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Overproduction of *Escherichia coli* D-Xylose Isomerase Using λP_L Promoter

PARK, HEUI-DONG, GIL-JAE JOO[1], AND IN-KOO RHEE*[1]

Department of Food Science and Technology, [1]Department of Agricultural Chemistry
Kyungpook National University, Taegu 702-701, Korea

In order to overproduce D-xylose isomerase, the *Escherichia coli* D-xylose isomerase (D-xylose ketol-isomerase, EC 5.3.1.5) gene (*xylA*) was fused to λP_L promoter. The promoterless *xylA* gene containing the ribosome binding site and coding region for D-xylose isomerase was cloned into a site 0.3 kb downstream from the λP_L promoter on a high copy number plasmid. An octameric *Xba*I linker containing TAG amber codon was inserted between 33rd codon of λN and the promoterless *xylA* gene. The resulting recombinant plasmid (designated as pPX152) was transformed into *E. coli* M5248 carrying a single copy of the temperature sensitive $\lambda cI857$ gene on its chromosomal DNA. When temperature-induced, the transformants produced 15 times as much D-xylose isomerase as that of D-xylose-induced parent strain. The amount of overproduced D-xylose isomerase was found to be about 60% of total protein in cell-free extracts.

D-Xylose isomerase (D-xylose ketol-isomerase, EC 5.3.1.5) is a very useful enzyme, which is great potential for carbon source in industrial applications. It converts non-fermentable D-xylose to fermentable D-xylulose for the production of alcohol by *Saccharomyces cerevisiae*. This enables *S. cerevisiae* to use hemicellulose hydrolysates containing D-xylose up to 60% of total sugar in the synthesis of ethanol (6, 23). It is well known that this enzyme has also the catalytic activity for converting D-glucose to D-fructose (22). Therefore, it has been effectively used in the production of high fructose corn syrup. By these reasons, many studies have been reported on D-xylose isomerase and its gene (*xylA*) (2, 9, 10, 13, 17-21, 23). The *xylA* gene has been isolated and characterized from various microorganisms such as *Escherichia coli* (9, 10, 17, 21), *Salmonella typhimurium* (2, 10), *Ampullariella* sp. (18), *Bacillus subtilis* (24) and so on. In a previous paper (13), we have reported on the isolation and characterization of the *E. coli xylA* gene. When cloned on a high copy number plasmid, the *E. coli xylA* gene failed to support high level expression, suggesting that *xylA* expression through its natural promoter is highly regulated (13). In order to overcome this problem, the *xylA* structural gene has been fused to other heterologous strong promoters such as *tac* (20) and λP_L (8). It is well known that λP_L promoter is a very strong promoter in *E. coli* (1, 15, 16). Also that, this promoter can be modulated very easily in an *E. coli* strain carrying the temperature sensitive $\lambda cI857$ gene (15). Lastick *et al.* has reported that the fusion of the *E. coli xylA* gene to a λP_L promoter resulted in overproduction of D-xylose isomerase up to 38% of total protein in cell lysates (8). However, they used an *E. coli* strain carrying the temperature sensitive $\lambda cI857$ gene on another plasmid, pRK248, as a host strain in order to control the λP_L promoter (8). This should affect the efficiency of the transcriptional activity of the λP_L promoter.

In this study, we describe the fusion of the promoterless *E. coli xylA* gene to the λP_L promoter and overproduction of D-xylose isomerase up to 60% of total protein in cell-free extracts from an *E. coli* strain carrying a single $\lambda cI857$ gene on its chromosomal DNA.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The bacterial strains used in this study were *E. coli* HB101 (F^-, *hsd*20, *rec*A13, *rpsL*20, *supE*44, *pro*A2, *mtl*-1, *ara*14, *lacY*1, *galK*2, *xyl*5, λ^-; 11), C600 (F^-, *supE*44, *thi*1, *lacY*1, *thr*1, *ton*A2, *leuB*6, λ^-; 11), C604 (*xylA* mutant of C600; 13), and M5248 ($\lambda cI857$, *bio*275, ΔH1; 15). Plasmids, pPL111 (12) and pEX13 (13) which were used as a cloning vector and a source of the *E. coli xylA* gene, respectively, are shown in Fig. 1.

