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Blue Ghosts: a New Method for Isolating Amber Mutants Defective in Essential Genes of *Escherichia coli*

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We describe a technique which permits an easy screening for amber mutants defective in essential genes of *Escherichia coli*. Using this approach, we have isolated three amber mutants defective in the *rho* gene. An extension of the technique allows the detection of ochre mutants and transposon insertions in essential genes.

We have been interested in isolating protein chain-terminating mutations in the *rho* gene of *Escherichia coli* (gene abbreviations are according to reference 1). This gene codes for the essential transcription termination factor, rho (12). For this purpose, we developed a replica plating method which allows us to identify amber (UAG) mutants defective in essential genes after mutagenesis. Using this method, we isolated three amber mutants defective in the *rho* gene after localized mutagenesis of the *ilv-rho* region of the bacterial chromosome. This method should be useful for isolating amber mutants defective in any essential gene.

Outline and principles of method. This method depends on the following steps and strain properties. (i) Localized mutagenesis (8) was used to obtain amber mutants defective only in the region of interest. In this case, we treated one *E. coli* strain (A) with a mutagen, made a P1 transducing lysate on this strain, and used the lysate to transduce an ilv^- recipient strain (B) to ilv^+. The *rho* gene is very closely linked to *ilv*, so that in this way we enriched for mutations in *rho* and nearby genes.

(ii) The donor and recipient strains for the transduction must carry an amber suppressor so that any amber mutants defective in essential genes will survive after the mutagenic treatment and in the transduction for ilv^+.

(iii) The amber suppressor (sup^+) must be present in a form such that it is lost at high frequency from strain B. This is necessary so that the lethality of any amber mutation can be easily tested. That is, amber mutants defective in essential genes are recognized as clones in which loss of the suppressor gene prevents growth. Strain B carries sup^+ on a transducing phage (ϕ80 p$supF^+$) which is integrated in the bacterial chromosome along with a second ϕ80 (wild-type) phage. This double lysogen is unstable, and ϕ80 p$supF^+$ is lost at high frequency via homologous recombination between ϕ80 sequences which flank it.

(iv) There must be selection for the loss of the Sup^+ character so that the effect of sup^- on each mutagenized clone can be observed. The selection we used was described by Berman and Beckwith (2) and involves a strain doubly mutant in the *ara* region. This strain carries an *araD* mutation, which makes a strain sensitive to arabinose in the growth medium (4). In addition, it carries an amber mutation in the positive regulatory gene for the arabinose system, *araC*. An *araC*(Am) *araD* strain will ordinarily be resistant to arabinose since the *ara* genes are not expressed. However, in a sup^+ strain, the bacteria will be arabinose sensitive. Thus, selection for growth in the presence of arabinose with an *araC*(Am) *araD* sup^+ strain yields (among others) derivatives which are sup^-. Since the strain we used loses the sup^+ character at high frequency, the vast majority of the arabinose-resistant derivatives of this strain were sup^- and were not due to mutations in the *ara* region.

(v) The presence of the sup^+ allele must be monitored by a color assay on a solid medium. By inclusion in the strain of an amber mutation in the *lacZ* gene, the indicator dye 5-bromo-3-chloro-indolyl-β-D-galactoside (XG) can be used (11). sup^+ bacteria form blue colonies and sup^- bacteria form white or very pale blue colonies on media containing XG.

(vi) The actual detection of lethal amber mutants involved replica plating from the P1 transduction plate onto a medium which selects against sup^+ and on which the presence of sup^+ could be observed by the XG color test.

