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Multiple-copy-gene integration on chromosome of *Escherichia coli* for beta-galactosidase production

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Abstract−Recombinant *E. coli* strains with 1-3 copies of *lacZ* genes on their chromosomes were constructed and their β-galactosidase (β-gal) expressions were examined. Serial dilution cultures were used to analyze the long-term genetic stability of the recombinant *lacZ* genes of the chromosomal or plasmid expression system. The strain with a 3-copy *lacZ* on the chromosome has a sustainable β-gal expression through 60 hours. However, the β-gal activity of the plasmid expression system lasted less than 36 hours under a no selection condition. Obviously, the genetic stability of the chromosomal expression system demonstrated in this study is better than that of the plasmid expression system under nonselective condition, such as a medium without antibiotics. The results demonstrated that the strains with a multiple-copy-gene on the chromosome are useful for protein production in industrial repeated fed-batch fermentation.

Key words: Genetic Stability, Homologous Recombination, Recombinant Protein Production

INTRODUCTION

Homologous recombination [1] is a useful tool for gene disruption and replacement on chromosomes. This causes an exchange of DNA fragments between two DNA molecules by crossing over in a region of identical sequence. In *Saccharomyces cerevisiae*, homologous recombination is very efficient [2]. Even with a very short region of homology (25-50 bp), 95% recombination efficiency was achieved in yeast. Based on this technique, multiple-copy gene integration on the chromosome was carried out in the yeast *Pichia pastoris*, which led to a gelatin production level as high as 14.8 g/*l* [3,4]. In the bacteria *Ralstonia eutropha*, a three-copy organophosphohydrolase (OPH) gene expression strain has been constructed to investigate the effect of chromosomal gene dosage on protein expression [5]. The recombinant gene stability and expression levels of chromosomal integration are better than a plasmid-based expression system in *Ralstonia eutropha* [5]. However, until now no work has focused on *E. coli*, the most popular host cell [6-8].

Homologous recombination is also called linear transformation in *E. coli* to distinguish it from the traditional transformation in which circular plasmids are involved [9]. The efficiency of homologous recombination in this organism has been quite low because linear DNA would be degraded by the host exonuclease, RecBCD [10]. However, during the last decade, significant advances have been made to improve the efficiency of linear recombination. The phage λ Red system, one of the developed techniques in linear recombination, enhanced the efficiency of gene replacement with a short region of homology (35-50 bp) [11,12]. The λ Red locus is composed of *bet*, *exo* and *gam* genes. Gam inhibits RecBCD exonuclease to attack linear DNA. Exo degrades the 5′ end of linear DNA and then Beta binds to the remaining 3′ strand for protection and subsequent recombination of target DNA. In *E. coli*, recombination efficiency could be improved to 0.1% of surviving cells with the λ Red system [11]. Further, Campellone et al. reported that a genetic size up to 45 kb of the genome could be replaced in enterohemorrhagic *E. coli* by a Red-promoted recombination [13].

To carry out linear transformation, we used a λ Red encoded plasmid pKD46 and an auxotrophic *E. coli* strain ZSC114 (Table 1) which cannot grow in media containing glucose, mannose, or lactose as the only carbon source. Based on this plasmid-mediated recombination system, two cloning strategies were used to construct recombinant strains. First, linear DNA *tet*^{R}-*lacZ* was prepared, transferred into ZSC114 and selected by its tetracycline resistance phenotype. Then *glk-lacZ* and *manXYZ-lacZ* were also recombined into the strain and selected by the complementation of its metabolism deficiency in glucose and mannose, respectively. In this study, we constructed *E. coli* strains that carry 1 to 3 copies of the *lacZ* gene on the chromosome. Beta-galactosidase (β-gal) expressions of these newly constructed strains were investigated to examine the effects of chromosomal gene dosage on protein expression. We also analyzed the genetic stability of HTC3Z, the 3-copy *lacZ* strain, in serial dilution culture that simulated repeated fed-batch culture [14] during protein overexpression.

