Monomer–dimer control of the ColE1 P_{cer} promoter

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XerCD-mediated recombination at *cer* converts multimers of plasmid ColE1 to monomers, maximizing the number of independently segregating molecules and minimizing the frequency of plasmid loss. In addition to XerCD, recombination requires the accessory factors ArgR and PepA. The promoter P_{cer} , located centrally within *cer*, is also required for stable plasmid maintenance. P_{cer} is active in plasmid multimers and directs transcription of a short RNA, Rcd, which appears to inhibit cell division. It has been proposed that Rcd is part of a checkpoint which ensures that multimer resolution is complete before the cell divides. This study has shown that ArgR does not act as a transcriptional repressor of P_{cer} in plasmid monomers. P_{cer} is unusual in that the –35 and –10 hexamers are separated by only 15 bp and this study has demonstrated that increasing this to a more conventional spacing results in elevated activity. An increase to 17 bp resulted in a 10- to 20-fold increase in activity, while smaller effects were seen when the spacer was increased to 16 bp or 18 bp. These observations are consistent with the hypothesis that P_{cer} activation involves realignment of the –35 and –10 sequences within a recombinational synaptic complex. This predicts that a 17 bp spacer promoter derivative should be down-regulated by plasmid multimerization, and this is confirmed experimentally.

Keywords: dimer resolution, *cer*–Xer recombination system, cell-cycle checkpoint, Rcd, plasmid stability

INTRODUCTION

Stable maintenance of bacterial plasmids requires efficient replication control and distribution to daughter cells. High-copy-number plasmids are distributed randomly at cell division and the probability of forming a plasmid-free daughter (p_0) is given by $p_0 = 2^{(1-n)}$, where n is the plasmid copy number of the dividing cell (Summers, 1991). Any factor which reduces the plasmid copy number increases the frequency of plasmid loss. Multimer formation by homologous recombination is an important cause of multicopy plasmid instability as multimer-containing cells contain fewer independent plasmids. Furthermore, dimers replicate at twice the rate of monomers and accumulate rapidly in the descendants of the cell in which they first appeared. This generates a sub-population of dimer-only cells from

which plasmid-free cells arise at high frequency (Summers *et al.*, 1993). The multicopy plasmid ColE1 counters the effects of multimerization through the activity of the *cer*–Xer recombination system (Summers & Sherratt, 1984). Recombination between *cer* sites in a plasmid multimer regenerates monomers and restores the normal copy number. Recombination at *cer* requires four chromosome-encoded proteins: ArgR (Stirling *et al.*, 1988a, b), PepA (Stirling *et al.*, 1989), XerC (Colloms *et al.*, 1990) and XerD (Blakely *et al.*, 1993).

cer-mediated dimer resolution is necessary, but not sufficient, for the stable maintenance of ColE1 and related plasmids. A mutation which inactivates the promoter, P_{cer} , within the *cer* site reduces plasmid stability despite having no detectable effect on plasmid dimer resolution (Summers & Sherratt, 1988; Patient & Summers, 1993). P_{cer} directs the synthesis of an approximately 75 nt transcript, Rcd, whose predicted secondary structure is reminiscent of small antisense RNAs involved in the control of plasmid replication and transposition (Sharpe *et al.*, 1999). The level of Rcd is

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Abbreviation: GalK, galactokinase.

Fig. 1. Sequences of P_{cer} and two mutant promoters. The -35 and -10 hexamers are underlined and the ArgR binding site in P_{cer} is shown by a box. The insertion site used to generate promoters with increased spacer length is indicated by a solid arrow. The nucleotide deleted to generate the 14 bp spacer mutant is indicated by a broken arrow. The putative alternative -35 hexamers for $P_{cer\Delta T}$ and the up-promoter mutant P_{cer}^+ (Patient & Summers, 1993) are shown by broken boxes. The *Thal* site used for subcloning the promoter fragments is indicated, as are the transcription start sites (*).

elevated in multimer-containing cells and it has been proposed that the transcript is part of a cell-cycle checkpoint which blocks division until multimer resolution is complete (Patient & Summers, 1993). An unusual feature of Rcd action is that growth and division appear to be stopped in a co-ordinated manner since cells recovered from solid medium after Rcd over-expression show cell-cycle arrest just before septation but do not filament (Patient & Summers, 1993). In broth culture septation is blocked by Rcd induction and moderate filamentation is apparent, with cells increasing to approximately three times their normal length (Rowe & Summers, 1999).

One of the most intriguing aspects of the Rcd checkpoint hypothesis is the activation of P_{cer} by plasmid multimerization. How might this be achieved? The separation between the -10 and -35 hexamers in P_{cer} is only 15 bp, compared to the consensus spacing for an *Escherichia coli* promoter of 17 ± 1 bp (Hawley & McClure, 1983; Harley & Reynolds, 1987). We have speculated that distortion of the promoter in a *cer-cer* synaptic complex might align the -10 and -35 regions for recognition by RNA polymerase (Summers, 1998). We refer to this as the 'local twisting hypothesis', although the distortion may conceivably include elements of both twist and writhe in the strict topological sense. Since recombination (and therefore synaptic complex formation) is limited to *cer* sites on the same molecule, this would ensure that Rcd was only made in multimer-containing cells. Support for the idea that P_{cer} can be activated by realignment of its -10 and -35 sequences came from the analysis of P_{cer}^+ , an up-promoter derivative of P_{cer} in which the -35 hexamer is replaced by a new sequence at a more conventional spacing from the -10 (Patient & Summers, 1993; Fig. 1). This derivative promoter is active even in the absence of multimers, suggesting that the short spacer is crucial to proper monomer-dimer control at P_{cer} .

In this paper we describe an investigation of factors which influence the activity of P_{cer} . The results lend support to the idea that realignment of the -10 and

-35 regions is indeed crucial to P_{cer} activation, and the mechanism by which this may be achieved in plasmid multimers is discussed.

