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Stability of Recombinant Plasmids Carrying the *stb* Locus of *E. coli* IncFII NR1 Plasmid in *E. coli* and Yeast

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The effect of *stb* locus of *E. coli* IncFII plasmid NR1 on the stability of chimeric plasmids was investigated. First, we have isolated the stability locus (*stb*) from *E. coli* NR1 plasmid and then inserted into the three different vectors, pUC8, YRp17 and YEp24. By examining their stability in *E. coli* and yeast, we showed that the recombinant plasmids containing *stb* locus were reasonably stable. Also, by comparing the amounts of the rDNA fragments per haploid genome with those of the plasmid fragments, we showed that copy number of recombinant plasmids was not increased. Consequently, the *stb* locus of *E. coli* IncFII plasmid NR1 stabilized the chimeric plasmids but did not affect the replication or copy number of plasmids.

KEY WORDS □ *E. coli* IncFII NR1 plasmid, plasmid stability, plasmid copy number, *Saccharomyces cerevisiae*

For a genetic element such as a chromosome or a bacterial plasmid to be inherited in a stable manner during many generations of cell growth and division, two major mechanisms must be regulated and coordinated with the cell division cycle (21). First, there must be a regular pattern of controlled replication to ensure that at least two copies of the element are present at the time of cell division. Second, during cell division at least one copy of the element must be inherited by each of the two daughter cells. For bacterial plasmid, this second process has been referred to as partitioning. Some naturally occurring plasmids may rely on random distribution of plasmid copies at cell division; if the number of plasmid copies is high enough the probability of a daughter cell receiving zero copy is very low. However, many natural plasmids are present at a low copy number per cell, comparable to the bacterial chromosome. If, for example, a low copy number plasmid fails to replicate during a complete cell cycle, this would, in some instances, lead to division of a cell harvouring only one copy of the plasmid resulting in a plasmid-free daughter cell (22). For such plasmids, random distribution clearly is inadequate to mediate stable inheritance. Low copy number plasmids, such as IncFII plasmids (18), F (22), and P1 (1) have specific plasmid-encoded genes that participate in partitioning. Mutations in these genes do not affect the replication or copy number control of the plasmids, but instead result in unstable inheritance characterized by the segregation of plasmid-free cells (24). Such plasmid-encoded genetic loci have been referred to as *stb*, for stable inheritance (24), *par*, for plasmid partitioning (17) and *sop*, for stability of plasmid maintenance (22). In any case, the division inhibition allows the plasmid to increase its copy number thereby ensured that cell harvouring the plasmid molecules can be partitioned properly by the independent *par* genes to the daughter cells. In addition to these stability loci, some plasmids encoded additional functions that may contribute to their overall stable maintenance.

The 95 kb IncFII group antibiotic resistance plasmid NR1 is composed of resistance transfer factor (RTF) and resistance-determinants components (6, 18). The NR1 plasmid is very stable (6, 18). The stability locus (*stb*) of NR1 is essential for stable inheritance of the plasmid in the absence of continuous antibiotic selection. However, NR1 containing mutated *stb* locus is unstable, but have not any change in plasmid copy number per cell. Therefore, instability of NR1 plasmid containing mutated *stb* locus has no relation to the replication (18).

In the yeast *Saccharomyces cerevisiae*, a range of high-copy number chimeric plasmids have been devised (23). They generally contain an origin of

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replication devised from either the region of the chromosome, ARS, believed to contain an origin of replication (i.e. YRp plasmid) or of the endogeneous 2 μm circle (i.e. YEp plasmid). Inspite of the advantages associated with a higher transformation efficiency and high copy number, YRp plasmids exhibit extreme mitotic and meiotic instability (26). On the other hand, the 2 μm yeast plasmid is a circular, duplex DNA molecule found in most naturally occuring and laboratory yeast strains. It is usually present at a copy number of about 60~100 per haploid genome (5). The plasmid confers no detectable selective advantage to cells in which it is resident. Nonetheless, the 2 μm plasmid is extremely stable. But, when *S. cerevisiae* cells have been transformed with 2 μm circle-based chimeric plasmid (i.e. YEp), the transformed phenotype has always been found to be unstable; a large proportion of the cells spontaneously lose the transformant phenotype and all traces of the plasmid (10).

