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Convenient and Reversible Site-Specific Targeting of Exogenous DNA into a Bacterial Chromosome by Use of the FLP Recombinase: the FLIRT System

LI-CHUN HUANG, ELIZABETH A. WOOD, AND MICHAEL M. COX*

Department of Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706

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We have created a system that utilizes the FLP recombinase of yeast to introduce exogenous cloned DNA reversibly at defined locations in the *Escherichia coli* chromosome. Recombination target (FRT) sites can be introduced permanently at random locations in the chromosome on a modified Tn*5* transposon, now designed so that the inserted FRT can be detected and its location mapped with base pair resolution. FLP recombinase is provided as needed through the regulated expression of its gene on a plasmid. Exogenous DNA is introduced on a cloning vector that contains an FRT, selectable markers, and a replication origin designed to be deleted prior to electroporation for targeting purposes. High yields of targeted integrants are obtained, even in a *recA* background. This system permits rapid and precise excision of the introduced DNA when needed, without destroying the cells. The efficiency of targeting appears to be affected only modestly by transcription initiation upstream of the chromosomal FRT site. With rare exceptions, FRTs introduced to the bacterial chromosome are targeted with high efficiency regardless of their location. The system should facilitate studies of bacterial genome structure and function, simplify a wide range of chromosomal cloning applications, and generally enhance the utility of *E. coli* as an experimental organism in biotechnology.

Site-specific recombination provides a vehicle to introduce exogenous DNA, delete DNA, or rearrange DNA at specific sites in a chromosome (41). Among the site-specific recombination systems characterized to date, the FLP system of the yeast 2μm plasmid and the Cre-*lox* system of bacteriophage P1 are among the most attractive for genomic manipulation because of their efficiency, simplicity, and demonstrated in vivo activity in a wide range of organisms. These systems have been used to construct specific genomic deletions and gene duplications, study gene function, promote chromosomal translocations, promote site-specific chromosome cleavage, and facilitate the construction of genomic libraries in organisms including bacteria, yeast, insects, plants, mice, and humans (2–5, 10–18, 24–26, 28, 30–35, 38–41, 44, 45, 47, 50). These studies have only begun to tap the potential of the approach.

Site-specific recombination catalyzed by the FLP and Cre recombinases occurs readily in bacterial cells (1, 5, 6, 21, 33). In principle, it could find wide application to studies of genomic structure and function as well as enhance the usefulness of *Escherichia coli* in biotechnology. Ironically, this approach has not been exploited in bacteria as it has been in eukaryotes, although bacteria were the first nonyeast cells in which FLP-mediated recombination was demonstrated (6). Even though gene targeting in bacteria can be achieved by homologous recombination, chromosomal targeting by site-specific recombination provides a new route to stable transformation with the advantages of very high efficiency, defined reproducible insertion sites in the chromosome, and controlled reversibility.

The yeast FLP system has been studied intensively (7, 8, 22, 36). The only requirements for FLP recombination are the FLP protein and the FLP recombination target (FRT) sites on the DNA substrates. The minimal functional FRT site contains only 34 bp. The FLP protein can promote both inter- and intramolecular recombination.

Previously, we reported the construction of a model system in *E. coli* using the FLP recombination system for chromosomal targeting and demonstrated the effectiveness of the general approach (21). Site-specific integration was absolutely dependent upon the expression of FLP protein and the presence of FRT sites in the chromosome. In some experiments, from 1 to 10% of the exogenous DNA molecules used, introduced on a modified bacteriophage λ vector, actually found their way into a cell and were integrated into the chromosome specifically at a chromosomal FRT.

Although we achieved a high integration frequency in our original targeting system, there were limitations inherent to the constructs that precluded detailed characterization as well as convenient application of the system to bacterial cloning and genomic studies. We have therefore modified the system so that we can regulate and monitor excision as well as integration, introduce FRTs virtually anywhere in the chromosome, and test a variety of additional parameters that might affect integration and/or excision. We have also constructed a system of plasmid constructs that is designed to allow very convenient use of the overall targeting scheme. This system is designated the FLIRT system (for FLP-mediated DNA integration and rearrangement at prearranged genomic targets).

MATERIALS AND METHODS

Media. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was from Indofine. IPTG (isopropyl-β-D-thiogalactopyranoside) was purchased from Bachem. Antibiotics were purchased from Sigma. Bacterial strains were grown at 37°C or 30°C in Luria (L) broth or on agar plates prepared as described previously (29) and supplemented with antibiotics as appropriate. Antibiotic concentrations were as follows: ampicillin (Amp), 100 mg/liter for cells containing multiple copies of the β-lactamase gene on plasmids or 20 mg/liter for cells containing a single copy of the β-lactamase gene on the chromosome; kanamycin

* Corresponding author. Mailing address: Department of Biochemistry, 420 Henry Mall, University of Wisconsin—Madison, Madison, WI 53706. Phone: (608) 262-1181. Fax: (608) 265-2603. E-mail: cox@biochem.wisc.edu.

(Kan), 40 mg/liter; tetracycline (Tet), 15 mg/liter; chloramphenicol (Cam), 25 mg/liter; X-Gal, 40 mg/liter. IPTG was used at 0.5 mM or 1 mM as indicated.

Bacterial strains. Key parental strains employed in this work were CSH26 [F$^-$ *ara* Δ(*lac pro*) *thi*] (29), RZ211 [F$^-$*ara* Δ(*lac pro*) *thi srl recA56*] (23), and MG1655 (F$^-$, wild-type) (19). Strains RZ211 and MG1655 were obtained from W. Reznikoff (University of Wisconsin) and G. Weinstock (University of Texas—Houston), respectively.

Enzymes and reagents. Restriction enzymes and bacteriophage T4 DNA ligase were obtained from New England Biolabs, Promega, or Boehringer Mannheim. FLP recombinase was purified and stored as described elsewhere (46). Avian myeloblastosis virus reverse transcriptase was from Life Sciences. Vent DNA polymerase, Klenow fragment, and linkers were purchased from New England Biolabs. Sequencing of DNA in experiments involving the FLIRT system was performed with the Sequenase version 2.0 DNA sequencing kit from Amersham Life Science. Bacterial alkaline phosphatase, the BioNick labeling system, and the PhotoGene nucleic acid detection system were from Gibco BRL. Linkers and biotinylated lambda *Hin*dIII digest were from New England Biolabs. All enzymatic reactions were performed essentially as described by Sambrook et al. (37) or as recommended by the suppliers. Radiolabeled deoxynucleotide triphosphates were obtained from Amersham Life Science. Oligonucleotides were prepared at the DNA synthesis facility in the University of Wisconsin Biotechnology Center or were from Operon Technologies Inc. SeaPlaque agarose was from FMC BioProducts. Geneclean was from Bio 101, Inc. Transformation and Bochner selection media were as previously described (9). All chemicals were of analytical grade or better and were purchased from common vendors.

