Article

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Reference

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The nucleotide sequence of replication and maintenance functions encoded by plasmid pSC101

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ABSTRACT

The nucleotide sequence of 1100bp around the origin of replication of the pSC101 plasmid has been determined. This segment of DNA is capable of replication in the presence of a helper plasmid. The sequence data reveal similarities between pSC101 and several other replicons. The origin of replication contains three direct repeats of an 18bp sequence associated with a segment exceptionally rich in A-T base pairs. A promotor that probably directs transcription of a gene encoding an essential plasmid replication function is associated with a region of extensive potential secondary structure. The sequence presented here includes the sequence of the par region involved in partitioning of plasmids at cell division.

INTRODUCTION

We have recently described (1) the isolation and genetic analysis of a series of insertions of the transposon Tn1000 (gamma-delta; 2) into the replication region of the plasmid pSC101 (3,4). These insertions were into a hybrid plasmid, pLC709, consisting of the HincIIA fragment of pSC101 ligated to a mini-colE1 plasmid and an antibiotic resistance marker (1). The HincIIA fragment has been shown to encode all the functions required for pSC101 replication (5). The leftward limit of the essential region (as drawn in figure 1 and 3) is the HaeII site (5; figure 1) and the rightward limit is before the RsaI site (1; figure 1). The plasmid replication genes identified by the analysis of the insertion mutations are shown in figure 3.

We were able to define a 200 bp locus responsible for the expression of pSC101-specific incompatibility (1). This locus lies within a larger region of approximately 450 bp

constituting an origin of replication that can function in the presence of a helper plasmid. This helper plasmid provides a function, rep, required for pSC101 replication and encoded by a segment of DNA adjacent to the origin of replication (1). Another function, par, responsible for the accurate partitioning of plasmids to daughter cells at division has been described and mapped to the other side of the replication origin from the rep locus (5). Examination of replicating molecules by electron microscopy shows that replication is unidirectional and proceeds towards the par locus (6; P. Linder, unpublished results).

We have used the insertions of Tn1000 to determine the nucleotide sequence of the origin region and adjacent DNA segments of pSC101. Chemical sequencing (7) from restriction sites located near to the ends of the transposon Tn1000 has generated a series of overlapping sequences that can be assembled to yield the sequence of the pSC101 replication origin and, simultaneously, locate each insertion within that region. From the properties of the insertion mutants we have assigned functions to certain features of the DNA sequence.

The pSC101 plasmid requires the product of the dnaA gene of E.coli for its replication (8,9). Since the product of this gene is essential for replication of the E.coli chromosome both in vivo (10) and in vitro (11), we have compared the sequence of the pSC101 replication origin with that of oriC (12). This comparison has revealed limited but possibly significant sequence homology between the two origins of replication. We have also compared the pSC101 sequence with that of other replication origins and the results of this comparison are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids and cloning procedures

All standard techniques have been described previously (1). The isolation and properties of the Tn1000 insertion mutants have been described (1). The insertions were isolated by conjugal mobilization of the pLC709 plasmid by the sex factor F (1,2). The vector plasmid pHP37, used for cloning

DNA fragments prior to sequencing, is similar to pHP34 (13) and is pBR322 (14) with an insertion of 14 base pairs at the EcoRI site, resulting in the sequence GAATTAATTCCCGGGAATTC, which contains a site for SmaI cleavage adjacent to an EcoRI site. Both these cleavage sites are unique in the plasmid and DNA fragments can thus be cloned into the SmaI site and sequenced by labelling the ends generated by EcoRI cleavage in a fashion analogous to that described for pHP34 (13).

To construct pHP37, the plasmid pKP6 (13), employed in the construction of pHP34 (13), was used. This plasmid is pBR322 carrying a DNA fragment specifying resistance to the antibiotics streptomycin and spectinomycin, flanked by sites for SmaI and EcoRI. The EcoRI site adjacent to the ampicillin resistance gene of pKP6 was destroyed after protection, with RNA polymerase (15), of the EcoRI site adjacent to the tetracycline resistance gene. Following digestion with EcoRI the cohesive ends were filled in, using the Klenow fragment of DNA polymerase I, then the DNA was recircularized with T4 DNA ligase and used to transform E.coli. A plasmid was recovered that retained only a single EcoRI site adjacent to the tetracycline resistance gene of pKP6. DNA of this plasmid was digested with SmaI and recircularized to remove the streptomycin resistance fragment of pKP6, resulting in pHP37.

