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Mutagenesis in the *lacI* gene target of *E. coli*: improved analysis for *lacI^d* and *lacO* mutants

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Abstract

The *lacI* gene of *E. coli* has been a highly useful target for studies of mutagenesis, particularly for analysis of the specificity (spectrum) of mutations generated under a variety of conditions and in various genetic backgrounds. The gene encodes the repressor of the *lac* operon, and *lacI*-defective mutants displaying constitutive expression of the operon are readily selected. DNA sequencing of the *lacI* mutants has often been confined to the N-terminal region of the protein, as it presents a conveniently short target with a high density of detectably mutable sites. Mutants in this region are easily selected due to their dominance in a genetic complementation test (*lacI^d* mutants). A potential complication in these studies is that constitutive expression of *lac* may also arise due to mutations in the *lac* operator (*lacO* mutants). Under some conditions, for example when analyzing spontaneous mutations, *lacO* mutants can comprise a very high fraction of the constitutive mutants due to a strong base-substitution hotspot in the *lac* operator. Such mutational hot spots diminish the return of the sequencing effort and do not yield significant new information. For this reason, a procedure to eliminate the *lacO* mutants prior to DNA sequencing is desirable. Here, we report a simple method that allows screening out of *lacO* mutants. This method is based on the lack of resistance of *lacO* mutants to kanamycin under conditions when the *kan* gene is expressed from a plasmid under control of the *lac* promoter-operator (*lacPO*). We show data validating the new approach with sets of known *lacI^d* and *lacO* mutants, and further apply it to the generation of a new collection of spontaneous mutations, where *lacO* mutants have historically been a significant contributor.

Keywords

Mutagenesis; *lacI* spectra; *lacO*; *lacI^d* mutants

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Conflict of interest statements

The authors declare that there are no conflicts of interest.

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1. Introduction

Studies investigating the precise mechanisms by which mutations occur have greatly benefited from studies of the specificity of mutation. In this approach large collections of mutants are sequenced to determine the precise nature of the mutations as well as the DNA sequence context in which they occur. Central to this approach is the realization that different mechanisms produce different types of mutation; hence, the mutational specificity serves as a fingerprint (or spectrum) for the underlying process.

One target that has been used productively in the bacterium *Escherichia coli* is the *lacI* gene, which encodes the repressor of the *lac* operon. Mutants in this gene may disable the repressor function, leading to constitutive expression of the *lacZ* (β -galactosidase), *lacY* (permease), and *lacA* (galactoside-acetyltransferase) genes that comprise the operon [1]. Such constitutive mutants can be conveniently selected on medium containing P-gal (phenyl- β -D-galactoside) as the sole carbon source. This sugar is a substrate for the β -galactosidase and permits growth, but is not an inducer for the operon. So, wild-type (*lacI*⁺) bacteria will not grow, whereas constitutive (*lacI*⁻) bacteria will form colonies. A large number of DNA sequencing studies on the *lacI* target have been conducted [2–8]. The resulting mutational spectra have provided information about the mutational processes that caused the mutations. In addition, these studies have provided important information about the nature and scope of the detectably mutable sites in the target. In addition to *E. coli*, the *lacI* gene has also been used as a mutational target for studies of mutagenesis in transgenic rodents [9].

A refinement of the system as used in *E. coli* has been a focus on mutations occurring in the early, N-terminal region of the protein, roughly amino-acid residues 1–70, which encode the DNA-binding region of the repressor [10, 11]. This region is highly sensitive to mutations and contains a high density of detectably mutable sites. Focus on this sub-target is further facilitated by the fact that mutations therein are genetically dominant over the wild-type *lacI*⁺ in a complementation test [10, 11]. This is due to the functioning of *lac* repressor as a tetramer [1], with the oligomerization site being located in the C-terminal region of the protein. Thus, the presence of both wild-type and DNA-binding-deficient repressors in the same cell leads to mixed wt/mutant tetramers that are defective, causing the dominant phenotype. These *lacI* mutants are generally referred to as *lacI*^d mutants. They have been observed at a total of 77 detectable base-substitution sites in the N-terminal region of the *lacI* gene, comprising a total 127 different base substitution pathways [3–7] (R.M.S., unpublished data). In addition, the *lacI*^d target allows detection of –1 frameshifts, and many larger deletion and duplication mutations [2–8].

