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Rekayasa Bakteri untuk Ternak dan Manusia: Pembuatan Mutan *Escherichia coli* Penghasil Protein Rekombinan

Bacterial Engineering for Cattle and Human: Construction of Escherichia coli Mutant for the Production of Recombinant Proteins

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ABSTRAK

Protein rekombinan seperti vaksin, antibodi, hormon, dan obat-obatan, semakin dibutuhkan oleh ternak dan manusia. Hambatan utama untuk menghasilkan protein rekombinan pada Escherichia coli sebagai inang yang digunakan paling luas adalah degradasi oleh enzim proteolitik. Hal ini disebabkan karena E. coli memiliki sejumlah enzim proteolitik yang tersebar di dalam sitoplasmanya. Untuk itu, lebih dari 90% degradasi protein terjadi di dalam sitoplasmanya. Pada penelitian ini, peneliti telah menghasilkan mutant E. coli BW25113 yang tidak memiliki gen penyandi enzim protease dengan menggunakan kombinasi metode pengerusakan kromosom dan metode transduksi phage P1. Pembuatan mutan tersebut dimulai dengan pengerusakan gen penyandi enzim protease pada kromosom bakteri dengan produk PCR yang memiliki bagian yang homolog dengan gen target. Mutan-mutan yang dihasilkan kemudian digunakan untuk menghasilkan mutan ganda dengan metode Transduksi phage P1. Analisis fenotif dan genotif menunjukkan bahwa kombinasi kedua metode tersebut sangat efektif untuk membuat lebih dari satu mutasi pada E. coli. Untuk itu, mutan E. coli yang telah diperoleh akan sangat bermanfaat untuk menghasilkan aneka protein rekombinan untuk ternak dan manusia.

ABSTRACT

Recombinant proteins, such as vaccines, antibodies, hormone, or drugs, are increasingly needed for cattle and human. The major constrain in the recombinant proteins production using Escherichia coli, the most widely used host strains, is their degradation by proteolytic enzymes. It is most likely that the E. coli possesses a number of proteolytic enzymes distributed in its cytoplasm. Therefore, more than 90% of the degradation process occurs in the cytoplasm. In this study, we constructed protease-deficient mutants in E. coli BW25113 using combination of chromosomal disruption and P1 phage transduction methods. The mutant construction began with the disruption of chromosomal gene encoding protease in E. coli by one-step disruption method in which PCR products provide the homology regions to the targeted gene(s).

These mutants were used to construct double mutants using P1 phage transduction. Phenotypic and genetic analysis showed that the combination of these methods were effective to construct more than one gene disruption in E. coli. Therefore, the obtained E. coli mutants would be absolutely useful for the generation of wide varieties of recombinant proteins for cattle and human.

In recent years, recombinant protein technology has become an important discipline spanning from scientific research to the pharmaceutical industry. It is considered so since recombinant proteins, such as vaccines, antibodies, hormone, or drugs, are increasingly needed for cattle and human health. The use of cowpox firstly as smallpox vaccine by Edward Jenner, followed by attenuated or killed virulent micro-organisms and recombinant proteins to fight disease has proven spectacularly successful. Appropriate administration of attenuated or killed *Bacillus anthracis* is very effective to prevent anthrax disease in farm animals. In addition, passive antibody therapies and immune sera could be used for treatment of certain infections in animal.

One of the challenges emerged in the biotechnology revolution to meet animals and humans demand is the development of techniques for the economical production of therapeutics recombinant proteins. Plant production system has been developed for the proteins production (Kersten *et al.*, 2003; Valdes *et al.*, 2003). Plants are potential "biofarming factories" because they are capable of producing unlimited number and amount of recombinant proteins safely and inexpensively. However, some of the current hurdles include a long growth time, regulatory elements uncertainties, and questions about the suitability of plant glycans for human therapeutics.

