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Amplification of Chloramphenicol Resistance Transposons Carried by Phage P1Cm in *Escherichia coli*

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Summary. We have characterized a number of P1Cm phages which contain the resistance genes to chloramphenicol and fusidic acid as IS1-flanked Cm transposons. Restriction cleavage and electron microscopic analysis showed that these Cm transposons were carried as monomers (M) or tandem dimers (D). Lysogens of P1Cm (D) are more resistant to chloramphenicol than those of its P1Cm (M) presumably as a result of an increased gene dosage. Amplification of the Cm transposons to tandem multimers was frequently observed in P1Cm (D) lysogens grown in the presence of high concentrations of chloramphenicol or fusidic acid and was also detected in P1Cm (M) lysogens. The degree of amplification varied in different clones which suggests that cells containing spontaneously amplified Cm transposons were selected by high doses of the antibiotics. The dimeric as well as the amplified Cm transposons carried in P1Cm lysogens grown in the absence of chloramphenicol displayed considerable stability. Mechanisms for the amplification of the IS1-flanked transposons are discussed.

Introduction

Gene amplification of plasmid-born functions, particularly drug resistance, has repeatedly been observed in *Proteus mirabilis* (Rownd and Mickel, 1971; Hashimoto and Rownd, 1975; Tanaka et al., 1976) and it is also described in *Streptococcus faecalis* (Clewell et al., 1975; Clewell and Yagi, 1977). In *Escherichia coli*, similar phenomena have recently been discovered and are described in this and other papers (Mattes et al., 1979; Chandler et al., 1979).

We have isolated several hybrid P1 phages (Mise and Arber, 1976; Iida and Arber, 1977) which have acquired drug resistance genes from the R plasmid NR1 (also called R100) and which still carry all essential genes for phage replication and lysogenization. Restriction enzyme cleavage and electron microscope analysis of their DNA revealed the presence of IS1-flanked transposons containing all or part of the r-determinant (r-det) of NR1 inserted at the unique IS1 site of P1 (Iida et al., 1978a) or at various other locations in the P1 genome (Arber et al., 1978). These hybrid phages may have evolved either by a transposition event or by cointegration of the P1 and NR1 plasmids by recombination between their IS1 elements and subsequent deletion formation (Iida et al., 1978b). Only a few of the resulting Cm[1] transposons have the same size as the Tn9 which is also an IS1-flanked Cm transposon (MacHattie and Jackowski, 1977).

Here we will present evidence that some of the Cm transposons are integrated in P1 DNA as tandem dimers of the structure IS1 – Cm – IS1 – Cm – IS1 and that further amplification to higher oligomeric repeats occurs and can be detected after growth of *E. coli* lysogenic for P1Cm in the presence of high concentrations of chloramphenicol or fusidic acid. Part of these results have been reported by Meyer et al. (1978).

Materials and Methods

Media, Bacteria and Bacteriophage

The media used were as described by Iida and Arber (1977). Bacteriophage P1 and its derivatives are listed in Table 1. Phage P1r-det is P1CmSmSuHg81*c*1ts225 described by Arber et al. (1978). The isolation and the characterization of Cm^r transducing P1 phages

[1] *Abbreviations.* Cm = chloramphenicol; Fa = fusidic acid; bp = base pairs; kb = kilobase pairs; P1Cm (M) = P1 phage carrying a monomer Cm transposon; P1Cm (D) = P1 phage carrying a tandem dimer Cm transposon; P1Cm (A) = P1 phage carrying a tandem multimer (amplified) Cm transposon

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Table 1. Phage P1 strains

Strain	Reference	Cm transposon carried		Number of Cm^r genes	Designation used in the text
		Insertion site on P1 DNA (map units)	Monomer size[a]		
P1*c1*ts225	Iida et al. (1978a)	–	–	–	
P1Cm0	new designation for P1CM isolated by Kondo and Mitsuhashi (1964)	20	2.7 kb=Tn9	1	
P1Cm0*c1*ts225	This study	20	2.7 kb	1	P1Cm0 (M)
P1Cm0*c1*ts100	Rosner (1972)	20	2.7 kb	2	P1Cm0 (D)
P1Cm13*c1*ts225	Iida et al. (1978a)	4	2.6 kb	1	P1Cm13 (M)
P1Cm89*c1*ts225	Iida et al. (1978a)	4	2.6 kb	1	P1Cm89 (M)
P1Cm88*c1*ts225	This study	4	2.6 kb	2	P1Cm88 (D)
P1Cm92*c1*ts225	This study	4	2.6 kb	2	P1Cm92 (D)
P1Cm80*c1*ts225	Arber et al. (1978)	47	2.9 kb	1	P1Cm80 (M)
P1Cm248*c1*ts225	Arber et al. (1978)	20	4.8 kb	2	P1Cm248 (D)

All P1Cm phages listed are plaque forming and belong to type A as defined by Iida and Arber (1977). P1Cm13 and P1Cm89 DNA gave the same restriction cleavage patterns and so did P1Cm88 and P1Cm92 DNA

[a] The monomer size gives the length of the Cm resistance determinant plus the two flanking IS1 elements. This corresponds to the size of the Cm insertion at map units 4 and 47. At map unit 20, however, the insertion is about 800 bp smaller because there is a resident IS1 at this location

has been described (Iida and Arber, 1977). *E. coli* WA921 (*thr leu met lac hsdS*$_k$) was used as a host for phage P1. The fusidic acid sensitive strain DB10 was obtained from Dr. J. Davies (Datta et al., 1974).

Selection for High Levels of Drug Resistance

Selection of lysogens resistant to high doses of chloramphenicol followed either of two protocols. *Method 1*: A single colony of cells lysogenic for P1Cm was suspended in LB containing 25 µg/ml Cm and grown at 30° C to saturation. After checking the structure of P1Cm DNA by restriction cleavage analysis (see next section) the culture was diluted 50-fold in LB containing 300 µg/ml Cm and grown at 30° C. It usually took 1–3 days to grow the culture to saturation. *Method 2:* A single colony of cells lysogenic for P1Cm was suspended in LB containing 25 µg/ml Cm and grown at 30° C to saturation. After checking the structure of P1Cm DNA the culture was diluted 100-fold in LB containing twice the concentration of Cm. The procedure was repeated until the final Cm concentration had reached 1 mg/ml. In both methods the cultures were then diluted 50-fold in LBMg, grown to 2×10^8 cells/ml and phage was induced. The selection of bacteria resistant to high doses of Fa followed Method 1.

