A thymidineless *Escherichia coli* strain useful for *in vivo* DNA labeling

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Key words: bromodeoxyuridine, *in vivo* DNA-labeling, thymidineless strain.

RESUMEN. Los métodos de detección no radiactivos eliminan las desventajas que representa el uso de la radioactividad en el diagnóstico médico. Tanto el ADN plasmídico como el cromosomal han sido marcados *in vivo* con el empleo de la 5-fluordesoxiuridina (FdUrd) como inhibidor de la timidilato sintasa (método FdUrd). Con el objetivo de evitar el empleo de FdUrd fue obtenida una cepa de *Escherichia coli* auxótrofa para la timidina (*thy*$^-$). Dicha cepa fue utilizada para el marcaje *in vivo* con BrdUrd de ADN plasmídico (método *thy*$^-$). La cepa de *E. coli*, DH5α fue mutagenizada con *N*-metil-*N*'-nitro-*N*–nitrosoguanidina. Se seleccionó una colonia mediante crecimiento selectivo en medio mínimo y medio mínimo con suplemento de timidina (0,6 g/L). La cepa mostró un índice de reversión menor que $3{,}93 \cdot 10^{-8}$ en medio mínimo con suplemento. Se realizó un estudio comparativo *in vivo* de estos sistemas de marcaje. La detección del ADN simple y doble cadena marcado con BrdUrd se llevó a cabo inmunoenzimáticamente. El valor límite de detección fue de 1 ng para el ADN simple cadena marcado por el método *thy*$^-$ y de 5 ng para el marcado por el método FdUrd. El valor de detección límite está relacionado con la cantidad de ADN, simple o doble cadena, disponible para la detección por anticuerpo anti-5-BrdUrd. La sensibilidad del sistema es comparable a la de otros sistemas no radioactivos de marcaje de ADN. Estos resultados demuestran la capacidad de la cepa *thy*$^-$ para el marcaje *in vivo* de ADN como un sistema capaz de producir grandes cantidades de sonda marcada.

ABSTRACT. Non-radioactive DNA detection methods have been used to overcome the disadvantages associated with radioactivity. Plasmid and chromosomal DNA have been labeled *in vivo* with thymidine analogs, using 5-fluorodeoxyuridine (FdUrd) as inhibitor of thymidylate synthase (FdUrd method). In order to avoid the use of FdUrd we have obtained a thymidineless (*thy*$^-$) *Escherichia coli* strain for *in vivo* labeling of plasmid DNA with BrdUrd (*thy*$^-$ method). *E. coli* DH5α strain was mutagenized with *N*-methyl-*N*'-nitro-*N*–nitrosoguanidine. A single colony was selected by screening colonies grown on properly supplemented minimal medium and supplemented minimal medium plus thymidine (0.6 g/L). The strain exhibited a mutation reversion index less than $3.93 \cdot 10^{-8}$ on supplemented minimal medium. The comparative study of *in vivo* labeling systems was carried out. The detection of single and double BrdUrd labeled DNA stranded was performed immunoenzymatically. The detection limit values of single stranded DNA by *thy*$^-$ method was 1 ng whereas by FdUrd method was 5 ng . The detection limit value is related to the amount of single or double stranded DNA available for detection by the anti-5-BrdUrd antibody. The sensitivity of the system is comparable to those of other non-radioactive nucleic acids labeling systems. These results demonstrate the capacity of the *thy*$^-$ strain for efficient *in vivo* DNA labeling as a system capable to produce large quantities of labeled probes.

INTRODUCTION

Nucleic acid hybridization and blotting techniques are essential methodologies in molecular biology. Hybridization probes originally labeled with radioactive isotopes as ^{3}H, ^{32}P, ^{35}S or ^{125}I have been used for sequence-specific nucleic acid detection.[1] These methods have been applied in the detection of infectious agents and the identification of genetic diseases. Nucleic acid hybridization, using radioactive probes, is being currently replaced by non-isotopic procedures.[1] They reduce the cost of the assay and do not depend on the decay of the radioactive isotope incorporated into the DNA probe.

