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New pSC101-derivative cloning vectors with elevated copy numbers

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Abstract

Mutations that increase the copy number of the pSC101 replicon have been used for construction of new cloning vectors. Replacement of glutamate at position 93 in RepA yields plasmids that replicate at medium (27 copies/cell) and high (~240 copies/cell) copy numbers. Based on the crystal structure of RepE, a structurally similar replication initiator protein from the F factor, the pSC101 *repA* mutants are predicted to be defective in dimerization. The cloning vectors permit increased expression of gene products along with the advantages of pSC101-derivative plasmids, including stable maintenance and compatibility with ColE1 plasmids. The plasmids also allow blue/white screening for DNA inserts and impart resistance to ampicillin, chloramphenicol and kanamycin. The vectors were used in a genetic assay to suppress temperature-sensitive mutants of *ffh*, encoding the protein component of the *E. coli* signal recognition particle, by overproduction of 4.5S RNA. While expression of 4.5S RNA from a wild type pSC101-derivative plasmid was not sufficient for suppression, use of the new vectors did suppress the temperature-sensitive phenotype.

Keywords

copy number mutants; gene expression; plasmid incompatibility

1. Introduction

In recombinant DNA technology, plasmids form the foundation for gene cloning and expression. While plasmid systems are available for a variety of prokaryotic and eukarytotic hosts, most vectors are used in combination with *E. coli* K-12 strains. *E. coli* plasmid vectors have been modified in numerous ways to enable selection and screening for DNA inserts, for regulation of gene expression by inclusion of a variety of promoters, to facilitate protein purification by addition of epitope tags to gene products, and allow the choice of multiple antibiotic resistance markers (Lu, 2004). Despite the large variety of plasmid derivatives, most vectors have been constructed from only a few different replicons. Most plasmids used for recombinant DNA utilize the replication origin from the ColE1-like plasmids pMB1 (including pBR322 and pUC18/19-derivatives) (Hershfield *et al.*, 1974) and p15A (Chang and Cohen, 1978), or pSC101 (Cohen *et al.*, 1973).

In contrast to the anti-sense RNA replication control used by ColE1 plasmids (Cesareni *et al.*, 1991; Polisky, 1988), pSC101 uses the RepA protein to initiate and regulate replication

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(Armstrong *et al.*, 1984) through binding repeated sequences known as iterons at the *ori* (Chattoraj, 2000; Churchward *et al.*, 1983; Vocke and Bastia, 1983). RepA also autoregulates its own expression by binding as a dimer to inverted repeats near the *repA* promoter (Linder *et al.*, 1985). pSC101-derivative plasmids are also stably maintained owing to the presence of the *par* locus (Tucker *et al.*, 1984). pSC101-derivative plasmids replicate at a relatively low copy number (<8 copies/cell) (Cabello *et al.*, 1976; Hasunuma and Sekiguchi, 1977) and numerous cloning vectors based on this replicon have been reported (Hashimoto-Gotoh *et al.*, 2000; Hasnain and Thomas, 1986; Hoang *et al.*, 1999; Lerner and Inouye, 1990; Phillips, 1999; Stoker *et al.*, 1982; Takeshita *et al.*, 1987; Wang and Kushner, 1991).

Another useful property of the pSC101 replicon is that it is compatible with ColE1 plasmids, allowing multiple vectors to be propagated in the same cell. Although there frequently is a need to introduce multiple plasmids to the same cell, the options can be limited. In addition to pSC101 plasmids, different ColE1 replicons can also be compatible with one another, such as pMB1 and p15A-derivative plasmids. However, like pSC101, p15A vectors, including pACYC177 and pACYC184, are relatively low copy number plasmids (Hiszczynska-Sawicka and Kur, 1997), which can limit their utility. Cloning vectors based on other ColE1-like plasmids have been reported, but they replicate at either relatively low or very high copy numbers (Phillips *et al.*, 2000). Despite these options for constructing strains of *E. coli* and other closely related bacteria transformed with multiple plasmids, we sought additional, well-characterized plasmids that can replicate at multiple copy numbers (low, medium and high) that are also compatible with all ColE1 vectors.

