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Method for the Isolation of *Escherichia coli* K-12 Mutants Deficient in Essential Genes

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We developed a general procedure for the induction and identification of mutations in chromosomal essential genes that are located in a diploid region of *Escherichia coli* K-12. The partial diploidy is conferred by an episome that is temperature sensitive for replication so that a mutant strain will form microcolonies at 42 C on complete media if an essential chromosomal gene in the diploid region is defective. Mutations identified by this procedure can be classified into cistrons by a complementation method devised for the purpose. To verify that the procedure works in practice, we fused an episome covering the *rif* region with an $F_{ts}lac^+$ and used the resulting temperature-sensitive episome to identify chromosomal mutations in essential functions near *rif*. As expected, a certain proportion of the mutations were in the *rif* gene, an essential gene that codes for the β subunit of ribonucleic acid polymerase.

Essentials genes of *Escherichia coli* are those whose functions are essential for growth even on complete or broth media (5, 22). Because of their obvious importance, it is often of interest to produce and analyze mutations in essential genes. In haploid organisms, the most common method for isolating mutants affected in essential genes has involved the use of conditionally lethal mutations. In a conditionally lethal strain, the mutant gene product functions only under certain (permissive) conditions and is inactive under other (nonpermissive) circumstances. It is likely, however, that some genes cannot be easily mutated to a form that will code for a product that functions only under experimentally desirable conditions. For instance, Edgar and Lielausis (11) found that, in a large sample of temperature-sensitive mutants of bacteriophage T4D, the mutations were not distributed randomly: some genes were mutated many times, and others were mutated only once. This suggests that there may be some genes in which conditionally lethal mutations cannot be induced. Furthermore, even when conditionally lethal mutations are obtained, low levels of activity of the gene product even under permissive conditions can obscure analyses (15).

Beckman and Cooper (5) used a strain of *E. coli* carrying a temperature-sensitive suppressor transfer ribonucleic acid (tRNA) (14) to obtain essential gene mutations on the chromosome. However, suppressors in general suppress with low efficiency, and the suppressor tRNA may also insert an amino acid unable to confer proper function on the protein product. Thus, the procedure of Beckman and Cooper also might not permit the isolation of mutations in some essential genes.

Murgola and Adelberg (27) used streptomycin suppression to isolate mutations in essential genes of *E. coli*. Streptomycin causes misreading and nonspecific suppression of all three nonsense codons (31, 38). Therefore, isolates with a lesion in an essential gene grew on complete medium to which streptomycin was added but not on complete medium without streptomycin. Although several mutants deficient in the syntheses of protein, lipid, and deoxyribonucleic acid (DNA) were isolated, most grew poorly even under the permissive condition because of the partial nature of the phenotypic suppression.

To avoid the problems inherent in the studies discussed above, we used a partially diploid strain of *E. coli* K-12 to isolate essential gene mutants. Because the F′ episome carries a duplicate set of genes, the functions of essential chromosomal genes in the diploid region can be completely abolished without loss of viability of the mutant strain. Partial diploids have been used, for example, to obtain recessive lethal mutations in ribosomal protein genes (28) and in the gene, called *rif* (3), that specifies the β-polypeptide of RNA polymerase (17, 30). In both of these cases, known phenotypic responses to particular drugs were used to select for the

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desired mutants. We are proposing a more general approach in which the wild-type alleles on the episome can be removed at will to unmask any recessive lethal mutation.

To do this, we chose to use an F′ factor that is temperature sensitive for its own replication. Cells with mutations in essential genes on the chromosome should then be dependent on the continued presence of the F′ factor for growth. At 28 C the cells can grow normally because the temperature-sensitive episome replicates efficiently. However, when episomal replication is inhibited by incubation at 42 C, the mutants should grow very poorly to form microcolonies. Nonmutant bacteria should grow normally at both temperatures, with the F′ factor being maintained at 28 C and lost at 42 C. The microcolonies formed by the mutant strains at 42 C should be analogous to the minute colonies produced during the unilinear transmission of bacteriophage in abortive transduction (29; B. A. D. Stocker, Heredity, 9:290–291, 1955).

To determine whether this method of mutant isolation would be successful, we selected an F′ factor that carried a known essential gene, so that we were certain that chromosomal mutants defective in at least this essential gene should be recovered. We chose the F′ factor F110 because it carries the essential gene *rif*. Strains deficient in the *rif* gene were characterized previously (4) and are known as *rif*^{0} mutants. *rif*^{0} mutants are inviable as haploids, a characteristic expected of an essential mutant.

To make F110 temperature sensitive, it was fused in vivo with an already well-characterized temperature-sensitive episome, $F_{ts62}lac^+$ (8). We mutagenized our strain with ICR-191, a frameshift mutagen that usually produces mutations that virtually abolish gene function (1, 32). We then isolated mutants defective in chromosomal essential genes covered by the temperature-sensitive episome. As predicted, some of the mutants are deficient in the chromosomal *rif* gene function.

We also developed a method to transfer the chromosomal mutant *rif* allele and other mutations in essential genes to an F′ factor by recombination. This procedure is important because it allowed us to perform complementation tests to classify the mutations into cistrons.

