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Cloning of the hepatitis B surface antigen gene (2): amplification, restriction enzyme digestion and ligation of HBsAg gene and yeast plasmid pPIC9K

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DNA cloning involves separating a specific gene or DNA segment from a larger chromosome, attaching it to a small molecule of carrier DNA, and then replicating this modified DNA thousands or millions of times through both the increase in cell number and the creation of multiple copies of the cloned DNA in each cell. The result is selective amplification of a particular gene or DNA segment. In a recent preliminary study, extraction and purification of the HBsAg gene from genomic DNA of *H. polymorpha* transformant cell, followed by ligation with pGEM-T vector and transformation into the competent *E. coli* DH5 alpha cells were successfully carried out. In this study, the HBsAg gene containing plasmid pGEM-T was amplified by using specific primers with restriction enzyme (RE) digestable nucleotide sequences followed by RE digestion with *EcoR* I and *Not* I to obtain sticky ends for an effective ligation. At the same time, the yeast expression plasmid, pPIC9K was digested with same RE to have sticky ends compatible with those of HBsAg gene containing plasmid. Then, these DNA segments with compatible nucleotide sequences were ligated, followed by successful transformation into the competent *E. coli* cells. The results from each and every step were confirmed by direct PCR identification, RE analysis followed by agarose gel electrophoresis determination and DNA sequencing analysis. These initial steps are important and essential for further transformation of the recombinant HBsAg gene into the host *Pischia pastoris* yeast cell which can be used as a more productive organism for production of recombinant hepatitis B vaccine in the near future.

INTRODUCTION

Recombinant DNA technology, which is also called gene cloning or molecular cloning, allows the recombinant of DNA molecules that would never or very rarely occur in nature, leading to transfer of genetic information from one organism to another. DNA cloning involves separating a specific gene or DNA segment from a larger chromosome, attaching it to a small molecule of carrier DNA, and then replicating this modified DNA thousands or millions of times through both the increase in cell number and the creation of multiple copies of the cloned DNA in each cell. The result is selective amplification of a particular gene or DNA segment [1].

For development of recombinant hepatitis B (HB) vaccines, HB virus recovered from plasma of a hepatitis B carrier has been used to prepare viral DNA that has been cloned in *E. coli*. Then the gene coding for HBsAg has been amplified and isolated. Prior to the transformation of the gene of interest into the yeast cells, plasmid construction has to be performed. Finally, the HBsAg gene has been inserted into yeast by means of appropriate expression yeast vectors. Purified HBsAg proteins expressed from

transformed yeast cells containing it have been formulated into HB vaccines and shown to induce protective antibody response in human [2].

The majority of currently available recombinant hepatitis B vaccines in the market have been produced by using appropriate expression plasmids in yeast cells. Since 1986 to date, *Sacchromyces cerevisiae* and *Hansenula polymorpha* have been used as host systems for commercial production of recombinant HB vaccines. Recently, an increasing number of publications attests to the favorable properties of methylotrophic yeasts as hosts for heterologous gene expression. Among them, *Pichia pastoris* yeast cell is emerging as the most significant industrial application for production of heterologous recombinant proteins [3, 4, 5, 6].

In a recent preliminary study, extraction and purification of the HBsAg gene from genomic DNA of *H. polymorpha* transformant cell, followed by ligation with pGEM-T and transformation into the competent *E. coli* DH5 alpha cells were successfully carried out [7]. In this study, attempts were made to extract the HBsAg gene containing plasmid from transformed *E. coli* cells followed by amplification, restriction enzyme digestion, ligation of the HBsAg gene with the yeast plasmid and transformation into competent *E. coli* cells for further transformation into final host, *Pichia pastoris* yeast cells with an aim to produce recombinant HB vaccine in the near future.