METHODS

Bacteria and plasmids. Most strains used in this work were derivatives of *Escherichia coli* DS941 (AB1157 *recF lacI^a lacZ* Δ M15). DS941 *argR* and DS941 *xerC* were deficient in *cer*-mediated recombination. The relevant gene had been inactivated by a transposon insertion (Stirling *et al.*, 1988a, b; Blakely *et al.*, 1993). JC8679 was a *recBC sbcA* derivative of AB1157 (Bachmann, 1972). Plasmids used in this study included pKS490 (Summers & Sherratt, 1988), pUC8 (Vieira & Messing, 1982) and pKO1 (McKenney *et al.*, 1981). Construction of the P_{cer} -*galK* transcriptional fusions based on pKO1 is described in the results. M13mp19 (Messing, 1983; Norrander *et al.*, 1983; Yanisch-Perron *et al.*, 1985) and M13mp19*cer* were prepared using TG1 (Sambrook *et al.*, 1989) as a host strain. M13mp19*cer* was constructed by inserting the *cer* site (as a 290 bp *EcoRI*-*HindIII* fragment excised from pKS490) between the *EcoRI* and *HindIII* sites of the M13mp19 multiple-cloning site.

Media and bacterial transformation. For the routine growth of bacteria L-broth (Kennedy, 1971) and Oxoid isosensitest agar were used (37 °C). Where appropriate, 50 μ g ampicillin ml^{-1} (Sigma) was added to the media. Plasmid transformation of calcium chloride-treated cells was by the method of Cohen *et al.* (1972). TG1 was maintained on minimal M9 agar (Sambrook *et al.*, 1989). For transformation TG1 cells were grown in 2YT broth (Sambrook *et al.*, 1989) and transfected cells were spread in soft agar overlays on L-agar plates. Enzymes were used according to the manufacturers' recommendations. All enzymes were obtained from Promega except *Thal*, which was obtained from Gibco-BRL.

Site-directed mutagenesis. Single-stranded M13mp19*cer* phage DNA was prepared by phenol extraction of a polyethylene glycol phage precipitate (Sambrook *et al.*, 1989) and used as a template for mutagenesis. Site-directed mutagenesis reactions were performed using the Sculptor *in vitro* mutagenesis system (Amersham). Oligonucleotides for mutagenesis were synthesized by the Protein and Nucleic Acid Chemistry Facility, Department of Biochemistry, University of Cambridge. Oligonucleotides used to introduce extra bases into the promoter (Fig. 1) were degenerate within the 1–4 base insert region. The oligonucleotide to generate a 14 bp intervening spacing (i.e. to remove 1 base) was synthesized with a T missing from the wild-type sequence (Fig. 1). Plaques were picked at random and sequenced to identify mutants. DNA sequences were determined using the Sequenase DNA sequencing kit (version 2.0, US Biochemicals) and an automated DNA sequencer (model 377; Applied Biosystems). Approximately 75% of the plaques obtained by this method were mutant.

Sequencing of P_{cer} derivative constructs. Plasmid templates for sequencing were isolated using a Qiagen plasmid mini kit (Qiagen tip-20). DNA sequences were determined using the ABI PRISM Dye Terminator cycle sequencing ready reaction kit with AmpliTaq DNA Polymerase FS (Perkin Elmer), and a model 377 DNA sequencer.

Assay of galactokinase activity. Transformant colonies were inoculated into L-broth containing 50 μ g ampicillin ml^{-1} and incubated in a 37 °C shaking water bath overnight. They were then subcultured into fresh medium and incubated at 37 °C

until an OD_{650} 0.2–0.3 was reached. GalK activity was assayed by the method of McKenney *et al.* (1981). Enzyme activity is expressed as the mean $\pm$ SD of a minimum of six independent assays.

Preparation of dimer plasmids and recombination analysis. Plasmid DNA was prepared from JC8679 by alkaline lysis (Sambrook *et al.*, 1989). DNA was electrophoresed on a 0.85% (w/v) agarose gel and dimer bands were cut out from the gel and eluted into TE buffer (pH 8.0) using the GeneClean kit (Bio 101). DS941 *xerC* was then transformed with dimer plasmid DNA and transformant colonies containing dimers were identified after preparation of plasmid DNA by standard techniques. Analysis of *cer*-mediated recombination was as described by Summers & Sherratt (1984, 1988).

Assay of host cell growth. A single colony of the host *E. coli* strain harbouring the test plasmid was picked from a selective plate and inoculated into 10 ml L-broth containing 100 μ g ampicillin ml^{-1} and incubated overnight at 30 °C in a shaking water bath. Cultures were diluted in L-broth to OD_{600} 0.005–0.01; a change in density of the culture at 37 °C was followed by measurement of OD_{600} . To prevent the accumulation of plasmid-free cells, antibiotic selection was maintained throughout. The overnight incubation was carried out at 30 °C to reduce Rcd growth inhibition of cells carrying the +CA P_{cer} derivative. If the overnight incubation was at 37 °C, cells carrying the +CA P_{cer} derivative became effectively non-viable.

Oligonucleotide probe preparation. For Northern blot and dot blot analysis oligonucleotide probes were used. Probes were end-labelled using T_4 polynucleotide kinase and [γ - ^{32}P]ATP (Amersham). The phosphorylation reaction contained Promega T_4 polynucleotide kinase buffer, 200 ng probe, and 10 units polynucleotide kinase in 20 μ l. After 30 min incubation at 37 °C, the mixture was heated to 90 °C to inactivate the kinase. The labelled probe was then purified using a S200-HR spin column (Pharmacia). To probe for Rcd an oligonucleotide complementary to a region just downstream of P_{cer} was used: 5'-GGTAAAAATGGCAACAAACC-3'. To probe for β -lactamase, an oligonucleotide probe with the sequence 5'-CGGGATAATACCGCGCC-3' was used.

Isolation of RNA. *E. coli* cells harbouring plasmids were cultured in L-broth containing 100 μ g ampicillin ml^{-1} , overnight in a shaking water bath at 30 °C. The cells were then diluted into fresh medium to an OD_{650} 0.01–0.02 and incubated at 37 °C until an OD_{650} 0.3 was reached. The cells were harvested and resuspended in 200 μ l of solution 1 (0.3 M sucrose, 0.01 M sodium acetate, pH 4.8). They were lysed by the addition of 200 μ l of solution 2 (2% SDS, 0.01 M sodium acetate, pH 4.8) preheated to 65 °C. RNA was isolated by three extractions with acid phenol (Bioline) at 65 °C. The initial phenol extraction was followed by rapid freezing in liquid nitrogen. The RNA was then extracted with phenol/chloroform at room temperature, followed by extraction with chloroform/isoamyl alcohol. Then 10% (v/v) 3 M sodium acetate was added and the RNA was precipitated by adding 2 vols of ethanol (at –20 °C, 90 min). The RNA pellet was washed with 70% ethanol and dissolved in water. DNA was removed by treatment with DNase I (Sigma); the RNA was extracted with phenol/chloroform, re-precipitated and resuspended in water. RNA concentrations were determined by measurement of A_{260} .

