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Cloning and Improving the Expression of *Pichia stipitis* Xylose Reductase Gene in *Saccharomyces cerevisiae*

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ABSTRACT

The intact *Pichia stipitis* xylose reductase gene (XR) has been cloned and expressed in *Saccharomyces cerevisiae*. The possible further improvement of the expression of the *Pichia* gene in the new host was studied. To improve the expression of the XR gene in yeast (*Saccharomyces cerevisiae*), its 5′-noncoding sequence containing the genetic elements for transcription and translation was systematically replaced by that from the yeast genes. It was found that the *Pichia* genetic signal for transcription of XR is more effective than the yeast TRP5 promoter, but is about half as effective as the yeast strong promoter of the alcohol dehydrogenase gene (ADC1). However, the nucleotide sequence immediately adjacent to the initiation codon of XR, which controls the translation of the gene product, seemed to be five times less effective than the corresponding sequence of the ADC1 gene. By totally replacing its 5′-noncoding sequence with that of the yeast ADC1 gene, the expression of XR in yeast was found to be nearly ten times higher. Furthermore, the cloned *Pichia* XR described in this article contains very little of its 3′-noncoding sequence. In order to study whether the 3′-noncoding sequence is important to its expression in *S. cerevisiae*, the intact 3′-noncoding sequences of the yeast xylulokinase gene was spliced to the 3′ end of the P_{ADC1}-XR structural gene. This latter modification has resulted in a twofold further increase in the expression of the *Pichia* XR in yeast.

Index Entries: Yeast alcohol dehydrogenase promoter; oligonucleotides; site-specific mutagenesis; 5′-noncoding sequence; 3′-noncoding sequence, yeast xylulokinase gene.

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INTRODUCTION

D-glucose and D-xylose are two major components of renewable plant biomass, and therefore, they are the most abundant sugar molecules in nature. Most microorganisms can readily use glucose as their sole carbon and energy source for growth; however, quite a few microorganisms cannot utilize xylose as the sole carbon and energy source for growth. For example, nearly half of the known yeast species, including *Saccharomyces cerevisiae*, cannot metabolize xylose either aerobically or anaerobically (*1*).

Microorganisms also metabolize xylose by different enzymatic mechanisms. Most bacteria, such as *E. coli* and *Bacillus subtilis*, convert xylose directly to xylulose by a single enzyme xylose isomerase, which requires no cofactor for its action (*2,3*). However, nearly all known xylose-metabolizing yeasts convert xylose to xylulose via an oxidoreductive mechanism, which relies on xylose reductase to oxidize xylose to xylitol and xylitol dehydrogenase to reduce xylitol to xylulose (*4*). Nevertheless, several yeasts, particularly *Pachysolen tannophilus* (*5*), *Candida shehatae* (*6*), and *Pichia stipitis* (*7*), have been found to be able to ferment xylose to ethanol. However, even the best xylose-fermenting yeast, *P. stipitis*, cannot ferment xylose to ethanol effectively. Furthermore, these xylose-fermenting yeasts are also very ineffective in fermenting glucose (*8*).

Ethanol is an excellent liquid fuel for transportation. Furthermore, it can be produced from renewable biomass. Since *S. cerevisiae* is still one of the most effective microorganisms for converting glucose to ethanol and has been traditionally used for alcohol fermentation for centuries, efforts have continuously been made to modify *S. cerevisiae* genetically so that it can also ferment xylose to ethanol with high efficiency.

One approach that may be able to make *S. cerevisiae* ferment xylose is to clone both the xylose reductase and xylitol dehydrogenase genes (XR and XD) from a xylose-fermenting yeast such as *P. stipitis* or *C. shehatae*, two of the best natural xylose-fermenting yeasts. Recently, Kotter et al. have shown that the cloned intact XR and XD from *P. stipitis* can be expressed in *S. cerevisiae* and the latter yeast that has acquired these cloned genes can also metabolize xylose, but with a much lower efficiency (*9*). In addition, the cloning of XR has also been reported by Takuma et al. (*10*)and Hallborn et al. (*11*), but neither of these two groups were able to make *S. cerevisiae* metabolize xylose by cloning the XR alone.

