Multiple gene expression by chromosomal integration and CRE-*lox*P mediated marker recycling in *Saccharomyces cerevisiae*

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Running title : Multiple gene expression in *Saccharomyces cerevisiae*

1. Introduction

Relatively few single gene deletions in *Saccharomyces cerevisiae* have any obvious phenotype, even fewer are harmful to the cell. This has been attributed to redundancy in the genome ***(1-3)*** i.e. more than one gene is capable of carrying out any given tasks in the cell. The hexose transporter gene family of *S. cerevisiae* is a good example of this. Although the twenty hexose transporter genes are expressed at different conditions, it took twenty consecutive gene disruptions to obtain a yeast strain incapable of hexose transport ***(4)***. The same dominant marker was recycled twenty times, since the number of genetic modifications exceeds the number of available markers.

Besides functional analysis of a redundant genome, Metabolic Engineering requires a system where many rounds of genetic engineering are possible ***(5)***. Metabolic engineering is defined as the directed change of the metabolism using genetic engineering ***(5)***. The theory of Metabolic Control Analysis (MCA) predicts that all enzymes along a metabolic pathway share the control of the metabolite flow through that pathway ***(6, 7)***. There are indeed few examples where overproduction of a single enzyme results in improvement of the desired trait. The overproduction of single glycolytic enzymes ***(8)*** did not result in higher glycolytic flux. Over production of multiple enzymes is usually necessary to attain significant improvements ***(9-12)***. This has also been predicted by theory ***(13, 14)***.

We used the pB3 PGK / pCRE3 system to over express four genes in the pentose phosphate pathway of a xylose fermenting *S. cerevisiae* strain ***(15)***. This chapter describes how to use the expression vector pB3 PGK and the CRE recombinase expression vector to change the expression level or regulatory characteristics of any number of genes in *S. cerevisiae*.

1.1 The pB3 PGK vector

The pB3 PGK ***(15)*** vector contains the strong *S. cerevisiae PGK1* promoter ***(16)***, a multi cloning site and the *S. cerevisiae* GCY1 terminator ***(17)***, the Amp gene for *E. coli* maintenance and the zeocin resistance gene (Invitrogen) for selection in both *E. coli* and *S. cerevisiae*. The zeocin gene is flanked by two *lox*P sites. The strategy to change the expression of any gene is a follows. A yeast gene (*YFG1*) is cloned between the promoter and terminator of the pB3 PGK, resulting in pB3 PGK *YFG1* (Figure 1A). The vector is linearized using a unique restriction site within the coding sequence of *YFG1*. The linear vector is used to transform yeast, directing the integration to the locus of *YFG1* (Figure 1B). The vector can also be cut in the ribosomal DNA sequence (rDNA; Figure 1A) to facilitate integration in the

repetitive rDNA sequences to obtain multiple copies ***(18)***. The *PGK1* promoter is excisable with *Sac*I and *Xba*I and can be exchanged for any of the following promoters, offering a wide range of promoters with diverse characteristics:

Promoters excisable with *Sac*I and *Xba*I.

Promoter	Characteristics	Reference
TDH3	Strong	***(19)***
TEF2	Strong, constitutive	---"---
ADH1	Intermediate	---"---
CYC1	Weak	---"---
CUP1	Copper induced	***(20)***
CTR1	Copper repressed	---"---
CTR3	Copper repressed	---"---
GAL1	Galactose induced	***(21)***

1.2 The pCRE3 vector

to further modify the strain with the integrated pB3 PGK vector, the zeocin resistance marker must be lost. This is accomplished by transformation with the vector pCRE3, that carries the CRE recombinase under the control of the GAL1 promoter ***(15)***. CRE catalyzes the specific recombination between genetic elements called the *lox*P sites. The *Lox*P site consists of two 13 bp inverted binding sites situated 6 bp apart. The pCRE3 is 2µ based and contains the URA3, AUR1-C and zeocin resistance genes. The URA3 or AUR1-C gene is used for selection of the vector. The zeocin marker facilitates simultaneous scoring of marker and pCRE3 loss by zeocin sensitivity.