Northern blot analysis. Twenty micrograms of total RNA was electrophoresed on polyacrylamide gels containing 7 M urea (Sambrook *et al.*, 1989). RNA was blotted onto Genescreen

Plus nylon membrane (DuPont) with a Bio-Rad electroblot apparatus (5 h at 75 V). The transfer buffer used was 0.5 $\times$ TBE (0.045 M Tris/borate, 0.001 M EDTA, pH 8.0). Pre-hybridization was at 37 °C for 1 h in a solution containing 6 $\times$ standard saline citrate (SSC), 0.5% SDS, 7.5 $\times$ Denhardt's solution and sonicated salmon sperm DNA (200 μ g ml^{-1}). Hybridization was at 37 °C for 24 h in pre-hybridization solution containing the labelled probe. Hybridized membranes were then washed for 4 $\times$ 10 min at 37 °C in 6 $\times$ SSC containing 0.1% SDS, followed by 2 $\times$ 10 min at 40 °C in 6 $\times$ SSC containing 0.1% SDS.

Dot blot analysis. Serial dilutions of RNA samples were prepared and dot blotted onto Genescreen Plus nylon membrane (DuPont) by the method of Sambrook *et al.* (1989). Filters were probed for Rcd as described above. Filters were probed for β -lactamase in the same pre-hybridization solution but the pre-hybridization and hybridization steps were at 40 °C and the washes were at 40 °C and 43 °C.

RESULTS

Site-directed mutagenesis of P_{cer}

It has been suggested that the distortion (bending or twisting) of P_{cer} in response to plasmid multimer formation may realign the –10 and –35 sequences and activate the promoter (Summers, 1998). Experimentally an alternative way to alter the relative positions of these promoter elements is to add or subtract bases in the spacer region. We therefore constructed a series of P_{cer} derivatives with altered spacer length, using single-stranded M13mp19 cer phage DNA as a template for site-directed mutagenesis. When inserting bases we used degenerate oligonucleotides so that a variety of insert sequences were obtained. This was necessary to disentangle the contributions of spacer sequence and spacer length to promoter activity. The site of insertion was central in the spacer, two bases before the 3'-end of the binding site for ArgR (the ARG box), which overlaps the –35 hexamer and spacer of P_{cer} (Fig. 1). Forty-one mutants with insertions in the spacer region were isolated (Table 1): four with a 16 bp spacer (+1); ten with a 17 bp spacer (+2); 12 with an 18 bp spacer (+3); and 15 with a 19 bp spacer (+4). Mutants which have an insertion beginning with CA reconstitute the ArgR binding site, and there was at least one such mutant in each of the groups where two or more bases were added. A promoter mutant with a 14 bp spacer (–1) was also generated by removing a T from the wild-type sequence just 3' of the end of the ArgR binding site (Fig. 1).

Dimer resolution assays

A plasmid dimer resolution assay was used to determine to what extent the addition of bases to the P_{cer} spacer affected recombination at *cer*. P_{cer} activity is not required for plasmid dimer-resolution (Summers & Sherratt, 1988), but the insertion of a non-integral number of helical turns between the binding-sites in *cer* for ArgR and XerCD has been shown to inhibit recombination, presumably by interfering with the formation of nucleoprotein recombination complexes (Guhathakurta *et al.*,

Table 1. Promoter activity of P_{cer} and derivatives with altered spacer length

Mutant†	Insert length (nt)	Spacer length (bp)	GalK units‡	SD	Relative expression§
Deletion	−1	14	151	9	1.9
Wild-type	−	15	80	16	1.0
A	+1	16	131	28	1.6
C	+1	16	163	25	2.0
G	+1	16	129	22	1.6
T	+1	16	140	27	1.8
AA	+2	17	1668	154	20.9
AG	+2	17	1339	265	16.7
AT	+2	17	1418	211	17.7
CA*	+2	17	1560	153	19.5
CC	+2	17	1122	107	14.0
CG	+2	17	1444	113	18.1
GA	+2	17	1532	95	19.2
GC	+2	17	1254	211	15.7
GG	+2	17	1182	110	14.8
TA	+2	17	1223	182	15.3
ACC	+3	18	489	64	6.1
ACT	+3	18	626	82	7.8
AGA	+3	18	687	78	8.6
AGC	+3	18	482	30	6.0
AGG	+3	18	616	142	7.7
CAT*	+3	18	481	62	6.0
CGC	+3	18	465	45	5.8
GGA	+3	18	747	101	9.3
GTG	+3	18	526	115	6.6
TCG	+3	18	380	71	4.8
TGG	+3	18	397	78	5.0
TTT	+3	18	170	35	2.1
AAAT	+4	19	22	2	0.3
ATTA	+4	19	28	3	0.4
CACA*	+4	19	19	6	0.2
CACT*	+4	19	20	5	0.3
CAGG*	+4	19	23	4	0.3
CATT*	+4	19	20	8	0.3
CCAA	+4	19	28	3	0.4
CGAA	+4	19	23	5	0.3
CGCA	+4	19	25	4	0.3
CGTT	+4	19	24	2	0.3
CTAT	+4	19	31	3	0.4
GATT	+4	19	22	3	0.3
TAAT	+4	19	20	4	0.3
TATT	+4	19	18	2	0.2
TTTT	+4	19	12	2	0.2

† An asterisk indicates those promoter derivatives in which the ARG box is regenerated.

‡ GalK units are expressed as nmol of galactose phosphorylated per minute per ml of cells at OD₆₅₀ 1.0. Stated enzyme activities are mean and SD of 6 independent assays.

§ Ratio of the activity of a given promoter derivative to P_{cer} .

