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Effect of *Escherichia coli* Plasmid DNA Sequences on Plasmid Replication in Yeast

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효모에서 plasmid의 복제에 대장균 plasmid DNA가 미치는 영향에 관한 연구

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The effect of *E. coli* plasmid DNA sequences contained by chimeric vectors on plasmid replication was investigated. We constructed YRp7- or 2μm circle-based plasmids containing *E. coli* plasmid DNA sequences and those not containing it. By examining their maintenance in yeast, we showed that plasmid without *E. coli* plasmid DNA sequences was more stable and presented higher copy number, and expressed higher level of hepatitis B viral surface antigen as a foreign gene. This result suggested that *E. coli* plasmid DNA sequences within chimeric plasmid somehow inhibited plasmid replication in yeast.

KEY WORDS□*E. coli* plasmid DNA sequences, yeast plasmid, replication.

The presence of certain bacterial sequences on hybrid plasmids has been shown to inhibit the replication of these hybrid plasmids in cultured mammalian cells (Lusky and Botchan, 1981). Whether the presence of extraneous bacterial sequences on yeast-*E. coli* chimeric plasmids inhibit their replication potential as well has not been established. It is certainly true that most chimeric plasmids do not attain the copy number or stability of the 2μm circle itself, even though they carry the sequences known to be involved in plasmid propagation and share the same replication machinery as the endogenous plasmid (Futcher and Cox, 1984). One explanation for this reduced stability and copy number is restriction of replication by *E. coli* plasmid DNA sequences within chimeric plasmids.

In this paper, we have constructed various plasmids without *E. coli* plasmid DNA sequences from YRp7 (Tshumper and Carbon, 1980) and 2 μm plasmid (Broach, 1982; 1983), and analyzed their stability, copy number and efficiency of expression of cloned Hepatitis B viral surface antigen gene in (cir$^+$) and (cir^0) yeast strains.

MATERIALS AND METHODS

Strains and growth condition

The *Escherichia coli* strain, HB101 (F$^-$, r$^-$, m$^-$, recA13) was used for all bacterial transformations. CSH28 (Δ(lac, pro), supF, trp, pyrF, his, strA, thi) and JA300 (thr, leuB6, thi, thyA, trpC 1117, hsr_k, hsm_k, str^r) were used for uracil and tryptophan selections. DPGM119 (dcm, dam, met,

lac, galK, galT) was used as a host for digestion of DNA containing a dam methylated Cla I site. *E. coli* strains were grown in rich (LB) medium (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl) or minimal medium containing glucose (2 mg/m*l*), thiamine (2 μg/m*l*) plus M9 salts (Miller, 1972) with appropriate amino acids (50 μg/m*l*).

Yeast strains, SHY4 (a, ste VC9, ura3-52, trp1-289, leu2-3, leu2-112, his3-Δ1 (cir$^+$)) and NNY1 (α, trp1-289, ura3-52, his3-1, gal2, gal10 (cir^0)) were used for yeast transformations. Yeast cells were grown in rich (YEPD) medium (2% bactopeptone, 1% yeast extract, 2% glucose) or minimal (SD) medium (0.67% yeast nitrogen base, 2% dextrose) supplemented with appropriate amino acids (100 μg/m*l*).

Transformation, DNA isolation and cloning procedures

Restriction enzymes and DNA modifying enzymes were purchased from BRL or Biolabs and used in accordance with the manufacturer's recommendations. *E. coli* was transformed by the method of Kushner (1981) and *S. cerevisiae* as described by Ito (1983). DNA was extracted from *E. coli* by an alkaline lysis method (Birnboim and Doly, 1979). Covalent closed circular DNA was purified from yeast according to the method described by Devenish *et al.* (1982).

Assay of segregational plasmid stability

Transformants were grown in selective media to a density of about 5×10^7 cells ml^{-1}. These cultures were diluted and plated onto both selective and nonselective media to determine the initial percentage of population containing the plasmid. Also these cultures were then transferred to YEPD (nonselective) media and grown for 20 hr. The proportion of plasmid-containing cells in cultures growing in nonselective media was measured by initially plating on nonselective media and then replica-plating onto selective media. All experiments were carried out with at least three individual transformants and for each sample at least 500 colonies were screened to ensure that results were statistically significant.

