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Construction of a Yeast Plasmid Cloning Vector with High Stability in *Saccharomyces cerevisiae* Strains Deficient in 2 μm *DNA***

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Plasmid *pCP1* was constructed from cloned 2 μm *DNA* of *Saccharomyces cerevisiae* and from plasmid *pJDB207*. Vector *pCP1* contains the complete *B* form of 2 μm *DNA* interrupted in the *FLP* gene, together with *DNA* derived from the *Escherichia coli* plasmid *pAT153* and a low expression variant of the *S. cerevisiae LEU2* gene. The new vector is lost at a low frequency from yeast cir^+ or cir^0 strains under non-selective growth conditions and is stable against rearrangements in cir^0 strains. Its usefulness for curing cir^+ strains from endogenous 2 μm *DNA* and for their conversion to cir^0 strains was demonstrated.

(Keywords: Cloning vectors)

Konstruktion eines Hefe-Plasmid-Cloningvektors mit hoher Stabilität in 2 μm DNA-defizienten Saccharomyces cerevisiae-Stämmen

Plasmid *pCP1* wurde ausgehend von klonierter *Saccharomyces cerevisiae*-2 μm *DNA* und Plasmid *pJDB207* konstruiert. Der Vektor enthält die vollständige, im *FLP*-Gen unterbrochene *B*-Form der 2 μm *DNA*, vom *Escherichia coli*-Plasmid *pAT153* abgeleitete *DNA* und eine wenig aktive Variante des *S. cerevisiae-LEU2*-Gens. Der neue Vektor weist unter nichtselektiven Wachstumsbedingungen in cir^+- und cir^0-Stämmen eine niedrige Verlustrate auf und ist in cir^0-Stämmen stabil gegen Umlagerungen. Seine Verwendbarkeit für die Umwandlung von cir^+-Stämmen in cir^0-Stämme durch Verdrängung endogener 2 μm-*DNA* wurde nachgewiesen.

** Dedicated to Professor Dr. *Karl Schlögl* on the occasion of his 60th birthday.

Introduction

Since the first description of methods for the transformation of yeast cells[1,2] a number of plasmid vectors have been developed which can be used in *Saccharomyces cerevisiae* or in other yeasts (see e.g. Refs.[3,4]). Most of these vectors are hybrid plasmids, which can be produced in larger quantities in *Escherichia coli* before they are used for yeast transformation. Those vectors capable of autonomous replication in yeast contain replication origins derived either from the endogenous 2 μm plasmid of *S. cerevisiae* or from yeast chromosomal sequences. Compared to 2 μm *DNA*, all these artificial plasmids exhibit markedly reduced stability[4,5]. In the absence of selective pressure, they are lost from yeast cells with frequencies ranging from 0.1 percent to over 10 percent per generation. One factor, but not the only important one influencing stability is the number of plasmid copies per cell. Copy numbers vary from over 10^2 for some plasmids derived from 2 μm *DNA* to 1 for plasmids containing a chromosomal centromer[4]. Plasmid copy number can be a relevant factor in studies of gene regulation and is obviously important in the development of systems for the expression of foreign genes with high efficiency. Studies of expression of genes present on plasmids or their use in biotechnology may be hampered in those cases where the structure of the plasmid used is unstable. In yeast cells containing the 2 μm plasmid (*cir*$^+$ cells) this circular *DNA* exists in two forms, which are quickly interconverted by homologous recombination[3]. At least in *cir*$^+$ cells, cloning plasmids derived from 2 μm *DNA* will also be altered with high frequency by recombination events[6]. On the other hand, many 2 μm plasmids are easily lost from cells free of endogenous plasmid (*cir*^{0} cells)[5,6].

In the course of studies aiming at the characterization of nonhistone proteins specifically bound to yeast plasmid chromatin[7] it became necessary to construct a high copy number plasmid that exhibited reasonable stability against loss and against changes in structure caused by recombinational events. The present paper describes a plasmid, which exhibits these properties when it is present in *cir*^{0} cells.