In this paper, we have constructed various chimeric plasmids containing the *stb* locus of NR1 plasmid (27) and examined the stability and/or copy number of the chimeric plasmids in *E. coli* and yeast *S. cerevisiae*, under the absence of continuous antibiotics or auxotrophics selection.

MATERIALS AND METHODS

Bacterial strains, yeast strains, and culture conditions

The *Escherichia coli* strain, KP245 (*met, trp, his, thy, lac, gal, tsx*) was used for NR1 plasmid isolation. Strains JM83 and HB101 were used for cloning with pUC plasmid vectors and for transformation with *E. coli*/yeast shuttle vectors (4). *E. coli* strains were grown in 2YT medium containing, per liter, 16g of tryptophan, 10g of yeast extract, and 5g of NaCl and minimal medium containing 2g of glucose, thiamine or thymine (20 μg/m*l*) plus M9 salt (19) with appropriate amino acids (50 μg/m*l*). 2YT plates contain Bacto-agar, 15 g/*l*. The HB101 strain was cultured in LB medium (19) containing, per liter, 10g of tryptophan, 5g of yeast extract, and 10g of NaCl. In some experiments, antibiotics were included in the medium; tetracycline hydrochloride, 12 mg/*l*; sodium ampicillin, 50 mg/*l*; chloramphenicol, 34 mg/*l*; streptomycin sulfate, 25 mg/*l*.

The yeast strains, DBY747 (*MATa, his3, leu2-3, ura3-52, trp-289*, cir^{+}) and SC3 (*MATα, trp1, ura3-52, his3, gal2, gal10*, cir^{o}) were used for yeast transformation and for uracil and tryptophan selection. Yeast cells were generally grown in YEPD medium containing, per liter, 1% yeast extract, 2% peptone, 2% dextrose, supplemented with 2% agar for plates or YNB medium containing, per liter, 0.67% yeast nitrogen base without amino acids and ammonium sulfate, 2% dextrose, 2% agar for plates, and supplemented with appropriate amino acid of 50 g/m*l*.

DNA manipulation, *E. coli* and yeast transformation

All restriction enzymes and DNA modifying enzymes were obtained from Promega (USA) and Bethesda Research Laboratories (USA). Bacterial plasmids were routinely prepared by the alkaline SDS lysis method (2). Transformation of *E. coli* with plasmid DNA was based on $CaCl_2$ method (16). *S. cerevisiae* cells were transformed by the procedure developed by Ito *et al.* (14) and DNA fragment was purified from agarose gel according to the method described by Girvetz *et al* (11). Yeast total DNA was extracted by the method of Cryer *et al* (8).

Measurement of plasmid stability

Stability of plasmids were tested by the method of Futcher and Cox (10). Transformants, in yeast, were grown in selective medium to density of about 5×10^7 cells per m*l*. These cultures were serially transferred from selective medium to nonselective medium to a density of 5×10^3 cells per m*l*. The cultures were diluted and plated onto nonselective media every day. This process was repeated for 10 days (some time longer). The sample usually yielded approximately 300 colonies on each of two plates. These 600 colonies were replica-plated on selective medium, and after 3 days, numbers of cells that contained plasmid were counted. All experiments were carried out with at least 3 individual transformants. In *E. coli*, stability of plasmids were determined by measuring the decrease in the fraction of antibiotic resistant cells during a period of nonselective growth. The cells were then repeatly subcultured by 106 fold dilution into medium lacking antibiotics followed by selective overnight culture. After each subculture, appropriate dilution of cultures was spread onto drug free 2YT agar plate and the antibiotic resistancy of colonies was tested by replica plating.