Among the most well documented and established systems used in various scales of recombinant protein production are the enterobacterium *Escherichia coli*. It is the most ubiquitous source of recombinant protein as it is simple, cheap, and its technology is mature (Kristensen *et al.*, 2005; Lombardi *et al.*, 2005). Recombinant protein from bacteria, archaeabacteria, and eukaryotes are in many cases efficiently expressed and accumulated in *E. coli* (Kristensen *et al.*, 2005). In addition, high production levels of recombinant proteins are usually attainable when *E. coli* is used as the host cells.

A major bottleneck in the proteins production using *E. coli,* is their degradation by proteolytic enzimes. It is due to the capacility of *E. coli* to produce a number of proteolytic enzymes distributed in its periplasm and cytoplasm. Previous studies demonstrated that endogenous proteases such as Lon, ClpP, DegP, and OmpT participated in rapid degradation of proteins *in vivo* (Vasilyeva *et al*., 2000; Weichard *et al*., 2002; Jones, 2002; Ignatova *et al*., 2003; Okuno *et al*., 2002). Therefore, more than 90% of the degradation occure in the cytoplasm.

One way to enhance the yield of recombinant proteins of interest in *E. coli* as a host is genetic manipulation. Ignatova *et al*., (2003) reported that the production of mature active penicillin amidase increased up to 10-fold when the protease-deficient strain *E. coli* BL21 (DE3) was used as the host. Therefore, the use of protease-deficient strains as the host is a successful strategy to achieve higher productivity of a proteolysis-susceptible target protein.

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Genetic manipulation in bacteria genome can be achieved by a variety of techniques including error-prone PCR, DNA shuffling, saturation mutagenesis, and family shuffling (Fuji *et al*., 2004). Error-prone PCR introduces random mutations during PCR by reducing the fidelity of DNA polymerase. The fidelity of DNA polymerase can be reduced by adding manganese ions or by biasing the dNTP concentration. The combination of error-prone PCR and saturation mutagenesis constitutes an efficient way to explore the protein mutation properties. However, the creation of mutants by the above methods has been limited partly by point mutations that can introduce only a limited range of amino acid substitutions. This limitation narrows the sequence space of mutant proteins that can be created.

In this work, we generated several single protease-deficient mutants of *E. coli* using one-step chromosomal disruption method (Datsenko *et al*., 2000). Parallel efforts were performed for the construction of double protease-deficient mutants using P1 Phage transduction method (Miller, 1972). The combination of one-step chromosomal disruption and P1 phage transduction methods was effective to construct more than one gene disruption in *E. coli* and would be widely useful for generating effective host of recombinant protein expression in other bacteria.

MATERIALS AND METHODS

Materials

Escherichia coli strains, plasmids, and phage used in this study are listed in Table 1. Luria-Bertani medium containing 1% of tryptone, 0.5% of yeast extract, and 1% of NaCl were used for cultivation. Super Optimal Broth (SOB) medium consisted of 2% tryptone, 0.5% yeast extract, 10 mM NaCl, and 2.5% mM KCl. SOC medium containing SOB medium plus 10 mM $MgCl_2$, 10 mM $MgSO_2$, and 20 mM Glucose. The solid medium containing 1.5% agar or 0.5% agar for soft-agar overlays. When required, the solid or liquid media were supplemented with antibiotics at the following concentrations i.e. 50 μg/ml kanamycin; 10 μg/ml chloramphenicol; and 50 μg/ml ampicilin.

Methods

Construction of Mutant Strains

The one-step chromosomal disruption method and P1 phage transduction method were prepared as previously described by Datsenko *et al*., (2000) and Miller, (1972) respectivelly. PCR products were generated using template plasmids (pKD3, pKD4, and pKD13) using primer with 60-nt extentions (Table 2). PCR mixture consisted of 10x *Ex taq* buffer, 0.2mM dNTP, 0.5μM each primer, 0.01 μg/μl of template plasmid, and *Ex Taq* DNA polymerase. The PCR program consisted of an initial step at 94°C for 5 min, followed by 30 cycle of 10 s at 94°C, 30 s at 55°C, and 1.5 min at 72°C, followed by the final extension step of 7 min at 72°C.

