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Effect of the Selection Marker on the Viability and Plasmid Stability of Two Human Proteins with Neurotrophic Action Expressed in *Escherichia coli*

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Most developed expression systems rely on the production of fusion proteins or the change of selection marker increasing genetic stability to avoid toxicity of heterologous proteins to *Escherichia coli* host cells. According to this, we analyzed the effect of the selection marker on the viability and plasmid stability of vectors pYMK5 and pYMK7 that codify neurotrophic factors brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF). We also analyzed the influence of two different lac promoter inducers on these parameters. We found that the addition of IPTG to culture medium produced a significant decrease of viability and plasmid stability for both expression vectors compared with values reached with lactose. There was no increase of both parameters when we changed the selection marker, so we can conclude that, in our case, a change of antibiotic does not solve the problem of low plasmid stability values. © 1999 Academic Press

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Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are members of a gene family termed neurotrophins that encodes structurally related proteins that promote the survival and differentiation of neurons in the vertebrate nervous system (1). BDNF is 55% homologous to NGF, and comparison of the two sequences demonstrates that there are several regions that are strongly conserved. These proteins have been cloned in *Escherichia coli* and their toxicity has been previously reported (2–4).

The expression of many eukaryotic proteins in bacteria often provokes a toxic effect on the host cell. This phenomenon usually results in the loss of plasmid from the culture by selective pressure, the reduction of the amount of recombinant protein being expressed or a decrease of culture viability. To prevent cell death due to toxicity they have been used some strategies such as the use of inducible promoters, the renewal of the amount of antibiotic in culture medium or the use of selection markers different from ampicillin. These strategies have allowed to increase the amount of recombinant protein in some cases (5, 6).

In this work, we study two genetic constructions, pYMK5 and pYMK7, that contain BDNF and NGF genes respectively, fused to the C-terminal region of *Streptococcus pneumoniae* amidase (cLyt) (7). This fragment cLyt has been fused to other proteins, improving their expression and purification (8). We compare viability and plasmid stability in presence of two different selection markers, ampicillin and kanamicin, and two different *lac* inducers, lactose and IPTG.

The objective of this work is to study whether the use of kanamicin as selection marker different from ampicillin, could increase viability and plasmid stability of both constructions, in presence of different *lac* promoter inducers.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains XL-1 Blue and W3110 (7) were used as host strains for DNA manipulation and protein expression, respectively. Plasmid pFGF-2 (8), generously given by Dr. José L. García, Biologic Research Center, C.S.I.C., Spain, was used for cloning.

DNA manipulations. hNGF and hBDNF genes were amplified from human genomic DNA (10). All DNA manipulations are described (Fig. 1) and were carried out by standard methods (11). Shaker-flask experiments were performed in a work volume of 50 mL LBA culture medium supplemented with lactose 2% or IPTG 1 mM during 6 h. Fusion proteins were detected by Western blot using a polyclonal anti-cLyt antibody developed in rabbit (generously given

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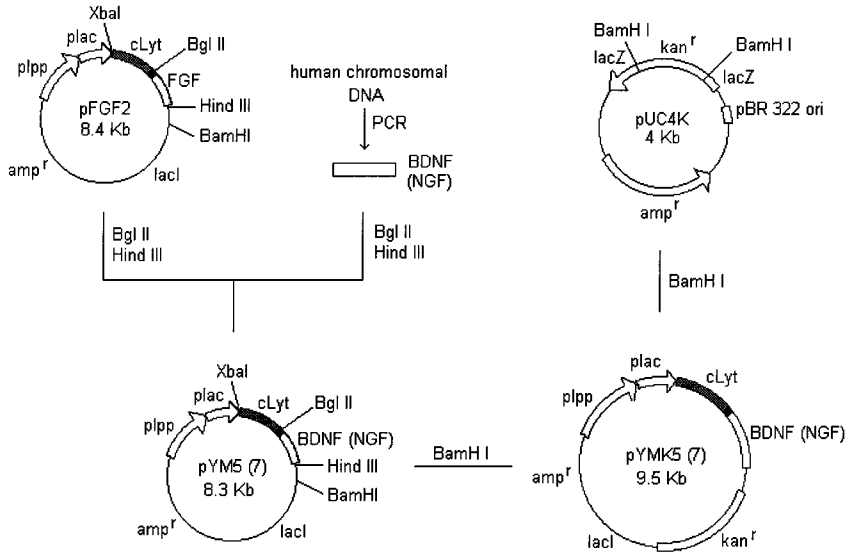

