Annals of Microbiology, **59 (1)** 179-182 (2009)

Use Pgal agarose minimal plate to screen *lac* constitutive mutation

Mingli TANG*, Li WANG, Pingping ZHANG, Pei HUANG, Lijun WU

Key Laboratory of Ion Beam Bioengineering, Institute of Plasma Physics, Chinese Academy of Sciences, Hefei 230031, P.O.Box 1126, Anhui, People’s Republic of China

Received 22 July 2008 / Accepted 9 December 2008

Abstract - The *lacI* gene has been used as a target gene in various mutation assays in bacteria and transgenic organisms. Pgal minimal plate is commonly used to select *lac* constitutive mutation. To simplify the preparation of the Pgal minimal plate, the agarose (1%) was used to replace agar (1.5%) in the Pgal minimal plate, omitting the process of spreading the *lac*$^-$ bacteria on the plate for scavenging the alternative carbon sources from the plate in advance of use. The results showed that the reducing sugar in agarose was extremely lower than that of others, the selective sensitivity and efficiency of the Pgal agarose was higher than those of the Pgal agar and Pgal agar scavenged. This might give the conclusion that Pgal agarose could be used conveniently and efficiently for the screening of *lac* constitutive mutation.

Key words: agar; agarose; *lac* constitutive mutatio; P-gal.

INTRODUCTION

The *lacI* gene had been used extensively for the analysis of mutations in bacteria and transgenic organisms with various DNA repair backgrounds (Glickman *et al.,* 1980; Sedwick *et al.,* 1986; Halliday and Glickman, 1991; Kohler *et al.,* 1991; Ono *et al.,* 1995; Murata-Kamiya *et al.,* 1995, 1997). There are two principal methods to select the *lacI* mutants, both of which use the presence or absence of β-galactosidase activity (Smith and Sadler, 1971; Miller, 1972, 1992). The first method relies on the colour of a plaque or a colony on the X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) indicator plate to screen mutants. But this method is not suitable for screening the mutant with large numbers or mutant with low mutation frequencies (MF). A second system is a selective assay (Smith and Sadler, 1971; Miller, 1972, 1992; Edelmann and Edlin, 1974; Shineberg, 1974) which uses P-gal (phenyl-β-D-galactopyranoside) as a sole carbon source. P-gal is not an inducer and the growth of the inducible wild type and low constitutive type on P-gal plate is autocatalytic depending on the initial level of β-galactosidase (Shineberg, 1974). The wild type which has very low constitutive expression is delayed several days, whilst constitutive cells having β-galactosidase levels 5-fold greater than the wild type, form visible colonies in 48 to 72 hours. This system has been used in the mutation studies in bacterial by most researchers (Kato *et al.,* 1985; Halliday andGlickman, 1991; Kohler *et al.,* 1991; Ono *et al.,* 1995; Murata-Kamiya *et al.,* 1995, 1997; Fujikawa *et al.,* 1998; Wijker *et al.,* 1996, 2000; Tang *et al.,* 2006).

In order to obtain precision frequency of constitutive mutation and efficient screen the *lacI* mutants, two precautions were necessary (Smith and Sadler, 1971): (1) the use of the purest grade agar, (2) the scavenging of alternative carbon sources from the plate by the spreading of the *lac*$^-$ bacteria on the plate several hours in advance of use. But the method to scavenge alternative carbon sources in advance is labouring and bothering. Therefore we used agarose to replace agar in P-gal minimal plates, and compared the content of their reducing sugar before or after autoclaving and their sensitivity, efficiency in mutant selection.

MATERIALS AND METHODS

Bacterial strain. Wild type strain W3110 ($F^-\lambda^-$) of *Escherichia coli* K12, generously donated by Prof Kaj Frank Jensen, was used for mutational research. *Escherichia coli* DH5α, used as *lacZ*$^-$ strain for the scavenging of alternative carbon sources from the minimal agar plate several hours in advance of use, was stored in our laboratory.

Reagents and media. Purified agar and agarose (molecular biology grade for lab use only) used in Pgal plate was purchased from Sigma and Genebase, respectively. LB medium and minimal A salts were prepared as described by Miller (Miller, 1972, 1992). Minimal glucose plates contained minimal A salts at a standard concentration, 1 mM $MgSO_4$, 5 μg/ml thiamine, and 0.2% glucose.

