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Efficient Introduction of Cloned Mutant Alleles into the *Escherichia coli* Chromosome

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An efficient method for moving mutations in cloned *Escherichia coli* DNA from plasmid vectors to the bacterial chromosome was developed. Cells carrying plasmids that had been mutated by the insertion of a resistance gene were infected with λ phage containing homologous cloned DNA, and resulting lysates were used for transduction. Chromosomal transductants (recombinants) were distinguished from plasmid transductants by their ampicillin-sensitive phenotype, or plasmid transductants were avoided by using a *recBC sbcB E. coli* strain as recipient. Chromosomal transductants were usually haploid when obtained in a nonlysogen because of selection against the λ vector and partially diploid when obtained in a lysogen. Pure stocks of phage that carry the resistance marker and transduce it at high frequency were obtained from transductant bacteria. The λ-based method for moving mutant alleles into the bacterial chromosome described here should be useful for diverse analyses of gene function and genome structure.

Escherichia coli K-12 is one of the best-known cellular organisms (28). About 1,400 of its genes have been identified by mutation, but many gaps are evident in its map, and more than half of its genome remains uncharacterized (3). Traditionally, most genes were identified by selecting mutations with specific phenotypes and then mapping each mutation. Open reading frames that often correspond to functional genes are now discovered during routine DNA sequencing, and often insights into how their protein products act can also be gleaned from the sequence. A third approach entails making mutations at predetermined locations (typically in cloned DNA), moving the mutations to the bacterial chromosome, and then testing the mutations for effects on phenotype. If made more efficient, this reverse genetic approach could potentially be used to identify all previously unknown genes that are essential or contribute significantly to growth.

Several methods for moving mutations marked by a resistance trait from the plasmids in which they were made to the bacterial chromosome have been developed, but none seems efficient enough for use on a large scale. For example, the resistance trait can be selected under conditions in which the vector plasmid does not replicate (16, 17). Although the desired haploid recombinants are found easily in many cases, heterozygous plasmid-chromosome cointegrates predominate in others. Consequently, a failure to isolate the desired recombinant could reflect either essentiality of the mutated gene or simply a low frequency of the second crossover. Alternatively, a *recD* or *recBC sbcB* strain can be transformed with genetically marked linear DNA fragments (18, 33, 38, 41). Only haploid recombinants are normally recovered by this method, but they are so rare that failure to recover them does not provide very convincing evidence that the mutated gene is essential. A third method entails subcloning mutated DNA segments to a λ *c*I857 vector (which encodes a temperature-sensitive repressor), selecting lysogens formed by crossing over between the phage-borne and chromosomal segments, and then selecting haploid mutant nonlysogens formed by a second crossover and loss of the excised prophage (8, 19), but this multistep method has not been much used.

Recent studies have shown that the λ *c*I mutant phage of the Kohara library of *E. coli* clones (20, 21) can be used effectively as a specialized transducing phage: a λ clone carrying a particular wild-type allele can typically transduce the corresponding mutant bacterial strain at a frequency of about 10^{-4} without need for a lysogenization step (26, 31). This formed the basis for a reverse genetic method of random insertion mutagenesis of individual phage clones with transposon Tn*5supF* followed by recombination of insertion mutant alleles into the *E. coli* chromosome (30, 31). Here, we present a method for moving preexisting plasmid-borne alleles to the *E. coli* chromosome that also entails use of a λ phage vector and homologous recombination (Fig. 1). Either haploid or partial diploid recombinant transductants and homogeneous stocks of phage bearing the mutant allele are easily obtained. The method is efficient and rapid, detects essential genes and sites, and seems well-suited for large-scale functional tests.

MATERIALS AND METHODS

General materials and methods. Standard molecular genetic methods (30, 31, 35) were employed. The bacterial strains, phage, and plasmids used in this study are listed in Table 1. The λ phage containing cloned *E. coli* DNA (20, 21) are *c*I mutants (repressor deficient) and tend to kill nonlysogens. The λ clone corresponding to a particular plasmid clone was identified by restriction mapping, DNA sequencing, genetic complementation (20, 26, 31), or recombination tests (present work) when there were relatively few candidates to screen. Medium E (40) was the defined minimal medium. N broth (1% NZ amine [Humko-Sheffield, Norwich, N.Y.], 0.5% NaCl) and LN broth (N broth plus 0.5% Difco yeast extract) were used for routine λ and bacterial growth, as previously described (8). Solid medium contained Bacto-Agar (Difco) at a concentration of 1.0% for λ plaques

