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Conditional-Replication, Integration, Excision, and Retrieval Plasmid-Host Systems for Gene Structure-Function Studies of Bacteria

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We have developed a series of powerful and versatile conditional-replication, integration, and modular (CRIM) plasmids. CRIM plasmids can be replicated at medium or high copy numbers in different hosts for making gene (or mutant) libraries. They can be integrated in single copies into the chromosomes of *Escherichia coli* and related bacteria to study gene function under normal physiological conditions. They can be excised from the chromosome, e.g., to verify that phenotypes are caused by their presence. Furthermore, they can be retrieved singly or en masse for subsequent molecular analyses. CRIM plasmids are integrated into the chromosome by site-specific recombination at one of five different phage attachment sites. Integrants are selected as antibiotic-resistant transformations. Since CRIM plasmids encode different forms of resistance, several can be used together in the same cell for stable expression of complex metabolic or regulatory pathways from diverse sources. Following integration, integrants are stably maintained in the absence of antibiotic selection. Each CRIM plasmid has a polylinker or one of several promoters for ectopic expression of the inserted DNA. Their modular design allows easy construction of new variants with different combinations of features. We also report a series of easily curable, low-copy-number helper plasmids encoding all the requisite Int proteins alone or with the respective Xis protein. These helper plasmids facilitate integration, excision ("curing"), or retrieval of the CRIM plasmids.

Multicopy plasmids have greatly facilitated gene structure-function studies. However, the use of such plasmids can lead to high-copy-number artifacts, especially in physiological studies. Thus, several methods have been developed for recombining genes on bacterial chromosomes in order to study their functions in single copies. Such methods are frequently used to construct novel *Escherichia coli* strains that stably express foreign genes for use in both basic research and biotechnology (5, 18, 27). However, the development of strains encoding complex metabolic or regulatory pathways poses special problems that often require manipulating many genes and expressing them individually at different levels or under separate regulatory controls. To address these concerns, we have developed a series of plasmid-host systems for the introduction of multiple genes into the same cell in single copies. Our approach is based on genome targeting systems that utilize plasmids carrying a conditional-replication origin and a phage attachment (*attP*) site (17). We refer to our plasmids as CRIM (conditional-replication, integration, and modular) plasmids. CRIM plasmids can be integrated into or retrieved from their bacterial attachment (*attB*) site by supplying phage integrase (Int) without or with excisionase (Xis) in *trans*.

Advantages of our CRIM plasmid-host systems include the use of alternative *attP* and *attB* sites (for phages λ, HK022, ϕ80, P21, and P22) and different selectable markers (for chloramphenicol, gentamicin, kanamycin, spectinomycin and streptomycin, tetracycline, and trimethoprim resistance) in conjunction with a polylinker or promoter (P_{araB}, P_{rhaB}, P_{rhaS}, P_{tac}, P_{syn1}, and P_{syn4}) for ectopic expression of the cloned gene(s). These CRIM plasmids have the γ replication origin of R6K, which requires the *trans*-acting Π protein (encoded by *pir*) for replication. So, they replicate at a medium (15 per cell) or high (250 per cell) plasmid copy number in *pir*$^+$ or *pir-116* (high-copy-number mutant) *E. coli* hosts (28), respectively. Int helper plasmids are used for integration of CRIM plasmids into the corresponding chromosomal *attB* sites of normal (non-*pir*) hosts, which are nonpermissive for CRIM plasmid replication. Xis/Int helper plasmids are used for excision ("curing") of the respective CRIM plasmids from the chromosome, e.g., to verify that phenotypes are due to their presence. Xis/Int helper plasmids are also used for retrieval (cloning) of CRIM plasmids from the chromosome, e.g., to recover a particular CRIM plasmid after screening of CRIM plasmid or mutant libraries.

Since integration and retrieval involve phage-site-specific recombination events, the original and recovered plasmids are identical. CRIM plasmids can therefore be used for the construction of gene (or mutant) libraries that can be directly integrated into bacterial chromosomes in single copies for screening or selection purposes. Afterwards, CRIM plasmids can be retrieved from individual cells or en masse. The recovered plasmids can then be propagated as plasmids for molecular analysis or integrated directly into the chromosomes of other hosts for subsequent processing without further in vitro manipulation steps. We previously found similar $oriR_{\gamma}$ att_{λ} plasmids to be extremely useful in mutagenesis studies, especially when it was important that the mutated gene be free of plasmid copy number effects (16). We also found them to be useful in studying genes from diverse bacteria, including gram-

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TABLE 1. Bacterial strains

Strain[a]	Genotype[b]	Pedigree[c]	Reference(s) and/or derivation[d]
BW37	IN(*rrnD-rrnE*)*I tna bglR*::IS[e] *trpR ilv rpsL rph-1*	W3110 via BW33	38, 40
BW5328	Δ(*lacIZYA argF*)*U169 rph-1 rpoS396*(Am) *recA1 robA1 creC510 hsdR514*	BD792 via BW5206	37
BW23473	Δ(*lacIZYA argF*)*U169 rph-1 rpoS396*(Am) *robA1 creC510 hsdR514* Δ*endA9 uidA*(Δ*Mlu*I)::*pir*(wt) *recA1*	BD792 via BW23438	15, 16
BW23838	*lacI*^{q} *rrnB3* Δ*lacZ4787* Δ*phoBR580* Δ*creABCD154 hsdR514* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) DE(*araBAD*)*567* DE(*rhaBAD*)*568 rph-1 rpoS396*(Am) *uidA*(Δ*Mlu*I)::*pir*(wt) Δ*endA9 recD1014 recA1*)	BD792 via BW23832	Srl$^+$ with P1*kc* on BW8078; 14
BW24249	*lacI*^{q} *rrnB3* Δ*lacZ4787* Δ*phoBR580* Δ*creABCD154 hsdR514* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) DE(*araBAD*)*567* DE(*rhaBAD*)*568 rpoS396*(Am) *rph-1*Δ*endA9 galU95 uidA*(Δ*Mlu*I)::*pir*(wt) *recA1*	BD792 via BW24217	Srl$^+$ with P1*kc* on BW8078
BW24304	*lacI*^{q} *rrnB3* Δ*lacZ4787* Δ*phoBR580* Δ*creABCD154 hsdR514* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) DE(*araBAD*)*567* DE(*rhaBAD*)*568 rph-1 rpoS396*(Am) Δ*endA9 galU95 uidA*(Δ*Mlu*I)::*pir-116 recA1*	BD792 via BW24296	Srl$^+$ with P1*kc* on BW8078
BW24320	*lacI*^{q} *rrnB3* Δ*lacZ4787* Δ*phoBR580* Δ*creABCD154* DE(*araBAD*)*567* DE(*rhaBAD*)*568 rph-1*	BD792 via BW24310	Cys$^+$ with P1*kc* on MG1655; 24
BW25113	*lacI*^{q} *rrnB3* Δ*lacZ4787 hsdR514* DE(*araBAD*)*567* DE(*rhaBAD*)*568 rph-1*	BD792 via BW25083	Pro$^+$ with P1*kc* on BW24321; 10, 24
BW25141	*lacI*^{q} *rrnB3* Δ*lacZ4787 hsdR514* DE(*araBAD*)*567* DE(*rhaBAD*)*568* Δ*phoBR580 rph-1 galU95* Δ*endA9 uid*A(Δ*Mlu*I)::*pir*(wt) *recA1*	BD792 via BW25140	Srl$^+$ with P1*kc* on BW8078; 10, 24
BW25142	*lacI*^{q} *rrnB3* Δ*lacZ4787 hsdR514* DE(*araBAD*)*567* DE(*rhaBAD*)*568* Δ*phoBR580 rph-1 galU95* Δ*endA9 uid*A(Δ*Mlu*I)::*pir-116 recA1*	BD792 via BW25137	Srl$^+$ with P1*kc* on BW8078
BW25695	*lacI*^{q} *rrnB3* Δ*lacZ4787 hsdR514 att*$_{P22(EcoB)}$ DE(*araBAD*)*567* DE(*rhaBAD*)*568 rph-1*	BD792 via BW25367	Pro$^+$ with P1*kc* on BW25676