Copy number measurement

Plasmid copy numbers in yeast transformants were estimated by extracting total DNA (Struhl, 1983) and comparing the staining intensity of ribosomal RNA gene-specific and plasmid-specific restriction fragments. The restriction enzymes used were Sma I for 2YΔS and 2YS; Sma I and Sal I for YARS; Sma I and Bam HI for YRp7. Digested DNA was running on agarose gels and photographed. The resultant photographs scanned with a microdensitometer. For all plasmids, several independent copy number determinations were made.

Hepatitis B viral surface antigen assay

The transformant was grown in 10 m*l* of minimal medium at 30 °C until the A_{600} reached 0.5. The cells were pelleted and suspended in 3 m*l* of Lyticase solution (100 μg/m*l*) in 1.2M sorbitol, 50 mM phosphate (pH 7.2) for 30 min at 30 °C. The spheroplast was collected and lysed in 1 m*l* of 0.1% Triton X-100, 50 mM phosphate (pH 7.2). The lysate was centrifuged and the supernatant was subjected to HBsAg radioimmunoassay kit from Abbott Labs.

RESULTS AND DISCUSSION

The effect of *E. coli* plasmid DNA sequences contained by chimeric yeast vectors on plasmid level and stability was investigated. Various plasmids (2YΔS(A), 2YΔS(B), 2YS(A), 2YS(B), YARS) were constructed and their maintenance was analyzed in (cir$^+$) and (cir^0) yeast strains.

Stability and copy number of YRp7-based plasmids

Natural vector (YARS) without *E. coli* plasmid DNA sequences was constructed as follows (Fig. 1). DNA fragment containing Tc marker gene isolated from YEp24 (Broach *et al.*, 1979) was ligated at the Pst I site of TRP-ARS region in pYCPADR. After transformation of HB101, the recombinant plasmid was isolated from Ap^r, Tc^r transformants and its structure verified. Digestion with Eco RI and self-ligation formed pURA. The pURA was then strengthened with ADH I promoter and HBsAg gene from YS 116. Finally, natural vector YARS was made by removing Pst I restricted pBR322 *E. coli* plasmid DNA sequences.

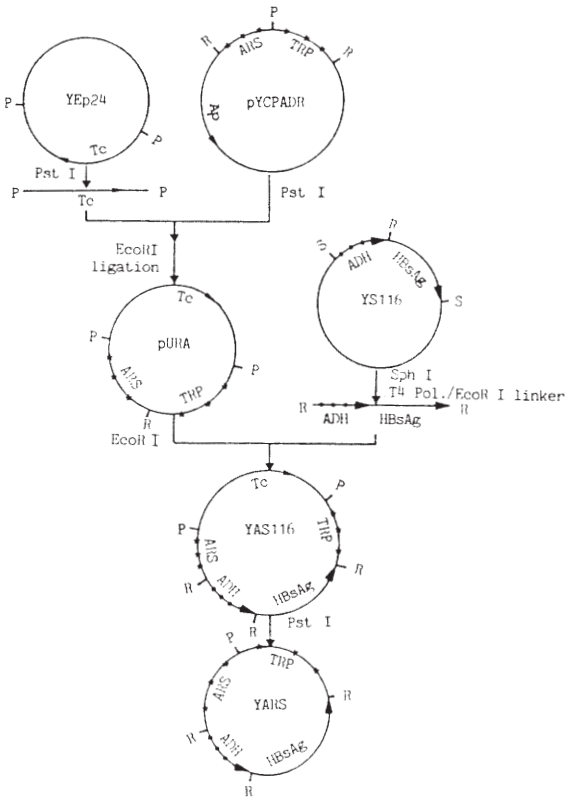

Fig. 1. *Construction strategy of natural plasmid based on YRp7.*
The resultant plasmid, YARS contains autonomous replicating sequences (ARS), tryptophan marker gene (TRP) and alcohol dehydrogenase I promoter (ADH)-hepatitis B viral surface antigen (HBsAg) as a foreign gene whereas chimeric plasmid, YRp7 retains *E. coli* plasmid DNA sequences (pBR322Δ) additionally.