Isolation and Restriction Cleavage Analysis of Phage DNA

Phage was induced from 200 ml of cells lysogenic for P1Cm and concentrated with polyethylene glycol (Iida and Arber, 1977). After treatment with 0.5 µg/ml each of pancreatic DNase and RNase A, phage particles were purified by a CsCl step gradient. Phage DNA was extracted with phenol.

In order to analyse rapidly a large number of samples, 15 ml of phage lysate were prepared by heat induction. After removing the cell debris by low speed centrifugation, the lysate was treated with DNase and RNase and phage particles were precipitated with polyethylene glycol. They were resuspended in 1 ml of phage buffer (10 mM Tris-HCl pH 7.5, 10 mM $MgSO_4$) to which EDTA pH 8.5 was added to a final concentration of 50 mM. After 5 min incubation at 37° C the mixture was extracted with phenol, DNA was concentrated by ethanol precipitation and redissolved in 200 µl of 10 mM Tris pH 7.4, 10 mM NaCl, 1 mM EDTA. Samples of 10 to 20 µl were digested with restriction enzymes and subjected to gel electrophoresis as described (Bächi and Arber, 1977).

Electron Microscopy

Heteroduplex molecules were prepared by denaturation and renaturation of a mixture of DNA fragments and mounted for electron microscopy by the formamide technique of Davis et al. (1971). Intrastrand reannealing of inverted repeats resulting from rapid renaturation was visualized as snap back structures. PM2 DNA (Espejo et al., 1969) and fd DNA (Beck et al., 1978) served as internal length standards for double-stranded and single-stranded DNA, respectively.

Results

1. Monomer and Tandem Dimer Forms of Differently Sized Cm Transposons are Found in Independent Isolates

A summary of studies on the location and size of the IS1-flanked Cm insertions carried in P1Cm phages is given by Arber et al. (1978). Detailed experimental data are presented here for the P1Cm derivatives listed in Table 1. Several of the Cm transposons studied locate at P1 map unit 4, others at P1 map unit 20. Both these locations are within restriction fragment *Bgl*II-2 of P1 DNA. None of the Cm insertions is cleaved by *Bgl*II restriction endonuclease.

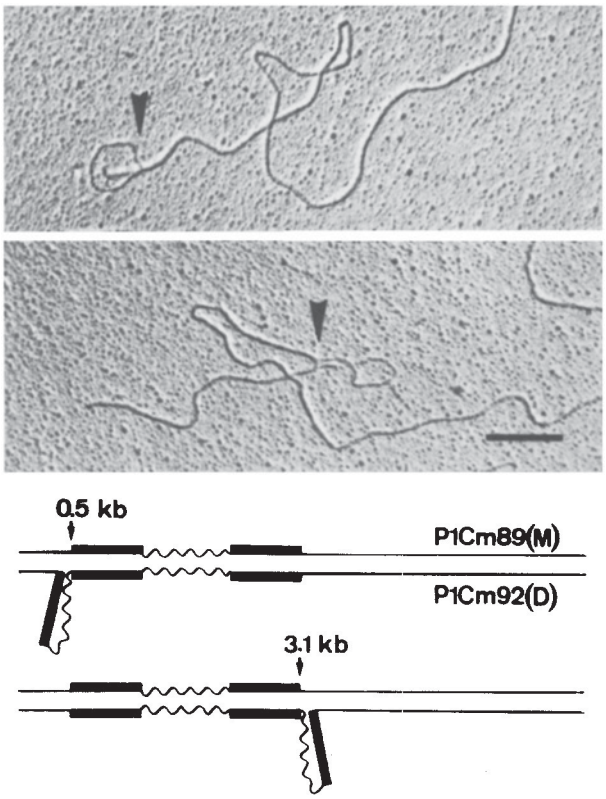

Fig. 1. Heteroduplex molecules between the *Bgl*II-2 fragments of P1Cm89 (M) and P1Cm92 (D) DNA. Only the relevant part is shown. The two electron micrographs give evidence for a tandem duplication by the presence of a single-stranded loop (arrows) of fixed size at variable positions between the boundaries of the monomer unit (principle outlined by Davidson and Szybalski, 1971). In the diagram the two extreme positions are shown. In this and all the following diagrams the straight lines represent P1 DNA, the black boxes identify IS1 elements and the Cm^r determinant is indicated by the wavy lines. The bars on electron micrographs represent 1 kb length of DNA

Electron microscope and restriction cleavage data reveal that some of these Cm transposons occur as monomers, others as tandem dimers.

a) Electron Microscopic Studies. Tandem repeats were detected in heteroduplex molecules between DNA fragments carrying the monomer and the dimer Cm transposon by the variable location of the single-stranded loop emanating from positions within the boundaries of the monomer unit. Figure 1 shows heteroduplex molecules between the *Bgl*II-2 fragments of P1Cm89 (M) and P1Cm92 (D) DNA. A single-stranded loop of 1.66 kb originates from positions varying from 0.53 kb to 3.08 kb distance from one end (27 molecules). By EM criteria the Cm transposons of P1Cm92 (D) and P1Cm89 (M) are inserted at the same site on P1 DNA and in the same orientation. The only difference is that P1Cm89 (M) carries a monomer and P1Cm92 (D) a dimer of the resistance determinant. In P1Cm92 (D) the insertion represents a tandem dimer of the structure IS1 – Cm – IS1 – Cm – IS1, with three directly repeated IS1 elements (Fig. 3). This structure was confirmed by the observation of single-stranded fragments of P1Cm92 (D) DNA which formed snap back structures between sequences about 800 bp long, as have been described for P1Cm89 (M) DNA (Iida et al., 1978a). The looped molecules fell into three classes which had the double-stranded part and one single-stranded end in common, but differed in the size of the loop and the other single-stranded end (Fig. 2, Table 2). The intramolecular reannealing occurred between the IS1 element at map unit 20 of the P1 genome and either one of three IS1 elements of opposite orientation present at both ends and in the centre of the insertion.

