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Physical and functional structure of a yeast plasmid, pSB3, isolated from *Zygosaccharomyces bisporus*

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ABSTRACT

The plasmid pSB3 of yeast Zygossacharomyces bisporus has been sequenced. It contains 6,615 base pairs, including a pair of inverted repeats (IR) consisting of 391 base pairs and 3 large open reading frames (ORF). One of the ORFs (A gene) participates in the recombination at the IRs and the other two (B and C genes) are necessary for the stable maintenance of this plasmid. The ARS sequence, which functions in a Saccharomyces cerevisiae host, was localized within 168 base pairs consisting of part of one of the IRs and a unique sequence contiguous to it. pSB3 can be maintained as stably in Z.rouxii as in the natural host Z.bisporus. In contrast, pSB3 is maintained fairly unstably in S.cerevisiae. The reason for this instability was found to be inefficient partitioning of pSB3 in S.cerevisiae. The molecular construction of pSB3 resembles that of 2-μm DNA, however, sequence homology at the DNA level was very poor.

INTRODUCTION

The 2-μm DNA is a yeast plasmid distributed widely among laboratory strains of S.cerevisiae(1-3).2-μm DNA is organized in chromatin structure with histones (4,5) and resides in the nucleus. This plasmid shares, at least in part, the same replication control as nuclear DNA (6,7). The advantage of the presence of this plasmid for yeast cells is not known,however, it is maintained stably at a rather high copy number (50 - 100 copies/cell, 2) and thereby shows non-Mendelian inheritance (3). The nucleotide sequence of the 2-μm DNA from Saccharomyces cerevisiae strain A364A D5 reveals that (i) the plasmid contains 6,318 base pairs, (ii) it has a pair of inverted repeats consisting of 599 base pairs, and (iii) there are 3 open reading frames or genes (A, B, and C) in this plasmid (8). The A gene, or FLP gene, participates in the site specific recombination between the two IRs (9,10). 2-μm DNA has one replication origin which is localized within the region spanning the junction between one of the IRs and the contiguous large unique region (9). The genes B and C are necessary for its stable maintenance (11,12).

In previous reports (13,14), we described circular DNA plasmids;pSR1 from Z.rouxii(S.rouxii), pSB1 and pSB2 from Z.bailii (S.bailii) and pSB3 and

pSB4 from one strain of Z.bisporus (S.bisporus IFO 1730). Although the gross structure of these plasmids resembles that of 2-μm DNA, no homology was detected among them by Southern hybridization except between pSR1 and pSB4. According to the restriction map along with the hybridization experiment, the nucleotide sequence of pSB4 is similar to that of pSR1. Comparative studies on these plasmids may shed some light on the mechanism of replication control, partitioning, and site specific recombination system as well as the significance of the presence of such plasmids in yeast cells, if any. To carry out such studies, information about nucleotide sequences of each plasmid is necessary. In this communication, we describe the nucleotide sequence of pSB3, from which we can predict the presence of 3 genes. One of them encodes a pSB3 specific FLP enzyme and the other two genes participate in the stable maintenance of this plasmid. The ARS functioning both in S.cerevisiae and Z.rouxii is located at the junction of one of the IRs and the contiguous unique sequence.

MATERIALS AND METHODS

Strains

Z.bisporus (Saccharomyces bisporus IFO 1730) contains two plasmids, pSB3 and pSB4 (14). pSB3 cloned into YIp5 (pBR322-URA3, 15) and derivatives from it were used for sequence analysis. Z.rouxii ME3 harbors pSR1 and is marked with a leucine requirement which can be complemented with the LEU2 gene of S.cerevisiae. To construct ME3 harboring pSB3, ME3 was first transformed to Leu^+ with a DNA mixture containing pAT286 (See Fig.6) and pSB3 and then each Leu^+ transformant was tested for the presence of pSB3 by agarose gel electrophoresis (Fig.1). The pAT286 plasmid was cured from the transformant and ME3[pSB3] was obtained. ME3[pSB3] could maintain pSB3 as stably as the natural host does. The absence of incompatibility between pSR1 and pSB3 was expected from the lack of sequence homology between these two plasmids. S.cerevisiae SHY3 (a steVC-9 leu2-3 leu2-112 ura3-52 trp1 his3Δ1 ade1-101 cir^+,15) and K12-2A (αleu2-3 leu2-112 ura3-1 ura3-2 cir^+) and cir^o derivatives from them were used as S.cerevisiae hosts to analyze the function of pSB3. Escherichia coli JA221 (F^- leuB trpΔE5 recA1 lacY hsdR, 16) was used for construction and purification of various recombinant plasmids.

