JOURNAL OF BACTERIOLOGY, Sept. 1989, p. 4617–4622
0021-9193/89/094617-06$02.00/0
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Vol. 171, No. 9

New Method for Generating Deletions and Gene Replacements in *Escherichia coli*

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Received 28 December 1988/Accepted 1 June 1989

We describe a method for generating gene replacements and deletions in *Escherichia coli*. The technique is simple and rapid and can be applied to most genes, even those that are essential. What makes this method unique and particularly effective is the use of a temperature-sensitive pSC101 replicon to facilitate the gene replacement. The method proceeds by homologous recombination between a gene on the chromosome and homologous sequences carried on a plasmid temperature sensitive for DNA replication. Thus, after transformation of the plasmid into an appropriate host, it is possible to select for integration of the plasmid into the chromosome at 44°C. Subsequent growth of these cointegrates at 30°C leads to a second recombination event, resulting in their resolution. Depending on where the second recombination event takes place, the chromosome will either have undergone a gene replacement or retain the original copy of the gene. The procedure can also be used to effect the transfer of an allele from a plasmid to the chromosome or to rescue a chromosomal allele onto a plasmid. Since the resolved plasmid can be maintained by selection, this technique can be used to generate deletions of essential genes.

With the development of recombinant DNA techniques, it has become relatively straightforward to generate point mutations and deletions of a cloned gene in vitro. The ideal host for the phenotypic characterization of a mutant allele is a strain in which the wild-type gene has been replaced with the mutant allele on the chromosome. Likewise, to facilitate the purification of a mutant protein, it is necessary to use a strain that has a null or deletion mutation of the gene on the chromosome or a strain in which the chromosomal wild-type gene has been replaced with the mutant allele. In *Escherichia coli*, such gene substitutions or specific deletions can be difficult to obtain.

Random chromosomal deletions can be generated by bacteriophage P2 eduction (33) or by imprecise excision of Tn*10* (5, 18, 22). Several other methods that can be used to make site-specific deletions of the chromosome have been reported. In some cases, these protocols can be modified to effect the replacement of a specific chromosomal gene with a mutant form of that gene. These include F′ homogenotization (27), the formation and resolution of ColE1 plasmid cointegrates in a *polA1* genetic background (10, 28), transformation of a *recBC sbcB* (14, 37) or a *recD* strain (29) with linear DNA, integration and induction of precisely engineered λ *c*I857 vectors (16), and transformation and mating via an Hfr cross (27a).

Balakrishnan and Backman (2) have developed a derivative of λ *c*I857 for use as an excision vector, primarily to make the (deletion) phenotype of a strain inducible in vivo. Another technique (17) depends on the intrinsic instability of multicopy plasmids that carry large insertions of chromosomal DNA to introduce mutations by homologous recombination (15, 32).

This communication describes a simple and rapid technique for carrying out gene replacements in *E. coli*. The technique proceeds by homologous recombination and can be used to generate specific deletions and gene replacements and to effect the in vivo cloning of a chromosomal locus. The method is applicable to any gene as long as some allele of the gene has been cloned and there is either some phenotypic or physical way of distinguishing between the cloned gene and the chromosomal copy of that gene.

MATERIALS AND METHODS

Bacterial strains and media. MC1061 [F⁻ *araD139* Δ(*ara-leu*)*7697* Δ(*lac*)*X74 galU galK strA*] was provided by R. L. Gourse. SK6775 has a *uvrD* (Km^r) deletion-insertion mutation (Δ*uvrD288*::Km^r) in the MC1061 genetic background and was constructed in this laboratory. Strain SR393 (*leuB19 thyA36 uvrD3*) was provided by K. Smith. SK707 [*argH1 hisG4 ilvD188 metE146 lacMS286*φdII*lacBK1*] carries a deletion-duplication construct of the lactose operon (40).

Growth conditions. Cells were grown in Luria (L) broth (19). For solid medium, 2% agar was added. L broth and L agar were supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (20 μg/ml) as needed.

