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Electron Microscopic Mapping of Deletions on a Streptococcal Plasmid Carrying Extraordinarily Long Inverted Repeats

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Deletions Δ101, Δ102, and Δ103 which occurred within the extraordinarily long inverted repeats of the self-ligated large *Eco*RI fragment of the streptococcal MLS (macrolides, lincosamides, streptogramin B)-resistance plasmid pSM19035 led to the formation of plasmids pDB101, pDB102, and pDB103. Their molecular lengths were determined by contour length measurements to be 17.8, 17.4, and 13.9 kb, respectively. Electron microscopic examination of self-annealed molecules revealed stem-loop structures with inverted repeats comprising 41 to 91% of the mass of plasmids. Two unique sequences (US_1 and US_2) separated the inverted repeats in the case of pDB101 and pDB103, while in pDB102 the repeats were joined at one end and separated at the other by a unique sequence (US_2). The size of the unique sequence US_2 was identical for all three plasmids, and the location of the resistance determinant was determined by electron microscopic examination of self-annealed molecules of the recombinant plasmid pDB201. Mapping of the deletion termini, accomplished by combining electron microscopic and *Hin*dIII restriction data, suggested that deletions may occur at preferential sites.

The 18-Mdal[3] streptococcal plasmid pSM-19035 (Behnke *et al.*, 1979a) specifies inducible resistance to macrolides, lincosamides, and streptogramin B-type antibiotics (MLS) (Malke, 1974). It has been shown by restriction enzyme analyses (Behnke *et al.*, 1979a) and electron microscopy (Boitsov *et al.*, 1979) that the plasmid has extraordinarily long inverted repeat sequences that comprise about 80% of the molecule. These sequences are separated by two nonrepeated segments equivalent to 14 and 5% of the pSM19035 genome. Cleavage of pSM19035 with *Eco*RI gave rise to two fragments of about 14 and 4 Mdal; with *Hin*dIII, 15 fragments were obtained, 12 of which represented six pairs of identical fragments derived from the inverted repeats (Behnke *et al.*, 1979a). In order to construct a potential streptococcal vector plasmid featuring a single *Eco*RI cleavage site, the purified large *Eco*RI fragment of pSM19035 was self-ligated and afterward introduced into the Challis strain of *Streptococcus sanguis* by transformation (Behnke *et al.*, 1979b). Subsequent analyses of plasmid DNA isolated from three such transformants revealed that the ligated fragment had suffered deletions of various extents, as a result of sequence rearrangements during or following the transformation process. As a result of deletions designated Δ101, Δ102, and Δ103, three smaller plasmids were obtained. Only one of these plasmids, pDB101, retained the single *Eco*RI cleavage site while the other two pDB102 and pDB-103, were devoid of this site. *Hin*dIII analyses of the deletions of the three plasmids, together with *Hin*dIII cleavage patterns of the purified *Eco*RI fragments of pSM19035, allowed the construction of *Eco*RI/*Hin*dIII restriction site maps for pSM-

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[3] Abbreviations used: Mdal, megadalton; MLS, macrolides, lincosamides, and streptogramin B-type antibiotics; kb, kilobase.

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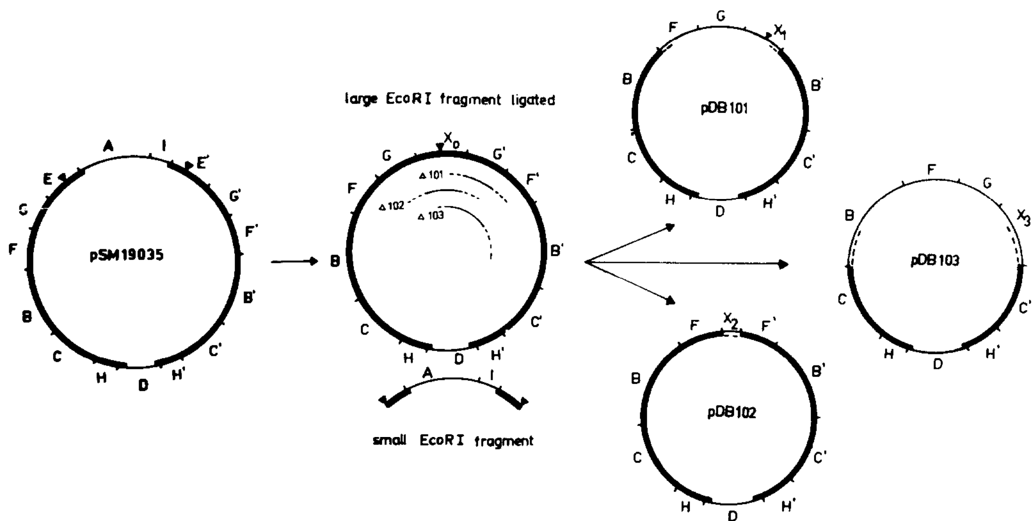


