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Multifunctional yeast high-copy-number shuttle vectors

(*Saccharomyces cerevisiae*; plasmid; YEp; cloning; recombinant DNA; polylinker; phagemid; multicopy vector; plasmid stability; segregation rate)

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SUMMARY

A set of four yeast shuttle vectors that incorporate sequences from the *Saccharomyces cerevisiae* 2μ endogenous plasmid has been constructed. These yeast episomal plasmid (YEp)-type vectors (pRS420 series) differ only in their yeast selectable markers, *HIS3*, *TRP1*, *LEU2* or *URA3*. The pRS420 plasmids are based on the backbone of a multifunctional phagemid, pBluescript II SK+, and share its useful properties for growth in *Escherichia coli* and manipulation in vitro. The pRS420 plasmids have a copy number of about 20 per cell, equivalent to that of YEp24. During non-selective yeast growth, pRS420 plasmids are lost through mitotic segregation at rates similiar to other YEp vectors and yeast centromeric plasmid (YCp) vectors, in the range of 1.5–5% of progeny per doubling. The pRS420 series provides high-copy-number counterparts to the current pRS vectors [Sikorski and Hieter, Genetics 122 (1989) 19–27].

INTRODUCTION

The first yeast shuttle vectors contained few useful restriction sites for cloning and lacked the versatility of current *E. coli* vectors. Yeast vectors incorporating more sophisticated features have been developed (Rose and Broach, 1991). Sikorski and Hieter (1989) constructed two series of yeast shuttle plasmids based on the backbone of the phagemid pBluescript (Stratagene, La Jolla, CA). These plasmids were termed pRS vectors, and they share with pBluescript the *lacZ* α-fragment for blue/white color screening, T7/T3 promoters for in vitro RNA transcription, and an f1 phage origin for production of single-stranded DNA. In addition, many of the 21 restriction sites in the polylinker are oriented to facilitate unidirectional exonuclease III digestion.

The four members of the pRS400 series (and pRS300 series) are yeast integrating plasmids (YIp) which contain the *HIS3*, *TRP1*, *LEU2* or *URA3* markers for selection and maintenance in yeast. The pRS410 (and pRS310) series are identical to the pRS400 series except for the addition of an autonomously replicating sequence (*ARS*) and a centromere. These YCp vectors replicate autonomously and have reasonable mitotic stability.

Yeast researchers also use yeast episomal plasmids (YEp), which replicate autonomously at high copy number in the yeast nucleus by virtue of sequences derived from the natural 2μ circular yeast plasmid. YEp vectors are multicopy in yeast, in contrast to the single copy YCp vectors. In this paper we describe the construction of a new series of pRS vectors that contain the 2μ origin of replication along with the *REP3* and *FRT* sequences necessary for high copy propagation in yeast.

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Abbreviations: bp, base pair(s); *FRT*, Flp recombination target site; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); *ori*, origin of DNA replication; YCp, yeast centromeric plasmid(s); YEp, yeast episomal plasmid(s); YEPD, 1% yeast extract/1% peptone/2% dextrose; YIp, yeast integrating plasmid(s).

EXPERIMENTAL AND DISCUSSION

(a) Construction of the vectors

A 1345-bp *Hpa*I-*Nde*I fragment containing the 2μ *ori* (A form) was removed from YEp24 (Hurley and Donelson, 1980; New England Biolabs catalogue, 1990–1991), its *Nde*I end was filled with PolIk, and the fragment was li-

gated into a unique *Aat*II site of pRSS56 (Sikorski and Hieter, 1989) that had been made blunt with T4 DNA polymerase treatment. The unique *Nde*I site of the resulting plasmid was filled with PolIk and ligated to a 1.8-kb *Bgl*II fragment (made blunt with T4 DNA polymerase) containing the *TRP1* gene isolated from pBR322/SC4128 (Stinchcomb et al., 1982) to make pJS92.

