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Purification and Characterization of a Regulatory Protein XylR in the D-Xylose Operon from *Escherichia coli*

SHIN, JAE-HO, DONG-HYUN ROH, GUN-YOUNG HEO, GIL-JAE JOO, AND IN-KOO RHEE*

Department of Agricultural Chemistry, Kyungpook National University, Daegu 702-701, Korea

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Abstract The D-xylose operon in *Escherichia coli* is known to be regulated by a transcriptional activator protein, XylR, which is responsible for the expression of both *xylAB* and *xylFGH* gene clusters. The XylR was purified to homogeneity by using the maltose binding protein fusion expression and purification systems involving two chromatography steps. The purified XylR protein was composed of two subunits of 45 kDa, which was determined by both sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel filtration. The purified XylR was specifically bounded to the *xylA* promoter, regardless of adding xylose to the reaction mixture, but binding of XylR to the *xylA* promoter was enhanced by adding xylose. The enhanced binding ability of XylR in the presence of xylose was not diminished by adding glucose. The presumed XylR binding site is located between 120 bp to 100 bp upstream from the *xylA* initiation codon.

Key words: D-xylose operon, *xylR*, transcriptional activator, xylose isomerase

Using the pentose phosphate pathway, *Escherichia coli* can actually grow on xylose as its sole source of carbon and energy [11, 20]. The *xylB*, *xylA*, *xylF*, *xylG*, *xylH*, and *xylR* genes are clustered on the chromosome in the order given. The divergent promoter is located between *xylF* and *xylA* genes [18]. Essential elements for the metabolism of xylose in *E. coli* are the isomerization of xylose to xylulose by xylose isomerase (*xylA*), and the phosphorylation of xylulose to xylulose-5-phosphate by xylulokinase (*xylB*). These conversions are required for xylose utilization in *E. coli* to occur [5]. The *xylF* gene encodes the xylose binding protein for a high affinity transport [21]. The role of the *xylGH* genes is still unknown, although the gene products have been induced by the xylose [19]. Supposedly, the regulation of the xylose catabolism is mediated by the *xyl* activator (XylR) that is a regulatory protein encoded by the *xylR* gene and involved in a pleiotropic response for the xylose utilization process [14, 15, 17, 19]. The *xylR* gene was initially cloned [14] and sequenced [18]. Song and Park [19] revealed that the XylR binds at least to two divergent promoters by *in vivo* footprinting analysis. In addition, they attempted to analyze the binding of XylR to the promoter region with the purified XylR, which turned out to be unsuccessful due to a loss of DNA-binding activity of the purified XylR.

Several mutants of the *xylR* gene were obtained from *E. coli* JM109 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment. It was found that in the gel mobility shift assay, the mobility of *xylA* promoter on the gel was retarded by the crude cell extracts of all mutants of *xylR*, as well as that of the wild-type strain. It is necessary to purify the regulatory protein, XylR, in order to investigate any differences between their crude cell extracts and purified XylR on the binding ability to the *xylA* promoter. Since the cell extracts of *xylR* mutants are also bound to *xylA* promoter in the gel mobility shift assay, an attempt was made to elucidate whether the XylR is specifically bound to the *xylA* promoter or not. In this paper, we purified the XylR protein and investigated the stability, binding activity, oligomeric structure, and the binding site of XylR in the *xylA* promoter.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

E. coli strain JM109 and its *xylR* mutant, strain DH60, were grown in Luria-Bertani (LB) medium at 37°C. *E. coli* JM109 was the source of DNA for construction of maltose-binding protein (MBP)-XylR fusion protein, and PCR template DNA for the *xylA* promoter region. The *xylR* mutant strain DH60 contains a single point mutation in its

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

xylR gene that results in replacing the glycine residue with serine at a position 84 in the amino acid sequence of XylR.

General Methods and Materials

Plasmid transformations were carried out as described by Sambrook *et al.* [16], using competent cells prepared by the method of Inoue *et al.* [9]. Chromosomal DNA was prepared as described in Ausubel *et al.* [1]. Plasmid DNA was prepared by the alkaline lysis procedure [16]. Restriction endonucleases, T4 DNA ligase, Klenow fragment, exonuclease III, mung bean nuclease, Taq DNA polymerase, and T4 DNA polymerase were obtained from Takara (Shiga, Japan) and used according to the manufacturer's instructions. Isotope [α-^{32}P]dCTP and [α-^{35}S]dATP were purchased from Amersham-Pharmacia Biotech (Buckinghamshire, England). Oligonucleotides used in PCR reaction were obtained from Bioneer Corporation (Chungwon, Korea).