[a] All strains are λ$^-$ and F$^-$.
[b] All known mutations are indicated.
[c] The ancestral strain from another lab and its immediate ancestor in our lab are given.
[d] Additional information is given in Materials and Methods.
[e] IS, insertion sequence.

negative and -positive cells (14, 15, 25, 34). Our versatile CRIM plasmid-host systems should be widely useful in gene structure-function studies. Here we describe our basic set of CRIM plasmids, the requisite helper plasmids, and how to use them.

MATERIALS AND METHODS

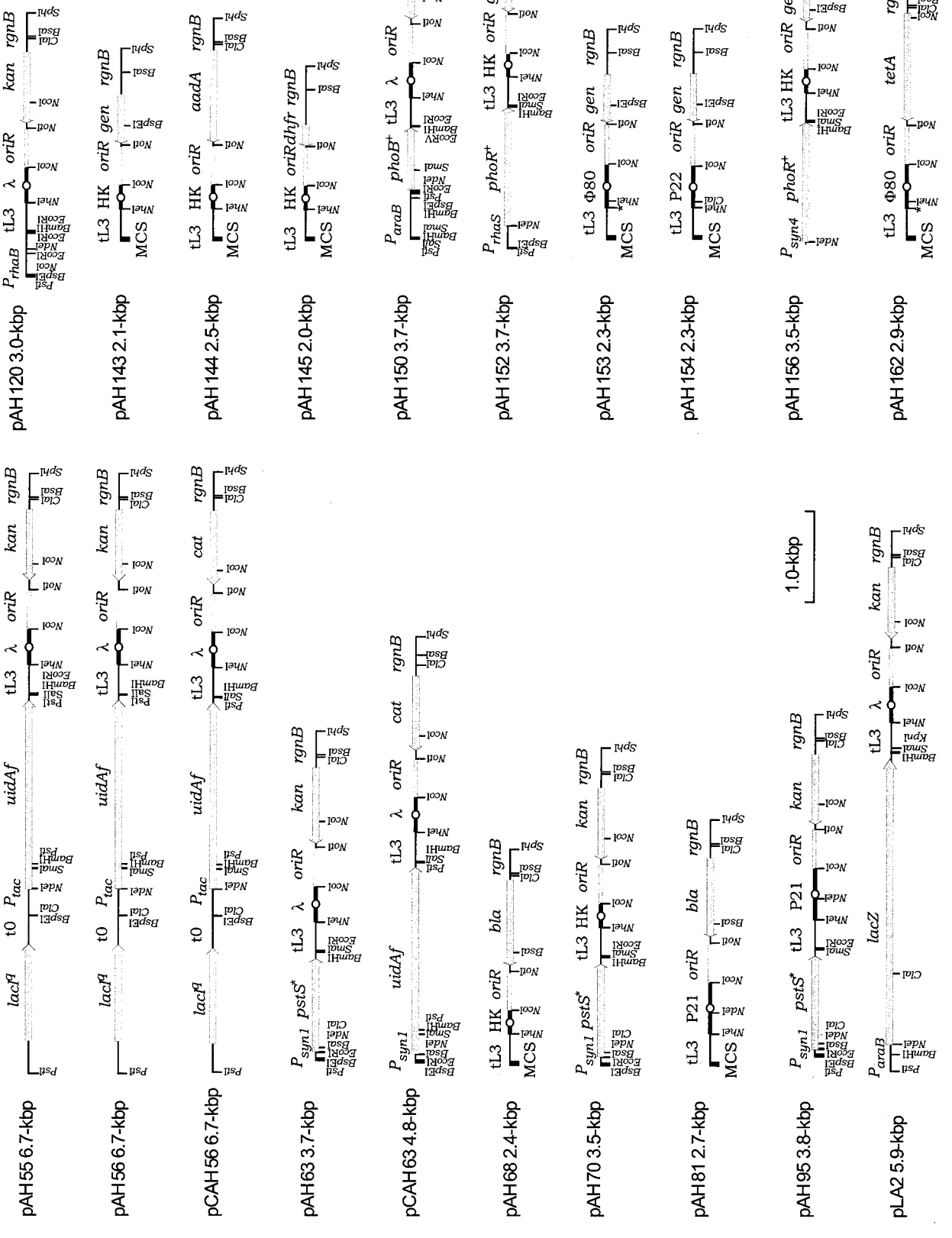
Media and culture conditions. Luria-Bertani (LB) broth (without glucose), tryptone-yeast extract agar (pH 7.0), and glucose M63 agar were used as complex and minimal media (38). SOB and SOC were prepared as described elsewhere (30). To maintain plasmids, antibiotics (from Sigma, St. Louis, Mo.) were added as follows: ampicillin at 100 μg/ml, chloramphenicol at 25 μg/ml, gentamicin at 15 μg/ml, kanamycin at 50 μg/ml, spectinomycin and streptomycin (together) at 100 μg/ml, trimethoprim at 300 μg/ml, or tetracycline at 12.5 μg/ml. Single-copy integrants were selected using chloramphenicol at 6 μg/ml, gentamicin at 5 μg/ml, kanamycin at 10 μg/ml, spectinomycin and streptomycin (together) at 35 μg/ml, trimethoprim at 25 μg/ml, or tetracycline at 8 μg/ml. We found that proper medium pH is especially important for selection of gentamicin-resistant (Gm^r) integrants. Complex media (tryptone-yeast extract agar) were used for selection of all forms of resistance except trimethoprim resistance, for which minimal media were used. Following primary selection, integrants were routinely maintained in the absence of antibiotics.