Media and Culture Conditions

Bacteria were grown in LB broth (1% Bacto-tryptone,

*Corresponding author
Phone: 82-53-950-5718. Fax: 82-53-953-7233.
E-mail: ikrhee@bh.kyungpook.ac.kr.
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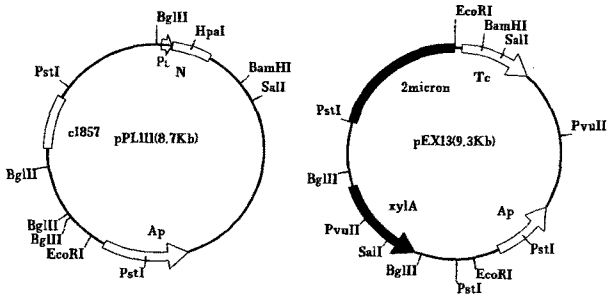

Fig. 1. Restriction maps of pPL111 and pEX13 plasmids used in this study.

Plasmid pPL111 used as a cloning vector carries λP_L promoter and $\lambda cI857$ gene on pBR322 backbone (12). Plasmid pEX13 used as a *xylA* gene source has been described in detail in our earlier study (13). Long and short arrows indicate the transcriptional directions of a gene and a promoter, respectively.

0.5% yeast extract, 0.5% NaCl) for cell growth and in Davis-Mingioli minimal media (4) composed of 0.5% K_2HPO_4, 0.3% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.05% Na-citrate $\cdot 3H_2O$, and 0.2% carbon source for D-xylose isomerase production. 0.2% D-glucose and D-xylose were used as carbon sources for the uninduced and xylose-induced conditions, respectively. To induce D-xylose isomerase by the λP_L promoter, bacteria were grown at 32°C in LB medium containing 50 μg/ml ampicillin. At the cell density of 1.0 of absorbance at 600 nm, the culture temperature was changed to 42°C by the addition of an equal volume of fresh LB medium prewarmed to 53°C with swirling. And then, cells were allowed to grow at 42°C for additional 5 h. Cell growth was monitored with a spectrophotometer by measuring the optical density at 600 nm.

Recombinant DNA Techniques

DNA manipulation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described by Maniatis *et al.* (11).

Protein Analysis

Cell-free extracts were prepared with sonication of cultured cells and centrifuged at 15,000 g at 4°C for 30 minutes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (7). The gel was stained with Coomassie brilliant blue R-250 and scanned using Densitron (Joosangyo Ltd., Japan).

D-Xylose Isomerase Activity Assay

D-Xylose isomerase activity was assayed with toluenized cell suspension as described earlier (13). The reaction mixture, composed of 50 μmol of Tris-maleate buffer (pH 7.5), 1 μmol of $MnCl_2$, 10 μmol of D-xylose, 1 μmol of $Na_2B_4O_7$, and the toluenized cell suspension in 0.7 ml of H_2O, was incubated at 37°C for 30 minutes. The D-xylulose formed in 50 μl of the reaction mixture was determined by measuring the absorbance at 540 nm by the cysteine-carbazole method (5). Activity was expressed with the increase of absorbance at 540 nm per ml of cell suspension whose cell density was 1.0 at 600 nm under the conditions described above.

