

STABILITY OF THE ARTIFICIAL CHIMERIC PLASMID IN YEAST

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Abstract—The Hepatitis B virus surface antigen gene was cloned into a yeast plasmid vector with a complete 2-micron origin and an *ars* origin, and the resultant recombinant plasmid, pMYN101, was transformed into several yeast strains, AH22, AH22 *cir*-0, A281, and LL20 *cir*-0. The recombinant plasmid was found to be very stable in all of these strains tested suggesting that the phenomenon of plasmid incompatibility due to the coexistence with another 2-micron plasmid does not occur. For comparison a few other kinds of yeast plasmids with an incomplete 2-micron origin or an *ars* origin were transformed into the same yeast hosts and their stability was studied in a batchwise operation. These plasmids showed an unstable yet interesting behaviour making careful prediction of plasmid stability based upon the complete characteristics of both the plasmids and the hosts, and their interaction an important necessity.

INTRODUCTION

In marked contrast to the partitioning of chromosomal DNA, plasmid segregation at cell division is sometimes irregular. Consequently, in a population of growing cells containing the plasmid, cell division can result in formation of progeny cells without plasmids. If no selection pressure for plasmid is applied to the population, the plasmid-free cells can outgrow the plasmid-containing cells, so that the fraction of plasmid-harboring cells should decline towards zero monotonically with time. Plasmid instability is clearly undesirable in genetic engineering applications involving synthesis of a cloned gene product.

Thanks to the intensive study in basic biological science the molecular biological properties on plasmid stability are fairly well known. Several plasmid- and host cell-related factors such as the *par* segment in an *E. coli* plasmid and the coding regions on the 2-micron plasmid of yeast [1-3] were taken responsible for the stability or instability of the plasmids in host cells. In bacteria, three main factors have been found to influence the stability under non-selective conditions. These are the activity of a *cis*-acting partition function which has been compared with the eukaryotic centromere [6], the plasmid copy number [7] and the impact of the plasmid on host growth rate [7].

The yeast is an important vehicle for the study of cloned eukaryotic genes and has been a major industrial microorganism for more than 8,000 years [8] and has been cited as one of the better hosts for recom-

binant plasmids. The two main types of the yeast plasmid replicons with good prospect for applications are the 2-micron origin and the *ars* origin [9-13]. In the case of 2-micron circles there are three classes of explanation for its near ubiquity. The first is that the 2-micron circle is of some benefit to the cell, and that (*cir*-0) cells are therefore at a selective disadvantage with respect to their (*cir* - +) counterparts. The second class of explanation is that 2-micron circle replication and partition is so regular and efficient that once a strain is 'infected' with 2-micron circles, the plasmid is not afterwards lost at any appreciable rate. The third of explanation is that 2-micron circles are infectious. If this was the case, then cells that have lost their 2-micron circles might regain them by being infected by other nearby cells [14, 15]. Also the presence or absence of endogenous 2-micron plasmid of yeast was found to have a great influence.

In contrast, the study on the stability behaviour of plasmid in large scale bioreactors is rather lacking and the results of research in such a direction are just coming to emerge [2,4,5]. Especially such a study with yeast plasmid is nonexistent and the current study is an attempt to circumvent such a situation in a bioreactor under batch-type operation. Although one could have extended the study to a continuous and/or multistage operation the main focus here was to use the self-made recombinant plasmid with both the 2-micron origin and the *ars* origin into which the HBsAg structural gene was cloned. Additionally several combinations of yeast plasmids and host cells were used for comparison and

also to demonstrate the plasmid-host interaction.

MATERIALS AND METHODS

Plasmids and hosts

Saccharomyces cerevisiae strains AH22, cir-0 derivative of AH22, AH22R-(APase constitutive derivative of AH22), A2, A281 (cir-0 derivative of A2), LL20 cir-+, and cir-0 derivative of LL20 cir-+ were used as recipient host cells in yeast transformations. Five of the plasmids used are pBR322, pAM82, pJDB219, pSZ93, and YEp13.

