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A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector

(Recombinant DNA; yeast; toxic product; inserts; copy number; complementation)

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SUMMARY

A set of genomic plasmid banks was constructed using the centromere-containing yeast shuttle vector YCp50. The centromere-containing vector is useful for the isolation of genes that are toxic to yeast when present in high copy number. Fourteen independent banks were prepared each with an average representation of two to three times the yeast genome. Any individual plasmid from a given bank is guaranteed to be of independent origin from plasmids obtained from each of the other banks. The banks were constructed from three different size classes of DNA fragments that resulted from varying conditions of partial digestion with *Sau*3A. This avoided the bias caused by differential sensitivity of sites to cleavage with *Sau*3A. Insert DNA is sufficiently large that most genes will be present in the set of plasmid banks at a frequency of about 0.1%.

INTRODUCTION

The simplest and most widespread method of identifying plasmids containing specific cloned genes from *Saccharomyces cerevisiae* is by the complementation of specific mutations in yeast. Several genomic plasmid banks have been constructed that use yeast shuttle vectors based on the *ARS1* element (Nasmyth and Reed, 1980) and the 2μ plasmid (Carlson and Botstein, 1982) for replication in yeast. These banks have been extremely successful but suffer from several limitations. The first is that these plasmids are present in high copy number in yeast. This can prevent the isolation of genes that are toxic when overexpressed due to the high copy number of the vector. Examples of such genes are the genes for actin (*ACT1*), α-tubulin (*TUB2*) (Thomas, 1984) and *KAR1* (Rose and Fink, 1987). In some cases, overexpression of genes other than the gene of interest can suppress the mutation used for selection

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Abbreviations: Ap, ampicillin; β-ME, β-mercaptoethanol; bp, base pair(s); kb, 1000 bp; LB, Luria-Bertani (broth); nt, nucleotide(s); PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; TE, see MATERIALS AND METHODS, section **a**; Tc, tetracycline; YCp, yeast centromere plasmid; YEPD, see MATERIALS AND METHODS, section **a**.

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(Hinnebusch and Fink, 1983; Kuo and Campbell, 1983; MacKay, 1983; Pringle et al., 1984; Natsoulis et al., 1986). Although the cross-suppressing genes are of interest, they introduce ambiguity into the identification of the gene. The second limitation is that the vectors are inherently unstable in yeast. Therefore, any transformant is a mixture of cells containing varying numbers of plasmids. The most plentiful class of cells in a transformant culture can contain those cells that have lost the plasmid. The stochastic distribution of copy number therefore complicates the analysis of the phenotype. Third, titration of regulatory factors by genes in high copy number may alter the pattern of gene expression as compared to the expression of the same genes present in single copy.

Centromere-containing plasmids are inherently low-copy-number (typically 1 or 2 per cell) and mitotically stable (less than 1% loss per cell per generation; Clarke and Carbon, 1980). Therefore, a genomic plasmid bank using the centromere-containing shuttle vector YCp50 circumvents the limitations associated with high-copy-number shuttle vectors.

