JOURNAL OF BACTERIOLOGY, Jan. 1979, p. 673-676
0021-9193/79/01-0673/04$02.00/0

Vol. 137, No. 1

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 λ*c*I857; (ii) a mutant of λ phage defective in integration and the establishment of repression (λ*b*$_2$*c*I); (iii) a virulent λ phage insensitive to the repressor activity. The final yield of spontaneously arising r_K^- m_K^+ and r_K^- m_K^- 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^{-3}) of bacteria receiving foreign DNA (23) fails 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 r_K^- m_K^+ or r_K^- m_K^- from this or from other strains (especially those with high $CaCl_2$-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 (9, 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 $MgSO_4$ and infected with λ*c*I857.0 (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 10^8) on Tr agar plates seeded with λ*b*$_2$*c*I·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 (r_K^-) or nonrestricting and nonmodifying (r_K^- m_K^-) mutants is based on the following assumptions. When a population of restriction-competent λ-sensitive cells of *E. coli* is infected with heterospecific λ*c*I857 (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

TABLE 1. *Bacterial strains*[a]

E. coli strain	Genetic character	Source
C600	$r_K^+ m_K^{+,b}$ *thr leu thi lac* $SupE^+$ (K-indicator)	Lynn Enquist
YMC *recA56*	$r_K^+ m_K^+ SupF^+$	Lynn Enquist
C	Prototroph, nonrestricting (C-indicator)	Reference 5 via P. H. Hofschneider
B	$r_K^+ m_K^{+,b}$ *his* F^+, λ^s (B-indicator)	*E. coli* B 2025 Arber collection via P. H. Hofschneider
D16[c]	$r_K^- m_K^{-,b}$ *thr leu thi lac* (λcI857)	My collection
D22[d]	$r_K^+ m_K^{+,b}$ *thr leu*, λ^s F^- "minicells"	My collection
KS805	(*cyl*) *trpR trpA9761 trpE9828*	Yanofski collection via N. Angerbauer

[a] Cells were grown at 37°C in Tr broth. Tr broth contains the following: tryptone (Difco), 10 g/liter; NaCl, 5 g/liter; supplemented with 1 μg of thiamine (B1) per ml. For cell plating, Tr broth was supplemented with 20, 12, and 7.5 g of agar (Difco) per liter, respectively, for Tr agar plates, bottom agar, and top agar.

[b] The following abbreviations defined by Arber and Linn (4) are used in this note: $r_K^+ m_K^+$ and $r_B^+ m_B^+$ designate the wild-type restriction and modification phenotypes of *E. coli* K-12 and B, respectively; $r_K^- m_K^+$ and $r_K^- m_K^-$ designate the phenotypes of restriction and restriction and modification *E. coli* K-12 mutants, respectively; λK, λB, or λC indicate bacteriophages carrying K-specific, B-specific, or C-specific modification, respectively; λcI857.0 designates bacteriophage derived from high-temperature induction of D16 $r_K^- m_K^-$ (λcI857).

[c] D16 was derived from C600 $r_K^- m_K^-$ (a gift of G. Hobom) and was lysogenized with λcI857.

[d] D22 is a spontaneous *mal*$^+$ λ^s revertant, isolated from K-12 strain P678-54 (1) (a gift of R. Curtiss).

DNA, leave the lytic pathway open. If the infection is carried out at a nonpermissive temperature (at which the λ repressor is inactive in λ*c*I857), 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 *c*I857 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 r_K^- cells survive. Low multiplicity of infection of λ*c*I857 facilitates the lysogenization (12). Thus, a population of *E. coli* cells, upon infection with λ*c*I857 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 ($r_K^+ m_K^+$) lysogenic for λ*c*I857 prophage; (3) bacteria ($r_K^- m_K^+$) lysogenic for λ*c*I857 prophage; (4) bacteria ($r_K^- m_K^-$) lysogenic for λ*c*I857 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 λb_2cI (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 $r_K^+ m_K^+$ (Fig. 1) is not lysogenic (does not lyse C-indicator) and is restrictive for heterospecific λ*vir*. On a background of C-indicator (Fig. 1A), all streaks, with the exception of those belonging to the control parental strain D22 $r_K^+ m_K^+$, are lysed. Thus, all bacteria surviving both steps of selection are lysogenic for λ*c*I857 prophage. On K-indicator (Fig 1B), all streaks, with the exception of type 4 colonies ($r_K^- m_K^-$) and the control strain, are lysed. From Fig. 1B and 1C it is possible to distinguish between $r_K^- m_K^-$ and $r_K^- m_K^+$ mutants among the survivors; the streaks of type 4 are composed of $r_K^- m_K^-$ cells. They produce λ phage restricted by K-indicator (absence of lysis on plate of Fig. 1B) and are lysed by λ*virC* (plate of Fig. 1C). Streaks of type 3 harbor $r_K^- m_K^+$ cells producing λ phage nonrestricted by K-indicator (presence of lysis on plate of Fig. 1B). They are lysed by λ*virC* (plate of Fig. 1C).

