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BACTERIAL PLASMIDS: REPLICATION OF EXTRACHROMOSOMAL GENETIC ELEMENTS ENCODING RESISTANCE TO ANTIMICROBIAL COMPOUNDS

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1. ABSTRACT

Plasmids are self-replicating extrachromosomal DNA molecules found in Gram-negative and Gram-positive bacteria as well as in some yeast and other fungi. Although most of them are covalently closed circular double-stranded DNA molecules, recently linear plasmids have been isolated from different bacteria. In general, plasmids are not essential for the survival of bacteria, but they may nevertheless encode a wide variety of genetic determinants, which permit their bacterial hosts to survive better in an adverse environment or to compete better with other microorganisms occupying the same ecological niche. The medical importance of plasmids that encode for antibiotic resistance, as well as specific virulence traits has been well documented and demonstrated the important role these bacterial genetic elements play in nature. Although they encode specific molecules required for initiation of their replication, plasmids rely on host-encoded factors for their replication. Plasmid replication initiates in a predetermined *cis*-site called *ori* and can proceed either by a rolling circle or a theta replication mechanism. Some of the plasmid-encoded elements required for their replication, such antisense RNA molecules and DNA repeated sequences located close to *ori*, determine plasmid attributes like copy number and incompatibility.

2. INTRODUCTION

Extrachromosomal replicons known as plasmids are found in Gram-negative and -positive bacteria as well as in some lower eukaryotes. Their existence was initially revealed as the "F factor" in *Escherichia coli* even before the double-helix structure of DNA was elucidated by Watson and Crick (1-3). Since then, molecular and genetic work on plasmids resulted in extraordinary contributions to the modern fields of molecular genetics and molecular biology (4). Molecular and genetic analysis of bacterial plasmids led to basic concepts such as "the operon" and "the replicon", and has provided essential information on DNA conjugation and fertility, control of gene expression, gene transfer and genetic recombination, and transposable elements. Studies of essential plasmid functions have resulted in important findings about basic aspects of initiation of DNA replication and its regulation, DNA partitioning, and plasmid copy number and incompatibility (5). In pathogenic microorganisms, plasmids that contribute directly to microbial pathogenicity in plants and animals, such as for instance iron transport in several pathogens or the presence of adhesins, invasins or antiphagocytic proteins, is well documented (6, 7). In addition, numerous studies have shown the role played by plasmids in bacteria of importance in other areas such as agriculture and plant molecular biology (8, 9). In a more applied vein, plasmids played a central role in the initial development of recombinant DNA technology, gene cloning, and the constant evolution of molecular biology (4).

One of the features that keep plasmids at the forefront of Microbiology is their ability to carry and transmit genes encoding resistance to antimicrobial compounds. This type of plasmids is widespread in

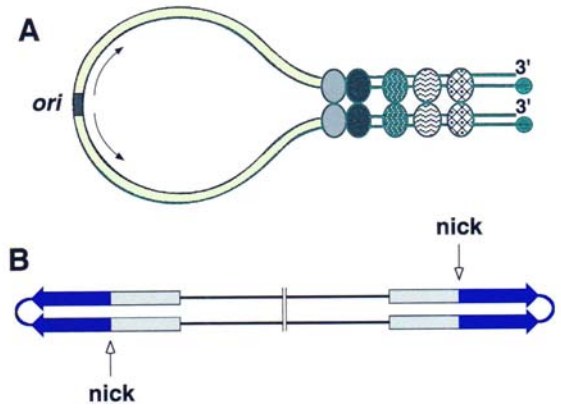

Figure 1. (A) Racket frame structure proposed for linear *Streptomyces* plasmids. The black circles represent the terminal protein attached to the 5' ends, which is required to protect the ends and complete the replication of both plasmid termini. The ovals represent juxtaposition proteins that bring together the plasmid termini by binding to specific regions of palindromic symmetry. The *ori* located near the center of the plasmid is depicted by the box and the arrows indicate the bidirectional DNA replication from this *ori*. (B) Typical structure of linear plasmids isolated from *Borrelia* containing terminal hairpin telomeric structures composed of inverted repeats (thick arrows) harboring the nick sites (vertical arrows) involved in DNA replication.

bacteria and can be transferred between different microorganisms, a genetic property that represents a very serious medical problem in human and animal medicine. These plasmids, called R plasmids, harbor a variety of genes encoding resistance to a wide spectrum of antimicrobial compounds, which include antibiotics, heavy metals, resistance to mutagenic agents like ethidium bromide, and even disinfectant agents such as formaldehyde (10, 11). Furthermore, genetic and molecular analysis of plasmids proved to be essential in understanding the structure of transposons and integrons and the role these genetic elements play in the transmission of resistance to antimicrobial agents (12-14). The presence of these mobile genetic elements in transmissible plasmids, some of them capable of replicating in bacterial strains belonging to different species, makes matters quite serious since they contribute to the transmissibility of resistance genes from strains to strains as well as between different replicons within any given strain.

The term plasmid was originally used by Lederberg to describe all extrachromosomal hereditary determinants, and it is currently used to describe autonomously replicating extrachromosomal DNA of bacteria (2, 3). While not essential for the survival of bacteria, plasmids may encode the wide variety of genetic determinants mentioned above which permit their bacterial hosts to survive better in an adverse environment or to compete better with other microorganisms occupying the same ecological niche. Plasmid size varies from a few to several hundred kilobases (kb) and bacterial cells can harbor more than one plasmid species (15). Plasmids are found in a wide variety of microorganisms, and it is as difficult to generalize about plasmids as it is to generalize about the microorganisms that harbor them. Plasmids also include the replicative forms of filamentous coliphages and the prophage state of phages such as P1 (16).

Although bacterial genomes have long been considered to contain only covalently closed circular (ccc) double-stranded DNA molecules, the presence of linear chromosome and plasmid molecules has been described in bacteria. The first linear plasmid was found in *Streptomyces rochei* in 1979 (17) and now they have been detected in several bacterial genera such as *Agrobacterium*, *Borrelia*, *Nocardia, Rhodococcus, Thiobacillus,* and even *Escherichia* (18). The *Streptomyces* linear plasmids range in size from nine to several hundred kb and all of them contain terminal inverted repeats of different length. The *Borrelia* plasmids are unique among extrachromosomal elements since some of them carry critical genes such as the *guaA* and *guaB* biosynthetic genes (19) as well as genes encoding the essential major outer surface Osp or Vmp lipoproteins (20-23). In addition, some of these linear plasmids are present stably in low copy number, about one per chromosome equivalent, and can be cured by exposing *Borrelia* cultures to the DNA gyrase inhibitor novobiocin (20-23). These genetic properties suggest that the replication and partition processes of linear plasmids are well controlled during cell division. All these facts together with the observation that the *Borrelia* chromosome has attributes of a linear DNA molecule has led to the idea that these linear plasmids might be regarded more properly as minichromosomes (24).

