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Plasmid Structural Instability Associated with pC194 Replication Functions

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The hybrid plasmid pJS37 is composed of the streptococcal plasmid pLS1, which confers tetracycline resistance, and the staphylococcal plasmid pC194, which confers chloramphenicol resistance. When gram-positive bacteria containing pJS37 were grown in the presence of chloramphenicol, four different deleted derivatives accumulated. The deletions in the plasmid enhanced resistance to chloramphenicol by placing the *cat* gene of pC194 near promoters of pLS1. All four deletions shared a common endpoint that corresponded to the putative target site for DNA strand nicking by the pC194 replication protein, RepH. At the other, variable endpoint, the DNA sequence was similar to the putative RepH target sequence. Alteration of the RepH protein, by in vitro modification of the gene encoding it, eliminated this class of deletions. By extending a previously proposed model for the generation of a different but related class of deletions (B. Michel and S. D. Ehrlich, EMBO J. 5:3691-3696, 1986), a comprehensive model that could generate both classes of deletions is suggested. It proposes that a nicking-closing activity of the plasmid replication protein at its normal target site and, aberrantly, at sites with similar sequence can generate deletions either proximal or distal to the aberrant site during rolling-circle replication of the plasmid.

The generation of deletions in recombinant plasmids by intramolecular rearrangement is a common phenomenon. Many of these deletions involve short direct repeats. Although some of them may arise by typical homologous recombination between the direct repeats, which in *Escherichia coli* requires *recA* function, others occur in *recA* mutants apparently as a result of other mechanisms for recombining DNA (1). Two kinds of intramolecular recombination events have been reported. In the first kind, the endpoints of deletions occur within short direct repeats (1, 3, 11, 20); in the second, deletions occur between nonidentical sequences. The latter kind has been observed in *Bacillus subtilis* (20) as well as in *E. coli* (15). Host cell mechanisms that are independent of *B. subtilis* RecE or *E. coli* RecA functions were thought to be responsible for both kinds of deletions. For *E. coli*, models involving slipped mispairing during DNA replication (1) and DNA gyrase subunit exchange (13) have been proposed. In the case of *B. subtilis*, Lopez et al. (20) postulated that a consensus sequence at the endpoints of some deletions constituted a recognition site for a topoisomeraselike enzyme that could generate deletions similarly to gyrase.

In addition to these mechanisms, the plasmid replication machinery may be responsible for generating deletions (11, 24). In *B. subtilis*, a hybrid plasmid composed of pUB110 and pSA2100 (itself composed of pC194 and pSA0501) showed a systematic *rec*-independent deletion between 18-base-pair (bp) directly repeated sequences (11) located at the plasmid origins of replication (9, 21). In *E. coli*, deletion formation was examined in hybrids between plasmids pC194 and pBR322 and either coliphage M13 or coliphage f1. When the triple hybrids carried M13, one of the deletion endpoints was located mainly at the site nicked by the phage gene II protein (23). A similar analysis, using hybrids with phage f1, suggested that a nick introduced in the postulated replication origin of pC194 by the plasmid replication protein served as a deletion endpoint (24). Since accumulation of single-stranded DNA during the replication of pC194 indicates that this plasmid replicates by a rolling-circle mechanism (30), there appears to be a correlation between deletion formation and replication through single-stranded intermediates.

We have previously shown that the hybrid plasmid pJS37, composed of plasmids pLS1 and pC194, which carries two origins of replication instead of three as in the examples cited above, undergoes systematic deletions in *Streptococcus pneumoniae* when adequate selection is applied (3). It was postulated that the deletions occurred spontaneously at a low frequency and that positive selective pressure allowed their detection. Here we report a correlation between generation of the deletions and activity of the replication initiation protein of pC194, RepH (1a, 14), which has also been called protein A (5). The hybrid plasmid pJS37 accumulates single-stranded intermediates similar in amount to those of pC194. Upon selection for high levels of chloramphenicol resistance, deleted derivatives could be obtained in *S. pneumoniae* and in *B. subtilis*. In four different derivatives, one of the deletion endpoints occurred at a fixed site located in the region of the replication origin of pC194. Drastic alteration of the gene encoding RepH in pJS37 eliminated this category of deletions. A model is presented for the generation of deletions in pJS37.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. pneumoniae* 708 (*end-1 exo-2 trt-1 hex-4 malM594*) carrying various plasmids was used throughout this work; it was grown in a casein-hydrolysate-based medium previously described (18). *B. subtilis* MB11 (*lys-3 metB10 hisH2*) was grown in L broth or, for development of competence for plasmid transformation, in minimal medium (7). Competence development and trans-