MATERIALS AND METHODS

(a) Preparation of yeast DNA

High-M_r yeast DNA was prepared by a modification of the method of Cryer et al. (1975). Strain GRF88 (MAT**a** *his4*-38, S288C background) was grown to 2×10^8 cells/ml in YEPD (Sherman et al., 1979). Cells were washed once in 1/5 vol. of ice-cold 50 mM Na_2EDTA and suspended in 50 mM Tris · HCl pH 9.5, 2% (v/v) β-ME for 15 min. Cells were spheroplasted in 1/50 vol. of 1 M sorbitol, 1 mM Na_2EDTA, pH 8.5, and 0.05 mg/ml of Zymolyase 60000 (Miles) at 37°C with gentle shaking until more than 95% of the cells burst upon dilution of a small sample into a solution of 1% sarkosyl. Spheroplasts were harvested by centrifugation and suspended in 1/200 vol. of lysis buffer (0.1 M Tris · HCl pH 9.5, 0.1 M Na_2EDTA, 0.15 M NaCl, 2% β-ME), frozen in liquid nitrogen and stored at −70°C. Thawed spheroplasts were lysed by three-fold dilution into lysis buffer made 4% (v/v) with sarkosyl and incubated at 45°C for 20 min. An equal volume of lysis buffer at pH 8.0 and containing sarkosyl was added and incubated at 70°C for 15 min. DNase-free RNase was added to 0.1 mg/ml and incubated at 45°C for 1 h. Pronase (Sigma, B grade) was added to a final concentration of 1.33 mg/ml in two aliquots at hourly intervals and the mixture was incubated at 45°C for a total of 2 h. After a 15-min incubation at 70°C, the mixture was extracted with an equal volume of chloroform–isoamyl alcohol (24 : 1) by gentle rocking until a white emulsion formed. Phases were separated by centrifugation at $20000 \times g$ and the aqueous supernatant was incubated at 45°C to remove traces of $CHCl_3$. High-M_r DNA was isolated by centrifugation on preparative sucrose gradients (5–20% sucrose, 20 mM Tris · HCl, pH 8.0, 20 mM Na_2EDTA, 0.2 M NaCl and 0.1% sarkosyl). Samples of 10 ml were layered onto 24-ml gradients over a 3-ml cushion of Angio-Conray made up to 20% sucrose. The gradients were run in a Beckman SW27 rotor at 13500 rev./min for 17 h. Samples of 1 ml were collected from the top of the gradients using a wide-bore pipette (> 1 mm orifice). Samples containing the viscous DNA were pooled and dialyzed against 0.15 M NaCl, 10 mM Tris · HCl pH 8.0, and 1 mM Na_2EDTA. CsCl was added to 10 g per 8 ml of solution (refractive index 1.400) and centrifuged at 50000 rev./min for 36 h in a type 50 Beckman rotor. DNA was collected by dripping through a 16-gauge needle. CsCl was removed by dialysis against 10 mM Tris · HCl, pH 8.0, 1 mM Na_2EDTA (TE). Yield was 300 to 400 μg per 10 g wet-cell pellet.

(b) Structure of YCp50

Plasmid YCp50 (Fig. 1) was constructed by M. Johnston and R.W. Davis (personal communication). The structure shown was deduced from the sequences of pBR322 (Sutcliffe, 1979), the *URA3* gene (Rose et al., 1984), *CEN4* (Mann and Davis, 1986), and *ARS1* (Tschumper and Carbon, 1980). Construction assumes the addition of 10 residues of G and 10 of C between nt residues 1428–1437 and residues 2601–2610, respectively, during the insertion of the *URA3 Hin*dIII fragment into the *Ava*I site of pBR322, to produce YIp5 (Struhl et al., 1979). The *Eco*RI site in YCp19 (Stinchcomb et al., 1982), between the *CEN4* and *ARS1* inserts, was blunted

with PolIk. The *Pvu*II-*Hin*dIII fragment containing the *CEN4* and *ARS1* fragments was cloned into the *Pvu*II site of YIp5. The *Pvu*II site was not regenerated. In addition, destruction of the *Eco*RI site in YCp19 produced a deletion of about 190 bp (R. Sapolsky, personal communication). This deletion has not been sequenced and so the bp numbering is provisional. Some sites near this deletion (at about bp 5000) may not be present (e.g., *Bal*I).

(c) Plasmid bank construction

Yeast DNA was partially digested with *Sau*3A using different concentrations of enzyme to avoid biasing the library due to preferential cleavage. Pooled DNA was run on preparative sucrose gradients as described in Maniatis et al. (1982). DNA fragments within the size ranges 10–15 kb, 15–20 kb and 20–30 kb were pooled and dialyzed against TE.

YCp50 vector DNA was cleaved with *Bam*HI, dephosphorylated with calf intestinal phosphatase (Boehringer-Mannheim), and mixed in roughly 2:1 ratio (by weight) with size-fractionated DNA. The 10–15-kb DNA was ligated at 30 μg/ml, the 15–20-kb DNA at 22.5 μg/ml and the 20–30-kb DNA at 15 μg/ml total DNA concentration (including vector DNA).

Strain HB101 (Boyer and Roulland-Dussoix, 1969) was transformed by the method of Mandel and Higa (1970) except that transformants were selected at one quarter the normal cell density on LB agar plates (Miller, 1972) containing 25 μg Ap/ml. Transformants were collected by washing cells from the surface of the plate using sterile LB broth and pooled. The suspended cells were concentrated by centrifugation and suspended in 50% sterile glycerol at a concentration of 1×10^9 cells/ml. Cells were stored at −70°C. Large-scale plasmid preparations (Maniatis et al., 1982) were made from pooled cells after diluting cells to approx. 2×10^7 cells/ml in LB broth and allowing the culture to grow to saturation.