Media

YPAD, SD, and various omission media were prepared as described previously (14) and were used for cultivation of yeasts. Nutrient broth and M9 medium with appropriate supplements were prepared according to Miller (17)

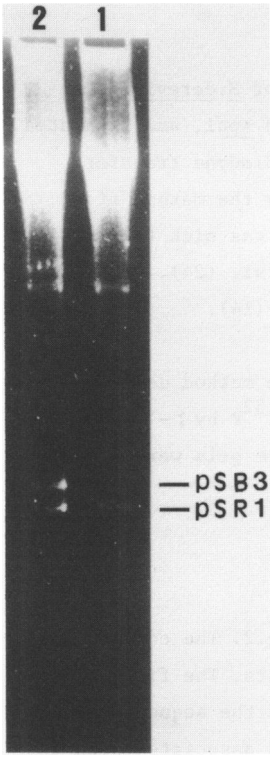

Figure 1. Construction of Z.rouxii containing pSB3. Z.rouxii ME3 was transformed to Leu$^+$ with a DNA mixture consisting of pAT286 and pSB3. Among 9 Leu$^+$ transformants thus obtained ,8 maintained pSB3. From one of the transformants the marker plasmid, pAT286, was cured and used as ME3[pSB3]. Total DNA was prepared from each of ME3 (lane 1) and Me3[pSB3] (lane 2) by the rapid method (21) and run on a 1% agarose gel.

and used for cultivation of E.coli. Yeasts were grown at 30 °C and E.coli at 37 °C.

Transformation

Yeast transformation was carried out by either the protoplast method (18) or the lithium acetate method (19). When Z.rouxii was used as a host, polyethyleneglycol 1000 was used instead of polyethyleneglycol 4000. E.coli transformation was done as described previously (14).

Preparation of plasmid DNA

The rapid extraction method (20) was followed through for preliminary characterization of recombinant plasmids. Yeast plasmids were prepared by the rapid method described by Cameron et al.(21).

Enzymes

T4-DNA ligase, T4-polynucleotide kinase, most of the restriction enzymes, and bacterial alkaline phosphatase were purchased from Takara Shuzo Co. (Kyoto,Japan). RsaI and NruI was from Nippon Gene (Niigata, Japan) and BanI, BanII, and BanIII were from Toyobo Biochemicals (Kyoto, Japan). Zymolyase

was from Kirin Brewry Co. (Takasaki, Japan).

Southern hybridization

Total DNA was prepared from the indicated strains of S.cerevisiae according to Hereford et al. (22), digested with BamHl and Kpnl, and separated on an agarose gel. The fragments were transferred to a Biodyne transfer membrane filter (Pall Ultrfine Filtration Corp.,N.Y.) by the method of Southern (23). The larger BamHl-Sall fragment of pBR322 was nick translated using ^{32}P-dATP (NEN,800Ci/mmole) according to Rigby et al. (24). Procedures for hybridization were the same as described previously (14).

Determination of DNA sequence

Nucleotide sequence was determined according to the method described by Maxam and Gilbert (25). 5' end of DNA was labelled with ^{32}P by γ-^{32}P-ATP (NEN,7000Ci/mmole) and T4-polynucleotide kinase. Sequence gels were 0.3mm thick, 6%, 8%, and 20% (30 x 40cm) polyacrylamide gels.

RESULTS

Nucleotide sequence of pSB3

Basic strategy for sequencing pSB3 was shown in Fig.2. The complete sequence is shown in Fig.3. pSB3 contains 6,615 base pairs. The first nucleotide of the Kpnl site is set as the first nucleotide of the sequence. The inverted repeats consist of 391 base pairs. IR1 which is associated with the ARS of pSB3 covers from 2,822 to 3,212, and IR2 from 5,878 to 6,268. There

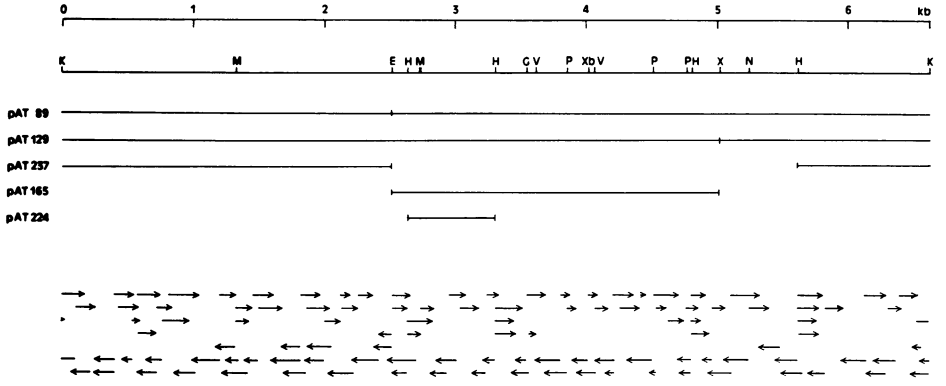

Figure 2. Subclones of pSB3 and sequence strategy. Arrows indicates the location, direction, and length of each sequence. ^{32}P-label is at each of arrow tail (5' end).
Symbols: E,EcoRl; G,BglII; H, HindIII; K, Kpnl; M, Mlul; N, Nrul;, P, Pstl; V, PvuII; X, Xhol; Xb,Xbal.