RESULTS AND DISCUSSION

Construction of λP_L Promoter-*xylA* Fusion Plasmids

The function of the *xylA* promoter has been shown to be tightly regulated by the *xylR* gene product, a D-xylose regulatory protein (9, 17). When the *xylA* gene including its natural promoter was cloned on a high copy number plasmid, it resulted in only 3-fold production of D-xylose isomerase (13). For the purpose of overproduction of D-xylose isomerase, the *xylA* gene was fused to the λP_L promoter which was known as a very strong promoter in *E. coli*. Transcriptional activity of the λP_L promoter has been reported to be 8 to 10 times stronger than that of the *lac* promoter (3). In addition, its activity can be regulated easily by controlling the culture temperature with an *E. coli* strain carrying the temperature sensitive $\lambda cI857$ gene (15). A 1.6 kb *Bgl*II fragment containing the *xylA* gene was isolated from plasmid pEX13, and ligated into the *Bam*HI site, 1.1 kb downstream from the λP_L promoter on the pPL111 plasmid on the same transcription orientation (Fig. 1). The resulting plasmid was designated as pPX15 (Fig. 2). In order to delete the *xylA* promoter and λt_L terminator (16) located upstream from the *xylA* structural gene, pPX15 was digested with *Hpa*I and *Sst*II, and then was made blunt-ended with T_4 DNA polymerase treatment. A large fragment (9.3 kb) was isolated from the agarose gel, ligated with 8-mer *Xba*I linker, and thereby a TAG termination codon between the residual 33 codons of the λN and the promoterless *xylA* gene containing the intact ribosome binding site could be inserted, yielding plasmid pPX151 (Figs. 2, 3). The $\lambda cI857$ gene was deleted from the high copy number plasmid, pPX151, with *Bgl*II digestion followed by isolation and self-ligation of the 6.2 kb fragment. The final plasmid, designated as pPX152 (Fig. 2), was transformed into *E. coli* M5248 (15) carrying the temperature sensitive $\lambda cI857$ gene on its chromosomal DNA. All those plasmids were confirmed by restriction enzyme analyses.

Overproduction of D-Xylose Isomerase Using the λP_L-*xylA* Fusion Plasmid

When temperature-induced, *E. coli* M5248 harboring pPX152 produced D-xylose isomerase even in the absence of D-xylose in LB medium. To investigate the λP_L dependence of the D-xylose isomerase induction in the recombinant *E. coli* cells, a set of experiment was carried out as follows; a batch of culture was induced by temperature shift to 42°C and kept for additional 6 h. The second batch was induced at 42°C for 1 h, and shift-

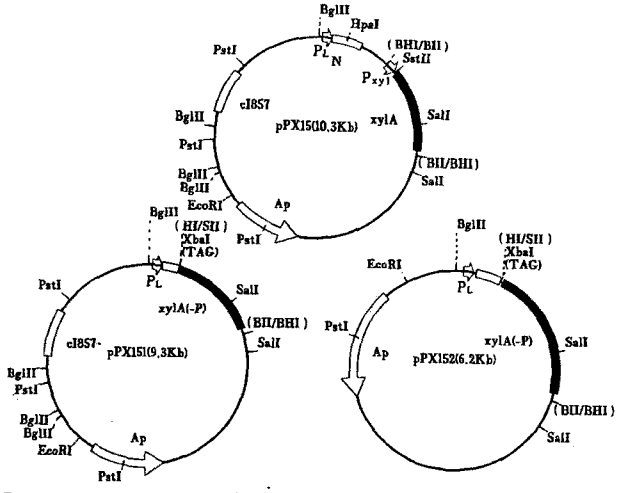

Fig. 2. Construction of λP_L promoter-*xylA* gene fusion plasmids for overproduction of D-xylose isomerase in *E. coli*.
Plasmid pPX15 was constructed by the cloning of 1.6 kb *Bgl*II fragment containing *E. coli xylA* gene into the *Bam*HI site, 1.1 kb downstream from the λP_L promoter on the pPL111 plasmid. In order to delete λt_L and *xylA* promoter, pPX15 was digested with *Hpa*I and *Sst*II, and then was made blunt-ended with T_4 DNA polymerase. A 9.3 kb fragment was isolated and ligated with 8-mer *Xba*I linker to insert TAG termination codon between residual 33 codons of the λN and the promoterless *xylA* gene (pPX151). The $\lambda cI857$ gene was deleted from pPX 151 with *Bgl*II digestion followed by self-ligation of the 6.2 kb DNA fragment to yield pPX152. Parentheses indicate destroyed restriction sites during the construction of the plasmids.

