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DNA SEQUENCES FROM A LIGNINOLYTIC FILAMENTOUS FUNGUS *Phanerochaete chrysosporium* CAPABLE OF AUTONOMOUS REPLICATION IN YEAST+

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Five different DNA sequences of *Phanerochaete chrysosporium* capable of supporting autonomous replication of yeast integration plasmid (YIp5) in *Saccharomyces cerevisiae* were isolated. These hybrid plasmids with the autonomous replication sequences from *P. chrysosporium* are maintained extra-chromosomally, are mitotically unstable and transform *Ura3* deletion mutant of *S. cerevisiae* to Ura+ phenotype with high frequency. The autonomous replication sequence in pRR2, one of the recombinant plasmids, was further characterized and was shown to be homologous to *P. chrysosporium* genomic DNA. Restriction analyses showed that this plasmid has unique PvuII and SalI restriction sites for cloning.

Specific DNA sequences called *ars* which when cloned into YIp5, confer on the latter plasmid the ability to autonomously replicate in yeast and transform yeast with high frequency have been isolated from *Saccharomyces cerevisiae* and a number of other eukaryotes (1-9). However, there have been no reports on the isolation of *ars* from *Phanerochaete chrysosporium*, a ligninolytic filamentous white-rot fungus, which is perhaps the most extensively used organism in studies on lignin biodegradation. We have described here the isolation of DNA sequences from *P.* chrysosporium, which are capable of autonomous replication in *S. cerevisiae*, to further our eventual objective of construction of a cloning system for *P. chrysosporium*.

MATERIAL AND METHODS

Strains, Plasmids and Media.

Plasmids YIp5,YRp12 and *S. cerevisiae* YNN27 (α *trp1-289 Ura3-52 gal2*) were supplied by R. W. Davis and *Escherichia coli* HB101 (*hsd proA2 thi lacYI recA13 rpsL20 ara-14 galK2 xyl-5 mtl-1 supT44*) and plasmid pBR322 were

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Abbreviations used: autonomous replication Sequence(s), *ars*.

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provided by P. T. Magee (7). P. chrysosporium (ATCC 34541) was obtained from T. K. Kirk and was maintained through periodic transfer on malt extract agar as previously described (10). E. coli was grown in L-broth (11), S. cerevisiae was grown in YEPD or YNBG medium (12) and P. chrysosporium was grown in malt extract broth (10).

DNA preparation, transformation of cells, and Southern hybridization.

Plasmid DNA from E. coli and total yeast DNA from transformed and untransformed cells was isolated as previously described (12,13). P. chrysosporium DNA was isolated as follows: wet mycelial mass (4 g) was mixed with 10 g of glass beads (0.1-0.2 mm), frozen at -70° C, powdered in a mortar and pestle, mixed with 20 ml of lysis buffer (12) and incubated at 65° C for 30 min. The supernatant was collected by centrifugation at 2,000 x g and extracted thrice with phenol (11) followed by ether extraction. The DNA was precipitated with ethanol at -20°C, the pellet was dissolved in TE buffer (11), and treated with RNase A (100 µg/ml) for 3 h at 37°C followed by proteinase K (100 µg/ml) treatment for 2 h at 37°C. Two additional phenol and ether extractions were then carried out, and the DNA was precipitated with ethanol at -20°C. The DNA pellet dissolved in TE buffer was used.

E. coli HB101 was transformed with plasmid DNA using the calcium chloride heat shock method (11). S. cerevisiae was transformed by using a modified Beggs method as described previously (12). The mitotic stabilities of hybrid plasmids in yeast were determined as previously described (7).

Southern hybridization procedure used was that described by Maniatis et al. (11). (^{32}P)dCTP was incorporated into plasmid DNA by nick translation with E. coli polymerase I (14). The procedure used for high stringency hybridization was described previously (15). Autoradiography was performed at -70°C with Kodack XAR5 film and intensifying screen (16).

RESULTS AND DISCUSSION

Isolation of ars.

