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Cloning the Yeast Xylulokinase Gene for the Improvement of Xylose Fermentation

Scientific Note

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INTRODUCTION

D-Xylose is a major constituent of the hemicellulose which makes up 20–30% of renewable biomass in nature (*1*). However, *Saccharomyces cerevisiae* and other related yeasts cannot ferment D-xylose (*2*). Nevertheless, they can ferment xylulose to ethanol with adequate efficiency (*2*). Furthermore, xylose can be converted to xylulose in vitro by the enzyme xylose isomerase, widely present in most bacteria and commercially known as glucose isomerase (*3*). This makes it possible for *S. cerevisiae* and other related xylose non-fermenting yeasts to ferment xylose by a two-stage "isomerization and fermentation" process (*4*).

Recently it has been shown that *E. coli* can be genetically engineered by recombinant DNA techniques to overproduce its xylose isomerase (*5,6*) by 20- to 50-fold. This will make the above described two-stage xylose fermentation process more economically feasible. Yeast xylulokinase has been reported to play an important role in yeast xylose or xylulose metabolism, possibly being the rate-limiting enzyme (*7*). Hence, it is de-

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sirable to study whether it is also possible to increase the xylulokinase activity in yeast by using recombinant DNA techniques. Recently, we have succeeded in the cloning of a 7.3 kb pair yeast DNA fragment which can complement *E. coli* xylulokinase mutations. In this paper we describe the characterization of the yeast DNA fragment to prove that it contains the nucleotide sequences coding for the synthesis of the yeast xylulokinase.

MATERIALS AND METHODS

Strains

S. cerevisiae AH22 (*a*, *leu*2-3, *leu*2-112, *his*4-529, *Can*1) was used for the isolation of the yeast DNA by the method of Cryer et al. (*8*). The *E. coli* xylulokinase mutants used for this study have been described previously (*9,10*). *E. coli* transformation was carried out according to Norgard et al. (*11*).

DNA Sequencing and Other General Methods

M13 cloning/dideoxy sequencing techniques were used for the analysis of the nucleotide sequence of the cloned yeast xylulokinase gene (*12,13*). Restriction endonucleases and other enzymes used for recombinant manipulation were from Bethesda Research Laboratories (BRL) [$\alpha - {}^{32}$p] and [$\alpha - {}^{35}$S] deoxyribonucleoside triphosphates used for DNA sequencing were obtained from Amersham.

RESULTS AND CONCLUSION

Isolation of the Xylulokinase gene from S. cerevisiae

Transformation of the *E. coli* xylulokinase mutant, SR14 (*9*), with a genomic bank of *S. cerevisiae* DNA in the yeast–*E. coli* shuttle vector, YEp13, resulted in the isolation of several Xyl^+ colonies. These positive transformants were detected as red colonies against a background of white colonies, on MacConkey-xylose–Amp indicator plates. Retransformation of SR14 with plasmid DNA from Xyl^+ transformants resulted in all of the colonies being Xyl^+. One of these plasmids, pLSK1 (structure not shown), was chosen for further analysis.

Mapping of the Yeast Xylulokinase Gene

The initial plasmid, pLSK1, consisted of a 7.3 kb yeast *Sau*3A fragment inserted into the *Bam*H1 site of YEp13. Restriction analysis of pLSK1 revealed that the yeast insert contained 3 *Hin*dIII restriction sites,

2 *Xho*I sites, 1 *Bam*H1, and *Bgl*II site, but no *Eco*RI site. Because the yeast insert did not contain any *Eco*RI restriction site, a simplified plasmid pLSK3, which still contained the intact insert, was constructed by the deletion of two *Eco*RI fragments (originally part of YEp13) to facilitate restriction mapping of the insert as shown in Fig. 1.

Through subcloning, the yeast xylulokinase gene, XYK-Sc, was localized on the two *Hin*dIII fragments near the unique *Eco*RI site in pLSK3 (Fig. 1). The 2 kb *Xho*I fragment was found also capable of complementing the *E. coli* xylulokinase mutant SR14, but only in one orientation, as shown in Fig. 2. Since yeast *TRP*5 promoter (P_{trp-5}) has been shown to be able to function in *E. coli* (*15*), the expression of the *Xho*I fragment is apparently a result of its orientation with respect to transcription initiated from the *TRP*5 promoter. This indicated that the *Xho*I fragment did not contain the genetic elements for gene expression in *E. coli*, but it contained the structural gene encoding yeast xylulokinase.

In order to be certain that the cloned yeast DNA fragment contains the yeast xylulokinase gene, pLSK3 was used to transform four additional transposon-induced *E. coli* xylulokinase mutants (*10*). pLSK3 was found also to complement these mutants.