DNA techniques. Restriction endonucleases, calf intestine alkaline phosphatase, T4 DNA ligase, and DNA polymerase I Klenow fragment was purchased from Boehringer Mannheim Biochemicals and used according to the specifications of the manufacturer. RNase A and lysozyme were purchased from Sigma Chemical Co.

E. coli chromosomal DNA was isolated as described by Silhavy et al. (30). Large-scale preparation of plasmid DNA was by the method of Birnboim and Doly (4). Rapid plasmid screens were done by a modification of the method of Ish-Horowicz and Burke (13) for small volumes of cell culture.

Isolation of restriction fragments for subsequent cloning was accomplished by elution from GTG agarose (Marine Colloids Co.) onto NA-45 paper (Schleicher & Schuell, Inc.) according to the instructions of the manufacturer.

Specific DNA probes were labeled with [α-^{32}P]dATP (Amersham Corp.) by the random-primer method of Fein-

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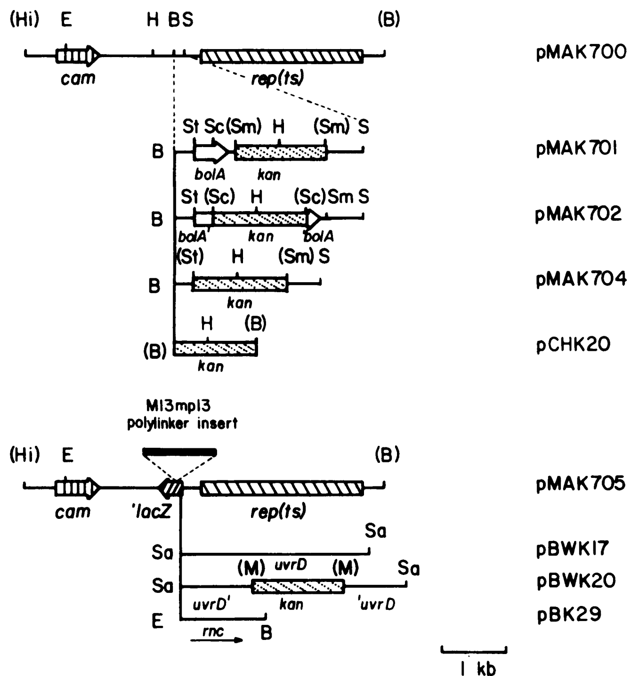

FIG. 1. Restriction maps of pMAK700, pMAK705, and their derivatives. pMAK700 is a derivative of pH01 (see Materials and Methods). Plasmids pMAK701, pMAK702, and pMAK704 are derivatives of pMAK700. pMAK701 and pMAK702 carry the *bolA* gene with the kanamycin resistance gene inserted at different sites in the gene. In plasmid pMAK704, the entire *bolA* gene has been replaced with the kanamycin resistance gene. pMAK705 is a derivative of pMAK700 constructed as described in Materials and Methods. pCHK20 has the kanamycin resistance gene inserted in the *Bam*HI site of pMAK700. Plasmids pBWK17 and pBWK20 are derivatives of pMAK705. pBWK17 carries the wild-type *uvrD* gene. pBWK20 carries the insertion-deletion mutation Δ*uvrD288*::Km^r. pBK29 contains the *rnc-sos* gene in pMAK705. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; Hi, *Hin*cII; H, *Hin*dIII; M, *Mlu*I; S, *Sph*I; Sc, *Sca*I; Sm, *Sma*I; St, *Stu*I. ( ), Restriction site used for plasmid construction that has been eliminated.

berg and Vogelstein (7). Southern blot hybridizations (31) were performed as described by Maniatis et al. (23).