FIG. 1. Derivation of plasmids pDB101, pDB102, and pDB103 and *Hin*dIII/*Eco*RI cleavage site maps of the plasmids involved. Inverted repeats are indicated by heavy lines; their extension into the terminal *Hin*dIII fragments are marked by dotted lines. Light lines represent unique sequences. Arrows label the positions of *Eco*RI cleavage sites while the letters refer to *Hin*dIII fragments. Within the self-ligated large *Eco*RI fragment, the extent of deletions Δ101, Δ102, and Δ103 leading to the formation of the respective plasmids are indicated.

19035 and its derivative plasmids (Behnke *et al.*, 1979b; Behnke and Ferretti, 1980a). The results are summarized in Fig. 1. The deletions included portions of one or both of the repeated sequences, and all of them overlap. (No difference in either the type or level of MLS resistance was detectable in the case of the molecules with deletions).

In this paper we report on electron microscopic analyses of the deletion-containing molecules, and combine the data with restriction enzyme analyses to map precisely the boundaries of the deletions. The data suggest that the deletions may involve preferential sites on the plasmid. In addition, the MLS resistance determinant was

TABLE 1

STREPTOCOCCAL STRAINS USED AS SOURCES FOR PLASMID DNA

Strain	Description of the plasmid	Reference
Challis (pDB101)	pDB101, a deletion mutant of the MLS-resistance plasmid pSM19035	Behnke *et al.*, 1979b; Behnke and Ferretti, 1980a
Challis (pDB102)	pDB102, a deletion mutant of the MLS-resistance plasmid pSM19035	Behnke *et al.*, 1979b
Challis (pDB103)	pDB103, a deletion mutant of the MLS-resistance plasmid pSM19035	Behnke *et al.*, 1979b
Challis (pDB201)	pDB201, a recombinant plasmid between pVA318 and the MLS-resistance determinant of pDB102	Behnke and Ferretti, 1980b
V318	pVA318, a 5.4-kb cryptic plasmid having a single *Hin*dIII restriction site	Macrina and Scott, 1978

observed to be located on one of the unique sequences of pSM19035.

MATERIALS AND METHODS

Bacteria. All bacterial strains which served as sources for the isolation of plasmid DNA are listed in Table 1. The *Streptococcus mutans* strain V318 was kindly provided by F. Macrina. Brain heart infusion broth from Difco was used to grow all strains.

Isolation of plasmid DNA. Growth in the presence of DL-threonine (Chassy, 1976) or glycine (Reider and Macrina, 1976) facilitated lysis of *S. sanguis* or *S. mutans*; respectively. Deproteinized cleared lysates were subjected to dye-buoyant density gradient centrifugation to isolate and purify plasmid DNA (Behnke and Ferretti, 1980a). All plasmid DNAs were kept at 4°C in 10 mM Tris–HCl (pH 7.4).

Electron microscopy. The techniques used for electron microscopy of native and self-annealed plasmid molecules were as described elsewhere (Davis *et al.*, 1971; Sharp *et al.*, 1972; Yagi and Clewell, 1976). An Hitachi HS-8 electron microscope was used throughout these experiments. Bacteriophage ϕX174 RFII DNA (5.386 kb) (Sanger *et al.*, 1977) served as a molecular length standard for both double- and single-stranded DNA. For the latter ϕX174 RFII DNA was subjected to the same denaturation–renaturation procedure as the plasmid DNAs. All measurements were made after projection on a Nikon profile projector (Model 60).

RESULTS

Molecular Length of Native Plasmids and Their Different Regions after Self-Annealing of Denatured Molecules

The molecular length of native plasmid DNA was determined by contour length measurement of open circular molecules [generated by heating at 95°C for 10 min in buffer (Yagi and Clewell, 1976)] to be 17.9 kb for pDB101, 17.4 kb for pDB102, and 14.0 kb for pDB103 (Table 2). These results corresponded to molecular lengths of 17.8, 16.9, and 14.5 kb for the respective plasmids, as calculated from restriction enzyme analyses (Behnke *et al.*, 1979b). The slight discrepancies between the two values for pDB102 and pDB103) are within experimental error.