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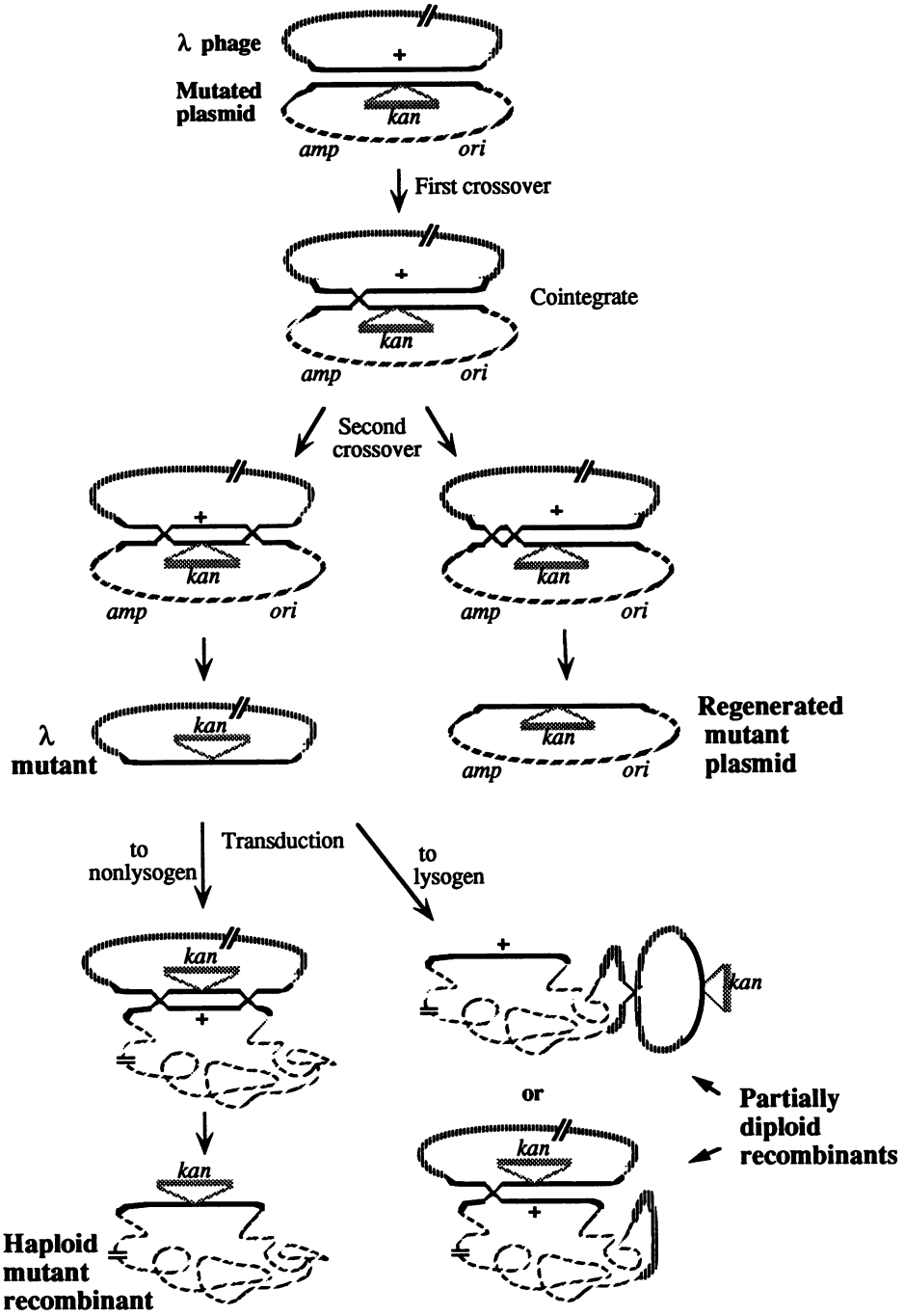

FIG. 1. λ-mediated transduction of plasmid-borne alleles. Symbols: ▬, bacterial sequences cloned in plasmid or λ phage, or corresponding segments in bacterial chromosome; ▬, *kan* insert sequences; ⋯, plasmid vector; ▬, λ phage vector; ∿, bacterial chromosome.

and 1.5% for bacterial colonies. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was added to plates at 40 μg/ml to score for *lacZ* expression. Glucose (0.2%) and sodium citrate (10 mM) were added to LN medium when needed to inhibit λ growth.

