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In vitro construction of deletion mutants of the bacteriocinogenic plasmid Clo DF13

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ABSTRACT

The isolation and characterization of deletion mutants of the bacteriocinogenic plasmid Clo DF13 is described. To construct these deletion mutants, DNA of Clo DF13::Tn*901* and Clo DF13-*rep*3::Tn*901* plasmids was digested with restriction endonucleases, ligated with T_4 ligase and introduced by transformation into *Escherichia coli*. The presence of the ampicilline transposon Tn*901* facilitated the selection of plasmids. The resulting Clo DF13::Tn*901* deletion mutants were analyzed by digestion with restriction endonucleases and electron microscopy. From the properties of the various deletion mutants it was concluded that a Clo DF13 DNA region, extending from 5 to 11.5% on the physical map, is essential for the replication of Clo DF13. This region, comprising about 600 base pairs, contains in addition to an origin of replication, DNA sequences which are involved in the regulation of Clo DF13 DNA replication. Furthermore it was observed that in case of the Clo DF13 copy mutant, Clo DF13-*rep*3, deletion of the 43% to 63% part of the plasmid genome, resulted in the generation of multimeric plasmid structures, accompanied with an impaired segregation of the plasmids to daughter cells.

INTRODUCTION

Plasmid Clo DF13, with a molecular weight of 5.75 MegaDaltons (MD), has been extensively used in our laboratory to study the replication, gene function and genetic organisation of bacterial plasmids (1-7). It was observed that this plasmid, which is present in *Escherichia coli* to the extend of about 10 copies per cell, directs the synthesis of at least four m-RNA's and eight proteins (2, 6). One of these proteins, the bacteriocidal protein cloacin DF13, kills sensitive cells by a specific cleavage of 16S ribosomal RNA (8). Another Clo DF13 specified protein, the immunity protein, abolishes the action of the cloacin protein by forming a complex with this protein (3, 9). Using chemical mutagenesis we have isolated different mutants of Clo DF13 e.g. mutants with an altered cloacin activity (7) or DNA replication (10). In addition, the transposition of transposons such as Tn*901* offered us another tool to isolate mutants of Clo DF13. In previous papers we have described the isolation of Clo DF13 plasmids to which Tn*901* had transposed (11, 12). This

transposon (M.W. 2.8 MD) mediates ampicillin resistance and is derived from the R plasmid pRI 30 (13). To study the expression of plasmids we employed both *in vivo* and *in vitro* systems developed previously (1, 6, 15). Analysis of Clo DF13::Tn*901* insertion mutants in these systems revealed that insertion of transposon Tn*901* within Clo DF13 might result in the disappearance or alteration of particular plasmid specified polypeptides (16). By correlating the site of Tn*901* insertion with the effect of these insertions on Clo DF13 gene expression, we were able to locate five genes on the Clo DF13 genome, covering 55% of the coding capacity of this plamid (16).

To get more insight in the genetic organisation of Clo DF13 we attempted to construct specific deletion mutants of Clo DF13. For this purpose we used Clo DF13::Tn*901* insertion mutants since the presence of the ampicillin resistance determinant facilitates the selection of plasmids. In this paper we describe the isolation procedure as well as the preliminary characterization of Clo DF13::Tn*901* deletion mutants. The use of Clo DF13::Tn*901* insertion and deletion mutants as tool in studies on replication and gene function of Clo DF13 will be discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *Escherichia coli* strain used in this study, was the minicell producing strain P678-54 (14), with the following markers: *thr*, *ara*, *leu*, *azi*, *tonA*, *LacY*, *minA*, *gal*, *minB*, *rpsL*, *malA*, *xyl*, *mtl*, *thi*, *supE*. The *Enterobacter cloacae* strain H478-02 (17) was used in cloacin immunity tests. Streptomycin (Str) and ampicillin (Amp) resistant derivatives of the *Klebsiella edwardsii* var. *edwardsii* strain NTCC 5054 were used to test cloacin production. The various Clo DF13::Tn*901* plasmids and some of their properties are listed in Table 1.

Media, chemicals and enzymes

Brain Heart Infusion medium (Difco) was normally used for growth in liquid. The composition of BHI agar and soft agar have been published previously (1) BSG medium (2) was used as standard diluent. Where specified, antibiotics were included in the agar at the following concentrations: Amp, 50 μg/ml; Strep, 100 μg/ml. Ethidium bromide and cytochrome c were purchased from Calbiochem. Agarose (type I, low EEO) was obtained from Sigma. The restriction endonucleases Hinc II, Pst I, Sal I and Hpa I were purchased from New England Biolabs. Sma I and Bam HI were obtained from Boehringer Mannheim. T_4 ligase and Hae II were purchased from Miles.

Determination of Minimal Inhibitory Concentration

Determination of the minimal inhibitory concentration (M.I.C.) of Amp was performed as follows: stationary cultures were pelleted, washed and subsequently diluted in BSG medium. Samples (100 µl) of an appropriate dilution were plated on BHI plates containing an increasing concentration of Amp. The minimal Amp concentration that inhibited growth of cells was taken as M.I.C.

Isolation of plasmid DNA and cleavage by restriction endonucleases

Procedures for the isolation of plasmid DNA from cleared lysates have been published previously by Veltkamp et al. (5) Incubations of plasmid DNA with restriction endonucleases were carried out in 30 µl reaction mixtures containing 0.5-3 µg of plasmid DNA and sufficient amounts of restriction endonucleases to obtain complete digestion of the DNA in 2 hours at 37°C. The composition of the reaction mixtures for different restriction endonucleases have been described (6). Incubation of DNA with Hpa I endonuclease was carried out in a mixture containing 10 mM Tris, 10 mM $MgCl_2$, 6 mM KCl, 1 mM DTT and 100 µg/ml gelatine (pH 7.4). The Hpa*I reaction mixture contained 15 mM Tris, 15 mM KCl and 6 mM $MgCl_2$ (pH 8.6). The reactions were stopped by either heating the mixtures for 10 min. at 70°C or by adding a ficoll-E.D.T.A. mixture, to a final concentration of respectively 5% and 25 mM.

Gel electrophoresis

Procedures for preparative and analytical gelelectrophoresis of plasmid DNA, and restriction fragments, have been described previously (6).