Comparison of the double-stranded length of the *Bgl*II-2 DNA fragments of a second pair of P1Cm derivatives P1Cm0 (M) and P1Cm0 (D) with the wild type fragment indicated additional sequences of 1.92 kb and 3.95 kb, respectively. The sizes and structures of the Cm insertions were also determined by heteroduplex mapping (Table 3). In heteroduplex molecules the variable location of the single-stranded loop within the IS1 element at map unit 20 or within the monomer Cm transposon is direct evidence for the presence of IS1-flanked Cm insertions at this site and for the dimer structure of the Cm transposon on P1Cm0 (D). Therefore, a similar monomer-dimer relation was demonstrated for P1Cm0 (M) and P1Cm0 (D) as for P1Cm89 (M) and P1Cm92 (D) (Fig. 3).

b) Restriction Cleavage Studies. Since the Cm transposons have no *Bgl*II site, as mentioned above, the *Bgl*II fragment of P1Cm DNA carrying the Cm insertion has a larger size. *Eco*RI cleaves the Cm transposons in question once (Arber et al., 1978), thus producing a fragment corresponding in size to one IS1 plus the Cm resistance determinant ("unit length" fragment) from tandem dimers, but not from monomers. *Pst*I cuts once within the IS1 element (Grindley, 1977; Othsubo and Ohtsubo, 1978; Iida et al., 1978a) creating one fragment from monomers and two fragments of the same length from dimers (Fig. 4C). *Eco*RI and *Pst*I restriction cleavage thus provides a rapid means for finding P1Cm derivatives carrying a tandem dimer of an IS1-flanked Cm transposon.

This situation is exemplified in Fig. 4: The *Bgl*II-2 DNA fragment of P1Cm13 (M) (Fig. 4A, slot b) is larger than the corresponding fragment of P1 (Fig. 4A, slot a) and the one derived from P1Cm88 (D) is even larger (Fig. 4A, slot c). *Pst*I cleavage of

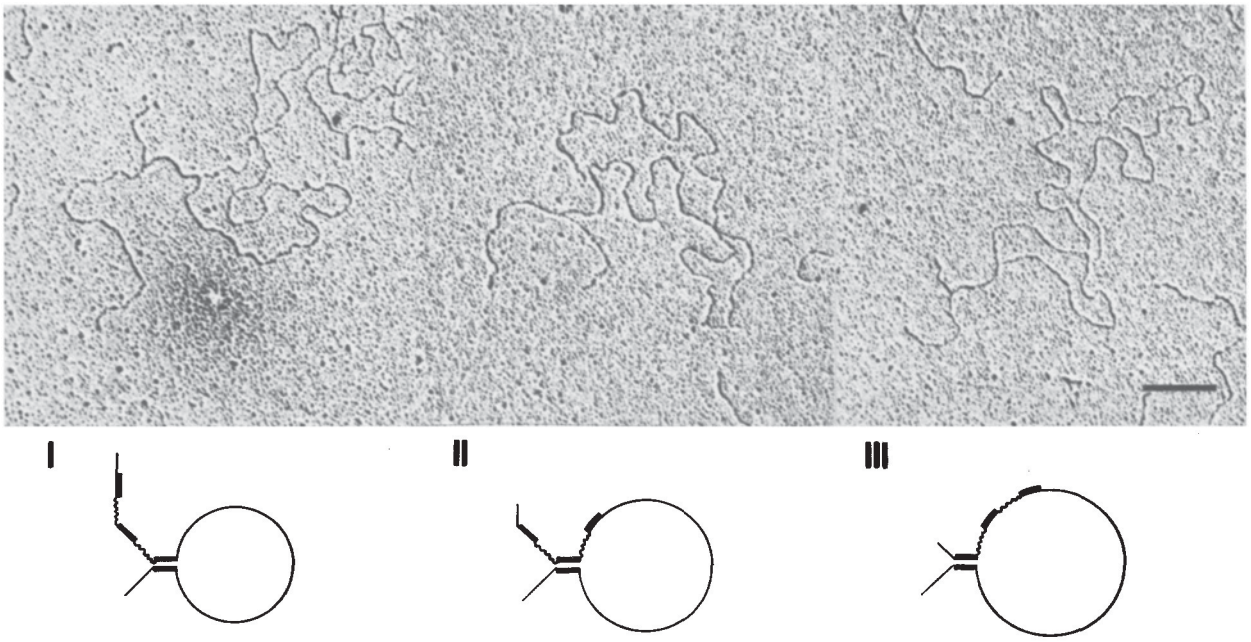

Fig. 2. Electron micrographs of snapback structures observed in single-stranded *Bgl*II-2 fragments of P1Cm92 (D) DNA. The diagram gives our interpretation of the structures: the intrastrand reannealing occurs between the IS1 at map unit 20 of the P1 genome and either of the three IS1 of the dimer Cm transposon. I, II and III refers to the class of molecules defined by the measurements given in Table 2

Table 2. Length measurements of single-stranded snap back *Bgl*II-2 fragments of P1Cm92 (D) DNA

Class	Number of molecules measured	Single-stranded loop	Single-stranded ends		Double-stranded part
			Constant	Variable	
I	14	12.74 ±0.1[a]	1.35 ±0.08	3.80 ±0.14	0.80 ±0.03
II	28	14.20 ±0.3	1.33 ±0.16	2.04 ±0.11	0.78 ±0.02
III	17	16.14 ±0.2	1.30 ±0.08	0.50 ±0.07	0.81 ±0.03

[a] All measurements are given in kb

Table 3. Analysis of heteroduplex molecules between the *Bgl*II-2 DNA fragments

Heteroduplex	Number of molecules	Size of loop	Location from right-hand end
P1Cm0 (M):P1	15	1.96 ±0.16[a]	1.2–2.2 kb (within IS1)
P1Cm0 (D):P1	20	4.01 ±0.24[b]	1.2–2.3 kb (within IS1)
P1Cm0 (M):P1Cm0 (D)	17	2.09 ±0.13	1.3–4.4 kb (within monomer unit IS1-Cm-IS1)

[a] All measurements are given in kb

[b] Two molecules had two loops of 2 kb instead, resulting from reannealing of the central IS1 element of the dimer Cm insertion of P1Cm0 (D) with the naturally occurring IS1 in the P1 DNA fragment

DNA of P1Cm13 (M) and P1Cm88 (D) (Fig. 4B, slots a and b) produces the "unit length" fragment once and twice, respectively. The same size DNA fragment is present in an *Eco*RI digest of P1Cm88 (D) (Fig. 4B, slot d), but is missing in that of P1Cm13 (M) (Fig. 4B, slot c). It should be noted here that P1Cm13 (M) and P1Cm88 (D) are indistinguishable from P1Cm89 (M) and P1Cm92 (D), respectively, used for the electron microscopic characterization.