2. Materials

1. *S. cerevisiae* / *E. coli* shuttle vector pB3PGK (***(15)***, see Note 9)

2. Zeocin (Invitrogen, Cat. nos. R250-01 or R250-05; CAYLA, Prod. no. ZEOCL0001)

3. Aureobasidin (TaKaRa, Prod. no. TAK 9000)

4. PEG 3350 MW 3350, Sigma cat. no. P3640 (50% w/v)

5. 1.0 M. LiAc

6. Salmon sperm DNA 2.0 mg/ml, Sigma Cat. no. D1626

7. YPD plates

8. YPD liquid medium

9. One air-tight plastic box (~2L volume, such as an ice-cream box)

10. General molecular biology reagents and equippment, such as restriction enzymes, PCR clean-up kit, agarose gel and PCR equipment.

3. Methods

3.1 Test host strain sensitivity to zeocin.

Make YPD (pH 7.5) plates with different zeocin concentrations (25; 50; 100; 150 mg/L). Note that the pH of the YPD medium for selective plates should be set to 7.5, since the zeocin is less active at lower pH. We routinely use 50 mg/L for the laboratory strain CEN.PK. Industrial and wild-type strains may require higher levels (Note 1 and 2). Resuspend a loopful of yeast cells in 1 mL sterile water, spread a loop of the suspension on each plate and check growth after two to three days at 30ºC.

3.2 Amplification of gene of interest by PCR and cloning in pB3 PGK

Amplify the gene of interest (*YFG1*) with a proofreading DNA polymerase with primers that incorporate a restriction site in the primer. We usually incorporate *BamH*I if possible, since this site is compatible with the *Bgl*II in pB3 PGK1. See Figure 1A for alternative enzymes. Purify the PCR product with a PCR clean-up kit. It is not necessary to perform agarose gel purification, given that the PCR was reasonably specific. Digest the restriction sites that were incorporated by the primers and do a second PCR clean-up. Alternatively phenol chloroform extraction can be used to remove the restriction enzymes. The PCR product is then ligated to the linearized pB3 PGK1 vector. The reverse primer used for cloning the gene can be used together with the BJ5756 primer to score correct *E. coli* clones. See Note 6 for the sequence of the BJ5756 primer.

3.3 Transform *S. cerevisiae* host strain with pB3 PGK *YFG1*

Digest approx. 0.3-1 µg pB3 PGK vector DNA with the appropriate unique restriction enzyme. The cut DNA can be used in yeast transformation without further purification or enzyme inactivation. The frequency of transformation and integration is high, so that there is no need for very high efficiency transformation. We use the "Quick and Easy TRAFO Protocol" as described by Dr. Daniel Gietz ***(22)*** with minor modification (see Note 7 for the www link to the original protocol).

Collect 25 µl yeast inoculum for EACH transformation from a fresh plate (less than 5 days) with a sterile loop.

Wash cells with 1mL water in an eppendorf tube.

Resuspend the cell pellet in 1 ml of 100 mM LiAc and incubate for 5 min at 30C.

Divide the cell suspension to the number of planned transformations

Spin cells at top speed in a microfuge for 5 sec.

Remove the supernatant with a pipet.

Loosen the pellet by vortexing briefly. This vortex step helps resuspend the cells after the heat shock (see below).

Add the following components into the tube on top of the cells:

240 µl of PEG (50% w/v)

36 µl 1.0 M. LiAc

50 µl SS-DNA (2.0 mg/ml)

5.0 µl of vector DNA (100 ng to 5 µg)

20 µl of sdd water.

Vortex the cell pellet for at least 1 min to resuspend the cell pellet in the transformation mix.

Incubate at 42ºC for 20 min.

Pellet the cells at top speed in a microcentrifuge for 10 sec.

Remove the supernatant with a pipet.

Wash the cells with 1mL water by slowly pipetting up and down.

Pellet the cells at top speed in a microcentrifuge for 10 sec.

Add 1mL non-selective YPD medium and resuspend carefully.

Transfer cells to a 15 mL or 50 mL Falcon tube or another large volume tube with a tight fitting cap. A large screw-cap tube will hold the cells safely, do not use Eppendorf tubes, since the lid may pop open by the pressure.