Several labeling methods have been developed for the non-radioactive detection of nucleic acids. Markers such as biotin, bromodeoxyuridine (BrdUrd) and digoxigenin are linked to nucleic acid precursors and subsequently incorporated into DNA probes, either by *in vitro* or *in vivo* labeling.[2] Most of the non-radio-

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active systems to detect DNA involve enzymatic, chemical or photochemical derivation of the nucleic acid molecule, followed by the detection of the modified nucleic acid via specific binding of a non-radioactive reporter system.[1] BrdUrd analogue can specifically replace thymidine nucleoside in DNA molecules using either *in vivo*;[3,4,5,6] or *in vitro*[7] enzymatic reactions. *In vivo* BrdUrd labeling has been reported for nuclear DNA of mammalian cells[6] and for bacterial plasmid DNA[3,5] by adding FdUrd as inhibitor of thymidylate synthase. Spontaneous bacterial *thy*$^-$ mutant strains obtained by trimethoprim selection methods[8] have been used for labeling of plasmid DNA.[4] In order to compare *in vivo* BrdUrd labeling methods (with FdUrd and with thymidineless strain) the authors obtained and characterized a thymidineless *E. coli* strain with the mutant agent *N*-methyl-*N*'-nitro-*N*–nitrosoguanidine.

MATERIALS AND METHODS

Escherichia coli DH5α strain

{*F, ϕ80dlacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17*(r_k^-, m_k^+), *supE44, relA1, deoR,* Δ(*lacZYA-argF)U169,* } was used.[9]

M9-t medium

Thiamin was added to M9[9] up to $1.66 \cdot 10^{-5}$ % final concentration.

Chemical mutagenic procedure to obtain a *thymidineless (thy$^-$) E. coli*

DH5α cells were grown in LB medium[9] at 37 °C with shaking until 0.40 $OD_{600\,nm}$ was reached. Cells were harvested by centrifugation (1 500 g) for 10 min and then suspended in 0.1 mol/L citrate buffer (pH 5.5). *N-methyl-N'-nitro-N–nitrosoguanidine* (Sigma) at 50 mg/mL dissolved in 0.1 mol/L citrate buffer (pH 5.5) was added to the culture and the suspension was incubated at 37 °C for 30 min . Every 10 min 100 μL aliquots were withdrawn, diluted with equal 0.1 mol/L phosphate buffer (pH 7.0) volume and plated on rich medium. The surviving cells were counted for different times of exposure to the mutagenic agent.[8]

Colonies were tested to detect the thymidineless mutants by replication in M9-t medium and M9-t medium with 0.6 g/L of thymidine (Sigma).

Reversion test

Mutants were grown in LB[9] plates to determine the number of colonies. Then, they were further plated on M9-t medium to count revertant colonies. The reversion index (number of revertants per mutagenized colony) was calculated as the ratio of revertant colonies to total count of colonies.[8]

Plasmid DNA labeling in thymidineless (*thy*$^-$) *E. coli* DH5α (*thy*$^-$ method)

E. coli DH5α *thy*$^-$ cells were transformed[9] with the plasmid pYAC (Amp., Sigma)[11] and growth in M9-t supplemented with 0.6 g/L of thymidine. Growth was done by triplicate at 37 °C with shaking until 0.40 $OD_{600\,nm}$ was reached. Chloramphenicol (Sigma) was added at 170 μg/mL to the bacterial cultures, which were additionally incubated for 80 min . Cells were harvested by centrifugation (1 500 g, 10 min) and washed twice with M9 medium to remove thymidine. Chloramphenicol was added at 170 μg/mL again to the three flasks. One received 0.6 g/ L of BrdUrd (Böehringer) and another 0.6 g/L of thymidine (positive growth control). Cultures were incubated at 37 °C with shaking for 16 h .

DNA isolation and purification

DNA molecules were isolated by the alkaline lysis procedure and purified by gel filtration in Sephacryl S-1000.[9]

Plasmid DNA labeling in *E. coli* DH5α by aided FdUrd (FdUrd method)

E. coli DH5α cells were transformed[9] with the plasmid pYAC (Amp, Sigma),[11] and growth in M9-t medium. Growth was done by triplicate at 37 °C with shaking until 0.40 $OD_{600\,nm}$ was reached. Chloramphenicol and 5-fluorodeoxyuridine (Fluka) were added at 170 μg/mL and 1 μmol/L, respectively, to the cultures, which were incubated further during 80 min .[5] One recived 20 μmol/L of BrdUrd (Böehringer) and another 20 μmol/L of thymidine (positive growth control). Cultures were incubated at 37 °C with shaking for 16 h .[5]

DNA molecules were isolated and purified as described previously.

