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Site-Specific Deletions of Chromosomally Located DNA Segments with the Multimer Resolution System of Broad-Host-Range Plasmid RP4

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The multimer resolution system (*mrs*) of the broad-host-range plasmid RP4 has been exploited to develop a general method that permits the precise excision of chromosomal segments in a variety of gram-negative bacteria. The procedure is based on the site-specific recombination between two directly repeated 140-bp resolution (*res*) sequences of RP4 effected by the plasmid-borne resolvase encoded by the *parA* gene. The efficiency and accuracy of the *mrs* system to delete portions of chromosomal DNA flanked by *res* sites was monitored with hybrid mini-Tn*5* transposons in which various colored (β-galactosidase and catechol 2,3 dioxygenase) or luminescent (*Vibrio harveyi* luciferase) phenotypic markers associated to *res* sequences were inserted in the chromosome of the target bacteria and exposed in vivo to the product of the *parA* gene. The high frequencies of marker excision obtained with different configurations of the *parA* expression system suggested that just a few molecules of the resolvase are required to achieve the site-specific recombination event. Transient expression of *parA* from a plasmid unable to replicate in the target bacterium was instrumental to effect differential deletions within complex hybrid transposons inserted in the chromosome of *Pseudomonas putida*. This strategy permits the stable inheritance of heterologous DNA segments virtually devoid of the sequences used initially to select their insertion.

Adaptation of bacteria to novel carbon sources or to changing environmental conditions is frequently accompanied by the acquisition or loss of single or multiple DNA segments which determine the functions required for survival (3, 30). One archtypical case of adaptation is the sequence of molecular events which lead to the assembly of metabolic pathways for the degradation of xenobiotic compounds (19, 36), in which analysis of the DNA sequences of the corresponding catabolic genes and operons suggests that they have resulted from joining DNA segments recruited from preexisting pathways (20, 22, 36, 39). Modularity of the genetic complement of bacteria implies the existence of natural genetic engineering systems to assemble the various sequence components of the different elements (3, 30). Adaptation determinants such as antibiotic resistance genes (3), high-affinity iron transport systems (37), and catabolic pathways (36) are frequently included within transposons which, at least in the case of the Tn*3*/Tn*21* family, have the capacity to exchange DNA segments through transposon-determined site-specific recombination systems (16).

We have constructed in the past a series of transposon vectors derived from Tn*5* which permit the insertion and stable inheritance of heterologous DNA elements into the chromosome of target bacteria (11, 21), a process which resembles to some extent the existing mechanisms of acquisition or loss of adaptation-related phenotypes. In a further step to develop tools for metabolic engineering (9, 10) inspired by natural processes of DNA insertion and excision, we have examined the capability of the multimer resolution system (*mrs*) of broad-host-range plasmid RP4 (13, 14, 17, 27) to generate predetermined deletions within DNA segments inserted in the chromosome of various gram-negative bacteria. The *mrs* system is one of the functions present within the *par* region, which determines the major plasmid stabilization system of RP4 (14, 27, 29). The product of the *parA* gene is a site-specific resolvase that catalyzes *recA*-independent intramolecular recombination between two directly oriented resolution (*res*) sites flanking any supercoiled DNA (13, 14, 27). The result of this process is the excision of the intervening nucleotide sequence. In this work, we show that transient expression of *parA* suffices to effect differential deletions within DNA segments inserted in the chromosome of *Pseudomonas putida* and other gram-negative strains. This permits the addition of heterologous DNA segments devoid of any phenotypic marker to the genome of the target bacterium.

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MATERIALS AND METHODS

Strains, plasmids, media, and general techniques. Bacteria and plasmids used in this study are listed in Table 1. *Escherichia coli* CC118λ*pir*, S17-1λ*pir*, and MV1190λ*pir* were used as hosts to propagate plasmids containing an R6K origin of replication (21). Complete LB medium (25) and AB-citrate (10 mM) minimal medium (6) were supplemented, where required, with ampicillin (100 μg/ml), kanamycin (50 μg/ml), rifampin (50 μg/ml), carbenicillin (1 mg/ml), piperacillin (50 μg/ml), streptomycin (50 μg/ml), chloramphenicol (30 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml), or isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 to 1 mM). All recombinant DNA techniques were carried out according to published protocols (25). Amplification of DNA sequences by PCR used the Vent-DNA polymerase (New England Biolabs, Beverly, Mass.), with annealing temperatures 5°C below the lower melting point of the two PCR primers used in each reaction. Bacterial chromosomal DNA was prepared as described by Grimberg et al. (18). Southern blots were made on Qiabrane nylon membranes (Diagen, Düsseldorf, Germany). The DNA probe specified in Fig. 2 was generated with PCR and then labeled with dioxygenin and revealed with a coupled alkaline phosphatase system (Boehringer GmbH, Mannheim, Germany).