Details of methods. A P1 lysate was grown on a mutagenized culture of the donor strain

MBGO (F$^-$Δ(*ara-leu*)*1119* Δ*lac-U169* *trp*(Am) *mal*(Am) *supF*$^+$) essentially as described by Hawrot and Kennedy (7). Media used in these experiments are described by Miller (11). This donor strain was chosen because it contained the *supF*$^+$ allele of *tyrT*, allowing viability of most amber mutants defective in *rho* during P1 growth. The strain was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Miller (11). The cells were harvested after the mutagenic treatment, washed with Luria broth (LB) plus 5 mM $CaCl_2$, and resuspended in LB medium containing 5 mM $CaCl_2$ to a density of 5×10^8 cells per ml. The resuspended cultures were next grown for 30 min at 37°C with shaking and then phage P1 *vir* was added at a multiplicity of infection of 2. After adsorption, the cultures were diluted fivefold into LB medium containing 0.2% glucose and 5 mM $CaCl_2$ and shaken at 37°C until lysis occurred. Cell debris was removed by centrifugation after addition of 20 mM $MgSO_4$ and $CHCl_3$.

Portions of recipient strain S27 (F$^-$ *araD* *araC*(Am) *lacZY14*(Am) *metB* *ilv*::Tn*10* *rpsL* (φ80) (φ80 p*supF*$^+$)) were adsorbed with the mutagenized P1 lysates (11), diluted with LB medium containing 50 mM sodium citrate, and grown for 1 h at 37°C with shaking. Reconstruction experiments with a known *rho* amber mutant (*nitA112*, reference 9) had shown this growing-out period to be advantageous for recovering *rho*(Am) transductants. The cultures with adsorbed phage were then harvested by centrifugation and spread on M63 agar medium containing 0.2% lactose, 25 μg of L-methionine per ml, and 10 mM sodium citrate to give about 300 colonies per plate. After 48 h, large colonies had developed. These were then replica-plated onto M63 agar medium containing 0.2% glycerol and 25 μg of L-methionine per ml and onto M63 agar medium containing 0.2% glycerol, 0.5% arabinose, 25 μg of L-methionine per ml, 40 μg of XG per ml, and 1 mM isopropyl-β-D-thiogalactoside to induce the *lac* operon. The replicas were incubated for 24 h at 37°C. Most transductants appeared as large, pale blue colonies when replicated onto the arabinose-containing plate. In fact, these colonies were the result of outgrowth of those frequent derivatives of the colony on the master plate which had lost φ80 *supF*$^+$, thus becoming arabinose resistant and Lac$^-$. Those transductant colonies which contained amber mutants defective in an essential gene (e.g., *rho*) appeared as hazy, dark blue "ghosts" of colonies on such a replica plate. Those derivatives within such a colony which were *sup*$^-$ could not grow because they had lost the expression of an essential gene. The blue color was due to the β-galactosidase present in the original colony which was replica-plated. The blue ghosts either had no growth or had small blue papillae on the arabinose-containing medium owing to mutations in the arabinose region which caused arabinose resistance.

Colonies which appeared to yield blue ghosts on replica plating (122 of about 20,000 transductants) were picked from the minimal glycerol replica plates and purified on M63 agar medium containing 0.2% glucose, 25 μg of L-methionine per ml, 40 μg of XG per ml, and 1 mM isopropyl-β-D-thiogalactoside. Fourty-four of these were false-positives, no longer yielding blue ghosts on arabinose-containing medium. To verify that the purified blue colonies carried amber mutations in genes essential for growth on rich medium, they were further screened by streaking on lactose-tetrazolium agar (11) spread with 2×10^9 plaque-forming units of a λ *c*I857-φ80 hybrid phage (14). This phage is a lambda-φ80 hybrid providing φ80 *int* and *xis* functions under lambda immunity control. Superinfection by this phage stimulates curing of φ80 lysogens. Those survivors cured of φ80 *supF*$^+$ were detected as red (Lac$^-$) colonies on the lactose-tetrazolium medium. Those colonies which were not curable of φ80 *supF*$^+$ on this medium (i.e., did not yield Lac$^-$ derivatives) were considered to carry amber mutations in an essential gene. Since, in addition to amber mutants defective in essential genes, *ilv*(Am) mutants would have been selected by this procedure, the purified blue colonies also were tested on the selective arabinose-containing agar medium with and without 0.4 mM L-isoleucine and 0.6 mM L-valine. We found, by these tests, 25 *ilv* amber mutants and 5 amber mutants defective in genes other than *ilv* which could apparently survive on rich medium in the absence of *sup*$^+$. There remained 43 mutants which failed to be cured of φ80 p*supF*$^+$ on rich medium.