Incubate the tube shaking at 30C for 3 hours to overnight

Transfer cells to a 1.5 mL eppendorf tube

Pellet the cells at top speed in a microcentrifuge for 10 sec.

Remove supernatant

Wash cells with 1mL water.

Resuspend the cells in 200µL water

Incubate on ice for 1 hour prior to spreading on selective plates. This step may lower the background, if high background is a problem.

Spread 20 µL and 100 µL on selective plates. The remainder may be saved in the fridge for several weeks. It is good to save an aliquot of transformants if the number of transformants on the plates are too many or too few.

3.4 Analytical colony PCR

The PCR master mix should be prepared and stored on ice so that the master mix can be immediately added to the cells.

PCR mix (50µL) :

1µM reverse primer for cloning of *YFG1*

1µM BJ5756

5U Taq Polymerase

100µM dNTPs

The background on zeocin is typically much lower than for geneticin. Most colonies that appear are true transformants. Pick a number of big, well grown colonies with a sterile pipet tip and streak a small amount of yeast cells on the side of a PCR tube, streak the remainder on a new selective plate. Incubate the PCR tube containing yeast cells in a microwave oven at full power for 1-2 min. Be careful not to take too much cells, as this will inhibit the PCR reaction. It is important to keep the reactions on ice after the addition of the master-mix. Otherwise the proteases from the lysed yeast may degrade the DNA polymerase. These are essentially the same methods as recommended by the EUROFAN project. Spot 10µL of the PCR product on an agarose gen with the appropriate size marker. See Note 7 for www link to the original protocol.

3.5 Transformation and curing of pCRE3

The same procedure for transformation as described above can be used to transform the correct integrants with the CRE expression vector pCRE3. The pCRE3 contains the selectable markers URA3, zeocin and AUR1-C. The selection of the vector must be done using the AUR1-C or the URA3 markers, since the strains are already zeocin resistant. When Aureobasidin resistant or uracile prototrophic clones have been obtained, the pCRE3 can be cured immediately. Note that it is not necessary to grow the cells in medium containing galactose, since the background activity of the CRE recombinase seems to bee efficient enough to obtain high frequency of looped out clones.

Inoculate 100 mL YPD with one colony (1 mm) from the pCRE3 transformed cells. Incubate 1-2 days or until stationary phase. Dilute 1µL of cell culture in 1 mL sterile water. Plate 10, 50 and 100 µL of the dilution on three YPD plates. This procedure should yield 100-500 cells on the plate with the least cells. Replica plate onto two fresh plates after two days, one YPD medium with zeocin and the other with YPD only, using sterile velvet. It is a good practise to replicate the YPD plate after the YPD/zeocin, since the amount of cells replicated on the second plate is usually lower. This practise assures that colonies are not mistakenly scored as zeocin sensitive, because insufficient number of cells were replicated onto the YPD/zeocin plate. Ten to twenty percent of the colonies are zeocin sensitive, following this procedure. Since the zeocin marker is present on both the chromosomal construct and the pCRE3 vector, zeocin sensitivity marks loss of both elements.

3.6 Simultaneous overexpression of the S. cerevisiae genes RKI1, RPE1, TAL1 and TKL1.

We used the pB3 PGK / pCRE3 system to create strains over-expressing the *S. cerevisiae* non-oxidative pentose phosphate pathway genes *RKI1*, *RPE1*, *TAL1* and *TKL1* ***(12)***. The genes were individually cloned in pB3 PGK and integrated in the locus of the respective gene, resulting in over-expression of the genes. The pCRE3 vector was used to create strains simultaneously over-expressing the *RKI1*, *RPE1*, *TAL1* and *TKL1* by successive integrations and removal of the *lox*P-zeocin-*lox*P cassette. Figure 2 shows an agarose gel loaded with the PCR products from the analytical colony PCR procedure, confirming the correct integrations. The *lox*P-zeocin-*lox*P cassette has also been used successfully to create xylose fermenting yeasts from industrial strains lacking auxotrophic markers ***(23, 24)***.