MATERIALS AND METHODS

Media. M63 minimal liquid medium and agar, lactose-tetrazolium agar (lac-tet), and Penassay broth were described previously (18, 19). Penassay agar contains 25.5 g of Antibiotic medium 2 (Difco) and 5 mg of thiamine per liter. Dilution medium, M63 buffer-salts, contained the following per liter: KH_2PO_4, 5.3 g; $K_2HPO_4 \cdot 3H_2O$, 13.93 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; and $FeSO_4 \cdot 7H_2O$, 0.5 mg. Plates made from complete media (lac-tet and Penassay agar) were stored in the dark before use to avoid lethality to RecA$^-$ strains (36). When required, media were supplemented with L-amino acids, purines, and pyrimidines, each at 20 μg/ml. The only exception was thymine, which was added at 50 μg/ml for strains requiring high concentrations of thymine. Rifampin and streptomycin were added to sterile media at final concentrations of 100 and 250 μg/ml, respectively. All media containing rifampin were stored in the dark.

Chemicals. ICR-191, {3-chloro-7-methoxy-9-(3-[chloroethyl]amino propylamino) acridine dihydrochloride} was the generous gift of H. J. Creech. Rifampin (B grade, Calbiochem) and rifamycin SV sodium salt (Schwarz/Mann) were both used in parts of this work. Since the results obtained with both drugs were the same, the designation rifampin will be used in the text to apply to either antibiotic. *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NG) was obtained from the Aldrich Chemical Co., and trimethoprim was from the Sigma Chemical Co.

Nomenclature. Abbreviations and symbols for genetic markers are in accordance with those proposed by Demerec et al. (10) and used by Taylor and Trotter (35). *ess* is used to signify a mutation in an essential gene. Alleles of the *rif* gene are denoted by the convention used by Austin and Scaife (3): *rif*^{s} confers sensitivity to the drug rifampin, *rif*^{r} confers resistance to the drug, and *rif*^{0} results in complete loss of the *rif* gene function.

Bacterial strains. The bacterial strains used in this study are listed in Tables 1 and 2.

Mutagenesis procedures. For the construction of various strains (Table 1), auxotrophs were induced by treating exponentially growing cultures with NG as described by Hoess and Herman (20). After mutagenesis, cells were collected by centrifugation, washed twice with M63 buffer-salts, and suspended in glucose minimal medium. When the culture reached stationary phase, penicillin selection (16) was performed to enrich for auxotrophs.

Mutations in essential genes were induced by treating an exponential culture of strain KAF1/KA17 (Table 2) with ICR-191 (19) for 4 and 8 h in lactose-threonine-tryptophan-methionine medium at 28 C using 0, 10, 15, and 20 μg of ICR-191/ml. After 4 and 8 h, cells were collected and washed as after NG mutagenesis and then suspended in M63 buffer-salts. The resuspended cells were diluted 1:100 into lactose-threonine-tryptophan-methionine medium and incubated with shaking at 28 C until stationary phase was reached.

The efficiency of the ICR-191 mutagenesis was monitored by examining the number of colonies of strain KAF1/KA17 that grew on minimal plates containing rifampin. For this purpose, the stationary cultures of segregated cells were diluted 500-fold before being plated and incubated at 28 C. The cultures that showed the highest frequency of rifampin-resistant colonies—this varied somewhat from experiment to experiment—were screened for mutants deficient in essential genes.

Thy$^-$ mutants. Spontaneous Thy$^-$ mutants were selected by using trimethoprim as described by Hoess and Herman (20).

TABLE 1. *Bacterial strains*

Strain	Sex	Genotype[a]	Derivation
F_{ts62}*lac*$^+$/200PS	F′	F_{ts62}*lac*$^+$/*lacZ*$^+$, *lacY*$^-$	F. Jacob (8)
F_{ts62}*lac*$^+$/KA9	F′	F_{ts62}*lac*$^+$/*argE, met, pur, str, thy*$^+$, *his, pro, lac, recA1*	F_{ts62}*lac*$^+$/200PS × KA9
JC5029	Hfr	*ilv-38, thr-300, recA*$^+$	CGSC[b]
JC5029 Thy$^-$	Hfr	*ilv-38, thr-300, recA*$^+$, *thy*	Spontaneous mutant of JC5029
JC5029 Thy$^-$ Leu$^-$	Hfr	*ilv-38, thr-300, recA*$^+$, *thy, leu*	NG-induced mutant of JC5029 Thy$^-$
KA1[c]	F$^-$	*argE, met*	NG-induced mutant of 254
KA2[c]	F$^-$	*argE, met, str*	Spontaneous mutant of KA1
KA3[c]	F$^-$	*argE, met, str, thy*	Spontaneous mutant of KA2
KA8[c]	F$^-$	*argE, met, pur, str, thy, his, pro, lac*	NG-induced mutant of KA3
KA9[c]	F$^-$	*argE, met, pur, str, thy*$^+$, *his, pro, lac, recA1*	KA8 × KL16-99 Thy$^-$
KA12[c]	F$^-$	*argE, met, pur, lac*	NG-induced mutant of KA1
KA13[c]	F$^-$	*argE, met, pur, lac, thy*	Spontaneous mutant of KA12
KA14[c]	F$^-$	*argE, met, pur, lac, thy*$^+$, *recA1*	KA13 × KL16-99 Thy$^-$
KA14 Rif^r [c]	F$^-$	*argE, met, pur, lac, recA1, rif*^{r}	Spontaneous mutant of KA14
KA14 Thy$^-$[c]	F$^-$	*argE, met, pur, lac, recA1, thy*	Spontaneous mutant of KA14
KA16[c]	F$^-$	*argE, met, pur, lac, thy, thr, trp*	NG-induced mutant of KA13
KA17[c]	F$^-$	*argE, met, pur, lac, thy*$^+$, *thr, trp, recA1*	KA16 × KL16-99 Thy$^-$
KL16-99 Thy$^-$	Hfr	*thy, recA1*	J. A. Clark (24)
KLF10/JC1553	F′	F110 *metB*$^+$/*argG6, metB1, his-1, leu-6, recA1, str-104, malB16*	CGSC[b]
KLF23/KL181	F′	F123 *trp*$^+$/*pyrD34, his-68, trp-45, recA1*	CGSC[b]
254	F$^-$	*argE*	P. Rogers (12)
254Rif^r	F$^-$	*argE, rif*^{r}	Spontaneous mutant of 254