Denaturation of open circular plasmid molecules followed by a short renaturation period allowed the formation of intrastrand duplex regions as a result of reannealing of inverted repeat sequences. As expected from restriction data stem-loop structures were observed for all three plasmids indicating the presence of inverted repeat se-

TABLE 2

MOLECULAR LENGTH OF NATIVE PLASMIDS AND THEIR DIFFERENT REGIONS AFTER DENATURATION AND INTRASTRAND ANNEALING[a]

Plasmid	Molecular length of native molecules	Self-annealed molecules: US_1	US_2	IR	Total
pDB101	17850 ± 890 (10)[b]	3050 ± 270	1440 ± 200	6870 ± 530	18230 ± 1530 (9)[b]
pDB102	17400 ± 540 (10)	—	1460 ± 170	7760 ± 560	16980 ± 1290 (12)
pDB103	13950 ± 1020 (8)	6320 ± 560	1310 ± 180	3420 ± 390	14470 ± 1520 (6)
pDB201	7160 ± 570 (6)	5280 ± 240	1530 ± 180	240 ± 30	7290 ± 480 (12)

[a] The molecular lengths are given in nucleotides or nucleotide pairs (bp). US, unique sequences; IR, inverted repeat.

[b] The figures in parentheses refer to the number of molecules measured.

