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Method for the Isolation of *Escherichia coli* Relaxed Mutants, Utilizing Near-Ultraviolet Irradiation

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Near-ultraviolet radiation (300 to 380 nm) induces a transient growth inhibition in "stringent" (*rel*$^+$) strains of *Escherichia coli*, whereas "relaxed" (*rel*$^-$) strains are only mildly affected. This difference permits rapid isolation of large numbers of relaxed mutants of *E. coli*.

During amino acid starvation, "stringent" (*rel*$^+$) strains of *Escherichia coli* curtail the accumulation of stable ribonucleic acid (RNA), whereas "relaxed" (*rel*$^-$) mutants permit RNA accumulation (8). Near-ultraviolet (near UV, 300 to 380 nm) irradiation simulates amino acid starvation in *E. coli* by inactivating transfer RNA species that contain 4-thiouridine (5–7). Thus, growth and RNA accumulation in *rel*$^+$ strains is temporarily inhibited by near UV, whereas RNA accumulation continues in *rel*$^-$ strains (which presumably replace the damaged transfer RNAs at a much higher rate than in *rel*$^+$ strains). Consequently, near-UV-irradiated *rel*$^-$ strains show no cessation of growth, but only a mild and temporary decrease in growth rate (6). The use of this difference in response of *rel*$^+$ and *rel*$^-$ strains to near UV in the isolation of relaxed mutants of *E. coli* is described.

(This research was performed in partial fulfillment of the requirements of the University of Texas at Dallas for the degree of Doctor of Philosophy.)

Technique. Cells were grown in M9 minimal medium (2) at 30°C. Growth was monitored by measuring the optical density in a 1-cm-light-path cuvette at 460 nm ($OD_{1.0}^{460}$) in a Zeiss PMQ II spectrophotometer. Two General Electric BLB 15T8 black-light lamps were the source of near UV. The irradiation procedure has been described (5, 6). The fluence rate was 35 ± 3 W/m^2 (6-cm source-to-sample distance).

Figure 1 shows a reconstruction experiment demonstrating the efficiency of near UV in enriching for *rel*$^-$ mutants. Stationary-phase cultures of strains B/r ATCC 12407 *lac*$^+$ *rel*$^+$ and B/r NC52 *lac*$^-$ *rel*$^-$ *valS*^{ts} were mixed, yielding different *rel*$^-$/*rel*$^+$ ratios. Each mixture was diluted 1:200 in M9 minimal medium and grown to an $OD_{1.0}^{460}$ of 0.3, and 20 ml was irradiated with near UV for 15 min (fluence 30 kJ/m^2). The irradiated cultures were diluted 1:9 in M9 and grown to stationary phase. On the next day, portions of dilutions were plated in triplicate on McConkey agar plates (4), the number of *lac*$^-$ (white) and *lac*$^+$ (red) colonies being determined after incubation at 30°C for 18 to 24 h. Each culture was again diluted 1:200, and the cycle of growth, irradiation, dilution, and overnight growth was repeated.

Since the *rel*$^+$ strain was *lac*$^+$, and the *rel*$^-$ strain was *lac*$^-$, the frequency of *rel*$^-$ cells in the mixed population was estimated by assaying for the fraction of *lac*$^-$ cells in the cultures. Testing of a number of *E. coli* strains with *lac* and *rel* alleles in different combinations showed that the state of the *lac* gene does not influence susceptibility to growth inhibition by near UV. Hence, any increase in the frequency of *lac*$^-$ cells induced by near UV in these mixed cultures results from its association with the *rel*$^-$ gene and reflects an increase in the frequency of *rel*$^-$ cells, provided near UV does not mutate the *lac*$^+$ cells to *lac*$^-$. Irradiation of the *lac*$^+$ strain alone showed that, even with seven cycles of near-UV irradiation, the frequency of *lac*$^-$ colonies induced was extremely low (~5 × 10^{-4}).

Figure 1 shows the increase in the proportion of *lac*$^-$ colonies with increasing number of near-UV enrichment cycles. Since only 300 to 600 total colonies were scored on a plate, the lower limit of detection of *lac*$^-$ colonies in the *lac*$^+$ background is approximately 2 × 10^{-3}. Hence, when input ratios are lower than this value (curves C and D), the increase in *lac*$^-$ cells is not detected for one to two enrichment cycles (broken lines). The initial proportion of *lac*$^-$ cells in each mixture was calculated from the titer of cultures used in the mixture.

