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A Method for Constructing Single-Copy *lac* Fusions in *Salmonella typhimurium* and Its Application to the *hemA-prfA* Operon

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This report describes a set of *Escherichia coli* and *Salmonella typhimurium* strains that permits the reversible transfer of *lac* fusions between a plasmid and either bacterial chromosome. The system relies on homologous recombination in an *E. coli recD* host for transfer from plasmid to chromosome. This *E. coli* strain carries the *S. typhimurium put* operon inserted into *trp*, and the resulting fusions are of the form *trp*::*put*::[Kan^r-*X*-*lac*], where *X* is the promoter or gene fragment under study. The *put* homology flanks the *lac* fusion segment, so that fusions can be transduced into *S. typhimurium*, replacing the resident *put* operon. Subsequent transduction into an *S. typhimurium* strain with a large chromosomal deletion covering *put* allows selection for recombinants that inherit the fusion on a plasmid. A transposable version of the *put* operon was constructed and used to direct *lac* fusions to novel locations, including the F plasmid and the *ara* locus. Transductional crosses between strains with fusions bearing different segments of the *hemA-prfA* operon were used to determine the contribution of the *hemA* promoter region to expression of the *prfA* gene and other genes downstream of *hemA* in *S. typhimurium*.

Genetic fusions are in widespread use for the analysis of gene regulation. Fusions joining interesting promoters or regulatory sites to the *lac* operon allow the geneticist to employ a battery of selections and screens for isolating mutants that may affect gene expression (24). Plasmid-borne fusions are preferred for many operations, including construction of fusions with precise endpoints, site-specific mutagenesis, and DNA sequencing, but they are often problematic for study of the regulatory behavior of mutants. A variety of artifacts related to variation in gene copy number and titration of regulatory proteins (9a, 13a) can be avoided by the use of single-copy fusions located in the bacterial chromosome.

Single-copy systems based on phage λ can be used to move fusions between a multicopy plasmid and the *Escherichia coli* chromosome (24–26). While this method is adaptable to other species such as *Salmonella typhimurium* and its phage P22 (e.g., reference 10), the prophage imposes limitations as well as advantages. We have devised a different method for isolation of strains that carry a *lac* fusion in single copy, based on transformation of an *E. coli recD* mutant host with linear plasmid DNA (19, 23). Fusions can be transferred simply to the *S. typhimurium* chromosome by P22 or P1 transduction, and probably also to other bacterial species by using a specially constructed F plasmid. Reassortment of the 5′ and 3′ ends of various fusions can be carried out by homologous recombination in transductional crosses using phage P22. Thus, mutagenesis of the promoter/regulatory region in the absence of *lac* may be followed by reassembly of the fusion and screening for *lac* expression. The technique should facilitate specific screening for mutants with decreased promoter function.

MATERIALS AND METHODS

Bacteria and phage. *S. typhimurium* and *E. coli* strains used in this study and their sources are listed in Table 1. All *S. typhimurium* strains were derived from the wild-type strain LT-2 (except TE296, as noted in Table 1). *S. typhimurium* wild type does not carry the *lac* operon.

Media and growth conditions. Nutrient broth (Difco) containing 0.5% NaCl and LB broth (16) were used as maximally supplemented media. NCE medium containing 0.2% glycerol was used as the minimal medium (1). Difco Bacto-agar was added at a final concentration of 1.5% for solid media. Supplementation with 5-aminolevulinic acid was at 2 μM in minimal medium and 150 μM in rich medium (7); amino acid supplementation was as described previously (6). Antibiotics were added to final concentrations in rich or minimal medium, respectively, as follows: sodium ampicillin, 30 or 15 μg/ml; chloramphenicol, 20 or 5 μg/ml; kanamycin sulfate, 50 or 100 μg/ml; and tetracycline hydrochloride, 20 or 10 μg/ml.

Genetic techniques. The high-frequency generalized transducing bacteriophage P22 mutant HT*105/1 int-201* (21) was used for most transductions. Phage P22 lysates of *S. typhimurium* and *E. coli* strains were prepared as described previously (6, 7). Details of P22 transduction methods were as previously described (8). Phage P1 *vir* was used for transduction between *E. coli* strains. The strain of P1 *vir* phage used was provided by Rolf Menzel and was originally from P. Bassford. P1 growth and transduction were carried out by standard methods (25).

Plasmid construction. Techniques for plasmid construction followed standard methods (3, 15). DNA fragments were purified from excised gel slices by using a GeneClean kit (Bio 101). Competent cells were prepared by standard techniques (17). Electroporation was performed with a Bio-Rad Gene Pulser according to the manufacturer's instructions (2.5 kV, 200 ohms, and 25 μF in 0.2-cm cuvettes).

Transformation with linearized plasmid DNA. *E. coli recD* mutant strains were used as recipients for transformation. These *recD* strains allow efficient recombination of linear (restriction enzyme-cleaved) DNA with homologous sequences present on the bacterial chromosome (19, 23). Plasmid DNA for these experiments was prepared by an alkaline lysis procedure (3) and sometimes was further purified by equilibrium cesium chloride density gradient centrifugation (15). Transformation experiments used restriction enzyme-digested plasmid DNA without further purification. To increase the yield of transformants, cells