MATERIALS AND METHODS

1. Cloning Strategy

Bacterial strains and plasmids used in this work are listed in Table 1. An illustration of our cloning strategy is shown in Fig. 1. To prepare for each linear cassette, we first constructed the recombinant plasmids pMIL, pTGL and pTML, which carry *tet*^{R}-*lacZ*, *glk-lacZ* and *manXYZ-lacZ*, respectively. The construction details are mentioned in the section Plasmids, the next paragraph. Using homologous primers (Table 2), these cassettes were amplified by PCR reaction with the relevant plasmid as template. Linear transformation was

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Table 1. Strains and plasmids used in this research

Strain	Genotype	Source or reference
ZSC114	*glk-7,manZ11, lacZ827(UGA),relA1*	CGSC[a]
HTC1Z	*glk-7,manZ11,relA1, tet*^{R}, 1-copy *lacZ*	This study
HTC2Z	*manZ11,relA1, tet*^{R}, 2-copy *lacZ*	This study
HTC3Z	*relA1, tet*^{R}, 3-copy *lacZ*	This study
VJS632	Wild Type	YP Chao [24]
Plasmid		
pKD46	*amp*^{R}*, beta,exo,gam*(lambda red),*ori*^{ts}	CGSC[b]
pMC1871	*tet*^{R},*lacZ*(without promoter)	CGSC[c]
pTRL01	*amp*^{R},*lacI*^{Q},*lacZ,rpoD*	This study
pJL	*amp*^{R},*lacI*^{Q},*lacZ*	This study
pTGL	*amp*^{R},*glk,lacZ*	This study
pTML	*amp*^{R},*manXYZ,lacZ*	This study
pMIL	*lacI*^{Q},*lacZ,tet*^{R}	This study

CGSC: *E. coli* Genetic Stock Center
[a]CGSC#: 5458; ID#: 5722; from W. Epstein [25].
[b]CGSC#: 7669; ID#: 64705; from B.L. Wanner [26].
[c]From H. Smith (Germino and Bastia 1984).

carried out as described in a later section. The *E. coli* strain ZSC114 that carries three auxotrophic gene mutations was used as the original host cell. ZSC114 is not resistant to tetracycline and it cannot grow on a tetracycline plate. We employed the λ Red linear transformation system to replace the *lacZ827*, the *lacZ* mutant on ZSC114, with the linear DNA *tet*^{R}*-lacZ*, and the transformant was selected by its tetracycline resistance phenotype. ZSC114 has other mutations, *glk-7* and *manZ11* (Table 1); hence it cannot grow on the plate that contains glucose or mannose as the sole carbon source. The mutated *glk* and *manZ* genes were replaced with functional DNA, *glk-lacZ* and *manXYZ-lacZ*. The transformants were selected on a glucose-only or mannose-only M9 plate. The order of linear transformation was first *tet*^{R}*-lacZ*, then *glk-lacZ*, and last *manXYZ-lacZ*. The constructed strains with one to three copies of the *lacZ* gene are listed in Table 1.

2. Plasmids

Plasmid pKD46, which carries λ *Red* genes (*exo*, *bet*, *gam*), was obtained from the *E. coli* Genetic Stock Center (CGSC, Yale University, New Haven, CT). Plasmids pMIL, pTGL and pTML were constructed to prepare linear DNA, and the cloning scheme was as follows (Fig. 1). Plasmids pMC1871 and pTRL01 were used to construct pMIL, which contains *tet*^{R}*-lacZ*. A 5,093-bp *lacI*^{Q}*-lacZ* fragment was amplified by PCR by using pTRL01 as template and lacp1 and lacp2 as primers (Table 2). The PCR product and plasmid pMC1871 were then cut with restriction enzymes PstI and ScaI, ligated with T4 ligase, and transformed into *E. coli* strain ZSC114. A tetracycline-containing LB plate was used to select for the transformant.

The *glk-lacZ*-containing plasmid, pTGL, was constructed by using plasmid pTRL01 and *E. coli* strain VJS632. An 1,169-bp *glk* fragment was amplified by PCR with VJS632 as template and glkp1 and glkp2 as primers (Table 2). The PCR product and plasmid pTRL01 were then cut with restriction enzymes NarI and SphI, ligated with T4 ligase, and transformed into *E. coli* strain ZSC114. An ampicillin-containing (200 μg/ml) LB plate was used to select for the transformant. Plasmid pTML containing *manXYZ-lacZ* was also constructed with plasmid pTRL01 and *E. coli* strain VJS632. A 3,058-bp *manXYZ* fragment was amplified by PCR with VJS632 as a template and manp1 and manp2 as primers (Table 2). The PCR product and plasmid pTRL01 were then cut with restriction enzymes NarI and SphI, ligated with T4 ligase, and transformed into *E. coli* strain ZSC114. An ampicillin-containing (200 μg/ml) LB plate was used to select for the transformant. Plasmid pJL, containing the wild-type *lacZ* gene from the chromosome of *E. coli* strain VJS632, was transformed into ZSC114 as a plasmid expression system for genetic stability comparison with HTC3Z.

