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Enhanced Transformation Efficiency of an Anticoagulant Hirudin Gene into *Saccharomyces cerevisiae* by a Double δ-Sequence

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Abstract Delta-integration vectors were constructed for the purpose of achieving homologous integration of the hirudin expression cassette into the chromosome of *Saccharomyces cerevisiae*. A double δ system truncated with the unnecessary bacterial genes, and consequently having a reduced insert size for integration, showed a four-fold increase in transformation efficiency at given DNA concentrations, and as a result, the constructed recombinant yeast strain had a 1.3-fold enhancement in hirudin expression level compared with a single δ system.

Key words: Hirudin, δ-integration, *Saccharomyces cerevisiae*, transformation efficiency

Hirudin is recognized as a protease inhibitor isolated from the salivary gland of bloodsucking leech, *Hirudo medicinalis* [9]. It is a potent thrombin-specific inhibitor with reported K_i value ranging between 10^{-14} and 10^{-11} M, and consequently, relatively low concentrations of hirudin effectively blocks thrombin-mediated conversion of fibrinogen to fibrin. Based on these properties, hirudin is recognized as a useful therapeutic agent for cardiovascular diseases, and thus, an abundant supply of highly purified and active hirudin is necessary for providing clinical studies and trials. However, the limited availability of natural hirudin from leech inevitably resulted in the development of recombinant cell fermentation processes for a large-scale production [3, 4, 12, 14, 18, 19].

Integration of a target gene into the *Saccharomyces cerevisiae* chromosome is an effective method for the stable expression of a foreign gene by overcoming the mitotic instability of an episomal expression system [13, 16]. A chromosomal integration vector such as YIp is not adequate for overproduction of recombinant proteins because the copy number ranging 1 to 2 copies is very low. To integrate more copies of a target DNA, repetitive chromosomal DNA sequences such as rDNA and δ-sequences as target sites have been used [1, 2, 5-8, 10, 11]. Types of host strains, and the amount and size of target DNA copies have been known to play important roles in the transformation/integration efficiency [6, 11, 15, 16, 20].

In this study, two different δ-integration vectors were constructed and evaluated for their transformation efficiency of the hirudin expression cassette into the genome of *S. cerevisiae*. Specific hirudin expression levels for the corresponding recombinant systems were also measured.

MATERIALS AND METHODS

Escherichia coli DH5α [*F⁻ lacZΔM15 hsdR17 (r⁻ m⁻) gyrA36*] was used for the propagation and preparation of plasmid DNA. *S. cerevisiae* 2805 [*Mata pep4:: HIS3 prb1 can1 his 3 ura 3-52*] was used as a host for hirudin expression. A hirudin expression cassette containing the *GAL10* promoter, *MFα1* leader sequence, structural hirudin gene, and *GAL7* terminator was isolated from the YEGαHIR525 plasmid [18]. Plasmid pδ-neo [1, 7] was used as a template vector for constructing both single and double δ-integration vectors. LB medium (1% NaCl, 1% tryptone, and 0.5% yeast extract) was used for *E. coli* cultivation. YPD plates (2% peptone, 1% yeast extract, 2% agar, and 2% glucose) which were supplemented with various amounts of filter-sterilized aminoglycosidic antibiotic (G418) were used for selecting yeast transformants. Hirudin expression experiments were carried out in 5-ml test tubes with YPG medium (2% peptone, 1% yeast extract, and 2% galactose). All yeast cultures were performed at 30°C and an initial pH of 5.5. DNA manipulation procedures were carried out as

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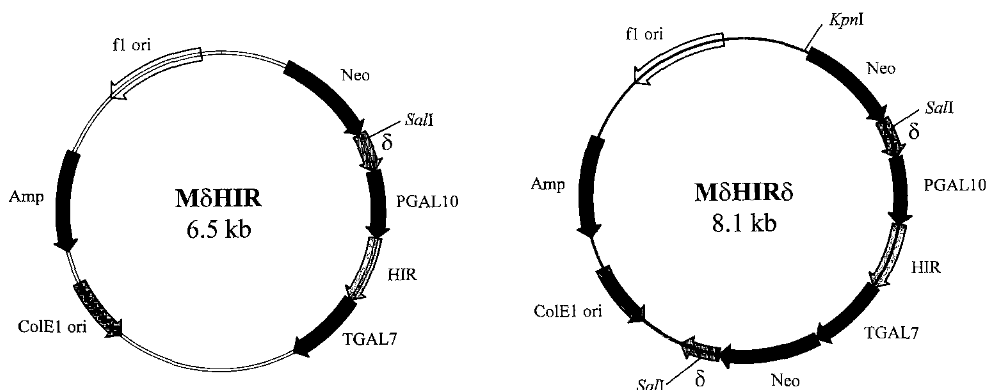