Construction of Overexpression Vectors for XylR Production

The *xylR* gene was fused to the *malE* gene by using the pMAL-c2 fusion expression vector (New England Biolabs, U.S.A.). The *xylR* gene was PCR amplified from chromosomal DNA of *E. coli* JM109 with Taq DNA polymerase by using the forward primer (ATGTTTACTA-AACGTCACCG) and reverse primer (ATGGATCCCTA-CAACATGACCTCGCT), which contained the engineered restriction site *Bam*HI just after the translational stop codon. The PCR solutions were overlaid with sterile mineral oil and inserted into a thermal cycler for 32 cycles under the following conditions: 94°C, 3 min (first cycle only); 94°C, 2 min; 50°C, 1.5 min; 72°C, 2 min; and 72°C, 8 min (final cycle only). After the reaction of PCR, the PCR products were blunt-ended with a Klenow fragment and digested with *Bam*HI to allow the directional ligation into pMAL-c2 to be linearized with *Xmn*I and *Bam*HI, yielding the plasmid pMALR8, in which the *xylR* structural gene was fused in-frame to the downstream of the *malE* gene. Plasmid pMALR8 was transformed into *E. coli* DH60, and the entire insert of the pMALR8 was sequenced to ensure the fidelity of PCR amplification and in-frame ligation with *malE*.

Purification of MBP-XylR and XylR Protein

To purify MBP-XylR fusion protein, the cultures of *E. coli* DH60 containing pMALR8 were grown to the mid-log-phase (OD 600 nm=1.0) at 37°C, after which isopropyl-β-D-galactopyranoside (IPTG; Sigma-Aldrich Co., U.S.A.) was added to a final concentration of 1 mM, and the bacterial cells were incubated at 37°C for a further 4 h. Cells were chilled on ice and harvested by centrifugation. The cell pellets from 500 ml of culture broth were washed twice with the column buffer (100 mM of Tris-HCl, pH 7.5; 250 mM of NaCl; 1 mM of dithiothreitol; 1 mM of 2-mercaptoethanol) and resuspended in a final volume of 15 ml. Cells were disrupted at 90 μA five times per one min with use of an ultra-sonicator (Ultrasonics Ltd., England). The resulting lysate was clarified by applying the centrifugation process for 30 min at 20,000 ×g at 4°C. The supernatant solution containing the fusion protein was diluted to 30 ml with a column buffer containing freshly prepared 50 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich Co., U.S.A.). The sample was loaded onto an affinity column (2.5×15 cm) containing 10 ml bed volume of amylose resin (New England Biolabs, U.S.A.). The bound fusion protein was washed with 150 ml of the column buffer and eluted to 3 ml fractions at a flow rate of 1 ml/min with the column buffer supplemented with 1 M of maltose. The fractions that contained MBP-XylR were determined by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The MBP-XylR fusion protein was eluted over a wide peak from a fraction number of 55 to 58 (Fig. 3A). The eluted fusion protein was resolved as a single band of approximately 90 kDa on SDS-PAGE (lanes 3 and 4 in Fig. 4A). To separate pure XylR from these fusions, MBP-XylR fusion protein was cleaved by a factor Xa protease (New England Biolabs, U.S.A.). In fact, this was accomplished by adding 1 μg of protease to 1 mg of the fusion protein in a 1 ml volume, and then, incubating it for 24 h at 0°C. Following cleavage, the sample was dialyzed against 1 liter of Q-Sepharose column buffer (10 mM of Tris-HCl, pH 7.4; 25 mM of NaCl). The dialysate was loaded onto a 15 ml Q-Sepharose column, and the column was washed with 150 ml of the same column buffer. The proteins were eluted with a 100 ml gradient of 0.1 to 1.0 M of NaCl in column buffer and the fractions with a binding activity were pooled, and freshly prepared 20 mM PMSF was added to the solution. The purified XylR protein was divided into an appropriate volume, and stored at −70°C. The thawed XylR protein was used immediately in a mobility shift assay, because its binding activity was completely lost within 30 min at 30°C (data not shown). During the purification steps, the protein concentrations were determined by the Bradford method [3].

Determination of Molecular Mass of the Purified XylR by Gel Filtration

The molecular mass and oligomeric structure of the purified XylR were determined by using the fast protein liquid chromatography (FPLC; Pharmacia Biotech, Sweden) mediated gel filtration with Superose 12 HR 10/30 prepacked column. The column was equilibrated with a column buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA) and loaded with 100 μl (10 μg of protein) volume of XylR and marker proteins. The proteins were eluted with the same buffer at a flow rate of 0.5 ml/min. The eluted

protein was detected by monitoring at A_{280}. The column was calibrated with three different size proteins, 180 kDa of glucose isomerase from *Streptomyces chibaensis* [10], 45 kDa of ovalbumin (Sigma-Aldrich Co., U.S.A.), and 14.3 kDa of lysozyme (Sigma-Aldrich Co., U.S.A.).

Construction of *xylA* Promoter Deletion Plasmids

Unidirectional truncations with exonuclease III were performed to make the successive deletion from the 5' region of the promoter in the *xylA* gene of *E. coli* by using the protocol as described by Henikoff [8] with slight modifications. Plasmid pUX30 contains the *xylA* structural gene and 209-bp *xylA* promoter region derived from pEX13 [13], which were inserted into the *Bam*HI site of plasmid pUC19 [12], where the 5' promoter region of the *xylA* gene is inserted proximally to the *Eco*RI site in the multiple cloning site. Deletions were initiated by the treatment with exonuclease III after cutting pUX30 with *Sac*I and *Sma*I to allow unidirectional deletions into the *xylA* promoter region. After being digested with exonuclease III and mung bean nuclease, the deleted DNA fragments were resuspended in 20 μl of ligation buffer (250 mM of Tris-HCl, pH 7.6; 50 mM of $MgCl_2$; 1 mM of dithiothreitol; 0.5 mM of ATP) and 1 unit of T4 DNA ligase was added. The ligation mixture was then incubated at 16°C for 12 h. The precise endpoints of each deletion at the 5' region of the *xylA* promoter were determined by the analysis of the DNA sequence. The resulting plasmids, named pUX31, pUX32, pUX33, and pUX34, contained 171, 166, 124, and 120-bp of the *xylA* promoter region (209-bp) in pUX30.