The linear structure of DNA molecules brings an interesting biological problem associated with the protection of the DNA ends from exonuclease degradation and the complete replication of these plasmids. The ends of linear *Streptomyces* plasmids have telomeric structures that are also found in adenoviruses and some prokaryotic phages as well as in almost all eukaryotic plasmids. An inverted terminal repeat was found in pSL2, a linear plasmid present in *S. rochei* (25, 26) (figure 1A). These terminal repeats, together with specific DNA binding proteins, are thought to be involved in the juxtaposition of the two plasmid termini containing identical or very similar nucleotide sequences. This structure, known as the racket frame-like DNA model includes a terminal protein covalently attached to the 5' DNA termini which is required to protect the DNA from degradation and complete the replication of the 3' overhanging ends. The structure of the 16- and 49-kb linear plasmids of *B. burgdorferi* consist of a double-stranded DNA chain connected at each end by a perfect palindromic AT-rich hairpin loop (figure 1B) (27, 28). In addition, each end contains a conserved 19-base pair (bp) inverted repeat sequence, a telomeric structural feature found in all linear plasmids analyzed in *Borrelia* to date (18, 20, 22). These

features have similarities to the telomeres of other linear double-stranded replicons, including among them viral genomes (29, 30) and the mitochondrial DNA of the yeast *Pichia* (31).

In general, bacterial plasmids replicate independently of the host chromosome, although usually they rely on some host-encoded factors for their replication. They are present in bacterial cells replicating at a specific number of copies per cell, which can range from one or two to several hundreds. However, replication is not enough for plasmids to be stably inherited in a cell line. Plasmid maintenance functions, particularly in low-copy number plasmids, such as F, fulfill an essential role in the equipartition of the molecules to the daughter cells (5). Plasmid partition systems, one of the maintenance mechanisms, are normally plasmid encoded, although host cellular functions may be required (32-34). The mechanism of partition apparently involves the recognition of pairs of plasmid molecules and membrane proteins followed by an active process of separation of the members of the pair to the opposite halves of the dividing bacterial cell (35). More recently, the partition mechanism of F and the role of plasmid-encoded proteins and plasmid DNA sequences in partition were examined by using the green fluorescent protein (36). This new approach allowed the analysis of plasmid partition while the cells were growing and dividing. Besides partition, plasmids have evolved other maintenance mechanisms such as site-specific recombination systems that resolve plasmid multimers. There are also anticlumping systems that destroy aggregates of plasmid molecules to allow them to diffuse freely in the cytoplasm. In addition, there are systems that kill cells that have lost the plasmid after division (5).

Since very early in the study of plasmids, attempts were made to develop a classification system based on properties such as copy number or their ability to survive in one or many different bacterial hosts. However, it was soon clear that those criteria were not suitable since unrelated plasmids could be grouped together for instance by their copy number and host range. Furthermore, genes encoding many specialized functions (such as resistance to antibiotics, production of toxins, synthesis or utilization of nutrients) were found to be highly mobile (included in transposable elements and integrons) and therefore plasmids could acquire those functions without changing their inheritance properties. Therefore, these criteria for classification were either too broad, grouping together unrelated plasmids, or were based on properties that could not be ascribed to the biological properties of the plasmid. In the early 1960s a property that is peculiar to plasmids was described: incompatibility. Plasmid incompatibility is the failure of two plasmids to be stably inherited, in the absence of selective pressure, in the same cell line. This phenomenon is a consequence of sharing elements of plasmid inheritance functions such as replication or partition (15, 37, 38). Therefore, incompatibility gives a better idea of closeness among plasmids, which can then be grouped together in what is called incompatibility (Inc) groups. Initially, these incompatibility groups were determined by using biological assays based on plasmid transformation and examination of plasmid stability by determining antibiotic resistance phenotypes and restriction map analysis; however a replicon typing system was recently proposed for plasmid classification (39). This system is more practical and it is based on the identification of sequence homologies between replicons using DNA hybridization techniques. A bank of replicon probes was prepared from replication regions of plasmids belonging to the different Inc groups.

In this review we describe the main plasmid replication systems and their role in plasmid incompatibility. In addition, we describe the structure and replication mechanisms of bacterial linear plasmids.

3. REPLICATION

Several host- and plasmid-encoded functions are required for plasmid replication. Initiation of plasmid replication is molecule specific and of great importance for the propagation process, copy number, and incompatibility properties of plasmids in both Gram-positive and -negative bacteria (40, 41). In general, plasmid replicons contain one or several origins (*ori*) of replication and one or more regulatory elements, located in a DNA fragment no larger than 4 kb. In addition, most plasmid replicons harbor a gene encoding either a protein or an RNA molecule that functions as a primer for DNA replication. The Rep proteins can often act in *trans* on a specific *ori*, but in some cases they may only function in *cis*. However, in all cases examined so far the preprimer RNA acts in *cis* with the replication initiation sequences. Composite multi-replicon plasmids were also described. One example is R6K, in which three origins are able to function *in vivo* independently, although the rate of initiation from each origin is different (42, 43). It was reported that many plasmid origins follow a molecular mechanism similar to *ori*C, the origin of replication of the *E. coli* chromosome (44). However, the major difference is that plasmids require an origin-specific plasmid-encoded protein for the initiation step, generally called Rep proteins. These plasmid-encoded Rep proteins act in place of or in combination with DnaA, the replication initiation protein for chromosomal DNA. Some plasmids require additional host-gene products such as *dam* methylases, integration host factor (IHF), and heat shock proteins to replicate (40, 45). Other plasmids, such as the ColE1-type encode an RNA-specific plasmid molecule and require the host-encoded DNA polymerase I (PolI or PolA), RNA polymerase, and ribonuclease H (RNase H) (46-48). Two types of mechanisms basically control the replication of plasmid DNA. One utilizes a series of repeated sequences, designated iterons, located at *ori* and capable of interacting