Procedure to obtain unlabeled DNA from *E. coli* DH5α strain (LB)

E. coli DH5α cells transformed with the plasmid pYAC (Amp)[11] were cultured in LB medium[9] at 37 °C with shaking for 16 h . DNA molecules were isolated and purified as described previously.[9]

DNA binding to nitrocellulose membrane

BrdUrd labeled DNA and unlabeled DNA solutions were heated at 100 °C for 10 min and immediately chilled on ice. Then, an equal volume of 1 mol/L NaOH was added to them and incubated at room temperature for 20 min . DNA samples were neutralized by adding 0.5 volumes of 1 mol/L NaCl, 0.3 sodium citrate, 0.5 mol/L Tris Cl (pH 8.0) and 1 mol/L HCl solution. Immediately, DNA samples were chilled on ice again. Ten microliters of denatured DNA samples were spotted on nitrocellulose membranes (Schleicher-Schuell, Germany) and air-dried. Filter was washed twice with 50 mL of 6 x SSC, dried 1 h at room temperature and baked at 80 °C, under vacuum, for 2 h . Membranes were kept in desiccators.

Hybridization procedure

The membranes containing denatured unlabed DNA were incubated for 2 h at 68 °C in prehybridization buffer (6 x SSC, 0.5 % SDS, 5 x Denhardt solution, 0.1 mg/mL heat-denatured salmon-sperm DNA), then overnight at 68 °C in fresh prehybridization buffer containing the labeled heat-denatured probe (BrdUrd-pYAC, 2 μg/mL). After hybridization, the membranes were washed three times at room temperature (5 min each) in 2 x SSC, 0.1 % SDS, then three times at 68 °C (15 min each) in 0.1 x SSC, 0.1 % SDS, and finally at room temperature in 0.1 x SSC.

Chromogenic signal detection

Filters were blocked with 5 % skimmed milk in PBS buffer, pH 7.2, for 90 min at 37 °C . Further, they were incubated with the 1F6F3 monoclonal antibody[12] (1 μg/mL, diluted in PBS buffer) against BrdUrd for 120 min at 37 °C . The filters were washed twice with 0.05 % Tween 80. Then they were, at 37 °C for 90 min, incubated with sheep anti-mouse peroxidase conjugate diluted in 0.05 % Tween 80-1 % skimmed milk in PBS buffer. The filters were rinsed twice with 0.05 % Tween 80. Finally, the signal was developed with aminoethylcarbimide solution (0.2 mg/mL, diluted in 5 mmol/L ammonium acetate) and H_2O_2 (8.8 mmol/L). The colored reaction was stopped placing the membranes in 0.05 % Tween 80.

RESULTS AND DISCUSSION

Isolation of thymidineless strain from *Escherichia coli*

E. coli DH5α survival data was obtained after performing mutagenesis in the presence of 50 μg/mL of *N*-methyl-*N*'-nitro-*N*–nitrosoguanidine. Survival cells decreased from 100 to 3.7 % as the duration of the exposure to the mutagenic agent increased from 0 to 30 min (Table 1).

Efficient mutagenesis has 10 % survival cells.[8] It was obtained 9.6 % of survival cells after 10 min of mutagenic treatment (Table 1, row 2).

Surviving DH5α cells after 10 min of mutagenic treatment were plated on LB[9], M9-t and M9-t medium supplemented with 0.6 g/L of thymidine after mutagenesis. Only, the 0.39 % of the survivor cells had mutation in the thymidine synthesis pathway. One of these cell was isolated (PBMS73) and stored.

PBMS73 efficiently grew in the M9-t medium supplemented with thymidine (Fig. 1 A), whereas it did not in M9-t alone (Fig. 1B). DH5α grew in both media (Fig. 1). This result indicated that PBMS73 displayed a thymidine dependent growth.

The reversion index[8] of PBMS73 strain in M9-t was $3.93 \cdot 10^{-8}$, thus the genetic stability of it must be warranted to further studies.

In vivo BrdUrd labeling DNA by FdUrd and thy⁻ methods

The yield of labeled plasmid DNA isolated from cultures was similar to those traditionally obtained from chloramphenicol-treated cultures.[9] Studies of the stability of obtained compounds showed similar characteristic as BrdUrd labeled DNA previously reported.[5]

Chromogenic dot blot assay

Immunobinding assays were done at different DNA concentrations for detecting the incorporation of BrdUrd label in pYAC plasmid DNA (single and double stranded) of *E. coli* DH5α by means of *thy⁻* and FdUrd labeling methods (Figures 2 and 3).