[a] Only genetic markers relevant to the current study are listed.
[b] *E. coli* Genetic Stock Center, Department of Human Genetics, Yale University, New Haven, Conn.
[c] The *met* and *pur* markers in these strains were selected because they could be complemented by the episome F110.

TABLE 2. *Construction of* F_{ts62}*lac*$^+$*-F110 rif*^{r} *met (KAF1)*

Strain	Sex	Genotype[a]	Derivation
F110 *rif*^{r}/254 Rif^r	F′	F110 *argE*$^+$, *rif*^{r}/argE, *rif*^{r}	KLF10/JC1553 × 254 Rif^r
F110 *rif*^{r}/KA14 Rif^r	F′	F110 *argE*$^+$, *rif*^{r}/*argE, met, pur, lac, recA1, rif*^{r}	F110 *rif*$_r$/254 Rif^r × DA14 Rif^r
F_{ts62}*lac*$^+$-F110 *rif*^{r}/KA9	F′	F_{ts62}*lac*$^+$-F110 *argE*$^+$, *rif*^{r}/*argE, met, pur, str*^{r}, *thy*$^+$, *his, pro, lac, recA1*	F110 *rif*^{r}/KA14 Rif^r × F$^-$ phenocopies of F_{ts62}*lac*$^+$/KA9
F_{ts62}*lac*$^+$-F110 *rif*^{r}/KA1	F′	F_{ts62}*lac*$^+$-F110 *argE*$^+$, *rif*^{r}/*argE, met, recA*$^+$	F_{ts62}*lac*$^+$-F110 *rif*^{r}KA9 × KA1
F_{ts62}*lac*$^+$-F110 *rif*^{r} *met*/KA1	F′	F_{ts62}*lac*$^+$-F110 *argE*$^+$, *rif*^{r}, *met*/*argE, met, recA*$^+$	Homogenote formation from preceeding strain
F_{ts62}*lac*$^+$-F110 *rif*^{r} *met*^{b}/KA9	F′	F_{ts62}*lac*$^+$-F110 *argE*$^+$, *rif*^{r}, *met*^{b}/*argE, met, pur, thy*$^+$, *his, pro, lac, recA1*	F_{ts62}*lac*$^+$-F110 *rif*^{r} *met*/KA1 × KA9
F_{ts62}*lac*$^+$-F110 *rif*^{r} *met*^{b}/KA17	F′	F_{ts62}*lac*$^+$-F110 *argE*$^+$, *rif*^{r}, *met*^{b}/*argE, met, pur, lac, thy*$^+$, *thr, trp, recA1*	F_{ts62}*lac*$^+$-F110 *rif*^{r} *met*^{b}/KA9 × KA17

[a] Only genetic markers relevant to the current study are listed.
[b] F_{ts62}*lac*$^+$-F110 *rif*^{r} *met* has been designated KAF1.

Identification of *recA*$^+$ and *recA* strains. The Rec$^-$ phenotype was detected by its sensitivity to ultraviolet light, using published procedures (20).

Preparation of F$^-$ phenocopies. Male strains were converted into F$^-$ phenocopies by a standard procedure (26).

Mating procedures. Aside from the exceptions described below, the standard mating procedure was to dilute overnight cultures of donors and recipients, grown in either minimal medium or broth, separately 1:40 into Penassay broth, to incubate with shaking at 37 C for 2.5 to 4 h, and then to mix in a

1:1 ratio. These mating mixtures were incubated without shaking for 1 to 2 h at 37 C.

Three other mating protocols were also employed because they involved either temperature-sensitive F′ factors or the need for screening large numbers of F′-containing colonies.

