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A set of temperature sensitive-replication/-segregation and temperature resistant plasmid vectors with different copy numbers and in an isogenic background (chloramphenicol, kanamycin, *lacZ*, *repA*, *par*, *polA*)

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Abstract

A set of plasmid vectors conferring chloramphenicol resistance (Cm^R), 3064 bp in size, or kanamycin resistance (Km^R), 2972 bp in size, were developed, having multiple cloning sites in *lacZ′* genes for α-complementation. pTH18cs1, pTH19cs1, pTH18ks1 and pTH19ks1 are temperature-sensitive (ts) in DNA replication (ts-Rep); pTH18cs5, pTH19cs5, pTH18ks5 and pTH19ks5 are ts in plasmid segregation (ts-Seg); and pTH18cr, pTH19cr, pTH18kr and pTH19kr are temperature resistant (tr) in both. They are based on the pSC101 replicon consisting merely of the replication origin and *repA* gene, compatible with ColE1/pMB1/p15-derived plasmids, and thus do not require *polA* function of host cells. The copy numbers of the ts-Rep, tr and ts-Seg plasmids were 14, 5 and 1 per chromosome at 30°C, respectively. These plasmids are fairly stable when inherited at 30°C, but not above 37°C or 41.5°C, depending on the *repA* mutations and host strains. They are isogenic apart from the ts mutations in the *repA* gene, and thus provide with useful tools for having appropriate controls in various experiments including bacterial gene-targeting, transposon mutagenesis, toxic gene expression, differential substitution on host functions, gene dosage analysis and so on.

1. Introduction

The plasmid pSC101 (Cohen and Chang, 1977), which requires the *dnaA* but not the *polA* function of host cells for its DNA replication, has been extensively studied as a model for stringently controlled plasmids in an attempt to elucidate the control mechanisms of DNA replication, partitioning and incompatibility (Hashimoto and Sekiguchi, 1976; Hashimoto-Gotoh and Sekiguchi, 1977; Hasunuma and Sekiguchi, 1977; Armstrong et al., 1984; Tucker et al., 1984; Gamas et al., 1986; Wahle and Kornberg, 1988; Manen et al., 1992; Sutton and Kaguni, 1995; Okubo and Yamaguchi, 1997; Datta et al., 1999). We have reported a series of temperature-sensitive (ts) mutants of pSC101 obtained by in-vitro mutagenesis of plasmid DNA with hydroxylamine (Hashimoto and Sekiguchi, 1976). Among them, two representative mutants, pHSG1 ($repA_{ts1}$) and pHSG5 ($repA_{ts5}$), were characteristic in their ts behavior in that the former exhibited abrupt cessation of plasmid DNA replication upon temperature shift, and the latter could replicate even at 43°C, but approximately 35% of new born cells produced from plasmid-carrying cells were plasmid-negative (Hashimoto-Gotoh and Sekiguchi, 1977; Hashimoto-Gotoh and Inselburg,

Abbreviations: *agp*, gene encoding aminoglucoside phosphotransferase responsible for Km^R; Ap, ampicillin; *bla*, gene encoding; β-lactamase responsible for Ap^R; bp, base pair(s); *cat*, gene encoding Cm acetyltransferase; Cm, chloramphenicol; *dnaA*, gene encoding a protein required for DNA replication of bacterial chromosome and pSC101; IPTG, isopropyl-β-D(−)-thiogalactopyranoside; Km, kanamycin; *lacZ′*, gene encoding α-peptide of β-galactosidase; LS, low-salt medium (10 g Bacto tryptone/5 g yeast extract/5 g NaCl, per liter, pH 7.4); MCS, multiple cloning site(s) (polylinker); nt, nucleotide(s); *polA*, gene encoding DNA polymerase I; ^{R}, resistance/resistant; repA, pSC101 plasmid-specified gene encoding a protein required for its DNA replication; ^{S}, sensitivity/sensitive; TE, 10 mM tris(hydroxymethyl)aminometan, pH 7.4, and 1 mM ethylenediamine tetraacetic acid; tr, temperature-resistant; ts, temperature-sensitive; ts-Rep, ts replication; ts-Seg, ts segregation; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

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1979). The copy number of pHSG5 was as low as one per chromosome versus four to six in pSC101 and pHSG1 at 30°C.