3. Preparation of Genomic or Linear DNA

Purification and manipulation of DNA (Miniprep Kit, Gene-Spin), along with genomic DNA preparation (DNeasy Tissue Kit, Qiagen), and transformation were executed by standard procedures [15,16] or the manufacturer's instructions. All restriction enzymes and Taq polymerase were from Promega Corp. T4 DNA ligase was from New England Biolabs. Standard PCR conditions were set to execute the amplification procedure. Plasmids pMIL, pTGL and pTML were used as PCR templates for *tet*^{R}*-lacZ*, *glk-lacZ* and *manXYZ-lacZ*, respectively. All primers used in this work are listed in Table 2. PCR products were gel-purified and stored at −20 °C.

4. Linear Transformation

The λ Red system used in this study is expressed from plasmid pKD46 (Table 1). λ Red genes (*exo*, *bet* and *gam*) are regulated by the P_{BAD} promoter, which is inducible by L-arabinose. Further, *repA101* makes pKD46 temperature sensitive, so it cannot exist at higher temperature (37-42 °C). In order to execute linear transformation, pKD46 was transformed into the following strains: ZSC114 (*lacZ* mutation), HTC1Z (1 copy of *lacZ*) and HTC2Z (two copies of *lacZ*). The *E. coli* strain carrying plasmid pKD46 was cultured overnight at 30 °C and then diluted 100-fold in 20 ml LB medium with ampicillin. Subsequently, the culture was grown at 30 °C in a shaking bath. When the cell culture reached an OD_{550} of 0.5, 1 mM L-arabinose was added to induce the *Red* genes expression. After induction for 1 hr, the cells were harvested by centrifugation and made