λ-mediated transduction. Stocks of the Kohara phage were prepared as plate lysates on bacterial strains carrying appropriate plasmid-borne or chromosomal mutations and were used to infect exponentially growing cells (2×10^8 cells per ml) at a multiplicity of one phage per cell. After 2 h of adsorption and expression of antibiotic resistance, the infected cells were plated on selective medium and incubated at 37°C (30°C when a lysogenic recipient strain was used).

Recovery of high-frequency-transducing phage lines. Phage were prepared by thermal induction of transductant derivatives of a λ *c*I857 *plac*5 *S*am7 lysogen (15 min at 42°C, then 120 min at 37°C), the lysates were plated on a lawn of strain MC4100 on X-Gal-containing medium, and single white

TABLE 1. Bacteria, phage, and plasmids used in this study

Strain, phage, or plasmid	Relevant traits	Reference or source
E. coli		
G13	*nusA supF*, contains Tn*10*	S. Adhya[a]
DB1434	*lac-3350 galT2 galK22 rpsL179* (λ p*lac*5 *c*I857 *S*am7)	Strain R594 in reference 2; 36
DB5648	HfrH *lac*(Am) *trp*(Am)	This work[b]
DB5678	Lac$^+$ revertant of DB5648	This work
DB5680	DB5648 *dam*-13::Tn*9*	This work, 30
DB6225	JC7623 (λ p*lac*5 *c*I857 *S*am7)	This work
DPB267	*recD*::Tn*10*	9
JC7623	*recB21 recC22 sbcB15 thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2 hisG4 rfbD1 rpsL21 kdgK51 xyl-5 mtl-1 argE3 thi-1*	13
MC1061	F$^-$ *araD139* Δ(*ara-leu*)*7697* Δ*lacX74 rpsL galU galK hsdR2*	12
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*205 flbB5301 ptsF25 relA1 rpsL150 deoC1*	11
λ phage clones[c]		
λ138/10A6	*lac*$^+$; position, 8 min; kb 362–380[d]	20, 21
λ452/6C8	*cysDNC*$^+$; position, 59 min; kb 2883–2903[d]	20, 21
λ460/3G1	*thyA*$^+$; position, 61 min; kb 2965–2984[d]	20, 21
λ656/5B5	*cysQ*$^+$; position, 96 min; kb 4512–4525[d]	20, 21
Plasmids[e]		
pBRG5450 ("p*cysQ*::*kan*")	*kan* from pUC4K in *Eco*RI site of *cysQ*, 1.3 and 0.37 kb from junctions with vector[f]	29
p5H 9.4-1 B1K ("p*cysD*::*kan*")	*kan* in *Bam*HI site of *cysD*, 5.6 and 3.8 kb from vector junction	23, 24
p5H 9.4-1 E12K ("pΔ(*cysD-N*)::*kan*")	Δ(*cysD-N*); replaced by *kan* insertion, 3.5 and 3.7 kb from junctions with vector	23, 24
p5H 9.4-1 E2K ("p*cysN/C*::*kan*")	*kan* between *cysN* and *cysC*, 5.7 and 3.7 kb from junctions with vector	23, 24
pBTAH 1.2 ("p*thyA*")	1.2 kb *thyA*$^+$ fragment in pUC9	5
pKOTAΔ283 K5 ("p*thyA*::*kan5*")	Deletion of 283 bp in *thyA*, replaced by *kan* insertion, 1.7 and 2 kb from junction with vector, orientation I	6
pKOTAΔ283 K6 ("p*thyA*::*kan6*")	Deletion of 283 bp in *thyA*, replaced by *kan* insertion, 1.7 and 2 kb from junction with vector, orientation II	6
pUC4K	*kan* inserted in *lacZ*α, 229 and 164 bp from junction with vector	39

[a] S. Adhya, Laboratory of Molecular Biology, National Cancer Institute, Bethesda, Md.

[b] DB5648 was derived from "DB6128" of D. Botstein (Department of Genetics, Stanford University). DB6128 grows poorly under anaerobic conditions, and DB5648 is a spontaneous mutant, selected for rapid growth under anaerobic conditions.

[c] λ138, λ452, λ460, and λ656 correspond to the systematic numbering system of the miniset in reference 20, while designations after the shill are from reference 21. These phages each lack λ *red* genes, and hence homologous recombination as in Fig. 1 is mediated by host *rec* functions.

