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Full Length Research Paper

Generation of *gua1* deletion using polymerase chain reaction (PCR)-mediated gene disruption method in fission yeast, *Schizosaccharomyces pombe*

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***Gua1* gene in *Schizosaccharomyces pombe* encodes inosine 5′-monophosphate dehydrogenase (IMPDH), which catalyzes the first step in *de novo* biosynthesis of guanosine monophosphate (GMP). Knock-out cassette was constructed with polymerase chain reaction (PCR)-based gene targeting technique for deletion of *gua1* gene in *S. pombe* and this knock-out cassette was transformed to *S.s pombe* wild type (*972h*$^-$). Knock-out cassette contains 653 and 285 bp sequences which are upstream and downstream of *gua1* gene, respectively, in *S. pombe* genome and kanamycin resistance gene obtained from *pFA6* plasmid. After transformation using lithium acetate method, knock-out cassette is aimed at replacing with the sequences of *gua1* gene via homologous recombination. The transformant *972h*$^-$ colonies which integrated knock-out cassette to the genome via homologous recombination are selected in YEA medium with antibiotic G418 after transformation and in this step, possible mutant colonies in *gua1* gene were determined. Finally, colony PCR techniques were performed to control whether the deletion is made in the right place or not. The results show that the *gua1* gene deleted strain was obtained.**

Key words: Fission yeast, deletion, *gua1* gene, knock-out cassette, inosine 5′-monophosphate dehydrogenase (IMPDH).

INTRODUCTION

Inosine 5′-monophosphate dehydrogenase (IMPDH) is the key enzyme in *de novo* biosynthesis pathway of purine nucleotides. The role of IMPDH at metabolic branch point in *de novo* biosynthesis pathway of purine nucleotides makes IMPDH a target for drug discovery for immunosuppression (Ratcliffe, 2006), cancer (Chen and Pankiewicz, 2007; Oláh et al., 2006), antiviral (Nair and Shu, 2007) and antimicrobial chemotherapy (Shu and Nair, 2008). *Schizosaccharomyces pombe* is a free living unicellular fungus which has common features with

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Table 1. Primer sequences and size of products.

Primer	Outside Primer	Inside Primer	Product size (bp)
Upstream primers	tgcagcaaggaaaccatatcactgg	***ttaacccggggatccgtcgac***tttaggccgcttggggtgtg	653
Downstream primers	ccgaagactcgacaaagcctcag	***atgaatcggccaacgcgcgg***tgcattacgaacgaattgtgcaag	285

*Sequences are homologous to kanamycin resistance gene (bold type).

higher eukaryotic organisms and characterized with properties of Ascomycetes (Wood et al., 2002). IMPDH is a product of *gua1* gene in *S. pombe* and this gene is located near the centromeric region of chromosome II (Karaer et al., 2006).

Knock-out technology is carried out successfully in different organisms from single-celled eukaryotic organisms to mammalian including human cells (Kim et al., 2010; Shen et al., 2015; Rong and Golic, 2000). This technique is based on gene targeting and it modifies the genome of the living organism via homologous recombination. In gene targeting with homologous recombination technique, exogen DNA fragments (plasmid, cassette) produced *in vitro* are transformed into the cell in order to generate homologous recombination between target sequence and exogen DNA fragment which causes desired genetic change (Sawitzke et al., 2013; Gardner and Jaspersen, 2014).

Knock-out technology has made feasible research initiatives such as *Saccharomyces* Genome Deletion Project (http://www-sequence.stanford.edu/group/ yeast_deletion_ project/deletions3.html) which aimed to bring about a complete set of yeast deletion strains. Assigning specific functions to all open reading frames (ORFs) in one organism through phenotypic analysis of the mutants is the most common purpose of knock-out studies.

Korean BIONEER Corporation attempted to create a knock-out library for all *S. pombe* genes (http://pombe.bioneer.co.kr). The study aimed to obtain haploid mutant *S. pombe* with *gua1* deletion, which does not take part in mutant collection of BIONEER. In this study, this cassette was similar to the modular kanMX6 cassette system described by Hentges et al. (2005) and Palabiyik et al. (2016). The lengths of homologous flanking sequences (60 to 80 bp) to the target gene were shorter in these studies, but our cassette had long sequences (653 to 285 bp) flanking to the *gua1* gene. The *gua1* gene deletion strain was obtained, although the strain is not viable for long time, as seen in other studies (Jiang et al., 2010).