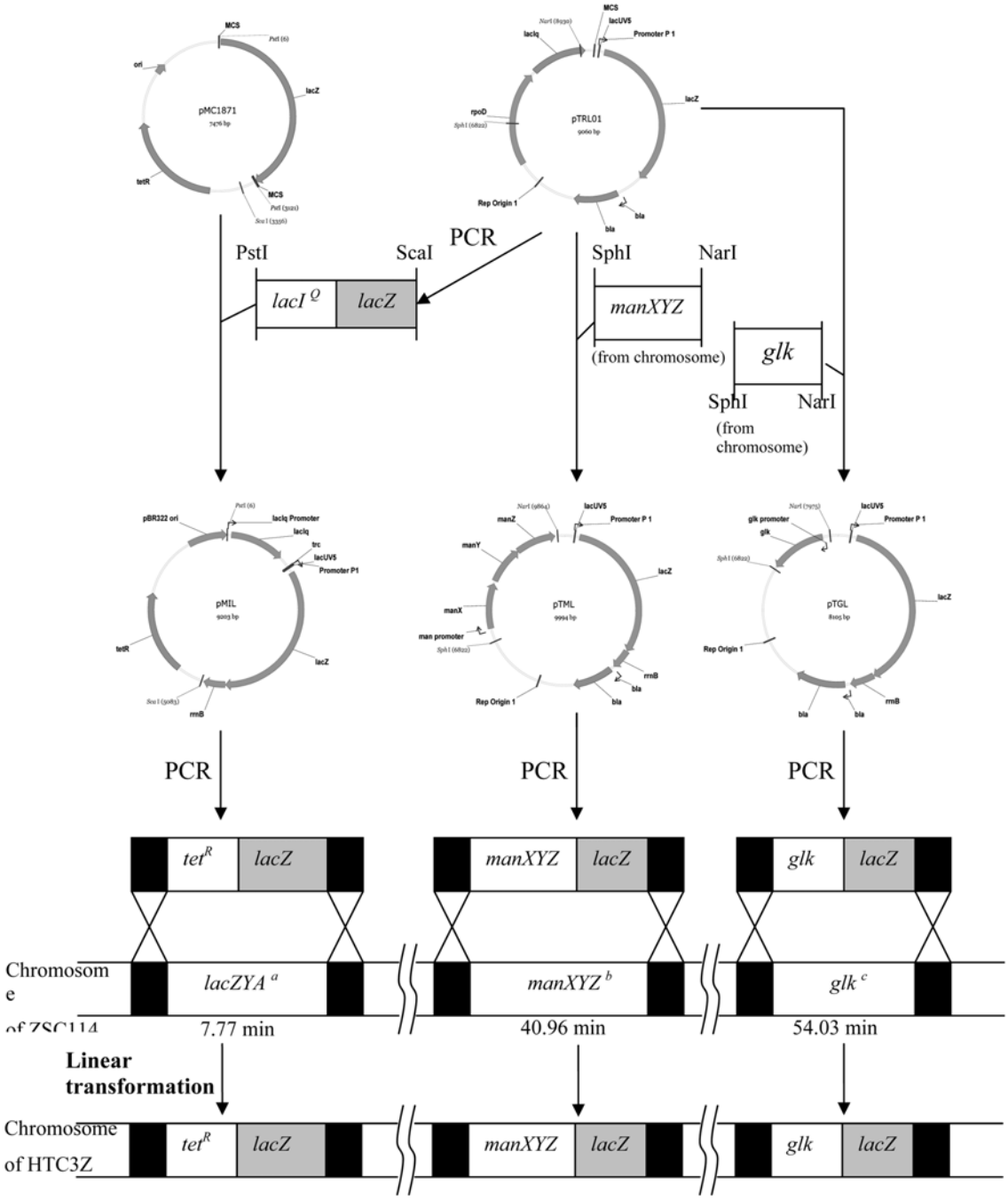

Fig. 1. The scheme of cloning strategy.
a. *lacZ* on the chromosome of ZSC114 is *lacZ827* (UGA). b. *glk* on the chromosome of ZSC114 is *glk-7*. c. *manZ* on the chromosome of ZSC114 is *manZ11*. d. "min" denotes the location at which the gene is situated on the chromosome.

electrocompetent by washing three times with deionized water. 2-5 μl of linear DNA was electroporated into the host cell. Electroporation was done under the conditions previously described [15]. Shocked cells were added to 1ml ice-cold LB and incubated at 37 ºC for 1-2 hr. The cells were centrifuged, resuspended and spread onto an LB plate containing tetracycline (50 μg/ml) or an M9 plate containing an appropriate sugar (0.4%) component to select for the transformants.

5. β-Galactosidase Assay

β-Galactosidase assay was performed as described before [17,18]. 100 μl of cell culture was mixed with 900 μl of Z buffer and 200 μl of chromogenic substrate O-nitrophenyl-β-D-galactopyranoside (ONPG). The intensity of the orange color was observed during the assay, and 500 μl of 1 M Na_2CO_3 was later added to stop the reaction. The enzyme activity of β-gal was measured in terms of Miller units.

6. Genetic Stability Analysis

Plasmid-containing cells were identified by replica plating as previously described [19]. Serial dilution culture was used to analyze the long-term genetic stability of the chromosome (HTC3Z) and

Table 2. Primers used in this research

Primer	Sequence	Product
gene cloning		
lacp1	AAGGCTGCAGTACGTTGACACCATCGA	$lacI^Q$,lacZ
lacp2	GGCGAGTACTGCGGATACATATTTGAA	$lacI^Q$,lacZ
glkp1	ACGTGCATGCTATGATTTAAAAGATTATCG	glk
glkp2	ATGTGGCGCCTGAAACGATAAAGT	glk
manp1	CCTTGCATGCTTAAACGGGAGTTAAAC	manXYZ
manp2	ATCAGGCGCCATTAATCCTCCAGAT	manXYZ
linear cassette		
tet-lacZ p1	CTGATAAGCGCAGCGTATCAGGCAATTTT TATAATGGTTTGACAGCTTATCA	tet^R-lacZ
tet-lacZ p2	ATGCATTTACGTTGACACCATCGAATGGCG CAAAAACATAATGGTGCAGGGC	tet^R-lacZ
glk-lacZ p1	AAGAAAAATTGAATAAACTGTATGATTTAA AAGATCGGTTCTGAGCTTTCCC	glk-lacZ
glk-lacZ p2	CGATAAAGTAATTGTGTGACCCAGATCGAT ATTTAACATATTTGAATGTATT	glk-lacZ
man-lacZ p1	ATCACATAAAATAATTTTTTCGATATCTAAA ATAACGGTTCTGAGCTTTCCC	manXYZ-lacZ
man-lacZ p2	AAAGAATCAGTACCAGGTCCGTGATTGTCA TTAATACATATTTGAATGTATT	manXYZ-lacZ