Measurement of plasmid copy number

The copy number of recombinant plasmids was measured by the method of Futcher and Cox (10). Cells from a single transformed clone were grown in selective medium to 2×10^7 cells per m*l*. DNA was extracted by the method of Cryer *et al* (8)., with a CsCl gradient as the final purification step. DNA was digested with *Eco*RI, run on agarose gels, and then stained with ethidium bromide. The gels were photographed by using exposure calculated to minimize the nonlinear response of the polaroid emulsion used. The negative were scanned with a TLC scanner. As the copy number of rDNA per haploid genome is constant (24), an estimate of change of the copy number of the various plasmids could be made by comparing the height of the peaks due to ribosomal fragments with those due to plasmid

fragments.

RESULTS AND DISCUSSION

In this work, we have investigated the effect of *stb* locus derived from *E. coli* IncFII plasmid NR1. We have constructed three chimeric plasmids, pFR820, pKS-1, and pKS-2. The detail procedure of construction of chimeric plasmids was published elsewhere (7). Their maintenance in *E. coli* and in yeast was analyzed and compared to that of NR1, YRp17, and YEp24, respectively. In addition, we have examined the copy number of recombinant plasmids in DBY747(cir$^+$) and SC3 (cir^0) yeast strains to determine whether stb locus affects the copy number control of the plasmid. The terms, cir$^+$ and cir^0, mean the presence and absence of 2 μm plasmid in yeast strains, respectively.

Stability of recombinant plasmid pFR820 containing *stb* locus

The *stb* locus in the largest *Eco*RI fragment of NR1 (12) was cloned according to the procedure as shown in Fig. 1. The resulting plasmid was named as pFR820. The stability of pFR820 was tested in *E. coli* as follows. After overnight culture, transformants was transferred from selective to nonselective 2YT medium. During 150 generations, 10^6 dilution of cultures was spreaded on a 2YT agar plate every day. The 600 colonies on nonselective plates were replica-plated on selective plates. The result shown in Fig. 3 indicated that pFR820 plasmid had high stability and its stability was equal to NR1 (27). NR1 has loss frequencies in the range of 10^{-6} to 10^{-7} per generation (21).

Stability and copy number of YRp17-based plasmid

The chimeric plasmid, named pKS-1, containing autonomously replicating sequence (ARS) and stb locus was constructed (Fig. 2). The plasmid was transformed into *E. coli* and its stability was tested. The stability of pKS-1 in *E. coli* was higher than that of YRp17 and almost the same as that of pFR820 (Fig. 3). In addition, the plasmid was introduced into the DBY747 (cir$^+$) and SC3 (cir^0) yeast strains by transformation. After Trp$^+$ transformants were subcultured from selective to nonselective media, the stability of plasmids was tested

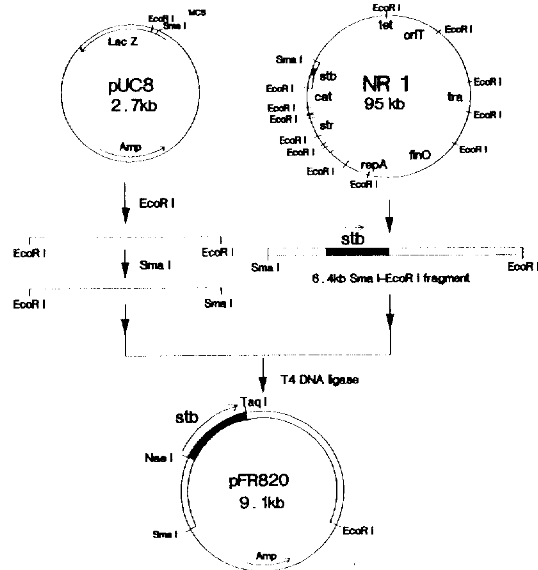

Fig. 1. *Construction of pFR820 recombinant plasmid.* pFR820 plasmid consists of pUC8, and 6.4kb *Sma*I-*Eco*RI fragment carrying the *stb* locus.

