Method for Isolating Restriction- and Modificationless Mutants of *Escherichia coli* K-12

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Received for publication 11 July 1978

A simple method is described for the selection and isolation of restriction- and modificationless mutants in *Escherichia coli* K-12 by using the following properties: (i) the temperature-sensitive repressor activity of phage λc1857; (ii) a mutant of λ phage defective in integration and the establishment of repression (λb2C1); (iii) a virulent λ phage insensitive to the repressor activity. The final yield of spontaneously arising rK⁻mk⁻ and rK⁻mK⁻ mutants from stationary-phase cultures was about 5% of the surviving cells.

DNA from bacteriophages, bacteria, and episomes, when transferred between two different strains of *Escherichia coli*, usually is subject to heterospecific host-controlled restriction, leading to degradation of incoming DNA by the recipient cell (6, 11). A small fraction (10⁻⁴ to 10⁻⁵) of bacteria receiving foreign DNA (23) fail to carry out the restriction process because the incoming DNA is modified to the homospecific type and therefore is no longer subject to heterospecific restrictive degradation. Work done in a number of laboratories (2-4, 7) has provided a reasonably comprehensive idea of the genetic and biochemical mechanism of DNA restriction and modification. However, some important questions still remain unanswered, and, in particular, very little is known about the regulation of restriction and modification activities (3).

Availability of large numbers of mutants defective in these systems will permit a fuller investigation of their mechanism and regulation. Furthermore, *E. coli* mutants with defective restriction and modification abilities would very likely be of particular value in the study of phage- or plasmid-mediated cloning of eucaryotic and procaryotic DNAs. The choice in this work of an *E. coli* strain producing minicells reflects this point of view. This system recently has been shown to be a powerful means for detecting gene products of plasmid and viral DNA (15, 18-21, 24). The isolation of rK⁻mk⁻ or rK⁻mK⁻ from this or from other strains (especially those with high CaCl₂-mediated transformation susceptibility) would be of considerable value.

Published methods for selecting restriction- and modification-negative mutants are based on the recovery of transductants, recombinants, or lysogens from a restricting host (8, 10, 13, 14, 16, 17, 23). However, all of these procedures, although convenient, are laborious and do not yield restriction- and modificationless mutants at high frequency. The presence in a bacterial population of *E. coli* K-12 of nonrestricting mutants at a level of about 10⁻⁵ (23) has stimulated the development of selective methods for isolating large numbers of these spontaneous mutants. Techniques described in this note utilize mutant enrichment and do not involve the use of any mutagenic agent, although they do not exclude it.

Spontaneous restriction and modification mutants of strain D22 were isolated by the following procedure. A tryptone broth (Tr broth; see Table 1, footnote a) overnight culture of bacteria (10 ml) was added to 20 ml of fresh Tr broth. After aeration for about 3 to 4 h at 37°C, the bacteria were starved in 10 mM MgSO₄ and infected with λc1857.0 (multiplicity of infection, 0.5) (see Table 1, footnote a).

The mixture was then placed in an ice bath for 2 min, followed by incubation at 30°C for 30 min with a low level of aeration. Bacteria were plated (final concentration, about 10⁶) on Tr agar plates seeded with λb2c1-K (multiplicity of infection, 100) and incubated at 30°C overnight.

Single colonies of surviving bacteria were streaked on Tr agar plates and screened for their restriction and modification phenotypes.

The basic idea for selecting nonrestricting (rK⁻) or nonrestricting and nonmodifying (rK⁻mK⁻) mutants is based on the following assumptions. When a population of restriction-competent λ-sensitive cells of *E. coli* is infected with heterospecific λc1857 (conditional temperature-sensitive phage at 37°C), most of the cells rapidly degrade the infecting DNA (11, 22). However, nonrestricting mutants present in the population, being unable to degrade the incoming DNA, are expected to inherit the phage DNA and to form plaques when infected with λb2c1-K.

DNA from the resulting phage lysates (see Table 1, footnote a) was extracted by the method of Maniatis et al. (12) and used to transform *E. coli* D22 as described in Table 1 (note b). This procedure should enrich for restriction-negative and modificationless mutants.
DNA, leave the lytic pathway open. If the infection is carried out at a nonpermissive temperature (at which the λ repressor is inactive in λcI857), this culminates in the production of new virus particles and cell lysis in such mutants (12). On the other hand, when the temperature is lowered to 30°C soon after the adsorption period, repression is rapidly restored (due to the conditional phenotype of the cl857 mutation of λ phage [8]), blocking the lytic cycle. The lysogenic pathway is established by the integration of λ DNA into the host DNA, and, consequently, the rK− cells survive. Low multiplicity of infection of λcI857 facilitates the lysogenization (12). Thus, a population of E. coli cells, upon infection with λcI857 at nonpermissive temperatures and growth at permissive temperatures, should be composed of the following classes of survivors: (1) noninfected bacteria sensitive to homospecific phage particles (the major class); (2) bacteria (rK+ mK+) lysogenic for λcI857 prophage; (3) bacteria (rK− mK−) lysogenic for λcI857 prophage; (4) bacteria (rK− mK−) lysogenic for λcI857 prophage; (5) λ-resistant bacteria.