Plasmid constructions. Derivatives of pMAK700 and pMAK705 relevant to this work are shown in Fig. 1. Plasmid pH01 (9, 11) is a mutant isolate of pSC101 (6) that is thermosensitive for DNA replication. Since pH01 was not a suitable vector for cloning purposes, the 2.7-kilobase-pair (kbp) *Hin*cII fragment of pBR325 (3) containing the gene for chloramphenicol resistance was ligated to the 2.9-kbp *Bam*HI fragment of pH01 containing the temperature-sensitive replicon. The protruding ends resulting from the *Bam*HI digestion were first treated with the Klenow fragment of DNA polymerase I. The resulting plasmid, designated pMAK700, contains unique *Bam*HI, *Hin*dIII, and *Sph*I restriction sites (Fig. 1).

Another vehicle, pMAK705, which contains additional cloning sites by incorporating the coding sequence for the α-peptide of β-galactosidase, was obtained by inserting the 0.6-kbp *Hae*II fragment from pUC19 (39) into pMAK700. pMAK700 plasmid DNA was digested with *Hin*dIII and *Sph*I and treated with Klenow fragment. The *Hae*II fragment of pUC19 contains the *lacZ* promoter, the M13mp19 polylinker, and the *lacZ* α-peptide. Accordingly, insertions into the polylinker can be easily identified in an appropriate indicator strain (26).

pMAK701 and pMAK702 contain the *bolA* gene (1), with two different insertions of the kanamycin resistance gene from pUC-4K (35; Pharmacia molecular biologicals catalog, 1986), cloned into the pMAK700 vector. pMAK701 has the kanamycin resistance gene inserted downstream of *bolA*, while pMAK702 has the kanamycin resistance gene inserted into the unique *Sca*I site located within the coding region of the *bolA* gene. In pMAK704, the kanamycin resistance gene replaces the entire *bolA* coding sequence (1). pCHK20 has the kanamycin resistance gene from pUC-4K inserted into the *Bam*HI site of pMAK700. This plasmid was the control for experiments involving Km^r insertion-deletion mutations.

pBWK17 has the wild-type *uvrD* gene on a 2.9-kbp *Sal*I fragment (24) inserted into the *Sal*I site of the polylinker of pMAK705. pBWK20 has the Km^r insertion-deletion mutation Δ*uvrD288*::Km^r (B. K. Washburn and S. R. Kushner, manuscript in preparation) inserted into the *Sal*I site of the polylinker of pMAK705.

pBK27 carries a wild-type *rnc* gene (36), modified in the 5' leader sequence to create a restriction fragment polymorphism, inserted in the *Bam*HI-*Eco*RI sites of the polylinker of pMAK705. pBK29 is identical to pBK27 except that it carries both the restriction site polymorphism and the *rnc-505* mutation (P. Babitzke and S. R. Kushner, manuscript in preparation).

Isolation of plasmid-chromosome cointegrates. The gene to be introduced into the chromosome was cloned into an appropriate plasmid vector that contained the temperature-sensitive pSC101 origin of replication. Because the plasmid replicates at 30°C but not at 44°C, it is possible to identify the integration of the plasmid into the chromosome of an appropriate host strain by selecting for Cm^r transformants at 44°C. These cointegrates appeared on the 44°C plates after about 24 h. Transformations were done by the method of Kushner (20).

This laboratory routinely uses 44°C as the nonpermissive temperature for temperature-sensitive mutations. The temperature-sensitive pSC101 replicon we used for these experiments was originally characterized by Hashimoto-Gotoh and Sekiguchi (11), using 43°C as the nonpermissive temperature. It seems likely that a nonpermissive temperature of 42°C (used in some laboratories) would also be effective.

The number of transformed cells per milliliter of culture was determined by plating several dilutions of cells onto selective media (L agar containing chloramphenicol) at 30°C. An estimated 10,000 to 50,000 transformed cells were plated onto 10 to 20 prewarmed chloramphenicol plates at 44°C to select for cointegrates. The total number of transformed cells plated at 44°C was determined from the number of transformed cells per milliliter observed at 30°C.