A series of vectors with the 2μ *ori* but otherwise similiar to the pRS300 vector series was made taking advantage of the two *Eco*O109I (*Dra*II isoschizomer) sites in pJS92. One of these sites is before the *Nde*I site and the other is located within the polylinker. We replaced the *TRP1* marker of pJS92 with similar marker-containing *Eco*O109I fragments from each of the four pRS300 plasmids. The plasmids pRS303, pRS305 and pRS306 each also contained a third *Eco*O109I site in the *HIS3*, *LEU2* and *URA3* genes, respectively. Partial digests of these three plasmids provided the appropriate fragments. The resulting pRS320 series of plasmids were further modified by changing the polylinker to that of pBluescript II SK+. This was done by fusing the appropriate *Pvu*I fragments as described in Sikorski and Hieter (1989). Maps of the resulting pRS420 series are shown in Fig. 1.

(b) Stability of the vectors in yeast

Although YEp and YCp vectors are more stable in yeast than yeast replicating plasmid vectors (YRp), they still require selection for maintenance. We compared the the mitotic stability of the pRS420 vectors to YEp24, a common YEp vector, and to pRS313, a YCp vector whose mitotic stability is known (Sikorski and Heiter, 1989). Yeast strain YPH252 *MAT*α *ura3 lys2 ade2 trp1 his3 leu2* (Sikorski and Heiter, 1989) was transformed with each plasmid type by the lithium acetate technique (Sherman et al., 1986; Ito