Transformation

Transformation of E. coli and Ent. cloacae cells was followed according to the procedure described in detail by van Embden et al. (11). Transformants were selected for ampicillin resistance, and tested for cloacin production as described by Andreoli et al. (7) using Klebsiella edwardsii as indicator strain.

In vitro construction of Clo DF13 deletion mutants

Plasmid DNA (3-4 µg) was completely digested with Bam HI restriction endonucleases in a reaction mixture of 30 µl. After incubation, the reaction was stopped by heating the mixture for 10 min at 70°C. Then DTT, $MgCl_2$ and ATP were added to a final concentration of 10 mM, 10 mM and 100 mM respectively. To the reaction mixture (50 µl), 1-2 units T_4-ligase were added and the mixture was incubated for at least 24h at 4°C. After ligation the reaction mixture was used to transform E. coli cells. The resulting Amp resistant colonies were tested for cloacin production. From those colonies, which were

Amp resistant and phenotypically cloacin minus (Clo$^-$), plasmid DNA was isolated and characterized by digestion with restriction endonucleases. The procedure for constructing deletion mutants with Hpa I or Hae II endonuclease is essentially the same as described for Bam HI endonuclease. In case Hpa I enzyme was used, the DNA was incubated with an excess of Hpa I enzyme in the Hpa* I reaction mixture.

Plasmid segregation tests

The segregation of plasmids into daughter cells was analyzed as follows. Ampicillin resistant colonies of isogenic E. coli or Ent. cloacae strains, harbouring different insertion or deletion mutants of Clo DF13, were inoculated in 5 ml BHI medium containing 20 µg Amp per ml and incubated overnight at 37°C. This culture was then diluted 1:20 into fresh BHI medium lacking Amp and grown at 37°C to an absorbance of 0.4 at 660 nm. The culture was subsequently diluted and kept in exponential phase for about 50 generations. The cells were then diluted and plated on BHI agar. At least 100 single colonies were tested for ampicillin resistance. The plasmid is denoted as phenotypically segregation minus (Seg$^-$), if the percentage of plasmid-less cells, after growth in the absence of Amp, was more than 5.

Electronmicroscopy of DNA

DNA was spread by the Kleinschmidt (1968) protein monolayer technique, as described previously (18). The protein-nucleic acid film was picked on pallodion-coated copper grids and dried in ethanol. The grids were rotary shadowed with Pt/Pd alloy (80/20) and examined in a Philips EM-300.

RESULTS

Mapping of Tn901 insertions on Clo DF13

In a previous paper (6) we have described the isolation procedure for Clo DF13 plasmids to which Tn901 had transposed. From the analysis of 33 Clo DF13::Tn901 plasmids, it was observed that transposon Tn901 is able to integrate at a large number of different sites within Clo DF13. However, no insertions of Tn901 were mapped within two large regions of the Clo DF13 genome. These areas are located between 99 and 11.5%, and between 46 and 63% on the Clo DF13 physical map. The absence of Tn901 insertions in these two area's might be due to the restricted number of insertion sites which have been mapped, or the lack of specific base sequences, which are involved in the transposition event. Alternatively, these two Clo DF13 regions might code for essential plasmid functions; in that case insertion of Tn901 within these area's will result in the loss of the plasmid. In view of these possibilities

we decided a) to extend our studies with other Clo DF13::Tn901 plasmids and b) to isolate deletion mutants of Clo DF13. For this purpose the physical maps of Clo DF13 and Tn901 DNA were further extended with respectively, the cleavage sites of Sma I and Hae II endonuclease. The cleavage maps of Clo DF13 and Tn901 DNA are represented in Figure 1 and 2. The Sma I restriction endonuclease cleaves Clo DF13 into five fragments, but does not cut into Tn901 DNA. Further Clo DF13 contains about 12 sites of Hae II, whereas Tn901 DNA is cut at 3 sites by this enzyme. Using the method which we have described previously (11) and which involes the analysis of cleavage patterns of Clo DF13::Tn901 DNA digested with different restriction endonucleases, we were able to determine the site and orientation of 12 additional Tn901 insertions. The results obtained from previous work (11) and this study are summarized in Figure 3 and Table 1. Figure 3, shows the site and orientation of 45 Tn901 insertions within the Clo DF13 plasmid. Although the sites of Tn901 insertions, are more or less scattered around the Clo DF13 genome, again no insertions were found in the Clo DF13 DNA regions from 99 to 11.5% and from 46 to 63%. Therefore it seems likely that these regions contain genetic information which is essential for the maintenance of Clo DF13. To obtain evidence for this hypothesis we attempted to isolate deletion mutants of Clo DF13.

Construction of Clo DF13::Tn901 deletion mutants using Bam HI

In a first approach to isolate deletion mutants of Clo DF13 we used the restriction endonuclease Bam HI. This enzyme cleaves Tn901 DNA at one single site which is located asymmetrically within Tn901 (see Figure 2). Clo DF13 however is digested into two fragments by Bam HI (see Figure 1). The two Bam HI cleavage sites are located within the cloacin gene (7), implicating that deletion of the small Bam B fragment of Clo DF13 will result in the deletion of a large part of the structural gene coding for cloacin DF13. The isolation procedure of Clo DF13::Tn901 deletion mutants, using Bam HI, is represented schematically in Figure 4. This figure shows a Clo DF13 plasmid to

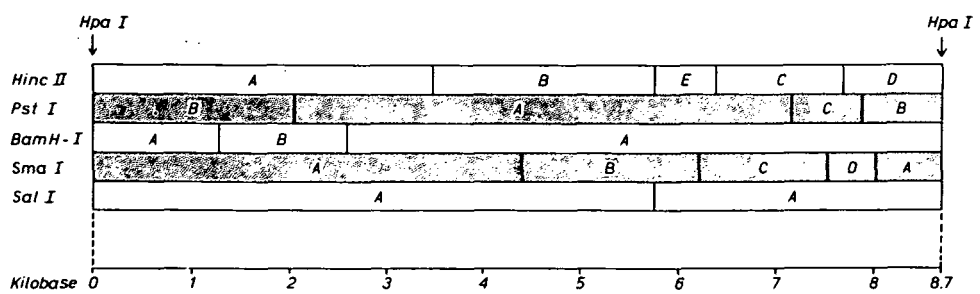

Figure 1. Cleavage map of the Clo DF13 plasmid.