Specific truncations in the *xylA* promoter were generated by PCR using forward primers P36 (CGAATTCGAATTATCTCAAT) and P37 (TGAATTCAATAGCAGT GTGA) with an *Eco*RI recognition site prior to the desired truncation point, and reverse primer P00 (GAGGATCCTGAGCCTTCATA) complementary to the 50 bases posterior to the *xylA* transcriptional initiation codon with a *Bam*HI recognition site. These oligonucleotides were annealed to chromosomal DNA of *E. coli* JM109 and amplified (30 cycles at 72°C with the Progene PCR system; Techne Ltd., England) to obtain the DNA fragments of truncated *xylA* promoter. The PCR products were digested with *Eco*RI and *Bam*HI and inserted into pUC19 that was linearized with the same enzymes. The resulting plasmids named as pUXP36 and pUXP37 were confirmed by the analysis of DNA sequences, which contained 110-bp and 100-bp of the *xylA* promoter region. All sequence analyses of DNA were performed with an ALF Express Auto Cycle Sequencing Kit and ALF Express auto-sequencer (Pharmacia Biotech, Sweden).

Gel Mobility Shift Assay

Gel mobility shift assays were performed by using the protocol as described by Ausubel *et al.* [1] with minor modifications. The *xylA* promoter truncated plasmids pUX30, pUX31, pUX32, pUX33, and pUX34 were digested by *Eco*RI and *Ssp*I, resulting in probe 1 (159-bp), probe 2 (121-bp), probe 3 (116-bp), probe 4 (74-bp), and probe 5 (70-bp), respectively. The pUXP36 and pUXP 37 were digested by *Eco*RI and *Bam*HI, yielding fragments of 160-bp (probe 6) and 150-bp (probe 7), respectively. The digested fragments were purified from 10% (w/v) polyacrylamide gel by following the crush-and-soak method [16] and labeled with [α-^{32}P]dCTP or [α-^{35}S]dATP by using the Klenow fragment. DNA binding reaction was performed in a 20-μl mixture containing 12 mM of HEPES-NaOH (pH 7.9), 4 mM of Tris-HCl (pH 7.9), 60 mM of KCl, 1 mM of EDTA, 1 mM of DTT, 300 μg of bovine serum albumin, 2 μg of poly(dI-dC), 1 ng of labeled *xylA* promoters (10,000 cpm) for probe, and suitable amounts of the crude extract or purified XylR. The reaction mixtures were incubated at 30°C for 15 min, loaded onto 6% (w/v) polyacrylamide gels, and separated by electrophoresis at 4°C and 15 V/cm for 90 min in low ionic strength running buffer containing 6.725 mM of Tris-HCl (pH 7.9), 3.3 mM of sodium acetate (pH 7.9), and 1 mM of EDTA (pH 8.0). The buffer was continuously circulated between the upper and lower buffer chamber. The gels were transferred to Whatman paper No. 2, dried, and subjected to autoradiography.

Results and Discussion

Binding of *E. coli* Cell Extracts to *xylA* Promoter

We have isolated *xylR* mutant DH60 from *E. coli* JM109 by treating with NTG. The *xylR* mutant *E. coli* DH60 was not able to produce either xylose isomerase or xylulokinase [14]. *E. coli* DH60 had a nonfunctional *xylR* gene, which contained a single point mutation in the 84th amino acid (Gly-84 to Ser-84) of the wild-type XylR, determined by DNA sequence analysis.

A [α-^{35}S]dATP-labeled 159-bp DNA fragment extending from −209 to −50 nucleotides from the *xylA* initiation codon (probe 1) was used in mobility shift assays to detect proteins which were binding to the *xylA* promoter region (Fig. 1). Crude extracts of *E. coli* JM109 (10 μg of total protein) induced with xylose caused a 159-bp fragment of the *xylA* promoter to have a distinct mobility shift on the gel (lane 3, Fig. 1), whereas the shifted band was very weak in cell extracts of *E. coli* JM109 without any induction (lane 2, Fig. 1).