The resultant plasmid YARS showed much higher stability and copy level than YRp7 (Tshumper and Carbon, 1980; Struhl *et al.*, 1979) containing *E. coli* plasmid DNA sequences additionally (Table 1 and 2). This suggested that *E. coli* plasmid DNA sequences somehow interfere with plasmid replication in yeast. Other possibility not yet discovered was not excluded; size of plasmid.

Stability and copy number of 2 μm circle-based plasmids

To assess previous result that removal of *E. coli* plasmid DNA sequences from chimeric vectors increases stability and copy number, we constructed another natural vector (2YS) from 2 μm plasmid (Fig. 2). Initially, 2 μm circles existed as A or B form in yeast were prepared from SHY4 (cir$^+$) containing the 2 μm plasmid and digested at its unique Cla I site, cloned into pBR322Δ. The URA3, selective marker gene was isolated by electroelution as a 1.1 kb DNA fragment from plasmid, BL URA. This fragment combined with Pst I linker was ligated into Pst I site in the 2 μm circle moiety that is nonessential for replication. A recombinant expression plasmid which directs the expression of HBsAg gene in yeast was construc-

Table 1. *Stability of constructed vectors in SHY4 (cir$^+$) and NNY1 (cir^0) yeast strains.*

Plasmid	Bacterial*** sequences	Strain**	Stability (% plasmid bearing cells)* selective	nonselective
YRp7	+	cir$^+$	27.4	10.0
YRp7	+	cir^0	28.3	13.7
YARS	–	cir$^+$	84.2	69.7
YARS	–	cir^0	57.4	40.8
2YΔS	+	cir$^+$	99.6	87.0
2YΔS(A)	+	cir^0	99.3	80.5
2YΔS(B)	+	cir^0	99.5	80.2
2YS	–	cir$^+$	99.8	93.5
2YS	–	cir^0	99.7	93.0

*Stability measurements are percentage of cells retaining plasmid 20 hr after a shift from selective to nonselective medium as described in materials and methods.
**cir$^+$: yeast strain containing 2 μm plasmid.
cir^0: yeast strain containing no 2 μm plasmid.
***+ and – denote that plasmid contains *E. coli* plasmid DNA sequences (3.5 kb) or not, respectively.

Table 2. *Copy number of constructed vectors in yeast.*

Plasmid	Strain*	Copy number
YRp7	cir$^+$	20
YRp7	cir^0	17
YARS	cir$^+$	50
YARS	cir^0	60
2YΔS	cir$^+$	30
2YΔS(A)	cir^0	40
2YΔS(B)	cir^0	40
2YS	cir$^+$	35
2YS	cir^0	50

*cir$^+$: yeast strain containing 2 μm plasmid.
cir^0: yeast strain containing no 2 μm plasmid.

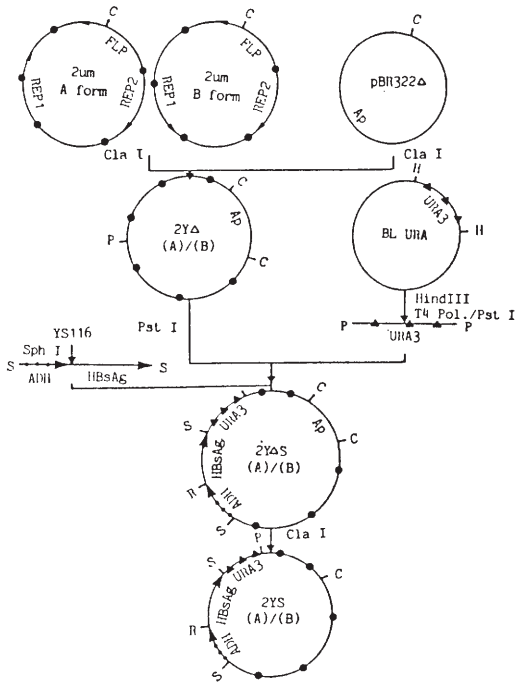

Fig. 2. *Construction strategy of natural plasmid based on 2 μm plasmid.*
The resultant plasmids, 2YS(A) and 2YS(B) contain complete genome of 2μm circle existed as A or B form, uracil marker gene(URA) and alcohol dehydrogenase I promoter **(ADH)-hepatitis** B viral surface antigen **(HBsAg) as a foreign gene,** whereas chimeric **plasmids,** 2YΔS(A) and 2YΔS (B) retain *E. coli* plasmid DNA sequences (pBR 322Δ) additionally.

