This is to certify that the

thesis entitled

CONSTRUCTION OF NEW CLONING
VECTORS FOR THE GENETIC
MANIPULATION OF YEASTS

presented by

WEN HWEI HSU

has been accepted towards fulfillment
of the requirements for

PH.D. degree in MICROBIOLOGY

C. Adinarayana Reddy
Major professor

Date 2/21/83

CONSTRUCTION OF NEW CLONING VECTORS FOR THE GENETIC MANIPULATION OF YEASTS

By

Wen Hwei Hsu

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1983

ABSTRACT

CONSTRUCTION OF NEW CLONING VECTORS FOR THE GENETIC MANIPULATION OF YEASTS

by

Wen Hwei Hsu

Genetic cloning systems for yeasts are largely restricted to Saccharomyces and Schizosaccharomyces. Therefore, studies were initiated to develop a broad host-range cloning vector for yeast. A yeast cloning vector, pHR40, which carries both 2μ DNA and an autonomous replication sequence (ars) from S. cerevisiae, and a kan^r determinant from Escherichia coli transposon Tn601, which codes for an aminoglycoside phosphotransferase that inactivates the antibiotics G418 and kanamycin. pHR40 transforms at high frequency a number of yeast genera besides S. cerevisiae into G418-resistance. This selection system eliminates the need for the construction of stable mutants to serve as the recipient strains. However, pHR40 does not appear to be the most desirable vector for gene transfer, because it is unstable in the host yeast. To study the factors which affect the stability of a cloning vector containing kan^r determinant, several new, relatively small, yeast cloning vectors were constructed carrying 2μ DNA, ars, and centromere (cen3) genes, either singly or in

combination, from S. cerevisiae and a kan^r determinant. All the vectors transform G418-sensitive S. cerevisiae to G418-resistance with a high frequency, and replicate autonomously in host yeast. The results suggested that the smaller the molecular size of the vector, the greater is its mitotic stability in S. cerevisiae. The presence of cen3 appears to enhance the mitotic stability of the vector. A new cloning vector (pHMR22) containing an ars from Candida utilis was constructed as a first step in developing a cloning system for C. utilis. pHMR22 is small in size (6.6 kb), and has several unique restriction enzyme sites for gene cloning. Therefore, pHMR22 should be a useful vector for cloning the desired genes in S. cerevisiae and perhaps also in C. utilis, and for the comparative study of ars in yeast.

To Huiyu, Suting, Suchi and my parents

ACKNOWLEDGMENTS

I would like to express my appreciation to Dr. C.A. Reddy for his valued counsel during the course of this study. I am also grateful to Drs. P. Gerhardt, P.T. Magee, H.L. Sadoff, J.M. Tiedje, and M.T. Yokoyama for serving as my Research Guidance Committee. The critical suggestions and assistance of Ms. B.B. Magee, Dr. M. Clancy, Dr. J.B. Dodgson, Mr. R.L. Kelley, and numerous others have been very useful and are gratefully acknowledged. I would like to thank Dr. P.C. Ma for his encouragement. Finally, I thank the Department of Microbiology and Public Health (Michigan State University), Food Industry Research and Development Institute (Republic of China), and National Science Council (Republic of China) for the financial assistance provided to me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

Appendix

General Introduction

Recombinant DNA methodology involves cleavage of a given host DNA by restriction enzymes and insertion of one or more host DNA fragments into a cloning vector, which is then used for transformation of a suitable recipient cell. Thus, genetic information from one organism may be transferred to and expressed in a related organism, or often in an unrelated organism as well. This powerful new technology allows one to understand the molecular mechanisms controlling the functions of the genes inside the cell and to alter the genetic make-up of an organism precisely according to plan so that one can create an organism tailored to produce a desired product inexpensively and efficiently. Genetic engineering techniques became available for the study of yeasts with the establishment of transformation system for Saccharomyces cerevisiae in 1978 (95). A great deal of progress has been made since then in developing many different type of yeast cloning vectors (13,14,21,27,119,149,188). However, at the present time, gene cloning systems are essentially restricted to Saccharomyces and Schizosaccharomyces pombe (9,13,106). Development of gene cloning techniques for other yeast genera has been hampered by the fact that suitable cloning vectors and transformation systems are not

available for these organisms.

Many yeasts, such as Candida utilis are important from the stand point of biotechnology. They can be used for the production of single cell protein, chemicals, enzymes and other products. To apply recombinant DNA methodology to these industrially important yeasts, a high frequency transformation system is prerequite. Such a system will allow the development and utilization of many different yeasts as host cells for foreign genes and will provide a means for the further analysis of structure and regulation of various yeast genes. Gene cloning will be particularly useful for genes such as xylose isomerase, which are industrially important but are absent in S. cerevisiae.

High frequency transformation in yeast has been accomplished primarily with two kinds of plasmids: 1) those containing fragments of 2 μ DNA, which clearly are capable of self-replication in yeast (13,14,21,27); and 2) those containing a certain yeast chromosomal sequence called autonomous replication sequence (ars), which presumably contains replication origin for DNA synthesis (21,111,119,188). Efficient replication and maintenance of the 2 μ chimeric vector in host yeast requires, in addition to the replication origin, the DNA regions rep1, rep2 and rep3 which almost span the entire 2 μ DNA and are implicated in copy number control. Two of these (rep1 and rep2) code for proteins that are active in trans (23). A third locus (rep3) is required in cis (G. Tschumper,

personal communication). This system maintains the 2 μ plasmid copy number at about 50-100 copies per diploid cell. Vectors containing the whole 2 μ DNA are relatively large and have too many restriction endonuclease sites to serve as ideal cloning vectors. Therefore, a number of smaller and more useful 2 μ plasmid vectors, which contain replication origin of the 2 μ DNA but do not have all three rep sequences, have been constructed (13,14,21,27). These plasmids have mostly been used to transform yeast strains containing endogenous 2 μ DNA, which supplies the rep gene functions. These type of plasmids, however, may not be suitable as cloning vectors for yeasts other than Saccharomyces and Schizosaccharomyce because 2 μ DNA or sequences complimentary to 2 μ DNA have not been demonstrated in other yeast genera (86,219).

Autonomous replication sequences, in contrast to 2 μ DNA, are reported to be widely distributed and have a broad host range. For example, the arss from several phylogenetically distant eukaryotes (121,200,232) as well as prokaryotes (80) were known to confer on yeast integrating plasmid (e.g. YIp5) the ability to replicate autonomously in S. cerevisiae. A question of considerable interest is whether a plasmid containing ars would serve as broad host range cloning vector for a wide variety of yeasts other than Saccharomyces.

Most selection systems used in yeast recombinant DNA methodology currently employ a suitable cloning vector,

which carries a gene responsible for a specific step in the biosynthesis of a nutrient such as an amino acid or nucleotide, and a corresponding auxotrophic recipient strain (12,95,100,138,200). Selection based on the use of auxotrophic mutants obviously has the disadvantage of requiring the construction of stable auxotrophs which is not always easy. With the exception of S. cerevisiae and S. pombe, stable auxotrophs suitable for genetic cloning are currently not available for most other yeasts. An antibiotic-sensitive yeast strain could be used as a recipient strain in place of nutritional auxotrophs, provided the yeast cloning vector carries the genetic determinant for that particular antibiotic. A number of antibiotic resistance determinants of bacterial origin have been known to be expressed in yeast (22,36,107,182), but most cells are relatively resistant to these antibiotics (45,106). One exception is G418, an aminoglycoside antibiotic, which was reported to inhibit the growth of Saccharomyces. Jimenez and Davies (114) recently described a transformation system for S. cerevisiae in which a cloning vector carrying kan^r determinant from Escherichia coli transposon Tn601 and a G418-sensitive recipient yeast strain were employed. Since selection based on drug resistance would eliminate the requirement for the construction of a stable yeast mutant of a specific genotype to serve as the recipient, it appeared that this type of selection system may be valuable for the genetic manipula-

tion of wild type strains of S. cerevisiae and perhaps other industrially important yeasts.

The main objectives of this study were as follows:

1. To isolate and characterize the ars sequences from C. utilis;
2. To determine whether a plasmid containing ars and kan^r would serve as a broad host-range cloning vector;
3. To analyze the factors controlling the mitotic stability of vectors containing kan^r and ars.

The results show that the cloned ars of C. utilis has no detectable homology to the S. cerevisiae genome but is fully functional in this yeast, and that the kan^r/G418 selection system coupled with LiCl transformation procedure is useful for the transformation of a number of yeast genera, besides Saccharomyces. The mitotic stability of kan^r-ars plasmids apparently depends on the molecular size of the vector. The smaller the molecular size of the vector, the greater is its mitotic stability in S. cerevisiae. The presence of a centromere element (cen3) appears to enhance the mitotic stability of a vector.

LITERATURE REVIEW

Gene Cloning in Yeasts

Introduction

Yeast, especially *Saccharomyces* species, have become important tools for genetic research in the past few years. This is partly due to the fact that the organization of the yeast genome is relatively less complex than that in most other eukaryotes (68,171,173). Further, genes from other eukaryotic organisms which are not selectable or compatible with bacterial systems can be cloned in yeasts. Furthermore, many yeasts can be propagated either in the haploid or diploid state with relative ease. The popularity of yeast as genetic tools also stems from the fact that many of them can be sporulated and that each spore can be germinated and studied individually. Studies on the genetics of yeasts have particularly mushroomed after the transformation system(s) which permitted the isolation of virtually every gene became widely available.

Yeast cloning technology provides a powerful tool for genetic studies because the effect of changes induced *in vitro* within the cloned gene can be monitored *in vivo* by transforming the recombinant plasmid back into a suitable recipient yeast cell (84,143,189,209). This has thus paved the way for extensive studies on the mechanism of gene

expression in yeast.

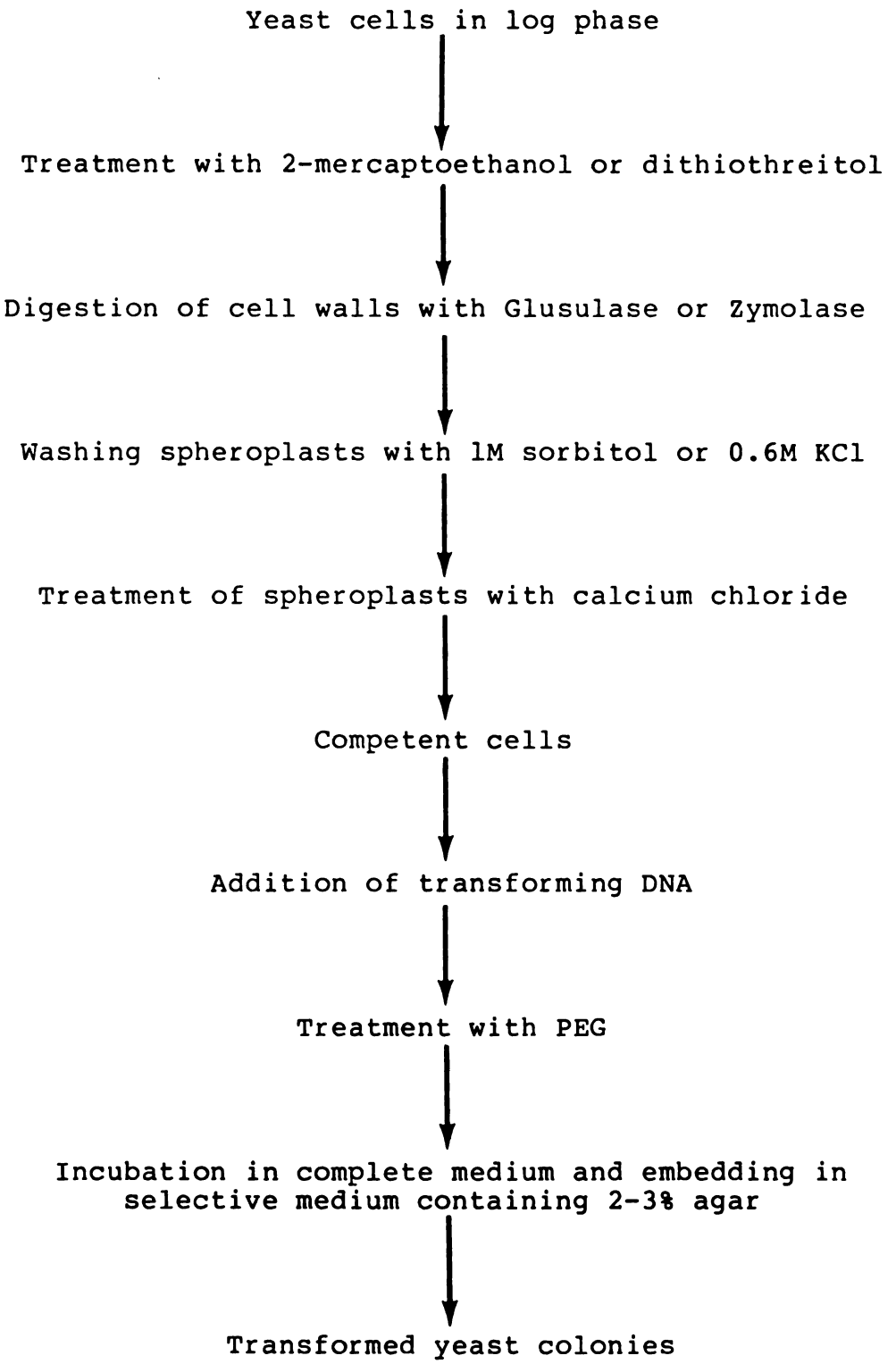
In this paper, I have reviewed the procedures for yeast transformation and described the properties of different types of cloning vectors currently available for yeast, especially S. cerevisiae. Considerable emphasis is placed on reviewing the isolation and expression of genes of yeast origin as well as foreign genes in yeast. Finally, some possible applications of yeast cloning technology and a few comments about future challenges in this field are presented. The current advances in yeast recombinant DNA technology are particularly emphasized. Readers who are interested in the general area of gene cloning in yeast are referred to several excellent recent reviews (13,96,106,163).

Transformation Methodology

Spheroplast method

Hinnen et al. (95) were the first to develop a Spheroplast method for the transformation of S. cerevisiae and several variations of this basic procedure have been reported subsequently (12,110,112). The basic steps (Fig. 1) involved in this procedure may be summarized as follows: cells in the logarithmic phase of growth are treated with a mixture of glucanases to produce yeast spheroplasts. The spheroplasts are extensively washed with 1M sorbitol and then treated with calcium chloride and polyethylene glycol (PEG; 4000 or 6000 M.W.) to promote the uptake of the transforming DNA. The transformed spheroplasts are

Fig. 1. Transformation of yeast: spheroplast method.


Yeast cells in log phase
Treatment with 2-mercaptoethanol or dithiothreitol
Digestion of cell walls with Glusulase or Zymolase
Washing spheroplasts with 1M sorbitol or 0.6M KCl
Treatment of spheroplasts with calcium chloride
Competent cells
Addition of transforming DNA
Treatment with PEG
Incubation in complete medium and embedding in selective medium containing 2-3% agar
Transformed yeast colonies

embedded in a selective medium containing a high percentage of agar, usually 2-3%, to facilitate the regeneration of the cell wall (225). In the final step, the spheroplasts are plated on a selective medium which would allow the growth of the transformed clones only.

Yeast cell walls are composed of a mixture of polysaccharides, such as glucans, mannans and chitin, and other components such as amino sugars, proteins, lipids and phosphates (3,4,7). The cell wall must be digested, at least partially, with a mixture of hydrolytic enzymes to generate spheroplasts and thus to promote the effective uptake of transforming DNA (13,95).

The effectiveness of various hydrolases is thought to be influenced by interactions between the polysaccharide and the other polymers (such as proteins) within the cell walls (7). Reducing agents such as, 2-mercaptoethanol (54,192), and dithiothreitol (192) are often used in transformation procedures because they reduce disulfide bridges in the cell wall proteins (67) and thus apparently renders the cell wall polysaccharides more susceptible to digestion by glucanases. However, it is still not known exactly how the reducing agents make the cell walls more vulnerable to hydrolysis. The pretreatment of yeast cells with reducing agent prior to the addition of hydrolytic enzymes is not always essential and its requirement probably depends on the yeast strain used and the physiological state of the cells to be treated (13). The

properties of lytic enzymes used for the preparation of yeast spheroplasts had been extensively reviewed by Kuo and Yamamoto (130) and by Hamlyn et al. (87). Most of the enzyme preparations used for making spheroplasts are rather crude mixtures of hydrolytic enzymes such as those in snail gut extracts and are capable of hydrolysing polysaccharide components such as chitin and β-glucans, which are known to play a major role in the cell wall mechanics of budding yeasts such as S. cerevisiae (3,63). Preparations such as Glusulase (87,130) and Zymolase (130), which contain high levels of β-D-glucanase and chitinase activities, generally give the best spheroplast yield from S. cerevisiae (87). The fission yeast Schizosaccharomyces pombe differs chemically from budding yeasts in that α-mannan and chitin are absent from its cell wall, and in that about one-third of the glucan contains (1→ 3)-α-linked glucose residues (7,28). This apparently accounts for the resistance of its cell wall to the extensive hydrolysis by (1→3) and (1→6) β-glucanases (58,118,217). Kopecka (126) using a combination of snail and Trichoderma viride enzymes, and Schwenke and Nagy (191) using a combination of snail enzymes and a (1→3) α-glucanase and a (1→3)-β-glucanase, have reported quantitative conversion of exponentially growing cells of S. pombe to spheroplasts. Dickinson et al. (60) described the use of Novozym 234, which contains high levels of α-D-glucanase and β-D-glucanase activities, to prepare spheroplasts from S. pombe. Both exponential growing and

stationary phase cells can be quantitatively converted to spheroplasts on a large scale by using this procedure. Beach and Nurse (9) have also successfully used Novozym 234 to develop an efficient transformation procedure for S. pombe.

Variations in susceptibility to attack by lytic enzymes has been observed between different yeast strains of the same species and between species (130). Even within a single strain, susceptibility is highly dependent on the physiological state of the yeast (130). This is undoubtedly due to the relative proportion of the different glucan components in the walls and, perhaps more importantly, their susceptibilities to hydrolysis by the particular glucanases in the digestion mixture. Generally, cell walls of exponentially growing yeast cells are more susceptible for digestion (56,64,184,185) and give a higher percentage of regeneration of spheroplasts than stationary phase cells. Therefore, exponential phase yeast cells, rather than stationary phase cells, are used for yeast transformation.

The extent of cell wall digestion must be carefully standardized to obtain an optimal level of yeast transformation because extensive cell wall removal will adversely affect the regeneration of spheroplasts and consequently lead to poor transformation efficiency (96). The extent of cell wall digestion can be monitored by lysing the spheroplasts with sodium dodecyl sulfate and viewing the

samples with a light microscope (192). The extent of cell wall digestion can also be monitored by adding four parts of water to one part of spheroplast solution and measuring the decrease in optical density at 800 nm (122,123,124) in comparison with that obtained when 1M sorbitol, instead of water, was added to the spheroplast suspension. Use of cells that are only partially converted into spheroplasts may be preferable because this will reduce spheroplast fusion occuring during transformation. Glusulase (95) and Helicase (13) are widely used for the transformation of S. cerevisiae, and S. carlsbergensis, since with these preparations overdigestion of the cell wall rarely, if ever, occurs. Although Helicase is a cruder preparation than glusulase, the tranformation efficiencies obtained with these preparations were very similar. In general, Zymolase treatment has to be timed more accurately because long exposure can extensively digest the cell wall and result in poor regeneration frequencies of spheroplasts (96).

Since spheroplasts are osmotically fragile, an osmotic stabilizer such as sorbitol must be added to the suspending medium. Although sorbitol at concentrations ranging from 0.9 to 1.2 M (106,130) has been recommended, 1.0 M sorbitol should be the best starting point in developing transformation for a new yeast species (4). In cases where sorbitol is not desired as an osmotic stabilizer, because the yeast species in question can use it as a carbon source, one can

use KCl (4,60,106,130). The recommended concentration for S. cerevisiae is 0.6 M KCl (4,106) because this is close to the osmolality which elicits incipient plasmolysis (5). The metabolic activities of the spheroplasts are influenced by the concentration and nature of the stabilizer (130,142,190). In some cases, potassium chloride leads to even better spheroplast regeneration frequencies than those observed with sorbitol (106).

DNA uptake by the spheroplasts is promoted by treatment with calcium chloride and by the addition of 20-40% PEG (13). Addition of PEG results in the fusion of some of the spheroplasts (92,225). Apparently, the function of the PEG is to induce better contact between cells (213,214). This in turn may result in more cell fusion during incubation in the regeneration medium (92,214).

To facilitate regeneration of the cell wall, spheroplasts are embedded in a solid matrix which contains 2-3% agar, is osmotically stabilized and contains certain specific nutrients required for the growth of the specific recipient yeast (12,95,192). Colonies develop in the agar in 2-7 d depending on the particular vector and recipient yeast strain used. Schizosaccharomyces spheroplasts can regenerate the cell wall even in liquid medium (154) and on the surface of agar (9).

The transformation efficiency (transformants/μg DNA) is influenced by several variables including the strain

(106,115), the extent of spheroplast formation and regeneration (106), the plasmid type (13) and the selection system (106). Genetic data from crosses between high-transformation and low-transformation strains suggested that the transformation frequency is a polygenic inheritance and high-frequency transformation is inherited in a recessive fashion in S. cerevisiae (115).

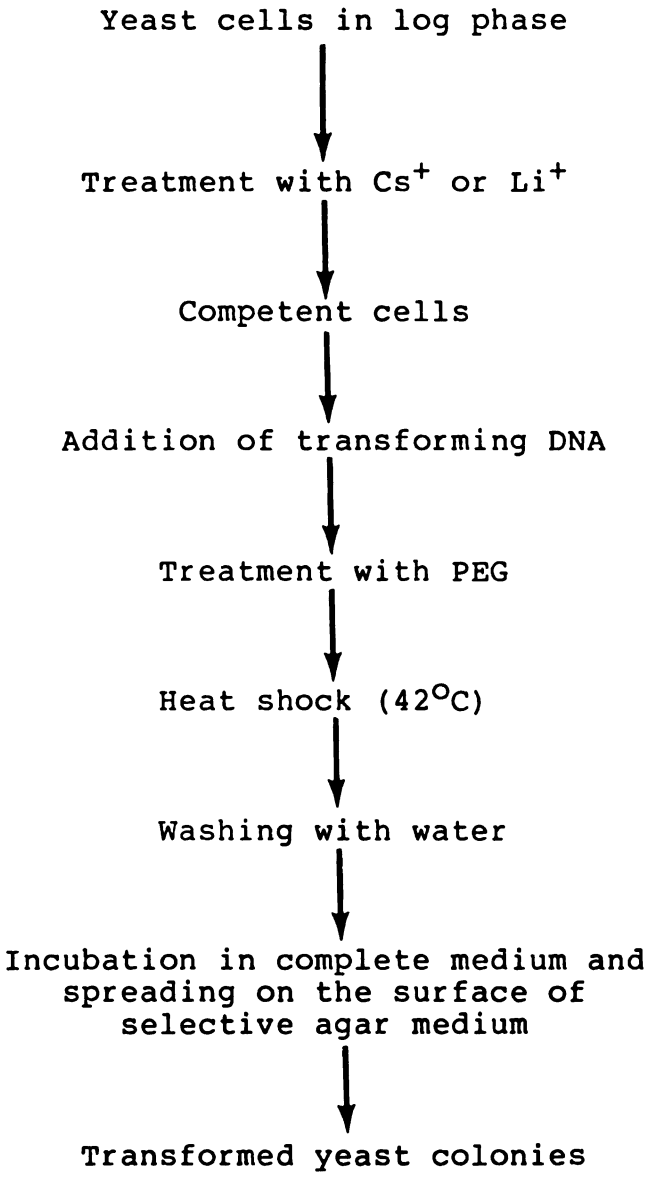
Alkali cations method

Ito et al. (113) recently described a new transformation procedure for S. cerevisiae in which alkali cations such as Li^+ and Cs^+, instead of glusulase, were used for preparing competent yeast cells (Fig. 2). This procedure does not require an osmotic stabilizer and regeneration agar. However, the transformation efficiency is 10-100 fold less than that obtained with conventional spheroplast procedure.

Selectable Genetic Markers for Transformation

Most selection systems used in yeast recombinant DNA methodology currently employ a suitable cloning vector, which carries a gene responsible for a specific step in the biosynthesis of a nutrient such as an amino acid, (12,95,110) or nucleotide (138,200), and a corresponding auxotrophic recipient strain. To eliminate background reversion of the selected marker in the transformation system, stable yeast mutants with double mutations such

Fig. 2. Transformation of yeast: alkali cations method.


Yeast cells in log phase
Treatment with Cs^+ or Li^+
Competent cells
Addition of transforming DNA
Treatment with PEG
Heat shock (42°C)
Washing with water
Incubation in complete medium and spreading on the surface of selective agar medium
Transformed yeast colonies

(e.g. leu2 locus) or deletion mutations (e.g. ura3 or his3 locus) are often used (188,200). These mutations have a reversion frequencies lower than 10^{-8} (96) and therefore background reversion is not a problem even when the integrating vectors are used.

Selection based on the use of auxotrophic mutants obviously has the disadvantage of requiring the construction of stable auxotrophs, which is not always easy. With the exception of S. cerevisiae and S. pombe, stable auxotrophs suitable for genetic cloning are currently not available for most other yeasts. An antibiotic-sensitive yeast strain could be used as a recipient strain in place of nutritional auxotrophs, provided the yeast cloning vector carries the genetic determinant for that particular antibiotic. However, at the present time drug-resistant genes of yeast origin which are dominant are not avialable as selective markers for transformation of wild type strains. Antibiotic resistant mutations which lead to alterations in the primary structure of ribosomal proteins are generally recessive. One such gene, encoding for trichodermin resistance in S. cerevisiae, has been cloned and shown to code for ribosomal protein L3 (74). A direct transformation selection for plasmids carrying the trichodermin-resistant gene was not possible.

With the knowledge that the genes of drug resistance, such as those arising from R-factors of Escherichia coli, are capable of expression in the most varied species of

bacteria (65), the expression of some of these bacterial antibiotic genes in yeast has been studied. Although the ampicillin determinant has been shown to be expressed in yeast (22,36,107,182), it is not useful as a selective marker for transformation, because S. cerevisiae is not sensitive to ampicillin (106). Jimenez and Davies (114) recently described a transformation system for S. cerevisiae in which a cloning vector carrying E. coli transposon Tn601/Tn903 (82,161) which contains the kan^r determinant (code for a phosphotransferase that inactivates the aminoglycoside antibiotics G418 and kanamycin) and a recipient yeast strain sensitive to G418 were employed. The yeast transformants obtained were resistant to high levels of antibiotic G418.

Growth and metabolism of yeasts on nonfermentable carbon sources (glycerol, alcohol, etc.) depend on the functioning of the mitochondria, which are inhibited by chloramphenicol. Yeast cells containing a plasmid bearing a chloramphenicol resistance gene (cam^r) from on E. coli plasmid can grow in a medium with a nonfermentable carbon source in the presence of small quantities (0.5 mg/ml) of antibiotic (45). However, since yeast cells are relatively resistant to chloramphenicol this antibiotic determinant is not very useful as a selective marker in transformation. A deletion of 120 bp immediately before the beginning of the structural part of the cam^r gene enhances expression of the gene and leads to a 50-fold increase in acetyltransferase

activity in yeast cells (44). Transformants containing this plasmid may be directly selectable on medium containing chloramphemicol but this fact has never been established. It is possible that with further research chloramphenicol resistance may prove to be a useful selectable marker, similar to G418 resistance. Nevertheless, based on the data available to date, it appears that antibiotic resistance determinants have the potential to serve as useful selectable markers for the transformation of agriculturally, medically or industrially important yeasts for which stable auxotrophic or other mutants are not available.

Cloning Vectors

Features of an ideal cloning vector

General purpose cloning vectors for yeast are genetically and structurally well-characterized and can be readily purified in large quantities. These plasmids are almost exclusively chimeric plasmids containing *E. coli* replicon and DNA sequences which include genes useful for selection in *E. coli* or in yeast transformation. In contrast to integrating vectors (e.g., YIp plasmid), the cloning vectors which are capable of autonomous replication in recipient yeast cells additionally carry DNA fragments containing a replication origin for 2 μ DNA (e.g., YEp plasmid) or autonomous replication sequence (*ars*; e.g., YRp plasmid). The properties of *S. cerevisiae* cloning vectors

described to date are listed in Table 1.

An ideal plasmid cloning vector for yeast should possess the following features: (a) should be relatively small so that high amounts of DNA can be cloned into it; (b) should contain a bacterial replicon such as the pBR322 origin which permits amplification of the plasmid in E. coli cells; (c) should carry selectable genetic markers for both yeast and E. coli transformation; (d) should contain unique restriction sites for as many of the commonly used restriction endonucleases as possible; (e) should possess one or more promoters that can actively transcribe cloned genes, (f) should have properties that permit the detection or selection of hybrid molecules easily, and (g) should contain an yeast replication origin and be stably maintained in the host yeast during cell propagation.

The type of yeast vector one chooses is clearly dictated by the specific cloning problem. If a high level of expression of the cloned gene is desired, a cloning vector with high copy number in the recipient yeast cell is advisable. However, with high copy number the potential deleterious effects from high gene dosage also have to be considered and might in some cases favor the use of a low copy number cloning vector such as the cloning vector containing a centromere gene and the replication origin from S. cerevisiae (7,110).

Table 1. *S. cerevisae* - *E. coli* shuttle vectors

Vectors	Size (kb)	Stabilizing[a] elements	Selective markers	Cloning sites	References or sources
YIp1	9.8	*ori*	Amp^r, *his3*	EcoRI,SalI XhoI	21
YIp5	5.5	*ori*	Amp^r, Tet^r *ura3*	EcoRI, BamHI SalI, HindIII SmaI	21,188, 201
YIp25	11.9	*ori*	Tet^r, *his4*	BamHI, HindIII	21
YIp26	7.8	*ori*	Amp^r, *leu2* *ura3*	BamHI, SalI SmaI	21
YIp27	7.8	*ori*	Amp^r *leu2* *ura3*	BamHI, SalI SmaI	21
YIp28	7.8	*ori*	Amp^r *leu2* *ura3*	BamHI, SalI SmaI	21
YIp29	7.8	*ori*	Amp^r *leu2* *ura3*	BamHI, SalI SmaI	21
YIp30	5.5	*ori*	Amp^r *ura3*	EcoRI, BamHI SalI, SmaI	21
YIp31	5.5	*ori*	Amp^r, *ura3*	EcoRI, BamHI SalI, SmaI	21
YIp32	6.7	*ori*	Amp^r, *leu2*	BamHI, SalI PstI, HindIII	21
YIp33	6.7	*ori*	Amp^r, *leu2*	BamHI, SalI PstI, HindIII	21
YRp7	5.7	*ars1*, *ori*	Amp^r, Tet^r *trp1*	BamHI, SalI	21,199
YRp12	7.0	*ars1*, *ori*	Amp^r, Tet^r *trp1*, *ura3*	BamHI, SalI HindIII	188
pLC544	10.3	*ars1*, *ori*	Amp^r, Tet^r *trp1*	BamHI, SalI	119
pLC1	8.7	*ars2*, *ori*	Tet^r, *arg4*	BamHI, EcoRI	111
pYe(*cen3*) 41	9.2	*ars1*, *cen3* *ori*	*leu2*	BamHI, HindIII SalI	41

Table 1 (cont'd)

Vectors	Size (kb)	Stabilizing[a] elements	Selective markers	Cloning sites	References or sources
YEp6	7.9	2μ, *ori*	Amp^r, *his3*	EcoRI, XhoI SalI	21
YEp13	10.7	2μ, *ori*	Amp^r, Tet^r *leu2*	BamHI, PvuII SstI	27
YEp16	10.7	2μ, *ori*	Amp^r, Tet^r *leu2*	BamHI, PvuII SstI, SalI	J. Hicks
YEp20	10.4	2μ, *ori*	Amp^r, *leu2*	BamHI, SalI PstI	21
YEp21	8.8	2μ, *ori*	Amp^r, *leu2*	BamHI, SalI	21
YEp24	7.6	2μ, *ori*	Amp^r, *ura3*	BamHI, SalI SmaI	21
pJDB207	6.9	2μ, *ori*	Amp^r, Tet^r *leu2*	BamHI, SalI PstI, HindIII	13,14
pJDB110	6.7	2μ, *ori*	Amp^r, *ura3*	BamHI, SalI EcoRI	13,14
pJDB210	7.9	2μ, *ori*	Amp^r, *leu2* *ura3*	BamI, SalI	13,14
pJDB211	7.9	2μ, *ori*	Amp^r, *leu2* *ura3*	BamHI, SalI	13,14
pDB248	10.1	2μ, *ori*	Amp^r, Tet^r *leu2*	BamHI, HindIII PstI	9
pFL 2	7.6	2μ, *ori*	Amp^r, Tet^r *ura3*	BamHI	37
pEYlurl	9.0	2μ, *ori*	Amp^r, Tet^r *ura1*	BamHI, SalI	141
pYT14-*kan5*	13.9	2μ, *ori*	Cam^r, Kan^r Tet^r	BamHI, SalI	J. Marmur
pDB262	9.8	2μ, *ori*	*leu2*	BamHI	D. Beach & P. Nurse

Table 1 (cont'd)

Vectors	Size (kb)	Stabilizing[a] elements	Selective markers	Cloning sites	References or sources
pYc1[b]	10.0	2μ, *ori*	Amp^r, *his3*	BamHI, EcoRI	100
pBT1-1[b]	11.1	2μ, *ori*	Amp^r, Tet^r *leu2*	BamHI	149
pBT1-10[b]	7.7	*ars1*, *ori*	Tet^r *trp1*	BamHI	149

a. DNA sequences capable of conferring mitotic stability on autonomously replicating vector in yeast (e.g., 2 μ, *ars* or *cen* 3) or *E. coli* (*ori*).
b. Cosmid.