Although Kotter et al. and others have shown that both the intact XR and XD from *P. stipitis* can be expressed in *S. cerevisiae*, the expression of these genes in *S. cerevisiae* by their natural genetic elements (promoters and ribosomal binding sites) for gene expression is not effective. This is evidenced by the fact that yeast containing these genes cloned on a high copy-number plasmid only produces the same level of xylose reductase and xylitol dehydrogenase as the wild-type *P. stipitis* (*9–11*). Overexpression of these genes in yeast might be able to improve the efficiency of the

yeast transformants containing these cloned genes for metabolizing xylose, particularly under anaerobic conditions. This is based on the results reported by Deng and Ho (*12*) on the cloning and overexpression of the yeast xylulokinase gene for improving yeast xylulose to ethanol conversion. Their results implied that yeast might require much higher enzyme activity produced by a gene cloned on a plasmid than the same gene located on a yeast chromosome to produce a product that requires multiple enzyme actions. One explanation of this phenomenon is that enzymes synthesized by the chromosomal genes might have been channeled to specific compartments in yeast cells where the enzymes can interact with the substrates more effectively. It has long been observed many yeast enzymes and their substrates are compartmentalized (*13*). In this article, it is reported that the expression of the *Pichia* XR in *S. cerevisiae* can be substantially improved by replacing its entire original noncoding sequence, particularly the nucleotide sequences immediately adjacent to the initiation codon ATG, with that of the yeast alcohol dehydrogenase gene (*14*).

MATERIALS AND METHODS

Strains and Plasmids

S. cerevisiae strain LSK1 (*15*) was used as the host for all yeast transformation. *E. coli* SR14 (*16*) or DH5α (obtained from BRL Life Technologies, Inc., Gaithersburg, MD) was used as the host for *E. coli* transformation. pUC18, pUC19, and phagemid pBluescript II KS (–) were obtained commercially. pUC18 or pUC19 was used for the cloning and amplification of gene fragments obtained via polymerase chain reaction. Phagemid was used for cloning DNA fragments for site-directed mutagenesis. pUCKm8 (*17*) and pUCKm10 are *E. coli–S. cerevisiae* shuttle vectors that were used to clone DNA fragment containing the xylose reductase gene for yeast (*S. cerevisiae*) transformation. pUCKm10 is a derivative of pUCKm8 with its *Eco*RI sites deleted.

Isolation of DNA

P. stipitis 5773 DNA was isolated by the method of Cryer et al. (*18*). A large preparation of plasmid DNA from *E. coli* transformants was carried out by the clear lysate method of Clewell and Kelinski (*19*). Miniprep of plasmids from *E. coli* transformants was carried out by the method of Homes and Quigley (*20*).

Polymerase Chain Reaction

The reaction conditions for PCR were according to the protocol described by Saiki et al. (*21,22*). The microprocessor-controlled Temperature Cycler from Ericomp (San Diego, CA) was used to carry out the reactions.

Transformation

E. coli transformation was by the $CaCl_2$ method of Lederberg and Cohen (23). Plates containing LB-xylose (1% tryptone, 1% NaCl, 05% yeast extract, and 0.1% xylose) with ampicillin (50 μg/mL) and X-gal (40 μg/mL) were used for the selection of transformants containing pUC18, pUC19, or pBluescript recombinants. Plates containing LB (1% trypsin, 1% NaCl, 0.5% yeast extract, and 0.1% glucose) with ampicillin (50 μg/mL) were used for the selection of *E. coli* transformants containing other recombinant plasmids, such as recombinant of pUCKm8, pUCKm10, pLX10-14 (24), and so forth.

Yeast transformation was carried out by the electroporation technique (25). After electroporation, cells were plated on plates containing YEPD (1% yeast extract, 2% peptone, and 2% glucose) with geneticin G418 (50 μg/mL) to select the putative transformants (the fast-growing colonies), which were further tested for the presence of plasmid by the penicillinase assay (26).