DNA sequencing

All sequences were determined by the chemical degradation procedures of Maxam and Gilbert (7), with modifications described by Smith and Calvo (16) and Will et al. (17). DNA fragments were end-labelled with ^{32}P by use of either T_4 polynucleotide kinase or the Klenow fragment of E. coli DNA polymerase I.

Two general sequencing strategies were used (figure 1). The restriction endonuclease RsaI cleaves 30bp from each end of Tn1000 (18). Each insertion plasmid was cleaved with RsaI and the two junction fragments, consisting of pSC101 sequences joined to 30bp of Tn1000 DNA, were purified by gel electrophoresis. After end-labelling with T4 polynucleotide kinase and secondary restriction enzyme cleavage, the resulting fragments, now labelled at one end, were sequenced.

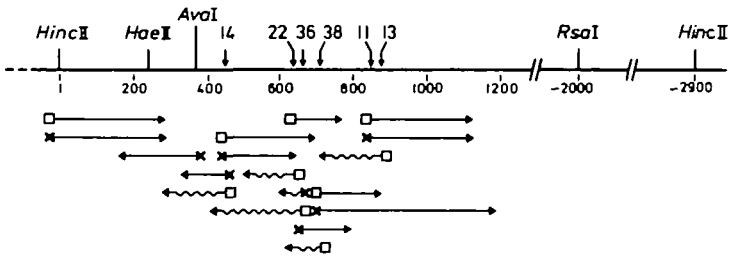

Figure 1.
Sequencing strategy. The restriction map and the sites of Tn1000 insertions (1) into the origin region of pSC101 are presented. Below are the sequences determined either from purified fragments (∿∿∿) or from fragments cloned in pHP37 (——). The DNA was labelled either with T4 polynucleotide kinase (□) or DNA polymerase I Klenow fragment (X).

The junction fragments were also cloned into the SmaI site of pHP37, in both orientations. The resulting plasmids were then digested with EcoRI, end-labelled and digested with ClaI. This resulted in a large fragment corresponding to pSC101 DNA and most of pBR322, and a 26bp EcoRI-ClaI pBR322 fragment. This mixture of fragments was sequenced without further purification if the first 26bp of the large fragment was Tn1000 DNA. Otherwise, the DNA was digested with a suitable restriction enzyme to cleave the large fragment and the appropriate labelled fragment was purified by gel electrophoresis.

RESULTS

Characteristics of the sequence

The nucleotide sequence of 1100bp around the origin of replication of pSC101 is presented in figure 2 and an interpretative drawing showing certain features is presented in figure 3.

A striking feature of the sequence around the replication origin is an 80bp stretch rich in A-T base pairs (84 %) beginning at coordinate 503, followed by 3 direct repeats of an 18bp sequence at coordinates 582, 603, and 635. The repeated sequences at coordinates 582 and 635 have an additional 6 base-pairs that are homologous. This segment of DNA between the sites of the Tn1000 insertions 14 and 36