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TABLE 1. Plasmids used

Plasmid	Size (bp)	Marker(s)	Reference(s)	Observations
pC194	2,907	Cm	5, 12	See Materials and Methods for sequence correction
pLS1	4,408	Tc	19	
pJS37	7,315	Tc, Cm	3	Hybrid pLS1-pC194
pJS370	6,260	Tc, Cm	This work	Deleted small *Pst*I fragment of pJS37
pJS38	7,317	Tc, Cm	This work	Filled-in *Acc*I site of pJS37 at 5657
pJS39	6,856	Tc, Cm	This work	Deleted small *Nsi*I fragment of pJS37
pJS1	4,390	Cm	This work	In vivo deletion of pJS37 in *B. subtilis*
pJS3	4,191	Cm	3	In vivo deletion of pJS37 in *S. pneumoniae*
pJS4	3,354	Cm, *repB*	3; this work	As above
pJS8	4,873	Cm	This work	As above
pJS12	4,989	Cm	This work	In vivo deletion of pJS39 in *S. pneumoniae*

formation procedures for the two bacterial hosts have been described (20). The plasmids used are described in Table 1. Selection with tetracycline was at 1 and 20 μg/ml for *S. pneumoniae* and *B. subtilis*, respectively. Chloramphenicol was used at the concentrations indicated in Results.

Plasmid DNA preparations. Crude plasmid extracts were prepared from *B. subtilis* by the procedure of Birnboim and Doly (4) and from *S. pneumoniae* by a modification of that method (29). Purification of plasmid DNA and isolation of circular covalently closed monomer plasmids have been described (3). For analysis of single-stranded plasmid DNA, 1.5-ml cultures were grown to 4×10^8 CFU/ml and washed twice with 0.15 M NaCl. *S. pneumoniae* cells were suspended in 100 μl of solution containing 25% sucrose, 0.15 M sodium citrate, 0.1% sodium deoxycholate, 0.01% sodium dodecyl sulfate, and 20 μg of pancreatic RNase and incubated at 20°C for 10 min. The lysed-cell extracts were vortexed for 3 min and subjected to three freeze-thaw cycles. The lysates were adjusted to 1% sodium dodecyl sulfate, and proteinase K was added to 300 μg/ml. After incubation at 20°C for 10 min, tracking dye was added, and the samples were immediately subjected to electrophoresis.

Detection and analysis of single-stranded plasmid DNA. The procedure used for single-stranded plasmid DNA detection and analysis was similar to that of te Riele et al. (30). Samples of cell extracts (50 μl) were loaded in 0.7% agarose gels in Tris-borate buffer containing ethidium bromide at 0.5 μg/ml. Electrophoresis was performed at 2 V/cm for 18 h. The gel fluorescence was photographed, and the position of the circular covalently closed monomer was recorded. DNA was transferred to nitrocellulose filters by diffusion without prior denaturation. Radioactive probes were prepared by the method of Feinberg and Vogelstein (8), using oligonucleotide primers and [α-^{32}P]dCTP. Filters were hybridized with the probe and exposed to X-ray films.

DNA manipulations. Restriction endonucleases were obtained commercially and used as specified by the suppliers. Analytical gel electrophoresis of plasmids and restriction fragments was carried out in 1% agarose or 5% polyacrylamide gels, which were stained with ethidium bromide. Plasmid constructions are described in Results.