The test for constitutive *lac* mutants will, in addition to defective repressor mutants, also select for *lac* operator mutants (*lacO*). These reside in the *lac* promoter-operator (*lacPO*) and result in a fully-proficient repressor that is unable to bind its intended target due to an altered operator sequence [11]. While the *lacO* target is small relative to the entire *lacI* or *lacI*^d target, it can nevertheless comprise a significant fraction of the overall constitutive mutants [4]. For example, in several spectra recorded in a wild-type strain some 30–50% of all mutations resided at *lacO* [4]. DNA sequencing revealed the existence of an A·T→G·C

substitution hot spot at position +6 of *lacO*. This site resides within the inverted repeat sequence of the *lac* operator [5]. Other base substitutions in this region were also detected, as well as a number of deletions that removed all or part of the operator [5, 8]. When studies were conducted in a DNA mismatch-repair defective (*mutHLS*) background, the relative incidence of the *lacO* hot spot had essentially disappeared [3, 6]. On this basis, we suggested that the *lacO* hot spot is not necessarily a mutation that is generated at very high frequency. Instead, its high incidence in the spectra reflects the fact that it is largely refractory to postreplicative DNA mismatch repair [7].

Regardless of the precise mechanism responsible for the *lacO* hot spot mutation(s), their repeated occurrence does not provide any new useful information and increases the DNA sequencing burden. Thus, in DNA mismatch-repair proficient strains or other strains where the mutant frequency is only modestly elevated over the wild-type level, a procedure to screen out *lacO* mutants would be helpful. In the present communication we present a simple extension of the standard procedures followed in the recording of a *lacI^d* mutant spectrum. We created a low-copy plasmid that contains the kanamycin resistance gene under control of the *lac* promoter (*lacPO*). In mutants expressing the *lac* operon constitutively due to a defective repressor, the kanamycin resistance will also be expressed constitutively, and the strain will be kanamycin resistant. However, in mutants that express *lac* constitutively due to a defective operator, the wild-type repressor present in this strain will prevent expression of the kanamycin resistance gene, resulting in kanamycin sensitivity. In this report, we first describe the overall approach, then demonstrate that the new method works as expected by using a set of known *lacI^d* and *lacO* mutants, and finally apply the procedure to generate a new mutational spectrum in a wild-type strain.

2. Materials and methods

2.1. *E. coli* strains and media

The *E. coli* strain used for *lacI* mutant isolation was NR17809. This strain is identical to strain NR9102 (*ara*, *thi*, *prolac*, *F'prolac*) described previously [7], but is a new conjugant. The strain carries the *F'prolac* (*F'*128-27) containing two silent base-pair substitutions in *lacI* that abolish an intragenic mutational hotspot [7]. Strains S90C and CSH52 used as collector strains for mutant *F'prolac* have been described [7]. Strain NR8057 (*ara*, *thi*, *recA56*, *prolac*) has been reported [12]. Strain NR17905 is NR8057, but streptomycin-resistant (spontaneously generated mutant). NR17993 is NR17905 but carrying plasmid pHSG576kan (see below).

LB broth and minimal media (MM) have been described [7, 12]. MM contained Vogel-Bonner salts [13], 0.2% glucose, 1 µg/ml thiamine, and 1 µg/ml D-panthothenic acid. P-gal plates were MM plates but containing 750 µg/ml phenyl-β-D-galactoside (P-gal) as carbon source instead of glucose. MM-strep plates contained 150 µg/ml streptomycin. MM strep-kan plates contained both streptomycin (150 µg/ml) and kanamycin (20 µg/ml). X-gal plates were MM plates containing 50 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

2.2 Construction of plasmid pHSG576-kan

Plasmid pHSG576 is a chloramphenicol-resistant low-copy plasmid [14]. The kanamycin-resistance gene of plasmid pKD4 [15] was PCR amplified and inserted into the multi-cloning site of pHSG576 between the HindIII and EcoRI sites. As a result, the kanamycin-resistance gene is under control of the *lac* regulatory region, including the *lac* operator (*lacPO*). The plasmid was constructed by Celplor, Raleigh, NC.