TABLE 1. Bacterial strains

Strain	Genotype[a]	Source or reference
E. coli		
DPB271	MG1655 (F⁻ λ⁻ *recD1903*::Tn*10d*-Tet	2
MH-1	*araD139* Δ(*lac*)*X74 galU galK hsdR* (Str^r)	12
RM443	F⁻ λ⁻ IN(*rrnD-rrnE*)*1* Δ(*lac*)*X74 rpsL galK2*	R. Menzel
TE1335	*trp* Δ(*lac*)*X74* Str^r /F′128 (P22 *HT105/1 int-201 sieA44*)	7
TE2680	F⁻ λ⁻ IN(*rrnD-rrnE*)*1* Δ(*lac*)*X74 rpsL galK2 recD1903*::Tn*10d*-Tet *trpDC700*::*putPA1303*::[Kan^s-Cam^r-*lac*]	This study
S. typhimurium		
SA990	*thrA49 leuBCD39 ara-7*/F⁺	K. Sanderson
MST1748	*pyrD121* Δ*put*(*PA*)*521*	S. Maloy
TE296	LT-7 Δ(*proAB*)*47*/F′128 *pro*⁺ *lacZ*⁺ *lacY476*::Tn*10d*-Cam	8
TE1902	*leuA414*(Am) *hsdL* (r_L^- m_L^+) (Fels2⁻)? *zde-1859*::Tn*10d*-Cam *hemA343* (Am)	Laboratory collection
TE2675	*pyrD121* Δ*put*(*PA*)*521*/pTE337	This study
TE2682	*putA1302*::Cam	This study
TE2684	*putPA1303*::[Kan^s-Cam^r-*lac*]	This study
TE2859[b]	*putPA1303*::[Kan^r-′*uorf1-hemA*′-*lac*] [op]	This study
TE2929	*zcc-6802*::Tn*10d*-Tet (80% linked to *put*⁺)	This study
TE2962	*putPA1304*::[′*prsA-uorf-hemA702*::Kan-*prfA-dorf-kdsA*′]	9
TE3416	*putPA1303*::[Kan^r-′*uorf1-hemA-prfA*′-*lac*] [pr]	This study
TE3420	*putPA1303*::[Kan^r-′*uorf1-hemA-prfA*′-*lac*] [op]	This study
TE4102	*putA1306*::Tn*10d*-Tet	This study
TE4120	*putP1305*::Tn*10d*-Tet	This study
TE4146	*putP1305*::Tn*10d*-Tet *putPA1303*::[Kan^r-′*uorf1-hemA*′-*lac*][op]	P22.TE4120 × TE2859
TE4148	*putPA1303*::[Kan^r-′*uorf1-hemA*′-*lac*] [op] *putA1306*::Tn*10d*-Tet	P22.TE4102 × TE2859
TE4166	*putP1305*::Tn*10d*-Tet *putPA1303*::Kan^s-Cam^r-*lac*	P22.TE4146 × TE2684
TE4180	*putPA1303*::[Kan^r-Δ33-′*hemA-prfA*′-*lac*] [pr]	This study
TE4203	*putP1305*::Tn*10d*-Tet *putPA1303*::[Kan^s-′*uorf1-hemA-prfA*′-*lac*] [op]	P22.TE4166 × TE3420
TE4217	*putPA1303*::[Kan^r-Cam^r-*lac*]	P22.TE2859 × TE2684
TE4239[c]	*putP1305*::Tn*10d*-Tet *putPA1307*::[Kan^s-′*uorf1-hemA-prfA-dorf-kdsA*′]	P22.TE4203 × TE2962
TE4249	*putPA1308*::[′*prsA-uorf-hemA*′-*lac*] [op] *putA1306*::Tn*10d*-Tet	P22.TE4128 × TE2962
TE4250	*putPA1303*::[Kan^r-′*uorf1-hemA-prfA*′-*lac*] [pr] *putA1306*::Tn*10d*-Tet	P22.TE4102 × TE3416
TE4251	*putPA1303*::[Kan^r-′*uorf1-hemA-prfA*′-*lac*] [op] *putA1306*::Tn*10d*-Tet	P22.TE4102 × TE3420
TE4252	*putPA1303*::[Kan^r-Δ33-′*hemA-prfA*′-*lacZ-lacY476*::Tn*10d*-Cam] [pr]	P22.TE296 × TE4180
TE4253[c]	*putPA1307*::[Kan^r-′*uorf1-hemA-prfA-dorf-kdsA*′]	P22.TE4217 × TE4239
TE4273[c]	*putPA1308*::[′*prsA-uorf-hemA-prfA*′-*lac*] [pr] *putA1306*::Tn*10d*-Tet	P22.TE4250 × TE2962
TE4274[c]	*putPA1308*::[′*prsA-uorf-hemA-prfA*′-*lac*] [op] *putA1306*::Tn*10d*-Tet	P22.TE4251 × TE2962
TE4275[c]	*putPA1307*::[Kan^r-Δ33-′*hemA-prfA-dorf-kdsA*′]	P22.TE4252 × TE4239
TE4338	*pyrD121* Δ*put*(PA)521/F⁺ *zzf-6807*::[Tn*10d-putA1301*::Kan]	This study
TE4351	*pyrD121* Δ*put*(*PA*)*521*/F⁺ *zzf-6807*::[Tn*10d-putA1302*::Cam]	P22.TE2682 × TE4338
TE4367	*putPA1303*::[Kan^r-′*uorf1-hemA-prfA*′-*lac*] [op] *putA1306*::Tn*10d*-Tet	P22.TE4253 × TE4274
TE4371	*putPA1303*::[Kan^r-Δ33-′*hemA-prfA*′-*lac*] [op] *putA1306*::Tn*10d*-Tet	P22.TE4275 × TE4274
TE4376	*putPA1304*::[′*prsA-uorf-hemA731*::Cam-*prfA-dorf-kdsA*′]	This study
TE4409	*putPA1303*::[Kan^r-′*prfA-dorf1*′-*lac*] [op]	This study
TE4410	*putPA1303*::[Kan^r-′*dorf1-dorf2-dorf3-kdsA*′-*lac*] [op]	This study
TE4412	*putPA1303*::[Kan^r-′*prfA-dorf1*′-lac] [op] *putA1306*::Tn*10d*-Tet	P22.TE4102 × TE4409
TE4413	*putPA1303*::[Kan^r-′*dorf1-dorf2-dorf3-kdsA*′-*lac*] [op] *putA1306*::Tn*10d*-Tet	P22.TE4102 × TE4410
TE4431	*putPA1308*::[′*prsA-uorf-hemA731*::Cam-*prfA-dorf1*′-*lac*] [op] *putA1306*::Tn*10d*-Tet	P22.TE4412 × TE4376
TE4432	*putPA1308*::[′*prsA-uorf-hemA731*::Cam-*prfA-dorf1-dorf2-dorf3-kdsA*′-*lac*] [op] *putA1306*::Tn*10d*-Tet	P22.TE4413 × TE4376
TE4442	*putPA1303*::[Kan^r-′*uorf1-hemA-prfA-dorf1*′-*lac*] [op] *putA1306*::Tn*10d*-Tet	P22.TE4253 × TE4431
TE4445	*putPA1303*::[Kan^r-Δ33-′*hemA-prfA-dorf1*′-*lac*] [op] *putA1306*::Tn*10d*-Tet	P22.TE4275 × TE4431
TE4446	*putPA1303*::[Kan^r-′*uorf1-hemA-prfA-dorf1-dorf2-dorf3-kdsA*′-*lac*] [op] *putA1306*::Tn*10d*-Tet	P22.TE4253 × TE4432
TE4448	*putPA1303*::[Kan^r-Δ33-′*hemA-prfA-dorf1-dorf2-dorf3-kdsA*′-*lac*] [op] *putA1306*::Tn*10d*-Tet	P22.TE4275 × TE4432
TR2279	Δ(*proAB*)*47 pyrB64 recA1 strA1*	J. Roth
TR5877	*hsdL6 hsdSA29* (r_{LT}^- m_{LT}^+ r_S^- m_S^+) *metA22 metE551 ilv-452 trpB2 xyl-404 rpsL120* (Str^r) H1-b H2-e,n,x (Fels2⁻) *nml*	B. A. D. Stocker

[a] The designation [op] or [pr] indicates that the strain carries a *lacZ* operon or protein fusion.