Fig. 1. Genetic map of plasmid MδHIR and MδHIRδ.

described by Sambrook *et al.* [17]. Yeast transformation was completed by using the Alkali-Cation Yeast Kit (BIO101 Inc., Palo Alto, CA, U.S.A.) according to the manufacturer's instruction with the following modifications to increase transformation efficiency [7]. The shock time for heating at 42°C was extended to 15 min, and the cells were resuspended in 0.2 ml of the SOS solution (BIO 101). The cell resuspension was mixed with 4.5 ml of YPD and 0.3 ml of the yeast extract (10%), and then it was incubated further at 30°C for 24 h. Dry cell mass concentration was measured with a spectrophotometer (Hitachi, Tokyo, Japan) at 600 nm. The hirudin activity in the culture broth was determined by measuring the antithrombin activity (ATU) using a chromogenic substrate, Chromozyme TH (Roche, Mannheim, Germany) [18-19]. The integrated copy number of the hirudin expression cassette was measured by using a Southern hybridization with the *GAL10* promoter gene as an internal standard. After Southern hybridization, the intensities of the band corresponding to the multicopy of the *GAL10* promoter in the transformants and the band corresponding to the single copy of the internal *GAL10* promoter were compared with a densitometer (Bio-Rad, Hercules, CA, U.S.A.) to estimate the copy number for the multiple integration vector. Copy number of YEGαHIR525 plasmid for the same host cell was measured for comparison with those of the integrating plasmids.

Results and Discussion

In order to possess a unique restriction site after construction, the *SalI* restriction sequences in YEGαHIR525 plasmid were removed by cleaving with *SalI*, end filling with the Klenow fragment, and ligating again. The *XbaI*-digested pδ-neo vector was treated with the Klenow fragment to make blunt ends and then digested again with *BamHI* and calf intestinal phosphatase (CIP). The 2.0-kb hirudin expression cassette was cut from the YEGαHIR525 plasmid by cleaving it with *BamHI* and *StuI* restriction enzymes and inserted into the δ-sequence downstream of the pδ-neo vector to make MδHIR plasmid.

To isolate the δ/*neo* fragment, pδ-neo vector was digested with *KpnI*, end-filled, and cleaved again with *SacI*. MδHIR plasmid was cut with *SacII*, blunt-ended, and further treated with *SacI* and CIP. The 1.6-kb δ/*neo* fragment that was cut from the pδ-neo vector was inserted at the 3'-end of the *GAL7* terminator of the single δ-integrating plasmid MδHIR to make the double δ plasmid, MδHIRδ (Fig. 1). MδHIR and MδHIRδ were linearized with *SalI* and *SalI*/*KpnI* restriction enzymes before transformation was made into *S. cerevisiae*, respectively. The same amount of the linearized hirudin expression cassette (74.0 nmole) was used to compare transformation efficiency. As reported elsewhere, the number of the transformants from both plasmids was dependent on when G418 concentration was added [1, 7]. In a case of MδHIRδ, unnecessary bacterial sequences containing the *bla* gene and *ori* were excised with the *SalI*/*KpnI* treatment that resulted in a smaller insert fragment (3.5 kb) for yeast transformation. MδHIRδ plasmid, with a 47% smaller insert size than the MδHIR plasmid, yielded a four-fold increase in transformation efficiency at the same concentration level of linear DNA, and this clearly suggests

Table 1. Transformation efficiencies of MδHIR and MδHIRδ plasmids as a function of G418 concentration.

G418 concentration (g/l)	Number of transformants (CFU/ml)*	
	Single δ system (MδHIR)	Double δ system (MδHIRδ)
0.5	+++**	+++
1.0	23	+++
2.0	16	+++
3.0	14	50
8.0	8	40
10.0	6	36

*Colony numbers were counted from the five independent YPD-G418 plates and averaged to evaluate transformation efficiency.
**YPD-G418 plates harboring colony numbers above 50 (CFU/ml) were designated as the '+++' sign.

the higher transformation efficiency for the double δ system (Table 1).