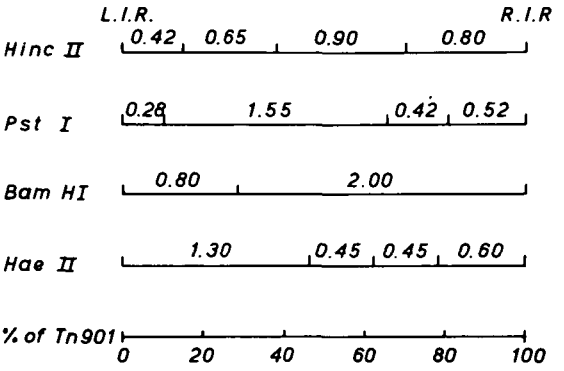

Figure 2. Cleavage map of transposon Tn901. The size of the restriction fragments is represented in Mega Dalton.

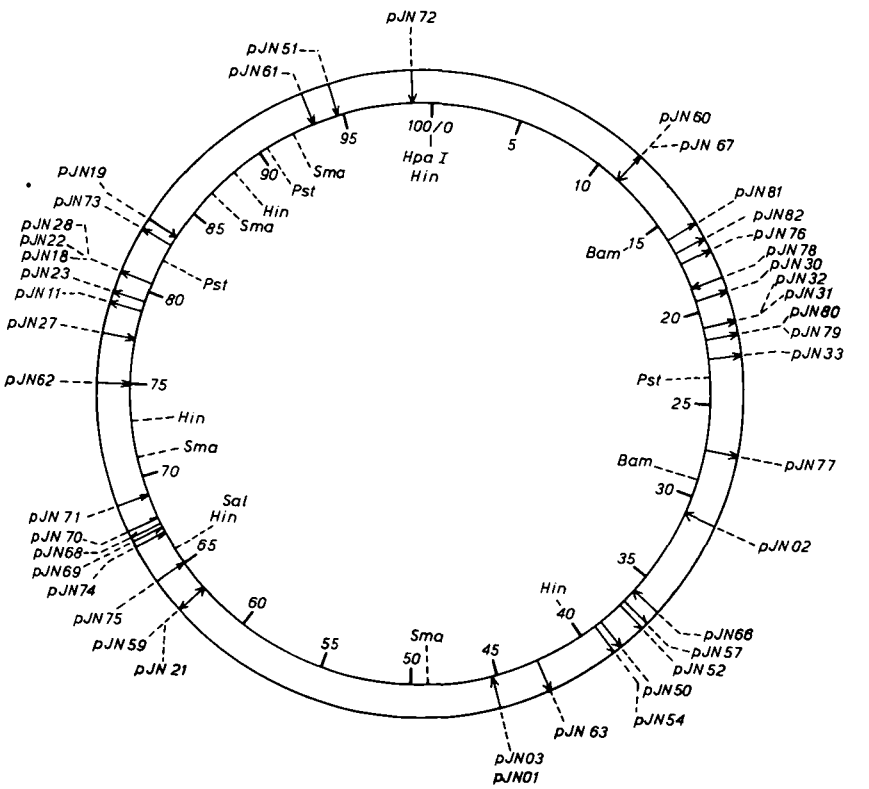

Figure 3. Map of site and orientation of Tn901 insertions into the Clo DF13 plasmid. The orientation of the insertion is represented by the direction of the arrow. When the arrow points towards the centre of the circle, the LIR of the transposon is orientated counterclockwise (R), whereas an arrow in the opposite direction indicates the LIR is orientated clockwise (L).

TABLE 1. Clo DF13::Tn*901* plasmids

Plasmid	Clo DF13 parental[a] plasmid	Map position[b]	Orientation of[c] transposon	Reference
pJN67	rep3	11.5	R	(11)
pJN60	rep3	11.5	L	(11)
pJN81	rep3	15.5	L	(11)
pJN82	rep3	16.5	L	(11)
pJN76	rep3	17.0	L	this paper
pJN78	rep3	19.0	R	(11)
pJN30	wt	19.5	L	this paper
pJN32	wt	21	L	this paper
pJN31	wt	21	L	this paper
pJN80	rep3	21.5	L	(11)
pJN79	rep3	21.5	L	(11)
pJN33	wt	22.5	L	(11)
pJN77	rep3	27.5	L	(11)
pJN02	wt	31	R	(11)
pJN66	rep3	37	R	this paper
pJN57	wt	37	L	(11)
pJN52	rep3	37.5	L	(11)
pJN50	rep3	38.0	L	(11)
pJN54	rep3	39	L	this paper
pJN63	rep3	43	L	this paper
pJN01	wt	45	R	(11)
pJN03	wt	45	R	(11)
pJN21	wt	63.5	L	this paper
pJN59	rep3	63.5	L	(11)
pJN75	rep3	65	R	(11)
pJN74	rep3	66.5	R	this paper
pJN69	rep3	67	R	(11)
pJN68	rep3	67	L	(11)
pJN70	rep3	68	R	this paper
pJN71	rep3	69	R	(11)
pJN62	rep3	75	R	(11)
pJN27	wt	77.5	R	(11)
pJN11	wt	79	L	(11)
pJN23	wt	79.5	L	(11)
PJN18	wt	79.5	L	this paper
pJN22	wt	79.5	L	this paper
pJN28	wt	79.5	L	this paper
pJN73	rep3	83	L	(11)
pJN19	wt	82.5	R	this paper
pJN61	rep3	93.5	R	(11)
pJN51	rep3	95	R	(11)
pJN72	rep3	99	R	(11)

a) Tn*901* insertions have been introduced in both the wt Clo DF13 plasmid and the Clo DF13-*rep*3 copy mutant.

b) The site of Tn*901* insertion is represented in percents of the Clo DF13 physical map.

c) The orientation of the Tn*901* insertion is indicated by the capital R or L. L represents that the left inverted repeat (LIR) is orientated clockwise, whereas R represents that the LIR is orientated anticlockwise (see also Figures 2 and 3).

which Tn*901* had transposed (the transposon is indicated by a waved line). Digestion of this plasmid with Bam HI generates three DNA fragments. After ligation of these DNA fragments with T_4-ligase, the DNA was used to transform *E. coli* cells to ampicillin resistance. Those colonies which were Amp^r and did not produce cloacin (Clo^-) were used in further experiments. Plasmid DNA