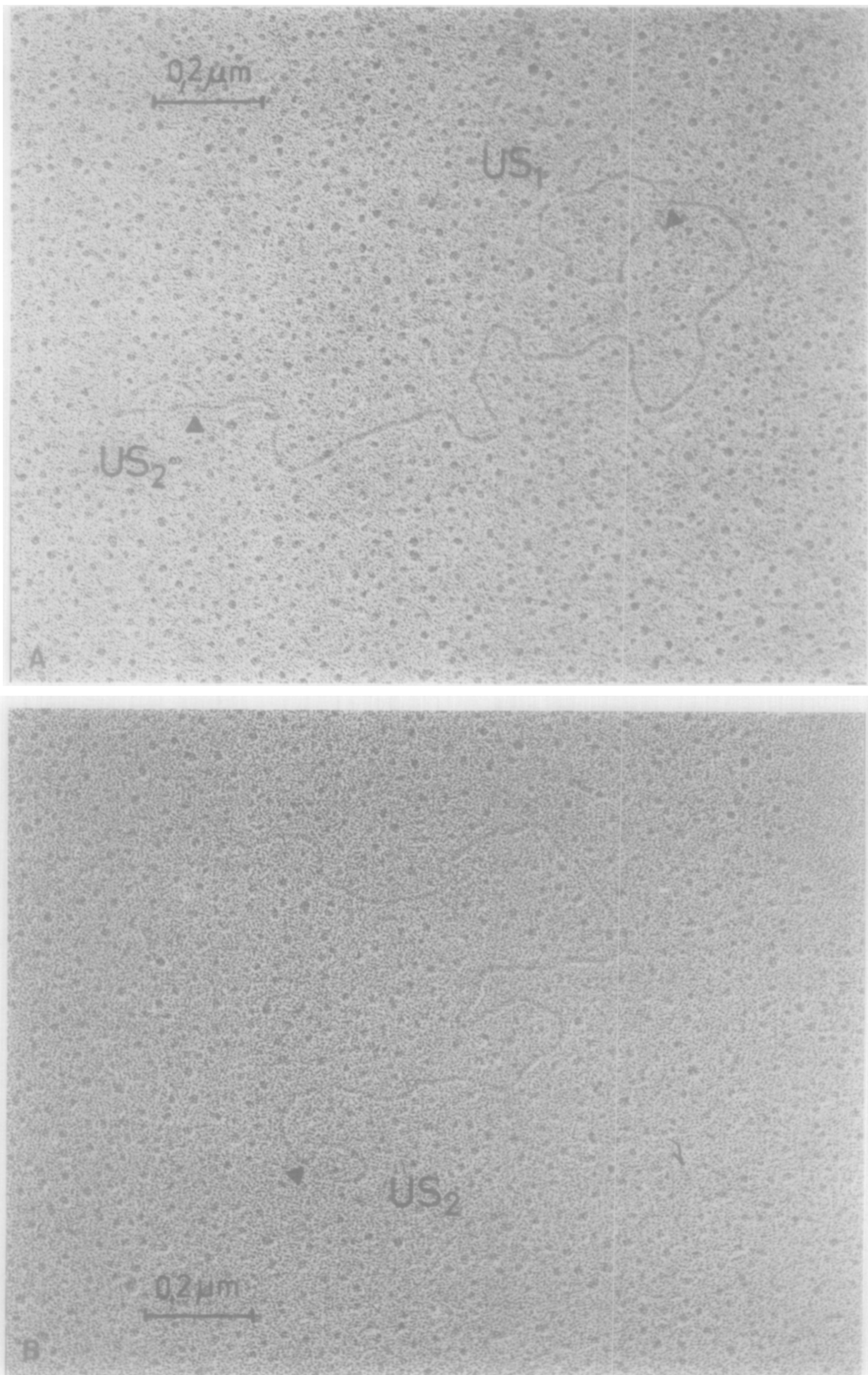
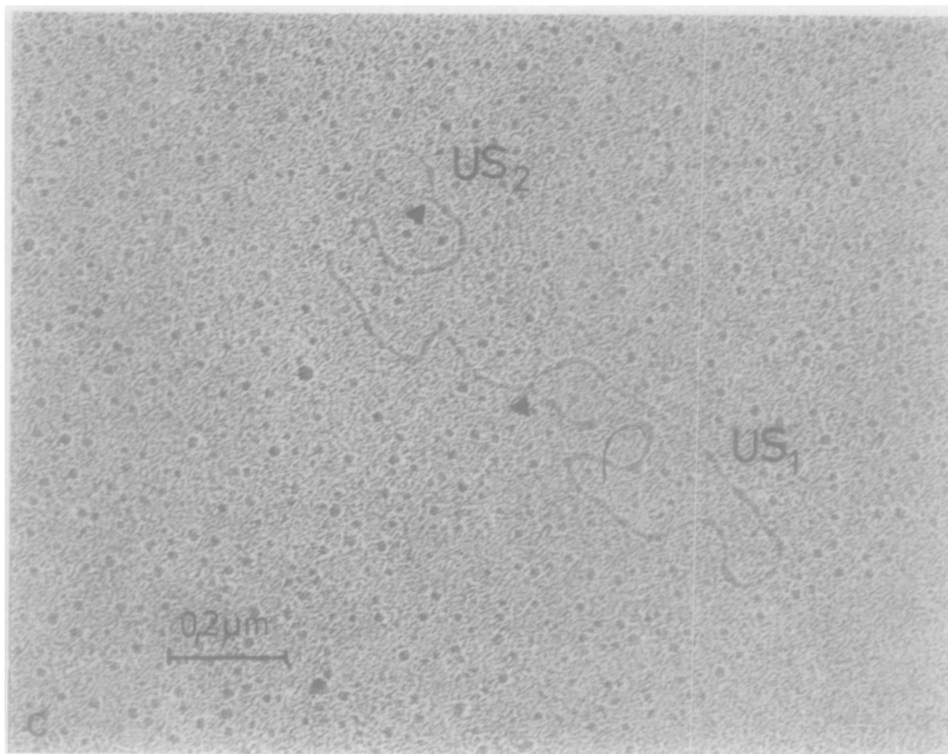


FIG. 2. Electron micrographs of denatured and self-annealed plasmid molecules. Junction points between double-stranded regions and single-stranded loops are marked by arrows. (A) pDB101; (B) pDB102; (C) pDB103; (D) pDB201.