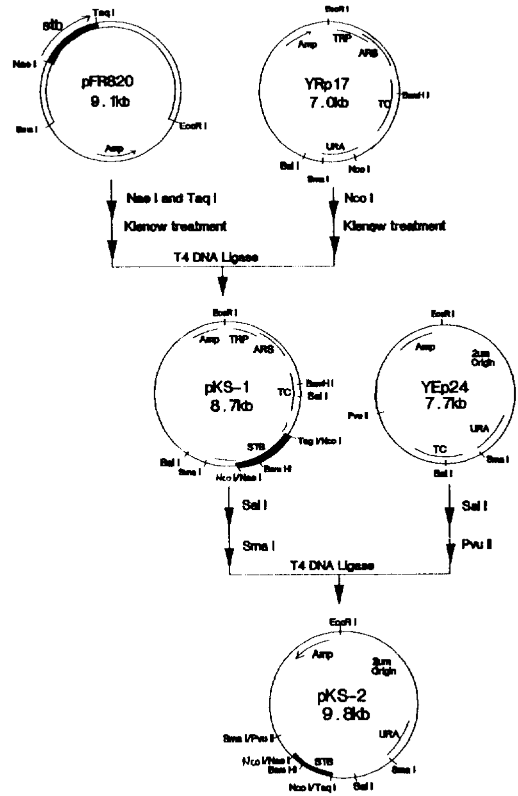

Fig. 2. *Construction of the E. coli/yeast shuttle plasmids, pKS-1 and pKS-2, which were derived from YRp17 and YEp24, respectively.* pKS-1 plasmid consists of YRp17 (thin line), and 1.7 kb fragment carrying the stb locus (thick line). pKS-2 plasmid consists of YEp24 (thin line), and 2.1 kb pKS-1 fragment carring the *stb* locus as indicated.

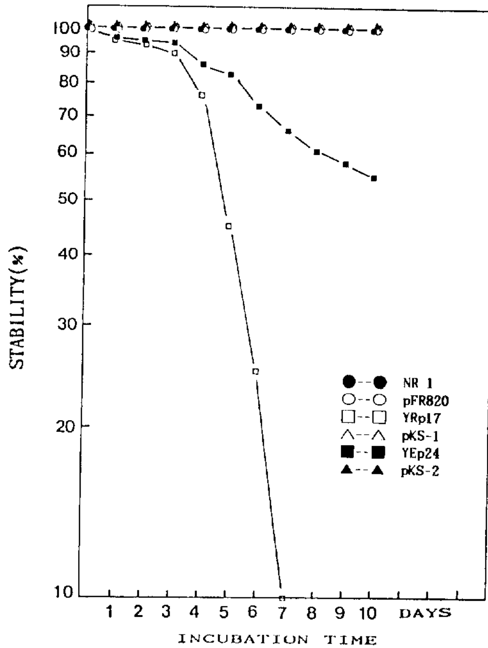

Fig. 3. *Stability of natural and recombinant plasmids in E. coli strain.*
The percent of cells that were resistant to ampicillin for 10 to 15 days after nonselective growth was determined by replica plated on selective plates once a day. The test plasmids were natural plasmid NR1 (●), commercial shuttle vector YRp17 (□) and YEp24 (■), recombinant plasmids pFR820 (○), pKS-1 (△), and pKS-2 (▲).

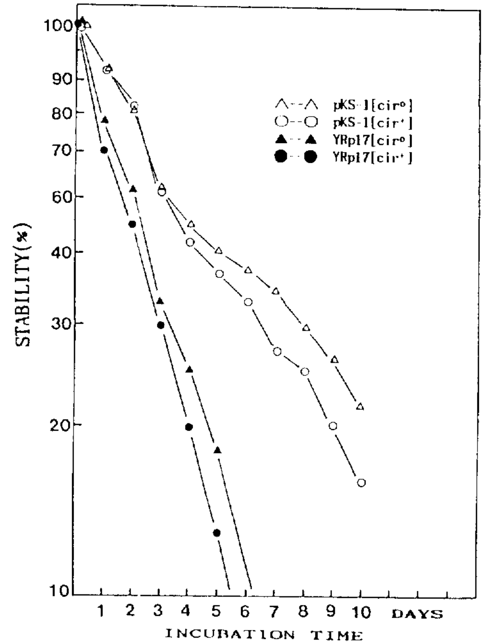

Fig. 4. *Stability of recombinant plasmid, pKS-1, in yeast strains DBY747[cir⁺] (circle) and SC3 [cir°] (triangle).*
Kinetics of loss of YRp17 (▲, ●) and pKS-1 (△, ○) was calculated based on YRp17. cir⁺, yeast strain containing 2 μm plasmid; cir°, yeast strain containing no 2 μm plasmid.

