Expression Analyses of Methylase Genes of Ppamc125 In Methylating Pbbre194 Prot-CM1 Plasmid In Escherichia Coli DH5α and TOP10

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Abstract. In molecular biological techniques, bacterial transformation is an essential process to strain improvement. However, transformation rate into wild type bacteria is commonly low because of the cell defense mechanism of the bacteria. Restriction modification (RM) in bacteria cells can prevent the introduction of recombinant plasmids into target bacteria. Previously, the transformation of recombinant shuttle vector pBBRE194 containing protease gene into wild type Bacillus halodurans CM1 (pBBRE194 prot-CM1) has been conducted. However, the transformation rate seemed low, and the stable recombinant clones could not be obtained. Therefore, in vivo methylation of this plasmid in E. coli has to be done before genetic transformation into the wild type bacterium, to obtain stable recombinant CM1 clones. In this study, a plasmid with artificial modification (pPAMC125) harboring genes encoding for the modification enzymes (methylases) from another strain, B. halodurans C-125, and a shuttle E.coli-Bacillus vector pBBRE194 prot-CM1 plasmid were transformed simultaneously into E. coli DH5α and TOP10, respectively. The expression of methylation enzymes genes carried by pPAMC125 in the presence of different L-arabinose concentration was observed via SDS-PAGE analyses. The analysis of the effect of these genes expression towards plasmid pBBRE194 prot-CM1 was performed by digesting the plasmid with the extracted cell of B. halodurans CM1. The digestion pattern was analyzed via DNA electrophoresis. As a result, compared to E. coli DH5α, E. coli TOP10 exhibited better condition for methylase genes expression with the result that pBBRE194 prot-CM1 plasmid was not be completely degraded by B. halodurans CM1 restriction endonuclease.

1. Introduction

Genetic engineering using the domesticated strain of bacteria such as Escherichia coli or Bacillus subtilis as hosts is routinely conducted in a laboratory to increase the target (recombinant) protein productivity. These domesticated bacteria are easy to be transformed by exogenous DNA plasmid and express the gene of interest [1][2]. However, in undomesticated wild type bacteria, genetic engineering is rarely done because there is no effective genetic transformation method yet. Commonly, wild type bacteria cannot maintain the presence of exogenous recombinant DNA plasmids in cells. This is due to
the presence of several factors inhibiting the transformation of foreign genes into wild type host bacteria, namely the presence of physical barriers in the form of cell membrane structures, cell walls, or exopolysaccharides, and low stability of recombinant DNA plasmids so that they are easily degraded by restriction enzymes in host cells [3][4].

Restriction modification (RM) protects host bacteria from foreign genes. RM recognizes and cuts 4-8 specific base pairs of foreign gene sequences so that it is degraded. The efficiency of recombinant plasmid transformation can be improved by manipulating the RM systems. Restriction sites in recombinant plasmids which modified by DNA methyltransferase (MTase) cannot be recognized and degrade by RM [4]. Degrading recombinant plasmid sequences by RM can be prevented by methylation. DNA methylation is the process of binding a methyl group with a nitrogen group in adenine and/or a carbon group in cytosine in a recombinant plasmid sequence with the help of S-adenosyl methionine as a donor of a methyl group [5].

To relieve the block of RM systems during the DNA introduction, in vivo methylation strategies have been developed to methylate DNA plasmid before the DNA transformation into wild type bacterium. The strategy usually contained a methylation plasmid carrying the MTase gene of the target bacterial and a shuttle plasmid used for genetic transformation [6][7]. After the co-transformation of the two plasmids into Escherichia coli, for example, the shuttle plasmid would be methylated by the MTase, which was encoded by the MTase gene from methylation plasmid. This strategy was successful to improve the genetic transformation of the exogenous plasmid into Bacillus sp. [8], or into Bacillus halodurans C-125 [7].

Bacillus halodurans CM1 is an indigenous local strain alkalotermophilic wild type of bacterium that isolated from Cimanggu Hot Spring, West Java, Indonesia; this bacterial strain is a very good producer of several industrially potential extracellular enzymes, and has not been commercialized. Bacillus halodurans CM1 can grow at temperatures of 40-55 °C and pH 7-11 [9]. In 2019, Rahmawati succeeded in inserted the alkalotermophilic protease gene from B. halodurans CM1 in the pBBRE194 plasmid, and transformed and expressed the protease gene in B. subtilis DB104, but had not yet succeeded in transformation the recombinant pBBRE194 plasmid into B. halodurans CM1 [10][11]. Therefore, in this study, to increase the genetic transformation efficiency into wild type CM1, we planned to conduct in vivo methylation as described by Wallace and Breaker (2011), using the same Plasmid Artificial Modification (PAM) called plasmid pPAMC125 [7].