The analogous situation is seen with P1Cm0 (M) and P1Cm0 (D) DNA. The *Bgl*II-2 fragments are larger (Fig. 4A, slots e and f). The "unit length" fragment appearing in *Pst*I digests (Fig. 4B, slots f

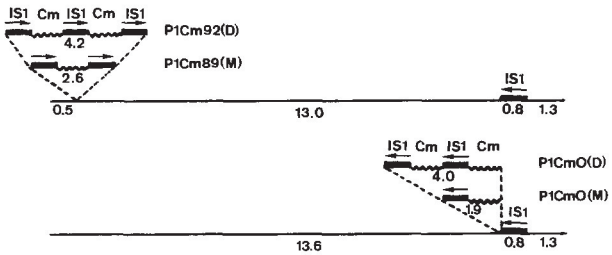

Fig. 3. Location, size and structure of monomer and dimer Cm transposons within the *Bgl*II-2 fragment of P1 DNA. The figures give the size in kb

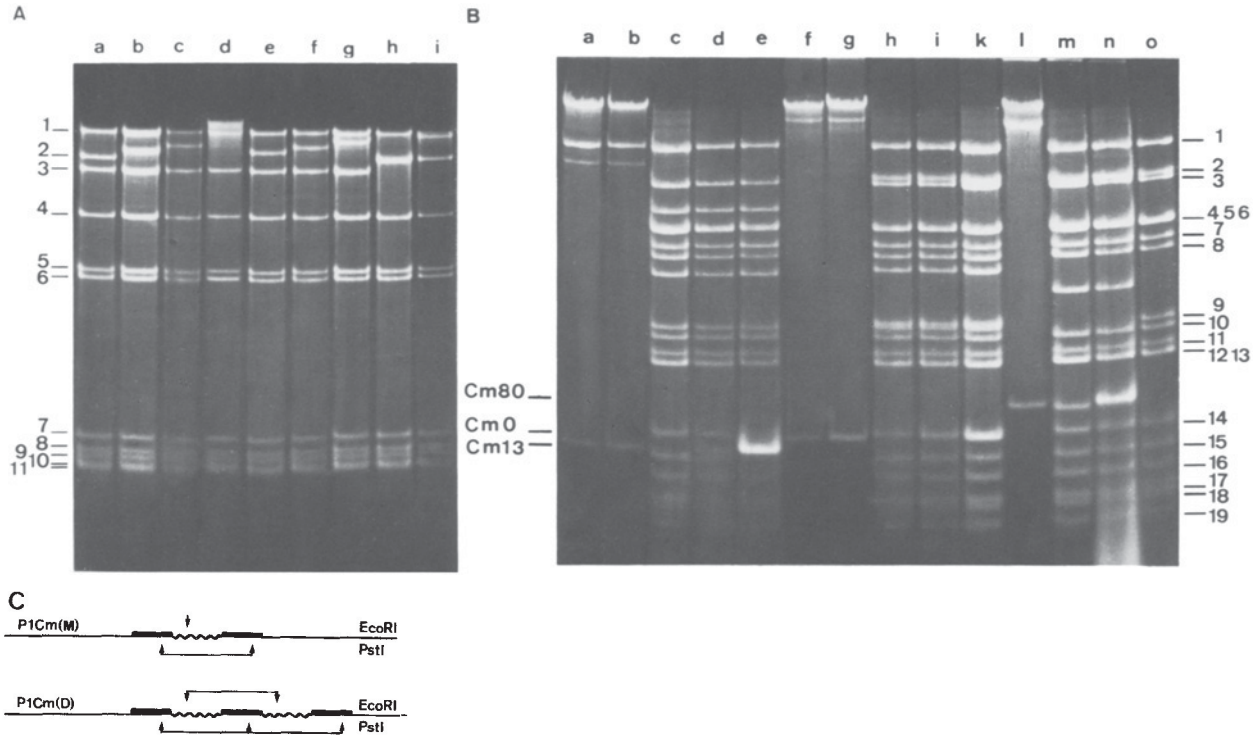

Fig. 4A–C. Monomer, tandem dimer and oligomer structure of Cm transposons revealed by agarose gel electrophoresis. See text for detailed explanation. **A** *Bgl*II digests of P1 and P1Cm DNA: (a) P1 DNA. The fragments are numbered to the left according to Bächi and Arber (1977); (b) P1Cm13 (M) DNA; (c) P1Cm88 (D) DNA; (d) P1Cm88 (A) DNA; (e) P1Cm0 (M) DNA; (f) P1Cm0 (D) DNA; (g) P1Cm0 (A) DNA; (h) P1Cm80 (M) DNA; (i) P1Cm80 (A) DNA. **B** *Pst*I and *Eco*RI digests of P1 and P1Cm DNA. (a) and (b) *Pst*I digests of P1Cm13 (M) and P1Cm88 (D) DNA. (c, d and e) *Eco*RI digests of P1Cm13 (M), P1Cm88 (D), and P1Cm88 (A) DNA. (f and g) *Pst*I digests of P1Cm0 (M) and P1Cm0 (D) DNA. (h, i and k) *Eco*RI digests of P1Cm0 (M), P1Cm0 (D), and P1Cm0 (A) DNA. (l) *Pst*I digest of P1Cm80 (M) DNA. (m to o) *Eco*RI digests of P1Cm80 (M), P1Cm80 (A) and P1 DNA. The numbers to the right identify the *Eco*RI fragments of P1 DNA according to Bächi and Arber (1977). The lines to the left give the positions of the "unit length" fragments (corresponding to the length of the Cm^r determinant plus one IS1, see Fig. 4C) of the respective P1Cm derivatives. **C** Schematic representation of the *Eco*RI and *Pst*I restriction cleavage of monomeric and dimeric Cm transposons on DNA of P1Cm13 (M), P1Cm88 (D), P1Cm0 (M), and P1Cm0 (D)

and g) is about 100 bp larger than that of P1Cm13 (M). Note that here the "unit length" fragment of P1Cm0 (D) almost comigrates in the gel with fragment *Eco*RI-14.