A considerable enrichment of restriction- and modificationless mutants can be obtained by a second step of selection; the bacterial culture is infected with λD22C (restriction- and modification-homospecific phage) at 30°C. This λ mutant does not integrate into the host chromosome and is unable to stabilize its own repression activity. Its lytic cycle, however, is blocked by the presence of repressor from λcI857. For this reason, the vast majority of nonlysogenic bacteria will be killed, whereas lysogenic bacteria survive. They belong to classes 2, 3, and 4 mentioned above. By curing techniques, it should be rather easy to overcome a minor drawback of this method, namely, the lysogenic state of the mutants obtained.

The rationale for this selective technique was verified by experimental data. The control parental strain D22 rK+mK+ (Fig. 1) is not lysogenic (does not lyse C-indicator) and is restrictive for heterospecific λvir. On a background of C-indicator (Fig. 1A), all streaks, with the exception of those belonging to the control parental strain D22 rK+mK+, are lysed. Thus, all bacteria surviving both steps of selection are lysogenic for λcI857 prophage. On K-indicator (Fig. 1B), all streaks, with the exception of type 4 colonies (rK− mK−) and the control strain, are lysed. From Fig. 1B and 1C it is possible to distinguish between rK− mK− and rK− mK+ mutants among the survivors; the streaks of type 4 are composed of rK− mK+ cells. They produce λ phage restricted by the K-indicator (absence of lysis on plate of Fig. 1B) and are lysed by λvirC (plate of Fig. 1C). Streaks of type 3 harbor rK+ mK− cells producing λ phage nonrestricted by K-indicator (presence of lysis on plate of Fig. 1B). They are lysed by λvirC (plate of Fig. 1C).

When the technique of two consecutive steps of selection described above was used, the yield of rK− mK− and rK− mK+ mutants was about 5%, with a predominance of the rK− mK− type. To verify the applicability of this technique to the isolation of restrictionless and restriction- and modificationless mutants from other E. coli strains, we recently isolated by this method rK−
mK\(^+\) and rK\(^-\)mK\(^-\) mutants from YMC recA56 and KS805, with final yields of 4 and 5%, respectively. Also in these two strains the rK\(^-\)mK\(^-\) class of mutants predominated.

Finally, the pattern of restriction and modification of the mutants was qualitatively and quantitatively assayed. To test for restriction ability, each mutant strain was used as an indicator to determine the efficiency of plating of \(\lambda\)vir with C, B, or K modification. C, B, and K are used as indicator controls. Table 2 shows that the loss of restriction activity of the D22 mutants is similar for all of the heterospecific \(\lambda\)vir utilized. In fact, the efficiency of plating (EOP) of each heterospecific \(\lambda\)vir on each mutant is comparable with their EOP on C-indicator. On the other hand, each heterospecific \(\lambda\)vir is restricted by restrictive indicators, their EOP being around \(10^{-4}\) to \(10^{-5}\).

To test for modification ability, stocks of \(\lambda\)Cl857 were prepared from each isolated mutant by high-temperature induction. The \(\lambda\)Cl857 phage stocks were assayed for EOP on the indicators C, B, and K. Table 3 shows that the \(\lambda\)Cl857 deriving from induction of the lysogenic mutant D22 rK\(^-\)mK\(^-\) (4) is not modified, the EOP on the restrictive indicator being about \(10^{-4}\) as compared with the EOP on C-indicator. In contrast, the \(\lambda\)Cl857 deriving from induction of the lysogenic mutant D22 rK\(^-\)mK\(^+\) (3) is modified, the EOP on K- and C-indicators thus being comparable, while the EOP on B is \(8 \times 10^{-4}\).

The technique described in this note, when compared with the method of Hubacek and Glover (16), offers the following advantages: (i)
Table 3. Plating efficiency on host strains

<table>
<thead>
<tr>
<th>Phage</th>
<th>Plating efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K</td>
</tr>
<tr>
<td>λ·D22 rK- mK+ type 3</td>
<td>1.0</td>
</tr>
<tr>
<td>λ·D22 rK- mK+ type 4</td>
<td>2 × 10^{-4}</td>
</tr>
</tbody>
</table>

*Modification test. Plating efficiency of λcI857 synthesized in strain D22 rK- mK+ (3) or in strain D22 rK- mK+ (4) on K-, B-, and C-indicators. Stocks of λcI857 derived from thermal induction of the lysogenic mutants of type 3 or 4 were assayed on the indicator hosts.

The low frequency of mutants resistant to λ phage among surviving bacteria renders it unnecessary to use a phage which binds to a second receptor (hybrid λcIb80 phage); (ii) an additional step of selection to enrich rK- mutants by conjugation with an F' lac+ donor strain is no longer included; (iii) the technique described allows a simple and rapid isolation of rK- mK+ and rK- mK- mutants with a high yield; (iv) the lysogens are easily cured of prophage, since they carry the λcI857 phage which is temperature sensitive.

I thank A. Campbell for kindly providing the λ phages. This work was carried out under a long-term EURATOM fellowship.

Literature Cited