* Corresponding Author. Phone: 86-551-5593145; Fax: 86-551-5591310; E-mail: tangmingli100@hotmail.com

X-gal minimal plates consisted of Minimal glucose plates supplemented with 45 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Vogel-Bonner salts (Ono *et al.,* 1995) were modified, contained 0.2 g $MgSO_4{\cdot}7H_2O$, 2.0 g $(NH_4)H_2PO_4$, 10.0 g K_2HPO_4, 2.8 g sodium citrate·$2H_2O$ per litre. Pgal agar and Pgal agarose plates used for the selection of *lac* constitutive mutants consisted of modified Vogel-Bonner salts supplemented with 75 mg P-gal and 15 g agar or 10 g agarose per litre. Pgal agar scavenged was spread 4 h in advance with approximately 10^6 cells of *E. coli* DH5α.

The reducing sugar assay of agar and agarose. The DNS method (Longxiang *et al.,* 1981) was used to test the reducing sugar in agar and agarose. Samples preparation were processed as follows: exactly made 100 ml 2% agar mix and 100 ml 2% agarose mix in flask, respectively. *Escherichia coli* DH5α culture (100 μl), grown in LB medium overnight at 37 °C and washed three times with 0.2 M PBS, was added to the agar mix and incubated at 37 °C with shaking for 4, 8, 12, 16 h, respectively. All these samples (100 ml 2% agar mix, 100 ml 2% agarose mix and 100 ml 2% agar scavenged for different times) were divided into two groups. Each group contains one of 100 ml 2% agarose, one of 100 ml 2% agar and 2% agar scavenged for 4, 8, 12, 16 h, respectively. One group was autoclaved routinely and the other group was filtrated. The content of reducing sugar was detected on a Beckman DU640 spectrophotometer.

Visible colonies test. A single colony of wild-type *E. coli* strain W3110 ($F^-\lambda^-$) on minimal glucose plate was replated on a X-gal minimal plate to confirm the *lacI*$^+$ genotype. One white colony on X-gal minimal plate was incubated in LB media at 37 °C to stationary phase, washed twice with 0.2 M PBS. 100 μl appreciated wild type cells from the sample were spread on different Pgal plates and incubated at 37 °C; the visible colonies were counted at different time.

The mutant frequency on different Pgal plates. Samples preparation was described as above. 100μl wild type cells were spread separately on different Pgal plates. After incubation for three days at 37 °C, spontaneous mutant colonies were counted. The surviving cells were determined by culturing the same samples on LB plate overnight at 37 °C. From these results mutant frequencies were obtained. The same method was used to determine the MF of *lacI* gene on the different Pgal plate induced by low-energy ion beam irradiation (Tang *et al.,* 2006).

The screening fidelity assay on Xgal indicator plate. To verify the screening fidelity, totally 180 colonies (each sixty) from different Pgal plates were picked up randomly and incubated in 0.1ml of the modified Vogel-Bonner salts containing Pgal 75 μg/ml overnight at 37 °C. The sample of the culture was grown overnight on an X-gal minimal plate at 37 °C. A deep blue colony on the X-gal minimal plate indicates *lacI*$^-$, a light blue colony indicates l*acO*^{c} mutant, a white colony indicates wild type.

The fidelity validation by sequencing. All the deep blue colonies from the Pgal agarose were picked up to LB medium, incubated over night at 37 °C. Chromsomal DNA was isolated from Bacteria Genomic DNA Mini-prep Kit (V-gene). The amplification of *lacI* gene was performed in a thermal cycler (Authourized Thermal Cycler, Eppendorf) and the primers were 5′-GACACCATCGAATGGCGC-3′(F) and 5′-TTCCCAGTCACGACGTTG-3′(R) (TaKaRa Dalian Corporation). DNA sequencing was performed in TaKaRa (Dalian Corporation) using a Perkin-Elmer Applied Biosystems Model 377 DNA Sequencer. The mutations were determined on line (http://www.ncbi.nih.gov/blast/) by a run of blastn (blast 2 sequences) between the sense strand sequence of the wild type W3110 and mutants. The subject sequence is the sequence of the same region as in *E. coli* K-12 wild type strain W3110 (GenBank ACCESSION: NC000913).

RESULTS AND DISCUSSION

The reducing sugar content of the agar and the agarose

The reducing sugar of the agar and the agarose was shown in Table 1. (1) The content in agarose solution was much lower than that of purified agar before and after scavenged, these results showed that agarose could be used in Pgal minimal plate for the screening of *lac* constitutive mutation. (2) The *lac*$^-$ bacteria could rapidly scavenge the reducing sugar in purified agar solution in several hours, declining 48.70% in purified agar filtrated solution at 4 h and 73.74% at 8 h respectively, 61.80% in the purified agar solution after autoclaving at 4 h, but all slowed down thereafter. These data were in accordance with Smith and Sadler (1971) who chose 2-4 h as the scavenging time. (3) The prominent increase of the reducing sugar after autoclaving might due to the thermal cleavage of polysaccharide or some sugar residues exposure. But the residues exposure did not completely equal to the available sugar. And in practice, the screening is on the surface of plate, some times on the top agar, thus the content of available sugar on top layer may become comprehensive lower.