2.3 Mutant selection

Mutants of strain NR17809 displaying constitutive expression of the *lac* operon (consisting of both *lacI* and *lacO* mutants) were isolated on P-gal plates, as described [7]. In this strain, the *lac* operon is located on the *F'**prolac*, which facilitates subsequent transfer of the constitutive mutants to certain tester strains, as described below. Overnight LB cultures of NR17809 were diluted in fresh LB medium to about 10^5 cells/ml, and 200 μ l aliquots were distributed over the wells of 96-well dishes and incubated overnight (ON) at 37°C on a shaking platform. Twenty-five μ l from each well were spread on a quarter-section of a P-gal plate and incubated for 60 hrs at 37°C. From each quarter section, four colonies were picked at random and placed in a gridded pattern on a P-gal plate. After 48 hrs, these plates were replicated using velvets on three LB plates, which were placed at 37°C. After overnight growth, these LB plates were replicated using velvets on three different plates: (i) a MM-strep plate containing 0.1 ml of an ON culture of strain CSH52, (ii) a MM-strep-kan plate containing 0.1 ml of an ON culture of strain NR17993, and (iii) a MM-strep plate containing 0.1 ml of an ON culture of strain S90C. This replication (replica-mating) step mediates transfer of the *F'**prolac* into the indicated streptomycin-resistant recipient strains. The plates were incubated ON. The CSH52 plate was further replicated on a MM X-gal plate and incubated overnight. Those *F'* conjugants that color blue on these plates were noted and recorded. The NR17993 plate allowed determination of the kanamycin-resistant colonies. Finally, the S90C plate was used to perform DNA sequencing on those mutants that were both blue on the CSH52 plate and kanamycin-resistant on the NR17993 plate. If more than one mutant for each original well (quarter plate) met the criteria, only one such mutant was submitted for DNA sequencing in order to preserve independence of all sequenced mutants (only one mutant per culture).

2.4 DNA sequencing

DNA sequencing was performed by the NIEHS Molecular Genomics Core facility. To obtain sequence information for the *lacI*^d region, the primers used were a 20-bp forward primer (5'-GGC GCT ACA ACG GGT AGC AA-3') corresponding to nucleotides -142 to -123 of *lacI*, and a 21-bp reverse primer (5'-CAG CTT CCA CAG CAA TGG CAT-3') corresponding to nucleotides 423 to 443 of *lacI*. To obtain sequence information for the *lacO* region, the primers used were a 25-bp forward primer (5'-GGT AGT GGG ATA CGA CGA TAC CGA A-3') corresponding to nucleotides 835 to 859 of *lacI*, and a 20-bp reverse primer (5'-GCA AAG CGC CAT TCG CCA TT -3') corresponding to nucleotides 204 to 223 of *lacZ*.

3. Results and discussion

3.1 Plasmid pHSG576kan and application to distinguishing *lacI^d* from *lacO* mutants

The analysis of *lacI^d* mutants has played an important role in improving our understanding of the mechanisms of mutagenesis [2–8, 10, 11, 16]. These mutants are genetically dominant in a complementation assay [10, 11]. However, one complication is that *lacO* mutants also show dominance in this same assay. While in many cases *lacO* mutants are a small minority, in some cases, in particular the case of spontaneous mutation in a wild-type strain, they can constitute a large proportion of all mutants. In particular one mutation, namely the A·T→G·C base substitution at position +6 of *lacO* is a major hotspot, likely because it is located in a potential hairpin structure recognized by the *lac* repressor [5, 7]. Hence, either due to secondary structure or due to repressor binding it is not accessible for postreplicative DNA mismatch repair.

Here, we have demonstrated a simple genetic method to distinguish these *lacO* mutants from the *lacI^d* mutants. It makes use of the presence of the plasmid pHSG576kan, which contains the kanamycin resistance gene inserted into the *lac* regulatory region, such that kanamycin resistance is now under control of the *lac* repressor. A scheme of the plasmid is shown in Fig. 1. The basic idea for our procedure is to transfer the *F'prolac* on which the mutations occur into a strain containing this plasmid. If the *F'prolac* contains a mutation in *lacI*, there will be no repressor activity in the conjugants, and the conjugant will be kanamycin-resistant. On the other hand, if the *F'* contains a *lacO* mutation, it will still produce a functional repressor, and this will prevent expression of the kanamycin resistance gene. Thus, our screen involves three stages (see the flow chart of Fig. 2). One is the selection of constitutive *lac* mutants. These consist of three classes: *lacI^d* mutants (these are dominant), other *lacI* mutants (these are recessive), and *lacO* mutants. Stage two is the scoring for dominant mutants (these are blue in the CSH52 test). Finally, stage three is the detection of kanamycin resistant mutants; this step will remove the *lacO* mutants from the consideration. Thus, in the end, we sequence only the mutants that are both blue (dominant) and kanamycin-resistant, representing the *lacI^d* mutants.