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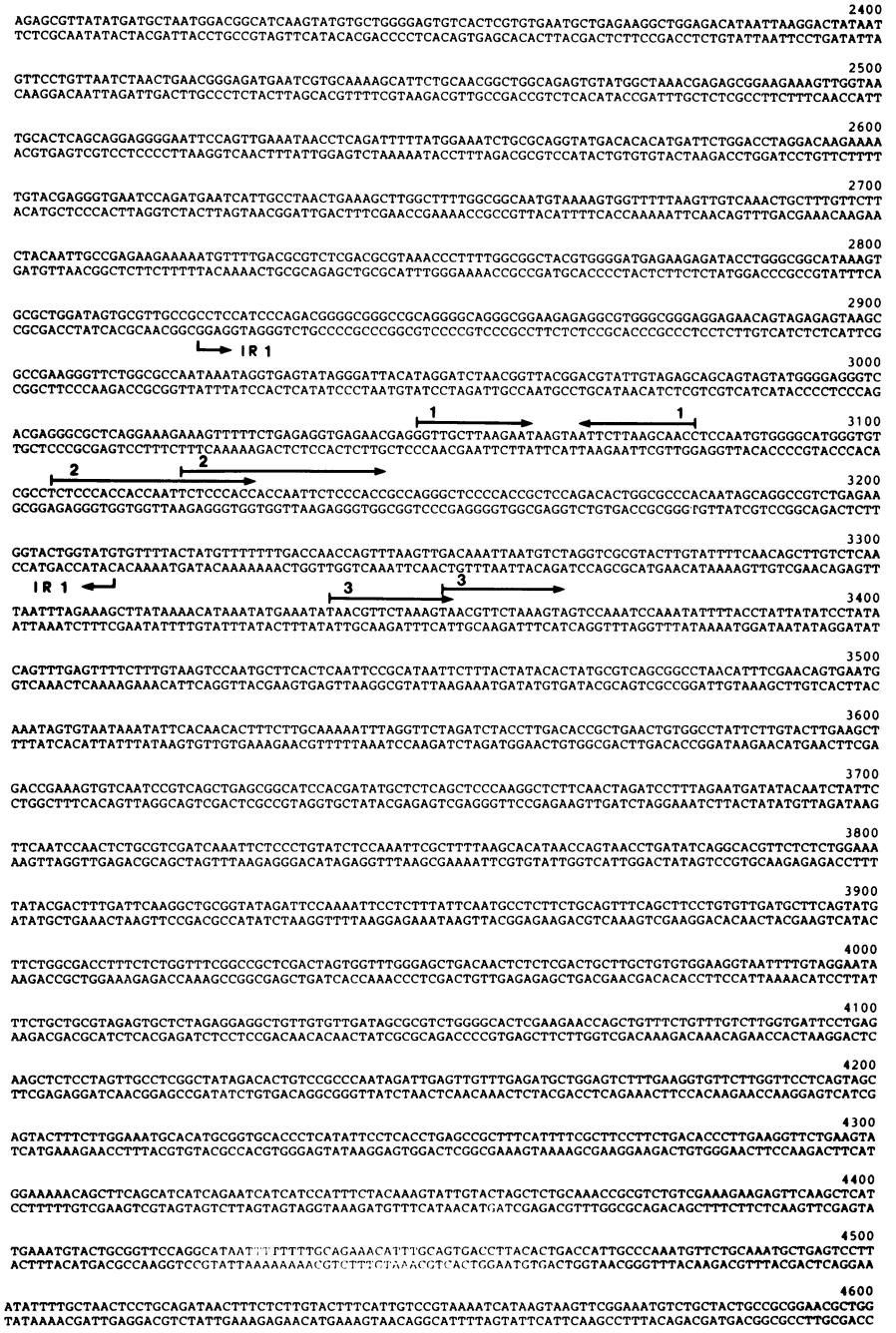
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1
1
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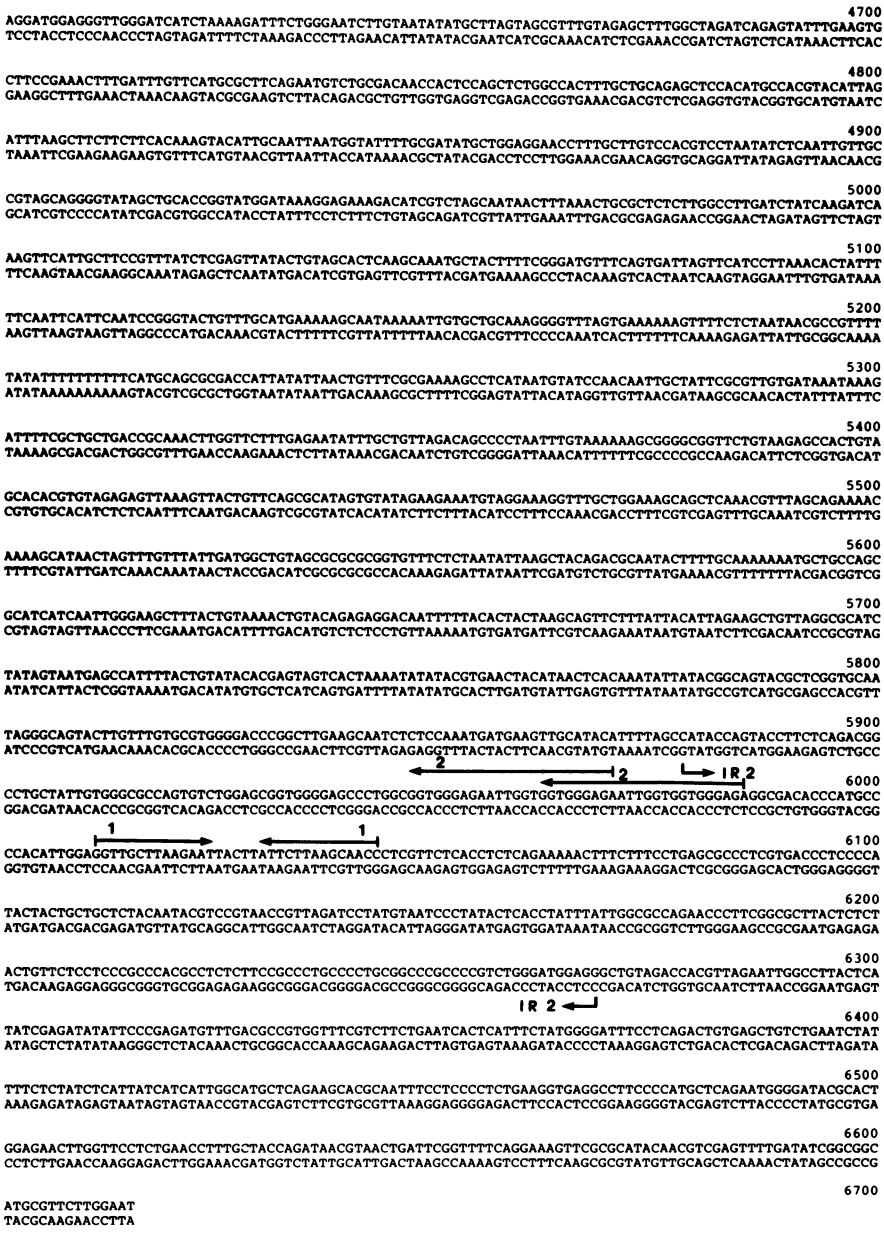