FIG. 1. Cloning strategy for the obtainment of plasmids pYMK5 and pYMK7.

by Dr. José L. García) and an anti-rabbit antibody conjugated to horseradish peroxidase (HRP) (Promega) (12).

Solid-medium experiments. Single colonies of transformed strains W3110[pYMK5] and W3110[pYMK7] were used to inoculate 3 mL of LBA medium and 3 mL of LBK medium and were grown overnight at 37°C with shaking. After this, 100-μL culture samples from each 3 mL culture were diluted with LB to yield 100 to 300 colonies per plate. Dilutions were plated in LB medium and LBK or LBA and IPTG 1 mM or lactose 2%. Plates were incubated overnight at 37°C. We calculated plasmid stability dividing the ratio of the average number of colonies from selective medium plates between the average number of colonies from non-selective medium plates. The number of colonies grown in non-selective medium defined the viability value. We compared the results using an Analysis of Variance (ANOVA) of two factors ($\alpha = 0.05$), for three experiments, each one comprising three replicas.

RESULTS AND DISCUSSION

We observed by immunoblotting (Fig. 2) that there was a marked difference between the expression level of proteins expressed in presence of lactose and those expressed in presence of IPTG. This may be supported by differences between these two metabolites as inducers of *lac* promoter. When we analyzed growing on solid medium, we found that, for both constructions, viability values were comparable for all culture conditions, except for those with IPTG. This metabolite, in presence or not of antibiotic, provoked a marked decrease of viability, making this parameter IPTG-dependent. We obtained that viability was antibiotic-independent (data not showed). We also found that plasmid stability of cultures, with or without selection markers, was similar for both clones, as shown in Fig. 3. The use of lactose did not vary plasmid stability values reached in control medium (LB). However, there was a significant decrease for both expression vectors in presence of IPTG (pYMK5 in LBA $7.8 \times 10^{-4} \pm 5 \times 10^{-5}$ and in LBK $7 \times 10^{-4} \pm 6 \times 10^{-5}$; pYMK7 in LBA $1.5 \times 10^{-2} \pm 7 \times 10^{-5}$ and in LBK $9 \times 10^{-4} \pm 1.3 \times 10^{-4}$). In all cases, we found that plasmid stability was IPTG-dependent and selection marker-independent.

In this work we analyzed viability and plasmid stability because they are primary affected when heterologous protein is toxic to the host cell. Besides, experiments in solid culture medium allowed us to simplify manipulation and to analyze a high number of culture conditions.

With our experiment, we wanted to verify whether the change of selection marker from ampicillin to kanamicin could solve the problem of low viability and plasmid stability levels obtained during the expression of fusion proteins cLyt-BDNF and cLyt-NGF. We used