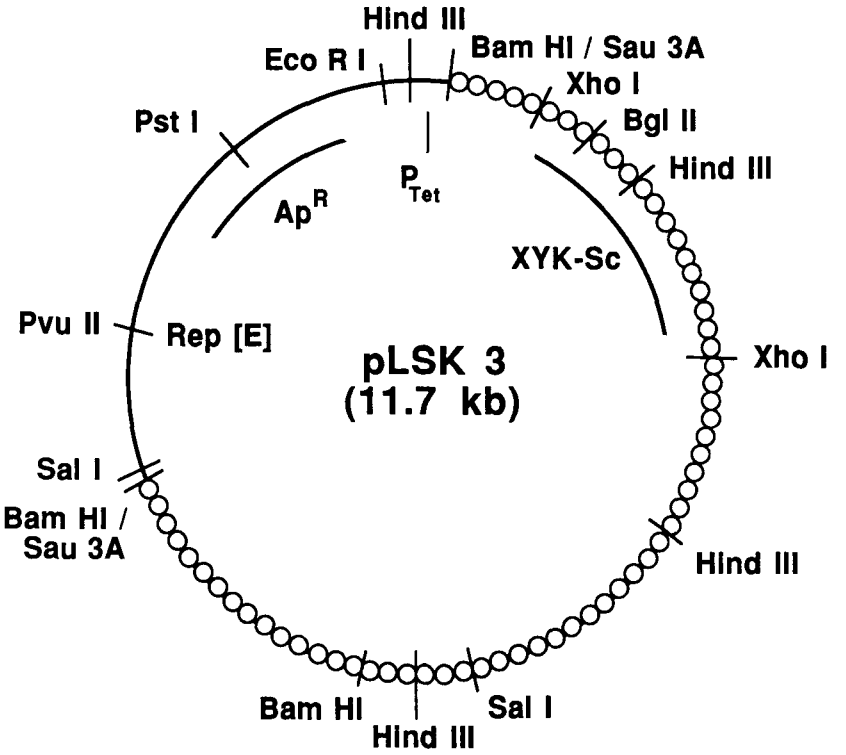

Fig. 1. Restriction map of plasmid pLSK3. The open circles represent the yeast DNA insert containing the xylulokinase gene. P_{Tet} represents the promoter of the tetracycline resistance gene.

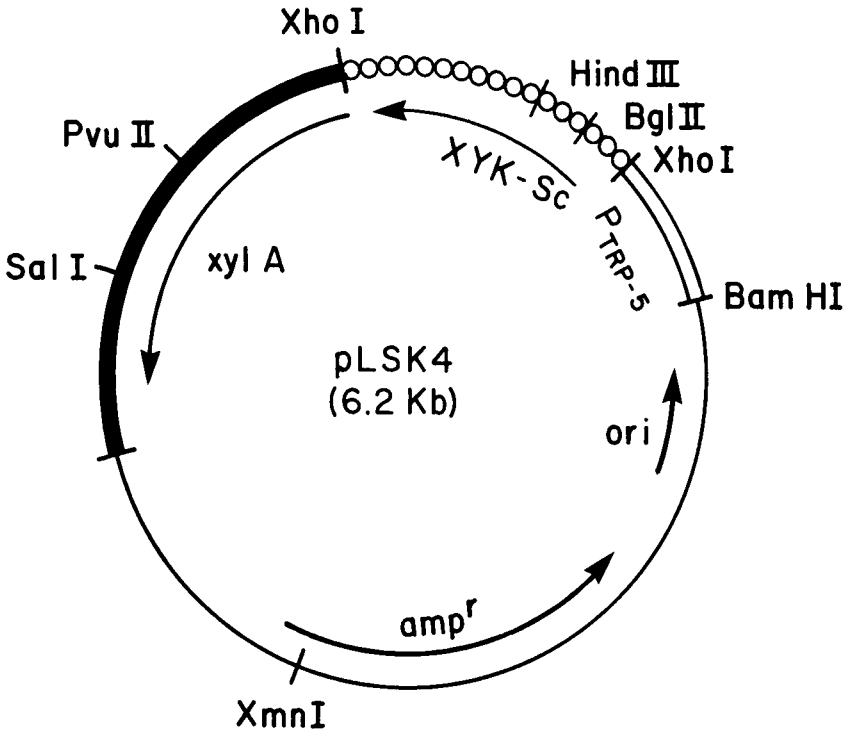

Fig. 2. Construction of plasmid pLSK4. The 2 kb *Xho*I fragment (open circles) was isolated from pLSK3 and ligated with the *Xho*I linearized plasmid pLX10-18 (*14*). The resulting ligation products were used to transform *E. coli* SR1 (a xylose isomerase mutant) (*9,14*) and the transformants were plated on MacConkey-xylose–Amp indicator plates. pLX10-18 is a direct selection vector. *E. coli* transformants harboring recombinant plasmid containing inserts at the *Xho*I site will form white colonies but those harboring the original pLX10-18 will form red colonies on the MacConkey-xylose–Amp indicator plates (*14*). Analysis of plasmids isolated from the white colonies indicated that two different recombinant plasmids pLSK4 and pLSK40 were obtained. pLSK40 is identical to pLSK4, except that the *Xho*I fragment was inserted in opposite orientation. Transformation of SR14 (the xylulokinase mutant) showed that only pLSK4 can complement the SR14 mutation.