MATERIALS AND METHODS

HBsAg containing plasmid

The HBsAg gene containing pGEM-T plasmid of ~3700 base pairs was obtained from the transformant *E. coli* DH5 alpha cells recently prepared in the laboratory of Research and Development Centre of Pharmaceuticals, Institute of Science and Technology, CJ Corporation, Ichon City, Republic of Korea [7].

Host strains

The E. coli DH5 alpha strain, grown in Luria-Bertani (LB) media supplemented with ampicillin and stored at –20^{o}C was used in cloning technique for plasmid amplification and transformation.

Expression vector

The closed circular vector, pPIC9K of 9276 base pairs (bp), containing the kanamycin resistance gene for *in vivo* screening of multicopy inserts, was commercially available from the Promega Co., USA (Fig.1).

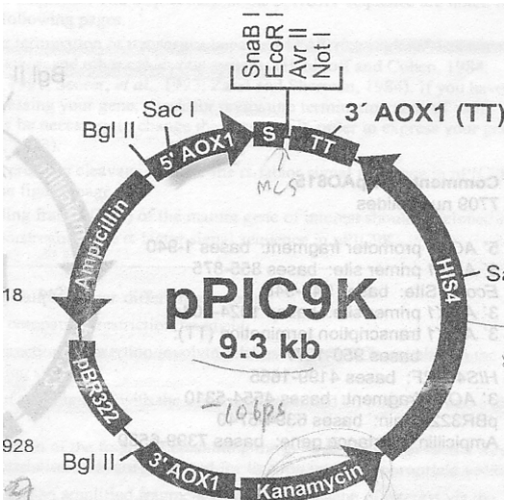


Fig. 1. Physical map of plasmid pPIC9K (976 bp)

Because of the presence of kanamycin (Kan) gene that confers resistance to geneticin in Pichia, only cloned gene integrated in this vector can survive on media with geneticin and it can be used as a tool to detect transformants that habours multicopies of the gene.

Ligation of the HBsAg gene with yeast plasmid vector

Preparation of the HBsAg gene for ligation

A single colony of *E. coli* transformant containing the HBsAg gene in pGEM-T vector (non-linearlized) was inoculated into LB media with ampicillin, followed by extraction of the closed circular recombinant plasmid using the AccuPrep plasmid extraction kit (Bioneer). The recombinant

gene was amplified by PCR using specific primers designed with the desired RE digestible nucleotide sequences :9S (5'-AAA GAA TTC ATG GAG AAC ATC ACA TCA GGA-3') and 35 A (5'-AAA GCG GCC GCT TAA ATG TAT ACC CAA AGA CA–3'). The amplified target recombinant DNA with specific nucleotide sequences was purified by using the PCR purification test kit from QIAGEN, USA [8]. The purified DNA segment was digested at specific nucleotide sequences by restriction enzymes (REs) *EcoR* I and *Not* I from Roche. The complete digestion was confirmed by determination on agarose gel electrophoresis. The specific and linearized RE digested DNA segment of interest with sticky ends was then extracted and purified by using the gel extraction kit (QIAGEN, USA), confirmed by agarose gel electrophoresis and quantified by spectrophotometric determination at the wavelength of 260 nm.

Preparation of yeast plasmid for ligation

A single colony of *E. coli* transformant containing yeast expression vector pPIC9K, which was commercially available from the Invitrogen Life Technologies, was inoculated into the LB media with ampicillin and extracted by using the method of the alkaline lysis with Sodium Dodecyl Sulfate (SDS) followed by RNase treatment [9]. These extracted yeast plasmids were then purified by the Phenol and Chloroform method [10].

The purified closed and circular plasmids containing the desired nucleotide sequences were digested by same RE, used in the treatment of the DNA segment of interest i.e *EcoR* I and *Not* I to obtain the linearized plasmid DNA with sticky ends, and the complete digestion was confirmed by agarose gel electrophoresis. The linearized plasmids were extracted by the gel extraction kit (QIAGEN, USA), followed by treatment with alkaline phosphatase for prevention of self ligation. This non-circularized plasmid was further purified by using the gel extraction kit (QIAGEN, USA) and confirmed by agarose gel electrophoresis and quantified by electrophoretic determination .