1996). The +C, +CA, +CAT and +CATT *cer* site-derivatives were transferred from phage M13mp19 into pUC8 as *EcoRI*–*HindIII* fragments. Dimers of pKS490 (which contained wild-type *cer*) and plasmids containing the +C, +CA, +CAT, and +CATT *cer* derivatives

were transformed into DS941 and DS941 *xerC* (the latter acting as a recombination-deficient control). Plasmid DNA from the transformant colonies was analysed on an agarose gel for the presence of monomers. As expected, there was no breakdown of plasmid

Fig. 2. (a) Assay of *cer*-mediated dimer resolution. Dimers of pK5490 (containing wild-type *cer*) or derivatives with modified P_{cer} were transformed into DS941. Plasmid DNA from transformant colonies was analysed on a 0.85% agarose gel. The positions of supercoiled monomers and dimers (1^m SC, 2^m SC) and relaxed monomers and dimers (1^m OC, 2^m OC) are indicated. Lane 1, monomer marker; lane 2, dimer marker; lane 3, wild-type pK5490 (w-t); lane 4, +C (+1); lane 5, +CA (+2); lane 6, +CAT (+3); lane 7, +CATT (+4). (b) Plasmid DNA from DS941 (lanes 1 and 2) and JC8679 (lanes 3 and 4), containing pK5490 (w-t) or the +CA promoter derivative (+CA). Supercoiled monomers (1^m SC), dimers (2^m SC), trimers (3^m SC) and tetramers (4^m SC) are indicated.

Fig. 3. Promoter activity of P_{cer} (in terms of GalK activity) and a range of promoter derivatives with altered spacer regions (mutants). Assays were carried out in DS941 (black bars) and DS941argR (hatched bars). The sequence inserted into the spacer is indicated below each pair of results. Error bars extend an equal distance above and below the blocks and indicate SD for six independent assays.

dimers in DS941 *xerC* (data not shown). In DS941, dimers of pK5490 were resolved efficiently to monomers. Dimers of plasmids containing the +C and +CA insertions showed partial resolution to monomers, but no resolution was observed for plasmids containing the +CAT or +CATT insertions (Fig. 2a). Analysis of dimer resolution with another 12 plasmids containing *cer* sites with 1–4 bp insertions confirmed the general

pattern of these results. Insertions of 1 or 2 bp reduced but did not completely abolish dimer resolution, while insertions of 3 or 4 bp abolished recombination completely (data not shown). Among the sites with 1 or 2 bp insertions the efficiency of dimer resolution varied with the insert sequence. The pattern for the 1 bp insertions was (with best recombination first) G > C > A > T, and CA > CG > AA > GC for the 2 bp insertions. Note that the CA insertion regenerates the consensus sequence for the ARG box.

Transcriptional activity of P_{cer} derivatives

P_{cer} and its mutant derivatives were cloned into plasmid pKO1, upstream of a promoter-less galactokinase gene (McKenney *et al.*, 1981). This was achieved by excising promoters from the pUC8 derivatives used for the recombination assay on a 157 bp *EcoRI*–*SalI* fragment and inserting them between the *EcoRI* and *SmaI* sites of pKO1. The *SalI* site lies immediately downstream of the P_{cer} transcription start (Fig. 1). Transcriptional activity of the P_{cer} –*galK* fusions was assayed in DS941 and the results are shown in Table 1 and Fig. 3. The wild-type promoter had an activity of 80 ± 16 GalK units. P_{cer} derivatives with a 16 bp spacer showed a small (1.6- to 2.0-fold) increase relative to the wild-type promoter, while derivatives with a 17 bp spacer showed a much more substantial (14- to 21-fold) increase. The majority of derivatives with an 18 bp spacer showed a 4.8- to 9.3-fold increase relative to the wild-type promoter: an exception was the TTT insertion, which gave only a 2.1-fold increase. Derivatives with a 19 bp spacer showed a three- to fivefold decrease in promoter activity relative to the wild-type. The mutant with a 14 bp spacer (ΔT) showed a 1.9-fold increase. The data suggest that the optimum spacer length for P_{cer} activity is 17 bp

Table 2. Activity of P_{cer} -*galK* fusions in monomer and dimer plasmids (host DS941 *xerC*)

Mutant	Spacer length (bp)	Monomer GalK units (mean $\pm$ SD)	Dimer GalK units (mean $\pm$ SD)	Ratio*
ΔT	14	102 $\pm$ 8	142 $\pm$ 15	1.39
Wild-type	15	62 $\pm$ 4	80 $\pm$ 5	1.29
+ C	16	120 $\pm$ 19	167 $\pm$ 18	1.39
+ CA	17	1221 $\pm$ 180	1389 $\pm$ 220	1.14
+ CAT	18	421 $\pm$ 77	482 $\pm$ 55	1.15
+ CATT	19	23 $\pm$ 5	26 $\pm$ 6	1.13

* Ratio of promoter activity in monomer and dimer plasmids.

and that the length of the spacer is a more important determinant of promoter activity than its sequence.

One complication in interpreting these data is that the *cer* ARG box overlaps the P_{cer} spacer (Fig. 1) and in some cases the insertion alters the ARG box sequence. It is therefore possible that changes in ArgR binding, as well as changes in spacer length, underpin the observed changes in promoter activity. To test this the activities of P_{cer} and 17 derivative promoters were reassayed in DS941 *argR*. The activity of these promoters was not substantially different in wild-type and *argR* backgrounds (Fig. 3), implying that spacer length rather than ArgR binding was the key determinant of promoter activity. This result also excludes the simple hypothesis that transcription from P_{cer} in plasmid monomers is repressed by ArgR alone.

An isolated P_{cer} promoter does not show monomer–dimer regulation

We next investigated the monomer–dimer control of P_{cer} and its derivatives. A key question was whether monomer–dimer regulation is exhibited by isolated promoters or whether the rest of the *cer* site plays an essential part. It is possible to formulate a plausible hypothesis in which isolated promoters would respond to plasmid dimerization. ArgR and PepA are involved in interactions between pairs of *cer* sites (Guhathakurta & Summers, 1995) and in the model of Hodgman *et al.* (1998) sites are brought together when PepA forms a bridge between ArgR hexamers bound to their respective ARG boxes. The ARG box overlaps P_{cer} so that the PepA/ArgR bridge provides a means of communication between promoters in a dimer: this might be necessary, or even sufficient, for their activation.