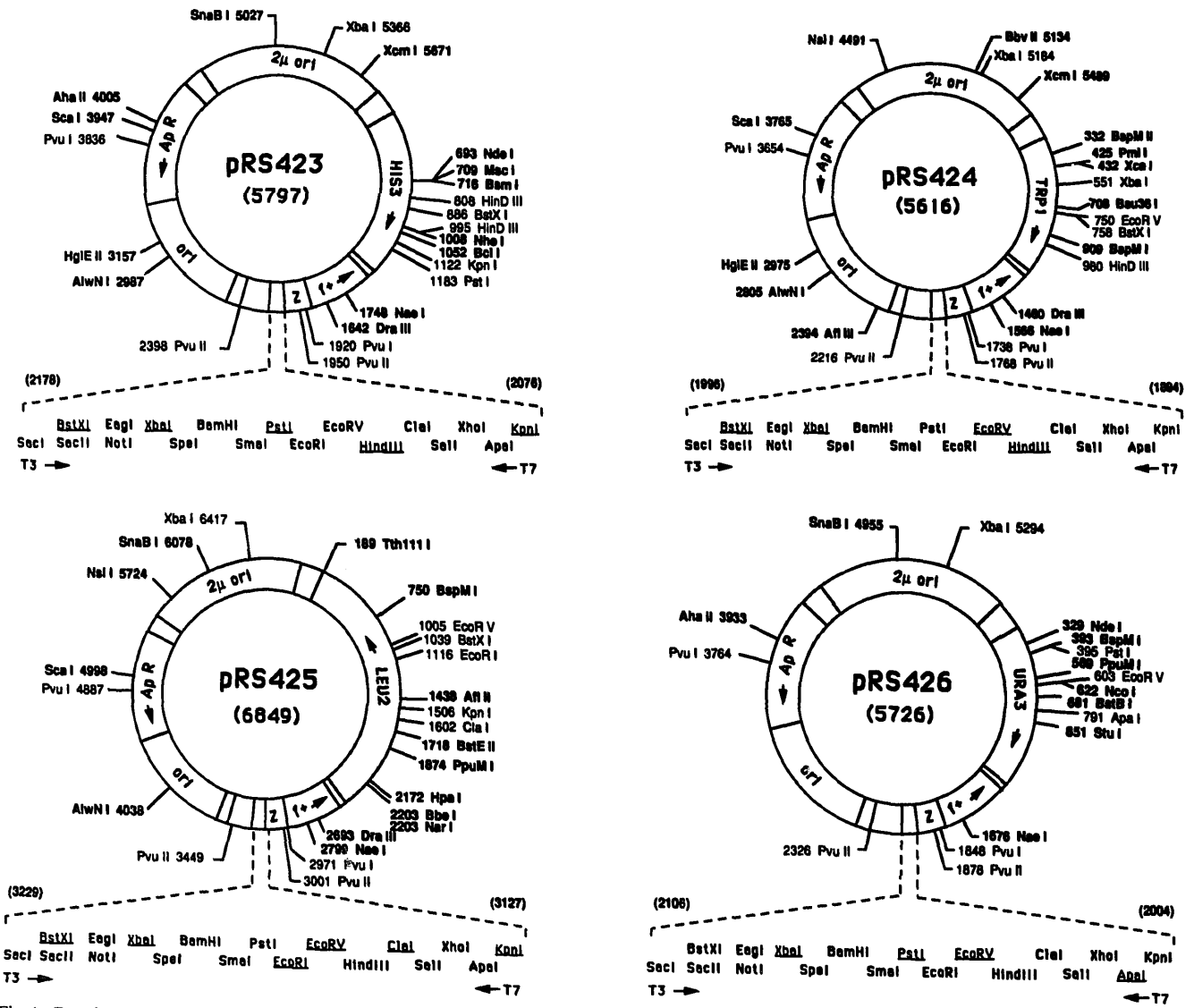

Fig. 1. Restriction maps of the pRS420 vectors. Unique restriction sites have bold labels. For emphasis, sites in each vectors' polylinker which are no longer unique are underlined. The T3 phage promoter transcribes in the *Sac*I to *Kpn*I direction and the T7 promoter transcribes in the opposite direction. Note that since the various pRS400 series of vectors are based on pBluescript II SK+, the orientation of the polylinker sites is reversed with respect to those in the pRS300 series of vectors, and the polylinker also has flanking *Bss*HII sites. Ap^R, ampicillin resistance marker.

et al., 1983). Five independent transformants from each vector were analyzed for stability (Table I).

The data in the table raise a number of interesting points. Even with selection, plasmid-free cells form about 10–30% of the total population. This has been previously observed (Futcher and Cox, 1984; Sikorski and Heiter, 1989); presumably this percentage represents a balance between new segregants arising and stasis or eventual loss of old plasmid-free segregants. In colonies on selective plates this proportion is even higher, ranging from 37–63% plasmid-free cells (data not shown), perhaps due to crossfeeding.

During non-selective liquid culture, we found plasmid-free segregants arise at rates typically ranging from 1.5–5% of progeny per doubling. This is similiar to the 2μ vector segregation rate of 2–6% reported by Futcher and Cox (1984) or to rates which can be calculated from published data (Gerbaud et al., 1979). Segregation rates for 12 and 24 generations of non-selective growth were similiar. Segregation rates (and therefore mitotic stability) vary among the different pRS420 vectors; pRS424 was the most stable. Insert fragment size and sequence are known to affect YEp vector copy number (Rose and Broach, 1991). Intriguingly, different transformants of the same vector in the same host often differed in stability, resulting in large standard deviations. The stability of pRS313 (YCp-type) was similiar to that reported by Sikorski and Hieter (1989); the data in the earlier paper can be used to calculate a segregation rate of 4.1%.

(c) Copy number of the vectors in yeast

Total yeast DNA was isolated from a transformant for each vector type, as well as the untransformed host strain YPH252. Southern-blot analysis (Southern, 1975; Sambrook et al., 1989) was performed in order to determine the plasmid copy number (Fig. 2). The filter was probed with