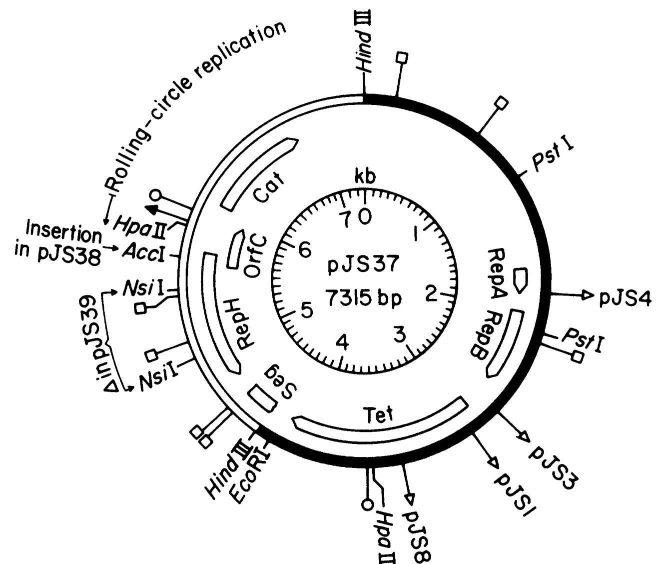

FIG. 1. Physical map of pJS37. Only relevant restriction sites are shown. Sites of the insertion in pJS38 and the deletion in pJS39 are indicated. Gene products: RepA and RepB, replication proteins of pLS1; Tet, protein conferring tetracycline resistance; Cat, chloramphenicol acetyltransferase; RepH, replication protein of pC194. Seg and OrfC are described elsewhere (1a). Symbols: ◆—, proposed RepH cutting site; ◁—, endpoints of deletions found in the plasmids indicated; □—, potential deletion sites corresponding to the consensus sequence for deletion endpoints found; ○—, deletion endpoints of pJS12.

DNA sequence determination. For nucleotide sequence determination, DNA restriction fragments were treated with calf intestinal phosphatase and labeled at the 5′ ends with [^{32}P]ATP and polynucleotide kinase. After subcutting with a second restriction enzyme, DNA sequences were determined by the chemical method of Maxam and Gilbert (22). From our determination of nucleotide sequences at the deletion endpoints, another correction was made to the published sequence of pC194 (12): between positions 1078 and 1079, a T was inserted. This correction and those of Dagert et al. (5) make the size of pC194 2,907 bp.

Computer analysis. The nucleotide sequence of pJS37 (7,315 bp in length) was numbered from the *Hin*dIII site proximal to the end of the chloramphenicol acetyltransferase (*cat*) gene of pC194 (Fig. 1). Searches for secondary structures, sequence similarity, and other features in the DNA sequence of pJS37 were performed with DNASTAR computer programs (DNASTAR, Inc., London, United Kingdom).

RESULTS

Structural instability. It was previously shown that the hybrid plasmid pJS37 undergoes systematic deletions in *S. pneumoniae* after cultures are shifted to chloramphenicol-containing medium (3; Fig. 1). Soon after the shift, analysis of plasmids within individual clones showed a 4.9-kilobase derivative, called pJS8, as well as plasmids pJS3 (4.1 kilobases) and pJS4 (3.4 kilobases). The latter two plasmids predominated after longer periods of growth. Ultimately, pJS4 was lost by segregation, presumably because it does not itself encode a replication protein (Fig. 1). This pattern of deletions was observed not only in wild-type strains but also in *rec-8* and *rec-19* (25) mutant strains of *S. pneumoniae*