The unlabeled pYAC and salmon sperm DNA were not stained, whereas positive control of BrdUrd label conjugated to BSA, containing 235 pmol of the nucleoside analog, gave a visible spot (Figures 2 and 3).

The incorporation of BrdUrd label in pYAC plasmid DNA is greater with the *thy⁻* labeling method than the FdUrd labeling method according to the colorimetric immunoenzymatic results (Fig. 2). The detection limit values of denatured labeled dsDNA by *thy⁻* method was 1 ng whereas by FdUrd method was 5 ng . Similar detection limited were obtained by López-Cánovas *et al.* (1994), but its not noteworthy to point out that chemiluminescent detection was used in that work which is one order more sensitive than colorimetric method used in this work.

The limit detection value for double stranded DNA is similar in both labeled methods (25 ng for hybridized dsDNA) according to the results obtained in the colorimetric immunoenzymatic (Fig. 3). The lower detection limit for denatured labeled dsDNA in relation to hybridized dsDNA, may be explained by the accessibility of the antibody 1F6F3 to labeled probe.

The results clearly demonstrate that a thymidineless strain can be used for the effective *in vivo* labeling of DNA with BrdUrd and further hybridization experiments. However, the detection limit value re-

Table 1. Survival data of *E. coli* DH5α cells exposed to 50 mg/mL *N-methyl-N'-nitro-N-nitrosoguanidine* diluted in 0.1 mol/L citrate buffer (pH 5.5) at 37 °C .

Exposure of cells to the mutagenic compound (min)	Number of Cells per mL	Survival percent (%)
0	$1.35 \cdot 10^5$	100
10	$\mathbf{1.30 \cdot 10^4}$	**9.6**
20	$6.00 \cdot 10^3$	4.4
30	$5.00 \cdot 10^3$	3.7

Fig. 1. *Growth of PBMS73 (left section) and DH5α (right section)* E. coli *cells in plates. (A): M9-t medium plates supplemented with 0.6 g/L of thymidine (Sigma). (B): M9-t medium plate.*

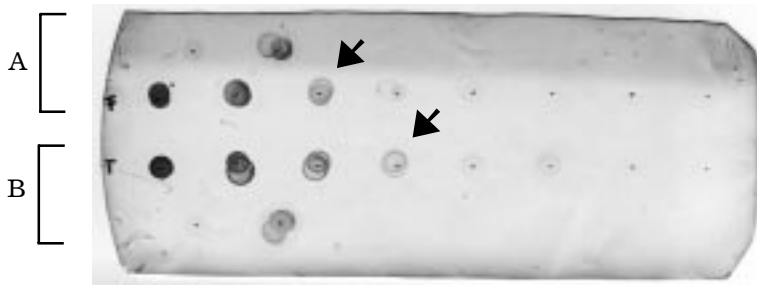

Fig. 2*. Colorimetric immunoenzymatic detection of BrdUrd-pYAC.* ***A-*** *Upper section: Negative control of unlabeled pYAC obtained from* E. coli *DH5α strain with thymidine (FdUrd method) and positive control of BrdUrd conjugated to BSA. Lower section: Serial dilutions (125 ng to 1.6 pg) of denatured labeled dsDNA obtained from* E. coli *DH5α strain with BrdUrd (FdUrd method) were spotted (10 mL) on nitrocellulose membranes (Schleicher and Shuell).* ***B-*** *Lower section: Negative control of unlabeled pYAC obtained from* E. coli *thy⁻ DH5α strain with thymidine (*thy⁻ *method) and positive control of BrdUrd conjugated to BSA. Upper section: Serial dilutions (125 ng to 1.6 pg) of denatured labeled dsDNA obtained from* E. coli *thy⁻ DH5α strain with BdUrd (thy⁻ method) were spotted (10 mL) on nitrocellulose membranes. BrdUrd label was detected with the anti-BrdUrd antibody (1F6F3).*[12]