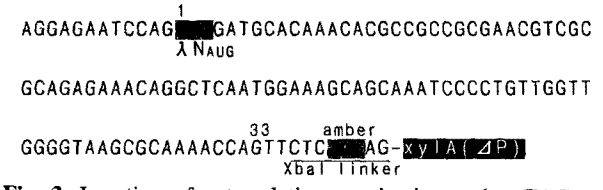

Fig. 3. Insertion of a translation termination codon TAG using 8-mer *Xba*I linker between 33 codons of λN and promoterless *xylA* gene in plasmid pPX151.

ed down to 32°C, and then grown for additional 5 h. The third batch was cultured at 32°C for 2 h after the temperature induction for 1 h at 42°C, and then its temperature was shifted up again to 42°C and kept on for 3 h. D-Xylose isomerase activities were assayed with cells from those 3 different cultures after toluenization, and expressed with units per ml of culture broth (Fig. 4). That the induction of D-xylose isomerase occurred when the temperature was shifted to 42°C, but not at 32°C, indicating that the induction was certainly dependent on the λP_L promoter. Maximum induction of the enzyme observed at 5 h culture after the temperature induction. No induction was observed at 32°C in M5248 harboring pPX152, suggesting that a single copy of the $\lambda cI857$ gene on its chromosomal DNA was enough to repress

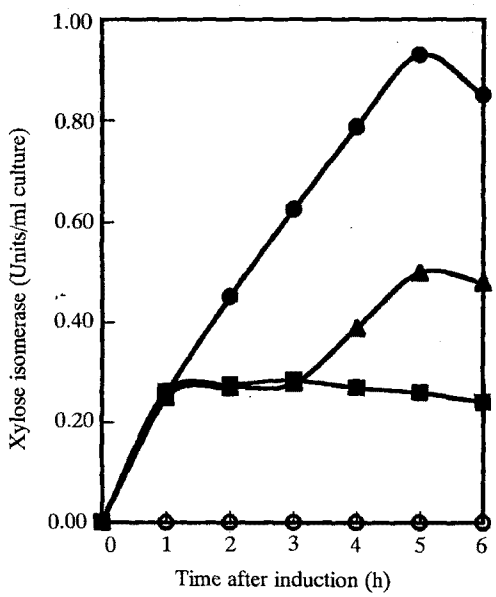

Fig. 4. Temperature dependent production of D-xylose isomerase in *E. coli* M5248 harboring plasmid pPX152.
●, culture kept at 42°C for additional 6 h after temperature induction; ■, culture kept at 42°C for 1 h after temperature induction, and then 32°C for additional 5 h; ▲, culture kept at 42°C for 1 h after temperature induction, 32°C for 2 h, and then 42°C again for 3 h; ○, culture kept at 32°C for 6 h without temperature induction.

the function of the λP_L promoter on the high copy number plasmid. The cells of strain M5248 harboring plasmid pPX152, and C604 harboring pPX15 or pPX151 were subjected to the temperature induction for 5 h at 42°C in LB media containing no D-xylose. No temperature induction of the synthesis of D-xylose isomerase was observed in strain C604 harboring pPX15. This reason has not been elucidated yet. While strain C604 harboring pPX151 showed an induction response but the expression level of the enzyme was very low compared with that obtained with the D-xylose-induced parent strain, C600. This is at least partly to be caused by the fact that the $\lambda cI857$ gene was located on the same high copy number plasmid carrying the λP_L-*xylA* gene. This result indicated that the temperature sensitive λcI repressor could be also produced too much because of gene dosage effect, and was not completely inactivated during the temperature induction. However, M5248 harboring plasmid pPX152 produced large amounts of D-xylose isomerase, which was about 15 times as much as those produced by the D-xylose-induced C600 cells (Fig. 5).

Analysis of D-Xylose Isomerase Overproduced

To analyze the overproduced D-xylose isomerase, cell-free extracts were prepared from the temperature-induced cells of M5248 harboring plasmid pPX152 at 42°C for 5 h and electrophoresed on 10% SDS-polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie

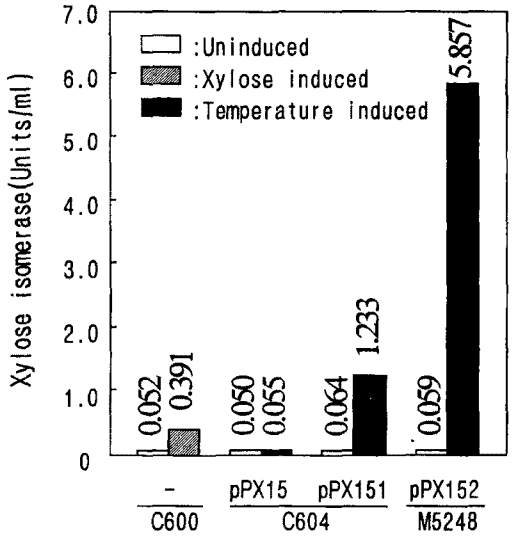

Fig. 5. D-Xylose isomerase activity of each strain harboring λP_L-*xylA* fusion plasmid.