Identification of resolution products. When the plasmid is integrated into the chromosome, replication from the plasmid origin is deleterious to the cell. Accordingly, when cointegrates identified at 44°C were subsequently grown at 30°C, the permissive temperature for replication of the plasmid, a second recombination event occurred, regenerating free plasmid in the cell. A pool of 6 to 10 individual colonies from the 44°C plates were used to inoculate a 100-ml culture of L broth containing chloramphenicol. This culture was then grown to stationary phase (overnight) at 30°C. Two more cycles of growth were generally carried out by diluting 0.1 ml of the overnight culture into 100 ml of fresh medium. Finally, single colonies were isolated by plating dilutions of the overnight culture onto L plates containing chloramphen-

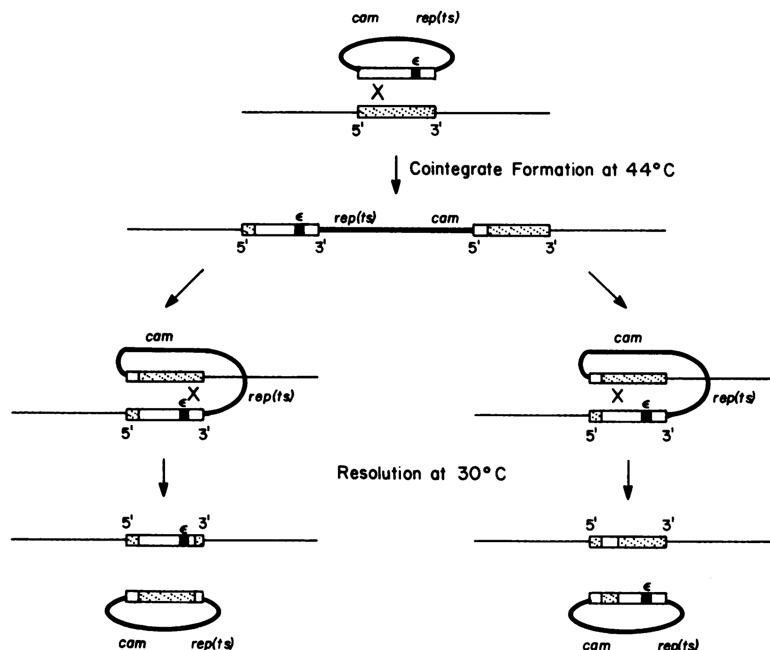

FIG. 2. General strategy for the gene replacement method. The initial recombination event occurs between homologous sequences of the plasmid and the chromosome and results in the formation of a cointegrate. Cointegrates are identified by plating transformed cells at 44°C (the nonpermissive temperature for plasmid DNA replication) onto medium that selects for the plasmid-encoded antibiotic resistance gene (chloramphenicol resistance). After growth at 30°C (the permissive temperature for plasmid DNA replication), the cointegrates undergo a second recombination event, regenerating plasmid in the cell. Depending on the site of the second recombination event, the marker on the incoming plasmid (ε) will either remain in the chromosome or reappear on the plasmid. As indicated, after integration and resolution, both copies of the gene will be composites.

icol at 30°C. Single colonies were tested for chloramphenicol resistance at 44°C by streaking on prewarmed L plates containing chloramphenicol. Colonies that were Cm^s at 44°C no longer had plasmid DNA integrated into the chromosome.

The results of rapid plasmid screens of these isolates indicated whether the plasmid that was present contained the original clone or the chromosomal copy of the gene. As long as a strain that had undergone a gene replacement was viable at 44°C (the nonpermissive temperature for replication of the plasmid), the resident plasmid was easily cured from a strain by growing the cells at 44°C in L broth without chloramphenicol.