Another gene which is linked by P1 transduction to *ilv* is *glmS* (13). This gene codes for the enzyme glucosamine phosphate isomerase. In its absence, *E. coli* requires glucosamine, even on rich media. Thus, we anticipated that some of our *ilv*-linked amber mutations in essential genes could be in the *glmS* gene. This turns out to be the case, as three of these amber mutants required glucosamine on YT medium (11).

The 40 remaining mutants that failed to lose the suppressor on rich medium were screened to determine whether they had amber mutations in or very close to the *rho* gene by complementation with a *rho*$^+$-carrying plasmid, pSB7. pSB7 is a pBR322 derivative containing *rho*, a tetracycline resistance gene, and the cohesive ends of

bacteriophage λ (λ *cos*). Plasmids containing λ *cos* are transducible by lambda (5). This fact allowed us to move the plasmid into our strains and thus to screen many candidates easily. Three of the mutants which failed to lose the suppressor on rich medium were complimented by the *rho*$^+$ plasmid for loss of φ80 p*supF*$^+$ on M63 agar medium containing 0.2% glycerol, 0.5% arabinose, 0.1% Casamino Acids, 25 μg of L-methionine per ml, 10 μg of tetracycline per ml, 40 μg of XG per ml, and 1 mM isopropyl-β-D-thiogalactoside. That is, in the presence of the *rho*$^+$ plasmid, these mutants no longer formed blue ghosts.

To determine whether these three amber mutations were in *rho*, we examined the Rho phenotype in the presence of the ochre suppressor *supC*$^+$, which only weakly suppresses amber mutations. We reasoned that low-level suppression of a *rho* amber mutation might allow growth but give a Rho$^-$ phenotype. The Rho$^-$ phenotype was assayed by measuring termination at the transcription terminator of the *trp* operon (*trp-t*) (6). All three amber mutations which were closely linked to or in the *rho* gene and the previously isolated *rho* amber mutation, *nitA112*, were found to relieve termination at *trp-t* in the presence of *supC*$^+$. Therefore, we feel that it is highly likely that these mutations are in the *rho* gene.

We have developed a new approach for isolating amber mutants defective in essential genes. Using this technique we have isolated 43 amber mutants defective in essential genes in the *ilv* region of the *E. coli* chromosome, including 3 in the *rho* gene and 3 in the *glmS* gene. Our collection should include other interesting amber mutants defective in essential genes in this region. The technique should be applicable to any region of the bacterial chromosome.

We have used the same approach to isolate ochre mutants defective in this region (E. R. Brickman, S. Brown, and J. Beckwith, unpublished data). Finally, an extension of this technique has allowed us to isolate insertions of transposons in the *rho* gene. This was done by screening for the inability of a plasmid containing the *rho* gene to complement a *rho* amber mutation after random insertion of Tn*5* in the plasmid (S. Brown and J. Beckwith, unpublished data).

The described method differs in several important ways from existing methods for the isolation of amber mutants defective in essential genes. Our method allows the harboring of the nonsense allele in the haploid state, facilitating mapping. (For instance, *rho*$^+$ recombinants can be selected, since they will be resistant to arabinose even when φ80 p*supF*$^+$ is lost.) Our method allows the isolation of the desired mutants in the presence of an efficient suppressor, placing different constraints on the survivors than screening procedures using the poorly suppressing, temperature-sensitive allele of *tyrT*, *supF6* (9, 10). In fact, Delcluve et al. (3) found that amber mutants which they had isolated in the presence of the *supF*$^+$ allele of *tyrT* (using a strategy similar to ours) were inviable in a strain containing the poorly suppressing, temperature-sensitive allele of *tyrT*, *supFA81*. Our method allows a greater difference in the degree of suppression between *sup*$^+$ and *sup*$^-$ states than that attained with temperature-sensitive suppressors. Finally, our method allows the identification of amber mutants by replica plating, permitting the rapid screening of many colonies.

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