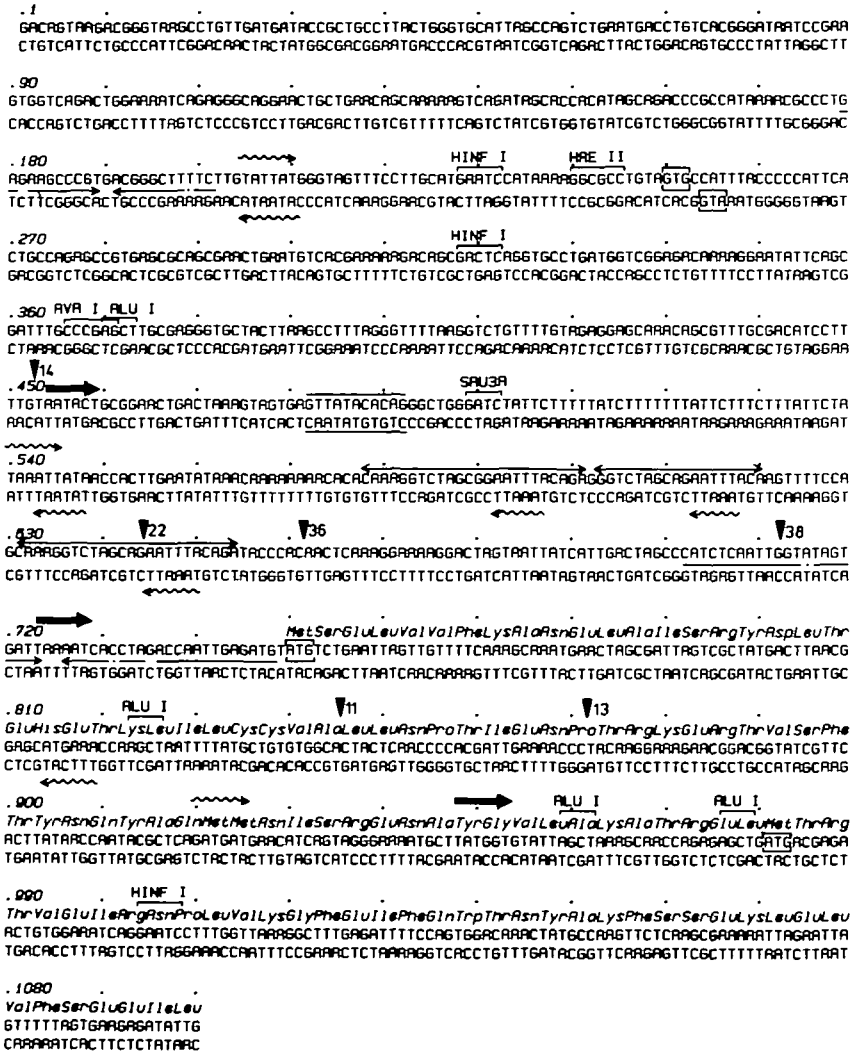

Figure 2. Nucleotide sequence of the pSC101 replication origin segment. ▼-sites of Tn1000 insertions. ⟷- direct repeats. ⟶- inverted repeat. ⟿- 10 regions of putative promotor sequences discovered by searching the sequence for possible - 10 regions and searching the surrounding sequences for homology with a consensus E. coli promotor sequence (49) ➡ -10 regions of functional promotors. The sequence indicated by lines at coordinate 482 is the 11 bp sequences repeated four times in oriC. The sequence between coordinates 757 and 1078 agrees with the sequence presented in reference 19. Coordinate 1 is the left HincII site in figure 1.

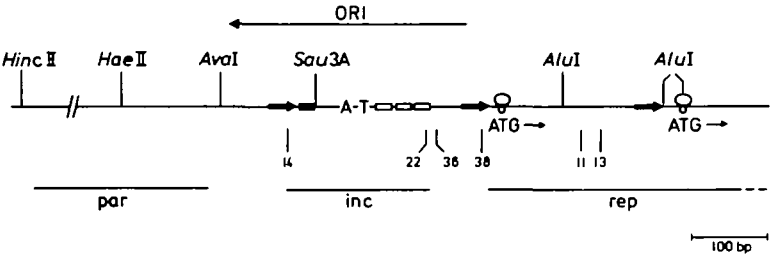

Figure 3. Features of the nucleotide sequence including restriction sites and Tn1000 insertions. The heavy arrows indicate promotor sequences known to function in vivo. The location of two sets of translation initiation signals, possibly coding for a function required for pSC101 replication (ATG) are shown. The A-T rich region and adjacent repeated sequences (1) are indicated as is the sequence repeated four times in oriC (■, figure 6). Replication initiates in the region marked ori, and procedes leftwards as indicated by the arrow. The location of the inc and rep loci are from reference 1 and the par region from reference 5.

(figure 2) has been shown to be responsible for the expression of pSC101-specific incompatibility and to contain the replication origin (1).

To the right of this segment, as drawn in figure 3, are inverted repeats of a 22bp sequence at coordinates 702 and 726. A potential secondary structure that could be formed by these repeated sequences is presented in figure 4. A second such feature is located around coordinate 190 in the par region (figure 2).