every day during 10 days. The 50 percent of the transformants have retained the Trp^+ phenotype after 40 generations of nonselective growth. On the studies of the mitotic stability of YRp17 plasmid by measuring the percentage of plasmid bearing cells in nonselective media, about 15 to 20% of cells harvor the plasmids (15, 25). Therefore, pKS-1 plasmid showed higher stability than YRp17 (Fig. 4). Also, we measured the copy number of recombinant plasmids to examine whether the replication affects the stability of plasmid. To do this, yeast total DNA was extracted by the method of Cryer *et al* (8)., with a CsCl gradient as the final purification step. The DNA was digested with *Eco*RI, run on agarose gels and the change of copy number of pKS-1 plasmids was scanned by TLC scanner. As shown in Table 1, the pKS-1 plasmid had nearly the same copy number as that of YRp17 in cir^+ and cir^0 yeast strains.

Stability and copy number of YEp24-based plasmid

To assess the above result that the *stb* locus on chimeric plasmids increases stability in *E. coli* and in cir^+ and cir^0 yeast strains, we constructed another chimeric plasmid, pKS-2, derived from YEp plasmid (Fig. 2). As if pKS-1 in *E. coli*, the stability of pKS-2 plasmid was higher than that

Table 1. *Investigation of copy number of various plasmids in yeast.*

Plasmids	Yeast strains	
	DBY747 (cir^+)	SC3 (cir^0)
	Ratio of plasmid/rDNA	
YRp17	0.472	0.545
pKS-1	0.509	0.510
	Ratio of plasmid/(plasmid+rDNA)*	
YEp24	1.213	1.006
pKS-2	1.127	1.108

*The ratio of plasmid and rDNA was calculated by the ratio of plasmid and plasmid+rDNA because the pick of plasmid overlapped with that of rDNA on a gel.

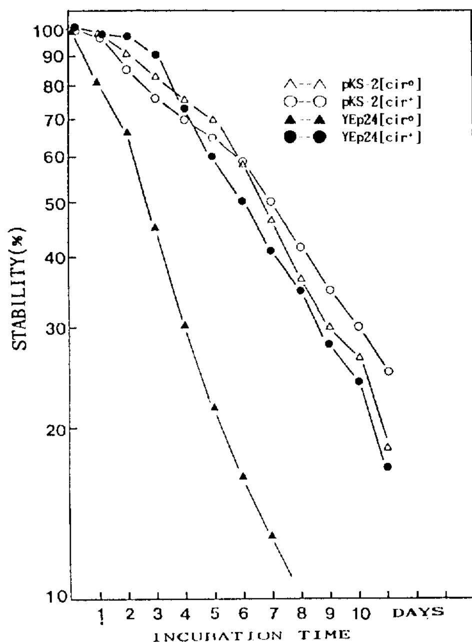

Fig. 5. *Stability of recombinant plasmid, pKS-2, in yeast strains DBY747[cir⁺] (circle) and SC3 [cir°] (triangle).*
Kinetics of loss of YEp24 (▲, ●) and pKS-2 (△, ○) was calculated based on YEp24.

of YEp24 and it was similar to that of pFR820 (Fig. 3). In the DBY747 (cir⁺) and SC3 (cir°) yeast strains, about 30~50% of the cell population of Ura⁻ transformants lost the selective marker after 40 generations of a nonselective culture (Fig. 5). However, the stability of YEp24 was similar to that of pKS-2 in a strain containing intact 2 μm circle (cir⁺). It is because the mitotic stability of 2 μm origin carrying plasmid, YEp24 was increased by trans-acting factor of 2 μm circle. But, in a strain lacking endogeneous 2 μm plasmid (cir°), YEp24 behaved just as an ARS plasmid (19), consequently, the stability of pKS-2 was higher than that of YEp24. The copy number of pKS-2 was alike to that of YEp24 (9) both in cir⁺ and in cir° yeast strains. Table 1 showed the copy number of each plasmids. Therefore, it could be suggested that the stb locus of NR1 may increase the stability of recombinant plasmids both in *E. coli* and in yeast strains, but stb locus has no relation to plasmid replication.