Electroporation

Electroporation-competent cells (10^{10}CFU/ml) were thawed quickly at room temperature and placed on ice. Disposable 0.1-cm electroporation cuvettes (Bio-Rad) were placed in an ice for 10 min prior to electroporation. PCR products (1-3 μl) were mixed with 50 μl of the competent cells. The mixture was pipetted into the precooled cuvettes (which were dried throughly) and electroporated at 20 kV and resistance of 200-ohm. Immediately after electroporation, 1 ml of SOC medium was added to the cuvette. The cells were transferred to a 15 ml polypropylene tube and allowed to recover for 1 h at 30°C with shaking at 200 rpm before plating on selective media containing chloramphenicol and kanamycin.

PCR Verification

Phenotypic selection using selective media and PCR analysis were used to verify strain construction and recombinant formation. Primers (20-mers) (Table 2) were used to verify kanamycin or chlorampehnicol-encoding gene replacement in protease-encoding gene site. PCR mixture contained 10x *Ex Taq* buffer, 0.2mM dNTP, 0.5μM each primer, 2.5 μl of mutant colony dilution, and *Ex Taq* DNA polymerase. The mixture was incubated at 95^{o}C for 5 min, followed by 30 cycles of 30 s at 94^{o}C, 30 s at 50^{o}C, 2 min at 72^{o}C and a final extension step of 7 min at 72^{o}C. PCR products were run on 1% agarose gel along with a λ/*Eco* T141 marker.

Elimination of Antibiotic Resistance Gene

Antibiotic resistance mutants were transformed with pCP20. Since the plasmid is an ampicilin plus chloramphenicol resistance plasmid, the results of transformation can be selected using selective media containing the antibiotic. Heat shock (43^{o}C) for inducing FLP synthesis was performed for antibiotic resistance gene elimination.

Table 1. Bacterial strains, plasmids and phage used in the experiment

Strain or phage	Chromosomal Markers/Description	Source or reference
E. coli BW25113	Δ(*araD-araB)567*, Δ*lacZ4787*(::rrnB-3), *lacIp-4000*(lacI^{Q}), λ^{-}, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*	Datsenko *et al*., 2000
Plasmid		
pKD3, pKD4 pKD13	Template for PCR product	Datsenko *et al*., 2000
pKD46	The Red helper plasmid	Datsenko *et al*. 2000
pCP20	FLP recombinase	Datsenko *et al*. 2000
Phage		
P1 phage		Laboratory stock

Table 2. List of primers used to verily kanamycein or chloramphonial encording gene replacement in protease encoding gene site

Primers	Sequence (5′ ⟶3′)
degP-H1	acagcaattttgcgttatctgttaatcgagactggaatacgtgtaggc tggagctgcttc
degP-H2	ggagaacccttcccgttttcaggaaggggttgagggagac ata tgaatatcctcctta
DegP-L	tatgaccgacctctatgcgt
DegP-R	cctgtgaaagtaccagcaat
Lon-H1	atctgattacctggcggaaattaaactaagagagagctctgtga ggctggagctgcttc
Lon-H2	tgccagccctgtttttattagtgcattttgcgcgaggtcaattcc ggggatccgtcgacc
Lon-L	cggtaattgatggtcaaagc
Lon-R	cacgctctttaacggcaaa
ompT-H1	cggggcgattttcacctcggggaaattttagttggcgttcgtgta ggctggagctgcttc
ompT-H2	tacatattcaatcattaaaacgattgaatggagaacttttcatat gaatatcctccttagt
OmpT-L	cccagaaatgtggctataac
OmpT-R	cagcgacaaaaagtgatgtg
clpP-H1	atgtcatacagcggcgaacgagataactttgcaccccataattc cggggatccgtcgacc
clpP-H2	gatgggtcagaatcgaatcgaccagaccgtattccaccgcgtgt aggctggagctgcttc
ClpP-L	tcaacgagctgatgaaccag
ClpP-R	tatacacggatggaccggca

RESULTS

PCR products, which consist of antibiotic resistance gene with homologous regions, were generated by PCR from plasmids template (pKD3, pKD4 and pKD13) using primers with 60-nt homologous extensions to the target. Each plasmid contains different antibiotic resistance genes that are flanked by directly repeated FRT site. pKD3 plasmid contain chloramphenicol resistance gene, pKD4 and pKD13 contain kanamycin resistance gene. The PCR products were purified, and digested with *Dpn I*. Transformation of the PCR products which contain a homologous region were then performed into *E. coli* BW25113 using electroporation method. After plating on selective media and incubating at 30^{o}C overnihgt, the growing colonies were then used as template for PCR confirmation.

