Plasmids Resembling 2- μ m DNA in the Osmotolerant Yeasts *Saccharomyces bailii* and *Saccharomyces bisporus*

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Five of eight strains of *Saccharomyces bailii* and one of 13 strains of *S. bisporus* were found to harbour DNA plasmids. pSB1 and pSB2 plasmids were isolated from *S. bailii* strains IFO 0488 and IFO 1047, respectively, and pSB3 and pSB4 from *S. bisporus* strain IFO 1730. All four plasmids resemble 2- μ m DNA of *S. cerevisiae* in that (i) their molecular sizes are about 6 kb, (ii) each molecule possesses a pair of inverted repeats, (iii) they exist as a mixture of two isomers and (iv) their copy numbers in the native host are similar. None of them showed homology with 2- μ m DNA or with each other by Southern hybridization under moderately stringent conditions, but pSB4 hybridized with the pSR1 DNA, which was found previously in a strain of *S. rouxii*. Each of the pSB plasmids has DNA sequence(s) effective for autonomous replication in *S. cerevisiae*. In *S. cerevisiae*, pSB3 and pSB4 showed intramolecular recombination but neither supported isomerization of 2- μ m DNA.

INTRODUCTION

In a previous study (Toh-e *et al.*, 1982), we surveyed various yeast strains other than *Saccharomyces cerevisiae* for plasmids. Of the 100 strains examined, two strains of osmophilic or halophilic yeast, *S. rouxii*, were each found to harbour a single species of plasmid, pSR1 or pSR2. No plasmids were found in the other 98 strains. The rarity of plasmids in yeasts other than *S. cerevisiae* was also noted by Gunge *et al.* (1981). They surveyed 57 strains of yeast other than *S. cerevisiae* and found two kinds of linear DNA plasmid in a strain of *Kluyveromyces lactis*, but none in the other 56 strains. The plasmids pSR1 and pSR2 have similar structural characteristics to 2- μ m DNA (Broach, 1981; Hartley & Donelson, 1980) and to each other in that (i) their molecular sizes are 6 kb, (ii) restriction analysis suggests each has a pair of inverted repeats and (iii) they have similar copy numbers in the native host. The plasmid DNAs of pSR1 and pSR2 were able to hybridize with each other but not with 2- μ m DNA by Southern hybridization. pSR1 has an autonomously replicating sequence (*ARS*) effective in *S. cerevisiae*.

Since we detected plasmids in two strains of *S. rouxii*, we surveyed 21 strains of similar osmophilic (or osmotolerant) yeasts for plasmids. This communication describes four plasmids, pSB1, pSB2, pSB3, and pSB4, resembling 2- μ m DNA; pSB1 or pSB2, or similar molecular species, were detected in five independent strains of *S. bailii*, and pSB3 and pSB4 in a strain of *S. bisporus*. None of the pSB plasmids showed DNA homology with 2- μ m DNA or pSR1, or each other, except for pSB4, which hybridized with pSR1. Each plasmid also possesses an *ARS* sequence effective in *S. cerevisiae* host.

Table 1. *Principal micro-organisms and plasmids*

Organism or plasmid	Genotype or characteristics	Source or reference*
<i>S. bailii</i>		
IFO 0468	Wild-type (pSB1, pSB2)	IFO
IFO 0488	Wild-type (pSB1)	IFO
IFO 1047	Wild-type (pSB2)	IFO
IFO 1137	Wild-type (one plasmid)†	IFO
IFO 1801	Wild-type (two plasmids)‡	IFO
<i>S. bisporus</i>		
IFO 1730	Wild-type (pSB3, pSB4)	IFO
Plasmid		
pSR1	Cryptic (6.2 kb)	Toh-e <i>et al.</i> (1982)
pSB1	Cryptic (6.0 kb)	This study
pSB2	Cryptic (5.4 kb)	This study
pSB3	Cryptic (6.4 kb)	This study
pSB4	Cryptic (5.8 kb)	This study

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† *S. bailii* strain IFO 1137 has a plasmid showing the same migration on an agarose gel as that of pSB1.

‡ *S. bailii* strain IFO 1801 has two species of plasmid which show the same migration on an agarose gel as those of pSB1 and pSB2.