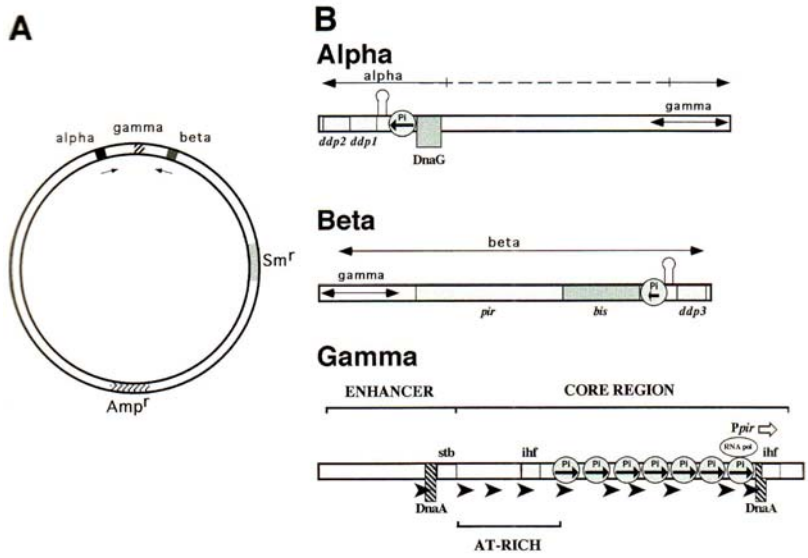

Figure 2. (A) Diagram of the R6K plasmid. The location of the three origins of replication and the genes encoding resistance to streptomycin (Sm) and ampicillin (Amp) are indicated. The arrows mark the *in vivo* direction of the initial replication from the alpha and beta origins. (B) Diagram of the alpha, beta, and gamma origins of replication (adapted from 74 and 77). Alpha, components of the active alpha origin. The double-headed arrows indicate the DNA fragments containing the alpha origin and the gamma core region required in *cis* for active replication. The dashed line represents the nonessential intervening sequences. The location of the long inverted repeat is indicated by the hairpin followed by the genes encoding the DDP1 and DDP2 proteins. The thick arrow and the large rectangle indicate the position of an iteron and a DnaG binding site, respectively. The circle indicates the replication protein Pi bound to the iteron. Beta, components of the active beta origin. The double-headed arrows indicate the DNA fragment containing the gamma core region and beta origin required in *cis* for active replication. The beta origin includes the *pir* and *bis* genes, a half iteron (half-thick arrow) bound by Pi (circle), the *ori* beta long inverted repeat (hairpin), and the gene encoding the DDP3 protein. Gamma, components of the enhancer and core regions of the gamma origin containing binding sites for the host-encoded proteins DnaA, IHF, and RNA polymerase. P*pir* and the open arrow represent the promoter site and the direction of transcription of the *pir* gene, respectively. The thick arrows represent the seven iterons that bind the Pi replication protein (circles). *stb* represents the locus involved in plasmid maintenance. The Fis-binding sites are indicated by the arrowheads.

with the replicator protein. In the other, small complementary RNA molecules (antisense) hybridize with the transcript responsible for the initiation process, either directly or indirectly by encoding the Rep protein.

3.1. Iteron-regulated plasmids: the R6K plasmid

Plasmid R6K belongs to the group of iteron-regulated replicons encoding an initiator protein that binds to repeated sequences located within *ori*. This group includes the *E. coli oriC* (44) and the plasmids F (49), pSC101 (50), P1 (16), pMJ101 (51, and manuscript in preparation), Rts1 (52), the REPI replicon of pColV-K30 (53), and the RK2-and RP-4 related plasmids (54). The conjugative plasmid R6K is a naturally occurring extrachromosomal element that codes for resistance to the antibiotics ampicillin and streptomycin (55) (figure 2A). It is about 38 kb in size and has a copy number of 13 to 40 per cell (56). These features together with a unique mode of replication made R6K an attractive system to study the genetic and molecular mechanisms involved in plasmid DNA replication. In addition, this plasmid and its replication components were among the first used in molecular biology to generate gene fusions, transcription enhancement, protein tagging, and site-specific proteolysis (57-59). Furthermore, R6K recombinant derivatives were instrumental in designing a series of suicide vectors successfully used to generate mutants by allelic exchange or transposition mutagenesis in Gram-negative bacteria (60, 61).

The alpha, beta, and gamma origins of R6K are all clustered within a 4-kb DNA fragment that contains also the *pir* and *bis* genes encoding the Pi and Bis replication proteins, respectively (figure 2). The main active origins *in vivo* are alpha and beta, while *ori* gamma tends to remain inactive (42) due to the synthesis of a silencer RNA encoded immediately downstream *ori* gamma (62). However, *ori* gamma can replicate autonomously when the other two origins are deleted and the *pir* gene is provided either in *cis* or in *trans* (63-67). Thus, *ori* gamma has served as the simplest model system derived from R6K to study the replication of an iteron-containing DNA molecule.

The gamma origin is the major binding-site for the Pi replication protein (57, 68, 69) and is required in *cis* for initiation of plasmid replication from the other two *ori* sites (70-72). Thus, the gamma origin behaves as a prokaryotic enhancer-type element since DNA-protein interactions at this site induce significant changes in DNA structure that facilitate initiation of DNA replication from the two other distantly located origins (73). The molecular organization of this origin is similar to other plasmid origins although it contains two functionally distinct segments: the enhancer and the core regions (figure 2B) (71, 74). The core is a 277-bp segment, which is common to all three origins and is essential for replication. It consists of three distinct regions: (i) the AT-rich region, bound by Pi and IHF, (ii) seven 22-bp repeats bound by Pi, and (iii) a region which interacts with DnaA, IHF, and RNA polymerase host proteins. The 106-bp enhancer lies immediately to the left of the core and includes a DnaA box and a small segment containing the *stb* locus (75). The enhancer region is required for stable maintenance of R6K derivatives containing only the gamma-origin harboring plasmids. The *stb* locus has some similarities with the *par* locus of pSC101, however the partition systems of these two plasmids differ from each other in several aspects (75). It has been proposed (75) that a host-encoded protein binds to the *stb* repeats, a hypothesis that is supported by the fact that R6K derivatives carrying all three origins bind *in vitro* to both inner and outer membrane fractions of *E. coli* (76). An alternative explanation is that *stb* mediates plasmid partition by altering the structure of gamma-origin containing plasmids (75). The host-encoded protein Fis binds to 10 sites in the gamma-origin that overlap the binding sites for the R6K-encoded Pi protein and the host-encoded DNA binding proteins DnaA, IHF, and RNA polymerase (77). The Fis protein appears to be required for plasmid replication only when Pi copy-up variants and the penicillin resistant marker are simultaneously used. This is an interesting observation since it demonstrates that plasmid genes encoding antibiotic resistance play a role not only in the ability of bacterial cells to survive in adverse environments, but also are important in the replication of plasmid DNA.

The functional alpha replicon contains two elements, separated by a nonessential 3-kb fragment, that must be present in *cis* and oriented as in the intact R6K: (i) a 580-bp fragment containing the alpha *ori* and a long 98-bp palindrome which serves as the recognition signal for initiation of DNA replication from this *ori*, and (ii) the 277-bp core segment located within the gamma *ori* (figure 2B). DNA homology analysis revealed the presence of a 23-bp sequence which resembles the seven 22-bp iterons found in the core segment (78) and plays a role in the Pi protein-mediated looping between the gamma and alpha sequences (73). In addition, this fragment contains a DnaG-binding site that can serve as a DnaB loading site by DnaB-DnaG interaction (79).

The minimal beta-replicon is a 2-kb fragment that encompasses: (i) the gamma core region, (ii) the *pir* gene encoding the Pi initiator protein, (iii) the *bis* gene encoding the 17.2 kDa Bis protein, and (iiii) the beta *ori* (72, 80-82) (figure 2B). The Bis protein is required only by this origin and its synthesis is coupled in *cis* to the expression of Pi protein from an unaltered *pir* gene (81). This origin also contains a half iteron and a 98-bp palindrome which has high homology with the hairpin located in the alpha origin (78). These two elements are required for the Pi-mediated looping between the beta and gamma sequences (73), and the initiation of DNA replication at the *ori* beta, respectively.