Autonomously replicating vectors

(I) 2μ plasmid vector

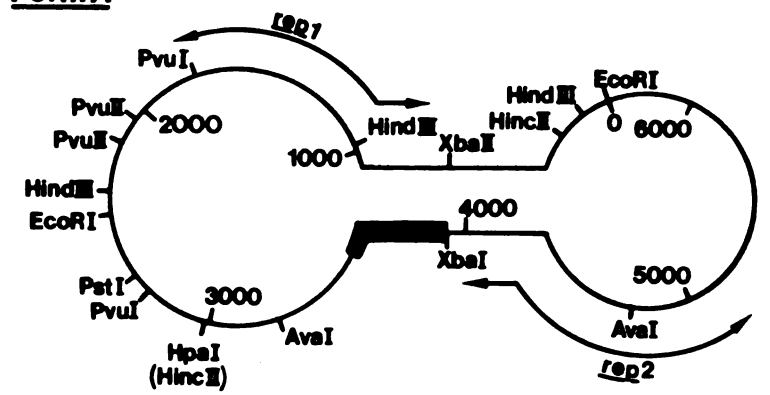
(i) Properties of 2μ DNA. The yeast 2μ plasmid, sometimes referred to as S. cerevisiae plasmid (Scpl), is a circular, double-stranded-DNA molecule of about 6.3 kb present in most S. cerevisiae strains at 50-100 copies per a diploid cell (43). In yeast species other than Saccharomyces and S. pombe 2μ DNA has not been found (86,219). The intracellular location of the plasmid is not clear, although many indirect arguments suggest that its actual location within the cell is in the nucleoplasm. The fact that autonomously replicating hybrid plasmid, consisting of a 2μ DNA and a yeast gene, can recombine with the homologous chromosomal DNA (12,62,78), that it is packaged into nucleosomes containing the normal composition of core histones (136,155), that it is extensively transcribed, presumably by RNA polymerase II (24), and that its replication is under the control of nuclear DNA (137,174), all support the idea that 2μ DNA resides in the nucleoplasm. The evidence against a nuclear location of 2μ DNA is that nuclei isolated by conventional methods contain less than 5-10% of the total cellular plasmids (43). However, it has recently been reported that using different lysis procedures, 90% of 2μ DNAs are associated with the nuclear matrix (216). Thus, it seems likely that the plasmid is associated with the nucleus, but the nature of this association is not known.

The function of the 2μ plasmid in yeast is not known, but its structure is well defined (23,25,26). The complete nucleotide sequence of this plasmid has been determined by Hartley and Donelson (88). The restriction map of 2μ DNA is depicted in Fig. 3. The plasmid consists of 2 unique segments of DNA (2774 bp and 2346 bp) separated by two copies of a 599bp sequence which are precise inverted repeats of each other. Reciprocal intramolecular recombination between the two inverted repeated sequences produces two forms of plasmid, form A and form B, that differ in the orientation of one unique region with respect to the other (23,25). At least two physical variants of the 2 μ DNA (Scp. 2 and Scp 3) have been reported as natural isolates from various strains of S. cerevisiae (30,135). Restriction analysis Scp 2 showed a 125 bp deletion, which removes HpaI restriction site in the large unique region (2774bp segment; Fig. 3). Scp 3 variant contain a 220 bp deletion, which removes both HpaI and AvaI restriction site, in the large unique region. Since the deletions in Scp 2 and Scp 3 are located at a site where a series of tandem repeats are present in 2μ DNA, it seems likely that these variants arose by equal or unequal intermolecular recombination at these repeats (23). The sizes of deletions in Scp 2 and Scp 3 are consistent with the removal of 2 and 4 copies of these repeat sequences, respectively.

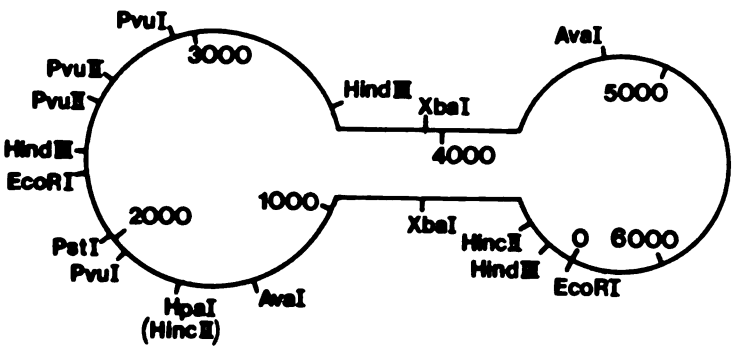
The replication of 2μ DNA is responsive to the

Fig. 3. Physical maps of the two forms of the 2μ DNA. The approximate location of the origin of replication of the 2μ circle is indicated by the heavy line in the schematic diagram of Form A. The regions rep1 and rep2 encode proteins that promote high copy levels of 2μ circle plasmid. (Adapted from reference 23).

Form A


rep1
PvuI
PvuII
PvuII
HindIII
EcoRI
PstI
PvuI
HpaI
(HincII)
2000
3000
AvaI
1000
HindIII
XbaII
HincII
EcoRI
0
6000
4000
XbaI
5000
AvaI
rep2

Form B


PvuI
PvuII
PvuII
HindIII
EcoRI
PstI
PvuI
HpaI
(HincII)
3000
2000
AvaI
HindIII
XbaI
4000
1000
XbaI
HincII
HindIII
0
6000
EcoRI
AvaI
5000

cellular control mechanism that limits chromosomal DNA replication to one round per cell division (137,175). The 2μ plasmid was shown by electron microscopy to have an active DNA replication origin (32,125). Using high frequency transformation of yeast as a criterion for origin function, Broach and coworkers (25,26) have identified a single 350 bp region of the 2μ plasmid which contains the replication origin. The region lies predominantly within one inverted repeat region but extending approximately 100 bp into the continuous large unique region (26).

(ii)Transformation with 2μ plasmid vectors. Yeast-E. coli chimeric plasmids, such as YEp containing part or all of the 2μ DNA can transform yeast cells with frequencies as high as 10^3-10^5 transformants/μg of DNA (12,13,110). Southern hybridization analysis clearly showed the presence of copies of the transforming plamid and genetic analysis revealed a 4^+: 0^- segregation pattern indicative of plasmid-mediated inheritance (92). These results reflected that the 2μ plasmid vector can autonomously replicate in host yeasts.

The transformation efficiency and mitotic stability of the 2μ DNA chimeric vector varies depending on which part of the 2μ sequence is carried by the vector and the presence of endogeneous 2μ DNA in the recipient yeast strain (26,92). The replication origin of 2μ DNA is required for the replication of the yeast -E. coli chimeric plasmid in recipient yeast. Plasmids containing the entire

2μ sequence transform both Cir^+ and Cir^o (2μ DNA-free) strains with high efficiency and the transformed phenotype is relatively stable (18,26,61). The 3.9 kb and 2.2 kb EcoRI fragments, from 2μ DNA forms A and B, respectively, contain the replication origin of 2 μ DNA (Fig. 3). Each of these fragments confers on the hybrid plasmid high efficiency of transformation and stable maintenance in a Cir^+ strain of S. cerevisiae. In contrast, plasmids carrying the 2.4 kb or the 4.1 kb EcoRI fragments (derived from 2 μ DNA form A and B, respectively) which do not contain the replication origin of 2μ DNA, have a relatively high transformation efficiency but are not stably maintained (26). However, if the recipient yeast is a Cir^o strain, the hybrid plasmid carrying the 2.2 kb or the 3.9 kb EcoRI fragment is not stably maintained in recipient yeast and those carrying the 2.4 kb or 4.1 kb EcoRI fragment fail to yield any transformants. These results indicated that efficient replication and maintenance of 2 μ chimeric vector in host yeast requires, in addition to the replication origin, the DNA regions rep1, rep2 and rep3 which almost span the entire 2 μ DNA and are implicated in copy number control. Two of these (rep1 and rep2) code for proteins that are active in trans (23) and a third locus (rep3) that is required in cis (G. Tschumper, personal communication). Under normal conditions, replication of 2 μ circles is strictly under cell cycle controls that limit the replication of 2μ DNA to one round per cell division

(23). Apparently rep proteins act to override cell-cycle control of 2μ DNA replication and to induce multiple rounds of replication during a single cell-division cycle.

Yeast-E. coli hybrid plasmids containing the whole 2μ DNA can transform both Cir^{o} and Cir^{+} strains of S. cerevisiae. However, many of these plasmids are relatively large and have too many restriction enzyme recognition sites to serve as convenient cloning vectors. Therefore, a number of smaller and more useful 2μ plasmid vectors (Table 1) which contain only the 2.2 kb EcoRI fragment or a similar fragment of 2μ DNA, are capable of high frequency transformation of yeast and have a high copy number in host yeast have been constructed. These plasmids which contain replication origin of the 2 μ DNA but do not have all three rep sequences have mostly been used to transform yeast strains containing endogeneous 2μ DNA which supplies the rep gene functions. These types of plasmids, however, are associated with two types of instability in Cir^{+} cells. First, they frequently recombine with endogeneous 2μ DNA and subsequently rearrange their DNA sequence (62,149). Secondly, under nonselective conditions, the endogenous 2μ DNA is, in most cases, retained at the expense of the transforming plasmid because the latter uses the same replication and transmission system as endogenous 2μ DNA (106).

Beggs (13) reported that the mitotic stability of the 2μ plasmid vector is associated with its copy number.

Plasmid pJDB207 has a copy number of about 50 in the transformed yeast whereas the copy number of pJDB110 is 5- to 6-fold lower than that of pJDB207. After propagation of the transformants for 20 generations on nonselective medium, 85% of the cells retained the pJDB207 while only 10-50% of cells contained pJDB110. This may be due to the fact that recombinant plasmids maintained at a high copy number per cell are segregated out less frequently than those with a low copy number. Endogenous 2μ DNA has a copy number of 50-100 and may be able to outcompete cloning vectors containing 2μ DNA sequences in a population of transformed cells under nonselective conditions. Therefore, Cir^{o} strains of S. cerevisiae are more desirable as recipients in gene cloning experiments if a useful 2μ plasmid vector which contains the replication origin and rep 1 and rep 2 sequences is available.

(II) ars vectors

(i) Properties of ars. Yeast transformation studies with recombinant DNA plasmids have identified a class of DNA sequences which promote high-frequency transformation and extrachromosomal maintenance of plasmid DNAs (10,34,200). These sequences are called autonomous replication sequences (arss) and their properties are believed to be due to their ability to serve as initiation sites for DNA replication (32). arss capable of high-frequency transformations have been reported to occur once in 30-40 kb in the S. cerevisiae (199) genome. The

frequency of one replication origin per 36 kb was independently confirmed by the average spacing of initiation sites in S. cerevisiae as detected by electron microscopy in small molecules of yeast DNA (156,157). The coincident frequency between the ars and replication origin in S. cerevisiae strongly suggest that the ars sequences isolated by high-frequency transformation contain the origins of replication of S. cerevisiae. Recently, Celniker and Campbell (32) developed an in vitro system for the replication of both 2μ circle and ars-containing plasmids. They found that only plasmids containing a functional yeast ars initiate replication at a specific site within the ars region in vitro. These data supported the view that ars sequences contain specific origins of chromosomal replication in yeast.

The DNA sequences of ars1, ars2 and ars3 of S cerevisiae have been studied in detail (198,221,222). Extensive homologies have not been observed among these three arss and 2μ DNA (198,222). A canonical shared sequence, TAAPyAPyAAPu, is present in ars1, ars3 and 2μ DNA but absent in ars2. Lack of sequence homology between the arss of S. cerevisiae was also indicated by their respective restriction enzyme maps. The essential region of the S. cerevisiae ars1 DNA resides at or near a PstI site (221,222), whereas a similar site is lacking in ars2 (198). These data indicate that the DNA sequences corresponding to ars function are very diverse. This

observation is in striking contrast to the ori sequences of different bacterial species which were shown to have remarkable sequence homologies (236).

Non-yeast DNA can allow autonomous replication of an integrating plasmid in S. cerevisiae. Stinchcomb et al. (200) found that DNA fragments from a wide variety of eukaryotes (Neurospora crassa, Dictyostelium discoideum, Caenorhabditis elegans, Drosophila melanogaster and Zea mays) are capable of conferring on yeast integrating plasmid (YIp) the ability to replicate autonomously in S. cerevisiae. The replication origins from both the DNA of Tetrahymena thermophila (121) and the mitochondrial DNA of Xenopus laevis (232) also promote high-frequency transformation and extra-chromosomal replication of the YIp plasmid. In addition to the DNA sequences from eukaryotes, a Staphylococcus aureus plasmid was found to act as an ars in S. cerevisiae (80). This evidence suggests that the ars sequences possess broad host-range specificity.

(ii)Transformation with ars vectors. Plasmids containing ars sequences, such as YRp, have been shown to be capable of high frequency transformation of yeast (10^3-10^4 transformants/ µg DNA) and were shown to be unstable during both mitotic and meiotic divisions of yeast transformants (110,119,211). In general, when yeast transformants are propagated in nonselective medium for 20 generations, 95-99% of the cells lose their selective phenotype and this in turn is associated with the loss of the entire hybrid

transforming plasmid. Kingsman et al. (119) grew the ars-vector transformants in selective medium and found that only 30-50% of the cells in transformed clones retain the selective phenotype.

The mitotic stability of the ars-vector appears to be affected by its size. Zakian and Scott (234) constructed a novel 1.45 kb yeast plasmid trp1 RI circle which contains ars plus trp1 and consists solely of yeast chromosomal DNA. The trp1 RI circle occurs at 100 to 200 copies/cell (57,234) and was shown to be stable during both the mitotic and the meiotic cell cycles. In contrast, YRp 7 plasmid which contains the 1.45 kb trp1 RI circle plus pBR322, has a relatively low copy number (20-30/cell) and is unstable. It is unlikely, however, that the stability of the trp1 RI circle can be attributed solely to its high copy number. For example, plasmid pXEY26 which is relatively unstable, similar to YRp 7 (232), is found at 200 copies per cell in some yeast strains (233). The mitotic instability of pXEY26, inspite of its high copy number suggests that ars-containing hybrid plasmids segregate nonrandomly. It is unlikely that the pBR322 sequence is responsible for the instability of YRp7. Stinchcomb et al. (198) constructed another vector, consisting only of yeast sequences, by circularizing an 8.0 kb XhoI/SalI fragment of S. cerevisiae chromosomal DNA carrying trp1 and ars1 (Sc4128), this fragment, when transformed into yeast, was also shown to be mitotically unstable. Therefore, the reasons for the

mitotic instability of YRp 7 are not clear. The stability of trp1 RI circle is on the other hand is most likely due to its small size which may permit it to escape the compartmentalization that causes asymmetric segregation of ars vectors.

The stability of ars vectors in transformed yeast can be enhanced by the addition of a cloned yeast DNA fragment containing a functional centromere (33,40,41,72,73). When the transformants are propagated in nonselective medium, 60-100% of the cells retain the centromere (cen) - containing ars vectors. The range of stability values obtained for the different cen-containing plasmids is not correlated with the size of the yeast DNA insert. For example, Fitzgerald-Hayes et al. (73) reported a similar range of mitotic stabilities for plasmids containing fragments of DNA subcloned from the yeast insert in pYe (met14) 2 (5.2 kb insert), pYe (cen11) 12 (1.6 kb insert) and pYe (cen11) 5 (0.9 kb insert). Thus, the factors which determine the stability of the ars - cen vectors are still unclear.

Hybrid plasmids containing centromeric DNA sequences in combination with a yeast ars exhibit typical Mendelian segregation ($2^+ : 2^-$) through meiosis (33,40,41,72,111). No specific class of chromosomal replicators appears to be required for proper functioning of the cen - containing mini-chromosomes. Hsiao and Carbon (111) found that the replicator function in a plasmid containing cen3 can be

supplied equally well by ars1, ars2 or even 2μ DNA. The ars - cen vectors allow the stable introduction of a single gene into a yeast cell and, therefore, may be an useful and reliable gene cloning vectors, especially where a low copy number is not a problem and high genetic stability is required.

Integrating vectors

(I) Circular vectors

A yeast integrating vector essentially consists of a bacterial cloning vector and a suitable yeast gene which can be used in the selection of yeast transformants and which provides homology to promote integration of the plasmid DNA into the chromosomal DNA of the recipient yeast cell (Table 1). Yeast transformation occurs as a result of the integration of the entire plasmid into the yeast genome. Such integrations can be confirmed by tetrad analysis in which the transformed gene shows Mendelian segregation patterns of $2^+:2^-$. Since the transformation process with an integrating vector includes the step of DNA integration (48,92,95,193,235), the efficiency of transformation is relatively low (1-10 yeast transformants per microgram of DNA; 98) and the transformed phenotype is relatively stable. After 20 generations of growth under nonselective conditions approximately 99% of the cells retain the selective marker (13). In the first transformation experiments described by Hinnen et al. (95), they

found that the LEU plasmid pYe leu10 occasionally integrated at a genomic site other than at the homologous leu2 region. Subsequently it was found that a subcloned fragment of pYe leu10 contains a repeated sequence (120). This subcloned plasmid integrated at the dispersed copies of the repeated sequence as well as at the leu2 locus (95). Thus, all integrations appear to result from recombination between homologous sequences. In about one - third of the transformants, yeast sequences on the plasmid are substituted for the chromosomal sequences without integration of the entire plasmid (95). The transformants with these substitution may arise either via a gene conversion or by a double - crossover event.

A model (Fig 4) for integration of a circular plasmid has been proposed by Orr-Weaver et al. (166). The pleiotropic recombination and repair mutation rad52-1 (140,178) does not block the integration of circular vectors. This suggests that repair synthesis is not required for the integration of circular plasmids. Strand invsion is initiated from a nick on either chromosome or plasmid DNA (166). In this case, it is not necessary to enlarge the resulting D-loop by repair synthesis and strand displacement (Fig. 4). If the D-loop is cut, a short region of symmetric heteroduplex can form, terminated by a classical Holliday junction (108). Resolution of the Holliday structure after isomerization can yield the reciprocally recombined form with an integrated plasmid.

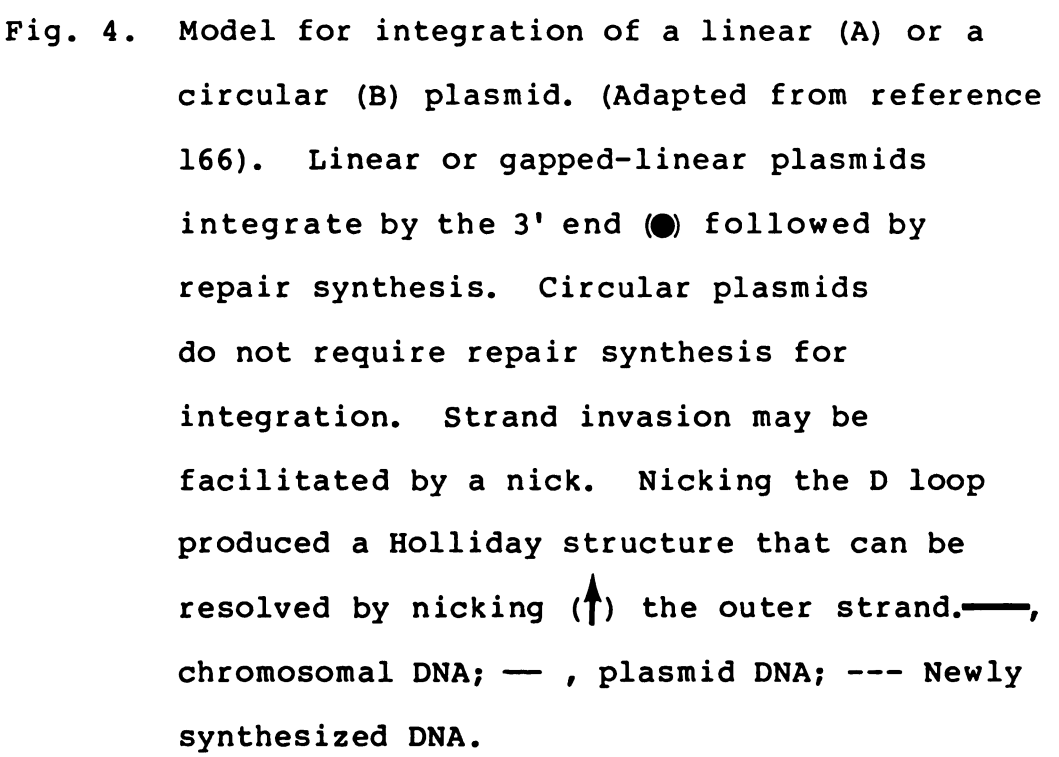
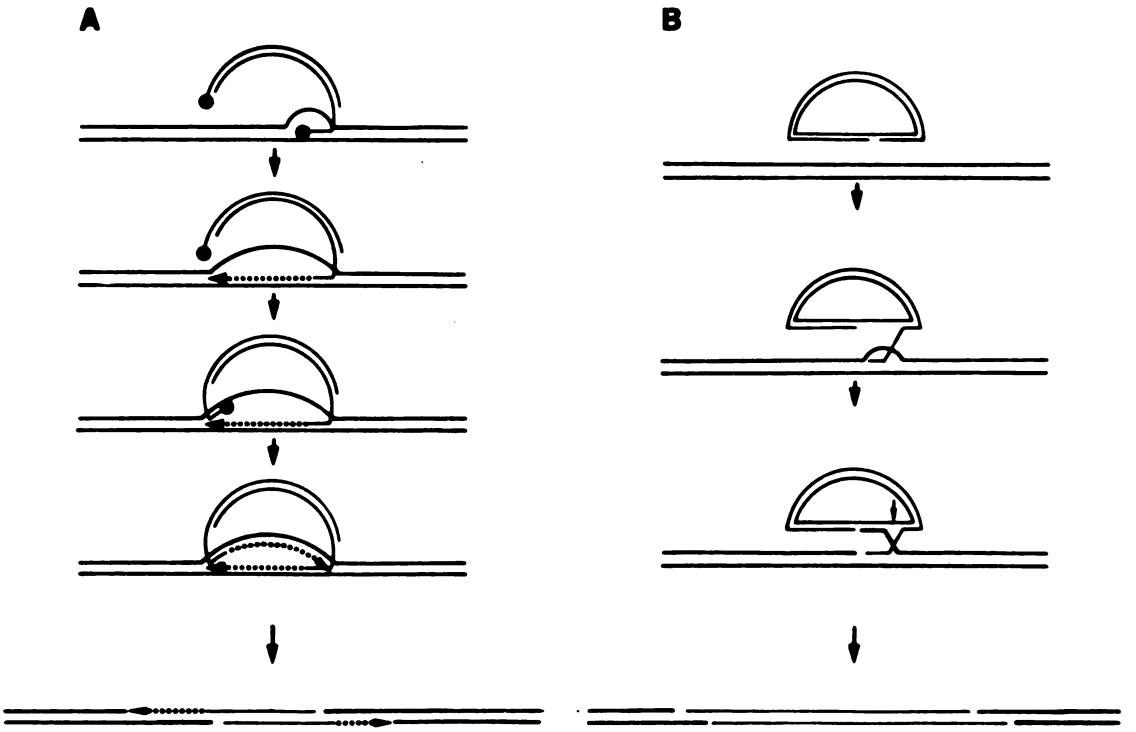


Fig. 4. Model for integration of a linear (A) or a circular (B) plasmid. (Adapted from reference 166). Linear or gapped-linear plasmids integrate by the 3' end (●) followed by repair synthesis. Circular plasmids do not require repair synthesis for integration. Strand invasion may be facilitated by a nick. Nicking the D loop produced a Holliday structure that can be resolved by nicking (↑) the outer strand. ▬▬, chromosomal DNA; — , plasmid DNA; --- Newly synthesized DNA.


A
B

To recover the integrated vector from yeast transformants, it is necessary to digest the yeast DNA with a restriction enzyme which is known to cut the plasmid DNA only once within or at the boundary of the yeast DNA insert. The mixture of DNA fragments is ligated at low DNA concentrations and the re-circularized plasmid is recovered by transformation of E. coli cells, selecting for a marker on the bacterial portion of the plasmid (166).

(II) Linear and gapped vector

Restriction enzyme digestion of a cloning vector within the yeast region which is homologous to yeast chromosomal DNA is known to greatly enhance the efficiency of integration. Hicks et al. (92) found that digestion of pYe leu10 in the yeast DNA sequence with a restriction enzyme resulted in a 5-to 20-fold increase in the efficiency of transformation, compared with the uncut plasmid. The nature of the cut (flush or sticky ends) does not appear to affect this stimulation (167). However, the increase in transformation frequency varies with the fragment. For example, Orr-Weaver et al. (166,167) observed a 2000-3000 fold stimulation of frequency when transforming linear plasmid contained a double - strand break in the his3 fragment, while a plasmid cut in the leu2 fragment showed only a 50-100 fold stimulation. If two restriction cuts are made within a region on the plasmid, homologous to chromosomal DNA, thereby removing an internal segment of DNA, the resulting deleted - linear molecules

(gapped plasmids) are still able to transform at a high frequency (166,167). Surprisingly, the gap is faithfully repaired from chromosomal information during the integration event (166). The requirement for the DNA repair system is suggested by the fact that the integration of linear and gapped-linear plasmids is blocked by the rad 52-1 mutation (167). A model for integration of gapped plasmids was also proposed by Orr-Weaver et al. and is shown in Fig. 4B. In this model, the two ends of a linear plasmid act cooperatively during integration; the repair synthesis initiated after strand invasion of the first end results in more efficient pairing of the second end. Homology must be present on both ends of the double - strand break to permit a recombination event. Hybrid plasmids that are made linear by cleavage within the bacterial portion do not increase the transformation frequency (92). Cuts on the junction give lower stimulation and result in an increased proportion of substitution events (166). Complex plasmids that contain more than one fragment of yeast DNA can be directed to integrate at one specific site by making a double - strand break in the corresponding region on the plasmid (166,167). The ability to direct a plasmid to integrate allows placement of an easily scored marker adjacent to a nonselectable locus to permit genetic mapping.

Cosmids

Cosmids are cloning vectors derived from plasmids which also contain the λ phage DNA cohesive end (cos). These vectors which were first developed by Collins and Hohn (47), are specifically designed for cloning large fragments (30 to 45 kb) of eukaryotic DNA. This cloning technique was adapted for yeast by Hohn and Hinnen (100). The yeast cosmid vector (Table 1) is a true hybrid between a yeast YEp or YRp vector and a cosmid (149). The presence of the cos site of bacteriophage allows this new plasmid to be packaged in vitro and thereby adds all of the advantages of the cosmid cloning technique (141) to the yeast cloning vector. Hinnen and Meyhack (96) had constructed a yeast EcoRI genome bank utilizing the yeast cosmid pYcl and successfully isolated two linked acid phosphatase structural genes by complementation in yeast. It is interesting to note that the pYcl hybrids, which are of high molecular weight (40 to 50 kb), show, on a molar basis, only a 50% reduction in transformation frequency if compared to pYcl or YEp6 (100). This is in striking contrast to the strong dependence on plasmid size of E. coli transformation frequencies (46,47). The recovery of hybrid cosmid DNA from transformed yeast does not seem to be easy. Both the packaging and the transformation efficiencies varied from one clone to the other (100). This result can be explained by the fact that the transformation efficiency in E. coli is dependent on plasmid size and that certain structural

properties of the DNA are known to affect the packaging efficiency (101).

Isolation and Expression of Cloned Genes in Yeast

Isolation techniques

The important breakthrough in the cloning of yeast genes is based on the selection of yeast sequences that complement a mutations in *E. coli*. Struhl, Cameron and Davis (206) constructed a pool of λ-yeast hybrid phage. This phage was used to infect a *his* B (imidazole glycerol phosphate (IGp) dehydratase) negative *E. coli* strain which was then His^+ phenotype were selected. The His^+ lysogen contained λ-yeast hybrids containing a unique yeast DNA fragment that was subsequently shown to contain the yeast *his*3 gene (IGp dehydratase). Several other yeast genes have been isolated by selecting for complementation of the corresponding bacterial mutation with a success ratio of about 25 percent (39).

The gene in yeast shotgun pools can be identified by screening or select for cloned sequences that will complement a mutation in yeast. The generality of this method was rapidly established following the development of autonomously replicating vectors which allow transformation of yeast at a high enough frequency to make possible the direct selection of individual genes out of total shotgun pools. Although the first application of complementation cloning was only published in 1978 (12,95), it has become

one of the most widely used methods for the cloning of most mutationally defined yeast genes as shown in Table 2.

Observation that a cloned sequence which complements a genetic defect in yeast or an analogous defect in E. coli is not sufficient to conclude that the clone contains the desired gene and additional supporting evidence is often needed. In the case of the leu2 gene, supporting evidence was obtained by physically mapping the complementing fragment to chromosome III. Hicks and Fink (91) found that the intensity of hybridization of the putative leu2 probe to DNA from haploid, diploid, chromosome III disomic and chromosome III monosomic (2n-1) strains was proportional to the copy number of chromosome III. Transformation of yeast by a plasmid capable of stable chromosomal integration with homologous DNA sequences, allowed a more stringent identification of desired gene in the clone (95,151). Nasmyth and Reed (151) showed that plasmids responsible for complementing the cdc 28^{ts} phenotype recombine specifically with the chromosomal cdc 28 locus, suggesting that these plasmids do, in fact, contain the cdc 28 gene.

It is also possible to isolate homologous mRNA sequence for the cloned genes (231). Such mRNAs should be translatable in vitro, directing the synthesis of the corresponding polypeptides, which can then be characterized (70,74). Fried and Warner (74) cloned a yeast gene for trichodermin resistance and the ribosomal protein L3 and

Table 2. Some Cloned yeast genes

Gene cloned	Gene product	Phenotype	Method of isolation	References
act1	actin	lethal	DNA-DNA hybridization	78,79,161
adc1	alcohol dehydrogenaseI	ally-alcohol-resistant; antimycin-A-sensitive on glucose	complementation in yeast	228
adr2	alcohol dehydrogenaseII	allyl-alcohol-resistant on glycerol	complementation in yeast	230
arg3[a]	ornithine carbamyl-transferase	arginine auxotrophy	complementation in yeast	49
arg4	arginino-succinate lyase	arginine auxotrophy	complementation in E. coli	39,110
can1	arginine permease	resistant to canavanine	complementation in yeast	27
car1	arginase	unable to use arginine as sole N source	complementation in yeast	212
cdc10	unknown	temperature-sensitive cell cycle arrest	complementation in yeast	41
cdc28	unknown	temperature-sensitive cell cycle arrest	complementation in yeast	154
cyc1	iso-1-cytochrome c	chloroacetate-resistant	DNA-DNA hybridization	20,148
cyc7	iso-2-cytochrome c	deficient in iso-2-cytochrome c	DNA-DNA hybridization	147

Table 2. (cant'd)

Gene cloned	Gene product	Phenotype	Method of isolation	References
gal1	galactokinase	unable to ferment galactose	DNA-DNA hybridization	201
gal4	transcriptional activator of *gal1*, *gal2*, *gal7* *gal10*, and *mel1*	unable to ferment galactose	complementation in yeast	116,133
gal7	galactose-1-phosphate uridyl -transferase	unable to ferment galactose	cDNA-DNA hybridization	201
gal10	UDP-glucose -4-epimerase	unable to ferment galactose	cDNA-DNA hybridization	201
gap	glyceraldehyde -3-phosphate dehydrogenase	Unable to grow on glucose	cDNA-DNA hybridization	102,103,104
his3	imidazole glycerol phosphate dehydratase	histidine auxotrophy	complementation in *E. coli*	179,206,207 208
$his^4{}_{ABC}$	phosphoribosyl -AMP cyclohydrolase phosphoribosyl -ATP pyrophosphorylase, histidinol dehydrogenase	histidine auxotrophy	complementation in yeast	94,180,181
HMLa	unknown		DNA-DNA hybridization	202
HML	unknown		DNA-DNA hybridization	93,152,202
HMRa	unknown		DNA-DNA hybridization	152,202

Table 2. (cont'd)

Gene cloned	Gene product	Phenotype	Method of isolation	References
HMR	unknown		DNA-DNA hybridization	202
hta1	histone H2A	lethal in an hta2 background	mRNA-DNA hybridization/ hybrid-selected mRNA translation	90
htb1	histone H2B	lethal in an htb2 background	mRNA-DNA hybridization/ hybrid-selected mRNA translation	90
rp	ribosomal proteins		mRNA-DNA hybridization/ hybrid-selected mRNA translation	19
ilv1	threonine deaminase	isoleucine auxotrophy	complementation in yeast	171
leu2	isopropylmalate dehydrogenase	leucine auxotrophy	complementation in E. coli	12,38,91 179
MATa	unknown		DNA-DNA hybridization	152,202
MAT	unknown		DNA-DNA hybridization	152,202
pgk1	3-phospho-glycerate kinase	glucose nonfermenter; able to grow on ethanol or glycerol	antigen production in E. coli	98
rad3	unknown	UV-and MM5 sensitive	complementation in yeast	153
rna	rRNA	defective in biosynthesis of rRNA	rRNA-DNA hybridization	71,128,129 177,195,223

Table 2. (cont'd)

Gene cloned	Gene product	Phenotype	Method of isolation	References
suf2	$tRNA^{Pro}$	frameshift suppressor (group III)	complementation in yeast	50,51
sup2	$tRNA^{Tyr}$	nonsense suppressor	tRNA-DNA hybridization	164,165
sup3	$tRNA^{Tyr}$	nonsense suppressor	tRNA-DNA hybridization	164,165,186
sup4	$tRNA^{Tyr}$	nonsense suppressor	tRNA-DNA hybridization	55,75,79 127,132,164 165
sup5	$tRNA^{Tyr}$	nonsense suppressor	tRNA-DNA hybridization	164,165
sup6	$tRNA^{Tyr}$	nonsense suppressor	tRNA-DNA hybridization	164,165
sup8	$tRNA^{Tyr}$	nonsense suppressor	tRNA-DNA hybridization	55,79,144
sup11	$tRNA^{Tyr}$	nonsense suppressor	tRNA-DNA hybridization	79,164,165
sup-RL1	$tRNA^{Ser}$	nonsense suppressor	tRNA-DNA hybridization	163
sug5	$tRNA^{Ser}$	nonsense suppressor	tRNA-DNA hybridization	163
?	$tRNA^{Leu}$	nonsense suppressor	tRNA-DNA hybridization	226
?	$tRNA^{Met}$	nonsense suppressor	tRNA-DNA hybridization	162
?	$tRNA^{Phe}$	nonsense suppressor	tRNA-DNA hybridization	224
tmp1	thymidylate synthetase	thymidylate auxotrophy	complementation in yeast	218

Table 2. (cont'd)

Gene cloned	Gene product	Phenotype	Method of isolation	References
*trp*1	phosphoribosyl-anthranilate isomerase	trptophan auxotrophy	complementation in *E. coli*	199,211
*trp*2	anthranilate synthatase	trptophan auxotrophy	complementation in yeast	2
*trp*3	indole-3-glycer-olphosphate synthetase	trptophan auxotrophy	complementation in yeast	2,168
*trp*5	tryptophan synthetase	trptophan auxotrophy	complementation in *E. coli*	31,227
*tyr*1	prephenate dehydrogenase	tyrosine auxotrophy	complementation in yeast	31,227
*ura*1[a]	dihydroorotate dehydrogenase	uracil auxotrophy	complementation in *E. coli*	138,139
*ura*2	carbamyl-phosphate synthetase asparate transcarbamylase	uracil auxotrophy	complementation in *E. coli* and in yeast	197
*ura*3[a]	orotidine-5-phosphate decarboxylase	uracil auxotrophy	complementation in *E. coli*	6

[a]Gene whose expression requires vector promoter

identified the gene by R loop-positive translation.