From pHSG1, we have developed a ts plasmid vector, pHSG415s, conferring triple antibiotic resistance against ampicillin (Ap^R), chloramphenicol (Cm^R) and kanamycin (Km^R) (Hashimoto-Gotoh et al., 1981), as well as its temperature-resistant (tr) revertant pHSG415r (*repA*$_{tr}$), from which Cm^R and *lacZ* α-complementation tr plasmids, pHSG575 and pHSG576, were developed (Takeshita et al., 1987). These plasmids are widely used for bacterial gene-targeting (Hamilton et al., 1989), transposon mutagenesis (Duchêne et al., 1992; Francia and Garcia-Lobo, 1996), transient expression of toxic genes (Naito et al., 1995; Cherepanov and Wackernagel, 1995), substitution analysis (Sweasy and Loeb, 1993; Kim and Loeb, 1995), and so on.

Since then, a number of inquiries have been made to one of the authors (T.H.-G.) about α-complementation plasmid vectors fulfilling the following criteria: (1) conferring a single drug resistance other than Ap^R, (2) being compatible with pBR322 derivatives or *polA* independent, and (3) having tr/ts allelic mutations or different low-copy-number mutations on an otherwise isogenic background. Here, we report a set of Cm^R or Km^R plasmid vectors based on the pSC101 replicon having *repA*$_{ts1}$, *repA*$_{ts5}$ or *repA*$_{tr}$, which would meet these criteria.

2. Materials and methods

2.1. Media, bacteria and plasmids

LS medium has been described (Hashimoto-Gotoh et al., 1993). The antibiotics Ap, Cm and Km were used at 50, 10 and 15 μg/ml, respectively, unless stated otherwise. TOP10 strain, *recA1* (Invitrogen, Carlsbad, CA), was used for plasmid construction, DNA preparation and copy number determination. HB101, *recA13* (Boyer and Roulland-Dussoix, 1969), JM109, *recA1* (Yanisch-Perron et al., 1985) and DH5α, *recA1* (Life Technologies, Rockville, MD) were used for a colony-forming ability test and/or ts segregation tests. HB101, first described as $r_B^- m_B^-$, F^-, *pro*$^-$, *gal*$^-$, *str*^{R}, *rec*$^-$, is referred to as $HB101L^+$ in this report, but we are aware of another HB101 strain (Takara Shuzo, Kyoto) circulated having an additional auxotrophic marker, *leu*$^-$, referred to as $HB101L^-$. We used $HB101L^+$ in this study, but no essential difference was observed between $HB101L^+$ and $HB101L^-$, as far as this work was concerned. JS295 (*rec*$^+$), described previously as SC18 (Witkin et al., 1982), was kindly provided by J.B. Sweasy and used for the segregation kinetics experiment. Plasmids used are listed in Table 1.

2.2. Segregation kinetics

The segregation kinetics was investigated as described previously (Hashimoto-Gotoh and Sekiguchi, 1977). From the kinetics, the plasmid copy number per chromosome (P) at 30°C and the duplication rate of plasmid DNA (k) at 41.5°C relative to that at 30°C were obtained by using the following equations,

$$P = \exp\left[\frac{-n}{N-n}\ln\left(b_N/c_N\right) - \ln C\right] \quad (1)$$

$$k = 2\exp\left[\frac{\ln\left(b_N/c_N\right)}{N-n}\right] - 1, \quad (2)$$

where n is the number of generations required for the appearance of plasmid negative cells after shift-up to 41.5°C; N is a given generation number ($N > n$); b_N and c_N are the numbers of Km^R and total cells per ml, respectively, at N generations after the temperature shift; and C is the average number of chromosomes in exponentially growing cells, assumed to be 2.5 (Hashimoto-Gotoh and Sekiguchi, 1977; Hashimoto-Gotoh et al., 1981).

2.3. Estimation of plasmid copy numbers

TOP10 harboring various plasmids was cultured in LS medium with Cm or Km at 30°C overnight. Two milliliters of the overnight culture were subjected to the standard alkaline/SDS lysis method (Maniatis et al., 1978), to extract RNA-free plasmid DNA dissolved in 20 μl of TE. A portion (5 μl) of DNA was taken and electrophoresed in 1% agarose gel with 0.1 μg/ml ethidium bromide and photographed. Then, the DNA bands were traced by an image scanner (Sharp JX-320M) and analyzed using NIH Image to determine the relative amount of plasmid DNA expressed as square pixels.