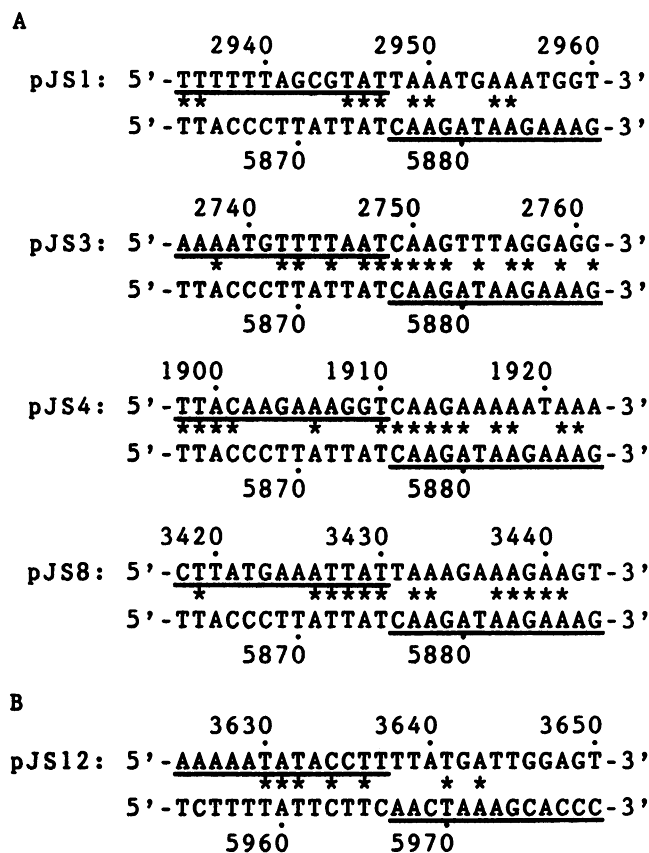

FIG. 2. Nucleotide sequences around the borders of the deletions. (A) Deletion endpoints in pJS1, pJS3, pJS4, and pJS8. (B) Deletion endpoints in pJS12, which was generated from pJS39. Upper strand, Sequence at the border in the pLS1 moiety; lower strand, sequence at the border in the pC194 moiety. Numbers indicate nucleotide positions in pJS37. Underlined segments of sequence remain in the deleted derivative. Deletion endpoints are aligned to show sequence similarities in the region of the deletions; asterisks indicate identical bases. The sequence of pJS3 was previously reported (3).

defective in recombination (data not shown). All of the deletions of plasmids isolated in this and previous work placed the *cat* gene immediately downstream of another promoter (either the *rep* or *tet* promoter of the pLS1 moiety), which led to higher expression of the *cat* gene (3). Since the *cat* gene of pC194 is expressed more poorly in *S. pneumoniae* than in *B. subtilis* (S. Ballester, P. Lopez, and M. Espinosa, manuscript in preparation), it might be expected that deletions leading to increased *cat* expression would be more readily obtained in the former than in the latter host. We therefore examined the stability of pJS37 in *B. subtilis* by growing a strain harboring pJS37 at various chloramphenicol concentrations. Whereas pJS37 exhibited structural instability in *S. pneumoniae* at chloramphenicol concentrations as low as 2.5 μg/ml, the plasmid was stable in *B. subtilis* even at 30 μg/ml. However, by raising the concentration to 60 μg/ml, one new deleted derivative, a 4.4-kilobase plasmid called pJS1, was isolated (Fig. 1).

Deletion endpoints. The nucleotide sequences of the deletion joints of all of the deleted derivatives of pJS37 were determined (Fig. 2A). In all cases, deletions occurred in regions of the pLS1 and pC194 moieties that showed some sequence similarity. All of the deleted plasmids shared a common endpoint located between nucleotides 5872 to 5881 of pJS37 (Fig. 2A). This region coincides with the postulated target site of the RepH nicking activity at 5872 and 5880 (25). When endpoints occur within a repeated sequence, the exact position of the deletion within the repeat is unknown. However, the four deletions could have occurred at the site shown in Fig. 2A, between nucleotides 5875 and 5876.

The variable endpoint of the deleted plasmids showed considerable similarity to the common endpoint. Identity in the 10 bases bordering the endpoints was greater than 50%: 5 of 10 bases for pJS1, 7 of 10 bases for pJS3, 7 of 10 for pJS4, and 7 of 10 for pJS8 (Fig. 2A). In all of the derivatives of pJS37, the deletion occurred between directly repeated sequences 3 to 6 bp long (Fig. 2A). However, as indicated in Discussion, we believe the similarity of sequence at the endpoints to be more important than the short repeats for generation of the deletions.

Alterations of RepH that affect the generation of deletions. The location of the fixed border of the deleted derivatives of pJS37 within the plasmid replication region of the pC194 moiety suggested involvement of RepH activity in generation of these deletions, as has been indicated for a different class of deletions (24). This idea was supported by the finding that pJS140, an *Acc*I-*Eco*RI-deleted derivative of pJS37 that lacks RepH, *seg*, and OrfC but conserves the RepH target sequence, generated only one deleted plasmid (pJS5), in which the deletion endpoint in the pC194 moiety was far from positions 5872 to 5880 in pJS37 (3).