(d) Other procedures

Yeast strains were transformed by the method of Hinnen et al. (1978) and by the method of Ito et al. (1983). Strain 8960-11b is MAT**α** *leu2*-3, *ura3*-52, *kar1*-1. Strain 8979-3A is MAT**α**, *leu2*-3, *leu2*-112, *ade2*, *his4*, *ura3*-52, *kar2*-1. Yeast strains were propagated as described in Sherman et al. (1979). Plasmid segregation experiments were carried out by inoculating 5 ml of YEPD broth with a single transformant colony, growing to saturation and plating serial dilutions of the culture on YEPD agar plates. Plates with isolated colonies were replica-plated to medium lacking uracil to score for loss of the plasmid.

RESULTS AND DISCUSSION

(a) Construction of the plasmid bank

High-M_r yeast DNA was partially digested with *Sau*3A and fractionated by size on preparative sucrose gradients. Different size classes of yeast DNA were ligated to YCp50 vector DNA (Fig. 1) that had been digested with *Bam*HI and dephosphorylated. A total of approx. 40000 *Escherichia coli* transformants were obtained with the ligated DNA. The transformants were collected into 14 independent pools (Table I) and stored.

The fraction of plasmids containing inserted DNA was estimated from the number of plasmids that had lost resistance to Tc. Samples of the pooled cells were diluted, plated onto LB containing Ap and plates containing well-isolated colonies were replica-plated to LB plates containing Tc. Of 1620 colonies tested only 163 were also still resistant to Tc, indicating that 90% of the plasmids were likely to contain inserts that disrupt the *tet* gene. This was confirmed by purifying plasmid DNA from 20 random transformants and determining that only two had a structure identical to the starting plasmid.

The plasmid pools have been separately maintained to reduce the loss of complexity that can arise from the differential growth rates of cells harboring plasmids with different sized inserts. Each pool contains from 1 to 3 times the minimum number of different plasmids required to contain one yeast genome equivalent. Therefore, the most efficient means of analyzing several independent isolates of a desired plasmid is to examine no more than one candidate per plasmid pool. A similar plasmid bank in YCp50 (Kuo and Campbell, 1983) used smaller insert DNA fragments. In that case, all of the plasmids were pooled into one bank and different