Figure 3. Sequence of pSB3. The first letter G of the KpnI site is the first nucleotide of the sequence. Direct repeats and inverted repeats are identified by numbers.

is a direct repeat consisting of 24 base pairs and an inverted repeat consisting of 14 base pairs in the IR. A direct repeat consisting of 15 base pairs can be seen near but outside of IR1. Three large open reading frames (ORF) are present in the sequence; ORF A (or gene A,1,704 base pairs) starts at 1,249 and ends at 6,161 on the bottom strand. ORF B (gene B, 966 base pairs) is from 4,425 to 3,460 on the bottom strand, and ORF C (gene C, 534 base pairs) from 1,999 to 2,532 on the top strand. Each gene has a unique restriction site; gene A contains a KpnI site, gene B contains a BglII site, and gene C contains an EcoR1 site. The XhoI site is also a unique site locating in the region where there is no coding frame.

ARS of pSB3

Since a competent host of Z.bisporus is not available at present, we used the S.cerevisiae host to localize the ARS of pSB3 and then the function of the ARS thus obtained was tested in the Z.rouxii host ME3. Z.rouxii is a better host to characterize the replication function of pSB3 than S.cerevisiae because (i) Z.bisporus is taxonomically more closely related with Z. rouxii and (ii) pSB3 is maintained in Z.rouxii stably.

Two plasmids, pAT89 and pAT129 (Fig.4), which contain the whole sequence of pSB3 can replicate in the S.cerevisiae host. pAT116 has been isolated by chance during cloning of the pSB3 sequence into YIp5 and it has two IRs but a part of unique sequence contiguous to IR1 has rearranged (sequence data not shown). pAT116 could not replicate in the S.cerevisiae host. This result indicates that IR1 and the unique sequence adjacent to it is necessary for the function of the ARS. The location of the ARS is narrowed down to within the HindIII fragment carrying IR1 since pAT224 consisting of the above Hind III fragment and YIp5 could replicate in S.cerevisiae. Refferring to the nucleotide sequence, we constructed a smaller plasmid, pAT299 harboring the 168 base pair BanII-HindIII fragment (Fig.4). This plasmid still retained the ARS function. These results are summarized in Fig.4.

Maintenance of the plasmids tested was unstable in the S.cerevisiae host. By using plasmid pAT299, we could succesfully transform the Z.rouxii host ME3 to Leu^+ (50 transformants/μg DNA in average). The resulting transformants lost the Leu^+ trait easily during cultivation in YPAD. These results suggest that the donor DNA used is maintained as a plasmid in Z.rouxii. Thus the 168 base pairs BanII - HindIII fragment contains sufficient information for the replication of pSB3 in both hosts, S.cerevisiae and Z.rouxii.