The use of antibody to detect clones carrying yeast genes that are not selectable in E. coli or that cannot function in E. coli has been described by clarke et al. (42). For example, a gene which codes for one subunit of complex enzyme can be detected by this way (97). The yeast pgk gene, coding for 3-phophoglycerate kinase, was cloned with this method (42,98). Since this method depends on a certain degree of expression of the cloned genes, the bacterial vectors used are always designed to maximize the synthesis of the proteins that are encoded by the cloned gene. This method should prove quite useful for the cloning of genes for which there are no available mutants and for which the mRNA is more difficult to purify than the proteins encoded by these genes.

Radioactively labeled RNA or DNA can be used to screen for those clones that contain corresponding homologous sequence (Table 1). Ribosomal RNAs (rRNAs) and nonspecific transfer RNA (tRNA) can be fairly readily purified and radioactively labeled for use as probes (11,17). Using such probes, many rRNA genes and tRNA genes were cloned (Table 2). Holland et al (102,103,104) partially purified the yeast mRNAs by size-fractionation, used these to make complementary DNA (cDNA) probes, and were able to identify a plasmid clone containing genes encoding glycolytic enzymes such as glyceraldelyde - 3 -phosphate dehydrogenase. In some cases, it is possible under special

growth conditions to induce the synthesis of specific mRNAs, this helps in synthesizing a specific cDNA as a probe. For example, mRNAs corresponding to the genes gal1, 7 and 10 are induced by growth on galactose but are reduced or absent in cells grown on glucose or acetate (109).

The sequence of a protein allows a prediction of the DNA sequence which codes for it. By using 13-base-pair (148) and 15-base-pair (215) synthetic probes, the genomic sequence coding for iso-1-cytochrome c has been identified from a lambda shotgun pool by plaque screening techniques. The feasibility of using a relatively short synthetic probe to locate genes in genomic DNA depends on the low complexity of the yeast genome. At a complexity of 1.5×10^7 base pairs, the largest random sequence that has 50% chance of occurring in yeast DNA is about 12 (163). On this basis, it was expected that the 13-base-pair synthetic fragment would be a marginal but adequate hybridization probe.

Another type of DNA-DNA hybridization screening is the use of a clone as a probe to identify a gene which is related but not identical to the genes in a previously - isolated clone. This approach has been applied to identify a number of cloned genes such as sup4 (79,131), his3 (207) cyc1, and cyc7 (147) and yeast mating type genes (93,152,202). There have been few efforts to identify cloned yeast genes using the evolutionarily homologous genes from another species as probes. The use of the cloned actin gene from the slime mold Dictyostelium as a

probe for detecting the cloned yeast actin gene is the only report in this area (76,77,158).

Gene expression in yeast

(I) Expression of foreign genes

The expression of a gene involves many steps including gene activation, RNA transcription, RNA processing, translation, protein maturation, and protein transport through membranes (1,52,53). All of these processes are subjected to specific control systems. The important question that is being investigated in this regard is how similar are these control systems in different organism. It apparently that in closely related species and even in the case of some more distantly related species the regulatory sequences for transcription and translation are very similar (52). The process of assembly of a polypeptide into a functionally active enzyme is evidently little subject to the influence of the intracellular environment (35,149,150,160). The mechanism of transport of a secretory proteins such as β- lactamase through membranes appear to be universal to prokaryotic and eukaryotic organisms (81,83,134,182). These commonly shared features suggest the possibility that even some of the most varied genes can be expressed under the control of the regulatory sequences of the recipient cell.

A number of *S. cerevisiae* genes are known to be expressed in *E. coli* (6,39,138,206,207). Similarly some

bacterial genes such as the genes for drug resistance, arising from R-factors, are shown to be expressed in S. cerevisiae. For example, the determinants for ampicillin (105,106), chloramphenicol (105), and kanamycin resistance (114) can be expressed in S. cerevisice. The ompA gene (106) which as the stuctural gene for the major outer membrane protein II, and the lac Z gene (169) which codes for encoding β-galactosidase of E. coli are also functionally expressed in yeast. Cohn et al. (44) reported that transformants containing a chimeric plasmid with a deletion of 120 bp immediately before the beginning of the structural gene of the cam^r determinant had a 50 - fold increase in the acetyltransferase activity in yeast. This same deletion actually reduced expression in E. coli, indicating that DNA sequences in this region function differently in yeast and E. coli. Possibly as a result of the deletion, an effective eukaryotic promoter is created.

Several studies indicated that interspecies and intergeneric expression of genes is possible in yeast, just as in bacteria. Thus, the β-galactosidase structural gene, lac4, from the yeast Kluyveromyces lactis is expressed in S. cerevisiae (59), the leu2 gene and cdc2 gene (cell cycle start gene) of S. cerevisiae function in cells of S. pombe (8) and the maltase structural gene, mal6, of S. carlsbergensis is expressed by transformation in S. cerevisiae (70).

Some genes from higher eukaryotic cells are also known

to be expressed in yeast. For example, when a purine auxotroph of yeast (ade 8) was transformed with a pool of recombinant plasmids containing fragments of the Drosophila genome (89), the recombinant plasmid isolated from an Ade^+ clone showed that it contains a 0.8 kb fragment of Drosophila DNA corresponding to the ade gene that is expressed in yeast. This fragment has no introns and is transcribed in yeast cells from its own promoter. However, the expression of a foreign eukaryotic gene containing introns in yeasts can be problematic as has been demonstrated with the rabbit β-globin gene (15). When cloned into yeast, the rabbit β-globin gene leads to the production of an altered globin protein. This may have been due to the formation of inadequate mRNA which is perhaps attributable to the absence of splicing of the globin mRNA in yeast cells. It is possible that cDNA clones which are under the control of yeast promoter are required for the expression of higher eukaryotic genes in yeast. This is supported by the finding that cDNAs for the avian ovalbumin (145), hepatits B virus surface gene (146) and leukocyte interferon D (99), under the control of a yeast promoter, were successfully expressed in S. cerevisiae. However, it is known that the boundary nucleotides of the intron in the actin gene of S. cerevisiae are the same as in higher organisms (77) and therefore, the possibility that at least some of the pre-mRNAs of higher eukaryotes might be subjected to splicing

in yeast cells can not be ruled out.

(II) Expression of yeast genes

It is frequently suggested that the TATA box is a site of DNA that is recognized specifically by eukaryotic RNA polymerase II (52,53). This suggestion was originally based on the ubiquitous presence of TATA box in front of eukaryotic genes, its relatively constant distance from the start of transcription and its sequence homology with the *E. coli* Pribnow's box. Several lines of evidence suggested that an additional DNA region upstream from the TATA box influences gene expression in *S. cerevisiae*. Struhl (204) found that simple inversion of a DNA fragment simultaneously alters both *trp*1 and *his*3 expression in yeast even though the inversion break point mapped more than 300 bp from either structural gene. A sequence located 112-155 bp upstream from the transcribed region of *his*3 structural gene is necessary for the wild-type expression and the TATA box is not sufficient for wild-type promoter function (203,205,209). An analysis of deletions in the upstream DNA suggests that the sequence required for the efficient transcription initiation of the iso-1-cytochrome (*cyc* 1) gene of *S. cerevisiae* lies within a DNA segment 250-270 bp upstream from the start of the *cyc*1 coding sequence (84). A promoter region of the *gal*10 gene was also identified at more than 130 bp upstream from *gal*10 transcriptional start site (85). Thus, the yeast promotor region appears large when compared with prokaryotic promoters, suggesting that

it may be more complex than being a simple site of interaction between RNA polymerase II and DNA.

The yeast transposable element Tyl has been observed to affect gene expression in S. cerevisiae. The presence of the Tyl element adjacent to the structural gene for cyc1 resulted in a 20-fold increase in expression of this gene (66). Constitutive expression of the glucose-repressible alcohol dehyodrogenase gene is reported to result from the insertion of a Tyl element near its 5' end of the structural gene (229,230). In contrast to these observation, insertion of a Tyl element into the 5' noncoding region of the his4 gene blocks the expression of the his4 - coding region (69). One might imagine that a new promoter and regulatory sequences are created by the insertion of a transposable element near the 5' - flanking region of a gene (189,229).

Gallwitz (76) has found that a functional yeast actin gene cannot be introduced into yeast on a high copy number 2u vector, since a deleterious effect results from high gene dosage. However, it was reported that yeast can use translational control to compensate for the extra copies of a ribosomal protein gene. Pearson et al. (170) have introduced into yeast an autonomously replicating plasmid carrying a trichodermin-resistance gene for ribosomal protein L3. Although the level of trancription of this gene is very high, equivalent to high gene dosage, the synthesis of the ribosomal protein L3 remains nearly equal

to that of the other ribosomal proteins. This balanced synthesis is established by decreasing the lifetime of this specfic mRNA and the efficiency of its translation.

Application of Gene Cloning in Yeast

The development of a cloning system for yeast has facilitated the isolation of several yeast genes which could not be obtained by cloning in E. coli. This technique has opened up an entirely new spectrum of possibilities for the investigation of the structure, expression, organization and mutational events of yeast genes at molecular level.

The construction of a plasmid vector carrying the gene of interest is a powerful tool for studying the regulatory regions of eukaryotic genes. Struhl (204,209) has cloned a fragment containing the his3 gene of S. cerevisiae on a plasmid vector and has deleted DNA sequences adjacent to the 5' end of the mRNA coding region of the his3 gene. The plasmids, after each specific deletion, was reintroduced into yeast cells and analyzed in their native physiological environment. The location of the yeast his3 promoter has been identified by this method (203,204,205). Similiar approach has also been used to study the regulatory region of S. cerevisiae genes gal4 (116), adr2 (16), arg3 (49). The construction of a chimeric gene is another powerful tool for studying the regulatory region of S. cerevisiae genes. Yeast - E. coli shuttle vectors carrying a hybrid gene between the E. coli lacZ gene and a specific gene of

S. cerevisiae (e.g., ura3 gene) have been constructed. When these hybrid vectors are introduced into yeast, they produce β-galactosidase activity, which is regulated in a way essentially identical to the regulation of the intact yeast gene (84,183). Deletion of a portion of the yeast DNA flanking sequence at the 5' end of the mRNA coding region from this plasmid will affect the levels of the β-galactosidase gene expressed in S. cerevisiae. The influence of deletion on the expression of the yeast gene can be followed by monitoring the β-galactosidase activity. S. cerevisiae has no endogenous β-galactosidase activity which would interfere with the assay for the activity encoded by lacZ. Moreover, extremely low levels of β-galactosidase activity can be measured (84). Thus this method has become important for the study of the regulatory regions of S. cerevisiae gene. Recently, the technique of gene fusion was employed for studying the regulatory sequences of the S. cerevisiae ura3 (183), cyc1 (84), his4 (194) and gal 10 (85) genes.

Yeast cloning experiments have also contributed to a greater understanding of the organization of certain regions of the yeast genome such as centromere gene (40,41, 72,73,111), ars sequences (198,199,200,221,222), repeated chromosomal elements (17,29,172,176) and intervening sequences (77,117,159,162,224). All of these will contribute to increase the knowledge of yeast differentiation at a molecular level.

Many cell cycle genes have been cloned in yeast (8,41,151) by complementation of cdc mutants under non-permissive growth conditions. The cdc2 gene of S. pombe, which is required for start of the cycle and for the control of mitosis, has been isolated from a S. pombe bank by complementation of a cdc2 mutation (8). This cdc2 gene and the cdc28 gene of S. cerevisiae do not have extensive homology but both of them perform homologous cell cycle control functions in S. pombe. DNA sequencing will be required to establish the precise relationship between cdc2 and cdc28. Moreover, the identification of the gene products and their functions may help to elucidate the mechanism by which the yeast cell division cycle is regulated.

Integrating plasmids have been used to induce site-specific mutagenesis of various chromosomal loci (187,188). Scherer and Davis (188) have been able to substitute an in vitro mutated gene on a plasmid for a chromosomal gene of a recipient yeast cell by transforming and integrating the plasmid into the corresponding chromosomal locus. Simlarly, linear and gapped plasmids can be employed to rapidly isolate and map the chromosomal mutations. Linear plasmids and gapped plasmids can often be targeted to the desired site by cutting with an appropriate restriction enzyme (166,167). The integration of the plasmid will take place at the site where the DNA ends are homologous. In this manner, an easily selectable marker can be placed

adjacent to a no selectable locus to permit genetic mapping (166). The repair of a gapped plasmid from chromosomal information can facilitate the cloning of mutant alleles from yeast chromosomes.

Although many desirable genes (e.g., the human gene for interferon) are likely to be nonselectable after yeast transformation, they can be introduced into a yeast cell by coupling them to a selectable marker. This can be accomplished *in vitro* by linking the nonselectable sequence to a plasmid (either autonomously replicating or integrating vector) carrying the selectable marker for yeast, or simply by cotransformation of two plasmids, one of which carries a nonselectable marker and the other a selectable marker. Zamir et al. (235) introduced the *nif* genes from *Klebsiella pneumoniae* into yeast by cotransformation with two plasmids. One plasmid contained a marker that was selectable in yeast and provided the necessary sequence homology with the yeast genome, while the second plasmid contained *nif* genes and an antibiotic-resistant determinant common to the first plasmid. Independent recombination events between the yeast sequences and the bacterial antibiotic resistance sequences resulted in integration of the nonhomologous *nif* genes into the yeast genome. Compton et al. (48) extended the above observation and found that multiple copies of the two plasmids, in both tandem and interspersed arrays, are inserted by this method. The introduction of foreign genes into yeast by autonomously

replicating vectors, integrating vectors or cotransformation may enable the use of yeast to produce many types of proteins which normally occur only in other organisms. This approach may be important for the production of industrially important products and for altering the fermentation behavior of the yeast cells.

Conclusion and Outlook

Cloning in S. cerevisiae was described for the first time in 1978 (95). A great deal of progress has been made since then in developing many different types of cloning vectors. Further construction of more versatile cloning vectors with high transformation efficiency, broad host range, and high mitotic stability should lead to the construction of the physical and functional map of the entire yeast genome. This will in turn facilitate the study of such phenomena as DNA replication, recombination, transposition, genetic instability and cell differentiation of eukaryotes at the moleculer level.

Further developments which are particularly important to achieve successful application of the recombinant DNA techniques in yeast are (1) new cloning vectors and mutant host strains which allow stable maintenance of cloned DNA with the desired copy number and (2) artificial transcription units which permit expression of the most varied genes, regardless of the presence of suitable trans-cription and translation signals on the cloned fragment, under

the control of the regulatory sequences of the recipient cell and/or the vector. The development of these methods is undoubtely of great practical value for the production of desired products useful in medicine, agriculture, commerce and industry.

Broad host-range vectors carrying a drug-resistant determinant as a selective marker for yeast transformation are particularly useful in that they can be transferred to and may be stably propagated in yeasts of many genera, and thereby enable the application of powerful recombinant DNA methodologies to a large number of poorly characterized but important yeast genera such as Candida. However, at the present time genetic cloning systems for yeasts are restricted only to Saccharomyces and Schizosaccharomyces, because suitable cloning vectors and selection systems are not available for other yeasts. Thus, the development of a generalized cloning system for wild-type yeast of different genera and species will be an important future goal.

Literature Cited

1. Abelson, J. A. 1979. RNA processing and the intervening sequence problem. Ann. Rev. Biochem. 48:1035-1069.

2. Aebi, M., P. Neiderberger and R. Hutter. 1982. Isolation of the TRP2 and TRP3 gene of Saccharomyces cerevisiae by functional complementation in yeast. Curr. Genet. 5:39-46.

3. Arnold, W.N. 1981. Physical aspects of the yeast cell envelope, p. 5-47. In W.N. Arnold (ed.), Yeast cell envelopes: biochemistry, biophysics, and ultrastructure, Vol.1. CRC Press, Inc., Florida.

4. Arnold, W.N. 1981 Protoplasts, P.:93-103, In W.N. Arnold (ed.), Yeast cell envelopes: biochemistry, biophysics, and ultrastructure. Vol. 2. CRC Press, Inc., Florida.

5. Arnold, W.N., and J.S. Lacy. 1977. Permeability of the cell envelope and osmotic behavior in Saccharomyces cerevisiae. J. Bacteriol. 121: 564-571.

6. Bach, M.L., F. Lacroute, and D. Botstein. 1979. Evidence for transcriptional regulation of orotidine-5'-phosphate decarboxylase in yeast by hybridization of mRNA to the yeast structural gene cloned in Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 76:386-390

7. Bacon, J.S.D., D. Jones, V.C. Farmer, and D.M. Webley. 1968. The occurence of α-(1→3) glucan in Cryptococcus, Schizosaccharomyces and Polyporus species and its hydrolysis by a Streptomyces culture filtrate lysing cell walls of Cryptococcus. Biochim. Biophys. Acta 158:313-315.

8. Beach, D., B. Durkacz, and P. Nurse. 1982. Functionally homologous cell cycle control genes in budding and fission yeast. Nature 300:706-709.

9. Beach, D., and P. Nurse. 1981. High-frequency transformation of the fission yeast Schizosaccharomyces pombe. Nature 290:140-142.

10. Beach, D., M. Piper, and S. Shall. 1980. Isolation of chromosomal origins of replication in yeast. Nature 284:185-187.

11. Beckmann, J.S., P.F. Johnson, and J. Abelson. 1977. Cloning of yeast transfer RNA genes in Escherichia coli. Science 196:205-208.

12. Beggs, J.D. 1978. Transformation of yeast by a replicating hybrid plasmid. Nature 275:104-108.

13. Beggs, J.D. 1981. Gene cloning in yeast. p. 175-203. In R. Williamson (ed.) Genetic engineering, Vol. 2. Academic Press. N.Y.

14. Beggs, J.D. 1981. Multiple-copy yeast plasmid vectors. Alfred Benzon symp. 16:383-395.

15. Beggs, J.D., J. van den Berg, A. van Ooyen, and C. Weissmann. 1980. Abnormal expression of chromosomal rabbit β-globin gene in Saccharomyces cerevisiae. Nature 283:835-840.

16. Beier, D.R., and E.T. Young. 1982. Characterization of a regulatory region upstream of the ADR2 locus of S. cerevisiae. Nature 300:724-728.

17. Bell, G.I.,L.J. DeGennaro, D.H. Gelfand, R.J. Bishop, P. Valenzuela, and W.J. Rutter. 1977. Ribosomal RNA genes of Saccharomyces cerevisiae. 1. Physical map of the repeating unit and location of the regions coding for 5S. 5.8C, 18S, and 25S ribosomal RNAs. J. Biol. Chem. 252:8118-8125.

18. Blanc, H., C. Gerbaud, P. Slonimski, and M. Guerineau. 1979. Stable yeast transformation with chimeric plasmids using a 2µm-circular DNA-less strain as a recipient. Mol. Gen. Genet. 176:335-342.

19. Bollen, G. H.P.M., L.H. Cohen, W.H. Mager, A.W. Klaasen, and R.J. Planta. 1981. Isolation of cloned ribosomal protein genes from the yeast Saccharomyces carlsbergensis. Gene 14:279-287.

20. Boss, J.M., M.D. Darrow, and R.S. Zitomer. 1980. Characterization of yeast iso-1-cytochrome c mRNA. J. Biol. Chem. 255:8623-8628.

21. Botstein, D., S.C. Falco, S.E. Stewart, M. Brennan, S. S. Scherer, D.T. Stinchcomb, K. Struhl, and R.W. Davis. 1979. Sterile host yeast (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene 8:17-24.

22. Breunig, K.D., V. Mackedonski, and C.P. Hollenberg. 1982. Transcription of the bacterial β-lactamase gene in Saccharomyces cerevisiae. Gene 20:1-10.

23. Broach, J.R. 1981. The yeast plasmid 2μ circle, p.445-470. In J.N. Strathern, E.W. Jones, and J.R. Broach (ed.). The molecular biology of the yeast Saccharomyces. I. Life cycle and inheritance. Cold Spring Harbor Laboratory, N.Y.

24. Broach, J.R., J.F. Atkins, C. McGill, and L. Chow. 1979. Identification and mapping of the transcriptional and translational products of the yeast plasmid 2μ circle. cell 16:827-839.

25. Broach, J.R., V.R. Guaracio, M.H. Misiewicz, and J. L. Campbell. 1981. Replication of the yeast plasmid, 2μ circle. Alfred Benzon Symp. 16:31-40.

26. Broach, J.R., and J.B. Hicks. 1980. Replication and recombination functions associated with the yeast plasmid, 2μ circle. Cell 21:501-508.

27. Broach, J.R., J.N. Strathern, and J.B. Hicks. 1979. Transformation in yeast: development of a hybrid cloning vector and isolation of the CAN1 gene. Gene 8:121-133.

28. Bush, D.A., M. Horisberger, I. Horman, and P. Wursch. 1974. The wall structure of Schizosaccharomyces pombe. J. Gen. Microbiol. 81:199-206.

29. Cameron, J.R., E.Y. Loh, and R.W. Davis. 1979. Evidence for transposition of dispersed repetitive DNA families in yeast. Cell 16:739-751.

30. Cameron, J.R., P. Philippson, and R.W. Davis. 1977. Analysis of chromosomal integration and deletions of yeast plasmids. Nucl. Acids Res. 4:1429-1448.

31. Carbon, J., B. Ratzkin, L. Clarke, and D. Richardson. 1978. The expression of cloned eukaryotic DNA in prokaryotes. p. 277-296. In C.W. Anderson (ed.), Genetic interaction and gene transfer. Brookhaven National Laboratory, Upton, N.Y.

32. Celniker, S.E., and J.L. Campbell. 1982. Yeast DNA replication in vitro initiation and elongation events mimic in vivo process. Cell 31:201-213.

33. Chan, C.S.M., G. Maine, and B.K. Tye. 1982. Functional components of the Saccharomyces cerevisiae chromosomes - replication origins and centromeric sequences. ICN-UCLA Symp. Mol. Cell. Biol. 22:455-472.

34. Chan, C.S.M., and B.K. Tye. 1980. Autonomous replicating sequences in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 77:6329-6333.

35. Chang, A., J. Nunberg, R. Kaufman, H. Erlich, R. Schimke, and S. Cohen. 1978. Phenotypic expression in E. coli of a DNA sequence coding for mouse dihydrofolate reductase. Nature 275:617-624.

36. Chevallier, M.R., and M. Aigle. 1979. Qualitative detection of penicillinase produced by yeast strains carrying chimeric yeast-coli plasmids. FEBS Lett. 108:179-180.

37. Chevallier, M.R., J.C. Bloch, and F. Lacroute. 1980. Transcriptional and translational expression of a chimeric bacterial-yeast plasmid in yeast. Gene 11:11-19.

38. Chinault, A.C., and J. Carbon. 1979. Overlap hybridization screening: isolation and characterization of overlapping DNA fragments surrounding the leu 2 gene on yeast chromosome III. Gene 5: 111-126.

39. Clarke, L., and J. Carbon. 1978. Functional expression of cloned yeast DNA in Escherichia coli: specific complementation of argininosuccinate lyase (argH) mutations. J. Mol. Biol. 120:517-532.

40. Clarke, L., and J. Carbon. 1980. Isolation of a yeast centromere and construction of a functional small circular chromosome. Nature 287:504-509.

41. Clarke, L., and J. Carbon. 1980. Isolation of the centromere-linked CDC 10 gene by complementation in yeast. Proc. Natl. Acad. Sci. U.S.A. 77:2173-2177.

42. Clarke, L., R. Hitzeman, and J. Carbon. 1979. Selection of specific clones from colony banks by screening with radioactive antibodies. Methods Enzymol. 68:436-442.

43. Clark-Walker, G.D., and G.L.G. Miklos. 1974. Localization and quantification of circular DNA in yeast. Eur. J. Biochem. 41:359-365.

44. Cohen, J.D., E. Abrams, T.R. Eccleshall, B. Buchferer, and J. Marmur. 1980. Expression of a prokaryotic gene in yeast: isolation and characterization of mutants with increased expression. p. 127. Abstr 10th Int. Conf. Yeast Genet. Mol. Biol. Louvain-ln-Neuve, Belgium.

45. Cohen, J.D., T.R. Eccleshall, R.B. Needleman, H. Federeff, B.A. Buchferer, and J. Marmur. 1980. Functional expression in yeast of the Escherichia coli plasmid gene coding for chloramphenicol acetyltransferase. proc. Natl. Acad. Sci. U.S.A. 77:1078-1082.

46. Collin, J. 1977. Gene cloning with small plasmids. Curr. Top. Microbiol. Immunol. 78:121-170.

47. Collins, J. and B. Hohn. 1978. Cosmid: a type of plasmid gene - cloning vector that is packageable in vitro in bacteriophage head. Proc. Natl. Acad. Sci. U.S.A. 75:4242-4246.

48. Compton, J.L., A. Zamir, and A.A. Szalay. 1982. Insertion of nonhomologous DNA into the yeast genome mediated by homologous recombination with a cotransformation plasmid. Mol. Gen. Genet. 188:44-50.

49. Crabeel, M., F. Messenguy, F. Lacroute, and M. Glansdorff. 1981. Cloning arg3, the gene for ornithine carbamyltransferase from Saccharomyces cerevisiae: expression in Escherichia coli requires secondary mutation; production of plasmid -lactamase in yeast. Proc. Natl. Acad. Sci. U.S.A. 78:5026-5030.

50. Cummins, C.M., and M.R. Culbertson. 1981. Molecular cloning of the SUF2 frameshift suppressor gene from Saccharomyces cerevisiae. Gene 14:263-278.

51. Cummis, C.M., T. F. Donahue, and M.R. Culbertson. 1982. Nucleotide sequence of the SUF2 frameshift suppressor gene of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 79:3565-3569.

52. Danilevskaya, D.N. 1982. Expression of cloned genes in pro- and eukaryotic cells. MOLBBJ 16:355-377.

53. Darnell, J.E. 1982. Variety in the level of gene control in eukarytotic cells. Nature 297:365-371.

54. Davis, R., and P.A. Elvin. 1964. The effect of β-mercaptoethanol on release of invertase and formation of protoplasts of Saccharomyce cerevisiae. Biochem. J. 93:8p.

55. DeRobertis, E.M., and M.V. Olson. 1979. Transcription and processing of cloned yeast tyrosine tRNA genes microinjected into frog oocytes. Nature 278:137-143.

56. Deutch, C.E., and J.M. Perry. 1974. Spheroplast formation in yeast during the transition from exponential phase to stationary phase. J. Gen. Microbiol. 80:259-268.

57. Devenish, R.J., and C.S. Newlon. 1982. Isolation and characterization of yeast ring chromosome III by a method applicable to other circular DNAs. Gene 18:277-288.

58. Devries, D.M.H., and J.G.H. Wessels. 1972. Release of protoplasts from Schizophyllum commune by a lytic enzyme preparation from Trichoderma viride. J. Gen. Microbiol. 73:13-22.

59. Dickinson, R. 1980. Expression of a foreign eukaryotic gene in Saccharomyces cerevisiae: β-galactosidase from Kluyveromyces lactis. Gene 10:347-356.

60. Dickinson, D.P. and I. Isenberg. 1982. Preparation of spheroplasts of Schizosaccharomyces pombe. J. Gen. Microbiol. 128:651-654.

61. Dobson, M., A.B. Futcher, and B.S. Cox. 1980. Control of recombination within and between DNA plasmids of Saccharomyces cerevisiae. Curr. Genet. 2:193-200.

62. Dobson, J.M., A.B. Futcher, and B.S. Cox. 1980. Loss of 2- μ DNA from Saccharomyces cerevisiae transformed with the chimeric plasmid pJDB219. Curr. Genet. 2:201-206.

63. Duffus, J.H., C. Leui, and D.J. Manners. 1982. Yeast cell-wall glucans. Adv. Microbiol. physiol. 23:151-181.

64. Eddy, A.A., and D.H. Williamson. 1957. A method of isolating protoplasts from yeast. Nature 179:1252-1253.

65. Ehrlich, S.D., B. Niaudet, and B. Michel. 1982. Use of plasmid from Staphylococcus aureus for cloning of DNA in Bacillus subtilis. Curr. Top. Microbiol. Immunol. 96:19-29.

66. Errede, B., T.S. Cardillo, F. Sherman, E. Dubois, J. Deschamps, and J.M. Wiame. 1980. Mating signals control expression of mutations resulting from insertion of a transposable repetitive element adjacent to diverse yeast genes. Cell 25:427-436.

67. Falcone, G., and W.J. Nickerson. 1956. Cell-wall mannan-protein of baker's yeast. Science 124:272.

68. Fangman, W.L., and V.A. Zakian. 1981. Genome structure and replication, p. 27-58. In J.N. Strathern, E.W. Jones, and J.R. Broach (ed.). The Molecular biology of the yeast Saccharomyces. I. Life cycle and inheritance. Cold Spring Harbor Laboratory, N.Y.

69. Farabaugh, P.J., and G.R. Fink. 1980. Insertion of the eukaryotic transposable element Tyl creates a 5-base pair duplication. Nature 286:352-356.

70. Federoff, H.J., J.D. Cohen, T.R. Eccleshall, R.B. Needleman, B.A. Buchferer, J. Giacalone, and J. Marmur. 1982. Isolation of a maltase structural gene from Saccharomyces carlsbergensis. J. Bacteriol. 149:1046-1070.

71. Ferguson, J., and R.W. Davis. 1978. A new electron microscopic technique for establishing the position of genes: an analysis of the yeast ribosomal RNA coding region. J. Mol. Biol. 123:417-430.

72. Fitzgerald-Hayes, M., J.M. Buhler, T.G. Cooper, and J. Carbon. 1982. Isolation and subcloning analysis of functional centromere DNA (CEN11) from Saccharomyces cerevisiae chromosome XI. Mol. Cell. Biol. 2:82-87.

73. Fitzgerald-Hayes, M., L. Clarke, and J. Carbon. 1982. Nucleotide sequence comparison and functional analysis of yeast centromere DNAs. Cell 29:235-244.

74. Fried, H.M., and J.R. Warner. 1981. Cloning of yeast gene for trichodermin resistance and ribosomal protein L3. Proc. Natl. Acad. Sci. U.S.A. 78:238-242.

75. Gafner, J., and P. Philippsen. 1980. The yeast transposon Tyl generates duplications of target DNA on insertion. Nature 286:414-418.

76. Gallwitz, D., and R. Seidel. 1980. Molecular cloning of the actin gene from yeast Saccharomyces cerevisiae. Nucl. Acids Res. 8:1043-1059.

77. Gallwitz, D., and I. Sures. 1980. Structure of a split yeast gene: complete nucleotide sequence of the actin gene in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 77:2546-2550.

78. Gerbaud, C., P. Fournier, H. Blanc, M. Aigle, H. Heslot, and M. Guerineau. 1979. High frequency of yeast transformation by plasmids carrying part of entire 2- μ yeast plasmid. Gene 5:233-253.

79. Goodman, H.M., M.V. Olson, and B.D. Hall. 1977. Nucleotide sequence of a mutant eukaryotic gene: The yeast tyrosine-inserting ochre suppressor SUP4-O. Proc. Natl. Acad. Sci. U.S.A. 74:5453-5457.

80. Goursot, R., A. Goze, B. Niaudet, and S.D. Ehrlich. 1982. Plasmids from Staphylococcus aureus replicate in yeast Saccharomyces cerevisiae. Nature 298:488-490.

81. Gray, O., and S. Chang. 1981. Molecular cloning and expression of Bacillus licheniformis β-lactamase gene in Escherichia coli and Bacillus subtilis. J. Bacteriol. 145:422-428.

82. Grindley, N.D.F., and C.M. Joyce. 1980. Genetic and DNA sequence analysis of the kanamycin resistance transposon Tn 903. Proc. Natl. Acad. Sci. U.S.A. 77:7176-7180.

83. Gruss, P., and G. Khoury. 1981. Expression of simian virus 40-rat preproinsulin recombinants in monkey kidney cells: use preproinsulin RNA processing signals. Proc. Natl. Acad. Sci. U.S.A. 78:133-137.

84. Guarente, L., and M. Ptashne. 1981. Fusion of Escherichia coli lacZ to the cytochrome c gene of Saccharomyces cerevisiae Proc. Natl. Acad. Sci. U.S.A. 78:2199-2203.

85. Guarente, L., R.R. Yocum, and P. Gifford. 1982. A GAL 10 - CYC 1 hybrid yeast promoter identifies the GAL 4 regulatory region as an upstream site. Proc. Natl. Acad. Sci. U.S.A. 79:7410-7414.

86. Gunge, N., A. Tasuko, F. Ozawa, and F. Sakaguchi. 1981. Isolation and characterization of linear deoxyribonucleic acid plasmids from Kluyveromyces lactis and the plasmid-associated killer character. J. Bacteriol. 145:382-390.

87. Hamlyn, P.F., R.E. Bradshans, F.M. Mellon, C.M. Santiago, J.M. Wilson, and J.F. Peberdy. 1981. Efficient protoplast isolation from fungi using commercial enzymes. Enzyme Microb. Technol. 3:321-325.

88. Hartley, J.L., and J.E. Donelson. 1980. Nucleotide sequence of the yeast plasmid. Nature 286:860-865.

89. Henikoff, S., K. Tatchel, B.D. Hall, and K.A. Nasmyth. 1981. Isolation of a gene from Drosophila by complementation in yeast. Nature 289:33-37.

90. Hereford, L.M., K. Fahrner, J. Woolford, Jr., M. Rosbash, and D.B. Kaback. 1979. Isolation of yeast histone genes H2A and H2B. Cell 18:1261-1271.

91. Hicks, J., and G.R. Fink. 1977. Identification of chromosomal location of yeast DNA from hybrid plasmid pYeleul0. Nature 269:265-267.

92. Hicks, J.B., A. Hinnen, and G.R. Fink. 1979. Properties of yeast tansformation. Cold Spring Harb. Symp. Quant. Biol. 43:1305-1313.

93. Hicks, J., J.N. Strathern, and A.J.S. Klar. 1979. Transposable mating type genes in Saccharomyces cerevisiae. Nature 282:478-483.

