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Molecular mechanisms of deletion formation in *Escherichia coli* plasmids

I. Deletion formation mediated by long direct repeats

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Summary. Derivatives of plasmid pBR327 with the *tet* gene interrupted by 165 pb or 401 bp direct repeats were constructed. In cells harboring these plasmids, deletions which restored the wild-type *tet* gene gave rise to tetracycline-resistant colonies, thereby allowing a simple phenotypic test for deletion formation. The frequencies of deletions in these plasmids were measured in *Escherichia coli* strains proficient or deficient in general recombination. The structure of plasmid DNA isolated from tetracycline-resistant transformants was analyzed by agarose gel electrophoresis, restriction mapping and sequencing. The data presented here demonstrate that deletion formation is always associated with dimerization of plasmid DNA. Dimeric plasmids were of two types. Those which carried both a deletion and a compensating duplication were the major type in a Rec^+ background and were rare in *recA*, *recF*, *recJ* and *recO* backgrounds. Dimers of the second type contained deletions, but no compensating duplications, and their formation was RecA-independent. The data presented demonstrate that deletion formation mediated by long direct repeats is mainly the result of unequal crossing-over between two plasmid molecules.

Key words: Deletion – Direct repeats – Plasmid recombination – Unequal crossing-over – RecA-dependence

Introduction

Substantial evidence indicates that long direct repeats mediate deletion of one of the repeats together with the DNA fragment in the region between them (Nisen et al. 1977; Chandler et al. 1979; Laban and Cohen 1981; Hill and Harnish 1982; Matfield et al. 1985; Timmons et al. 1986). Due to the evolutionary importance of deletion formation, the role of direct repeats has been widely discussed (Abastado et al. 1987; Widom et al. 1988; Brunier et al. 1989). Investigations on the molecular mechanism of deletion formation have often used specially designed plasmids (Jones et al. 1982; Laban and Cohen 1981; Matfield et al. 1985; Yi et al. 1988). These studies have suggested that, in the case of long direct repeats, deletions can arise through homologous intraplasmidic recombination (Laban and Cohen 1981; Matfield et al. 1985).

Models have been offered to explain deletion formation in terms of intraplasmidic recombination (Cramer et al. 1977; Cohen et al. 1978; Laban and Cohen 1981). An alternative model of deletion formation postulates mispairing due to slippage during DNA replication (Albertini et al. 1982; Brunier et al. 1989). Although first proposed to explain the formation of deletions between short repeats, the slipped mispairing model may also be applied to deletions mediated by long repeats.

With recent advances in molecular genetics, an appreciable number of studies on the mechanism of plasmid recombination and deletion formation have been performed. It has been observed that homologous plasmid recombination is inhibited by mutations in the *recA*, *recF*, *recJ* and *recO* genes (Hobom and Hogness 1974; Bedbrook and Ausubel 1976; Potter and Dressler 1977; Laban and Cohen 1981; James et al. 1982; Kolodner et al. 1985), but not by mutations in *recB* and *recC* genes (Willetts 1975; Laban and Cohen 1981; Fishel et al. 1981; James et al. 1982; Cohen and Laban 1983). In our experimental approach to study the molecular mechanism of deletion formation, we manipulated the *tet* gene of plasmid pBR327 so that it ultimately contained duplications of lengths from 13–401 bp, which inactivated the *tet* gene. Recombination between the resulting direct repeats restored the primary structure of the *tet* gene and conferred tetracycline-resistance to cells harboring the

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plasmids. We analyzed the structure of the deleted plasmids and determined the frequencies of their formation in strains carrying different *rec* mutations. In this paper we present data for plasmids with repeats of 401 bp or 165 bp; in the accompanying paper (Mazin et al. 1991) for plasmids with repeats of 42 bp, 21 bp or 13 bp. It is demonstrated that the formation of two types of deleted plasmid is consistently associated with plasmid dimerization. The data show that 85–90% of the deletions in a Rec^+ *Escherichia coli* strain are formed through unequal crossing-over. About 10% of the dimeric plasmids with deletions were found to be of a non-conservative nature, suggesting their generation via a pathway involving complete loss of deleted material. Mutations in the *recB* and *recC* genes stimulated the formation of dimeric plasmids by this second mechanism.