To investigate monomer–dimer control of isolated promoters we used the P_{cer} -*galK* fusion plasmids whose construction was described in the previous section. These plasmids contain the *cer* ARG box and P_{cer} , but the *cer* sequences downstream of the Rcd transcription start (including the XerCD binding site) have been deleted. Transcription from P_{cer} and its derivatives was compared in strains containing monomers or dimers of the P_{cer} -*galK* constructs. Plasmids used in the assay contained either wild-type P_{cer} , the ΔT deletion or the

+ C, + CA, + CAT and + CATT insertions. These four insertion derivatives were chosen because they reconstitute the ARG box. Dimers of all the plasmids showed a small (13–39%) increase in transcription compared to the monomers (Table 2). The fact that this increase was small and that similar increases were seen for the wild-type promoter and its up-regulated derivatives suggests that the effect is unlikely to represent a biologically significant activation of P_{cer} . It seems more likely to result from differences in the *galK* dosage in monomers and dimers. This interpretation was supported by the observation that even in an *argR* mutant strain (where a PepA/ArgR bridge would be impossible) the wild-type P_{cer} -*galK* fusion plasmid showed an increased GalK activity of 15% between monomers and dimers (55 ± 10 and 63 ± 13 GalK units, respectively). We noticed that the monomer plasmids all showed slightly lower GalK activity in the *xerC* strain than in the wild-type (compare Tables 1 and 2). This may be due to the reduced viability of *xerC* cells because XerC is required for the resolution of chromosome dimers formed during replication.

Analysis of Rcd expression from P_{cer}

The experiments described so far involved promoters which were excised from their normal sequence environment and fused to a promoter-less *galK*. However, within the *cer* site P_{cer} is likely to be affected by the assembly of a nucleoprotein complex which contains XerC, XerD, PepA and ArgR (Hodgman *et al.*, 1998; Sträter *et al.*, 1999). To obtain a more realistic picture of P_{cer} regulation it was necessary to investigate its activity when it was part of an intact *cer* site. Northern blot analysis was used to detect Rcd produced from pKS490 (wild-type *cer*) or plasmid derivatives containing *cer* sites with mutated P_{cer} . To ensure that the structures of nucleoprotein complexes which assemble at the mutant sites were not radically different from the wild-type, the analysis was restricted to cells containing plasmids with + C and + CA insertions in P_{cer} . These remained capable of dimer resolution, albeit at a reduced rate (Fig. 2a), implying that functional recombination complexes still formed at these sites. As expected, very little Rcd was detected in the pKS490-containing strain since the plasmid is predominantly monomeric. There was a slight increase for the strain containing the + C mutant

Fig. 4. Northern blot analysis of Rcd expression from P_{cer} and its derivatives. Total RNA was prepared from strain DS941 carrying the appropriate plasmids. Equal amounts of total RNA were subjected to electrophoresis and probed for Rcd. Lane 1, DS941 pKS490 (wt); lane 2, DS941 pKS496 (P_{cer}^+); lane 3, +C derivative (+C); lane 4, +CA derivative (+CA). The Rcd transcript is indicated by an arrow.

promoter and a very large increase for the +CA mutant (Fig. 4). Transcription from the +CA promoter was higher than from P_{cer}^+ , the up-promoter mutant of P_{cer} (Fig. 1; Patient & Summers, 1993). The Northern blot data are consistent with the results of the GalK fusion experiment, where the increases for the +C and +CA mutants were twofold and 20-fold, respectively.

Mechanism of P_{cer} activation and the local twisting hypothesis

We have seen that in isolation P_{cer} is insensitive to plasmid dimerization. It seems likely that the assembly of a nucleoprotein complex at *cer* is required to activate the promoter. We have developed this idea in the local twisting hypothesis (Fig. 5), which proposes that P_{cer} is inactive in the nucleoprotein complex formed in a plasmid monomer (the single-site complex) but is activated when it finds itself in the synaptic complex formed between sites in a dimer. We envisage that in the single-site complex the -35 and -10 sequences are incorrectly aligned for RNA polymerase recognition (Fig. 5a). In the synaptic complex, distortion of the promoter brings these sequences into the correct alignment for transcription to occur. For simplicity the model invokes only twisting of the promoter, with no change in writhe of the DNA. Since the -35 and -10 sequences are separated by 15 bp, twisting must introduce a relative rotation of approximately 69° , which is equivalent to an insertion of 2 bp. This model is supported by both our *galK* fusion and Northern blot data which demonstrate that promoter derivatives with a 2 bp insertion are extremely active in plasmid monomers. On

Fig. 5. Local twisting hypothesis for P_{cer} activation. (a) Wild-type P_{cer} . In the single-site complex (which predominates in strain DS941) P_{cer} is inactive because the -35 and -10 boxes are misaligned. Plasmid multimerization (stimulated in strain JC8679) leads to synaptic complex formation. This twists the promoter, aligns the -10 and -35 boxes and stimulates transcription. (b) +CA P_{cer} derivative. In the single-site complex (in strain DS941) the -10 and -35 boxes are already aligned and P_{cer} is active. Plasmid multimerization (stimulated in strain JC8679) leads to synaptic complex formation. Associated twisting of the promoter misaligns the -10 and -35 boxes and down-regulates the promoter.

its own this might be regarded as a trivial result since it is well known that the optimum $-35/-10$ separation is 17 bp. However, our model makes the important prediction for the regulation of a promoter derivative with a 17 bp spacer (e.g. the +CA derivative). Twisting in the synaptic complex should move the -35 and -10 out of alignment and down-regulate the promoter (Fig. 5b).

To test this prediction we compared Rcd production from wild-type P_{cer} and its +CA derivative in strains DS941 and JC8679. In DS941 plasmid monomers predominate, while in JC8679 plasmid multimerization is stimulated (Fig. 2b). Comparison of Northern blots of RNA from the two strains revealed that less Rcd was produced from the +CA promoter in JC8679 than DS941 (data not shown). However, such comparisons are at best semi-quantitative and may be influenced by changes in plasmid copy-number and the efficiency of RNA isolation from the two strains. To exclude these variables, quantitative dot blot analysis was carried out. Samples of total RNA prepared from DS941 or JC8679 containing either pKS490, the +C plasmid or the +CA plasmid were spotted onto duplicate filters. One was probed for Rcd and the other for β -lactamase mRNA. The plasmid-borne β -lactamase gene is transcribed constitutively and can be used to correct for variations in plasmid copy-number and RNA extraction efficiency. Autoradiography was carried out using film pre-flashed according to the manufacturer's instructions. The autoradiographs were analysed using the densitometry package NIH Imaging 1.52, and the ratios of Rcd to β -lactamase mRNA were calculated for each combination

Table 3. Effect of plasmid multimerization on Rcd expression from P_{cer} and mutant promoters

Transcript	Relative expression*					
	DS941			JC8679		
	pKS490	+C†	+CA‡	pKS490	+C†	+CA‡
Rcd	1.0	0.9	7.3	2.2	3	4.7
<i>bla</i> mRNA	1.0	0.5	0.6	1.4	1.9	1.0
Ratio Rcd : <i>bla</i> mRNA	1.0	1.8	12.2	1.6	1.6	4.7

* RNA expression level normalized to pKS490 in strain DS941.