The Pi initiator protein is a homodimer with a 36-kDa molecular weight for the monomeric form that is lysine rich and weakly basic (63). It binds to the seven iterons in the gamma origin and to an eighth iteron and a smaller inverted pair of repeats located in the operator-promoter region of the *pir* gene (57, 59, 68, 69, 83) (figure 2B). In addition, Pi interacts with the iterons located in the alpha and beta iterons, however, these contacts are weak and require the enhancing effect of Pi already bound to the seven gamma iterons (73, 84). This protein is essential for replication from each R6K origin and although it can be provided in *cis* or *trans* to regulate the activity of the gamma and alpha origins, it is required in *cis* for activation of the beta origin (65, 72, 78, 82, 85). Thus, the Pi protein has a positive role (86) in the replication of R6K, which is displayed by its ability to enhance replication of *ori* alpha and *ori* beta. This activity is mediated by promoting DNA unwinding and bending and looping out of intervening sequences located between the *ori* gamma core region and the alpha and beta origins (84, 87). These DNA conformational changes lead to the activation of these two *ori* sites. Site-directed mutagenesis showed that the Pi protein is necessary but not sufficient for activation of *ori* beta and probably *ori* alpha (73). The DNA looping process is also required for the transfer of a multiprotein complex capable of initiating DNA replication. It was recently reported that Pi specifically interacts with the host-encoded helicase DnaB replication protein (73, 79). This observation indicates that DnaB is initially recruited by Pi bound to *ori* gamma and then delivered by the Pi-induced DNA looping to the alpha and beta origins.

Three additional proteins required for the distortion of the DNA structure of the R6K origins were recently described (88). Two of them, designated DDP1 and DDP2, are encoded by two tandem genes located at the 5' end of the long inverted repeat of the alpha origin. The other protein, designated DDP3, is encoded by a gene mapped at the 3' end of the beta origin long inverted repeat (figure 2B). Although the distortions caused by these proteins are potentially linked to R6K replication, they are not equivalent to those described in other replication regions previously characterized (44). It was also suggested (88) that this distortion system serves to synchronize the initiation of replication and establishes the direction of replication from the alpha and beta origins.

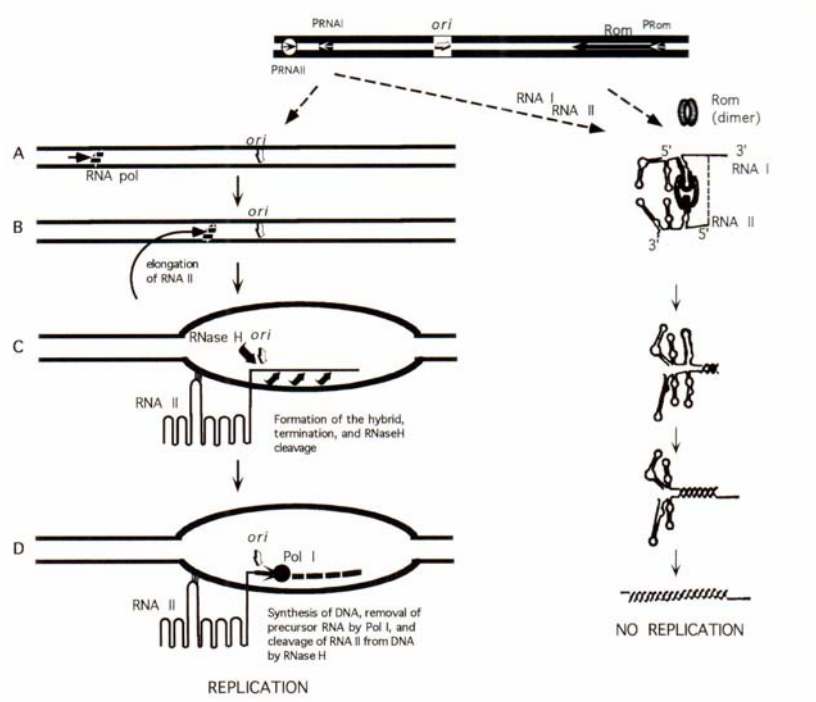

Figure 3. Mechanism of initiation of replication of ColE1 and its regulation. The diagram in the top shows a genetic map of the initiation of replication region of ColE1. The left portion of the diagram depicts the mechanism of initiation of replication. The right portion shows the action of RNA I and the Rom protein. The arrows inside black or white circles indicate initiation of transcription locations for RNA II and RNA I. The origin of replication (*ori*) site (nucleotide 555 of RNA II) and the site of action of RNase H are indicated. A through D represents different steps in the initiation of replication. The proposed mechanism of the succession of events involved in the regulation of the initiation of replication of ColE1 by RNA I and Rom (adapted from 40) are shown on the right portion. The interaction between RNA I and RNA II leads to the inhibition of DNA synthesis is shown. The first interaction between RNA I and RNA II (kissing) is reversible and stabilized by the Rom protein. A more detailed scheme of the possible interactions between the RNA and Rom species has been reported elsewhere (40).

3.2. RNA-regulated plasmids: the ColE1-type plasmids

A large number of naturally occurring plasmids as well as many of the most commonly used cloning vehicles replicate their DNA using a common mechanism: the synthesis of an RNA molecule that forms a double stranded structure with the template DNA followed by digestion with RNase H and initiation of replication by DNA polymerase I (5, 40, 89-95). These plasmids include the naturally occurring ColE1, pMB1, p15A, pJHCMW1, as well as cloning vehicles such as pBR322 and related vectors, the pUC plasmids, the pET series, the pBluescript series and several others (91-93, 96-103). Of all these plasmids, ColE1 initiation of replication and its regulation has been the most thoroughly studied (47, 101, 102, 104-139).

