

Development of Autonomously Replicating Plasmids for *Candida albicans*

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A pool of *Candida albicans* *RsaI* fragments cloned onto a vector containing pBR322 sequences and the *Candida ADE2* gene was used to transform a *Candida ade2* mutant to adenine prototrophy. A potential autonomously replicating sequence (ARS) in *Candida* DNA was identified by two criteria: (i) instability of the selectable marker in the absence of selection and (ii) the presence of free plasmid in total DNA preparations. Plasmids carrying the ARS transformed *C. albicans* at a high frequency (200 to 1,000 ADE⁺ transformants per µg of DNA), and Southern hybridization analysis of these transformants indicated that multiple copies of the plasmid sequences were present and that, although they were present in high-molecular-weight molecules, these sequences had not undergone rearrangement. Orthogonal field alternation gel electrophoresis indicated that the high-molecular-weight transforming sequences were not associated with any chromosome. The simplest interpretation to account for these data is that the transforming sequences are present as oligomers consisting of head-to-tail tandem repeats. The transformed strains occasionally yield stable segregants in which the transforming sequences are integrated into the chromosome as repeats. The *Candida* sequence responsible for the ARS phenotype was limited to a single 0.35-kilobase *RsaI* fragment which is present in one copy per haploid genome.

Candida albicans is the major fungal pathogen of man (34). With increased use of immunosuppressive therapy and the increased incidence of immunosuppressive disease, *C. albicans* infections are a rapidly growing cause of nosocomial infections, morbidity, and mortality (20). Several laboratories have pursued genetic studies in *C. albicans*, in part as a means to understand the nature of pathogenic determinants in *C. albicans* and to develop improved therapies for candidal infections. Genetic analysis in *C. albicans* is frustrated by the fact that the organism has a diploid genome and no known sexual cycle (40). New techniques are continuously being developed to facilitate genetic studies in *C. albicans*. Parasexual analysis after fusion of protoplasts has been simplified by the observation that diploids can be selected from tetraploid fusion products by heat shock (18) or by recessive fluorocytosine resistance (46, 47). Recombinant DNA technology can also be applied to *C. albicans* by using an integrative transformation system that we have described previously (25).

In several fungal species, autonomously replicating sequences (ARSs) have been identified subsequent to the development of protocols for integrative transformation (10, 11, 14, 24, 38, 43, 44, 45). Vectors carrying such sequences transform at higher frequencies and facilitate cloning of nuclear genes by mutant complementation. Sequences on autonomously replicating vectors are generally present at high copy numbers and provide the potential to study the effects of gene dosage on gene expression.

This report describes the isolation of such a sequence and its behavior in *C. albicans* transformants. Similar to ARS elements in *Saccharomyces cerevisiae*, the *Candida* ARS element produces an increased transformation rate and unstable transformants which show a high copy number of transforming DNA sequences. Unlike chromosomal ARSs

from *S. cerevisiae*, the cloned *C. albicans* ARS promotes the formation of head-to-tail tandem repeats which are either present as unstable free plasmids or integrated stably into the chromosome. We also show that a *C. albicans* gene can be overexpressed by cloning onto these high-copy-number vectors.

MATERIALS AND METHODS

Materials. β-Glucuronidase (type H-2), D-sorbitol, polyethylene glycol 4000, trimethoprim, methotrexate, sulfanilamide, and dihydrofolic acid were obtained from Sigma Chemical Co. (St. Louis, Mo.). [α-³²P]deoxynucleotide triphosphates (7,600 Ci/nmol) were from New England Nuclear Corp. (Boston, Mass.), BioTrace RP was from Gelman Sciences, Inc. (Ann Arbor, Mich.), Elutip-d was from Schleicher & Schuell, Inc. (Keene, N.H.), and restriction enzymes and DNA-modifying enzymes were from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) or New England BioLabs, Inc. (Beverly, Mass.).

Strains. *Escherichia coli* RR1 (5), a *recA*⁺ strain, was used for routine amplification of plasmids. When a *recA* strain was desired *E. coli* HB101 was used (6). To detect the function of the *C. albicans* *URA3* gene, *E. coli* DB6656 (*pyrF*::*μ trp lacZ hsdR*) was transformed to antibiotic resistance and tested on defined medium lacking uracil.

The *ade2-1*-containing *S. cerevisiae* strain, W343-4A, kindly provided by R. Rothstein, has the following markers: *mata ade2-1 ura3-11 leu2-3 leu2-112 lys2-1 his3-11 his3-15 can1-100*. Strain A-3 (*mata leu2-3 leu2-112 his3-11 his3-15*) was used as the recipient for some YEpl3 constructions. Strain S288C (*mata SUC2 mal mel gal2 CUP1*) was used as the *S. cerevisiae* wild type for chromosome separations.

A spontaneous MET⁺ derivative (strain SGY-129) of *C. albicans* hOG300 (35) has the markers *ade2 pro*. SGY-129 was used as the recipient for most of the transformation experiments described below. The other independently isolated *ade2 C. albicans* strains employed in the ADE2

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subcloning were A81-Pu (*ade2*) (26) and hOG480 (*ino ade2*) kindly provided by R. Poulter. Strain SC5314 is a clinical isolate and is the source for wild-type DNA for library construction. Strain SGY-243, a homozygous *ura3* deletion mutant that lacks a 4-kilobase (kb) fragment encompassing the entire ~1.2-kb *URA3* coding region and flanking DNA, has been described elsewhere (23a).