Note: Letters underlined denote homologous sequence

plasmid (ZSC114/pJL) expression systems. Overnight culture from an isolated colony of each strain was subcultured at an initial OD_{550} as 0.05 at 37 °C. One hour after subculture, IPTG was added. Samples were taken and β-galactosidase activities were measured at every 2 h after IPTG addition. The culture was diluted back to OD_{550} as 0.1 every four hours by using the exact same medium with the same concentration of IPTG. The process lasted 60 hr to examine the long-term genetic stability of HTC3Z and ZSC114/pJL.

RESULTS

1. Confirmation of Recombinants

The cloning strategy is demonstrated in Fig. 1, and the construction of *lacZ*-carrying strains is described in the section on Materials and Methods. To verify the constructions, the chromosomes of three *lacZ*-carrying strains were severed as templates for PCR with suitable primers (Table 2). The sizes of linear DNA *tet*^{R}*-lacZ*, *glk-lacZ* and *manXYZ-lacZ* were 6,118, 5,492, and 7,381, respectively. The PCR products for each strain were at the proposed position of the DNA gel after electrophoresis (Fig. 2A). All of the strains were tested by using the proper plates. For example, the strain HTC2Z can grow on a glucose-only M9 plate but not on a mannose-only M9 plate (data not shown). For further verification, SDS-PAGE gel electrophoresis was performed, and the protein production of each strain is shown in Fig. 2B. For the control, a *lacZ*-containing plasmid pJL (Table 1) was transformed into the original host ZSC114 as a plasmid expression system. If necessary, 300 μM IPTG was added to each shaking cell culture after 1hr subculture. Samples were harvested 4 hours after induction. Without induction, there is no significant LacZ-production from each strain. This indicates that the regulation is tight. With 300 μM IPTG induction, the expression of β-galactosidase (116 kDa) increased greatly. Based on these data, we infer that the 1-3 copy *lacZ*-carrying recombinant strains was successfully constructed.

2. The β-Galactosidase Production in Batch Culture

To observe the effect of genetic copy number on protein expression, the strains ZSC114, HTC1Z, HTC2Z, and HTC3Z were cultured separately, and β-galactosidase assay was carried out for each strain (Fig. 3). The initial optical density at 600 nm (OD_{600}) for each of the mentioned strains was 0.05 in Luria-Bertani (LB) medium [15]. IPTG (300 μM) was added one hour after subculture. The growth and productivity curves of typical ZSC114, HTC1Z, HTC2Z, and HTC3Z cultures are shown in Fig. 3. Reasonably, there is no β-gal activity in the original host strain ZSC114. After induction, strains HTC1Z (1-copy *lacZ*) and HTC2Z (2-copy *lacZ*) produced around 650 and 1,200 Miller units of β-galactosidase, respectively. However, the β-gal expression of the strain HTC3Z (3-copy *lacZ*) continued to accumulate to more than 10,000 Miller units with a maximum of 13,000. Fig. 3 also shows the growth curve of strain HTC3Z in the experiment. The growth curves of strains HTC1Z and HTC2Z are the same as that of strain HTC3Z (data not shown). The OD_{600} was measured per hour as the concentration of living cells. The β-gal activity was the highest for HTC3Z when the cells entered the stationary phase. It has been known that the host/plasmid system, on the basis of a high copy number (10 or more), has a high level of protein expression. Interestingly, based on Fig. 3, it appears that the multi-copy-gene expression from the chromosome, even at a low copy number like that of 3-copy *lacZ*, may cause the accumulation of gene products and may also be an effective production strategy.