FLIRT system vectors. (i) Vectors for delivery of exogenous DNA. A cloning vector for convenient targeting of exogenous DNA to the bacterial chromosome was pEAW116. To create pEAW116, pJFS36 (42) was digested with *Bam*HI, the ends were filled in, and *Cla*I linkers were ligated at this site. The resulting construct was digested with *Cla*I and *Sph*I. A synthetic DNA fragment, with *Cla*I and *Sph*I ends and containing a short polylinker with *Hin*dIII, *Pst*I, and *Sal*I sites and an FRT in the same orientation as the FRT already present in pJFS36, was ligated to the prepared vector. The resulting plasmid (now with two *Sal*I sites) was partially digested with *Sal*I and the ends were filled in. The tetracycline resistance gene from Tn*10* was ligated to the filled-in *Sal*I site, and a construct in which the Tet^r gene had been inserted in the polylinker between the FRTs was chosen. This plasmid was then isolated and digested at *Hin*dIII. A short synthetic polylinker containing one *Hin*dIII sticky end, sites for *Kpn*I, *Sma*I, *Not*I, *Nhe*I, and *Bgl*II, and another end compatible with *Hin*dIII but that does not regenerate a *Hin*dIII site after ligation (so that *Hin*dIII appears at one but not both ends of the fragment added to the vector) was ligated into the cleaved vector. This plasmid was pEAW116. A variant of pEAW116 was constructed by digesting it at the polylinker *Sma*I and *Nhe*I sites and filling in the ends and then inserting the wild-type *recA* gene and its promoter to generate pEAW118.

(ii) Plasmids for introducing FRT sites on the *E. coli* chromosome. Plasmid pEAW127 contains an FRT, a selectable marker, and a polylinker between 56-bp Tn*5* outside end sequences, 19 of which are required for Tn*5* transposition. The ampicillin resistance gene, origin of replication, *lacI*^{q}, and Tn*5* transposase are located in the region that is not transposed. The FRT was the minimal wild-type FRT derived from pJFS36 (42). The starting point was plasmid pRZ4828 (35a), constructed by inserting a mini-Tn*5* element containing 56 bp of the Tn*5* outer ends and *Bam*HI sites flanking the Tn*903* kanamycin resistance gene into the filled-in *Bam*HI site of pRZ4825 (48). The Tn*903* Kan^r gene of pRZ4828 was first deleted by digestion with *Bam*HI. This was replaced with a *Bam*HI fragment with bases 1052 to 2262 of Tn*903*, containing the kanamycin resistance gene, a short polylinker containing *Not*I, *Kpn*I, and *Dra*III sites, and an FRT, ligated to the *Bam*HI-digested pRZ4828. This placed all of the sequences between the Tn*5* outer ends. In order to facilitate the deletion of the replication origin prior to electroporation, *Eco*RI sites were placed on either side of the plasmid origin (*Eco*RI linkers were placed at the filled-in *Nde*I site and at the *Dra*I site that is not included in the Amp^r gene).

(iii) Plasmids for FLP expression. Plasmid pLH29 provides for expression of FLP recombinase, regulated by p*lac* along with an integral *lac*I gene. Construction of this plasmid is described elsewhere (20).

Construction of target strains. MG1655 *srl*::Tn*10* Δ*recA1398* was transformed with pLH29 and selected for chloramphenicol resistance. Tet^s mutants were then selected by using Bochner medium (9). These were designated MG1655 Δ*recA* Tet^s/pLH29. Plasmid pEAW127 (10 μg) was digested with *Eco*RI to remove the origin of replication. In order to separate any contaminating undigested pEAW127, the digested DNA was incubated at 65°C for 10 min with an equal volume of 1.5% SeaPlaque low-melting-point agarose. This was then loaded in the wells of a horizontal, 20-cm long, 0.8% agarose gel and allowed to cool for 5 min before 1× TAE (0.04 M Tris acetate [pH 8], 1 mM EDTA) buffer was added and the gel was run. The low-melting-point agarose matrix trapped the circular DNA in the wells (FMC BioProducts) (8a). The large *Eco*RI fragment without the origin was excised from the gel, and DNA was eluted with Geneclean. The DNA was self-ligated to circularize for 1 h at room temperature in a volume of 65 μl. The ligation mix was extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitated. The resulting circular DNA was resuspended and digested with *Bsp*LU11I for 1 h at 37°C in a volume of 100 μl. This linearizes any contaminating pEAW127 that cut only once with *Eco*RI, since the *Bsp*LU11I site is between the *Eco*RI sites. The *Bsp*LU11I digest was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitated.

The resulting pEAW127 Δ*ori* DNA was resuspended in 30 μl of H_2O. The DNA concentration was determined from the optical density at 260 nm, and 0.2, 0.4, and 0.6 μg of DNA were electroporated into 40 μl of electrocompetent MG1655 Δ*recA* Tet^s/pLH29 cells. Electrocompetent cells were grown in 0.5 mM IPTG and 25 mg of Cam/liter and prepared according to the procedure from Bio-Rad. Electroporations were performed at 25 μF, 2.5 kV, and 200 Ω in an ice-cold cuvette with a 0.2-cm gap by a Bio-Rad Gene Pulser. The cells were plated on 40-mg/liter Kan plates and incubated at 37°C overnight. Twenty-four Kan^r colonies were picked and screened on Amp plates. Kan^r Amp^s colonies indicated that a transposition event occurred to insert the FRT and Kan^r gene onto the chromosome. Small-scale plasmid DNA preparations were done to confirm the presence of pLH29 as the only plasmid in the cells.

Target strains containing FRT sites located at predefined sites in the *lac* operon were generated by homologous recombination. These strains were used to study the effect of transcription on targeting efficiency, with transcription regulated by IPTG. In these experiments, FLP expression was provided by plasmid pEAW38, in which the FLP gene was subject to temperature induction (21). For better control of the timing of the IPTG-mediated transcription, a *lacY* strain was preferred in the study. To obtain the *lacZ*::FRT *lacY* construct, strain RR1 (*lacZ*$^+$ *lacY*) was first transduced to *recD*::Tn*10* by bacteriophage P1 grown on MG1655 *recD*::Tn*10* (from P. Kiley, University of Wisconsin). Then strains RR1 *recD* and MG1655 *recD* were transformed with *Sca*I-linearized pLH20 and pLH32, respectively, by electroporation with a Bio-Rad Gene Pulser and the protocol recommended by the manufacturer. On plasmids pLH20 and pLH32, the FRT sites were cloned within the *lac* operon so the lac-FRT constructs could replace the original *lac* sequence on the *E. coli* chromosome by homologous recombination. The construction of these plasmids is described elsewhere (20). In the case of pLH20 transformation, LacZ$^-$ colonies were screened on IPTG–X-Gal–Luria-Bertani (LB) plates; in the case of pLH32 transformation, LacZ$^+$ LacY$^-$ colonies were screened on *lac*-MacConkey plates. The colonies with the desired phenotypes were picked, and the chromosomal FRT sites were transduced to wild-type RR1 (for pLH20) and MG1655 (pEAW38) (for pLH32). The colonies were selected for Kan^r and screened for the Lac$^-$ phenotype. The pLH20 FRT site in RR1 was further transduced to wild-type MG1655 (pEAW38). The final strain derived from pLH20 transformants with the FRT site at *lacZ* is called MG1655lacZ::FRT (pEAW38), and the final strain derived from pLH32 transformants with the FRT site at *lacY* is called MG1655lacY::FRT (pEAW38). Phenotypically, MG1655lacZ::FRT is LacZ$^-$ LacY$^-$ and MG1655lacY::FRT is LacZ$^+$ LacY$^-$. The *lac* locations of the FRT sites were confirmed by Southern analysis (data not shown).