Bacteria. All strains are derivatives of *E. coli* K-12. Normal (self-replicating) plasmids were propagated in DH5α (12), BW5328, or BW25141. Strains from this laboratory are fully described Table 1. The DE(*araBAD*)*567* and DE(*rhaBAD*)*568* mutations correspond to the Δ*araBAD*$_{AH33}$ and Δ*rhaBAD*$_{LD78}$ alleles (14), respectively. The adjacent *rrnB3* Δ*lacZ4787* mutations were previously called *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ (14). The Δ*endA9* allele corresponds to the Δ*endA8*::*tetAR* mutation (from BT333 [9]) after Flp-mediated excision of *tetAR* with pCP20 (9). The *recD1014* mutation originated from V355 (from G. C. Walker [33]). The *att*$_{P22(EcoB)}$ allele refers to the *att*$_{P22}$ site of *E. coli* B, which had been introduced into BW25368 (*proA*::Tn*10*) by using P1*kc* grown on NC3 (*E. coli* B/r *hsdR*, also called BW9688 [39]) by selecting proline-independent transductants to make BW25676. Our standard wild-type *E. coli* K-12 strain is BD792 (36), which is a direct F$^-$ descendant of W1485 (2). The *rph-1* allele refers to the *rph* frameshift mutation (19), which is also present in *E. coli* BD792 (data not shown). Strain BD792, like both its parent, W1485, and wild-type *E. coli* K-12 EMG2 (20), carries the *rpoS396*(Am) allele (data not shown). Several strains were therefore made *rpoS*$^+$. This was done in two steps. A strain was first made tetracycline resistant (Tc^r) and kanamycin resistant (Km^r) by using P1*kc* grown on ZK1001 (*cysC95*::Tn*10 rpoS*::*kan*; from R. Kolter). A resultant Cys$^-$ transductant was then made cysteine independent and kanamycin sensitive by using P1*kc* grown on MG1655. *E. coli* K-12 strains BW25113, BW25141, BW25142, and BW25695 are descendants of BD792 derivatives that were made *rpoS*$^+$.

CRIM (*ori*R_γ) plasmids were propagated at a medium copy number in BW23473, BW24249, or BW25141 or at a high copy number in BW23474, BW24304, or BW25142. As standard wild-type hosts, we used BW25113 and BW25695 [like BW25113, except with *att*$_{P22(EcoB)}$]. CRIM plasmids were retrieved from integrants of BW24320 by using helper plasmid transformants of BW23473 (*pir*$^+$ *recA*) or BW25141 (*pir*$^+$ *recA*) when we used P1*kc* transduction (P1-Int-Xis [PIX] cloning [16]) or helper plasmid transformants of BW23838 (*pir*$^+$ *recD*) when we used transformation (see below). Strain BW37 was used as the recipient for selection of Ilv$^+$ transductants when we determined the Ilv$^+$ transducing titers of P1*kc* lysates.

Molecular biology methods. PCR fragments for cloning were generated by using Vent (New England Biolabs, Beverly, Mass.) or *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) and oligonucleotide primers (from IDT Inc., Coralville, Iowa). Other enzymes were from New England Biolabs or Promega (Madison, Wis.). Qiagen (Hilden, Germany) products were used for the isolation of plasmid DNA, extraction of DNA fragments from agarose gels, and purification of PCR fragments.

Plasmids. CRIM (Fig. 1) and CRIM helper plasmids (Fig. 2; Table 2) were assembled by standard techniques. pLA2 was constructed by L. Avramova and


pAH120 3.0-kbp
pAH143 2.1-kbp
pAH144 2.5-kbp
pAH145 2.0-kbp
pAH150 3.7-kbp
pAH152 3.7-kbp
pAH153 2.3-kbp
pAH154 2.3-kbp
pAH156 3.5-kbp
pAH162 2.9-kbp
pAH55 6.7-kbp
pAH56 6.7-kbp
pCAH56 6.7-kbp
pAH63 3.7-kbp
pCAH63 4.8-kbp
pAH68 2.4-kbp
pAH70 3.5-kbp
pAH81 2.7-kbp
pAH95 3.8-kbp
pLA2 5.9-kbp
1.0-kbp

B. L. Wanner (unpublished). Various fragments were subcloned directly or cloned as PCR-generated fragments containing restriction site extensions (Table 3). The *bla*, *cat*, *kan*, *tet*, and *oriR*$_{\gamma}$ segments were from pANTSγ, pCANTSγ, pKANTSγ, and pTANTSγ (32) (from M. Koob); the *att*$_{\lambda}$ (with a destroyed *Nde*I site) and *lacI*^{q}-*P*$_{tac}$ segments were from pCANTSγΔ*Nde*I and pCANTSγ*lacI*^{q}SLP (26) (both from A. S. Lynch); the *lacZ* cassette in pAH125 was from pCS3 (29); the promoterless *uidAf* (the *uidA2* fusion in pSK49Δ::*uidA2*) cassette was from pWM3 (29); the *P*$_{araB}$ fragment in pAH150 was from pAH31 (14); and *P*$_{rhaB}$ in pAH120 was from pLD78 (14). The *lacZ* gene in pLA2 was constructed in a series of steps that involved introducing an *Nde*I site overlapping its Met start codon and eliminating a native *Nde*I site; the *lacZ* gene was generated using pOD (31) as the template, so it has a mutated *lacO2* region; and the *P*$_{araB}$ fragment in pLA2 was generated as a PCR fragment (Table 3). Our initial CRIM plasmid pAH55 is a derivative of pKANTSγ in which we introduced a mutated *att*$_{\lambda}$ segment (lacking its native *Nde*I site), *lacI*^{q}, and *uidAf* segments in sequential steps. The CRIM helper plasmids were assembled using pINT-ts (17) as the backbone. PCR fragments were generated by using as templates pHH7013 and pHH7009 (from J. C. Hu) for *P*$_{syn1}$ and *P*$_{syn4}$; a derivative of pLD78 (14) called pLD81 (from L. Daniels) for *P*$_{rhaS}$; p34E-Tp (11) (from D. E. Woods) for *dhfr*; pBBR1MCS-5 (22) (from M. Kovach) for *gen*; λ (from R. Somerville) for *xis*$_{\lambda}$; pBAD33 (13) (from L.-M. Guzman) and pBS1*att*$_{P6\text{-}1}$ (6) (from A. Campbell) for *att*$_{P21}$, *xis*$_{P21}$, and *int*$_{P21}$; pEY109 (42) (from E. Yagil) for *att*$_{HK022}$, *xis*$_{HK022}$, and *int*$_{HK022}$; pJL10 and pJL110 (23) (from A. Landy) for *att*$_{\phi 80}$, *xis*$_{\phi 80}$, *int*$_{\phi 80}$, and *att*$_{P22}$; and P22 (from S. Maloy) for *xis*$_{P22}$ and *int*$_{P22}$.