94. Hinnen, A., P. Farabaugh, C. Ilgen, and G.R. Fink. 1979. Isolation of a yeast gene (his4) by transformation of yeast. ICN-UCLA sym. Mol. Cell. Biol. 14:43-50.

95. Hinnen, A., J.B. Hicks, and G.R. Fink. 1978. Transformation of yeast. Proc. Natl. Acad. Sci. U.S.A. 75:1929-1933.

96. Hinnen, A., and B. Meyhack. 1982. Vectors for cloning in yeast. Curr. Top. Microbiol. Immunol. 96:101-117.

97. Hitzemann, R.A., A.C. Chinault, A.J. Kingsman, and J. Carbon. 1979. Detection of E. coli clones containing specific yeast genes by immunological screening. ICN-UCLA Symp. Mol. Cell. Biol. 14:57-68.

98. Hitzeman, R.A., L. Clarke, and J. Carbon. 1980. Isolation and characterization of the yeast 3-phospho glycerokinase gene (pGK) by an immunological screening technique. J. Biol. Chem. 255:12073-12080.

99. Hitzeman, R.A., F.E. Hagie, H.L. Levine, D.V. Goeddel, G. Ammerer, and B. D. Hall. 1981. Expression of a human gene for interferon in yeast. Nature 293:717-722.

100. Hohn, B., and A. Hinnen. 1980. Cloning with cosmids in E. coli and yeast. p. 169-183. In J.K. Setlow, and and methods. Vol. 2. Plenum Press. N.Y.

101. Hohn, T., and J. Katsura. 1977. Structure and assembly of bacteriophage lambda Curr. Top. Microbiol. Immunol. 78:69-110

102. Holland, J.P., and M.J. Holland. 1979. The primary structure of a glyceraldehyde-3-phosphate dehydrogenase gene from Saccharomyces cerevisiae. J. Biol. Chem. 254:9839-9845.

103. Holland, M.J., and J.P. Holland. 1979. Isolation and characterization of a gene coding for glyceraldehyde-3-phosphate dehydrogenase from Saccharomyces cerevisiae. J. Biol. Chem. 254:5466-5474.

104. Holland, J.P., and M.J. Holland. 1980. Structural comparison of two nontandemly repeated yeast glyceraldehyde-3-phosphate dehydrogenase genes. J. Biol. Chem. 255:2596-2605.

105. Hollenberg, C.P. 1979. The expression in Saccharomyces cerevisiae of bacterial -lactamase and other antibiotic resistance genes integrated in A 2-µm DNA vector. ICN-UCLA Symp. Mol. Cell. Biol. 15:325-338.

106. Hollenberg, C.P. 1982. Cloning with 2µm DNA vectors and the expression of foreign genes in Saccharomyces cerevisiae. Curr. Top. Microbiol. Immunol. 96:119-144.

107. Hollenberg, C.P., B. Kustermann-Kuhn, V. Mackedonski, and E. Erhart. 1980. The expression of bacterial antibiotic resistance genes in the yeast Saccharomyces cerevisiae. Alfred Benzon Symp. 16:109-120.

108. Holliday, R. 1964. A Mechanism for gene conversion in fungi. Genet. Res. 5:282-304.

109. Hopper, J.E., J.R. Broach, and L.B. Rowe. 1978. Regulation of the galactose pathway in Saccharomyces cerevisiae: induction of uridyl transferase mRNA and dependency on GAL4 gene function. Proc. Natl. Acad. Sci. U.S.A. 75:2878-2882.

110. Hsiao, C., and J. Carbon. 1979. High frequency transformation of yeast by plasmids containing the cloned yeast ARG4 gene. Proc. Natl. Acad. Sci. U.S.A. 76:3829-3833.

111. Hsiao, C.L., and J. Carbon. 1981. Characterization of a yeast replication origin (ars2) and construction of stable minichromosomes containing cloned yeast centromere DNA (CEN3). Gene 15:157-166.

112. Ilgen, C., P.J. Farabaugh, A. Hinnen, J.M. Walsh, and G.R. Fink. 1979. Transformation of yeast. p. 117-132. In J.K. Setlow, and A. Hollaender (ed.), Genetic engineering principles and methods, Vol.1. Plenum Press. N.Y.

113. Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153:163-168.

114. Jimenez, A. and J. Davies. 1980. Expression of a transposable antibiotic resistance element in Saccharomyces cerevisiae: a potential selection for eukaryotic cloning vectors. Nature 287:869-871.

115. Johnston, J., F. Hilger, and R. Mortimer. 1981. Variation in frequency of transformation by plasmid YRp7 in Saccharomyces cerevisiae. Gene 16:325-329.

116. Johnston, S.A., and J.E. Hopper. 1982. Isolation of the yeast regulatory gene GAL4 and analysis of its dosage effects on the galactose / melibiose regulon. Proc. Natl. Sci. U.S.A. 79:6971-6975.

117. Johnson, J.D., R. Ogden, P. Johnson, J. Abelson, P. Dembeck, and K. Itakura. 1980. Transcription and processing of a yeast tRNA gene containing a modified intervening sequence. Proc. Natl. Acad. Sci. U.S.A. 77:2564-2568.

118. Kanetsuna, F., L.M. Carbonell, R.E. Moreno, and J. Rodriguez. 1969. Cell Wall composition of the yeast and mycelial forms of Paracoccidioides brasiliensis. J. Bacteriol. 97:1036-1041.

119. Kingsman, A.J., L. Clarke, R.K. Mortimer, and J. Carbon. 1979. Replication in Saccharomyces cerevisiae of plasmid pBR313 carrying DNA from the yeast TRP 1 region. Gene 7:141-153.

120. Kingsman, A.J., and R.L. Gimlich, L. Clarke, A.C. Chinault, and J. Carbon. 1981. Sequence variation in dispersed repetitive sequences in Saccharomyces cerevisiae. J. Mol. Biol. 145:619-632.

121. Kiss, G.B., A.A. Amin, and R.E. Pearlman. 1982. Regions of Tetrahymena rDNA allowing autonomous replication of plasmids in yeast. ICN-UCLA Symp. Mol. Cell. Biol. 22:607-614.

122. Kitamura, K. 1982. A protease that participates in yeast cell wall lysis during zymolase digestion. Agric. Biol. Chem. 46:2093-2099.

123. Kitamura, K., and K. Tanabe. 1982. Effect of culture medium on susceptibility of yeast cells to zymolyase. Agric. Biol. Chem. 46:553-554.

124. Kitamura, K., and Y. Yamamoto. 1981. Lysis of yeast cells showing low susceptibility to zymolase. Agric. Biol. Chem. 45:1761-1766.

125. Kojo, H., B.D. Greenberg, and A. Sugino. 1981. Yeast 2- μ plasmid DNA replication in vitro:origin and direction. Proc. Natl. Acad. Sci. U.S.A. 78:7261-7265.

126. Kopecka, M. 1975. The isolation of protoplasts of the fission yeast Schizosaccharomyces by Trichoderma viride and snail enzyme. Folia Microbiol. 20:273-276.

127. Koski, R.A., and S.G. Clarkson. 1980. Mutations of the yeast SUP4 $tRNA^{Tyr}$ locus: transcription of the mutant genes in vitro cell 22:415-425.

128. Kramer, R.A., J.R. Cameron, and R.W. Davis. 1976. Isolation of bacteriophage containing yeast ribosomal RNA gene: Screening by in situ RNA hybridization to plaques. Cell 8:227-232.

129. Kramer, R.A., P. Philippsen, and R.W. Davis. 1978. Divergent transcription in the yeast ribosomal RNA coding region as shown by hybridization to separated strands and sequence analysis of cloned DNA. J. Mol. Biol. 123:405-416.

130. Kuo, S.C., and S. Yamamoto. 1975. Preparation and growth of yeast protoplasts. Methods cell Biol. 11:169-183.

131. Kurjan, J., and B.D. Hall. 1980. Mutations at the yeast SUP4 $tRNA^{Tyr}$ locus:DNA sequence changes in mutants lacking suppressor activity. Cell. 20:701-709.

132. Kurjan, J. and B.D. Hall. 1982. Mutation at the Saccharomyces cerevisiae SUP4 $tRNA^{Tyr}$ locus: isolation, genetic fine-structure mapping, and correlation with physical structure. Mol. Cell. Biol. 2:1501-1513.

133. Laughon, A., and R.F. Gesteland. 1982. Isolation and preliminary characterization of the GAL4 gene, a positive regulator of transcription in yeast. Proc. Natl. Acad. Sci. U.S.A. 79:6827-6831.

134. Lee, N., K. Nakamura, and M. Inouye. 1981. Expression of the Serratia Lipoprotein gene in Escherichia coli. J. Bacteriol. 146:861-866.

135. Livingston, D.M. 1977. Inheritance of 2 μm DNA plasmid from Saccharomyces. Genetics 86:73-84.

136. Livingston, D.M., and S. Hahne. 1979. Isolation of condensed intracellular form of the 2 μm DNA plasmid of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 76:3727-3731.

137. Livingston, D.M., and D.M. Kupfer. 1977. Control of Saccharomyces cerevisiae 2 μm DNA replication by cell division cycle genes that control nuclear DNA replication. J. Mol. Biol. 116:249-260.

138. Loison, G., and R. Jund. 1981. Expression of a cloned Saccharomyces cerevisiae gene (URA1) is controlled by a bacterial promoter in E. coli and by a yeast promoter in S. cerevisiae. Gene 15:127-137.

139. Loison, G., R. Jund, M. Nguyen-Juilleret, and F. Lacroute. 1981. Evidence for transcriptional regulation of dihydroorotic acid dehydrogenase in Saccharomyces cerevisiae. Curr. Genet. 3:119-123.

140. Malone, R., and R. Esposito. 1980. The RAD52 gene is required for homothallic interconversion of mating types and spontaneous mitotic recombination in yeast. Proc. Natl. Acad. Sci. U.S.A. 77:503-507.

141. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

142. Matern, H. 1979. Catabolite inactivation of phosphoenolpyruvate carboxykinase in spheroplasts from Saccharomyces cerevisiae. Biochim. Biophys. Acta 586:258-265.

143. Mckmight, G.L., T.S. Cardillo, and F. Sherman. 1981. An extensive deletion causing over production of yeast iso-2-cytochrome c. Cell 25:409-419.

144. Melton, D.A., E.M. DeRobertis, and R. Cortese. 1980. Order and intracellular location of the events involved in the maturation of a spliced tRNA. Nature 284:143-148.

145. Mercereau-Puijalon, O., F. Lacroute, and P. Kourilsky. 1980. Synthesis of a chicken ovalbumin-like protein in the yeast Saccharomyces cerevisiae. Gene 11:163-167.

146. Miyanohara, A., A. Toh-e, C. Nozaki, F. Hamada, N. Ohtomo, and K. Matsubara. 1983. Expression of hepatitis B surface antigen gene in yeast. Proc. Natl. Acad. Sci. U.S.A. 80:1-5.

147. Montgomery, D.L., D.W. Leung, M. Smith, P. Shalit, G. Faye, and B.D. Hall. 1980. Isolation and sequence of the gene for iso-2-cytochrome c in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 77:541-545.

148. Montgomery, D.L., B.D. Hall, S. Gillam, and M. Smith. 1978. Identification and isolation of the yeast cytochrome c gene. Cell 14:673-680.

149. Morris, D.W., J.D. Noti, F.A. Osborne, and A.A. Szalay. 1981. Plasmid vectors capable of transferring large DNA fragment to yeast. DNA 1:27-36.

150. Nagahari, K., T. Koshikawa, and K. Sakaguchi. 1980. Cloning and expression of the leucine gene from Thermus thermophilus in Escherichia coli. Gene 10:137-145.

151. Nasmyth, K.A., and S.I. Reed. 1980. Isolation of genes by complementation in yeast: Molecular cloning of a cell-cycle gene. Proc. Natl. Acad. Sci. U.S.A. 77:2119-2123.

152. Nasmyth, K.A., and K. Tatchell. 1980. The structure of transposable yeast mating type loci. Cell 19:753-764.

153. Naumovski, L., and E.C. Friedberg. 1982. Molecular cloning of eukaryotic genes required for excision repair of UV-irradiated DNA:isolation and partial characterization of the RAD3 gene of Saccharomyces cerevisiae. J. Bacteriol. 152:323-331.

154. Necas, O., and A. Svoboda. 1981. Morphogenesis in protoplasts, P.:105-127. In W.N. Arnold (ed.), yeast cell envelopes: biochemistry, biophysics, and ultrastructure. Vol. 2. CRC Press, Inc., Florida.

155. Nelson, R.G., and W.L. Fangman. 1979. Nucleosome organization of the yeast 2 μm DNA plasmid: A eucaryotic minichromosome. Proc. Natl. Acad. Sci. U.S.A. 76:6515-6519.

156. Newlon, C.S., and W. Burke. 1980. Replication of small chromosomal DNAs in yeast. ICN-UCLA symp. Mol. Cell. Biol. 19:399-409.

157. Newlon, C.S., T.D. Petes, L.M. Hereford, and W.L. Fangman. 1974. Replication of yeast chromosomal DNA. Nature 247:32-35.

158. Ng, R., and J. Abelson. 1980. Isolation and sequence of the gene for actin in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 77:3912-3916.

159. Ogden, R.C., J.S. Beckman, J. Abelson, H.S. Kang, D. Soll, and O. Schmidt. 1979. In vitro transcription and processing of a yeast tRNA gene containing an intervening sequence. Cell 17:399-406.

160. O'Hare, K., C. Benoist, and R. Breathnach. 1981. Transformation of mouse fibroblasts to methotrexate resistance by a recombinant plasmid expressing a pro karyotic dihydrofolate reductase. Proc. Natl. Acad. Sci. U.S.A. 78:1527-1531.

161. Oka, A., H. Sugisaki, and M. Takanami. 1981. Nucleotide sequence of the kanamycin resistance transposon Tn903. J. Mol. Biol. 147:217-226.

162. Olah, J., and H. Feldman. 1980. Structure of a yeast non-initiating methionine-tRNA gene. Nucl. Acid. Res. 8:1975-1986.

163. Olson, M.V. 1981. Application of molecular cloning to Saccharomyces. p. 57-88. In J.K. Setlow, and A. Hollaender (ed.), Genetic engineering principles and methods, Vol. 3. Plenum Press. N.Y.

164. Olson, M.V., B.D. Hall, J.R. Cameron, and R.W. Davis. 1979. Cloning of the yeast tyrosine transfer RNA genes in bacteriophage lambda. J. Mol. Biol. 127:285-295.

165. Olson, M.V., K. Loughney, and B.D. Hall. 1979. Identification of the yeast DNA sequences that correspond to specific tyrosine-inserting nonsense suppressor loci. J. Mol. Biol. 132:387-410.

166. Orr-Weaver, T.L., J.W. Szostak, and R.J. Rothstein. 1981. Yeast transformation a model system for the study of recombination. Proc. Natl. Acad. Sci. U.S.A. 78:6354-6358.

167. Orr-Weaver, T.L., J.W. Szostak, and R.J. Rothstein. 1983. Genetic applications of yeast transformation with linear and gapped plasmids. Methods Enzymol. (In press).

168. Paluh, J.L., and H. Zalkin. 1983. Isolation of Saccharomyces cerevisiae TRP3. J. Bacteriol. 153:345-349.

169. Panthier, J.J., P. Fournier, H. Heslot, and A. Rambach. 1980. Cloned β-galactosidase gene of Escherichia coli is expressed in the yeast Saccharomyces cerevisiae Curr. Genet. 2:109-113.

170. Pearson, N.J., H.M. Fried, and J.R. Warner. 1982. Yeast use translational control to compensate for extra copies of a ribosomal protein gene. Cell 29:347-355.

171. Petes, T.D. 1980. Molecular genetics of yeast. Ann. Rev. Biochem. 49:845-76.

172. Petes, T.D., L.M. Hereford, and K.G. Skryabin. 1978. Characterization of two types of yeast ribosomal RNA genes. J. Bacteriol. 134:295-305.

173. Petes, T.D., C.S. Newlon, B. Byers, and W.L. Fangman. 1974. Yeast chromosomal DNA: size, structure, and replication. Cold Spring Harbor Symp. Quant. Biol. 38:9-16.

174. Petes, T.D., and D.H. Williamson. 1975. Fiber autoradiography of replicating yeast DNA. Expt. Cell. Res. 95:103-110.

175. Petes, T.D., and D.H. Williamson. 1975. Replicating circular DNA molecules in yeast. Cell 4:249-253.

176. Philippsen, P., R.A. Kramer, and R.W. Davis. 1978. Cloning of the yeast ribosomal DNA repeat unit in SstI and Hind III lambda vectors using genetic and physical size selections. J. Mol. Biol. 123:371-386.

177. Philippsen, P., M.J. Thomas, R.A. Kramer, and R.W. Davis. 1978. Unique arrangement of coding sequences for 5S, 5.8S, 18S and 25S ribosomal RNA in Saccharomyces cerevisiae as determined by R-loop and hybridization analysis. J. Mol. Biol. 123:387-404.

178. Prakash, S.L. Prakash, W. Burke, and B. Montelone. 1980. Effects of the RAD5 gene or recombination in Saccharomyces cerevisiae. Genetics 94:31-50.

179. Ratzkin, B., and J. Carbon. 1977. Functional expression of a cloned yeast gene in E. coli. Proc. Natl. Acad. Sci. U.S.A. 74:487-491.

180. Roeder, G.S., P.J. Ferabaugh, D.T. Chaleff, and G.R. Fink. 1980. The origins of gene instability in yeast. Science 209:1375-1380.

181. Roeder, G.S., and G.R. Fink. 1980. DNA rearrangements associated with a transposable element in yeast. Cell 21:239-249.

182. Roggenkamp, R., B. Kustermann-Kunh, and C. Hollenberg. 1981. Expression and processing of bacterial β-lactamase in the yeast Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 78:4466-4470.

183. Rose, M., M.J. Casadaban, and D. Botstein. 1981. Yeast genes fused to β-galactosidase in Escherichia coli can be expressed normally in yeast. Proc. Natl. Acad. Sci. U.S.A. 78:2460-2464.

184. Rost, K., and H. Venner. 1965. Enzymatische zellwandverdauung bei durch schneckenenzym. Arch. Mikrobiol. 51:122-129.

185. Rost, K., and H. Venner. 1965. Wachstum von hefeprotoplasten und ihre reversion zu intakten hefezellen. Arch. Mikrobiol. 51:130-139.

186. Rothstein, R. 1979. Deletions of tyrosine tRNA gene in S. cerevisiae. Cell 17:185-190.

187. Rothstein, R.J. 1983. One-step gene disruption in yeast. Methods Enzymol. (In press).

188. Scherer, S., and R.W. Davis. 1979. Replacement of chromosome segments with altered DNA sequence constructed in vitro. Proc. Natl. Acad. Sci. U.S.A. 76:4951-4955.

189. Scherer, S., C. Mann, and R.W. Davis. 1982. Reversion of a promoter deletion in yeast. Nature 298:815-819.

190. Schlenk, F., and J.L. Dainko. 1965. Action of ribonuclease preparations on viable yeast cells and spheroplasts. J. Bacteriol. 89:428-436.

191. Schwenke, J. and M. Nagy. 1978. Preparation of protoplasts of Schizosaccharomyces pombe. Methods cell Biol. 20:101-105.

192. Sherman F., G.R. Fink, and J.B. Hicks. 1981. Methods in yeast genetics. Cold Spring Harbor Loboratory, Cold Spring Harbor, N.Y.

193. Shortle, D., J.E. Haber, and D. Botstein. 1982. Lethal disruption of the yeast actin gene by integrative DNA transformation. Science 217:371-373.

194. Silverman, S.J., M. Rose, D. Bostein, and G.R. Fink. 1982. Regulation of HIS4-lacz fusion in Saccharomyces cerevisiae. Mol. Cell. Biol. 2:1212-1219.

195. Skryabin, K.G., A.M. Maxam, T.D. Petes, and L. Hereford. 1979. Location of the 5.8S rRNA Gene of Saccharomyces cerevisiae. J. Bacteriol. 134:306-309.

196. Smith, M., W.L. David, S. Gillam, and C.R. Astell. 1979. Sequence of the gene for iso-1-cytochrome c in Saccharomyces cerevisiae. Cell 16:753-761.

197. Souciet, J.L., J.C. Hubert, and F. Lacroute. 1982. Cloning and restriction mapping of the yeast URA 2 gene coding for the carbamyl phosphate synthetase aspartate-transcarbamylase complex. Mol. Gen. Genet. 186:385-390.

198. Stinchcomb, D.T., C. Mann, E. Selker, and R.W. Davis. 1982. DNA sequences that allow the replication and segregation of yeast chromosomes. ICN-UCLA Symp. Mol. Cell. Biol. 22:473-488.

199. Stinchcomb, D.T., K. Struhl, and R.W. Davis. 1979. Isolation and characterization of a yeast chromosomal replicator. Nature 282:39-43.

200. Stinchcomb, D.T., M. Thomas, J. Kelly, E. Selker, and R.W. Davis. 1980. Eukaryotic DNA segments capable of autonomous replication in yeast. Proc. Natl. Acad. Sci. U.S.A. 77:4559-4563.

201. St. John, T.P., and R.W. Davis. 1979. Isolation of galactose-inducible DNA sequences from Saccharomyces cerevisiae by differential plaque filter hybridization. Cell 16:443-452.

202. Strathern, J.N., E. Spatola, C. McGill, and J.B. Hicks. 1980. Structure and organization of transposable mating type cassettes in Saccharomyces yeast. Proc. Natl. Acad. Sci. U.S.A. 77:2839-2843.

203. Struhl, K. 1981. Deletion mapping of a eukaryotic promoter. Proc. Natl. Acad. Sci. U.S.A. 78:4461-4465.

204. Struhl, K. 1981. Position effects in Saccharomyces cerevisiae. J. Mol. Biol. 152:569-575.

205. Struhl, K. 1982. The yeast his3 promoter contains at least two distinct elements. Proc. Natl. Acad. Sci. U.S.A. 79:7385-7389.

206. Struhl, K., J.R. Cameron, and R.W. Davis. 1976. Functional genetic expression of eukaryotic DNA in Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 73:1471-1475.

207. Struhl, K., and R.W. Davis. 1977. Production of a functional eukaryotic enzyme in Escherichia coli: cloning and expression of the yeast structural gene for imidazole glycerol phosphate dehydratase (his-3). Proc. Natl. Acad. Sci. U.S.A. 74:5255-5259.

208. Struhl, K., and R.W. Davis. 1980. A physical, genetic and transcriptional map of the cloned his3 gene region of Saccharomyces cerevisiae. J. Mol. Biol. 136:309-332.

209. Struhl, K., and R.W. Davis. 1981. Promoter mutants of the yeast his3 gene. J. Mol. Biol. 152:553-568.

210. Struhl, K., R.W. Davis, and G.R. Fink. 1979. Suppression of a yeast amber mutation in Escherichia coli. Nature 279:78-79.

211. Struhl, K., D.T. Stinchcomb, S. Scherer, and R.W. Davis. 1979. High frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. U.S.A. 76:1035-1039.

212. Sumrada, R.A., and T.G. Cooper. 1982. Isolation of the CARI gene from Saccharomyces cerevisiae and analysis of its expression. Mol. Cell. Biol. 2:1514-1523.

213. Svoboda, A. 1980. Morphogenesis during regeneration and reversion of dimorphic yeasts. Biologia 35:157-165.

214. Svoboda, A. 1981. Polyethylene glycol induced membrane fusion in yeast protoplasts. Acta Histochem. Suppl. 23:211-217.

215. Szostak, J.W., J.I. Stiles, B.K. Tye, P. Chiu, F. Sherman, and R. Wu. 1979. Hybridization with synthetic oligonucleotides. Methods Enzymol. 68:419-428.

216. Taketo, M., S.M. Jazwinski, and G.M. Edelman. 1980. Association of the 2μm DNA plasmid with yeast folded chromosome. Proc. Natl. Acad. Sci. U.S.A. 77:3144-3148.

217. Tanaka, H., and H.J. Phaff. 1965. Enzymatic hydrolysis of yeast cell walls. 1. Isolation of Wall-decomposition organisms and separation and purification of lytic enzymes. J. Bacteriol. 89:1570-1580.

218. Taylor, G.R., B.J. Barclay, R.K. Storms, J.D. Friesen, and R.H. Haynes. 1982. Isolation of the Thymidylate Synthetase gene (TMP1) by complementation in Saccharomyces cerevisiae. Mol. Cell. Biol. 2:437-442.

219. Tah-e, A., S. Tada, and Y. Oshima. 1982. 2 μ DNA-like plasmids in the osmophilic haploid yeast Saccharomyces rouxii. J. Bacteriol. 151:1380-1390.

220. Toh-e, A., and R.B. Wickner. 1981. Curing of the 2 μ DNA plasmid from Saccharomyces cerevisiae. J. Bacteriol. 145:1421-1424.

221. Tschumper, K., and J. Carbon. 1980. Sequence of a yeast DNA fragment containing a chromosomal replicator and TRP1 gene. Gene 10:157-166.

222. Tschumper, G., and J. Carbon. 1982. Sequencing and subcloning analysis of autonomously replicating sequences from yeast chromosomal DNA. ICN-UCLA Symp. Mol. Cell. Biol. 22:489-500.

223. Valenzuela, P., G.I. Bell, A. Venegas, E.T. Sewell, F.R. Masiary, L.J. DeGennaro, F. Weinberg, and W. J. Rutter. 1977. Ribosomal RNA genes of Saccharomyces cerevisiae. II. Physical map and nucleotide sequence of the 5S ribosomal RNA gene and adjacent intervenic regions. J. Biol. Chem. 252:8126-8135.

224. Valenzuela, P., A. Venegas, F. Weinberg, R. Bishop, and W.J. Rutter. 1978. Structure of phenylalanine-tRNA genes: Intervening DNA segment within region coding for tRNA. Proc. Natl. Acad. Sci. U.S.A. 75:190-194.

225. van Solingen, P., and J.B. van der Plaat. 1977. Fusion of yeast spheroplasts. J. Bacteriol. 130:946-947.

226. Venegas, A., M. Quiroga, J. Zaldivar, W.J. Rutter, and P. Valenzuela. 1979. Isolation of yeast $tRNA^{Leu}$ genes: DNA sequence of a cloned $tRNA^{Leu}$ gene. J. Biol. Chem. 254:12306-12309.

227. Walz, A., B. Ratzkin, and J. Carbon. 1978. Control of expression of a cloned yeast (Saccharomyces cerevisiae) gene (trp5) by a bacterial insertion element (1S2). Proc. Natl. Acad. Sci. U.S.A. 75:6172-6176.

228. Williamson, V.M., J. Bennetzen, E.T. Young, K. Nasmyth, and B.D. Hall. 1980. Isolation of the structural gene for alcohol dehydrogenase by genetic complementation in yeast. Nature 283:214-216.

229. Williamson, V.M., D. Cox, E.T. Young, D.W. Russell, and M. Smith. 1983. Characterization of transposable element-associated mutations that alter yeast alcohol dehydrogonase II expression. Mol. Cell. Biol. 3:20-31.

230. Williamson, V.M., E.T. Young, and M. Ciriacy. 1981. Transposable elements associated with constitutive expression of yeast alcohol dehydrogenase II. Cell 23:605-614.

231. Woolford, J.L., Jr., L.M. Hereford, and M. Rosbash. 1979. Isolation of cloned DNA sequences containing ribosomal protein genes from Saccharomyces cerevisiae. Cell 18:1247-1259.

232. Zakian, V.A. 1981. Origin of replication from Xenopus laevis mitochondrial DNA promotes high-frequency transformation of yeast. Proc. Natl. Acad. Sci. U.S.A. 78:3128-3132.

233. Zakian, V.A., and D. Kupfer. 1982. Replication and segregation of an unstable plasmid in yeast. Plasmid 8:15-28.

234. Zakian, V.A., and J.F. Scott. 1982. Construction, replication and chromatin structure of TRPI R1 circle, a multiple-copy synthetic plasmid derived from Saccharomyces cerevisiae chromosomal DNA. Mol. Cell. Biol. 2:221-232.

235. Zamir, A., C.V. Maina, G.R. Fink, and A.A. Szalay. 1981. Stable chromosomal integration of the entire nitrogen fixation gene cluster from Klebsiella pneumoniae in yeast. Proc. Natl. Acad. Sci. U.S.A. 78:3496-3500.

236. Zyskind, J.W., N.E. Harding, Y. Takeda, J.M. Cleary, and D.W. Smith. 1982. The DNA replication origin region of the Enterobacteriaceae. ICN-UCLA Symp. Mol. Cell. Biol. 22:13-25.

CHAPTER I

Construction of a New Yeast Cloning Vector Containing Autonomous Replication Sequences from *Candida utilis*

ABSTRACT

There has been no publication to date regarding isolation of autonomous replication sequences (*ars*) in *Candida utilis*, an industrially important yeast. As a first step toward a genetic cloning system for this yeast, we have isolated *ars* from *C. utilis* and have cloned it into a yeast integration plasmid (YIp5) which contains the *ura*3 gene of *Saccharomyces cerevisiae* and cannot replicate in a *ura*3 deletion mutant of *S. cerevisiae* YNN27. Any DNA fragment from *C. utilis* which is capable of conferring on YIp5 the ability to replicate autonomously in *S. cerevisiae* should contain an *ars* of *C. utilis*. Several plasmids which transform *S. cerevisiae* YNN27 to Ura3$^+$ with an efficiency of 2 x 10^3 transformants per µg DNA were obtained. The existence of autonomously replicating plasmids was demonstrated by Southern gel hybridization, transformation of *Escherichia coli*, and the recovery of plasmid DNA. One of the hybrid plasmids, pHMR22 (6.6 kb) contains an *ars* which is homologous with two different DNA fragments of the *C. utilis* genome but has no detectable homology to total DNA from *C. albicans*, *Pachysolen tannophilus* or *S. cerevisiae*. The fact that the *ars* has no detectable homology to the *S. cerevisiae* genome but is still fully functional in this yeast is consistent with an earlier report that homology with the host DNA is not a prerequisite for the function of *ars*. Restriction and subcloning analysis of pHMR22 showed that Sau3A destroys the functions

of the cloned *ars* whereas there are no BamHI, PstI, SalI, HindIII, EcoRI and PvuII sites in the region of the *ars* which is required for its functional integrity. Thus, pHMR22 appears to be a useful vector for cloning desired genes in *S. cerevisiae* and for the comparative study of *ars* in yeast.

INTRODUCTION

Candida utilis is an industrially important yeast which is widely used for the production of single cell protein from biomass-derived sugars (15,24) and a number of other carbohydrate sources (16,26,31). This organism is also used for the production of L-glutamic acid (U.S. Pat. 3,220,929), L-serine (U.S. Pat. 3,755,081) and degradation of hydrocarbons (U.S. Pat. 3,769,164). However, genetic studies on *C. utilis* have been relatively sparse. In particular, little is known concerning the nature and organization of the *C. utilis* genome and no cloning vector is presently available for this organism. Genetic analysis of the chromosome structure and replication of *C. utilis* will facilitate the genetic manipulation of this yeast for industrial uses.

High frequency transformation in yeast has been accomplished primarily with two kinds of plasmids: 1) those containing fragments of 2 μ DNA which has clearly been shown to be capable of self replication in yeast (1,3,9) and 2) those containing certain yeast chromosomal sequences called autonomous replication sequences (*ars*) which presumably contain initiation sites for DNA synthesis (1,2,9,21,38). The plasmid called 2 μ DNA has been demonstrated only in *Saccharomyces* (5,10,13,19,35,40) and *Schizosaccharomyces pombe* (12) and has never been demonstrated in *Candida* (14,40). Autonomous replication sequences responsible for high frequency transformation may

be present in the eucaryotic chromosome either as multiple copies (7,8) or as unique sequences or as sequences which share limited homology with other replicator sequences in the chromosome (37,42). Several *ars* have been described in *S. cerevisiae* (7,8,37,42). Also, *ars* from a wide variety of eucaryotes, but not *Escherichia coli*, have been shown to exhibit high frequency transformation in *S. cerevisiae* (38). These observations raised the possibility that replication origins are similar or identical in all eukaryotes, although definitive information is still lacking. To date, there have been no reports on the isolation of *ars* from *C. utilis* or other candida species. Identification of an *ars* from *C. utilis* that is capable of autonomous replication in *S. cerevisiae* would have several advantages. It would: 1) open up new approaches to the study of *C. utilis* DNA replication; 2) serve as a defined template for *in vitro* replication studies; 3) help identify and isolate factors required for chromosomal DNA replication; and 4) serve as a potential vector for cloning desired yeast genes. The objective of this study, therefore, was to isolate *ars* from *C. utilis*.

In this paper we have described the isolation and characterization of an *ars* from the *C. utilis* genome that allows autonomous replication of yeast integration plasmid (YIp5; 38) in *S. cerevisiae*. The results indicate that the *ars* contains a replication origin from the *C. utilis* genome. We also found that the cloned *ars* is homologous

with two different fragments of the C. utilis genome but does not have detectable homology with the genome DNA of S. cerevisiae, C. albicans or P. tannophilus.

MATERIALS AND METHODS

Strains, plasmids and media. The yeast and bacterial strains and plasmids used in this study are listed in Table 1. Yeast media were those described by Sherman et al. (34). Types of yeast media included YPD (1% yeast extract, 2% Bacto-peptone, and 2% glucose) and minimal medium (0.67% Difco yeast nitrogen base without amino acids, and 2% glucose). Minimal medium was supplemented when necessary with amino acids, purines, and pyrimidines at concentrations given by Sherman et al. (34). LB medium used for growth of E. coli was described by Maniatis et al. (25).

DNA preparation. Plasmid DNA was prepared from E. coli by the rapid boiling method as described by Holmes and Quigley (20). Total yeast DNA from both transformed and untransformed cells was isolated according to the procedure of Sherman et al. (34) except that Novozym 234 was used for cell wall digestion instead of Zymolase. DNA fragments to be labeled by nick translation and recloned into other vectors were isolated from agarose gels by electroelution as described by Maniatis et al. (25).

Transformation of yeast and bacteria. Yeast strains were transformed by using a modification of Begg's method (34). Bacteria were transformed with vector DNA using the calcium chloride-heat shock method as described by Maniatis et al. (25).