Activity assays. Expression of the *luxAB* luciferase genes of *Vibrio harveyi* was qualitatively detected by exposing the colonies grown on LB agar to traces of *n*-decanal, followed by blotting on a nitrocellulose filter and exposure to X-ray

TABLE 1. Bacteria and plasmids used

Bacterial strain or plasmid	Relevant genotype or characteristics	Reference or origin
Strains		
Escherichia coli		
CC118λ*pir*	*E. coli* K-12 Δ(*ara-leu*) *araD lacX74 galE galK phoA thi-1 rpsE rpoB argE*(Am) *recA1*, lysogenized with λ*pir* phage	21
MV1190λ*pir*	*E. coli* K-12 Δ(*lac-proAB*) *thi supE* Δ(*srl-recA*)::Tn*10* F′::[*traD proAB lacI*^{q}*Z M15*], lysogenized with λ*pir* phage	21
S17-1λ*pir*	*E. coli* K-12 Tp^r Sm^r *recA thi hsdRM*$^+$ RP4::2-Tc::Mu::Km Tn*7*, λ*pir* phage lysogen	11
TG1	*E. coli* K-12 *hsdM mcrB* Δ(*lac-proAB*) *thi supE* F′::[*traD proAB lacI*^{q}*Z M15*]	27
HB101	*E. coli* K-12/B hybrid, Sm^r *recA thi pro leu hsdRM*$^+$	23
Pseudomonas putida KT2442	*hsdR* Rif^r	21
P. fluorescens R2fRif	Rif^r derivative of strain R2f	D. van Elsas
Enterobacter cloacae SM1302	Nal^r	S. Molin
Serratia liquefaciens MG1	Ap^r Tc^r	15
Plasmids		
RK600	Cm^r ColE1*oriV* RP4*tra*$^+$ RP4*oriT* helper plasmid in triparental matings	23
pUC18Not	Ap^r, same as pUC18 (38) but with polylinker flanked by *Not*I sites	21
pUC18Sfi	Ap^r, same as pUC18 (38) but with polylinker flanked by *Sfi*I-*Avr*II sites	21
pUT *lacZ1*	Km^r Ap^r, transposon delivery vector for mini-Tn*5 lacZ1*	11
pUT *luxAB*	Tc^r Ap^r, transposon delivery vector for mini-Tn*5 luxAB*	11
pUT *Sm*	Sm^r Ap^r, transposon delivery vector for mini-Tn*5 Sm*	11
pCK152	Ap^r, pUC18Not with 3.5-kb *Not*I insert *lacI*^{q} $P_{A1/04/03}$::*parA* (element 7; Fig. 1)	13
pCK155	Ap^r Km^r, pUC18Not with 2.1-kb *Not*I insert *res-npt-res* (element 1; Fig. 1)	13
pCK183	Ap^r Sm^r, delivery vector for hybrid transposon mini-Tn*5 Sm lacI*^{q}$P_{A1/04/03}$::*parA*	This work
pCK199A	Ap^r Km^r, same as pUT *lacZ1* but with Km^r marker flanked by *res* sites as in pCK155, delivery vector for mini-Tn*5 lacZ1 res-npt-res* (element 4; Fig. 1)	This work
pCK217	Ap^r Km^r, pUC18Sfi with 2.1-kb *Sfi*I insert *res-npt-res* (element 1; Fig. 1)	This work
pCK218	Ap^r Km^r, same as pUT *luxAB* but with Tc^r gene substituted by the Km^r marker flanked by *res* sites as in pCK217, delivery vector for mini-Tn*5 luxAB res-npt-res* (element 5; Fig. 1)	This work
pXYLE10	Km^r, source of *xylE* gene	32
pRR41-5	Ap^r, RP4 derivative, *parA*$^+$ *tra*$^+$ *mob*$^+$	27
pJMSA8	Ap^r, 3.7-kb R6K*oriV*, RP4 *oriT*, derived from pUT *Km* (11) deleted of internal *Not*I and *Sal*I fragments	This work
pJMSB8	Ap^r R6K*oriV* RP4 *oriT* pJMSA8 derivative inserted with the *Not*I cassette *lacI*^{q} $P_{A1/04/03}$::*parA* of pCK152 (element 7; Fig. 1)	This work
pJMSA9	Ap^r Km^r, pUC18Sfi with 2.6-kb *Sfi*I insert *res1-npt-xylE-res2* (element 3; Fig. 1)	This work
pJMS09	Ap^r Km^r, pUC18Sfi with 2.6-kb *Sfi*I insert *res1-xylE-npt-res2* (element 2; Fig. 1)	This work
pJMS10	Ap^r Km^r, same as pUT *luxAB* but with Tc^r gene substituted by the *Sfi*I cassette of pJMSA9, delivery vector for mini-Tn*5 luxAB res-npt-xylE-res* (element 6; Fig. 1)	This work

film. Specific light intensities were measured in exponentially growing cells. Light emission was measured quantitatively, using a Turner TD-20e (Turner Designs, Sunnyvale, Calif.) apparatus. Values for luminescence represent averages of two independent experiments that differed by less than 20%. Catechol 2,3-dioxygenase (C2,3O) activity was detected by spraying 1% catechol on top of colonies grown on a petri plate; the C2,3O$^+$ clones acquired a bright yellow color resulting from production of a semialdehyde from the *meta*-ring cleavage of the aromatic substrate.