c) Cm Transposon Tn9 in P1Cm0. The Cm transposon Tn9 was physically characterized in λcam phages as an IS1-flanked Cm^r determinant (MacHattie and Jackowski, 1977) which was derived from phage P1Cm0 (Gottesman and Rosner, 1975). Phage P1Cm0 originated from growth of phage P1 in cells carrying an R plasmid now called pSM14 (Kondo and Mitsuhashi, 1964). We have determined that the Cm transposon carried by P1Cm0 is integrated into the IS1 site of P1 DNA (Iida et al., 1978a; Iida and Arber, 1979; this study) and that by *Pst*I restriction cleavage analysis it has the same size as Tn9 in λcam (data not shown).

2. Level of Resistance to Chloramphenicol of P1Cm Lysogens Containing a Monomer or a Dimer Cm Transposon

P1Cm lysogens were grown in the absence of chloramphenicol and then plated on agar containing increasing concentrations of the drug. The number of colonies formed was scored after incubation at 30° C for 48 h and the efficiency of colony formation was plotted as a function of the Cm concentration (Fig. 5). P1Cm lysogens carrying a dimer Cm transposon displayed a slightly higher degree of resistance to chloramphenicol than those with a monomer Cm transposon of the same size and inserted at the same location. Presumably, the increase in gene dosage allows faster inactivation of the drug. However, the location and/or size of the Cm transposon seem to be additional parameters influencing the level of resistance.

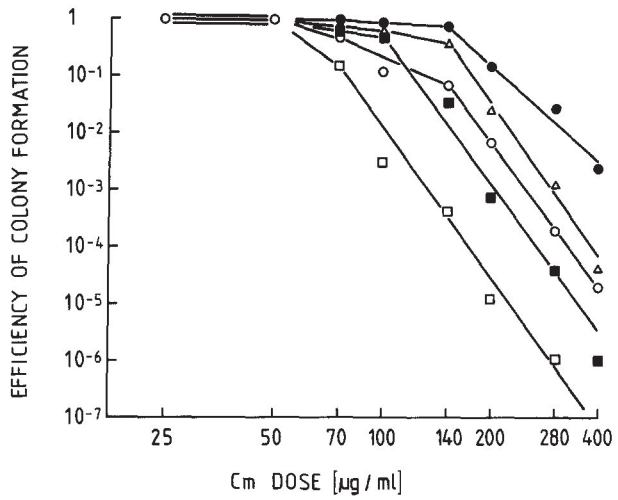

Fig. 5. Level of resistance to chloramphenicol of two pairs of P1Cm lysogens carrying monomer or dimer Cm transposons, compared to P1r-det lysogens. The efficiency of colony formation is plotted as a function of the chloramphenicol concentration in the plate. Lysogens – □ – P1Cm13 (M), – ■ – P1Cm88 (D), – ○ – P1Cm0 (M), – ● – P1Cm0 (D), – △ – P1r-det

An analysis of the Cm transposons in some of the P1Cm lysogens surviving 200 µg/ml Cm indicated that dimer Cm transposons were further amplified. Since the amplification of the r-determinant of R plasmids has been observed in *Proteus mirabilis* but not in *E. coli*, the phenomenon of duplication of Cm transposons carried on phage P1 DNA in the *E. coli* host was further studied.

3. *Higher Oligomeric Forms of Cm Transposons*

a) Populations of Increased Resistance to Chloramphenicol. P1Cm lysogens were grown to saturation in the presence of 300 µg/ml Cm. Subcultures were grown in the absence of the drug, phage P1 was induced and the phage DNA analysed by *Bgl*II and *Eco*RI restriction cleavage. Amplification of the Cm transposons was frequently observed when P1Cm lysogens with tandem dimer Cm transposons were grown in medium containing high concentrations of chloramphenicol (Table 4). The phenomenon could also be detected in lysogens of P1Cm (M), but in most experiments resistant populations produced P1 still carrying the original monomeric Cm transposon. The mechanism by which these cells may have acquired the higher resistance is discussed later.

Tandem oligomerization of the Cm transposons was revealed in restriction cleavage analysis by the following observations documented in Fig. 4. The "unit length" fragment produced once by *Pst*I cleavage of PlCm(M) DNA or by *Eco*RI cleavage of PlCm(D) DNA (Fig. 4C, Fig. 4B, slots a, d, f, i and l) is generated several times from P1Cm(A) DNA. The corresponding band thus appears much more intense (Fig. 4B, slots e, k and n). In *Bgl*II digests of P1Cm (A) DNA, the fragment carrying the multimeric Cm transposon is further enlarged. In P1Cm88 (A) there are several new *Bgl*II-2 bands, some even larger than the *Bgl*II-1 band (Fig. 4A, slot d) and the *Bgl*II-2 band corresponding to that of the original P1Cm88 (D) has faded. In P1Cm0 (A) there is also a faint *Bgl*II-2 band at the position of that of P1Cm0 (D) and a larger new *Bgl*II-2 band (Fig. 4A, slot g). In P1Cm80 the Cm transposon is inserted in the *Bgl*II-3 fragment which in P1Cm80 (M) results in an increase of its size to just below that of the *Bgl*II-2 fragment (Fig. 4A, slot h). In P1Cm80 (A) this fragment is further enlarged to almost the size of *Bgl*II-1 (Fig. 4A, slot i). Tandem oligomerization of the Cm transposons on P1 DNA was also confirmed by digestion with *Bam*HI and double digestion with *Bam*HI : *Pst*I and *Bgl*II : *Pst*I restriction endonucleases (data not shown).