Wallace and Breaker (2011) used the B. halodurans C-125 PAM system and succinate nutrient agar to greatly improve the efficiency and speed of protoplast transformation of B. halodurans C-125 by shuttle vector plasmid [7]. The pPAMC125 plasmid harboring the methylase genes from B. halodurans C-125 which could methylate and protect the shuttle vector plasmid, so that the structure of plasmids can be maintained. Based on this report, we are planning to employ this pPAMC125 to improve the genetic transformation of pBBRE194 prot-CM1 plasmid into our wild type CM1 bacterial strain, that previously could not be obtained. Hence, before the transformation into this wild type CM1, we have to modify this shuttle plasmid by the C125 PAM system firstly using the E. coli expression system. As a first step of the whole study, we have to find the optimal condition for in vivo methylation.

In this study, we cotransformed pPAMC125 and pBBRE194 prot-CM1 plasmid into E. coli DH5α and E. coli TOP10, respectively. We also conducted observing the expression of modification enzymes of pPAMC125 in the presence of different L-arabinose concentration via SDS-PAGE analyses and analyzing the effect of these methylase genes expression towards plasmid pBBRE194 prot-CM1 by digesting the plasmid with the extract of B. halodurans CM1 cells.

2. Material and Methods
2.1 Bacterial strains and plasmids
E. coli DH5α, E. coli TOP10, and Bacillus halodurans CM1, which were deposited in The Agency of Assessment and Application of Technology (BPPT)-Culture Collection (BPPT-CC), LAPTIAB-BPPT, Puspiptek-Serpong, were used for transformation and detection of methylation effect. pBBRE194
prot-CM1 was used as a shuttle plasmid harboring alkalotermophilic protease genes from *B. halodurans* CM1 and resistant to tetracycline, as described previously [11]. pPAMC125 was used as a methylation plasmid harboring genes encoding for the modification enzymes (methylase) from *B. halodurans* C-125 [7] and resistant to chloramphenicol. *E. coli* DH5α containing pPAMC125 was purchased from Bacillus Genetic Stock Center, Ohio State University, USA.

2.2 Preparation of Competent Cells and Transformation

Super optimal broth (SOB) and super optimal broth with catabolite repression (SOC) media were prepared as described by Hanahan (1983) [12]. A single colony of *E. coli* DH5α and *E. coli* TOP10 in LB plate was used. TB buffer contained 10 mM PIPES, 55 mM MnCl₂, 15 mM CaCl₂, 250 mM KCl, all the components except for MnCl₂ were mixed and the pH was adjusted to 6.7. Then, MnCl₂ was dissolved, the mixture was sterilized by filtration through a 0.45 µM filter unit and stored at 4 °C (Inoue et. al. 1990) [13]. Preparation of competent cells were prepared as described by Inoue et. al. (1990) [13]. *E. coli* DH5α and *E. coli* TOP10 were grown in Luria Bertani (LB) plate. One to two colonies of overnight culture was inoculated to 50 mL SOB containing 0.01% Mg solution and were inoculated in a shaker at 37 °C and 50 rpm until the optical density of suspension at 600 nm reached in the range of 0.5-0.6. Then the suspension was kept on ice for 30 min. Following this step, the bacteria were pelleted using HITACHI high-speed refrigerated centrifuge at 3000 rpm for 15 min at 4 °C. The bacterial pellet was re-suspended in 16.75 mL cold Transformation Buffer (TB) and placed on ice for 10 min. Next, the cell suspensions were centrifuged as above and pellets were dissolved in 1 mL cold TB and 150 µL dimethylsulfoxide (DMSO). The suspensions were kept on ice for 10 min. The suspension was transferred into microtubes of 50 µL each and stored at -80 °C.