Reagents for growth media

Bacto-agar, yeast extract, peptone, tryptone, neopeptone, YNB w/o amino acids, and polypeptone were purchased from Difco. Adenine sulfate, dextrose, sorbitol, L-histidine, L-leucine, uracil, L-tryptophane, L-methionine, L-arginine, L-tyrosine, L-isoleucine, L-lysine, L-valine, and L-phenylalanine were purchased from Sigma. Riboflavin, biotin, calcium pantothenate, inositol, niacin, pyridoxin HCl, and thiamine HCl are all GR grade.

Chemicals and supplies

Trizma base, tris-hydrochloride, sodium chloride, Na₂EDTA, magnesium chloride, calcium chloride, ammonium chloride, sodium acetate, ammonium hydroxide, boric acid, potassium acetate, potassium phosphate (monobasic and dibasic), sodium phosphate (mono- and dibasic), ammonium sulfate heptahydrate, sodium carbonate, potassium tartarate, SDS, copper sulfate pentahydrate, sucrose, potassium iodide, ferric chloride, manganese sulfate, sodium molybdate dihydrate, zinc sulfate heptahydrate, lithium sulfate, ammonium molybdate tetrahydrate, and ascorbic acid were purchased from Sigma, Kanto, Wako, or Hayashi.

2-ME, Nonidet P-40, Triton X-100, DTT, PMSF, ethidium bromide were from Sigma.

Polyethylene glycol #4,000 is from Katayama Chem. and Hanawa Chem. Co.. Oxydiformic acid diethyl ester, a nuclease inhibitor, is from Kanto Chemical Co.. Bromophenol blue and xylene cyanol FF are from Hanawa Chemicals and Nakarai Chemicals. Fresh distilled water was used for preparation of all media and reagents. Membrane and filters were purchased from Millipore and Toyo. Polaroid type 667 film and 084 are from Kodak. Siliconization compound, dimethyldichlorosilane, is from Nakarai Chemicals. Agarose type I (low EED) is from Sigma.

The inside of eppendorf tubes used for the DNA work, was siliconized by soaking in a 5% solution of dichlorodimethylsilane in chloroform and then rinsed many times with sterilised distilled water.

Yeast plasmid DNA isolation

Yeast plasmid DNA can be prepared by the pro-

cedure of Cryer et al. [16]. Cells were grown in 5ml YPAD liquid medium overnight. 1 liter broth of YPAD contained 10g of yeast extract, 20g of bacto-peptone, 20g of dextrose, and 400mg of adenine sulfate. The cells were centrifuged for 7 min at 3,000 rpm, washed once with 4ml of distilled water and centrifuged again. 400 μ l of zymolyase solution (100ml solution contains 2M sorbitol 60ml, 1M potassium phosphate buffer pH 7.5 5ml, 2-ME 40 μ l, zymolyase 60,000 20mg and 35ml of distilled water.) It was incubated in water bath with shaking at 30°C for 30 minutes and transferred to eppendorf tube. Then 0.04ml of 0.5M sodium EDTA (pH8.5), 0.02ml of 2M Trisbase, and 0.02ml of 10% SDS were added and mixed. 1 μ l of oxydiformic acid diethyl ester (nuclease inhibitor) was added to each tube and mixed. It was put on standing on desk for 30 min with the lid of eppendorf tube open. 0.1ml of 5M KOAc (potassium acetate) was added to each tube and after mixing the tubes were placed on ice bath for 60 min. It was centrifuged for 10 min in microcentrifuge, the supernatant was transferred to a new eppendorf tube, and 2 volumes of ethanol was added. It was mixed and incubated for 10 min at room temperature, and microcentrifuged for 10 min at 10,000 rpm, and the precipitate was taken and vacuum dried for 30 min. The product was dissolved in 100 μ l of 1mg/ml RNase solution and incubated at 37°C for 30 min. The precipitate was swirled with a small glass hook which can be easily fashioned by melting the tip of a pasteur pipette. It must not be spinned. Part of the sample was loaded onto gel and the bands were checked.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed in a slab gel apparatus, a modification of R.W. Davis' system [27]. Either 0.7 or 1.0% agarose type I was used depending on whether whole plasmids or small DNA fragments were to be resolved. Usually TBE buffer was recommended, but the capacity of TAE buffer was enough for almost all cases and because of the ease of its use, TAE buffer was chosen. Agarose was melted briefly (5-10min) in electrophoresis buffer by autoclaving. Electrophoresis was carried out at 12V/cm and usually 2 hours were necessary for bromophenol blue tracking dye in the samples to migrate 10cm. Yeast plasmid detection by agarose gel electrophoresis was performed according to the procedure by Livingston and Kelvin [18]. The gel (about 5mm thick) was stained with 5g of ethium bromide per ml for 15-20 minutes at room temperature. Photograph was taken of gels that were positioned over a middle wave UV light source (UV transilluminator, UV Product Inc.). Polaroid film (type 667, 084 ASA 3,000) was exposed to UV through a wratten gelatin filter (Kodak CAT 1495506).