With a few exceptions, the plasmid loss can be described by straight line plots on semi-logarithmic graph paper (10). This has several implications. First, instability is constant from generation to generation. Second, (plasmid⁺) and (plasmid°) cells grow essentially at the same rates in nonselective medium since selection either for or against (plasmid⁺) cells would inevitably lead to curved lines (3). Third, stable integration of the plasmids is rare, since such integration would produce traces of phenotypically stable transformants. The plasmid system is briefly represented that copy number is maintained at same number n in each cell, that the number of plasmids doubles to $2n$ before cell division, and that these $2n$ copies are randomly distributed between the daughter cells. (plasmid°) varients would then segregate at the rate of 2^{-2n}. If instability were mainly to random plasmid partition, then instability would satisfy the equation of stability (plasmid⁺ cell numbers/total cell numbers)$=2^{-2n}$ (10, 25). However, plasmid instability is far higher than predicted. This discrepancy has only one readily apparent explanation: cells which have lost a plasmid and become genetically autotrophic can nevertheless undergo several generations of residual growth on selective medium, and so increase the proportion of auxotrophs (10). Although the instabilities of 2 μm circle-based plasmids are higher than expected from random partition, their instability nevertheless much lower than those of ARS-based plasmids. The high instabilities of ARS-based plasmids are not due to low copy numbers (20, 25). Various studies have shown that in a selectively grown culture, about 80~90% proportion of transformants carrying the ARS plasmid (28) have average plasmid copy number of 30 to 90. Therefore, almost of transformants do contain many copies of plasmid (13). Despite their high copy number, the chimeric plasmid shows segregational instability (6), suggesting that they lack a partition function. When joined to other unstable replicons, such as YRp17 and YEp24 plasmid, the *stb* segment stabilized them. That has no relation to any change of plasmid copy number per cell in *E. coli* and in yeast because both pKS-1 and pKS-2 plasmids were more stable than its counterparts but their copy numbers were similar to those of YRp17 and YEp24, respectively. Therefore, the *stb* locus may affect the increase of stability of recombinant plasmids both in *E. coli* and in yeast strains, and the effect of *stb* locus was not related to the plasmid replication.

Because the control of copy number and instability of chimeric plasmids is complex, finely balanced, and easily upset, it seems unlikely that any simple rule could explain the array of results obtained, or that a complete set of rules could be derived without more experimenal data.

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초 록: 대장균과 효모에서 *Escherichia coli* IncFII NR1 플라스미드의 *stb* 좌위를 포함하는 재조합 플라스미드의 안정성에 관한 연구
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E. coli IncFII NR1 플라스미드의 *stb* 좌위를 분리하여 pUC8, YRp17, YEp24 벡터에 삽입시켜 재조합 플라스미드 pFR820, pKS-1, 그리고 pKS-2를 생성하였다. 이 재조합 플라스미드들을 *E. coli*와 yeast에 형질전환시킨 후, 이들의 안정성을 조사한 결과, *stb* 좌위가 없는 벡터에 비해 그 안정성이 크게 증가하였다. 그러나 이 재조합 플라스미드들의 세포내 숫자는 그대로 유지되고 있었다. 그러므로 플라스미드수의 증가에 의한 플라스미드 안정성의 증가는 아님을 알 수 있다. 따라서, *E. coli* IncFII NR1 플라스미드의 *stb* 좌위가 재조합 플라스미드의 안정성에 직접적으로 관련되었음을 추정할 수 있다.