Construction of *res-npt-res* and *res-npt*/*xylE-res* cassettes. A *Sfi*I restriction fragment containing the kanamycin resistance (Km^r) gene of Tn*5* (*npt*) flanked by tandem multimer resolution sequences of RP4 (*res-npt-res*) was constructed by transferring the 2.1-kb *Eco*RI-*Hin*dIII insert of pCK155 (13) to the corresponding sites of pUC18Sfi, thus giving rise to pCK217 (Fig. 1). This cassette was added with a promoterless *xylE* gene sequence (encoding C2,3O) by digesting pCK217 with *Xma*I and *Bgl*II and ligating it to the purified 0.8-kb *Bam*HI-*Xma*I fragment of pXYLE10 (32). This ligation gave rise to two equally useful *res-npt*/*xylE-res* DNA segments (elements 2 and 3 in Fig. 1). These *Sfi*I cassettes were then used to replace the tetracycline resistance (Tc^r) gene of plasmid pUT/mini-Tn*5 luxAB* (pUT *lux* [11]). This resulted in hybrid minitransposons containing either a sequence of segments *luxAB res-npt-res* (element 5; Fig. 1) in plasmid pCK218 or *luxAB res-npt*/*xylE-res* (element 6; Fig. 1) in plasmid pJMS10, flanked in both cases by the terminal I and O sequences of Tn*5* (Fig. 1; see below). Similarly, the *Not*I cassette *res-npt-res* of pCK155 was replaced by the Km^r marker of pUT/mini-Tn*5 lacZ1* (pUT *lacZ1* [11]), producing the mobile sequence *lacZ-res-npt-res* (element 4; Fig. 1) in plasmid pCK199A.

Mobilization and transposition. Insertion of the various gene cassettes into the chromosome of gram-negative strains indicated was achieved using the mini-Tn*5* transposon delivery system described in detail elsewhere (21) and generally known as the pUT system. When the hybrid transposon used involved one or more *res* sites, triparental matings (11) were used to mobilize the delivery plasmids from *E. coli* λ*pir* hosts into the recipient strains. For this, the donor λ*pir* lysogen strain (*E. coli* CC118λ*pir* or *E. coli* MV1190λ*pir*), the recipient, and the helper strain *E. coli* HB101(RK600) were separately grown overnight in LB with antibiotics, washed with fresh medium, mixed in a proportion 1:5:1, and applied as 50-μl drops on LB agar plates. After 4 to 6 h of incubation at 30°C, the cells were scraped off the surface, dispersed in 1 to 5 ml of sterile 0.9% NaCl, diluted as required, and plated on citrate minimal medium with antibiotics for counterselection of the donor and helper *E. coli* cells. In the cases where hybrid transposons did not carry *res* sites, donor plasmids (Table 1) were transformed into a λ*pir* lysogen of *E. coli* S17-1 which contains a chromosomally integrated RP4 derivative providing conjugal transfer functions (31). Biparental matings between *E. coli* S17-1λ*pir* derivatives and the recipient strain indicated in each case were then set on nitrocellulose filters as described previously (11).

In vivo expression of the resolvase gene. Depending on the specific application (see Results and Discussion), the *parA* gene was expressed in different configurations. In one case, transcription of *parA* was driven by its native, autoregulated promoter in the RP4 derivative pRR41-5 (27). In other cases, *parA* translation and transcription were controlled from an IPTG-inducible promoter ($P_{A1/04/03}$ [23]) to which the *parA* gene sequence is adapted, through its first ATG codon, to an improved ribosome binding sequence (13). This *parA* expression cassette (element 7; Fig. 1) was assembled as a *Not*I restriction fragment and further introduced at the corresponding site in pUT *Sm* (11), thus generating the hybrid transposon mini-Tn*5 Sm lacI*^{q}$P_{A1/04/03}$::*parA* present in delivery plasmid pCK183 (Table 1). Alternatively, the *parA* expression cassette was cloned at the same site of the suicide plasmid pJMSA8 (Table 1), resulting in pJMSB8. This plasmid was