[b] The temporary name *uorf* (upstream open reading frames) refers to two open reading frames of unknown function that lie upstream of *hemA*. These are referred to as *uorf1* and *uorf2* or, collectively, *uorf*. These genes are transcribed divergently from the *hemA* operon, toward *prsA*. Similarly, *dorf* refers to three downstream open reading frames of unknown function which lie between *prfA* and *kdsA* and are transcribed in the same direction as *hemA*, *prfA*, and *kdsA*.

[c] Allele numbers refer to the site of insertion rather than to the added material, which is described following the double colon. Recombinants carrying the left (Kan^r) half of *putPA1303* and the right (*kdsA*′) half of *putPA1304* are designated *putPA1307*; the reciprocal class is *putPA1308* (′*prsA* to *lac*).

were first plated on nutrient agar, allowed to form a light lawn, and then replica printed to selective plates.

Nomenclature for insertions. The nomenclature used to describe constructs is based on standard conventions. Genes are listed in the order in which they appear on the chromosome of the strain being described, but only those genes and open reading frames of interest or known to be mutant are shown. Use of a double colon indicates that the material following is a true insertion and implies that the sequence of the interrupted gene or operon resumes after the insertion. Genes which lie at the same level and are not inserted one into the other are separated by a hyphen. Complex insertions or extended deletions are indicated by brackets or parentheses enclosing all of the elements involved. Finally, the notation 'gene *X* or gene *X*' indicates that part of gene *X* is missing on the side labeled by the prime. To make the nomenclature less cumbersome, primes are not always shown. Complete genotypes for all strains are listed in Table 1.

For simplicity, the genus of origin of the *E. coli trp* and *S. typhimurium put* DNA fragments used here is not usually specified, since only one construction was used (see below).

Elements derived from Tn*10* are described here as Tn*10d*-Tet or Tn*10d*-Cam (8, 29). The Tn*10d*-Tet insertions in *putP* and *putA* (Table 1) were isolated from a pool of insertions (prepared as described in reference 8) by transduction into a *lac* fusion strain, selection for Tet^r, and screening for Lac$^-$ and Kan^s, and then for Put$^-$. Insertion strains were classified as *putP* or *putA* mutants on the basis of their response to azetidine-5-carboxylic acid (18).

Construction of strain TE2680 (*trp*::*put*::[Kan^s-Cam^r-*lac*]). Strain TE2680 was constructed in several steps. A diagram of the relevant DNA fragments is presented in Fig. 1, and a flowchart for the construction is shown in Fig. 2. Plasmids used in the sequential linear transformations used for the construction are described in more detail in Table 2. The basic design was to first construct a *trp*::*put*::Kan strain (TE2628) by using a Kan^r insertion in *put* as the selective marker, then switch the insertion to *put*::Cam, and finally insert a *put*::Kan^r-*lac* segment (TE2631). Transformation of strain TE2631 with pTE335 gave *trp*::*put*::[Kan^s-Cam^r-*lac*]. At each step in this construction, inheritance of a plasmid marker was selected (either Kan^r or Cam^r), followed by a screen for loss of the appropriate chromosomal marker.

Construction of *hemA-lac* and *hemA-prfA-lac* fusions. The *hemA-lac* fusions contain a fragment extending from a *Bam*HI site 731 bp upstream of the *hemA* AUG codon to codon 17 of *hemA*, inserted into either pRS550 (operon fusion) or pRS577 (protein fusion) (see reference 7 for the *hemA* sequence). As indicated in Table 1, this fragment includes part of the upstream open reading frame designated *uorf1*. The *hemA-prfA-lac* fusions contain a fragment extending from the same *Bam*HI site to an *Mlu*I site at codon 111 of the *prfA* gene, inserted into pRS550 and pRS577. These *hemA-prfA-lac* fusions were constructed with a mutant *hemA* gene made by filling in the *Mlu*I site early in *hemA*. The *Mlu*I site was later restored in vivo (see Results). The deletion mutant Δ33 has been described previously (7); it removes the promoter and the first 23 codons of *hemA*. The *lac* fusion using Δ33 starts at a *Bam*HI site at codon 23 of *hemA* and extends to codon 111 of the *prfA* gene.

Construction of Tn*10d-put* and isolation of F-plasmid derivatives carrying Tn*10d-put*::Kan^r-*lac* fusions. Construction of Tn*10d-put* started with plasmid pNK862 (29). A *Bam*HI fragment derived from pNK862 that carries the ends of Tn*10* closely flanking the *Bam*HI site, and which also carries a

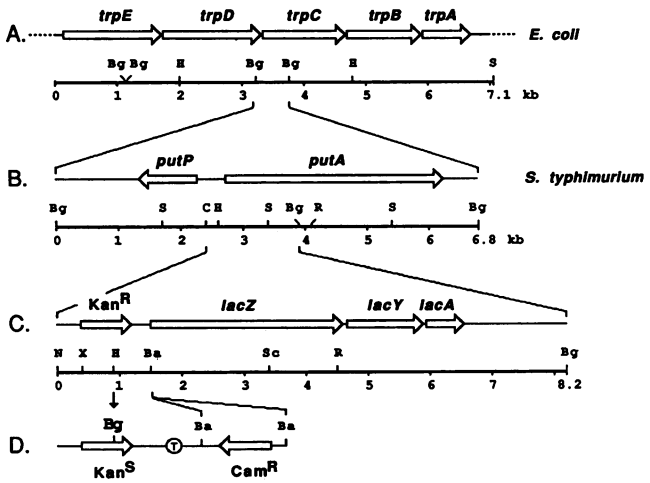

FIG. 1. Scale drawing of the DNA fragments used. (A) Map of the *E. coli trp* operon. pTE307 contains the 2.8-kb *Hin*dIII fragment (2.0 to 4.8 kb on this map) as described in Table 2. (B) Map of the *S. typhimurium put* operon which was inserted into *trp* by using *Bgl*II linkers. (C) Map of the Kan^r-*lac* segment from pMC931, which was inserted into *trp*::*put* to give *trp*::*put*::[Kan^r-*lac*]. The *putP* promoter lies to the right of the *Cla*I site and is not included in the resulting construct. (D) Map of the Kan^r segment in pTE335, derived from pRS577, including a *Bgl*II linker inserted at the *Hin*dIII site which inactivates Kan^r (labeled Kan^s), a Cam^r fragment inserted at the *Bam*HI site, and four tandemly repeated copies of the *E. coli rrnB* T1 transcriptional terminator, indicated by an encircled T. Restriction sites: Ba, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; N, *Nhe*I; R, *Eco*RI; S, *Sal*I; Sc, *Sac*I; X, *Xho*I.