Materials and methods

E. coli strains and plasmids. The *E. coli* strains and plasmids used are listed in Table 1. All the strains were isogenic derivatives of *E. coli* AB1157 (Bachmann 1972), and the plasmids were derived either from pBR327 (Soberon et al. 1980) or from pSU2718 (Martinez et al. 1988).

Media and growth conditions. The bacterial cultures were grown in LB broth. Platings were made on solid medium containing 1.0 g NH_4Cl, 1.5 g KH_2PO_4, 6.3 g $Na_2HPO_4 \cdot 12\,H_2O$, 0.1 g $Mg_2SO_4 \cdot 7\,H_2O$, 2.0 g glucose, 10.0 g peptone, 5.0 g yeast extract per l, and 2% (w/v) agar. When necessary media were supplemented with 50 μg/ml ampicillin, 10 μg/ml tetracycline or 10 μg/ml chloramphenicol.

Purification of plasmid DNA. Plasmid DNA was extracted from cells by the alkaline lysis procedure (Birnboim and Doly 1979) and used directly for agarose gel electrophoresis or restriction analysis. When needed for sequencing, the plasmid preparations were further purified by CsCl/ethidium bromide centrifugation (Clewell and Helinski 1969).

Recombinant DNA methods. Molecular cloning, agarose and polyacrylamide gel electrophoresis, transformation and nucleotide sequence analysis were all performed according to standard procedures (Maniatis et al. 1982; Maxam and Gilbert 1980).

Construction of plasmids with direct repeats. Three different deletions were generated in the 5′ region of the *tet* gene of pBR327 by successive digestion of *Bam*HI-linearized plasmid DNA with exonuclease III and S1 nuclease (Henikoff 1984) and subsequent recircularization. They were designated d(3–402), d(10–525) and d(15–554). The numbers in parenthesis indicate the leftmost and rightmost boundaries of the deletions using pBR327 coordinates. A fourth deletion d(27–166) was constructed by partial digestion of pBR327 with *Rsa*I followed by cleavage with *Cla*I, filling-in of protruding ends and blunt-end ligation. Then, an *Eco*RI fragment of 609 bp was constructed by joining the *Eco*RI-*Sph*I fragment of pBR322 and the *Sph*I-*Eco*RI fragment from the polylinker sequence of pUC18. This 609 bp *Eco*RI fragment was inserted into the unique *Eco*RI site of the modified plasmid. The resulting constructs with direct repeats of 401 bp, 165 bp, 42 bp and 13 bp were designated, respectively, pK401–469, pK165–209, pK42–94 and pK13–69. Similarly, an *Eco*RI fragment of 201 bp was constructed from the *Eco*RI-*Eco*RV fragment of pBR322 and the *Sma*I-*Eco*RI fragment from the polylinker of pUC18. Upon insertion of this fragment into the unique *Eco*RI site of d(27–166), plasmid pK21–61, bearing direct repeats of 21 bp, was obtained. Finally, the *Apa*LI-*Ava*I fragment of 1962 bp from pK165–209, containing the *tet* gene, was inserted into the *Hin*dIII site of pSU2718 by blunt-end ligation. The resulting plasmid with the p15A replicon was designated pM165–209. The structures of the plasmids constructed were confirmed by restriction analysis and sequencing. They

Table 1. Characteristics of *Escherichia coli* strains and plasmids used

Strain	Relevant genotype[a]	Reference
AB1157	Rec^+	Bachmann (1972)
JC 10287	d*(srlR-recA)304*	Czonka and Clark (1979)
JC 5519	*recB21 recC22*	Willetts et al. (1969)
JC 9239	*recF143*	Horii and Clark (1973)
JC 13031	*recJ153*	Horii and Clark (1973)
RDK 1541	*recO1504*::Tn*5*	Kolodner et al. (1985)

[a] In all cases, other mutations were: *thr-1 ara-14 leuB6 del(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1*

Plasmid	Repeat length (bp)	Distance between repeats (bp)	Native replicon	Parental plasmid	Reference
pK401–469	401	68	pMB1	pBR327	Soberon et al. (1980)
pK165–209	165	44	pMB1	pBR327	Soberon et al. (1980)
pM165–209	165	44	p15A	pSU2718	Martinez et al. (1988)