To characterize pSC101 replication, mutations that increase copy number have been found. These mutations, in general, resulted from changes at several amino acid positions in the RepA protein (Furuno *et al.*, 2000; Xia *et al.*, 1993; Xia *et al.*, 1991). Although these mutations have proven useful for understanding plasmid replication control, they have not been developed for use in recombinant DNA. We, therefore, used a *repA* mutant altered at glutamate (Glu) 93, which results in a 4-5 fold increase in copy number (Xia *et al.*, 1991), to construct a new series of cloning vectors that replicate at various copies/cell and that also offer the advantages of the well-characterized pSC101 replicon. We further reasoned that additional amino acid substitutions of Glu 93 might further disrupt the normal regulation of replication initiation by RepA yielding new classes of copy number mutants. Indeed, through this strategy we isolated a new pSC101 replicon mutant that replicates at high copy number (~240 copies/cell) and also incorporated it into cloning vectors. We demonstrated the usefulness of these plasmids in an assay of genetic suppression of signal recognition particle mutants in *E. coli*. These vectors should be useful for any number of studies where stably maintained ColE1-compatible pSC101-derivative plasmids are required at elevated copy numbers.

2. Materials and methods

2.1. Generation of copy number mutants by site-directed mutagenesis

The original *cop* mutation isolated by Xia *et al.* (1991) along with four additional mutations that changed the Glu at position 93 of the RepA protein were generated by PCR mutagenesis. To facilitate construction of the different copy number mutants, a *Bgl*II restriction site was first introduced to *repA* corresponding to residue 93 in the pSC101-derivative plasmid pCL21 (Lerner and Inouye, 1990).

Primers *repABgl*IIS: 5′-AC*AGATCT*TCCAGTGGA CAAACTATGCCAAGTTC-3′ and *repABgl*IIAS: 5′-TGAGGA*AGATCT*CAAAGCCTTTAACCAAAGGATTCCTG-3′ (the *Bgl*II site is italicized) were used in an inverse PCR reaction (Stemmer and Morris, 1992) using the reaction conditions: 95°C for 1 m; 95°C for 30 s, 55°C for 30 s and 72°C for 2 m for 25 cycles; 72°C for 10 m. PCR products of the appropriate size (~4.5 kb) were agarose gel purified

and treated with *Dpn*I at 37°C for 1 h. before digestion with *Bgl*II and self-ligation. The ligation reactions were used to transform DH5α and the resulting plasmids were purified using a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) and confirmed by DNA sequencing. The resulting construct was named pCL21-*Bgl*II and used in subsequent PCR mutagenesis reactions.

PCR was used to generate DNA fragments containing specific alterations that were used to replace the *Bgl*II-*Eag*I fragment from pCL21-*Bgl*II. Using pCL21-*Bgl*II as a template, PCR reactions using the conditions: 95°C for 1 m; 95°C for 30 s, 55°C for 30 s and 72°C for 1 m for 30 cycles; 72°C for 5 m. The sense primer EagI-S, 5′-CT*CGGCCG*TCGCGGCGC-3′ (*Eag*I site is italicized), was used in combination with the following anti-sense primers containing a *Bgl*II or *Bam*HI (italicized) restriction site and the desired mutation (underlined):

*Bgl*II-E93K: 5′-CTGGA*AGATCT*T<u></u>AAAGCCTTTAACCAAAGGATTCCTG-3′;

*Bgl*II-E93N: 5′-CTGGA*AGATCT*G<u></u>AAAGCCTTTAACCAAAGGATTCCTG-3′; *Bam*HI-E93R:

5′-CT*GGAGGATCC*G<u></u>AAAGCCTTTAACCAAAGGATTCCTG-3′; *Bam*HI-E93W: 5′-

CT*GGAGGATCC*A<u></u>AAAGCCTTTAACCAAAGGATTCCTG-3′; *Bam*HI-E93G: 5′-

CT*GGAGGATCC*C<u></u>AAAGCCTTTAACCAAAGGATTCCTG-3′.

Following PCR, 1.3-kb fragments were gel purified from each reaction, digested with *Eag*I and *Bgl*II or *Bam*HI and ligated with pCL21-*Bgl*II digested with *Eag*I and *Bgl*II. Plasmid DNA was isolated from transformants of each ligation reaction to determine the relative yield of each construct. Mutations were confirmed by DNA sequencing.

Two mutations (E93K and E93R) were used to construct new cloning vectors by cloning a ~1.1-kb *Nde*I to *Spe*I fragment into the pSC101-derivative plasmid pWSK29 (Wang and Kushner, 1991) to generate pJPA12 and pJPA13 (Fig 1). *Bsp*HI fragments encoding resistance to chloramphenicol (Cam^R) and kanamcyin (Kan^R) were isolated from pDHC29 and pDHK29 (Phillips *et al.*, 2000), respectively, and used to replace *bla*, encoding ampicillin resistance (Amp^R) from pJPA12 and pJPA13, generating pJPC12 and pJPK13 (Fig 1).