replication origin and encodes Tn*10* transposase, was ligated to the *Bgl*II fragment of pTE316 which carries a complete *put* operon, including *putA1301*::Kan. The resulting plasmid (pTE453) was introduced into *S. typhimurium* TR5877 and then into SA990 by electroporation. Pooled transformants of the F$^+$ strain SA990 were mated with TR2279 (*recA* Str^r), with selection for Kan^r and Str^r and screening for Amp^s. The resulting exconjugants were shown to carry F$^+$ *zzf-6807*::Tn*10d*-[*putA1301*::Kan] by the following criteria: (i) they failed to donate Kan^r in transductional crosses with the recipient strain MST1748 (Δ*put*), indicating that Kan^r no

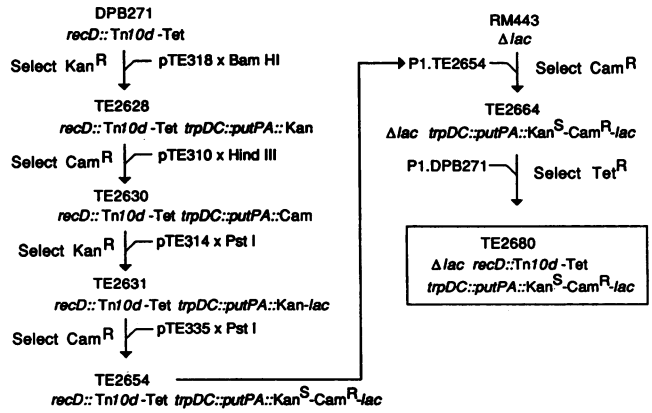

FIG. 2. Flowchart of strain construction. All strains are *E. coli*. The starting genotype is shown at the top. Each subsequent modification to this genotype is shown, along with the selection used to introduce the new genetic marker.

TABLE 2. Plasmid construction

Plasmid	Method of construction
pTE307	Ligation of the *Hin*dIII fragment containing bp 1998 to 4776 of the *E. coli trp* operon from pVH5 (*trp* coordinates from reference 31) into the pBR322 *Hin*dIII site. The insert includes the C-terminal 531 codons of *trpD*, all of *trpC*, and the N-terminal 30 codons of *trpB*; oriented with *trpB* next to the *Eco*RI site of pBR322.
pTE309	Ligation of the 1.3-kb *Bam*HI fragment encoding Kan^r from pUC4K (28) into the *Bgl*II site of pPC6 (11), disrupting *putA*. Kan^r is transcribed toward the *putPA* promoter region. The insertion is *putA1301*::Kan.
pTE310	Ligation of the 1.35-kb *Bam*HI fragment encoding Cam^r (originally from pCJ89 [13]) into the *Bgl*II site of pPC6. Cam^r is transcribed toward the *putPA* promoter region. The insertion is *putA1302*::Cam.
pTE312	Ligation of a 12-bp *Bgl*II linker into the *Hin*dIII site of pRS577, disrupting the Kan^r element. This disrupted gene is referred to here as Kan^s.
pTE314	Ligation of the *Nhe*I (filled in)-*Bgl*II fragment of pMC931 (8.2 kb, encoding Kan^r and *lac* [4]) into *Cla*I (filled in)-*Bgl*II-digested pPC6. Kan^r and *lac* are transcribed in the same direction as *putA*. The insertion is *putPA1303*::[Kan^r-*lac*].
pTE316	Sequential conversion of the *Aat*II and *Pvu*II sites flanking the *put* operon in pTE309 to *Bgl*II sites by using *Bgl*II linkers.
pTE318	Ligation of the *Bgl*II fragment carrying *putA1301*::Kan from pTE316 into *Bgl*II-digested pTE307. The *putA* gene is transcribed in the same direction as the *trp* genes. The *put* insert replaces the C-terminal 29 codons of *trpD* and the N-terminal 120 codons of *trpC*. This insertion is *trpDC700*::[*putP*$^+$ *putA1301*::Kan].
pTE335	Ligation of the 1.35-kb *Bam*HI fragment encoding Cam^r from pCJ89 into the *Bam*HI site of pTE312. Cam^r is transcribed away from *lac* and toward the nonfunctional Kan^s element.
pTE337	Self-ligation of the large fragment resulting from *Eco*RI and *Sac*I digestion of pTE312, resulting in deletion of the N-terminal two-thirds of *lacZ*.
pTE453	Ligation of the large *Bgl*II fragment carrying *putP*$^+$ *putA1301*::Kan from pTE316 into a *Bam*HI fragment derived from pNK862, carrying an origin of replication and expressing Tn*10* transposase. The *Bam*HI site is closely flanked by Tn*10* ends, allowing transposition of the inserted segment as Tn*10d-put*.

longer resides on a small, transducible plasmid; (ii) when used as donors in transductional crosses with the recipient strain TE2682 (*putA1302*::Cam), they showed 100% linkage of Kan^r to Cam^s, demonstrating that Kan^r is still inserted in *put*; and (iii) they can efficiently transfer the F plasmid and the Kan^r insertion into MST1748 (Δ*put*) by conjugation. The strain resulting from this cross was TE4338 {*pyrD121* Δ*put* (*PA*)*521*/F$^+$ *zzf-6807*::Tn*10d*-[*putA1301*::Kan]}. Its *putA*::Cam derivative is TE4351 {*pyrD121* Δ*put*(*PA*)*521*/F$^+$ *zzf-6807*::Tn*10d*-[*putA1302*::Cam]}.

TE4351 carries the *put* operon on an F′ plasmid, while the chromosomal copy of *put* is deleted, and the plasmid-borne *putA* gene is marked with a Cam^r insertion. Any *lac* fusion can be moved onto the F plasmid in a simple transductional cross, using a P22 phage lysate grown on a *lac* fusion as the donor into TE4351 as the recipient. In such crosses, all Kan^r transductants become Cam^s and carry a *lac* fusion on the F plasmid, as shown by the ability to act as donors of Kan^r and *lac* in subsequent conjugation crosses.