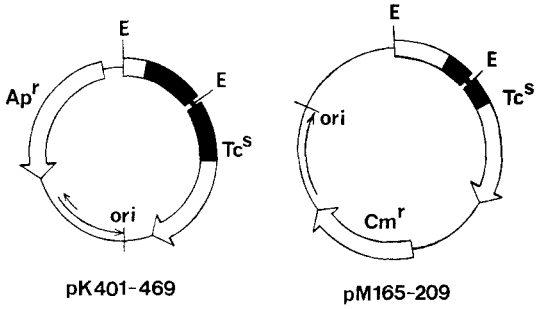

Fig. 1. Schematic representation of plasmids pK401–469 and pM165–209. The *filled* portions indicate repeats of the *tet* gene sequence separated by the polylinker from pUC18. The two *Eco*RI sites (E) used in plasmid construction are indicated. Tc, tetracycline; Ap, ampicillin; Cm, chloramphenicol

are depicted in Fig. 1 and their main features are summarized in Table 1.

Measurement of spontaneous deletion frequencies. The deletions mediated by direct repeats restored the structure of the *tet* gene and, as a consequence, a reversion to tetracycline resistance occurred. The procedure used for measuring spontaneous deletion frequencies was as follows. *E. coli* strains carrying a plasmid with direct repeats were plated onto plates containing ampicillin to obtain individual colonies. To purify the isolates young colonies were restreaked on the ampicillin-containing plate. Entire young isolated colonies from the second plate were inoculated into 2 ml of LB without antibiotics. The cultures were shaken until the cell density attained an OD_{550} of 0.5–2.0, and the cells were plated onto medium containing ampicillin or tetracycline and incubated at 37° C for 18–36 h. The frequency of deletion formation was expressed as the ratio of the number of tetracycline-resistant to ampicillin-resistant colonies.

Results

Characterization of plasmid constructs

To study the molecular events underlying the formation of deletions mediated by direct repeats, we constructed derivatives of pBR327 in which the *tet* gene was inactivated by a duplication of 165 bp (plasmid pK165–209) or 401 bp (plasmid pK401–469) (Fig. 1). Deletions restored the functional *tet* gene by eliminating the duplications, and resulted in the appearance of tetracycline-resistant colonies.

The frequency of spontaneous deletion formation in the wild-type *E. coli* strain (AB1157) was about 2.5-fold greater for the repeat length of 401 bp than for the repeat length of 165 bp (Tables 2, 3).

A relative increase of tetracycline-resistant revertants during the growth of the culture might have been expected. However, the frequencies of reversions for each plasmid in strain AB1157 were constant during the whole of log phase growth and slightly decreased when stationary phase was reached (data not shown). Similar observations for tetracycline-resistant reversions have been previously reported (Laban and Cohen 1981; James et al. 1982). This phenomenon could probably be explained by the decreased viability of cells containing the Tet protein (James et al. 1982).

Frequencies of deletion formation in recombination-deficient strains

Homologous recombination of plasmids in *E. coli* requires the products of the *recA*, *recF*, *recJ* and *recO* genes (Hobom and Hogness 1974; Bedbrook and Ausubel 1976; Potter and Dressler 1977; Laban and Cohen 1981; James et al. 1982; Kolodner et al. 1985), and there is evidence that the formation of deletions between long repeats may be mediated by these recombination events (Chandler et al. 1979; Laban and Cohen 1981; Matfield et al. 1985; Timmons et al. 1986). The present data support these findings: in the RecA$^-$ *E. coli* strain JC10287, the deletion frequencies decreased 8-fold for a repeat length of 401 bp, and 10-fold for a repeat length of 165 bp when compared to the wild type (Tables 2, 3). Mutations in the *recF*, *recJ* and *recO* genes also lowered the deletion frequencies approximately 6-, 8- and 17-fold, respectively, for pK165–209 (Table 3). Judging from these data, the enzymes of general recombination are involved in the formation of the majority of the deletions between repeats of 165 bp or 401 bp long.

In contrast, in an *E. coli* strain with mutations in the *recB* and *recC* genes, the frequency of deletion formation between 401 bp repeats was about 40% of the wild-type level and that between 165 bp repeats did not differ from the wild-type level (Tables 2, 3).