[d] The chromosomal positions of the cloned DNA segments in minutes and kilobases refer to those in references 3 and 21, respectively.

[e] Plasmids were maintained in strain MC1061.

[f] Estimated position of *kan* insert relative to left and right junctions of cloned DNA with plasmid sequence, and thus the extent of homology for crossing over between phage and plasmid.

(Lac$^-$) plaques were toothpicked to lawns of strains MC4100 or G13. Patches of confluent phage growth, which also contained some surviving cells, were toothpicked to medium containing kanamycin to score transduction and thereby identify single phage clones that carried the resistance marker. Strain G13 gave severalfold more transductants in these patch tests than strain MC4100, possibly because the *nusA* mutant allele in G13 interferes with lytic phage growth and cell killing (7, 14). Alternatively, transductant colonies from initial selection plates, where they are chronically infected with small numbers of phage, were suspended in buffer and chloroform treated to kill bacteria, and aliquots of the suspension were plated with indicator bacteria for single phage plaques.

RESULTS

λ phage-mediated transduction of plasmid-borne alleles. An initial test of how efficiently λ phage clones could mediate transfer of mutations from plasmid clones to the bacterial chromosome was carried out with lysates of *lac*$^+$ phage λ138 grown on cells carrying the *lacZ*::*kan* plasmid pUC4K. These lysates transduced a nonlysogenic recipient (DB5678) to Kan^r at a frequency of about 10^{-7} (Table 2). Almost 90% of transductants were Amp^s and Lac$^-$, indicating that they were plasmid free, haploid, and did not carry λ, and they probably arose by pairs of crossovers as diagrammed in Fig. 1. Plasmid-free partial diploids, formed by single crossovers between phage-borne and chromosomal sequences, were not recovered among transductants of the nonlysogen, because of killing of nonimmune cells by λ *c*I mutant phage (31). The high frequency of plasmid-free Kan^r transductants was unexpected, since the *kan* insert in pUC4K is bracketed by only 229 and 164 bp of *E. coli* sequence. Reconstruction experiments, however, indicated that pUC4K is unstable in this strain (70% Amp^s segregants after overnight growth without selection versus <1% segregants for a *cysQ*::*kan* control plasmid in this strain or pUC4K in another strain [DB1434]). The cause of this unexpected instability is not known, but either selection against plasmid-carrying cells or inefficient plasmid replication could greatly increase the yield of haploid mutant recombinants among transductants.

TABLE 2. λ-mediated replacement of chromosomal by plasmid-borne alleles

Kohara phage	*kan* insertion	DB5648 (nonlysogen)		DB1434 (lysogen)	
		Transduction frequency (10^{-7})	Fraction (%) Amp^s	Transduction frequency (10^{-7})	Fraction (%) Amp^s
λ138	*lacZ*[a]	0.8	27/31 (87)	50	2/185 (1)
λ452	*cysD*	1.5	14/38 (37)	43	4/94 (4)
λ452	*cysN/C*	0.2	44/76 (58)	90	15/79 (19)
λ452	*cysΔ(D-N)*	14	83/178 (47)	340	19/104 (18)
λ460	*ΔthyA5*	0.9	26/53 (49)	130	37/98 (38)
λ460	*ΔthyA6*	0.9	22/59 (37)	120	29/100 (29)
λ656	*cysQ*	5	5/204 (2)	5,000	1/304 (0.3)

[a] In this cross, the recipient was DB5678, a Lac^+ derivative of DB5648.

The other 10% of Kan^r transductants from the λ138 infection of cells carrying pUC4K were Amp^r and did not carry λ, and most (>95%) were Lac^+. Each of six Amp^r Lac^+ colonies tested carried a plasmid the size of pUC4K. Thus, these colonies resulted from plasmid transduction and loss of the λ vector, not from recombination between phage-borne and chromosomal sequences.

Selectable (*kan* insertion and substitution) mutations in biosynthetic genes from three different chromosomal regions were transduced from their parental plasmids to the chromosome of a nonlysogen at frequencies ranging from $\sim 10^{-8}$ to 10^{-6} (Table 2). With six of seven plasmids tested, more than one-third of transductants were Amp^s. Each Amp^s transductant exhibited the expected auxotrophy, and, conversely, most Amp^r transductants were prototrophic and contained the expected plasmid (12 of 12 tested). The rarity of haploid mutant recombinants in the case of transduction with the *cysQ*::*kan* plasmid (2% of Kan^r transductants) might reflect both proximity of the *kan* insert to one vector junction (370 bp) and relatively infrequent homologous recombination in certain regions (25, 27, 37).

