Cloning of the *Escherichia coli* dapD gene via fragment amplification by PCR method*)

Sudjadi1, Zupriza1, M. Nur Cahyanto2 and Tety Harto2

1) Faculty of Pharmacy, Gadjah Mada University, Yogyakarta 55281, Indonesia. Tel: 62-274-90660.
2) Inter University Center for Biotechnology, Gadjah Mada University, Yogyakarta 55281, Indonesia.
3) Faculty of Agriculture Technology, Gadjah Mada University, Yogyakarta 55281, Indonesia.

Abstract

In *Escherichia coli*, there are 9 genes responsible for L-lysine biosynthesis from aspartate. One of them is the dapD gene encoding N-succinyl diaminopimelate aminotransferase. This enzyme is not found in birds so they are unable to synthesize lysine naturally. To study transformation of the dapD gene into birds, therefore the gene has to be cloned.

The main problem of genomic library approach in cloning genes is time consuming. On the other hand, there is a direct cloning approach using amplification fragment by the Polymerase Chain Reaction (PCR) method. The nucleotide sequence of the gene has been known. Based on available data of the *E. coli* dapD gene sequence, the priming primers have been made with the addition of BamHI and EcoRI sites, respectively. The fragment was then amplified by the PCR method. The PCR product was digested with BamHI and EcoRI and then ligated into BamHI-EcoRI digested pUC19. Six out of eight clones analyzed were able to convert *E. coli* AT982 dapD to growth without diaminopimelate (DAP). It can be concluded that the six clones carry the dapD gene.

Keywords: Cloning - dapD - PCR.

Introduction

The biosynthesis of L-lysine has been studied since 1950. It was found that bacteria as well as the higher plants synthesize lysine via diaminopimelate (Nakayama, 1972). In *E. coli* the 9 genes involved in the pathway are scattered in the chromosome (Reverend et al., 1982). Two genetic loci were identified in the 4 minute region of the chromosome and were called the dapC and dapD (Bukhari and Taylor, 1971).

Birds are unable to synthesize lysine due to lack of the dapD gene encoding N-succinyl diaminopimelate aminotransferase (Labier and Leclercq, 1992). Love et al. (1994) have successfully transferred and expressed the *E. coli* lacZ gene into chicken by DNA microinjection technique. If the *E. coli* dapD gene carried in an appropriate vector was injected into chicken egg, it hopefully would produce transgenic chicken which is capable to synthesize lysine so the feed does not need added lysine.

Richaud et al. (1984) reported that the *E. coli* dapD has been cloned into pBR322 from lambda transducing phage; its complete nucleotide sequence was established. This sequence shows that the *dapD* gene is composed of a single coding sequence encoding a 274 amino acid polypeptide, M 30,040. Cloning via this approach is time consuming especially when producing phage carrying the interested gene. The direct cloning can be carried out via amplification the dapD gene fragment by Polymerase Chain Reaction (PCR) using flanking primers made based on nucleotide sequence available. The PCR product is then cloned into a vector. This type of approach is quicker than the genomic library approach. Lysine biosynthesis pathway is illustrated in Figure 1.

Materials and Methods

**Media, strains and plasmids.** Bacterial strains and plasmids used are listed in Table 1. Bacteria were grown in Luria Bertani medium. For *E. coli* AT982, 80 μg/ml diaminopimelate (DAP) was added to the medium. For plasmid harbouring bacteria, 50 μg/ml ampicillin was added.

**DNA Manipulation.** Isolation of *E. coli* K-12 chromosome was carried out according to the method of Chow et al. (1977). DNA plasmid was isolated by alkaline lysis method and purified by lithium chloride precipitation as described by Sambrook et al. (1989). Transformation of *E. coli* was carried out according to Hanahan (1985). Restriction enzymes (BRL) were used. Ligations were performed with T4 DNA ligase (BRL); incubation was done overnight at room temperature in 10 μl. Analytical electrophoresis was done in 40 mM Tris, 10 mM NaCl and 1 mM EDTA on agarose.

**Fragment amplification by PCR.** Two primers, SUD1 5'-AGGACATCACTAAGGGTGACAGAG3' and SUD2 5'-TGGAAATCTCGGCCGCGGCTGTCG3', were made according to the dapD sequence reported by Richaud et al. (1984). Two recognition sites, BamHI and EcoRI, were added into each primer. The primers were provided by Research Instrument Sdn. Bhd., Selangor Malaysia. Amplification was carried out using Taq DNA polymerase (Boehringer Mannheim) with denaturation at 94°C, annealing at 55°C and polymerization at 72°C.

Cloning strategy: PCR product was digested with BamHI and EcoRI and then ligated into BamHI-EcoRI digested pUC19. Ligation mixtures were used to transform

![Figure 1. The lysine biosynthesis pathway via aspartate. The numbers show the responsible enzymes for the reactions: 1. aspartokinase; 2. L-aspartate dehydrogenase; 3. DHP synthetase; 4. DHP reductase; 5. THDP synthetase; 6. N-succinyl diaminopimelate aminotransferase; 7. deacetylase; 8. DAP epimerase; 9. DAP decarboxylase.](image-url)
E. coli DH5α. Transformants were selected on Luria Bertani medium containing 100 μg/ml ampicillin, 3 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 30 μg/ml IPTG (isopropyl thio-β-D-galactoside). Plasmid DNA of white colonies was isolated and used to transform E. coli AT982. Transformants were selected on Luria Bertani containing ampicillin and DAP. The single colonies were then tested in the ability to grow on Luria Bertani ampicillin without DAP.