Structure of plasmids with deletions

Two events may lead to the formation of plasmids with deletions and thereby to the appearance of tetracycline-resistant bacteria: intramolecular recombination between repeats in the same molecule or intermolecular recombination between repeats in different molecules of plasmid DNA. The expected products of intramolecular recombination are plasmid monomers, whereas intermolecular recombination would produce plasmid dimers.

To see what is really taking place in our case, we subjected preparations of plasmid DNA from tetracycline-resistant colonies to 1% agarose gel electrophoresis (Fig. 2) and found that they were virtually all dimers and, much less frequently, trimers. We analyzed more than 1500 individual plasmid preparations and failed to find even a single monomeric deleted plasmid. At the same time, approximately 150 individual plasmids isolated from ampicillin-resistant (teracycline-sensitive) cells were exclusively monomers.

To determine the structure of the *tet* genes in the dimeric derivatives of pK165–209, we digested their DNA with restriction endonucleases *Eco*RV and *Bgl*I simultaneously (Fig. 3). This analysis revealed the presence of two structurally different types of dimers

Table 2. Frequencies of formation of dimers 1+2 and 1+3 from pK401–469 in Rec$^+$, *recA* and *recB recC* backgrounds

Relevant genotype	Total frequency of deletion formation ($\times 10^4$)	Efficiency of deletion formation[a]	Calculated frequencies of dimer formation ($\times 10^4$)	
			1+3 dimer	1+2 dimer
Rec$^+$	4.8±0.6	1.00	4.10	0.60
d(srlR-recA)304	0.6±0.1	0.13	0.35	0.25
recB21 recC22	2.5±0.6	0.52	2.10	0.24

[a] Efficiency was calculated as a ratio of the frequency of deletion formation in this background to the frequency of deletion formation in a Rec$^+$ background

Table 3. Frequencies of formation of dimers 1+2 and 1+3 from pK165–209 in Rec$^+$ and also in different Rec$^-$ backgrounds

Relevant genotype	Total frequency of deletion formation ($\times 10^5$)	Efficiency of deletion formation[a]	Calculated frequencies of dimer formation ($\times 10^5$)	
			1+3 dimer	1+2 dimer
Rec$^+$	17.0±0.1	1.00	15.0	2.0
d(srlR-recA)304	1.7±0.3	0.10	0.1	1.6
recF143	2.9±0.5	0.17	0.8	2.1
recJ153	2.4±0.5	0.14	1.1	1.3
recO1504	1.0±0.2	0.06	0.1	0.9
recB21 recC22	16.0±0.2	0.94	4.0	12.0

[a] Efficiency was calculated as a ratio of the frequency of deletion formation in this background to the frequency of deletion formation in a Rec$^+$ background

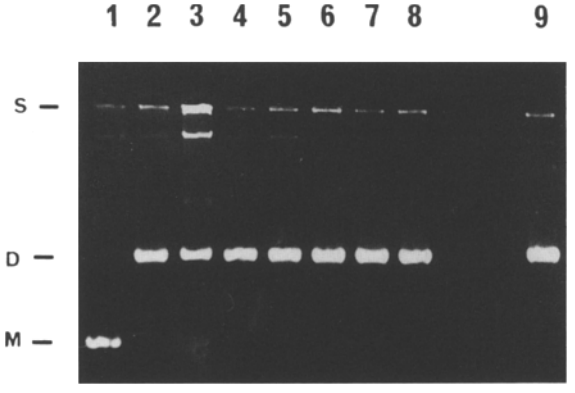

Fig. 2. Analysis of deleted plasmids by 1% agarose gel electrophoresis. S, origin; D, dimers; M, monomers. Lane 1, original construct, monomer of pK165–209; lanes 2–8, plasmids from tetracycline-resistant clones; lane 9, dimer of pK165–209

(Fig. 3B). Each dimer contained two copies of the *tet* gene: one wild-type copy (represented by a 744 bp long fragment) and a copy containing repeated sequences. The two dimeric types differed in the structure of the second copy of the *tet* gene. In the first type of dimer, the second *tet* gene carried two repeats (original construction), and it yielded a fragment of 954 bp; the corresponding dimer was designated 1+2 (Fig. 3C). In the second type of dimer, the number of repeats carried by the second *tet* gene was three, and it yielded a fragment of 1164 bp; the dimer was, hence, designated 1+3. The DNA sequence of the different *tet* genes in 1+2 and 1+3 dimers confirmed their proposed structures (data not shown).