Experimental

Strains

E. coli K-12 strain *HB101*[8] was used in all bacterial cloning experiments. *S. cerevisiae* strain *13S-7A* (*a leu2 his3 ura3 trp1 ctt1 cir*$^+$) was derived from a cross of strains *WS13-9A* (*a leu2 his3 ura3 ctt1*)[9] and *SHU32* (*a leu2 ura3 trp1*) (kindly donated by *G. Ammerer*, Seattle). Strain *15A* (*a pep4 hem1 ura3 leu2 trp1 cir*$^+$) was obtained by crossing *13S-7A* with strain *5B* (*a pep4 hem1 ura3 leu2 trp1 cir*$^+$), which was obtained by a cross of strains *MIC-3C* (*a pep4 ura3 leu2*)

(obtained from *E. Mattes*, this laboratory) and *RG3-13B* (*a trp*1 *ura*3 *leu*2 *hem*1 *cyc*1)[10].

Media

Untransformed yeast strains were grown on *YPD* medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose), which was supplemented with Tween 80 (2.6 g/l), ergosterol (12 mg/l) and δ-aminolevulinate (50 mg/l) in the case of *hem*1 mutants. Transformants were usually grown on selective media as previously described[9].

Plasmids

E. coli plasmid *pBR322*[11] was used for cloning yeast 2 μm *DNA*. The yeast-*E. coli* hybrid plasmid *pJDB207*[3] was obtained from J. D. Beggs, London. Yeast 2 μm plasmid was isolated from strain *WS13-9A* as described by *Zakian* and *Scott*[12], using agarose gel electrophoresis instead of cesium chloride gradient centrifugation for the purification of the plasmid. Other plasmid *DNA*s were isolated from *E. coli* transformants as described previously[9]. Yeast 2 μm plasmid content of strains was assayed by isolation of a fraction enriched in plasmid *DNA*[13] followed by *RNA*ase treatment and gel electrophoresis.

Restriction endonuclease digestions, ligations

Restriction enzymes and *T4 DNA* ligase were purchased from BRL, Neu Isenburg, Germany. Digestion with restriction enzymes was carried out as recommended by the supplier. Electrophoretic separation and reisolation of restriction fragments was carried out as described before[9]. Ligations of *DNA* fragments were carried out with *T4 DNA* ligase.

Transformation procedures

E. coli and *S. cerevisiae* transformation was carried out essentially as described previously[9]. Ampicillin or tetracycline resistance was used for selection of *E. coli* transformants, yeast transformants were selected on leucine-deficient medium.

Results and Discussion

A yeast vector with high stability, especially in *cir*^{0} cells, should consist of the following components: 1. The complete sequence of the yeast 2 μm plasmid; this plasmid carries genes coding for components necessary for the maintenance of 2 μm-derived vectors in yeast cells[14,15]. These genes should therefore be present on the vector to ensure its stability in cells that do not contain any endogenous 2 μm plasmid. However, one of the gene products of 2 μm *DNA* (*FLP*)[14] catalyzes the interconversion of *A* and *B* forms of 2 μm sequences by homologous recombination within a 599 basepair inverted repeat, and should therefore be inactivated so that no such interconversion occurs in *cir*^{0} cells. 2. An allele of the *LEU2* gene with low efficiency of expression[16]; it has been demonstrated previously that vectors bearing this *LEU2* allele are maintained in high copy numbers in *leu*2 mutants transformed with such a vector when grown in leucine-free medium. 3. For technical reasons the vector should contain sequences