Strains that facilitate isolation of plasmid-free transductants. Although with most mutations, haploid recombinants were found by testing just a few transductants, the rarity of haploid *cysQ*::*kan* recombinants (Table 2), despite the dispensability of *cysQ*, illustrated the potential weakness of relying only on screening for plasmid loss when searching for essential genes. We therefore sought to identify bacterial strains which exhibited higher relative frequencies of chromosomal recombinants.

A severalfold increase in the yield of chromosomal recombinants among transductants was obtained with a Dam^- (DNA adenine methylation deficient) strain (Table 3). This may reflect the replication deficiency of ColE1-type plasmids when transferred from Dam^+ to Dam^- cells (34). A more-than-10-fold increase in yield was obtained by using bacteria with a *recD* mutation, which is known to cause some plasmid instability during prolonged growth (9). The most dramatic result, however, was obtained by using a *recBC sbcB* strain: all Kan^r transductants were Amp^s and plasmid free in both the nonlysogen and in its λ-lysogenic derivative (Table 3). Transductants of the nonlysogen were mutant in phenotype, and those of the lysogen were prototrophic or Lac^+, as expected of heterozygous partial diploids. The lack of plasmid transductants is probably due to an extreme instability of ColE1-type plasmids in this strain, which may reflect their tendency to replicate in it as long concatemers (4, 13).

TABLE 3. Effect of genotype on yield of haploid *cysQ*::*kan* recombinants[a]

Recipient strain and relevant traits	Transduction frequency (10^{-7})	Fraction (%) Amp^s among transductants
DB5648; *rec*$^+$ *dam*$^+$[b]	5	5/204 (2)
DB1434; *rec*$^+$ *dam*$^+$(λ)[b]	5,000	1/304 (0.3)
DB5680; *dam*	0.1	9/105 (9)
DPB267; *recD*::Tn*10*	20	27/100 (27)
JC7623; *recBC sbcB*	9	166/166 (100)
DB6225; *recBC sbcB* (λ)	3	12/12 (100)

[a] Kan^r transductants were obtained by using lysates of λ656 grown on the strain p*cysQ*::*kan* DB5450.
[b] Data for this strain is from Table 2.

The ease of obtaining haploid *cysQ*::*kan* mutant phage when using a *recBC sbcB* strain for transduction led us to test whether plasmid transformation of this strain would also generate many haploid bacterial recombinants. Kan^r transformants made with the p*cysQ*::*kan* and pUC4K plasmids were found to grow slowly, and each of them was Amp^r (50 transformants tested). Overnight growth of several of the Kan^r transformants without antibiotic selection resulted in overgrowth by Kan^s Amp^s segregants. The rare ($<10^{-6}$) Kan^r bacterial colonies recovered from these cultures were still Amp^r, and in the case with pUC4K, still Lac^+ (blue), and thus carried the plasmid. Apparently, plasmid-chromosome recombination in this *recBC sbcB* strain is quite inefficient; λ-mediated transduction thus remains our preferred method for moving plasmid-borne alleles to the *E. coli* chromosome.

Transduction of chromosomal alleles. For many experiments, it is desirable to move mutant chromosomal alleles to new genetic backgrounds. We found that λ lysates prepared on *rec*$^+$ or *recBC sbcB* strains containing chromosomal *kan* insertions transduced the *kan* marker to recipient cells at frequencies of $\geq 10^{-7}$. In each of the seven cases, >98% of transductants exhibited the expected mutant phenotype (auxotrophic or Lac^-) (data not shown).

Isolation of partial diploids by using a lysogenic recipient. Heterozygous partial diploid transductants of the type that would be useful in analyzing essential genes were obtained by using the lysogenic strain DB1434. Transduction was 30- to 1,000-fold more efficient with this strain than with the nonlysogen (Table 2), probably in large part because of its immunity to killing by superinfecting λ. The largest enhancement of transduction was seen with phage λ656, which routinely grew to higher titers than the other Kohara phage we used; a relationship between vigor of phage growth and efficiency of cell killing would explain much of the variability in relative frequencies of transduction of lysogenic and nonlysogenic strains seen in Table 2.