2.2. Copy number determination

The copy number of each of the copy number mutants was performed using qPCR similar to a method described previously (Whelan *et al.*, 2003). Two genes, the plasmid encoded *bla* and *ffh*, located on the *E. coli* chromosome, were selected as targets for qPCR analysis. Copy number was determined by first measuring the absolute amounts of plasmid DNA by comparison with a standard curve. Next, the relative amounts of plasmid DNA were reported as a ratio of plasmid copies/chromosome.

A standard curve was generated to establish the relationship of the threshold cycle values (C_T) with the absolute copy number of the target sequences. The plasmid pBAD-*ffh* (lab collection), a 5.5-kb, pBR322-derivative plasmid carrying both target genes (*bla* and *ffh*), was used to generate the standard curve. Serial dilutions of a known quantity of pBAD-*ffh* were subjected to qPCR using a BioRad MyiQ single color detection system (Bio-Rad, Hercules, CA). Reactions were performed using the DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes, Woburn MA) using the following conditions: 95°C for 10 s, 62°C for 10 s and 72°C for 10 s, for 40 cycles. The primers used for probing *bla* on the plasmids were:

*blaQ*f : 5′CTACGATACGGGAGGGCTTA-3′; and

*blaQ*r : 5′-ATAAATCTGGAGCCGGTGAG-3′ (Lee *et al.*, 2006). The primers used for probing

ffh were: *ffh*-S : 5′-TAAACTCGGTAAGTTCCTGCGCGA-3′; and *ffh*-AS : 5′-AGCGCCGCGTTAA CAATATCAACC-3′.

pBAD-*ffh* was purified and its concentration measured using a NanoDrop ND-1000 Spectrophotometer. The concentrations were used to calculate the absolute number of copies of plasmid in the sample using the equation (Whelan *et al.*, 2003):

$$\text{Copies of plasmid} = [6.02 \times 10^{23}\ (\text{copy/mol}) \times \text{amount (g)}] / [\text{length (bp)} \times 660\ (\text{g/mol/bp})]$$

Plasmid DNA was serially diluted from 7.4×10^5 to 7.4×10^9 molecules/μl and the qPCR reactions were performed in triplicate. The C_T values were calculated using the BioRad MyiQ software version 3.032 and were plotted against the log of the calculated total copies of plasmid in each sample (Fig 2). From these standard curves, the efficiency for each target (E in Fig 2) was calculated by the MyiQ software and was found to be near ideal.

To use the standard curve for copy number determination, each pSC101-derivative plasmid, as well as pBR322, was introduced to DH5α and transformants were grown to mid-log phase. Total DNA was extracted using a MasterPure Complete DNA & RNA Purification kit (Epicentre, Madison WI). The samples were then run in a standard qPCR reaction in triplicate probing separately for both *bla* (plasmid encoded) and *ffh* (chromosomal encoded) to determine C_T values.

All C_T values were then converted to total copy number using the respective standard curve for each probe. The plasmid copy number per chromosome was then determined as the ratio of the copy number of *bla*, representing the total copy number of plasmids, to the copy number of *ffh*, representing the total copy number of the chromosome. The procedure was repeated with DNA extracted from two independent samples.

2.3. Determination of plasmid stability and compatibility

A control plasmid was constructed by replacing the *bla* gene from pWSK29 with a *Bsp*HI fragment encoding *cam* from pDHC30 (Phillips *et al.*, 2000). To test plasmid stability, pJPC11, pJPC12 and pWSK29-*cam* were used to transform NEB5α and the cells grown in LB overnight in the absence of antibiotic selection. One overnight culture was considered the result of 10 generations of growth. Each overnight culture was diluted by 1000 (5 μl into 5 ml) and grown again for a total of 80 generations. Cultures were diluted after every 20 generations of growth and plated onto LB plates both with and without Cam. Colonies were counted after an overnight incubation. Stability was reported as the number of colonies growing on the antibiotic plates/ the total number of colonies on the non-selective plates.

Compatibility with ColE1 replicon plasmids was tested by transforming pJPC12, pJPC13 and pWSK29-*cam* into DH5α containing pBR322. The strains were grown for multiple generations, as described above. For this study, Amp was included in the growth medium to select for pBR322. Cultures were diluted after every 20 generations of growth and plated onto LB + Amp plates both with and without Cam. Plasmid compatibility was reported as the number of colonies growing on the Amp + Cam plates/the total number of colonies on the Amp plates.