3. Results and discussion

3.1. Construction of tr, ts-Rep and ts-Seg plasmid vectors

The maps of tr, ts-Rep and ts-Seg plasmid vectors are shown in Fig. 1. The pSC101 replicon (Frag. I) was taken as *Hae*II–*Nde*I fragments from pHSG415r for pTH18cr, pTH19cr, pTH18kr and pTH19kr, from pHSG415s for pTH18cs1, pTH19cs1, pTH18ks1 and pTH19ks1, and from pHSG5 for pTH18cs5, pTH19cs5, pTH18ks5 and pTH19ks5. The region covering the *PO*$_{lac}$, MCS and N-terminal coding portion for α-peptide of β-galactosidase (Frag. II) was taken as *Ase*I–*Bgl*I fragments from pKF18c for pTH18kr, pTH18ks1 and pTH18ks5, from pKF19c for pTH19kr, pTH19ks1 and pTH19ks5, from pKF18k for pTH18cr, pTH18cs1 and pTH18cs5, and from pKF19k for pTH19cr, pTH19cs1

Table 1
Plasmids used in this work

Plasmids	Relevant markers	Origin of replication	Sources (Accession Nos[a])
pHSG5	*tet*, $repA_{ts5}$	pSC101	Hashimoto-Gotoh and Sekiguchi (1977)
pHSG367	*bla1*	pMB1	Honkawa et al. (1987)
pHSG415r	*bla*, *agp*, *cat*, $repA_{tr}$	pSC101	Takeshita et al. (1987)
pHSG415s	*bla*, *agp*, *cat*, $repA_{ts1}$	pSC101	Hashimoto-Gotoh et al. (1981)
pHSG576	*cat1*, *lacZ′*, $repA_{tr}$	pSC101	Takeshita et al. (1987)
pHSG1337	*cat1*	pMB1	Hashimoto-Gotoh et al. (1993)
pHSG1339	*agp*	pMB1	Takeshita et al. (1987)
pKF4	*agp6*	pMB1	Hashimoto-Gotoh, (1995)
pKF18c	*cat1*, *lacZ′*	pMB1	Hashimoto-Gotoh et al. (1995a)
pKF19c	*cat1*, *lacZ′*	pMB1	Hashimoto-Gotoh et al. (1995a)
pKF18k	*agp3*, *lacZ′*	pMB1	Hashimoto-Gotoh et al. (1995b)
pKF19k	*agp3*, *lacZ′*	pMB1	Hashimoto-Gotoh et al. (1995b)
pKF299	*agp3*, *lacZ′*	pMB1	Hashimoto-Gotoh et al. (1995b)
pTH18cr	*cat1*, *lacZ′*, $repA_{tr}$	pSC101	This work [AB019609]
pTH18cs1	*cat1*, *lacZ′*, $repA_{ts1}$	pSC101	This work [AB019610]
pTH18cs5	*cat1*, *lacZ′*, $repA_{ts5}$	pSC101	This work [AB019611]
pTH19cr	*cat1*, *lacZ′*, $repA_{tr}$	pSC101	This work [AB019612]
pTH19cs1	*cat1*, *lacZ′*, $repA_{ts1}$	pSC101	This work [AB019613]
pTH19cs5	*cat1*, *lacZ′*, $repA_{ts5}$	pSC101	This work [AB019614]
pTH18kr	*agp5*, *lacZ′*, $repA_{tr}$	pSC101	This work [AB019603]
pTH18ks1	*agp5*, *lacZ′*, $repA_{ts1}$	pSC101	This work [AB019604]
pTH18ks5	*agp5*, *lacZ′*, $repA_{ts5}$	pSC101	This work [AB019605]
pTH19kr	*agp5*, *lacZ′*, $repA_{tr}$	pSC101	This work [AB019606]
pTH19ks1	*agp5*, *lacZ′*, $repA_{ts1}$	pSC101	This work [AB019607]
pTH19ks5	*agp5*, *lacZ′*, $repA_{ts5}$	pSC101	This work [AB019608]
pUC18	*bla*, *lacZ′*	pMB1	Yanisch-Perron et al. (1985)

[a] The entire nucleotide sequence data of plasmids reported in this work will appear in the GSDB, DDBJ, EMBL and NCBI nt sequence databases with the Accession Nos indicated.