Competent cells suspension thawed at room temperature. 1 µL pBBRE194 prot-CM1 (11 µg/µL) and 1 µL pPAMC125 (0.5 µg/µL) were added to 50 µL *E. coli* DH5α and *E. coli* TOP10 competent cells and pipetted gently. The suspensions were kept on ice for 30 min then heated at 42 °C for 2 min. Following heat shock step the tubes were kept on ice for another 2 min. Then 0.8 mL SOC was added then inverted. The suspension was incubated in a shaker at 150 rpm for 2.5 hours at 37 °C. The cells were centrifuged at 6000 rpm for 5 min at 4 °C. About 650 µL supernatant was discarded, and the pellet was re-suspended in about 200 µL supernatant by pipetting. Each 100 µL suspension was inoculated by the spreading method on LB containing 10 µg/mL chloramphenicol and 12.5 µg/mL tetracycline-resistant plates and incubated at 37 °C for 17 hours.

2.3 Analyses of Methylase Gene Expression

Methylase gene expression in pPAMC125 was prepared as described by Suzuki and Yasui (2011) [6]. The methylase genes in pPAMC125 were expressed using L-arabinose. A positive colony of each *E. coli* DH5α and *E. coli* TOP10 harboring pBBRE194 prot-CM1 and pPAMC125 plasmids were inoculated in 20 mL LB broth containing 10 µg/mL chloramphenicol and 12.5 µg/mL tetracycline, then incubated in a shaker at 150 rpm for 17 hours at 37 °C. Methylase genes in pPAMC125 were induced by L-arabinose in the range of concentration from 0.00002% to 2%, respectively. The culture then incubated in an incubator shaker at 190 rpm for 4 hours at 37 °C.

2.4 Polyacrylamide gel electrophoresis

SDS-PAGE was performed using the Nowakowski et. al. (2015) protocol with modification [14]. Five mL of the *E. coli* DH5α and TOP10 culture from different concentration of L-arabinose was used to SDS-PAGE analyses. 5 mL culture of each L-arabinose concentration was added 500 µL Na-Fosfat 20 mM pH 8 + mercaptoethanol then resuspended. The suspensions were lysed by sonication at 750 W for 15 min and centrifuge at 12000 rpm for 10 min at 4 °C. The supernatant was stored as the whole crude enzyme then stored at 4 °C.

In brief, 8 µL of protein sample was mixed with 2 µL of 5X protein sample buffer and heated at 100 °C for 10 min. Samples were then loaded into 12.5% separating gel. Then, 5 µL of Spectra™ Multicolor Broad Range Protein Ladder. Electrophoresis was performed at room temperature for
approximately 60 min using a constant voltage (140 V) in the 1X running buffer until the dye front reached the end of the gel.

A 5X protein sample buffer contained 0.25 M Tris-HCl buffer pH 6.8, 10% (w/v) sodium deoxycholate (SDS), 50% (w/v) glycerol, 0.2% (w/v) bromophenol blue, 0.1 M diethiothreitol (DTT). 10X running buffer contained 1.92 M glycine, 1% (w/v) SDS, and 0.25 M Tris-HCl. 12.5% separating gel contained 2.4 mL ddH2O, 1.5 mL 1.5 M Tris-HCl buffer pH 8.8, 60 µL 10% SDS, 3 mL 30% polyacrylamide, 30 µL 10% ammonium persulfate (APS), and 7 µL TEMED. Stacking gel contained 1.5 mL ddH2O, 625 µL 0.5 M Tris-HCl buffer pH 6.8, 25 µL 10% SDS, 334 µL 30% polyacrylamide, 15 µL 10% ammonium persulfate (APS), and 7 µL TEMED.

2.5 Protein staining
Discard the gel from the glass plate and washed by water for 2-3 times. Move it to a container for staining and destaining process. Add staining solution (0.5 g coomassie brilliant blue, 40% (v/v) methanol, 40% (v/v) acetic acid) until the gel is submerged. Incubate for 60 min at room temperature by gently shaking. Discard the staining solution and wash the gel with water 2-3 times. Gels were destained by destaining solution (33% (v/v) methanol, 33% (v/v) acetic acid). Gels soaked in the destaining solution overnight then washed by water for 2-3 times.