Replication of ColE1-type plasmids is initiated at a unique *ori* site (140, 141) (figure 3) and unlike other plasmid families, a plasmid-encoded protein does not mediate initiation of replication. However, ColE1 requires the host's DNA Polymerase I (PolI) enzyme (46), a host-encoded RNA polymerase and RNase H (46-48). The replication process is initiated by the synthesis of an RNA molecule, called RNA II, which is initiated 555 nucleotides upstream of *ori* (figure 3, step A). This RNA II extends about 700 nucleotides from its initiation (figure 3, steps B and C) and its 3' end forms a duplex with the template plasmid DNA location close to *ori* (figure 3, step C) (47, 130). This process, known as coupling, is strongly dependent on the formation of a specific secondary structure at the 5' end of the RNA II molecule that results in an interaction between the RNA portion on the *ori* and an upstream region of this molecule with the template DNA (see figure 3, step C) (111, 113, 114, 116, 142). RNase H, which digests the RNA II at the replication origin, recognizes this RNA II-DNA duplex. As a consequence a free 3'-hydroxyl group is generated that serves as primer for DNA synthesis catalyzed by PolI (figure 3, steps C and D) (47). Mutagenesis analysis on RNA II showed that some point mutations prevent this molecule from adopting the right spatial conformation that in turn affects hybridization with the template DNA (111,

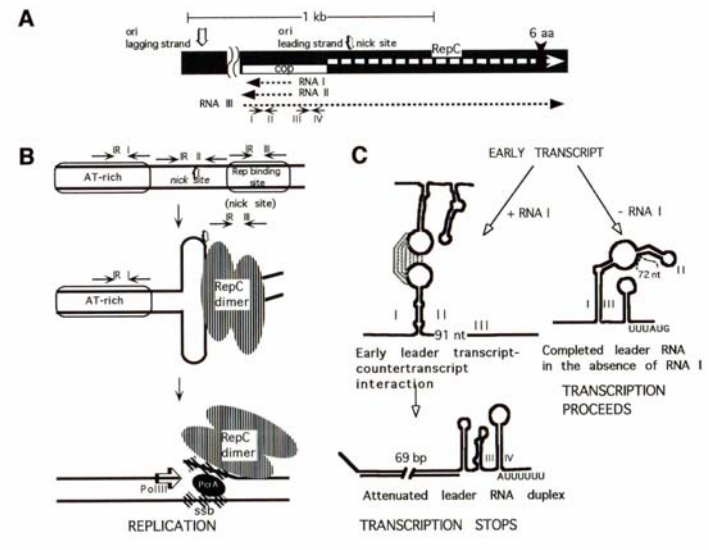

Figure 4. Mechanism of initiation of replication of the plasmid pT181 and its regulation (adapted from 5, 162, 170). (A) Diagram of the pT181 replication region showing the two origins of replication (leading and lagging strands). The broken line arrow represents the RepC coding region. The six amino acid region of RepC recognized by *ori* are indicated as a black square. The location of the *cop* region is indicated by a box. The three RNA species are indicated showing the inverted repeats I, II, III, and IV. For the sake of clarity, these inverted repeats are not at scale. (B) Model for initiation of replication of pT181. The first diagram shows the region encompassing the origin of replication of the leading strand and the three inverted repeats. This region is also referred to as the double stranded origin (DSO). The second diagram shows the formation of the cruciform structure after binding of the RepC homodimer, the bending of the DNA at the binding region, and the change in structure of RepC. The AT-rich region that includes IR I facilitates the melting process. The third diagram shows the assembly of a replisome after the nicking took place. Presumably DNA polymerase III initiates replication in the presence of the helicase PcrA and single-strand binding protein (ssb). (C) Mechanism of regulation of expression of RepC by antisense RNA (adapted from 41, 218). The early RNA III transcript can interact with antisense RNA (RNA I). This interaction leads to the formation of a stem-loop between inverted repeats III and IV that results in a transcription termination signal. In the absence of RNA I, the inverted repeat III is sequestered by inverted repeat I, inhibiting formation of the transcription termination signal and leading to completion of the *repC* mRNA.

113, 114). Once PolI begins the addition of deoxynucleotides, the remaining portion of RNA II which is still hybridized to the template DNA is digested at other sites by RNase H and by the 5'-3' exonuclease activity of PolI (figure 3, step D) (125). ColE1 DNA replication proceeds unidirectionally with the initiation of the lagging strand synthesis at specific ColE1 sites.

Two alternative mechanisms that seem not to be used in wild-type cells and that may be adaptations to specific host mutations were described for replication in the absence of either RNase H or RNase H and PolI (109, 115, 143, 144). In these two mechanisms, RNA II is required to be in the correct three dimensional configuration to act as the primer for DNA replication (109, 144, 145). In the case of a double mutant lacking PolI and RNase H, RNA II synthesis is extended because of the lack of RNase H. This fact allows the formation of a single stranded DNA region that can extend to a length that is adequate for assembly of a replisome and initiation of synthesis on the opposite DNA strand (lagging strand) (40, 115). In the case of RNase H deficient mutants, a mechanism dependent of PolI was described. The extended RNA II species can be recognized by PolI and used as a primer (109). However, *in vitro* experiments suggested that this mechanism of initiation is rather inefficient (47).

3.3. Rolling-circle replicating plasmids: the pT181-type plasmids

A number of small, high copy number plasmids from Gram-positive bacteria have been initially shown to replicate using the rolling circle mechanism of replication and later some plasmids of Gram-negative bacteria were identified that use this mechanism of replication (41, 146-153). The molecular mechanisms of replication of plasmids pT181, pC221, pUB110, pC194 and pMV158 are the best known to date (43, 53, 154-158).

Based on sequence comparisons and genetic organization of the replication regions of plasmids of Gram-positives, five replication systems have been defined (5, 41, 147, 154), the rolling circle, the theta mechanism (two different types: those using a short RNA primer and those using a long RNA primer), and the *Streptomyces* and

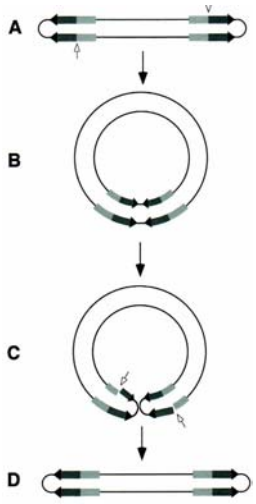

Figure 5. Replication model for linear plasmids in *Borrelia* (adapted from 24). (A) The replication process begins by the nicking (open-arrowhead arrows) of the initiation site located within the telomeric inverted repeats (shaded arrows). (B) The open linear plasmid circularizes due to the presence of complementary sequences at its termini and replicates as a circular replicon. (C) The newly replicated plasmids are nicked within the telomeric inverted repeats. (D) The free single-stranded ends pair back with their complementary copies located in the same monomer, reconstituting the hairpins at the plasmid termini. Two copies of the original linear plasmid are generated after DNA ligation.

Borrelia linear plasmids (discussed in section 3.4). The most common replication system among the Gram-positives plasmids is the rolling circle (5). They replicate using an asymmetric rolling circle pathway similar to that of the single-stranded filamentous bacteriophages (5, 41, 159). Four groups of rolling circle replicating plasmids have been recognized. The pT181, the pMV158, the pC194, and the pSN2 groups (41, 43, 53, 146, 147, 150, 154-156). Here we will review the replication mechanism of pT181, one of the best understood rolling circle replication mechanisms.