The mobility of *xylA* promoter on the gel was retarded by binding the crude cell extracts of *xylR* mutant as well as that of JM109. The binding intensity of cell extracts of XylR mutants was very weak, as compared with that of JM109 (Fig. 1). But the intensity of shifted band by cell extracts of *xylR* mutant did not show any differences

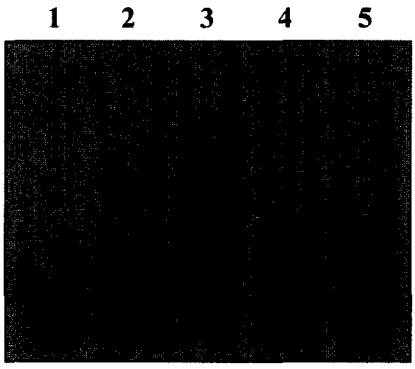

Fig. 1. Mobility shift of the [α-^{35}S]dATP-labeled *xylA* promoter region with cell extracts of *E. coli* JM109.
Cell extracts (10 μg) were incubated with 1 ng of labeled probe 1 (10,000 cpm) and 2 μg of poly (dI-dC) as described in Materials and Methods. Lanes; 1, probe 1 (159-bp *xylA* promoter) only without cell extract; 2, cell extract from *E. coli* JM109 without induction; 3, cell extract from *E. coli* JM109 induced with xylose; 4, cell extract of *E. coli* DH60 (*xylR* mutant) without induction; 5, cell extract of *E. coli* DH60 induced with xylose.

between the cell extracts induced with and without xylose (lane 4 and 5, Fig. 1). It is still unknown whether the mutant XylR protein was induced by xylose or not. The mutant XylR protein from DH60 was able to bind to the promoter, but it could not activate binding of XylR to the promoter by xylose.

In order to prove the specificity of binding of XylR in the cell extract to the [α-^{32}P]-labeled probe 1 (159-bp *xylA* promoter), the intensity of shifted bands was investigated (Fig. 2), using the same amount of cold probe 1 that was added to the reaction mixture. The shifted bands almost disappeared by adding a 5-fold molar excess of the

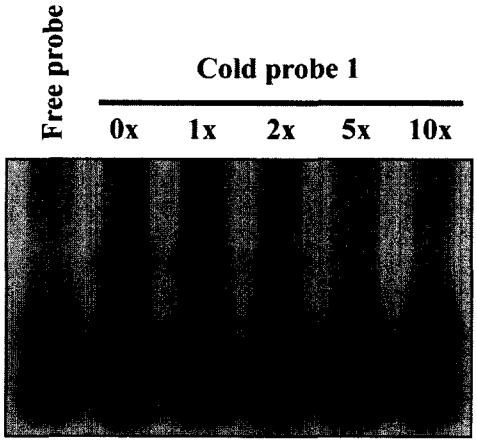

Fig. 2. Competition of [α-^{32}P]dCTP-labeled probe 1 (159-bp *xylA* promoter) with the unlabeled probe for the binding of XylR in cell extract of *E. coli* JM109 induced with xylose.
The molar excess of cold probe was added to the mobility shift assay mixture containing 10 μg of extract, 1 ng of labeled probe 1, and 2 μg of poly (dI-dC).

cold probe, indicating the cold probe 1 competing with the hot probe in binding to XylR. This means that the shifted band was made by specific binding of XylR to *xylA* promoter.

Purification of XylR

Because the molar concentration of XylR was very low in a cell, the crude extract was not suitable for detailed investigation of the interaction between the XylR protein and *xylA* promoter on the mobility shift assay. In order to remove the effects of other DNA binding proteins, a purified XylR was necessary. At first, we constructed a XylR overexpressed plasmid by using a T7 expression system of Novagen Inc. (U.S.A.). The recombinant plasmid was able to complement the *xylR* mutant *in vivo*. Then, an attempt was made on a mobility shift assay with the XylR-overproduced cell extract. Unfortunately, any detectable shifted band under the assay condition using 1 μg of the cell extracts could not be observed, and this was most likely due to the inactivation by the aggregation and instability of XylR in the cell.

Won *et al.* [25] applied an MBP-fusion system to solubilize and overproduce AgfA protein of *Salmonella enteritidis*, which had an extremely aggregative nature, since MBP can facilitate the folding of fimbriae and increase the stability in the cell, resulting in a soluble form of the protein. Therefore, we tried to apply the MBP-fusion expression system for overexpression and purification. We constructed an overexpression vector pMALR8 in which the *xylR* gene was fused in-frame to the downstream of the *malE* gene as described in Materials and Methods.

The expressed MBP-XylR protein was found to be active on the transcription like the wild-type of XylR. The *xylR* mutant *E. coli* DH60 containing pMALR8 could actually produce xylose isomerase and xylulokinase and regulate the production of both enzymes as well as *E. coli* JM109, which is the parent strain of the *xylR* mutant. The *malE-xylR* fusion gene in pMAL-c2, which can produce MBP-XylR fusion protein, complements *xylR* mutation in *E. coli* DH60. It is not clear whether a few fusion proteins are cleaved to MBP and XylR by a specific protease *in vivo* or if the fusion protein itself acts as a regulatory protein for the expression of *xylA* gene in *E. coli* DH60. But *E. coli* DH60 containing pMALR8 is shown to be slow on the catabolite repression by addition of glucose to the media, as compared with *E. coli* JM109 (data not shown).

Using this MBP-XylR fusion expression system, XylR was purified through two different chromatographic steps. Crude cell extract of *E. coli* DH60 containing pMALR8 was loaded on to the amylose affinity column equilibrated with column buffer. Most of the proteins passed through by washing the amylose affinity column. MBP-XylR immediately emerged after loading the buffer containing maltose (Fig.

