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Construction of a Suitable Vector for Lacz Gene Expression in *Hansunela polymorpha*

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Abstract: In the present study *Hansunela polymorpha* plasmid was constructed to express lacZ gene under yeast gene promoters. Hpleu2 gene was used as marker and to target the integration of plasmids into the corresponding *Hansunela polymorpha* genome locus. LacZ and Hpleu2 genes insert into polylinker site of pBluscript II ks. Expression was obtained with regulatable MOX promoter. Utility of the vector was illustrated by expressing the bacterial lacZ gene. The vector constructed are potentially useful for the construction of efficient producers of heterologous proteins in *Hansunela polymorpha*.

Key words: *Hansunela polymorpha*, LacZ, plasmid, gene expression, yeast

Introduction

A wide variety of microorganisms have been used as hosts for expression of heterologous proteins. Initially, *Escherichia coli* provided the best conditions as a productive strain (Stader, 1995). But for some more complex eukaryotic proteins, *Saccharomyces cerevisiae* became a preferable host, as it generates the folding and glycosylation patterns of proteins from higher eukaryotes. *Saccharomyces cerevisiae* also enables heterologous proteins to be secreted into the medium, which reduces further purification steps (Sha *et al*., 1997). At present, other yeasts species, like *Pichia pastoris* and *Hansunela polymorpha* have been developed as more advantageous yeast expression system (Gatzke *et al*., 1995).

Methylotrophic yeasts comprise a group of microorganisms able to utilize methanol as carbon source and energy. This system results in high level expression of heterologous proteins, high cellular density and high product concentration (Rodriguez *et al.,* 1996). *Hansunela polymorpha* has been described as particularly efficient hosts for high level heterologous gene expression when using the strong promoter (Agaphonov *et al*., 1995). The methanol utilization pathway, localized in the peroxisomes begins with the oxidation of methanol to formaldehyde, a reaction catalysed by the enzyme methanol oxidase. The methanol oxidase gene can be induced by methanol and is repressed by glucose. Under fully induced conditions the cellular protein content consists of at least 40% methanol oxidase, so the use of this strong methanol oxidase promoter, in combination with the efficient growth and induction on the relatively inexpensive methanol, makes *Hansunela polymorpha* a good option for the production of heterologous protein (Dijik *et al*., 2000). *Hansunela polymorpha* has some specific advantages over other methylotrophic yeasts (*Pichia pastoris* and *Candida boidinii),* being more thermotolerant and capable to grow at higher rates on simple, defined media. The relatively high optimal growth temperature for *Hansunela polymorpha* (37-43°C) may be favourable for the production of mammalian (including human) proteins and furthermore has the advantage that it allows a better management and reduces the risk of contaminations in large scale fermentations (Gellissen, 2000). *Hansunela polymorpha* also proved an excellent model by which to study the nitrate assimilation pathway and its transcriptional regulation (Brito *et al*., 1999). All these facts have promoted important development of the genetic analysis and molecular biology tools for *Hansunela polymorpha*. In this way auxotrophic mutant strains have been obtained Leu2 and URA3 genes have been isolated and used as selective plasmid markers (Agaphonov *et al*., 1999). Moreover, replicative plasmids containing *Saccharomyces cerevisiae* Leu2 genes as a replicon like sequence and *Hansunela polymorpha* ARS have been constructed. As a contribution to enriching the tools for the study of gene expression in this yeast, a integrative plasmids for *Hansunela polymorpha* to produce lacZ fusions has been constructed. Although other reporter genes have been used in *Hansunela polymorpha,* the lacZ gene has the advantage that numerous tools have been developed for it. Moreover, *Hansunela polymorpha* strains containing the lacZ gene fused to a given promoter can be used to isolate mutants in genes involved in the transcriptional regulation of the fusioned promoter such as have been used in *Saccharomyces cerevisiae.*

An essential tool for yeast cell biology is vectors for the expression of endogenous of foreign genes (Schneider and Guarente, 1991). Expression vector have been developed for *Hansunela polymorpha*, but many of them

have some problems. Here we construct a compact expression vectors that contain a pBluscript II KS derived polylinker for the insertion of cloned genes. Different vectors were designed to allow for either constitutive or regulated gene expression. Strong regulatable expression can be obtained with the MOX promoter.

Materials and Methods

Strains and media: *Escherichia coli* DH5α was used for bacterial transformation and plasmid propagation. *E.coli* was grown in LB medium. When needed, ampicillin was added to a final concentration of 100 mg ml^{-1}.

Yeast strain used in this study was *H. polymorpha* DL-1 (Leu2), a derivative of *H. polymorpha* DL-1 (ATCC 26012). *H. polymorpha* was grown in defined medium containing 0.68% yeast nitrogen base (YNB) without aminoacids supplemented with 20 mg ml^{-1} leucine and 2% glucose or in 1% yeast extract, 2% bactopepton (YP).