MATERIALS AND METHODS

Strains, media, growth conditions

S. pombe Linder str. *Liquefaciens* haploid wild type (*972h-*) and *Escherichia coli DH5α* strain containing *pFA6-KanMX4* plasmid were used and these organisms were obtained from Istanbul University Department of Molecular Biology and Genetics Laboratory collection.

In this study, media which were described by Gutz et al. (1974) were used for production of *S. pombe* cells. *S. pombe* cells were produced in yeast extract with supplements (YES) media (supplemented with adenine, histidine, leucine, uracil, lysine and guanine at the concentration of 50 mg/L for each), yeast extract agar (YEA), minimal media agar (MMA). Luria Bertani (LB) media with ampicillin (50 mg/L) was used for *E. coli* containing *pFA6-KanMX4* plasmid.

Isolation of *pFA6-KanMX4* plasmid from *E. coli DH5α* strain and genomic DNA from *S. pombe*

E. coli DH5α strain containing *pFA6-KanMX4* plasmid was grown overnight using LB media broth with ampicillin (50 mg/L) at 37°C and 150 rpm. Isolation of *pFA6-KanMX4* plasmid was performed according to the manufacturer's instructions (DSBIO Eco Friendly Plasmid Miniprep Kit).

972h- was grown in yeast extract agar plate 2 days at 30°C. Then, 1x 10^6 cells/mL were incubated overnight in yeast extract media at 30°C and 180 rpm. The isolation of genomic DNA from these cells was performed with DSBIO Quick Yeast Genomic DNA Extraction Kit according to the manufacturer's instructions. Mechanical disruption was carried out with 0.3 g 0.45-0.55 mm glass beads in dismembrator (Sartorius Mikro-Dismembrator S) at 3000 rpm for 1 min and this step is repeated 5 times for each sample.

Production of knock-out cassette

Primers which include both homologous sequences for downstream and upstream sequences of *gua1* gene and short sequences that are homologous to kanamycin resistance gene are designed as shown at Table 1.

Knock-out cassette was obtained in two steps. In the first PCR, genomic DNA of *S. pombe* was used as a template and DNA fragments sized 652 and 285 bp were obtained. After purification, DNA fragments were used together for second PCR in which *pFA6-KanMX4* plasmid was used as a template and outside primers. Knock-out cassette was observed by using agarose gel electrophoresis and gel extraction of cassette was carried out by Roche High Pure PCR Product Purification Kit.

Control of knock-out cassette with DNA sequence analysis

Upstream-outside primer of knock-out cassette and a reverse primer (inside primer) which complements kanamycin resistance gene (Table 2) were used in DNA sequence analysis. Sequence analysis was carried out with Sanger method by using 3500XL Genetic Analyzers (Applied Biosystems).

Transformation of knock-out cassette to *S. pombe*

Transformation of knock-out cassette to *S. pombe 972h-* wild type

Table 2. Primers which was used in nucleotide sequence analysis of knock-out cassette.

Primer	Sequences of primer
Upstream-outside primer	tgcagcaaggaaaccatatcactgg
İnside primer	ttgcccgacattatcgcgag

Table 3. Primers which was used in colony PCR and size of products.

	Forward primer	Reverse primer	Product size
gua1	gggctgcagatgtctgcctttaagcc	gaagatctagtaaagacgcttttc	~ 1.5 kb
kan^r	atgggtaaggaaaagactcacg	ttagaaaaactcatcgagca	~1 kb

strain was carried out using lithium acetate method according to the method of Gregan et al. (2006). Solutions in transformation procedure (LiAc solution (0.1 M LiAc, 1X TE (pH 7.5)), LiAc-PEG solution (0,1 M LiAc, 40% PEG 3350, 1X TE (pH 7)) were sterilized by autoclave for 20 min at 121°C and 1.2 atm pressure.