Donors containing $F_{ts62}lac^+$ or the fused episomes derived from this F′ factor were grown to stationary phase at 28 C in either Penassay broth ($F_{ts62}lac^+$) or liquid minimal medium (the temperature-sensitive, fused episomes). They were then diluted 1:20 into Penassay broth, shaken at 34 C for about 5 h, and mated without shaking for 2 h at 34 C as described above. Plates spread with these mating mixtures were incubated at 28 C.

Replica matings were used to screen large numbers of isolates for their ability to donate an episome with normal replication machinery to another strain carrying an episome that was temperature sensitive in replication. Stationary donor cultures were grown in minimal medium at 37 C and were diluted and plated on minimal plates to give approximately 100 colonies per plate. The plates were incubated at 37 C until the colonies were approximately 1.5 mm in diameter (about 48 to 56 h) and then replica plated onto selective plates spread with 0.15 ml of a culture of the recipient strain containing the temperature-sensitive episome. The recipient had been grown in Penassay broth at 28 C for 5 to 7 h. The selective plate contained a minimal medium selecting against the growth of both parents but permitting the growth of recipients that had received the non-temperature-sensitive episome. It was crucial for the success of this procedure that both the donor plates and replica mating plates be fresh, i.e., dried either overnight or for 2 days at room temperature.

Spot matings were used to test for the ability of one strain to transfer episomes to a recipient by conjugation. Bacteria used in these crosses were grown in minimal medium at 37 C to stationary phase if the cells did not contain a temperature-sensitive episome. If the bacteria did possess a temperature-sensitive episome, the cells were grown at 28 C in Penassay broth for the matings. Approximately 0.01 ml of a culture of one strain was spotted onto a selective plate. After the spot had dried, 0.01 ml of the other strain was deposited on top, and the mixed spot was permitted to dry before the plate was incubated.

RESULTS

Outline of construction of the $F_{ts62}lac^+$-F110 *rif*^{r} *met* episome. As discussed above, we wished to develop a method for isolating mutants deficient in essential genes of *E. coli*. Our plan was to induce the mutations in strains that carried a duplicate copy of the essential gene on an F′ factor temperature sensitive for replication. As a test of our procedure, we chose to determine whether we could isolate a mutant deficient in a specific chromosomal essential gene, *rif*. One reason for choosing the *rif* gene was that simple tests could be used to determine if the chromosomal *rif*^{s} gene, which is the wild-type allele, had been mutated. We thus began our experiments with the F′ factor F110 because it carries the *rif* gene (3).

An F′*rif*^{r}/*rif*^{s} strain is usually sensitive to rifampin (3). However, if the chromosomal *rif*^{s} allele is mutated either to *rif*^{r} or *rif*^{0}, the resulting F′*rif*^{r}/*rif*^{0} or F′*rif*^{r}/*rif*^{r} strain is phenotypically rifampin resistant (3). Thus, if we construct an F′*rif*^{r}/*rif*^{s} strain, we can use the appearance of the Rif^r phenotype to score directly for chromosomal mutations to *rif*^{r} or *rif*^{0}; moreover, we can use the frequency of appearance of Rif^r mutants to estimate the success of the mutagenesis with ICR-191. Since F110 carries the *rif*^{s} allele, our first step was to convert this allele to *rif*^{r}. This was done by transferring F110 into a cell carrying a *rif*^{r} gene on its chromosome and by selecting for homogenate formation.

Our identification of mutations in a chromosomal essential gene required that the episome carrying the gene be temperature sensitive for replication so that microcolony formation at 42 C could be used to identify the mutants. Therefore, our second step was to fuse F110 *rif*^{r} with $F_{ts62}lac^+$ to obtain an episome with the *rif*^{r} gene from F110 and the temperature-sensitive replication machinery from $F_{ts62}lac^+$. The technique of fusing these two F′ factors (37) to obtain $F_{ts62}lac^+$-F110 *rif*^{r} allowed us to isolate a temperature-sensitive episome relatively easily compared with the alternative of mutagenizing and isolating temperature-sensitive mutants of F110 *rif*^{r}. $F_{ts62}lac^+$ was used because its replication has been shown to be more thermolabile than that of $F_{ts114}lac^+$, another episome commonly used (8, 26).

Finally, we wanted our F′ factor to carry an auxotrophic marker also carried by the chromosome so that selection could be used to replace the resident F′ factor by another F′ factor carrying the wild-type allele of the marker. Such replacement is important both for the complementation tests and the mapping of mutations in chromosomal essential genes (see below and the accompanying paper [2]). We chose a *met* marker for this purpose and used homogenate formation to transfer the *met* marker from KA1 onto our episome. Strain KA1 is *recA*$^+$ and carries the same *met* marker as KA17, which has the chromosome that will be used in the search for *ess* mutants.

In summary, the episome construction necessary for the test of our procedure for isolating mutants deficient in essential genes involved: (i) isolating an F110 episome carrying a *rif*^{r} mutation, (ii) fusing this F′ factor with $F_{ts62}lac^+$, and (iii) isolating a *met* variant of the

fused episome in which the *met* allele was the same as the one on the chromosome. These procedures are outlined in Table 2 and are discussed in detail below.