Curve A shows that the proportion of *lac*$^-$(*rel*$^-$) cells in the culture remains constant

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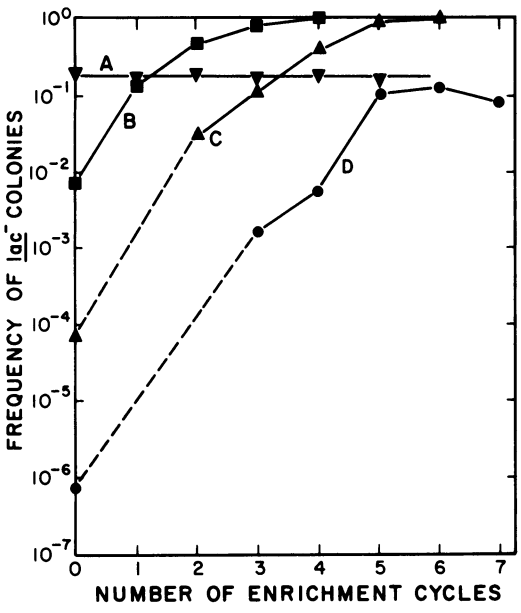

FIG. 1. *Increase in the frequency of lac⁻, rel⁻ cells after cycles of treatment with 30 kJ of broad-band near-UV radiation per m² in mixed cultures containing E. coli B/r NC52 lac⁻ rel⁻ valS^ts and E. coli B/r ATCC 12407 lac⁺ rel⁺. The strains were mixed in various proportions to yield A, 2 × 10⁻¹; B, 7 × 10⁻³; C, 7 × 10⁻⁵; and D, 7 × 10⁻⁷ frequencies of lac⁻ rel⁻ cells. Cultures B, C, and D were enriched by near-UV treatment. Culture A went through all steps of the procedure (see text) except near-UV irradiation.*

in the absence of near-UV irradiation, indicating that the enrichment seen in curves B, C, and D is not due to factors such as differences in growth rate or stationary-phase titers of the two strains. In curve B a complete takeover of the culture by the *lac*⁻ cells is evident. In curves C and D, the frequency of *lac*⁻ cells reaches saturation values of 0.9 and 0.1, respectively, after five cycles of enrichment treatment.

This selection process will enrich all mutants that respond like *rel*⁻ strains to near-UV radiation, i.e., by not showing growth inhibition. The *lac*⁺ *rel*⁺ strain alone subjected to seven cycles of near-UV enrichment showed a growth response after near-UV irradiation characteristic of *rel*⁻ strains (6), whereas all the cells retained the *lac*⁺ character, indicating that *rel*⁻-like mutants are enriched by near UV. Thus, in mixed cultures, after several cycles of enrichment, most of the *lac*⁺ cells would be *rel*⁻-like, and further cycles would no longer result in a change in the frequency of *lac*⁻ cells; therefore, a saturation *lac*⁻ frequency would be reached.

Use of the technique. As a test for the isolation of new *rel*⁻ mutants, four identical (unmutagenized) cultures of *E. coli* B/r NC32 *lac*⁻ *rel*⁺ *valS*^ts were subjected to cycles of near-UV enrichment involving 40 kJ of black-light radiation per m² (20 min) in each cycle. The $OD^{460}_{1.0}$ of the irradiated cultures was followed for 120 to 200 min after each irradiation. With increasing cycles of enrichment, the growth pattern of the irradiated cultures changed progressively from that of *rel*⁺ strains to that of *rel*⁻ strains (6), no further change being seen after seven cycles. At this stage, the incorporation of [¹⁴C]uridine by these cultures subjected to amino acid starvation is similar in magnitude to that of *E. coli* B/r NC52 *rel*⁻ (6), indicating that a large fraction of the cells in the enriched cultures was *rel*⁻.