RESULTS AND DISCUSSION

General description of the method. The strategy for the gene replacement technique is shown in Fig. 2. One of the advantages of this technique is that it can be carried out in a wild-type genetic background. Since it is not necessary to use a strain that is *polA*, *recBC sbcB*, or *recD* deficient, this technique may also be useful for carrying out gene replacements in enteric bacteria other than *E. coli*. The use of a wild-type genetic background also prevents any problems that might occur when uncharacterized mutations or deletion constructs are introduced into strains that are *polA*, *recBC sbcB*, or *recD* deficient. The only requirements for the host strain are that it be recombination proficient and be able to support the replication of pSC101. The *dnaA* gene is the only known (*E. coli*) host gene that is absolutely required for pSC101 replication (8, 12). Because this method uses covalently closed plasmid DNA for transformation, the low efficiency of transformation observed with linear DNA is avoided.

This method works because the presence of the pSC101 replication origin in the *E. coli* chromosome dramatically reduced the growth rate of the cell; pSC101 cointegrates grew very poorly at 30°C on L-agar plates containing chloramphenicol. Cells in which the plasmid had been resolved from the cointegrate demonstrated normal growth rates. Similar observations have been made with ColE1 cointegrates (38).

Factors affecting cointegrate formation. By using the series of plasmids described in Fig. 1, it was possible to show that the frequency of cointegrate formation correlated with the size of the DNA homology present (Table 1, group A). These results support the previous observations of Liljestrom et al. (21) and Teifel-Greding (34). When homologous sequences were interrupted by nonhomologous sequences that approached the size of the total homology, the integration frequency was most closely correlated to the average size of the flanking homologies (Table 1, group B). If the nonhomologous sequences were less than the total homology present, the integration frequency resembled that of uninterrupted sequences (Table 1, group B). In the one case where the region of nonhomology was on the chromosome rather than on the plasmid, the insertion frequency was reduced 50% compared with the value for the inverse experiment (data not shown).

Resolution of cointegrates. After approximately 48 h of log-phase growth at 30°C, 60 to 80% of the single-cell isolates that were originally identified as cointegrates contained freely replicating plasmid. Cells carrying plasmid resolution

TABLE 1. Chromosomal insertion frequencies

Group	Plasmid	Strain	Insert/homology	Insert/5′ homology + 3′ homology	Insertion frequency (10^{-4})[a]
A[b]	pH01	MC1061	None		0.03
	pMAK700	MC1061	None		0.03
	pMAK705	MC1061	None		0.05
	pMAK705	SK707	*lacZα*/150 bp		0.05
	pCHK20	MC1061	Km^r/insertion sequences		0.14
	pBK27	MC1061	*rnc*/1.3 kbp		6.25
	pBWK17	MC1061	*uvrD*/2.9 kbp		17.0
B[c]	pMAK701	MC1061		*bolA*/0.85 kbp + 0.6 kbp	2.81
	pMAK702	MC1061		*bolA*/0.6 kbp + 0.85 kbp	2.79
	pMAK704	MC1061		*bolA*/0.3 kbp + 0.55 kbp	1.13
	pBWK20	SR393		*uvrD*/1.0 kbp + 1.1 kbp	16.0
	pBWK20	MC1061		*uvrD*/1.0 kbp + 1.1 kbp	20.0

[a] Number of cointegrates at 44°C/total number of transformed cells plated at 44°C.
[b] DNA homology (where present) is continuous.
[c] Homologous sequences flanking a region of nonhomology (1.4-kbp kanamycin resistance gene from pUC-4K) on the plasmid.

products could be easily distinguished from unresolved cointegrates by their sensitivity to chloramphenicol at 44°C. Resolution frequencies were in part dependent on the gene or type of gene construction involved. When strains that did not resolve well were subjected to further growth at 30°C, the percentage of cell isolates that contained resolution products did not increase (data not shown).

Construction of deletions-insertions in the *bolA* gene. MC1061 was transformed with pMAK701, and cointegrates were identified at 44°C. Plasmid DNA from 16 resolved isolates was analyzed by agarose gel electrophoresis. All 16 clones showed the presence of a plasmid, and 6 of them contained a plasmid smaller than pMAK701, indicating that the original *bolA* deletion-insertion construct on pMAK701 had been transferred to the chromosome. Restriction enzyme analysis of these six plasmids indicated that they carried the wild-type copy of the *bolA* gene (data not shown).