Dimers of exclusively types 1+2 and 1+3 appeared also in plasmid pK401–469, with the two types differing markedly in their mobility in 1% agarose gels (data not shown).

Effects of mutations in the recA, recF, recJ, recO *and* recB recC *genes on the frequencies of formation of 1+3 and 1+2 dimers*

We examined the effect of mutations in the different *rec* genes on the formation of the 1+2 and 1+3 dimeric types. For this purpose, we determined the ratios of the dimeric types among the revertants recovered from the wild-type strain and also from the different Rec$^-$ strains. For both pK165–209 and pK401–469, almost 90% of the dimers had the 1+3 structure in the wild-type background (Table 3). Mutations in the *recA*, *recF*, *recJ* and *recO* genes caused a drop of about 160-, 16-, 15- and 160-fold in the frequencies of 1+3 dimer formation, respectively (Table 3).

In contrast, the *recB recC* mutations had a moderately inhibitory effect on the frequency of formation of 1+3 dimers and decreased their occurrence 2- to 4-fold (Table 3). From these data, it may be inferred that 1+3 dimers result mainly from homologous recombination. Dimers of the 1+2 type constituted somewhat greater than 10% of all the revertants for both pK165–209 and pK401–409 (Table 3). *RecA*, *recJ* and *recO* mutations weakly affected their formation, whereas the *recF* mutation did not affect it at all. However, mutations in *recB* and *recC* caused a 6-fold rise in the formation of 1+2 dimers in pK165–209 relative to the wild-type level (Table 3).

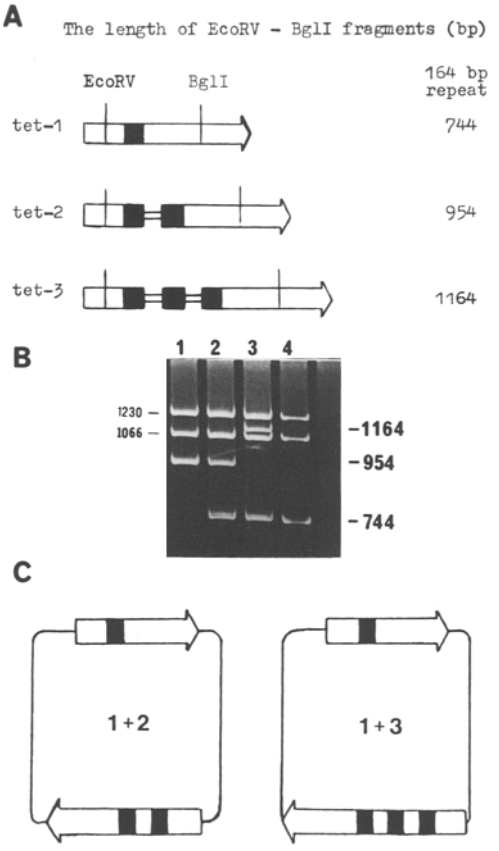

Fig. 3. **A** A scheme for the restriction analysis of the *tet* genes in dimeric plasmids arising from pK165–209. *Filled rectangles* represent direct repeats. *tet*-1 is the wild-type allele; *tet*-2 is the original construct; *tet*-3 contains three repeats separated by two polylinkers. **B** Polyacrylamide gel electrophoresis (3.5%; acrylamide:bisacrylamide = 19:1) of the products of restriction reactions. Lane 1, pK165–209 monomer, lane 2, dimer 1+2; lane 3, dimer 1+3; lane 4, pBR327 monomer. Fragments of 1230 bp and of 1066 bp are from regions of the plasmids not involved in recombination events. **C** The structures of the two types of dimers deduced from the restriction analysis of the deletion-containing plasmids

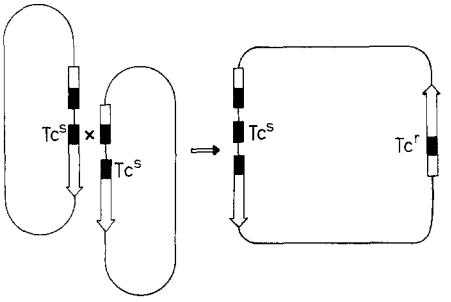

Fig. 4. A schematic representation of the model that accounts for the origin of dimers 1+3. *Filled rectangles* represent the direct repeats

Discussion

We have constructed from pBR327 plasmids bearing direct repeats of 165 bp and 401 bp in the sequence of the *tet* gene. Deletions restoring the structure of the *tet* gene resulted in the appearance of tetracycline-resistant colonies.