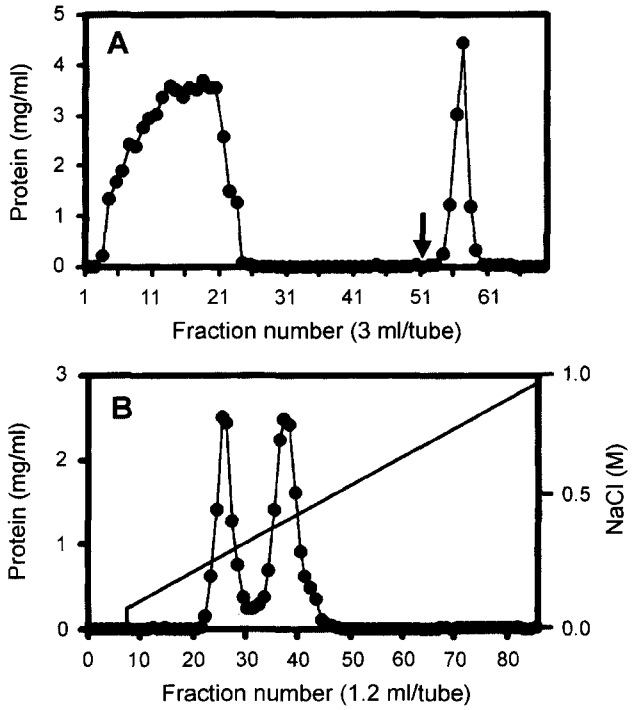

Fig. 3. Purification of XylR with column chromatography.
(A) Amylose affinity column chromatography with cell extract of *E. coli* DH60 containing pMALR8. Crude extract was loaded on to the column (2.5×15 cm). The MBP-XylR fusion proteins were eluted with the buffer containing maltose at the fraction number 51 (arrow). Fractions from 55 to 58 were pooled for further purification. (B) Q-Sepharose column chromatography with MBP-XylR fusion protein digested by factor Xa protease. The active fractions from the amylose affinity column were treated by 1% factor Xa protease and loaded on to the column (2.5×15 cm). Proteins were eluted with a 100 ml linear gradient of 0.1 M to 1.0 M of NaCl.

3A). Amylose affinity column was able to remove most of the other proteins from the crude cell extract.

To cut out MBP from this MBP-XylR fusion protein, the MBP-XylR protein was digested by incubating with 1% (w/w) factor Xa protease for 24 h at 0°C. The cleavage between MBP and XylR was confirmed by SDS-PAGE (lane 5, Fig. 4A) and mobility shift assays (lane 2, Fig. 4B). Under this condition, the fusion protein was digested completely. In order to remove the MBP residues, the digested proteins were applied to the Q-Sepharose column equilibrated with a column buffer. Two major peaks emerged in a gradient of 0.1 to 1 M of sodium chloride on the Q-Sepharose column (Fig. 3B). Binding activity was eluted in the second major peak (lane 4, Fig. 4B) of Q-Sepharose column chromatography and the same peak showed the distinct band of approximately 45 kDa on SDS-PAGE (lane 7, Fig. 4A), which was found to be in good agreement with the molecular mass of 44.9 kDa predicted from the DNA sequence of the *xylR* gene.

Figure 4 shows the SDS-PAGE and the corresponding mobility shift assays for the fractions eluted from amylose affinity column and Q-Sepharose column after being digested with factor Xa. The mobility of *xylA* promoter on the gel was shifted by the specific binding of the purified MBP-XylR fusion and XylR protein (Fig. 4B). In lanes 2 and 4 of Fig. 4B, a minor band was observed only when a very high concentration of XylR fraction was used in the assay, while the minor band was not detected on the gel of the undigested fusion protein (Fig. 4B, lane 1) and crude cell extracts (Figs. 1 and 2). Since most of their binding activity was lost during the complete digestion process with factor Xa for the preparation of the pure form of XylR, a partially digested XylR (more than 90%) was used