Mapping the genomic location of FRT sites in target strains. Genomic DNA from the target strains was isolated as described previously (51), and 5 μg was digested with either *Nhe*I, *Pvu*II, or *Sph*I for 2 h at 37°C. There are no sites for these enzymes in the FRT-containing DNA transposed to the chromosome. The digested genomic DNA was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitated. The genomic DNA was ligated to pUC119 digested with *Xba*I, *Sma*I, or *Sph*I, which generate ends compatible with *Nhe*I, *Pvu*II, and *Sph*I, respectively. The ligated DNA was transformed into competent DH5α cells [*supE44* Δ*lacU169* (φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*], plated on 40-mg/liter Kan plates, and incubated at 37°C overnight. Kan^r colonies should contain inserts of transposed DNA and flanking genomic DNA. Plasmid DNA from the selected colonies was purified and sequenced with a primer consisting of bases 1091 to 1074 of Tn*903*. This reads through the FRT and 56-bp Tn*5* outer ends and into the flanking genomic DNA. The Fasta program (Genetics Computer Group) was used to compare the genomic DNA sequences with those in GenBank and identify the precise locations of the FRT-containing sequences in seven target strains. These strains were designated MG1655 Δ*recA* Tet^s 127FRT#1/pLH29 to MG1655 Δ*recA* Tet^s 127FRT#7/pLH29 (#1 to #7).

The insert for #1 maps next to base 67128 of the lambda clone, accession no. U29579, comprising the *E. coli* chromosomal region from 61 to 62 min. This is between two unidentified open reading frames (ORFs), o191 and f297. #2 transposed next to base 228222 of the lambda clone with accession no. U14003, at 92.8 to 00.1 min. This is in unidentified ORF f326b. #3 transposed next to base 2165 of the *nagC* gene, which is at 15.5 min on the *E. coli* chromosome. #4 transposed next to base 49628 of the lambda clone with accession number U00039, comprising the *E. coli* chromosomal region from 76 to 81.5 min. It is in unidentified ORF o383. #5 transposed next to base 11640 of the clone with accession number D90699, at 12.6 to 12.9 min on the chromosome. It is in the unidentified ORF o110. #6 maps to next to base 51888 of the lambda clone with accession number U18997, containing the region from 67.4 to 76.0 min. It is in the unidentified ORF f408. #7 maps next to base 19887 in the lambda clone with accession number U28379, at approximately 68 min. It is in the unidentified ORF f168.

Targeting trials. Plasmid pEAW116 or pEAW118 was first linearized by *Fsp*I digestion. Contaminating undigested plasmid DNA was separated by trapping the circular DNA in 1.5% SeaPlaque low-melting-point agarose as described above for pEAW127. The linearized DNA (16.8 μg) was then recircularized by intramolecular FLP-mediated site-specific recombination. The reaction mixture contained 25 mM *N*-Tris (hydroxymethyl) methyl-3-aminopropanesulfonic acid

(TAPS) buffer (pH 8), 1 mM EDTA, 2.5 mg of bovine serum albumin/ml 7.5% polyethylene glycol 8000, 10.8% glycerol, 180 mM NaCl, and 145 nM FLP recombinase in a total reaction volume of 400 μl, and the reaction was carried out at 30°C for 10 min. The reaction was stopped by the addition of 50 μl of a solution containing 30% glycerol, 0.03% bromphenol blue, 30 mM EDTA, and 4% sodium dodecyl sulfate. The reaction was loaded on a 0.8% agarose gel at 20 μl/lane and run in 1× TAE (27). The circular deletion product of the FLP reaction was eluted from the agarose (Geneclean) in 20 μl of H_2O. The DNA concentration was determined by absorption at 260 nm. This DNA (0.03 to 0.1 μg) was electroporated into electrocompetent MG1655 Δ*recA* Tet^s 127FRT#3/pLH29 or MG1655 Δ*recA* Tet^s 127FRT#4/pLH29 cells as described above for processed pEAW127. Electroporated cells were selected for Tet^r and then picked and screened for Tet^r and Amp^r. Tet^r Amp^s colonies indicated targeting. To demonstrate the dependence of targeting on the presence of the FLP protein and a chromosomal FRT, the same procedure was used to electroporate electrocompetent cells of MG1655 Δ*recA* Tet^s 127FRT#4/pLH29 or MG1655 Δ*recA* Tet^s/pLH29 and MG1655 Δ*recA* Tet^s 127FRT#4 with processed pEAW118.

Reversibility of targeting. In a typical experiment, single colonies resulting from targeted integration were isolated. Overnight cultures of these isolates were diluted 100-fold in L broth containing 1 mM IPTG to induce excision or no IPTG as a control. The cells were kept at 30°C. Once the culture reached stationary state (about six generations), an aliquot of the culture was transferred to fresh media with 1:100 dilution to resume growth. At the same time, 0.1 ml of the culture was plated on Amp-selective medium to determine the number of the cells which still kept the phenotype of an integrant. Also, the same volume of the cultures was plated on LB plates to determine the total number of cells. The ratio of Amp^r surviving integrants to total cells was determined. The excision rate (X) was calculated from the equation $X = 1 - e^{\ln(r)/n}$, where r is the number of Amp^r colonies divided by the total number of colonies and n is the number of generations.

Testing for the presence of a functional *recA* gene by exposure to UV light. Overnight-cultured cells to be tested were diluted 1/100 in LB media and grown for ~1.5 h to mid-log phase. Cultures (10 ml) were then spun at 2,000 × *g*, washed, and resuspended in M9 medium at an optical density at 600 nm of 0.054. Aliquots (2 ml) were placed on sterile uncovered 35-mm plates and shaken gently beneath a UV light source (254 nm). Irradiation was conducted under a photographic red light to prevent photoreactivation. Irradiation was carried out for an appropriate time at a fluence rate of 0.8 or 1.6 J/m^2 · s. The lamp was calibrated before each experiment with a J-225 shortwave UV meter. The exposed (or unexposed control) cells were serially diluted and spread on TYE-Cam20 plates. The plates were then wrapped in tinfoil and incubated overnight at 37°C. Colonies were counted the following day. Each data point represents an average from two experiments.

Southern analysis. Genomic DNA (5 μg) was digested with 20 U of *Pvu*II for 2 h at 37°C in a final volume of 50 μl. The DNA was ethanol precipitated and resuspended in 10 μl of Tris-EDTA. Each digested DNA was loaded on a 1% agarose gel along with 1 μl of a 100-μg/ml biotinylated *Hin*dIII-digested lambda DNA marker. The gel was run at 50 mA in 1× TAE and then photographed after staining in ethidium bromide. The DNA was transferred to PhotoGene nylon membranes, and Southern analysis was performed by the procedure specified by Gibco BRL. Probes were made by excising the FRT, kanamycin resistance marker, and flanking genomic DNA from the subclones in pUC119 used for mapping the FRTs on the MG1655 Δ*recA* Tet^s chromosome. *Eco*RI and *Hin*dIII were used for digestion, and the DNA fragments were separated on a 0.8% agarose gel. The DNA to be used as a probe was eluted from the gel with Geneclean, and 1 μg of DNA was labeled with the Gibco BRL BioNick labeling system.