Construction of F110 *rif*^{r}. F110 was transferred by conjugation into strain 254 Rif^r (*argE rif*^{r} *recA*$^+$; Table 2). Rif^r merodiploids were formed by recombination during subsequent growth and then identified by their ability to grow on rifampin-containing plates. To prevent the occurrence of further changes in the episome by recombination with chromosomal markers, it was transferred to KA14 Rif^r, a *recA* strain. The resulting strain was resistant to rifampin, as expected. Proof that the F′ factor now carried a *rif*^{r} allele (and not *rif*^{0}) was the segregation of rifampin-resistant recombinants after the episome was transferred to KA2, a *rif*^{s} *recA*$^+$ strain.

Fusion of F110 *rif*^{r} with F_{ts62}*lac*$^+$. F110 *rif*^{r}/KA14 *rif*^{r} *recA* was grown to stationary phase in glucose minimal medium, diluted 1:40 into Penassay broth, grown to exponential phase at 37 C, and mated at 34 C for 2 h with F$^-$ phenocopies of F_{ts62}*lac*$^+$/KA9 grown to stationary phase at 28 C. Undiluted aliquots of the mating mixture were spread onto lactose plates containing streptomycin to select against the donor. The plates were also supplemented with histidine and proline, two amino acids required by the recipient. Colonies that grew after incubation at 28 C were candidates for containing an episome produced by the fusion of F110 *rif*^{r} and F_{ts62}*lac*$^+$ because growth on this medium required the *argE*$^+$, *met*$^+$, and *pur*$^+$ genes from F110 *rif*^{r} as well as the *lac*$^+$ gene from F_{ts62}*lac*$^+$, and also because only one F′ factor can exist stably in a cell (9, 33). Although deletion of some DNA seems to occur during episomal fusion, probably because of the incompatibility of two sets of F replication genes (37), the *rif*^{r} gene should be on the fused episome because *rif*^{r} lies between *argE*$^+$ and *pur*$^+$, two genes required for growth on the selective plates. The putative F_{ts62}*lac*$^+$-F110 *rif*^{r}/KA9 strains that grew on the minimal plates were purified twice by streaking onto the same selective medium and then tested by mating at 34 C with KA17, an F$^-$ strain. The mating mixtures were plated onto selective medium to test for the transfer by F_{ts62}*lac*$^+$-F110 *rif*^{r}/KA9 of the genes *lac*$^+$, *argE*$^+$, *met*$^+$, and *pur*$^+$. In this way we obtained an episome that did indeed have all four of these genes and was also temperature sensitive for replication. The fused episome F_{ts62}*lac*$^+$-F110 *rif*^{r} carried by KA9 (*his pro*) was transferred to KA1 (*argE met recA*$^+$) by conjugation. Dilutions of the mating mixture were spread onto glucose plates containing methionine. During subsequent growth, recombination could occur to produce *met* homogenates, which were identified as follows. Single-colony isolates from the selective plates were grown to stationary phase at 28 C in liquid medium containing glucose and methionine. To prevent further recombination from occurring between the episome and chromosome, the episome was transferred to KA9, an F$^-$*recA* strain that carries the same *met* allele as strains KA1 and KA17. In the conjugation for the transfer, streptomycin was used to select against the donor. Also, the selection medium contained lactose and was deficient in arginine and adenine to select against KA9. After incubation at 28 C, the plates were replicated to similar plates lacking methionine, and colonies that didn't grow on the replica plates were identified as *met* homozygous merodiploids. The colonies were purified twice by streaking to obtain single colonies on selective plates containing methionine at 28 C. These isolates now contained the desired episome F_{ts62}*lac*$^+$-F110 *rif*^{r} *met*, which we have designated KAF1. Any mutations in essential genes identified by using this fused episome will be in chromosomal genes that are duplicated on F110 because the chromosomal genes carried by F_{ts62}*lac*$^+$ are all dispensable (7).

Chromosomal genotype of the strain used for isolating mutations in essential genes. The chromosomal genotype we constructed for our experiments (Table 1) contained the following mutations: *argE, met, pur, thr, trp*, and *recA1*. The *recA* mutation was needed to prevent the loss of *ess* mutations on the chromosome by recombination with the episome, which carries *ess*$^+$ alleles. Also, it was necessary for the chromosome of this strain to carry auxotrophic alleles of genes carried on the episome so that selection for the continued presence of the episome could be exerted. KAF1 is often lost unless episomal genes are required during growth. The *argE, pur*, and *lac* mutations served this purpose. The functions of other chromosomal markers (*met, thr*, and *trp*) will become apparent later when the complementation procedure is discussed. Thus, the final strain we used for our mutant isolation was $KAF1_{ts}$*lac*$^+$ *argE*$^+$ *rif*^{r} *met pur*$^+$/*argE met pur lac thr trp recA1*. This strain was obtained by conjugating KAF1/KA9 with KA17 (Table 2).