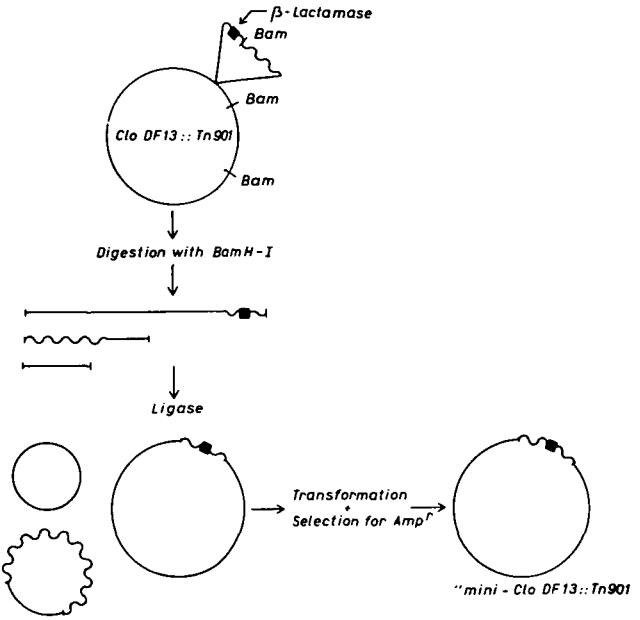

Figure 4. Schematic presentation of the construction of Clo DF13::Tn901 deletion mutants.

was isolated from these colonies and analyzed by digestion with restriction endonucleases. By comparing the cleavage pattern of these DNA's with that of the original Clo DF13::Tn901 DNA's the deleted DNA fragments were easily determined. An example of such an restriction analysis is demonstrated in Figure 5. Figure 5, track e, shows Clo DF13 DNA cleaved by Bam HI into two fragments, Bam A and Bam B. The Clo DF13::Tn901 plasmid pJN60 is cleaved by Bam HI into three fragments because this plasmid contains an additional Bam HI cleavage site located on the Tn901 DNA (Figure 5, track g). It can be deduced from this cleavage pattern that insertion of Tn901 had occured within the Clo DF13 Bam A fragment, since the mobility of this fragment is altered. Analysis of the Bam HI cleavage pattern of the deletion mutant pEV2, derived from pJN60, revealed that in this case the small Bam B fragment of Clo DF13 is deleted (Figure 5, track f). The relative orientation of the two remaining Bam fragments of pEV2 was determined by digestion of pEV2 DNA with other restriction endonucleases e.g. Pst I (figure 5, track c). This analysis demonstrated that the relative orientation of the two remaining Bam fragments of pEV2 DNA was identical to the orientation of these fragments within the original plasmid pJN60. It is obvious that the part of the Clo DF13 DNA which can be deleted by this method, directly depends on the site and orientation of the

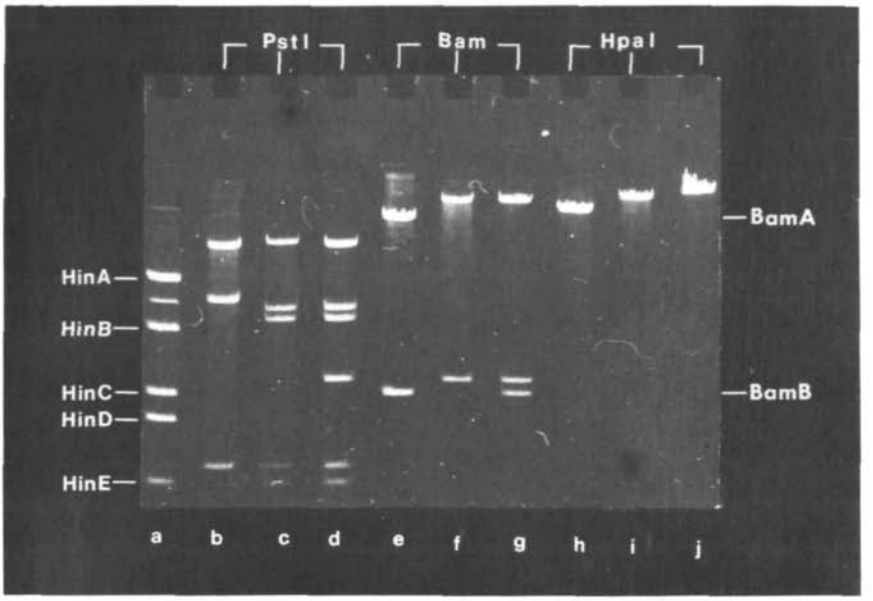

Figure 5. Agarose gelelectrophoresis of plasmid DNA fragments. Track a, Clo DF13 DNA fragments generated by Hinc II endonuclease digestion. Clo DF13, pEV2 and pJN60 plasmid DNA, was digested with Pst I endonuclease (resp. track b, c and d), Bam HI endonuclease (c, f and g) and Hpa I endonuclease (h, i and j).

Tn901 insertion within the Clo DF13 plasmid. Using different Clo DF13::Tn901 plasmids we were able to isolate various miniplasmids of Clo DF13; the physical maps of some of these miniplasmids are represented in figure 6. The results demonstrated that the structural gene coding for β-lactamase is located on the small Bam fragment of Tn901. Furthermore we observed that large parts of the Clo DF13 plasmid can only be deleted if Tn901 is inserted within Clo DF13 in the R orientation (the R orientation is defined in the legend of Figure 3). All miniplasmids, which were constructed with Bam HI, contain the 99-11.5% region of the Clo DF13 genome; a region in which no insertions of Tn901 have been mapped. However, the region between 43 and 63% on the Clo DF13 physical map was deleted in some miniplasmids. The smallest miniplasmid constructed in vitro with Bam HI, plasmid pEV26, was derived from the Clo DF13::Tn901 plasmid pJN73 (Figure 6), and lacks the 15-83% part of the Clo DF13 genome. Since the number of different deletion mutants of Clo DF13, which can be isolated using Bam HI endonuclease, is restricted, we employed other restriction endonucleases to delete specific parts of the Clo DF13 genome.

Construction of Clo DF13::Tn901 deletion mutants using Hpa I and Hae II endonuclease.