A typical yeast integration plasmid, YIp5 (1,17), which contains plasmid pBR322 DNA (18) and URA3 gene of S. cerevisiae was employed as the cloning vector. P. chrysosporium DNA was partially digested with MboI and the restriction fragments were ligated to BamHI-digested YIp5 plasmid DNA. This pool of hybrid DNA molecules was used to transform ura3 deletion mutant of S. cerevisiae YNN27 to the Ura$^+$ phenotype. Five Ura$^+$ transformants (PC1-PC5) were picked randomly for further study. If all the Ura$^+$ transformants possessed autonomously replicating plasmids, they should be detectable in total DNA isolated from these transformants by hybridization with (^{32}P) labeled pBR322 DNA and by transformation of E. coli. Total DNA from Ura$^+$ transformants (described above) contained bands homologous to pBR322, with electrophoretic mobility corresponding to the supercoiled, open circular and multimeric forms of the transforming plasmids (Fig. 1). DNA from untransformed YNN27 (control) did not show hybridization with pBR322 DNA. DNA

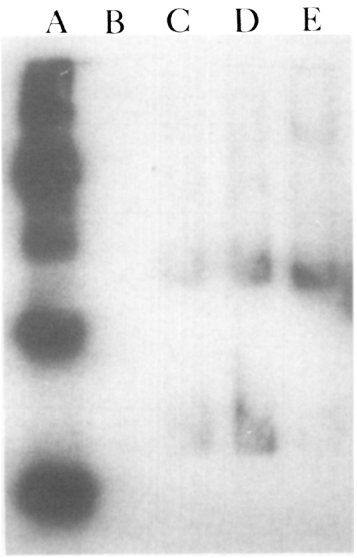

Figure 1. Probing of total DNA from Ura$^+$ transformants of S. cerevisiae YNN27 with (^{32}P) labeled pBR322 DNA. About 10 µg of DNA, isolated from yeast transformants grown on YNBG containing tryptophan, was fractionated by electrophoresis on 0.7% agarose gel. Blot hybridization was carried out under high stringent conditions. YIp5 plasmid DNA (lane A) served as positive control and DNA from untransformed S. cerevisiae YNN27 (lane B) served as negative control. Lanes C, D and E contained DNA from Ura$^+$ transformants PC1, PC2 and PC5, respectively.

preparations from all the five Ura$^+$ transformants (PC1-PC5) transformed E. coli HB101 with a high frequency yielding 100 to 1000 ampicillin-resistant and tetracycline-sensitive bacterial clones. The five plasmids isolated from E. coli were designated pRR1, pRR2, pRR3, pRR4 and pRR5, corresponding, respectively, to transformants PC1, PC2, PC3, PC4 and PC5. Each of the five plasmids could transform S. cerevisiae YNN27 to Ura$^+$ phenotype with high frequency (Table 1). YIp5 alone does not transform the Ura3-52 deletion mutant strain of S. cerevisiae YNN27 to Ura$^+$ phenotype because the mutant contains a small deletion so that URA3 gene in YIp5 cannot recombine with its genomic counterpart at a frequency sufficient to observe transformants (1,17). Therefore, transformation of S. cerevisiae YNN27 to Ura$^+$ phenotype occurs only when the DNA fragment inserted into YIp5 confers on it the ability to autonomously replicate in yeast (1-9,17). Thus the high-frequency transformation of S. cerevisiae YNN27 to Ura$^+$ phenotype observed with the

Table 1. Properties of recombinant plasmids (YIp5 plus *ars* from *P. chrysosporium*) isolated from Ura$^+$ transformants of *S. cerevisiae*[a]

Plasmids	Insert size (kb)	Transformation efficiency (Transformants per µg DNA)	Generation time (h) of transformants on: Select. medium[b]	Non-select. medium[b]	Mitotic stability[c] Select. medium[b]	Non-select. medium[b]
pRR1	1.55	55	4.1	2.8	23.3	4.2
pRR2	1.50	153	4.1	2.5	22.0	2.2
pRR3	12.00	261	5.4	3.0	13.9	0.7
pRR4	1.50	95	4.1	2.8	35.3	4.8
pRR5	1.50	34	4.0	2.6	29.6	1.7
YRp12	1.50	2164	4.0	2.6	30.7	0.7
YIp5		0				

[a]All mitotic stability experiments were performed on plasmids transformed into *S. cerevisiae* YNN27. Each stability figure is an average value from three separate experiments.

[b]Selective medium refers to minimal medium supplemented with tryptophan (50 µg/ml) and non-selective medium refers to selective medium supplemented with Uracil (50 µg/ml).

[c]Mitotic stability is expressed as the percentage of cells remaining Ura$^+$ after 20 generations on selective or non-selective medium.

hybrid plasmid can be considered to be inherent property of the *P. chrysosporium* DNA sequences inserted into YIp5.