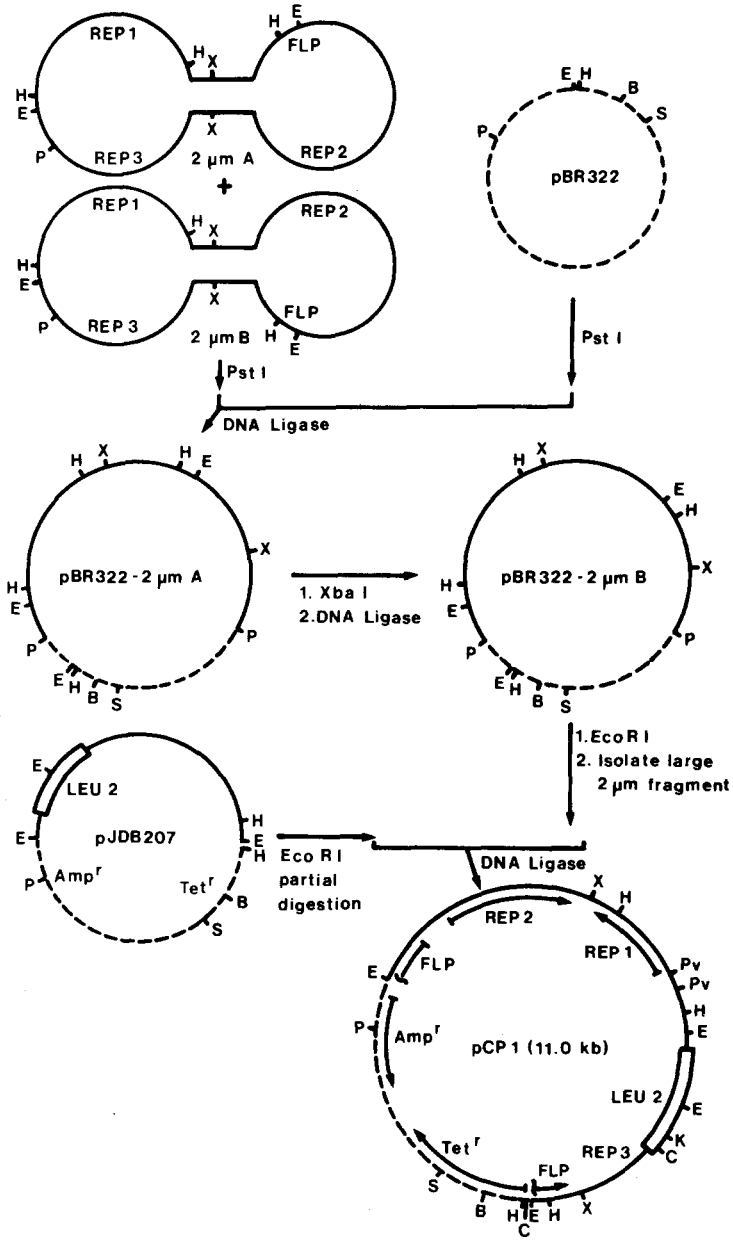
allowing its replication and selection in *E. coli* and unique restriction cleavage sites. To avoid unnecessary bacterial sequences, plasmid *pAT153*[17], a smaller derivative of *pBR322*, appears to be a good source of such sequences.

Since some of the components of the plasmid to be constructed are combined already in *pJDB207*[3], an *E. coli*-yeast hybrid plasmid, this vector was used as a starting material for our construction (Scheme 1). Plasmid *pJDB207* contains the *pAT153* sequences and the *LEU2* allele discussed above. It lacks, however, a major part of the 2 μm plasmid since it contains only the smaller of the two *Eco RI* fragments of the *B* form of the 2 μm plasmid. It was attempted therefore to isolate the larger 2 μm-*B Eco RI* fragment and to ligate it with *pJDB207* to reconstitute the complete 2 μm *DNA* sequence. For technical reasons it appeared easier to clone the whole 2 μm sequence into an *E. coli* vector as a first step.

As outlined in Scheme 1, plasmid isolated from an untransformed *cir*$^+$ yeast strain was cloned into the *Pst I* site of *pBR322*. Since all clones obtained happened to contain the *A* form of 2 μm *DNA*, one of the plasmids was converted to the *B* form *in vitro* by cutting with *Xba I* and religation. Partial *Eco RI* digestion of *pJDB207*, ligation with the large *Eco RI* fragment of 2 μm *DNA* isolated from *pBR322-2 μmB* and cloning of ligation products in *E. coli* resulted in the formation of plasmid *pCP 1*, which was characterized by restriction analysis. This new recombinant plasmid carries the *REP1*, *REP2* and *REP3* loci[15], which are necessary for stable maintenance of a 2 μm *DNA*-derived plasmid in *cir*^{0} cells. It contains an *FLP* gene inactivated by insertion of bacterial *DNA*. Accordingly, no rearrangement was observed in *pCP1* plasmids present in transformants derived from *cir*^{0} cells. The plasmid contains three unique cloning sites (*BamHI*, *SalI* and *PstI*).