The plasmids used to generate the three different *bolA* deletions were pMAK701, pMAK702, and pMAK704 (Fig. 1 and 3A). The wild-type *bolA* plasmid was cured from each of the deletion strains after growth at 44°C, indicating that the *bolA* gene was not essential. To confirm that each of the Km^r deletion-insertion constructs of the *bolA* gene had been transferred to the chromosome, chromosomal DNA was isolated from the strains and subjected to Southern analysis. The probe used in the DNA-DNA hybridization experiments was the 1.4-kbp *Bam*HI-*Pst*I fragment which includes the *bolA* gene (Fig. 3A). The hybridization patterns were as expected if the wild-type copy of the *bolA* gene had been replaced by the deletion constructs (Fig. 3B).

Identification of a gene replacement by restriction fragment polymorphisms. A mutant allele of the *rnc* gene (RNase III), *rnc-505*, which was identified after in vitro mutagenesis of pBK27 (Babitzke and Kushner, in preparation), was inserted into the chromosome of MC1061 by using pBK29 (*rnc-505*) and the method described above. Although there was no positive selection for the *rnc-505* mutation, the gene replacement was identified by a restriction enzyme polymorphism engineered into the upstream nontranslated region of the mutant gene (data not shown).

Recovery of a chromosomal Km^r insertion-deletion mutation. A chromosomal deletion-insertion mutation, Δ*uvrD 288*::Km^r, was rescued from the chromosome of SK6775 onto pBWK17 (Fig. 1), a plasmid that carries the wild-type *uvrD* allele. In this case, since there was a positive selection for identification of the chromosomal allele on the plasmid, the protocol was modified as follows. After growth of the cointegrates at 30°C to generate resolution products, plasmid DNA was isolated. Analysis of transformants obtained with this plasmid DNA indicated that both of the possible reso-

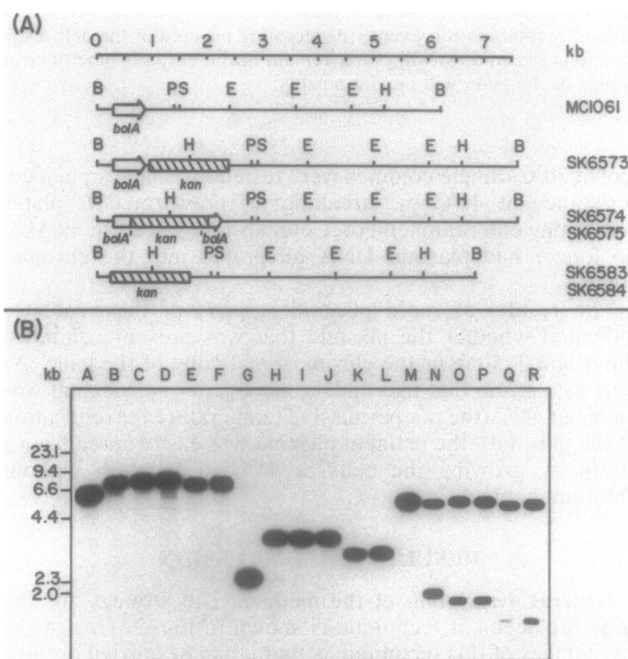

FIG. 3. (A) Restriction map of the 6.2-kbp *Bam*HI chromosomal fragment from MC1061 that contains wild-type *bolA* gene and comparison of the wild-type *bolA* restriction map with that of *bolA* deletion strains SK6573, SK6574, SK6575, SK6583, and SK6584. The *bolA* deletions strains were constructed as described in Materials and Methods. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sph*I. (B) Southern analysis of *bolA* deletion strains. Chromosomal DNA samples were digested with *Bam*HI (lanes A to F), *Bam*HI plus *Eco*RI (lanes G to L), and *Bam*HI plus *Hin*dIII (lanes M to R). The 1.4-kbp *Bam*HI-*Pst*I fragment containing the *bolA* gene was used as the probe. Lanes contained MC1061 (A, G, and M), SK6573 (B, H, and N), SK6575 (C, I, and O), SK6575 (D, J, and Q), SK6583 (E, K, and Q); and SK6584 (F, L, and R).

lution products were present. Of 100 transformants that were identified as Cm^r at 30°C, 70 were also Km^r at 30°C. Thus, 70% of the resolved plasmids carried the Δ*uvrD288*::Km^r allele that had originally been present on the chromosome.