Plasmid and library construction. Standard recombinant DNA techniques were used to subclone the 5.2-kb *ClaI* *Candida* fragment containing the *ADE2* gene from pMC2 (25). pSM7, the plasmid used for library construction, contains the left-hand *EcoRV* insert fragment of pMC2 (which includes 190 base pairs of pBR322 sequence from *EcoRV* to the *BamHI-Sau3A* junction) inserted into the *EcoRV* site of pBR322. Small *C. albicans* DNA fragments of ~1.5kb were prepared by digesting strain SC5314 total genomic DNA to completion with *RsaI* which produces blunt ends. The fragments were ligated to *PvuII*-cleaved pSM7 DNA, and the reaction mix was used to transform *E. coli* RR1 cells to ampicillin resistance. A total of 24,000 individual transformants were recovered. Plasmid from 16 colonies chosen at random was isolated by the rapid boiling procedure of Holmes and Quigley (19). The restriction nuclease pattern of the plasmids indicated that at least 40% had inserts. DNA was isolated from a pool of these 24,000 *E. coli* transformants. Subcloning p56 and its derivatives was accomplished by standard recombinant DNA techniques. A description of the plasmids is provided in the Results section. The integrative *URA3*-containing plasmids, pET5 and pUR3, have been described previously (6, 23a). pET5 contains a 15-kb insert of the *C. albicans Ura3* gene region in YEpl3. pUR3 has a 6.1-kb *PstI* fragment containing the *URA3* region in pBR322. Isolation and subcloning the *C. albicans* dihydrofolate reductase (DHFR) gene is described in the Results section. The gene was isolated from a library of *Sau3A* fragments in YEpl3 (13).

Media. A rich medium, YEPD (2% Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 1% yeast extract, 2% glucose), was used to grow *C. albicans* and *S. cerevisiae*. Synthetic media were prepared by addition of the appropriate amino acids or nucleic acid bases to 0.7% yeast nitrogen base (without amino acids) and 2% glucose (41). Media were solidified with 1.5% agar. Transformation media contained 1 M sorbitol. To test for DHFR overexpression in *C. albicans*, we used the selective medium of Miyajima et al. (32). This medium contains 5 mg of sulfanilamide per ml and 10 μ g of methotrexate per ml to inhibit the dihydrofolate pathway. To test for DHFR expression in *E. coli*, we grew cells in Luria broth or minimal medium containing the appropriate antibiotic for plasmid maintenance (31).

DNA isolation and hybridization. Plasmid DNAs in *E. coli* were amplified and isolated by standard procedures (28). Plasmid DNA from *S. cerevisiae* was isolated by the small-scale rapid method of Sherman et al. (41). Total genomic DNA from *S. cerevisiae* or *C. albicans* was prepared by standard methods (41). CsCl gradient purification was sometimes omitted. Digestion of genomic or plasmid DNA with restriction enzymes was carried out as described by Maniatis et al. (28). DNA fragments were separated by electrophoresis on 0.5 to 0.7% agarose slab gels in 89 mM Tris borate buffer, pH 8.0. For orthogonal field alternating gel electrophoresis, the conditions described by Carle and Olson (9) were reproduced with a 1.5% agarose gel, a 300-V field, and a 20-s switch interval. Gel-fractionated DNA restriction fragments were transferred to BioTrace RP by the procedure recommended by the manufacturer. Hybridizations with

DNA labeled by nick translation were performed by standard procedures (28).

Transformation. Calcium chloride-treated *E. coli* cells were transformed by the method described by Maniatis et al. (28). *S. cerevisiae* spheroplasts were transformed by the method of Beggs (4). *C. albicans* spheroplasts were transformed by the *Saccharomyces* protocol described by Kurtz et al. (25). *Candida* transformants were selected on the surface of selective medium with 1 M sorbitol as osmotic support.

DHFR assay. The spectrophotometric DHFR assay of Baccanari et al. (1) was used to determine enzyme activity of crude extracts of *E. coli* cells. One enzyme unit is defined as the amount of enzyme required to reduce 1 μ mol of dihydrofolate per min and is expressed per milligram of total protein. Yeast cell extracts were prepared and assayed by a variation of the above method described by Nath and Baptist (33) except that cells were broken by vortexing with glass beads. In all cases, values reported are the average of two independent experiments.

Plasmid copy number determination. Total DNA from *C. albicans* transformants was digested with *HindIII*, and Southern blots of the serially diluted, cut DNA were hybridized with ^{32} P-labeled pSM7. The two genomic *HindIII* fragments of the *ADE2* gene served as a single-copy gene standard. The intensity of the hybridization signal from vector sequences was quantified by visual inspection of the dilution series as compared with the single-copy control. These estimations were based on the assumptions that all the DNA fragments transferred with equal efficiency and that hybridization was proportional to the homology of the probe to the DNA fragment. No correction was made for the fact that a large fraction of cells do not have plasmid even under selective pressure. In two determinations, the appropriate region of the gel was identified on the autoradiogram, cut out, and counted. A blank region of the gel of equal size was used for background determinations. The counts were corrected to account for the different homology length of the probe and compared with the single-copy *ADE2* gene. Essentially the same results were obtained by visual inspection and by counting the fragments.

RESULTS

Isolation of putative ARS sequence from *C. albicans*. We had previously used a 5.2-kb *C. albicans* fragment carrying the *ADE2* gene in pBR322 or YEpl3 as an integrative vector. Subcloning experiments indicated that a 2.3-kb region of this fragment was sufficient to produce *ade2* complementation in both *Candida* mutants tested (see Materials and Methods and Fig. 1). This region along with 190 base pairs of pBR322 sequence was subcloned as an *EcoRV* fragment into the *EcoRV* site of pBR322 to produce plasmid pSM7. This vector was then used to construct a library of *Candida* DNA *RsaI* fragments as described in Materials and Methods.

Based on the characteristics of chromosomally derived ARS plasmids in *S. cerevisiae*, two criteria were chosen to screen potential ARS plasmids in *C. albicans*: (i) instability of the selectable marker (*ADE*⁺) in the absence of selection and (ii) the presence of free plasmid in total yeast DNA preparations. *C. albicans* SGY-129 (*ade2 pro*) was transformed with the *RsaI* library, and 12 unstable *ADE*⁺ transformants were selected from 260 adenine prototrophs representing 70 to 90 kb of insert. Total DNA from these 12 unstable transformants was used to transform *E. coli* to ampicillin resistance to test for free plasmid.