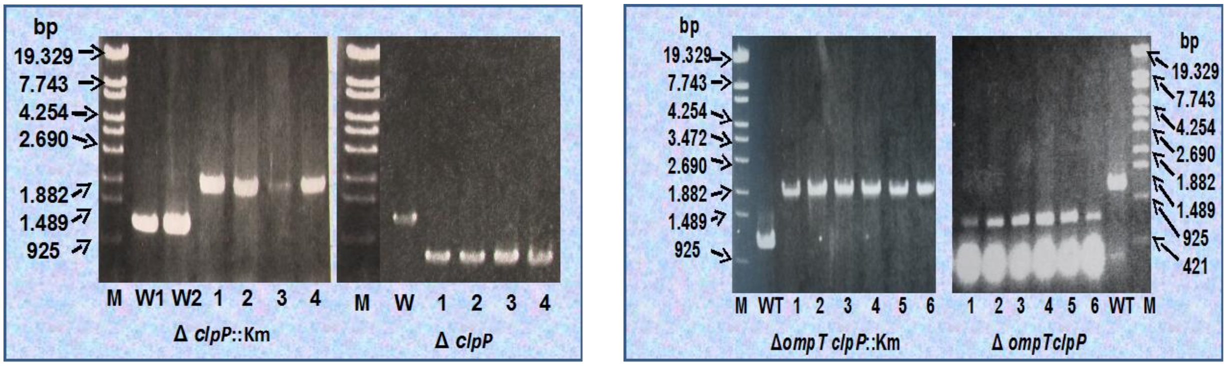
The generated single mutants were Lon-deficient mutant (Δ *lon*), ClpP-deficient mutant (Δ *clpP*), DegP-deficient mutant (Δ*degP*), and OmpT-deficient mutant (Δ*ompT*). Electrophoresis result of PCR amplification of ClpP-deficient mutant (Δ *clpP*::Km^{r}) were shown in Figure 1 A. The band size of ClpP protease-deficient mutant (Δ*clpP*::Km^{r}) was higher than that of wild type (W1-W2) because of antibiotic resistance gene insertion between the homologous regions.

All of the single mutant that were still containing antibiotic resistance gene were subsequently used as donor cells for double mutant construction by means of P1 phage transduction. Receptor cells were prepared from these mutants following isolation of the antibiotic resistance gene. FRT sites placed in the same chromosome will lead to a deletion or inversion of the antibiotic resistance segment by FLP recombinase produced by the strain with an easy curable FLP-expressing plasmid (pCP20) (Cherephanov *et al.*, 1995). After excision of the antibiotic-resistance determinant, as shown in electrophoresis results of Δ *clpP* (Figure 1A), the band size of the mutant was lower than that of wild type.

Double-protease deficient mutant produced in this study were DegP + OmpT-deficient mutant (Δ*degP-ompT*), DegP + Lon-deficient mutant (Δ*degP-lon*), Lon + OmpT-deficient mutant (Δ*lon-ompT*), and OmpT+ClpP-deficient mutant (Δ*ompT-clpP*). We also tried to construct triple mutant, but no colony was obtained.

Electrophoresis result of PCR amplification of OmpT and ClpP-deficient mutant were shown in Fig. 1 B. Replacement of protease gene by PCR products (antibiotic resistance segment) was increased the band size of these mutants (Δ*ompTclpP*::Km^{r}) comparing to wild type. As occurred in single mutant, the band size become lower than wild type after elimination of the antibiotic resistance cassette (Δ*ompTclpP*).