ted by cloning a DNA fragment containing ADH I promoter-HBsAg gene from YS 116. After purification of 2YΔS(A) and 2YΔS(B) from dam⁻ *E. coli* strain, natural vectors, 2YS(A) and 2YS(B) were made by removing Cla I restricted pBR322Δ *E. coli* plasmid DNA sequences.

The resultant another natural plasmid (2YS) was more stable and presented higher copy number than 2YΔS retaining pBR322Δ sequences (Table 1 and 2). Constructed vector by removing ADH I promoter and HBsAg (nonyeast DNA) from 2YS showed same maintenance with 2YS itself. This result showed that the *E. coli* plasmid DNA sequences (pBR322) not ADH I promoter-HBsAg sequences somehow interfere with plasmid replication in yeast. Lusky and Botchan (1981) have shown that in cultured mammalian cells, specific bacterial sequences between the pBR322 origin of replication and Pvu II site inhibit the replication of hybrid plasmid. They have proposed that modification such as methylation in these sequences may occur in the simian cell which may influence replication elongation rates. The similar modification may also influence a plasmid's ability to re-establish in *E. coli.* However, these inhibitory sequences are removed in pBR322Δ by deletion between the pBR322 Bal I site and Acc I site. Thus, some other sequences or mechanisms restrict replication potential of plasmids. We think that two factors may influence the stability and copy number of constructed plasmids in yeast: replication and partitioning to daughter cells. Inactivation of replication functions (such as *E. coli* plasmid DNA sequences may effect on overall replication elongation rates) will result in the phenotype of plasmid instability and reduced copy number. However, it is also possible that defects in segregation functions due to *E. coli* plasmid DNA sequences or some other factors may lead to the same result.

Table 3. *Expression of HBsAg as a foreign gene in yeast.*

Plasmid	Strain	Reactivity with anti-S Ab. specific activity (cpm)*	P/N ratio**
None	cir⁺	225	1
None	cir⁰	220	1
YARS	cir⁺	1950	8.67
YARS	cir⁰	2600	11.82
2YΔS	cir⁺	1600	7.11
2YΔS(A)	cir⁰	1700	7.73
2YΔS(B)	cir⁰	1700	7.73
2YS	cir⁺	2200	9.78
2YS	cir⁰	2370	10.77

*Yeast cells carrying each plasmid were grown to 2×10^7 cells ml^{-1} in 10 ml of minimal medium at 30 °C, lysed with 1 ml of lytic mixture and cleared by centrifugation as described in materials and methods, and assayed by AUSRIA-II kit (Abbott) after three fold dilution with PBS buffer.
**P/N ratio: cpm of samples divided by cpm of negative control.

Expression of a foreign gene by constructed plasmids

We constructed vectors free of *E. coli* plasmid DNA sequences with high stability and copy number to increase expression of HBsAg as a foreign gene in yeast. Quantitation of HBsAg levels by immunoassay indicated that constructed natural vectors (2YS, YARS) expressed the highest level of HBsAg in yeast (Table 3). These results were consistent with the data of maintenance of vectors previously shown.

It is necessary to elucidate the inhibition mechanism by *E. coli* plasmid DNA sequences as well as expression of other foreign gene in suitable host system for the purpose of feasibility of our constructed natural vectors as a general high efficiency expression plasmid in yeast.

적 요

효모 유전자 운반체의 복제에 대장균 플라스미드 DNA 가 어떤 영향을 미치는지 알아보았다. YRp7과 2μm circle로부터 대장균 플라스미드 DNA 가 제거된 새로운 유전자 운반체들을 각각 재조합하여 그들의 안정도, 복제수와 HBsAg 발현 정도를 (cir^+)와 (cir^0) 효모균주에서 조사해본 결과 대장균 플라스미드 DNA 가 재조합 유전자 운반체의 복제에 억제효과를 나타낸다는 사실을 알았다.

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