CRIM plasmid integration. Cells carrying a CRIM helper plasmid were grown in 5 ml of SOB cultures with ampicillin at 30°C to an optical density of 600 nm of ca. 0.6 and then made electrocompetent. Following electroporation, cells were suspended in SOC without ampicillin, incubated at 37°C for 1 h and at 42°C for 30 min, and then spread onto selective agar and incubated at 37°C. Colonies were purified once nonselectively and then tested for antibiotic resistance for stable integration and loss of the helper plasmid and by PCR for copy number (see below).

CRIM plasmid excision. Cells were transformed with the respective Xis/Int CRIM helper plasmid and then spread on ampicillin agar media at 30°C. Colonies were purified once or twice nonselectively on plates that were incubated for 1 h at 42°C and overnight at 37°C. They were then tested for antibiotic sensitivities and by PCR for loss of the integrated plasmid.

CRIM plasmid retrieval. P1*kc* lysates were made on integrants by using standard procedures. Recipient cells carrying the corresponding Xis/Int CRIM helper plasmid were grown in LB agar with ampicillin at 30°C to early stationary phase and then infected with a P1*kc* lysate. Following phage absorption, centrifugation, and resuspension as described elsewhere (38), the infected cells were incubated for 1 h at 37°C, 30 min at 42°C, and an additional hour at 37°C and then spread onto selective media (without ampicillin) for the CRIM plasmid and incubated at 37°C. To recover plasmids by transformation, chromosomal DNAs were isolated from integrants and subjected to shearing by sonication or DNase I digestion in the presence of divalent manganese (1). Recipient cells carrying a helper plasmid were grown in 5-ml SOB cultures with ampicillin at 30°C to an optical density at 600 nm of ca. 0.6 and then made electrocompetent. Following electroporation, cells were suspended in SOC without ampicillin, incubated at 37°C for 1 h and at 42°C for 30 min, and then spread onto selective agar and incubated at 37°C.

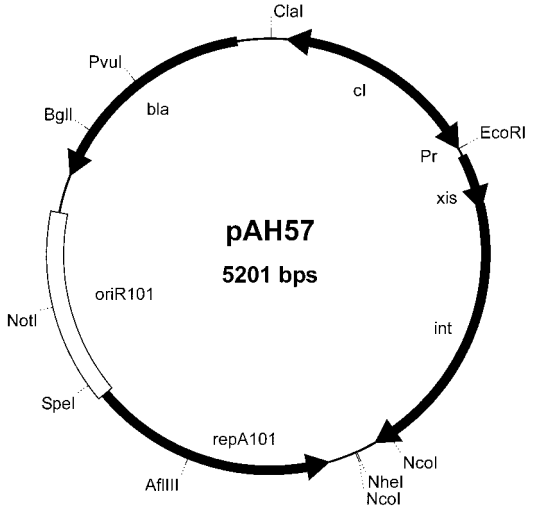


FIG. 2. CRIM helper plasmid. All helper plasmids express *int* alone or with *xis* from λ*p*$_{R}$ under *c*I857 control, which is also borne by these plasmids, and are temperature sensitive for plasmid replication. As described in Materials and Methods, all are derivatives of pINT-ts, whose complete DNA sequence we determined in this study. With one exception, the *xis-int* plasmids express these genes in the same orientation as that found naturally. The construction of pAH129 resulted in placing *xis*$_{\phi 80}$ upstream of *int*$_{\phi 80}$ to create an *xis-int*$_{\phi 80}$ operon (data not shown). We also deliberately destroyed the native *Bam*HI site in *int*$_{\lambda}$ during the construction of pAH57 by introducing a silent mutation with the *xis*$_{\lambda}$-5′ primer (Table 2). Pr, phage λ promoter.

PCR verification of integrant copy number. Isolated colonies were picked up with a plastic tip and suspended in 20 μl of water. Five microliters of the cell suspension, 10 pmol of each primer (P1 to P4 together), and 0.5 U of *Taq* DNA polymerase (New England Biolabs) were combined in 1× PCR buffer–2.5 mM $MgCl_2$ with deoxynucleoside triphosphates in a final volume of 25 μl. PCR was carried out for 25 cycles with denaturing for 1 min at 94°C, annealing for 1 min (Table 4), and extension for 1 min at 72°C.

DNA sequencing. The DNA sequences of all CRIM and CRIM helper plasmids were deduced in their entirety by verifying the sequences of all modules used in their constructions. PCR-amplified segments were verified by automated DNA sequencing of both strands after initial cloning. Many were initially cloned into *Sma*I-digested pSPORT1 (from Gibco BRL, Gaithersburg, Md.) or *Eco*RI- and *Nco*I-digested pLITMUS29 (from New England Biolabs). Others were cloned directly into a CRIM plasmid and then sequenced. Several additional regions were also sequenced to permit generation of detailed maps of all CRIM and CRIM helper plasmids.

FIG. 1. Structures of CRIM plasmid series. Gene designations include *aadA* (aminoglycoside adenyltransferase for spectinomycin and streptomycin resistance), *bla* (β-lactamase for ampicillin resistance), *cat* (chloramphenicol acetyltransferase), *dhfr* (dihydrofolate reductase for trimethoprim resistance), *gen* (gentamicin-3-acetyltransferase for gentamicin resistance), *kan* (aminoglycoside 3′-phosphotransferase for kanamycin resistance), *pstS*∗ (a mutant *pstS*, P_i-specific binding protein), *tetA* (tetracycline resistance), and *uidAf* (the *uidA2* fusion in pSK49Δ::*uidA2* [16]). The multiple cloning site (MCS) is from pUC18 (44). Unique sites within the MCS of pAH68 include, from left to right, *Sph*I, *Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Sac*I, and *Eco*RI. All sites are illustrated for the enzymes shown. Sites destroyed during construction are marked with an asterisk. Modules are flanked by *Sph*I, *Eco*RI (*Bam*HI or *Nde*I), *Nhe*I, *Nco*I, *Not*I, and *Cla*I (and/or *Bsa*I) sites. Plasmids with *aadA*, *bla*, or *gen* facilitate certain constructions as they have a unique *Nco*I site. Due to the manner in which these plasmids were constructed, the *P*$_{araB}$ region of pAH150, but not of pLA2, encodes an N-terminal portion of AraC as a fusion protein. As a consequence, *P*$_{araB}$ is expressed at a normal level in pLA2 but at a much reduced level in pAH150 (see the text). All *attP* sites were designed taking into account information on important DNA binding sites and structure (23, 35, 43). Accordingly, the *att*$_{P21}$ sequence encodes the C terminus of *icd* and the *att*$_{P22}$ sequence includes sequences for the *thrW* tRNA gene (6). Unexpectedly, we found that CRIM plasmids carrying *att*$_{P22}$s or *att*$_{\phi 80}$s (Table 3) failed to integrate or gave very few integrants, respectively, suggesting that additional *att* sequences are required (data not shown). Plasmids carrying a longer *att*$_{\phi 80}$ site (such as pAH153 and pAH162) integrated efficiently, while those carrying the longer *att*$_{P22}$ site (such as pAH154) integrated less frequently than others (see the text). Primers routinely used to verify cloned inserts by PCR or DNA sequencing include rgnB-f (TTGTCGGTGAACGCTCTCCT), ParaB-f (CACATTGATTATTTGCACGG), PrhaB-f (CGTTCATCTTTCCCTGGT), and tL3-r (AGGATGCGTCATCGCCATTA). Priming sites rgnB-f and tL3 are common to all CRIM plasmids and are useful for sequencing inserts in all CRIM plasmids, except those containing *lacI*^{q}, in which only tL3-r remains useful.