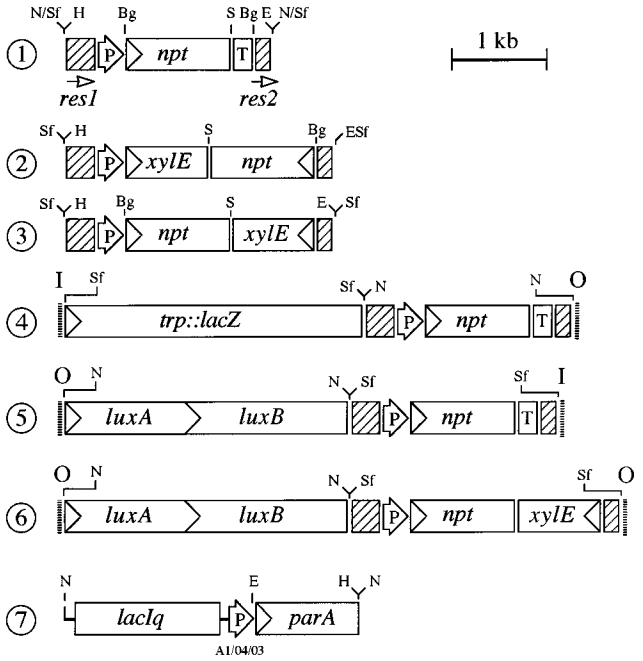

FIG. 1. Organization of chromosomal inserts carrying *mrs* elements of RP4. Each of the DNA segments used in this work is shown with its reference number indicated to the left (Table 1). The two tandem core *res* sites included in elements 1 to 6 (hatched boxes) are two identical DNA sequences of 140 bp, although because of the pedigree of element 1 (13), they are inherited included in a longer (*res1*, 305 bp) or a shorter (*res2*, 158 bp) restriction fragment. Relevant restriction sites: N, *Not*I; Sf, *Sfi*I; H, *Hin*dIII; Bg, *Bgl*II; S, *Sma*I-*Xma*I; E, *Eco*RI. The genes and functions determined by the different portions of the DNA segments are specified in each case: *npt*, Km^r gene of Tn*5*; *xylE*, gene encoding C2,3O; *trp*::*lacZ*, reporter gene encoding a hybrid protein with β-galactosidase activity; *luxAB*, luciferase genes of *V. harveyi*; *lacI*^{q}, gene encoding the *lac* repressor; *parA*, gene of the RP4 resolvase; T, transcriptional terminator of *rpoC*. The 19-bp I and O termini of Tn*5* are represented in elements 4 to 6 as vertical hatched lines at the extremes of the DNA segments. The promoters (P) included within the elements are indicated as boxed arrows, while the orientation of each of the promoterless genes is marked with a triangle within the relevant box. The promoter $P_{A1/04/03}$, which drives expression of *par*A in element 7, is a superinducible/superrepressible *lac* promoter derivative (24).

used for transient expression of the resolvase in target bacteria as explained below.

RESULTS AND DISCUSSION

The RP4 resolvase excises chromosomal DNA segments flanked by *res* sites. Previous work on the multimer resolution system of broad-host-range plasmid RP4 (13, 14, 27) has shown that heterologous DNA segments of ca. 2 kb flanked by tandem *res* sites and cloned in a multicopy vector can be precisely deleted in vitro and in vivo by the product of the *parA* gene. On this basis, we asked whether this property could be applied as a system to generate predetermined chromosomal deletions in a variety of gram-negative bacteria. To address this question, we constructed a collection of hybrid transposons determining phenotypic indicators resulting from marker genes associated to *res* sites (Fig. 1). After insertion in the chromosome of target bacteria, these gene cassettes provided a convenient genetic assay to study the performance of the system on chromosomal DNA segments.