Construction of an *ara*::Tn*10d-put*::[Kan^r-*hemA-lac*] insertion. Pooled transformants of *S. typhimurium* TR5877 carrying pTE453 (Tn*10d-put*::Kan), described above, were screened for mutants with an Ara$^-$ phenotype on MacConkey arabinose plates. Putative *ara*::Tn*10d-put*::Kan insertions were transduced into the recipient MST1748 [*pyrD121* Δ*put*(*PA*)*521*], with selection for Kan^r and screening for Ara$^-$. One such strain, TE4417, was then transduced to Cam^r with a phage P22 lysate grown on TE2682 (*putA1302*::Cam), giving strain TE4418. TE4418 was then transduced to Kan^r, using a donor phage lysate grown on the *hemA-lac* fusion strain TE2859. The resulting strain, TE4428, carries the Kan^r-*hemA-lac* fusion as an insertion in *ara* as shown by the following experiment. When TE4428 was transduced with donor phage P22 grown on TE1076 (*leu*::Tn*10d*-Cam), all Cam^r transductants were either Ara$^-$ Kan^r Lac$^+$ or Ara$^+$ Kan^s Lac$^-$, demonstrating complete linkage of the fusion (Kan^r Lac$^+$) and insertion (Ara$^-$) phenotypes.

Transductional disassembly and reassembly of *lac* fusions. Parent strains used for construction of the various *lac* fusion strains are given in Table 1, and the methods used are also discussed in Results. Details of some transductional crosses are given here.

(i) P22.TE4203 × TE2962 → TE4239. Selection was for Tet^r, with a screen for Kan^s. Another class of Tet^r Kan^s transductants that had lost the N-terminal half of *hemA* was also found. These were recognized in a subsequent cross with a *hemA* mutant recipient (TE1902) because, unlike the TE4239 donor, they failed to show linkage of Hem$^+$ when Tet^r was selected.

(ii) P22.TE4217 × TE4239 → TE4253. Selection was for Kan^r, with a screen for Tet^s and Cam^s.

(iii) P22.TE4252 × TE4239 → TE4275. Selection was for Kan^r, with a screen for Tet^s and Cam^s. Two classes of Kan^r Tet^s Cam^s transductants were recovered: those carrying the Δ33 promoter deletion and those with a wild-type *hemA* promoter. These were distinguished by subsequent transductional crosses into strain TE1902 (*hemA*) as described above.

(iv) P22.TE4251 × TE2962 → TE4274. Selection was for Tet^r, with a screen for Lac$^+$ and Kan^s. Lac$^-$ Tet^r transductants were not recovered, which suggests that the *putA1306*::Tn*10d*-Tet insertion lies between the *Bgl*II site at kb 3.9 and the *Sal*I site at kb 5.4 of *putA* (Fig. 1). A similar cross using TE4250 as the donor gave TE4273 (a protein fusion).

(v) P22.TE4412 × TE4376 → TE4431. Selection was for Tet^r and Cam^r, with a screen for Lac$^+$ and Kan^s.

β-Galactosidase assays. Cultures for β-galactosidase assays were grown in minimal glycerol medium at 37°C with shaking to A_{600} = 0.5. Cells were harvested by centrifugation and resuspended in Z buffer (16) at an appropriate concentration (up to 40-fold concentrated). Cell suspensions were made permeable by treatment with sodium dodecyl sulfate and chloroform, and assays were performed as described previously (16). Assay mixtures were centrifuged before the A_{420} was measured. Replicate samples were incubated for

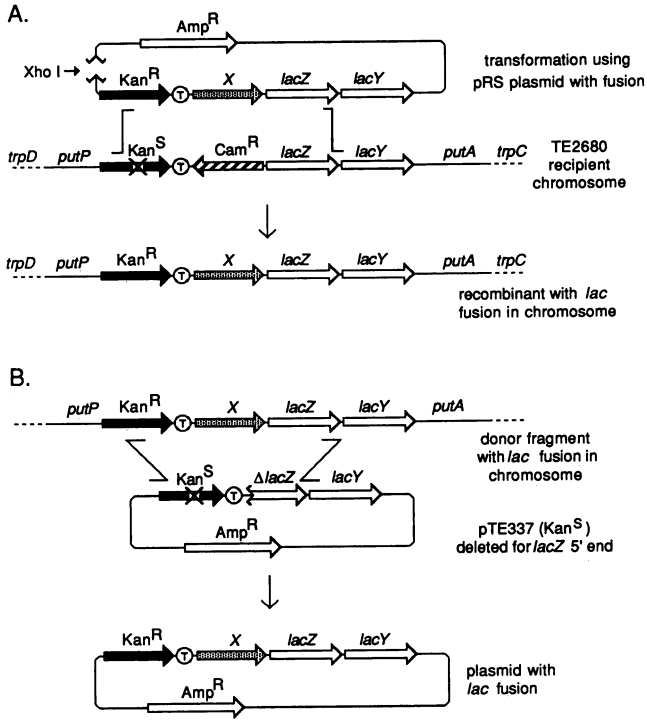

FIG. 3. Schematic diagram showing construction and recovery of single-copy *lac* fusions. *S. typhimurium hemA-prfA* operon fragments were used as the promoter-bearing segments in these experiments. (A) Homologous recombination between linearized plasmid DNA and the bacterial chromosome, after transformation of *E. coli* TE2680, selection for Kan^r, and screening for Cam^s. (B) Recovery of a *hemA-lac* fusion from the *S. typhimurium* chromosome by transduction into a recipient (TE2675) bearing a nontransducible deletion of *put* and carrying pTE337. Selection for Kan^r and screening for Lac$^+$ allows recovery of plasmids indistinguishable from the original (with the exception of a restriction site polymorphism discussed in the text).

various times up to 1 h, and enzyme activity was calculated from the slope of a plot of A_{420} versus time. Values for β-galactosidase activity are expressed according to Miller (16).

RESULTS

Method for transfer of *lac* fusions to the *E. coli* chromosome. I have developed a general method for reducing *lac* fusions formed on multicopy plasmids to single-copy insertions in the *E. coli* chromosome. It requires only that the fusion be constructed in a plasmid carrying Kan^r upstream of the fusion and in the correct orientation. A special recipient strain (TE2680; Table 1) is transformed with linearized plasmid DNA, selecting for Kan^r (Fig. 3A). A double crossover between the fusion plasmid and the bacterial chromosome generates a single-copy insertion of the *lac* fusion. The desired recombinant can be recognized easily by its Cam^s phenotype. In experiments with a dozen different fusions, between 10 and 40% of Kan^r transformants were of the desired type. The method should be applicable to any *lac* fusion.

The fusions used were generated in a set of plasmids constructed by Simons et al. (26). The pRS series plasmids carry a Kan^r gene from Tn*903* placed upstream of the *E. coli lac* operon, separated from *lac* by a transcriptional terminator. Convenient restriction sites between the terminator and *lac* can be used to insert promoters or gene fragments whose expression is to be measured. As illustrated in Fig. 3A, the transformation recipient strain TE2680 contains homology to the pRS plasmids, including an upstream segment with a Kan^r gene which has been inactivated by the insertion of a linker into the *Hin*dIII site (marked Kan^s) and a downstream segment consisting of the *lac* operon. These two segments are separated by a Cam^r fragment. TE2680 also bears a *recD*::Tn*10d*-Tet insertion to allow efficient recombination with linearized plasmid DNA. The recombination event illustrated in Fig. 3A gives Kan^r Cam^s recombinants which have transferred the fusion to the bacterial chromosome.