To test the role of RepH, a gross modification of its gene was made by removal of a 459-bp *Nsi*I fragment of pJS37 to give pJS39 (Fig. 1). Plasmid pJS39 would encode a protein of 161 amino acids, RepH*, in which the first 80 residues are identical to those of RepH, whereas the remaining 81 amino acids are different. This drastic alteration makes it unlikely that RepH* could function as an initiator of replication.

The chloramphenicol-dependent generation of deletions was compared in *S. pneumoniae* harboring pJS39 and pJS37, respectively. With chloramphenicol at 10 μg/ml, pJS37 yielded pJS3 and pJS4, whereas cells carrying pJS39 failed to grow. With chloramphenicol at 5 μg/ml, the pattern obtained for pJS37 was the same, but a new deleted derivative (pJS12) was generated from pJS39. The nucleotide sequence at the ends of the deletion in pJS12 is depicted in Fig. 2B. Very little sequence similarity was observed at the borders of the deletion. The deletion endpoint in the pC194 moiety occurred 90 bp from the common end of the deletions generated in pJS37. No deletions that shared the common end were generated from pJS39 or from the plasmid missing RepH (pJS140; 3), which supports the hypothesis that RepH plays a direct role in the instability of plasmids carrying the pC194 replicon.

In another attempt to modify RepH, we filled in the unique *Acc*I site of pJS37 (position 5658; Fig. 1) to construct plasmid pJS38. The 2-bp insertion (AT) was confirmed by DNA sequencing. We do not know how this change affects the *repH* product, but the frequency of deletions was greatly reduced. To compare the frequency of deletions in strains harboring pJS37 or pJS38, 2×10^7 cells were plated in medium containing 10 μg of chloramphenicol per ml. An average of 46 clones (10 independent assays, none containing an obvious "jackpot" event) were obtained from pJS37-harboring cultures, and all of those examined (40 clones) contained deleted plasmids. No colonies were found in the plates of the pJS38 strain. However, after 2×10^{10} cells of the latter strain were plated, 36 colonies, all containing deletions, were found. Restriction analysis showed the de-

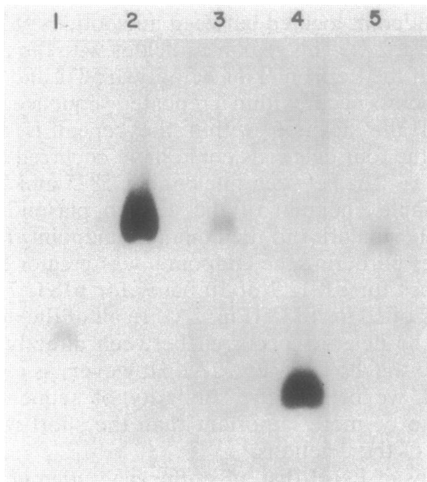

FIG. 3. Generation of single-stranded plasmid DNA in *S. pneumoniae*. Lanes: 1, pLS1; 2, pJS37; 3, pJS38; 4, pC194; 5, pJS39. See Materials and Methods for procedural details.

leted plasmids to be similar, if not identical, to pJS3, pJS4, and pJS8. Consequently, the frequency of generation of deletions for pJS37 was 2.3×10^{-6}, whereas for pJS38 the frequency dropped to 1.8×10^{-9}, or 1,300-fold less.

Single-stranded intermediates in replication of the hybrid plasmids. Plasmids pC194 and pLS1 appear to replicate through single-stranded intermediates in a rolling-circle mechanism initiated by a plasmid-encoded protein (6, 9, 27, 30). In *S. pneumoniae*, pLS1 accumulates single-stranded intermediates to a much lesser degree than does pC194 in the same host (6). To examine the effect of RepH alteration in the replication of pJS38 and pJS39, the generation of single-stranded DNA by these plasmids was compared with that in pJS37 (Fig. 3). Parental plasmids pLS1 (lane 1) and pC194 (lane 4) served as controls. The amount of single-stranded DNA generated by pJS37 (lane 2) was similar to that of pC194, which indicates that the normal RepH protein encoded by pJS37 functions at a high level. In contrast, little single-stranded intermediate accumulated with pJS39 (lane 5), which indicates that only the pLS1 replication system functions and is consistent with the lack of activity of RepH*.