In a first round of experiments, we inserted hybrid transposon mini-Tn*5* *lacZ1 res-npt-res* into the chromosome of *P. putida* KT2442 (Table 1). This hybrid transposon (element 4; Fig. 1) carries a Km^r marker (determined by the *npt* gene of Tn*5*) flanked by directly repeated *res* sites. For its insertion into the chromosome of the target strain, we set up a triparental mating between donor strain *E. coli* MV1190λ*pir*(pCK199A), the *Pseudomonas* recipient cells, and the helper strain *E. coli* HB101(RK600) as described in Materials and Methods. It should be noted that strains carrying plasmid RP4 integrated into the chromosome (like *E. coli* S17-1λ*pir*; Table 1) cannot be used as donors of *res*-containing constructions, since they express an active *parA* product (not shown). The operational frequency of insertion of the hybrid transposon (number of insertions/number of recipients [11]) was 5.0×10^{-7}. Of 100 Lac$^+$ Km^r exconjugants examined, 98 were also piperacillin sensitive, indicating that the DNA segment of interest had been properly inserted into the chromosome as such and not as a plasmid cointegrate (11). These frequencies were identical to those observed when *E. coli* CC118λ*pir* (pUT *lacZ1*) was used as the donor strain in the matings. Plasmid pUT *lacZ1* (11) harbors a mini-Tn*5* *lacZ1* transposon which is functionally identical to mini-Tn*5* *lacZ1 res-npt-res*, except that the Km^r selection determinant is not flanked by *res* sites. Ten Lac$^+$ Km^r independent exconjugants containing either of the transposons were then subjected to a second round of insertion mutagenesis using mini-Tn*5* *Sm lacI*^{q}$P_{A1/04/03}$::*parA* (Table 1), which expresses the *parA* resolvase gene under the control of an IPTG-inducible promoter. Of 100 streptomycin-resistant (Sm^r) exconjugants examined, resulting from *Pseudomonas* cells pre-inserted with mini-Tn*5* *lacZ1 res-npt-res*, 92 were Km^s regardless of the addition of IPTG to the medium. All 100 colonies examined were also Lac$^+$, as detected in medium with X-Gal. There were also ca. 10% Sm^r Km^r Lac$^+$ exconjugants, which may have arisen from undefined insertions or mutations resulting in spontaneous resistance to streptomycin, since they maintained their phenotype in the presence of IPTG.

Acquisition of mini-Tn*5* *Sm lacI*^{q}$P_{A1/04/03}$::*parA* by cells pre-inserted with mini-Tn*5* *lacZ1* (i.e., with the *npt* gene but without the *res* sites) resulted in exconjugants which were Sm^r Km^r Lac$^+$ in all 100 cases examined. This proved that the loss of the Km^r marker in cells carrying mini-Tn*5* *lacZ1 res-npt-res* was due to the presence of *res* sites flanking the *npt* gene, since in their absence, the Km^r marker remains unaffected by ParA. These observations provided a first indication of the functionality of the *mrs* system for excision of chromosomal sequences. They suggested also that a moderate expression of *parA* resulting from either leakiness of the superrepressible $P_{A1/04/03}$ promoter (24) or the transient transcription of the gene in the recipient cells before synthesis of sufficient LacI repressor could trigger the site-specific recombination event with an efficiency close to 100%.

We further examined the effect of *parA* expression on other strains and with a different source of the *parA* gene. Since various gram-negative bacteria often express an intrinsic β-galactosidase activity, we resorted to the *luxAB* genes of *V. harveyi* as a phenotypic marker for the genetic analysis of the strains under study. *Pseudomonas fluorescens* and *P. putida* strains (Table 1) were subjected to transposition mutagenesis with the hybrid transposon mini-Tn*5* *luxAB res-npt-res* carried by donor plasmid pCK218 (Table 1). The frequencies of insertion of the hybrid transposon were roughly within the same range, namely, 6.7×10^{-7} for *P. putida* and 1.2×10^{-6} for *P. fluorescens*. Six Km^r luminescent *P. putida* exconjugants and three Lux$^+$ Km^r colonies of *P. fluorescens* were then mated with *E. coli* TG1(pRR41-5) and selected for carbenicillin or piperacillin resistance (Cb^r or Pip^r) as a marker for acquisition of the *parA*$^+$ plasmid pRR41-5 (an RP4 derivative that carries a β-lactamase gene; Table 1). Transcription of *parA* in pRR41-5 occurs through the native, autoregulated *parCAB*