In spite of the cryptic nature of pSB3, it is possible to introduce this plasmid into S.cerevisiae cells by using cotransformation with a plasmid

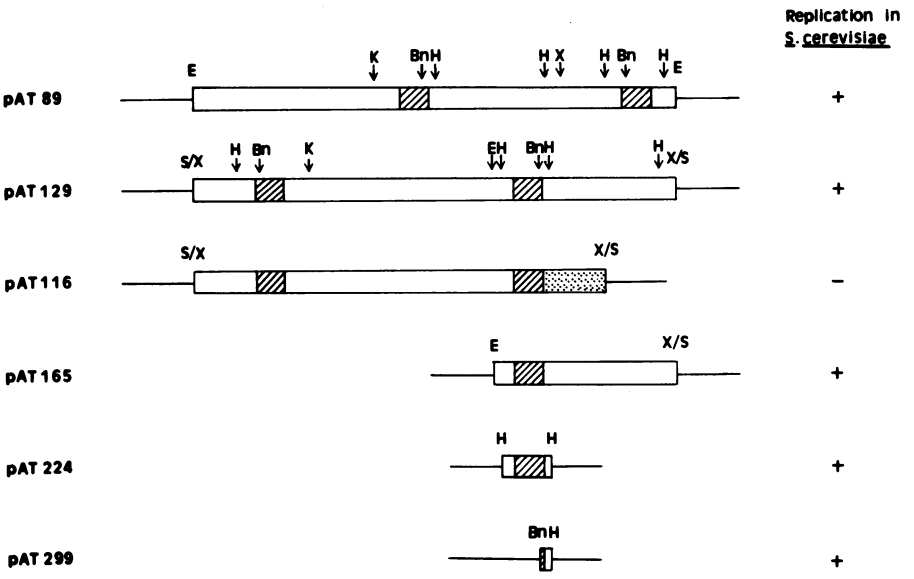


Figure 4. Localization of the ARS of pSB3 functioning in S.cerevisiae. Sequence derived from pSB3 was shown by boxes. Hatched arears represent IR1 and IR2. Thin lines contain sequence of pBR322 and the LEU2 and URA3 genes. pAT89 and pAT129 contain the whole sequence of pSB3. pAT165 was constructed by deleting the EcoR1 fragment carrying IR2 from pAT129. pAT224 was constructed by inserting the HindIII fragment carrying IR1 into the HindIII site of YIp5. Sequence rearranged in pAT116 was shown by a dotted area. pAT116 has full length of IR1 and IR2. pAT299 was constructed by substituting BanII-HindIII fragment of YIp5 by BanII-HindIII fragment (about 170 base pairs) of pSB3. ARS activity was assayed by transforming appropriate S.cerevisiae host. High frequency of transformation and instability of the transformed trait shown by the transformants are criteria for the presence of the ARS function. Symbols: Bn, BanII; S, SalI. Others are the same as those described in the legend to Figure 2.

carrying an appropriate marker. The fact that S.cerevisiae carrying pSB3 thus constructed easily lost this plasmid (50% retention after overnight growth in YPAD) indicates that instability of recombinant plasmids containing the ARS of pSB3 in S.cerevisiae is due to an intrinsic character of pSB3. Instability of pSB3 and its derivatives in S.cerevisiae is due either to inefficient replication or to inefficient partitioning. To test this possibility, the BglII fragment containing the CEN4 of S.cerevisiae was excised from YCp19(26) and then inserted into the BamH1 site of pAT89 and the resulting plasmid (pAT295) was used to transform K12-2A cir^{o} to Ura^{+}. Stability of the Ura^{+} trait was compared among Ura^{+} transformants carrying different plasmids. As shown in Table 1, CEN4 greatly improves the stability of pAT89. Since pAT295

Table 1. CEN4 stabilizes plasmids carrying the ARS of pSB3 in S.cerevisiae.

Plasmid	Relevant characteristics	%Ura^{+} b)
pAT129	ARS of pSB3	16.2
pAT89	ARS of pSB3	10.9
pAT295 a)	ARS of pSB3, CEN4	82.0
YCp19	ARS1, CEN4	80.0

a) pAT295 was constructed by inserting the BglII fragment containing the CEN4 excised from YCp19 into the BamH1 site of pAT89.
b) The indicated plasmid was introduced into the SHY3 host by protoplast transformation. 4 independent Ura^{+} transformants from each experiment were grown overnight in YPAD and tested for their retension of the Ura^{+} trait.

shows the same level of stability as that of YCp19, the heterologous ARS derived from pSB3 functions as efficiently as the ARS1 does in S.cerevisiae. The BglII fragment carrying CEN4derived from YCp19 does not harbor an intact ARS since this fragment could not support stable autonomous replication of YIp5 (Data not shown). This result indicates that the instability of pSB3 and its derivatives in S.cerevisiae is due to inefficient partitioning. The CEN4 did not function in Z.rouxii (Data not shown).