DISCUSSION

Spontaneous generation of deletions may result from different and unrelated mechanisms. Prior studies deduced three mechanisms: (i) the presence of short direct repeats leading to slippage (1), (ii) erroneous exchange of topoisomerase subunits (13), and (iii) erroneous termination of plasmid replication (23, 24). From previous (3) results and those presented here, we propose that deletions in the *S. pneumoniae* pJS37 plasmid model system are generated by at least two mechanisms, to give end products with greater expression of chloramphenicol acetyltransferase. The first mechanism depends on an active replication protein, RepH, and gives rise to plasmids pJS3, pJS4, and pJS8. The mechanisms giving rise to pJS5 (3) and pJS12 (this work) are independent of RepH and are not discussed here.

Single-stranded DNA coliphages such as φX174 and fd replicate by a rolling-circle mechanism (reviewed in reference 2). Many small gram-positive plasmids, such as pC194 (30), pT181 (10), and pLS1 (6), appear to replicate by a similar mechanism. The general mechanism can be outlined as follows. A replication protein nicks one strand of the covalently closed double-stranded replicative form or plasmid at a particular site in the recognition sequence of the viral or plus-strand origin. Secondary structure near this site in the supercoiled form may facilitate this interaction. The protein remains bound to the 5′ end of the nicked strand, as has been shown for φX174 (28) and surmised for pT181 from the blocking of 5′-OH at the nicked site (17). That strand then peels off, as a DNA polymerase, primed by the 3′ end and, with the closed complementary strand as template, replicates the strand being displaced. When the replication point completes the cycle and returns to its origin, the replication protein attaches again to its recognition sequence, joins the 5′ end currently attached to the protein to the appropriate point (3′ end) of the displaced strand, thereby releasing it as a single-stranded circle, and, at least in the case of φX174, nicks the double-stranded circle again to repeat the cycle. The number of such cycles may be limited, especially for plasmids, since pT181 copy number can be proportional to RepC activity in the cell (26). We hypothesize that the strand closure and renicking are parts of the same reaction and require less DNA specificity than does the original nicking of the supercoiled form. The single-stranded circle is then replicated from its minus-strand origin.

The RepC protein of pT181 exhibits DNA-binding and strand-nicking and -closing activities (17) at a specific recognition sequence (16). It was proposed that RepH of pC194 similarly involves nicking of DNA at a specific sequence (9, 24). In accordance with the model of Michel and Ehrlich (24) for generation of deletions distal to putative RepH recognition sites, and their suggestion that pJS3 (misprinted in reference 24 as pLS3) may represent a complementary class of deletions, we propose that most of the deletions observed in pJS37 result from action of the RepH protein (encoded by the pC194 component) nicking at its normal recognition sequence, which corresponds to the fixed border of the deletions, and at other sites which resemble that sequence. Deleted plasmids produced in this manner would accumulate under the selective conditions of the experiment. Comparison of the endpoints of the 4 deletions shown in Fig. 2A and the 16 reported by Michel and Ehrlich (24) revealed the 9-base consensus sequence shown in Table 2. (Note that positions 5 and 8 contain either T and A or C and G, respectively.) The consensus sequence presumably corresponds to a secondary RepH target site. To see whether all such sites give rise to deletions, we searched plasmid pJS37 for all instances of this consensus sequence. Eleven sites were found. Table 2 shows these sites represented in the strand that carries the sense message for RepA, RepB, and Tet (Fig. 1), that is, in the strand opposite the one nicked. Six of those sites, if used to generate deletions, would yield plasmids either without a primary replication origin (at positions 203 and 776) or without any selective advantage for the expression of chloramphenicol acetyltransferase (at positions 4520 through 5876) because no promoter would be coupled to the *cat* gene (3), inasmuch as a transcription terminator appears to be present near the *Eco*RI site in the pLS1 segment (19). Except for the site at position 2207, the rest of the sites coincided with the variable borders of the deletions found in this work (Fig. 2A). Although it appears that RepH is involved in the generation of these deletions, erroneous termination of replication cannot account for them because that mechanism would give deleted plasmids resulting from pJS37 that are precisely the complement of