A Southern blot analysis for twenty randomly selected transformants from both plasmids were undertaken to measure the copy number of the hirudin expression cassette that was integrated into the yeast genome using the *GAL10* promoter gene as the internal standard probe. The average copy number of the YEGαHIR525 plasmid was estimated to be about four copies per cell. In both single and double δ systems, the pattern for the single integration site with tandem array integrants seemed to be more dominant than single or multiple integrations along the yeast chromosome, as reported elsewhere [5]. Lee and Da Silva [7] reported a greater variety of integration patterns for the reduced insert size in yeast transformation. However, in this study, it was very interesting to note that the double δ system with a smaller insert showed more tandemly arrayed integration patterns for the hirudin expression cassette than the single δ system. The maximum copy number of the hirudin expression cassette that was integrated was estimated at 10 for MδHIR and 15 for MδHIRδ, as shown in Fig. 2.

To compare specific hirudin expression levels for the two recombinant yeast strains, twenty independent transformants from the MδHIR and MδHIRδ plasmids were randomly selected and grown in 5 ml of YPG medium for 24 h. Experimental errors caused by the difference in inoculum size were minimized by dividing the hirudin concentration by the dry cell mass concentration. A specific average hirudin expression level of the double δ system was 35% higher than that of the single δ system, which corresponds to over a two-fold enhanced value compared to that of the episomal hirudin expression system (Table 2). The MδHIRδ plasmid resulted in a linear DNA fragment with reduced size for chromosome integration. The exact cause of enhanced transformation efficiency and concomitant elevation of a hirudin expression level by using MδHIRδ plasmid is unclear right now, and thus, more research is necessary to elucidate the causes.

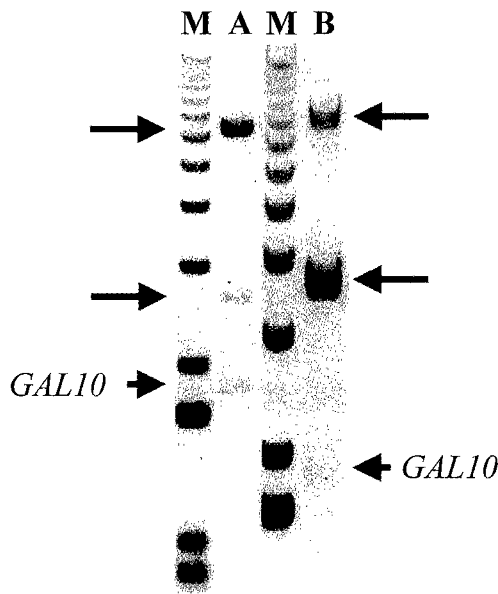

Fig. 2. Southern hybridization to measure the copy number of MδHIR (A) and MδHIRδ (B) with the *GAL10* promoter gene as an internal standard probe.
Arrows indicate the band of the hirudin gene integrated into the *S. cerevisiae* genome. M denotes the size marker.

Table 2. Dependencies of specific hirudin expression levels on the copy number of the hirudin expression cassette in recombinant *S. cerevisiae* strains.

Strain	Copy number	Expression context	Specific hirudin expression level (mg hirudin/g cell)
YIpHIR	1	Integrated	0.26
YEGαHIR525	4	Episomal	0.90
MδHIR	10	Integrated	1.89
MδHIRδ	15	Integrated	2.45

As a result, a recombinant yeast strain with a higher specific hirudin expression level was successfully obtained by enhancing the transformation efficiency. More investigations are now in progress to reveal the relationship between the copy number of the hirudin expression cassette and specific hirudin expression level, and thereby to isolate a highly efficient recombinant strain for a large-scale production of hirudin.

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REFERENCES

1. Cho, K. M., Y. J. Yoo, and H. S. Kang. 1999. δ-Integration of endo/exo-glucanase and β-glucosidase genes into the yeast chromosomes for direct conversion of cellulose to ethanol. *Enz. Microb. Technol.* **25:** 23-30.
2. Cho, K. M. and Y. J. Yoo. 1999. Novel SSF process for ethanol production from microcrystalline cellulose using the δ-integrated recombinant yeast, *Saccharomyces cerevisiae L2612dGC. J. Microbiol. Biotechnol.* **9:** 340-345.
3. Choi, C. M., M. D. Kim, S. K. Rhee, and J. H. Seo. 1996. Effects of medium composition on hirudin production in recombinant *Saccharomyces cerevisiae. Biotechnol. Lett.* **18:** 1129-1132.