TABLE I

Mitotic stability and segregation rates

Vector[a]	Marker scored	Mean values for five transformants after twelve doublings				Single transformants after 24 doublings			
		Percent (P) plasmid-carrying cells				Percent (P) plasmid-carrying cells			
		After 10 doublings selective[b]	After g doublings nonselective[c]		Segregation rate[d]	After 10 doublings selective	After g doublings nonselective[e]		Segregation rate
		P_1	g	P_2	m	P_1	g	P_3	m
pRS423	*HIS3*	73 ± 5	12.5 ± 0.4	42 ± 7	4.3 ± 1.3	80	24.7	21	5.3
pRS424	*TRP1*	82 ± 8	12.6 ± 0.2	68 ± 8	1.5 ± 1.7	80	23.9	69	0.6
pRS425	*LEU2*	73 ± 6	11.9 ± 0.3	42 ± 4	4.6 ± 0.6	73	23.5	29	3.8
pRS426	*URA3*	78 ± 5	12.8 ± 0.3	45 ± 11	4.4 ± 1.4	82	24.6	37	3.2
YEp24	*URA3*	88 ± 3	12.7 ± 0.3	69 ± 4	2.0 ± 0.7	88	24.0	55	2.0
pRS313	*HIS3*	76 ± 5	12.3 ± 0.5	51 ± 8	3.2 ± 1.4	77	24.4	36	3.0

[a] Vector maps: for pRS420 vectors, see Fig. 1; for YEp24, see New England Biolabs catalogue 1990–1991, and for pRS313, see Sikorski and Hieter (1989).

[b] Five individual transformants for each vector were inoculated into selective minimal media (Sherman et al., 1986) containing appropriate supplements. After growth to stationary phase, this culture was diluted 1:1000 into non-selective YEPD broth. Five microliters of this dilution were immediately plated onto YEPD agar. The colonies which appeared on the YEPD agar were counted to determine the viable cell concentration of the dilution (N_1) and then scored for plasmid retention by replica plating onto selective agar. The mean and standard deviation of the % of plasmid-carrying cells in the inoculum was calculated (P_1).

[c] When the YEPD cultures grew to stationary phase, a 1:4000 dilution (stationary density in YEPD was about fourfold higher than in minimal) into sterile water was made, and 5 μl of the dilution was immediately plated onto YEPD agar. The culture's viable cell concentration (N_2) allowed us to calculate with formula *1* the number of doublings (g) during non selective growth. Replica plating provided the % of plasmid-carrying cells (P_2).

$$g = \frac{\ln (N_2/N_1)}{\ln 2} \qquad (1)$$

[d] To determine plasmid stability, we then used these figures to calculate the segregation rate (m), which we define as the % of plasmid-free segregants appearing in the final population after a single doubling. Formula *2* incorporates a number of considerations. First, part of the inoculum already consists of plasmid-free cells. Second, the cultures were grown through many doublings, with segregants appearing and subsequently reproducing in each. Third, since the segregation rate is high, the % of plasmid-carrying cells declines every doubling, reducing the number of cells able to segregate in future doublings. Fourth, we assumed that plasmid-carrying and plasmid-free cells had the same doubling time in non-selective media; this has been shown to be the case by Futcher and Cox (1984). The terms P_1, P_2 and g are described in footnotes b and c.

$$m = 1 - e^{\frac{\ln (P_2/P_1)}{g}} \qquad (2)$$

[e] For each of the vector types, one (of the five) was selected for another round of growth. After a 1:4000 dilution with YEPD, the culture was grown to stationary, diluted 1:4000 with sterile water, and 5 μl was plated. After counting and replica plating, P_3 (the % of plasmid-carrying cells after the second round of growth) was used to determine g and m by using formulas *1* and *2*.

nick-translated pRS90, a plasmid that contains 2.7 kb of single-copy yeast DNA (from the *CDC23* locus; R.S. S., W.A.M. Michaud and P. H., in preparation) inserted into the 2961-bp pBluescript vector. This probe therefore hybridized to the pBluescript based pRS420 vectors and the chromosomal *CDC23* gene with similiar specific activity, enabling us to estimate plasmid copy number relative to a single copy gene by comparing hybridization intensity.