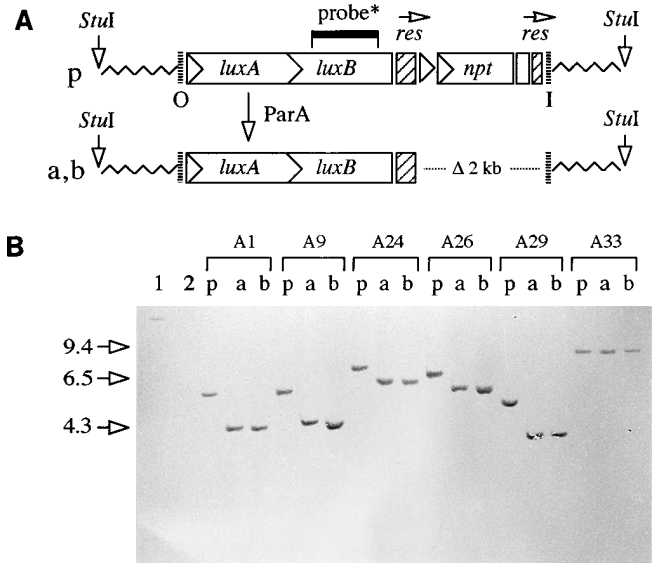

FIG. 2. ParA-mediated excision of chromosomal mini-Tn*5 luxAB res-npt-res* insertions. (A) Scheme of the site-specific deletion events examined in the Southern blot shown in panel B. Six *P. putida* KT2442 exconjugants carrying independent chromosomal insertions of hybrid transposon mini-Tn*5 luxAB res-npt-res* were provided in vivo with the *parA*$^+$ plasmid pRR41-5. Chromosomal DNA was isolated from the six parental Km^r Lux$^+$ strains (lanes p) as well as from two Km^s Lux$^+$ derivatives of each of them after acquisition of pRR41-5 (lanes a and b). The DNA was then digested with *Stu*I (which does not cleave inside the hybrid transposon), run in an agarose gel, blotted onto a nylon membrane as indicated in Materials and Methods, and hybridized to a labeled 592-bp DNA probe spanning nucleotides 2129 to 2721 of the *V. harveyi luxAB* sequence (GenBank access number M10961). Lane 1 was loaded with undigested pCK218 (which has no *Stu*I sites); lane 2 is a control of *Stu*I-digested chromosomal DNA from *P. putida* KT2442 not inserted with the hybrid transposon. The serial number of each of the strains examined before (lanes p) and after (lanes a and b) exposure in vivo to *parA*$^+$ is indicated on above the gel. The sizes of DNA markers in kilobases are specified to the left. Note that in each case, parental strains give rise, after acquisition of pRR41-5, to corresponding *Stu*I fragments which are ca. 2 kb smaller than the original DNA segments, as predicted for the site-specific recombination event. In sample A33, the deletion may not be as evident because of the lower resolution of the gel for high molecular sizes.

promoter of RP4, under which, expression of resolvase is extremely weak (7, 11). However, similarly to what had been observed with the mini-Tn*5 lacZ1 res-npt-res* insertions in *P. putida* (see above), all of the 100 Cb^r Pip^r transconjugants in each strain which had received pRR41-5 became Km^s but were still luminescent. These data provided genetic evidence for the activity of the RP4 resolvase system in *trans*, even when expression of the *parA* gene is kept at near native levels and stoichiometry.

Analysis of the site-specific deletions caused by resolvase expression. The genetic results described above on the loss of the phenotype encoded by a DNA segment flanked by *res* sites were directly confirmed through Southern blot experiments. Figure 2 shows the analysis of six independent *P. putida* KT2442 derivatives containing chromosomal insertions of mini-Tn*5 luxAB res-npt-res* at different locations, before and after receiving the *parA*$^+$ plasmid pRR41-5. The Southern blot data indicate that in all cases, expression of the resolvase resulted in the deletion of 2 kb of each of the *Stu*I restriction fragments spanning the hybrid transposons, as was predicted for the loss of the DNA segment encoding the Km^r gene flanked by the two *res* sites. Since the frequency of *parA*-mediated deletions in each case was virtually identical (>95%) and the sizes of the deleted products appear to be always the same, we concluded that the resolvase-mediated excision is independent of the chromosomal location of the *res* sites. Similar results were obtained in the Southern blot analysis of mini-Tn*5 lacZ1 res-npt-res* insertions in *P. putida* KT2442 and *P. fluorescens* R2fRif before and after receiving pRR41-5 (data not shown).

TABLE 2. Influence of ParA-mediated marker excision on nearby promoter activity[a]

P. putida strain	Specific light emission (RLU, 10^3) min^{-1}	
	Primary insertion mini-Tn*5 luxAB res-npt-res*	Excised insertion Δ*res-npt-res*
A1	0.25	0.21
A9	25.1	27.4
A24	20.9	21.4
A26	0.11	0.18
A29	12.9	9.16
A33	11.5	10.9

[a] Measured as light emission per optical density at 450 nm from different *P. putida* KT2442 exconjugants inserted with mini-Tn*5 luxAB res-npt-res* before and after acquisition of *parA*$^+$ plasmid pRR41-5. Luminescence is expressed in arbitrary units (relative light units [RLU]) after integration of emission of 100 μl of an exponentially growing culture in LB medium of each of the strains for 90 s, 30 s after supplying the cells with 1 μl of *n*-decanal.

The *P. putida* strains used in the Southern blot of Fig. 2 were also used to examine whether deletion of the intervening DNA between the *res* sites had any effect on the functionality of neighboring sequences. For this analysis, the level of light emission of each of the strains was measured before and after the excision event (Table 2). Since the *luxAB* genes carried by the transposon are promoterless (11), the luminescence is an indication of the strength of external promoters adjacent to the hybrid transposon. The data summarized in Table 2 indicate that the deletion of the DNA segments adjacent the *luxAB* sequences was phenotypically neutral with respect to the activity of nearby promoters.