Conditions for transformation of TE2680 have not been optimized. Typically, about 1 μg of plasmid DNA prepared by alkaline lysis and partially purified (Stratagene PlasmidQuik) was digested with *Xho*I, and the resulting mixture used directly to transform TE2680. Ten to 100 Kan^r colonies were obtained, of which 10 to 40% were Cam^s. Transformation efficiency can be increased by starting with plasmid DNA purified on cesium chloride gradients. Efficiency might also be improved by purifying the digested DNA and by substituting electroporation for transformation.

The fusions are embedded in a promoterless *S. typhimurium put* operon which, in turn, has been inserted into the *E. coli trp* operon (Fig. 1). P22 transducing phage grown on an *E. coli* fusion strain by zygotic induction (7) can be used to transduce the fusion into *S. typhimurium*, where it replaces the resident *put* operon. Phage P1 or a specially constructed F plasmid may also be used for this step.

A variety of physical and genetic tests demonstrate that the model for the recombination events shown in Fig. 3A is correct. Strain TE2680 was constructed in a series of linear transformations as described in Materials and Methods. The intermediate strains in this construction and TE2680 all had the predicted structures when analyzed by *Hin*dIII digestion and Southern blotting (data not shown). Genetic tests included transductional crosses in which the loss of gene function associated with the insertions (Trp$^-$ in *E. coli*; Put$^-$ in *S. typhimurium*) was 100% linked to Kan^r. Kan^r also showed appropriate linkage to transposon insertions near *put* in *S. typhimurium*.

Recovery of *lac* fusions on a plasmid. Transductional crosses with the *lac* fusion strains as donors into *S. typhimurium* recipients which are deleted for *put*, but also carry a plasmid with appropriate homology, allow selection of rare recombinants in which a fusion has been crossed back onto the plasmid (Fig. 3B). Thus, interesting regulatory mutations can be recovered easily for sequencing.

Transfer of *lac* fusions from the *S. typhimurium* chromosome back to a plasmid invariably gave a plasmid with the predicted structure and was used routinely for verifying that strains carried the correct *lac* fusion. Transfer was accomplished in transductional crosses using a phage P22 donor lysate grown on a fusion strain into a recipient, TE2675, that carries a large nontransducible deletion of the *put* region and also carries a plasmid, pTE337. This plasmid was derived from pRS577 by insertion of a *Bgl*II linker into the *Hin*dIII site (inactivating Kan^r) followed by deletion of the 5′ half of the *lacZ* gene between the *Eco*RI and *Sac*I sites (Fig. 3B). The recombination events shown in Fig. 3B lead to reconstruction of a plasmid indistinguishable from the parent fusion plasmid.

As in the plasmid-to-chromosome crosses described above, linkage of the fusion segment to Kan^r is less than 100% (typically 10 to 25%). The desired class of Lac$^+$

recombinants was screened by pooling Kan^r colonies obtained by transduction, preparing plasmid DNA from them, and using the DNA to transform an *E. coli* Δ*lac* strain to Kan^r on plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Two additional points are important for interpreting the structures of these reconstructed plasmids. First, the pRS plasmids lack an *Eco*RI site present in the wild-type *lacZ* gene, while that site is present in the pMC931 parent of the *lac* operon used to construct strain TE2680 (4). Thus, only some individual transformants with a chromosomal fusion carry the *Eco*RI site, depending on the site of the crossover; similarly the *Eco*RI site may or may not be inherited in a chromosome-to-plasmid recombination event. Second, if a dimer or higher multimer of the pTE337 plasmid exists in the TE2675 recipient, the resulting fusion plasmid may be heterozygous for the region of the fusion insert.

Interaction between the fusion insert and its resident homolog. P22 transductional crosses can be used for mapping of putative regulatory mutations and assembly of fusions joining different endpoints. Use of fusion strains in mapping crosses requires that recipients be Rec$^+$, allowing the possibility of genetic exchange between the fusion segment and its resident homolog. This point is illustrated by a fusion that joins a segment of the *S. typhimurium hemA-prfA* operon to *lac*. The fusion contains a complete *hemA* gene and part of *prfA* and carries a frameshift mutation at an *Mlu*I site early in the *hemA* gene. The frameshift was necessary to make the starting plasmids more stable. The fusion was transferred to the chromosome of the wild-type *S. typhimurium* strain LT-2, in which it expressed *lac* only weakly as assayed on X-Gal indicator plates. Low expression is due to a polar effect of the *hemA* frameshift on the *prfA-lac* fusion. But upon transduction from an otherwise wild-type (LT-2) donor background into a wild-type (LT-2) recipient, rare dark blue colonies that expressed *lac* strongly were observed. Repair of the frameshift was confirmed by recovery of the Lac$^+$ fusions onto a plasmid followed by restriction enzyme digestion. The frequency of dark blue colonies observed after transduction averaged 2% and was substantially higher than the spontaneous reversion frequency. Whether repair was due to gene conversion or a double crossover was not determined.

Recombination between a fusion insert and its resident homolog will result in a duplication when the two sequences lie in the same orientation in the chromosome (or possibly a deletion if the homolog is closely linked to *put*). An inversion will occur when the interacting DNA segments lie in opposite orientation. All of the fusions described here contain segments from the *hemA-prfA* operon and nearby genes, which have the same orientation as *hemA*. Since the resident *hemA-prfA* operon is transcribed in the opposite direction to *putA*, an exchange between the *hemA* fusion segment and the resident *hemA-prfA* operon will lead to an inversion.

Inversions are easily recognized by linkage disruption (22). The Tn*10d*-Tet insertion carried by strain TE2929 is about 80% linked to *put*$^+$. If a strain carries an inversion formed by using the fusion insert, linkage of the fusion to Tet^r when strain TE2929 is used as a transduction donor should drop to 5% or less (7a). In addition, inverted fusions should not be transducible into a wild-type noninverted chromosome except at very low efficiency. Thus, any fusion which has been backcrossed against wild type is likely to be in the standard orientation. No inversions of these *lac* fusions have been observed in the absence of additional selection.