Generation of large chromosomal deletions. This technique is especially useful in cases where the necessary recombination events must occur across small regions of DNA homology. Thus, it was possible to create a 7-kbp chromosomal deletion with only 600 bp of flanking homologous sequences. If necessary, transformed cells can be grown for many generations at 44°C to permit cointegrate formation and subsequently at 30°C to generate resolution products.

A DNA fragment containing the hydrogenase I structural gene of *E. coli* was replaced by the kanamycin resistance gene from pUC-4K, leaving approximately 600 bp of chromosomal homology on either side of the insert in pMAK705 (K. M. Nanda, H. B. Peck, and A. E. Pryzbyla, manuscript in preparation). With only 600 bp of homology, the expected insertion frequency was on the order of 2×10^{-4} (Table 1, group B). However, when more than 40,000 transformed cells were plated at 44°C, no cointegrates were observed. Thus, to generate cointegrates, 10 transformants obtained at 30°C were grown at 44°C in L broth containing kanamycin. After approximately 48 h of growth in log phase at 44°C, analysis of several hundred single colonies indicated that 100% of them were cointegrates. The 44°C culture was then used to inoculate L broth containing kanamycin, and log-phase growth was carried out for approximately 48 h at 30°C. Of 500 Km^r single colonies derived from this culture, three were Cm^s at 30°C, indicating that the wild-type plasmid was lost and that the gene was not essential. The expected 7-kbp chromosomal deletion was verified by Southern analysis (data not shown).

Construction of deletions of essential genes. Since the resolved plasmid can be maintained by selection, this is a powerful technique for generating deletions of and identifying essential genes. As long as the deleted gene can be complemented in *trans*, this method can be used to generate a chromosomal deletion of an essential gene. The chromosomal deletion will be complemented by the wild-type gene on the plasmid at 30°C but not at 44°C because of the temperature-sensitive nature of the plasmid replicon. Deletions in essential genes will appear conditionally lethal at 44°C in the absence of chloramphenicol. This phenotype provides direct evidence that a gene is essential. When there is a positive selection for the desired gene replacement event and the gene is not essential, both recombination events and the loss of the plasmid may be identified at 44°C. A temperature-sensitive pSC101 replicon has been used in this way to construct a deletion mutant of *micF* (25).

Comments on the method. There are some caveats to be considered before using this protocol. Since there is no way of determining the precise location of the recombination events that effect the gene replacement, it is possible for a mutation not to be transferred as expected because the two copies of the gene obtained after resolution are composites (Fig. 2). It may be necessary to look at a series of isolates that have undergone gene replacements in order to find one carrying the desired mutation. When asymmetric regions of homology flank a nonhomologous insert, the frequency of one resolution product may be greater than 50%. It is also likely that in addition to gene replacements, some gene conversion events occur.

The temperature-sensitive plasmid vector used for these experiments is a derivative of pSC101 that has a copy number of 6 to 10 per cell (8, 12). It is possible that for some genes there will be a gene dosage problem with 6 to 10 copies per cell. Finally, this method may also have limitations when applied to genes which themselves are involved in the processes of DNA replication or genetic recombination.

ACKNOWLEDGMENTS

We thank G. Churchward for the gift of plasmid pH01.

This work was supported by a Public Health Service grant GM27997 to S.R.K. from the National Institutes of Health. C.M.H., B.K.W., and P.B. were supported by Public Health Service predoctoral training grant GM07103 from the National Institutes of Health.

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