Transient expression of ParA suffices for site-specific deletion of chromosomal sequences in various gram-negative strains. Since the *mrs* system seems to be extremely efficient in causing chromosomal deletions even at very low levels of expression, we investigated whether transient activity of the resolvase (i.e., without stable inheritance of the *parA* DNA sequence) would be sufficient to obtain excision events at significant frequencies. To do so, we used a double-reporter system for easy monitoring of the site-specific deletion events. A mini-Tn*5 luxAB res-npt-xylE-res* transposon organized as shown in Fig. 1 (element 6) was constructed. Insertion of this element into the chromosome of target bacteria provides the double phenotype Lux$^+$ (detected as luminescence) C2,3O$^+$ (which gives rise to yellow colonies upon spraying with catechol). The latter (C2,3O$^+$) is determined by the *xylE* gene sequence, which is flanked by the *res* sites along with the Km^r marker (*npt*), while the emission of light is caused by the *luxAB* genes outside the excisable cassette (Fig. 1). To obtain a transient expression of the ParA resolvase, we used a *lacI*^{q}*P*$_{A1/04/03}$::*parA* cassette carried in a plasmid (pJMSB8; Table 1 and Fig. 1) that can be mobilized with the RP4 transfer system at high frequencies into a variety of gram-negative target bacteria but which is unable to replicate in them.

To carry out the in vivo *parA* transient expression experiments, we first generated various insertions of the mini-Tn*5 luxAB res-npt-xylE-res* module into the chromosome of *P. putida* KT2442 through triparental matings described as above.