The pT181 plasmid was isolated from *Staphylococcus aureus* (160) and a diagram showing its replication region and the mechanism of initiation of replication is shown in figure 4. The plasmid pT181 encodes a 38-kDa initiator protein, RepC, that has sequence-specific endonuclease and topoisomerase I-like activities (161). This protein induces a nick in one of the pT181 DNA strands (leading strand) at a specific site signaling the initiation of replication. The nick generates a free 3'-OH end that is used as primer for DNA synthesis (162). The functional conformation of the RepC protein is a homodimer that recognizes and binds to a specific site (Rep binding site) that encompasses an inverted repeat (IR III in figure 4) (161). A domain of six amino acids in RepC plays an important role in the recognition and interaction of the Rep binding site (figure 4) (162). The binding efficiency of RepC is increased by the presence of *cmp*, a 100-bp *cis*-acting replication enhancer located about 1 kb from the nicking site (163, 164). It has been recently demonstrated that a *S. aureus* protein, CBF1, binds *cmp* and increases distortion of the already bent *cmp* locus (165). Whether this binding is associated with the enhancing activity of *cmp* is still not known.

Binding of the RepC homodimer to IR III triggers bending of DNA in this region (166) followed by a change in structure of RepC, DNA melting, and formation of a cruciform structure at the IR II region (figure 4) (162). The melting step is facilitated by the presence of an AT-rich inverted repeat region (IR I) located upstream of IR II (5). This induces the formation of a cruciform structure that may help in approximating the nicking site of the leading strand to the active site of RepC. This process involves a tyrosine residue that appears to facilitate the generation of the nick. After the endonuclease attack, the RepC protein remains covalently bound to the 5' end of the DNA by a phosphotyrosine bond (155). It is probable that RepC remains covalently attached to the DNA throughout the replication of the leading strand. However, although this seems to be the case for pT181, it was recently shown that in a derivative of pMV158 the initiator protein does not remain covalently bound to the DNA after nicking (156). After generation of the 3'-OH terminal end by RepC nicking of pT181 DNA, an initiation complex is formed with DNA polymerase III, the helicase PcrA and single strand binding protein (figure 4) (5, 167, 168). Following (or during) replication, there is an addition of a 10-12-mer oligodeoxynucleotide identical to the sequence located immediately 3' to the origin of the leading strand to the RepC molecule resulting in a modified protein known as RepC*. This modification process leads to the loss of the two enzymatic activities of RepC (169, 170). As a consequence, the active initiator RepC/RepC homodimer becomes the inactive RepC/RepC* heterodimer after it has been used for replication of pT181 (171).

Replication of the leading strand does not require any protein encoded by the plasmid (172). Initiation of replication of the lagging strand is initiated at a different location than the leading strand known as SSO (single-strand origin) or *palA* (173). This locus comprises a stretch of about 160-bp palindromic DNA sequence (5, 41). As a consequence of the synthesis of the leading strand a displaced single-stranded DNA is generated which allows the initiation of replication region of the lagging strand to adopt the appropriate conformation to serve as a priming site. It has been previously shown that some rolling circle replicating plasmids have different origins of replication for their lagging strands when replicating in different hosts, which suggests that lagging strand origins of replication may be important in determining the host range of plasmids (5).

Termination of pT181 synthesis of the leading strand occurs at the nick site by a strand transfer RepC-mediated mechanism (174). Once the nick site has been replicated and extended a few nucleotides beyond this site, one of the subunits of the RepC dimer contacts the growing strand. This interaction initiates a strand transfer reaction resulting in the formation of a single stranded monomer (old strand), a double stranded molecule where one of the strands is newly synthesized, and a dimer in which one of the monomers is attached to the oligonucleotide resulting from the extension of replication beyond the nick site (5, 41).

3.4. Replication of linear plasmids: the *Streptomyces* and *Borrelia* plasmids

It was proposed initially that the *Streptomyces* linear plasmids replicate by a protein-primed replication mechanism (18, 175), where the telomere is the origin of replication. This region is recognized by specific DNA binding proteins that promote the unwinding of the double helix and serve as the primer for a specific DNA polymerase. However, it was latter observed that the pSCL *S. clavuligerus* linear plasmid can replicate as a circular DNA molecule when the telomeres are removed and the ends are ligated (176). This observation suggested that linear plasmids such as pSLA2 must contain an internal site capable of promoting DNA replication. Agarose gel electrophoresis analysis showed that linear plasmids are indeed replicated primarily bidirectionally from an internal origin, located near the center of the plasmid, toward the ends, rather than by full-length strand displacement initiated at the telomeres. However, pSLA2 still requires a protein-primed strand displacing mechanism to complete the replication of a 280-nucleotide segment at both plasmid termini. It was postulated (175) that the synthesis of the 5' terminal segment of the lagging strand of this linear plasmid uses the 3' overhang of the leading strand as a template and is primed by the protein covalently attached to the 5' end of the mature plasmid.

The knowledge on the replication mechanisms of linear plasmids in *Borrelia* has been extended by a series of very recent publications (24, 177, 178). Some of the *Borrelia* linear plasmids can exist and replicate either as a linear or a monomeric circular element (24), a process that involves circular intermediates. These intermediates are formed by a head-to-tail junction, after a nick introduced in the inverted repeat opens the plasmid termini (figure 5, steps A and B), which can replicate as any other well characterized circular replicon. The linear double-stranded copies are generated from the circular intermediate by a second nick within the terminal inverted repeats (figure 5, steps C and D). More recently, it was found that *B. burgdorferi* sensu lato contains atypical large linear plasmids ranging from 92 to 105 kb (178). These plasmids carry *p27* and *ospAB*, genes that were also detected in other isolates on the 50-kb linear plasmid pAB50. A more detailed analysis of the larger plasmids demonstrated that they are formed by tail-to-tail dimerization of pAB50. The presence of such a dimers can explain the unusual plasmid variability observed among different isolates and may provide new information regarding the replication mechanism of these linear replicons. It was postulated (178) that these linear dimers are the result of failed segregation after DNA replication by a mechanism similar to that described for vaccinia viruses (179). In this model proposed by Marconi *et al*. (178), initiation of plasmid replication proceeds from one of the termini, after the head hairpin loop in this particular case is nicked, allowing the formation of tail-to-tail dimer intermediates. Alternatively, these dimers can arise by DNA replication initiated from an *ori* located within a linear monomer that results in a circular replication intermediate. In the normal replication process, the circular intermediate is resolved into two linear monomers by independent cleavage events at each telomere junction. Cleavage failure at the tail hairpin by a specific DNA cleavage system results in two monomers linked tail to tail, a possibility that was confirmed experimentally by restriction analysis and Southern blot DNA hybridization experiments (178). However, this same analysis demonstrated that not all *Borrelia* large plasmids were originated via dimer formation and alternative DNA replication systems still uncharacterized must exist.