The pRS420 series vectors have hybridization signals similiar to or stronger than YEp24 (lanes 2, 4, 6, 8 and 10), and therefore have copy numbers equal to or greater than this standard 2μ vector (YEp24 has all pBR322 sequences included in pBluescript). In each of these lanes, higher bands of less intensity are present. Since these bands are absent in lane 12, which has untransformed YPH252 yeast DNA and lane 1, which has pRS313 transformed YPH252 DNA, the bands are specific for yeast transformed with plasmids with a 2μ origin. These bands may be due to recombination of the vectors with the endogenous 2μ plasmid. In Fig. 2, some of the higher bands overlap the estimated 9-kb *CDC23* band; this chromosomally derived band is unobstructed in lanes 1 and 12. The plasmid origin of the higher bands was demonstrated by reprobing with nick translated pBluescript (data not shown).

The *CDC23* band and the yeast single copy pRS313 vector had similiar signal intensities, as expected for a YCp vector. In the 5 ng lanes, the plasmids pRS423 and pRS425 had a signal intensity approximately equal to the single copy standards, while pRS425 had a greater signal intensity and pRS424 and YEp24 had less (Fig. 2, panel B). These various yeast vectors therefore have a copy number

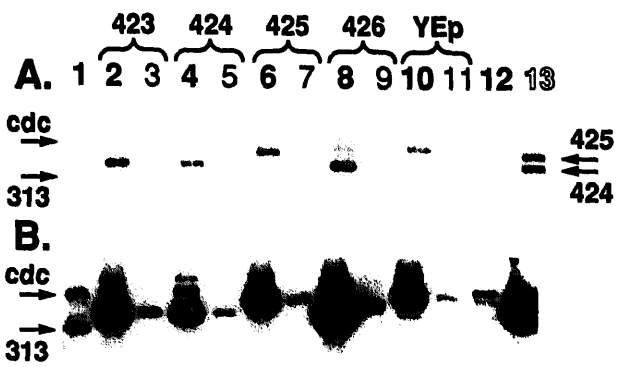

Fig. 2. Southern blot determination of copy number. All DNA was digested with *Bam*HI, which cuts each plasmid only once, and loaded onto a 0.8% agarose gel. Lane 13 was loaded with 1 ng each of purified pRS424 and pRS425 plasmid DNA as markers; the arrows on the right margin mark their positions. All other lanes were loaded with total YPH252 yeast DNA, either 100 ng (bold numbers) or 5 ng (a 1:20 dilution). Lane 12 contained DNA from untransformed YPH252, lane 1 was from a pRS313 transformant (pRS313 band marked on left with arrow labeled "313"), and lanes 2–11 contained DNA from YEp24 or pRS420 series transformants as designated by the brackets. Panels **A** and **B** are different exposures of the blot hybridized with pRS90; the position of the yeast chromosomal *CDC23 Bam*HI band is marked on the left margin with arrows labeled "cdc".

of about 10–30 per haploid yeast genome. This is within the range of 10–40 copies per cell reported for a number of YEp-type vectors (Futcher and Cox, 1984; Rose and Broach, 1991).

(d) Conclusions

The pRS420 series of plasmids provides a high copy number counterpart to the pRS400 and pRS410 series of yeast vectors. All of these shuttle vectors share the useful properties of pBluescript for growth in *E. coli* and in vitro manipulation. In yeast, the pRS420 plasmids are about as stable as YEp24 and centromeric vectors. The four yeast markers available in the pRS400, pRS410 and pRS420 series of vectors provide valuable flexibility for transformation and restriction site selection. The vectors pRS423, pRS424, pRS425 and pRS426 are deposited with the American Type Culture Collection (ATCC Rockville, MD) under the respective Nos.: ATCC77104, ATCC77105, ATCC77106 and ATCC77107.

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The vectors described in this paper are available on request from T. C. Please include a self-addressed envelope of about 23 × 30 cm (9 × 12 inches); for U.S. requests, please affix 75 cents postage.

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