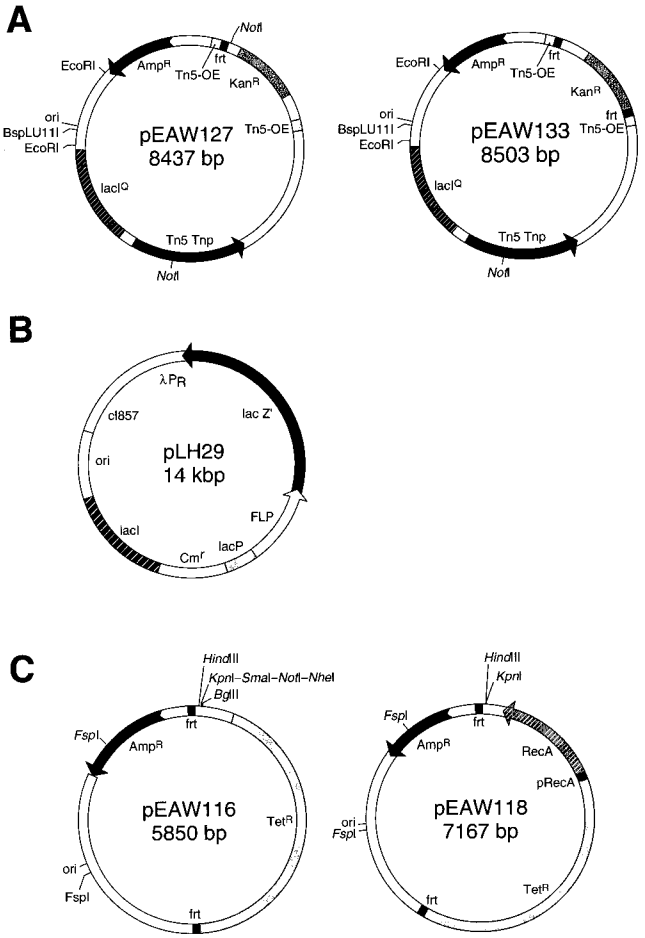

FIG. 1. The FLIRT system. (A) Plasmids used to introduce FRT sites into the chromosome. Important features are described in the text. Plasmid pEAW133 differs from pEAW127 only in the addition of a second FRT site flanking the Kan^r marker. After introduction of the transposed segment to the chromosome and mapping, the additional FRT permits the easy deletion of the Kan^r marker so that Kan selection can be used for other purposes. (B) FLP expression plasmid pLH29. Unlike the other components of the system, plasmid pLH29 contains sequences left over from earlier constructs that are not necessary for (but do not interfere with) its function. (C) Plasmids used to introduce exogenous DNA into bacteria and target it to chromosomal FRT sites. Plasmid pEAW116 is designed as a general cloning vector, with a polylinker containing a number of unique restriction sites. Plasmid pEAW135, described in the text, is essentially the same as pEAW116 but lacks *Xba*I sites other than those within the FRT sites. With pEAW135, the part of the plasmid containing Tet^r and any cloned DNA can be removed circularized with *Xba*I plus ligation, eliminating any requirement for purified FLP recombinase.

RESULTS

We previously described a method for chromosomal targeting of exogenous DNA in *E. coli* (21). The present work was undertaken to determine the effects of parameters such as transcription, chromosomal location, and the host homologous recombination system on integration efficiency and to refine the system both to facilitate these experiments and to make the system convenient for general use. The system consists of three elements: (i) *E. coli* target strains, each with a single FRT site located on the chromosome; (ii) a plasmid permitting the regulated expression of FLP protein; and (iii) a delivery vector for exogenous DNA containing a drug resistance gene marker as well as an FRT site compatible with the FRT site on the chromosome. We describe here the FLIRT system, which is entirely plasmid based and designed for general use, and then briefly summarize some results obtained with a variant of the bacteriophage λ-based system we reported on previously (21), which investigated some of the parameters that might affect targeting (20).

The FLIRT system. The FLIRT system is presented in Fig. 1. For regulated FLP protein expression, the FLIRT system uses pLH29, which was described in earlier work (20, 21). The improvements in the FLIRT system involve the methods used to introduce FRT sites into the *E. coli* genome and to target exogenous DNA to those sites. The system also makes economical use of the most common selectable markers.

Generating *E. coli* strains with FRT sites in the genome. Plasmid pEAW127 includes one FRT site and a selectable marker (Kan^r) located between the two outside ends of transposon Tn*5* (FRT segment). The Tn*5* transposase is encoded elsewhere on the plasmid and is not transferred to the bacterial chromosome with the FRT site. The site is thus stable and

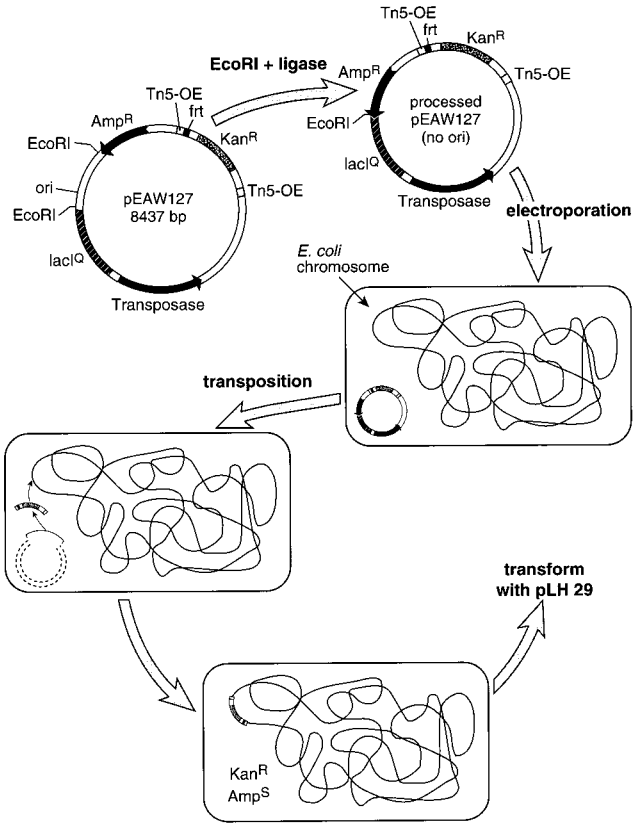

FIG. 2. Scheme for introducing FRT sites into the bacterial chromosome. Plasmid pEAW127 (or pEAW133) is processed to remove the replication origin. The recircularized DNA is then electroporated into prepared cells. Without the replication origin, Kan^r is conferred on the cell only if a transposition event occurs transferring the FRT and Kan^r-containing segment to the chromosome. The remainder of the electroporated DNA molecule, including the transposase gene and the Amp^r marker, is lost. The transposed segment is structured to facilitate the sequencing of the chromosomal DNA flanking the segment after transposition, allowing the target to be mapped to base pair precision within the *E. coli* genome database. OE, outside end.

permanent once it is transferred. The replication origin is removed from the plasmid prior to electroporation. Since the plasmid DNA is introduced into the cell only transiently, stable transformation to Kan^r requires the transposition of the FRT segment to the bacterial chromosome or some other replicating DNA element. A second selectable marker that is not part of the FRT segment (Amp^r) provides a means to detect suboptimal plasmid preparation or anomalous recombination events.

The scheme for generating target strains with pEAW127 is described in Fig. 2, with the details presented in Materials and Methods. The plasmid origin is first removed by cleaving with *Eco*RI and religating. Cleaving again with *Bsp*LU11I linearizes any plasmids that retain the origin segment. The circularized plasmids are electroporated into prepared *E. coli* cells. Transposition of the FRT segment to the chromosome (or a replicating extrachromosomal element) is detected by selection for Kan^r. Anomalous recombination events or transformation with intact pEAW127 can be eliminated by screening for Amp^s. These generated a total of 6,847 Kan^r colonies. Of 214 Kan^r colonies screened (24 each from 9 different electroporations), only 9 (4%), were Amp^r. The Amp^r colonies almost invariably arose from pEAW127 DNA from which the *ori* sequences had not been removed.

The FRT segment that is retained after transposition has short Tn*5* ends (56 bp, including the 19 bp required to direct transposition). The short length of these repeated end sequences facilitates the sequencing of flanking DNA in order to locate the transposed FRT site with base pair precision. Sequencing primers can be directed at unique sequences in the transposed segment, with sequencing directed outward across the outside ends. A number of the chromosomal segments containing FRT sites were subcloned and sequenced, and the chromosomal positions of the FRT in seven independently chosen target strains are given in Fig. 3.