Ligation of the HBsAg gene and yeast plasmid pPIC9k

The linearized HBsAg gene and plasmid, both with compatible sticky ends were incubated in the 16^{o}C water bath for 6 hours followed by incubation at 4 ^{o}C over night by using the T4 DNA ligase from Promega, USA for specific DNA ligation [11].

Transformation of the recombinant HBsAg gene containing plasmid into E. coli cells

The above linearized recombinant DNA segment pGMT-T plasmid containing the HBsAg gene was transformed into the competent *E. coli* DH5 alpha cells which had already been prepared in the laboratory, by using the heat-shock procedure i.e. 42 ^{o}C for exactly 90 seconds without shaking [12]. The transformant containing non-linearized recombinant plasmid was identified as appearance of white colonies in a culture plate containing LB medium with ampicillin. A single colony of *E. coli* transformant containing plasmid-insert DNA construct was inoculated into LB media with ampicillin, followed by extraction using the alkaline lysis with SDS method and further purified by the Phenol-Chloroform method. The extracted and purified recombinant DNA was confirmed by RE analysis with *EcoR* I and *Not* I, followed by agarose gel electrophoresis. The presence of the HBsAg structural gene was also confirmed by direct colony PCR (using specific primers 9S and 35A, annealing temperature of 50^{o}C, repeated cycles of 30), followed by agarose gel electrophoresis determination and DNA sequencing analysis by using the ABI Prism 3100 Genetic Analyzer [13].

RESULTS

Fig. 2 represents the electrophoretic determination of the HBsAg DNA segment and the plasmid pPEM-T transformed into *E. coli* cell after restriction enzyme digestion with

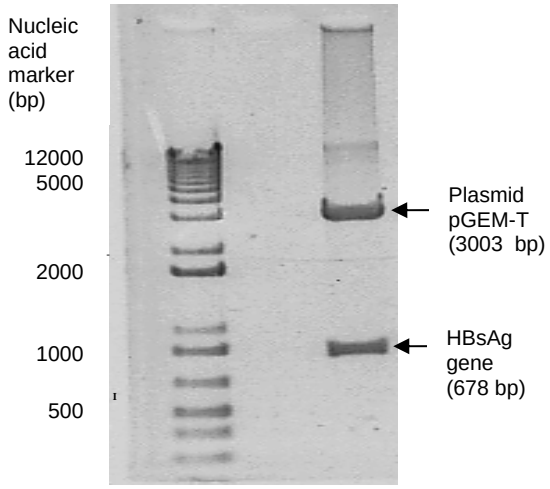
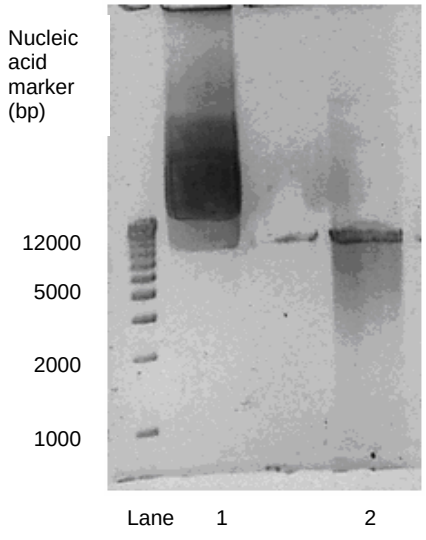


Fig. 2. Electrophoretic determination of HBsAg gene and the plasmid pGEM-T after RE digestion with *EcoR* I and *Not* I

EcoR I and *Not* I. Two distinct DNA bands of the HBsAg gene and pGEM-T vector were detected at levels of ~700 bp and ~3000 bp, respectively, indicating the complete digestion of the plasmid-insert DNA construct by the specific enzyme. Electrophoretic identification of extracted and purified plasmid pPIC9K, before and after digestion with *EcoR* I and *Not* I is shown in Fig. 3.