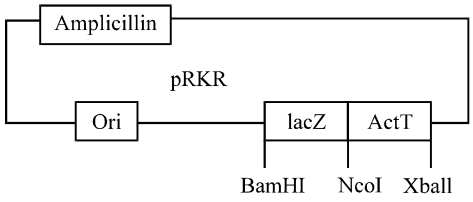

Fig. 1: pRKR is *E.coli*-yeast shuttle vectors based on pBluscript II Ks. The plasmid contain lacZ gene from YEp353 and Actin terminator

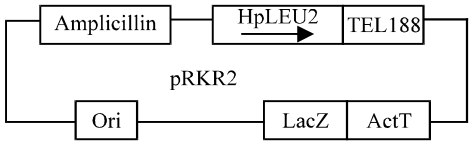

Fig. 2: Plasmid pRKR2 was constructed with subcloning of HpLeu2Tel in plasmid of pRKR

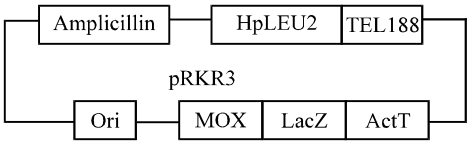

Fig. 3: Plasmid pRKR3 was constructed with fusion of MOX promoter in lacZ gene

General DNA techniques: General DNA manipulations were performed with general method (Sambrook *et al*., 2001). DNA fragments required for subcloning were gel-purified using a QiaEx kit (Qiagen). Oligonucleotides for PCR were obtained from the custom service of Korea Biotech Co. (Taejon, Korea). PCR was done with amplitaq DNA polymerase (perkin-Elmer) using an automatic thermal cycler (gene AMP PCR system 2400, perkin-Elmer).

Plasmid construction: Plasmid pRKR was derived by the subcloning of lacZ fragment and actin terminator into BamHI-XbaI site of pBleuscript II KS (Fig. 1). Actin terminator was amplified by PCR from *H. polymorpha* actin gene (HpActin). The oligonucleotides used as primers were HactF (CGGCCATGGATTCTGTCGGGTCTATTC) and HactR (CCCTCTAGACCAGTGTGC AGGATAAGAC). The amplified fragment was subcloned into pBluscript II KS with NcoI-XbaI site. The lacZ fragment was obtained as a single 3.87 kb BamHI-NcoI fragment from the plasmid YEp353.

Plasmid of pRKR2 was constructed by subcloning of 4.1 kb fragment of lac Zactin Terminator into the XbaI-BamHI site of pCTEL188 (Fig. 2). pCTEL188 has HpLEU2TEL188 fragment.

Twoprimers (CCCGTCGACGCGAAGAACGATCTCCTC) and (CCCAAGCTTATTCAA GCATTTGTTTTTG) were used to amplify MOX promoter. Plasmid of pRKR3 was constructed by subcloning of amplified fragments into the HindIII-SalI site of pRKR2 (Fig. 3).

Transformation and stabilization of *H. polymorpha*: *H. polymorpha* was transformed by the modified lithium acetate method (Bogdanova *et al*., 1995). Stabilization was carried out by transferring small transformants to a selective plate three or four times until most of the colonies appeared in a uniformly large size. Subsequently, they were transferred to a nonselective medium and incubated at 37°C for 2 days. Individual colonies were checked for protrophy on a selective plate. After 100% stability in the Leu+ phenotype of the stabilized colony was confirmed, the colonies were stored under selective conditions.

Culture conditions: In shake-flasks culture, triple-baffled 250 ml capacity shak flasks were used to ensure adequate mixing and oxygen transfer. Duplicate flasks containing 25 ml growth medium were inoculated with pre-cultured cells in YP and incubated with vigorous shaking at 37 °C. The MOX promoter was induced with 2% methanol and repressed by 2% glucose.

ß-galactosidase assay: *H. polymorpha* was grown in YPD with vigorous shaking at 37°C until an optical density $(OD)_{595}$ of 1.0-1.5 was reached. 100 µl of sample were

added directly to 0.9 ml of Z buffer (L^{-1}, 16.1 g of $Na_2HPO_4.7H_2O$, 5.5 g of $NaH_2PO_4.H_2O$, 0.75g of KCl, 0.246 g of $MgSO_4.7H_2O$ and 2.7 ml 2-mercaptoethanol; pH adjusted to 7.0) to make a total volume of 1 ml. Choloroform 100 µl and 50 µl of 0.1% SDS are added and cells are vortexed at high speed for 10 sec. *o*-Nitrophenyl-ß-D-galactoside (ONPG) hydrolysis is measured as described. Briefly, 0.2 ml of a 4 mg ml^{-1} solution of ONPG (dissolved in H_2O) is added to samples preincubated at 28°C. The reaction is stupped by adding 0.5 ml of 1 M Na_2CO_3 and the cell debris is spun out. The OD_{420} is measured. Assays are normalized to the OD_{600} of the culture and to the assay time. Protein concentration in the extract measure by using the dye-binding assay of Bradford (Bradford and Gibson, 1976). The ß-galactosidase activity of the extract calculate according to the following formula:

$$\text{ß-galactosidase activity} = \frac{(OD_{420})\,1.4}{0.0045\,(\text{protein})\,(\text{extract volum})(\text{Time})}$$