TABLE 2. Nucleotide sequences in pJS37 corresponding to the consensus RepH target site

Nucleotide position[a]	Sequence[b]	Predicted nature of resulting plasmid
203	5'-ATAT | TAAAA-3'	No replication origin
776	5'-TAAT | TAAAA-3'	No replication origin
1911	5'-AGGT | CAAGA-3'	pJS4
2207	5'-AGAT | TAAAT-3'	Not observed
2749	5'-TAAT | CAAGT-3'	pJS3
2948	5'-GTAT | TAAAA-3'	pJS1
3431	5'-TTAT | TAAAG-3'	pJS8
4520	5'-AAAT | TAAAA-3'	No enhanced Cm[r c]
4557	5'-GGGT | TAAAA-3'	No enhanced Cm[r]
5018	5'-TTAT | TAAAT-3'	No enhanced Cm[r]
5390	5'-AAGT | TAAAA-3'	No enhanced Cm[r]
5876	5'-TTAT | CAAGA-3' / 3'-AATA | GTTCT-5'	Putative recognition site of RepH
Consensus[c]	5'-DDRT | TAAAD-3' or 5'-DDRT | CAAGD-3'	

[a] At the right of the broken line.
[b] Broken line indicates point of nicking in opposite strand by proposed terminating-nicking activity of RepH.
[c] Cm[r], Chloramphenicol resistance.
[d] D, Not C; R, purine (International Union of Biochemists code).

the ones found; that is, they would have lost the *cat* gene of pC194 and the replication proteins of pLS1.

However, if erroneous initiation were coupled to erroneous termination, as in the model described below, the observed deletions would be obtained. A search for potential secondary structure in the DNA surrounding the putative RepH nicking site revealed two possible hairpin structures in the region required in *cis* position for replication using RepH (9). No inverted repeats that could give rise to such structures were found in the vicinity of the variable deletion endpoints. It is conceivable that the secondary DNA structure at the primary RepH recognition site, which would be more likely to form in a supercoiled plasmid, is required only for the initial attachment of RepH to the supercoiled form; subsequent termination and reinitiation on the nicked, relaxed plasmid might require only the recognition sequence or its analog. The ϕX174 gene A protein requires more sequence specificity for initial nicking than it does for subsequent rounds of replication (31).

Plasmids pC194 and pJS37 both give accumulation of large amounts of single-stranded replication intermediate in comparison with pLS1 (Fig. 3). Presumably, this results from the greater activity of RepH than of the RepB protein of the pLS1 replicon. The plasmid with altered RepH, pJS39, accumulates single-stranded circles only to the extent of the accumulation by pLS1, which indicates that the RepH activity has been reduced at least 10-fold. The inability of RepH*, encoded by pJS39, to generate deletions of the type under discussion suggests that the replication activity of this protein was abolished.

On the basis of the considerations presented above, the model depicted in Fig. 4 for generation of deletions from pJS37 can be proposed. The RepH protein binds specifically to its target site at the origin of replication of pC194 (1a) on the supercoiled plasmid form (Fig. 4A). After it nicks the DNA, the 3'-OH end generated is used by a DNA polymerase to start a rolling-circle replication (Fig. 4B). In normal replication, the rolling circle ends when RepH returns to the origin of pC194 (Fig. 4C), and the final products of this step are a double-stranded plasmid and a circular single-stranded plasmid intermediate (Fig. 4D). Replication from the minus-strand origin converts single-stranded plasmid molecules to double-stranded plasmids (6) (Fig. 4E). Occasionally, in the course of replication, RepH associated with the displaced strand may recognize a secondary site similar to its genuine nicking site (Fig. 4F). If RepH has a nicking and closing activity similar to that of RepC of pT181 (17), recognition of a secondary site leads to closing of the displaced strand (Fig. 4G) and, in the case of pJS37 deletions, generation of a nonreplicative single-stranded deleted form (Fig. 4H). Replication then resumes from this site (Fig. 4H). Termination of replication at the RepH primary recognition site (Fig. 4I) generates the parental plasmid (Fig. 4J) and a deleted single-