The size of the *P. chrysosporium* DNA inserts in plasmids pRR1, pRR2, pRR4 and pRR5 was approximately 1.5 kb whereas that in pRR3 was 12.0 kb. The doubling time for Ura$^+$ transformants in selective and non-selective media (Table 1) was similar to that of YRp12 transformants (positive control). All the Ura$^+$ transformants were unstable in selective and non-selective media (Table 1), similar to other *ars* plasmids previously described (1-9, 17, 19, 20).

Restriction analysis of the *ars* plasmid pRR2 (Fig. 2a) indicated that the *ars* does not contain restriction site for PvuII or SalI and that this plasmid still contains a restriction site for BamHI after BamHI-MboI ligation (Fig. 2a). Double enzyme digestion indicated that BamHI site is near or at one of the ends of *ars*, close to the SalI site (Fig. 2b). Restriction map of plasmid pRR2 (Fig. 3) shows that this plasmid has unique PvuII and SalI restriction sites for cloning. Hybridization experiments showed that (^{32}P) labeled pRR2 DNA, but not (^{32}P) labeled YIp5 DNA, is homologous to *P.*

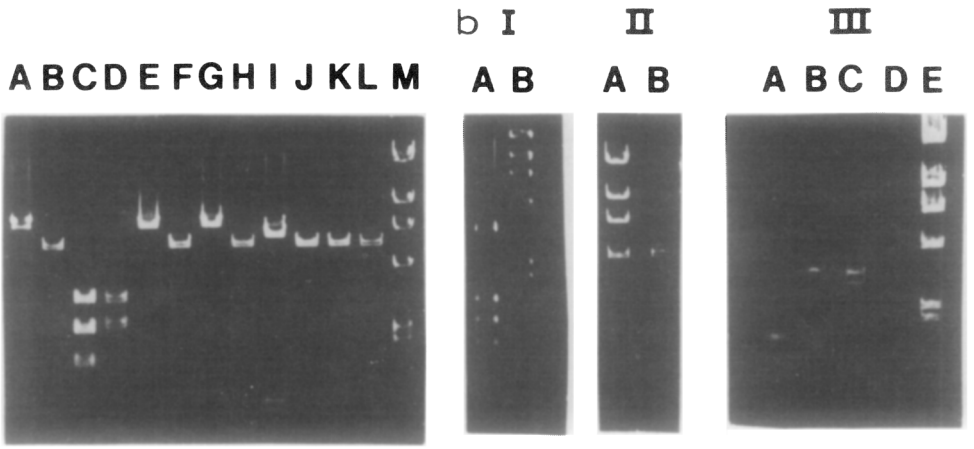

Figure 2. Restriction mapping of plasmid pRR2 containing ars from P. chrysosporium
a. Single digestion of pRR2 and YIp5, respectively, with BamHI (lane A and B), PstI (lane C and D), PvuII (lane E and F), SalI (lane G and H), HindIII (lane I and J) and EcoRl (lane K and L). Lane M contained size markers of λ DNA-HindIII fragments. The DNA, after restriction was electrophoresed on agarose gel 0.7%. b. Double digestion of pRR2 with BamHI-PstI (b.I. lane A), PvuII-PstI (b.II. lane B), PvuII-BamHI (b.III. lane A), PvuII-HindIII (b.III. lane B) PvuII-EcoRl (b.III. lane C) and PvuII-PstI (b.III. lane D). Lanes B, A and E in I, II and III in this figure are size markers of λ DNA HindIII fragments.

chrysosporium genomic DNA (Fig. 4). These results indicate that the ars in pRR2 originated from P. chrysosporium genome.

The results of this study show that several DNA sequences of P. chrysosporium which promote autonomous replication of plasmid YIp5 in S.