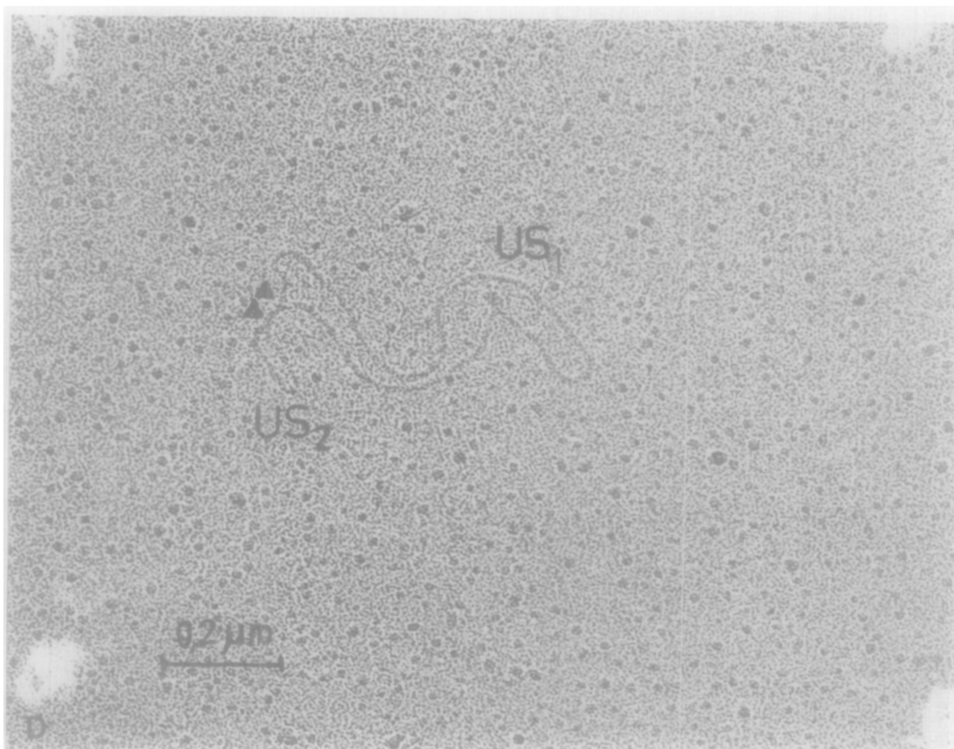


FIG. 2—*Continued.*

quences of different lengths for pDB101, pDB102, and pDB103. Electron micrographs of typical molecules are shown in Figs. 2A–C. In self-annealed molecules of plasmids pDB101 and pDB103 single-stranded loops were observed on both ends of the continuous central double-stranded region (Figs. 2A and C), thus indicating the presence of two interstitial unique sequences (US_1 and US_2) that separate the inverted repeat stretches in these plasmids. In contrast, only one terminal single-stranded DNA loop was detectable in self-annealed molecules of pDB102 (Fig. 2B). Plasmid pDB102, therefore, consisted of two inverted repeats which were connected with each other at one end and separated at the other by a short intervening sequence (US_2). (A second interstitial sequence shorter than 50–75 base pairs may have escaped detection by electron microscopic techniques.)

The molecular length of inverted repeats and nonrepeated sequences were determined by measuring the different regions of denatured and self-annealed plasmid molecules (Table 2). Plasmid pDB103 was the result of the largest deletion (Δ103) reducing the inverted repeat regions to only 47% of the plasmid genome and generating a large unique sequence (US_1) that comprised 44% of the molecule. The symmetrical deletion Δ102 led to the formation of pDB102 which had an extreme of more than 91% of duplicated DNA in its genome. Relations of inverted repeat to nonrepeated sequences of pDB101 were similar to those observed for the parental plasmid pSM-19035. For all three plasmids the smaller of the two unique sequences (US_2) was similar in size, averaging a length of 1.4 kb (Table 2). This value was consistent with the one determined previously for US_2 in self-annealed pSM19035 molecules (Boitsov *et al.*, 1979).

Localization of the Resistance Determinant on US_2

Based on restriction data it was deduced that US_2 consisted of the *Hin*dIII fragment D into which part of the inverted repeats extended (Fig. 1). Conclusive evidence for this was derived from electron microscopic examination of pDB201—a recombinant plasmid consisting of the cryptic *S. mutans* plasmid pVA318, which has a single *Hin*dIII site, and the inserted pSM19035 *Hin*dIII fragment D which carried the resistance functions (Behnke and Ferretti, 1980b). Self-annealed pDB201 molecules showed a short double-stranded region (~240 base pairs) and two single-stranded loops (Fig. 2D), the smaller of which was identical in size with US_2 of pSM19035 and its derivatives (Table 2). The larger loop corresponded to the molecular length of pVA318 (5.4 kb) (Macrina and Scott, 1978). No stem-loop structures were observed when denatured pVA318 molecules were allowed to self-anneal (data not shown). Therefore, the small loop (resembling US_2) together with the short double-stranded region of ~240 base pairs found with pDB201 originated from the cloned *Hin*dIII fragment D—the location of the resistance determinant of pSM19035 and its derivatives.