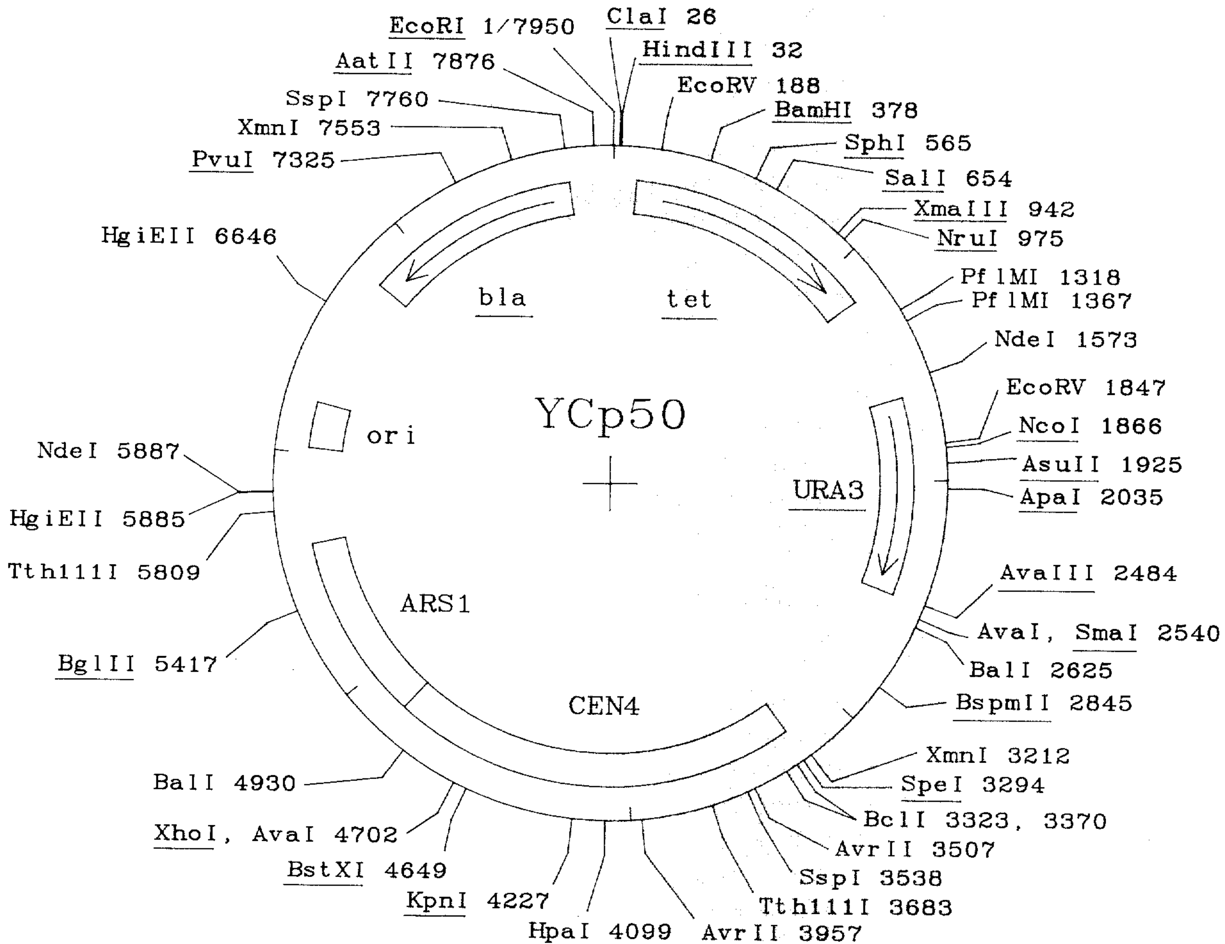

Fig. 1. Restriction map of yeast shuttle vector YCp50. Numbers indicate the first bp of the recognition site in all cases. Sites are indicated for enzymes that cleave once or twice. Single cutters are underlined. Type II enzymes that do not cut are *Afl*II, *Bst*EII, *Not*I, *Sac*I, *Sac*II, *Sna*BI, *Mlu*I, *Pvu*II, *Sau*I, *Xba*I, *Bss*HII, *Mst*II, and *Sfi*I. Enzymes *Acc*I, *Acy*I, *Afl*III, *Aha*III, *Cfr*I, *Eco*P15, *Gdi*II, *Hae*I, *Hae*II, *Hgi*AI, *Hgi*CI, *Hgi*JII, *Hin*dII, *Mst*I, *Nae*I, *Nar*I, *Nsp*(7524)I, *Nsp*BII, *Nsp*CI, *Sca*I, *Sna*I, *Stu*I, *Tth*111II, and *Xho*II all cut three or more times. For the genes *bla*, *tet* and *URA3* the boxes containing arrows indicate the extent and direction of the coding sequence. The *URA3* yeast DNA fragment begins at bp 1438 and ends at bp 2601. For *CEN4* and *ARS1*, the box indicates the extent of the yeast DNA fragment in the vector. The symbol *ori* represents the origin of DNA replication of the ColE1 plasmid derived from pBR322.

isolates of complementing plasmids may not be of independent origin.

(b) Frequency of complementing plasmids in yeast

Plasmid DNA prepared from each pool of *E. coli* transformants was used to transform yeast by the spheroplast transformation method. A total of 21 100 Ura$^+$ transformants of strain 8979-3A were recovered and replica-plated to test whether any had become Ade$^+$ as a result of transformation with a plasmid carrying the intact *ADE2* gene. A total of 31 transformants had become Ade$^+$ indicating that an average of 0.15% of the plasmids carried the *ADE2* gene. Similarly, the *KAR1* gene was found at a frequency of 0.14% among transformants of strain 8960-11b. Molecular analysis of six independent plasmids harboring the *KAR1* gene demonstrated a range of insert sizes from 8.5 kb to 19 kb with a mean of 14.7 kb. Transformation using the lithium acetate procedure (Ito et al., 1983) yields a somewhat lower frequency of complementing plasmids apparently due to the lower frequency of multiple plasmids per transformed cell (F. Winston, personal communica-

TABLE I

Plasmid bank structure

Pool number[a]	Estimated insert size[b]	Number of independent plasmids in pool[c]
A1	10–15	1500
A2	10–15	1500
A3	10–15	4900
A4	10–15	4900
A5	10–15	4900
A6	10–15	4900
B1	15–20	1400
B2	15–20	2600
B3	15–20	2600
B4	15–20	2600
C1	20–30	1400
C2	20–30	2400
C3	20–30	2400
C4	20–30	2400

[a] Each pool was censtructed independently and maintained as a separate collection.