2.4. Suppression of temperature sensitive *Ffh*

To test for suppression of the temperature sensitivity of an *ffh* mutant, plasmids pWSK30*ffs*, pJPA12*ffs* and pJPA13*ffs* were constructed by cloning a 0.6–kb *Eco*RI-*Bam*HI fragment from pSB832 (Park *et al.*, 2002) into each plasmid digested with the same enzymes. Plasmids were used to transform the strains SKP1101 (ffh^{TS}) and SKP1102 (ffh^{+}) (Park *et al.*, 2002) and the reactions were plated at 30°C and 42°C overnight. After incubation for 24 hours, colonies were counted. Suppression of the temperature sensitivity of SKP1101 was calculated by dividing

the number of colonies on the 42°C plates by the number on the 30°C plates. Also, colonies were streaked from the 30°C plates at 30°C and 42°C to confirm suppression of the TS phenotype.

3. Results and discussion

3.1. Isolation of copy number mutants

Previous studies revealed that a change of Glu to Lys in the RepA initiator protein resulted in a 4-5 fold increase in plasmid copy number of pSC101 (Xia *et al.*, 1991). We reasoned that replacement of Glu93 with other amino acids could result in further changes to the copy number of the pSC101 replicon and that these new *repA* mutations could be used to construct useful cloning vectors. We used PCR mutagenesis to generate multiple *repA* mutants by changing the triplet GAG (encoding Glu93) to CGG (Arg), AAG (Lys), TGG (Trp), CAG (Gln) and GGG (Gly). These amino acids represent a diversity of side chain sizes and are encoded by bases that are compatible with the *Bgl*II restriction site engineered into *repA* of pCL21-*Bgl*II (Table 1). Mutants were constructed as described in *Materials and methods* and all resulted in elevated plasmid DNA yields in comparison to the wild-type *repA* parent, pCL21-*Bgl*II (data not shown).

Quantification of the plasmid DNA from the different *repA* mutants revealed two classes of high copy number mutants. As reported, *repAE93K* yielded a modest increase in copy number (Xia *et al.*, 1991) (Table 1). We observed a similar increase in plasmid copy number with the *rep*AE93G mutation. When the *repAE93R*, *repAE93W* or *repAE93N* mutants were characterized, even higher plasmid DNA yields were observed. We chose to continue to further characterize the *repAE93K* and *repAE93R* variants, which represented both classes of increased copy number mutants (Table 2).

3.2. Plasmid copy number determination

Quantitative PCR (qPCR) was used to measure the copy number of the *rep*AE93K and *rep*AE93R high copy mutants, using a method reported by Whelan *et al.* (Whelan *et al.*, 2003). To enable the use of primers specific for the plasmid encoded *bla* gene (Whelan *et al.*, 2003), we cloned the two *repA* mutant alleles into pWSK29, a low copy number, pSC101-derivative plasmid (Wang and Kushner, 1991), as described in Materials and methods.

A standard curve was first generated to determine the relationship between threshold cycle value (C_T) and copy number of *bla* and *ffh* carried on the same recombinant plasmid. As shown in Fig 2, the standard curves for both sequences were generated with near ideal efficiency with values of 95% and 94.4% respectively. Efficiency refers to the increase of signal per cycle at the signal threshold used to calculate the C_T values. An ideal efficiency is achieved when all of the double stranded templates are denatured, each strand anneals to a primer, and the polymerase replicates the each template. Under these ideal conditions the amount of signal should double with each cycle of the PCR or have an efficiency (E) of 1.0. Also, the standard curves showed consistency over the range of dilutions, with a linear regression of greater than 0.99. These results indicated that the standard curve could be used to obtain an accurate determination of plasmid copy number.

The copy number of each pSC101-derivtive plasmid and a control plasmid was determined by first using qPCR to measure the C_T values of both *bla* and *ffh* from samples of total DNA isolated directly from *E. coli* transformants. The absolute copy number was then found for each target gene from the standard curve. The results are reported as plasmid copy number per chromosome, since a single copy of *bla* is on the plasmid and a single copy of *ffh* is on the chromosome.

The copy number measurements are summarized in Table 2. As a control for the qPCR method, we also measured the copy number of the ColE1 plasmid pBR322 and of the pSC101-derivative pWSK29. The qPCR method yielded a copy number of 16.5 plasmids/chromosome for pBR322, consistent with a range of 15-20 plasmids per chromosome (Lee *et al.*, 2006;Lin-Chao and Bremer, 1986). In addition, pWSK29 was found to have a copy number of 6.7 plasmids/chromosome, closely matching previous measurements for pSC101 of ~6 plasmids/chromosome (Cabello *et al.*, 1976;Hasunuma and Sekiguchi, 1977). Measurements of the high copy number mutants showed the *repAE93K* mutation resulted in a copy number of 27 plasmids/chromosome. We categorized this mutant as a medium copy number plasmid. This value is consistent with the published report the E93K substitution increased the copy number of pSC101 4-5 fold (Xia *et al.*, 1991). In contrast, the E93R replacement yielded a plasmid that replicated to 237 copies/chromosome and was designated as a high copy number plasmid.