3. Long-Term Expression of LacZ Protein

Serial dilution culture was used to analyze the long-term genetic stability of the chromosome (HTC3Z) and plasmid (ZSC114/pJL)

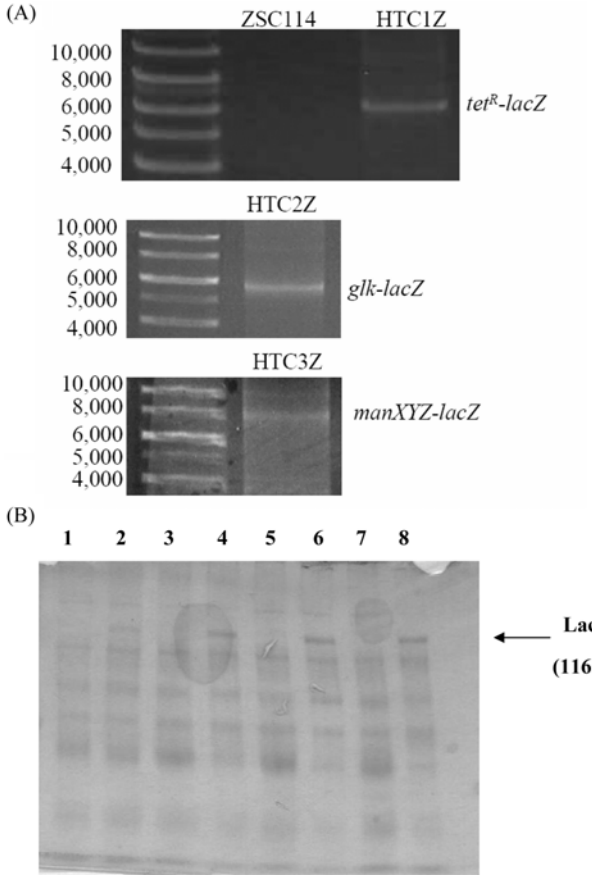

Fig. 2. (A) Agarose-gel analysis and (B) SDS-PAGE analysis for cloning verification. (A) Eectrophoresis was performed in 0.7% agarose gel. The primers used for each linear DNA fragment are listed in Table 2. (B) Lanes 1, 2: strain HTC1Z; Lanes 3, 4: HTC2Z; Lanes 5, 6: HTC3Z; Lanes 7, 8: ZSC114/pJL. Laness 1, 3, 5, and 7 correspond to a sample without induction, and lanes 2, 4, 6, and 8 correspond to a sample with 300 μM IPTG induction. Culture medium: LB; Harvest point: 4 h after induction.

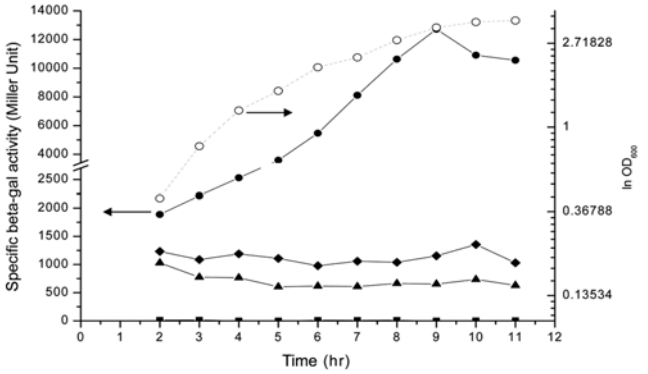

Fig. 3. β-Galactosidase activity assay. Solid symbols stand for β-galactosidase activity, and empty symbols stand for OD_{600} value (the right y-axis). One hour after subculture, 300 μM IPTG was added. Symbol: ■: ZSC114; ▲: HTC1Z; ◆: HTC2Z; ●: HTC3Z. Culture medium: LB broth. Culture temperature: 37 °C.

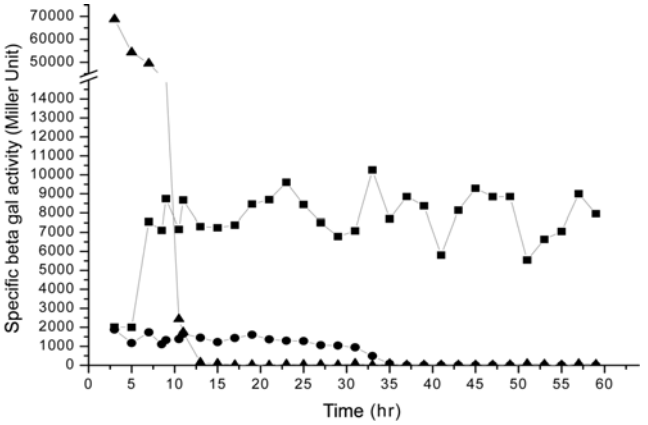

Fig. 4. Genetic stability of ZSCGMtZ and ZSC114/pJL. Symbol: ■: HTC3Z with 300 μM IPTG induction; ●: ZSC114/pJL with 5 μM IPTG induction; ▲: ZSC114/pJL with 300 μM IPTG induction. Culture medium: LB broth; Culture temperature: 37 °C.