Some of the proteins involved in the replication of the linear plasmids in *Borrelia* were identified by determining the complete nucleotide sequence of the 16-kb plasmid lp16.9 isolated from *B. burgdorferi* B31 (177). This study revealed the presence of 15 open reading frames (Orf), named A to O. The predicted proteins encoded by OrfM and N showed homology to proteins involved in plasmid replication and cell division. OrfM is homologous to MinD, a cytoplasmic membrane protein with ATPase activity required for the correct placement of the division site (180, 181). OrfM has also homology with plasmid partition proteins, such as ParA or SopA (182, 183), and the RepB protein encoded by the pheromone-responsive plasmid pAD1 in *Enterococcus faecalis* and involved in the control of the copy number of this extrachromosomal element (184). The predicted features and the primary sequence of the OrfN hypothetical polypeptide are similar to those of the CopB DNA-binding proteins, also known as RepB or RepA2, involved in the control of plasmid copy number in Gram-negative bacteria (185). This sequence analysis also led to the hypothesis that OrfN may interact with the short repeated sequence located within the promoter region of OrfM and, thus, controlling the expression of the latter (177). In summary, sequence analysis of *Borrelia* plasmids showed that the few proteins identified are similar to prokaryotic proteins involved in plasmid replication and maintenance in Gram-positive and -negative bacteria.

4. PLASMID COPY NUMBER AND INCOMPATIBILITY

Several of the plasmid replication elements described above are also involved in the expression of functions controlling the initiation of plasmid DNA

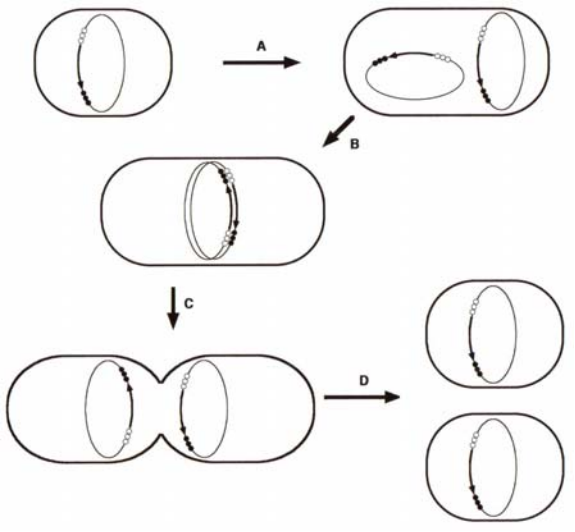

Figure 6. Model for control of plasmid replication by the handcuffing mechanism (adapted from 221, 222). (A) At low copy number replication is initiated by the interaction of the Rep protein with the iterons located within *ori*. (B) Further plasmid replication is blocked by antiparallel pairing when the plasmid reaches the appropriate copy number or a different plasmid harboring the same replication system is introduced into the cell. (C) The plasmid copies are pulled apart into the new daughter cells by a partition and segregation mechanism (D), where the replication cycle is then reiterated. The curved arrows depict the *rep* gene while the open and closed circles represent the Rep protein bound to the *inc* and *ori* determinants, respectively.

replication. These control mechanisms are ultimately responsible for the copy number and incompatibility properties of different plasmids. The two main mechanisms controlling plasmid replication and incompatibility are based on antisense RNAs and repeated sequences located near the origin of replication. There are also plasmids such as R1, pMV158 and pIP501 in which the control of copy number involves both, antisense RNA and protein. In the following sections we will concentrate on the description of some antisense RNA- and iteron-mediated control of copy number mechanisms. Control of copy number of other plasmids not presented here have been thoroughly reviewed recently (5, 15, 37, 40, 147, 154, 158, 186-188).

4.1. Antisense RNAs

Antisense RNA molecules are responsible for regulation of replication of several plasmids by different mechanisms: in some cases they control the synthesis of a replication protein and in some others they inhibit the activity of the RNA primer (186, 189). In the case of IncFII plasmids, which include R1, R100 or R6-5 as well as other related elements belonging to the IncFIIa, IncFIFc, IncFIII, and IncFVII group, an antisense RNA of about 90 nucleotides regulates initiation of replication (186). The best known of these plasmids is R1 that requires the RepA protein for initiation of replication (190, 191). The RepA mRNA is known as CopT (Cop *t*arget) and the regulator antisense RNA is called CopA. CopA controls initiation of R1 replication by interacting with CopT resulting in posttranscriptional inhibition of RepA synthesis (192-200). The concentration of CopA in the cytosol is influenced by the PcnB and RNase E proteins (201). On the other hand, in the case of ColE1-type plasmids the antisense RNA acts by interacting with the RNA primer.

The mechanism of regulation of initiation of replication of ColE1 is among the best understood (5, 37, 40, 117, 187, 188, 202-204). It is worthy to mention that the study of replication of the ColEl plasmid yielded the discovery of antisense RNA and the first demonstration that antisense RNA can control gene expression. Regulation of initiation of replication of ColE1 is mediated by the action of a 108-nucleotide antisense RNA transcript, called RNA I. This RNA specie is encoded in a region that overlaps the coding region for RNA II (figure 3) (47, 205). Therefore, RNA I is an antisense molecule with respect to RNA II. RNA I inhibits initiation of replication by binding to nascent RNA II by complementary base pairing. This process results in a conformational change in RNA II that prevents coupling with DNA (101, 114, 130, 133, 206). There are several stem-loops within the secondary structures of both RNA species (figure 3). The first interaction between both RNA species occurs at complementary loops. This is a reversible process denominated "kissing" that results in the generation of an unstable initial complex (figure 3) (110, ,131, 132, 135, 207). After this initial contact takes place, an irreversible process leads to the formation of an RNA duplex between RNA I and RNA II (figure 3) (102, 132, 133). For RNA I to exert its inhibitory effect it must be present during a short, specific interval in the synthesis of RNA II (133, 208). However, this situation occurs very often because RNA I initiation of transcription proceeds as much as five times more often than transcription of RNA II. As a consequence only one out of about 20 RNA II molecules that are initiated results in a productive DNA replication. Some other factors can have an influence in the available amount of RNA I in the cell: polyadenylation (209, 210) and bacterial RNase E which inactivates RNA I by cleavage of its 5' end (211).

Another factor involved in the control of initiation of replication of some, but not all, ColE1-type plasmids is a 63 amino acid protein called Rom (RNA one modulator) or Rop (Repressor of primer) (figure 3) (5, 131-133, 205, 212). The gene encoding this short protein is located downstream of *ori*. Rom binds to the stem-loop portions of the unstable initial complex (110, 207), reducing the dissociation constant of the complex. Therefore, its presence results in the creation of a pathway for a stable binding of RNA I and RNA II which reduces the number of productive initiation of replication rounds (figure 3) (132, 136). As a consequence, although the presence of Rom is not needed for viability of the plasmid, ColE1 deletion derivatives that lack *rom* present a higher

copy number than plasmids that have the complete ColE1 replication region (5, 131, 213). RNA I is the main incompatibility determinant in ColE1-type plasmids. Two plasmids that depend on the same RNA I species for regulation of initiation of replication can not coexist in the same cell (15, 37, 38). It has been shown that even single nucleotide changes can have profound effects in the incompatibility properties of ColE1-type plasmids (101, 206).