3.3 Model for copy number control in repA mutants

Since the crystal structure of RepA is not available, we used the three-dimensional structure of the highly similar RepE initiator protein from the F factor to understand how substitutions at E93 resulted in elevated plasmid high copy number. In addition to being similar to pSC101 RepA, RepE is highly similar to initiator proteins from other replicons, including plasmids R6K, pCU01, pPS10 and bacteriophage P1 (Sharma *et al.*, 2004). For example, all of the proteins contact independent half sites of single iterons by specific α-helices and use a series of β-sheet structures near the amino terminus for dimerization (Sharma *et al.*, 2004). Fig 3 shows that the Glu side chain from RepE, which is in the identical location in RepA, is located in one of these β-sheets comprising the dimerization domain (β2b in Fig 3).

Initial characterization of the high copy number mutant E93K originally isolated by Xia, *et al.* (1991) concluded that the mutant RepA protein was found at a higher protein concentration and had an increased affinity for repeated sequences located at replication origin (Xia *et al.*, 1993). Given the understanding of pSC101 replication control (Chattoraj, 2000), it is likely that mutations altering Glu93 would reduce the efficiency of initiator protein to form dimers. Since RepA autoregulates its own synthesis by preferentially binding to sites near the *repA* promoter as a dimer, *repAE93* mutants express higher levels of the monomeric form of the protein that are more active in initiating replication.

In isolating the original *repAE93K* mutant, Xia *et al.* (1991)(Xia *et al.*, 1991) selected a spontaneous mutant that was able to grow on elevated levels of antibiotic. This copy number mutant resulted from a commonly found G to A transition (Glu_{GAG} to Lys_{AAG}). The *repAE93R* mutation, however, results from two base pair changes, including a rarer G to C tranversion (Glu_{GAG} to Arg_{CGG}). Likewise, the other high copy mutants described in this study would have been difficult to isolate by conventional random mutagenesis since they require either a double mutation (Glu_{GAG} to Trp_{TGG}) or a single transversion (Glu_{GAG} to Gln_{CAG}).

3.4. Plasmid stability and compatibility

To determine if the increased copy number plasmids retain the stability properties of pSC101, we measured the frequency of loss of each plasmid after growth for multiple generations in the absence of antibiotic selection. As shown in Table 3, essentially 100% of the cells transformed with the low (pWSK29) or medium- (pJPA12) copy number plasmids retained the plasmids even after 80 generations without Amp. The high copy number plasmid pJPA13 exhibited decreased stability, however, with nearly half of the cells having lost the plasmid after 60 generations without selection. Nearly all of the cells retained the plasmid after 20 generations, however. The decreased stability of JP13 could be due to a combination of the increased metabolic load placed on *E. coli* imposed by the high copy number plasmid and

disrupted dispersion of plasmid clusters in the cell (Weitao *et al.*, 2000). Continual selective pressure by antibiotics is necessary to ensure this replicon is maintained in the culture.

To determine if the high copy number plasmid maintain their compatibility with ColE1 plasmids, *E. coli* was transformed with pBR322 (ColE1 replicon) and pJPC12, pJPC13, or pWSK29-*cam*, a Cam^R derivative of pWSK29 (Wang and Kushner, 1991) constructed for this study. Cells were grown in the presence of Amp to select for pBR322 for up to 80 generations. Following growth, loss of each plasmid was determined by testing sensitivity to Amp (pBR322) or Cam. As shown in Table 4, pJPC12 and the control pWSK29-*cam* were fully maintained, even after 80 generations, while increased loss of pJPC13 was observed. Since the pJPC13 was lost from the culture at a similar rate as shown in Table 3, we conclude that plasmid loss was due to increased instability of the high copy number plasmid and not altered incompatibility with ColE1 plasmids.

3.5. Construction of new cloning vectors

The mutant *repA* alleles were used for construction of new cloning vectors (Fig 1). As described in Materials and methods, derivatives of the Amp^R plasmids pJPA12 (medium copy) and pJPA13 (high copy) were constructed. The resulting plasmids impart resistance to Cam^R (pJPC12/13) and Kan^R (pJPK12/13). The plasmids allow inserts to be identified by blue/white screening on 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). They further offer the high stability and ColE1 compatibility characteristic of pSC101 plasmids.