† pKS490 derivative with a 1 bp (C) insertion into P_{cer} .

‡ pKS490 derivative with a 2 bp (CA) insertion into P_{cer} .

of plasmid and host (Table 3). The data show that moving pKS490 from DS941 to JC8679 resulted in a 60% increase in Rcd expression, consistent with the higher level of plasmid multimerization in the latter. The +C plasmid showed a similar level of Rcd expression in both hosts. However, moving the +CA plasmid from DS941 to JC8679 resulted in a 2.6-fold decrease in Rcd expression.

As an independent (albeit less direct) measure of Rcd production we compared the growth rates of DS941 and JC8679 carrying pKS490 and the +CA insertion. In DS941 the +CA mutation increased the generation time more than fivefold, compared with the same strain containing pKS490 (164 ± 43 min and 32 ± 43 min, respectively; mean $\pm$ SD, $n \geq 4$). The high SD in the generation time of DS941 carrying the +CA plasmid reflected the difficulty in culturing this very unfit strain. However, in JC8679 the +CA insertion had little effect on growth (generation time 51 ± 9 min), causing an increase in generation time of only 25% when compared to pKS490 (42 ± 3 min). Assuming that increased Rcd production is the cause of the increased generation time, these data are consistent with the results of the Northern blot analysis. Theoretically it is possible that the results of the growth rate experiment were due to a difference between DS941 and JC8679 which is unrelated to plasmid multimerization. However, this is excluded by the observation that, as expected if the +CA promoter is down-regulated within the synaptic complex, the growth of JC8679 *pepA* is severely inhibited by the +CA promoter plasmid (data not shown).

DISCUSSION

A very clear result of this study is that P_{cer} activity is dependent upon spacer length. The +2 derivatives (spacer length 17 bp) had activities 14- to 21-fold higher than the wild-type promoter, and approximately three times stronger than induced P_{lac} or P_{gal} (McKenney *et al.*, 1981). More modest increases were observed with +1 and +3 derivatives, while derivatives with a 19 bp spacer showed a three- to fivefold decrease in activity.

These results are consistent with many previous observations that maximum promoter activity and open complex formation by RNA polymerase occurs with a spacer length of 17 bp and that lengthening or shortening the spacer leads to a decrease in promoter activity (Berman & Landy, 1979; Ackerson & Gralla, 1982; Mandecki & Reznikoff, 1982; Stefano & Gralla, 1982; Aoyama *et al.*, 1983; Mandecki *et al.*, 1985). A less consistent observation was that the P_{cer} derivative with a 14 bp spacer showed a 1.9-fold increase in promoter activity relative to wild-type P_{cer} , and was similar to P_{cer} promoter derivatives with a 16 bp spacer. It is conceivable that this promoter uses alternative -10 or -35 sequences with a more conventional spacing. Inspection of the promoter sequence suggests that RNA polymerase may recognize the normal P_{cer} -10 hexamer and a weaker TGGTTG -35 hexamer 17 bp upstream (Fig. 1).

Although the activity of P_{cer} derivatives was determined primarily by spacer length there was some variation within each group, reflecting the fact that the sequence of the spacer region also plays a role. The interaction between RNA polymerase and the spacer is largely non-specific, but if the sequence is intrinsically bent, or has the necessary flexibility to bend in the appropriate orientation, this may assist promoter activation (Collis *et al.*, 1989; Lozinski *et al.*, 1991). Equally, bends in the spacer that mis-orientate the DNA and disrupt protein-DNA interactions can decrease promoter activity. The P_{cer} mutant promoter with an 18 bp spacer created by insertion of TTT shows considerably lower promoter activity than the other promoter mutants with an 18 bp spacer. The insertion of TTT at the insertion site generates a run of five Ts. Such a T-tract is a rigid structure and will distort the DNA (Koo *et al.*, 1986; Hagerman, 1990). Presumably this T-tract bending misplaces the protein-DNA interfaces, thereby reducing promoter activation.

In isolation, our observation that a +2 bp derivative of P_{cer} shows high, constitutive activity was unremarkable. It did, however, suggest a mechanism by which the wild-

type promoter might be activated: instead of inserting extra bases, the effect of a non-optimal spacer length might be counteracted by twisting or bending the promoter. Inserting 2 bp into the spacer is equivalent to a 69° relative rotation of the -10 and -35 sequences; something which could perhaps be achieved by appropriate protein-DNA interactions. A precedent for promoter activation by protein-mediated twisting is provided by the *mer* operon of Tn501, which encodes the mercuric ion detoxification system. The promoter of the *mer* operon has a 19 bp spacer region which is essential for induction of the operon in the presence of mercuric ions and for repression in their absence. Induction is controlled by binding of MerR at the *mer* operator sequence, which is a 7 bp perfect dyad sequence between the -35 and -10 hexamers (Parkhill & Brown, 1990). The MerR protein binds to this sequence in the presence or absence of mercuric ions. In the absence of mercuric ions MerR acts as a repressor, but in the presence of mercuric ions the MerR protein is allosterically modified. This modified form of MerR causes underwinding of the DNA at the operator site, which realigns the -35 and -10 hexamers into the orientation found in a promoter with an 18 bp spacer (O'Halloran *et al.*, 1989; Ansari *et al.*, 1992).