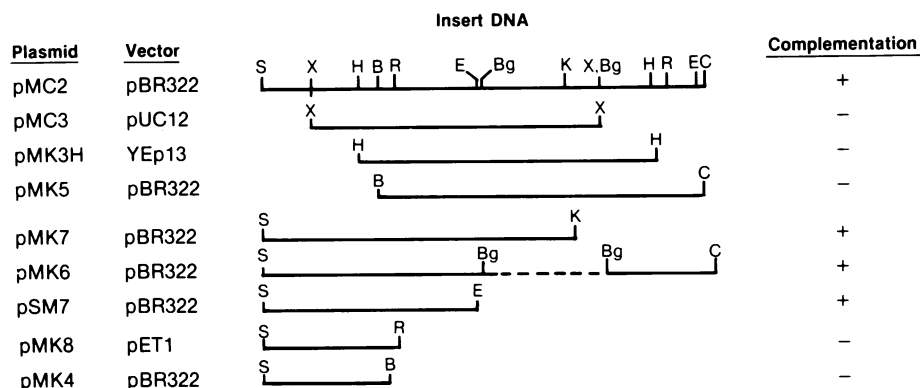


FIG. 1. Subcloning of *C. albicans* insert DNA and localization of the *ade2*-complementing region. Details of plasmid construction are given in Materials and Methods. Selected restriction sites are shown: S, *Sau3A*; X, *XbaI*; H, *HindIII*; R, *EcoRI*; Bg, *BglII*; K, *KpnI*; and C, *Clal*. Complementation was determined by the appearance of ADE⁺ transformants of *C. albicans* SGY-129, A81-Pu, and hOG480. Cloning vehicles pET1 (13), pBR322 (5), YEp13 (7), and pUC12 (30) have been described previously.

The plasmids from each of the *E. coli* transformants were isolated and used to retransform *C. albicans*. Only one of these plasmids, p56, transformed at a high frequency (200 to 1,000 ADE⁺ transformants per μ g of plasmid DNA) compared with the parent plasmid, pSM7 (0.5 to 5/ μ g). The remaining plasmids transformed at the same frequency as the parent plasmid. Plasmid p56 also conferred the unstable adenine prototrophy expected of an autonomously replicating vector. After eight generations in nonselective medium, only 1 to 2% of the progeny were still ADE⁺. We define the sequence(s) producing these properties as *Candida* ARS 1 (CARS1).

Characterization of CARS1. The unstable adenine prototrophs produced by p56 transformation are small pink colonies on selective medium, while stable integrated pSM7 transformants produce white, vigorously growing colonies. The pink color of these prototrophic colonies is probably due to the unstable nature of the chromosomal ARS. In *S. cerevisiae*, the *C. albicans* ADE2 gene on an *S. cerevisiae* ARS1 plasmid resulted in pink colonies, while the same gene on the more stable 2 μ m plasmid gave white colonies. Transformation of *C. albicans* with p56 also produces a small number (5/ μ g) of stable, white transformants, probably the result of plasmid integration into the *Candida* chromosome. The pink colonies are unstable on selective medium as well as on adenine-containing medium, producing white fast-growing papillae after extended incubation. These white prototrophs are presumably due to integration of the autonomously replicating plasmid into the *Candida* genome as they are stable.

To confirm the presence of free plasmid in p56 transformants, total uncut DNAs from the original *Candida* isolate (SGY-159), three pink retransformants (R-1, R-2, R-3), and one white colony (W-1) were isolated and analyzed by hybridization in gel blots. Unexpectedly, the results of this experiment showed that hybridization with pBR322 (or p56 [data not shown]) occurred at the position of chromosomal DNA in all cases (Fig. 2A). However, the high intensity of the hybridization bands compared with that of the control strains with pSM7 integrated into the chromosome suggested that multiple copies of the plasmid sequences were present (compare lanes 8 and 9 with lanes 2 to 5). These integrated pSM7 transformants were chosen on the basis of having the strongest hybridization to vector sequences in colony hybridization experiments. Southern blot analysis indicated the presence of multiple (at least two) integrated copies of the

vector. Three *PvuII* bands (10, 8, and 4.8 kb) are expected when DNA from a transformant carrying a single integrated copy of pSM7 is probed with vector sequences. These two transformants, however, contained four or five hybridizing bands respectively (Fig. 2C, lanes 8 and 9.), indicating the presence of additional integrated plasmid copies. This therefore demonstrates a bona fide increase in copy number produced by p56 relative to what can be achieved via simple integrative transformation. The untransformed recipient, SGY-129, does not hybridize to the pBR322 probe.

The unexpected hybridization of vector sequences as a high-molecular-weight band suggested that most, if not all, of the p56 sequence was not present as a simple, monomeric plasmid. To determine whether the p56 sequence had been rearranged in the transformants, total DNA from several transformants was digested with *HindIII*, and Southern blots were probed with ³²P-labeled pSM7. All the pink transformants (Fig. 2B, lanes 2, 3, and 5) showed the pattern expected for unrearranged p56 DNA (1.3- and 7.2-kb bands), as did the white transformant (lane 4). As expected, all the transformants have the 3.4- and 4.0-kb *HindIII* fragments produced by hybridization to the chromosomal ADE2 sequences. The intensity of the 1.3- and 7.2-kb plasmid bands indicates that they are present in greater copy number than the genomic ADE2 gene. In addition to the plasmid and genomic copy bands, some of the transformants (Fig. 2B, lanes 5, 6, 11, and 13) display other fragments which hybridize to pSM7. One of these fragments (2.45 kb) was also seen in strain SGY-416, a pSM7 transformant (lane 9), and may indicate the existence of another potential site for plasmid integration.