Analysis of the Xylose Reductase Activity

The NADPH- and NADH-dependent xylose reductase activity was analyzed as described by Bolen et al. (27). Protein determinations were performed according to Lowry. One unit (U) of the enzyme is defined as the amount of the enzyme catalyzing the oxidation of 1 νmol NADPH (or NADH) min^{-1}. The xylose reductase activity of the transformants containing different recombinant plasmids was analyzed at least twice—once immediately after each recombinant plasmid was constructed and its LSK1 (15) transformants were isolated, and once after all the desired plasmids were isolated and transformants containing each plasmids were constructed. The activities listed in Table 1 are from the latter analysis. Nevertheless, the results of the earlier analyses for each different transformant are very similar to those reported in Table 1.

Synthesis of Oligonucleotide Primers

The oligonucleotide primers used for PCR or for site-directed mutagenesis were synthesized by using an oligonucleotide synthesizer (Applied Biosystems, Model 380). The nucleotide sequences of the four primers used for this study are:

Primer I, a 21 mer: 5′-GGATCCACAGACACTAATTGG-3′
Primer II, a 24 mer: 5′-GCAACCTTCTTAGACGAAGATAGG-3′
Primer III, a 29 mer: 5′-GGCTCGAGGAGTTTTCTTTTCATTTCTCC-3′

Site-Directed Mutagenesis

This was carried out according to the procedure of Kunkel et al. (28). Phagemid DNA prepared from recombinant pBluescript II KS (−) was the source of the single-stranded template DNA. Colonies containing plasmid

Table 1
Xylose Reductase Activity
of *Pichia stipitis* and *S. cerevisiae* Transformants

Strain	Plasmid	Xylose reductase NADPH-dependent activity, 10^{-2} μ/mg protein	Structural modification of XR cloned in pUCKm8
P. stipitis CBS5773	-	52.4	-
S. cerevisiae LSK1	pUCKm8	1.3	-
LSK1	pLXR1	23	None, the original *Pichia* XR
LSK1	pLXR2	12	XR fused to yeast Trp5 promoter
LSK1	pLXR10	42	XR fused to ADC1 promoter
LSK1	pLXR13	192	XR fused to ADC1 promoter and ADC1 ribosomal binding site
LSK1	pLXR14	97	Same as pLXR13, but the modified XR is located in opposite orientation
LSK1	pLXR15	489	XR fused to ADC1 promoter, ribosomal binding site, and to the intact terminal sequence of the yeast xylulokinase gene

with the correct mutation were verified by nucleotide sequence analysis, using a primer that annealed 50–70 nucleotides upstream to the site of mutation.

RESULTS

Amplification of *Pichia* Xylose Reductase Gene by Polymerase Chain Reaction (PCR)

Recent advances in PCR technology have made it possible to copy and amplify genes directly from the chromosomes of any organism (*21,22*). By Using this technique, the *Pichia* XR was amplified as shown in Fig. 1. To accomplish this, two oligonucleotide primers (Primer I and Primer II, *see* Materials and Methods) were synthesized according to the published DNA sequence of *Pichia* XR (*10*). The amplified gene is a 1.3-kb DNA fragment that contains the structural gene of XR and the first 256 bp (−1 to −256) of the original 5′-noncoding sequence adjacent to the structural gene. For convenience, the amplified XR gene is designated as P_{XR}-T_{XR}-XR according to its genetic organization. P_{XR} represents the original 5′-noncoding sequences containing the *Pichia* genetic elements, such as the promoter, that control the transcription of XR in *P. stipitis*. T_{XR} represents the original 5′-noncoding sequence of XR immediately adjacent to the XR structural gene and controlling the translation of the gene product. P_{XR}-T_{XR}-XR was then cloned into plasmid pUC18 at its *Sma*I site, and the