A number of yeast strains were transformed with plasmid *pCP1*. As with other plasmids, transformation frequency varies dramatically from strain to strain and with the details of the transformation procedure. Therefore the results obtained are not documented in detail. It can be generalized, however, that numbers of transformants obtained with *pCP1* were consistently higher than those observed with the parent plasmid *pJDB207* and that they were in the range observed with the widely used plasmid *YEp13*[18]. When *cir*$^+$ strains are transformed with yeast plasmids derived from 2 μm *DNA*, most plasmids are present in transformants in copy numbers lower than that of the endogenous 2 μm *DNA*[16]. Some plasmids containing the partially defective *LEU2* allele present in *pJDB207* and *pCP1* have recently been reported to reach higher copy numbers than 2 μm *DNA* when transformants are grown in leucine-free medium[16]. Under such conditions these vectors compete efficiently with the 2 μm plasmid and after a number of generations on selective medium,

Scheme 1



Restriction sites: E: *Eco RI*, H: *Hind III*, X: *XbaI*, C: *ClaI*, B: *BamHI*, P: *PstI*, K: *KpnI*, Pv: *PvuII*.
ClaI, *KpnI*, and *PvuII* sites are given only in *pCP1*

transformants are observed that have lost the endogenous plasmid. When these are grown on nonselective medium, some of the cells will also loose the hybrid vector so that completely plasmid-free *cir*^{0} cells arise.

Therefore, *pCP1* was tested for its efficiency in curing *cir*$^+$ strains from their endogenous plasmid. Strain *13S-7A* was transformed with *pCP1* and transformants were selected on leucine-free medium. Starting from a single transformant colony, cells were grown on leucine-free medium, followed by growth on *YPD* and then on synthetic medium supplemented with leucine. Cells that had lost the *LEU2* gene were subsequently enriched by treatment with nystatin on leucine-deficient medium[19]. Among the leucine auxotrophs obtained one colony was tested for its plasmid content and was found to be plasmid-free (strain *HP01*, α *leu2 his3 ura3 trp1 ctt1 cir*^{0}). A similar experiment was carried out with strain *15A*. Cells were, however, grown on *YPD* medium for a longer period of time (80 generations) in this case so that no nystatin selection was necessary. Among the *cir*^{0} clones obtained, one (*HP03, a leu2 ura3 trp1 hem1 pep4 cir*^{0}) was kept for further experiments.

The stability of plasmid *pCP1* under nonselective conditions was tested in a *cir*$^+$ and a *cir*^{0} transformant. Strains *WS13-9A* (*cir*$^+$) and *HP01* (*cir*^{0}) were transformed with the plasmid and grown on complete medium. From time to time, aliquots of cells were plated on *YPD* medium. Colonies arising were replica-plated to leucine-deficient medium and the percentage of colonies unable to grow in the absence of leucine was determined. No significant difference in stability of the plasmid in the two strains was observed. After an initial period of higher stability, plasmid loss was in the range of 0.5 percent per generation.

The results obtained show that the vector *pCP1* can be used in a number of ways: 1. Strains containing the 2 μm plasmid can be cured from this endogenous plasmid.

2. Vector *pCP1* and plasmids derived from it by insertion of *DNA* fragments (not documented) are present in transformants in high copy number and can therefore be used in experiments where a high gene dosage is desirable.

3. Since, in *cir*^{0} strains, *pCP1*-type plasmids are stable against the interconversion of *A* and *B* forms of 2 μm *DNA* and fairly stable against plasmid loss during nonselective growth they can be used in experiments where a high copy number as well as integrity and stability of the plasmid are important, e.g. in the characterization of plasmid chromatin (*Bachmair A., Mattes E.*, unpublished results) or in some experiments on gene regulation.

4. Because of its high copy number and its relative stability under nonselective conditions, *pCP1* is potentially useful in the industrial production of foreign proteins in yeast cells.

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