TABLE 4. Transduction by λ *thyA*::*kan* in the presence or absence of thymine

Donor phage	Frequency of transduction[a] with the following recipient strain:					
	DB5648		DB5648 [p*thyA*$^+$]		DB1434	
	−Thymine	+Thymine	−Thymine	+Thymine	−Thymine	+Thymine
λ460 Δ*thyA5*	1×10^{-8}	3×10^{-5}	2×10^{-5}	5×10^{-5}	6×10^{-4}	7×10^{-4}
λ460 Δ*thyA6*	1×10^{-8}	1×10^{-5}	3×10^{-5}	4×10^{-5}	6×10^{-4}	6×10^{-4}

[a] Numbers indicate Kan^r transductants obtained per added phage.

Some Kan^r transductants were Amp^s in each case, although these Amp^s isolates were less frequent with the lysogenic than with the nonlysogenic recipient. More than 98% of such Kan^r Amp^s transductants were prototrophic and were probably partial diploids formed by crossing over in either λ or bacterial sequences (Fig. 1).

Isolation of high-frequency-transducing phage lines. Homogeneous stocks of mutation-bearing phage give relatively high transductant yields, and would thus be useful in testing interpretations that particular DNA segments are essential and in facilitating transduction between strains or species that differ in restriction and/or modification systems or that have diverged in sequence (10, 22, 32). To obtain pure stocks, we induced phage development in Kan^r Amp^s transductant derivatives of the *rec*$^+$ strain DB1434 or *recBC sbcB* strain DB6225, both of which carry λ p*lac*5 (Lac$^+$) as a prophage, plated aliquots of the resultant phage lysates on strain MC4100, and tested Lac$^-$ plaques for transduction. Overall, about half of the phage tested from many different transductant clones carried the *kan* marker. Considerable clone-to-clone variation was also noted, however. Much of this probably reflects the position of the initial crossover in λ or cloned sequences, which affects the relative yields of the two phage types after prophage induction (Fig. 1).

An alternative method for isolating pure stocks of mutation-bearing phage was based on the persistent low-level infections that are characteristic of transductant colonies on initial selection plates. About half of the plaques obtained by suspending such transductants in buffer, killing the bacteria with chloroform, and then streaking the suspension on an indicator bacterial lawn also carried the *kan* marker.

Test strategy for isolating phage that are mutant in an essential gene. *thyA* was used as a model essential gene because *thyA* mutant cells undergo rapid cell death during thymine starvation (15). Efficient transduction of the *thyA*::*kan* allele to a nonlysogenic plasmid-free strain (DB5648) was dependent on added thymine, whereas transduction of strains carrying either a *thyA*$^+$ plasmid or a resident prophage was not (Table 4), as expected. The very rare (0.1%) Kan^r Thy$^+$ transductants of the nonlysogenic plasmid-free strain segregated Thy$^+$ Kan^s and Thy$^-$ Kan^r derivatives at a high frequency and had thus probably resulted from rare tandem duplications prior to transduction (1). Electrophoresis of DNA from pools of plasmid-carrying transductants indicated that less than 5% of plasmids contained the *kan* insert. Similarly, more than 99% of Amp^r transformants of a *thyA* strain made with these pooled DNAs were Kan^s and Thy$^+$. Thus, recombination events after λ infection were targeted primarily to the bacterial chromosome. Most Thy$^+$ Kan^r transductants of the lysogen were heterozygous, as expected (Fig. 1), and pure stocks of *thyA*::*kan* mutant phage were readily obtained from them. In conclusion, these reconstruction experiments illustrate an efficient way of obtaining pure stocks of phages with null mutations in essential genes.

DISCUSSION

We have developed an efficient method for moving mutations in cloned *E. coli* DNA from plasmid vectors to the bacterial chromosome. It depends on having a selectable marker in or closely linked to the mutant allele in the plasmid clone, infection by a λ phage carrying cloned homologous DNA, and recombination between the plasmid and the phage and later between the phage and the recipient bacterial chromosome. The major advantages of this method relative to other allelic replacement methods stem from the technical ease of λ phage growth and of isolating haploid recombinants after infection with a transducing phage. The large numbers of haploid recombinants obtained when using pure stocks of mutant phage in the case of nonessential genes mean that any failure to isolate such recombinants can serve as evidence of an essential gene or site. In one application, we have begun constructing deletions that remove previously uncharacterized *E. coli* DNA and moving these deletions from plasmids to the *E. coli* chromosome in order to efficiently scan large regions of the genome for loci that are essential or contribute to bacterial growth. This method should also speed functional tests focused on individual genes, help in the engineering of special-purpose *E. coli* strains, and facilitate molecular genetic analyses of related bacteria.

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