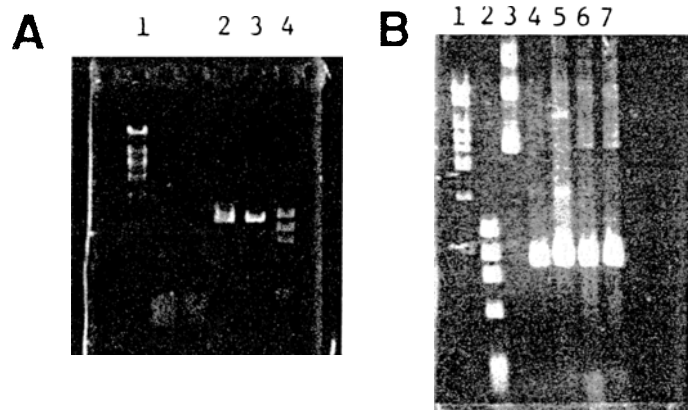

Fig. 1. Amplification of *Pichia stipitis* reductase gene (XR) by polymerase chain reaction (PCR). A. Direct amplification of the intact XR (with its original *Pichia* promoter) from *P. stipitis* chromosomal DNA. Lanes 1 and 4: Molecular markers, the first band of lane 4 contains a DNA fragment approx 1.4 kb. Lanes 2 and 3: The amplified XR. B. Direct amplification of the promoterless XR from pLXR1, a plasmid containing the intact XR directly amplified from *Pichia* chromosomal DNA as shown in A. Lanes 1 and 2: Molecular markers, the second band in lane 2 contains a DNA fragment approx 1 kb. Lane 3: pLXR1. Lanes 4 to 7: Amplified promoterless XR.

resulting plasmid is designated pUC18- (P_{XR}-T_{XR}-XR). The *Bam*H1-*Eco*RI fragment from the latter plasmid containing the intact XR (P_{XR}-T_{XR}-XR) was subcloned at the *Bam*H1 and *Eco*RI sites of pUCKm8 (Fig. 2), and the resulting plasmid is designated pLXR1. pLXR1 was confirmed to contain the intact XR (P_{XR}-T_{XR}-XR) by restriction analysis (Fig. 5), PCR analysis, and the analysis of the xylose reductase activity as shown in Table 1.

Fusion of *Pichia* XR to Yeast TRP5 Promoter

Although the TRP5 promoter of *S. cerevisiae* is not considered to be very strong, it has been able to improve the expression of the yeast xylulokinase gene nearly 50-fold (*29*). Hence, this promoter was used first to modify the XR gene to improve its expression in *S. cerevisiae*. For fusion of XR to the yeast TRP5 promoter (P_{TRP5}), a promoterless XR was amplified from the *P. stipitis* chromosomal DNA by PCR (it can also be amplified from any of the plasmids the authors constructed containing the XR gene). To do this, a new oligonucleotide primer (Primer III) was synthesized according to the published nucleotide sequence of XR (*10*). Primers II and III were used to synthesize and amplify the promoterless XR as shown in Fig. 1. The oligonucleotide primer III was specially designed so that the resulting amplified XR contained only -1 to -50 bp of its original 5′-noncod-

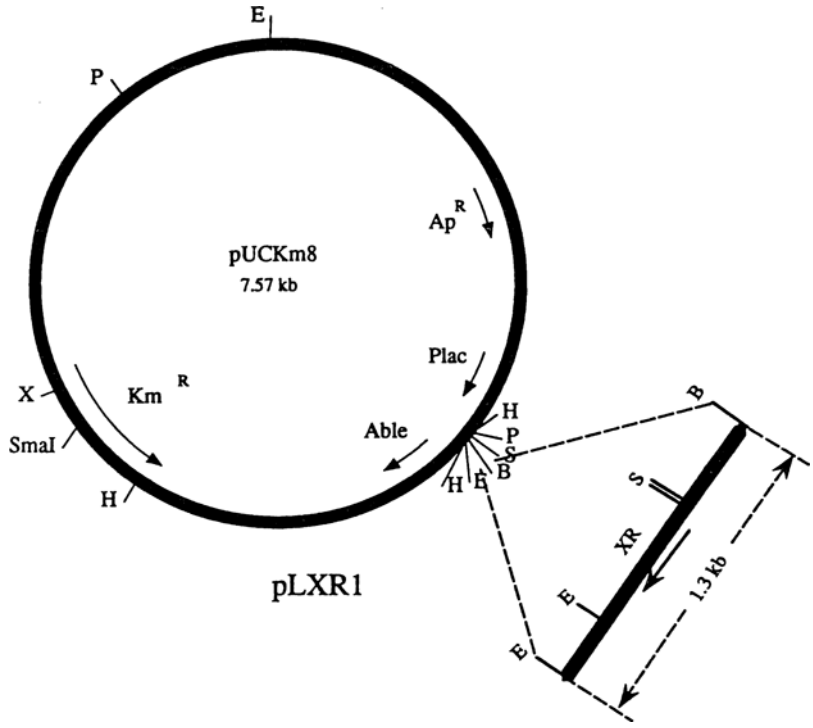

Fig. 2. Restriction map of pLXR1.