Mapping of the Deletion Termini in pDB101, pDB102, and pDB103

Mapping of the deletion termini was accomplished by combining *Hin*dIII restriction data with results obtained from electron microscopic examination of self-annealed plasmid molecules. To facilitate comprehension, Table 3 and Fig. 3 give a compilation of the data and an example of the logic on which the location of the deletion termini was based. Plasmids pDB101, pDB102, and pDB103 contained the new *Hin*dIII fragments X_1, X_2, and X_3 that were fusion products of the nondeleted remainders of those two fragments in which the deletions terminated (Figs. 1 and 3). Since all of the deletions include part of at least one of the inverted repeats, this fusion point can be located by determining the length of that portion of the X fragment that contains repeated sequences. In the case of pDB101 (see Fig. 3), this portion should anneal with

TABLE 3

COMPILATION OF THE DATA USED TO LOCATE THE DELETION TERMINI OF Δ101, Δ102, AND Δ103[a]

Plasmid	Deleted *Hin*dIII fragments[b]	Unique *Hin*dIII fragments	Molecular length of the unique *Hin*dIII fragments[c] (A)	Length of US_1 as determined by electron microscopy (B)	Extension of the inverted repeats into X fragments[d] $\left(\frac{A-B}{2}\right)$
pDB101	(X_0), G′, (F′)	F, G, X_1, D	4810	3050	880 (F or F′)
pDB102	(G), X_0, (G′)	X_2, D	680	—	340 (G, G′)
pDB103	(X_0), G′, F′, (B′)	B, F, G, X_3, D	7140	6320	410 (B or B′)

[a] All molecular lengths are given as nucleotides or nucleotide pairs (bp).

[b] The fragments indicated formed the deletions Δ101, Δ102, and Δ103 yielding plasmids pDB101, pDB102, and pDB103, respectively. The nondeleted remainders of the termination fragments (indicated by parenthesis) fused to the *Hin*dIII fragments X_1, X_2, and X_3.

[c] These values do not include the unique fragment D since it has been shown to form the unique sequence US_2. All molecular lengths were calculated from restriction data (Behnke *et al.*, 1979b; Behnke and Ferretti, 1980a).

[d] The extentions of the inverted repeats into the X fragments are identical with the nondeleted remainder of one of the termination fragments (the particular fragment is given in parenthesis), thus, yielding their fusion point which allows the location of the map positions of the deletion termini in the original *Hin*dIII fragments.

an equivalent portion of fragment F. The length of this annealed portion corresponds to one-half of the difference between the sum of the unique *Hin*dIII fragments (ignoring fragment D) and the length of US_1 determined by electron microscopy (Table 3). In this manner the fusion points were located, and the map positions of the deletion-termini on the original *Hin*dIII fragments were calculated from the length of their nondeleted residues contributing to the X fragments.

Detailed map positions for Δ101, Δ102, and Δ103 are shown in Fig. 4. One deletion

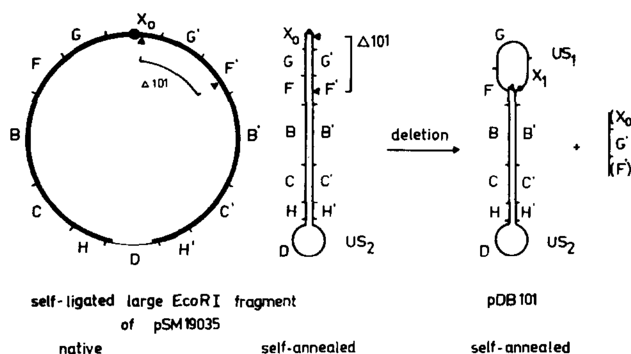


FIG. 3. Example of the logic on which the localization of deletion termini was based. *Hin*dIII fragment X_1 was the fusion product of the nondeleted remainders of those *Hin*dIII fragments where Δ101 terminated. One of these residues (belonging to F′) formed the extension of the inverted repeat of pDB101 into fragment X_1 and could, therefore, be determined as half of the difference between the total length of fragments F, G, and X_1 (covering US_1) and the actual size of US_1. The position of the fusion point then allowed determination of the map positions of the deletion termini on the original *Hin*dIII fragments. Solid circles indicate *Eco*RI cleavage sites and arrows point to the deletion termini. The heavy line in the native ligated *Eco*RI fragment represents inverted repeats and the light line the interstitial sequence.