Results and Discussion

At present a range of heterologous expression system is available to cover the need for various proteins to be used for fundamental structure/function analysis and for biotechnological and farmaceutical purposes. Among these system, *H. polymorpha* is now recognized as a very suitable one. The first recombinant products produced in *H. polymorpha* have now passed the clinical examinations and are being launched on the market (e.g. a novel Hepatitis B vaccine) and other will soon follow (Lepetic *et al*., 1996). but experiment use of this organism has been limited by a lack of convenient expression vectors. We succeeded in synthesizing the bacterial enzyme ß-galacotsidase by *H. polymorpha* under of the methanol oxidase (MOX) promoter, the expression plasmid, pRKR3, contained the ß-galactosidase gene sandwiched between the methanol oxidase promoter and actin terminator (Fig.3). The expression plasmid pRKR3, a derivative of pBleuscript II KS, contain ori of ColE1 gene as the origin of replication in *E.coli*, the sequence of HpLeu2Tel188 as the origin of replication and the ß-iso-propylmalate dehydrogenase gene (Leu2) as a selection marker for transformation to the Leu2-deficient *H. polymorpha* strain A16. Vectror for generating recombinant methylotrophic yeasts are integrated in to the genome of the various hosts. As pointed out above, integration occurs either randomly or by homologous recombination, targeting the heterologous DNA to particular genomic loci. Accordingly, plasmid structure is generally designed either harboring or lacking sequences for targeted integration (Gellissen, 2000). In *P. pastoris* targeting is directed to one of two alchol oxidase genes or to the HIS4 locus. In *H. polymorpha* foreign DNA is either randomly integrated or directed to defined genomic loci, i.e. by distruption/replacement into the MOX/TRP3 locus (Agaphonov *et al*., 1995), or by targeting to an ARS sequence (Sohn *et al.,* 1996) or the leu2 or URA3 gene (Brito *et al.* 1999). In general, transformation vectors are hybrids of yeast-derived and bacterial sequences. The prokaryotic portion of the vectors have an ori and an antibiotic resistance for propagation and selection in a bacterial host. Similarly, the yeast portion contains the elements for selection of yeast transformants. The selection sequences are designed to complement the respective deficiencies of the hosts, i.e. HIS4 in *P. pastoris,* ADE2 in *P.methanolica,* Leu2 and URA3 in *H. polymorpha (*Gellissen, 2000). In the constructed vector, Leu2 was used for selection sequence. Transformation procedures for methylotrophic hosts employ methods similarly described for other yeasts, including *S.cerevisiae* (Dohmen *et al.,* 1991). They can be transformed using whole-cell methods, with addition of PEG, or by electroporation.

The lacZ gene from *Escherichia coli* has proven to be useful reporter for measuring promoter activity of organisms (Serebriiskii and Golemis, 2000). We compared lacZ expression in present of glucose and methanol. The results show that lacZ expression is repressed with glucose and induce by methanol. The bacterial lacZ gene, encoding the enzyme ß-galactosidase, is a matter reagent applied to the study of problems in genetics, cell and molecular biology, development and recently emerging fields of proteomics. Because ß-galactosidase activity is readily assessed *In vitro* and *In vivo*. Like other methylotrophic yeasts, *H. polymorpha* possesses several strong promoters, which are induced during growth on methanol. The promoters of methanol oxidase, dihydroxyacetone synthase and formate dehydrogenase gene are most commonly used to control heterologous gene expression (Dijik *et al.,* 2000). By using the above promoters various attractive induction strategies can now be designed. For instance, in case of harmful recombinant proteins, first biomass can be generated, followed by an alteration in composition of the growth medium, thereby inducing the expression of the heterologous gene. The MOX gene is regulated at the transcriptional level and belongs to the most strongly expressed and tightly regulated genes known in yeast. For example, in *H. polymorpha* cells growing on methanol MOX can make up to 40% of the soluble cell protein, whereas in cells growing in glucose or ethanol virtually no MOX protein

is detectable. In addition, the regulation of the MOX gene seems to be correlated with the proliferation of proxisomes (Godecke *et al.,* 1994).

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