and pTH19cs5. For the Cm^R vectors, the remaining part containing the entire *cat* gene (Frag. III) was taken as a *Bgl*I–*Bbe*I fragment from pHSG576, whereas for the Km^R plasmids, the *agp* gene was divided into two portions, one proximal to the C-terminus and the other to the N-terminus (Frags. IV and V, respectively). Frag. IV was taken as a *Bgl*I–*Cfr*10I fragment from pKF299 carrying *agp3*, and Frag. V as a *Cfr*10I–*Dsa*I fragment from pKF4 carrying *agp6*. Then, Frags. IV and V were fused at the *Cfr*10I site, resulting in *agp5*. The *Bbe*I, *Hae*II and *Dsa*I protruding ends were polished with T4 DNA polymerase prior to ligation. These fragments were ligated step by step to create the Cm^R or Km^R, tr, ts-Rep and ts-Seg plasmids.

3.2. Segregation kinetics, copy number determination, and ts mutations

Tucker et al. (1984) have reported that pSC101 plasmids lacking the *par* site containing the three polynucleotide segments ccataaaacgccctg, acgggctttt and ccataaaaggcgcctg, located approximately 230–320 bp downstream from the pSC101 *ori*, behave as so-called *super-par*$^-$ mutants, in which the entire pool of non-replicating plasmid molecules is segregated to daughter cells as a single unit but not randomly. If this is the case, in our ts-Rep plasmids that also lack the three segments, 50% of the daughter cells resulting from the first cell division after the shift-up to non-permissive temperatures must be plasmid-free. Then, we examined the segregation kinetics of pTH18ks1 at 41.5°C in JS295 (*rec*$^+$) cells (Fig. 2). The plasmid copy number per chromosome (P) at 30°C and the relative rate of DNA duplication (k) at non-permissive temperatures were obtained by applying Eqs. (1) and (2) in Section 2.2.

From Fig. 2, one obtains $n=5.0$, $b_{10}=4.10\times10^5$ and $c_{10}=1.46\times10^7$, and then $P=14$ and $k=0$. According to the single segregation unit model, n should be 0, and P must be 1 when $k=0$ (the shaded line in Fig. 2). Therefore, the results did not fit their model but rather support the random distribution model. As we pointed out previously (Hashimoto-Gotoh and Timmis, 1981; Hashimoto-Gotoh and Ishii, 1982), it is important to use *rec*$^+$ host cells to study the plasmid segregation or incompatibility kinetics, because only 50–60% of all the cells in an exponentially growing culture of *recA*$^-$ bacteria are viable. In other words, 10–25% of new born cells cannot divide any further in *recA*$^-$ strain, and thus the generation numbers, calculated on the basis of the doubling time of viable cells, are underestimated. This could explain, at least in part, the discrepancy between their and our observations. Furthermore, according to