To verify the effect of protease gene deletion on *E. coli* growth, some mutants and control (wild type) were grown in LB medium at 37 ^{o}C. Figure 2 shows that *E. coli* protease-deficient mutants were grown slowly than did the wild type (control). Each of single mutants has the same tendency to grow faster than double mutant, but was slower than the wild-type.



A B

Fig. 1. Electrophoresis results of PCR confirmation of *E. coli* BW25113 protease-deficient mutants. A = single mutants (Δ*clpP*::Km = ClpP-deficient mutants containing kanamycin-encoding gene; Δ*clpP* = ClpP-deficient mutants without kanamycin-encoding gene; W1, W2, W = wild type, 1-4 = single mutants). B = double mutants (Δ*ompTclpP*::Km = OmpT and ClpP-deficient mutant containing kanamycin-encoding gene; Δ*ompTclpP* = OmpT and ClpP-deficient mutant without kanamycin-encoding gene; WT = wild type, 1-6 = double mutants). M = λ DNA marker.

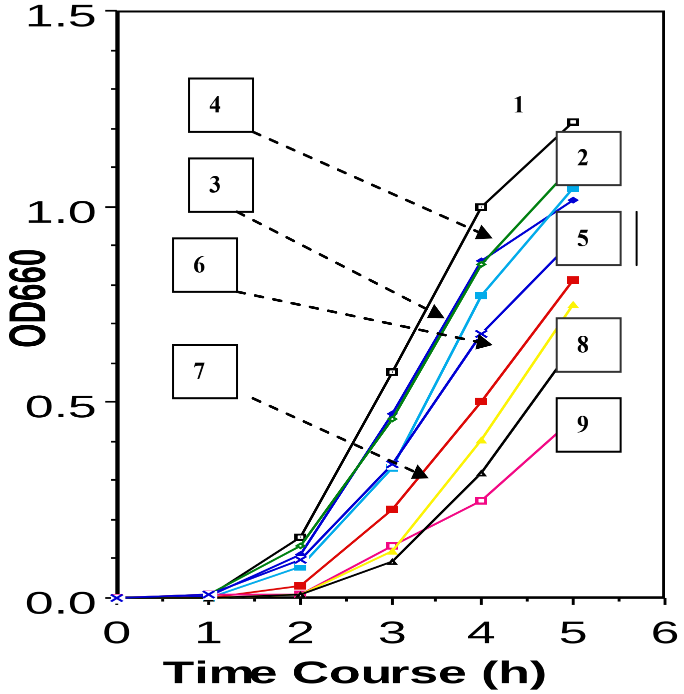


Fig. 2. Time course of *E. coli* mutant growth Cells were grown at 37 °C. 1 = wild type/control, 2 = Δ*ompT*, 3 = Δ*clpP*, 4 = Δ*degP*, 5 = Δ*clpP-ompT*, 6 = Δ*lon*, 7 = Δ*degP-lon*, 8 = Δ*lon-ompT*, 9 =Δ*degP-ompT*.