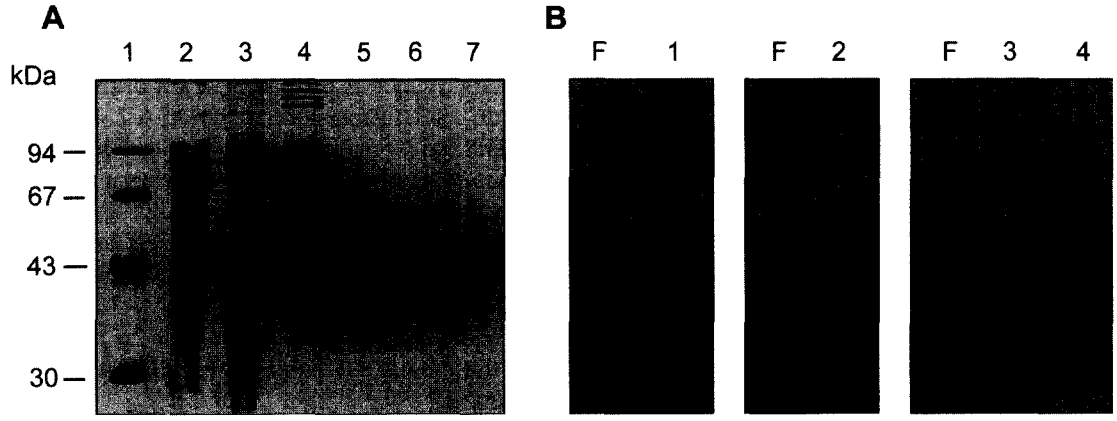

Fig. 4. SDS-PAGE and mobility shift assays of the purified MBP-XylR and XylR proteins.
(A) SDS-PAGE gel at different states of purification. Cell extract (10 μg) of *E. coli* DH60 containing pMALR8 induced with IPTG (lane 3) and without IPTG (lane 2), the active fractions (1 μg) from amylose affinity column (lane 4), MBP-XylR fusion protein digested with factor Xa (lane 5), and peak 1 (0.5 μg; lane 6) and peak 2 (0.5 μg; lane 7) fractions of Q-Sepharose column were loaded on to SDS-PAGE gel of 10% polyacrylamide. Lane 1 is the marker protein for molecular mass. Numbers indicate molecular mass in kDa. (B) Mobility shift assays of *xylA* promoter (probe 1) with proteins purified from *E. coli* DH60 containing pMALR8. One hundred ng of protein was incubated with probe 1 (10,000 cpm) for 15 min at 30°C, as described in Materials and Methods, before the mobility shift was analyzed. Lanes; F, probe 1 only without any proteins; 1, MBP-XylR fusion protein; 2, MBP-XylR fusion protein digested with factor Xa; 3, MBP (lane 6 in A; peak 1 fraction in Fig. 3B); 4, XylR protein (lane 7 in A; peak 2 fraction in Fig. 3B).

in the gel mobility shift assays. As a result, it can be assumed that a minor band above the major shifted band was due to contamination of MBP-XylR protein.

Characterization of Purified XylR

The purified XylR protein is extremely labile in heat and proteolysis. Its binding ability was completely lost within 60 min at 30°C. The binding ability was much accelerated by adding the crude cell extract, even in the presence of PMSF. The XylR protein could possibly be degraded rapidly in the cell, but MBP-XylR fusion protein was rather resistant to proteolysis. Unfortunately, the overproduction of XylR by the T7 expression system with the pET vector was not achieved.

The effects of xylose and glucose in the reaction mixture were investigated by the mobility shift assay with XylR and the *xylA* promoter (probe 1). When 300 mM of xylose was added to the reaction mixture (lane 3, Fig. 5), the intensity of the shifted band increased about two-fold, compared with the intensity of the shifted band in reaction mixture without xylose (lane 2, Fig. 5), which was calculated by using the Bio-Profil image analyzer system (Vilber Lourmat, France). However, there was no effect on the intensity of the shifted band by adding 150 mM of glucose (lane 4, Fig. 5). In the presence of xylose, the increased band intensity was not changed by adding glucose (lane 5, Fig. 5). The binding of XylR to *xylA* promoter is stimulated by xylose, and the glucose may not disturb the binding of xylose to XylR and XylR to *xylA* promoter. XylR protein possesses a region closely matching to a consensus sequence of binding proteins,

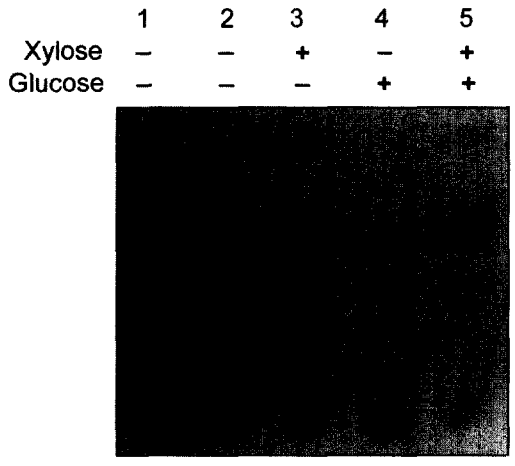

Fig. 5. Effect of xylose and glucose on binding affinity between XylR and *xylA* promoter.
Plus and minus symbols indicate the reaction mixture (20 μl) for the mobility shift assay with and without xylose or glucose, respectively. Lanes: 1, 1 ng of probe 1 (159-bp fragment of *xylA* promoter) without XylR protein; 2, 20 ng of XylR; 3, 20 ng of XylR with 300 mM of xylose; 4, 20 ng of XylR with 150 mM of glucose; 5, 20 ng of XylR with 300 mM of xylose and 150 mM of glucose.