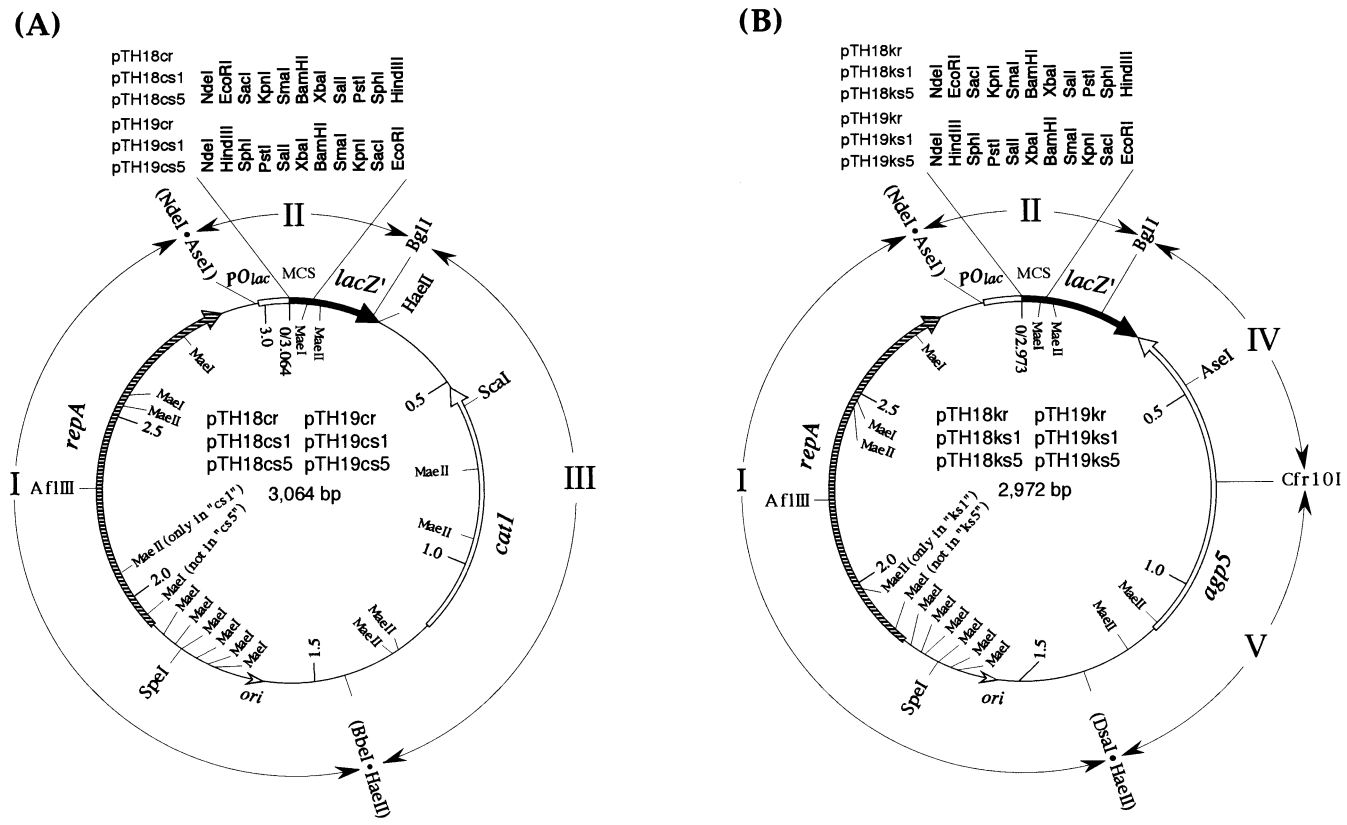

Fig. 1. Restriction maps and construction strategies of (**A**) Cm^R and (**B**) Km^R, tr, ts-Rep and ts-Seg plasmids. MCS are shown on the top of respective maps. Restriction sites flanked by closed arrowheads outside plasmid circles are those used for isolation of Frags. I, II, III, IV and V, among which *Nde*I, *Ase*I, *Bbe*I, *Hae*II and *Dsa*I sites in parentheses were not reconstituted after ligation (see Section 3.1). Restriction sites without parentheses shown outside the plasmid circles cut the molecules only once, and *Mae*I (CTAG) and *Mae*II (ACGT) shown inside plasmid circles differentiate $repA_{ts1}$, $repA_{ts5}$ and repAtr genes. As indicated, *Mae*I cuts the $repA_{ts5}$ twice, but cuts the repAtr and $repA_{ts1}$ three times, whereas *Mae*II cuts the repAtr and $repA_{ts5}$ once, but cuts the $repA_{ts1}$ twice. Symbols: ori (≻) represents the origin and direction of pSC101 DNA replication; closed, hatched and open arrows on plasmid circles indicate the approximate extent and direction of transcription in *lacZ'*, *repA* and (**A**) *cat1* or (**B**) *agp5*, respectively. Open boxes shown by PO_{lac} indicate the lac promoter/operator regions.

Fig. 4 of Tucker et al. (1984), some 10% of PM191 (*recA*$^-$) cells lost pCM302 (ts-Rep and *super-par*$^-$) already within 2 h at 30°C prior to the temperature shift, but the rate of plasmid loss in pCM301 (a *par*$^+$ derivative of pCM302) was faster after the lag period at 42°C than that in pCM302. Therefore, it seems that their data may reflect merely the extraordinary instability of pCM302 in their particular strain, PM191, but may not necessarily support the single unit segregation model. In any case, it is clear from our data that a lack of these three polynucleotide segments at the *par* site is not causative per se for the single unit segregation of non-replicating plasmid copies.