To test whether multiple single copies of the plasmid are integrated at various sites in the genome, we cut total DNA from transformants with *PvuII*. Plasmid p56 has no internal *PvuII* sites as a result of the *RsaI* cloning. Thus, for integrated plasmids, the fragment size generated by cleavage of transformant DNA with *PvuII* would depend on the chromosomal location of the plasmid and the nearest *PvuII* sites. Multiple single copies would produce many different-sized bands hybridizing to the pSM7 probe in addition to the known chromosomal *ade2* locus. Integrated tandem repeats would appear as a single very large fragment since there would be no *PvuII* sites within the entire repeated unit. Oligomeric free plasmid would also hybridize as a single large fragment. The Southern hybridization results in Fig. 2C indicate that the plasmid sequences are present as a large

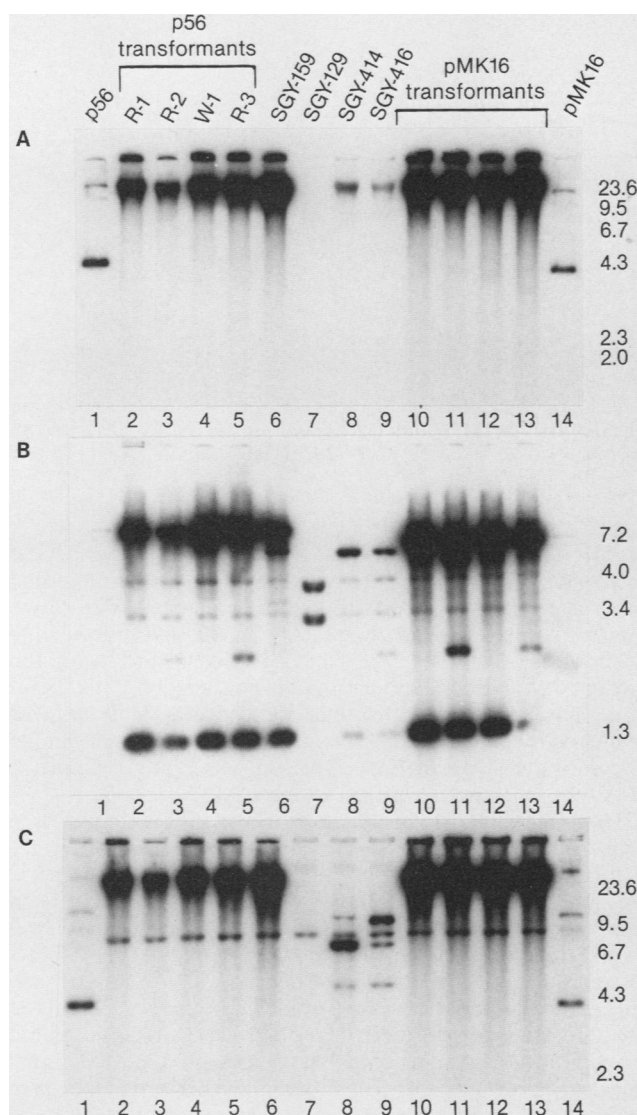


FIG. 2. Autoradiograms of Southern blot hybridizations of genomic DNAs from p56 and pMK16 transformants of SGY-129. Numbers on the right indicate size in kilobases. (A) Equivalent amounts of uncut genomic DNA were run in each lane on a 0.7% gel, and blots were probed with ^{32}P -labeled pBR322. Lanes: 1, uncut p56; 2 to 5, p56 transformants; 6, original p56-containing isolate, SGY-159; 7, untransformed SGY-129; 8 and 9, pSM7 transformants SGY-415 and SGY-416; 10 to 13, pMK16 transformants; 14, uncut pMK16. (B) *Hind*III-cut genomic DNAs were run on a 0.7% gel, and blots were probed with ^{32}P -labeled pSM7. Equivalent amounts were used in each lane except for lane 7, in which seven times as much DNA was used to visualize clearly the position of the genomic *ADE2* fragments. The slightly greater mobility of the *ADE2*-hybridizing bands compared with that of the other lanes is probably due to relative overloading. Lanes are as in panel A. Hybridization to pure plasmid is extremely faint in lanes 1 and 14 and was visualized by longer exposures. (C) Equivalent amounts of *Pvu*II-cut genomic DNAs were run in each lane on a 0.5% gel, and blots were probed with ^{32}P -labeled pSM7. Lanes are as in panel A.

unresolved smear above 30 kb in size, consistent with tandem multimers. The single-copy *Pvu*II fragment of the *ADE2* gene (8 kb) is clearly resolved in this gel, and *Pvu*II-treated p56 DNA runs as covalently closed circles (compare lanes 1 in Fig. 2A and C). Last, to rule out

concatenated circles as a mechanism to produce these results, DNA from p56 transformants was digested with topoisomerase II and run on gels. No change in hybridization pattern was observed relative to untreated DNA (data not shown).

Additional evidence favoring the presence of large multimeric plasmids comes from *E. coli* transformation experiments. In general, *Candida* DNA preparations transformed *E. coli* cells poorly, and this was at least in part due to an inhibitor of transformation in the preparations. Transformation was especially poor with *recA* cells (HB101) when compared with otherwise largely isogenic *recA*⁺ cells (RR1). The lower transformation rate observed with *recA* cells could potentially be due to the inability to convert large oligomeric plasmids to monomers which are inherited more efficiently. In one rare HB101 transformant, a large oligomeric plasmid was isolated after transformation with total *Candida* DNA. When partially digested with *Cla*I, the oligomeric plasmid produced fragments of ~26, 17, and 8.5 kb indicating a tandem repeat of three or more (data not shown). Large plasmids were transiently seen in *recA*⁺ cells, but were rapidly converted to the monomer, presumably by homologous recombination. Similar difficulties in recovering multimeric plasmids from *Schizosaccharomyces pombe* cells in *E. coli* have been reported (38).