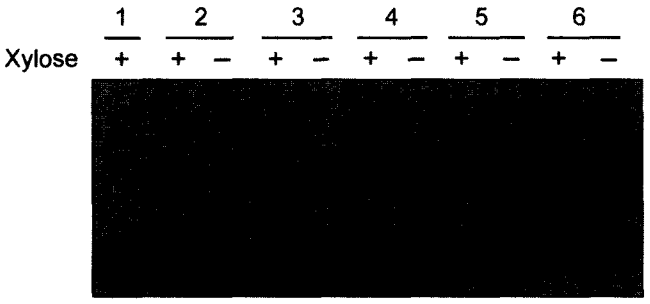

Fig. 6. Estimation of the binding affinity of XylR and the *xylA* promoter by mobility shift assay with increasing amounts of purified XylR protein.
Lane 1 contains 1 ng of probe 1 without XylR protein; Lanes 2 to 6 contain 1, 5, 10, 20, and 50 ng of XylR protein with the probe 1, respectively. Plus and minus symbols indicate the reaction mixture (20 μl) for the mobility shift assay with and without the addition of 300 mM of xylose, respectively.

which are specific for hexoses and pentoses. This consensus sequence was defined as $K(LIVFAG)_3IX_3D(SGP)X_3(GS)X(LIVA)_2X_2A$ in lactose, galactose, and fructose repressors of *E. coli* [22]. A motif in XylR that is similar to this consensus sequence is revealed on the amino acid sequence alignment (KLCVIGIDNEELTRYLSRVA). Thus, it is presumed that XylR contained a specific xylose-binding motif. Since the binding of XylR to the *xylA* promoter was stimulated by the xylose, it is clear that XylR acts as a positive regulator on the *xylA* promoter.

Using the image analyzer system, each intensity level of the free and shifted DNA bands was estimated from Fig. 6. The binding ratio for the interaction of XylR with the *xylA* promoter was calculated. When the ratio of the shifted DNA to the total DNA was 0.5 (50% shift), a 40.8-fold molar excess of XylR protein was needed to cause the

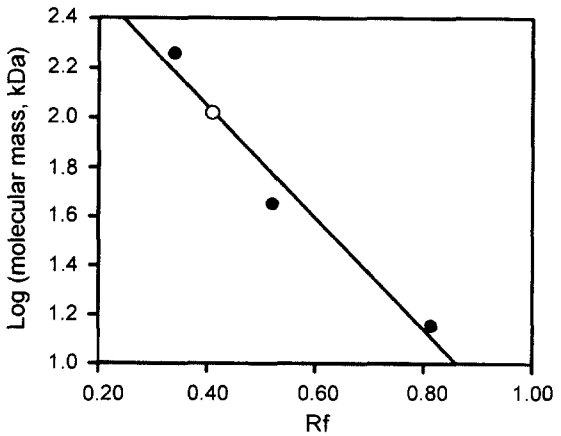

Fig. 7. Estimation of molecular mass of native XylR by gel filtration on Superose 12 HR column.
The size marker proteins (closed circles) were glucose isomerase (180 kDa), ovalbumin (45 kDa), and lysozyme (14.3 kDa). The open circle indicates the elution position of purified XylR protein.

shift. Alternatively, 734 fmol of XylR (as dimer) was needed to cause 50% of 18 fmol of the DNA probe 1 to actually shift. The XylR protein was needed only at 5.5-fold by adding 300 mM of xylose to the reaction mixture; 99 fmol of XylR was needed to cause 18 fmol of DNA probe 1 to 50% shift on the assay gel. The binding ratio was remarkably decreased from 40.8 to 5.5 by adding xylose to the reaction mixture. The binding of XylR to *xylA* promoter and increase of the binding affinity by xylose provide evidence of this protein for the positive regulation of the expression of *xylA* gene as an activator.

Molecular mass of the purified XylR protein was determined to be 100 kDa by FPLC on a Superose 12 gel filtration column (Fig. 7). As the purified protein showed an apparent molecular mass of 45 kDa on SDS-PAGE (lane 7, Fig. 4A), the native XylR might be composed of a dimer, which is a common characteristic of many DNA binding proteins [6]. Hendrickson and Schleif [7] reported that AraC protein in *E. coli* was found to bind with the *araI* and $araO_I$ regulatory sites as a dimer. RhaR and RhaS, which are recognized as transcriptional activator proteins of the L-rhamnose operon in *E. coli*, were also bound to the promoter region as a dimer [23].

Binding Site for XylR on *xylA* Promoter

Mobility shift assays with the probes of the truncated promoter were conducted in order to identify the binding site of the purified XylR on the *xylA* promoter. The serial deletion of the 5' region of promoter will restrain binding of XylR to the promoter. Song and Park [19] proposed that the XylR binding area was located at −120 bp to −76 bp upstream from the *xylA* initiation codon, by using the *in vivo* footprinting method. However, they did not demonstrate the direct interaction of purified XylR and specific site of *xylA* promoter. It is necessary to investigate the direct interaction of purified XylR and binding site of the *xylA* promoter. The binding region of XylR on *xylA* promoter is gradually limited by the successive digestion of the 5' region of the promoter with exonuclease III. The mobility shift assay was attempted with the DNA fragments of *xylA* promoter truncated with the exonuclease III. From the mobility shift assay, the binding site of XylR was assumed to be a 70-bp segment between the −120 bp and −50 bp at the *Ssp*I site (Fig. 8). Two additional DNA probes were synthesized by PCR (probes 6 and 7) to define the binding site in detail. XylR caused a mobility shift with probe 6 (−110 bp to +50 bp) but not with probe 7 (−100 bp to