ing sequence designated as (the T_{XR} region) as well as an *Xho*I restriction site at its 5′ terminal. This ensured that the resulting XR did not contain any *Pichia* genetic elements for transcription (P_{XR}), but it still relied on its *Pichia* signal for translation (T_{XR}) of its protein product. This was because the yeast TRP5 promoter to be used for this construction did not contain the 5′-noncoding region for the control of the translation of the gene product. Furthermore, this also allowed the study of the effectiveness of the *Pichia* T_{RX} in controlling the translation of the gene product in *S. cerevisiae*. The addition of an *Xho*I site at the 5′ terminal of the promoterless XR was to facilitate the fusion of XR with the yeast TRP5 promoter, which is located on a 0.4-kb *Bam*H1–*Xho*I fragment cloned in pLX10-14 (*24*). The construction of $P_{TRP\,5}$-T_{XR}-XR fusion and the cloning of the resulting fusion into pUCKm10 are outlined in Fig. 3. pUCKm10-containing $P_{TRP\,5}$-T_{XR}-XR is designated pLXR2. The presence of $P_{TRP\,5}$-T_{XR}-XR in pLXR2 was confirmed by restriction analysis (Fig. 5) and by the analysis of the xylose reductase activity of yeast transformants containing pLXR2 as shown in Table 1.

Somewhat surprisingly, it was found that yeast transformants containing pLXR1 had higher xylose reductase activity than those containing pLXR2 (*see* Table 1). These results have made it especially desirable to fuse XR to stronger yeast promoter.