METHODS

Organisms and plasmids. Principal strains of *S. bailii* and *S. bisporus* used and plasmids detected are listed in Table 1. Strains of *S. bailii* (IFO 0519, IFO 1098, and IFO 1611) and *S. bisporus* (IFO 0467, IFO 0723, IFO 1055, IFO 1131, IFO 1249, IFO 1250, IFO 1615, IFO 1732, IFO 1733, IFO 1734, IFO 1736, and IFO 1737) were screened for plasmids, but with negative results (not listed). *Saccharomyces cerevisiae* strain SHY3 (*MATa steVC9 ura3-52 trp1-289 leu2-3,112 his3 ade1-10 can1-100*; Botstein *et al.*, 1979) harbouring 2-µm DNA, i.e. the *cir*⁺ strain, was obtained from D. Botstein. The *cir*⁺ derivative (i.e. a strain without harboured plasmids) of strain SHY3 was constructed by the method of Toh-e & Wickner (1981), and provided by Y. Kikuchi. These *cir*⁺ and *cir*⁰ strains of SHY3 were used for the detection of *ARS* function and intramolecular recombination of the plasmids with pJDB219 (Beggs, 1978), a chimeric plasmid of 2-µm DNA and pMB9, in which the gene necessary for intramolecular recombination of 2-µm DNA is inactivated. For detailed analysis of yeast plasmids, a yeast integrative vector, YIp5 (Botstein *et al.*, 1979), which has the *URA3* gene of *S. cerevisiae* and ampicillin and tetracycline resistance genes of pBR322 but is unable to replicate in a *S. cerevisiae* host, was employed for construction of hybrid plasmids. *Escherichia coli* strain JA221 (F⁻ *leuB6 ΔtrpES lacY hsdR⁻ hsdM⁺ recA*) was used for the propagation of YIp5 and hybrid plasmids, as described previously (Toh-e *et al.*, 1982).

Electron microscopy of DNA. Fold-back molecules were prepared by the method of Davis *et al.* (1971). Electron micrographs of the DNA molecules were taken after shadowing with a Pt-Pd cathode at a magnification of 8000 under an electron microscope (model HS70; Hitachi, Tokyo, Japan). Contour lengths of DNAs were measured with a Numonics graphic calculator (model 1250; Numonics Co., Lansdale, Pa., USA) after enlargement of the image with a Nikon profile projector (model V-12; Nihon Kogaku Co., Tokyo, Japan). For calibration of single-stranded circles of M13mp7 (Messing *et al.*, 1981) (7238 bases according to the Catalog 1982-1983, New England Biolabs, Beverly, Mass., USA) and double-stranded circles of pBR322 (4362 bp; Sutcliffe, 1978) were used. M13mp7 plasmid DNA was purchased from Bethesda Research Laboratories, and pBR322 DNA was prepared from cleared lysates of *E. coli* JA221 (Davis *et al.*, 1971).

Media, genetic and biochemical methods. The compositions of nutrient and minimal media for yeast and *E. coli*, and the general methods of cultivation, genetics, biochemistry, Southern hybridization (Southern, 1975), and restriction analysis of DNA were as described previously (Toh-e *et al.*, 1982).

RESULTS

DNA plasmids in S. bailii and S. bisporus

Crude DNAs were prepared from eight strains of *S. bailii* and 13 strains of *S. bisporus* by the same rapid method (Cameron *et al.*, 1977) as employed for the *S. rouxii* strains (Toh-e *et al.*, 1982) and analysed by agarose gel electrophoresis. Although the detailed data are not shown, five strains of *S. bailii* and one strain of *S. bisporus* listed in Table 1 showed satellite DNA bands besides the heavy band of chromosomal DNA. The intensity of fluorescence of the bands on the