Restriction mapping. DNA digested with restriction enzymes was fractionated by horizontal agarose gel

Table 1. Microbial strains and plasmids used and their sources

Strains	Plasmids	Plasmid markers	Chromosomal markers	Sources or references
E. coli HB101	pBR322	*tet*^{r}, *amp*^{r}	*hsd*, *proA2* *thi*, *lacYI* *recA13*, *rpsL20*, *ara-14*, *gal K2*, *xyl-5*, *mtl-1* *supt44*, λ^-	4,25
E. coli PNN33	YRp12	*trp1*$^+$, *ura3*$^+$ *tet*^{r}, *amp*^{r}		33,38
E. coli PNN36	YIp5	*ura3*$^+$, *tet*^{r} *amp*^{r}		38
S. cerevisiae YNN27			*ura3-52*, *gal2* *trp1-289*	38
S. cerevisiae AH22			a *leu 2-3*, *leu 2-112* *his4-517*, *can1*	18
Candida utilis NRRL Y-900			wild type	NRRL[a]
C. albicans FC18			wild type	P.T. Magee
Pachysolen tannophilus NRRL Y-2460			wild type	NRRL

[a]Northern Regional Research Laboratory, Peoria, Illinois.

electrophoresis in Tris-acetate buffer (0.04 M Tris, 0.02 M Na acetate and 2.0 mM Na_2EDTA, pH 7.8). The size of DNA fragments was estimated by comparing their migration distances on gels to the migration of DNA fragments of known molecular weight generated by digesting λ phage DNA with Hind III (36). The linear order of restriction sites was determined by analyzing restriction fragments generated by multiple enzyme digestions.

Southern transfer and hybridization to nitrocellulose paper. DNA was fractionated by electrophoresis on 0.7% agarose gels, stained with ethidium bromide, photographed and blotted to nitrocellulose BA85 (Schleicher and Schuell) as described by Maniatis et al. (25). α-^{32}PdATP was incorporated into plasmid DNA by nick translation with E. coli polymerase I (32). For high stringency hybridization, the blotted filter paper was prehybridized for a minimum of 4 hr at 42°C with hybridization buffer containing 50% (vol/vol) formamide, 1 M NaCl, 10 mM Tris.HCl at pH 8.0, 1 mM EDTA, 0.05 M $Na_4P_2O_7$ at pH 6.8, 5X Denhardt's solution, 10 µg/ml of poly (rA), and 8 µg/ml of heat-denatured salmon sperm DNA. ^{32}P-labeled probe was then added to the hybridization solution and hybridized for 40 hr at 42°C. The hybridized filters were washed twice at ambient temperature for 5 min in 10 mM Tris.HCl, pH 7.5/1 mM EDTA/0.1% sodium dodecyl sulfate/0.1% $Na_4P_2O_7$/50 mM NaCl, followed by four 15 min washes with the same solution at 65°C. In case of low stringency hybridization, the

procedures were performed identically, except that 1.5 M NaCl was used in the hybridization buffer, 0.3 M NaCl was added to the wash buffer, and the hybridized filters were washed at 50°C instead of 65°C (11). Autoradiography was performed at -70°C with Kodak XAR-5 film and intensifying screen (23).

Determination of mitotic stabilities of hybrid plasmids in yeast. The yeast transformants were grown from a single colony in yeast minimal medium supplemented with tryptophan and uracil (50 ug/ml) for 15-20 generations at 600 nm. Cell suspensions were diluted in sterile distilled water to yield 100-500 colonies when suitable inoculum volumes were spread on YPD agar plates. After overnight incubation at 30°C, the colonies were tested for the Ura^+ phenotype by replica plating onto plates of yeast minimal medium plus tryptophan with or without added uracil.

Enzymes and chemicals. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories (Gaithersburg, Maryland); polyethylene glycol 4000 was from BDH Chemicals (Poole, England); and Glusulase was from Endo Laboratories (Garden, New York). Novozym 234 was a gift from Novo Laboratories (Wilton, Connecticut). Deoxyadenosine-5'-(α-^{32}P) triphosphate was purchased from New England Nuclear (Boston, Massachusetts); pronase and ribonuclease were from Calibiochem-Behring (La Jolla, California), calf alkaline phosphatase was from Boehringer Mannheim Biochemicals (Indianapolis, Indiana) and poly(rA)

was from P-L Biochemicals (Milwaukee, Wisconsin). The other chemicals and enzymes were purchased from Sigma Chemical Co. (Saint Louis, Missouri). Buffers and reaction conditions were those specified by the respective vendors.

RESULTS

Isolation of ars. The scheme for isolating an ars from C. utilis is shown in Fig. 1. A typical yeast integration plasmid, YIp5, which contains the plasmid pBR322 DNA (4) and the ura3 gene of S. cerevisiae, was employed as the cloning vector. This chimeric plasmid cannot replicate autonomously in a ura3 deletion mutant of S. cerevisiae YNN27 (38). Any fragment of C. utilis DNA capable of conferring on YIp5 the ability to replicate autonomously in S. cerevisiae should contain an ars of C. utilis.

C. utilis DNA was partially cleaved with MboI and then ligated to BamHI-restricted YIp5 plasmid DNA. This pool of hybrid DNA molecules was used to transform S. cerevisiae YNN27 to the Ura^+ phenotype. About 300 to 400 Ura^+ transformants per µg of DNA were obtained. Since YIp5 does not transform the ura3 deletion mutant of S. cerevisiae YNN27 to Ura^+, the results indicate that high-frequency transformation of YNN27 to Ura^+ is an inherent property of the ars DNA inserted into YIp5. Three Ura^+ transformants (CU81, CU82 and CU22) were picked randomly for total DNA isolation. Total DNA from these three transformants contained bands homologous to pBR322 with an electrophoretic mobility corresponding to the supercoiled, open circular and multimer forms of the transforming plasmids (Fig. 2). DNA from untransformed YNN27 (control) did not show hybridization with pBR322 DNA. When total DNA isolated from CU22 transformant was completely digested

Fig. 1. Scheme for the isolation of *ars* from *C. utilis*. *C. utilis* DNA was prepared as described by Sherman et al. (1981). DNA was partially digested with MboI and then ligated to BamHI restricted YIp5. The pool of hybrid DNA molecules (2 µg) were used to transform a *ura3* deletion mutant of *S. cerevisiae* YNN27 to Ura^+. YIp5 DNA was used as a control in this experiment.

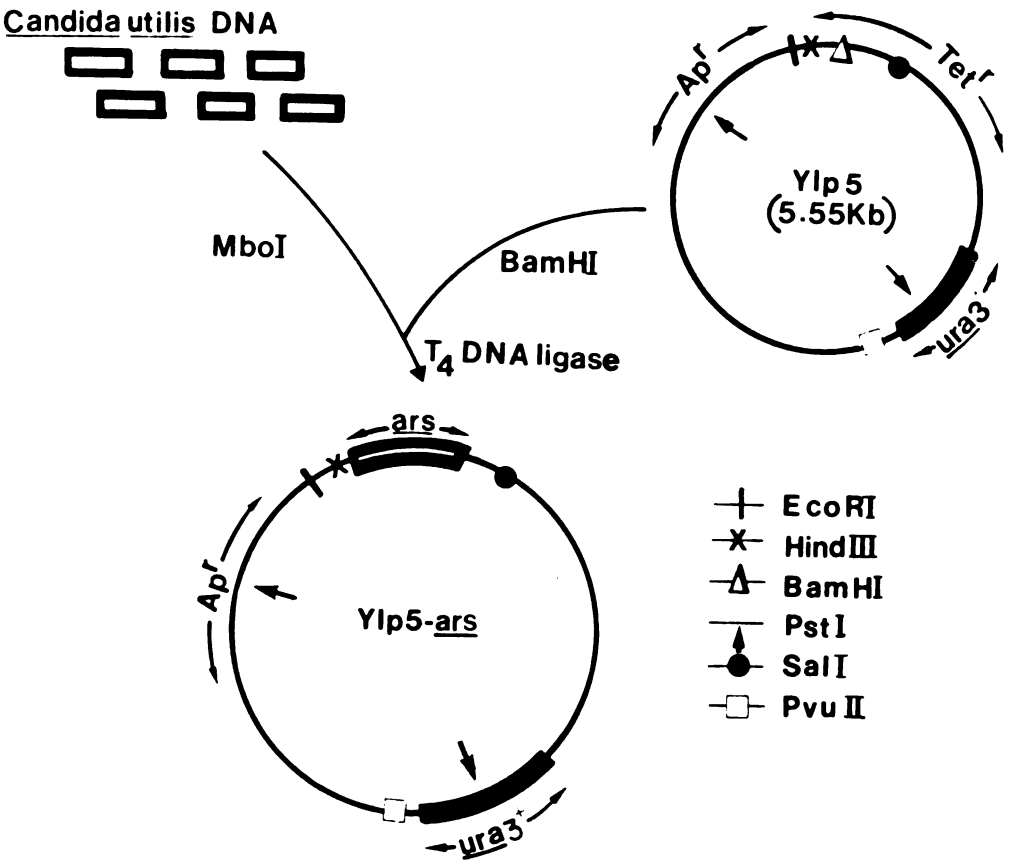

Candida utilis DNA
MboI
BamHI
T4 DNA ligase
Apr
Tetr
Yip 5
(5.55Kb)
ura3
ars
Apr
Yip5-ars
ura3+
EcoRI
HindIII
BamHI
PstI
SalI
PvuII

Fig. 2. Analysis of the transforming plasmids of *S. cerevisiae* YNN27 in Ura$^+$ transformants. Transformants were grown on minimal medium containing tryptophan. Yeast DNA was prepared as described by Sherman et al. (34), and 7 µg of this preparation was fractionated by electrophoresis on 0.7% agarose gel. Blot hybridization was carried out under high stringent conditions as described in Materials and Methods. Untransformed strain YNN27 DNA (lane A), transformant strain CU81 DNA (lane B), transformant strain CU82 DNA (lane C), transformant strain CU22 DNA (lane D), BamHI restricted CU22 DNA (lane E), and EcoRI restricted CU22 DNA (lane F).

A B C D E F

with BamHI or EcoRI only a single band of hybridization was observed with pBR322 DNA indicating that the ars plasmid replicates autonomously and is not integrated into the S. cerevisiae chromosomal DNA.

Transformation of bacteria and Yeast. DNA from the three Ura$^+$ transformants was extracted by the rapid procedure described by sherman et al. (34). These DNA preparations were used to transform E. coli HB101. Ten to one hundred ampicillin-resistant and tetracycline sensitive bacterial clones were obtained, indicating that the bacterial plasmid sequences encoding drug resistance and replication function (ori) are not altered. The three types of plasmids isolated from E. coli were designated pHMR22, pHMR81 and pHMR82 corresponding respectively, to transformants CU22, CU81 and CU82. Each of the three plasmids could transform YNN27 to Ura$^+$ at a high frequency (2×10^3 transformants/μg DNA), similar to YRp12 (Table 2). Thus, the YIp5 portion of the hybrid plasmid appears to be intact after the genetic manipulations described above.

Phenotype of Ura$^+$transformants. The doubling time of transformants, CU81 and CU22, was similar to that of a YRp12 transformant (positive control) grown in selective medium (Table 2). However, the doubling time for transformant CU83 was higher than that of CU81 and CU22. All of the Ura$^+$ transformants were unstable both in selective and nonselective media (Table 2) which is rather characteristic of the ars plasmid of yeast (2,38). After

Table 2. Properties of the ars-YIp5 plasmids isolated from Ura$^+$ transformants[a]

Plasmids	Insert size (kb)	Transformation efficiency (Transformants per ug DNA)	Generation time (hr) of transformants on[b] nonselective medium	Generation time (hr) of transformants on[b] selective medium	Percent remaining Ura$^+$ after 20 generations on[b] nonselective medium	Percent remaining Ura$^+$ after 20 generations on[b] selective medium
PHMR81	4.1	1800	2.5	4.0	> 1	36
PHMR82	4.5	1100	2.5	6.0	> 1	14
pHMR22	1.0	2000	2.5	4.0	> 1	25
YRp12	1.5	2200	2.5	4.0	> 1	30

[a]All mitotic stability experiments were performed on plasmids transformed into S. cerevisiae YNN27. Stability value is indicated for an average of three separate experiments.

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

20 generations in nonselective medium, 99% of the transformants lost the Ura^+ phenotype.

Frequency of ars loci. To obtain a rough estimate of the frequency of ars in C. utilis genome, E. coli HB101 was transformed with the pool of YIp5 C. utilis hybrid DNAs. Twelve clones, each containing inserted YIp5 (ampicillin-resistant and tetracycline-sensitive), were isolated, and the plasmid DNA isolated from each individual clone was used to transform S. cerevisiae YNN27. One of these 12 hybrid plasmids allowed high-frequency transformation of YNN27. The average size of the MboI inserts in 12 hybrid plasmids is about 2.5 kb. This frequency of one ars per 30 kb is consistent with the demonstrated frequency of one ars per 30-40 kb in S. cerevisiae genome (2,8).

Restriction mapping of ars plasmid pHMR22. pHMR22 was first digested with various enzymes and the fragment patterns compared to those of YIp5. Single enzyme digestion indicated that the ars does not have a site for PstI, SalI, HindIII, EcoRI and PvuII (Fig. 3A). Also, the ars plasmid still contains a cleavage site for BamHI after BamHI/MboI ligation (Fig. 3A, Lane C). Double enzyme digestion indicated that the BamHI site is near or at one of the ends of the ars close to the SalI site of the YIp5 sequences (Fig. 3B). The restriction map of plasmid pHMR22 containing the ars of C. utilis is shown in Fig. 4.

Subcloning of ars in pHMR22. To determine whether the BamHI site is located in that part of the ars essential for

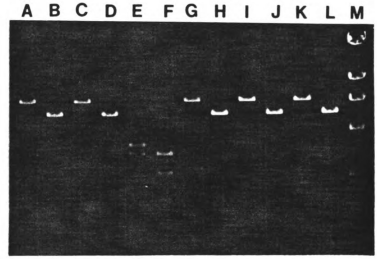
Fig. 3. Restriction mapping of plasmid, pHMR22, containing ars of C. utilis.

a. Single digestion of pHMR22 and YIp5, respectively, with EcoRI (lane A and B), BamHI (lane C and D), PstI (lane E and F), PvuII (lane G and H), HindIII (lane I and J) and SalI (lane K and L). The DNAs, after restriction, are electrophoresed on 0.7% agarose gel. Lane M contains size markers of λ DNA-HindIII fragments.

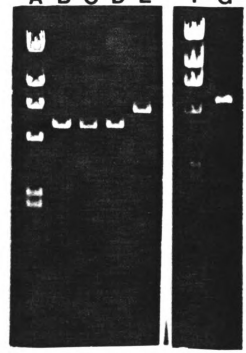
b. I. Double digestion of YIp5 and pHMR22, respectively, with HindIII/BamHI (lane B and C) and SalI/BamHI (lane D and E).

II. Double digestion of pHMR22 with HindIII/SalI (lane G). Lanes A and F contain size markers of λ DNA-HindIII fragments.

a.



b.

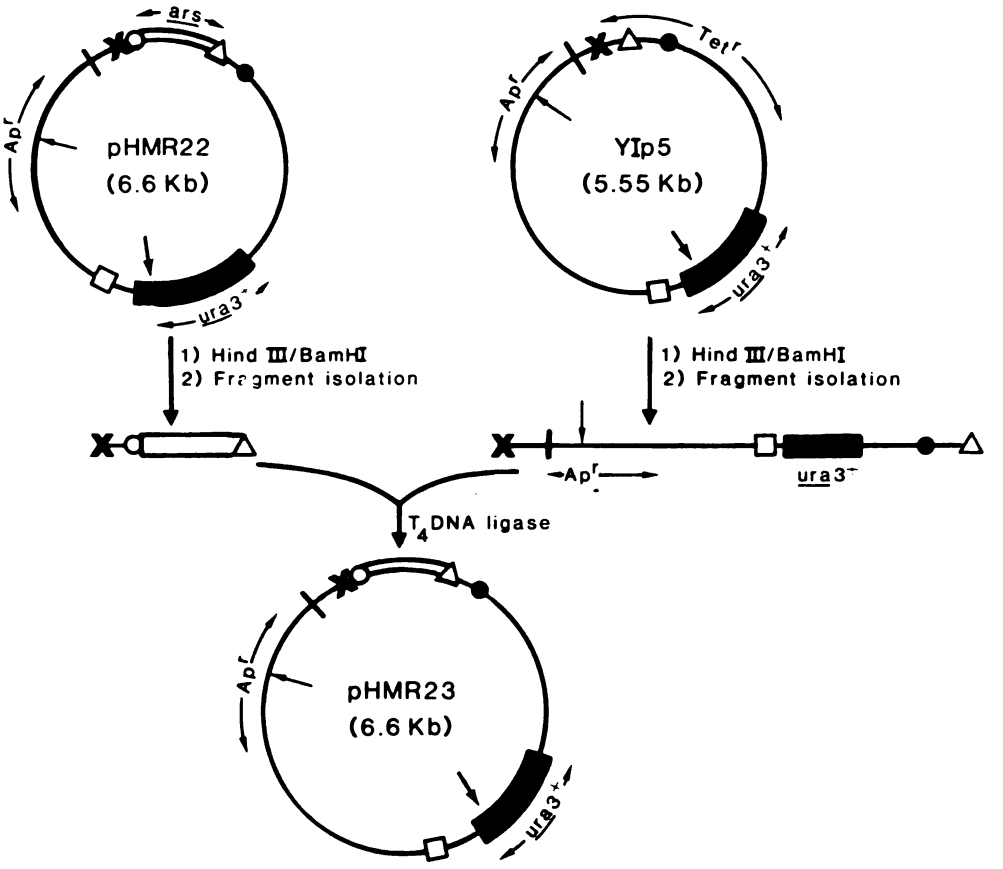


function, we constructed a new plasmid (pHMR23) containing a 1.35 kb HindIII/BamHI fragment of pHMR22 and a 5.25 kb HindIII/BamHI fragment of YIp5 (Fig. 4A) and examined the autonomous replication of this plasmid in S. cerevisiae YNN27. If the BamHI site is not located in the essential portion of the ars, the 1.35 kb HindIII/BamHI fragment of pHMR22 should confer on YIp5 the ability to replicate autonomously in S. cerevisiae. pHMR22 and YIp5 were cleaved with HindIII/BamHI and the resulting fragments were separated by agarose gel electrophoresis. The small DNA fragment (1.35 kb) of pHMR22 and the large DNA fragment (5.25 kb) of YIp5 were isolated from the agarose gel by electrophoretic elution and then ligated with T4 DNA ligase. The pool of hybrid DNA was transformed into E. coli HB101 and ampicillin-resistant and tetracycline-sensitive clones were selected. We isolated three plasmids containing the desired DNA fragment from pHMR22 and YIp5. All of these three plasmids were capable of transforming S. cerevisiae YNN27 to Ura^+ with high efficiency similar to pHMR22 (data not shown). These results show that the BamHI site is not located in the essential portion of the ars.

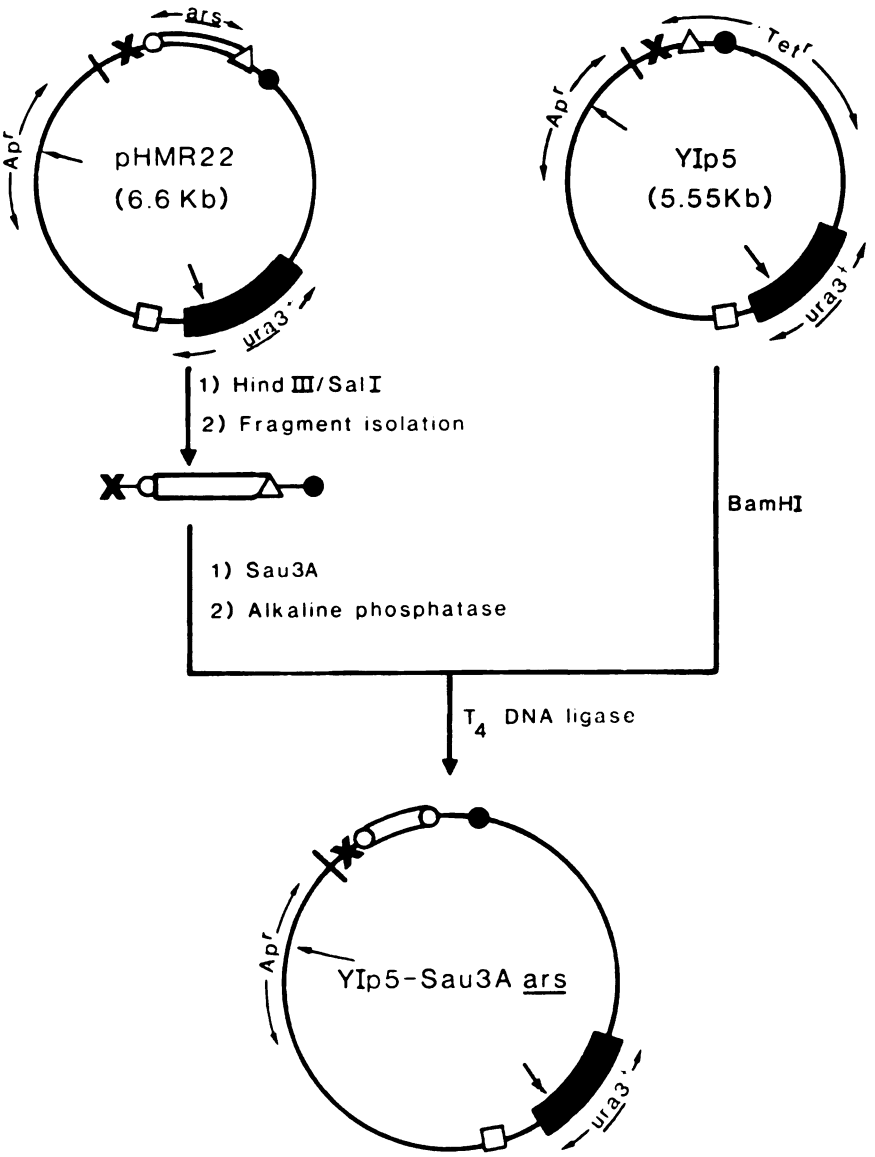
The ars in pHMR22 was subcloned further to localize the chromosomal replicator (Fig. 4B). pHMR22 was digested with HindIII/SalI and separated on an agarose gel. The ars fragments were isolated from agarose gel and subjected to Sau3A digestion. To prevent the self-ligation of Sau3A fragments, the pool of Sau3A fragments was treated with

Fig. 4. Subcloning analysis of *ars* DNA from *C. utilis*. Plasmid pHMR22 is YIp5 plus a 1.05 Kb MboI restricted fragment of *C. utilis* which includes *ars*. A new plasmid, pHMR23, was constructed using 1.35 kb HindIII/BamHI fragment of pHMR22 and a 5.25 kb HindIII/BamHI fragment of YIp5 (Fig. 4a). Subcloning of the *ars* in pHMR22 after Sau3A digestion is shown in Fig. 4b. Restriction enzyme sites are as follows: EcoRI (+), HindIII (X), BamHI (Δ), PstI (↑), SalI (O), PvuII (□), and Sau3A (O). Not all Sau3A sites are shown.

a


ars
Ap^r
pHMR22
(6.6 Kb)
ura3^+
Tet^r
Ap^r
YIp5
(5.55 Kb)
ura3^+
1) Hind III/BamHI
2) Fragment isolation
1) Hind III/BamHI
2) Fragment isolation
Ap^r
ura3^-
T4 DNA ligase
Ap^r
pHMR23
(6.6 Kb)
ura3^+

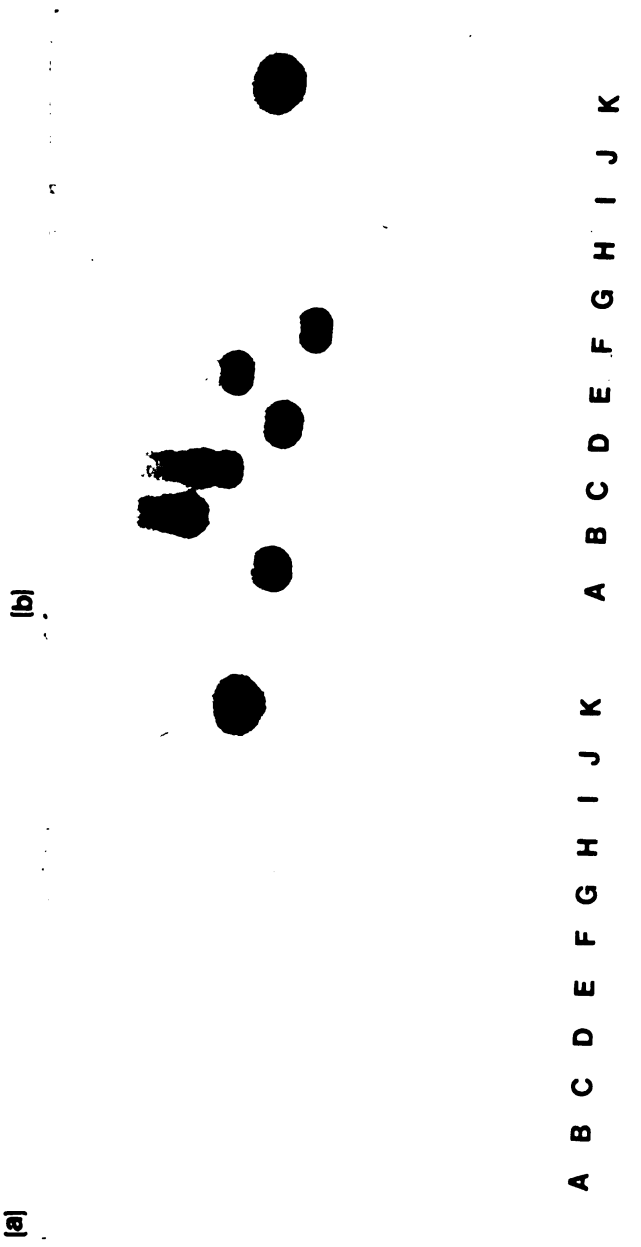
b


ars
Apr
pHMR22
(6.6 Kb)
ura3'
Tetr
Apr
YIp5
(5.55Kb)
ura3'
1) Hind III/Sal I
2) Fragment isolation
BamHI
1) Sau3A
2) Alkaline phosphatase
T4 DNA ligase
Apr
YIp5-Sau3A ars
ura3'

calf alkaline phosphatase to remove the terminal 5'-phosphates from the DNA (6). After the ligation of the Sau3A fragments with BamHI-restricted YIp5 plasmid DNA, the pool of hybrid plasmids was transformed to E. coli HB101. About 500 of the tetracycline-sensitive and ampicillin-resistant clones of transformed E. coli were pooled together for total plasmid isolation. A pool of resulting hybrid plasmids (10 μg) was used to transform S. cerevisiae YNN27. No Ura^+ transformant harboring plasmid was isolated. These results indicated that the Sau3A site is located in the essential portion of cloned ars.

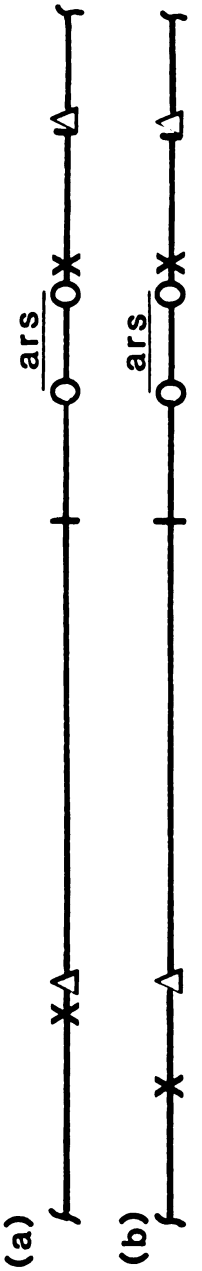
Yeast sequences homologous to the cloned ars of pHMR22. The results of hybridization of the ^{32}P-labeled ars fragment with total yeast DNA under low stringency conditions are shown in Fig. 5. We also conducted an experiment under high stringency hybridization conditions and found that the hybridization spectrum was the same as that shown in Fig. 5 (data not shown). We then asked the question whether DNA complementary to the ars was present elsewhere in the C. utilis genome or in the genome of other yeasts. Total DNAs of C. utilis, C. albicans, S. cerevisiae and P. tannophilus were isolated and subjected to restriction enzyme digestion. After separation of DNAs by agarose gel electrophoresis, the DNAs were transferred to nitrocellulose paper and hybridized with ^{32}P-labeled HindIII/SalI ars DNA or ^{32}P-labeled pBR322 DNA. As shown in Fig. 5a, pBR322 did not hybridize to yeast DNA even at

Fig. 5. Probing of total yeast DNA with ^{32}P-labeled pBR322 DNA (a) and SalI/HindIII *ars* fragment of pHMR22 DNA (b). About 5 µg of each DNA preparation was digested with restriction enzymes and electrophoresed in 0.7% agarose gel. *C. utilis* DNA was digested with EcoRI (lane A). BamHI (lane B), HindIII (lane C), EcoRI/BamHI (lane D), HindIII/BamHI (lane E) and EcoRI/BamHI/HindIII (lane F). *S. cerevisiae* (lane G), *C. albicans* (lane H),and *P. tannophilus* (lane I) DNAs were restricted with EcoRI. Lane J was blank and lane K contained 1 µg of BamHI restricted pHMR22. Blot hybridization was carried out under low stringent conditions as described in Materials and Methods.


A B C D E F G H I J K
A B C D E F G H I J K
[a]
[b]

very low stringency hybridization conditions. This result precluded the possibility of the pBR322 sequences which flank the *ars* cross-hybridizing with yeast chromosomal DNAs. Tracks G, H and I of Fig. 5B are EcoRI cleaved DNAs of *S. cerevisiae*, *C. albicans*, and *P. tannophilus*; none of these showed homology to the cloned *ars* of *C. utilis* even under conditions that favor the formation of mismatched hybrid. Tracks A to F are the total DNA of *C. utilis* cleaved with different restriction enzymes. Surprisingly, there were two bands seen in lane C, i.e. when total DNA was cleaved with HindIII, and only one band was present in lanes A, B, D, E and F (Fig. 5B) where the DNA was treated with other restriction enzymes individually or in combination. These results indicated that the cloned *ars* hybridized with two different fragments of *C. utilis* genome DNA (see Fig. 6 and Discussion below).

Fig. 6. Restriction maps of C. utilis DNA flanking cloned ars DNA in pHMR22. (a) and (b) show two different fragments of C. utilis DNA containing ars. Restriction maps are deduced from the hybridization experiments (Fig. 5). The distance between restriction enzyme sites shown is relative. Restriction enzyme sites are as follows: EcoRI (+), HindIII (X), BamHI (Δ), and Sau3A (O). Not all Sau3A sites are shown.


(a)
ars
(b)
ars

DISCUSSION

The results of this study show that several DNA fragments of C. utilis have been cloned into YIp5 and that these DNA fragments confer on YIp5 the ability to replicate autonomously in S. cerevisiae. All of these hybrid plasmids transformed S. cerevisiae (Table 2) and E. coli at high frequency and could be reisolated from transformants without any detectable change in plasmid structure or function. These results suggested that the cloned DNA fragments fit description of ars previously isolated from other eukaryo-tic cells (1,2,21,38,39). To the best of our knowledge this is the first report describing the isolation of an ars from C. utilis.

Ars capable of high-frequency transformations have been reported to occur once in 30-40 kb in the S. cerevisiae chromosome (2,8) and once in 15 kb in the Drosophida melanogaster chromosome (38). The frequency of one replication origin per 36 kb was independently confirmed by the average spacing of initiation site in S. cerevisiae as detected by electron microscopy in small molecules of yeast chromosomal DNA (27). Our results, suggesting the presence of one ars in 30 kb, are consistent with the demonstrated frequency of arss in S. cerevisiae genome, but the number of clones analyzed by us is too small to consider the data conclusive. The observations of high-frequency transformation, extrachromosomal copies of the ars-YIp5 plasmid, and coincident frequency between the

ars in *C. utilis* and the replication origin in *S. cerevisiae* strongly suggest that the *ars* sequences we isolated by high-frequency transformation include the origins of replication of *C. utilis* genome. This conclusion is supported by the recent finding of Celniker and Campbell (5) that *ars*1 and *ars*2 in *S. cerevisiae* are specific origins of chromosomal replication.

Initiation of DNA replication occurs at multiple internal sites in the chromosomes, and these sites may be activated at different times during the replication phase of the cell cycle (28). If the origins share DNA sequence homology, then the *ars* should hybridize to several yeast chromosomal DNA fragments. We have shown that the cloned *ars* from *C. utilis* only hybridizes to two fragments of *C. utilis* genome even under low stringency hybridization conditions. This suggests that the cloned *ars* is unique or shares limited homology with other replicator sequences. The nucleotide sequence responsible for the replication of several *S. cerevisiae ars* has been studied in detail (37,41,42). Extensive homologies have not been observed among *ars*1 (42), *ars*2, *ars*3 and 2 μ DNA (17,42). A canonical shared sequence, TAAAPyAPyAAPu, is present in *ars*1, *ars*3 and 2 μ DNA but absent in *ars*2. The significance of this canonical sequence is, therefore, unclear. This is in striking contrast to the comparison between *ori* sequences of different bacterial species where remarkable sequence homologies are evident (44). Apparently, the DNA

sequences corresponding to *ars* function are very diverse. This conclusion is supported by the findings of this study that little homology exists between the present cloned *ars* and other replicator(s) in *C. utilis* genome. Sequence diversity may reflect many different classes of replicators in one yeast which are temporally and coordinately activated by respective signals during S phase. Although definitive information is not available at this time, results of several previous investigators suggested that, in *S. cerevisiae*, all DNA initiation sites are not activated simultaneously (28,29,30) and some of the *ars* are reiterated in the genome (7,8).

Non-yeast DNA can allow autonomous replication of a yeast integration plasmid in *S. cerevisiae* (22,38,43). Sequence homology between one of these DNAs (the *ars* of *Tetrahymena* found in rDNA) and *S. cerevisiae* *ars*1, *ars*2, and the replication origin of 2 μ circle have been investigated (22). These results showed that no extensive homology exists among these DNA replicators. Extensive homology between the cloned *ars* of *C. utilis* and sequences in the *S. cerevisiae* genome was not detected by hybridization experiments in our study (Fig. 5B, Track G), even though the *ars* exhibits high-frequency transformation in the latter yeast. Lack of sequence homology between the cloned *ars* of *C. utilis* and the *ars* of *S. cerevisiae* was also indicated by their respective restriction enzyme maps. The essential region of the *S. cerevisiae* *ars*1 DNA resides

at or near a PstI site (37,41,42), whereas cloned ars of pHMR22 does not have this site (Fig. 4). The only similarity between ars1 and the cloned ars described in this paper is that the functional DNA sequences in both these arss contain a Sau3A site. In contrast, ars2 (42) does not have a Sau3A site in the region required for its function. However, neither ars2 nor the ars cloned in pHMR22 have a PstI site. All of these data indicate that there is too little homology between DNA sequences corresponding to the ars of C. utilis and S. cerevisiae to be detected by standard hybridization procedures. These results extend the recent findings of Kiss et al. (22) that S. cerevisiae "initiation proteins" can recognize sequences corresponding to heterozygous replication origins.

