0273-2289/91/2829—0369$02.00

Genetic Transformation of Xylose-Fermenting Yeast *Pichia stipitis*

Scientific Note

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ABSTRACT

A plasmid-mediated transformation system has been developed for the xylose-fermenting yeast *Pichia stipitis*. We found that plasmid vectors containing the *Saccharomyces cerevisiae* 2 μ replicon and the kanamycin resistance gene (Km^R) could be introduced into the *Pichia* cells and maintained as extrachromosomal elements. *Pichia* transformants containing such vectors will be resistant to the antibiotic geneticin that can be inactivated by the protein product of Km^R. Plasmids identical to those used for transformation can be recovered from the *Pichia* transformants. Protocols for transformation of *P. stipitis* by the $CaCl_2$-polyethylene glycol-protoplast process or by direct electroporation of intact *Pichia* cells have both been developed.

Index Entries: *Pichia stipitis* genetic transformation system; electroporation; *Saccharomyces cerevisiae* 2 μ replicon; kanamycin resistance gene; geneticin.

INTRODUCTION

D-Xylose is a major constituent of hemicellulose, which makes up 20–30% of renewable plant biomass in nature (*1*). Up to now, xylose has not been used for the production of industrial products, such as ethanol. This is partly because most known yeasts, including *S. cerevisiae*, cannot ferment xylose to ethanol, and some of them cannot even utilize xylose for growth (*2*). Recently, a number of new yeasts have been found that can directly ferment xylose to ethanol (*3*), and *P. stipitis* is one of such

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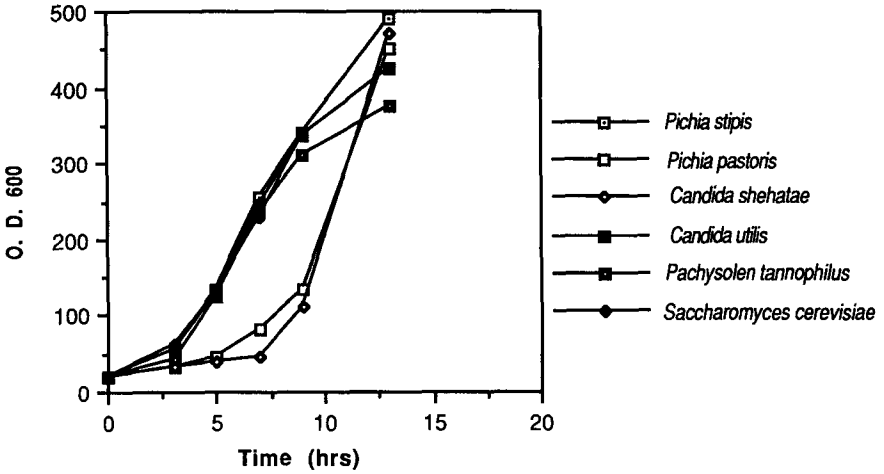

Fig. 1. Growth curves of yeasts in medium with glucose as the sole carbon source.

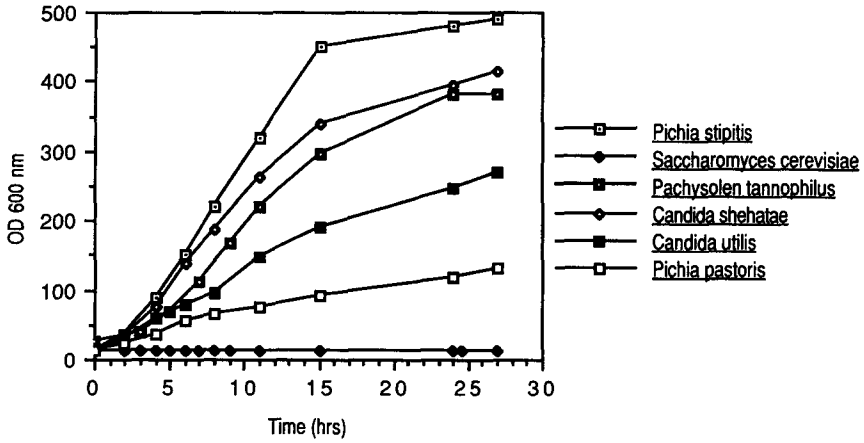

Fig. 2. Growth curves of yeasts in medium with xylose as the sole carbon source.

yeasts that can ferment xylose more effectively (4). However, it has been reported that even the best xylose-fermenting yeasts, such as *P. stipitis*, still only ferment both glucose and xylose with low efficiency (4). In order to be able to effectively convert xylose to liquid fuel ethanol, strain improvement is very much desired.