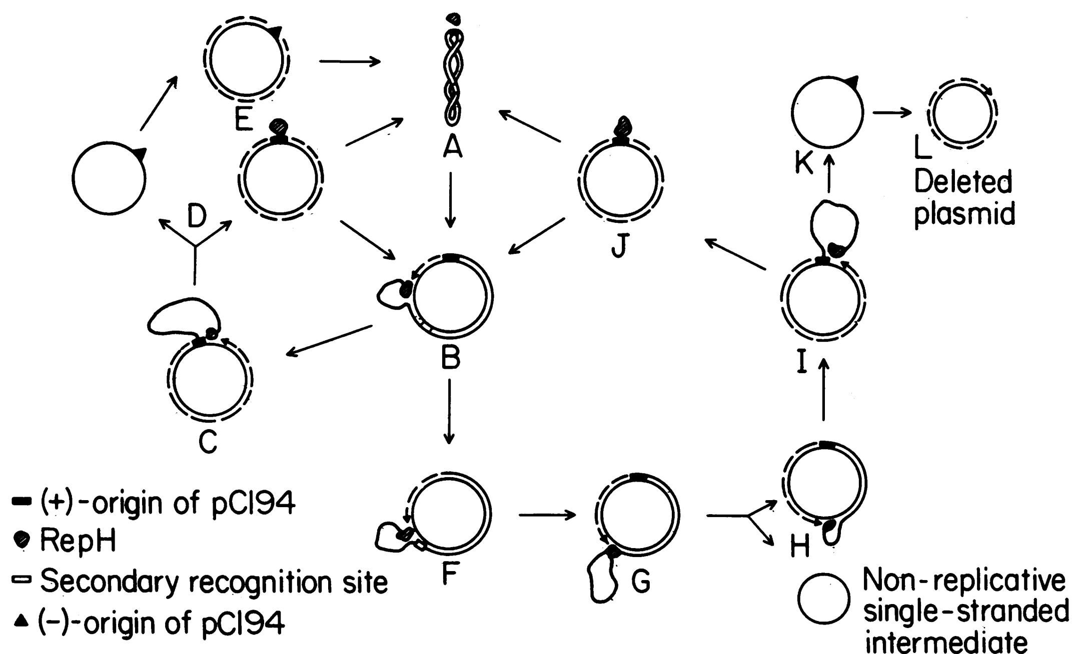

FIG. 4. Model for the generation of deletions by aberrant nicking-closing activity of a replication protein. See text for details.

stranded intermediate (Fig. 4K) that can be converted to a double-stranded plasmid by use of the pC194 minus-strand origin (Fig. 4L). Subsequent rounds of replication of these deleted plasmids would use the pLS1 replicative machinery.

The frequency of the class of deletions observed here appears to be quite low, on the order of 10^{-7} per plasmid. According to the data presented above, 46 clones per 2×10^7 cells with a copy number of 20 plasmids per cell calculates to 1.2×10^{-7} events per plasmid. The deleted forms are detected only because they confer higher resistance to chloramphenicol. Therefore, it is not surprising that this class of deletions was not observed by Gros et al. (9). The complementary class, which they did observe, may occur more frequently or may be subject to positive selection of an unknown sort.

The comprehensive model that we propose for the generation of deletions by action of nicking-closing replication proteins at sites homologous to their specific recognition sequences extends the erroneous termination model of Michel and Ehrlich (24) to account for deletions both proximal and distal to the homologous site. It also explains the observation of plasmid deletions in a hybrid of pSA2100 (itself a hybrid of pSA0501 and pC194) and pUB110 by Hahn and Dubnau (11). In that case, deletions occurred between 18-bp direct repeats, which can now be seen to include, respectively, the RepH recognition sequence of the pC194 component and the homologous RepU recognition sequence of the pUB110 component (9, 21). These two component plasmids show considerable homology between their replication origins, although RepH cannot use the pUB110 origin for replication (21), perhaps because that origin lacks the requisite DNA secondary structure for initiation of replication by RepH. With respect to the observations by Hahn and Dubnau, both deleted derivatives generated in steps G and H of Fig. 4 would be able to replicate, and both deleted plasmids were in fact observed. The distinctive feature of our model is that the same aberrant nicking-closing event is able to generate plasmids with proximal deletions (this work), distal deletions (24), or both (11), depending on the presence of appropriate replicative machinery in the deleted form.

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