expression systems. The procedures are described in the section on Materials and Methods. As shown in Fig. 4, at the beginning, a high activity of about 70,000 Miller units was observed in ZSC114/pJL with 300 μM IPTG induction, but it soon plunged to zero at the 13th hour, and the ratio of plasmid-containing cells dropped to zero at the same time (data not shown). ZSC114/pJL with 5 μM induction had a longer duration of expression at a lower level of 1,500 Miller units, and also dropped to nothing at the 34th hr. The initial specific β-gal activities are about the same when ZSC114/pJL is induced by 5 μM IPTG and HTC3Z is induced by 300 μM IPTG. The 3-copy *lacZ* strain HTC3Z, however, maintained a stable expression at 6,000-8,000 Miller units through at least 60 hours. While protein overexpression is widely known as the main cause of plasmid instability [19], these data suggest that it may not be a problem in the chromosomal expression system.

DISCUSSION

Since the first report by Murphy [20], the λ Red system has been an efficient homologous recombination technique and has been extensively used in *Escherichia coli*. This procedure is applicable to any *E. coli* strain and is inducible by L-arabinose [21]. For application, this genetic technique has been used to create genetic mutants for analysis of functionally unknown genes [22]. In the genetic engineering field, the development of such a highly efficient system is very crucial because it overcomes the difficulty in executing gene replacement in *E. coli*. Here, we adopted the λ Red system to build a multicopy *lacZ* expression *E. coli* strain and observed the effect of the genetic copy number on protein expression by chromosomes. Our results show that the 3-copy *lacZ*-carrying strain had a sustainable β-gal expression at 6,000-8,000 Miller units.

The specific β-galactosidase activities of strains containing a different copy number of *lacZ* are very different as shown in Fig. 3. The specific activity of the 3-copy *lacZ* strain HTC3Z increased along with time when the specific activities of the 1-copy *lacZ* strain HTC1Z and the 2-copy *lacZ* strain HTC2Z remained the same. This

phenomenon indicates that the LacZ protein is accumulated in the 3-copy *lacZ* strain HTC3Z. In the study presented by Srinivasan et al. [5], all of the 1-3 copy chromosomal OPH strains showed OPH accumulation. It has been known that recombinant protein would be accumulated in the host cell if it had been greatly induced. Many high copy number plasmids possess a great capacity for protein overexpression. With a strong promoter, a single-copy plasmid causes 47,500 Miller units of β-gal activity [23]. Furthermore, the maximum β-gal activity of the 3-copy *lacZ* strain (13,000 Miller units) is much lower than that of the plasmid expression system, ZSC114/pJL (70,000 Miller units) whose plasmid copy number is 20. Hence, the production level may be further enhanced by more *lacZ* gene integration or a stronger promoter replacement for *lacZ*.

In protein production, another topic to be addressed is genetic stability. As shown in Fig. 4, with the same induction concentration (300 μM of IPTG), the expression level of HTC3Z is not as high as that of the common host/plasmid system at the beginning, but it has a significantly longer duration time for LacZ protein expression in the serial dilution experiment. With lower IPTG concentration, the expression level of the host/plasmid system is lower at the beginning, but it lasts longer compared with higher IPTG concentration. However, it drops to zero while the expression level of HTC3Z still remains the same. In other words, the recombinant strain HTC3Z has better performance in long-term genetic stability. The HTC3Z strain could maintain a stable expression at 6,000-8,000 Miller units through at least 60 hours. This indicates that the chromosomal expression system established in the present study has a great potential to be used in long-term, repeated fed-batch protein production.

In summary, the present work shows that the multiple-copy gene integrated strain constructed by λ Red homologous recombination caused an enhanced and durable expression of recombinant protein β-galactosidase. Hence, based on these results, we are now attempting to build more copies of the recombinant gene into the bacterial chromosome to further investigate the effect of gene dosage on recombinant protein production and to establish an optimum copy number expression system in *Escherichia coli*.

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