Recent advances in recombinant DNA techniques have demonstrated that recombinant manipulation can play a significant role in improving the capability of microorganisms. Yet, none of the xylose-fermenting yeasts has an established plasmid-mediated genetic transformation system. In addition to being one of the best xylose-fermenting yeasts, we found that *P. stipitis* also has a number of other outstanding properties. For example, mutants are relatively easily isolated from *P. stipitis;* it also grows extremely well aerobically on both glucose and xylose, as shown in Figs. 1 and 2. This may make *P. stipitis* a potential host for the conversion

of glucose and xylose to various recombinant products, if a host-vector system can be established for this yeast. These are the reasons that make it extremely desirable to develop a genetic transformation system for the yeast *P. stipitis.*

MATERIALS AND METHODS

Strains

P. stipitis CBS 7126, obtained from the Centraalbureau voor Schimmelcultures, Yeast Division, Delft, the Netherlands, was used for the development of the plasmid-mediated transformation system. *E. coli* HB101 was used for preparing shuttle vectors, as well as for recovery of plasmids from yeast transformants.

Bacterial Transformation

E. coli transformation was carried out according to Norgard et al. (*5*).

Pichia Transformation

Transformation of *P. stipitis* was carried out by a procedure similar to that developed for the transformation of *S. cerevisiae* (*6*) and *Pichia pastoris* (*7*), except that 0.6 *M* KCl instead of 1.2 *M* sorbitol was used as the stabilizer for the protoplasts.

Recovery of Plasmids from *Pichia* Transformants

To prove that shuttle vectors were introduced and maintained in the *Pichia* transformants, plasmids were recovered from the *Pichia* transformants. This was accomplished by inoculating a 5 mL *P. stipitis* culture, incubated at 30°C overnight. Plasmids were isolated from the resulting cells according to the procedure described by Struhl et al. (*8*). The recovered plasmids were subsequently used to retransform *E. coli* HB101, and *E. coli* transformants resistant to both Amp and Km were selected. Plasmids were then isolated from the *E. coli* transformants, and the resulting plasmids were analyzed by agarose gel and compared with the original shuttle vectors used for transformation of *P. stipitis.*

RESULTS

Attempt to Isolate Cryptic Plasmids from *P. stipitis*

A vector that can be used to transform a specific host must contain at least two essential elements. One is a replication origin that can be used

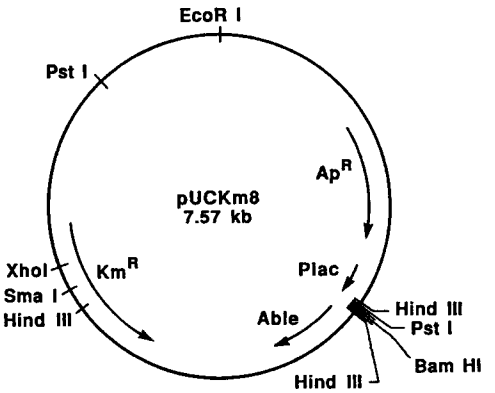

Fig. 3. High copy-number shuttle vector. The plasmid was constructed by the insertion of a 4.6 kb EcoRI fragment containing the 2 μ replicon and the Km^R, isolated from plasmid pKan5 (*15*) into plasmid pUC9. The plasmid transforms *E. coli*, *S. cerevisiae*, and *P. stipitis* very effectively.

by the host DNA polymerase to initiate autonomous replication of the plasmid. The other is a selection marker that allows the distinction of transformants from nontransformants. The replication origin from a cryptic plasmid of the host should be the most desirable replication origin for the construction of the cloning vectors for the host. Efforts have been made to isolate cryptic plasmids from *P. stipitis* according to the procedure described by Devenish and Newlon (*9*). The circular DNA isolated from *P. stipitis* by the latter procedure is identical to the *Pichia* mitochondrial DNA isolated by the procedures (*10,11*) used for the isolation of mitochondrial DNA from other yeasts. Hence, the only extrachromosomal element present in *P. stipitis* is its mitochondrial DNA.