Table 4. Amplification of Cm transposons in P1Cm lysogens grown in 300 µg/ml Cm

Prophage	Number of experiments	Number of phage populations with amplified Cm transposon
P1Cm13 (M)	5	1[a]
P1Cm88 (D)	7	6
P1Cm0 (M)	4	1[b]
P1Cm0 (D)	4	3

[a] Aberrant (see Discussion)
[b] Dimer

Furthermore, electron microscope analysis of 75 single-stranded *Bgl*II-2 DNA fragments derived from a population of P1Cm88 (A) carrying amplified Cm transposons revealed snapback structures analogous to those shown in Fig. 2. The length of the loops fell into distinct size classes, beginning with classes I – III and further increasing by steps of about 1.8 kb up to a size of 23 kb (indicating the presence of a tandem hexamer, 3 molecules). One molecule had an exceptionally large loop of 32 kb (suggesting a tandem undecameric Cm transposon). The degree of oligomerisation is higher than indicated by the size of the loops in these snapback molecules, and it can be determined from the total length of the *Bgl*II-2 fragments. The majority of the *Bgl*II-2 fragments had a size corresponding to the presence of a trimer, tetramer and pentamer Cm transposon.

Thus restriction cleavage and electron microscope analysis of P1Cm (A) DNA reveal mixtures of oligo-

mers of Cm transposons with a variable number of units. In many preparations the P1Cm (D) carrying the original dimer Cm transposon was still observed.

The plaque forming capacity of the P1Cm (A) phage particles was tested for a few clones only. The results confirm earlier findings (Iida and Arber, 1977; Arber et al., 1978; Iida and Arber, 1979) that insertions in the P1 DNA which are larger than the terminal redundancy (about 10 kb) decrease the efficiency of plaque-formation. However, due to the circular permutation of the phage DNA, an oversized genome can be reconstituted by recombination after infection of a cell by several phage particles, each carrying less than a complete genome.

b) Populations of Increased Resistance to Fusidic Acid. The R plasmid NR1 carries the Fa resistance determinant closely linked to and in the same operon as the Cm resistance gene (Lane and Chandler, 1977; Miki et al., 1978), possibly as overlapping genes (Marcoli, Iida and Bickle, in preparation). All of our P1Cm studied here also confer resistance to fusidic acid. In order to determine whether amplification of Cm transposons on P1 could be selected for by growing lysogens in the presence of high doses of fusidic acid, the Fa^s strain *E. coli* DB10 (Datta et al., 1974) was lysogenized with P1Cm88 (D). The resulting strain DB10 (P1Cm88 (D)) was grown in LB medium containing 300 μg/ml Fa or on LA plates containing 100 μg/ml Fa. Phages produced from these cultures also carried an oligomeric Cm transposon. The result was the same as for Cm selected populations. The degree of oligomerization of Cm transposons on P1Cm phage DNA was higher in phages obtained from colonies formed on plates with 100 μg/ml Fa than in those derived from cells grown in LB medium with 300 μg/ml Fa. Plates provide a stronger selection than liquid cultures containing equal concentrations of Fa (and we obtained similar results with Cm), presumably due to a slower diffusion of the drugs.

c) Amplification is Not Induced by the Selective Agent. The degree of amplification and the proportion of P1Cm genomes showing amplification differed from experiment to experiment. A typical example is given in Fig. 6 (also compare to Fig. 4A, slot d). Three single colonies of P1Cm88 (D) lysogens were grown in the presence of 25 μg/ml Cm and subcultures were made in medium containing 300 μg/ml Cm. Phage was induced and its DNA analysed as described. Judged from the mobility of the *Bgl*II-2 fragments containing the oligomeric Cm transposons, they contain predominantly trimeric (Fig. 6, slot c), tetrameric (slot b) or more than octameric (slot e) Cm transposons. However, when several subcultures derived from

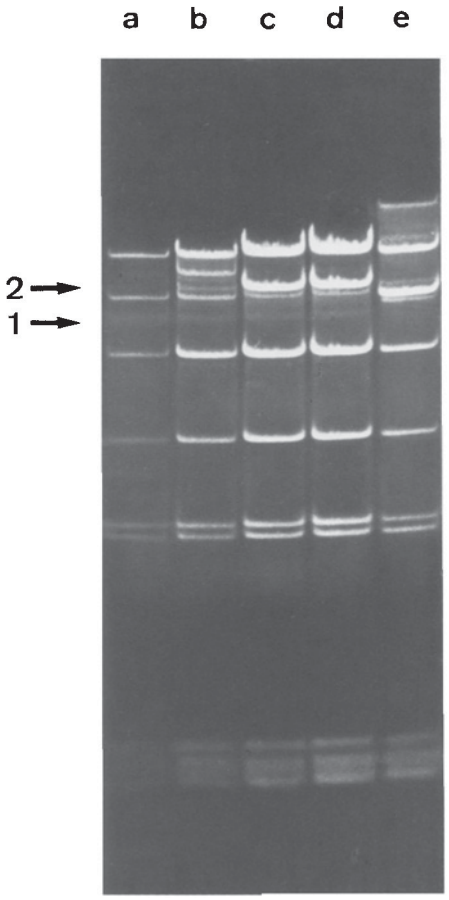

Fig. 6. Degree of amplification of the Cm transposon of P1Cm88 (A) shown by agarose gel electrophoresis of *Bgl*II digests. (a) P1Cm88 (D) DNA; (b to e) P1Cm88 (A) phage DNA derived from different cultures grown in the presence of 300 μg/ml Cm. See text for experimental details. Arrows give the positions of the *Bgl*II-2 band of P1 (arrow 1) and of P1Cm88 (D) (arrow 2) DNA

the same culture in medium containing 25 μg/ml Cm (originating from a single colony) were grown in the presence of 300 μg/ml Cm, they resulted in P1Cm with amplified Cm transposons which gave indistinguishable restriction cleavage patterns (typical example shown in Fig. 6, slots c and d). These observations suggest that the amplification occurs spontaneously and the lysogens carrying amplified Cm transposons outgrow those containing non-amplified Cm transposons in the selective medium.