The performance of the system is demonstrated in Fig. 3 with a set of known *lac* mutants. We used three previously established *lacI^d* mutants and three previously established *lacO* mutants (see Legend). As one can see, the three *lacI^d* mutants are blue and kanamycin resistant, whereas the three *lacO* mutants are blue, but kanamycin sensitive, as expected.

3.2 Validation of the plasmid test for a set of unknown mutants

In the next set of experiments, we chose to isolate a number of new *lac* mutants from a wild-type *E. coli* strain on P-gal plates, and subjected them to the plasmid test. They were then sequenced for both the *lacI^d* and *lacO* regions to validate the assignments made by the plasmid system. Table 1 shows the results for 20 mutants that were predicted to be *lacO* and 20 that were predicted to be *lacI^d*. The results showed that each of the 20 predicted *lacO* mutants had a mutation in the operator region: 7 were G→A at position +5, 12 were T→C at +6, and one was a 14-bp deletion, while no mutation was found for any of these mutants in the *lacI^d* region.

For the 20 mutants predicted to be in the *lacI^d* region, a mutation was found for 16 of them in this region, while no mutation was found for any of them in the *lacO* region. For the remaining four mutants no mutation was found in either region. These may be due to mutations outside the sequenced areas. Overall, on the basis of this experiment, we conclude that the use of the plasmid pHSG576kan is an effective tool for distinguishing between the two classes of constitutive mutants and therefore screening out *lacO* mutants.

3.3. Generation of a spectrum of spontaneous *lacI^d* mutants

To generate a spectrum of independent spontaneous mutations in the *lacI^d* region, we initiated a large number of LB cultures, grown in 96-well microtiter dishes (see Materials and Methods). For each culture four colonies were picked at random and tested using the plasmid assay described above. The number of dominant mutants in this collection, as tested by the CSH52 assay, was about 50 %. However, this number was reduced to near 10 % of total mutants upon applying the plasmid test. After accounting for some DNA losses and sequences without a mutation in the *lacI^d* region, we obtained sequence information on 150 *lacI^d* mutants as described in Table 2.

In aggregate, the new spectrum conforms well to the previously recorded *lacI^d* spectra [2, 4, 7]. As before, the spectrum is dominated by base-pair-substitution mutations (73%), followed by single frameshifts (8%), larger deletions (18%) and two duplications (1.3%). Among the base-pair substitutions, both transitions and transversions contribute. Among the transitions, the G·C→A·T outnumber the A·T→G·C by several fold. Among the transversions all four classes were found. Finally, a significant fraction of the mutants are larger deletions ranging in size from 4 to 175 bases, also consistent with previous results [2, 4, 7].

3.4 Concluding remarks

In this work we demonstrate the feasibility of an improved procedure for obtaining spectra of mutations in the *lacI* gene of *E. coli*, specifically the *lacI^d* region, which has proven most informative in prior studies of the specificity of mutagenesis. While new technical approaches using high-throughput DNA sequence analysis have become available, the *lacI^d* system remains valuable for detailed studies of the specificity of mutation, especially when a high density of mutations within a given target is desired. The extensive knowledge regarding the detectable mutable sites within this target is also an advantage.

Our validation studies clearly indicate that *lacO* mutants are readily excluded based on their kanamycin sensitivity in the plasmid pHSG576kan test. Indeed, among 20 kanamycin-sensitive candidates, all carried a *lacO* mutation, while among 20 kanamycin-resistant candidates, no *lacO* mutations were observed. Instead, the large majority of them (16/20) contained an expected *lacI^d* mutation.

The new approach was subsequently used to obtain a large collection of *lacI^d* mutants in a wild-type background, in which *lacO* mutants historically were shown to make a major contribution. Upon sequencing, a spectrum of mutations was obtained that very closely matches previous recordings of a spectrum of *lacI^d* mutants in this background. We intend to

pursue this new approach for the analysis of *lacI*^d mutational spectra in a number of *E. coli* strains where mutator effects are observed but a strong contribution of *lacO* mutants is expected.

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Highlights

- A plasmid is constructed with the *kan* gene under control of the *lac* promoter-operator
- A method is described for separating *lacO* from *lacI^d* mutants
- A spectrum of spontaneous *lacI^d* mutants is described

Fig. 1.