Direct proof for free plasmids in the p56 transformant was obtained by separating the CARS plasmid from the *Candida* chromosomes on pulsed orthogonal field gels. Conditions were chosen (Fig. 3A) so that all the large chromosomes of *C. albicans* remained near the well. Under these conditions some of the smaller *S. cerevisiae* chromosomes were resolved (compare lanes 1 to 3 with lane 4). When blots of these gels were probed with ^{32}P -labeled pBR322 for plasmid sequences, strain SGY-415, a pSM7 integrative transformant, hybridized at the position of the *Candida* chromosomes. In contrast, DNA from SGY-417, a p56 transformant, hybridized intensely both at the well and at a position well below the *Candida* chromosomes. Since free plasmids run anomalously in these gels, the size of the plasmid could not be determined. We consistently observed significant hybrid-

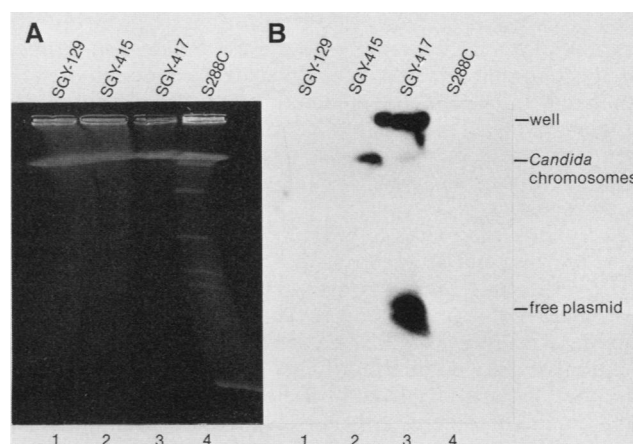


FIG. 3. Orthogonal field alternation gel electrophoresis gels of *C. albicans* transformed with integrative and CARS vectors. (A) Ethidium bromide-stained gel. Lanes: 1, SGY-129, untransformed control; 2, SGY-415, an integrative pSM7 transformant; 3, SGY-417, a pink p56 transformant; 4, S288C, a wild-type *S. cerevisiae* strain included to show chromosome separation. (B) Autoradiogram of gel in panel A blotted and probed with ^{32}P -labeled pBR322.

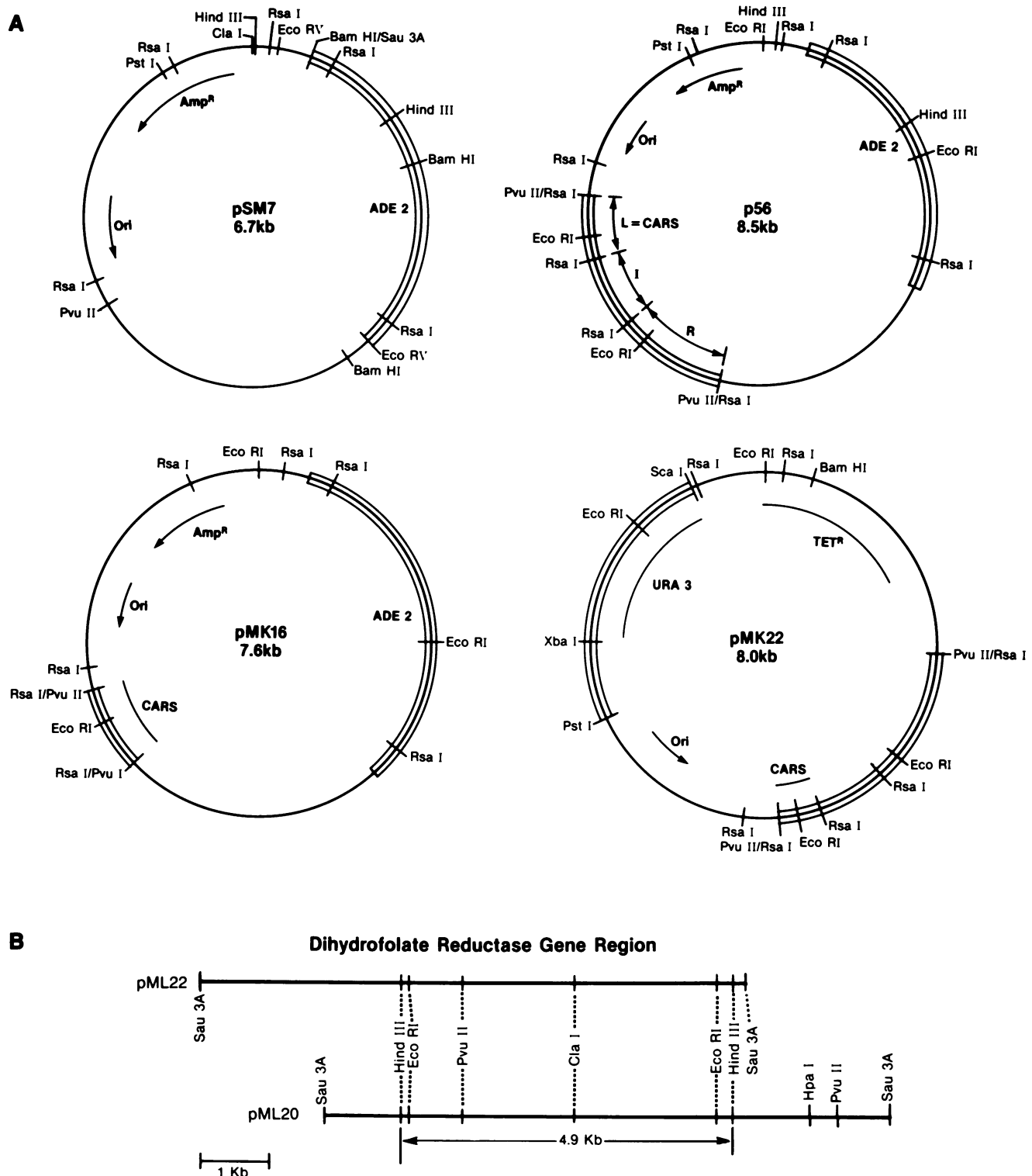


FIG. 4. (A) Restriction maps of some plasmids used in this study. Bold lines, pBR322 sequences; boxed lines, *C. albicans* sequences; L, left-hand *Rsa*I fragment; I, internal *Rsa*I fragment; R, right-hand *Rsa*I fragment. Selected restriction sites are shown for each plasmid which are referred to in the cloning manipulations discussed in the text. Ori, Origin. (B) Restriction map of *C. albicans* inserts which contain the DHFR gene region.