The MerR system is relatively simple, with a single protein activating the promoter by inducing a change in its structure. By analogy it seemed possible that P_{cer} activation in a plasmid dimer might involve only the two promoters and ArgR hexamers bound to their ARG boxes (the 'isolated promoter' model). Alternatively it might require the formation of full nucleoprotein complexes at *cer*. A previous observation that dimerization of a plasmid containing a P_{cer} -*galK* fusion resulted in increased GalK activity (H. Withers & D. Summers, unpublished data) seemed to support the isolated promoter model but the more detailed study reported here suggests that this is not the case. The small increase in GalK activity observed for fusion plasmid dimers in this work appears likely to reflect a copy-number effect (i.e. dimers of the fusion plasmid had slightly more than half the copy number of monomers) and in any case was not ArgR-dependent. A slight increase in *galK* gene dosage for dimers is not unexpected because the copy-number control of ColE1-like plasmids exhibits exponential kinetics (Summers, 1998).

The lack of support for the isolated promoter model implies that monomer-dimer regulation of P_{cer} is likely to require the full nucleoprotein complexes which form at *cer* during dimer resolution. We have published a structural hypothesis for *cer*-Xer recombination (Hodgman *et al.*, 1998) which proposes that nucleoprotein complexes form initially at individual *cer* sites in plasmid monomers. These single-site complexes are able to pair via an ArgR/PepA bridge which can form between two sites in a dimer or in separate monomers. However, only in the dimer is the paired structure sufficiently long-lived to isomerize into a recombination-competent synaptic complex. Some of the basic concepts

of the Hodgman model were included in a model proposed by Sträter *et al.* (1999). However, the latter does not discuss structures for single-site complexes but envisages that the synaptic complex forms *de novo* on a plasmid dimer. In either case, the synaptic complex is the only nucleoprotein assembly unique to a plasmid dimer so we presume that it is within this that P_{cer} activation must take place.

If P_{cer} is activated by distortion within the synaptic complex, the +CA derivative is likely to be subjected to similar forces, but they may have different consequences for promoter regulation. For the +CA derivative the -10 and -35 sequences in an isolated promoter are positioned appropriately for recognition by RNA polymerase, and the local twisting model predicts that in the synaptic complex they should be moved out of alignment, deactivating the promoter (Fig. 5). To test this hypothesis we needed to generate a high level of synaptic complexes in the host cells. One possible approach would be to use recombination-deficient mutants of the XerC to trap the synaptic complex. However, there may be subtle differences in structure between a 'frozen' synaptic complex and its recombination-proficient counterpart; in any case we have no idea whether P_{cer} is activated before or after the XerC-mediated strand exchange. Given our poor understanding of the system, we decided it was more appropriate to increase the frequency of synaptic complexes by stimulating recombination. We used the *sbcA* strain JC8679, in which plasmid multimers are generated continuously by homologous recombination, thus providing a constant supply of substrate for Xer-*cer* recombination. The demonstration by both Northern blot analysis and growth rate experiments that the +CA promoter derivative is less active in JC8679 than in DS941 (where the plasmids are mostly monomeric) is consistent with the predictions of the local twisting hypothesis. We would not expect complete de-activation of the +CA promoter since only about 50% of the plasmids are multimeric at any time. Furthermore, the synaptic complex modelled by Hodgman *et al.* (1998) is asymmetric, with only one of the recombining sites subject to distortion during synaptic complex formation (the other site retaining the structure seen in the single-site complex). This would mean that only one wild-type promoter in a synaptic complex would fire and only one +CA derivative promoter would be down-regulated.

Finally it is interesting to contrast our analysis of P_{cer} with a study of *proU* promoter regulation. The *proU* promoter responds to changes in extracellular osmolarity and, like P_{cer} , has a suboptimal (16 bp) spacer (Jordi *et al.*, 1995). Jordi *et al.* (1995) predicted that if the *proU* promoter is activated by twisting, increasing the spacer length from 16 to 18 bp would reverse the effect of increased osmolarity on its activity. However, a *proU* promoter derivative with an 18 bp spacer showed essentially normal osmoregulation. Jordi *et al.* (1995) discounted the hypothesis that changes in DNA twist activate the *proU* promoter and proposed instead that changes in promoter flexibility, facilitating writhe, were

responsible. In contrast to the *proU* results, we have found that a P_{cer} derivative with an 18 bp spacer does indeed show a reversed response to plasmid multimerization, consistent with promoter activation involving twist. Monomer-dimer control of P_{cer} therefore appears to represent a distinct and novel mechanism of transcriptional control.