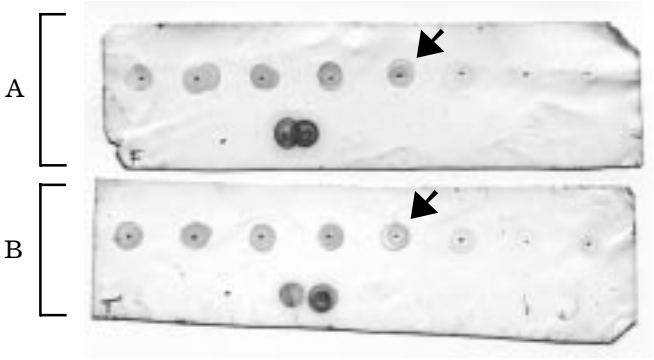

***Fig. 3**. Colorimetric immunoenzymatic detection of immobilized unlabeled pYAC DNA hybridized with denatured BrdUrd-pYAC (2 µg/mL) from **A**-* E. coli *DH5a strain (FdUrd method) **B**-* E. coli *thy⁻ DH5a strain (*thy⁻ *method). **A-B**- Upper section: Serial dilutions (800 ng to 6.25 ng) of unlabeled denatured dsDNA obtained from* E. coli *DH5a strain (LB) were spotted on nitrocellulose membranes (Schleicher and Shuell) (10 µL). Lower section: Negative control of salmon sperm DNA (1 mg) and positive control of BrdUrd conjugated to BSA on nitrocellulose membranes. Hybridized dsDNA was detected with the anti-BrdUrd antibody (1F6F3).*[12]

ported for denatured labeled dsDNA and hybridized dsDNA in this work is larger than other values reported for 5-BrdUrd-labelled M13 single and double strained DNA.[3,4] The detection limit value would be decreased by means of chemiluminescent detection according to previous report.[5]

CONCLUSIONS

The results demonstrate the possibility of using the PBMS73 strain for *in vivo* labeling of DNA with BrdUrd. The detection limit value is related to the amount of single or double stranded DNA available for detecting by the anti-5-BrdUrd antibody. The sensitivity of the system is comparable to those of other non-radioactive nucleic acids labeling systems, but this system is a cheaper and easier way of producing large quantities of labeled probes. Optimal labeling conditions and probe isolation are easy enough to be reproduced in any laboratory.

BIBLIOGRAPHY

1. Kessler C., Holtke H-J., Seibl R., Burg J. and Muhlegger K. Non-radioactive labeling and detection of Nucleic Acids. **Biol. Chem.**, **371**, 91, 1990.
2. Le Brun S., Duchange N., Namane A., Zakin M., Huynh-Dinh, T. and Igolen, J. Simple chemical synthesis and hybridization properties of non-radioactive DNA probes. **Biochemie, 71**, 319, 1989.
3. Traincard F., Terynck T., Danchin A. and Avrameas S. Une technique inmunoinzymatique puor la mise en evidence de I´hybridation moleculaire entre acides nucleiques. **ANN Inst. Pasteur Immunology, 134**, 399, 1983.
4. Sakamoto H., Traincard F., Vo-Quang T., Ternynck T., Guesdont J.L. and Avrameas S. 5-Bromodeoxyuridine *in vivo* labeling of M13 DNA, and its use as a non-radioactive probe for hybridization experiments. **Molecular and Cellular Probes, 1**, 109, 1987.
5. López-Cánovas L., *et al*. Evaluation of Chemiluminescent detection of Bromodeoxyuridine-labeled DNA. **Archives of Medical Research, 25**, 189, 1994.
6. Gratzner H., Leif R., Ingram D. and Castro A. The use of antibody specific for Bromodeoxyuridine for the immunofluorescent determination of DNA replication in single cells and chromosomes. **Experimental Cell Research, 95**, 88, 1975.
7. Miltenburg R., Rüger P., Grünewald-Janho S., Leons M., Schröder C. The DIG System User's Guide for Filter Hybridization, **Böehringer Mannheim**, 12, 1995.
8. Miller J. Experiments in molecular genetics. Nitrosoguanidine Mutagenesis, Unit III, 3rd Ed, Cold Spring Harbor Laboratory Press, New York, 121-189, 1972.
9. Sambrook J., Fritsch E.F. and Maniatis T. Molecular cloning. A laboratory Manual. 2nd Ed., Cold Spring Harbor Laboratory Press, New York, 1989.
10. Zinsser. Microbiología, Chapter I, 2nd Ed. Médica Panamericana., Buenos Aires, Argentina, 78-109, 1994.
11. Mathew C. Protocols in human molecular genetics. Chapter 19, Volume 9, Humana Press, New Jersey, 97-217, 1991.
12. Rojas A. and Sarracent J. Generación de hibridomas secretores de anticuerpos monoclonales anti-5-bromodeoxiuridina. **Biotecnologia Aplicada, 10**, 190, 1993.