A variant of pEAW127 (designated pEAW133 [Fig. 1]) which includes a second FRT site (FRT^2 segment) on the opposite end of the Kan^r element from the first has been developed. Once the FRT^2 segment is transposed to the chromosome and the Kan^r element has been used to facilitate selection and sequence-based mapping, the two FRT sites permit the deletion of the Kan^r element while retaining the chromosomal FRT. This simply requires induction of FLP recombinase with IPTG, growth for a few generations without Kan, and screening for a Kan^s colony. This feature should be useful in some applications, in that it preserves Kan^r selection for subsequent cloning steps. FLP has previously been used in a similar strategy to remove selectable markers after gene disruption (5).

Targeting trials. Plasmid pEAW116 features a selectable marker (Tet^r) and a polylinker for cloning in a segment flanked by FRT sites. An Amp^r element in the remaining DNA again functions as a marker for anomalous events. Use of this plasmid (Fig. 4) is similar to that outlined above for pEAW127. The plasmid can first be linearized with *Fsp*I. This step, not shown in Fig. 4, removes a fragment containing the origin of replication and part of the Amp^r element and ultimately reduces the background of cells transformed with unprocessed plasmid. Incubation of the larger linear fragment with FLP recombinase in vitro leads to product circles containing only one FRT along with the polylinker and Tet^r marker. These circles are then electroporated into the prepared target cells. Since the circles lack a replication origin, they are not retained unless they are integrated into a replicating DNA molecule, and the potential complexity of introducing a second replication origin into the chromosome is avoided. To illustrate the use of pEAW116, a variant (pEAW118) in which the *recA*$^+$ gene and its regulatory elements were cloned into the pEAW116 polylinker was constructed.

Virtually all of the Tet^r colonies arose from FLP-mediated

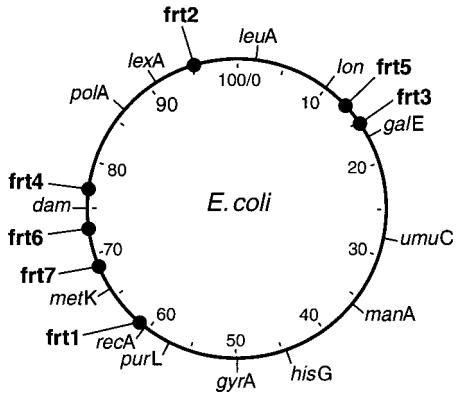

FIG. 3. Mapped locations of some FRT targets generated by pEAW127. The base pair locations of these targets are given in Materials and Methods. The bacterial strain used was MG1655 Δ*recA* Tet^s/pLH29.

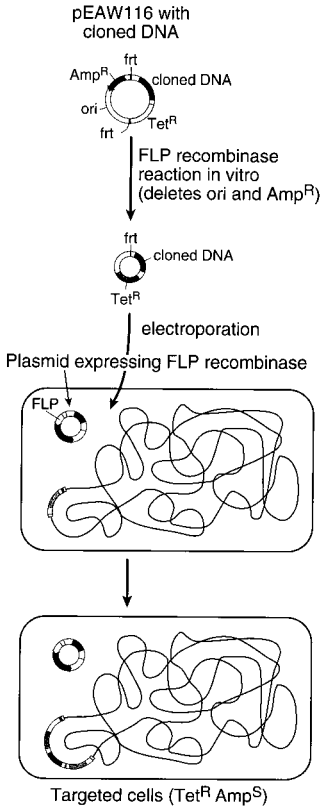

FIG. 4. Scheme for targeting exogenous DNA to the chromosomal FRTs with pEAW116 and derivatives. Plasmid pEAW116 (or a derivative like pEAW118) is processed to remove the replication origin and Amp^r marker. The recircularized DNA is then electroporated into prepared cells. Without the replication origin, Tet^r is conferred on the cell only if the electroporated circle is integrated into the chromosomal FRT in an FLP-mediated reaction. A targeted integrant should be Amp^s. Events resulting from improperly prepared DNA or anomalous recombination are generally detected as Amp^r.

targeted integration. In trials with pEAW118, the production of Tet^r colonies was reduced by at least two orders of magnitude if either the chromosomal FRT or the FLP-expressing plasmid was not present (Table 1). Of 265 Tet^r colonies screened over the course of four independent targeting trials, 100% were also Amp^s, indicating that the inclusion of unwanted plasmid sequences or transformation by unprocessed pEAW118 did not constitute a significant problem. The cells used in these trials contained a deletion of the *recA* gene. Interestingly, the targeting trials showed that target #3 (Fig. 3), located in the *nagC* gene, exhibits lower than normal targeting efficiency. This may define a relatively "cold" spot for FLP-mediated targeting in the *E. coli* genome. Even here, however, it was not difficult to obtain significant numbers of targeted integrants with pEAW118.

The site specificity of targeting is illustrated in the Southern blots in Fig. 5. A probe was directed at genomic sequences adjacent to the chromosomal FRT. Upon introduction of the chromosomal FRT by transposition, the labeled fragment was seen to increase in size by an increment consistent with the introduction of the 1,430-bp element derived from pEAW127 (including the FRT plus the Kan^r marker) (Fig. 5A and B, lanes 2). The use of pEAW118 as the source of exogenous DNA adds another 4,270 bp when the chromosomal FRT is targeted (Fig. 5A and B, lanes 3). Targeting was efficient and reliably site specific. Six colonies in which processed pEAW118

TABLE 1. The FLIRT system: targeting efficiency with pEAW118[a]

Experiment	FLIRT system		
	Complete	No chromosomal FRT	No FLP recombinase
1	97	0	1[b]
2	63	0	1[c]
3	23	0	0
4	82	0	0

[a] Bacterial strain MG1655 Δ*recA* Tet^s/pLH29 was used with FRT #4 (Fig. 3). The four electroporation experiments were all done on different days. Each used 0.03 μg of pEAW118 DNA, processed to remove the replication origin and Amp^r marker as described in Materials and Methods. For each experiment, separate side-by-side electroporation trials were done with bacterial strains identical to MG1655 Δ*recA* Tet^s/pLH29 except that they lacked either the chromosomal FRT or pLH29 (which expresses FLP recombinase). Values are the total number of Tet^r colonies obtained.
[b] Colony was Amp^r.
[c] Colony was Amp^s.

was integrated into target #4 were selected at random and examined by Southern analysis. The targeting occurred at the same location in each case (Fig. 5C). The targeted integration was also reversible (Fig. 5A and B, lanes 4). As shown in Fig. 5D, the introduction of the DNA from pEAW118 introduces a degree of resistance to UV irradiation that is consistent with the introduction of the *recA* phenotype into the cell. This phenotype is lost when the targeted DNA is excised.