FLP function

As previously reported (14), the FLP function of pSB3 is expressed in S. cerevisiae and the FLP enzyme of pSB3 does not use the IR of 2-μm DNA as substrate. pAT287 contains the A gene of pSB3 which has been destroyed by inserting a 2.1kb Kpn1 fragment derived from YEp13 at the unique Kpn1 site of pSB3. DNAs were prepared from transformants carrying pAT129 (the parent of pAT287) or pAT287, digested with BamH1 and Kpn1, and analyzed by Southern hybridization by using ^{32}P-labelled pBR322 (larger BamH1 - Sal1 fragment) as probe. As shown in Fig.5, DNA containing pAT129 gave rise to two hybridization bands which were expected from the FLP function, whereas only one band could be seen in the lane having the DNA sample containing pAT287. This result indicates that pAT287 does not undergo intramolecular recombination in this host. The defect of the FLP function of pAT287 was complemented by the coexistence of pSB3 (Fig.5, lane 6). We concluded that the A gene codes for the FLP enzyme of pSB3.

Stability

Since S.cerevisiae was not a suitable host to analyze the mechanism of stable maintenance of pSB3, we decided to use Z.rouxii for this purpose.

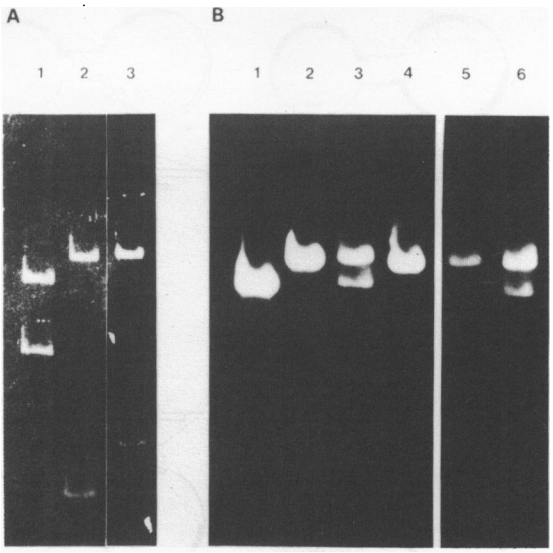
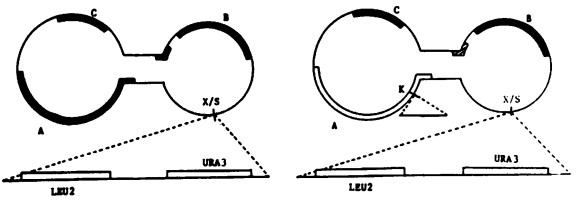


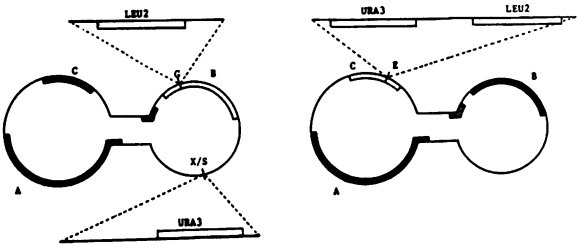
Figure 5. Identification of the gene encoding FLP function of pSB3. Total DNA was isolated from each of K12-2A[pAT129] or K12-2A[pAT287], or K12-2A[pAT287, pSB3], and processed for Southern analysis. Since the probe was the larger BamHl - Sall fragment of pBR322, only the largest fragment of each digest could be hybridized. Panel A: Ethidium bromide staining; lane 1, intramolecular recombinant derived from pAT129; lane 2, pAT129; lane 3,pAT 287. Digestion patterns of genomic DNA were omitted from this figure. Panel B: Autoradiogram. lane 1,intramolecular recombinant derived from pAT129; lane 2, pAT129, lane 3, K12-2A[pAT129]; lane 4, pAT287; lane 5, K12-2A[pAT287]; lane 6, K12-2A[pAT287, pSB3].

Since the Leu^- of ME3 can be complemented by the LEU2 gene of S.cerevisiae, a series of plasmids containing the LEU2 gene of S.cerevisiae were constructed as shown in Fig.6. pAT288 retains all 3 ORFs intact, the B gene is inactivated in pAT293, the C gene was inactivated in pAT286 and the A gene was inactivated in pAT302. Each plasmid DNA was introduced either into ME3 or ME3[pSB3]. Four independent Leu^+ transformants selected from each transformation experiment were grown under non-selective conditions overnight (6 - 7 generations) and plated on a YPAD plate after an appropriate dilution. Colonies developing on the plates were tested for their retention of the Leu^+ phenotype by replica-plating onto complete minus leucine medium. pAT288 as well as pAT302 were maintained as stably in ME3 as in ME3[pSB3]. This result indicates that the FLP enzyme is not necessary for maintenance of pSB3 under this condition. When the B gene or the C gene was inactivated, the stability of the plasmid in ME3 was greatly reduced. This destabilization was cancelled



pAT288 pAT302

HOST	Leu$^+$ (%)	Leu$^+$ (%)
ME3	43.9	62.0
ME3[pSB3]	47.5	55.6



pAT293 pAT286

HOST	Leu$^+$ (%)	Leu$^+$ (%)
ME3	0	1.9
ME3[pSB3]	58.9	49.4

Figure 6. pSB3 genes required for its stable maintenance. pAT302 was constructed by inserting the SalI - XhoI fragment containing the LEU2 gene at the SalI site of pAT287. pAT293 was constructed by inserting the BglII fragment containing the LEU2 gene into the BglII site locating within the B gene. pAT286 was constructed by inserting the Sal - XhoI fragment containing the LEU2 gene at the SalI site of pAT89. Indicated plasmid was introduced into Z.rouxii ME3 or ME3[pSB3] by transformation via protoplasts. The stability of the Leu$^+$ trait was tested and the rate of retention of the Leu$^+$ trait was expressed in percent.