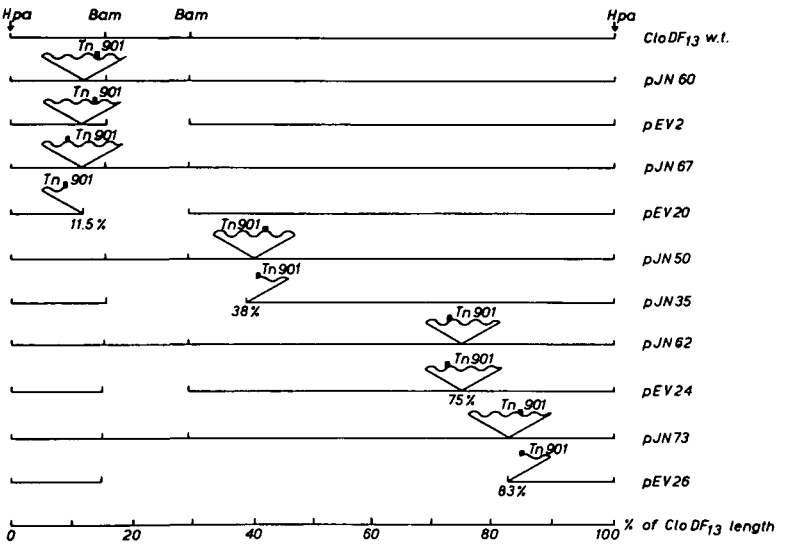

Figure 6. Schematic presentation of "mini" plasmids constructed with Bam H-I endonuclease. The upper line represents a linear Clo DF13 DNA molecule. The Hpa I cleavage site is used as reference point (0/100%). The location of the Bam HI cleavage site on the Tn901 transposon (waved line) is indicated by ■.

As described in the previous section, all miniplasmids constructed with Bam HI contained the Clo DF13 DNA region from 83 to 11.5%. To investigate whether or not this region can be deleted we attempted to construct deletion mutants using other restriction endonucleases. For this purpose we have used the Hpa I and Hae II restriction endonucleases. The Hpa I enzyme cleaves Clo DF13 DNA, under standard conditions (see Materials and Methods), at one single site; this site is used as reference point (0%) on the Clo DF13 physical map. Tn901 DNA is not cut by Hpa I under these conditions. However we observed a remarkable change in the specificity of the Hpa I enzyme, when the conditions of incubation were altered. It appeared that, at a pH of 8.6 and a Mg^{++} concentration of 6 mM, the Hpa I enzyme cleaves Clo DF13 DNA not only at 0%, but also at a number of additional sites, whereas Tn901 is cut under these condition at at least three sites (gels not shown). Figure 7 shows the physical map of plasmid pJN67. For convenience, these additional Hpa I cleavage sites are indicated by Hpa I^*. The site of Tn901 insertion in this particular plasmid is located at about 11.5% relative to the Hpa I reference point. Those Hpa I^* cleavage sites which could be located on the physical maps of Clo DF13 and Tn901, are presented in Figure 7A. For the construction of deletion mutants, pJN67 DNA was digested with Hpa I in the Hpa I^* incubation mixture. Sub-

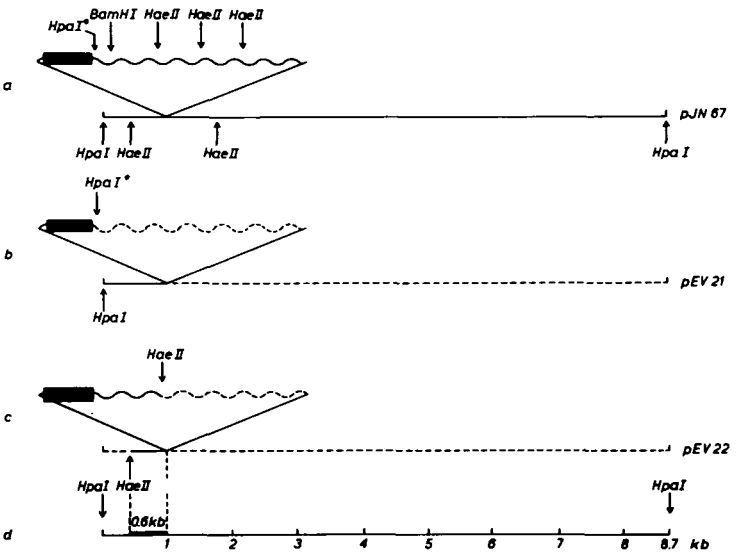

Fig. 7. Physical maps of the Clo DF13::Tn901 plasmid pJN67 and deletion mutants derived from this plasmid. The deleted area's are indicated by dotted lines.

sequently, the DNA fragments were ligated and used to transform E. coli cells. Plasmid DNA was isolated from the resulting transformants and analyzed by digestion with restriction endonucleases. In this way the miniplasmid pEV21 was isolated and characterized; the physical map of this plasmid is shown in Figure 7B. This particular miniplasmid has a molecular weight of about 1.3 Mega Dalton and contains only the 0-11.5% (about 1 Kb) region of the Clo DF13 genome. The restriction endonuclease Hae II cleaves the Clo DF13 genome at about 12 different sites. From the analysis of Hae II digestion patterns of both Clo DF13::Tn901 insertion mutants and miniplasmids constructed with Bam HI, we were able to locate two of the Hae II cleavage sites on the Clo DF13 physical map. These sites were mapped at about 5% and 20% respectively (see Figure 7C). Furthermore this analysis enabled us to locate the three Hae II cleavage on the transposon Tn901 (Figure 2 and Figure 7). None of these Hae II cleavage sites are located within the 0.8 Mega Dalton Bam fragment of Tn901. Since this fragment contains the structural gene coding for β-lactamase, digestion of Tn901 DNA with Hae II will not interfere with the β-lactamase gene. To determine whether the 0-5% region of Clo DF13 contains sequences which are essential for plasmid replication, DNA of plasmid pJN67 was digested with Hae II enzyme, ligated and introduced by transformation into E. coli. From one of the resulting Amp^r, Clo^- transformants, we

isolated a miniplasmid, designated pEV22, that only contains the 5-11.5% part of the Clo DF13 genome (corresponding to about 0.6 Kilobase). As shown in Figure 7C, this miniplasmid also contains a large fragment of Tn901 DNA which comprises approximately 2 Kb. This pEV22 miniplasmid contains one single site for Pst I, Bam HI, and Hae II whereas two Hinc II cleavage sites are present on this plasmid.