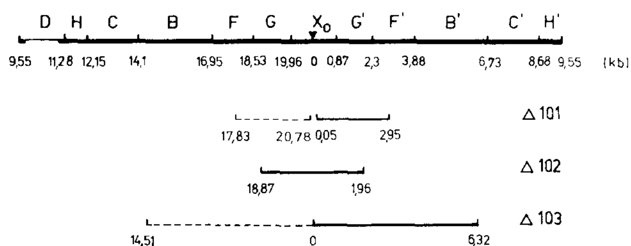


FIG. 4. Map locations of the deletions Δ101, Δ102, and Δ103 in relation to *Hin*dIII cleavage sites on the map of the linear large *Eco*RI fragment of pSM19035. The single *Eco*RI cleavage site (arrow) represents the junction point between the two inverted repeats (heavy lines). Two locations are possible for deletions 101 and 103 (solid or dotted lines) because of the internal symmetry of the molecule.

terminus of Δ101 was located ~50 base pairs away from the single *Eco*RI site leaving it unaffected. In contrast, Δ103 removed the single *Eco*RI site; thus, the deletion extended beyond this point. This Δ103 endpoint has been mapped close to the *Eco*RI site and it would appear to be in a similar position as the Δ101 terminus only on the opposite side of the *Eco*RI site. No real significance can be assigned to the ~50 base pair deviation of the positions of these two deletion termini, since the method used to calculate their locations does not yield such resolution. Deletion Δ102 extended into both repeats appearing to have symmetrically located end points.

DISCUSSION

Introduction of the self-ligated large *Eco*-RI fragment of pSM19035 into Challis by transformation is accompanied by deletions of DNA segments from its extraordinarily long inverted repeats. These repeats cover about 92% of the total molecule (Behnke *et al.*, 1979b). Analyses of single-stranded loops and double-stranded regions in self-annealed molecules of plasmids pDB101, pDB102, and pDB103 (Figs. 2A–C; Table 2) established the location and the extent of the deletions. Mapping of the deletion termini suggests the interesting possibility that preferential sites may be located on the *Hin*dIII fragments G or G′ and X_0. One terminus of the deletions Δ101 and Δ103 was localized close to, although on different sides of, the single *Eco*RI site. It is conceivable that these sites are identical distances from the EcoRI site and represent identical points in the two inverted repeats. Deletion Δ102 extended into both of the inverted repeats with apparently symmetrical endpoints on the sister *Hin*dIII fragments G and G′. The possible presence of preferential sites for deletion termini could reflect hot spots for internal recombinational events. However, as reciprocal recombination between symmetrical sites in a pair of inverted repeat sequences will result in inversion and not deletion of the intervening segment, it is difficult to account for Δ102 on this basis.

Electron microscopic analysis of a recombinant plasmid consisting of the small cryptic plasmid pVA318 (Macrina and Scott, 1978) and the unique *Hin*dIII fragment D cloned from pDB102 (Behnke and Ferretti, 1980b) showed that the latter fragment gives rise to the unique sequence US_2. This fragment contains the MLS resistance determinant and is present in all three of the deleted plasmids, as well as the original pSM19035.

Insofar as the US_2 sequence (1.4 kb) is relatively small (room for only one or a few genes), it probably contains genetic information related only to MLS resistance. This brings up the very interesting question regarding the location of genes related to replication. Since the remainder of the pDB101, pDB102, and pDB103 molecules consists of sequences that are repeated in pSM19035, the replication functions must be located on these sequences. Thus, all

replication genes must be present in duplicate and arranged in reverse orientation with respect to each other. It also follows that there are probably two origins of replication. Two replication origins have been reported in the case of the R6K (Crosa *et al.*, 1975; Lovett *et al.*, 1975) and NR1 (Perlman and Rownd, 1976). There is no evidence, however, for the involvement of extensive duplicate segments of origin-containing DNA in these plasmids. Analyses of the replication of pSM19035 should yield information on whether two origins are in fact used, and whether replication from the origin(s) is unidirectional or bidirectional. Analysis of replication might also shed some light on why pSM19035 stably maintains such extraordinarily long duplicate sequences.

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