The salient finding was that deletion formation was invariably associated with plasmid dimerization. We have examined more than 1500 plasmid DNA preparations purified from tetracycline-resistant cells of different *E. coli* strains. The results documented that they all contained dimeric and, rarely, trimeric plasmids (Fig. 2). A small amount of the monomeric form, not exceeding 10% of the total plasmid DNA, appeared in plasmid DNA preparations only after passaging the bacterial cells for more than 50 generations.

It is pertinent to note that in previous studies using similar constructs, deleted plasmids were mainly monomers (Laban and Cohen 1981; Matfield et al. 1985). Recently, however, Kobayashi and co-workers (Yamamoto et al. 1988) have shown that gene conversion mediated by long inverted repeats in *E. coli* plasmids occurs through the formation of an unstable dimer that is subjected to rapid resolution into monomers. In the present experiments, we observed that deletion formation mediated by long direct repeats is also associated with plasmid dimerization. We offer the following explanations for the discrepancy between our data and those in the literature. The first explanation concerns the procedure; we have analyzed plasmid DNA only from young colonies and deliberately omitted the step of retransformation of plasmids from tetracycline-resistant colonies used in previous studies (Laban and Cohen 1981; Doherty et al. 1983; Matfield et al. 1985). Secondly, our parental plasmid, pBR327, presumably has no specific sequences responsible for plasmid demultimerization, which may be present on pBR322 or the other plasmids previously used (Laban and Cohen 1981; James et al. 1982; Doherty et al. 1983; Matfield et al. 1985; Yamamoto et al. 1988). We have obtained support for this suggestion using a plasmid bearing a combination of the *tet* gene plus the p15A replicon. The dimeric derivatives of plasmid pM165–209 resolve into monomers in 40–50 cell generations (data not shown).

The multimeric structure of the tetracycline-resistant plasmid is evidence that deletions may arise as a result of either interplasmidic events or, perhaps, of the interaction of the incompletely synthesized daughter strands (Yagi and Clewell 1977).

The frequencies of deletion formation were reduced in *rec*-deficient strains (Tables 2, 3), suggesting that the deletions might have arisen by interplasmidic homologous recombination involving unequal crossing-over. Figure 4 schematically represents a mechanism of deletion formation by interplasmidic recombination. The deletion results from mispairing of two plasmid molecules at the direct repeats, and a compensating duplication is expected (Yagi and Clewell 1977). Indeed, some of the dimers comprise a copy of the *tet* gene restored by the deletion, and its other copy with the expected duplication (Fig. 3). Dimers of this 1+3 version made up about 90% of all the deletion derivatives in a wild-type background (Table 3). Evidence for reciprocity of recombination between direct repeats was previously reported by Mahan and Roth (1988).

The finding of 1+2 dimers, in which deletions in one copy of the *tet* gene were not compensated for in the other copy, was quite unexpected. Furthermore, mutations in the genes inhibiting the formation of 1+3 dimers had no significant effect on the frequencies of formation of 1+2 dimers (Table 3). One finding relevant to the question of their origin, was the appreciable increase in the frequencies of their formation observed in a *recB recC* background. Thus, the two types of dimeric plasmids with deletions in the *tet* gene differ in their structure and also in response to mutations in the *rec* genes. The present data indicate that deletions between long direct repeats in plasmid DNA are formed predominantly via unequal crossing-over during homologous recombination (Yagi and Clewell 1977). However, in more than 10% of all cases, the formation of deletions follows some other pathway that is independent of the *rec* genes. As will be shown in the accompanying paper, (Mazin et al. 1991) the 1+2 dimers are the major products among plasmids with deletions between short repeats, since their formation is not concealed by production of 1+3 dimers as in the case of long repeats.

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