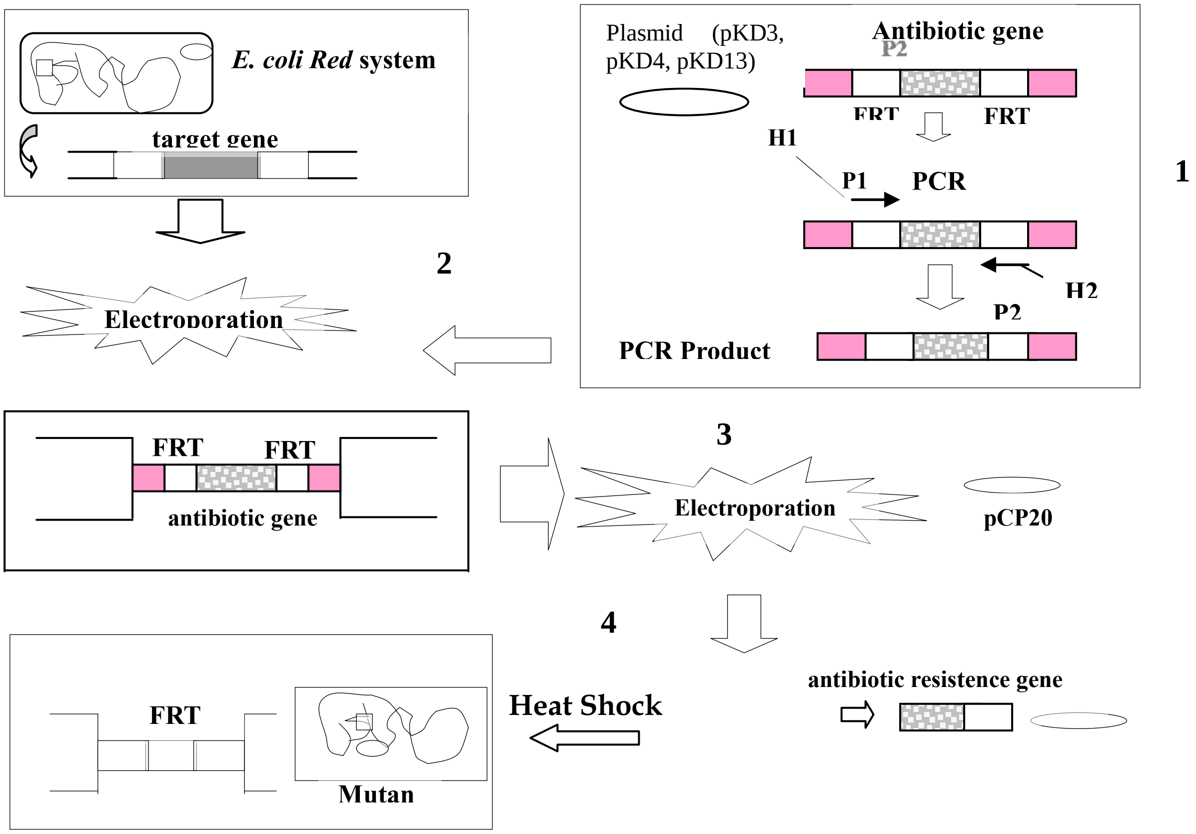


Fig. 3. A schematic showing simple steps of gene disruption strategy for mutant construction. 1 = PCR products were generated from plasmid (pKD3, pKD4 and pKD13) using primer with 60-nt extentions, 2 = Electroporation of PCR products into *E. coli* Red system for gene target replacement, 3 = electroporation of FLP-expressing plasmid (pCP20) into *E. coli* mutants for antibiotic resistance gene elimination, 4 = elimination of antibiotic resistance gene using heat-schock method. H1 and H2 refer to the homology regions, P1 and P2 refer to priming site. FRT = FLP recognition target.

DISCUSSION

Since *E. coli* posseses a number of proteolytic enzyme, many recombinant proteins are rapidly degraded when expressed in the bacteria. Lon is the primary protease degrading abnormally folded proteins in *E. coli*. Outer membrane protease, OmpT, can cleave many cytoplasmic protein after lysis cells. In the periplasm, DegP is protease which has a serine active site. In addition, ClpP can associate with ATPase subunit to form protease (Gottesman, 1996; Hengge and Bukau, 2003).

The procedure for the construction of protease-deficient mutants using one-step chromosomal disruption method are shown diagrammatically in Figure 3 The basic principle of the chromosomal disruption method is to replace a target gene with a selectable antibiotic resistance gene and homologous regions. Linear DNA containing antibiotic resistance gene flanked by homologous regions of bacterial chromosome is then transformed or electroporated into recombination-proficient *E. coli* BW25113 strain. Recombination between both ends of the linear DNA fragment and the bacterial chromosome results in gene replacement. The recombinations can be easily selected by the presence of the antibiotic resistance marker.

The antibiotic resistance gene was generated by PCR from plasmids template (pKD3, pKD4 and pKD13) using primers with 60-nt homologous extensions to the target. Each plasmid contains different antibiotic resistance genes that are flanked by directly repeated FRT site and homologous regions. pKD3 plasmid contain chloramphenicol resistance gene, pKD4 and pKD13 contain kanamycin resistance gene. The homologous regions were provided by primers with 60-nucleotides homologous extensions.