4. Notes

1. Zeocin and aureobasidin are expensive. Using less medium in the selective plates is a way of cutting costs. Petri dishes with only 5 mL medium can be used if some measure is taken to prevent over drying of the plates (see Note 2).

2. Plates with 5 mL medium can be incubated in an airtight container with moist air. Put the plates upside down in an empty ice cream box at some distance from the bottom. Pour 10 mL boiling water in the box and tighten the lid. In this way the plates can be incubated for many days without drying.

3. The frequency of correct integrants is very high with the pB3 PGK vector as is the frequency of marker loss with the pCRE3 vector. Therefore it may not be necessary to

perform the analytical PCR after the integration, but to wait until after picking zeocin negative cells after transforming and curing of the pCRE3 vector. The advantage of doing this is that the positive identification of correct integration is done in the end of the process. The probability of having incorrect contaminating clones is much reduced.

4. The simultaneous disruption of a wild-type gene and its expression under the control of a different promoter has been demonstrated with the CRE-*lox*P system. Underproduction of a protein, i.e. production at lower levels than the wild-type, requires that the wild-type promoter gene is removed or silenced. The pB3 PGK vector can be used to simultaneously silence the copy of the gene that is controlled by the wild-type promoter. This is achieved by cloning the 5' part of the gene (about 80%) in the pB3 PGK vector. Integration with this construct disrupts the gene expressed under the original promoter, while the gene controlled by the promoter in the pB3 PGK vector remains intact. This strategy was successfully used to create strains based on TMB3001, with under or conditionally expressed ZWF1 gene ***(25)***.

5. If a heterologous gene is to be expressed, the gene itself cannot be used as a target of integration. If the yeast already contains an integrated vector containing the bacterial amp gene, this can be used as integration target. The pB3 PGK may be linearized in the amp gene with *Nde*I. Alternatively, the integration can be targeted to the rDNA sequences with pBR3PGK linearized with *Sna*BI.

6. The sequence of the PGK promoter specific primer BJ5756 is 5'-CAT CAA GGA AGT AAT TAT CTA CT-3' Tm 51C.

7. WWW links to protocols and suppliers. Transformation protocols: The Gietz Lab Yeast Transformation Home Page www.umanitoba.ca/faculties/medicine/biochem/gietz/Trafo.html; The EUROFAN colony PCR protocols mips.gsf.de/proj/eurofan/eurofan_1/ b0/home_requisites /guideline/sixpack.html

Suppliers: CAYLA www.cayla.com; Invitrogen www.invitrogen.com; TaKaRa bio.takara.co.jp

8. The pB3 PGK vector is also available with the AUR1-C marker instead of the zeocin marker (pB1 PGK). This vector is useful if a particular yeast strain shows zeocin insensitivity.

9. To obtain the vectors described in this chapter, contact Bärbel Hahn-Hägerdal, Department of Applied Microbiology, Lund University, P.O. Box 124, 221 00 Lund, Sweden, barbel.hahn-hagerdal@tmb.lth.se

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References

1. Oliver, S. G. (1996) From DNA sequence to biological function. *Nature* **379,** 597-600.

2. Mewes, H. W., Albermann, K., Bahr, M., Frishman, D., Gleissner, A., Hani, J., Heumann, K., Kleine, K., Maierl, A., Oliver, S. G., Pfeiffer, F., and Zollner, A. (1997) Overview of the yeast genome. *Nature* **387,** 7-65.

3. Wolfe, K. H., and Shields, D. C. (1997) Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* **387,** 708-13.

4. Wieczorke, R., Krampe, S., Weierstall, T., Freidel, K., Hollenberg, C. P., and Boles, E. (1999) Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. *FEBS Lett* **464,** 123-28.

5. Bailey, J. E. (1991) Toward a science of metabolic engineering. *Science* **252,** 1668-75.

6. Kacser, H., and Burns, J. A. (1973) The control of flux. *Symp Soc Exp Biol* **27,** 65-104.

7. Heinrich, R., and Rapoport, T. A. (1974) A linear steady-state treatment of enzymatic chains: General properties, control and effector-strength. *Eur J Biochem* **42,** 89-95.