3.6. Use of new elevated copy number cloning vectors for suppression analysis

Previous studies have shown that the temperature sensitivity of the *ffh* mutant SKP1101 can be suppressed by overproduction of 4.5S RNA, the interactive partner of Ffh in forming the signal recognition particle (Park *et al.*, 2002). To better understand the SRP in *E. coli*, we were in need of a ColE1-compatible plasmid that would express 4.5S RNA in sufficient levels to suppress the *ffh*Ts mutant. Previous attempts to use wild type pSC101 plasmids for suppression failed due to the insufficient levels of 4.5S RNA.

To test the high copy pSC101-derivative plasmids we inserted *ffs*, the structural gene for 4.5S RNA, into pWSK30, pJPA12 and pJPA13. The plasmids were used to transform SKP1101 and the ffh^+ control strain SKP1102 (Park *et al.*, 2002) and suppression was measured by comparing the efficiency of plating at 30°C and the non-permissive temperature of 42°C. The results are summarized in Table 5. All of the copy number mutants suppressed the temperature-sensitive phenotype (plating efficiencies were greater than 0.88). Consistent with our previous results, pWSK30*ffs* failed to suppress the temperature-sensitivity of SKP1101 (plating efficiency of less than 0.01). There was no significant difference in plating efficiencies between pJPA12 and pJPA13-derivative plasmids, as shown by the SKP1102 (ffh^+) transformants (Table 5). The pJPA12 and 13-derivative plasmids expressing 4.5S RNA will be useful to study the interaction between RNA and the Ffh protein.

In summary, new cloning vectors based on elevated copy number mutants of the pSC101 replicon have been constructed. These plasmids will facilitate expression studies where ColE1-compatible plasmids that replicate at medium and high copy numbers are required. These new vectors offer the advantage of pSC101 replicon plasmids, while overcoming the limitations of relatively low copy number plasmids.

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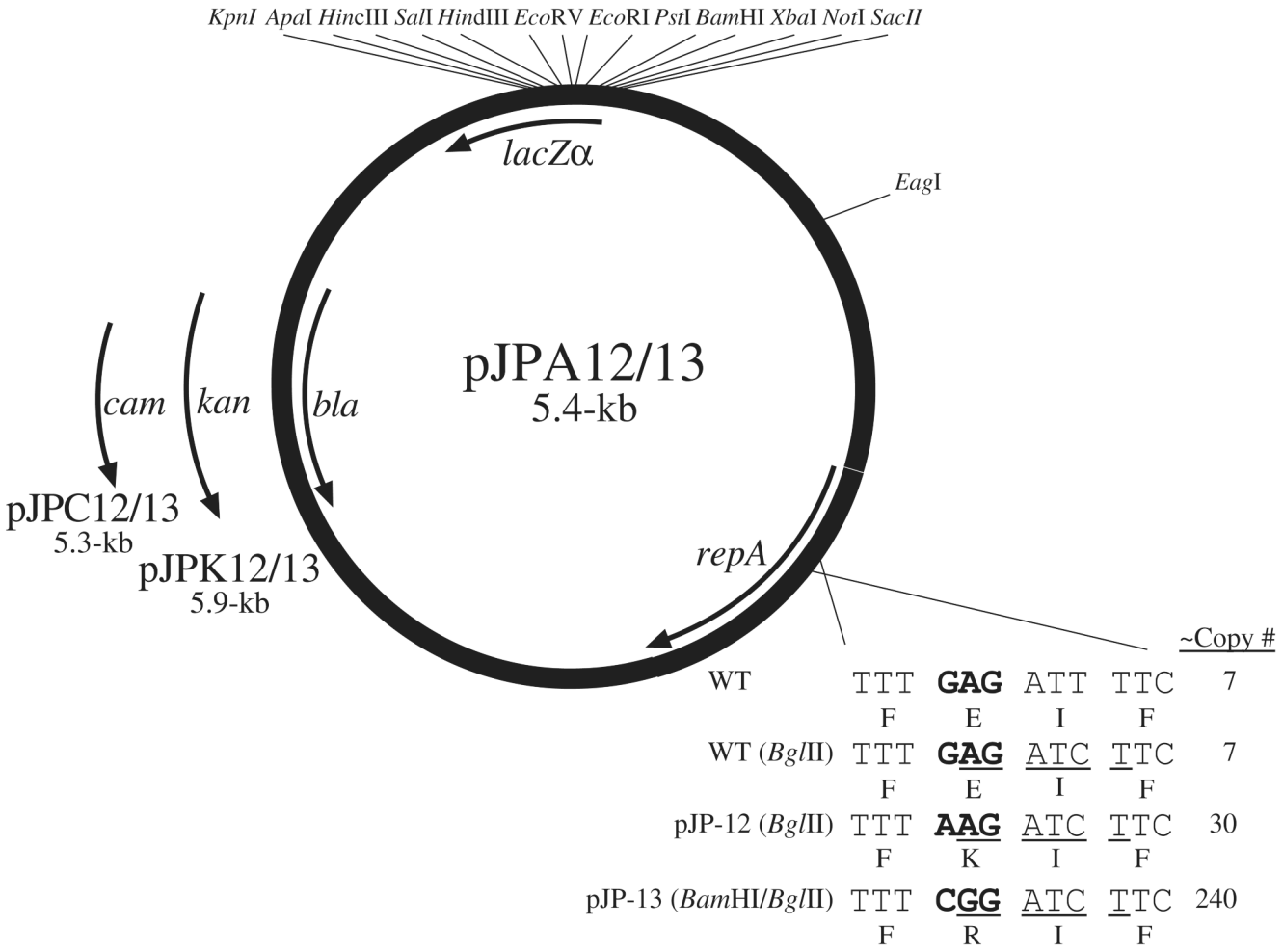