This view is supported by the following experiment: *E. coli* DB10(P1Cm88 (D)) grown in 15 μg/ml Cm was separately grown in 300 μg/ml Cm and 300 μg/ml Fa. The lysogens grown in the two different selective media produced P1Cm with the same degree of amplification (data not shown).

d) Stepwise Increase of Cm Concentration. The proportion of P1 with an amplified Cm transposon exist-

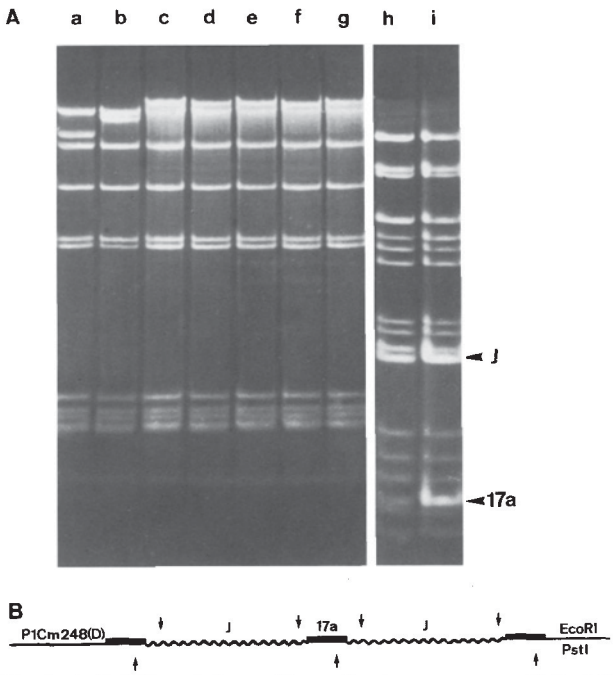

Fig. 7. A Stability of the multimer Cm transposon on P1Cm248 (A) DNA shown by agarose gel electrophoresis of *Bgl*II digests. (a) P1 DNA; (b) P1Cm248 (D) DNA (c) P1Cm248 (A) DNA derived from a population of cells adapted to 1 mg/ml Cm. (d) P1Cm248 (A) DNA derived from a population of cells grown in the absence of Cm for more than 40 generations. (e to g) P1Cm248 (A) DNA derived from cells grown from three individual colonies picked from the population of cells analysed in (d). Detection of the tandem multimer Cm transposon on P1Cm248 (A) DNA by agarose gel electrophoresis of *Eco*RI digests. (h) P1Cm248 (D) DNA. (i) P1Cm248 (A) DNA. The intensified bands J and 17a are labeled according to Arber et al. (1978). **B** Schematic representation of the *Eco*RI and *Pst*I restriction cleavage of P1Cm248 (D) DNA

ing prior to the treatment with high doses of Cm is too small to be detected by restriction cleavage or electron microscope analysis. It may even be so small that one culture step in the presence of 300 µg/ml Cm is not sufficient to enrich them to a detectable level, but cultures with stepwise increasing doses of Cm could allow their accumulation. Indeed, we did not observe P1Cm80 (M) derivatives with an amplified Cm transposon after treatment with 300 µg/ml Cm, but we did obtain such phages after gradual increase of the Cm concentration to 1 mg/ml (Fig. 4B, slot n).

Phage P1Cm248 (D), a plaque forming deletion derivative of P1CmSmSuHg81, carries a large dimeric Cm transposon with two *Eco*RI but no *Bgl*II sites per monomer unit (Arber et al., 1978). Amplified transposons were detected on P1Cm248 (A) obtained from cells adapted to increasing concentrations of chloramphenicol. Restriction cleavage analysis revealed a longer *Bgl*II-2 fragment containing the multimeric Cm transposon and two intensified *Eco*RI fragments (Fig. 7B; Fig. 7A, slots c, and i). We did not observe an amplification of a larger monomeric Cm transposon on P1CmSm81-1 or of the monomeric r-determinant on P1r-det (Arber et al., 1978), even after growing lysogens in medium with gradually increasing doses of Cm or streptomycin up to 1 or 2 mg/ml, respectively.

e) Stability of Oligomer Cm Transposons on P1 DNA. Single colonies of P1Cm0 (D) lysogens formed on plates without chloramphenicol were inoculated and grown in LB broth. All 25 colonies produced P1Cm still carrying the original dimer Cm transposon as determined by restriction cleavage analysis. Even after storage of lysogens in airtight stab bottles containing nutrient agar for three years, 8 out of 8 individual subclones derived from 4 different stabs produced P1Cm with the dimeric Cm transposon.

The stability of amplified Cm transposons was examined in lysogens of P1Cm248 (A) grown in the absence of Cm. Analysis of phage DNA revealed that the majority of induced P1Cm carried the original oligomeric Cm transposon even after growth in the absence of Cm for as many as 40 generations (Fig. 7A, slot d, compared to slot c). This finding was extended by the analysis of single clones derived from this population: 7 out of 10 colonies formed on LA plates produced P1Cm phage with the Cm transposon still amplified to the original level (Fig. 7A, slots e and g), one carried the Cm transposon amplified to a lower degree (Fig. 7A, slot f), and the remaining two colonies did not produce P1 phage (and were not further examined). The procedure was repeated with independently obtained lysogens of P1Cm248 (A): 3 out of 10 single colonies produced P1Cm carrying the unchanged amplified Cm transposon and the other 7 colonies did not produce P1 phage. We conclude therefore that amplified Cm transposons carried by P1Cm in rec^+ cells show considerable stability.

Discussion

P1Cm derivatives have been isolated which have acquired the r-determinant together with the two flanking IS1 from the R plasmid NR1. By IS1-mediated deletion formation smaller transposons evolved comprising the part of the r-determinant carrying genes for resistance to chloramphenicol and fusidic acid between the two IS1 elements. Most of the plaque forming P1Cm phages carry the Cm transposon in a monomeric form, some, however, carry a tandem dimer of the structure IS1 – Cm – IS1 – Cm – IS1. The cells harbouring a P1Cm plasmid with a dimer

Cm transposon were isolated as those resistant to the commonly used concentration of 25 μg/ml of chloramphenicol (as were those with a monomer), suggesting that the duplication occurred spontaneously.