The pHSG576kan plasmid used in this study to distinguish between *lacI^d* and *lacO* mutants. The plasmid was created by inserting the kanamycin gene (*kan*) from plasmid pKD4 into the multiple-cloning site of plasmid pHSG576 [14]. Abbreviations are: *ori* (plasmid replication origin), *cat* (chloramphenicol acetyltransferase), *lacPO* (*lac* promoter-operator), *lacZα* (α -complementation part of *lacZ* gene). See Materials and methods for details.

Fig. 2.

Flow chart depicting the method to obtain *lacI^d* mutants for DNA sequencing. Bacterial cultures were grown overnight and then diluted to 10^5 cells/ml and distributed in 200 µl aliquots into the wells of 96-well dishes. (A) After incubating at 37°C overnight, 25 µl from each well was put on a quarter of a P-gal plate and grown overnight at 37°C. Four isolates are then randomly picked from each quarter plate and placed in a grid formation on a new P-gal plate, which was grown overnight at 37°C. (B) The P-gal plate was then replicated onto three individual LB plates using velvets. (C) The LB plates were replicated onto three different plates for transfer of the F'*prolac* into three different strain backgrounds, as follows: (a) MM + Strep plate containing a lawn of strain CSH52, (b) MM + Strep + Kan plate containing a lawn of strain NR17993 (this strain contains plasmid pHSG576kan), and (c) MM + Strep plate containing a lawn of strain S90C. In (D), the CSH52 plate was then

replica plated onto an X-gal plate to test for blue color development. The final selection for mutants to be sequenced is for colonies that are blue on the X-gal plate and kanamycin resistant (see Materials and methods for details). In a few cases, we have added boxes to emphasize that four colonies were taken from each culture (quarter plate). When more than one candidate was identified per group of four, only one was taken for DNA sequencing to ensure independence of all sequenced mutants.

Fig. 3.

Visual demonstration of the pHSG576kan-mediated discrimination between established *lacO* and *lacI^d* mutants. Three different previously characterized *lacO* and *lacI^d* mutants [8] as well as a wild-type control strain were transferred by replica-mating into strains CSH52 and NR17993 and subjected to the procedures outlined in the Fig. 2. It is shown that the wild-type control is white in the CSH52 background and kanamycin sensitive. In contrast, the three *lacO* mutants (all containing the T→C mutation at position +6 of *lacZ*) are blue and kanamycin sensitive, while the three *lacI^d* mutants [(91–177), G→A at 185, and C→T at 75, respectively] [8] are blue but kanamycin resistant.

Table 1DNA sequencing results for predicted *lacO* and *lacI^d* mutants^a

Predicted <i>lacO</i> mutants		
<i>lacI</i> sequence	<i>lacO</i> sequence	
	change	position
none	G→A	+5
none	G→A	+5
none	G→A	+5
none	G→A	+5
none	G→A	+5
none	G→A	+5
none	G→A	+5
none	T→C	+6
none	T→C	+6
none	T→C	+6
none	T→C	+6
none	T→C	+6
none	T→C	+6
none	T→C	+6
none	T→C	+6
none	T→C	+6
none	T→C	+6
none	T→C	+6
none	T→C	+6
none	14 bp	−9 to +5

Predicted <i>lacI^d</i> mutants		
<i>lacI</i> sequence		<i>lacO</i> sequence
change	position	
T→C	54	none
T	72	none
A→T	81	none
C→T	92	none
C→T	92	none
G→A	93	none
T→A	96	none
C→T	104	none
C→T	104	none
T	153	none
T→A	167	none

Predicted <i>lacI</i> ^d mutants		
<i>lacI</i> sequence		<i>lacO</i> sequence
change	position	
C→T	198	none
C→A	210	none
12bp	34–45	none
duplication, 58 bp	138–195	none
14bp	193–206	none
none	none	none
none	none	none
none	none	none
none	none	none

^a Predictions as described in the text. “None” indicates that no sequence change was found in the indicated area.

Table 2Results of DNA sequencing of *lacI*^d mutants

Mutation	Number observed
Transitions	63
A·T→G·C	13
G·C→A·T	50
Transversions	46
A·T→T·A	19
A·T→C·G	12
G·C→C·G	7
G·C→T·A	9
-1 frameshifts	12
runs	5
non-runs	7
Larger deletions	27
Duplications	2
Total	150