TABLE 2. CRIM helper plasmids

Plasmid[a]	Function(s)
pINT-ts	Int_{λ}
pAH57	Xis and Int_{λ}
pAH69	Int_{HK022}
pAH83	Xis and Int_{HK022}
pAH121	Int_{P21}
pAH122	Xis and Int_{P21}
pAH123	$Int_{\phi 80}$
pAH129	Xis and $Int_{\phi 80}$
pAH130	Int_{P22}
pAH131	Xis and Int_{P22}

[a] The structure of an example is shown in Fig. 2.

Nucleotide sequence accession numbers. GenBank accession numbers for the CRIM plasmids are AY048713 (pAH55), AY048714 (pAH56), AY048716 (pAH63), AY048717 (pAH68), AY048719 (pAH70), AY048720 (pAH81), AY048722 (pAH95), AY048723 (pAH120), AY054372 (pAH125), AY048730 (pAH143), AY048731 (pAH144), AY048732 (pAH145), AY048733 (pAH150), AY048734 (pAH152), AY048735 (pAH153), AY048736 (pAH154), AY048737 (pAH156), AY048738 (pAH162), AY048739 (pCAH56), AY048740 (pCAH63), and AY054373 (pLA2). GenBank accession numbers for the CRIM helper plasmids are AY048715 (pAH57), AY048718 (pAH69), AY048720 (pAH83), AY048724 (pAH121), AY048725 (pAH122), AY048726 (pAH123), AY048727 (pAH129), AY048728 (pAH130), AY048729 (pAH131), and AY048741 (pINT-ts).

RESULTS AND DISCUSSION

General description. Our basic CRIM plasmids are shown in Fig. 1. Each has four general regions in common: a polylinker or a cloning region consisting of a promoter for ectopic expression with or without a regulatory gene, a phage attachment (*attP*) site, a conditional-replication origin ($oriR_{\gamma}$), and a selectable marker. Several already contain an *E. coli* gene (*lacZ*, *phoB*, *phoR*, *pstS*, or *uidAf*) within the cloning region; however, these genes act solely as replaceable ("stuffer") fragments in new constructions. In addition, all CRIM plasmids have bacterial (*rgnB*) and phage λ (t0, tL3) terminators flanking their cloning region to protect other segments from transcriptional readthrough. The CRIM plasmids were designed so that stan-

TABLE 3. Oligonucleotide primers used for plasmid constructions

Region	Template	Primer, sequence[a]
aadA	pWM5	*aadA*-5′, GCA*ATCGAT*ACGGATGAAGGCACGAACC; *aadA*-3′, GCA*GCGGCCGC*TCGGCTTGAACGAATTGTTAG
dhfrIIb	p34E-Tp	*dhfrIIb*-5′, GCA*GGCGCC*ACGAACCCAGTTGACATAAGC; *dhfrIIb*-3′, GCA*GCGGCCGC*TTAGGCCACACGTTCAAGT
gen	pBBR1MCS-5	*gen*-5′, GCA*GGCGCC*CACGAACCCAGTTGACATA; *gen*-3′, GCA*GCGGCCGC*CTTGAACGAATTGTTAGG
P_{araB}	pBAD33	P_{araB}-5′, AA*CTGCAG*CGCCATTCAGAGAAGAAACCAA; P_{araB}-3′, GCA*GTCGAC*CATATGAATTCCTCCATCCAAAAAAACGGGTATGGAGA
P_{rhaS}	pAH151	P_{rhaS}-5′, GCA*TCCGGA*AATTCGCGACCTTCTCG; P_{rhaS}-3′, GCAT*CTAGAGCATATG*GGCCTCCTGATGTCGTC
P_{syn1}-*pstS*∗	pLD81	P_{syn1}-5′, GCA*TCCGGAACTAGT*GTCTTCAAGAATTCTAGG; *pstS*-3′, CG*GGATCCACGCGT*TTACAAAGTC
xis_{λ}	λ	xis_{λ}-5′, CCG*GAATTC*TTGCGTGTAATTGCGGAGAC; xis_{λ}-3′, GGA*AGATCT*CCTTCGAAGGAAAGACCTGATGC
$attP_{HK022}$	pEY109	$attP_{HK022}$#1, GCA*GCTAGC*TAATGCTCTGTCTCAGGTC; $attP_{HK022}$#2, GCA*CCATGG*GACAAAATTGAAATCG
int_{HK022}	pEY109	int_{HK022}-5′, GCA*CCATGG*TAAGTAGGTCATTATTAGTC; int_{HK022}-3′, GCA*GAATTC*GTAGCCTTTTGAAGAGG
xis-int$_{HK022}$	pEY109	xis_{HK022}-5′, GCA*GAATTC*TGCGGAGACTTTGC; int_{HK022}-3′, see above
$attP_{P21}$	pBS1*attP6*-1	$attP_{P21}$#1, GCA*CCATGG*AATGACCGACCGATA; $attP_{P21}$#2, GCA*GCTAGC*ATA*AGGCCT*CGCAA
int_{P21}	pBS1*attP6*-1	int_{P21}-5′, GCA*GAATTC*AGAACCGCAACTCCCAA; int_{P21}-3′, GCA*CCATGG*ATAACGGGCGTATAACA
xis-int$_{P21}$	pBS1*attP6*-1	xis_{P21}-5′, GCA*GAATTC*GCAGCTAAGAGGAGGAC; int_{P21}-3′, see above
$attP_{P22s}$	pJL110	$attP_{P22}$#1, GCA*GCTAGC*CGTTGTTACCGATCAAT; $attP_{P22}$#2, GCA*CCATGG*AAGGCACGAATAATACG
$attP_{P22}$	P22	$attP_{P22}$#3, GCA*GCTAGC*CATTATGAAAATCAGCGGATTCGGA; $attP_{P22}$#4, CATG*CCATGG*AATCACCTGACTGAACATGCTCGAC
int_{P22}	P22	int_{P22}-5′, GCA*GAATTC*CACCACACGACAAGCCT; int_{P22}-3′, GCA*CCATGG*ACTCCTATTATCGGCAC
xis-int$_{P22}$	P22	xis_{P22}-5′, GCA*GAATTC*CTACGACATGCCTAACG; int_{P22}-3′, see above
$attP_{\phi 80}$s	pJL10	$attP_{\phi 80}$#1, A*ACCGGT*GAATCACGAC; $attP_{\phi 80}$#2, GCA*CCATGG*ACATCCTTGAAAGCCTG
$attP_{\phi 80}$	pJL10	$attP_{\phi 80}$#3, GCA*TCTAGA*GGTGAATCACGACAAAGCGTATC; $attP_{\phi 80}$#4, CATG*CCATGG*GCCCGTGCGAATCAGAAATAAT
$int_{\phi 80}$	pJL10	$int_{\phi 80}$-5′, GCA*GAATTCGCTAGC*AACCCTTATCCAGCA; $int_{\phi 80}$-3′, GCA*CCATGG*CAATCAAAGAGTGGGAG
$xis_{\phi 80}$	pJL10	$xis_{\phi 80}$-5′, GCA*GAATTC*GGCAACTGGAGAGAGCTAT; $xis_{\phi 80}$-3′, GCA*GCTAGC*AAGAGATCATCGGAGAG