Selection of transformant colonies

After the transformation of knock-out cassette to wild type *S. pombe,* for the detection of the transformant cells, these cells were inoculated into yeast extract agar plate with antibiotic G418 (200 mg/ml) (Sigma G418 disulfate salt) and incubated at 32°C for 5 days. Then resistant *972h*$^-$ cells to antibiotic G418 were inoculated separately into selective media (YEA, YEA with G418, MMA and MMA with guanine) at the same time and incubated at 32°C for 5 days.

Control of transformant colonies

Colonies which grown with G418 were used as templates in PCR in order to determine the homologous recombination and colony PCR was separately performed with primers which are suitable for kanamycin resistance and *gua1* genes (Table 3).

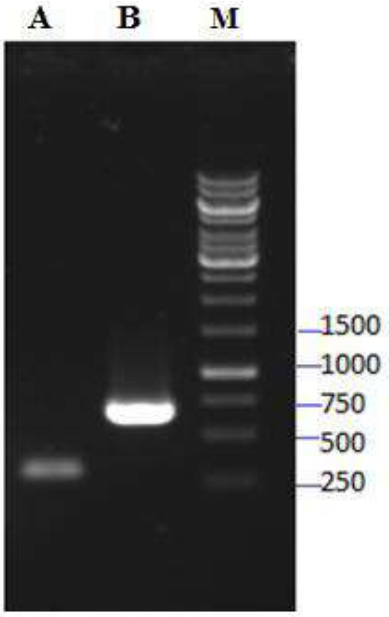

Figure 1. A. Downstream flanking region of *gua1* (285 bp). **B.** Upstream flanking region of *gua1* (653bp). M. Thermo Scientific™ O'GeneRuler 1 kb DNA Ladder.

RESULTS

Production of knock-out cassette

In the genome of *S. pombe* 653 and 285 bp homologous sequences which is located upstream and downstream flanking regions of gua1 gene respectively were amplified by two separate PCR (Figure 1). Knock-out cassette (2.5 kb) which includes kanamycin resistance gene was obtained at the second PCR (Figure 2).

Control of knock-out cassette with DNA sequence analysis

Results of DNA sequencing was confirmed with BLAST analysis in National Center for Biotechnology Information (NCBI). Sequencing results of knock-out cassette were compared with *S. pombe 972h*$^-$ genome and *pFA6-KanMX4* plasmid. Outcomes of sequencing which carried out with upstream-outside forward primer were compared with genome of *S. pombe 972h*- and it was observed that this regions showed at the rate of 95% identification with downstream and upstream regions of *gua1* gene.

Sequencing with inside reverse primer of kanamycin resistance gene was compared with *pFA6-KanMX4* plasmid. According to the result of BLAST analysis, it was observed at the rate of 99% identification to kanamycin resistance gene and expectation value was found to be “0.0”.

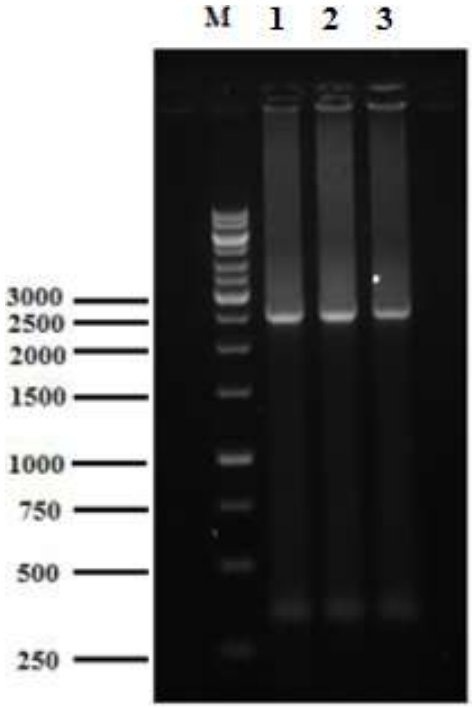

Figure 2. Agarose gel electrophoresis of knock-out cassette. 1, 2, 3, Knock-out cassette. M, Thermo Scientific™ O'GeneRuler 1 kb DNA ladder.