Separating the two halves of *hemA-lac*. Frequently one would like to isolate mutations that damage an element required for gene expression, such as the promoter or a binding site for a positive effector. When *lac* fusions are used, such mutants cannot be distinguished easily from polar mutations in the *lacZ* gene, which are very common. The scheme described below is designed to eliminate *lacZ* mutants. This can be accomplished by separately mutagenizing a segment that includes Kan^r and the promoter of interest but does not contain *lac* and then recombining this with another segment that carries the 3′ half of the fusion including the *lac* sequences, to test the level of *lac* expression.

I took advantage of a strain constructed previously that carries a 7.5-kb *Hin*dIII fragment including the entire *hemA-prfA* operon inserted in *put* (TE2962; 9). The orientation of *hemA* in this construct is the same as in the *lac* fusions. Figure 4A diagrams the cross used to construct strain TE4239, which carries the left junction of the fusion segment (Kan^s-*hemA*) but does not carry *lac*. Figures 4B and C show the crosses used to construct Kan^r derivatives of TE4239 which carry either the wild-type *hemA* promoter region or a deletion (Δ33) which removes the *hemA* promoter and the first 23 codons of the *hemA* gene (7). Figure 4D shows the cross used to construct strain TE4274, which carries the right junction of the fusion segment (*hemA-prfA-lac*) but does not carry Kan^r.

Figures 5A and 5B show the reconstruction crosses. Donor phage lysates grown on a strain carrying the wild-type *hemA* promoter (TE4253) or the Δ33 promoter deletion (TE4275) were used to transduce recipient strains TE4273 and TE4274, which carry a 3′ fusion half including *lac*. Only the crosses into the TE4274 recipient are illustrated. Recombinants were selected as both Kan^r and Tet^r; these have recombined in the interval between the left and right joints of the fusion segment to regenerate the fusion.

The results of the complete experiment are given in Table 3. When the donor carried a functional *hemA* promoter, all the Kan^r Tet^r transductants formed blue colonies on minimal glycerol–X-Gal plates, whereas when the donor carried the Δ33 promoter deletion, the Kan^r Tet^r transductants were all white. As expected, the type of fusion was determined by the *lac* half. When TE4273 was used as the recipient, the Kan^r Tet^r recombinants were light blue on minimal X-Gal plates and white on MacConkey lactose medium, characteristic of the lower level of expression of a *prfA-lac* protein fusion. Finally, formation of Tet^r Kan^r recombinants required homology within the fusion segment, since a *lac* fusion joint early in *hemA*, which lies upstream of the Δ33 endpoint, gave Kan^r Tet^r recombinants only with the wild-type promoter and not with Δ33.

Contribution of the *hemA* promoter to expression of genes downstream of *prfA*. The experiments described above show that the region upstream of the *hemA* gene, which carries two promoters serving *hemA* (27), is required for expression of the *prfA* gene. (It is also possible that *hemA* and *prfA* are translationally coupled.) Another set of *lac* fusions carrying DNA segments originating downstream of *hemA* was constructed; one spans the start of the first gene downstream of *prfA*, which is termed *dorf1* (downstream open reading frame 1), and a second extends from the middle of *dorf1* to the *kdsA* gene (30). Derivatives of these fusions bearing their *lac* junctions, and containing in addition the *'prsA-uorf-hemA-prfA* upstream DNA, were constructed as illustrated in Fig. 6. Crosses similar to those in Fig. 5 were then used to

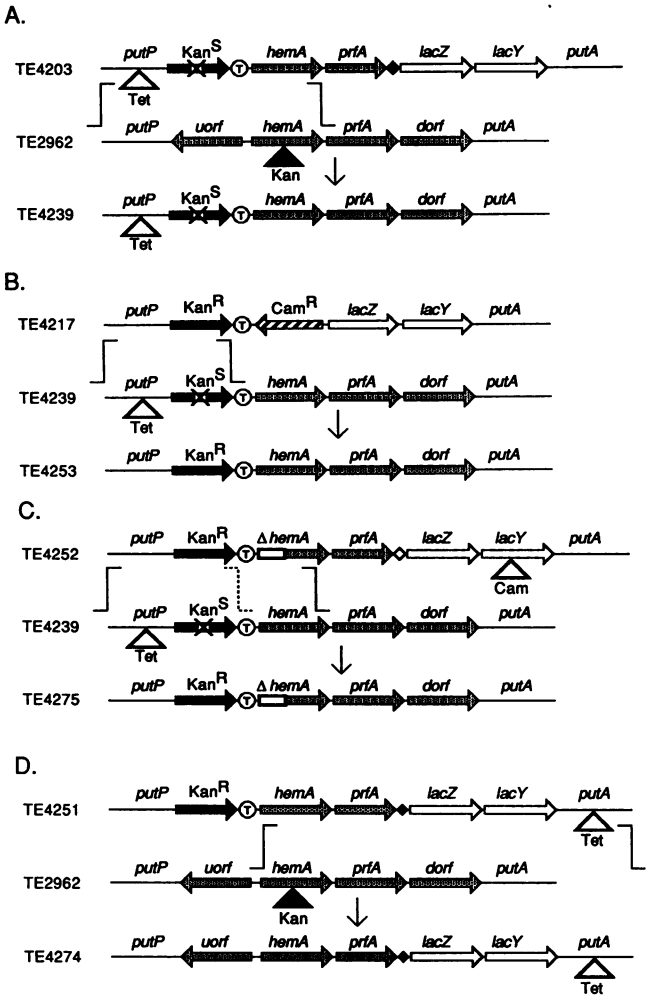

FIG. 4. Disassembly of Kan^r-*hemA-prfA-lac* fusions into the 5′ half (A and B), the 5′ half with a promoter deletion (Δ33) (C), and the 3′ half (D). For each cross, the donor fragment is shown above the recipient chromosome, and the recombinant is shown below the arrow. Some progeny of the cross in panel C inherited a wild-type promoter; the crossover point is indicated by the dotted line. On this diagram, two upstream open reading frames and three downstream open reading frames are collectively labeled *uorf* and *dorf*, respectively. Open triangles indicate defective transposons derived from Tn*10*, filled triangles indicate a Kan^r fragment from Tn*903*, an encircled T represents the tandem transcriptional terminators, and filled or open diamonds indicate operon or protein fusions, respectively. See Table 1 for complete genotypes and Table 2 and Materials and Methods for details of the transductional crosses.

construct isogenic derivatives bearing either a wild-type *hemA* promoter region or the Δ33 deletion.

The activity of β-galactosidase in these strains is shown in Table 4. The data show that the *hemA* promoter region is required for essentially all transcription of the *prfA* gene and nearly three-fourths of the transcription of *dorf1*, but it does not contribute significantly to expression of *kdsA*. Thus, the *hemA-prfA* operon includes at least one additional downstream gene, *dorf1*, but ends before *kdsA*. The *dorf1* gene is also served by an additional promoter located within about 435 bp upstream of its initiation codon.