by the coexistence of pSB3. These results indicate that both B and C genes of pSB3 participate in stabilization of pSB3 by producing trans-acting factors. pAT299 which has the 168 base pairs fragment containing the ARS of pSB3 was found to be unstable in ME3[pSB3]. This result suggests that the presence of a certain cis-acting DNA sequence is also required for the stable maintenance.

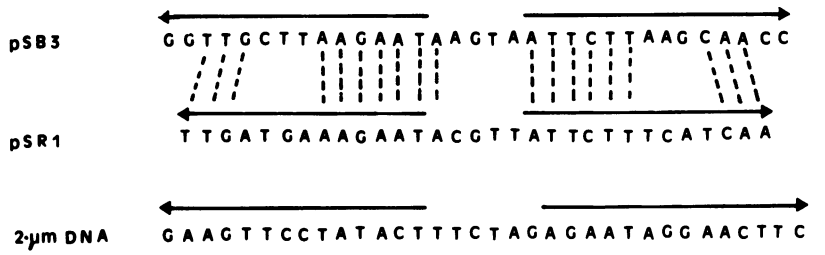


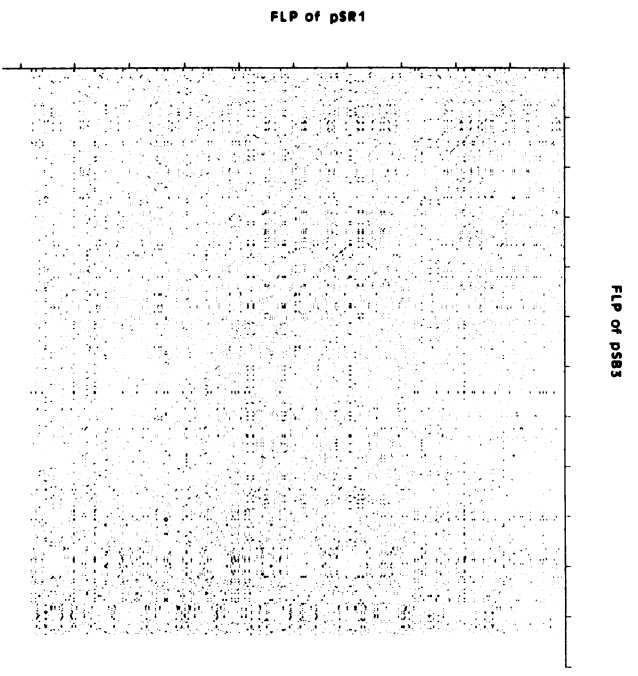
Figure 7. Comparison of nucleotide sequence of putative cross-over sites of pSB3 with those of 2-μm DNA or pSR1. Two arrows drawn in each sequence represent a pair of inverted repeats surrounding a 5 or 6 base pairs central region. Direct sequence homologies are indicated by the dashed lines.

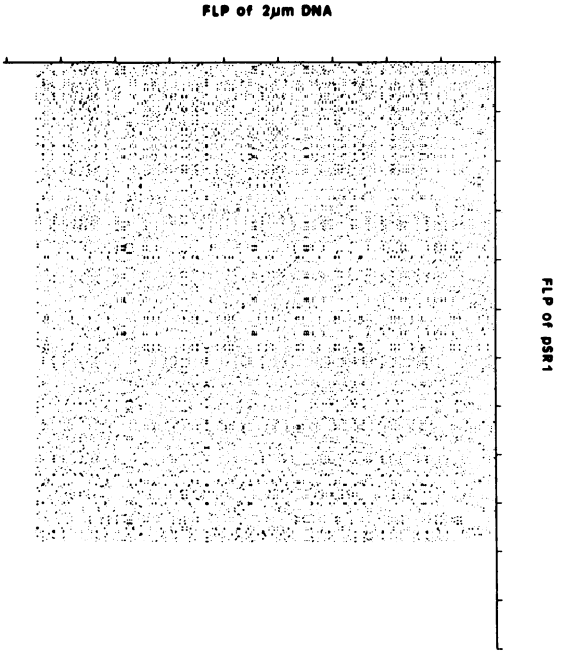
DISCUSSION

Comparing nucleotide sequences between pSB3, pSR1 (27), and 2-μm DNA (8), there is no extensive homology among them. pSB3 shares three sequences consisting of 12 base pairs each with 2-μm DNA and one sequence consisting of 14 base pairs with pSR1. This observation supports our previous result (14). However, gene organization in these plasmids are rather similar;(i) each plasmid has 3 large ORFs, (ii) one of them participates in the FLP function, (iii) the other two are essential for the stable maintenance of the plasmid itself, at least in the case of 2-μm DNA and pSB3.