TABLE 1. Transformation frequencies of various CARS-containing derivatives

Plasmid	CARS insert		Transformation frequency ^a	
	<i>RsaI</i> fragment	No. of copies	ADE ⁺	URA ⁺
pMK16	L	1	900	
pMK17	L	2	1,300	
pMK13	L	3	470–1,500	
pMK15	I	2	14	
p56	L, I, R	1 each	666–1,400	
pSM7	None	0	24	
pMK22	L, I, R	1 each		250
pET5	None	0		1.1
pUR3	None	0		50

^a Transformation frequency was determined by calculating the number of ADE⁺ or URA⁺ colonies per microgram of DNA. The number of revertants was subtracted when appropriate.

ization at the well with the p56 transformant but not with other strains, suggesting that either the CARS plasmid does not enter the gel efficiently or it is present in two forms, one of which is unable to enter the gel.

A rough estimate of copy number was achieved by a series of dilutions of *HindIII*-cut transformant DNA. Gel blots were probed with pSM7, and the relative intensities of plasmid and chromosomal bands were compared visually. An overall average copy number of 5 to 10 per diploid genome was determined as in Materials and Methods. An alternative method which counted the radioactivity in a band directly gave similar results.

Subcloning the insert of p56. Restriction mapping of p56 indicated that the insert region had three *RsaI* fragments (Fig. 4A). The three fragments may be the result of incomplete *RsaI* digestion of *Candida* DNA used to construct the library and therefore represent three contiguous fragments or may be a cloning artifact of fragment excess and represent three nonadjacent fragments. It was therefore of interest to determine whether one or more of the fragments was sufficient for ARS activity. Initially, genomic blots of *RsaI*-cut DNA were probed with a p56 subclone lacking the *ADE2* gene (pMK12) which hybridized with three bands of 0.37, 0.61, and 3.3 kb (data not shown). The smaller bands correspond in size to the two smaller *RsaI* fragments in p56. The largest band is much larger than any of the insert fragments, indicating that a rearrangement must have occurred either during cloning or in vivo. Southern gel analysis of *HindIII*-, *EcoRV*-, and *ScaI*-cut *Candida* DNA probed with p56 or pSM7 showed three bands in addition to the fragment derived from the *ADE2* region (data not shown). The intensity of the bands indicated that the *RsaI* fragments are not reiterated relative to the single-copy *ADE2* gene.

In view of the fact that the *RsaI* fragments are likely derived from nonadjacent DNA sequences, the two complete *RsaI* fragments (I and L, Fig. 4A) were subcloned and used to transform SGY-129 to adenine prototrophy to test for ARS activity. The data in Table 1 show that the L fragment but not the I fragment confers ARS activity. Furthermore, increasing the number of L tandem repeats from one to three does not increase transformation frequency within the accuracy of this determination (Table 1). The majority of the pMK16 (one L fragment) transformants were pink unstable ADE⁺ colonies identical to p56 transformants. Growth rate in minimal selective media was the same for all pink transformants tested (data not shown). The third *RsaI* fragment present in p56 was not subcloned.

HindIII- or *PvuII*-digested and undigested total DNAs from pMK16 transformants were analyzed by hybridization of pSM7 to gel blots. As in the p56 transformants, the pMK16 plasmid sequences were reiterated and present as oligomers (Fig. 2). No free monomer band was seen (Fig. 2A, lanes 10 to 13). Although the activity of the right-hand (R) fragment remains undetermined, we can define the L fragment as CARS1, since the L fragment was necessary and sufficient to confer all the properties of the p56 plasmid.

CARS1 function with the *Candida URA3* gene. The unusual properties of CARS1 made it desirable to determine the general usefulness of the cloned CARS. Construction of autonomously replicating vectors with a different selectable marker, the *URA3* gene of *C. albicans*, was accomplished by first deleting the *ADE2*-containing *EcoRV* fragments of p56 to generate pMK12 (Fig. 4A). A 1.5-kb *PstI*-*ScaI* fragment containing the *URA3* gene was inserted into the *PstI*-*ScaI* sites of pMK12. The resultant plasmid, pMK22, was used to transform *C. albicans* SGY-243, a *ura3* deletion mutant which lacks all but a 0.6-kb region of homology to the *URA3* insert (23a), to uracil prototrophy. The frequency of appearance and characteristics of pMK22 transformants were compared with those of integrative plasmids pET5 and pUR3. The transformation of the CARS-containing plasmids was much higher than that of the integrating plasmids (Table 1). The latter, however, produced numerous pinpoint colonies most of which did not grow when subcultured to medium lacking uracil. Plasmid pMK22 transformants were unstable; only 5% were still URA⁺ after 10 generations on uracil-containing media, while the pET5 and pUR3 transformants were stable. Southern analysis of uncut DNA showed a pattern consistent with a multicopy plasmid present as an oligomer (Fig. 5).

Uncut total DNA from three pMK22 transformants hybridized to the *PstI*-*ScaI* fragment of pMK22 (the *URA3* region) at the position of chromosomal DNA (Fig. 5, lanes 5 to 7). Since the probe contains a small piece of DNA homologous to the chromosome in this deletion strain, the faint band seen in the untransformed control SGY-243 indi-

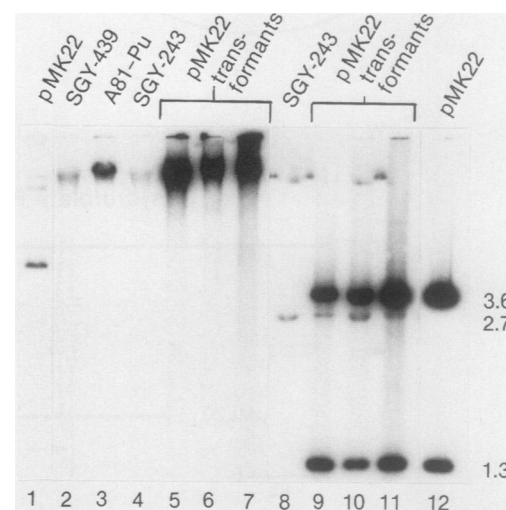


FIG. 5. Autoradiograms of Southern blot hybridizations of genomic DNAs from URA⁺ transformants probed with the *ScaI*-*PstI* fragment of the *URA3* gene (Fig. 4A). Lanes: 1 to 7, uncut DNAs; 8 to 12, *EcoRI*-digested DNAs. Equivalent amounts of DNA were run in each lane except for purified plasmids. Numbers on right are in kilobases.