[a] Restriction sites are italicized. Bases complementary to the template are underlined.

TABLE 4. PCR tests for integration of CRIM plasmids[a]

attP phage	Primer P1 sequence	Primer P4 sequence	Temp (°C)	Predicted size(s) of PCR fragment(s) for *attB* with[b]: No integrant with P1 and P4	Single integrant with P1 and P2, P3 and P4	Multiple integrant with P1 and P2, P3 and P2, P3 and P4
λ	GGCATCACGGCAATATAC	TCTGGTCTGGTAGCAATG	63	741	577, 666	577, 502, 666
HK022	GGAATCAATGCCTGAGTG	GGCATCAACAGCACATTC	59	740	289, 824	289, 373, 824
ϕ80	CTGCTTGTGGTGGTGAAT	TAAGGCAAGACGATCAGG	63	546	409, 732	409, 595, 732
P21	ATCGCCTGTATGAACCTG	TAGAACTACCACCTGACC	57	506	568, 620	568, 682, 620
P22	AAGTGGATCGGCATTGGT	TTCGTATCGACAGGAAGG	63	735	376, 926	376, 568, 926
e14*attR*	CGCTTGAAGATGTGTGGT	GTTACGGTCTTGGCATTG	57	862	1,226, 389	1,226, 682, 389
P22(EcoB)	AAGTGGATCGGCATTGGT	CGATTGAACCGCAGATTACG	63	609	376, 801	376, 568, 801

[a] Primers P2 (ACTTAACGGCTGACATGG) and P3 (ACGAGTATCGAGATGGCA) are the same for all *attP* sites. "Temp" is the annealing temperature. The P4 priming sites for att_{P21} and att_{P22} lie within prophage elements of wild-type *E. coli* K-12 (Fig. 2). An att_{P21} CRIM plasmid integrates to the left (counterclockwise) of e14. The e14*attR* primers were used to test for integration to the right (clockwise) of e14. *E. coli* K-12, but not *E. coli* B, has an uncharacterized prophage in att_{P22}. An att_{P22} CRIM plasmid integrates adjacent (counterclockwise) to this prophage in *E. coli* K-12. Primers for $att_{P22(EcoB)}$ were used to test for integration of att_{P22} CRIM plasmids in *E. coli* K-12 strains (BW25695 and others) with an "empty" att_{P22} site from *E. coli* B.

[b] Sizes are in nucleotides.

dard cloning methods can be used for making new ones with various combinations of these and other features, as necessary.

CRIM plasmid integration. CRIM plasmids can be simply integrated into the chromosome by direct transformation of normal (non-*pir*) hosts carrying a CRIM helper plasmid synthesizing the respective Int (Fig. 2; Table 2). Int synthesis from the helper plasmids is induced at elevated temperatures. Since the helper plasmids are also temperature sensitive for replication (see Materials and Methods), the resulting transformants are nearly always simultaneously cured of the helper plasmid. Upon integration at the respective *attB* site, all CRIM plasmids lie in the same relative orientation on the *E. coli* chromosome (Fig. 3). Therefore, even though they have sequences in common (tL3, $oriR_{\gamma}$, and *rgnB*), homologous recombination among them does not lead to instability because essential chromosomal genes lie between these *attB* sites. Due to the high efficiency of site-specific recombination, these homologies also do not interfere with integrating multiple CRIM plasmids at different *attB* sites in the same strain.

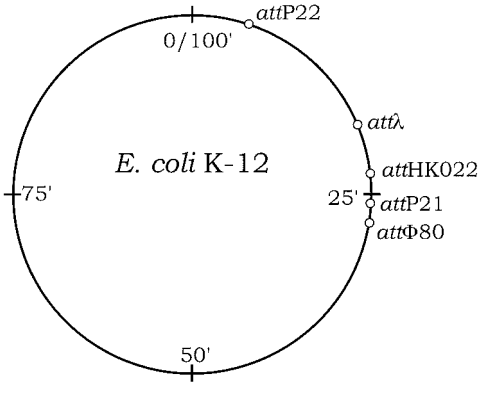


FIG. 3. Locations of chromosomal *attB* sites. Wild-type *E. coli* K-12 contains the prophage element e14 adjacent (clockwise) to att_{P21} (6). Sites are based on the linkage map (3) and the positions of the appropriate *attB* core sequences (for att_{λ}, gCTTttTtatActAA; for att_{HK022}, CTTTaggtgaa; for att_{P21}, tGCtGCgcCATAT; for att_{P22}, ATTcgtAATGcGAAG; for $att_{\phi 80}$, AACAcTTTcttAAAt; lowercase letters indicate bases that differ from the consensus [6]), in the *E. coli* K-12 genome sequence (4). *E. coli* K-12 has two att_{P22} sites separated by ca. 34 kb (at nucleotides 262125 and 296433 of the genome), which is consistent with their being separated by an uncharacterized phage. Furthermore, by using PCR and P22(EcoB) primers (Table 2), we have shown that the intervening sequences is absent from *E. coli* B, so it lacks this phage (data not shown).