The BamHI site almost always disappears after a MboI/BamHI ligation (25). However, we did find a BamHI site in pHMR22 which was constructed by MboI/BamHI ligation. The restriction map of C. utilis genome DNA in the area of the ars (Fig. 6) based on hybridization experiments (Fig. 5) revealed that there is no BamHI site in the ars before cloning. Thus, we believe that the BamHI site in the cloned ars was created from a MboI/BamHI ligation and should be at the junction of YIp5 and cloned DNA (Fig. 4).

Availability of unique cloning site(s) is one of the requirements for an ideal cloning vector. In addition, the smaller the cloning vector the greater is the amount of foreign DNA that can be cloned into it, at least on

theoretical grounds. The constructed plasmid, pHMR22, offers several advantages. It is small in size (6.6 kb) and therefore a significant amount of DNA can be cloned into it. It has unique restriction sites for BamHI, SalI, HindIII, EcoRI and PvuII restriction endonucleases and these sites are not located in the region of the ars which is essential for its function. These features make pHMR22 a useful vector for cloning desired genes in S. cerevisiae and perhaps in C. utilis when a selectable marker for this yeast becomes available.

We observed that the chromosomal DNA of C. utilis digested with HindIII gives two bands which hybridized with the cloned ars (Fig. 5B, track C) even though there is no HindIII site in the ars (Fig. 6). This result is consistent with the suggestion that the organism is diploid, and with HindIII site polymorphism on the two homologues bearing the ars. However, if the ars is randomly repeated in the genome, either tandemly with a site for HindIII between the two copies, or separately as a part of a larger homologous sequence, the same result might be obtained. In principle, probing with a number of separate clones might give evidence for a ploidy higher than one if all gave two or more bands.

In conclusion, we have for the first time constructed a new cloning vector (pHMR22) containing an ars from C. utilis. The cloned ars is fully functional in S. cerevisiae although there is no demonstrable homology

between the *ars* DNA and *S. cerevisiae* genome DNA. The fact that pHMR22 is relatively small in size (6.6 kb) and that its *ars* has unique sites for several restriction enzymes frequently used in genetic cloning work makes it a useful vector for cloning the desired genes both in *S. cerevisiae* and *C. utilis* and for the comparative study of *ars* in yeasts.

ACKNOWLEDGMENTS

This research was supported in part by a grant from the Michigan Agricultural Experiment Station. We acknowledge Drs. Jerry Dodgson and Mary Clancy for helping with the techniques, R. W. Davis for supplying the plasmids YIp5 and YRp12 and S. cerevisiae YNN27, and Novo Laboratories for donating Novozym.

LITERATURE CITED

1. Beach, D., and P. Nurse. 1981. High-frequency transformation of the fission yeast Schizosaccharomyces pombe. Nature 290:140-142.

2. Beach, D., M. Piper, and S. Shall. 1980. Isolation of chromosomal origins of replication in yeast. Nature 284:185-187.

3. Beggs, J. D. 1978. Transformation of yeast by a replicating hybrid plasmid. Nature 275:104-108.

4. Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:75-93.

5. Celniker, S. E., and J. L. Campbell. 1982. Yeast DNA replication in vitro: initiation and elongation events mimic in vivo processes. Cell 31:201-213.

6. Chaconas, G., and J. H. van de Sande. 1980. 5'^{32}P-labeling of RNA and DNA restriction fragments. Methods Enzymol. 65:75-85.

7. Chan, C.S.M., G. Maine, and B. K. Tye. 1982. Functional components of the Saccharomyces cerevisiae chromosomes - replication origins and centromeric sequences. ICN-UCLA Symp. Mol. Cell. Biol. 22:455-472.

8. Chan, C.S.M., and B. K. Tye. 1980. Autonomous replicating sequences in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 77:639-6333.

9. Chevallier, M. R., J. C. Bloch, and F. Lacroute. 1980. Transcriptional and translational expression of a chimeric bacterial-yeast plasmid in yeast. Gene 11:11-19.

10. Clark-Walker, G. D. 1972. Isolation of circular DNA from a mitochondrial fraction from yeast. Proc. Natl. Acad. Sci. USA 69:388-392.

11. Engel, J. D., and J. B. Dodgson. 1981. Histone genes are clustered but not tandomly repeated in the chicken genome. Proc. Natl. Acad. Sci. USA 78:2856-2860.

12. Giudice, L. D., K. Wolf, P. Sassone-Corsi, and A. Mazza. 1979. 2 μm covalently closed non-mitochondrial circular DNA in the petite-negative yeast Schizosaccharomyces pombe. Mol. Gen. Genet. 172:165-169.

13. Guerineau, M., C. Grandchamp, C. Paoletti, and P. Slonimski. 1971. Characterization of a new class of circular DNA molecules in yeast. Biochem. Biophys. Res. Commun. 42:550-557.

14. Gunge, N., A. Tasuko, F. Ozawa, and F. Sakaguchi. 1981. Isolation and characterization of linear deoxyribonucleic acid plasmids from Kluyveromyces lactis and the plasmid-associated killer character. J. Bacteriol. 145:382-390.

15. Han, Y. W., and A. W. Anderson. 1975. Semisolid fermentation of rye grass straw. Appl. Microbiol. 30:930-934.

16. Hang, Y. D., D. F. Splittstoesser, and R. L. Lanschoot. 1972. Sauerkraut waste: a favorable medium for cultivating yeasts. Appl. Microbiol. 24:1007-1008.

17. Hartley, J. L., and J. E. Donelson. 1980. Nucleotide sequence of the yeast plasmid. Nature 286:860-865.

18. Hinnen, A., J. B. Hicks, and G. R. Fink. 1978. Transformation of yeast. Proc. Natl. Acad. Sci. USA 75:1929-1933.

19. Hollenberg, C. P., P. Borst, and E.F.J. van Bruggen. 1970. Mitochondrial DNA. V. A 2 μ closed circular duplex DNA molecules in wild-type yeast mitochondria: structure and genetic complexity. Biochim. Biophys. Acta 209:1-15.

20. Holmes, D. S., and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114:193-197.

21. Hsiao, C., and J. Carbon. 1979. High frequency transformation of yeast by plasmids containing the cloned yeast ARG4 gene. Proc. Natl. Acad. Sci. USA 76:3829-3833.

22. Kiss, G. B., A. A. Amin, and R. E. Pearlman. 1982. Regions of Tetrahymena rDNA allowing autonomous replication of plasmids in yeast. ICN-UCLA Symp. Mol. Cell. Biol. 22:607-614.

23. Laskey, R. A., and D. Mills. 1977. Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitized film. FEBS Letts. 82:314-316.

24. Lawford, G. R., A. Kligerman, and T. Williams. 1979. Production of high-quality edible protein from Candida yeast grown in continuous culture. Biotechnol. Bioeng. 21:1163-1174.

25. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

26. Moreton, R. S. 1978. Growth of Candida utilis on enzymatically hydrolyzed potato waste. J. Appl. Bacteriol. 44:373-382.

27. Newlon, C. S., and W. Burke. 1980. Replication of small chromosomal DNAs in yeast. ICN-UCLA Symp. Mol. Cell. Biol. 19:399-409.

28. Newlon, C. S., T. D. Petes, L. M. Hereford, and W. L. Fangman. 1974. Replication of yeast chromosomal DNA. Nature 247:32-35.

29. Petes, T. D., C. S. Newlon, B. Byers, and W. L. Fangman. 1974. Yeast chromosomal DNA: size, structure, and replication. Cold Spring Harbor Symp. Quant. Biol. 38:9-16.

30. Petes, T.D., and D. H. Williamson. 1975. Fiber autoradiography of replicating yeast DNA. Expt. Cell. Res. 95:103-110.

31. Phillipchuk, G. E., and H. Jackson. 1979. Rapeseed oil meal as a nitrogenous substrate for microbial fermentation. J. Gen. Appl. Microbiol. 25:117-125.

32. Rigby, P. W., M Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling DNA to high specific activity in vitro by nick translation with DNA polymerase 1. J. Mol. Biol. 113:237-251.

33. Scherer, S., and R. W. Davis. 1979. Replacement of chromosome segments with altered DNA sequence constructed in vitro. Proc. Natl. Acad. Sci. USA 76:4951-4955.

34. Sherman, F., G. R. Fink, and J. B. Hicks. 1981. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

35. Sinclair, J. H., B. J. Stevens, P. Sanghavi, and M. Rabinowitz. 1967. Mitochondrial-satellite and circular DNA filaments in yeast. Science 156:1234-1237.

36. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

37. Stinchcomb, D. T., C. Mann, E. Selker, and R. W. Davis. 1982. DNA sequences that allow the replication and segregation of yeast chromosomes. ICN-UCLA Symp. Mol. Cell. Biol. 22:473-488.

38. Stinchcomb, D. T., M. Thomas, J. Kelly, E. Selker, and R. W. Davis. 1980. Eukaryotic DNA segments capable of autonomous replication in yeast. Proc. Natl. Acad. Sci. USA 77:4559-4563.

39. Struhl, K., D. T. Stinchcomb, S. Scherer, and R. W. Davis. 1979. High frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA 76:1035-1039.

40. Tho-E, A., S. Tada, and Y. Oshima. 1982. 2 μm DNA-like plasmids in the osmophilic haploid yeast Saccharomyces rouxii. J. Bacteriol. 151:1380-1390.

41. Tschumper, K., and J. Carbon. 1980. Sequence of a yeast DNA fragment containing a chromosomal replicator and TRP1 gene. Gene 10:157-166.

42. Tschumper, G., and J. Carbon. 1982. Sequencing and subcloning analysis of autonomously replicating sequences from yeast chromosomal DNA. ICN-UCLA Symp. Mol. Cell. Biol. 22:489-500.

43. Zakian, V. A. 1981. Origin of replication from Xenopus laevis mitochondrial DNA promotes high-frequency transformation of yeast. Proc. Natl. Acad. Sci. USA 78:3128-3132.

44. Zyskind, J. W., N. E. Harding, Y. Takeda, J. M. Cleary, and D. W. Smith. 1982. The DNA replication origin region of the Enterobacteriaceae. ICN-UCLA Symp. Mol. Cell. Biol. 22:13-25.

CHAPTER II

Development of a New Generalized Transformation System for Yeasts.

Abstract

We have described here for the first time a generalized lithium chloride transformation procedure and *kan*^{r}-G418 selection system suitable for the transformation of a number of yeasts belonging to several different genera. This system is useful for the transformation of any G418-antibiotic sensitive yeast and consequently eliminates the need for the construction of stable mutants of known geno -type (e.g., auxotrophs) to serve as the recipient strains. We constructed a broad host-range yeast cloning vector, pHR40, which carries both a 2 μ DNA replication origin and an autonomous replication sequence (*ars*) from *Saccharomyces cerevisiae*, and a *kan*^{r} determinant from *Escherichia coli* transposon Tn601 (Tn903), which codes for an aminoglycoside phosphotransferase that inactivates antibiotics G418 or kanamycin. pHR40 transforms a number of G418-sensitive yeast strains, belonging to several different genera, to G418 resistance with a high frequency.

INTRODUCTION

At the present time genetic cloning systems for yeasts are restricted only to *Saccharomyces cerevisiae*[1,2,3,4,5], *S. carlsbergensis*[6] and *Schizosacharomyces pombe*[7], because suitable cloning vectors and selection systems are not available for the other yeasts. Most genetic cloning systems in yeasts currently employ a cloning vector which carries a gene responsible for a specific step in the biosynthesis of an amino acid[1,2,5,7] or nucleotide[8] and a corresponding auxotrophic recipient strain. However, stable auxotrophs with the desired genotype are generally not available for yeasts other than *Saccharomyces* and *Schizosaccharomyces*. An antibioticsensitive yeast strain could be used as a recipient strain in place of nutritional auxotrophs, provided the yeast cloning vector carries an dominant genetic determinant for an enzyme which inactivates that particular antibiotic.

A number of antibiotic resistance determinants of bacterial origin have been known to be expressed in yeast, but most yeast cells are relatively resistant to these antibiotics[9,10]. On exception is G418, an aminoglycoside antibiotic, which is reported to inhibit the growth of *Saccharomyces*[11]. Jimenez and Davies recently described a transformation system for *S. cerevisiae* in which a cloning vector carrying *kan*^{r} determinant from *Escherichia coli* transposon Tn601 and a recipient yeast strain sensitive to G418 were employed[12]. The yeast transformants obtained

were resistant to high levels of G418. Resistance to G418 in these transformants was shown to be due to the presence of an aminoglycoside phosphotransferase encoded by the *kan*^{r} determinant. These results suggested the possibility that the *kan*^{r}/G418 combination might serve as a generalized selection system for yeasts other than *S. cerevisiae*, especially since this selection should work with any G418-sensitive yeast strain as the recipient, there being no need to construct an appropriate genotype.

High frequency transformation in yeast has been demonstrated with cloning vectors containing 2 μ DNA[2,4,6] or certain yeast chromosomal sequences called autonomous replication sequences (*ars*)[3,5]. Both types of DNA were shown to contain a yeast replication origin[13]. Since *ars* have been shown to have a relatively broad host specificity[8,14,15], we felt that a plasmid containing *ars*, 2 μ DNA sequences and the *kan*^{r} determinant from Tn601 might serve as a generalized cloning vector for transforming G418-sensitive yeasts. The results presented here show that this is indeed the case. The cloning vector constructed, pHR40, has a broad host-range and transforms a wide variety of different G418-sensitive yeast strains to G418 resistance at a high frequency.

Construction of a broad host-range cloning vector containing *kan*^{r}, and 2 μ DNA

Plasmid YRp12 carries a 1.45 kb EcoRI fragment from the *S. cerevisiae* chromosome which contains the *trp*1 and

the *ars1*[6]. The portion of *ars1* which is essential for replica-tion is located within a PstI/EcoRI fragment close to the tetracycline resistance determinant of YRp12[17,18]. Thus, to construct a yeast-*E. coli* "shuttle vector" carrying the 2 μ DNA sequence, *ars1*, *kan*^{r}, and the replication origin of *E. coli*, plasmids YRp12 and pTY14-*kan5* (a plasmid carrying the *kan*^{r} determinant from Tn601 and 2 μ DNA sequences; a gift from Dr. Julius Marmur, Department of Biochemistry, Albert Einstein University, New York) were digested with PstI and the resulting fragments were ligated with T4 DNA ligase (Fig. 1). Total hybrid DNA was used to transform *E. coli* HB101[19]. The transformants resistant to G418 and tetracycline and sensitive to chloramphenicol and ampicillin were selected. A 13.5 kb hybrid plasmid, pHR40, isolated from one of the transformants, has a unique site for the restriction endonuclease BamHI, and transformed *E. coli* HB101 to tetrancycline resistance or kanamycin resistance with equal efficiency (10^5 transformants per μg DNA).

Generalized transformation procedure for yeasts

Eleven yeast strains belonging to several different genera were tested for their sensitivity to G418 and were shown to be highly sensitive to this antibiotic at a concentration of 100 ug/ml (Table 1). This concentration is considerably lower than that used previously for *S. cerevisiae*[12]. This result indicated that the *kan*^{r}-G418

Fig. 1. Construction of the broad host-range yeast cloning vector, pHR40. Plasmids YRp12 and pTY14-kan5 were digested with PstI and then ligated with T4 DNA ligase. The pool of hybrid plasmids was used to transform E. coli HB101. Transformants which were resistant to tetracycline and kanamycin and sensitive to ampicillin and chloramphenicol were selected. The structure of the resulting plasmid, pHR40, was deduced by restriction endonuclease digestion followed by agarose gel electrophoresis (unpublished data). Symbols: ~~~, yeast 2 μ DNA; —|—, EcoRI; —×—, HindIII; —Δ—, BamHI; —↑—, PstI; —●—, SalI; —□—, PvuII. Not all HindIII and SalI sites in pHR40 are shown.

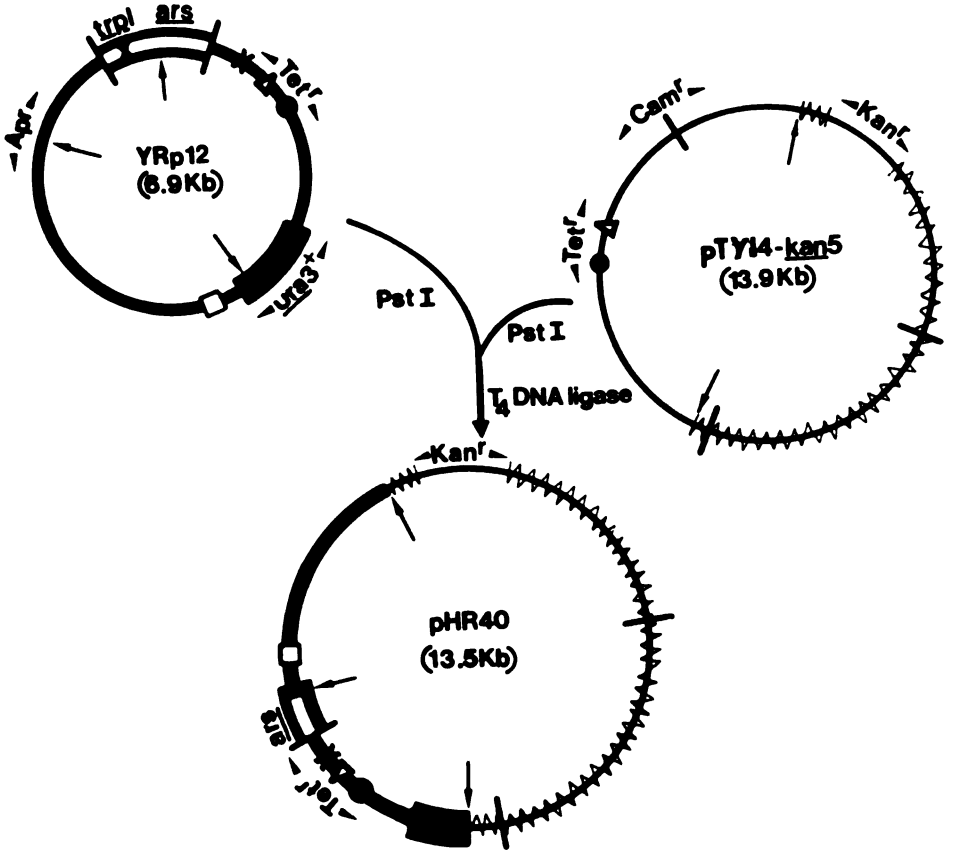

trp1
ars
Ter
Amp
YRp12
(6.9Kb)
ura3
Pst I
Pst I
T4 DNA ligase
Cam
Kan
Ter
pTY14-kan5
(13.9Kb)
Kan
pHR40
(13.5Kb)
ars
Ter

Table 1. Sensitivity of various yeast strains to antibiotic G418[a]

Yeast strains	Concentration of G418 (μg/ml) 0	25	50	100	150	200	300	Recommended[b] G418 concentration (μg/ml)
Candida utilis NRRL Y-900	+	-	-	-	-	-	-	60
C. utilis NRRL Y-1084	+	+	-	-	-	-	-	60
C. lusitaniae NRRL Y-5395	+	+	+	+	+	-	-	200
Torulopsis molischiana NRRL Y-2237	+	+	-	-	-	-	-	60
T. wickerhamii NRRL Y-2564	+	+	+	-	-	-	-	100
Schwanniomyces castellii NRRL Y-2477	+	+	+	-	-	-	-	100
Kluyveromyces cicerisporus NRRL Y-8277	+	+	+	-	-	-	-	100
Pachysolen tannophilus NRRL Y-2460	+	+	-	-	-	-	-	60
Rhodosporidium toruloides ATCC26194	+	+	-	-	-	-	-	60
Schizosaccharomyces pombe NRRL Y-164	+	+	-	-	-	-	-	60
S. cerevisiae YNN27	+	+	+	+	+	+	-	350

[a]Cell were grown at 30°C until stationary phase (5 x 10^8 cells ml^{-1}) in YPD medium[20] and were harvested by centrifugation. One ml of cell suspension was mixed with 20 ml of melted YPD agar (47-50°C), containing various concentrations of G418 as indicated and poured immediately into a sterile Petri dish. The plates were scored for the appearance of G418-resistant colonies after 3 d incubation at 30°C. +, growth; -, no growth.
[b]Desired concentration of antibiotic G418 in the medium for obtaining optimal results in the transformation experiment (Table 2).

Table 2. Transformation of various G418-sensitive yeast strains to G418 resistance with chimeric plasmids harboring *kan*^{r} determinant[a].

Yeast strains	No. of G418-resistant colonies[b]		
	pHR40	pTY14--*kan*5	no DNA
C. utilis NRRL Y-900	5000 (1667)	375 (13)	0
C. utilis NRRL Y-1084	300 (100)	22 (8)	0
C. lusitaniae NRRL Y-5394	89 (30)	14 (5)	30
T. molischiana NRRL Y-2237	3000 (1000)	806 (269)	7
T. wickerhamii NRRL Y-2564	1300 (433)	225 (75)	0
S. castellii NRRL Y-2477	1300 (433)	300 (100)	20
K. cicerisporus NRRL Y-8277	4800 (1600)	94 (31)	8
P. tannophilus NRRL Y-2460	800 (267)	7 (3)	0
R. Toruloides ATCC26194	550 (183)	300 (100)	46
S. pombe NRRL-164	3000 (1000)	600 (200)	0
S. cerevisiae YNN27	3600 (1200)	2531 (844)	60

[a]Cell to be transformed were grown in YPD medium to late exponential phase (1-2 x 10^8 cells per ml), collected by centrifugation, washed with TE buffer (10 mM Tris plus 1mM EDTA, pH 7.5) and resuspended in TEL buffer (TE plus 1 M LiCl). After incubation at 30°C for 1 hr, the cells were centrifuged and resuspended in the appropriate volume of TEL to give 10^9 cells per ml. Plasmid DNA (3µg) was added to 200ul of the cell suspension and after

incubating the mixture at 30°C for 30 min, 1.5 ml of 40% polyethylene glycol 4000 (BDH Chemicals Ltd., Poole, England) was added and the mixture reincubated for 1 hr at 30°C. The cells in the incubation mixture were then heat shocked at 42°C for 5 min, washed twice with sterile distilled water, and reincubated in 200 µl YPD at 30°C for 2 hr. Cells were plated directly on the surface of YPD plate with or without G418. The concentrations of G418 used for each organism is shown in Table 1. G418-resistant transformants on the plates were counted after 2-4 d of incubation.

[b]The number of transformants given was for 3 µg of DNA of the respective plasmid. Data shown in parentheses represent transformation efficiencies (transformants per µg plasmid DNA).

selection system might be useful for the transformation of yeasts other than S. cerevisiae. Plasmids pHR40 and pTY14-kan5 were used to transform yeast cells to G418 resistance. Since the glusulase method[20] gave either a low transformation efficiency or no transformation at all for most of the yeast strains (S. cerevisiae was the exception), we evaluated the suitability of the lithium chloride procedure (K. Murata, personal communication) for yeast transformation.

Our results showed that the use of 0.1 M LiCl in the procedure recommended by Murata works only for S. cerevisiae and S. pombe but not for the transformation of other yeast strains tested (data not shown). Hence, we used 1.0 M LiCl in all the transformation experiments (Table 2). All yeasts, except Candida lusitaniae, transformed with pHR40 gave a much higher number of G418-resistant transformants than that either obtained with pTY14-kan5 DNA or the number of spontaneous mutants observed in the "no DNA" control experiments. These results indicated that the kan^r/G418 selection system coupled with the LiCl transformation procedure is useful for the transformation of a wide-range of G418-sensitive yeasts.

The higher frequency of transformation obtained with pHR40, which contains both ars and 2μ DNA, as compared to pTY14-kan5, which contains only 2μ DNA, presumably reflects the relatively narrow host-range for 2μ DNA whcih has so

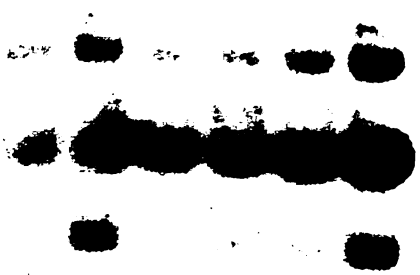
far been found only in Saccharomyces and Schizosaccharomyces[2122,23,24]. The lower transformation efficiency observed with pTY14-kan5 perhaps is also an indication of the instability of this plasmid in organisms phylogenetically distant from Saccharomyces. In contrast to 2μ DNA, arss have been shown to have a broader host specificity. For example, non-yeast DNA containing the arss from a wide variety of eukaryotes as well as the ars from C. utilis were shown to confer on the yeast integration plasmid (YIp5) the ability to replicate autonomously in S. cerevisiae[8,25]. The broad host specificity of the ars is further supported by the finding that pHR40 containing the ars1 of S. cerevisiae is able to transform a wide variety of yeast strains with high efficiency.

Demonstration of pHR40 in yeast transformants

Total DNA was extracted from untransformed C. utilis, Torulopsis molischiana and Pachysolen tannophilus and the same yeasts transformed with pHR40. All the DNAs were fractionated individually by agarose gel electrophoresis, blotted to nitrocellulose paper and hybridized with ^{32}P-labeled pBR322 probe[19]. The results (Fig. 2) show that the labeled pBR322 does not hybridize with untransformed yeast DNA (lane G, H, and I), but hybridized only with the DNA isolated from transformed yeasts (lanes A to F). Since the pHR40 contains part of the pBR322 sequence, these results indicate that the resistance of the transformed yeast to G418 is due to the continued presence of the transforming

Fig. 2. Hybridization of total yeast DNA to ^{32}P-labeled pBR322 DNA. Untransformed yeast strains were grown in YPD medium and G418-resistant transformant strains were grown in YPD medium containing G418 at a concentration recommended for each organism (see Table 1). DNA was extracted from yeast as described by Beach and Nurse[26]. Total DNA preparations (10 ug each) from two transformants each of C. utilis (lanes A and B). T. molischiana (lanes C and D), and P. tannophilus (lane E and F), and from untransformed C. utilis (lane G), T. molischiana (lane H) and P. tannophilus (lane I) were fractionated by electrophoresis on 0.7% agarose gels, stained with ethidium bromide, photographed and blotted onto nitrocellulose BA85 (Schleicher and Schuell) as described by Maniatis et al.[19]. α-^{32}P dATP was incorporated into pBR322 DNA by nick translation with E. coli polymerase 1. Blotted filter paper was prehybridized for a minimum of 4 hr at 42^{o}C with hybridization buffer containing 50% (vol/vol) formamide, 1 M NaCl, 10 mM Tris.HCI at pH 8.0, 1 mM EDTA, 0.05 M $Na_4P_2O_7$ at pH 6.8, 5X Denhardt's solution, and 10 µg of poly(rA), and 8 ug of heat-denatured salmon sperm DNA per ml[19]. ^{32}P-labeled pBR322 probe was then added to the hybridization solution and hybridized for 40 hr at 42^{o}C. The hybridized filters were washed twice at ambient temperature for 5 min in a wash buffer (10 mM Tris.HCl, pH 7.5/1 mM EDTA/0.1% sodium dodecyl sulfate/0.1% $Na_4P_2O_7$/ 50 mM NaCl) followed by four 15 min washes with the same buffer at 65^{o}C. Autoradiography was performed at -70^{o}C with Kodak XAR film and intensifying screen[27].

A B C D E F G H I



plasmid pHR40.

To determine whether the plasmid pHR40 can be reisolated from yeast transformants, we transformed E. coli HB101 with the total DNA isolated from C. utilis, T. molischiana and P. tannophilus transformants. Transformed cells were plated on L agar, L agar containing tetracycline (10 μg ml^{-1}) and L agar containing kanamycin (50 μg ml^{-}) [1,19]. No antibiotic-resistant transformants were obtained. There are at least two possible explanations for our failure to isolate plasmid pHR40 from G418-resistant transformants. First, the copy number of the transforming plasmid in host yeasts may be too low to be isolated. The average copy number of pHR40 in host cells may have decreased dramatically due to spontaneously occurring G418-resistant mutants among the transformants. A second possibility is that a recombination has occurred between the transforming plasmid and the yeast genome and as a result the recombinant plasmid lost its ability to transform E. coli. These possibillities are currently being investigated.

In conclusion, we have described here for the first time a generalized transformation system which utilizes kan^{r}-G418 selection and the LiCl transformation procedure. This system is useful for the transformation of any G418-sensitive yeast and consequently eliminates the need for the construction of stable mutants of known genotype (e.g., auxotrophs) to serve as the recipient strains. The data

indicate that pHR40 transforms a number of different yeast genera. However, in spite of its broad host-range, pHR40 does not appear to be the most desirable vector for gene transfer because it is unstable in the host yeast. We do not know whether deletion of some part of the pHR40 (e.g., 2 μ DNA) could be carried out without affecting the transformation efficiency. If this can be done, the determination of stability and copy number of the resulting plasmid would be of considerable interest.

Acknowledgements

We acknowledge P.T. Magee for his invaluable counsel, R.W. Davis for supplying YRp12, J. Marmur for supplying pTY14-*kan*5, the Northern Regional Research Laboratory, Peoria, Illinois, U.S.A. for supplying the yeast cultures, and Schering Corporation (60 Orange St., Bloomfield, N.J.) for donating the antibiotic G-418 sulfate.

REFERENCES

1. Hinnen, A., Hicks, J.B. & Fink, G.R. Proc. Natl. Acad. Scil. USA 75, 1929-1933 (1978).

2. Beggs, J.D. Beggs, J.D. Nature 275, 104-108 (1978).

3. Beach, D., Piper, M. & Shall, S. Nature 284, 185-187 (1980).

4. Broach, J.R., Stralhern, J.N. & Hicks, J.B. Gene 8, 121-133 (1979).

5. Hsiao, C. & Carbon, J. Proc. Natl. Acad. Sci. USA 76, 3829-3833 (1979).

6. Broach, J.R. & Hicks, J.B. Cell 21, 501-508 (1980).

7. Beach, D. & Nurse, P. Nature 290, 140-142 (1981).

8. Stinchcomb, D.T., Thomas, M., Kelly, J., Selker, E., & Davis, R. W. Proc. Natl. Acad. Sci. USA 77, 4559-4563 (1980).

9. Cohen, J.D., Eccleshall, T.R., Needleman, R.B., Federeff, H., Buchferer, B.A. & Marmur, J. Proc. Natl. Acad. Sci. USA 77, 1078-1082 (1980).

10. Hollenberg, C.P., Kustermann-Kuhn, B., Mackedonski, V. & Erhart, E. Alfred Bengon Symp. 16, 109-120 (1980).

11. Daniels, P.J.L., Yehaskel, A.S. & Morton, J.B. 13th Interscience Conf. Anti-Microbial Ag. Chemother., Washington, Abstr. 137 (1973).

12. Jimenez, A. & Davies, J. Nature 287, 869-871 (1980).

13. Celniker, S.E. & Campbell, J.L. Cell 31, 201-213 (1982).

14. Kiss, G.B., Amin, A.A. & Pearlman, R.E. ICN-UCLA Symp. Molec. Cell Biol. 22, 607-614 (1982).

15. Zakian, V.A. Proc. Natl. Acad. Sci. USA 78, 3128-3132 (1981).

16. Struhl, K., Stinchcomb, D.T., Scherer, S. & Davis, R.W. Proc. Natl. Acad. Sci. USA **76**, 1035-1039 (1979).

17. Tschumper, G. & Carbon, J. Gene **10**, 157-166 (1980).

18. Stinchcomb, D.T., Stuhl, K. & Davis, R.W. Nature **282**, 39-43 (1979).

19. Maniatis, T., Fristsch, E.F. & Sambrook, J. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

20. Sherman, F., Fink, G.R. & Hicks, J.B. Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1981).

21. Grerineau, M., Grandchamp, C., Paoletti, C. & Slonimski, P. Biochem. Biophys. Res. Commun. **42**, 550-557 (1971).

22. Toh-E. A., Tada, S. & Oshima, Y. J. Bacteriol. **151**, 1380-1390 (1982).

23. Gunge, N., Tasuko, A., Ozawa, F. & Sakaguchi, F. J. Bacteriol. **145**, 382-390 (1981).

24. Giudice, L.D., Wolf, K., Sassone-Corsi, P. & Mazza, A. Mol. gen. Genet. **172**, 165-169 (1979).

25. Hsu, W.H., Magee, P.T., Magee, B.B. & Reddy, C.A. J. Bacteriol. (Submitted, 1983).

26. Beach, D. & Nurse, P. Nature **290**, 140-142 (1981).

27. Laskey, R.A. & Mills, D. FEBS Lett. **82**, 314-316 (1977).

CHAPTER III

Construction of New Cloning Vectors for Gene Transfer in *Saccharomyces cerevisiae*

Abstract

We have constructed several new, relatively small, yeast-*Escherichia coli* shuttle vectors which carry the 2 u DNA replication origin, an autonomous replication sequence (*ars*), and a centromere gene (*cen*3), either singly or in combination, from *Saccharomyces cerevisiae*. Each of the vectors additionally carries a kanamycin determinant from the *E. coli* transposon Tn601 (Tn903) which codes for an aminoglycoside phospotransferase that inactivates the antibiotics G418 and kanamycin. All the newly constructed vectors contain a unique restriction site for BamHI, transform G418-sensitive yeast strains to G418 resistance with a high frequency and replicate autonomously in the host yeast. The transformation efficiency was 10^3-10^4 transformants per µg DNA. It appears that the smaller the molecular size of the vector the greater is its mitotic stability in *S. cerevisiae*. The presence of a centromere element (*cen*3) appears to further enhance the mitotic stability of the vector (pHR2).

INTRODUCTION

The recent advances in recombinant DNA methodology have led to a rapid increase in investigations on yeast genetics and have made possible the detailed analysis and manipulation of a number of genes in *Saccharomyces cerevisiae* at the molecular level (1,3,4,9,12,15). These cloning techniques have been used to isolate and amplify the desired DNA sequences for basic genetic studies (12,21,24) and for cloning industrially important genes (11). However, most of the yeast cloning systems are presently restricted to specific yeast genotypes (mutants) which have an appropriate genetic marker for yeast transformation (10,12). The lack of stable mutants of known genotype for many industrially important yeasts has limited the application of recombinant DNA methodology, in improving the yield and productivity of various fermentation processes or in constructing new yeast strains capable of producing new products. Therefore, the development of cloning systems suitable for wild type yeast would be very useful.

