of base pairs in the outside end of insertion sequence IS*50* that are needed for IS*50* and Tn*5* transposition. Proc. Natl. Acad. Sci. USA **84**: 9118–9122.
33. **Raibaud, O., M. Mock, and M. Schwartz.** 1984. A technique for integrating any DNA fragment into the chromosome of *Escherichia coli*. Gene **29**:231–241.
34. **Richards, E.** 1987. Preparation of genomic DNA from bacteria. Miniprep of bacterial genomic DNA, p. 1–2, unit 2.4.1. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
35. **San Francisco, M. J. D., L. S. Tisa, and B. P. Rosen.** 1989. Identification of the membrane component of the anion pump encoded by the arsenical resistance operon of R-factor R773. Mol. Microbiol. **3**:15–21.
36. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Silver, S., and T. K. Misra.** 1988. Plasmid mediated heavy metal resistances. Annu. Rev. Microbiol. **42**:717–743.
38. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host-range

mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1**:784–791.

39. **Stark, M. J. R.** 1987. Multicopy expression vectors carrying the *lac* repressor gene for regulated high-level expression of genes in *Escherichia coli*. Gene **51**:255–267.
40. **Strauch, E., W. Wohlleben, and A. Pühler.** 1988. Cloning of a phosphinotricin *N*-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Streptomyces lividans* and *Escherichia coli*. Gene **63**:65–74.
41. **Taylor, R. K., C. Manoil, and J. Mekalanos.** 1989. Broad host range vectors for the delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. J. Bacteriol. **171**:1870–1878.
42. **Thompson, C. J., N. Rao Movva, R. Tizard, R. Grameri, J. E. Davies, M. Lauwereys, and J. Botterman.** 1987. Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. EMBO J. **6**:2519–2523.
43. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153**:3–11.
44. **Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner.** 1984. New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene **32**:369–379.
45. **Wohlleben, W., W. Arnold, I. Broer, D. Hillemann, E. Strauch, and A. Pühler.** 1988. Nucleotide sequence of the phosphinothricin *N*-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Nicotiana tabacum*. Gene **70**:25–37.
46. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–109.