Fig. 1. Restriction analysis of pSB plasmids. Each pSB DNA was digested with the indicated restriction endonuclease(s) and electrophoresed on an agarose gel. *Hind*III-digested λ DNA was run in parallel as a size marker (Szybalski & Szybalski, 1979). (a) pSB1 (lanes 1 to 5) digested with *Eco*RI (lane 1), *Hind*III (lane 2), *Xho*I (lane 3), *Sal*I (lane 4) or *Pst*I (lane 5) and pSB2 (lanes 7 to 11) digested with *Eco*RI (lane 7), *Hind*III (lane 8), *Xho*I (lane 9), *Sal*I (lane 10) or *Pst*I (lane 11). Lane 6 is the λ DNA digested with *Hind*III. (b) pSB1 digested with *Hind*III (lane 1), *Sal*I (lane 2), *Sal*I and *Hind*III (lane 3), *Xho*I (lane 4), *Sal*I and *Xho*I (lane 5), *Pst*I (lane 7), *Pst*I and *Sal*I (lane 8), *Xho*I and *Pst*I (lane 9), and *Hind*III and *Pst*I (lane 10). Lane 6 is the λ DNA digested with *Hind*III. (c) Plasmid DNA fraction from *S. bisporus* IFO 1730 containing both pSB3 and pSB4. Lane 1, undigested DNA; lane 2, digested with *Xho*I; lane 3, with *Eco*RI; lane 4, with *Xho*I and *Eco*RI; lane 5, with *Pst*I; lane 6, with *Pst*I and *Eco*RI; and lane 7, λ DNA digested with *Hind*III.

gel indicated that the copy numbers of the plasmids in *S. bailii* and *S. bisporus* are roughly the same as that of 2- μ m DNA in *S. cerevisiae*. The migration patterns suggested two classes of *S. bailii* plasmids. The DNA preparations from strains IFO 0488 and IFO 1137 had open and closed circular DNA molecules of single species showing the same migration patterns as the corresponding molecules of 2- μ m DNA; and the plasmid found in IFO 1047 was smaller than 2- μ m DNA, as it migrated faster than 2- μ m DNA. We designated as pSB1 the plasmid found in *S. bailii* IFO 0488, and as pSB2 the one found in *S. bailii* IFO 1047. Two species of plasmid were found in the DNA preparations of *S. bailii* IFO 0468 and IFO 1801. In their migration on agarose gel, one species matched pSB1, the other pSB2. The identity of the plasmids in *S. bailii* IFO 0468 was supported by analysis with restriction endonucleases (described later).

The plasmids found in *S. bailii* IFO 1137 seem to be the same species as pSB1, while *S. bailii* IFO 1801 carries both pSB1 and pSB2, as does IFO 0468. These plasmids were, however, not investigated in detail in this study.

The migration pattern of DNAs on agarose gel indicated that *S. bisporus* strain IFO 1730 contains two different species of plasmids (data not shown). One of them has almost the same molecular size as 2- μ m DNA and the other is a little larger. We designated as pSB3 the larger species in *S. bisporus* IFO 1730, and as pSB4 the smaller one.

Structure of pSB plasmids

pSB1 DNA has a unique site for each of *Pst*I and *Sal*I, and two (or more) closely linked sites for both *Hind*III and *Xho*I (Fig. 1a). The molecular size of pSB1 was estimated to be approximately 6 kb from the migration distance of the *Pst*I and *Sal*I fragments. *Eco*RI digestion of pSB1 gave rise to four bands; the sum of the molecular sizes of the largest and the smallest and that of the two intermediates were both approximately 6 kb. This fact strongly suggests that pSB1 has a pair of inverted repeats, and that the two *Eco*RI sites are located on separate unique sequences. This is also supported by the results of double digestion with combinations of *Hind*III, *Pst*I, *Sal*I, and *Xho*I (Fig. 1b). One of the unique sequences has a single *Sal*I site and two closely linked *Hind*III sites, while the other has one *Pst*I site and two closely linked *Xho*I sites.

Table 2. Size of unique and repeated sequences of the pSB plasmids

Plasmid	No. of molecules measured	Length (bp)		
		Short unique	Long unique	Inverted repeat
pSB1	10	2326 $\pm$ 234	2913 $\pm$ 278	674 $\pm$ 66
pSB2	14	2111 $\pm$ 142	2522 $\pm$ 174	383 $\pm$ 40
pSB3	10	2628 $\pm$ 446	3042 $\pm$ 525	365 $\pm$ 81
pSB4	12	1741 $\pm$ 198	2670 $\pm$ 324	953 $\pm$ 87
pSR1*		1679	2654	959
2- μ m DNA†		2346	2774	599

* Our unpublished results.

† Molecular sizes are those described by Hartley & Danelson (1980).