Lane 1 = Plasmid pPIC9K (linearized) (9276 bp)

Lane 2 = Plasmid pPIC9K (closed circular) (9276 bp)

Fig. 3. Electrophoretic identification of the extracted and purified plasmid pPIC9K before and after RE digestion with *EcoR* I and *Not* I

Both DNA bands were observed at the level of ~9000 bp but the electrophoretic mobility of linearized plasmid was found to be slightly faster than that of the close and circular plasmid. The linearized plasmid with sticky ends was used for further recombination with compatible DNA segment.

Electrophoretic identification of non-circularized plasmid pPIC9K with desired nucleotide sequences after digestion with alkaline phosphatase is shown in Fig. 4. A distinct DNA band was detected at the level of ~10,000 base pairs, indicating the efficient transformation of non-circularized pPIC9K into *E. coli* cells.

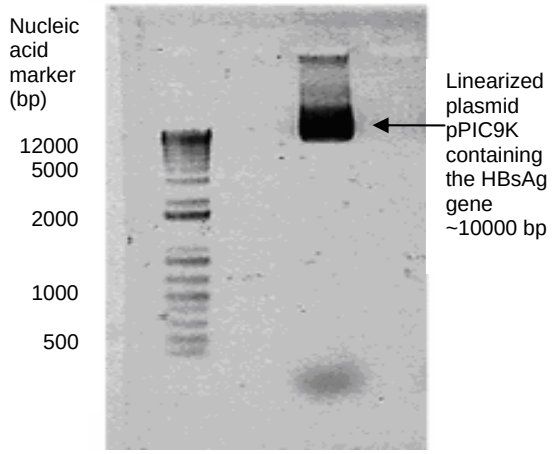


Fig. 4. Electrophoretic identification of linearized plasmid pPIC9K with desired nucleotide sequences after digestion with alkaline phosphatase

Fig. 5 represents the electrophoretic identification of the plasmid-insert DNA construct before and after digestion with *EcoR* I and *Not* I. Only a single band was observed at the level of ~10,000 base pairs before RE digestion whereas two distinct bands were observed at the levels of ~10,000 bp and ~700 bp, thus indicating the presence and complete digestion of the cloned DNA.

Fig. 6 demonstrates agrose gel electrophoresis pattern of the HBsAg gene after the direct colony PCR identification of the HBsAg DNA segment cloned with plasmid pPIC9K vector in the *E. coli*. transformant. The nucleotide sequence of the purified

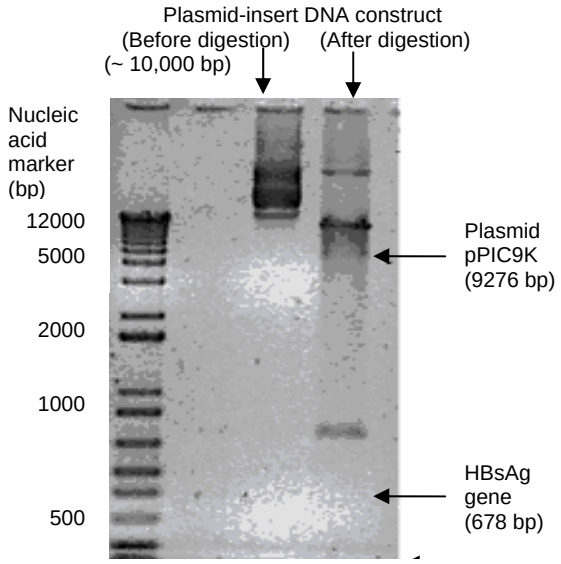


Fig. 5 . Electrophoretic identification of the plasmid-insert DNA construct before and after RE digestion with *EcoR* I and *Not* I