Promotor-like sequences

The DNA sequence was examined by computer for sequences resembling promotors for transcription (figure 2). In figure 3 are shown three sequences which have been shown to promote transcription either in vivo, by genetic evidence (1) and the construction of gene fusions with β-galactosidase, or by transcription in vitro (P. Linder, unpublished results). One promotor is situated at coordinate 454 and is interrupted by Tn1000 insertion 14. A second promotor (coordinate 723) overlaps the region of strong potential secondary structure (figure 4) and is interrupted by insertion 38. A third promotor is located at coordinate 948 and has been previously

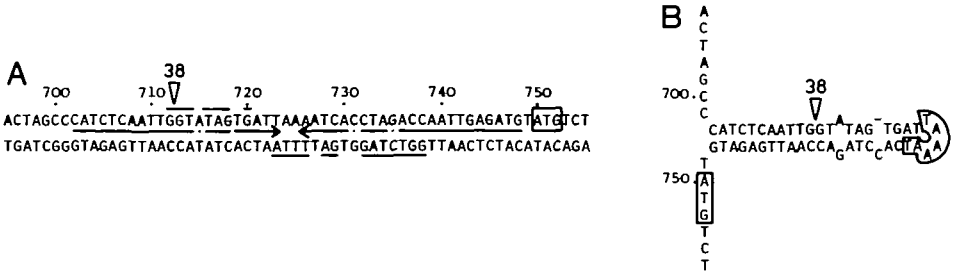

Figure 4. Potential secondary structure in the origin region.
a) ▽ site of Tn1000 insertion 38. ⟶ inverted repeats. —— partial homology with the three direct repeats. The boxed ATG codon is the initiation codon of the open reading frame in the rep region.
b) One strand is shown folded into the most stable hairpin loop structure (50). The boxed bases show the -10 region of a promotor directing transcription of the open reading frame.

characterized and shown to function in vivo (19). These promotors all direct transcription to the right as drawn in figure 3. The possible role of transcription from these promotors in the replication of pSC101 is discussed below (see Discussion).

The three direct repeats at coordinate 582, 603 and 635 all could be contained within promotor sequences directing transcription towards the left.

Open reading frames

Downstream of the promotor at coordinate 723 is an ATG initiation codon and a 117 codon reading frame open to the end of the region of DNA that we have sequenced. The Tn1000 insertions 11 and 13 interrupt this reading frame. The promotor at coordinate 948 lies within the reading frame and downstream of this second promotor is a second potential ATG initiation codon (coordinate 981). Both initiation codons are preceded by potential ribosome binding sites and have been shown to be capable of initiating translation by the construction of appropriate gene fusions to β-galactosidase (P. Linder, unpublished results). They are in the same phase. This suggests that two proteins, one 77 amino acids longer than the other, but otherwise identical, could be synthesized from this region. Experiments to detect these proteins using

an in vitro system (20) have revealed the presence of one protein of 34,000 D that is not synthesized by a plasmid carrying Tn1000 insertion 13 (P. Linder, unpublished results). Such a protein could be encoded by the DNA between either initiation codon and a termination codon situated before the RsaI site which identifies the rightward end of the minimal replication region indicated in figure 1. Genetic evidence (1) shows that this region encodes a function, rep, that is required for pSC101 replication.

One 49 codon open reading frame that begins with ATG is located between coordinates 256 and 109, reading leftwards.

There are also two short reading frames that begin with GTG. One, reading rightwards from coordinate 250 to 397 (49 codons), is associated with a potential promotor sequence at coordinate 205. The other reads leftwards from coordinate 901 to 799 (34 codons). A third reading frame starting leftwards from GTG at coordinate 152 continues to the end of the sequence.

DISCUSSION

We have previously shown that a 450bp segment of pSC101, terminated at its rightward boundary by the site of insertion 38 (figure 2), contains all the functions required in cis for plasmid replication (1). Examination of replicating molecules by electron microscopy (6; P. Linder, unpublished observations) reveals that an origin of replication is located within the segment and that replication proceeds leftwards, as shown in figure 3, unidirectionally towards the par locus. This replication origin segment of pSC101 contains three direct repeats of an 18bp sequence, adjacent to a region of high A-T base pair content. A similar arrangement has been found in several other replication origins, such as R6K (21), RK2 (22), phage λ (23) and F (24). The number of direct repeats varies from 3 in the case of pSC101 to 9 in the case of mini-F (24). The repeated sequence of pSC101 shows no homology to those of any of these replicons.