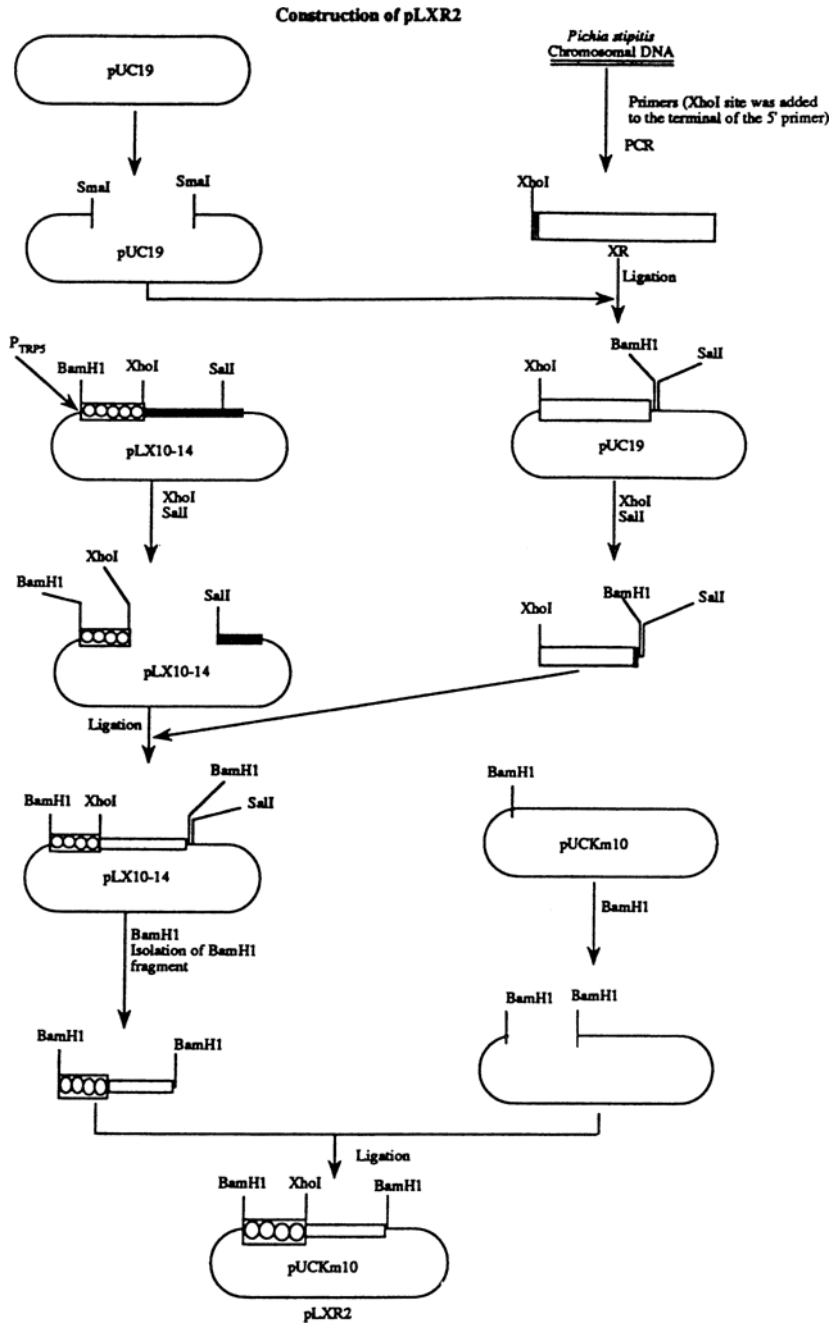

Fig. 3. Construction of pLXR2.

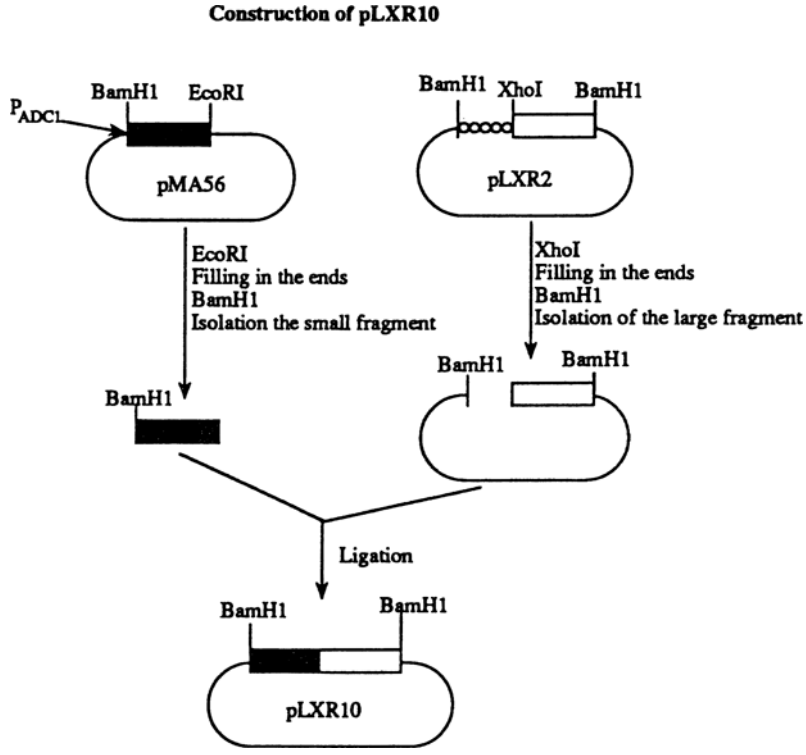

Fig. 4. Construction of pLXR10.