Jimenez and Davis (18) recently developed a new cloning system for *S. cerevisiae* in which a cloning vector carrying a *kan*^{r} determinant from *Escherichia coli* transposon Tn601 and a G418-sensitive recipient yeast strain were employed. The *kan*^{r} determinant codes for an aminoglycoside phosphotransferase that inactivates the aminoglycoside antibiotics G418 and kanamycin. Since

selection based on drug resistance would eliminate the requirement for the construction of a stable yeast mutant of a specific genotype to serve as the recipient, it appeared that this type of selection system may be valuable for the genetic manipulation of wild type strains of S. cerevisiae and perhaps other industrially important yeasts. Hollenberg (12) constructed a plasmid, pMp81, which carries Tn601 and has been used for the direct transformation of G418-sensitive S. cerevisiae to G418 resistance. However, the usefulness of this plasmid as a vector for cloning yeast genes has not been demonstrated. A plasmid pTY14 which carries a kan^r determinant form the transposon Tn601 has recently been constructed by J. Marmur (unpublished data). However, plasmids carrying the kan^r determinant to date are relatively large in size (12 kb) and, therefore, would permit cloning of only a relatively small amount of foreign DNA. Additionally, these types of plasmids were reported to be mitotically unstable (12,18).

In this paper we describe the construction of several new, relatively small and stable yeast cloning vectors for wild type S. cerevisiae which carry kan^r from Tn601 and stabilizing elements (segments of DNA capable of conferring mitotic stability on the autonomously replicating vectors in yeast) 2 μ DNA, an autonomous replication sequence (ars) and a centromere gene (cen3), either singly or in combination.

MATERIALS AND METHODS

Strains, plasmids and media. The yeast and bacterial strains and plasmids used in this study are listed in Table 1. The yeast medium (YPD) was described by Sherman et al. (22). The LB medium used for growing E. coli was described by Maniatis et al. (20).

DNA preparation. Plasmid DNA was prepared from E. coli as previously described (14). Total yeast DNA from both transformed and untransformed cells was isolated as described earlier (17). DNA fragments to be labeled by nick translation and recloned into other vectors were isolated from agarose gels by electroelution as described by Maniatis et al. (20).

Transformation of yeast and bacteria. Yeast strains and E. coli were transformed as previously described (22). Yeast transformants were selected on plates of YPD containing 350 ug of antibiotic G418 per ml.

Southern transfer and hybridization to nitrocellulose paper. The procedures used for Southern hybridization were described previously (17).

Determination of mitotic stabilities of the hybrid plasmids in yeast. Cells from a single transformant colony were grown in YPD medium for 20 generations at 30°C. Cell density was measured by the absorbance at 600 nm and the cultures were diluted in sterile distilled water to yield 100-500 colonies when suitable aliquots were spread on YPD agar plates. After overnight incubation at 30°C, 96

randomly picked colonies were tested for the presence of the transforming plasmid using the yeast colony hybridization technique (22). Yeast DNA immobilized on nitrocellulose paper was hybridized with ^{32}P-labeled pBR322 DNA by the method described previously (17).

Table 1. Microbial strains and plasmids used and their sources

Host Strains	Plasmids	Plasmid markers	References or source
E. coli HB101	pTY14-*kan*5	*kan*^{r}, *tet*^{r}, *cam*^{r}	Marmur[a]
E. coli JA221	pYE(*cen*3)41	*leu*2+	5
E. coli HB101	YRp12	*trp*1$^+$, *ura*3$^+$	21,23
E. coli HB101	pBR322	*tet*^{r}, *amp*^{r}	2
E. cerevisiae YNN27			23

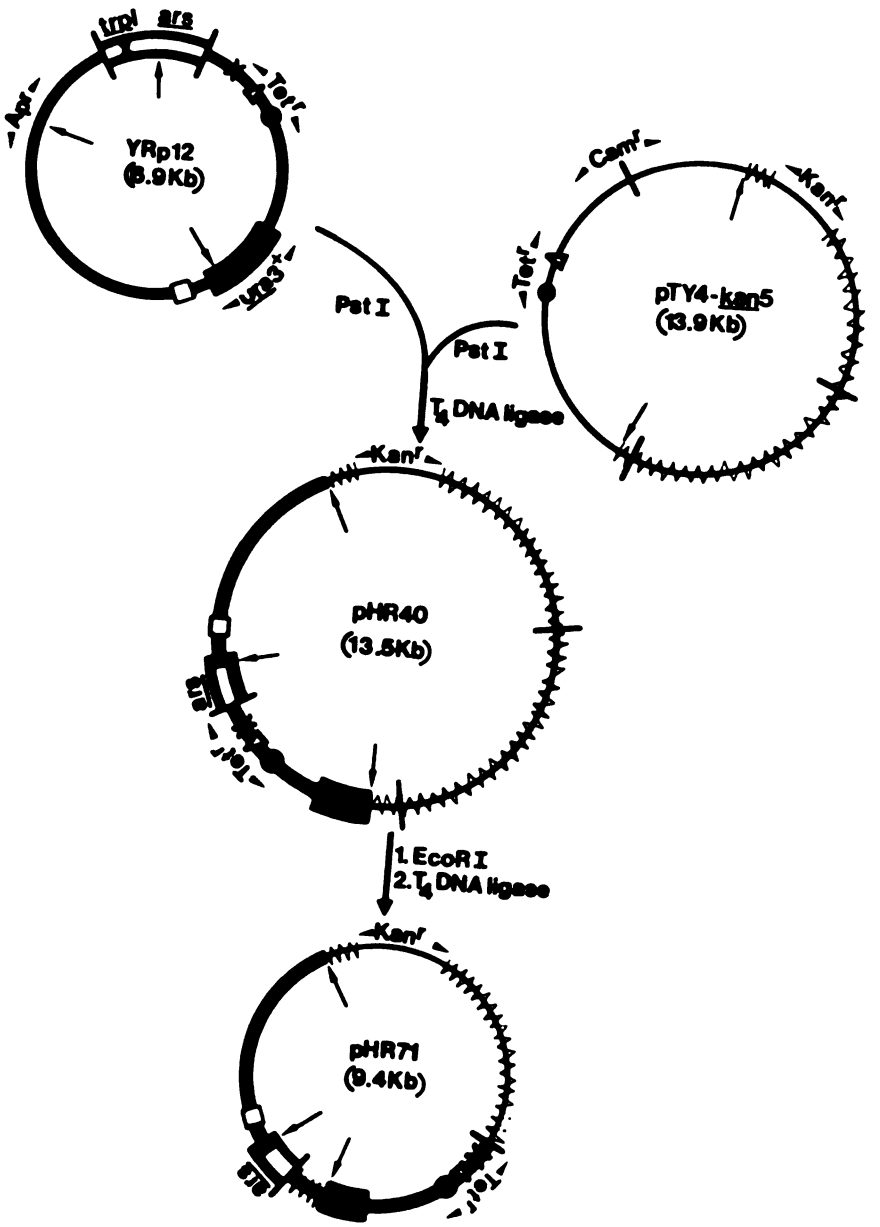
[a]Marmur, J., Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY.

RESULTS

Construction of cloning vectors. Both plasmid pTY14-kan5 and YRp12 (21,23) are S. cerevisiae-E. coli shuttle vectors. pTY14-kan5 contains the E. coli plasmid pBR325, the kan^r determinant from transposon Tn601, and yeast 2 μ DNA (Fig. 1). YRp12 carries functional $ura3^+$, $trp1^+$ and ars1 sequences from S. cerevisiae and the E. coli plasmid pBR322. To construct a new yeast-E. coli shuttle vector containing ars1, 2 μ DNA, the E. coli replication origin and a kan^r determinant, plasmids pTY14-kan5 and YRp12 were digested with restriction endonuclease PstI and then ligated with T4 DNA ligase (Fig. 1). The pool of hybrid DNA was then used to transform E. coli HB101 and transformants resistant to tetracycline and kanamycin and sensitive to chloramphenicol and ampicillin were selected. One of the plasmids, pHR40 (Fig. 1), containing DNA fragments from both pTY14-kan5 and YRp12 was isolated and its restriction enzyme map was determined. pHR40 (13.5 kb) was subsequently reduced in size by deleting a 4.1 kb EcoRI fragment which does not contain DNA sequences essential for the selection and replication of this plasmid either in yeast or in E. coli. The resulting smaller plasmid, pHR71 (9.4 kb), carries all the useful determinants originally present in pHR40 (Fig. 1).

It had been reported earlier that the addition of a centromere gene from S. cerevisiae effectively stabilizes the ars plasmid through both mitosis and meiosis in yeast

Fig. 1. Construction of the yeast cloning vectors pHR40 and pHR71. Symbols: ~~~, yeast 2 μ plasmid DNA; —|—, EcoRI; —x—, HindIII; —△—, BamHI; —↑—, PstI; —●—, SalI; —□— pvuII.


trp1
ars
Ampr
Terr
YRp12
(8.9Kb)
trp3+
Pst I
Pst I
T4 DNA ligase
Camr
Kanr
Terr
pTY4-kan5
(13.9Kb)
Kanr
pHR40
(13.5Kb)
ars
Terr
1. EcoR I
2. T4 DNA ligase
Kanr
pHR71
(9.4Kb)
ars
Terr

(5,8,16). Therefore, we constructed a new yeast cloning vector (pHR2; Fig. 2) which contains *ars1*, *cen3* and the *E. coli* replication origin from plasmid pYE(*cen3*)41 (5), and *kan*^{r} and 2 μ DNA from plasmid pTY14-*kan5*. Plasmid pYE(*cen3*)41 and pTY14-*kan5* were digested with EcoRI and then ligated with T4 DNA ligase. The hybrid DNA was used to transform *E. coli* HB101 and transformants resistant to kanamycin and sensitive to tetracycline were selected. One of the several plasmids isolated from *E. coli* transformants was designated pHR2. It contained the desired sequences from pYE(*cen3*)41 and pTY14-*kan5*. To confirm that pHR2 carries a *cen3* element from pYE(*cen3*)41, both these plasmids were cleaved with EcoRI and PstI individually and the resulting fragments were separated by agarose gel electrophoresis. Two DNA sequences, a 7.1 kb EcoRI fragment (Fig. 3, lanes D and F) and a 5.0 kb PstI fragment (Fig. 3, lanes G and H), both of which contain the *cen3* element were generated from the restriction enzyme digestion of either pYE(*cen3*)41 or pHR2. These results clearly indicated the pHR2 carries *cen3* element from pYE(*cen3*)41.

Plasmid pHR31 (9.8 kb) was constructed by deleting a 4.1 kb EcoRI fragment of 2 μ DNA from pTY14-*kan5* (Fig. 2). The orientation of the EcoRI fragment carrying the tetracycline determinant was reversed in the plasmid pHR31, resulting in inactivation of the chloramphenicol determinant. All of the newly constructed plasmids, which contain one or more of the stabilizing elements 2 μ DNA,

Fig. 2. Construction of the yeast cloning vectors pHR2 and pHR31. Symbols are the same as in Fig. 1.

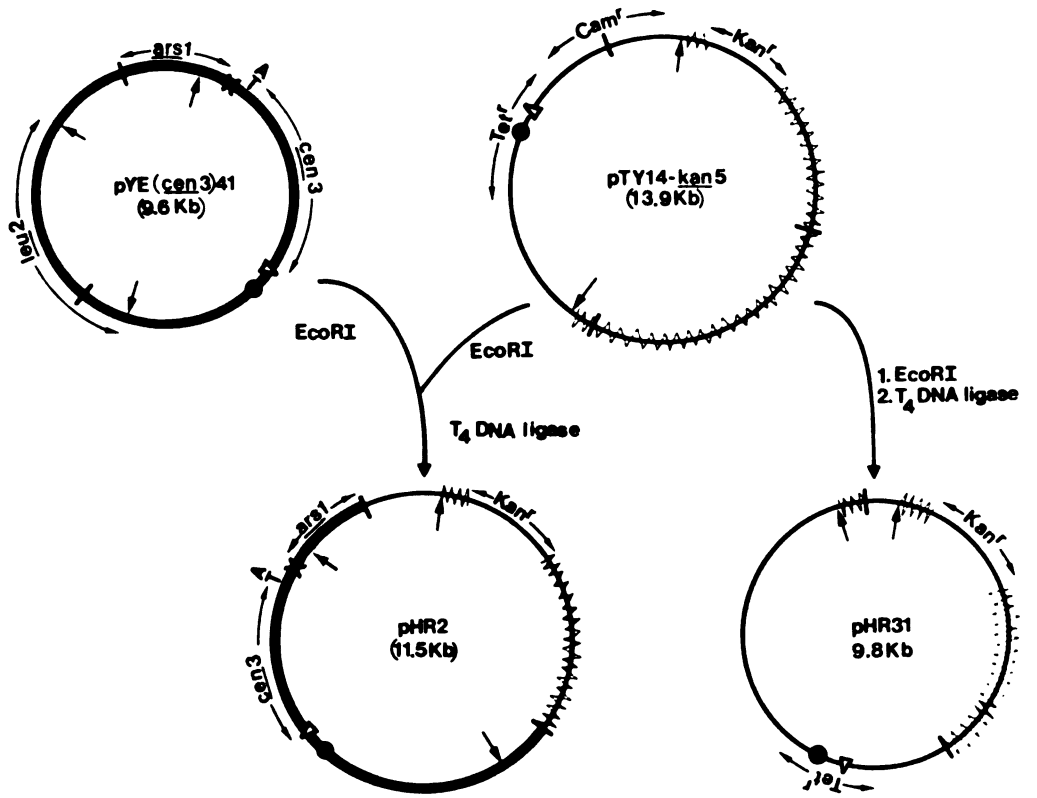
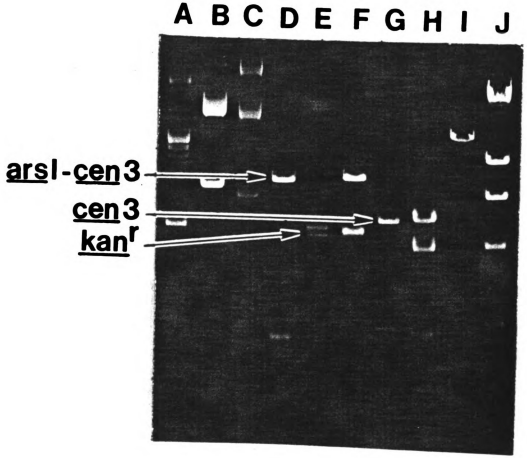

ars1
cen 3
leu2
pYE(cen3)41
(9.6 Kb)
Cam^r
Kan^r
Tet^r
pTY14-kan5
(13.9Kb)
EcoRI
EcoRI
T4 DNA ligase
1. EcoRI
2. T4 DNA ligase
ars1
Kan^r
cen 3
pHR2
(11.5Kb)
Kan^r
pHR31
9.8Kb
Tet^r

Fig. 3. Restriction analysis of the pHR2 DNA containing cen3 gene of S. cerevisiae. pHR2 DNA digested with the indicated restriction enzymes was run on agarose gel parallel to similarly digested pYE(cen3)41 and pTY14-kan5. Lanes A [pYE(cen3)41], lane B (pTY14-kan5), and lane C (pHR2), contained undigested DNAs; lane D [pYE(cen3)41], lane E (pTY14-kan5), and lane F (pHR2), contained DNAs digested with EcoRI; lane G [pYE(cen3)41], and lane H (pHR2), contained DNAs digested with PstI; lane I (pHR2), contained DNA digested with BamHI. Lane J contained size markers of λ DNA-HindIII fragments.


A B C D E F G H I J
arsI-cen3
cen3
kanr

*ars*1 and *cen*3, transform G418-sensitive *S. cerevisiae* (strain YNN27) to G418-resistance with high efficiency (10^3-10^4 transformants per μg DNA).

Mitotic stability of new cloning vectors in S. cerevisiae. The data on mitotic stability of the newly constructed plasmids and that for pTY14-*kan*5 are presented in Table 2. The results suggest that mitotic stability is influenced by the size of the transforming plasmids. Plasmid pTY14-*kan*5 (13.9 kb) which carries a 2 μ stabilizing element is relatively unstable in that 34% of the host cells retain this plasmid after growth on a nonselective medium for 20 generations. Presence of both *ars*1 and 2 μ DNA in the same plasmid, as in pHR 40 (13.5 kb), resulted in no detectable increase in mitotic stability in comparison with that of pTY14-*kan*5. However, plasmids pHR31 and pHR71, which were obtained, respectively, by the deletion of a 4.1 kb EcoRI fragment from pTY41-*kan*5 and pHR40 (Fig. 1 and 2), displayed greater mitotic stability than the parent plasmids, with about 50% of the cells harboring the transforming plasmid after 20 generations (Table 2). Although plasmid pHR2 (11.5 kb), which carries a *cen*3 gene besides *ars*1 and 2 μ DNA elements, is larger than pHR71 (9.4 kb) and pHR31 (9.8 kb), its mitotic stability is comparable to these two plasmids and considerably higher than of pHR40 and pTY14-*kan*5. This result suggested that *cen*3 gene does confer a certain degree of mitotic stability on the plasmid carrying it.

Table 2. Mitotic stability of different cloning vectors in S. cerevisiae[a]

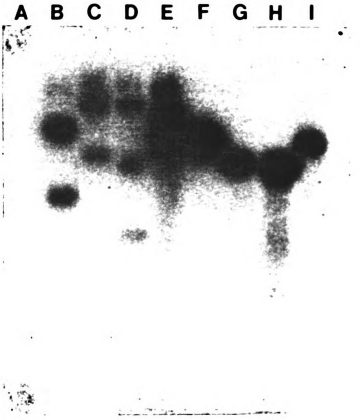
Vectors	Size (kb)	Stabilizing elements	Mitotic stability[b] (%)
pTY14-kan5	13.9	2 μ	34
pHR31	9.8	2 μ	48
pHR40	13.5	ars1, 2 μ	31
pHR71	9.4	ars1, 2 μ	52
pHR2	11.5	cen3, ars1, 2 μ	51

[a]Mitotic stability was determined by the yeast colony hybridization technique (22) as described in Materials and Methods.

[b]Stability is expressed as the precentage of cells containing the transforming plasmid after 20 generations in a non-selective medium.

Autonomous replication of the new cloning vectors in yeast. Several lines of evidence indicate that all the new cloning vectors described here replicate autonomously without integrating into yeast genome. High frequency transformation of S. cerevisiae YNN27 to G418-resistance with the cloning vectors indicates autonomous replication because integration of the plasmids into the yeast chromosome is known to result in a low frequency transformation (9). Furthermore, the pHR plasmids were easily recoverable from E. coli transformed with total DNA isolated from the yeast transformants. Yeast integration plasmids, such as YIp5 (23), are not expected to behave in this way (9). The restriction map of pHR plasmids recovered from E. coli revealed that these plasmids are structurally intact after yeast transformation. Southern blot hybridization was then employed to demonstrate the presence of autonomously replicating pHR plasmids in the G418-resistant yeast transformants. Total yeast DNA was prepared from the transformed and untransformed S. cerevisiae YNN27 and each sample was completely digested with BamHI. All the pHR plasmids have a single restriction site for BamHI. The digested DNA samples were fractionated on an agarose gel, blotted onto nitrocellulose paper and probed with ^{32}P-labeled pBR322 (Fig. 4). DNA from untransformed YNN27 (control) did not show hybridization with the pBR322 DNA (lane A). However, DNA from the transformants contained bands homologous to pBR322 with electrophoretic mobility

Fig. 4. Hybridization of total yeast DNA to ^{32}P-labeled pBR322 DNA. Total DNA preparation (10 µg each) from untransformed yeast (lane A) and yeast transformed with pHR40 (lane B and F), pHR71 (lane C and G), pHR31 (lane D and H), and pHR2 (lane E and I) were fractionated by electrophoresis on an agarose gel, blotted onto nitrocellulose and hybridized with ^{32}P-labeled pBR322 DNA under high stringency conditions. Lanes B, C, D and E represent uncut DNA and lanes F, G, H and I represent DNA completely digested with BamHI.


A B C D E F G H I

corresponding to the supercoiled, open circular and multimer forms of the transforming plasmids (lane B, C, D and E). Restriction of DNA from the transformed yeast with BamHI showed a single band of hybridization with pBR322 DNA indicating that the plasmids are not integrated into the S. cerevisiae chromosomal DNA (lane F, G, H and I).

DISCUSSION

The mitotic stability of a composite plasmid under nonselective conditions is reported to be an indication of the copy number of that plasmid (1,12), because composite plasmids maintained at a high copy number per cell are segregated out less frequently that those that have a low copy number. The copy number of a composite plasmid, carrying *ars*1 or 2 μ DNA as the stabilizing elements, was shown to increase by decreasing the size of this plasmid (12,27). Thus, the higher stability of pHR31 and pHR71, as compared to pTY14-*kan*5 and pHR40, may probably be attributed to their relatively small size (Table 2) and consequently higher copy number.

Plasmids which contain *ars* and *cen*3 as stabilizing elements were previously shown to be stably maintained in host yeast for many generations even in the absence of selection pressure (5,8,16). pHR2 which was shown to contain stabilizing elements 2 μ, *ars*1 and *cen*3, as evidenced by restriction enzyme digestion and agarose gel electrophoresis (Fig. 3), was relatively stable in host yeast, with 51% of the cells carrying this plasmid after 20 generations in a nonselective medium (Table 2). This stability value is somewhat lower than that previously reported (5,8) for *ars*-*cen* plasmids (60-100% of stability). The reason for the lower mitotic stability of pHR2 is not

clear, since it has been shown previously that cen3 plays a dominant role in determining the mitotic stability of a cloning vector containing either ars-cen3 or 2 μ-cen3 (16). Earlier studies (1,25) showed that certain DNA sequences on a plasmid may be responsible for the lower mitotic stability of that plasmid (e.g., pBR325 sequence on the plasmid pMP78). Thus, the relatively lower mitotic stability of pHR2 may also be due to certain DNA sequences (such as the kan^r determinant) on this plasmid. Additional features like strain specificity of the host yeast (5,12) and structures of the chimeric plasmid have been reported to be of importance in determining mitotic stability (1,12). It would be of interest to delete certain nonessential DNA sequences from pHR2 and look for the stability of the plasmid obtained in several different strains of G418-sensitive S. cerevisiae.

Drug resistance determinants such as kan^r, are capable of functioning in a wide variety of bacterial (7) and yeast species (6,12,13,18). The ars element of eukaryotes was also reported to be widely distributed and have a broad host range. For example, the arss from several phylogenetically distant eukaryotes were known to confer on a yeast integration plasmid (YIp5) the ability to replicate autonomously in S. cerevisiae (19,23,26). A question of considerable interest is whether plasmids pHR71 and pHR2 which contain ars1 and kan^r would serve as broad host-range cloning vectors for a wide variety of G418-sensitive yeasts

other then S. cerevisiae. Studies are being undertaken to test this possibility.

The new cloning vectors, pHR31, pHR71 and pHR2 are relatively small in size, contain a unique BamHI site, transform G418-sensitive yeast strains with a high efficiency, replicate autonomously in the host yeast without losing any of the useful functional determinants and are relatively stably maintained. These properties make the pHR plasmids useful vectors for cloning genes in industrially important G418-sensitive S. cerevisiae strains.

LITERATURE CITED

1. Beggs, J.D. 1981. Multiple-copy yeast plasmid vectors. Alfred Benzon Symp. 16:383-395.

2. Bolivar, F., R.L. Rodriguez, P.J. Greene, M.C. Betlach, H.L. Heynecker, H.W. Boyer, J.H. Crosa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:75-93.

3. Broach, J.R., and J.B. Hicks. 1980. Replication and recombination functions associated with the yeast plasmid 2 μ circle. Cell 21:501-508.

4. Broach, J.R., J.N. Strathern, and J.B. Hicks. 1979. Transformation in yeast: development of a hybrid cloning vector and isolation of the CAN1 gene. Gene 8:121-133.

5. Clarke, L., and J. Carbon. 1980. Isolation of a yeast centromere and construction of a functional small circular chromosome. Nature 287:504-509.

6. Cohen, J.D., T.R. Eccleshall, R.B. Needleman, H. Federoff, B.A. Buchferer, and J. Marmur. 1980. Functional expression in yeast of the Escherichia coli plasmid gene coding for chloramphenicol acetyl transferase. Proc. Natl. Acad. Sci. USA 77:1078-1082.

7. Ehrlich, S.D., B. Niaudet, and B. Michel. 1982. Use of plasmids from Staphylococcus aureus for cloning of DNA in Bacillus subtilis. Curr. Top. Microbiol. Immunol. 96:19-29.

8. Fitzgerald-Hayes, M., L. Clarke, and J. Carbon. 1982. Nucleotide sequence comparisons and functional analysis of yeast centromere DNAs. Cell 29:235-244.

9. Hinnen, A., J.B. Hicks, and G.R. Fink. 1978. Transformation of yeast. Proc. Natl. Acad. Sci. USA 75:1929-1933.

10. Hinnen, A., and B. Meyhack. 1982. Vectors for cloning in yeast. Curr. Top. Microbiol. Immunol. 96:101-117.

11. Hitzeman, R.A., F.E. Hagie, H.L. Levine, D.V. Goeddel, G. Ammerer, and B.D. Hall. 1981. Expression of a human gene for interferon in yeast. Nature 293:717-722.

12. Hollenberg, C.P. 1982. Cloning with 2 um DNA vectors and the expression of foreign genes in Saccharomyces cerevisiae. Curr. Top. Microbiol. Immunol. 96:119-144.

13. Hollenberg, C.P., B. Kustermann-Kuhn, J. Mackedonski, and E. Erhart. 1980. The expression of bacterial antibiotic resistance genes in the yeast Saccharomyces cerevisiae. Alfred Benzon Symp. 16:109-120.

14. Holmes, D.S., and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114:193-197.

15. Hsiao, C., and J. Carbon. 1979. High frequency transformation of yeast by plasmids containing the cloned yeast ARG4 gene. Proc. Natl. Acad. Sci. USA 76:3829-3833.

16. Hsiao, C., and J. Carbon. 1981. Characterization of a yeast replication origin (ars2) and construction of stable minichromosome containing cloned yeast centromere DNA (CEN3). Gene 15:157-166.

17. Hsu, W.H., P.T. Magee, B.B. Magee, and C.A. Reddy. 1983. Construction of a new yeast cloning vector containing autonomous replication sequences from Candida utilis. J. Bacteriol. (Submitted).

18. Jimenez, A., and J. Davis. 1980. Expression of a transposable antibiotic resistance element in Saccharomyces cerevisiae: a potential selection for eukaryotic cloning vectors. Nature 287:869-871.

19. Kiss, G.B., A.A. Amin, and R.E. Pearlman. 1982. Regions of Tetrahymena rDNA allowing autonomous replication of plasmids in yeast. ICN-UCLA Symp. Mol. Cell. Biol. 22:607-614.

20. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY.

21. Scherer, S., and R.W. Davis. 1979. Replacement of chromosome segments with altered DNA sequence constructed in vitro. Proc. Natl. Acad. Sci. USA 76:4951-4955.

22. Sherman, F., G.R. Fink, and J.B. Hicks. 1981. Methods in yeast genetics, Cold Spring Harbor Laboratory. Cold spring Harbor, NY.

23. Stinchcomb, D.T., M. Thomas, J. Kelly, E. Setker, and R.W. Davis. 1980. Eukaryotic DNA segemts capable of autonomous replication in yeast. Proc. Natl. Acad. Sci. USA 77:4559-4563.

24. Struhl, K., D.T. Stinchcomb, S. Scherer, and R.W. Davis. 1979. High frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA 76:1035-1039.

25. Toh-e, A., P. Guerry-Kopecko, and R.B. Wickner. 1980. A stable plasmid carrying the yeast LEU2 gene and containing only yeast deoxyribonucleic acid. J. Bacteriol. 141:413-416.

26. Zakian, V.A. 1981. Origin of replication from Xenopus laevis mitochondrial DNA promotes high-frequency transformation of yeast. Proc. Natl. Acad. Sci. USA 78:3128-3132.

27. Zakian, V.A., and J.F. Scott. 1982. Construction, replication and chromatin structure of TRP1 R1 circle, a multiple-copy synthetic plasmid derived from Saccharomyces cerevisiae chromosomal DNA. Mol. Cell. Biol. 2:221-232.

APPENDIX

APPENDIX

Alkaline Hydrogen Peroxide Pretreatment of Wheat Straw for Enhancing Cellulase Hydrolysis and Ethanol Production

Summary

The effectiveness of the H_2O_2-NaOH pretreatment (0.5% NaOH plus 0.5% H_2O_2) in enhancing the susceptibility of wheat straw to cellulase digestion was investigated. H_2O_2-NaOH pretreatment of wheat straw for 8 hr at 30°C was very effective in giving saccharification equivalent to 86% of the theoretical maximum. Glucose yields were considerably lower when wheat straw was pretreated with 0.5% NaOH alone. There was no appreciable increase in the efficiency of pretreatment when H_2O_2-NaOH was supplemented with 0.44 mM $FeSO_4$. When wheat straw pretreated with NaOH alone or with H_2O_2-NaOH was saccharified with Trichoderma reesei cellulase, and the resulting hybrolysates were fermented anaerobically with Saccharomyces cerevisiae, ethanol production was appreciably higher from the H_2O_2-NaOH treated wheat straw compared to that treated with NaOH alone. This result in turn indicates that more cellulose is converted to glucose when wheat straw is treated with H_2O_2-NaOH than when treated with NaOH alone. In conclusion, H_2O_2-NaOH pretreatment of wheat straw appears to be relatively faster, more effective and allows enhanced saccharification and greater ethanol yields than those obtained with NaOH treatment alone.

INTRODUCTION

Lignocellulosic biomass is the most abundant renewable resource in the biosphere and is composed of approximately 40-50% cellulose, 20-30% hemicellulose and 15-30% lignin[1]. An estimated 2-3 billion tons of lignocellulosic residues (wet weight) such as wheat straw are produced annually in the United States[2]. Bioconversion of lignocellulosic materials to sugars for the production of fuels and chemical feed stocks have been receiving increasing attention in recent years[3]. However, lignin, which is recalcitrant to biological degradation, exists in close physical and chemical association with cellulose and limits the efficiency and extent of its utilization in various bioconversion processes[4]. Chemically, lignin-carbohydrate bonds form metabiolic blocks that greatly limit the action of microbial cellulases and physically lignin forms a barrier suppressing the penetration by cellulases. To some extent, the degree of crystallinity of the cellulose also limits the extent of its hydrolysis by cellulases[5,6]. Therefore, to increase the yield of sugars from the enzymatic hydrolysis of lignocellulosic materials these substrates must be pretreated to depolymerize or remove lignin and to reduce the crystallinity of cellulose so that more cellulase would be accessible for cellulase action.

Physical, chemical and biological pretreatment processes, either singly or in combination have been used to increase the efficiency of utilization of

lignocellulosic materials. These methods included ball milling[7,8], wet milling[9,10], steam-explosion[11,12], irradiation[13], alkali swelling[14,15], partial acid hydrolysis[16,17], ozonation[18], solvent extraction[19,20] and biological delignification[21]. Many of these processes are, however, relatively expensive, energy intensive or inefficient.

Hydrogen peroxide in alkaline medium is widely used as a bleaching agent in the pulping industry[22,23]. In the absence of a stabilizing agent such as sodium silicate, $NaOH-H_2O_2$ is known to result in extensive delignification and depolymerization of cellulose[23]. Most recently, Forney et al[24] reported that hydroxyl radical, derived from H_2O_2 in a Fenton-type reaction, plays an integral role in lignin degradation by Phanerochaete chrysosporium (a white-rot fungus). Koenig (1975) also reported that hydrogen peroxide plus $FeSO_4$ alters the structure of lignin and also depolymerizes cellulose[25]. These results indicate that H_2O_2 pretreatment may be effective in reducing the lignin content and crystallinity of the cellulose in lignocellulosic biomass. However, there has been little published information to date on utilizing $NaOH-H_2O_2$ for pretreating lignocellulosic materials to enhance the enzymatic hydrolysis of these substrates for obtaining greater yield of sugars or ethanol.

In this paper we investigated the effectiveness of H_2O_2-NaOH pretreatment on the enzymatic hydrolysis of wheat

straw, one of the major lignocellulosic residues generated in the U.S.A. and worldwide[26]. The efficiency of this process is compared with that of NaOH pretreatment process which is one of the more efficient and widely used pretreatment procedures[26,27]. Our results showed that pretreatment with H_2O_2-NaOH will yield more glucose in a shorter time and under milder conditions, for ethanol production as compared to the pretreatment with NaOH alone.

MATERIALS AND METHODS

Substrate

Wheat straw which was chopped to 2.5-5.0 cm length and Wiley-milled to about 40 mesh, prior to pretreatment process, was used as the substrate. The average composition of wheat straw was 10% moisture, 37% cellulose, 65% total carbohydrate and 20% lignin. Sigma Cell, a microcrystalline cellulose was purchased from Sigma Chemical Co. (St. Louis, Missouri) and was used as a substrate in the control experiments for evaluating the efficiency of different preatreatment processes.

Pretreatment and Enzymatic Hydrolysis

Cellulase was prepared by the method of Mandels and Sternberg utilizing *Trichoderma reesei* QM 9414[28]. Wheat straw was pretreated with H_2O_2-NaOH which contained 0 to 1% NaOH and 0 to 0.5% H_2O_2 by weight in different experiments as noted in the results. In some experiments H_2O_2-NaOH was supplemented with 0.44 mM $FeSO_4$. The cellulosic substrate was suspended in the H_2O_2-NaOH solution (0.5 g/45 ml) at 30°C for 24 hr, unless otherwise indicated. Citrate buffer (2.5 ml of a 1 M solution; pH 4.8), containing 0.1% azide as a preservative, was added to this pretreated wheat straw which was adjusted to pH 4.8 with 6 N H_2SO_4. Cellulase (1 filter paper unit/ml) was then added and saccharification was carried out at 50°C for 24 hr.