Transformation of *P. stipitis* with a 2 μ-Based Shuttle Vector

The replication origin of the *S. cerevisiae* 2 μ plasmid was reported to function in some yeasts (*7,12*) besides *S. cerevisiae*, although the 2 μ replicon was found not necessarily to function as a replication origin in all the yeasts (*13,14*). We decided to study whether the 2 μ replicon could also function effectively in *P. stipitis*. Recently, we have constructed the 2 μ-based shuttle vector pUCKm8 (Fig. 3), which is a high copy-number plasmid for *S. cerevisiae* (Ho, unpublished results). pUCKm8 contains the kanamycin resistance gene (Km^R), which might be able to serve as a selection marker for *P. stipitis*, since we found that the latter yeast is at least partially resistant to the antibiotic geneticin (Table 1). Geneticin is known to be inactivated by the same enzyme encoding by Km^R. By using a modified protocol developed for the transformation of *S. cerevisiae* (*6*) and *P. pastoris* (*7*), we found that *P. stipitis* can be transformed to geneticin resistant. The fact that plasmids identical to pUCKm8 can be recovered from

Table 1
Pichia Stipitis Resistant to Antibiotic Geneticin in Different Media

Medium	Intact Cells	Protoplast Regenerated Cells
YEPD[a] with 50 μg/ml Geneticin	++++[c]	++++
YEPD with 100 μg/ml Geneticin	++[d]	++++
YEPGE[b] with 50 μg/ml Geneticin	----[e]	++
YEPGE with 100 μg/ml Geneticin	----	

[a]YEPD contains 1% yeast extract, 2% peptone, and 2% glucose.
[b]YEPGE contains 1% yeast extract, 2% peptone, 3% glycerol, and 2% ethanol.
[c]Cells grow very well.
[d]Very few cells can grow.
[e]No cell can grow for more than seven days.

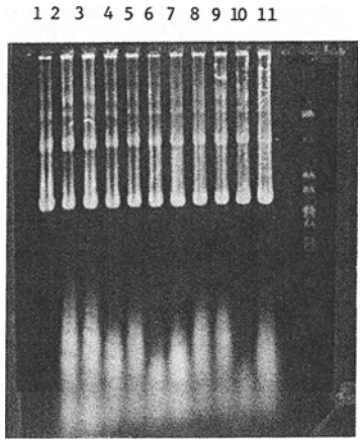

Fig. 4. Analysis of plasmid DNA recovered from *P. stipitis* transformed with plasmid pUCkm8. Lane 1: pUCKm8 used for *Pichia* transformations; lanes 2–11: plasmids recovered from *Pichia* transformants.

P. stipitis transformants, as shown in Fig. 4, has provided the crucial evidence that the plasmid vector can be introduced and maintained in *P. stipitis* by the procedure described here. Furthermore, pLC11, an *E. coli-Candida utilis* shuttle vector (*15*) that has similar construction to pUCKm8, except that it contains a *C. utilis* autonomous replication origin instead of the 2 μ fragment, was also used to transform *P. stipitis* under identical

to avoid this complication, we are currently developing a protocol for direct transformation of intact *P. stipitis* cells by electroporation. Preliminary results indicate that suitable conditions for transformation of intact *P. stipitis* could be developed. Furthermore, after electroporation, the only geneticin resistant cells were those containing pUCKm8.

DISCUSSION AND CONCLUSION

In this paper, we have shown that a plasmid-mediated genetic transformation system can be established for *P. stipitis* and the 2 μ replicon can be used as a replication origin for the *Pichia* DNA polymerase. Based on the number of plasmids that can be rescued from the *Pichia* transformants, the plasmid vector pUCKm8 probably is a high copy-number plasmid for *P. stipitis*.

Although Km^R is not an ideal selection marker for the transformation of *Pichia* protoplasts, it does allow the selection of true transformants when transformation is carried out by electroporation of intact cells (data not shown). Nevertheless, we found that *Pichia* mutants are relatively easy to isolate. For example, we have already isolated a number of xylose reductase mutants from *P. stipitis* (Ho, unpublished results). With recent advances in gene cloning techniques, it is relatively easy to clone any gene that complements a specific mutation. The *Pichia* mutants and the genes that are capable of complementing the mutations will be able to serve as excellent selection mechanisms for the establishment of improved transformation systems for *P. stipitis*.

ACKNOWLEDGMENTS

This work was supported by the Solar Energy Research Institute subcontract XX-8-77245, and by USDA grant 89-37233-4890.

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