8. Schaaff, I., Heinisch, J., and Zimmermann, F. K. (1989) Overproduction of glycolytic enzymes in yeast. *Yeast* **5,** 285-90.

9. Niederberger, P., Prasad, R., Miozzari, G., and Kacser, H. (1992) A strategy for increasing an *in-vivo* flux by genetic manipulations. The tryptophan system of yeast. *Biochem J* **287,** 473-79.

10. Hauf, J., Zimmermann, F. K., and Müller, S. (2000) Simultaneous genomic overexpression of seven glycolytic enzymes in the yeast *Saccharomyces cerevisiae*. *Enzyme Microb Technol* **26,** 688-98.

11. Smits, H. P., Hauf, J., Müller, S., Hobley, T. J., Zimmermann, F. K., Hahn-Hägerdal, B., Nielsen, J., and Olsson, L. (2000) Simultaneous overexpression of enzymes of the lower part of glycolysis can enhance the fermentative capacity of *Saccharomyces cerevisiae*. *Yeast* **16,** 1325-34.

12. Johansson, B., and Hahn-Hägerdal, B. (2002) The non-oxidative pentose phosphate pathway controls the fermentation rate of xylulose but not of xylose in Saccharomyces cerevisiae TMB3001. *FEMS Yeast Research* **2,** 277-82.

13. Kacser, H., and Acerenza, L. (1993) A universal method for achieving increases in metabolite production. *Eur J Biochem* **216,** 361-67.

14. Fell, D. A., and Thomas, S. (1995) Physiological control of metabolic flux: the requirement for multi-site modulation. *Biochem J* **311,** 35-39.

15. Johansson, B., and Hahn-Hägerdal, B. (2002) Overproduction of pentose phosphate pathway enzymes using a new CRE-loxP expression vector for repeated genomic integration in *Saccharomyces cerevisiae*. *Yeast* **19,** 225-31.

16. Mellor, J., Dobson, M. J., Roberts, N. A., Tuite, M. F., Emtage, J. S., White, S., Lowe, P. A., Patel, T., Kingsman, A. J., and Kingsman, S. M. (1983) Efficient synthesis of enzymatically active calf chymosin in *Saccharomyces cerevisiae*. *Gene* **24,** 1-14.

17. Hermann, H., Hacker, U., Bandlow, W., and Magdolen, V. (1992) pYLZ vectors: *Saccharomyces cerevisiae/Escherichia coli* shuttle plasmids to analyze yeast promoters. *Gene* **119,** 137-41.

18. Nieto, A., Prieto, J. A., and Sanz, P. (1999) Stable high-copy-number integration of *Aspergillus oryzae* alpha-AMYLASE cDNA in an industrial baker's yeast strain. *Biotechnol Prog* **15,** 459-66.

19. Mumberg, D., Müller, R., and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156,** 119-22.

20. Labbé, S., and Thiele, D. J. (1999) Copper ion inducible and repressible promoter systems in yeast. *Methods Enzymol* **306,** 145-53.

21. Güldener, U., Heck, S., Fielder, T., Beinhauer, J., and Hegemann, J. H. (1996) A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24,** 2519-24.

22. Gietz, R. D., and Woods, R. A. (1994) *in* "Molecular genetics of yeast: practical approaches" (Johnston, J. A., Ed.), pp. 121-34, Oxford University Press, Oxford.

23. Johansson, B. (2001) Metabolic Engineering of the Pentose Phosphate Pathway of Xylose Fermenting *Saccharomyces cerevisiae*, Lund University, Lund, Sweden.

24. Zaldivar, J., Borges, A., Johansson, B., Smits, H. P., Villas-Boas, S. G., Nielsen, J., and Olsson, L. (2002) Fermentation performance and intracellular metabolite patterns in laboratory and industrial xylose-fermenting Saccharomyces cerevisiae. *Appl Microbiol Biotechnol* **59,** 436-42.

25. Jeppsson, M., Johansson, B., Hahn-Hägerdal, B., and Gorwa-Grauslund, M. F. (2002) Reduced by-product formation from xylose fermentation by modulation of the glucose 6-phosphate dehydrogenase gene of recombinant xylose fermenting *Saccharomyces cerevisiae*. *Manuscript*.