The role of direct repeats in plasmid replication

It seems that these directly repeated sequences play a

role in plasmid replication. Insertion of a transposon into one of the repeats (insertion 22, figure 2) abolishes pSC101 replication activity (1). Similar results have been obtained with R6K using the transposon Tn5 (25) and deletion of four or more of the repeated sequences of R6K has been shown to abolish origin function (25).

The repeated sequences are also involved in the expression of plasmid incompatibility. The same transposon insertion, insertion 22 (figure 2), that abolishes replication activity, also abolishes the expression of incompatibility towards a pSC101 plasmid. It has previously been postulated that pSC101 incompatibility function is due to a trans-acting repressor of plasmid replication (6). Our results thus suggest that such a repressor is encoded by the region of pSC101 containing the direct repeats. There are no obvious open reading frames for a protein in this segment but the repressor could be a small RNA molecule, as has been found for the plasmids colE1 (26, 27) and R1 (28). Each of the three repeated sequences could be part of a promotor sequence directing transcription leftwards, as shown in figure 3. As yet we have no evidence that any of these putative promotor sequences functions in vivo.

The involvement of directly repeated sequences in the expression of plasmid incompatibility is not restricted to pSC101. It has been reported that cloning a 58bp fragment, carrying two direct repeats from the incC region of F, onto a normally compatible plasmid results in incompatibility between F and the plasmid carrying the fragment (29). Similar results have been obtained with R6K (30).

The A-T rich segment

Adjacent to the three direct repeats of pSC101 is a stretch of 80 base pairs exceptionally rich in A and T residues (coordinates 503-581, figure 2). Such a segment may be preferentially denatured during the initiation of DNA replication but no function can be attributed to this sequence at present. However, deletion of a similar DNA segment in the origin of replication of phage λ completely abolishes replication activity (31). The arrangement of the

pSC101 direct repeats and its A-T rich segment is similar, with respect to the direction of replication, to that found in RK2 (22, 32).

Transcriptional activation

We have previously discussed evidence that insertion 14 inactivates a transcriptional event essential for pSC101 replication (1). The nucleotide sequence demonstrates that the site of insertion 14 is within a putative promotor, and preliminary *in vitro* transcription experiments suggest that this promotor sequence is functional. The role of this transcription in pSC101 replication is not clear, but since it is opposed to the direction of replication, it may be involved in "transcriptional activation" of the origin. This phenomenon has previously been described for phage λ (33,34).

Proteins involved in pSC101 replication

Around the site of insertion 38 (figure 2) is a region of strong potential secondary structure (figure 4). The insertion 38 reduces, but does not completely abolish, pSC101 replication origin activity (1). The efficiency of transformation of a *polA*$^-$ strain by pLC709 harboring insertion 38 is reduced to 1%, compared with pLC709 (1). The DNA sequence of this region contains a number of interesting features. As indicated in figure 4, a promotor sequence is present that would direct transcription towards the right, as depicted in figure 3; it is followed by an ATG initiation codon and an open reading frame. This putative promotor is overlapped by sequences having partial homology to the sequence of the direct repeats (figure 4). This raises the possibility that the direct repeats may be recognition sequences for a plasmid-encoded replication protein that also regulates its own synthesis. We have shown that a function required for plasmid replication, *rep*, is encoded by the segment of DNA to the right of insertion 38 (1). It has been shown for R6K that such autoregulation of a plasmid encoded replication protein, the π protein, does occur (35), possibly by interaction of the protein with a single copy of the repeated sequence (36). In this case the copy of the repeated sequence associated with the protein is in the same

orientation as the other direct repeats (21).

Such an interpretation is complicated by the observation that a second set of transcription and translation initiation signals is located to the right of this site (figure 3) and that two overlapping proteins might be encoded by the rep region. It is possible that both proteins are synthesized and recognize the region of potential secondary structure. It could be imagined that a truncated protein produced from a transcript initiating at the downstream promotor acts as a repressor, inhibiting the synthesis of the longer protein. The longer protein would bind to the same site on the DNA but would be involved in the initiation of replication. Obviously a considerable effort will be required to elucidate the molecular mechanisms responsible for pSC101 replication and its regulation but this type of speculation is suggested by an analogy with the transposon Tn5. A truncated form of the transposase protein is responsible for the negative regulation of the synthesis of the complete transposase (37).