A potential problem with the use of pEAW116 is the need

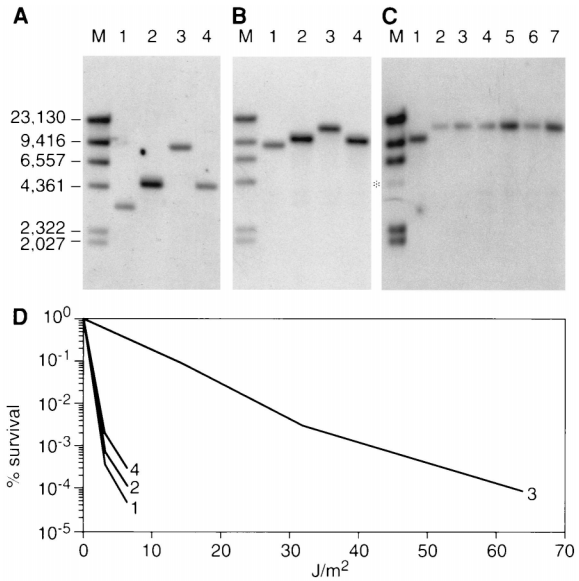

FIG. 5. Demonstration of FLP-mediated site-specific chromosomal targeting with the FLIRT system. The bacterial strain used was MG1655 Δ*recA* Tet^s/pLH29. (A and B) Southern analyses of successive steps in targeting. The probe in each case is directed at genome sequences immediately adjacent to the FRT target. Genomic DNA was digested with *Pvu*II in both cases. Lanes: M, markers generated from a *Hin*dIII digest of bacteriophage λ DNA (from New England Biolabs) with DNA fragment sizes indicated in base pairs; 1, bacterial strain MG1655 Δ*recA* Tet^s/pLH29 without an FRT; 2, after introduction of FRT #3 (A) or #4 (B); 3, after targeting with pEAW118; 4, after FLP-mediated excision of pEAW118-derived DNA from the chromosome. (C) Southern analysis of six colonies selected at random (lanes 2 to 7) in which processed pEAW118 was integrated into target #4. ∗, a 4,361-bp marker band that is not as prominent as in panels A and B. (D) Sensitivity to UV irradiation of the strains analyzed in panel B (numbered curves correspond to lanes 1 to 4). Note the elevated resistance observed for the strain from lane 3, reflecting the introduction of a wild-type *recA* gene.

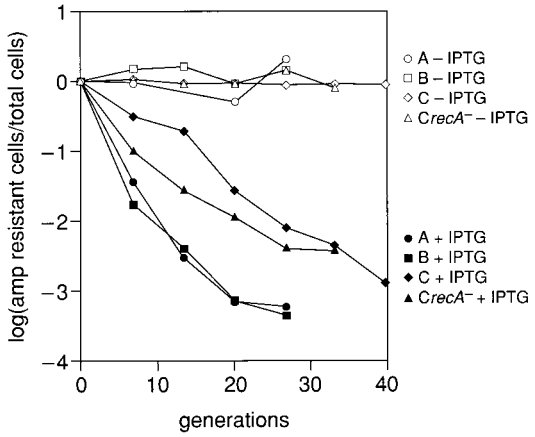

FIG. 6. Rates of FLP-mediated excision of targeted DNA from a chromosomal FRT site. Experiments were carried out as described in Materials and Methods. The presence (+) or absence (−) of IPTG is indicated. Targets A, B, C, and C*recA*$^-$ each have a single chromosomal FRT in CSH26, with the last of these transduced to *recA56*. The FRT sites were introduced on bacteriophage λ Tn*5* vectors by strategies described elsewhere (20, 21). Targets A, B, and C correspond to targets 3601, 3602, and 3621, respectively, as described previously (20, 21). FLP expression was provided by pLH29. All of the strains were originally targeted with λFRT36 (21), and the precise excision of this DNA element is monitored in this experiment.

for processing with FLP recombinase, which is not yet commercially available. (Strains expressing FLP recombinase and suitable purification protocols are available from us. Small amounts of the purified protein may also be occasionally available for trials.) We have constructed a variant of pEAW116 in which all *Xba*I restriction sites, other than those present in the FRT sites, have been removed. The plasmid is pEAW135 (not shown; essentially identical to pEAW116) and it allows the removal of the replication origin by cleavage with *Xba*I followed by circularization (by ligation) of the fragment containing the Tet^s element plus any cloned DNA. If the cloned DNA does not contain an *Xba*I site, this alternative eliminates the need for FLP recombinase.

Integrant stability. To study the reversibility of the site-specific integration event in more detail, integrants obtained with a precursor of the FLIRT system (20, 21) were examined to determine the rate of integrant excision. Colonies resulting from a λFRT36 (21) × chromosomal FRT integration reaction were isolated and grown in L broth with 1 mM IPTG. The FLP recombinase was expressed by using pLH29, which is also used in the FLIRT system. At intervals, cells recovered were plated on both LB plates and selective media. A typical result is shown in Fig. 6. We also ran controls in which no IPTG was added. The ratio of surviving integrants/total cells decreased sharply in the first six generations of growth in IPTG-containing media. Later, the decrease seemed to slow down. The estimated excision rate during the first six generations was about 30% per generation, with a range of 25% to 40% per generation for six independent excision experiments. There was no detectable excision when the integrants were grown in media without IPTG (Fig. 6).

To determine if integrants that survived after 25 generations were resistant to excision, the cells that remained Amp^r were isolated and grown in fresh media with 1 mM IPTG. The integrated DNA in these cells was excised as efficiently as the original integrants, again with an excision rate of about 30% per generation. We found no evidence for a subclass of cells in which excision was reduced or did not occur. In addition, cells in which the integrated DNA had been excised were capable of being subjected to targeting trials again, and the apparent integration frequency of these cells was about the same as the apparent integration frequency of the parental target strains (data not shown). This result shows that the targeting system based on FLP site-specific recombination reactions is fully reversible and indicates that the FRT sites in the chromosome remain intact during repeated integration and excision. We also compared the excision rate of RecA$^+$ and RecA$^-$ integrants. The rate was about the same in two independent trials whether the integrants had RecA$^+$ or RecA$^-$ phenotypes (Fig. 6). Rates of excision were very similar in trials carried out with integrants generated by the FLIRT system.

Effects of transcription from a nearby promoter. It is known that transcription affects the topology of the DNA template, generating positive supercoils ahead of the RNA polymerase and negative supercoils behind (49). In addition, RNA polymerase might at least transiently block a chromosomal FRT site in its path during transcription. To determine how transcription might affect the efficiency of site-specific targeting into the *E. coli* chromosome, we introduced our chromosomal FRT constructs at a fixed position on the chromosome within the *lac* operon. We made two constructs. In the strain designated MG1655lacZ::FRT, the FRT site is located within the *lacZ* gene, about 80 nucleotides downstream of the transcription initiation site. In the second construct, designated MG1655lacY::FRT, the FRT site is located within the *lacY* gene. In this construct, the *lacZ* gene remains intact and the transcription from the *lac* promoter can be assessed by the expression of the *lacZ* gene product. The positions of FRT sites in these strains were confirmed by Southern analysis (data not shown). To mediate targeting, both of the strains contain the FLP expression plasmid pEAW38 (21). The expression of the FLP recombinase on this plasmid is heat inducible. This allowed us to independently induce the *lac*-FRT operon by adding IPTG to the medium and FLP gene transcription by shifting the culture to high temperature as needed.

Our results showed that transcription from the *lac* promoter did not have a dramatic effect on targeting frequency (Table 2). The apparent integration frequency of MG1655lacZ::FRT increased slightly when 1 mM IPTG was added. When the FRT site was moved farther downstream of the *lacY* region, there was no detectable difference in integration frequency with or without IPTG. These experiments were repeated at least eight times for the *lacZ*-FRT construct and three times for *lacY*-FRT construct. A modest effect of transcription (two- to threefold) was always observed for *lacZ*-FRT. We conclude that FLP-mediated integration is only moderately sensitive to transcription at the *lac* locus.

TABLE 2. Effect of targeting efficiency of transcription initiation from an upstream promoter[a]

Strain	No. of colonies per plate[b]		Ratio of +IPTG/−IPTG (mean ± SD)[c]
	+IPTG	−IPTG	
Nontarget	1	0	
MG1655lacZ::FRT	139	39	2.42 ± 0.71 (n = 8)
MG1655lacY::FRT	206	247	1.11 ± 0.25 (n = 3)

[a] Introduction of exogenous DNA was accomplished by phage infection rather than by electroporation. Approximately 2×10^3 phage and 10^8 to 10^9 cells per plate were used. Cells, with an FRT site positioned in either the *lacZ* or *lacY* genes, as noted, were targeted with λFRT36, a modified λ phage with an FRT site and selectable marker (21).