Properties of Clo DF13::Tn901 deletion mutants

In the preceeding sections we have described the isolation of various Clo DF13::Tn901 deletion mutants. Using different Clo DF13::Tn901 plasmids we were able to construct a set of Clo DF13::Tn901 miniplasmids (pEV plasmids) with overlapping deletions. We have shown previously (7), that insertion of Tn901 within Clo DF13 might result in the alteration or dissappearance of particular Clo DF13 gene products. To determine if deletion of specific parts of Clo DF13 is accompanied with the loss of particular Clo DF13 gene functions, we examined some properties of the pEV miniplasmids. The characteristics of pEV plasmids and some of their properties are listed in Table 2. The pEV plasmids are ordered in this table to the size of the Clo DF13 DNA which is deleted. Since all Clo DF13::Tn901 deletion mutants lack the Clo DF13 DNA region between 15 and 29%, which is part of the cloacin gene (7), it is obvious that cells harbouring these plasmids showed a Clo^- phenotype (Table 2). We also examined whether deletions of specific parts of the Clo DF13 genome results in an alteration of the number of plasmid copies per cell. In this respect it is necessary to mention that some miniplasmids were derived from the wildtype Clo DF13 plasmid whereas others were derived from the copy mutant of Clo DF13, Clo DF13-rep3 (see Table 2). Cells harbouring the Clo DF13-rep3 plasmid contain about 7 times more plasmid DNA than cells harbouring the wild type Clo DF13 plasmid (10). To determine the number of plasmid copies in E. coli cells harbouring Clo DF13::Tn901 insertion or deletion mutants, we used the procedure described previously (10). From these experiments we observed that insertion of Tn901 within Clo DF13 or Clo DF13-rep3 had no significant effect on the number of plasmid copies per cell. However, when we analyzed the amount of plasmid DNA, present in cells harbouring various pEV miniplasmids, we observed that in case of particular miniplasmids (pEV7, pEV10, pEV26, pEV21 and pEV22), the amount of plasmid DNA, present in cell cultures, was extremely low. This phenomenon could be abolished if the cells were grown in the presence of ampicillin. To study this phenomenon in more detail, cells harbouring the pEV plasmids, were grown in BHI-medium in the absence of ampicillin. After about 50 generations, the percentage of cells

TABLE 2. Properties of Clo DF13::Tn*901* deletion mutants

pEV plasmid	pJN parental plasmid	wild type or rep3	molecular weight	Clo DF13 deletion	Tn*901* deletion	Cloacin phenotype	segregation phenotype	MIC[a] (mg/ml)
pEV2	pJN60	rep3	7.75	15-29%	-	Clo^-	Seg^+	6
pEV14	pJN03	wt	7.75	15-29%	-	Clo^-	Seg^+	1.5
pEV24	pJN62	rep3	7.75	15-29%	-	Clo^-	Seg^+	6
pEV20	pJN67	rep3	5.55	11.5-29%	29-100%	Clo^-	Seg^+	6
pEV35	pJN50	rep3	5.15	15-39%	29-100%	Clo^-	Seg^+	6
pEV32	pJN63	rep3	4.95	15-43%	29-100%	Clo^-	Seg^-	6
pEV10	pJN59	rep3	3.75	15-63%	29-100%	Clo^-	Seg^+	6
pEV29	pJN21	wt	3.75	15-63%	29-100%	Clo^-	Seg^-	1.5
pEV7	pJN68	rep3	3.55	15-67%	29-100%	Clo^-	Seg^+	6
pEV4	pJN11	wt	2.90	15-79%	29-100%	Clo^-	Seg^-	1.5
pEV26	pJN73	rep3	2.65	15-83%	29-100%	Clo^-	Seg^-	6
pEV21	pJN67	rep3	1.30	11.5-100%	23-100%	Clo^-	Seg^-	6
pEV22	pJN67	rep3	1.67	11.5-100-5%	46-100%	Clo^-	Seg^-	6

[a] Minimal inhibitory concentration of AMP.

sensitive to ampicillin was determined. It turned out that when this experiment was carried out with cells, harbouring the plasmids pEV7, pEV10, pEV26, pEV21 and pEV22, at least 40% of the cells were sensitive to ampicilline, whereas cells harbouring other pEV plasmids all remain resistant to ampicillin. Since all pEV miniplasmids are capable of replication, these results indicate that during cell division the segregation of the miniplasmids pEV7, pEV10, pEV26,pEV21 and pEV22 to daughter cells is affected (indicated as Seg^-). From Table 2 it can be deduced that this phenomenon is restricted to those pEV plasmids which lack the DNA region between 15 and 63% of the Clo DF13 genome and are derived from the copy mutant Clo DF13-rep3. Since the miniplasmids pEV32 and pEV35 are Seg^+ and lack the 15-43% part of the Clo DF13 genome, we concluded that the Clo DF13 DNA region between 43 and 63% contains genetic information which is involved in plasmid replication and/or segregation. The determination of the number of plasmid copies present in cells harbouring Seg^- pEV miniplasmids, by centrifugation of plasmid DNA in CsCl-ethidium bromide or sucrose gradients, is complicated due to the generation of plasmid-less cells. Therefore we employed another procedure, namely the determination of the minimal inhibitory concentration (M.I.C.) of ampicillin for cells harbouring different plasmids. We have shown previously (11) that there seems to exist a linear relationship between the M.I.C. of Amp and the plasmid copy number. Transposition of Tn901 to the copy mutant Clo DF13-rep3, which is present in about 70 copies per cell, resulted in a 4-6 times higher M.I.C. of Amp compared to cells carrying wild type Clo DF13::Tn901 plasmids (present in about 10 copies per cell). The M.I.C. of Amp was determined for cells harbouring pEV plasmids (see Table 2). The results revealed that these M.I.C.'s were almost identical to those of cells, carrying the Clo DF13::Tn901 plasmids from which these miniplasmids were derived. This means that for cells carrying pEV miniplasmids derived from the copymutant Clo DF13-rep3 , the M.I.C. of Amp is 4-6 times higher than for cells carrying pEV plasmids derived from the wild type Clo DF13 plasmid. From these results it seem likely that pEV miniplasmids, derived from Clo DF13-rep3::Tn901 plasmids, still contain the rep3 mutation. Another interesting property of the Seg^- pEV plasmids was observed when the DNA of these plasmids, isolated by dye buoyant density centrifugation, was analyzed by agarose gelectrophoresis. An example of such an analysis is presented in Figure 8. As shown in Fig. 8, track C through E, the DNA of Seg^- pEV plasmids (e.g. pEV10, pEV26 and pEV21), does not only exists in the open circular or supercoiled monomeric forms, but also a large number of DNA bands with a higher molecular weight can be observed. That this phenomenon is restric-