After seven cycles of enrichment, single colonies were isolated and tested for parental *lac*⁻ and *valS*^ts markers and then tested for stringency. Mass screening for the *rel*⁻ genotype was done by a modification of the autoradiographic technique of Martin (3), as described in the legend to Fig. 2. Amino acid starvation was induced by incubating the cells at 42°C, inactivating the valyl synthetase. Four single-colony isolates from each of the four enriched cultures were screened, and a spot of the parent strain NC32 was also included for comparison (Fig. 2). Barring two spots in the first column (NC32 and C1), all the spots show darker images, indicating higher incorporation of [¹⁴C]uridine during amino acid starvation, a behavior characteristic of relaxed mutants.

Although the above assay permits rapid screening, its success depends on having nearly the same number of cells at each spot at the time of amino acid starvation, so that the incorporation of label is a function of RNA accumulation alone. Differences in spotting titers, variable lag before resumption of growth, or differences in growth rate could lead to misidentification. Hence, all the cultures in Fig. 2 were tested for incorporation of [³H]uridine during amino acid starvation: all except the parent NC32 and spot C1 behaved as *rel*⁻. Thus, 15 out of the 16 colonies isolated from the near-UV-enriched cultures were *rel*⁻ mutants. The remaining one was a mutant lacking 4-thiouridine and will be described elsewhere.

Since the cultures were not mutagenized before the enrichment procedure, the mutants enriched may have arisen spontaneously or been induced by near UV. Black light is mutagenic for *E. coli* and induces mutations to phage T5 resistance at a frequency of 1.3 ×

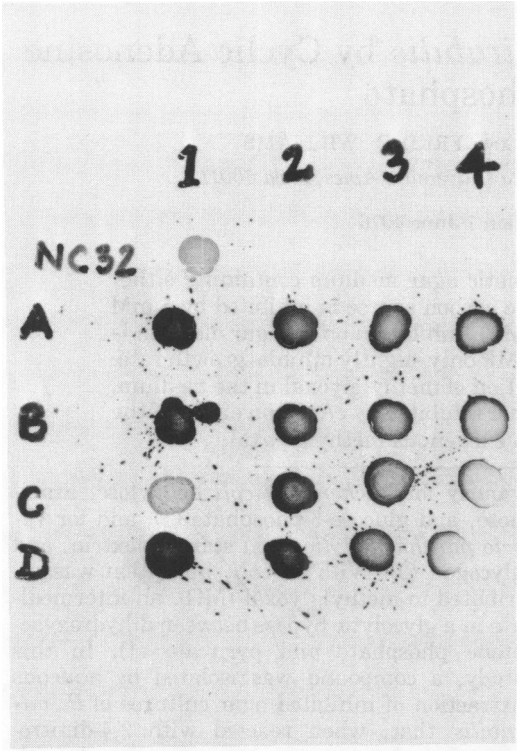

FIG. 2. *Autoradiogram ([^{14}C]uridine) of the membrane filter used in screening of rel$^-$ mutants. A, B, C, D represent four different enriched cultures. 1, 2, 3, 4 represent single-colony isolates from each of these four cultures. Stationary-phase cultures derived from single-colony isolates from enriched cultures were diluted 1:100 in M9 medium and 2 μl of each was spotted onto a 47-mm membrane filter (Millipore Corp., 0.45 μm). The filter was (i) incubated on M9 minimal agar for 14 to 16 h at 30°C to permit growth of bacteria, (ii) transferred to a prewarmed M9 minimal agar plate and incubated at 42°C for 1 h, (iii) transferred to a prewarmed M9 minimal plate containing [^{14}C]uridine (0.5 μCi/ml, 55 Ci/mol in 5 ml of medium) and incubated for 4 h at 42°C, (iv) incubated for 1 h at 42°C on an M9 minimal plate containing 10 μM nonradioactive uridine. Subsequently the filter was dried, mounted, and autoradiographed for 10 to 12 h with Kodak RP54/R54 medical X-ray film.*

10^{-12} mutants/cell per J/m^2 (9). However, since neither the spontaneous nor the near-UV-induced mutation rates at the *rel* locus are known, the origin of the mutants isolated by this technique is uncertain.

The only other selection technique reported for isolation of *rel*$^-$ mutants (1) involves a mutagenesis step and many cycles of a procedure involving amino acid starvation, penicillin treatment, etc. The method reported here is easier. Separation of the enrichment treatments by overnight growth of the cultures was done merely for convenience. It should be possible to achieve a faster selection using two to three enrichment cycles per day.

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