Transformation of yeast

Yeast protoplasts for transformation were prepared by lytic enzyme treatment [19]. Protoplasts and DNA were mixed and treated with polyethylene glycol to promote DNA uptake.

Protoplasts gave rise to colonies on supplemented minimal agar, in 3 to 4 days. To detect the presence of the transformed plasmids in yeast colonies, extracts of yeast cells were analyzed by gel electrophoresis.

Production of (cir-0) strains

The leu2 strain was transformed to Leu⁺ with pJDB219 DNA using the standard transformation protocol [19]. Cells from a single transformant were resuspended in selective media (about 2ml of synthetic complete medium: Table 1) minus leucine and grown overnight at 30°C with shaking. A sample of this culture was then diluted 1,000 times in YPAD medium. An appropriate dilution of this culture is spread on solid YPAD medium to give about 200 colonies per plate, and colonies auxotrophic for leucine are identified by replica plating to synthetic complete medium minus leucine.

Determination of stability of the recombinant plasmids in yeast.

Table 1. Synthetic complete medium.

Constituent	Final mg per 1 of medium	Stock mg per 200ml	Solution ml for 600ml of medium
YNB w/o a. a.			4 g
Dextrose			12 g
Bacto-agar			12 g
adenine sulfate	20	240	10
uracil	20	480	5
L-tryptophan	20	480	5
L-histidine-HCl	20	480	5
L-arginine-HCl	20	480	5
L-methionine	20	480	5
L-tyrosine	30	180	20
L-leucine	30	720	5
L-isoleucine	30	720	5
L-lysine-HCl	30	720	5
L-phenylalanine	50	600	10
L-glutamic acid	100	1.2 g	10
L-aspartic acid	100	800	15
L-valine	150	3.6 g	5
L-threonine	200	4.8 g	5
L-serine	375	9.0 g	5

Cells grown in YNBD (Difco yeast nitrogen base without amino acids, and dextrose) minimal medium were inoculated to YNBD minimal medium and YPAD complex medium. 0.1ml samples of each selected (in minimal medium) culture and non-selected (in complex medium) culture were diluted and spreaded on YPAD plate (2% agar). Colonies formed on the plate were transferred to YNBD plate by toothpick to select non-segregated colonies.

RESULTS AND DISCUSSION

Stability of recombinant plasmid pMYN 101

The HBsAg structural gene was subcloned into a yeast plasmid vector, pAM82, with both of the 2-micron origin and the ars origin, and the resulting recombinant plasmid was named pMYN 101. This plasmid was transformed into each of the 4 yeast strains, AH22, AH22 cir-0, A281, and LL20 cir-0. The results shown in Figure 1 demonstrate that pMYN 101 is extremely stable, that is, it does not require the coexistence with the endogenous 2-micron plasmid for its enhanced stability. Also the difference in the genetic background of the different host cells does not make any significant influence.

Stability difference between cir-+ strain and cir-0 strain, and among several plasmids

Not like pMYN 101, when one starts with less stable or less complete plasmids one can immediately notice a marked difference in the ability to stably maintain the plasmid depending upon different host cell strains and also on different plasmid species even with the same