Digestion of PCR products using *Dpn I* was done to eliminate methylated (unamplified) template DNA. The linear DNA (PCR product) was then electroporated into transformants (*E. coli* BW25113) carrying the Red helper plasmid. In one-step chromosomal disruption method, target gene replacement with antibiotic resistance gene was conducted based on the Red system. Transformants, which carrying pKD46, have three genes (*exo*, β and λ) that facilitate and are necessary for recombination (Datsenko *et al*., 2000). The *exo* gene encodes the Redα protein, a 5′ to 3′ exonuclease that processively degrades the 5′-ended strand of a linear double-stranded DNA (dsDNA) fragment to produce 3′-ended single-stranded DNA (ssDNA) overhangs. The β gene encodes a pairing protein (Redβ) that binds to the 3′-ended ssDNA overhangs created by the Redα protein and promotes renaturation of complementary strands, and is capable of mediating strand annealing and exchange reaction *in vitro* (Li and Wilkinson, 1998). The recombination function of Redα and Redβ proteins are further assisted by the λ-encoded Gam protein, which inhibits the host RecBCD exonuclease V, an intracellular exonuclease that degrade the linear pieces of DNA in *E. coli*, in order to linear DNA (PCR products) transformable.

All obtained mutants were verified by PCR colony wich tested for the presence of new locus- and junction-specific fragments. As shown in Figure 1, The presence of new locus (antibiotic resistance gene) was appeared by increasing size of band in single and double mutants, Δ*clpP*::Km^r and Δ*ompTclpP*::Km^r.

The resistance gene was then eliminated by using a helper plasmid (pCP20) expressing the FLP recombinase, which act on the directly repeated FRT (FLP recognition target) sites flanking the resistance gene. FRT sites placed in the same chromosome will lead to a deletion or inversion of the antibiotic resistance segment by FLP recombinase that produced by the strain with an easy curable FLP-expressing plasmid (pCP20) (Cherephanov *et al*., 1995). After excision of the antibiotic-resistance determinant such a sequence would be left in the chromosome at the site of the initial cassette insertion. Elimination of the antibiotic resistance gene from these mutants reduce the size of band.

In mutant growth condition, as shown in Figure 2, *E. coli* protease-deficient mutants were grown slowly than did the wild type. The data presented here demonstrate that the deletion of protease gene has effects on the growth of *E. coli*. Each of single mutants has the same tendency to grow faster than the double mutant, but was slower than the wild-type. In other word, the double mutants were the slowest grown, indicating that deletion of more than one protease gene has more harmful effects on the *E. coli* growth. However, Lon mutant grow slower than several some double mutants. It is due to that this protease catalyzes an initial rate-limiting step in the degradative pathway that is necessary for *E. coli* growth (Goff and Goldberg, 1987; Surpuran *et al*., 2002).

Several double mutants were also difficult to survive in a viable condition especially on solid growth media. These mutants show a variety of other phenotypic alteration which seem to be resulted from their decreased ability to degrade certain short-lived protein (Gottesman, 1996). We had attempted to make triple mutants, however all of such trials were unsuccessful, suggesting that deletion of double or more certain protease genes might give harmful effects on the growth and viability of *E. coli* cells. This suggestion is based on the role of these proteases in the rapid turnover of short-lived regulatory protein for balanced growth of *E. coli* (Hengge and Bukau, 2003). Moreover, as reported previously, deletion of the above protease in *E. coli* leads to mucoidy and reduced strain fitness (Goff and Goldberg, 1987; Jones *et al*., 2002; Weichart *et al*., 2002). Jiang *et al*., (2001) also reported that deletion of protease gene in *E. coli* made the cell growth quite poor.

CONCLUSION

In conclusion, we have constructed four single and double protease-deficient mutants in *E. coli* using combination of one-step chromosomal disruption and P1 phage transduction methods. Using the methods, desired mutations can be made in any part of the DNA, independent of the presence of appropriate restriction enzyme sites. The combination of these methods allowed us to make more than one mutation, suggesting that the method is useful to create molecular diversity to other bacteria.

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