Sequence homology with phage G4

The role of the rep protein could be to direct priming of leading strand DNA synthesis (leftwards in figure 3). The region adjacent to the site of insertion 38 shows striking homology (figure 5) with the segment of phage G4 that is involved in priming of complementary strand DNA synthesis by the E. coli dnaG gene product (38, 39).

Similarities with oriC

The same segment contains the longest region of homology with oriC, the origin of replication of the E. coli chromosome (figure 5). Otherwise there are few features in common, despite the fact that both oriC and pSC101 are dependent upon the activity of the dnaA gene product for replication. An 11bp sequence, GTTATACACAG, is present in pSC101 at coordinate 482 and is repeated four times in the sequence of oriC (figure 6). These sequences are conserved in all the origins of replication of the Enterobacteriaciae sequenced to date (12) and mutations in this sequence can abolish replication activity of oriC (40). One of these sequences is present in the segment of oriC showing homology

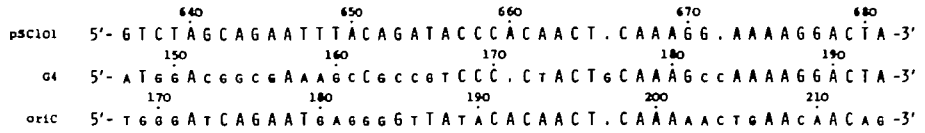

Figure 5. Sequence homology between pSC101 (upper line), G4 (middle line) and oriC (lower line). The sequences are drawn to maximise homology. The coordinates for pSC101 are as in figure 2, for G4 from references 38, 39 and for oriC from reference 12. Dots indicate gaps and homologous bases are in large letters. In G4 the primer transcript for complementary strand replication starts at base 174 and procedes leftwards, the same direction as pSC101 replication.

to pSC101 (oriC coordinate 185; figure 5) and, thus, the sequence GATACCCACAA at coordinate 653 of pSC101 may also be of particular significance. A copy of the 11 bp sequence is also located between the two promotors of the dnaA gene (41). This 11bp sequence might therefore be required for the action of the dnaA gene product in pSC101 and oriC replication and in the autoregulation (42) of the dnaA gene itself. However, a computer search has revealed the presence of this sequence 85 bp downstream of the origin of replication of colE1 (43) and there is no evidence that suggests that the dnaA gene product is involved in colE1 replication (9).

Sites susceptible to methylation by the dam methylase of E. coli, GATC, occur frequently in oriC and in the promotor region of the dnaA gene (12, 41). Only one such site is found in the origin region of pSC101.

On a functional level the only similarity between oriC

GTTATACACAG	pSC101
GTTATACACAA	oriC
GTTATCCACAG	oriC
GTTATCCACAG	oriC
GTTATCCAAAG	oriC
TTTATCCACAG	dnaA

Figure 6. An 11 bp sequence present in pSC101 and oriC. The 11 bp sequence at coordinate 482 of pSC101 is shown, compared with the four repetitions of the sequence in oriC (12) and in the dnaA gene (41).

and pSC101, apart from the requirement for dnaA gene product, is the observation that, like the origin of pSC101, the minimal replication origin segment of oriC encodes an incompatibility determinant (44).

The par region

The segment between the HincII and AvaI sites (figure 2, coordinates 1 to 368) has previously been shown to be required for the accurate partitioning of pSC101 plasmids to daughter cells at division (5). The only obvious feature of this region is a potential hairpin structure that could be formed by inverted repetition of sequences at coordinates 179 and 191. The segment also contains one short open reading frame. It is not known whether or not the potential protein encoded by this reading frame plays any role in the function of the par region.

Conclusion

It seems that pSC101 belongs to a class of plasmids whose origins of replication are characterized by the presence of an A-T rich segment associated with direct repeats. The mechanism of replication and regulation has yet to be elucidated for any of these plasmids, in contrast to those plasmids, such as colE1 or R1, whose replication is much better understood. In colE1, a transcript initiated by RNA polymerase serves as a primer of replication (45). Replication is regulated by the action of a small RNA molecule, complementary to the primer RNA, that affects processing of the primer transcript and which is responsible for colE1 incompatibility (46), and by a protein that regulates primer transcription (47). R1 is similar except that the presumptive primer transcript also encodes a protein apparently required for plasmid replication (48). Whether or not the negative regulation of plasmid replication by small RNA molecules is a general feature of plasmid replication remains to be established.

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