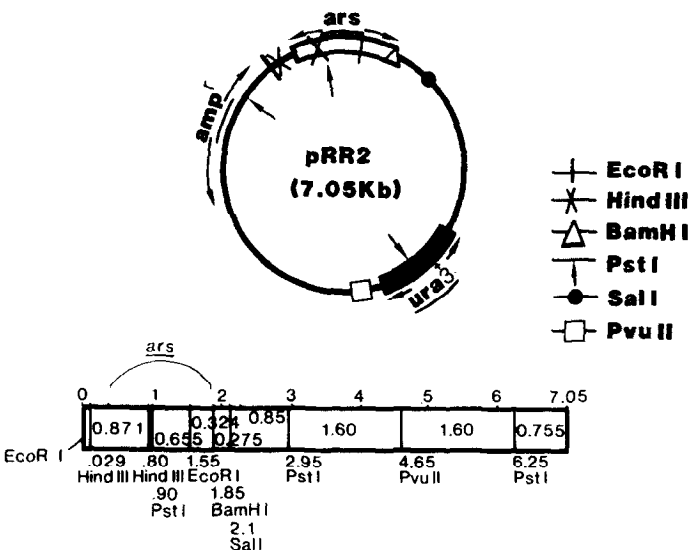

Figure 3. Restriction map of plasmid pRR2.

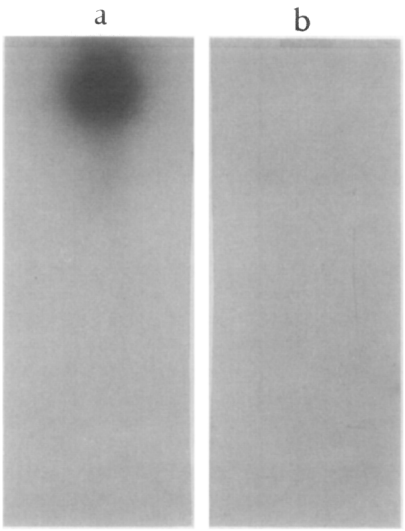

Figure 4. Probing of total P. chrysosporium DNA with (^{32}P) labeled pRR2 (a) and (^{32}P) labeled YIp5. Blot hybridization was carried out under high-stringency conditions.

cerevisiae have been isolated. Hybridization experiments showed that the hybrid plasmids contain inserts of P. chrysosporium DNA. All of these hybrid plasmids transformed S. cerevisiae and E. coli at high frequency and could be reisolated from transformants without any detectable change in plasmid structure or function. These results suggest that the cloned DNA fragments of P. chrysosporium fit the description of ars previously isolated from other eukaryotic cells (1-9, 17).

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REFERENCES

1. Struhl, K., Stinchcomb, D. T., Scherer, S., and Davis, R. W. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1035-1039.
2. Hsiao, C., and Carbon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3829-3833.
3. Stinchcomb, D. T., Thomas, M., Kelly, J., Selker, and Davis, R. W. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4559-4563.
4. Lazdins, I. B., and Cummings, D. J. (1982) Curr. Genet. 6, 173-178.
5. Zakian, V. A. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 3128-3132.
6. Banks, G. R. (1983) Curr. Genet. 7, 79-84.
7. Hsu, W. H., Magee, P. T., Magee, B. B., and Reddy, C. A. (1983) J. Bacteriol. 154, 1033-1039.
8. Henry, B. E., Raab-Traub, N. J., and Pango, J. S. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 1096-1100.
9. Roth, G. E., Blanton, H. M., Hager, L. J., and Zakian, V. A. (1983) Mol. Cell. Biol. 3, 1893-1908.

10. Kirk, T. K., Schutlz, E., Connors, W. J., Lorenz, L. F., and Zeikus, J. G. (1978) Arch. Microbiol. 117, 227-285.
11. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular cloning: a laboratory manual, pp. 98-478, Cold Spring Harbor Laboratory. N.Y.
12. Sherman, F., Fink, G. R., and Hicks, J. B. (1981) Methods in yeast genetics, pp. 61-64, and pp. 113-119, Cold Spring Harbor Laboratory. N. Y.
13. Holmes, D. S., and Quigley, M. (1981) Anal. Biochem. 114, 193-197.
14. Rigby, P. W., Dieckmann, M., Rhodes, C., and Berg, P. (1977) J. Mol. Biol. 113, 237-251.
15. Engel, J. D., and Dodgson, J. B. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 2856-2860.
16. Laskey, R. A., and Mills, D. (1977) FEBS Lett. 82, 314-316.
17. Scherer, S., and Davis, R. W. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4951-4955.
18. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynecker, H. L., Boyer, H. W., Crosa, and Falkow, S. (1977) Gene 2, 75-93.
19. Beach, D., Piper, M., and Shall, S. (1980) Nature (London) 284, 185-187.
20. Beach, D., and Nurse, P. (1981) Nature (London) 290, 140-142.