Visible colonies changed with incubation time

The results were shown in Fig. 1. Generally, the wild type *E. coli* cannot form visible colonies on Pgal plate in 72 h, except there is enough other available sugar on Pgal plate. When the same samples are plated on Pgal plates, excessive visible colonies in 72 h represent that the wild type cells have formed visible colonies. The method was firstly used to test the effect of scavenger density on constitutive plating efficiency by Smith and Sadler (1971). We used the method to verify the selection precision on different Pgal plates containing different reducing sugar and to evaluate the influence of the incubation time. The visible colonies on Pgal agarose at 66-72 h were lower than

TABLE 1 - The content of reducing sugar in agar and agarose detected by the DNS method

Group	Agar					Agarose
	0 h	4 h	8 h	12 h	16 h	
Filtrated	12.6 ± 0.46	6.5 ± 0.55	3.3 ± 0.48	3.1 ± 0.36	2.5 ± 0.61	0.4 ± 1.0
Autoclaved	205.7 ± 2.7	78.6 ± 1.8	73.6 ± 1.7	71.6 ± 2.7	64.9 ± 1.6	36.4 ± 3.9

Values were from at least three repeated experiments.

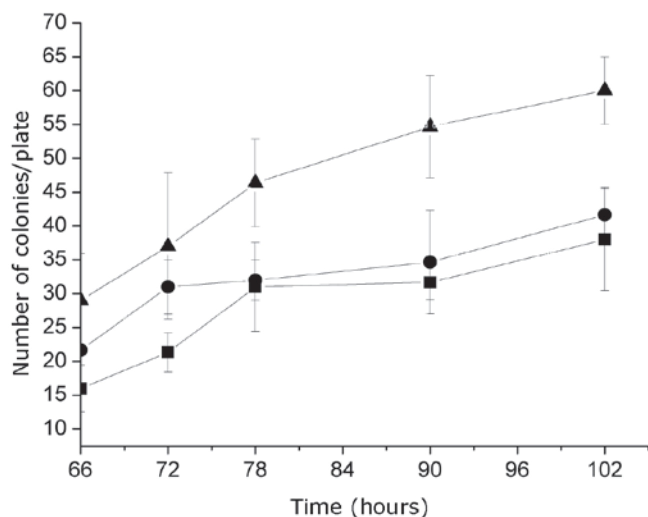

FIG. 1 - The mutant colonies on Pgal plates at different incubation time. Values were from at least three plates. ▲: Pgal agar, ●: Pgal agar scavenged, ■: Pgal agarose.

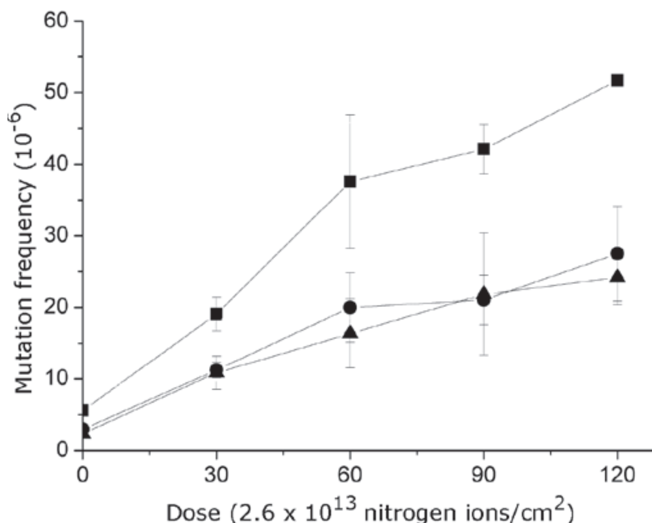

FIG. 2 - The MF of induction on different Pgal plates at different dose of low-energy ion beam irradiation. Values were from at least three repeated experiments. ▲: Pgal agarose, ●: Pgal agar scavenged, ■: Pgal agar.

that of Pgal agar scavenged and Pgal agar (Fig. 1), this might due to the low level available sugar in agarose; after 72 h, the colonies on the Pgal agarose were similar to that of Pgal agar scavenged but significantly different from that of Pgal agar. The data suggested that using Pgal agarose could increase the precision of the mutant selection.