Once dimers of Cm transposons are carried on P1Cm DNA further amplification to tandem multimers is readily observed in lysogens grown in the presence of high doses of chloramphenicol or fusidic acid. Our data bearing on the degree of oligomerization support the concept put forward by Hashimoto and Rownd (1975) that a small fraction of cells harbouring P1Cm with a spontaneously amplified Cm transposon have a growth advantage and therefore accumulate under highly selective conditions. Since chloramphenicol is bacteriostatic, lysogens carrying a nonamplified Cm transposon may also replicate after the drug has been inactivated. This results then in the mixed populations observed.

Spontaneous amplification occurs more frequently in P1Cm carrying a tandem dimer than in those with a monomeric Cm transposon. All 4 tandem dimer Cm transposons tested were amplifiable to higher oligomers. The size of the monomer unit varied between 2.6 kb (P1Cm88 (D)) and 4.8 kb (P1Cm248 (D)) and they were carried at two different loci of the P1 genome (map units 4 and 20).

On several occasions we detected the duplication and amplification of monomeric Cm transposons on phage P1 DNA (Fig. 4, Table 4). Amplification of the Cm transposon Tn9 carried by plasmids was also observed by Chandler et al. (1979) and by R. Mattes and R. Schmitt (personal communication). Some limitations in detecting oligomerisation of Cm transposons could be due to the fact that we analyzed phage DNA. All amplifications of Cm transposons on P1Cm plasmid DNA which abolished lytic phage functions would have remained unnoticed in our experimental procedures. It should be mentioned in this context that in many experiments a fraction of cells originally lysogenic for a P1Cm (M) did not produce phage particles after treatment with a high dose of chloramphenicol. But the majority of P1 phages obtained from the heterogenous population of cells in such cultures were the original P1Cm (M).

Besides the tandem amplification of the Cm^r gene of P1Cm DNA several other mechanisms were probably involved in the development of the high level of drug resistance. Some of them may be accompanied by DNA rearrangements. For example, it is conceivable that the Cm^r gene is transposed into the host chromosome and then further amplified (we have not tested this possibility). However, we have observed amplification of a Cm transposon on P1Cm DNA accompanied by DNA rearrangement. Preliminary structural analysis indicate that an additional Cm transposon was transposed into a different site on the same P1Cm DNA followed by amplification of these two Cm transposons together with the P1 DNA segment between them. This complicated duplication was observed in phage induced from cells which had been lysogenic for P1Cm13 (M) and grown in the presence of high concentration of chloramphenicol (Table 4). In contrast to findings in *Proteus mirabilis* (Rownd and Mickel, 1971; Tanaka et al., 1976) amplification of the r-determinant in NR1 or R100 (Chandler et al., 1979) or on P1CmSmSuHg81 has not been observed in *Escherichia coli* (this paper).

Both dimeric and amplified Cm transposons are rather stable even in recombination proficient host cells grown in the absence of the drug. This suggests that the frequency of homologous recombination leading to less amplified structures is not high enough to be detected by our procedures, and that in drug free medium there is no significant difference in growth rate between cells harbouring Cm transposons amplified to various degrees. In *Proteus mirabilis* instability of the amplified r-determinant has been reported (Hashimoto and Rownd, 1975).

Two types of models have been proposed to explain the tandem amplification of drug resistance genes on R plasmids (Hu et al., 1975; Ptashne and Cohen, 1975; Clewell and Yagi, 1977; Anderson and Roth, 1977). One model involves excision of resistance transposons followed by reintegration into a second R plasmid molecule. In the R plasmid NR1 excision and reintegration occurs by reciprocal recombination between two IS1 elements (Hu et al., 1975; Ptashne and Cohen, 1975). In the other type of model recombination between the daughter chromosomes after replication gives rise to tandem duplication and deletion (Anderson and Roth, 1977; Clewell and Yagi, 1977). According to this model duplication of a monomeric Cm transposon carried on a P1Cm plasmid must occur by recombination between two IS1 (Fig. 8A). Further amplification of a dimer repeat may result from recombination either between homologous Cm^r genes or again between IS1 elements (Fig. 8B).

If excision and reintegration contributed significantly to the occurrence of amplified Cm transposons at locations other than map unit 20 on P1 DNA, reintegration would be expected to occur not only within the resident Cm transposon but also into the IS1 locus of P1 DNA. This has not been observed. Beside the one derivative mentioned, all amplified Cm transposons resided at the insertion site of the original Cm transposon. Therefore we favour the second model, but our data do not rule out the first one.

Recombination between two IS1 elements was

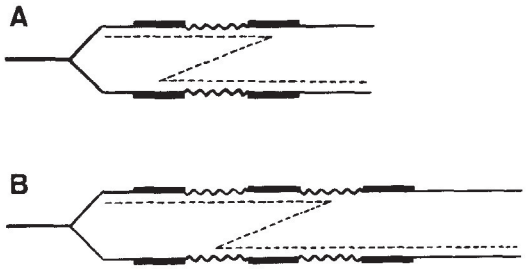

Fig. 8A and B. A model for the tandem oligomerisation of the IS1-flanked Cm transposon. **A** explains how a tandem dimer could result from recombination, after replication, between IS1 elements of the two daughter monomer Cm transposons. **B** indicates further amplification by recombination between the homologous Cm^r determinants resulting in triplication. Recombination could also occur between IS1 elements leading to tandem trimer or tetramer Cm transposon

observed under *rec*$^-$ condition but it occurred more frequently in *rec*$^+$ cells (MacHattie and Jackowski, 1977; Chandler et al., 1978; S. Iida, unpublished results). Therefore the amplification of Cm transposons is expected to be enhanced by the *recA* dependent recombination pathway and tandem dimers provide higher probability of recombination. Indeed, Chandler et al. (1979) could detect amplification of a monomeric Tn9 under *rec*A$^-$ condition only at a very low level.

A high level of drug resistance due to an amplification of resistance gene(s) has been observed repeatedly in the laboratory. The examples studied (Tanaka et al., 1976; Clewell and Yagi, 1977; Chandler et al., 1979; this study) indicate that resistance genes flanked by directly repeated DNA sequences may readily form tandem multimers thus generating an increase in gene number. Whether amplification phenomena also occur during antibiotic therapy remains to be determined.

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