Location of the ARS of pSB3 functioning in *S.cerevisiae* has been narrowed down to 168 base pairs (3,315 - 3,148) which consists of part of an IR and the contiguous unique region. Although this region does not contain a typical consensus ARS sequence for *S.cerevisiae*, $\frac{T}{A}TTTAT\frac{A}{G}TTT$, it works as efficiently in the *S.cerevisiae* host as the *ARS1* does. ARS selected in a *S. cerevisiae* host among heterologous DNA does not necessarily work as ARS in the orginal organism (28). However, the ARS sequence of pSB3 was effective as an ARS in *Z.rouxii* and most probably in *Z.bisporus*. It should be emphasized that ARSs of yeast plasmids are located near or within an IR.

The cross-over site mediated with the FLP enzyme of 2-μm DNA has been limitted to a sequence of less than 65 base pairs (10), within which there is an incomplete dyad symmetry consisting of 14 base pairs. This structure is most probably the cross-over site. A dyad symmetry can be also seen in the IRs of pSB3 as well as in the IRs of pSR1. There are precise 14 base pairs and 13 base pairs inverted repeats separated by 5 base pairs, in pSB3 and pSR1, respectively.Sequence similarity between the cross-over site of 2-μm DNA and


FLP of pSR1
FLP of pSB3


FLP of 2μm DNA
FLP of pSR1

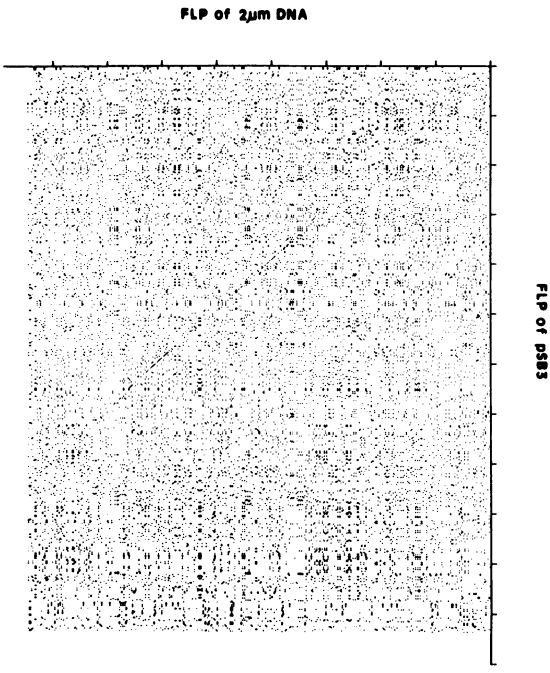


Figure 8. Dot matrix analysis of amino acid sequence of the putative FLP enzyme of pSB3 with that of 2-μm DNA or pSR1. Amino acid sequence was deduced from the FLP gene of each plasmid. A dot was plotted when the same amino acid was encountered. A computer program developed in our laboratory was used for this analysis.

loxP of P1 phage has been pointed out by Vetter et al. (29). Inspecting sequence similarity between these putative cross-over sites, there is no clear homology between 2-μm DNA and pSB3 or pSR1. However, remarkable sequence similarity between the putative cross-over sites of pSR1 and pSB3 can be seen (Fig.7). When amino acid sequence was compared between putative FLP enzymes of these 3 plasmids by dot matrix analysis, there was significant homology between them (Fig.8). In spite of these similarities, the function of each FLP gene is specific to the homologous plasmid.

Stable maintenance of 2-μm DNA in S.cerevisiae requires 3 plasmid encoded factors; 2 protein factors and one cis-acting site or STB site (11,12). A similar mechanism is working in the stable maintenance of pSB3. We found that two genes, B and C,were required for stabilization of pSB3. Our result also suggested the presence of a cis-acting site which receive the signals from the B and C genes, however, it remains to be localized on the plasmid yet.

Direct tandem repeats can be seen around the STB site of 2-μm DNA, however, there is no such unique sequence on plasmid pSB3. Furthermore, the difference of stability of pAT288 expressed in S.cerevisiae and in Z.rouxii suggests that some host factor(s) is needed for the stabilization of the plasmid. pAT288 is less stable than pSB3 in ME3. This is probably because (i) the size of the recombinant plasmid is too large, or (ii) the interruption at the XhoI site inactivates a function which may play some role in stabilization of the plasmid.

S.cerevisiae is tolerant of the presence of a large number of replication origins; it has approximately 400 ARSs distributed over 17 chromosomes and it still has room for another 100 copies of 2-μm DNA. This may be also true for Z.rouxii and Z.bisporus, in which two different species of high copy number plasmids can coexist. From these observations, we infer that incompatibility of yeast plasmids, if present, is due to competion for partitioning mechanism rather than for replication machinery.

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