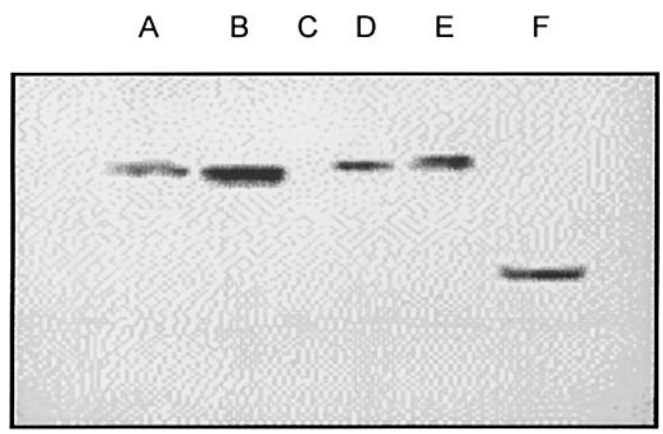

FIG. 2. Immunological detection of fusion proteins cLyt-BDNF and cLyt-NGF. A, induction of cLyt-BDNF with lactose 2%. B, induction of cLyt-BDNF with IPTG 1 mM. C, W3110 strain (negative control). D, induction of cLyt-NGF with lactose 2%. E, induction of cLyt-NGF with IPTG 1 mM. F, cLyt protein (positive control).

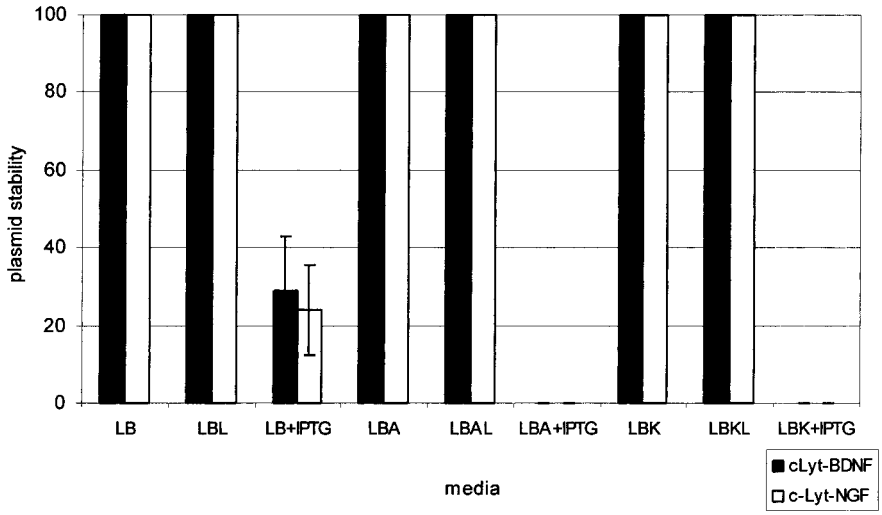

FIG. 3. Plasmid stability values of W3110[pYMK5] and W3110[pYMK7] in different culture media. Figure represents the means of three experiments with three replicas each one and their standard deviations ($\alpha = 0.05$).

lactose at 2% since we had previous references about the work of Sánchez-Puelles *et al.* (13). On the other hand, we also wanted to use IPTG since it is the most commonly used *lac* promoter inducer.

In our case, we think that there is an evident relationship between the type of inducer and the viability and plasmid stability values. Protein expression in the presence of each inducer could be inciding directly on the number of colonies that grows and in the stability of expression vectors. IPTG is a synthetic product, therefore, its levels in culture medium remain constant during growth, producing high protein expression levels. If the given protein is toxic to the host, as it has been reported for neurotrophic factors (2–4), we should expect that high expression levels affect viability and plasmid stability. For the contrary, lactose is an inducer that can be use as carbon source, therefore its levels in the culture medium could diminish during growing process, producing lower expression levels than those reached with IPTG. Results of ANOVA indicate that viability and plasmid stability are IPTG-dependent, since eliminating IPTG of statistical analysis, significant differences between the different culture media disappear (data not shown). According with these results, there is an inversely proportional relationship between expression level of proteins and viability and plasmid stability values. As we did not detect significant differences of parameters taking the antibiotic as source of variation, we can conclude that changing selection marker does not increase plasmid stability levels of pYMK5 and pYMK7. On the other hand, we proved that our methodology of growing on solid medium could be a powerful tool to analyze the best culture and expression conditions for each particular protein, taking into consideration its rapidity and confiability.

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