To test for single-copy integration, we routinely use a single PCR with four primers (P1, P2, P3, and P4 in Fig. 4A; see Materials and Methods). Single-copy integrants are revealed as integrants that have lost the fragment corresponding to the respective *attB* site (the P1 to P4 fragment) and simultaneously gained two new fragments that are characteristic of the *attL* (BOP′; the P1-to-P2 fragment) and *attR* (POB′; the P3-to-P4 fragment) junctions. Recombinants with two (or more) CRIM plasmids at the *attB* site are also easily distinguishable. Such multiple integrants also gain a third fragment characteristic of the *attP* site of the integrating plasmid (the P2-to-P3 fragment). Recombinants resulting from integration elsewhere on the chromosome yield instead PCR products for both the *attB* (the P1-to-P4 fragment) and *attP* (the P2-to-P3 fragment) sites, provided that such integration occurs via homologous recombination or otherwise outside the *attP* region. Based on these criteria, we have shown that integration occurs primarily at the respective *attB* site and always requires the corresponding Int. The most common undesirable events are the occurrence of multiple-copy integrants; however, these integrants seldom represent more than a few percentages of the antibiotic-resistant transformants. With one exception, this is true for all CRIM plasmids (data not shown).

The exception concerns the att_{P22} CRIM plasmids. These plasmids differ in two ways. First, the att_{P22} plasmids integrate ca. 100-fold less efficiently than the others. Second, one-half or more of the resulting att_{P22} plasmid integrants are often incorrect and appear to occur via recombination events that do not involve the att_{P22} site. Since wild-type *E. coli* K-12 apparently has an uncharacterized prophage occupying the chromosomal att_{P22} site (unpublished results), we considered the possibility that this prophage interferes with site-specific integration at this site. However, we obtained similar results with an otherwise isogenic host lacking this prophage, suggesting that an additional factor or sequence is required for efficient att_{P22} recombination. Nevertheless, att_{P22} CRIM plasmids have still been quite useful for constructing strains that have multiple

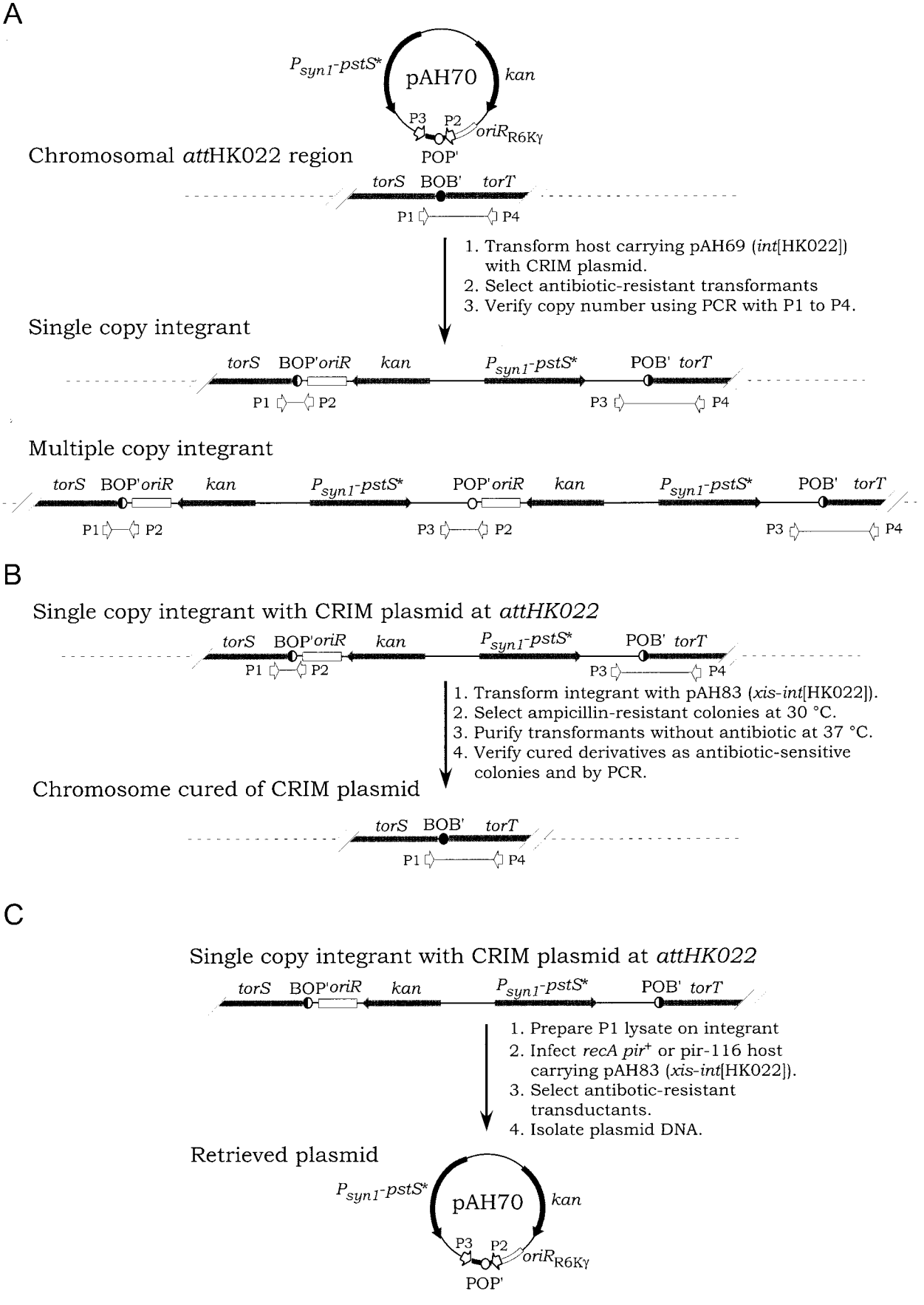


FIG. 4. Integration (A), excision (B), and retrieval (C) of CRIM plasmids from att_{HK022}. POP′ and BOB′ are sites for phage site-specific recombination according to the Campbell model (7, 41). P1, P2, P3, and P4 are priming sites used in PCR tests (see Materials and Methods).

CRIM plasmids. In these cases, we have usually integrated att_{P22} CRIM plasmids before integrating others to prevent the att_{P22} plasmid from recombining with others via homologous recombination, which can also occur at low frequency. The att_{P22} CRIM plasmids are therefore less valuable as vectors for library construction or other uses requiring high integration efficiency.

CRIM plasmid excision. Integrated CRIM plasmids can also be excised very efficiently. CRIM plasmid excision is carried out by using CRIM helper plasmids encoding both Xis and Int (Fig. 4B). We found that all CRIM plasmids were easily eliminated from a specific *attP* site when using the respective Xis/Int helper plasmid but not when using an Xis/Int helper plasmid for a different *attP* site. In most cases, 100% of the transformants were cured of the respective CRIM plasmid after a single colony purification step (see Materials and Methods). No aberrant (nonspecific) excision events were detected when we used cells containing multiple CRIM plasmids integrated at different sites (data not shown). We have used excision as a simple way to verify that novel phenotypes result from the presence of particular CRIM plasmids. We have also found excision to be useful in certain strain constructions. For example, when studying complex metabolic or regulatory pathways, it has often been necessary to make strains containing multiple CRIM plasmids in various combinations. In such cases, it has occasionally been more convenient to excise a single CRIM plasmid from a strain containing a combination of different CRIM plasmids in order to introduce an alternative one than to construct an entirely new strain containing most of the same CRIM plasmids by integrating each individually.