Fusion of Promoterless XR to the Strong Promoter of Yeast Alcohol Dehydrogenase Gene ADC1

The promoter for the yeast alcohol dehydrogenase gene, P_{ADC1}, is considered to be a strong promoter for the expression of genes in *S. cerevisiae*, and it has been cloned on plasmid pMA56 as a 1-kb *Bam*H1–*Eco*RI fragment (*30*). The cloned P_{ADC1} contains most of the 5′-noncoding sequence of the ADC1 gene, except for the first 14 bp (-1 to -14, designated the T_{ADC1} region) immediately adjacent to the structural gene of ADC1. Since the readily available P_{ADC1} did not contain the T_{ADC1} region (-1 to -14 bp 5′-noncoding region) of the ADC1 gene, the T_{XR} region present in the P_{TRP5}-T_{XR}-XR was not deleted for the construction of the fusion between $P_{ADC1}$and the promoterless XR.

The fusion was constructed using an isolated *Bam*H1–*Eco*RI fragment containing the P_{ADC1} from pMA56 to replace the *Bam*H1–*Xho*I fragment containing the TRP5 promoter fused to XR cloned in pLXR2 as shown in Fig. 4. The resulting plasmid containing the P_{ADC1}-T_{XR}-XR is designated pLXR10. The pLXR10 was confirmed to contain the P_{ADC1}-T_{XR}-XR fusion

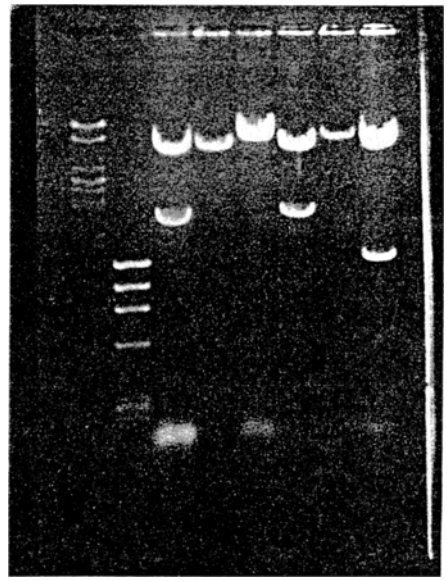

Fig. 5. Analysis of restriction enzyme digestion of pUCKm8 (or pUCKm10) derivatives containing *Pichia* xylose reductase gene fused to different promoters. Lanes 1 and 2: Molecular markers. Lane 3: pLXR13/*Bam*H1. Lane 4: pUCKm8 (or pUCKm10). Lane 5: pLXR1/*Bam*H1. Lane 6: pLXR11/*Bam*H1. Lane 7: pLXR11/*Eco*RI. Lane 8: pLXR2/*Bam*H1.

by restriction analysis (Fig. 5) and also by the analysis of the xylose reductase activity of yeast transformants containing pLXR10 (Table 1). Although it was found that yeast transformants containing pLXR10 had higher levels of xylose reductase activity than those containing pLXR1, replacing P_{XR} by P_{ADC1} only improved the expression of XR not more than two- to threefold (Table 1). This made it highly desirable to investigate whether replacing the -1 to -50 *Pichia* T_{XR} sequence present in the P_{ADC1}-T_{XR}-XR fusion with the 14 bp (-1 to -14) 5′-noncoding sequence of ADC1 would substantially improve the expression of XR in *S. cerevisiae*. In this manner, the XR is expressed entirely by the 5′-noncoding sequence of ADC1.

Fusion of XR to the Intact 5′-Noncoding Sequence of the Yeast ADC1 Gene

In order to express XR solely by the genetic elements of ADC1 for gene expression, the authors proceeded to fuse the XR structural gene to the intact 5′-noncoding sequence of ADC1. There are several ways to accomplish this. The chosen method was to replace the *Pichia* DNA sequence within the 5′-noncoding region of XR, which includes the -1 to -50 bp original *Pichia* 5′-noncoding sequence for XR and the linker sequence containing the restriction sites, with the 14-bp (-1 to -14) 5′-noncoding sequence of T_{ADC1}. This was accomplished by the synthesis of a 48-mer