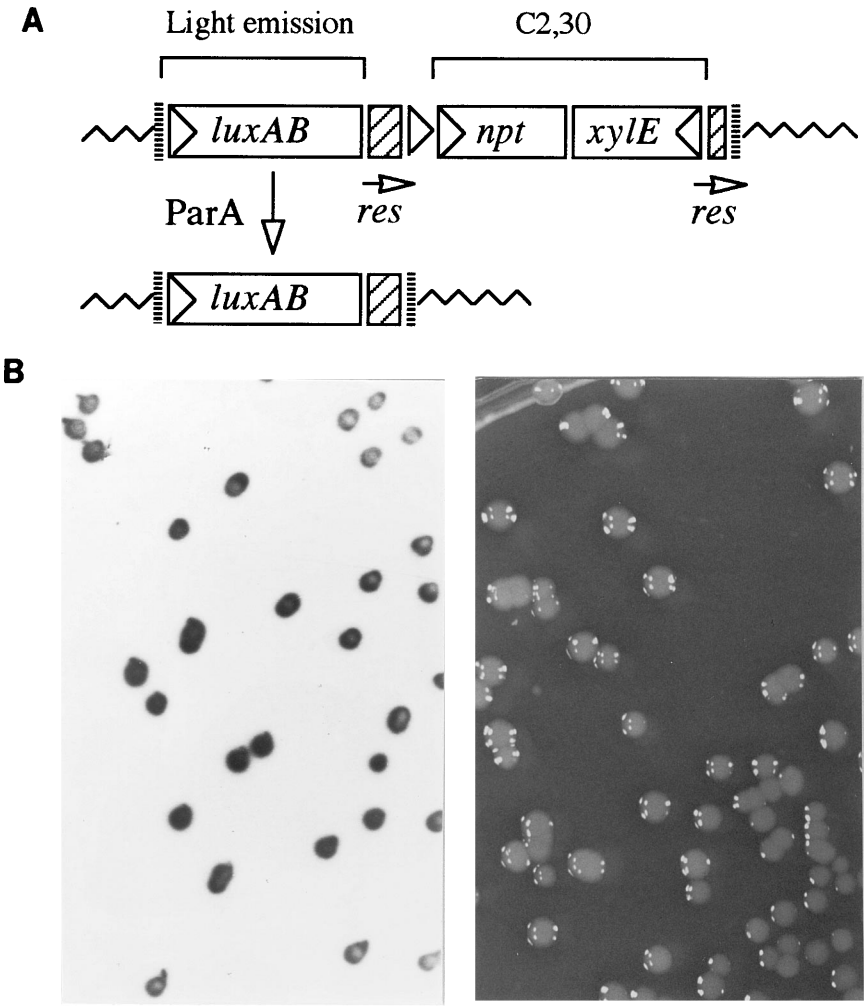

FIG. 3. Phenotypic monitoring of ParA transient expression. (A) Rationale of the experiment, which was repeated in the same conditions on 10 luminescent and C2,3O$^+$ *P. putida* KT2442 strains carrying independent chromosomal insertions of hybrid transposon mini-Tn*5 luxAB res-npt-xylE-res*. Each of these strains was mated with *E. coli* CC118λ*pir*(pJMSB8) and strain *E. coli* HB101(RK600) by mixing, in 5 ml of 10 mM $MgSO_4$, 5 μl of an overnight culture of the recipient *Pseudomonas* strain with 50 μl of cultures of each donor and helper *E. coli* strains, all of them pregrown in LB. This suspension was then passed through a nitrocellulose filter as described previously (11) and placed on top of an LB agar plate for 2 h at 30°C. The same filter was subsequently transferred to another plate with LB supplemented with 0.1 mM IPTG and further incubated for an additional 3 h. The membrane was then passed through a nitrocellulose filter as described previously (11) and placed on top of an LB agar plate for 2 h at 30°C. The same filter was subsequently transferred to another plate with LB supplemented with 0.1 mM IPTG and further incubated for an additional 3 h. The membrane was then vortexed in 5 ml of 10 mM $MgSO_4$, and dilutions of the cell suspension were plated on minimal citrate medium for counterselection of the donor. Plates were either sprayed with catechol for revealing the maintenance or loss of the *xylE* gene or briefly exposed to *n*-decanal and lifted on a nitrocellulose filter for detection of light emission (see Materials and Methods). One typical result is shown in panel B. While 100% of the resulting *Pseudomonas* colonies were still luminescent (left), about 15% of them had become Km^s and C2,3O$^-$ (right). The Lux$^+$ white colonies were Km^s Pip^s and had no traces of C2,3O$^-$ activity as measured in cell extracts (not shown).

All of the 100 Km^r exconjugants of the corresponding triparental mating were also luminescent (but with different emission intensities) and turned yellow when sprayed with catechol. Ten independent Km^r C2,3O$^+$ Lux$^+$ *P. putida* exconjugants colonies were then subjected to a second triparental mating with *E. coli* CC118λ*pir*(pJMSB8) and *E. coli* HB101(RK600), as specified in the legend to Fig. 3, followed by simple counterselection of the donor and helper strains. As shown in Fig. 3, about 15% of the colonies resulting from each of the second matings were still luminescent but remained white upon spraying with catechol and had become Km^s as well. These data indicate that expression of the resolvase during the short residence of pJMSB8 in the *Pseudomonas* recipient is sufficient to effect the predicted excision of the DNA segment that is flanked by the *res* sites without any inheritance of the *parA* gene itself, as evidenced by the sensitivity to piperacillin of all the Km^s exconjugants. Furthermore, since the frequency of transfer of RP4 is in the same range as the frequency of marker excision (not shown), it is likely that every recipient which received pJMSB8 underwent a ParA-mediated deletion. Although we initially sought to stimulate excision events by transferring the mating filter onto an IPTG-containing plate (see the legend to Fig. 3), we have noticed that even without that step, the site-specific deletions occur at equally significant frequencies.

These results allowed us to examine the efficiency of the system in bacterial species other than *P. putida*. Insertions of mini-Tn*5 luxAB res-npt-res* were generated also in *P. fluorescens*, *Enterobacter cloacae*, and *Serratia liquefaciens*. Similarly to the procedure described above, Km^r Lux$^+$ exconjugants of each strain were subjected to a second round of matings for transient acquisition of pJMSB8. Depending on the strain and

the insertion, 1 to 10% of the resulting exconjugant colonies became Km^s while they remained Lux^+, predictably, as a consequence of the site-specific deletion event within the chromosomal DNA of the insertion. We have observed also that optimizing mating and selection conditions (i.e., donor/recipient/helper ratios, mating time, and temperature) for each recipient strain may increase marker excision frequencies up to ≥90% (not shown).

Applications of the ParA-*res* system. We have shown that the *mrs* system of plasmid RP4 is an efficient genetic asset to generate predetermined deletions of chromosomal DNA segments in various gram-negative strains. Although we have examined the performance of the system with relatively short intervening DNA segments, the mechanism of multimer resolution (which works over 60 kb in RP4) makes our strategy generally applicable to DNA fragments of virtually any size, even large chromosomal portions. The broad-host-range plasmid RP4 is known to be stably maintained in a wide range of gram-negative genera (35), and the *par* locus has been shown to be functional in a variety of hosts (27, 29). Therefore, we believe that the ParA-*res* system should function in virtually any bacteria able to support the stable inheritance of RP4. Obviously, other site-specific recombination systems are eligible for the same purpose, in particular the Cre-*lox* resolvase system of the phage/plasmid P1 (33, 34) or the TnpR-*res* system of the γδ transposon (5). DNA segments flanked by directly repeated *lox* sites in plasmids have been shown to be efficiently excised in vivo and in vitro when exposed in *trans* to the action of the phage Cre protein (1, 2, 4). This property has been exploited, for instance, to develop transgenic plants devoid of antibiotic genes (7, 26, 28).

Although various applications of the ParA-*res* system can be easily envisioned, it seems to be particularly useful for generating chromosomal insertions of heterologous DNA segments eventually devoid of any selection marker, as is currently required for metabolic engineering of novel phenotypes for environmental applications and development of live vaccine strains (9, 10).

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