4. Chung, B. H., W. K. Kim, K. J. Rao, C. H. Kim, and S. K. Rhee. 1999. Downstream processing of recombinant hirudin produced in *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* **9:** 179-183.
5. Kim, Y. S., S. Y. Kim, J. H. Kim, and S. C. Kim. 1999. Xylitol production using recombinant *Saccharomyces cerevisiae* containing multiple xylose reductase genes at chromosomal δ-sequences. *J. Biotechnol.* **67:** 159-171.
6. Lee, F. W. F. and N. A. Da Silva. 1996. Ty1-mediated integration of expression cassettes: Host strain effects, stability and product synthesis. *Biotechnol. Prog.* **12:** 548-554.
7. Lee, F. W. F. and N. A. Da Silva. 1997. Improved efficiency and stability of multiple cloned gene insertions at the δ sequences of *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **48:** 339-345.
8. Lopes, T. S., C. A. J. Hakkaart, B. L. Koerts, H. A. Rau, and R. J. Planta. 1991. Mechanism of high-copy-number integration of pMIRY-type vectors into the ribosomal DNA of *Saccharomyces cerevisiae*. *Gene* **105:** 83-90.
9. Markwardt, F., J. Hauptmann, G. Nowak, C. Kless, and P. Walsmann. 1982. Pharmacological studies on the antithrombotic action of hirudin in experimental animal. *Thromb. Haemostasis* **47:** 226-229.
10. Pack, S. P., K. M. Cho, H. S. Kang, and Y. J. Yoo. 1998. Development of cellulose-utilizing recombinant yeast for ethanol production from cellulose hydrolyzate. *J. Microbiol. Biotechnol.* **8:** 441-448.
11. Parekh, R. N., M. R. Shaw, and K. D. Wittrup. 1996. An integrating vector for tunable, high copy, stable integration into the dispersed Ty δ-sites of *Saccharomyces cerevisiae*. *Biotechnol. Prog.* **12:** 16-21.
12. Park, J. B., Y. E. Kweon, S. K. Rhee, and J. H. Seo. 1995. Production of hirudin by recombinant *Saccharomyces cerevisiae* in a membrane-recycle fermentor. *Biotechnol. Lett.* **17:** 1031-1036.
13. Park, J. N., D. J. Shin, H. O. Kim, D. H. Kim, H. B. Lee, S. B. Chun, and S. Bai. 1999. Expression of *Schwanniomyces occidentalis* α-amylase gene in *Saccharomyces cerevisiae* var. *diastaticus*. *J. Microbiol. Biotechnol.* **9:** 668-671.
14. Rao, K. J., C. H. Kim, B. H. Chung, J. H. Sohn, and S. K. Rhee. 1998. Effect of galactose feeding on the improved production of hirudin in fed-batch cultures of recombinant *Saccharomyces cerevisiae*. *Bioprocess Eng.* **19:** 385-388.
15. Reddy, A. and F. Maley. 1993. Dithiothreitol improves the efficiency of yeast transformation. *Anal. Biochem.* **208:** 210-212.
16. Romanos, M. A., C. A. Scorer, and J. J. Clare. 1992. Foreign gene expression in yeast: A review. *Yeast* **8:** 423-488.
17. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual.* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
18. Sohn, J. H., S. K. Lee, E. S. Choi, and S. K. Rhee. 1991. Gene expression and secretion of the anticoagulant hirudin in *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* **1:** 266-273.
19. Sohn, J. H., M. Y. Beburov, E. S. Choi, and S. K. Rhee. 1993. Heterologous gene expression and secretion of the anticoagulant hirudin in a methylotrophic yeast *Hansenula polymorpha*. *J. Microbiol. Biotechnol.* **3:** 65-72.
20. Wilson, J. H., W. Y. Leung, G. Bosco, D. Dieu, and J. E. Haber. 1994. The frequency of gene targeting in yeast depends on the number of target copies. *Proc. Natl. Acad. Sci. USA* **91:** 177-181.