The DNA of pSB2 has a single cutting site for *Eco*RI, giving rise to a 5.4 kb fragment (Fig. 1a). Digestion with *Xho*I gave rise to four bands, which can be explained by the presence of a pair of inverted repeats and each one of the two *Xho*I sites being located on each unique sequence. No *Sal*I and *Pst*I sites exist on pSB2. The five bands resulting from *Hind*III digestion of pSB2 can be explained by the presence of three *Hind*III sites, two of them on one unique sequence and the other on another.

With the structures of pSB1 and pSB2 in mind, we analysed the *Eco*RI and *Xho*I digests of the plasmid DNA from IFO 0468, which was suggested by agarose gel electrophoresis to contain two species of plasmids with the same mobilities as pSB1 and pSB2. The *Eco*RI digest of the plasmid DNA generated five bands, one with the same migration as the linear molecule of pSB2 and four others that matched those of the *Eco*RI digest of pSB1 DNA (data not shown). The *Xho*I digest also produced five bands, one migrating the same distance as the linear form of pSB1 and four other bands corresponding to those observed in the *Xho*I digest of pSB2.

Since pSB3 and pSB4 were isolated as a mixture from *S. bisporus* IFO 1730, restriction analysis of the plasmid DNA afforded limited information. The data in Fig. 1(c), however, indicate that each plasmid has one *Eco*RI and one *Xho*I site. When the plasmid DNA was digested with *Eco*RI and *Xho*I simultaneously, at least seven fragments were visible on the gel. These restriction patterns suggest that both the pSB3 and pSB4 plasmids have two isomers, as do pSB1 and pSB2.

To confirm physically the presence of inverted repeats in the pSB plasmids, the plasmid DNAs were denatured with alkali then allowed to renature. Under the electron microscope, these samples showed characteristic dumb-bell-shaped DNA molecules consisting of a double-stranded region at the centre and two single-stranded loops at the terminals (photomicrographs not shown). Measurements of 10 to 14 dumb-bell-shaped molecules for each plasmid species were made using pBR322 and M13mp7 as internal markers (Table 2).

To construct a more detailed map, composite plasmids were constructed by connecting each of the pSB plasmids with YIp5. Purified DNA samples of the composite plasmids were prepared from the *E. coli* host and analysed by digestion with various restriction enzymes. Structures of the pSB plasmids were determined as illustrated in Fig. 2 with the results of the above analysis (data not shown) together with the data of Fig. 1 and Table 2.

DNA homology between pSB, 2- μ m DNA, and pSR1 plasmids

To test whether the pSB plasmids share a common DNA sequence with 2- μ m DNA or pSR1, or with each other, DNA homology was examined by Southern hybridization under moderately stringent conditions as described previously (Toh-e *et al.*, 1982) by using ³²P-labelled DNA of pSR1 or that of 2- μ m DNA prepared by nick translation (Rigby *et al.*, 1977) as probe. None of DNAs of the intact pSB plasmids and the chimeric plasmids (Fig. 2) showed hybridization signals with 2- μ m DNA (Fig. 3b). In contrast, the DNA sample from *S. bisporus* IFO 1730, which has pSB3 and pSB4, hybridized with the pSR1 probe (Fig. 3c). Since in other experiments using the chimeric plasmids, ³²P-pAT89 (a probe for pSB3; Fig. 2) could not hybridize with

Fig. 2. Structure of pSB plasmids in composite plasmids constructed with YIp5. Open boxes indicate the DNA sequence of the pSB plasmid, and the thin lines are YIp5 (Botstein *et al.*, 1979). In the native state, pSB plasmids exist as circular molecules generated by ligation of the two ends of the pSB DNA sequence. Arrows indicate approximate size and position of the inverted repeats in each pSB molecule. Restriction sites: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sal*I; V, *Pvu*II, and X, *Xho*I.

Fig. 3. Southern hybridization of pSB plasmids. (a) Ethidium bromide staining of the gel. (b) ³²P-labelled 2-µm DNA probed hybridization. (c) ³²P-pSR1 probed hybridization. Lane 1, pSR1; lane 2, pAT71 (pSB1) digested with *Sal*I; lane 3, pSB2; lane 4, pSB3 and pSB4; lane 5, pAT68 (an uncharacterized plasmid found in the preparation of pSB1 DNA); and lane 6, 2-µm DNA. Two sets of these samples were run on the same agarose slab gel and transferred to a membrane filter. One of the photographs showing ethidium bromide staining was omitted from this figure since the print was the same as that seen in (a).

pSR1 (data not shown), the hybridization signals detected with the DNA sample of IFO 1730 were considered due to the pSB4 DNA. Thus, the pSB plasmids, pSR1, and 2-µm DNA are independent DNA species from each other, except that pSB4 has some homology in the DNA sequence with pSR1.