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REFERENCES

- Ackerson, J. W. & Gralla, J. D. (1982). *In vivo* expression of *lac* promoter variants with altered -10 , -35 , and spacer sequences. *Cold Spring Harbor Symp Quant Biol* **47**, 473–476.
- Ansari, A. Z., Chael, M. L. & O'Halloran, T. V. (1992). Allosteric unwinding of DNA is a critical step in positive control of transcription by Hg-MerR. *Nature* **355**, 87–89.
- Aoyama, T., Takanami, M., Ohtsuka, E., Taniyama, Y., Marumoto, R., Sato, H. & Ikehara, M. (1983). Essential structure of *E. coli* promoter: effect of spacer length between the two consensus sequences on promoter function. *Nucleic Acids Res* **17**, 5855–5864.
- Bachmann, B. J. (1972). Pedigrees of some mutant strains of *Escherichia coli* K-12. *Bacteriol Rev* **36**, 525–557.
- Berman, M. L. & Landy, A. (1979). Promoter mutations in the transfer RNA gene *tyrT* of *Escherichia coli*. *Proc Natl Acad Sci USA* **76**, 4303–4307.
- Blakely, G., May, G., McCulloch, R., Arciszewska, L. K., Burke, M., Lovett, S. T. & Sherratt, D. J. (1993). Two related recombinases are required for site-specific recombination at *dif* and *cer* in *E. coli* K12. *Cell* **75**, 351–361.
- Cohen, S. N., Chang, A. C. Y. & Hsu, L. (1972). Non-chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc Natl Acad Sci USA* **69**, 2110–2114.
- Collis, C. M., Molloy, P. L., Both, G. W. & Drew, H. R. (1989). Influence of the sequence-dependent flexure of DNA on transcription in *E. coli*. *Nucleic Acids Res* **17**, 9447–9468.
- Colloms, S. D., Sykora, P., Szatmari, G. & Sherratt, D. J. (1990). Recombination at ColE1 *cer* requires the *Escherichia coli* *xerC* gene product, a member of the lambda integrase family of site-specific recombinases. *J Bacteriol* **172**, 6973–6980.
- Guhathakurta, A. & Summers, D. K. (1995). Involvement of ArgR and PepA in the pairing of ColE1 dimer resolution sites. *Microbiology* **141**, 1163–1171.
- Guhathakurta, A., Viney, I. & Summers, D. K. (1996). Accessory proteins impose site selectivity during ColE1 dimer resolution. *Mol Microbiol* **20**, 613–620.
- Hagerman, P. J. (1990). Sequence-directed curvature of DNA. *Annu Rev Biochem* **59**, 755–781.
- Harley, C. B. & Reynolds, R. P. (1987). Analysis of *E. coli* promoter sequences. *Nucleic Acids Res* **15**, 2343–2361.
- Hawley, D. K. & McClure, W. R. (1983). Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Res* **11**, 2237–2255.
- Hodgman, T. C., Griffiths, H. & Summers, D. K. (1998). Nucleo-protein architecture and ColE1 dimer resolution: a hypothesis. *Mol Microbiol* **29**, 545–558.
- Jordi, B. J. A. M., Owen-Hughes, T. A., Hulton, C. S. J. & Higgins, C. F. (1995). DNA twist, flexibility and transcription of the osmoregulated *proU* promoter of *Salmonella typhimurium*. *EMBO J* **14**, 5690–5700.
- Kennedy, C. K. (1971). Induction of colicin production by high temperature or inhibition of protein synthesis. *J Bacteriol* **108**, 10–19.
- Koo, H.-S., Wu, H.-M. & Crothers, D. M. (1986). DNA bending at adenine thymine tracts. *Nature* **320**, 501–506.
- Lozinski, T., Adrych-Rozek, K., Markiewicz, W. T. & Wierchowski, K. L. (1991). Effect of DNA bending in various regions of a consensus-like *Escherichia coli* promoter on its strength *in vivo* and structure of the open complex *in vitro*. *Nucleic Acids Res* **19**, 2947–2953.
- McKenney, K., Shimatake, H., Court, D., Schmeisser, U., Brady, C. & Rosenberg, M. (1981). A system to study promoter and terminator signals recognized by *Escherichia coli* RNA polymerase. In *Gene Amplification and Analysis*, vol. 2, pp. 383–415. Edited by J. G. Chirikjian & T. S. Papa. Amsterdam: Elsevier.
- Mandecki, W. & Reznikoff, W. S. (1982). A *lac* promoter with a changed distance between -10 and -35 regions. *Nucleic Acids Res* **10**, 903–912.
- Mandecki, W., Goldman, R. A., Powell, B. S. & Caruthers, M. H. (1985). *lac* up-promoter mutants with increased homology to the consensus promoter sequence. *J Bacteriol* **164**, 1353–1355.
- Messing, J. (1983). New M13 vectors for cloning. *Methods Enzymol* **101**, 20–78.
- Norlander, J., Kempe, T. & Messing, J. (1983). Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26**, 101–106.
- O'Halloran, T. V., Frantz, B., Shin, M. K., Ralston, D. M. & Wright, J. G. (1989). The MerR heavy metal receptor mediates positive activation in a topologically novel transcription complex. *Cell* **56**, 119–129.
- Parkhill, J. & Brown, N. L. (1990). Site-specific insertion and deletion mutants in the *mer* promoter-operator region of Tn501; the nineteen base-pair spacer is essential for normal induction of the promoter by MerR. *Nucleic Acids Res* **18**, 5157–5162.
- Patient, M. E. & Summers, D. K. (1993). ColE1 multimer formation triggers inhibition of *Escherichia coli* cell division. *Mol Microbiol* **9**, 1089–1095.
- Rowe, C. D. & Summers, D. K. (1999). The quiescent-cell expression system for protein synthesis in *Escherichia coli*. *Appl Environ Microbiol* **65**, 2710–2715.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sharpe, M. E., Chatwin, H. M., Macpherson, C., Withers, H. L. & Summers, D. K. (1999). Analysis of the ColE1 stability determinant Rcd. *Microbiology* **145**, 2135–2144.
- Stefano, J. E. & Gralla, J. D. (1982). Spacer mutations in the *lac* p^s promoter. *Proc Natl Acad Sci USA* **79**, 1069–1072.
- Stirling, C. J., Stewart, G. & Sherratt, D. J. (1988a). Multicopy plasmid stability in *Escherichia coli* requires host-encoded functions that lead to plasmid site-specific recombination. *Mol Gen Genet* **214**, 80–84.
- Stirling, C. J., Szatmari, G., Stewart, G., Smith, M. C. M. & Sherratt, D. J. (1988b). The arginine repressor is essential for

plasmid stabilizing site-specific recombination at the ColE1 *cer* locus. *EMBO J* 7, 4389–4395.

Stirling, C. J., Colloms, S. D., Collins, J. F., Szatmari, G. & Sherratt, D. J. (1989). *xerB*, an *Escherichia coli* gene required for plasmid ColE1 site-specific recombination, is identical to *pepA*, encoding aminopeptidase A, a protein with substantial similarity to bovine lens leucine aminopeptidase. *EMBO J* 8, 1623–1627.

Sträter, N., Sherratt, D. J. & Colloms, S. D. (1999). X-ray structure of aminopeptidase A from *Escherichia coli* and a model for the nucleoprotein complex in Xer site-specific recombination. *EMBO J* 18, 4513–4522.

Summers, D. K. (1991). The kinetics of plasmid loss. *Trends Biotechnol* 9, 273–278.

Summers, D. K. (1998). Timing, self-control and a sense of direction are the secrets of multicopy plasmid stability. *Mol Microbiol* 29, 1137–1145.

Summers, D. K. & Sherratt, D. J. (1984). Multimerization of high copy number plasmids causes instability: ColE1 encodes a

determinant essential for plasmid monomerization and stability. *Cell* 36, 1097–1103.

Summers, D. K. & Sherratt, D. J. (1988). Resolution of ColE1 dimers requires a DNA sequence implicated in the three-dimensional organization of the *cer* site. *EMBO J* 7, 851–858.

Summers, D. K., Beton, C. W. H. & Withers, H. L. (1993). Multicopy plasmid instability: the dimer catastrophe hypothesis. *Mol Microbiol* 8, 1031–1038.

Vieira, J. & Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 19, 259–268.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33, 103–119.

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