Isolation of mutants defective in essential genes. Strain KAF1/KA17 was mutagenized with ICR-191 and grown in minimal medium at 28 C to allow chromosomal segregation. Stationary cultures of the segregated cells were

diluted in M63 buffer-salts to give about 100 colonies per plate on lac-tet agar. The plates were incubated at 28 C for 32 to 34 h and then replicated to the same medium that had been prewarmed to 42 C. The replica plates were incubated at 42 C for approximately 50 h. Most colonies, including those formed by unmutagenized bacteria, grew normally on both the master and replica plates. Rare colonies were formed, however, that grew normally on the 28 C master plate but that formed tiny clusters of microcolonies, which we called spotty colonies, on the 42 C plate. These colonies we tentatively identified as having mutations in essential genes on the chromosome. Such mutants would be dependent upon the presence of the episome KAF1 for continued growth, and the replication of the episome is temperature sensitive.

To verify that the bacteria in the spotty colonies were indeed defective in a chromosomal essential gene in the diploid region, cells were purified from the corresponding colony on the 28 C master plate and were spot-mated with a strain carrying the F123 *trp*$^+$ episome (strain KLF23/KL181, Table 1). Trp$^+$ merodiploids were selected on glucose-arginine-methionine-adenine-threonine minimal plates incubated at 37 C. The failure of an isolate to form viable Trp$^+$ colonies after this mating suggested that KAF1 could not be replaced by F123 *trp*$^+$, since two F′ factors cannot be stably maintained in the same cell; therefore, each such isolate presumably required the continued presence of KAF1 because the isolate contained a lesion in some essential chromosomal gene whose function was coded for by this episome.

An alternative possibility was that a strain that formed a spotty colony at 42 C could no longer accept any F′ factor by conjugation. To ascertain that this was not true, each strain forming a spotty colony at 42 C was mated with a strain containing the F110 *met*$^+$ episome. F110 *met*$^+$ carries all of the genes on KAF1 and, therefore, should be able to replace KAF1 even if the latter episome carried a gene essential for growth. Replacement of KAF1 was accomplished by mating strain KLF10/JC1553 with spotty colony isolates of KAF1/KA17 and selecting for Met$^+$ merodiploids. The existence of Met$^+$ colonies after this mating indicated that the inability to form Trp$^+$ colonies after mating with F123 *trp*$^+$ was because F123 *trp*$^+$ did not code for the wild-type allele of a mutant chromosomal essential gene and not because KAF1/KA17 was unable to accept F′ factors during mating.

Control matings using the unmutagenized parent strain as the recipient consistently gave numerous Trp$^+$ merodiploids in spot matings with strain KLF23/KL181. Any mutant isolates that showed the parental phenotype of formation of viable Trp$^+$ merodiploids were concluded not to be defective in chromosomal essential genes and were not examined further.

Isolation of chromosomal *rif*^{0} mutants. Since KAF1 carries the *rif*^{r} gene, we expected some of the chromosomal mutations in essential genes to be *rif*^{0} mutations. Theoretically, it was possible to have mutated the chromosomal *rif*^{s} gene to either *rif*^{r} or *rif*^{0}, but we would have identified only the *rif*^{0} type as an essential mutant because only the *rif*^{0} class of mutants would not accept F123 *trp*$^+$ in place of KAF1. The F′*rif*^{s}*rif*^{0} mutants should be easily identified by their rifampin-resistant phenotype. Therefore, all mutant strains that were unable to form Trp$^+$ merodiploids were transferred onto minimal plates with and without rifampin to test for rifampin resistance. The plates were incubated at 28 C. Two rifampin-resistant strains were found out of nine independently isolated mutants in which KAF1 could be replaced by F110 *met*$^+$ but not by F123 *trp*$^+$. When KAF1 was replaced by F110 *met*$^+$ *rif*^{s}, the merodiploids were sensitive to rifampin as expected for a *rif*^{0} mutation in the chromosomal *rif* gene. Furthermore, the mutations in those two strains have been mapped to the *rif* portion of the diploid region (2). We conclude that these two mutant strains are of the type that we had predicted we could isolate. The other seven mutants will be discussed in the accompanying paper (2).

Construction of F110 episomes carrying essential mutations. To be able to classify the essential gene mutations into complementation groups, we wanted to transfer them onto F110 *met*$^+$ episomes. We shall illustrate our procedure (Fig. 1) for this using one of the *rif*^{0} mutations. As already described, one of our tests for identification of mutations in essential genes involved the replacement of KAF1 by F110 *met*$^+$. The resulting strain has the following genotype: F110 *met*$^+$ *ess*$^+$/*argE met pur lac thr trp ess recA1*, designated strain C in Fig. 1. Our basic procedure was to introduce a *recA*$^+$ gene into this strain so that the episome could pick up the *ess* mutation from the chromosome by recombination: the F110 *met*$^+$ *ess*$^+$/*met ess recA1* heterogenotes (strain C) were made into F$^-$ phenocopies and then mated with a *recA*$^+$ Hfr strain, JC5029 Thy$^-$ Leu$^-$ (Table 1). It was found that these matings were as much as 100-fold more efficient if the recipient was grown at 28 C instead of 37 C in liquid minimal medium