ARS sequence of pSB plasmid effective for the *S. cerevisiae* host

The chimeric plasmids (Fig. 2) were used to transform *S. cerevisiae*. In all cases tested, Ura⁺ transformants were obtained (Table 3), irrespective of the presence or absence of 2-µm DNA in the host, whereas the YIp5 DNA gave no Ura⁺ transformants. Several Ura⁺ yeast

Table 3. Yeast transformations with composite plasmids consisting of *YIp5* (*URA3*) and one of the *pSB* plasmids

Donor DNA*	No. of Ura ⁺ transformants per µg DNA†	
	<i>cir</i> ⁺	<i>cir</i> ^o
pAT71 (pSB1)	295, 467	119, 33
pAT73 (pSB1)	316, 454	97, 69
pAT63 (pSB2)	593, 1039	287, 51
pAT65 (pSB2)	222, 632	259, 48
pAT89 (pSB3)	1670, 695	578
pAT115 (pSB3)	221	98
pAT129 (pSB3)	403	112
pAT165 (pSB3)	955	NT
pAT88 (pSB4)	1883, 513	1492
YIp5	0	0
pJDB219 (2-µm DNA)‡	454, 108	71

NT, Not tested.

* Identity of the *pSB* plasmids is shown in parentheses.

† *cir*⁺ and *cir*^o strains of *S. cerevisiae* strain SHY3 (*ura3 leu2-3,112*) were used as the hosts. Numbers in the table indicate the number of transformants per µg DNA in each independent experiment.

‡ Transformants with pJDB219 were selected by the Leu⁺ phenotype.

transformants in each experiment were randomly selected and tested for the stability of the Ura⁺ trait during vegetative cell growth. Each yeast transformant clone was inoculated into nutrient medium, and the cultures were incubated at 30 °C overnight, then diluted and spread on a nutrient plate. About 100 colonies formed after incubation for 2 d at 30 °C were replicated onto minimal medium with and without uracil to score the frequency of Ura⁻ segregants. Both the *cir*⁺ and *cir*^o hosts carrying pAT71 (constructed with pSB1; Fig. 2), pAT65 (pSB2), pAT115 (pSB3), and pAT88 (pSB4) showed mitotic instability of the Ura⁺ phenotype: Ura⁺ cells amounted to 0 to 30% of the population after the overnight cultivation on nutrient medium, i.e. approximately six to seven generations of cell division. A high frequency of ampicillin resistant transformants of *E. coli* was obtained with all the DNA samples prepared from the yeast Ura⁺ transformants by the rapid method (Cameron *et al.*, 1977) (data not shown). All these observations accord well with the idea that each *pSB* plasmid has an *ARS* sequence effective for its autonomous replication in *S. cerevisiae*.

Intramolecular recombination of pSB plasmids in S. cerevisiae

Plasmid DNAs recovered by rapid alkali extraction (Birnboim & Doly, 1979) from the final *E. coli* transformants in the above experiments were digested with an appropriate restriction endonuclease. The original composite plasmids used for yeast transformation were similarly digested with the restriction endonucleases and run in parallel on the gel. When composite plasmids constructed with pSB3 (pAT89 and pAT115; Fig. 2) or with pSB4 (pAT88 and pAT126) were analysed, two different molecular species were found in the samples from the final *E. coli* transformants: one showed the same migration pattern as the original composite plasmid DNA, the other appeared to be the result of intramolecular recombination at the inverted repeats in the original composite plasmid molecule. This was confirmed by restriction analysis (data not shown). On the other hand, no isomerization was observed in either the *cir*^o or *cir*⁺ host of *S. cerevisiae* with the composite plasmids derived from pSB1 (pAT71 and pAT73) or pSB2 (pAT63 and pAT65) in 25 and 26 ampicillin resistant transformants tested, respectively.