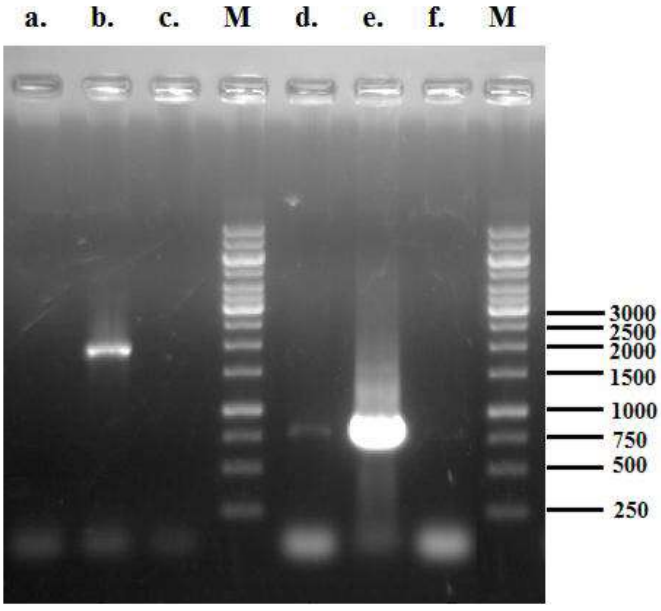

Figure 3. Colony PCR images of *gua1* mutant. a. PCR product with primers of *gua1* gene in *gua1* mutant colony; b. PCR product with primers of *gua1* gene by using *972h*$^{-}$ genomic DNA; c. No template with primers of *gua1* gene; d. PCR product with primers of *kan*^{r} gene in *gua1* mutant colony; e. PCR product with primers of *kan*^{r} gene by using *pFA6-KanMX4* plasmid DNA; f. No template with primers of *kan*^{r} gene; M. Thermo Scientific™ O'GeneRuler 1 kb DNA Ladder.

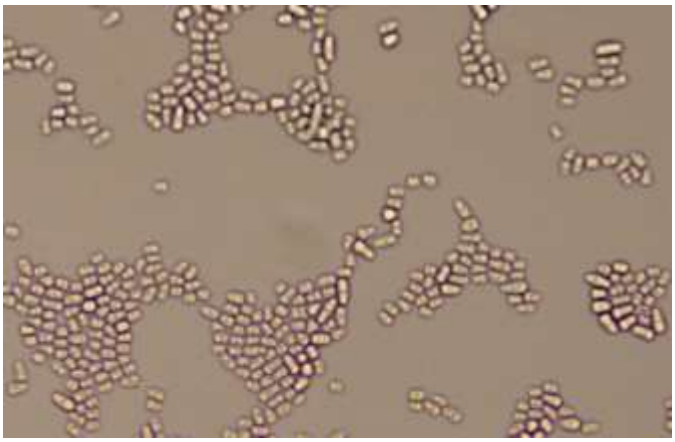

Figure 4. Images of putative mutant cells under fluorecent microscope.

Observation of null *gua1* mutant of *S. pombe*

24 independent transformation experiments with the knock-out cassette yield, 3604 positive colonies which were obtained according to kanamycin resistance, was carried out. Subsequently, colonies were inoculated into YEA, MMA, YEA with G418 and MMA with guanine in order to confirm whether cassette was inserted to *gua1* gene region or not. 56 colonies which could not live in media without guanine were detected after incubation for 5 days at 32°C.

After colony PCR with these 56 transformant colonies and primers which are appropriate to *gua1* and *kan*^{r} gene, samples were analyzed on agarose gel electrophoresis. In 55 colonies, it was observed that insertion of knock-out cassette into the location of *gua1* gene was unsuccessful. *Gua1* gene fragment does not exist in only one colony but a band with primers of *kan*^{r} gene in the PCR of this colony (Figure 3).

Observation of *gua1* deletion mutant cells using the phase-contrast microscope

When *gua1* deletion mutant cells (Figure 4) and wild type *S. pombe* cells (Figure 5) were investigated under the fluorescent microscope, it was seen that mutant cells were smaller than wild type and mutant cells tend to be aggregate.