Fig. 1.

New cloning vectors constructed from pSC101 *repA* elevated copy number mutants. Shown are unique restriction enzyme recognition sites; *lacZα*, encoding the α peptide from β-galactosidase; *bla* (Amp^R); *kan* (Kan^R); and *cam*, (Cam^R). The sizes of each plasmid in kb are also shown. The expanded region below *repA* shows the relevant sequences of the gene and product from wild type pSC101 along with the *repA* (*Bgl*II) mutation. Also shown are the two high copy number mutations. The restriction sites used to construct the vectors are underlined and the sequences encoding amino acid position 93 are shown in bold. The copy number of each replicon is also shown. The position of the *Eag*I site used in constructing the vectors is shown, however, this site is not unique.

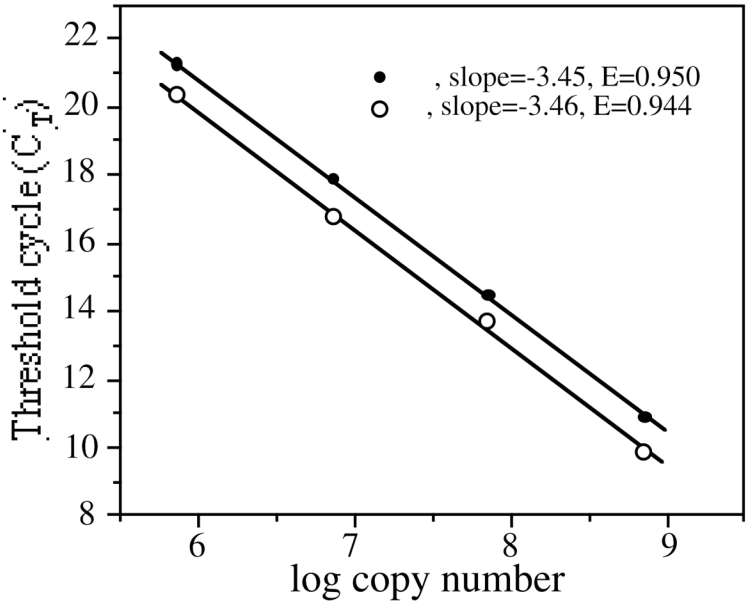

Fig. 2.

Standard curves for *bla* and *ffh*. The curves were constructed by serially diluting a sample of pBAD-*ffh*, as described in Materials and methods. Each dilution was analyzed by qPCR probing for both *bla* and *ffh*. The threshold cycle values (C_T), defined as the cycle number where exponential increase of PCR product ends, for each sample were plotted against the log of the copy number. E represents the efficiency of the PCR reaction corresponding to the number of cycles needed for duplication of signal and was calculated by the MyiQ software.

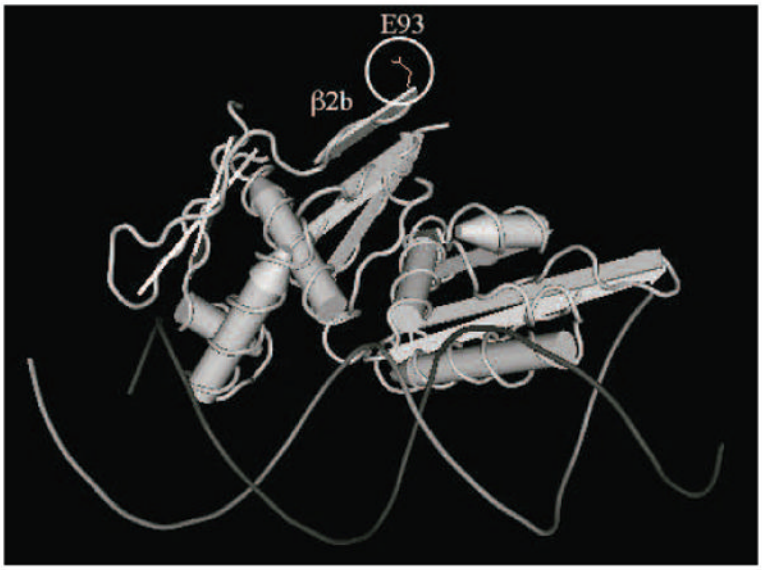

Fig. 3.