To test whether the enzyme(s) responsible for the isomerization of *pSB* plasmids is also effective for that of 2-µm DNA, the *cir*^o host of *S. cerevisiae* was co-transformed with a pSB3 derivative (pAT89 or pAT115) or with a pSB4 derivative (pAT88 or pAT126), in combination with pJDB219, which bears the tetracycline resistance gene for the *E. coli* host and the *LEU2* gene for the *S. cerevisiae* host. Transformants were obtained by selecting for Leu⁺ Ura⁺

colonies. Since pJDB219 has lost the gene activity of 2- μ m DNA necessary for intramolecular recombination [*FLP* (Broach *et al.*, 1982) or *Able* (Hartley & Donelson, 1980)] by insertion of the pMB9 molecule at the *EcoRI* site on the *FLP* locus but is still able to serve as substrate for the *FLP* enzyme, it should undergo isomerization in the co-transformants if the homologous enzyme of the pSB plasmids reacts with the inverted repeats of 2- μ m DNA. DNAs were prepared from four independent Leu⁺ Ura⁺ yeast co-transformants selected from each different transformation experiment, and used for *E. coli* transformation. Ten tetracycline resistant *E. coli* transformants were picked up randomly from each transformation experiment. All 40 plasmids from the Leu⁺ Ura⁺ transformants tested, however, showed the same pattern as pJDB219 on gel electrophoresis after *EcoRI* digestion (data not shown). This fact suggests that the gene products of pSB3 and pSB4 do not affect the recombination of 2- μ m DNA.

DISCUSSION

Plasmids are rare in yeasts other than the hybridization group of *S. cerevisiae* (Gunge *et al.*, 1981; Toh-e *et al.*, 1982). In contrast, they appear to be common in *S. bailii*, since five of the eight strains tested harboured DNA plasmids. All of these yeast plasmids have common features with 2- μ m DNA and pSR1: (i) a molecular size of around 6 kb; (ii) a pair of inverted repeats, although of different length in different plasmids (Table 2); (iii) a high copy number (roughly the same as that of 2- μ m DNA in *S. cerevisiae*) in their natural host cells; (iv) two isomeric forms in the host cells; and (v) an *ARS* function effective in the *S. cerevisiae* host. It remains to be seen whether the *ARS* sequences found in the pSB plasmids function as the origin in the natural host. None of the plasmids found in this study showed DNA homology with 2- μ m DNA or pSR1, except for pSB4, which strongly hybridized with pSR1. Thus, pSB1, pSB2, and pSB3 were new species of yeast plasmid.

It was suggested that pSB4 and pSR1 have closely similar structures in that they both have a pair of inverted repeats, short and long unique sequences of similar lengths (Table 2), similar restriction maps (Fig. 2 and Toh-e *et al.*, 1982), and are able to hybridize with each other (Fig. 3). This fact further suggests a close phylogenetic relationship between *S. rouxii* and *S. hisporus*; they may have once been able to interbreed. It should be noted that these two species of yeast live in similar ecological niches with high salt or sugar content, such as the mashes for soy sauce and miso paste making, or jam, syrup and honey (van der Walt, 1970).

At least two factors are needed for site-specific recombination of 2- μ m DNA: one is a protein factor, probably an enzyme, which is encoded by the *Able* (Hartley & Donelson, 1980) or *FLP* (Broach, 1981; Broach *et al.*, 1982) gene of 2- μ m DNA; the other is the inverted repeats. Broach *et al.* (1982) delimited the sequence necessary for site-specific recombination to a 65 bp region in the middle of the inverted repeats. By analogy with the 2- μ m DNA system, it can be assumed that each pSB plasmid carries a similar gene to *FLP*. Derivatives of pSB3 (pAT89 and pAT115) and pSB4 (pAT88 and pAT126) undergo site-specific recombination in the *S. cerevisiae* *cir⁺* host. The fact that the co-existence of pAT88, pAT89, pAT115, or pAT126 with pJDB219 does not restore the ability of site-specific recombination to pJDB219 strongly suggests that the site-specific recombination systems of 2- μ m DNA and pSB3 or pSB4 are independent of each other. The inability of pAT71 (constructed with pSB1; Fig. 2) and pAT65 (with pSB2) to express the *FLP* function in the *S. cerevisiae* host can be interpreted in alternative ways: (i) the interruptions at the *SalI* site of pSB1 and at the *EcoRI* site of pSB2 inactivate the genes responsible for site-specific recombination; or (ii) *FLP* genes of pSB1 and pSB2 cannot express themselves in the *S. cerevisiae* host. These alternatives remain to be clarified.

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