Strains and plasmids used were shown below the figure. D-Xylulose formed in the D-xylose isomerase reaction mixture was determined at 540 nm by the cysteine-carbazole method (5). Activity was expressed with the increase of absorbance at 540 nm per ml of cell suspension whose cell density was 1.0 at 600 nm under the conditions of this study.

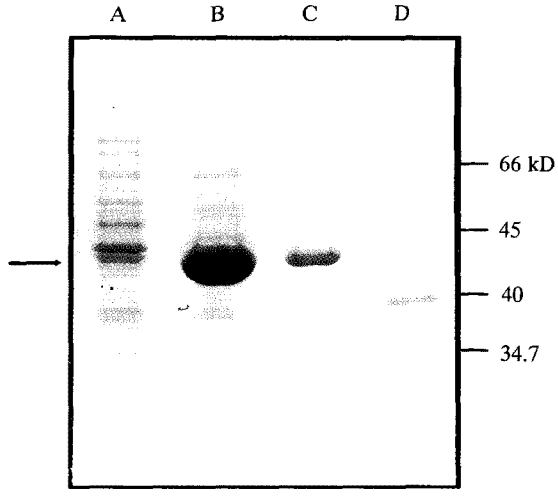

Fig. 6. SDS-PAGE analysis of cell-free extracts of *E. coli* M 5248 harboring plasmid pPX152.

150 μg of protein in the extracts of uninduced cells (A), temperature-induced cells (B), and 1/5 amount of protein (C) of that used on lane B were electrophoresed on 10% SDS-polyacrylamide gel by the method of Laemmli (7). The numbers represent molecular weight (kDa) of the standard marker proteins in lane D. Arrow indicates overproduced *E. coli* D-xylose isomerase.

brilliant blue. Large amounts of D-xylose isomerase was observed from the cell-free extracts from temperature-induced cells (Fig. 6, lane B), even when the extract actually loaded had been diluted by 5 fold (Fig. 6, lane C). When the stained gel was scanned with densitometer, the amount of overproduced D-xylose isomerase was estimated to be about 60% of the total protein in the cell-free extracts. Molecular weight of the overproduced D-xylose isomerase was about 44 kDa on SDS-PAGE (Fig. 6), which is consistent with the *E. coli* D-xylose isomerase (19). The overproduction of various heterologous proteins in *E. coli* using the λP_L promoter has been reported in many papers. For instance, Remaut *et al.* has reported the overproduction of β-lactamase in *E. coli* up to 33% of total cellular protein using a λP_L promoter (14). *E. coli* tryptophan synthetase A (14) and T_4 DNA ligase (15) have been expressed up to 39 and 20% of total protein by the λP_L promoter, respectively. The first attempt to overproduce *E. coli* D-xylose isomerase using a λP_L promoter was carried out by Lastick *et al.* (8). They cloned the promoterless *xylA* gene into the *Hpa*I site, 0.3 kb downstream from the λP_L promoter on a high copy number plasmid. This recombinant plasmid was transformed into an *E. coli* strain carrying the λ*cI*857 gene on another plasmid, pRK248 (8). When temperature-induced, this transformant has been found to produce D-xylose isomerase up to 38% of total protein which was lower than the level obtained in this study. Similar results were observed in the study of Bernard *et al.* (1) who studied the expression of the *trpA* gene under the control of a λP_L promoter. They used two different host strains carrying the λ*cI*857 gene, one on its chromosomal DNA and the other on a plasmid, pRK248. When the tryptophan synthetase activities were compared in both strains transformed with the λP_L promoter-*trpA* gene fusion plasmid, higher level expression was obtained in the strain carrying the λ*cI*857 gene on its chromosomal DNA than that obtained in the other strain (1).

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