To estimate the copy numbers of other plasmids, we measured relative DNA amounts of tr, ts-Rep and ts-Seg plasmids per culture volume by the alkaline/SDS lysis method. Then, the copy numbers of other plasmids were calculated by assuming the $P=14$ for pTH18ks1 (Table 2). Rather unexpectedly, the copy number of ts-Rep plasmids like pTH18cs1 and pTH18ks1 was increased compared to that of their parental plasmid, pHSG415s, whereas no significant changes of copy number were observed in tr and ts-Seg plasmids. We sequenced the entire pSC101 region (Frag. I) of pTH18kr, pTH18ks1 and pTH18ks5, but found no mutations other than the original mutations such as ^{56}Ala[GCT]→^{56}Val[GTT] in $repA_{ts1}$ and ^{13}Ala-[GCG]→^{13}Thr[ACG] in $repA_{ts5}$. The $repA_{tr}$ was found to be the authentic reversion to the wild type, namely ^{56}Val[GTT]→^{56}Ala[GCT]. Therefore, we concluded that the $repA_{ts1}$ mutation is responsible for this copy number increase, but this is expressive only when the 3′-untranslated region of *repA* from the *Nde*I site (see Section 3.1) is deleted.

3.3. Plating efficiency and segregation frequency

The colony forming ability of cells carrying the newly constructed plasmids was examined in HB101L$^+$ and JM109 at various temperatures (Fig. 3). The relative colony-forming ability was reduced by four orders of magnitude at 41.5°C compared to that at 30 or 37°C in

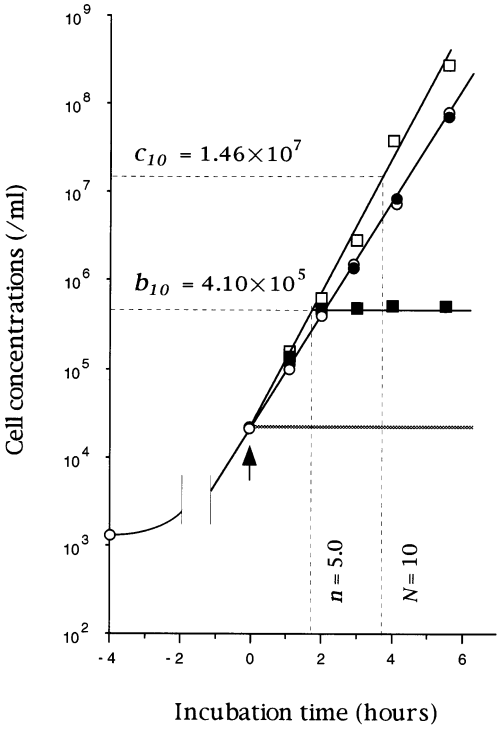

Fig. 2. Growth curves of cells harboring pTH18ks1. JS295 (*rec*$^+$) cells carrying pTH18ks1 were grown in LS medium at 30°C overnight with Km. The overnight culture was diluted 4×10^6-fold in a fresh LS medium without drugs and grown for 4 h at 30°C, then divided into two portions. One was grown further at 30°C and the other at 41.5°C with vigorous aeration. Concentrations of the plasmid-carrying (Km^R) and total (Km^S+Km^R) cells were periodically determined by titrating on LS agar plates with and without kanamycin, respectively. An arrow indicates the time point (0 h) at which the culture was divided into two portions. For *n*, *N*, b_{10} and c_{10}, see Sections 2.2 and 3.2. The generation times at 30 and 41.5°C were 28 and 22 min, respectively. Symbols: □, total cells at 41.5°C; ■, Km^R cells at 41.5°C; ○, total cells at 30°C; ●, Km^R cells at 30°C. A shaded line indicates an expected curve for Km^R cells at 41.5°C according to the single segregation unit model (see Section 3.2).

HB101L$^+$ with ts-Rep plasmids, whereas no essential differences were observed with tr or ts-Seg plasmids among these temperatures. The temperature dependence of this effect was not influenced by the drug-resistance marker used, but influenced by the genetic background of the host cells. Namely, the ts-Rep plasmids did not confer antibiotic resistance to JM109 even at 37°C (Fig. 3). This was also the case in DH5α (data not shown). One of the plausible explanations may be that the *recA* mutations, namely *recA13* in HB101 versus *recA1* in JM109/DH5α, are probably responsible for the different extents of temperature sensitivity.