The mutant frequency on different Pgal plates

The spontaneous mutant frequency in *lacI* gene had been investigated in different bacteria with various background, the results were different, from 10^{-3} to 10^{-8} (Miller, 1972; Glickman *et al.,* 1980; Schaaperand Dunn, 1987, 1991; Hallidayand Glickman, 1991; Sargentini and Smith, 1994; Murata-Kamiya *et al.,* 1997; Stuart *et al.,* 2000). In this work, the spontaneous MF screened on Pgal agarose was 1.479 x 10^{-6}, 1.75 x 10^{-6} on Pgal agar scavenged and 2.338 x 10^{-6} on Pgal agar respectively. The former was quite close to the result of Murata-Kamiya et al and Schaaper et al (Schaaperand Dunn, 1987, 1991; Murata-Kamiya *et al.,* 1997) with the same strain, which was 1.3 x 10^{-6} and 1.5 x 10^{-6}, respectively. The mutant frequency induced by low-energy ion beam was shown in Fig. 2 (0 dose is the control sample of vacuum and desiccation). The MF of induction selected on Pgal agarose at different dose was slight lower than that of Pgal agar scavenged, but much lower than those of Pgal agar. In general, Pgal agar scavenged was a standard method in the screening of *lac* constitutive mutation in bacteria. Our data of spontaneous and induction MF on Pgal agarose was quite close to those of Pgal agar scavenged, indicating Pgal agarose could be used as a standard method.

The selection fidelity

Lac constitutive mutation includes *lacI*$^-$ and *lacO*^{c}. *lacO* is the binding site of the *lacI* product (repressor) and consists of 28 bp only. The *lacO* mutation can reduce the affinity to the repressor and result in the *lac* operon partially expression (Smith and Sadler, 1971; Miller, 1972, 1992; Edelmann and Edlin, 1974; Shineberg, 1974; Sorensen *et al.,* 2003; Anthony *et al.,* 2004). Thus the phenotype of *lacO*^{c} on X-gal indicator plate is light blue. The *lacO*^{c} in all *lac* constitutive mutation is about 5% to 15% (Schaaperand Dunn, 1987, 1991; Sargentini and Smith, 1994). Our data was about 8-14% (Table 2), very close to their results.

The initial level of other available sugar in Pgal minimal plate is a determinative element for the successful screen of *lac* constitutive mutation. High available sugar excluding P-gal results in high false positive rate, and affect the precision of the MF determining (Smith and Sadler, 1971; Miller, 1972, 1992; Edelmann and Edlin, 1974; Shineberg, 1974). The reason is that the wild type *E. coli* can use them to form colonies. The false positive rate selected by Pgal agarose (5%) was lower than that of Pgal agar scavenged (6.67%) and Pgal agar (13.33%) (Table 1). In order to further conform the screen fidelity, the *lacI* gene of all mutants from Pgal agarose were sequenced. The results were as follows: all the deep blue colonies picked up from the Pgal agarose had one or more mutation site in the *lacI* gene, including base substitutions (33%), deletions (8%), insertions (11%) including two *IS*1 insertion, +TGGC at hot spot (48%), data was not shown in this article (Tang *et al.,* 2006). All these data including the lower reducing sugar content, the lower false positive rate and high screen fidelity illustrated Pgal agarose could be precisely and

TABLE 2 - The phenotype of the 180 colonies on the Xgal indicator plates

Colonies from	Total	Deep blue colonies	Light blue colonies	White colonies	Positive rate (false positive)
Pgal agarose	60	49 (81.67%)	8 (13.33%)	3 (5%)	95%
Pgal agar scavenged	60	50 (83.33%)	6 (10%)	4 (6.67%)	93.33%
Pgal agar	60	47 (78.33%)	5 (8.33%)	8 (13.33%)	86.67%

efficiently used in the screening *lac* constitutive mutation, and furthermore, the plate preparation is very convenient.

Acknowledgements
We would like to thank Prof. Kaj Frank Jensen (Department of Biological Chemistry, Institute of Molecular Biology, University of Copenhagen) who kindly present the *Escherichia coli* strain W3110. This research was supported by National Nature Science Foundation of China under Grant No. 10225526 and Hundred Talents Programme of The Chinese Academy of Sciences.

REFERENCES

Anthony L.C., Suzuki H., Filutowicz M. (2004). Tightly regulated vectors for the cloning and expression of toxic genes. J. Microbiol. Methods, 58: 243-250.