Structure of RepE initiator protein from F factor, a protein structurally similar to RepA of pSC101. The Glu at position 93 (E93) of RepE is identical to RepA. The side chain of E93 is circled and is located in β2b, a β-sheet important for protein dimerization. The structure of RepE (Diaz-Lopez *et al.*, 2003) was obtained from PDB file 1REP and imaged with the program Cn3D. Helical regions are represented as cylinders and β-sheets as ribbons. The initiator protein is shown contacting DNA.

Table 1

The plasmids and strains constructed and used in this work

Plasmid or *E. coli* Strain		Relevant Genotype	Source or Reference
Plasmids			
	pWSK30	*repA*$^+$	(Wang and Kushner, 1991)
	pCL21	*repA*$^+$	(Lerner and Inouye, 1990)
	pCL21-*Bgl*II	*repA-Bgl*II (silent mutation)	This work
	pBR322	*bla*	Lab collection
	pBADffh	*bla*, *ffh*	Lab collection
	pJPA12	*repAE93K*, *bla*	This work
	pJPA13	*repAE93R*, *bla*	This work
	pJPC12	*repAE93K*, *cam*	This work
	pJPC13	*repAE93R*, *cam*	This work
	pJPK12	*repAE93K*, *kan*	This work
	pJPK13	*repAE93R*, *kan*	This work
	pWSK*ffs*	*repA*$^+$*ffs+*	This work
	pJPA11*ffs*	*repA-62*, *ffs+ bla*	This work
	pJPA12*ffs*	*repA*E93K, *ffs+ bla*	This work
	pJPA13*ffs*	*repA*E93R, *ffs+ bla*	This work
Strains			
	NEB5α	*fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* (general cloning host)	New England Biolabs
	SKP1101	*ffh*^{TS}, *cam*	(Park *et al.*, 2002)
	SKP1102	*ffh*$^+$, *cam*	(Park *et al.*, 2002)

Table 2

Copy numbers of plasmids as determined by qPCR

Plasmid	*repA* Genotype	Copy Number*
pWSK30	*repA*$^+$	6.7±0.2
pJPA12	*repAE93K*	27.0±1.5
pJPA13	*repAE93R*	232.7±9.8
pBR322	NA	16.5±0.6

*Copy numbers were calculated as described in Materials and methods. NA, not applicable.

Table 3

Stability of pSC101 vectors in the absence of antibiotic selection

Plasmid	Plasmid stability*				
	Number of generations				
	1	20	40	60	80
pWSK30	1.0	0.97	1.0	1.0	1.0
pJPA12	1.0	1.0	1.0	1.0	1.0
pJPA13	1.0	0.97	0.63	0.45	0.45

*Plasmid stability reported as number of colonies appearing on LB + Amp/number of colonies on LB alone after growth for the indicated number of generations without antibiotic.

Table 4

Compatibility of high copy pSC101 vectors with the ColE1 replicon pBR322

Plasmid	Plasmid stability*				
	Number of generations				
	1	20	40	60	80
pWSK29-*cam*	1.0	0.98	0.98	0.97	0.89
pJPC12	1.0	1.0	0.94	0.93	0.91
pJPC13	1.0	0.74	0.68	0.65	0.56

*Plasmid stability reported as number of colonies appearing on LB + Cam/ number of colonies on LB + Amp (pBR322 selection) after growth for the indicated number of generations without antibiotics.

Table 5

Efficiency of plating of S1101 and S1102 transformed with the differing copy number plasmids carrying *ffs*

Plasmid	SKP1101 (*ffh*^{ts})	SKP1102 (*ffh*$^{+}$)
pWSK*ffs*	<0.03±0.02	1.21±0.15
pJPA12*ffs*	0.89±0.08	0.95±0.14
pJPA13*ffs*	0.89±0.07	0.85±0.05
pJPA13	<0.01±0.01	0.94±0.03

The table shows the efficiency of plating shown as number of colony forming units at 42°C/number of colony forming units at 30°.