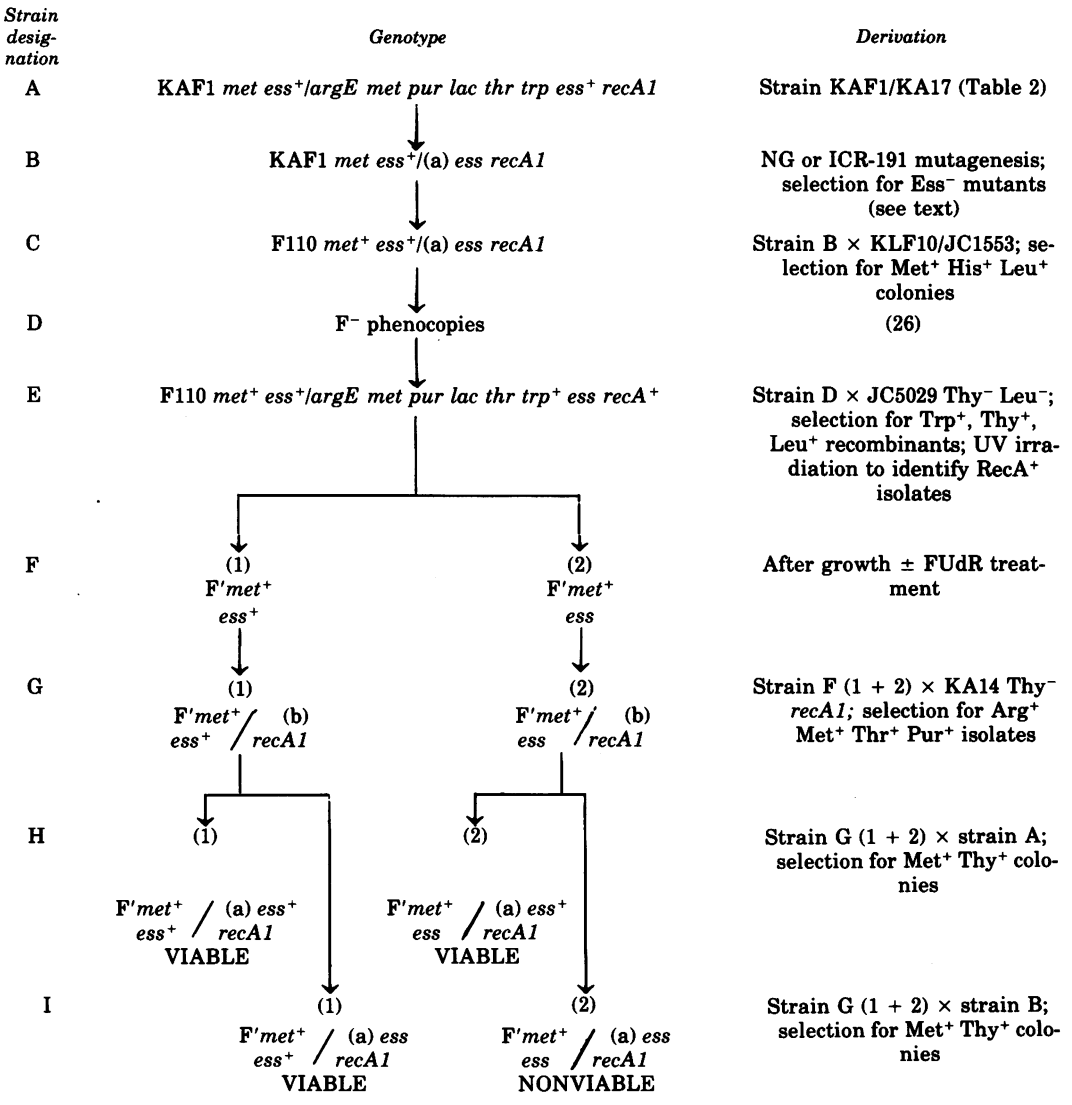

FIG. 1. *Procedure for transfer of chromosomal ess mutations to F110 episomes. The symbol (a) denotes the markers argE met pur lac thr trp, and (B) denotes argE met pur lac thy.*

before being made into F⁻ phenocopies. After mating at 37 C for 2 h, Trp⁺ Leu⁺ Thy⁺ recombinants were selected on minimal agar at 37 C. Since the *recA*⁺ gene enters before *trp*⁺ in the transfer of DNA by the Hfr strain (6), approximately 50% of the Trp⁺ recombinants inherit the *recA*⁺ gene from the Hfr. These Trp⁺ recombinant strains were twice purified by streaking onto the same type of selective plates, grown in minimal liquid medium at 37 C, and irradiated with ultraviolet light (UV) to identify UV-resistant (RecA⁺) isolates (strain E).