Edelmann P.L., Edlin G. (1974). Regulation of the synthesis of the lactose repressor. J. Bacteriol., 657-665.

Fujikawa K., Kamiya H., Kasai H. (1998). The mutations induced by oxidatively damaged nucleotides, 5-formyl-dUTP and 5-hydroxy-dCTP, in *Escherichia coli.* Nucleic Acids Res., 26: 4582-4587.

Glickman B.W., Rietveld K., Aaron C.S. (1980). γ-ray induced mutational spectrum in the *lacI* gene of *Escherichia coli*, comparison of induced and spontaneous spectra at molecular level. Mutat. Res., 69: 1-12.

Halliday J.A., Glickman B.W. (1991). Mechanisms of spontaneous mutation in DNA repair-proficient *Escherichia coli.* Mutat. Res., 250: 55-71.

Kato T.Y., Oda Y., Glickman B.W., (1985). Randomness of base substitution mutations in the *lacI* gene of *Escherichia coli* by ionizing radiation. Radiat. Res., 101: 402-406.

Kohler S.W., Proyost G.S., Fieck A. (1991). Spectra of spontaneous and mutagen-induced mutations in the *lacI* gene in transgenic mice. Proc. Natl. Sci. USA, 88: 7958-7962.

Longxiang Z.H., Tingfang Z.H., Lingyuan L. (1981). Method and Technique for Biochemistry. Higher Education Press, BJ.

Miller J.H. (1972). Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Miller J.H. (1992). A Short Course in Bacteria Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Murata-Kamiya N., Kamiya H., Iwamoto N., Kasai H. (1995). Formation of a mutagen, glyoxal from DNA treated with oxygen free radicals. Carcinogenesis, 162: 251-2253.

Murata-Kamiya N., Kamiya H., Kaji H., Kasai H. (1997). Mutational specificity of glyoxal, a product of DNA oxidation, in the *lacI* gene of wild type *Escherichia coli* W3110. Mutat. Res., 377: 255-262.

Ono T., Negishi K., Hayatsu H. (1995). Spectra of superoxide-induced mutations in the *lacI* gene of a wild type and a mutM strain of *Escherichia coli* K-12. Mutat. Res., 326: 175-183.

Sargentini N.J., Smith K.C. (1994). DNA sequence analysis of spontaneous and γ-Radiation (anoxic)-induced *lacI*^{d} mutations in *Escherichia coli* umuC122::Tn5:differential requirement for umuC at G..C vs A.T sites and for the production of transversions vs. transitions. Mutat. Res., 311: 175-189.

Schaaper R.M., Dunn R.L. (1987). Spectra of spontaneous mutations in *Escherichia coli* strains defective in mismatch correction: The nature of *in vivo* DNA replication errors. Proc. Natl. Sci. USA, 84: 6220-6224.

Schaaper R.M., Dunn R.L. (1991). Spontaneous mutation in the *Escherichia coli lacI* gene. Genetics, 129: 317-326.

Shineberg B. (1974). Mutations partially inactivating the lactose repressor of *Escherichia coli.* J. Bacteriol., 500-507.

Sedwick W.D., Brown O.E., Glickman B.W. (1986). Deoxyuridine misincorporation causes site-specific mutational lesions in the *lacI* gene of *Escherichia coli.* Mutat. Res., 162: 7-12.

Smith T.F., Sadler J.R. (1971). The nature of lactose constitutive mutations. J. Mol. Biol., 59: 273-305.

Sorensen S.J., Sorenson A.H., Hansen L.H. (2003). Direct detection and quantification of horizontal gene transfer by using flow cytomertry and *gfp* as a reporter gene. Curr. Microbiol., 47: 129-133.

Stuart G.R., Oda Y., de Boer J.G., Glickman B.W. (2000). No change in spontaneous mutation frequency or specificity in dietary restricted mice. Carcinogenesis, 21: 317-319.

Tang M.L., Wang S.C., Wang T., Zhao S.G., Wu Y.J., Wu L.J., Yu Z.L. (2006). Mutational spectrum of the *lacI* gene in *Escherichia coli* K12 induced by low-energy ion beam. Mutat. Res., 602: 163-169.

Wijker C.A., Lafleur M.V.M., Steeg H.V. (1996). γ-Radiation-induced mutation spectrum in the episomal *lacI* gene of *Escherichia coli* under oxic conditions. Mutat. Res., 349: 229-239.

Wijker C.A., Slotman B.J., Lafleur M.V.M. (2000). Mutagenic effect by phenylalanine during γ-irradiation in aqueous solution under oxic conditions. Mutat. Res., 454: 71-76.