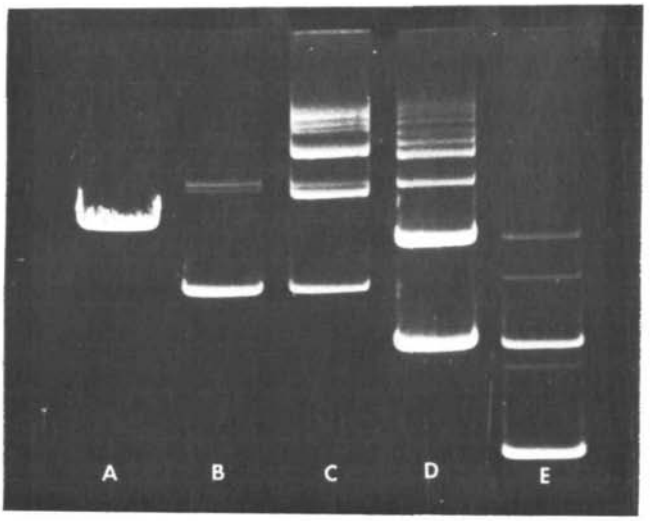

Fig. 8. Analysis of plasmid DNA on agarose gels. Supercoiled DNA of plasmids pEV10, pEV29, pEV26 and pEV21 was isolated by dye buoyant density centrifugation and subjected to electrophoresis on a 0,7% agarose gel. (A) pEV10 DNA digested with Hpa I endonuclease; (B) pEV29 DNA; (C) pEV10 DNA; (D) pEV26 DNA and (E) pEV21 DNA.

ted to Seg^- miniplasmids is demonstrated in Figure 8, track B and C. These tracks represent agarose gelelectrophoresis of respectively pEV_{29} DNA and pEV_{10} DNA. Although these miniplasmids lack the same Clo DF13 and Tn*901* DNA regions (Table 2), the DNA preparation of plasmid pEV10 Seg^- contains additional DNA bands, whereas such bands are hardly detectable in DNA preparations of plasmid pEV29 (Seg^+). The difference between these two miniplasmids is that pEV29 was derived from a wild type Clo DF13::Tn*901* plasmid (pJN21; see also Table 1 and Figure 3), while a Clo DF13-*rep*3::Tn*901* plasmid (pJN59) was used for the construction of pEV20. To determine whether the large number of additional DNA bands, observed in DNA preparations of Seg^- miniplasmids, is due to the presence of contaminating DNA, the DNA was cleaved with restriction endonucleases. For this purpose we used restriction endonucleases, with a single recognition site on pEV miniplasmids, e.g. Hpa I. Cleavage of a heterogenic DNA preparation will result in the generation of number of different DNA fragments. From Figure 8, track A, it is clear that cleavage of the pEV10 DNA preparation (Figure 8, track C), with Hpa I, results in the generation of the single DNA fragment. Similar results were obtained with DNA preparations of other Seg^- pEV plamids, indicating that the additional DNA bands represent pEV plasmid DNA which is in the catenated or multimeric form. To discriminate between catenated or multimeric forms, the DNA preparations were also examined

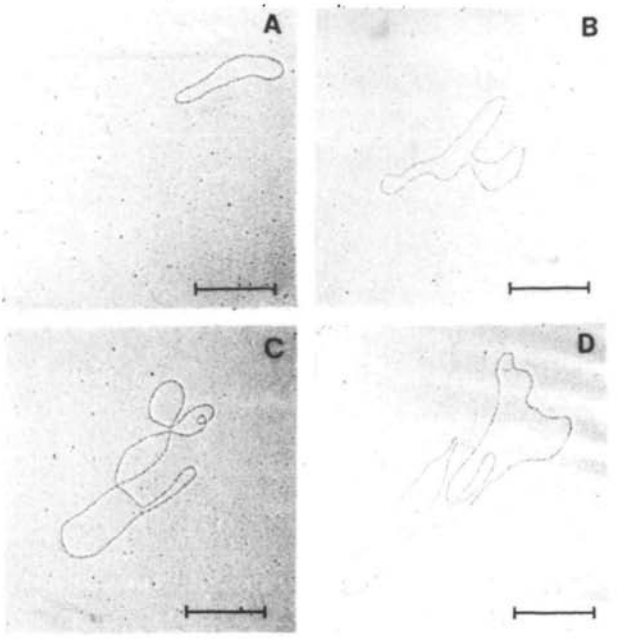

Figure 9. Electronmicroscopy of open circular monomeric (A); dimeric (B); trimeric (C) and tetrameric (D) pEV26 DNA molecules. The bar represents 0.5 ,um.

by electronmicroscopy. About 95% of the 500 molecules examined, appeared to be supercoiled, while 5% of the DNA molecules were found in the open circular form. No catenated forms were observed in these DNA preparations. From measurements of the open circular molecules it was evident that a large number of these molecules represent multimeric forms. Figure 9a through d, shows electromicrographs of monomeric (a), dimeric (b), trimeric (c) and tetrameric (d) pEV 26 DNA molecules.

DISCUSSION

Previously we have described the isolation of Clo DF13 plasmids harbouring transposon Tn901 that mediates ampicillin resistance (11). Analysis of integration sites of 42 Tn901 insertions within Clo DF13 revealed that no insertions had occurred within two large Clo DF13 DNA regions, namely from about 99 to 11,5% and from about 45 to 63% on the Clo DF13 physical map. To get more insight in the genetic organization of Clo DF13, and in particular the regions mentioned above, we attempted to delete specific parts of the plasmid genome. For this purpose we used different Clo DF13:: Tn901 plasmids, since the presence of the ampicillin transposon facilities

the construction and selection of deletion mutants. Upon digestion of these plasmid DNA's with Bam HI, Hae II or Hpa I endonuclease, followed by ligation with T4 ligase and transformation of E. coli, a large number of different "mini" Clo DF13::Tn901 plasmids was obtained. In the course of this study it was observed that the specificity of the Hpa I enzyme can be changed by alteration of the incubation conditions. Similar effects have been described for the endonucleases EcoR I and Bsu I (21, 22). Under normal incubation conditions Clo DF13 is cleaved by Hpa I at one single site (0% on the physical map). However, by alteration of the incubation conditions additional DNA sequences are recognized by the Hpa I enzyme and cleaved at a low rate. This phenomenon enabled us to construct different types of Clo DF13 deletion mutants. (This paper: Andreoli et al, manuscript submitted.) In a first approach to characterize the Clo DF13::Tn901 deletion mutants, we examined the DNA regions that were deleted and the phenotypic properties such as cloacin production and stability of the plasmids in the bacterial cells.