Alcohol Fermentation

Saccharomyces cerevisiae ATCC 26603, used for

producing ethanol, was maintained on YM agar (0.3% yeast extract, 0.3% malt extract, 0.5% Bacto-peptone, 1% glucose and 2% agar). S. cerevisiae was cultivated in 100 ml of YM broth in a 250 ml foam-stoppered Erlenmeyer flask. The flasks were shaken at 200 rpm on a Gyrotory Water Bath Shaker (Model G76, New Brunswick Scientific Co., N.J.) at 30°C for 24 hr (10^8 cells per ml). The yeast cells were harvested by centrifugation, and were resuspended in sterile distilled water to give 2 x 10^9 cells per ml. This cell suspension was used as the inoculum. The wheat straw hydrolysate was concentrated by a vacuum evaporator to contain 4% (wt/vol) reducing sugars. Fermentation was carried out in a total volume of 50 ml concentrated hydrolysate supplemented with 0.67% yeast nitrogen base (Difco) in 125 ml Erlenmeyer flasks. Each of the flask was covered with a one-holed rubber stopper and a pasteur pipette plugged with cotton was inserted through the hole. The flasks were sterilized by autoclaving, inoculated and were shaken at 200 rpm on a Gyrotory Water Bath Shaker at 30°C for 24 hr. The initial pH was 5.6 and initial cell density was 1 x 10^8 cells per ml.

Analytical Methods

Lignin was determined by a modified Klason technique[29]. Cellulose was determined as described by Updegraff[30] except that the phenol sulfuric acid reagent[31], instead of Anthrone reagent, was used for determining the yield of reducing sugar. Hydrolysis of cellulosic

materials, for total sugar analysis was performed as described by Hrubant et al[32] and the sugar yield was measured as glucose by the dinitrosalicylic acid (DNS) procedure[33]. Glucose concentration was measured using the glucostat diagnostic kits commercially available from Worthington Biochemical Co. (Freehold, New Jersey). Ethanol concentration was determined by a gas chromatograph (Varion Aerograph Series 2400, Varian Co., Sunnyvale, California) with a stainless steel column packed with Chromosorb W (acid washed and 80/100 mesh; Supelco Co., Bellefonte, Pennsylvania) and a flame ionization detector. The carrier gas was N_2 (30 ml/min) and column temperature was set at 80°C. To determine the dry weight loss, samples were filtered through a pre-weighed glass microfibre filter (GF/C, Whatman, New Jersey) and then rinsed with water. The filter with the wheat straw residue was oven-dried at 80°C for 48 hr and reweighed. Delignification as evidenced by increase in absorbance at 280 nm[34] of the treated slurries was measured using a Varian Model 634 spectrophotometer. Percent saccharification was defined for each sample as previously described by Mandels and Sternberg[28]:

$$\% \text{ Saccharification} = \frac{\text{mg glucose (produced)/ml slurry}}{\text{mg substrate/ml slurry}}$$

Cellulase activity was determined by the filter paper assay described by Mandels and Sternberg[28]. One filter paper unit (FPU) is equivalent to one micromole of glucose (determined as reducing sugar) released per min.

RESULTS

Effectiveness of H_2O_2-NaOH Pretreatment as Influenced by Varying H_2O_2 and NaOH Concentration

The effectiveness of the pretreatment of wheat straw was evaluated by determining the percent loss of dry weight, delignification as evidenced by the change in absorbance at 280 nm[34], and the percent saccharification. In a control experiment, wheat straw was soaked in distilled water, instead of alkaline H_2O_2 solution, at 30°C for 24 hr and then treated with cellulase. The dry weight loss, absorbance at 280 nm and percent saccharification of this control sample were 1.5% 1.7 and 2% respectively. As shown in Fig.1a, the delignification of treated materials increased with increasing NaOH concentration as well as with increasing H_2O_2 concentration. In general, delignification was better with H_2O_2-NaOH pretreatment than with NaOH treatment alone. At a given H_2O_2 concentration, the delignification increased at NaOH concentrations between 0.1% and 0.5% but leveled off at concentrations higher than 0.5%. Since delignification with H_2O_2-NaOH has been reported to increase in the presence of heavy metal ions such as copper (II), Mn (II) and Fe (II)[23,35], $FeSO_4$ (final concentration 0.44 mM) was added to the H_2O_2-NaOH reaction mixture. However, no increase in delignification was evident at any of the NaOH or H_2O_2 concentrations tested (data not shown).

One of the common criteria used for evaluating the

Fig. 1. H_2O_2-NaOH pretreatment of wheat straw: effect of NaOH and H_2O_2 concentration on (a) delignification as shown by increase in absorbance at 280 nm, (b) loss of dry weight, and (c) % saccharification. The values were measured after cellulase digestion. Hydrogen peroxide concentrations used were 0.5% (O), 0.10% (▲), 0.01% (●), and none (Δ). Each value plotted in the figures represents mean of three samples.

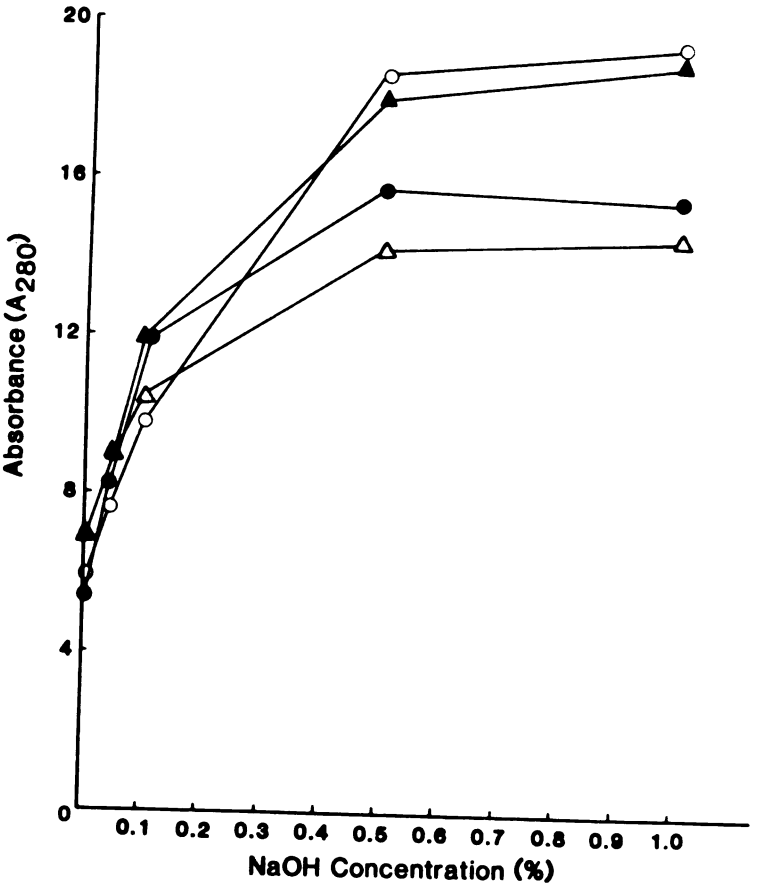

20
16
12
8
4
0
Absorbance (A_{280})
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
NaOH Concentration (%)

Fig. 1a.

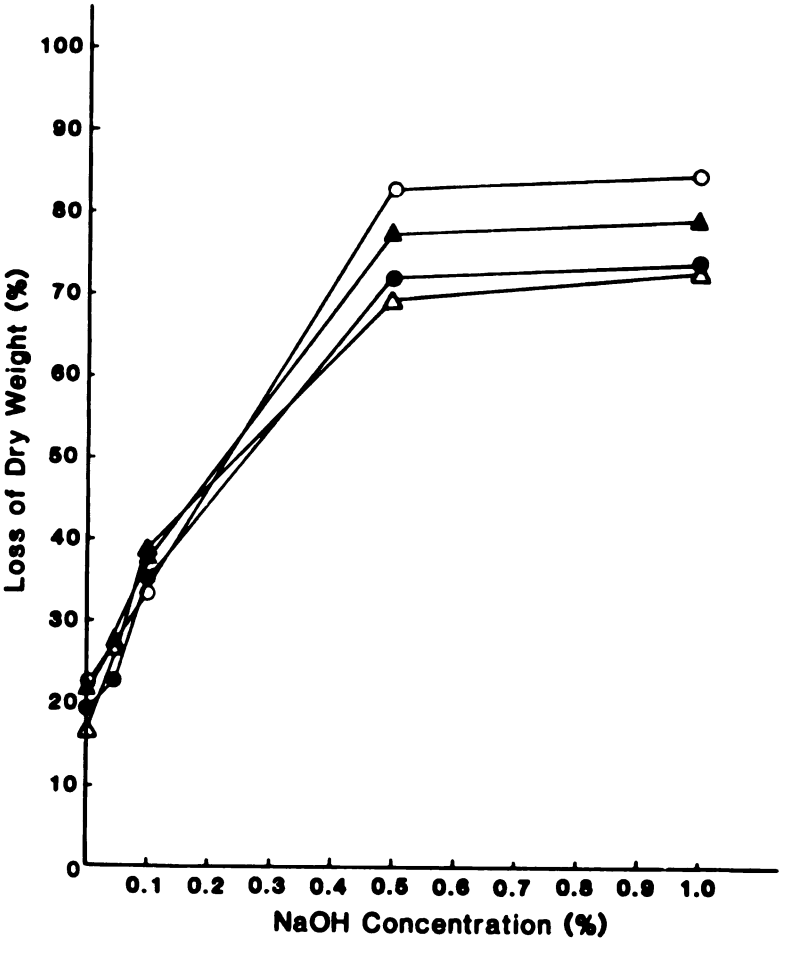

100
90
80
70
60
50
40
30
20
10
0
Loss of Dry Weight (%)
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
NaOH Concentration (%)

Fig. 1 b.

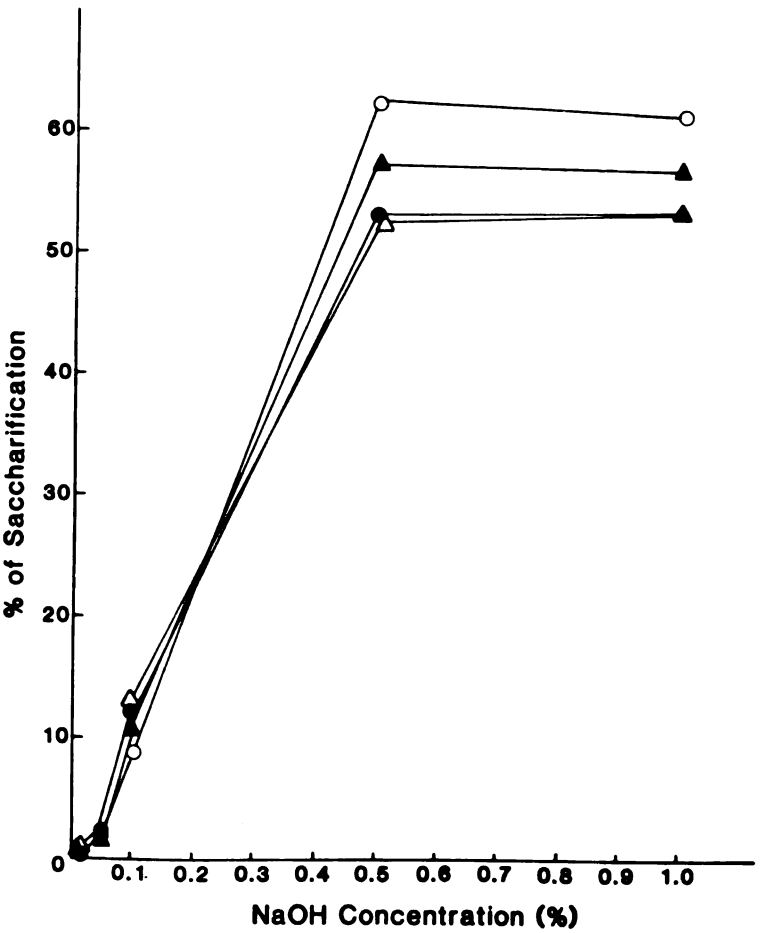

% of Saccharification
0
10
20
30
40
50
60
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
NaOH Concentration (%)

Fig. 1C.

effectiveness of a pretreatment process is the loss in dry weight of the lignocellulosic substrate after cellulase digestion. As shown in Fig. 1b there was greater solubilization of wheat straw with increasing NaOH and H_2O_2 concentrations. However, the effect of H_2O_2 was appreciable only when the concentration of NaOH higher than 0.1% was employed. Exposure of wheat straw to 0.5% NaOH increased dry weight loss to 67% whereas with 0.5% H_2O_2 and 0.5% NaOH the loss in dry weight increased to 83%. In contrast to its effect on delignification, addition of $FeSO_4$ to H_2O_2-NaOH solution increased loss in dry weight significantly at all concentrations of H_2O_2 tested (data not shown) and reached a maximum of 90% at 0.5% H_2O_2-0.5% NaOH.

The extent of saccharification on adding cellulase to H_2O_2-NaOH pretreated wheat straw increased with increassing alkali concentration as well as with increasing H_2O_2 concentration (Fig. 1c). The saccharification pattern was similar to that of delignification and dry weight loss. Maximum saccharification obtained with H_2O_2-NaOH treated wheat straw was 63% (equivalent to 97% of the theoretical maximum) which was about 10% higher than that obtained with NaOH alone. Added $FeSO_4$ did not increases the extent of saccharification. On the contrary, at high H_2O_2 concentration (0.5%), added $FeSO_4$ resulted in substantial decrease in percent saccharification (data not shown).

Influence of Reacting Time on the Effectiveness of H_2O_2-NaOH Pretreatment

The effect of pretreatment time on delignification (Fig. 2a), dry weight loss (Fig. 2b) and percent saccharification (Fig. 2c) of wheat straw after cellulase digestion was investigated. NaOH and H_2O_2 concentrations, temperature, and agitation were kept constant during these experiments. The results revealed that the most profound changes in the absorbance at 280 nm, dry weight loss and percent saccharification occurred in the first 8 hr of pretreatment. H_2O_2-NaOH treatment resulted in higher extent of delignification (OD_{280} = 22.7), saccharification (56%; equivalent to 86% of the theoretical maximum) and dry weight loss (81%) as compared to 20, 48%, and 64%, respectively, observed on pretreatment with NaOH alone. Only 50% saccharification was obtained with NaOH alone even after 24 hr pretreatment. The results also showed a more pronounced change in absorbance at 280 nm in samples treated with cellulase (Fig. 2a) as compared to samples not treated with cellulase. These results indicate that greater solubilization of lignin is obtained when pretreated straw is digested with cellulase.

Effect of Temperature on the Efficiency of H_2O_2-NaOH Pretreatment

Since H_2O_2-NaOH pretreatment is a chemical reaction, we examined the effect of pretreatment temperature on the susceptibility of wheat straw to cellulase digestion (Table

Fig. 2. H_2O_2-NaOH pretreatment of wheat straw: effect of pretreatment time on (a) delignification as shown by increase in absorbance at 280 nm, (b) loss of dry weight, and (c) saccharification. Pretreatments were carried out with 0.5% NaOH (▲ and △) or 0.5% NaOH plus 0.5% H_2O_2 (○ and ●) and the treated straw samples were digested with cellulase. Open symbols represent values before cellulase digestion. Values in the figure are averages for three samples.

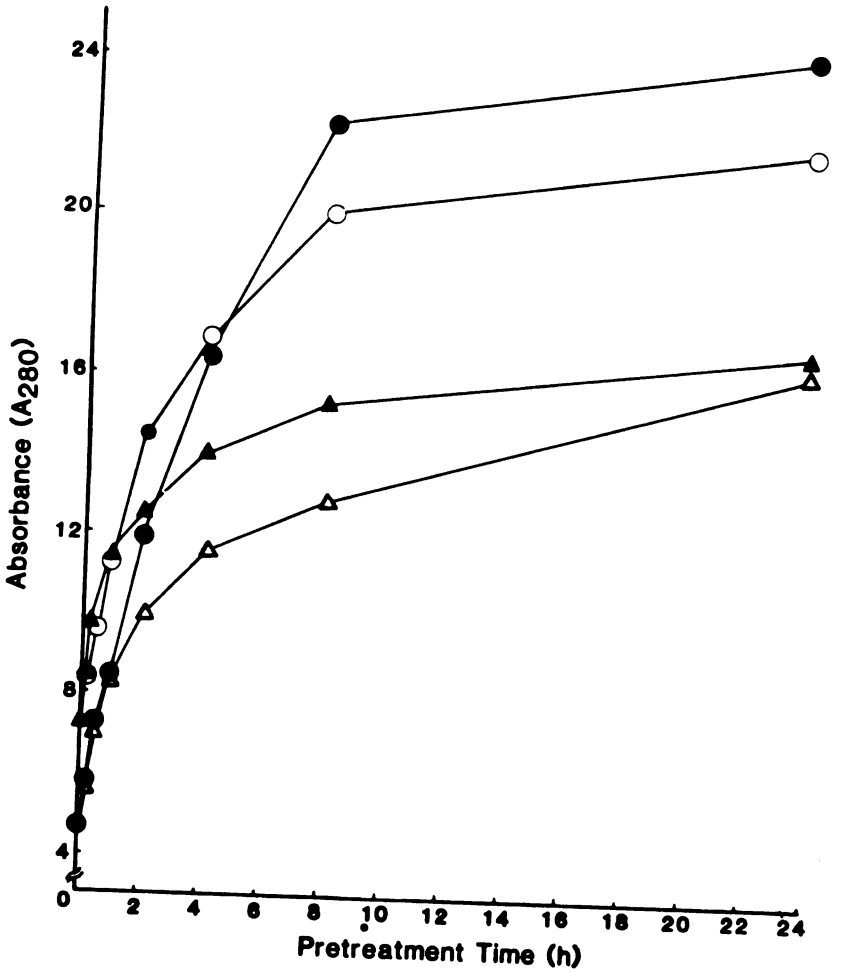

Absorbance (A_{280})
24
20
16
12
8
4
0
2
4
6
8
10
12
14
16
18
20
22
24
Pretreatment Time (h)

Fig. 2a.

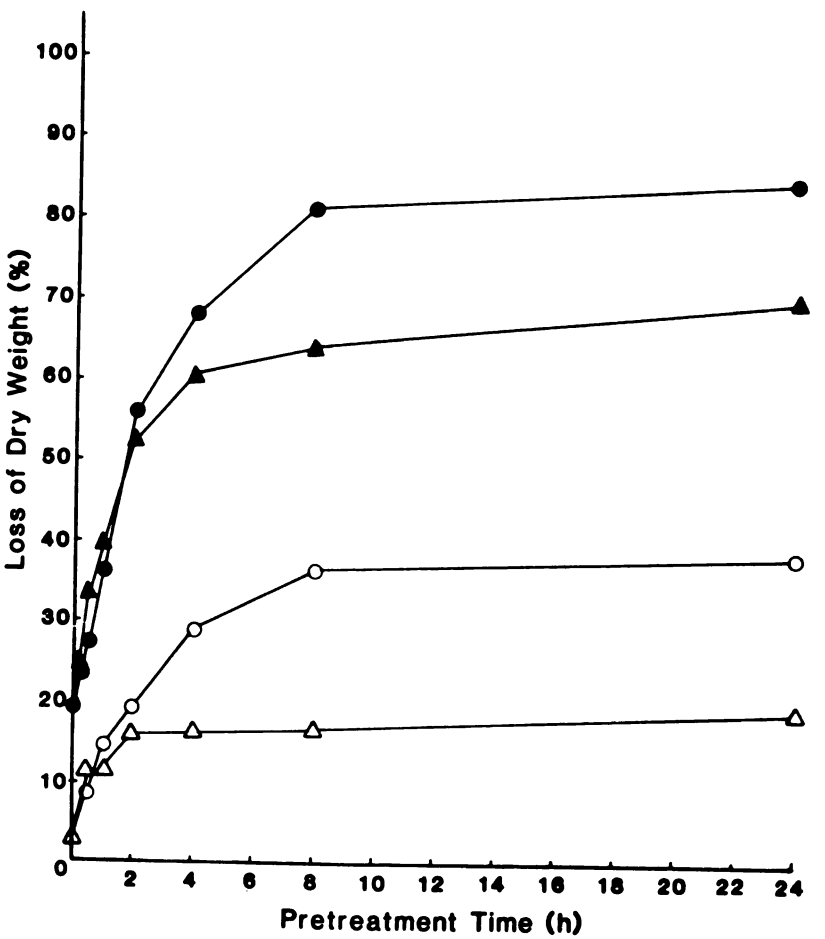

Loss of Dry Weight (%)
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
22
24
Pretreatment Time (h)

Fig. 2b.

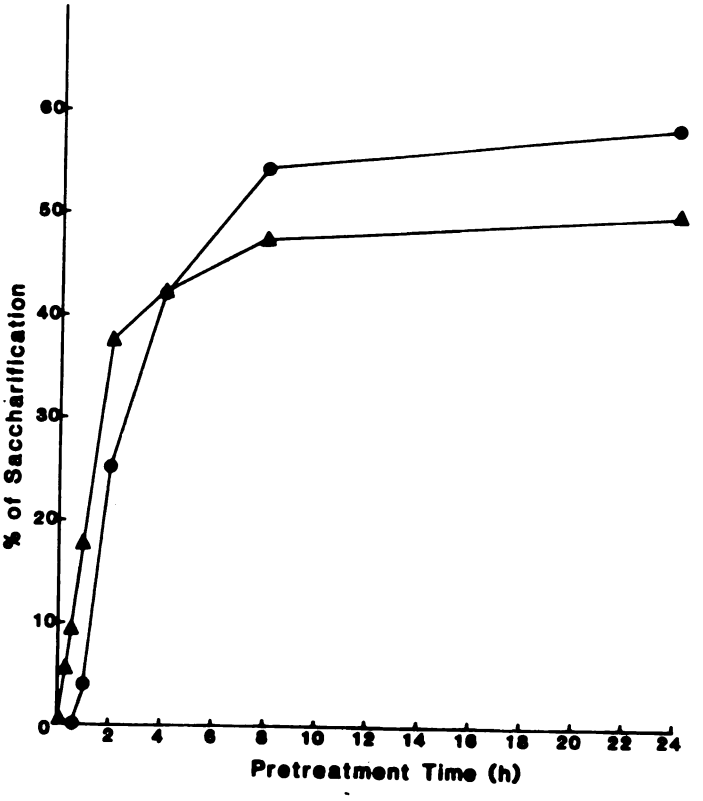

% of Saccharification
0
10
20
30
40
50
60
2
4
6
8
10
12
14
16
18
20
22
24
Pretreatment Time (h)

Fig. 2c.

1). Wheat straw (1%, w/w) was treated with 0.5% NaOH or 0.5% NaOH plus 0.5% H_2O_2 at 30°C for a period of 24 hr and then exposed to T. reesei cellulase for a further 24 hr at 50°C. The effectiveness of H_2O_2-NaOH pretreatment as evidenced by higher dry weight loss was considerably enhanced at 50°C than at 30°C. However, H_2O_2-NaOH pretreatment at 50°C resulted in a substantial decrease in the extent of saccharification compared to that observed at 30°C.

Suitability of Wheat Straw Hydrolysate as a Substrate for Alcohol Fermentation

Wheat straw and Sigma Cell were pretreated with H_2O_2-NaOH at 30°C for 8 hr and digested with T. reesei cellulase. The yield of glucose on H_2O_2-NaOH pretreatment of wheat straw or Sigma Cell was relatively high compared to that obtained on treating the same materials with NaOH only (Table 2). Ethanol yields, a reflection of the concentration of glucose in the respective hydrolysate, were also higher when the substrates were pretreated with H_2O_2-NaOH than with NaOH alone.

Table 1. Effect of Incubation Temperature During NaOH and H_2O_2-NaOH Pretreatments on the Susceptibility of Wheat Straw to Cellulase[a]

Temp.	% Saccharification		% Loss of dry weight	
	NaOH	H_2O_2+NaOH	NaOH	H_2O_2+NaOH
30°C	50	62	67	84
50°C	58	56	80	82

[a]Values are averages for three samples. Wheat straw (1%, w/v) was treated with 0.5% NaOH plus 0.5% H_2O_2 for 24 hr at a given temperature and then exposed to T. reesei cellulase at 50°C for an additional 24 hr.

Table 2. Effect of NaOH and H_2O_2-NaOH Pretreatment on Glucose and Ethanol Production from Wheat and Sigma Cell[a]

Substrates and pretreatments[b]	Glucose in hydrolysate[c] (g/100 ml)	Ethanol produced (g/100 ml)	Yield of ethanol[d] (g of ethanol/g of glucose consumed
Wheat straw			
0.5% NaOH	2.00±0.075[e]	0.59±0.015	0.29 (57)
0.5% NaOH + 0.5% H_2O_2	2.32±0.043	0.64±0.010	0.30 (59)
Sigma Cell			
0.5% NaOH	2.12±0.055	0.71±0.018	0.33 (65)
0.5% NaOH + 0.5% H_2O_2	2.60±0.090	0.85±0.015	0.33 (65)

[a]Values are averages for three samples.

[b]Each pretreatment was carried out at 30°C for 8 hr.

[c]Hydrolysate contains 4% (w/v) reducing sugar.

[d]Figures in parentheses represent the percent of theoretical yield.

[e]Standard deviation.

DISCUSSION

Since the efficiency of enzymatic hydrolysis of cellulose in lignocellulosic substrates is known to be profoundly affected by the extent of lignification[27] and crystallinity of the cellulose[5,6], the increased saccharification observed with H_2O_2-NaOH pretreatment may be a reflection of the extensive delignification and depolymerization of cellulose obtained with this pretreatment. Decomposition of H_2O_2 under alkaline conditions, in the presence of heavy metals (part of the ash in lignocellulosic materials), leads to the production of hydroxyl radicals, superoxide ions and oxygen as shown in reactions 1 to 5[36-40]. The reduced and oxidized states of the metal are denoted as M and M^+, repectively in the following reactions.

$$H_2O_2 + OH^- \rightarrow OOH^- + H_2O \quad (1)$$

$$H_2O_2 + HO_2^- \rightarrow HO. + O_2^-. + H_2O \quad (2)$$

$$H_2O_2 + M \rightarrow M^+ + HO. + HO^- \quad (3)$$

$$H_2O_2 + M^+ + 2OH^- \rightarrow M + O_2^-. + 2H_2O \quad (4)$$

$$O_2^-. + HO. \rightarrow O_2 + HO^- \quad (5)$$

The hydroxyl radicals and/or other oxygen radicals produced from H_2O_2 decomposition under alkaline conditions may cause oxidative degradation and depolymerization of lignin[22,23] and cellulose[41].

Previous investigators have found that high alkalinity accelerates the decomposition of hydrogen peroxide to give oxygen[23]. The partial pressure of oxygen derived from the

decomposition of H_2O_2 also increased with increasing H_2O_2 concentration in the reaction mixture. Moreover, high alkalinity and H_2O_2 favor the formation of carbanions, the substrates for the oxygenation reaction[22]. Accordingly, the extent of delignification appears to increase with increasing alkalinity and H_2O_2 concentration and subsequently increase the accessibility of cellulose in wheat straw to cellulase action.

Pretreatment with H_2O_2-NaOH and $FeSO_4$ significantly increases the weight loss of wheat straw after cellulase digestion. However, this resulted in a decrease in sugar yield but no change in the extent of delignification, as compared with H_2O_2-NaOH pretreatment without $FeSO_4$. It has been demonstrated that the presence of $FeSO_4$ increases the rate of decomposition of hydrogen peroxide, and the formation of both hydroxyl radicals and superoxide ions (equation 3 and 4) in H_2O_2-NaOH reaction[37,38,42]. The results reflected that the addition of the $FeSO_4$ does accelerate the reaction as shown by the increase in loss of dry weight. An increase in temperature also results in an enhanced rate of decomposition of H_2O_2 to produce various oxygen radicals[23,43]. Increased dry weight loss obtained on H_2O_2-NaOH pretreatment at elevated temperature (50^{o}C) is consistent with this explanation. However, both in the presence of added $FeSO_4$ and at elevated temperature, H_2O_2-NaOH pretreatment followed by cellulase digestion resulted in decreased sugar yield, which may be due to the oxidative

breakdown of the carbohydrates by oxygen radicals[44].

As glucose is the common substrate desired for various industrial processes such as the fermentative production of alcohol, any system which can result in a greater conversion of the cellulose in the substrate to glucose is more desirable. The results of this study (Table 2) show that pretreatment of wheat straw with H_2O_2-NaOH results in more release of glucose from cellulose and consequently more ethanol production than that treated with NaOH alone. Recently Detroy et al. reported relatively low alcohol yields on fermentation wheat straw that was pretreated with NaOH and hydrolysed with cellulase[15,45]. These low yields are perhaps attributable to the generation of inhibitory substances during the pretreatment process or due to other reasons not clear at this time. In this study we obtained ethanol yield of about 0.3 g per g of glucose consumed (59% of the theoretical yield) which is comparable to that reported by other investigators[15,45]. It should be emphasized that H_2O_2-NaOH pretreatment does not decrease ethanol yields compared to those obtained with NaOH pretreatment. In fact, ethanol production was somewhat higher with the NaOH-pretreated wheat straw.

In summary, H_2O_2-NaOH pretreatment provides a rapid, effective method for enhancing the enzymatic saccharification of lignocellulosic substrates. The resulting sugars contain higher amounts of glucose for ethanol production by S. cerevisiae.

Acknowledgement

We would like to acknowledge Dr. M.T. Yokoyoma for the use of Wiley Mill, and a grant from the Michigan Agricultural Experiment Station.

References

1. G.T. Tsao, M.R. Ladish, M. Woloch, and P. Bienkowski, Proc. Biochem., **17(5)**, 34 (1982).

2. W.D. Bellamy, Biotechnol. Bioeng., **16**, 869 (1974).

3. H.R. Bungay, Science, **218**, 643 (1982).

4. L.J. Forney, and C.A. Reddy, Dev. Ind. Microbiol., 20, 163 (1979).

5. L.T. Fan, Y.H. Lee, and D.H. Beardmore, Avd. Biochem. Eng., **14**, 101 (1980).

6. L.T. Fan, Y.H. Lee, and D.H. Beardmore, Biotechnol. Bioeng., **22**, 177 (1980).

7. J.M. Nystrom and R.K. Andren, AIChe Symp. Ser., **72**, 91 (1976).

8. M. Mandels, L. Hontz, and J. Nystrom, Biotechnol. Bioeng., **16**, 1471 (1974).

9. R.G. Kelsey and F. Shafizadah, Biotechnol. Bioeng., **22**, 1025 (1980).

10. M.J. Neilson, R.G. Kelsey, and F. Shafizadah, Biotechnol. Bioeng., **24**, 293 (1982).

11. N. Neese, J. Wallick, and J.M. Harper, Biotechnol. Bioeng., **19**, 323 (1977).

12. D.G. MacDonald and J.F. Mathews, Biotechnol. Bioeng., **21**, 109 (1979).

13. Y.M. Han, J. Timpa, A. Ceigler, J. Courtney, W.F. Curry, and E.N. Lumbrement, Biotechnol. Bioeng., **32**, 2525 (1981).

14. R. Datta, Biotechnol. Bioeng., **23**, 61 (1981).

15. R.W. Detroy, R.L. Cunningham, R.J. Bothast, and M.D. Bagby, Biotechnol. Bioeng., **24**, 1105 (1982).

16. D. Knappert, H. Grethlein, and A. Converse, Biotechnol. Bioeng., **22**, 1449 (1980).

17. M. Tanaka, T. Morita, M. Taniguchi, R. Matsumo, and J. Kamikubo, J. Ferment. Technol., **58**, 517 (1980).

18. A. Binder, L. Pelloni, and A. Fiechter, Appl. Microbiol. Biotechnol., **11**, 1 (1980).

19. M.R. Ladisch, C.R. Ladisch, and G.T. Tsao, Science, **201**, 743 (1978).

20. J. Krieger, Chem. Eng. News, **60**, 38 (1982).

21. K.E. Erikson, Biotechnol. Bioeng., **22**, 363 (1980).

22. J. Gierer and F. Imsgard, Svensk Papperstidn, **80**, 510 (1977).

23. R. Agnemo and G. Gellerstedt, Acta Chem. Scan., **B33**, 337 (1979).

24. L.J. Forney, C.A. Reddy, M. Tien, and S.D. Aust, J. Biol. Chem., **257**, 11455 (1982).

25. J.W. Koenigs, Biotechnol. Bioeng. Symp., **5**, 151 (1975).

26. Y.W. Han, Adv. Appl. Microbiol., **23**, 119 (1978).

27. C.E. Dunlap, J. Thomson, and L.C. Chiang, AlChE Symp. Ser., **72**, 58 (1978).

28. M.Mandels and D. Sternberg, J. Ferment. Technol., **54**, 267 (1976).

29. W.E. Moore and D.B. Johson, Procedures for the Chemical Analysis of Wood and Wood Products (Forest Products Laboratory Forest Service, U.S. Department of Agriculture, 1967).

30. D.M. Updegraff, Anal. Chem., **32**, 420 (1969).

31. M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. rebers, and F. Smith, Anal. Chem., **28** 350 (1956).

32. G.R. Hrubant, R.A. Rhodes, and J.H. Sloneker, Specific Composition of Representative Feedlot Wastes: A Chemical and Microbial Profile (Science and Education Administration, U.S. Department of Agriculture, 1978).

33. G.L. Miller, Anal. Chem., **31** 426 (1959).

34. D.B. Johnson, W.E. Moore, and L.C. Zank, Tappi, **44**, 793 (1961).

35. G. Gellerstedt and R. Agnemo, Acta Chem. Scan., **B34**, 275 (1980).

36. J.L. Roberts, M.M. Morrison, and D.T. Sawyer, J. Am. Chem. Soc., **100**, 329 (1978).

37. C. Walling, Acc. Chem. Res., **8**, 125 (1975).

38. M.E. Bodini and D.T. Sawyer, J. Am. Chem. Soc., **98**, 8366 (1976).

39. A.U. Khan, J. Am. Chem. Soc., **99**, 370 (1977).

40. H.J. Guiraud and C.S. Foots, J. Am. Chem. Soc., **98**, 1984 (1976).

41. L. Olm and T. Teder, Tappi, **62**, 43 (1979).

42. N. Kitajima, S. Fukuzumi, and Y. Ono, J. Phys. Chem., **82**, 1505 (1978).

43. C.J. Alfthan, E. Andersson, S. Andersson, and J.E. Hook, Svensk Papperstidn., **80**, 480 (1977).

44. H.S. Isbell, E.W. Park, and R.G. Naves, Carbohydr. Res., **45**, 197 (1975).

45. R.W. Detroy, L.A. Lindenfelser, S. Sommer, and W.L. Orton, Biotechnol. Bioeng., **23**, 1527 (1981).