Table 1. E. coli strains and plasmids used

<table>
<thead>
<tr>
<th>Strains/Plasmids</th>
<th>Characterization</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5a</td>
<td>lacZM15</td>
<td>Hanahan, 1981</td>
</tr>
<tr>
<td>E. coli AT982</td>
<td>ampR, lacZ</td>
<td>Bukhari and Taylor, 1971</td>
</tr>
<tr>
<td>pUC19</td>
<td>AmpR, lacZ</td>
<td>Yasuzaki, et al., 1983</td>
</tr>
<tr>
<td>pTH1001</td>
<td>AmpR, ampD</td>
<td>This work</td>
</tr>
</tbody>
</table>

Results and Discussion

Amplification of the dapD fragment. Sequence of the E. coli dapD gene and the flanking regions (Richaud et al., 1984) was run on the DNA Strider 1.0 program and seeks the restriction map. It shows that there is no BamHI and EcoRI sites on the gene. That is why the primers, SUD1 and SUD2, were made with BamHI and EcoRI sites. Melting points of SUD1 and SUD2 are 88°C and 76°C, respectively. As indicated that the annealing temperature should be around 25°C below the melting point. Therefore in this experiment, annealing was carried out at 55°C. PCR products were then run on agarose 1% and showed 1 kb band whereas the negative control showed no band (Figure 2). These results are in agreement to the data of the DNA Strider showing the amplified fragment should be 954 bases.

Cloning of the dapD gene. Plasmid pUC19 was chosen as the cloning vector because it has polycloning sites. When the PCR product was cloned into BamHI-EcoRI site, the dapD gene orientation should be the same as the lac promoter of pUC19. The transcription of the dapD gene would be enhanced by the lac promoter. In addition, pUC19 is a high copy number plasmid, this would make further manipulation easier.

PCR product was digested with BamHI-EcoRI and then ligated into BamHI-EcoRI digested pUC19. Ligation mixtures were used to transform E. coli DH5. Transformants were selected with a complementation on Luria Bertani containing ampicillin, X-gal and IPTG. It was found 72 white colonies and 924 blue colonies. It seems that the ratio of the PCR product to plasmid is low and the plasmid digestion did not work well. Eight white colonies chosen randomly were grown separately on Luria Bertani containing ampicillin and then the plasmids were isolated. The plasmid were digested with BamHI-EcoRI and the present of inserted DNA were seen by electrophoresis. Figure 3 shows that the 2.7 kb band is the vector size and the 1 kb is the inserted DNA. However, there are two inserted DNA with the size of 1.3 and 1.7 kb, respectively. These show that the primers have annealed to non-specific target due to the low annealing temperature.

Concluding the research, the dapD gene was successfully cloned into BamHI-EcoRI sites of pUC19. The direct approach cloning via PCR is quicker than the genomic library.

Acknowledgment

This work is supported by the RUT II Project Contract No. 023/SP-KD/PPIT/IV/94. We are indebted to Dr. B.J. Bachmann for providing E. coli AT982. We wish to thank Dr. A. Yuswantyo for helpful discussion and critical reading the manuscript.
Development of probes for detection of betaine genes in rhizobacteria using PCR-amplified betaine-encoding DNA sequences of *Escherichia coli*

Triswibowo Yuwono, Maya Shovitri, E. Mursyanti and Joedoro Soedarsono

1) Laboratory of Microbiology, Inter University Center for Biotechnology, Gadjah Mada University, Yogyakarta, Indonesia
2) Faculty of Agriculture, Gadjah Mada University, Yogyakarta, Indonesia

**Abstract**

Probes for detection of betaine genes have been created by PCR approach using genomic DNA of *Escherichia coli* as template. Primers were designed to amplify both betA and betB-encoding DNA sequences. The PCR fragments were then cloned into pUC19 and subsequently labeled with digoxigenin. The probes have been successfully used for hybridisation with genomic DNA of *E. coli*. Following hybridisation with genomic DNA of *Rhizobium*, however, no significant signal has been detected. The possible factors contributing to this failure of detection is discussed.

**Keywords**: Betaine - rhizobacteria - probe

**Introduction**

Glycine betaine is an osmolyte synthesised by many microorganisms and plants which is used as a protective substance against osmotic stress, such as seawater, as well as drought condition encountered in soil (Murao et al., 1989; Kunin and Rudy, 1991; Peter et al., 1996). It has been demonstrated that staphylococci and *Escherichia coli* require choline or glycine betaine to achieve maximal salt tolerance (Kunin and Rudy, 1991; Gauthier and Rudulier, 1990). Restoration of colony-forming activity of osmotically-stressed *E. coli* has also been achieved by exposing the cells to betaine (Roth et al., 1988).

Glycine betaine is synthesised through the conversion of choline to betaine aldehyde, which is subsequently converted to glycine betaine. In *E. coli*, the oxidation of choline to betaine aldehyde is catalysed by choline dehydrogenase whereas the conversion of betaine aldehyde to glycine betaine is catalysed by betaine aldehyde dehydrogenase. Complete nucleotide sequences encoding choline dehydrogenase (betA) and betaine aldehyde dehydrogenase (betB) have been characterised (Lamarck et al., 1991). Recently, three genes (gbsA, gbsB, and gpdD) whose products were essential for glycine betaine biosynthesis, transport and osmo-protection in *Bacillus subtilis* have also been reported (Boch et al., 1996; Kappes et al., 1996).

The study of osmoregulation is of great significance in agriculture, since the availability of water in soil is a limiting factor for crop growth and productivity. It has been

**References**