These studies revealed that all miniplasmids, isolated so far, share the Clo DF13 DNA region from about 5% to 11,5% on the physical map. This result was not surprising since, recently, we obtained evidence by electron microscopic studies of Clo DF13 replicating intermediates, that an origin of replication is located in this region (Stuitje et al, manuscript in preparation). Replication of Clo DF13 initiates at about 5% on the Clo DF13 physical and proceeds unidirectionally counter clockwise (see Figure 10). Assuming that Clo DF13 does not contain "silent" origins of replication, deletion of the 5 to 11,5% part of the plasmid genome will result in the loss of the plasmid. Furthermore it was observed that all miniplasmids, derived from the copy mutant Clo DF13 - rep3, still showed the mutant phenotype, namely the MIC of Amp of cells harbouring these miniplasmids is comparable to that of cells harbouring Clo DF13 - rep3::Tn901 plasmids. Since the Clo DF13 - rep3, plasmid is mutated in a DNA sequence involved in the control of initiation of Clo DF13 DNA replication (10), the data strongly suggest that this genetic information is also located within the 5 to 11,5% part of the plasmid genome. With respect to the location of the structural gene coding for β-lactamase gene within the ampicillin transposon TnA (24). Deletion of the 43 to 63% part of the Clo DF13 genome (indicated in Figure 10, as replication*), resulted in case of miniplasmids derived from Clo DF13 - rep3, in an impaired segregation of plasmids to daughter cells. In addition we observed, in cells harbouring these miniplasmids a remarkable

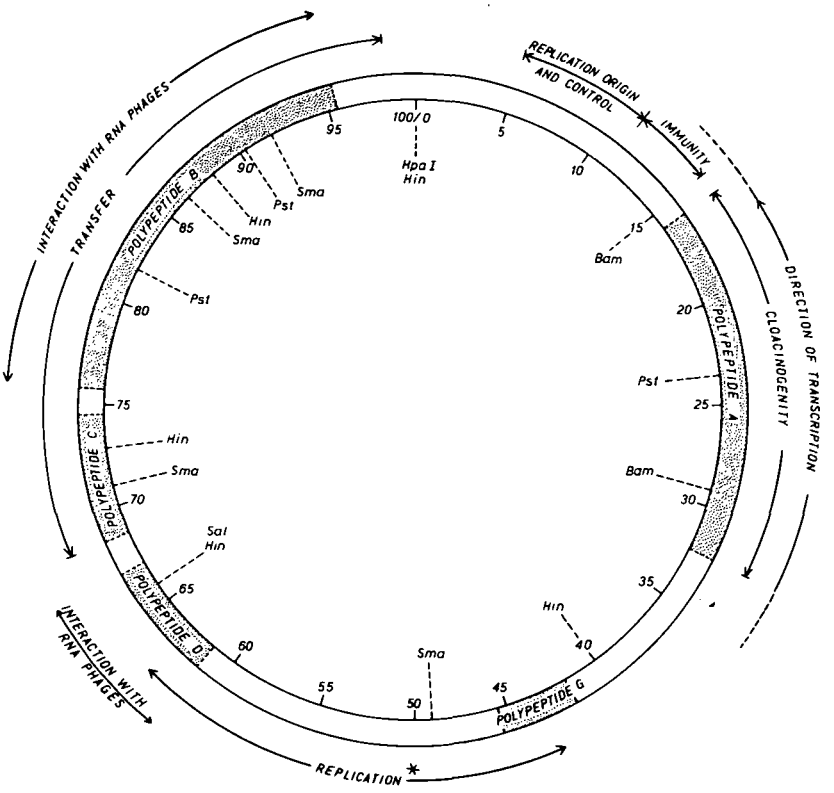

Figure 10. The figure shows the localization of the genes A, B, C, D and G as well as the genetic information which in involved in the mobilization and replication of Clo DF13. Furthermore the genetic information that is involved in the interaction with male specific RNA phages is indicated. This figure is based on data obtained by Andreoli et al (16), van Embden et al (11), van der Pol et al (27, manuscript in preparation) and data described in this paper.

increase in the number of multimeric plasmid molecules. We found earlier that cells, harbouring Clo DF13 - rep3 plasmids, contain a large amount of catenated DNA molecules which presumably is due to the increased frequency of initiation of plasmid replication (Stuitje et al, manuscript in preparation). It has been postulated by Sogo et al (25) that these interlocked DNA molecules can be processed to monomeric DNA molecules by some "dissociation function" which is independent of a recombination system. Furthermore the catenated DNA molecules can be converted to multimeric DNA structures by recombination. The observation that deletion of the 43 to 63% part of Clo DF13 - rep3 plasmids, in an increased amount of multimeric DNA molecules can

be explained in different ways. Firstly, it could be that this part of the plasmid codes for a gene product that inhibits recombination, by interacting with one of the hosts recombination enzymes. Such an interaction has been predicted for the λ gam gene product (26), and E. coli rec BC enzyme. Deletion of the gene coding for this inhibitor will result in an enhanced recombination. Since cells harbouring Clo DF13 - rep3 plasmids contain a large amount of catenated DNA molecules, the enhanced recombination will, in turn, result in the generation of multimeric molecules.
Alternatively, it could be that the 43 to 63% part of Clo DF13 is involved in the dissociation of interlocked DNA molecules to monomeric molecules. A block in the dissociation by deletion of this part of the plasmid genome will result in a accumulation of catenated structures, which in turn can be converted to multimers by recombination. We speculate that in both models only a small fraction of multimeric DNA molecules is converted by recombination to monomers. In any way the generation of multimers might reduce the actual number of physically separated plasmid copies per cell which, in turn, can result in the formation of plasmid-less cells during cell division. In summary, the construction of deletion mutants of Clo DF13, described in this paper, offered us a powerfull tool to study the plasmid DNA replication process. Furthermore these miniplasmids are usefull in studies on the gene function and the genetic organization of the Clo DF13 plasmid (12, 27).

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