[b] Approximate size of each purified fraction of partially digested yeast DNA used to construct the set of plasmid pools. Sizes based on electrophoretic mobility of DNA after fractionation on preparative sucrose gradients.

[c] Total number of independent *E. coli* transformants that were collected into each plasmid pool. Approximately 90% of the plasmids contain inserted yeast DNA.

tion; M. Fedor and M.D.R., unpublished observation).

Due to the small number of plasmids in each pool, any given pool may have 0, 1, 2, 3 or more independently derived plasmids bearing any given gene. Therefore the frequency of acquiring complementing plasmids from any single pool may vary from 0 to as high as 4×10^{-3}, depending on the specific gene.

(c) Plasmid stability in yeast

One proof that a given transformant contains a plasmid that harbors a complementing gene (as opposed to being a revertant containing an unrelated plasmid) is the demonstration that the complementing activity cosegregates with the plasmid-selectable gene. The frequency of mitotic segregation of centromere-containing plasmids varies inversely with plasmid size (Hieter et al., 1985). Therefore, the plasmids containing larger inserts are more stable (e.g., 1 to 6% loss of plasmids carrying *KAR1* in 30 generations) in yeast than the YCp50 vector without an insert (50% loss in 30 generations). One consequence of the great stability of these plasmids is that simultaneous transformation by multiple plasmids can complicate the demonstration of cosegregation. The spheroplasting method of transformation (Hinnen et al., 1978) frequently results in transformants that have taken up several different plasmids (Kunes et al., 1984). In such cases, loss of the complementing plasmid will be much more frequent than loss of all of the plasmids. For example, in such transformants loss of the plasmid marker *URA3* gene occurred at less than 0.1%, whereas the *KAR1* gene was lost at a frequency of several percent. From each such yeast transformant several unrelated plasmids were recovered by transformation into *E. coli*. Only one of these plasmids turned out to be structurally related to plasmids obtained from other transformants. These common plasmids subsequently proved to carry the *KAR1* gene after retransformation into yeast strain 8960-11b. Although the lithium acetate procedure (Ito et al., 1983) can give rise to transformants containing multiple plasmids, this has not proved to be a significant problem since the frequency of cotransformation is lower (F. Winston, personal communication). Alternatively, 5-fluoro-orotic acid (Boeke et al., 1984) can be used to select for cells that have lost all of their plasmids. The resulting cells can be used to show the loss of any plasmid-derived complementing activity.

(d) Copy number effects

One demonstration of the utility of the low-copy-number vector is that plasmids carrying the *KAR1* gene could be isolated from the YCp50 library by complementation in yeast but could not be isolated from any of the existing 2μ-vector-based libraries (M.D.R. and G.R.F., unpublished observations). Subsequent experiments indicate that the *KAR1* gene is toxic to the yeast cell when present on high-copy-number plasmid vectors (Rose and Fink, 1987).

Although the low copy number of the plasmids should prevent most cross-suppression, in some cases a single extra copy of a wild-type gene can suppress mutations in other apparently functionally related genes. For example, the *SEC4* gene isolated

from this plasmid bank is capable of suppressing mutations in *SEC15*, *SEC8* and *SEC2* (Salminen and Novick, 1987).

Some yeast genes may be difficult to isolate from this library. Any gene which is toxic to *E. coli* may be under-represented. For example, the *KAR2* gene was found at a frequency of 1×10^{-4}, about ten-fold lower than other genes in the bank. Subsequent experiments indicate that this gene or a sequence associated with it is toxic to *E. coli* when present on high-copy-number plasmids (M.D.R., unpublished observation). Similarly, genes that are located near the telomeres of chromosomes may be present at a reduced frequency. Genes located near chromosomal centromeres may be present in the bank but show reduced transformation efficiency in yeast due to the formation of dicentric plasmids (Mann and Davis, 1983). In addition, DNA that contains structures that interfere with plasmid replication (e.g., large inverted repeats; Collins, 1981) may have been lost or rearranged during amplification in *E. coli*. Similarly, repeated sequences may be subject to residual recombination in *recA* hosts (Albertini et al., 1982) giving rise to faster growing deletion derivatives.

Both the vector YCp50 and the plasmid banks have been deposited with the American Type Culture Collection. YCp50 is acquisition 37419 and the plasmid banks are entered as acquisition 37415.

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