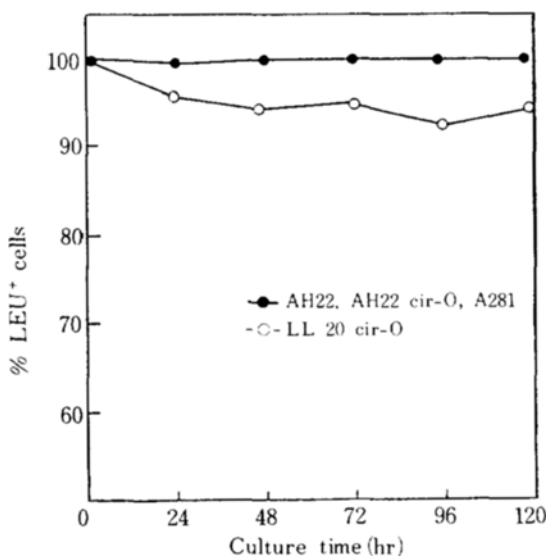


Fig. 1. Stability of pMYN101 in batch culture.
Medium: YPAD

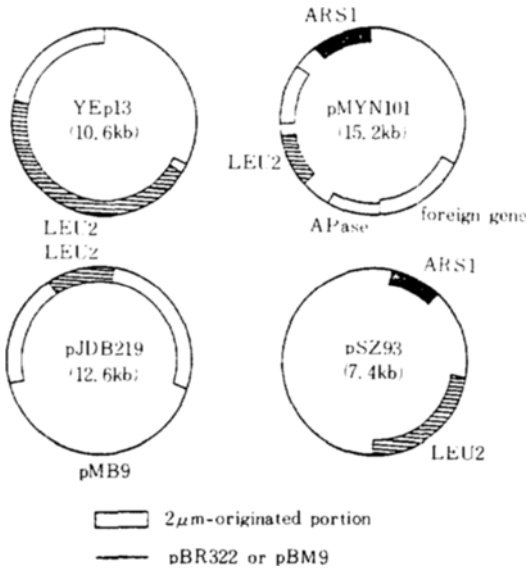


Fig. 2. Schematic diagram of the artificial chimeric plasmids.

host cell.

In order to help understand the structural differences among the different plasmids used in the following experiments their gene maps are shown in Figure 2. YEp13 and pJDB219 are shown to contain a rather incomplete portion of the 2-micron plasmid whereas pSZ93 contains ars origin alone. The data in Figure 3 show the significant instability of pSZ93. The LL20 strains are relatively unsuitable as hosts to stably

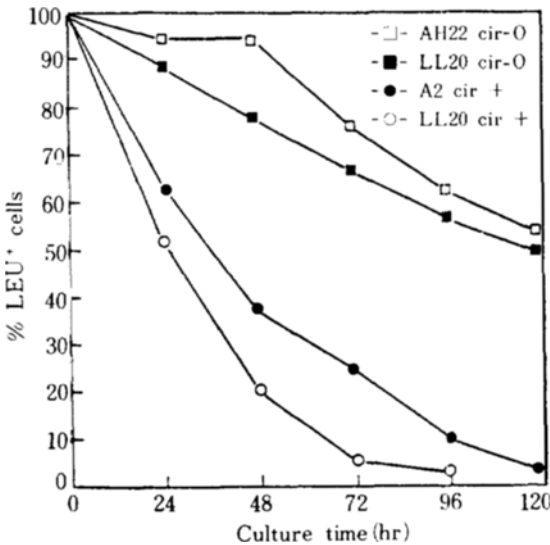


Fig. 3. Stability of pSZ93 in batch culture.
Medium: YPAD

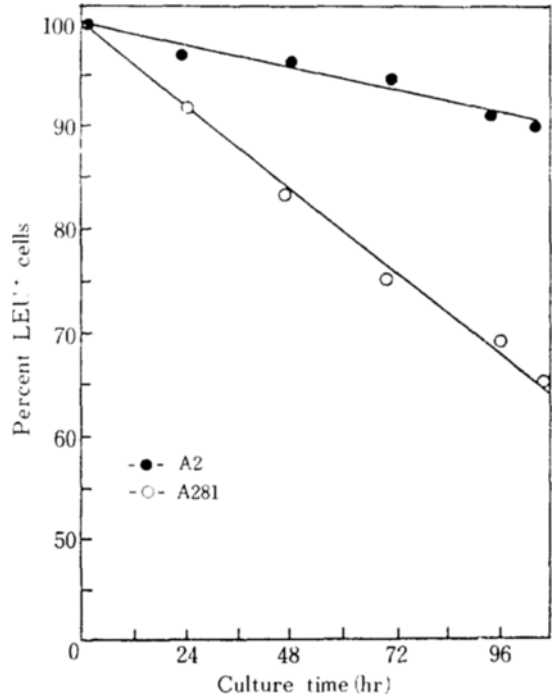


Fig. 4. Stability of YEp13 in batch culture.
Medium: YPAD

keep the pSZ93 plasmid. Also the strains with the endogenous 2-micron plasmids gave lower stability toward pSZ93, which signifies that the ars origin is of different kind than the 2-micron origin so that one can not observe the typical mutually cooperative effect