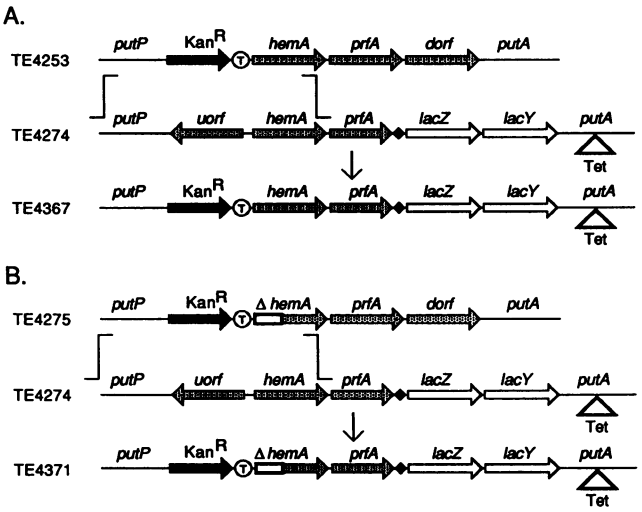

FIG. 5. Reassembly of *hemA-prfA-lac* fusions using a wild-type promoter 5′ half (A) or the Δ33 promoter deletion together with an operon fusion forming the 3′ half (B). Symbols are described in the legend to Fig. 4.

DISCUSSION

I have described the construction and use of an *E. coli* strain which yields chromosomal insertions when transformed with linearized plasmids carrying *lac* fusions. The *lac* fusion plasmids are those of Simons et al. (26). The resulting *E. coli* strains have insertions of the form *trp::put::Kan*^{r}*-X-lac*, where *X* is the promoter or gene fragment under study. The *put* operon DNA flanking *lac* is derived from *S. typhimurium*, and this homology allows the transfer of fusions into *S. typhimurium*. The existence of *S. typhimurium* strains with large deletions of *put* permits recovery of fusions back onto a plasmid by a standard transductional cross.

Choice of the *put* operon as the site for the fusions in *S. typhimurium* was based on the following considerations. (i) The *put* operon is transcribed divergently (11, 14); hence, fusions which replace the divergent *put* promoters should be insulated from other transcription units. (ii) Large deletions of *put* have been isolated. (iii) Both Put$^+$ and Put$^-$ phenotypes can be positively selected (18). (iv) The *put* operon is relatively close to the terminus of replication (about 10 min

TABLE 3. Reassembly of Kan^r-*lac* fusions by transduction

Recipient[a]	No. of Kan^r Tet^r transductants[b]			
	Donor[a] = TE4253 (*putPA*::[Kan^r-*hemA-prfA-dorf-kdsA'*]		Donor = TE4275 (*putPA*::[Kan^r-Δ33-′*hemA-prfA-dorf-kdsA'*]	
	Blue	White	Blue	White
TE4249 *hemA-lac* [op]	17, 20	0, 0	0, 0	0, 0
TE4273 *hemA-prfA-lac* [pr]	97, 109	0, 0	0, 0	61, 52
TE4274 *hemA-prfA-lac* [op]	116, 112	0, 0	0, 0	39, 61

[a] See Table 1 for complete genotypes.

[b] Selection for only Kan^r gave >1,000 transductants for each cross. Most of these were white on X-Gal, for crosses which gave blue Kan^r Tet^r colonies; otherwise, all Kan^r transductants were white. The results of two experiments are reported for each cross.

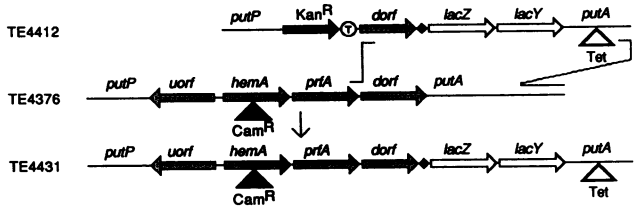

FIG. 6. Disassembly of a Kan^r-*dorf1-lac* fusion into a 3′ half. The donor fragment is shown above the recipient chromosome, and the recombinant is shown below the arrow. Symbols are described in the legend to Fig. 4.

away), minimizing the effect of generation time on gene dosage and protecting against some classes of duplication events. (v) An F′ plasmid carrying *put* has been isolated (5).

The existing F′ *put* plasmid is temperature sensitive for replication (which may be a virtue for some applications). In this work, a transposable version of the *put* operon, Tn*10d-put*, was constructed, and this transposon was used to isolate a derivative of the F$^+$ plasmid which carries *put* and can replicate normally at 42°C (strains TE4338 and TE4351; Table 1). Similar methods might be used to construct *put* insertions in conjugal plasmids that can replicate in bacterial species in which the F plasmid will not. The Tn*10d-put* transposon can also direct *lac* fusions to sites within the *S. typhimurium* or other bacterial genomes. Tn*10d-put* may be useful as a transposon in its own right, given a positive selection for Put$^-$.

The fusion strains do not carry a λ prophage, which simplifies P22-mediated transductional crosses involving them. The versatility of this system, in combination with a strain carrying an insertion of the wild-type *hemA-prfA* region in *put*, is shown by disassembly of *lac* fusions into their right and left halves. This allows the reassembly of fusions from their component halves. The purpose of this experiment is to facilitate screening for mutations which damage promoters and binding sites for positively acting factors. Such mutations may be phenotypically similar to polar mutations in *lacZ*, which are very common and must be distinguished from them by additional genetic manipulations. The system described here should permit mutagenesis of the promoter region in the absence of *lac*, followed by reassembly and screening for the level of *lac* expression to identify promoter mutants.

Previous experiments have demonstrated by indirect means that the *hemA* and *prfA* genes of *S. typhimurium* are organized into an operon. This conclusion is based on the existence of genetic polarity, such that nonsense mutants in *hemA* show a PrfA$^-$ phenotype (9), while insertions in *hemA* are lethal due to a failure to express *prfA* (7). Here, I show that transcription of the *prfA* gene, as assayed by expression of a *prfA-lac* fusion, requires the *hemA* promoter. The *hemA-prfA* operon also includes at least one additional gene whose function is unknown (*dorf1*), but the operon does not extend as far as *kdsA*.

TABLE 4. Determination of the extent of the *hemA-prfA* operon with *lac* fusions

Strain	Gene fused to *lacZ*	*hemA* promoter	β-Galactosidase activity (U)
TE4367	*prfA*	Wild type	960
TE4371	*prfA*	Deletion	6
TE4442	*dorf1*	Wild type	520
TE4445	*dorf1*	Deletion	120
TE4446	*kdsA*	Wild type	725
TE4448	*kdsA*	Deletion	650

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