TABLE 2. Enzymatic assay of DHFR activity in strains transformed with the *Candida* DHFR gene

Strain	DHFR activity (mU/mg of protein)	Trimethoprim-resistant DHFR activity (mU/mg of protein)	% Trimethoprim resistant	Ratio pMK18/p56
<i>E. coli</i>				
pML23	2.22	1.17	53	
pBR325	1.47	0.24	16	
<i>C. albicans</i>				
pMK18	12.84			
p56	1.95			6.6

cates the migration of uncut chromosomal DNA (lane 4). As expected, the intensity of the pMK22 transformant bands was much greater than that of a URA^+ transformant with an integrated plasmid, pUR3, strain SGY-439 (lane 2), or a strain with a wild-type *URA3* region (lane 3). When gel blots of *EcoRI*-digested DNAs were probed with the pMK22 *URA3* fragment, intense bands of 3.6 and 1.3 kb were obtained as predicted for reiterated plasmid sequences. The faint band of 2.7 kb is the single-copy chromosomal band homologous to 0.6 kb of the probe and can be used as an internal control (see SGY-243 in lane 7).

If pMK22 had integrated at the *URA3* locus in any of the transformants, the *EcoRI* digests would generate new bands owing to the flanking sequence. For example, if integration had occurred at the 0.6-kb region of homology at the left-hand portion of the *URA3* locus, new *EcoRI* bands of 2.0, 1.3, and 4.0 kb should be seen (23a). Unlike the p56 and pMK16 transformants, the URA^+ transformants with pMK22 (Fig. 5) had no unidentified hybridizing bands in addition to the predicted free plasmid fragments. The limited homology between the plasmids and chromosomal sequences may reduce integration and rearrangement.

Cotransformation. The reported ability of several yeast species to produce new plasmid constructions in vivo by cotransformation (12, 17, 21, 39, 44) led us to test this in *C. albicans*. As a model system, pMK12, which has no selectable marker for *C. albicans* but does have the *C. albicans* ARS, and pSM7, an *ADE2* integrative plasmid without ARS activity, were used to cotransform SGY-129 (*ade2*) to adenine prototrophy. Equal amounts of plasmid DNA were used. The cotransformation frequency was 400/ μ g of total plasmid DNA or 800/ μ g of pSM7 plasmid DNA. The frequency was very similar to that of p56 (800/ μ g) in this experiment. Adenine prototrophs arose at 10/ μ g of DNA for pSM7 alone, while no transformants were seen with pMK12. The vast majority of the cotransformants were pink, slow-growing colonies which were unstable for the ADE^+ phenotype. White, stable prototrophs appeared with the same frequency as for pSM7. Southern analysis of total DNA from these transformants indicated the presence of reiterated copies of the *ADE2* gene fragment on the plasmid (data not shown). Free plasmids were obtained from transformants by transforming *E. coli* to ampicillin resistance with total DNA. Restriction enzyme analysis showed that these plasmids were larger than either pSM7 or pMK16. However, a simple recombinational event between the homologous regions of pSM7 and pMK16 would not be expected to generate the structure of the plasmids obtained.

Cloning of DHFR gene from *C. albicans*. To demonstrate

overexpression of genes on the p56-derived plasmids, we cloned the *Candida* DHFR gene and assayed the level of the *C. albicans* DHFR enzyme activity in a strain carrying the cloned gene on p56.

The cloning of the DHFR gene was based on the observation that trimethoprim, a dihydrofolate analog, inhibits DHFR from *E. coli* but not from *C. albicans* (8). The differential action of trimethoprim has been used successfully to screen an *S. cerevisiae* DNA library in *E. coli* for the *S. cerevisiae* DHFR gene (33). On the assumption that the *Candida* gene would also be expressed in *E. coli*, a *Sau3A* partial library of *Candida* DNA in YEpl3 was screened for expression of trimethoprim resistance (*Tmp^r*) in *E. coli* RR1 cells. Colonies resistant to 50 μ g of trimethoprim per ml were scored for plasmid-mediated resistance by plasmid-curing experiments on nonselective media. Four strains were obtained which showed linkage between ampicillin resistance (the YEpl3 plasmid marker) and trimethoprim resistance. Restriction enzyme analysis of the plasmids obtained indicated that three different plasmids had been isolated with inserts of 8.1, 8.3, and ~18 kb. The two smaller plasmids (pML20 and pML22) were analyzed in detail and found to have an ~6-kb region in common (Fig. 4B). A 4.9-kb *HindIII* fragment central to this region was subcloned into plasmid pBR325 (to yield pML23) and indicated that the trimethoprim resistance determinant was wholly contained by this fragment. (A 4.9-kb *HindIII* fragment was also present in the third, larger plasmid which produced trimethoprim resistance.) That the 4.9-kb *HindIII* fragment was derived from *C. albicans* DNA was confirmed by Southern analysis with ^{32} P-labeled pML23 as a probe. No specific hybridization was observed with either *E. coli* or *S. cerevisiae* DNA, while strong specific hybridization was observed to *C. albicans* DNA in a pattern consistent with the restriction map of the probe (data not shown).

Additional evidence that the cloned fragment encoded *Candida* DHFR was obtained from enzyme studies. DHFR levels in *E. coli* cells carrying pML23 were compared with those in control vector-carrying cells. Cells carrying pML23 produced an average of 51% greater specific activity when compared with the vector-containing control strain (Table 2). Since the DHFR activity produced by *C. albicans* is not inhibited by trimethoprim (8), assays were also performed after the addition of trimethoprim to 80 nM. This level of trimethoprim was chosen because lower concentrations of trimethoprim produced greatly less inhibition of *E. coli* DHFR, while higher concentrations of trimethoprim produced little increase in inhibition. As shown in Table 2, 53% of the DHFR activity of pML23-containing cells was trimethoprim resistant under conditions in which DHFR in control cells was inhibited to 16% of the original level. This demonstrates the production of trimethoprim-resistant DHFR activity in cells containing the cloned *Candida* fragment.