When the technique of two consecutive steps of selection described above was used, the yield of $r_K^- m_K^-$ and $r_K^- m_K^+$ mutants was about 5%, with a predominance of the $r_K^- m_K^-$ 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 r_K^-

m_K^+ and r_K^- m_K^- mutants from YMC *recA56* and KS805, with final yields of 4 and 5%, respectively. Also in these two strains the r_K^- m_K^- 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 λ*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 λ*vir* utilized. In fact, the efficiency of plating (EOP) of each heterospecific λ*vir* on each mutant is comparable with their EOP on C-indicator. On the other hand, each heterospecific λ*vir* is restricted by restrictive indicators, their EOP being around 10^{-4} to 10^{-5}.

To test for modification ability, stocks of λcI857 were prepared from each isolated mutant by high-temperature induction. The λcI857 phage stocks were assayed for EOP on the indicators C, B, and K. Table 3 shows that the λcI857 deriving from induction of the lysogenic mutant D22 r_K^- m_K^- (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 λcI857 deriving from induction of the lysogenic mutant D22 r_K^- m_K^+ (3) is modified, the EOP on K- and C-indicators thus being comparable, while the EOP on B is 8×10^{-4}.

The technique described in this note, when compared with the method of Hubacek and Glover (16), offers the following advantages: (i)

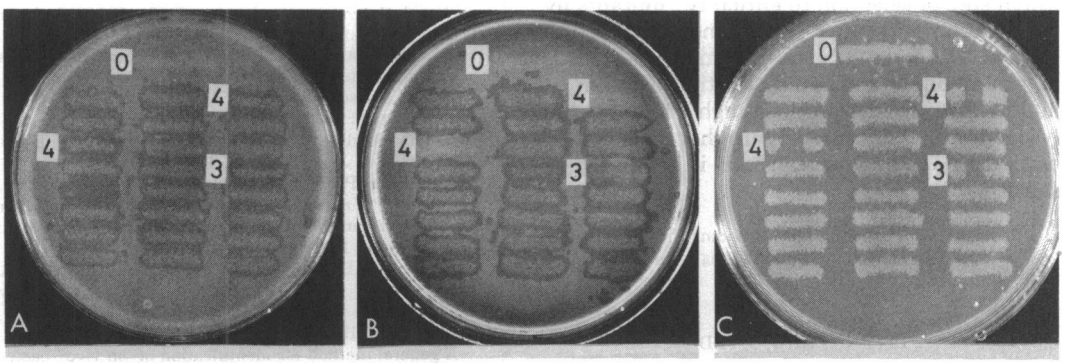

FIG. 1. *Detection and differentiation of r_K^- m_K^+ and r_K^- m_K^- mutants of E. coli K-12 strain D22. Streaks on a mother plate from colonies grown from cells which had undergone the two successive steps of selection described in the text were replica plated on lawns of two E. coli indicator strains or streaks of a λ phage suspension, respectively. (A) Test for lysogeny: replica plating on Tr agar plates containing 10^8 starved cells of E. coli C (C-indicator) and successive incubation for 2 h at 42°C and then at 37°C overnight. The presence of lysis proves all mutants, in contrast to the control parental strain D22 r_K^+ m_K^+ (marked 0), to be lysogenic for λcI857. (B) Modification test: replica plating on Tr agar plates containing 10^8 starved cells of E. coli strain C600 (K-indicator) and successive overnight incubation at 37°C. The absence of lysis proves streaks of type 4 to be composed of cells of a nonmodifying (m_K^-) mutant producing phages restricted on K-indicator. (C) Restriction test: 10^7 particles of phage λvirC, per ml were streaked across a Tr agar plate, allowed to dry, and replica plated with the bacterial streaks of the mother plate. Successive incubation was at 30°C and overnight. Lysis of streaks 3 and 4 proves them to be composed of nonrestricting (r_K^-) mutants. Thus, cells of streak 3 are r_K^- m_K^+, and those of streaks marked 4 are r_K^- m_K^-.*

TABLE 2. *Plating efficiency on host strains*[a]

Phage	Plating efficiency				
	Type 3 r_K^- m_K^+ derived from strain D22	Type 4 r_K^- m_K^- derived from strain D22	K	B	C
λ·K	1.0	1.0	1.0	5×10^{-5}	1.0
λ·B	1.0	1.0	2×10^{-4}	1.0	1.0
λ·C	1.0	1.0	3×10^{-4}	4×10^{-5}	1.0

[a] Restriction test. Plating efficiency of λ*vir* with C, B, or K modification on restrictionless host strains of types 3 and 4 of Fig. 1 and on K-, B-, and C-indicators. Stocks of λ*vir* with C, B, or K modification were assayed on the various hosts. The temperature of incubation was 30°C.

TABLE 3. *Plating efficiency on host strains*[a]

Phage	Plating efficiency		
	K	B	C
λ·D22 r_K^- m_K^+ type 3	1.0	8×10^{-4}	1.0
λ·D22 r_K^- m_K^- type 4	2×10^{-4}	1×10^{-4}	1.0

[a] Modification test. Plating efficiency of λ*c*I857 synthesized in strain D22 r_K^- m_K^+ (3) or in strain D22 r_K^- m_K^- (4) on K-, B-, and C-indicators. Stocks of λ*c*I857 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 λ*c*I:h80 phage); (ii) an additional step of selection to enrich r_K^- mutants by conjugation with an F′ lac^+ donor strain is no longer included; (iii) the technique described allows a simple and rapid isolation of r_K^- m_K^+ and r_K^- m_K^- mutants with a high yield; (iv) the lysogens are easily cured of prophage, since they carry the λ*c*I857 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.

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