CRIM plasmid retrieval. The ease of retrieving CRIM plasmids from the chromosome is an especially valuable attribute. Because Xis and Int catalyze the excision and circularization of molecules from the corresponding *att* sites, CRIM plasmids can be retrieved simply by introducing chromosomal DNAs from an integrant into permissive (*pir*$^+$) hosts that synthesize Xis and Int from a helper plasmid. We have usually done this by using the generalized transducing phage P1*kc* in a process that we have called PIX cloning (Fig. 4C) (16).

PIX cloning is done using recipients that are *pir*$^+$ for replication of the CRIM plasmids and *recA* to avoid homologous-recombination events and carry the appropriate Xis/Int CRIM helper plasmid. We measured PIX cloning efficiencies by determining the number of antibiotic-resistant transductants per infectious phage in standard phage P1 crosses (Table 5). We assayed the transducing titer of the same P1*kc* lysates by determining the number of Ilv$^+$ transductants. As shown in Table 5, PIX cloning is an extremely efficient process. Efficient retrieval occurs only in the presence of the proper CRIM helper plasmid (data not shown). The recovered plasmids have always been correct, based on restriction enzyme analysis of plasmid DNAs isolated from several representative transductants in numerous such crosses. We have also used PIX cloning to recover plasmids for direct DNA sequence analysis (16; unpublished results). In addition, we have shown that CRIM plasmids can be recovered following transformation of a *recD pir*$^+$ host carrying the appropriate helper plasmids with chromosomal DNA (Materials and Methods). Accordingly, CRIM plasmids are also retrievable from bacteria that are insensitive to phage P1*kc*.

TABLE 5. PIX cloning efficiencies

attP Phage	CRIM plasmid	No. of Gm^r or Km^r transductants per PFU[a]
λ	pAH63	2.3×10^{-5}
HK022	pAH70	1.6×10^{-5}
ϕ80	pAH153	1.9×10^{-4}
P21	pAH95	3.9×10^{-5}
P22	pAH154	1.8×10^{-7}

[a] Values are normalized to the I1v$^+$ transducing titers. The same P1*kc* lysates yielded ca. 10^{-4} to 10^{-3} I1v$^+$ transductants per PFU.

Using CRIM plasmids. Although CRIM plasmids can be used with most ordinary (non-*pir*) *E. coli* strains, we have made standard hosts for their use. These hosts have defined deletions of *araBAD*, *rhaBAD*, and *lacZ* and are *lacI*^{q}. Hence, they cannot catabolize arabinose or rhamnose and yet encode the regulatory proteins (AraC, RhaR, and RhaS) required for ectopic expression of foreign genes from the corresponding promoters (P_{araB}, P_{rhaB}, and P_{rhaS}, also called P_{BAD} or *araBp*, *PrhaB*, and *PrhaS*, respectively). These hosts provide tight regulation of these and LacI-controlled promoters. They can also be used with *lacZ* fusions generated using our standard CRIM *lacZ* transcriptional fusion vector pAH125 (Fig. 5). Expression levels of P_{araB} in pLA2, P_{rhaB} in pAH120, and P_{rhaS} in pAH152 were similar to those reported elsewhere (14). We have also shown elsewhere that P_{rhaB} is an especially tightly regulated promoter (14). The synthetic (P_{syn1} and P_{syn4}) promoters provide for low-level unregulated gene expression. These promoters are juxtaposed to a ribosome binding site and AUG start codon that is contained within an *Nde*I site for convenient construction purposes. With the exception of plasmids carrying att_{P21}, the *Nde*I site is unique (14).

Unexpectedly, we have recently found that P_{araB} expression is much lower in pAH150 than in pLA2, which shows a normal level of expression (14; L. Avramova and B. L. Wanner, unpublished). Lower expression in pAH150 results from interference by an N-terminal AraC′ fusion protein that is encoded by the P_{araB} segment in pAH150 but not in pLA2. Nevertheless, both of these P_{araB} CRIM plasmids have been useful as they both show arabinose-regulated promoter expression. pAH150 has been especially useful for conditional expression of regulatory genes, such as *phoB*, requiring low-level expression, while pLA2 has been more useful for expression of structural genes requiring high-level expression. Elsewhere, we have recently described *E. coli* hosts that show homogeneous expression of genes under P_{araB} control which constitutively synthesize the low-affinity AraE transporter from the chromosome (21).

We have also shown that att_{P22} and att_{λ} CRIM plasmids integrate into the appropriate *attB* sites of *Salmonella enterica* serovar Typhimurium. Others were not tested. Since phages tend to exploit highly conserved and sometimes essential genes (e.g., tRNA genes) as sites for integration (8), several CRIM plasmids can probably integrate into chromosomes of other bacteria, especially in other members of the family *Enterobacteriaceae* and related families. CRIM plasmids should therefore be useful in many applications involving bacteria other than common laboratory strains.

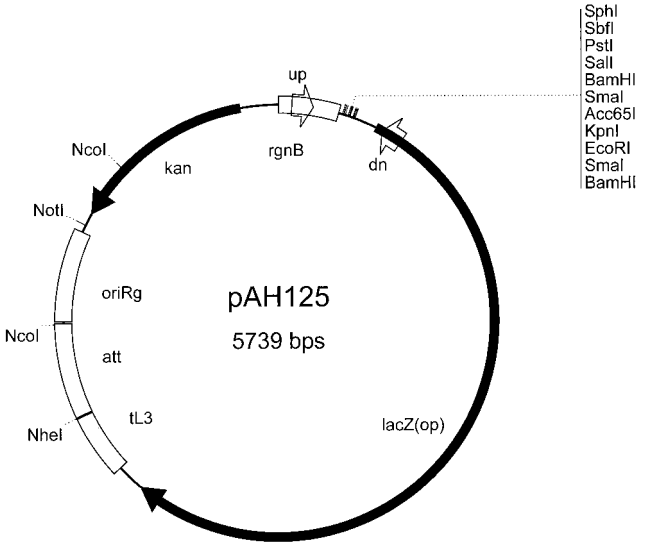


FIG. 5. CRIM reporter plasmid for construction of *lacZ* transcriptional fusions. Primer sites routinely used to sequence inserts are indicated as "up" (TTGTCGGTGAACGCTCTCCT, same as rgnB-f in Fig. 1) and "dn" (down) (AAGTTGGGTAACGCCAGG).

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