Overexpression of the DHFR gene on a CARS1 plasmid. To test the overexpression of the DHFR gene on a CARS vector in *C. albicans*, the 4.9-kb *HindIII* fragment from pML23 was blunt ended and ligated into the *SalI* site of p56 to produce pMK18. White and pink pMK18 transformants of A81-Pu were tested for increased copy number of the DHFR gene by in situ colony hybridization with the labeled *HindIII* fragment. One of the white transformants which had integrated multiple copies of the plasmid as judged by colony hybridization (SGY-304) and a control strain with multiple integrated copies of p56 (SGY-298) were assayed for DHFR activity. The data (Table 2) demonstrate that there was an

approximate 6.6-fold increase in DHFR specific activity in the pMK18 transformant. This result confirms the identity of the *Hind*III fragment as the *C. albicans* DHFR gene and indicates that the CARS plasmid increases both copy number and gene expression.

DISCUSSION

ARSs have been sought in a variety of fungal species having integrative transformation systems. The behavior of such sequences is quite varied when different fungal species are compared. Vectors carrying chromosomal ARS sequences in *S. cerevisiae* transform at high frequency, are propagated as plasmid monomers which partition inefficiently, and are thus present in high copy number in some cells and are lost from others. Integration is rarely observed, and partition efficiency is improved in vectors carrying 2 μ m DNA sequences (reviewed in reference 48). Transformation in the fission yeast *S. pombe* is in some ways similar to that in *S. cerevisiae*: ARS plasmids transform at increased rates and are unstable (3, 29). However, a wide variety of cloned DNAs which show these two qualities in *S. pombe* are found to be rearranged when reisolated from the transformed cell (29). These sequences do not have ARS activity as commonly defined. In other experiments, true ARSs have been identified, and these elements replicate in *S. pombe* as multimers which may integrate via homologous or nonhomologous recombination at a low rate (29, 38, 49).

A potential ARS has been identified for *Aspergillus nidulans* on the criterion of increased transformation rate. This reiterated *Aspergillus* sequence (*ansI*) was described as producing stable, high-frequency transformation, and although plasmid monomers were never observed, plasmids could be recovered from total DNA of transformants via transformation of *E. coli* and selection for plasmid markers (2). *E. coli* transformants are thought to be produced from rare plasmids excised from the *Aspergillus* genome (2, 22). Grant et al. (14) described ARSs in *Neurospora crassa* which increase transformation rate and are unstable, replicate as oligomeric or modified plasmids or both, and can also integrate at multiple sites in a deletion host, but in a nonfunctional manner.

The most striking feature of ARS behavior in *C. albicans* is the formation of multimeric, head-to-tail repeats. This has been most clearly described in *S. pombe* in which autonomously replicating oligomers were estimated to be 60 to 80 kb in size and consist of 10 to 20 repeats (38). *Hansenula polymorpha*-derived sequences also produce multiple tandem repeats but are stable transformants in this yeast (36). Similarly, recombinant plasmids containing the cloned glutamate dehydrogenase gene of *N. crassa* have been reported to replicate extrachromosomally as oligomers (14). Polymeric plasmids have been described for a small derivative of an *S. cerevisiae* plasmid (15). Transformants with oligomeric plasmids have also been observed for the dimorphic fungus *Yarrowia lipolytica*, although in this case no evidence for ARS activity was obtained (12). Formation of tandem repeats is a feature of some *A. nidulans* plasmids, as discussed above. The mechanism for generating the tandem repeats will require further studies.

The *Candida* ARS described here does not promote extrachromosomal replication in *S. cerevisiae* (unpublished observations). This lack of function indicates, as shown in other studies, that screening for ARS activity in *S. cerevisiae* does not predict utility in the homologous host (29, 48). Conversely, *C. albicans* has several fragments which will

serve as ARSs in *S. cerevisiae*, but these DNA sequences do not replicate autonomously in *C. albicans* (unpublished data). We had previously shown that the portion of 2 μ m in YEp13 does not replicate autonomously in *C. albicans* (25), and we now have data which indicate that neither the entire 2 μ m circle nor *S. cerevisiae* ARS 1 replicates autonomously in *C. albicans* (unpublished data). Further screening of the *Rsa*I or an analogous library may identify a sequence that is functional in both hosts.

Cotransformation is not unusual and has been observed for several fungal species (12, 17, 21, 23, 27, 39). Apparently, recipient spheroplasts competent to take up exogenous DNA generally take up many DNA molecules. The mechanism for generating the composite plasmids found after the pSM7-pMK12 cotransformation is not simply due to homologous recombination. Further studies will be required to understand this phenomenon. Mechanism notwithstanding, the high frequency of cotransformation of an integrating vector with a selectable marker and a CARS vector with no selectable marker suggests that this technique will be very useful. It should now be possible to introduce any foreign DNA incapable of replicating in *C. albicans* into this yeast at high frequency. New techniques with cotransformation include improved methods for one-step gene replacement (37, 42) and identification of complementing sequences in a cosmid without subcloning (44). These techniques should also be applicable to *C. albicans*.

In applications in which the integrative ability of p56 would be a disadvantage, the *URA3*-containing plasmid can be used with a *URA3* deletion strain. The difficulty in obtaining stable *URA*⁺ colonies in this case indicates that the small homology left in the *URA3* region is not sufficient to promote rapid integration. Further modification of pMK25 is in progress to eliminate all homology at the *URA3* locus and to construct convenient cloning vehicles. In addition, we are attempting to clone other *C. albicans* sequences which will allow more equal plasmid partition during cell division. We anticipate that sequences such as the *stb* fragment from *S. pombe* (16) should enhance plasmid stability and utility.

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