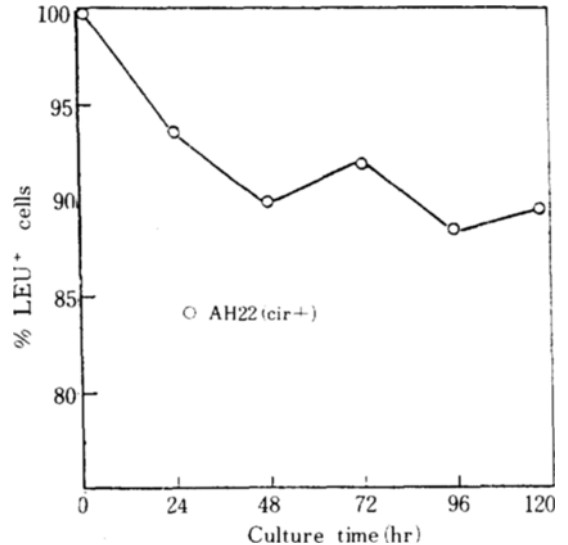
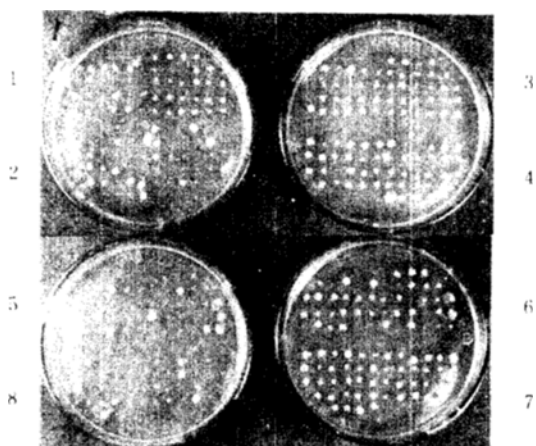


Fig. 5. Stability of pJDB219 in batch culture.
Medium: YPAD



1. LL20 cir-O pSZ93 w/ 2. LL20 cir-O pSZ93 w/o
 3. LL20 cir-O pMYN101 w/ 4. LL20 cir-O pMYN101 w/o
 5. LL20 cir-O pSZ93 w/ 6. LL20 cir-O pSZ93 w/
 7. A281 cir-O pMYN101 w/ 8. A281 cir-O YEp13 w/

★ w/ : with selection pressure.

★ w/o : without selection pressure.

Fig. 6. Comparison of plasmid segregation rates.

among the 2-micron plasmids. Here one can simply see the prototype plasmid incompatibility. Similar experiments were done with YEp13 which does need help from the endogenous 2-micron plasmid for its stable maintenance. The instability in this case is less severe than the ars plasmid pSZ93. The first-order decay feature of the plasmid is more vividly shown by the curvature of the data in Figure 3 than in Figure 4. The data in Figure 5 show that after the initial sharp drop in the plasmid-harboring population it stays rather constant. This signifies that there might be some kind of equilibrium between the frequency of plasmid loss, the reinfection, and the cell growth rate. This result can be compatible with the peculiar behaviour of the particular plasmid, pJDB219, in being used to kick out the endogenous plasmid after which the pJDB219 itself can be induced to be cured. This kind of behaviour can also be host-cell dependent as shown by the lower curve of Figure 1.

In order to help readers get a more direct and physical grasp of the graphical data presented in Figures 1-5, various hosts harboring different kinds of recombinant vectors are plated on the petri dishes with or without selection pressure, i.e. the leucine require-

ment, and the resulting colonies appear as shown in Figure 6. For example if one compares the upper, 1, and lower, 2, segments of the upper left plate, the lower segment has less population density showing the instability of the particular plasmid, pSZ93, under consideration.

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