Incidentally, a 50–60% reduction of the colony forming ability was reproducibly observed in HB101L$^+$ with pUC18 relative to that with its PO_{lac}-less derivative, pHSG367, at 37 and 41.5°C (Fig. 3). This PO_{lac} effect was more severe (>90% reduction) in DH5α having Δ*lacI*, and suppressed in JM109 having *lacI*^{q} (data not shown). Moreover, the colonies of HB101L$^+$ or DH5α cells harboring pUC18 were irregular and small above 37°C compared to those at 30°C. These results indicate that the over-expression from this promoter in pUC18 may be toxic to host cells, implying that a cDNA library using pUC18 in DH5α may not quantitatively reflect the frequency of mRNA molecules in a cell. Interestingly, this effect was not observed with pKF18c and pKF18k in HB101L$^+$ (Fig. 3) and DH5α (data not shown), even though they are also of a high copy number. In this context, it may be worth pointing out the nt sequence differences such as AGGAAACA-GCTATGG (pUC18) versus AGGAAAACATATGG (pKF18c/pKF18k) between the Shine–Dalgano sequences (underlined) and the initiation codon (underlined) for the *lacZ′* gene. Consequently, none of the newly constructed vectors exhibited such a PO_{lac} toxicity, probably because they are of a low copy number and their PO_{lac} was derived from pKF18c/pKF18k. The ts-Seg plasmids in particular may be suitable for constructing random libraries due to their low copy number characteristics, namely one per chromosome.

3.4. Segregation frequency test

Since no apparent differences were observed in the relative colony-forming ability between the tr and ts-Seg

Table 2
Plasmid copy numbers per chromosome at 30°C

Plasmids	Relative DNA amounts[a] (square pixels)	Copy numbers per chromosome[b]	Origins of *repA* gene	Parental copy numbers[c]
pTH18cr	865	5.0	pHSG415r	4∼6
pTH18cs1	2289	13.3	pHSG415s	4∼6
pTH18cs5	173	1.0	pHSG5	1.0
pTH18kr	1021	5.9	pHSG415r	4∼6
pTH18ks1	2410	14.0	pHSG415s	4∼6
pTH18ks5	181	1.1	pHSG5	1.0

[a] Relative amounts of plasmid DNA were quantitated as described in Section 2.3.
[b] The copy numbers were calculated as 14 (underlined) for pTH18ks1 obtained on the basis of the segregation kinetics experiments as shown in Fig. 2 (see also Section 3.2).
[c] The copy numbers for the parental plasmids, pHSG415r, pHSG415s and pHSG5, are based on unpublished data (Hashimoto-Gotoh), Hashimoto-Gotoh et al. (1981), and Hashimoto-Gotoh and Sekiguchi (1977), respectively.