oligonucleotide primer (structure not shown) to carry out the site-specific mutagenesis of the P_{ADC1}-T_{XR}-XR fusion. The latter was first subcloned from pLXR10 to phagemid PBluescript II KS (−) to facilitate the conversion of P_{ADC1}-T_{XR}-XR to P_{ADC1}-T_{ADC1}-XR. The latter mutated gene was then isolated from the Bluescript phagemid as a 2-kb *Bam*H1 fragment and reinserted into pUCKm10 at its *Bam*H1 site. Two recombinant plasmids, designated pLXR13 and pLXR14, were obtained. Both pLXR13 and pLXR14 contain the insert P_{ADC1}-T_{ADC1}-XR, but in opposite orientations. pLXR13 and pLXR14 were confirmed to contain the proper insert P_{ADC1}-T_{ADC1}-XR by restriction analysis (Fig. 5), by DNA sequence analysis (to verify the results of site-directed mutagenesis), and by the analysis of the xylose reductase activity of the yeast transformants containing pLXR13 or pLXR14 (Table 1). The details for the construction of pLXR13 and pLXR14 will be reported elsewhere.

By comparison of the results of the xylose reductase activity of the yeast transformants containing pLXR1, pLXR10, and pLXR13 (or pLXR14), shown in Table 1, it seems that the replacement of the original *Pichia* 5′-noncoding sequence next to the initiation codon of XR with the −1 to −14 bp sequence of ADC1 has tremendously improved the expression of XR in *S. cerevisiae*.

Splicing the P_{ADC1}-XR with the 3′-Noncoding Sequence of the Yeast Xylulokinase Gene

The published *Pichia* XR DNA sequence (*10*) contains 256 bp of its 5′-noncoding sequence, but only contains < 20 bp of its 3′-noncoding sequence. Although the gene is expressed in *S. cerevisiae*, the absence of adequate 3′-noncoding sequence may affect the efficiency of its expression in the new yeast host. Recently, the authors have cloned (*31*) and sequenced the yeast xylulokinase gene (XK) (Ho et al., unpublished results). In addition to the coding sequence of XK, more than 350 bp of its 3′-noncoding sequence were also analyzed. Based on the authors' recent study on the overexpression of XK in yeast, it was found that the yeast XK seemed to contain very effective genetic signals in its 3′-noncoding region for polyadenylation, mRNA termination, and so on. Thus, the authors chose to fuse the intact 3′-noncoding sequence of XK to the XR structural gene to improve its expression in *S. cerevisiae*.

This was accomplished by cloning both the P_{ADC1}-XR and the PCR-amplified XK 3′-noncoding sequence on a Bluescript plasmid. Recombinant plasmid containing the P_{ADC1}-XR and the 3′-terminal region of XK (designated Ter_{XK}) in the correct orientation was isolated. The site-directed mutagenesis technique was used to remove all the undesired sequences between the XR structural gene and the XK 3′-noncoding sequence. The resulting P_{ADC1}-XR-Ter_{XK} was subcloned on pUCKm8, designated pLXR15. Yeast transformants containing pLXR15 were found to produce two times higher XRtase activity than those containing pLXR13 (Table 1).

DISCUSSION

These results demonstrate that the expression of *Pichia* XR can be improved nearly 20-fold by fusion of the XR structural gene to the 5′-noncoding sequence of yeast ADC1 containing the intact genetic elements for gene expression and the 3′-noncoding sequence of yeast xylulokinase gene. These results imply that the natural promoter for *Pichia* XR functions very well in *S. cerevisiae*, but its natural 5′-noncoding sequence that controls the translation process does not.

Furthermore, XR cloned in pUCKm10 is expressed more effectively when XR is oriented in the opposite direction of the *lac* promoter. Since the *lac* promoter has been shown to be able to function in *S. cerevisiae* (*32*), this is probably because of partial expression of the XR gene through the *lac* promoter, and the resulting transcripts are unable to produce functional enzymes. Among the constructs listed in Table 1, only pLXR1 and pLXR14 contain the XR gene in the same orientation with the *lac* promoter cloned on the same plasmid.

This work also demonstrates that the recent advances in site-specific mutagenesis and PCR techniques have made in vitro manipulation of a gene much easier to accomplish. Without these techniques, the cloning of XR, the deletion of its promoter, the deletion of the -1 to -50 noncoding sequence of XR, and the addition of -14 to -1 of the 5′-noncoding sequence of ADC1 to XR, as well as the addition of the 3′-noncoding sequence to the latter will require tremendously more effort to accomplish.

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