*Sequence Analysis of the Hind*III Fragments

The two *Hind*III fragments (1.2 kb and 2.3 kb) adjacent to the unique *Eco*RI site in pLSK3 were subcloned into the RF form of M13mp19 virus (*13*) for the analysis of their DNA sequence. The strategies for the analysis of the DNA sequence of the two fragments are shown in Fig. 3. The DNA sequence data (not shown) revealed that an initiation codon ATG is located 27 bp upstream from the *Bgl*II site.

Downstream from this translation initiation site, there exists a large open reading frame (ORF) coding for the amino acid sequence of a protein (enzyme). Upstream from the same initiation codon are mostly AT rich regions with a TATAAA sequence approximately 200 bp from the

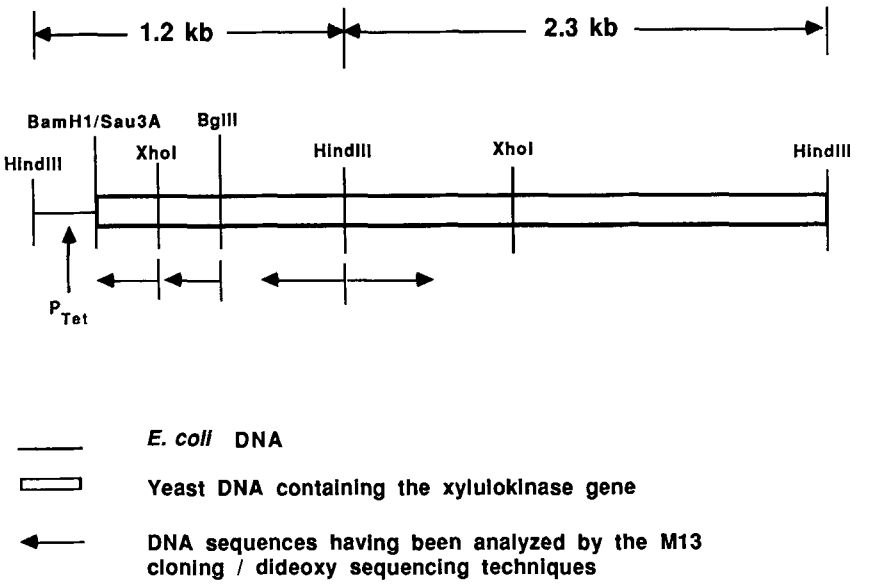

Fig. 3. Strategy for determining the nucleotide sequences of the *Hin*dIII fragments containing the yeast xylulokinase gene. The arrows indicate the direction of sequence determined from a specific restriction site.

initiation codon. Hence, the sequence data and the expression of the enzyme in *E. coli* suggest that the necessary genetic elements for the synthesis of the enzyme in yeast (*S. cerevisiae*) are present within the two *Hin*dIII fragments.

In this paper, we have demonstrated that pLSK1 or pLSK3 containing a yeast DNA fragment can complement *E. coli* xylulokinase mutations as well as that the cloned yeast DNA probably contains all the necessary structural elements of a yeast gene encoding a yeast protein (enzyme). Furthermore, we have found that *E. coli* xylulokinase mutants harboring either pLSK1 or pLSK3 will synthesize xylulokinase in the absence of xylose induction as well as in the presence of glucose (insensitive to glucose inhibition) (data not shown). We conclude that the yeast DNA fragment cloned on pLSK1 or pLSK3 at least contains the structural gene encoding *S. cerevisiae* xylulokinase.

DISCUSSION

The cloning of the yeast xylulokinase gene in *E. coli* will facilitate the subsequent determination of the highest possible level of overproduction of the xylulokinase in *S. cerevisiae* via recombinant DNA techniques and the effect of such high levels of xylulokinase activity on yeast xylulose fermentation.

It also allows the further exploration of the molecular mechanism controlling xylose metabolism in yeast (*S. cerevisiae*), and it provides a model system to identify the genetic elements controlling the expression

of genes related to xylose fermentation. In addition, the yeast xylulokinase genes from various yeasts can be used as model genes for the comparison of the genetic elements controlling xylose metabolism in various yeasts. Finally, the cloned gene can be used as a selection marker for the construction of ideal vectors for gene cloning in yeast.

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