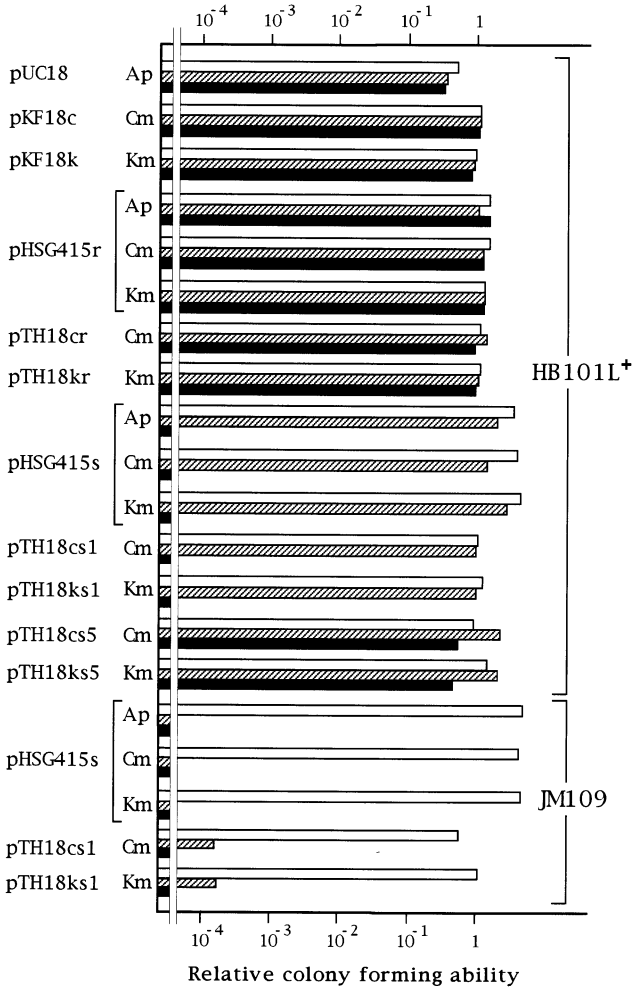

Fig. 3. Relative colony forming ability of cells with PO_{lac}-carrying/-lacking plasmids at 30, 37 and 41.5°C. HB101L$^+$ or JM109 cells carrying various plasmids were cultured in LS medium with Ap, Km and/or Cm overnight at 30°C. The overnight culture was titrated at 30°C (□) 37°C (▨) and 41.5°C (■) on LS agar plates containing Ap, Km or Cm. As controls for PO_{lac}-carrying plasmids, pUC18, pKF18c and pKF18k were used for Ap, Cm and Km selections. Relative colony forming ability was defined as the number of colonies of cells harboring respective plasmids divided by that of cells harboring pHSG367 (Ap^R), pHSG1337 (Cm^R) or pHSG1339 (Km^R), all lacking PO_{lac}, whose cell concentrations were $4.0–5.3 \times 10^9$/ml and $2.7–4.1 \times 10^9$/ml in JM109 and HB101L$^+$ overnight cultures, respectively. The colony size of HB101L$^+$ cells carrying a $repA_{ts5}$ plasmid formed at 37 or 41.5°C was smaller than that at 30°C (1–1.5 mm or 0.5–1.0 mm in diameter, respectively, versus 2–3 mm at 30°C after two nights).

plasmids at 41.5°C (Fig. 3), the segregation frequency was examined semi-quantitatively at various temperatures in HB101L$^+$. As can be seen in Fig. 4, the ts-Seg plasmids (pTH18cs5 and pTH18ks5) were lost at both 37 and 41.5°C, whereas the ts-Rep plasmids (pTH18cs1 and pTH18ks1) were lost only at 41.5°C, and the tr plasmids (pTH18cr and pTH18kr) were stable at all the temperatures.

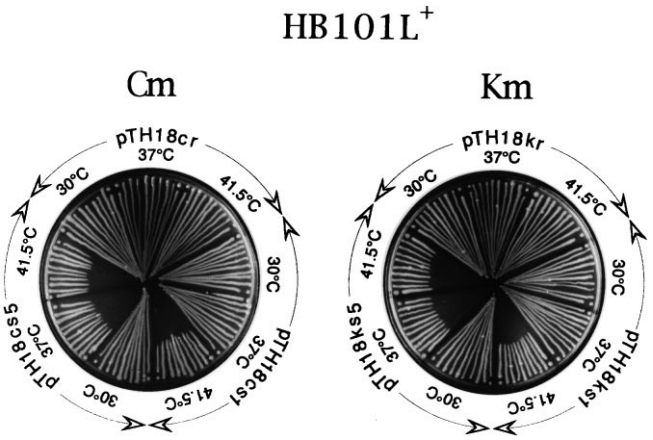

Fig. 4. Segregation tests of tr, ts-Rep and ts-Seg plasmids. HB101L$^+$ cells harboring respective plasmids were picked from colonies formed on LS agar plates containing Cm or Km, and spread for single colony isolation on drug-free LS agar plates in triplicates. The plates were then incubated at 30, 37 or 41.5°C for two nights. Ten single colonies were randomly picked from each plate by toothpicks and streaked on LS agar plates from edge to center, on which 50 μl of Cm (1 mg/ml in 70% ethanol) or Km (1.5 mg/ml in TE) were applied in a 3 cm diameter area at the center with the aid of a turn table.

3.5. Conclusions

1. A set of tr, ts-Rep and ts-Seg plasmid vectors conferring Cm^R or Km^R were developed in an isogenic background, selectable by blue/white colony assay in the presence of IPTG and X-gal.
2. The ts-Rep plasmids exhibited abrupt cessation of their DNA replication at 41.5°C, and the ts-Seg plasmids were lost from daughter cells, even at 37°C, whereas the tr plasmids were stable at these temperatures.
3. The copy numbers of the ts-Rep, tr and ts-Seg plasmid vectors were 14, 5 and 1, respectively, per chromosome at 30°C.
4. The ts-Rep plasmids were more ts in JM109 and DH5α than in HB101L$^+$, probably because they could not replicate even at 37°C in the former strains.
5. The ts-Rep plasmids without the *par* site are distributed in a random fashion to the daughter cells at non-permissive temperatures.

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