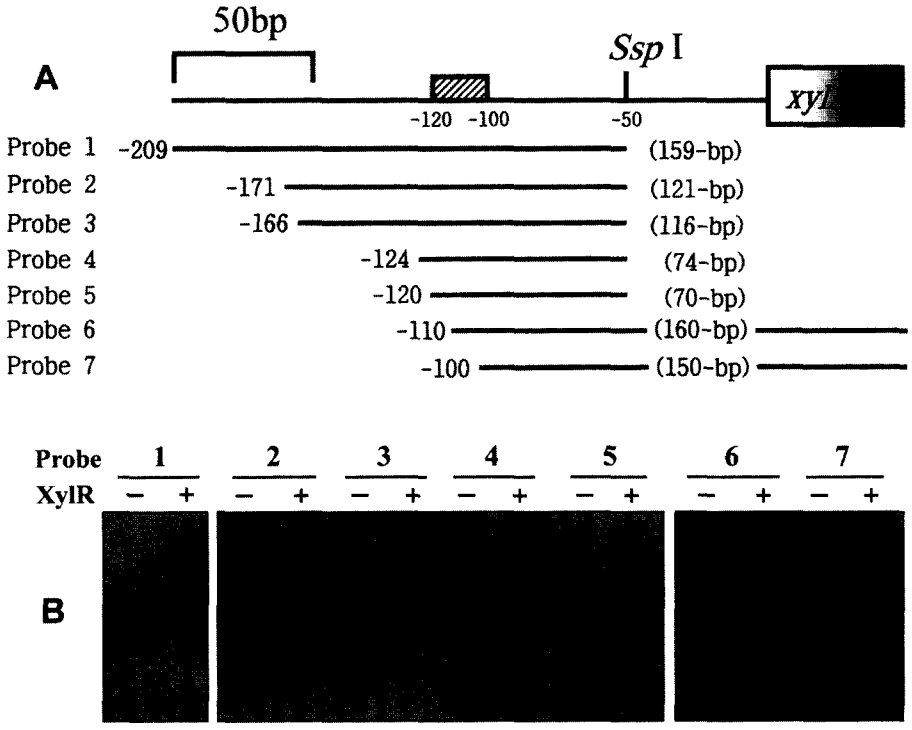

Fig. 8. Mobility shift assay with different sizes of *xylA* promoter (probes 1 to 7) for identification of the XylR binding region on the *xylA* promoter.

(A) Schematic diagram of the XylR binding region (hatched box) and the probes (*xylA* promoter region) used for mobility shift assays. Probe 1 was the 159-bp fragment used for monitoring the DNA binding activity during the protein purification process. Probes 2 (121-bp), 3 (116-bp), 4 (74-bp), and 5 (70-bp) were generated by the digestion with exonuclease III to define the binding region of XylR. Probes 6 (160-bp) and 7 (150-bp) were generated by PCR. (B) Electrophoresis of probes 1, 2, 3, 4, 5, 6, and 7 in the presence (+) and absence (−) of 100 ng of purified XylR. Numbers indicate base pairs upstream from the initiation codon.

+50 bp). According to these results, a 20-bp AT-rich region located between −120 bp and −100 bp upstream from the *xylA* initiation codon (hatched box, Fig. 8), were presumed to be important for carrying out the XylR binding to the *xylA* promoter.

The *xylA* promoter contained 16-bp of the direct-repeat sequence between −113 and −77 bp from the initiation codon, which were spaced by 5-bp in the middle of the sequence. The sequences were composed of TGTGAAtTAtCtcAAT (−113 bp to −98 bp) and TGTGAAaTAaCatAAT (−92 bp to −77 bp) [19]. The result suggested that the directly repeated 16-bp sequence TGTGAAt/aTAt/aCt/ac/tAAT (Capital letters are consensus bases; underlined letters are conserved sequences) is known to be a critical consensus sequence for the interaction with XylR.

Many of the transcriptional activators in *E. coli* binding to repeated sequences were closely spaced. An inspection of the 26 known full CRP binding sites has yielded two closely spaced 11-bp palindromic repeated consensus sequences [2]. L-Arabinose regulatory protein, AraC, also binds *araI* with 4-bp spaced two direct repeat elements, which are composed of TAGCA and TCCATA in the 17-bp sequence [4]. The purified RhaR protein, which is an activator protein in L-rhamnose operon of *E. coli*, binds to an inverted repeat sequence located within the *psr* promoter, which is the promoter for the *rhaS* and *rhaR* genes. A property of the binding site in the *psr* promoter is that the two half-sites of the inverted repeat are separated from one another by a 17-bp of uncontacted DNA [24]. Thus, as closely spaced DNA repeated sequences can be implicated in the binding of regulatory proteins in some *E. coli* transcription systems [2, 4, 24], it is possible that the TGTGAA and AAT repeats in the 16-bp sequence might be a recognition sequence for XylR. The purification of mutant XylR and crystallization of XylR will be attempted for further investigation in order to identify and characterize its biological roles as well as genetic and biochemical properties.

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