DISCUSSION

Over the past 60 years, *S. pombe* has become an important model organism along with *S. cerevisiae* to study the molecular biology of eukaryotes (Hoffman et al., 2015). Yeasts help to improve comprehension of basic cellular processes and metabolic pathway in human. This

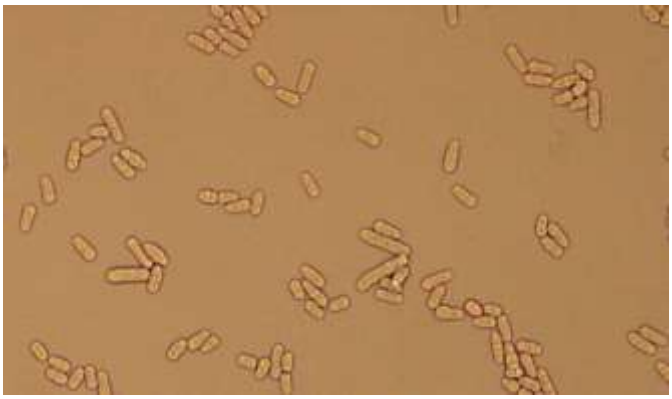

Figure 5. Images of wild type *S. pombe* cells under fluorescent microscope.

organism makes easier molecular analysis of several genes related to diseases (Suter et al., 2006). *S. pombe* is a very convenient model organism for investigating specific cancer pathway, owing to its cell cycle and cell control point mechanisms (Wood et al., 2002).

Deletions of *S. pombe'*s ORFs were carried out at the rate of 98.4% (Kim et al., 2010; Spirek et al., 2010). *Gua1* gene in which deletion was performed in this study was not yet deleted within the scope of the "*S. pombe* Genome Deletion Project". Obtaining a viable *gua1* gene deleted strain will make a contribution regarding the determination of the effect of IMPDH on the various cellular pathway such as cancer and immunosuppression.

In this study, the deletion of *gua1* gene was carried out by creating knock-out cassette via homologous recombination according to Kim et al. (2010). Before transformation knock-out cassette, it was analyzed with sequencing.

Colonies which were phenotypically selected were confirmed by colony PCR. In the results of colony PCR, one single candidate transformant colony from *972h-* showed the expected band profile on the agarose gel electrophoresis. PCR was performed in the candidate *972h-* colony using primer set of *kan*^{r} gene, the band of interest at the correct size was observed. However, no band was shown when PCR was carried out with same colony but using primer sets of *gua1* gene. After the transformation, 56 colonies were reproduced at selective media and it was observed that only one colony was inserted knock-out cassette to the target region in genomes.

When these mutant cells were analyzed microscopically, it was observed that the cells were small and displayed aggregation similar to the results of Fernández-Álvarez et al. (2009). In this study, these mutant colonies were phenotypically more flattened than wild type colonies. Jiang et al. (2010) studied on human fungal pathogen *Candida albicans* and aimed at the deletion of *gua1* gene in *Candida albicans*. They reported that *gua1/gua1* diploid mutant colonies could not live longer than a week at 4°C. Similar to the results of Jiang et al. (2010), it is also observed that these mutant cells with *gua1* deletion are not viable at 4°C for a long time. The authors passaged this mutant cells six times in every other day and inoculated at 30°C, then it was observed that the mutant cells can survive for six or seven generations. This study could be maintained with glycerol stocks of the single colony which was obtained after the transformation. Although, mutant cells with deletion of g*ua1* gene have no long life span, the deletions in their genome are permanent and after some optimization, this mutant strain could be used in other studies.

Decottignies et al. (2003) carried out a pilot gene deletion project in *S. pombe* to determine the number of essential genes. They reported that, obtaining systematic deletion of all *S. pombe* ORFs is more difficult than *S. cerevisiae* when similar studies are compared. The reason is that the homologous recombination efficiency of *S. pombe* is lower than *S. cerevisiae* (Kaur et al., 1997).

In addition, they reported that the genome of *S. pombe* includes some regions which contain inadequately transcribed genes and these genes have closed chromatin structure. The other possible reason for low efficiency may be the deletion cassette integrated into genome but kanamycin resistance gene was deficiently expressed by the reason of heterochromatin structure (Decottignies et al., 2003). The location of a gene in the centromeric region reduces the homologous recombination frequency. In this context, location of the *gua1* gene makes the deletion to be more difficult than the other genes which are in euchromatic region. Finally, data presented here support the fact that the obtained colony is the first mutant strain of *S. pombe* with deletion of *gua1* gene.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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