2.6 Analyses of PAM effect on pBBRE194 prot-CM1 by digesting with whole-cell extract
*B. halodurans* CM1
Preparation of the *B. halodurans* CM1 whole-cell extract for restriction enzymes activity detection was described as prepared by Gao et. al (2011) [8]. *Bacillus halodurans* CM1 was grown in 50 mL Horikoshi pH 9 broth to reach to OD600 of 2.0. Then the culture was collected by centrifugation at 9000 rpm for 10 min at 4 °C, the pellet was re-suspended in 5 mL K-phosphate buffer pH 7.5. The suspension was lysed by sonication at 750 W for 15 min and centrifuge at 8000 rpm for 3 min at 4 °C. The supernatant was stored as the whole-cell extract CM1. The whole-cell extract CM1 was used for the detection of the RM system in *Bacillus halodurans* CM1. The reaction system of the detection contained 1 µg plasmid DNA, 8 µL of the whole-cell extract CM1 and 2 µL CutSmart buffer in a total volume of 20 µL. And the reaction was performed at 37 °C for 16 h. The detection result was visualized by electrophoresis using 0.8% (w/v) agarose and running at 100 V for 25 min and gel documentation.

3. Result and Discussion
3.1 Co-transformation into *E.coli* (DH5α and TOP10) and plasmid verification
To select a positive transformant colony with the correct target insert plasmid, *E. coli* DH5α and *E. coli* TOP10 transformants were grown in selective antibiotics (chloramphenicol and tetracycline) LB medium agar. Chloramphenicol was used as a selectable marker for pPAMC125 (Figure 1), tetracycline was used as a selectable marker for pBBRE194 prot-CM1 (Figure 2). Therefore, positive colonies that are resistant to chloramphenicol and tetracycline were predicted to contain both plasmids. However, the sequence of the plasmid must be verified further by restricting the enzyme.

Successful transformation of *E. coli* DH5α (Figure 3A) and *E. coli* TOP10 (Figure 3B) by these two plasmids were confirmed by analyzes using the restriction enzymes of the extracted plasmids. These strains are efficient for plasmid transfection [13][15]. The *KpnI* and *PstI* restriction endonuclease enzymes were used to digest the plasmids, pPAMC125 digested into 3 DNA fragments which are 5.3 kb, 1.9 kb, and 0.9 kb and pBBRE194 prot-CM1 digested into 2 DNA fragments which are 8.4 kb and 1.4 kb. The recombinant *E. coli* containing the sequence of the correct plasmid was then chosen for further qualitative methylation gene expression assays.
Figure 1. The Map of Recombinant pPAMC125 plasmid harboring three methylase genes (dcm, damI, damII) of B. halodurans C-125 [7].

Figure 2. The Map of Recombinant pBBRE194 prot-CM1 plasmid harboring protease genes of B. halodurans CM1 [10][11].
Figure 3. Plasmid pBBRE194 prot-CM1 and pPAMC125 Verification of Positive transformant using KpnI and PstI as the Digesting Enzymes. (A) Plasmid Verification of positive E. coli DH5α transformant. (B) Plasmid Verification of Positive E. coli TOP10. (M) GeneRuler 1 kb DNA ladder. (1) Plasmid Extraction. (2) Plasmid after digested by KpnI and PstI.

3.2. Analyses of methylase genes expression
The qualitative assay was performed to ensure the expression of methylase genes of pPAMC125 in E. coli DH5α and E. coli TOP10 after induced by L-arabinose. Different concentration of L-arabinose were performed to get the optimal concentration. The method used to visualize the expression of methylase was SDS-PAGE. The SDS-PAGE analyses in E. coli DH5α did not show any difference between the uninduced and induced methylase gene (Figure 4). However, pPAMC125 in E. coli TOP10 that induced by 0.02% L-arabinose indicated good expression of methylase genes (Figure 5). A 0.02% L-arabinose is a sufficient amount to induced methylase genes in E. coli TOP10. It depends on the ability of cells to take up the inducer [16]. Over-production of methylase genes leads to cell death or inhibit the growth rate of the PAM host [17]. The weight of protein of three methylase genes of pPAMC125 which BH3508 (dcm), BH4003 (damI), and BH4004 (damII) are 36 kDa, 30 kDa, and 42 kDa, respectively [18].

