Cloning of the *Pachysolen tannophilus* Xylulokinase Gene by Complementation in *Escherichia coli*

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Received 20 April 1987/Accepted 1 September 1987

The gene coding for xylulokinase has been isolated from the yeast *Pachysolen tannophilus* by complementation of *Escherichia coli* xylulokinase (xylB) mutants. Through subcloning, the gene has been localized at one end of a 3.2-kilobase EcoRI-PstI fragment. Expression of the cloned gene was insensitive to glucose inhibition. Furthermore, the cloned gene did not cross-hybridize with *E. coli* and *Saccharomyces cerevisiae* xylulokinase genes.

*Pachysolen tannophilus* is a *d*-xylose-utilizing yeast isolated from wood sulfite liquor by Wickerham (14). Although this organism has been studied extensively relative to its ability to ferment Xylose to ethanol (3, 4, 9, 11), very little is known about its genetic organization and gene expression.

Unlike prokaryotes, such as *Escherichia coli* and *Salmonella typhimurium*, which isomerize xylose directly to xylulose (2, 10), *P. tannophilus* utilizes *d*-xylose reductase (*d*-xylose → xylitol) and xylitol dehydrogenase (xylitol → *d*-xylulose) to achieve this conversion. Xylulokinase then converts xylulose to xylulose-5-phosphate, an intermediate of the pentose phosphate pathway. Lachke and Jeffries (6) found that xylulokinase is critical for the aerobic and anaerobic utilization of xylene. In this report we describe the isolation of the *P. tannophilus* xylulokinase gene (XYK-Pa) by complementation of *E. coli* xylB mutants.

A genomic bank was constructed for *P. tannophilus* by the insertion of *Sac3A*-digested *P. tannophilus* DNA onto the *BamHI* site of the yeast-*E. coli* shuttle vector YEpl3 (1). Transformation of the *E. coli* xylulokinase mutant strain SR14 (8) with the genomic bank resulted in the isolation of five Xyl*+* colonies. These positive transformants were detected as red colonies on MacConkey-xylose fermentation indicator plates against a background of nonpigmented (white) colonies. Digestion by *Sal*I of plasmid minipreparations isolated from the Xyl*+* colonies indicated the presence of additional *Sal*I fragments in these plasmids other than those in YEpl3. Retransformation of strain SR14 with plasmid DNA from Xyl*+* transformants resulted in all colonies being Xyl*+*. One of these plasmids, pLPK1 (structure not shown), was chosen for further analysis.

The initial plasmid, pLPK1, consisted of a 9-kilobase (kb) *Sac3A* fragment inserted into the *BamHI* site of YEpl3. To facilitate restriction site mapping, a 4.5-kb *EcoRI* fragment from pLPK1 was subcloned into pBR322, yielding pLPK2 (Fig. 1). In addition to complementing SR14, pLPK2 also complemented four transposon-induced *E. coli* xylulokinase mutants (XK104, XK106, XK201, and XK202) which were generated with the Tn0 derivatives described by Way et al. (13). This indicates that the cloned gene is not a *Pachysolen* suppressor tRNA gene. Partial digestion of pLPK2 by *PstI* followed by religation and transformation of strain SR14 resulted in the isolation of pLPK3 (Fig. 1). The latter plasmid conferred a Xyl*+*, Tc*, Ap* phenotype on SR14. This analysis localized the gene to a 3.2-kb *PstI*-PstI-*EcoRI* fragment distal to the gene for Ap* in pLPK2 (Fig. 1). Deletion of the 1.4-kb HindIII fragment from pLPK3 abolished the ability of pLPK3 to complement strain SR14. The 1.4-kb HindIII fragment from pLPK3 subcloned into pUC9 also failed to complement SR14. Furthermore, the 2.3-kb *PstI* fragment cloned into pUC9 also did not complement SR14. In addition, removal of the HindIII or *PstI* sites, spaced 450 base pairs apart on pLPK2 and pLPK3, disrupted the ability of the cloned fragment to complement the xylB mutants.

The *EcoRI* fragment derived from pLPK2 was also subcloned into pUC9 in both orientations. The orientation which placed the large (approximately 1.3-kb) *Ava* fragment proximal to the *lac* promoter yielded transformants on minimal medium with 0.4% xylose after a 12-h incubation. Transformants containing the plasmid in the opposite orientation yielded smaller colonies after 48 h.

In addition to XYK-Pa of *P. tannophilus*, the xylulokinase genes of *E. coli* (7, 8) and *Saccharomyces cerevisiae* (S. F. Chang and N. W. Y. Ho, Appl. Biochem. Biotechnol., in press) have also been isolated by complementation of *E. coli* xylulokinase mutants. The cloned xylulokinase genes from these three organisms have different restriction enzyme patterns and do not cross-hybridize with each other (data not shown). The identity of the *XYK-Pa* gene was further established by the fact that the cloned gene hybridized to *P. tannophilus* cellular DNA and not to other yeast DNA (Fig. 2). The xylulokinase assay results (Table 1) demonstrate that the expression of the cloned *XYK-Pa* gene in *E. coli* is insensitive to glucose inhibition, while the expression of the *E. coli* chromosomal *xylB* gene is repressed in the presence of glucose. This further indicates that the cloned gene is not the *E. coli* *xylB* gene and that it does not code for a *P. tannophilus* suppressor tRNA.

A preliminary analysis showed that yeast (*S. cerevisiae*) transformants harboring pLPK1 or pLPK4 (pLPK3 with a fragment containing TRPI-ARS [12] cloned into the *EcoRI* site) did not exhibit increased rates of xylose utilization. Furthermore, xylulokinase assays of the yeast transformants harboring pLPK4 did not reveal the presence of any plasmid-borne xylulokinase activity. The absence of plasmid-borne xylulokinase activity could be due to differences in the transcription or translation mechanism or both in the two
organisms or to the absence in the cloned gene of the intact control elements for gene expression in yeasts.

The XYK-Pa gene is, to our knowledge, the first gene isolated from P. tannophilus. Analysis of the nucleotide sequence of the cloned gene will provide the necessary data for comparing the mechanisms of gene expression in P. tannophilus and S. cerevisiae. Such analysis is important because the former organism is one of the few yeasts capable of directly fermenting xylose, while the latter organism, a major industrial microorganism for ethanol production, is not able to utilize xylose. This is especially relevant to the utilization of renewable biomass, of which xylose is a major component. Furthermore, there is no cloning system available for P. tannophilus at present. The cloned XYK-Pa gene can also serve as a selection marker in establishing such a system.

This work was supported in part by U.S. Department of Agriculture grant 85-FSTY-9-0109 and National Science Foundation grant DBM-8305043.

LITERATURE CITED


11. Slininger, P. J., R. J. Bothast, J. E. Van Cawenberg, and C. P.