[b] Results of a typical experiment are shown. +, with; −, without.

[c] n, number of experiments.

DISCUSSION

FLIRT is a plasmid-based system for FLP-mediated chromosomal targeting and genome rearrangement in *E. coli*. FRT sites can be introduced into the chromosome of almost any *E. coli* strain and mapped to base pair precision. The chromosomal FRT sites are stable once introduced, since the Tn*5* transposase gene is not included in the DNA transposed to the chromosome. The FRT can, in principle, be introduced at any chromosomal location where Tn*5* can transpose. Once on the chromosome, the FRT becomes an integration site for exogenous DNA. The procedure for bringing in exogenous DNA makes use of a plasmid cloning vector, processed prior to electroporation to remove the replication origin. Chromosomal integration is efficient and reliably site specific. The targeted integrants are stable as long as FLP recombinase is not induced. However, integration is reversible in the presence of FLP recombinase. Transcription initiation at an upstream promoter (Lac) had only a modest effect on targeted integration frequencies, although we cannot rule out the possibility that other promoters might affect targeting to a greater extent.

A number of parameters that might affect targeting have been explored previously, in some cases with FLP-based systems that are precursors of FLIRT (20, 21). (Some of these results are published only in thesis form. A copy of a manuscript detailing the results of experiments described in this paragraph is available upon request.) All of the recombination reactions are *recA* independent, and the FLP-mediated processes were several orders of magnitude more efficient and reliable than events mediated by homologous genetic recombination (20). There is no detectable pseudo-FRT site in the *E. coli* genome that could react with a normal FRT site, helping to ensure that the background of anomalous recombination events is low. A survey of 88 independently selected strains with chromosomal FRTs, placed on the chromosome as randomly as can be done with Tn*5* transposition, indicates that FLP-mediated chromosomal targeting is largely independent of the chromosomal location of the FRT site (20). However, we have detected at least two apparent "cold spots" in the genome that always give 10- to 100-fold lower targeting frequencies than the others. First, 4 of the 88 surveyed sites, all located within a 10,000-bp region encompassing the *cyo* operon (20), gave lower-than-normal targeting frequencies. The other 84 surveyed target sites were not mapped. Of the seven precisely mapped chromosomal FRTs used in the current study (Fig. 3), one located in the *nagC* gene (#3) also gave lower-than-normal targeting frequencies. We do not know why these few chromosomal FRTs were less efficient in targeting trials than normal, but targeting efficiency even with these was high enough that targeted integrants were easily obtained.

A few features of the FLIRT system or applications of site-specific recombination have been previously developed in other bacterial systems. First, a method for deleting a plasmid ColE1 origin in vivo by placing it between two phage f1 replication origins has been described (43). The result is a kind of suicide vector that can be used for chromosomal allele replacements. In the FLIRT system, provision has been made to remove plasmid replication origins enzymatically in vitro where necessary. The Cre-*loxP* system has been used to generate precise chromosomal deletions. Homologous recombination was used to position *loxP* sites on either side of the DNA to be deleted, followed by induction of the Cre recombinase on a suitable expression plasmid (1). Controlled deletion can be used to study gene function, and a similar approach has been used in eukaryotic organisms in a range of studies, as enumerated in the introduction. The FLIRT system could expand the range of experiments accomplished with such a precise deletion construct. If FRT sites were similarly positioned on either side of a chromosomal bacterial gene or regulatory site (perhaps using the allele replacement strategy of Slater and Maurer [43]), the FLIRT plasmid pLH29 could be adapted to delete the DNA and pEAW116 or derivatives could be used to target variants of the same or different DNA segments to the single FRT that would be left behind at the same chromosomal location. Precise deletion with FLP has also been coupled to a conditional replication origin to permit the excision and amplification of large chromosomal segments in vivo, permitting their isolation as large plasmids (33). The FLIRT system generally complements these applications.

Site-specific recombination may be usefully applied when the exogenous DNA has no homology to the bacterial genome, more precise control or higher efficiency is required in the integration reaction to facilitate the independent introduction of several alleles of a gene into an isogenic background, the exogenous DNA is required only transiently to facilitate one step in strain construction, or a *recA* background is required for genetic complementation tests. Allelic or other genetic comparisons can be made without the complication of chromosomal position effects. FLIRT simplifies the task of placing any DNA sequence directly onto the chromosome. Parts or all of the system should be adaptable for use in other bacterial species.

The technique should also facilitate the study of broader genome structure. Sequences that readily take up altered DNA structures can be positioned at a variety of locations in the chromosome, and their effects on DNA or cellular metabolism can be studied. New replication origins, promoters, or termination sites for replication or transcription could be introduced. Although the use of Tn*5* introduces the FRT sites into more or less random locations in the chromosome, the FRTs can be placed more precisely if a selection exists for the disruption of a particular gene. Note that the use of pEAW133 to introduce FRTs into the chromosome allows for the simple removal of the Kan^r selection marker once the FRT site is mapped. This would set the stage for introducing a second FRT site somewhere else in the same genome. Expression of FLP recombinase would then lead to the inversion or deletion of the intervening genomic DNA. There are obviously many other possibilities.

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REFERENCES

1. **Ayres, E. K., V. J. Thomson, G. Merino, D. Balderes, and D. H. Figurski.** 1993. Precise deletions in large bacterial genomes by vector-mediated excision (VEX). The trfA gene of promiscuous plasmid RK2 is essential for replication in several gram-negative hosts. J. Mol. Biol. **230:**174–185.
2. **Baubonis, W., and B. Sauer.** 1993. Genomic targeting with purified Cre recombinase. Nucleic Acids Res. **21:**2025–2029.
3. **Bayley, C. C., M. Morgan, E. C. Dale, and D. W. Ow.** 1992. Exchange of gene activity in transgenic plants catalyzed by the Cre-lox site-specific recombination system. Plant Mol. Biol. **18:**353–361.
4. **Bernet, A., S. Sabatier, D. J. Picketts, R. Ouazana, F. Morle, D. R. Higgs, and J. Godet.** 1995. Targeted inactivation of the major positive regulatory element (HS-40) of the human alpha-globin gene locus. Blood **86:**1202–1211.
5. **Cherepanov, P. P., and W. Wackernagel.** 1995. Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene **158:**9–14.

6. **Cox, M. M.** 1983. The FLP protein of the yeast 2-micron plasmid: expression of a eukaryotic genetic recombination system in Escherichia coli. Proc. Natl. Acad. Sci. USA **80:**4223–4227.
7. **Cox, M. M.** 1989. DNA inversion in the 2μm plasmid of *Saccharomyces cerevisiae*, p. 661–670. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
8. **Craig, N. L.** 1988. The mechanism of conservative site-specific recombination. Annu. Rev. Genet. **22:**77–105.

8a. **Daum, H., III.** Personal communication.

9. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **DiSanto, J. P., W. Muller, G. D. Guy, A. Fischer, and K. Rajewsky.** 1995. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. Proc. Natl. Acad. Sci. USA **92:**377–381.
11. **Fiering, S., E. Epner, K. Robinson, Y. Zhuang, A. Telling, M. Hu, D. I. Martin, T. Enver, T. J. Ley, and M. Groudine.** 1995. Targeted deletion of 5′HS2 of the murine beta-globin LCR reveals that it is not essential for proper regulation of the beta-globin locus. Genes Dev. **9:**2203–2213.
12. **Fiering, S., C. G. Kim, E. M. Epner, and M. Groudine.** 1993. An "in-out" strategy using gene targeting and FLP recombinase for the functional dissection of complex DNA regulatory elements: analysis of the beta-globin locus control region. Proc. Natl. Acad. Sci. USA **90:**8469–8473.
13. **Fukushige, S., and B. Sauer.** 1992. Genomic targeting with a positive-selection lox integration vector allows highly reproducible gene expression in mammalian cells. Proc. Natl. Acad. Sci. USA **89:**7905–7909.
14. **Fuse, T., H. Kodama, N. Hayashida, K. Shinozaki, M. Nishimura, and K. Iba.** 1995. A novel Ti-plasmid-convertible lambda phage vector system suitable for gene isolation by genetic complementation of Arabidopsis thaliana mutants. Plant J. **7:**849–856.
15. **Gage, P. J., B. Sauer, M. Levine, and J. C. Glorioso.** 1992. A cell-free recombination system for site-specific integration of multigenic shuttle plasmids into the herpes simplex virus type 1 genome. J. Virol. **66:**5509–5515.
16. **Golic, K. G.** 1994. Local transposition of P elements in Drosophila melanogaster and recombination between duplicated elements using a site-specific recombinase. Genetics **137:**551–563.
17. **Golic, K. G., and S. Lindquist.** 1989. The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. Cell **59:**499–509.
18. **Gu, H., Y. R. Zou, and K. Rajewsky.** 1993. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell **73:**1155–1164.
19. **Guyer, M. S., R. R. Reed, J. A. Steitz, and K. B. Low.** 1981. Identification of a sex factor affinity site in *E. coli* as γσδ. Cold Spring Harbor Symp. Quant. Biol. **45:**135–140.
20. **Huang, L. C.** 1992. A bacterial model system for chromosomal targeting. Ph.D. thesis. University of Wisconsin—Madison, Madison.
21. **Huang, L. C., E. A. Wood, and M. M. Cox.** 1991. A bacterial model system for chromosomal targeting. Nucleic Acids Res. **19:**443–448.
22. **Jayaram, M.** 1994. Phosphoryl transfer in Flp recombination: a template for strand transfer mechanisms. Trends Biochem. Sci. **19:**78–82.
23. **Johnson, R. C., J. C. Yin, and W. S. Reznikoff.** 1982. Control of Tn5 transposition in Escherichia coli is mediated by protein from the right repeat. Cell **30:**873–882.
24. **Lakso, M., B. Sauer, B. J. Mosinger, E. J. Lee, R. W. Manning, S. H. Yu, K. L. Mulder, and H. Westphal.** 1992. Targeted oncogene activation by site-specific recombination in transgenic mice. Proc. Natl. Acad. Sci. USA **89:**6232–6236.
25. **Lakso, M., J. G. Pichel, J. R. Gorman, B. Sauer, Y. Okamoto, E. Lee, F. W. Alt, and H. Westphal.** 1996. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc. Natl. Acad. Sci. USA **93:**5860–5865.
26. **Leslie, N. R., and D. J. Sherratt.** 1995. Site-specific recombination in the replication terminus region of Escherichia coli: functional replacement of dif. EMBO J. **14:**1561–1570.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Medberry, S. L., E. Dale, M. Qin, and D. W. Ow.** 1995. Intra-chromosomal rearrangements generated by Cre-lox site-specific recombination. Nucleic Acids Res. **23:**485–490.
29. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Morris, A. C., T. L. Schaub, and A. A. James.** 1991. FLP-mediated recombination in the vector mosquito, Aedes aegypti. Nucleic Acids Res. **19:**5895–5900.
31. **O'Gorman, S., D. T. Fox, and G. M. Wahl.** 1991. Recombinase-mediated gene activation and site-specific integration in mammalian cells. Science **251:**1351–1355.
32. **Osborne, B. I., U. Wirtz, and B. Baker.** 1995. A system for insertional mutagenesis and chromosomal rearrangement using the Ds transposon and Cre-lox. Plant J. **7:**687–701.
33. **Posfai, G., M. Koob, Z. Hradecna, N. Hasan, M. Filutowicz, and W. Szybalski.** 1994. In vivo excision and amplification of large segments of the Escherichia coli genome. Nucleic Acids Res. **22:**2392–2398.
34. **Qin, M., E. Lee, T. Zankel, and D. W. Ow.** 1995. Site-specific cleavage of chromosomes in vitro through Cre-lox recombination. Nucleic Acids Res. **23:**1923–1927.
35. **Ramirez, S. R., P. Liu, and A. Bradley.** 1995. Chromosome engineering in mice. Nature **378:**720–724.

35a. **Reznikoff, W.** Unpublished data.

36. **Sadowski, P. D.** 1995. The Flp recombinase of the 2-micron plasmid of Saccharomyces cerevisiae. Prog. Nucleic Acid Res. Mol. Biol. **51:**53–91.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Sauer, B.** 1992. Identification of cryptic lox sites in the yeast genome by selection for Cre-mediated chromosome translocations that confer multiple drug resistance. J. Mol. Biol. **223:**911–928.
39. **Sauer, B.** 1993. Manipulation of transgenes by site-specific recombination: use of Cre recombinase. Methods Enzymol. **225:**890–900.
40. **Sauer, B.** 1994. Recycling selectable markers in yeast. BioTechniques **16:** 1086–1088.
41. **Sauer, B.** 1994. Site-specific recombination: developments and applications. Curr. Opin. Biotechnol. **5:**521–527.
42. **Senecoff, J. F., R. C. Bruckner, and M. M. Cox.** 1985. The FLP recombinase of the yeast 2-micron plasmid: characterization of its recombination site. Proc. Natl. Acad. Sci. USA **82:**7270–7274.
43. **Slater, S., and R. Maurer.** 1993. Simple phagemid-based system for generating allele replacements in *Escherichia coli*. J. Bacteriol. **175:**4260–4262.
44. **Smith, A. J., S. M. De, A. B. Kwabi, P. A. Heppell, H. Impey, and P. Rabbitts.** 1995. A site-directed chromosomal translocation induced in embryonic stem cells by Cre-loxP recombination. Nat. Genet. **9:**376–385.
45. **Van, D. J., M. Fornerod, R. B. Van, and G. Grosveld.** 1995. Cre-mediated site-specific translocation between nonhomologous mouse chromosomes. Proc. Natl. Acad. Sci. USA **92:**7376–7380.
46. **Waite, L. L., and M. M. Cox.** 1995. A protein dissociation step limits turnover in FLP recombinase-mediated site-specific recombination. J. Biol. Chem. **270:**23409–23414.
47. **Wang, P., M. Anton, F. L. Graham, and S. Bacchetti.** 1995. High frequency recombination between loxP sites in human chromosomes mediated by an adenovirus vector expressing Cre recombinase. Somatic Cell Mol. Genet. **21:**429–441.
48. **Weinreich, M. D., H. Yigit, and W. S. Reznikoff.** 1994. Overexpression of the Tn5 transposase in *Escherichia coli* results in filamentation, aberrant nucleoid segregation, and cell death: analysis of *E. coli* and transposase suppressor mutations. J. Bacteriol. **176:**5494–5504.
49. **Wu, H. Y., S. H. Shyy, J. C. Wang, and L. F. Liu.** 1988. Transcription generates positively and negatively supercoiled domains in the template. Cell **53:**433–440.
50. **Xu, T., and G. M. Rubin.** 1993. Analysis of genetic mosaics in developing and adult Drosophila tissues. Development **117:**1223–1237.
51. **Yu, G.-L., and E. H. Blackburn.** 1990. Amplification of tandemly repeated origin control sequences confers a replication advantage on rDNA replicons in *Tetrahymena thermophila*. Mol. Cell. Biol. **10:**2070–2080.