Figure 4. SDS-PAGE of Methylase Gene Expression in pPAMC125 in E. coli DH5α. (1) Uninduced methylase gene. (2) Induced methylase gene by 0.02% L-arabinose. (3) Induced methylase gene by 0.05% L-arabinose. (4) Induced methylase gene by 0.1% L-arabinose. (M) Spectra™ Multicolor Broad Range Protein Ladder.
2.7 Analyses of PAM effect on pBBRE194 prot-CM1 using the whole extract of B. halodurans CM1

Methylase genes from *B. halodurans* C-125 were used as a reference in this study because of the lack of whole-genome sequence information of *B. halodurans* CM1. Based on the 16S rDNA sequence, *B. halodurans* CM1 had 99% similarity to *B. halodurans* C-125 [9]. Perhaps, methylase genes from C-125 could work and the shuttle vector might be protected from restriction endonuclease of *B. halodurans* CM1.

After L-arabinose induced the methylase gene in pPAMC125, methylase would be expressed and methylation process occurred. Therefore, pBBRE194 prot-CM1 would be methylated at a specific site. The analysis of the effect of methylase genes expression towards plasmid pBBRE194 prot-CM1 was performed by digesting the plasmid with the extracted cell of *B. halodurans* CM1. Methylated pBBRE194 prot-CM1 from *E. coli* DH5α and *E. coli* TOP10 extracted and treated with the CM1 whole-cell extract. Digested the methylated plasmid by whole-cell extract is performed as described by Gao et al. [8].

As shown in Figure 6A, methylated plasmid pBBRE194 prot-CM1 by varied concentrations in *E. coli* DH5α were digested with whole-cell extract into discrete DNA fragment, which indicated the methylation process didn’t work well. *E. coli* DH5α is suitable for cloning, plasmid maintenance and propagation of the plasmid, but not the expression of recombinant protein. This strain optimal for stable amplification of the DNA [1].

In Figure 6B, methylated plasmid pBBRE194 prot-CM1 by varied concentrations in *E. coli* TOP10 was treated by the whole-cell extract. Plasmid pBBRE194 prot-CM1 that methylated by methylase that induced by 0.02% L-arabinose could maintain the structure from restriction endonuclease activity. It because the pattern has similarities to undigested pBBRE194 prot-CM1. Plasmid pPAMC125 harboring *araBAD* promoter then compatible with *E. coli* TOP10 genotype which has *araD139* promoter [17]. *E. coli* TOP10 is being used as a cloning and expression host in many research to express pBAD33 plasmid [6][17][19]. This strain is capable of transporting L-arabinose and not metabolizing it then the level of L-arabinose will remain inside the cell and not decrease. The suitable strain to express pBAD should be *araBADC* and *araEFGH* strain [20].

![Figure 5. SDS-PAGE of Methylase Gene Expression in pPAMC125 in E. coli TOP10. (1) Uninduced methylase gene. (2) Induced methylase gene by 0.00002% L-arabinose. (3) Induced methylase gene by 0.0002% L-arabinose. (4) Induced methylase gene by 0.002% L-arabinose. (5) Induced methylase gene by 0.02% L-arabinose. (6) Induced methylase gene by 0.2% L-arabinose. (7) Induced methylase gene by 2% L-arabinose. (M) Spectra™ Multicolor Broad Range Protein Ladder.](image-url)
**Figure 6.** Methylated pBBRE194 prot-CM1 treated with CM1 whole-cell extract. (A) performed in *E. coli* DH5α. (B) performed in *E. coli* TOP10. (1) pBBRE194 prot-CM1. (2) pBBRE194 prot-CM1 treated by whole-cell extract. (3) pBBRE194 prot-CM1 and pPAMC125 treated by whole-cell extract. (4) pBBRE194 prot-CM1 methylated by 0.02% L-arabinose treated by whole-cell extract. (5) pBBRE194 prot-CM1 methylated by 0.05% L-arabinose treated by whole-cell extract. (6) pBBRE194 prot-CM1 methylated by 0.1% L-arabinose treated by whole-cell extract. (M) GeneRuler 1 kb DNA ladder.

4. **Conclusion**

*E. coli* TOP10 exhibited the optimal condition for methylase genes expression of pPAMC125 with the result that pBBRE194 prot-CM1 might be methylated by methylases that was induced by 0.02% L-arabinose. This pBBRE194 prot-CM1 was not completely degraded by CM1 restriction endonuclease. Hence, using this methylated recombinant plasmid, further genetic transformation into wild type bacterial strain *B. halodurans* CM1 will be conducted.

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5. **References**

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