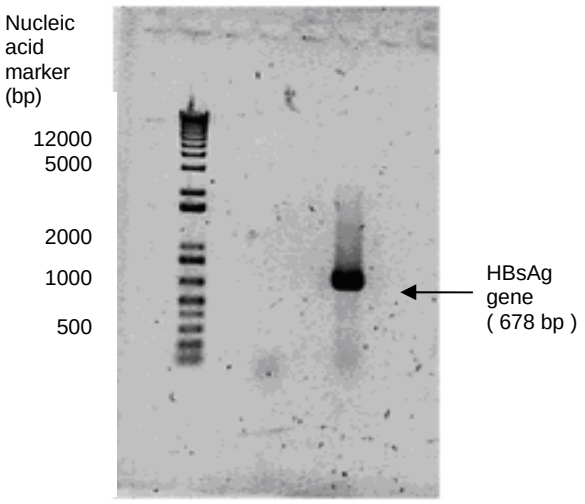


Fig. 6. Direct colony PCR identification of HBsAg gene integrated in plasmid pPIC 9K in *E. coli* transformant

HBsAg segment, extracted and purified from transformat *E. coli* cell was analyzed by the ABI Prism 3100 Genetic Analyzer. It was found that the nucleotide sequences obtained were totally identical to the theoretical sequences of the HBsAg gene, indicating the successful transformation of the HBsAg gene into competent *E. coli* cell after ligation with pPIC9K.

DISCUSSION

Recombinant DNA is an altered DNA due to the insertion of a sequence of deoxy-ribonucleotide not present in an existing molecule of DNA by enzymatic or non-enzymatic means. These DNA fragments, obtained by restriction endonuclease treatment of chromosome, can be cloned . In order to obtain a large amount of a particular fragment of DNA, cloning vehicle plasmid is required. Plasmid is a small extra-chromosomal circular molecule of DNA that replicates independently of the host DNA. Virtually all bacteria genera have plasmids. Some plasmids carry information for their own transfer from one cell to another; others encode resistance to antibiotics; others carry specific sets of genes for the utilization of unusual metabolites; and some have no apparent functional coding genes. Plasmids can range in size from less than 1 kb to more than 500 kb. Each plasmid has a sequence that functions as an origin of DNA replication; without this site, it cannot replicate in a host cell. Replication and amplification of the plasmid containing a gene of interest can be performed in microorganisms. However, some plasmids, because of the specificity of their origin of replication, can only replicate in one specific species. They can be introduced into the bacterial cells by a process called transformation [14].

In this study, the HBsAg gene containing plasmid pGEM-T was amplified by using specific primers with RE digestable nucleo-tide sequences followed by RE digestion with *EcoR* I and *Not* I to obtain a sticky end for an efficient ligation. At the same time, the yeast expression plasmid, pPIC9K was digested with same RE to have sticky ends compatible with those of HBsAg gene containing plasmid. Then, these DNA segments with compatible nucleo-tide sequences were ligated, followed by successful transformation into thecompetent *E. coli* cells. The results obtained from each step were confirmed by direct PCR identi-fication, RE analysis followed by agarose

gel electrophoresis determination and DNA sequencing analysis. Since the yeast plasmid amplification can never be achieved in yeast cells, an initial plasmid construction was performed in *E. coli* cells before transferring into the ultimate host, *Pichia pastoris* yeast cells.

Our study described the effective amplification of the HBsAg gene, complete restriction enzyme digestion, ligation of the HBsAg gene with yeast plasmid, and successful transformation of the plasmid-insert DNA construct into the competent *E. coli* cells. These steps are important and essential for further transformation of the recombinant HBsAg gene into the ultimate host, *Pischia pastoris* yeast cell which can be used for production of recombinant hepatitis B vaccine in the near future. Further research works on transformation of the recombinant HBsAg gene into the *Pichia pastoris* yeast cells are in progress at the laboratory of Research and Development Centre of Pharmaceuticals, Institute of Science and Technology, CJ Corporation, Ichon City, Republic of Korea.

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