To increase recombination between chromosome and episome further, the Trp⁺ RecA⁺ strains were usually treated with fluorodeoxyuridine (FUdR) for 90 and 150 min by the method of Gallant and Spottswood (13). Before the recombinant *ess* episomes could be identified, a sample of episomes from each culture was transferred to F⁻KA14 Thy⁻ (*recA1*) by conjugation to prevent further recombination. These crosses were done immediately after any FUdR treatments. After 60 to 90 min of mating at 37 C, the mating mixtures of cells of strains F-1 and F-2 and KA14 Thy⁻ *recA* were diluted 1:100 into liquid minimal glucose-thymine medium

to enrich for KA14 Thy⁻ *recA* cells that had received an episome from the donor strain (strains G-1 and G-2). After growth to stationary phase at 37 C, the culture was diluted and spread onto glucose-thymine plates to form single colonies. Each colony of strains G-1 and G-2 was then tested for the presence of an F'*argE*⁺ *met*⁺ *pur*⁺ *ess* episome. This was done by mating each with the original *ess* mutant parent: KAF1 *met ess*⁺/*argE met pur lac thr trp recA1 ess* (strain B) and selecting for Met⁺ Thy⁺ (Arg⁺ Pur⁺) merodiploids. Nonrecombinant (*ess*⁺) episomes carried by strain G-1 can replace the resident episome of the recipient to give viable Met⁺ Thy⁺ merodiploids (strain I-1), but *ess* episomes carried by strain G-2 cannot (strain I-2). Because *ess* episomes were rare (1 to 3% in some FUdR-treated cultures and less than 0.1% in untreated cultures), replica matings were used first to screen large numbers of colonies for the inability to produce Met⁺ Thy⁺ merodiploids. Candidates for carrying an *ess* episome identified by this test were twice purified on selective plates and then spot-mated with both the chromosomal *ess*⁺ parent (strain A), KAF1 *met ess*⁺/*ess*⁺ KA17, and its chromosomal *ess* mutant derivative (strain B). Isolates that gave Met⁺ Thy⁺ colonies when mated with the chromosomal *ess*⁺ strain, but no Met⁺ Thy⁺ colonies when mated with the chromosomal *ess* strain, were deduced to have the following desired genotype: F'*argE*⁺ *met*⁺ *pur*⁺ *ess*/*argE met pur lac recA1 thy*.

By this method, we have constructed F'*met*⁺ *rif*ᵘ episomes from KAF1/*met rif*ᵘ strains. As predicted, these episomes form Met⁺ Thy⁺ merodiploids in matings with the *ess*⁺ parent KAF1/*met rif*ˢ but not in matings with the *rif*ᵘ mutant parent.

DISCUSSION

In this paper, we have delineated a procedure for isolating mutants deficient in chromosomal essential genes in a partially diploid strain of *E. coli* K-12. Knowing that the episome that conferred partial diploidy carried the essential gene *rif*, we predicted that some of the essential mutants we isolated would be defective in the chromosomal *rif* gene. Indeed, two mutants of this type were isolated.

A feature of the method we developed is that it concentrates on a limited region of the genome–the region covered by the F' used. Because the method enables one to classify the mutations into genes by complementation tests, it should be feasible to saturate a particular chromosomal region with mutations in essential genes. The idea of localized mutagenesis was described by Hong and Ames (21), although their method involved temperature-sensitive mutations, whereas an advantage of the method we have used is that the mutations can simply lead to inactive gene products. F' factors are available for almost any desired region of the *E. coli* chromosome (25), so the applicability of the method can obviously be extended.

Naturally, one would like to be able to identify the biochemical defects in the essential gene mutants. We can suggest three possible approaches for doing this. All involve first making a guess as to particular proteins that might be involved. Such guesses could be based on genes that are already known to map in the region being studied. One procedure involves assaying a specific protein in a mutant strain before and after a shift to 42 C. At 42 C the differential rate of synthesis of the protein should be reduced compared with other essential proteins if the chromosomal gene for that protein is inactive. At 42 C a culture of mutant cells does not completely lack the essential gene function. Some cells of the culture contain an unreplicating episome, and presumably in these cells the *ess*⁺ gene product continues to be synthesized. F⁻ cells are continually segregated, however. These cells should contain a nearly normal amount of *ess*⁺ gene product when they are segregated, but the growth of these cells and their descendants should gradually slow down as the *ess*⁺ gene product becomes diluted and rate-limiting for growth.

A second type of biochemical analysis that could be applied to the mutants we isolated is an immunological technique described by Horvitz (23). Briefly, a supernatant extract is obtained from radioactively labeled cells and incubated with antibody to a purified protein. The antigen-antibody complex is then analyzed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. Horvitz (23) used this procedure to demonstrate sequential phosphorylations of the α subunit of RNA polymerase. In extracts of our mutants, we could look for polypeptide fragments, since frameshift mutations frequently lead to premature termination of translation (32). The gel pattern of the extract from the mutant strain should show an extra band, the polypeptide fragment, that would not be present in the gel pattern from the wild-type strain. This approach would, of course, not work if the polypeptide fragment were so altered that it no longer could be precipitated by antibody.

A third possible approach involves the isolation of partial revertant strains that are viable in the absence of the fused episome. These hap-

loid strains would be assayed for enzyme activity and should exhibit a specific activity lower than that of the wild-type F^- parent. These partial revertants, which could carry either intragenic or intergenic suppressor mutations, would be analogous to the leaky mutants described by Schlessinger (34).

In conclusion, we believe that the method we have developed could be used to isolate mutations in almost any essential gene in *E. coli*. In addition to the chromosomal *rif* mutations discussed above, we have isolated several other mutants in essential genes mapping in the diploid region of our strain. The mutants will be discussed and characterized in the accompanying paper (2).

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