Regulation of the synthesis of the RepC protein is the main mechanism of control replication of pT181 (5, 214-216). The organization of pT181 replication regulation region (*cop*) is shown in figure 4. The *repC* mRNA (RNA III) includes a leader sequence typical of genes subjected to regulation by attenuation (217). This leader sequence has four interacting sequences (I - IV). Sequence I, a 9-nucleotide sequence called "preemptor" can form duplexes with sequences II and III (figure 4). In addition to RNA III, there are two antisense RNA species (RNA I and RNA II). These RNAs are complementary to the leader sequence of RNA III. In the presence of antisense RNA, a stem-loop structure between inverted repeats III and IV, which is followed by an AUUUUUU sequence, is formed in the leader portion of RNA III. This structure acts as a transcription termination signal (figure 4) (218). In the absence of antisense RNA, the interaction between inverted repeats I (the preemptor) and III generates a structure that prevents the formation of the III-IV stem-loop allowing transcription of *repC* to proceed (figure 4) (215, 218). Thus, the main incompatibility elements in pT181-type plasmids are the antisense RNA and the origin of replication of the leading strand (5, 215, 218). The origin of replication of the leading strand directly binds RepC, titrating the protein that is present at limiting levels in the cytosol due to the controlling action of the antisense RNAs.

4.2. DNA iterons

In the case of the iteron-regulated plasmids, the clusters of repeated sequences located at *ori*, and the copy control and autoregulation regions are the main incompatibility determinants (5). Initially, it was proposed that the replication protein was titrated by the iterons, limiting the rounds of initiation, however, further studies on plasmid replication suggested the existence of other mechanisms involved in copy number control and incompatibility (38, 219). The structural analysis of P1 RepA protein-DNA complexes revealed that the *ori* and copy control iterons can compete with the autoregulation iterons for RepA binding. A close examination of those RepA-DNA complexes showed the formation of a DNA loop between the *incA* determinant and the origin of replication (220). This DNA looping between *incA* and *ori*, mediated by RepA, can occur intra- as well as intermolecularly (220, 221), producing a steric blockage of replication initiation that causes plasmid incompatibility and affects negatively the plasmid copy number.

More recently, a similar negative control mechanism was proposed for P1 (221, 222), RK2 (54), and R6K (223). This model, known as "handcuffing" or coupling, which is based on the interaction between the replication protein and the origin of replication, proposes two alternative pathways for the Rep-bound iterons depending on the intracellular concentration of these complexes (figure 6). At low concentration, the Rep-iteron complexes stimulate the initiation of replication until the regular copy number is achieved. However, when the copy number is increased beyond the normal levels or a second plasmid harboring the same replication system is introduced into the cell, the Rep-iteron concentration is high enough to produce the binding of almost all protein-bound iterons. This direct interaction between the plasmid molecules produces the coupling or handcuffing of the origins, making them unavailable for the initiation of replication. Thus, iteron-containing plasmids regulate their copy number and express incompatibility by sensing the formation of coupled origin sequences mediated specifically by their cognate initiation proteins (224).

In the case of R6K, the control of copy number and incompatibility functions are due to the two negative activities of the Pi replication protein. One of them is as an autorepressor at the level of transcription and involves its binding to iteron sequences located within the operator-promoter site of the *pir* gene (figure 2B). These interactions act by either preventing the binding of the RNA polymerase to the promoter or displacing the RNA polymerase from promoter-enzyme complexes (66, 83, 225) leading to a reduction in the synthesis of Pi. The other negative role was detected when increased intracellular levels of Pi either lowered the plasmid copy number or completely prevented its replication (225). This negative regulatory activity of Pi was explained by molecular models involving either direct Pi-DNA interactions or association of Pi with either other Pi molecules or with host encoded proteins (86, 223). The R6K "handcuffing" model is based on the observation that Pi has the ability to associate two DNA molecules containing gamma *ori* sequences and to enhance the DNA ligase-catalyzed multimerization of a single DNA fragment carrying this origin of replication (223). In addition, it was shown that the negative domain of Pi is located in the N-terminal region of this protein and that the Pi-mediate inhibition of R6K replication does not require direct binding to DNA (226). Consequently, the origins located within these DNA-protein complexes are unable to initiate replication most likely because Pi-induced DNA structure alterations in the origins or potential Pi-host protein interactions are prevented. In summary, all these observations proved that Pi has positive and negative functions in the replication of R6K. This well controlled replication process is the result of a competition between positive gamma-alpha and gamma-beta interactions and the inhibitory aggregation of gamma-containing molecules, all mediated by the Pi replication protein.

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6. REFERENCES

1. Hayes, W.: Observations on a transmissible agent determining sexual differentiation in *Bacterium coli. J Gen Microbiol* 8, 72-88 (1953)

2. Lederberg, J., L.L. Cavalli-Sforza, & E.M. Lederberg: Sex compatibility in *Escherichia coli. Genetics* 37, 720-730 (1952)

3. Lederberg, J.: Personal perspective. Plasmid (1952-1997) *Plasmid* 39, 1-9 (1998)

4. Cohen, S.N.: Bacterial plasmids: their extraordinary contribution to molecular genetics. *Gene* 135, 67-76 (1993)

5. D Helinski, A Toukdarian, & R Novick: Replication control and other stable maintenance mechanisms of plasmids. In: *Escherichia coli and Salmonella*. Cellular and Molecular Biology. Ed: Neidhardt F, American Society for Microbiology Press, Washington, DC 2295-2324 (1996)

6. Crosa, J.H.: Genetics and molecular biology of siderophore-mediated iron transport in bacteria. *Microbiol Rev* 53, 517-530 (1989)

7. Falkow, S., The evolution of pathogenicity in *Escherichia coli, Shigella, and Salmonella*. In: *Escherichia coli and Salmonella*. Cellular and Molecular Biology. Ed: Neidhardt F, American Society for Microbiology Press, Washington, DC 2723-2729(1996)

8. Baron, C. & P.C. Zambryski: Plant transformation: a pilus in *Agrobacterium* T-DNA transfer. *Curr Biol* 6, 1567-1569 (1996)

9. Kado, C.: Promiscuous DNA transfer system of *Agrobacterium tumefaciens*: role of the *virB* operon in sex pilus assembly and synthesis. *Mol Microbiol* 12, 17-22 (1994)

10. Foster, T.J.: Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiol Rev* 47, 361-409 (1983)

11. Kummerle, N., F. Heinz-Hubert, & P.M. Kaulfers: Plasmid-mediates formaldehyde resistance in *Escherichia coli*: characterization of a resistance gene. *Antimicrob Agents Chemother* 40, 2276-2279 (1996)

12. Hall, R. & C. Collins: Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol Microbiol* 15, 593-600 (1995)

13. Kleckner, N.: Transposable elements in prokaryotes. *Annu Rev Genet* 15, 341-404 (1981)

14. Tolmasky, M.E. & J.H. Crosa: Genetic organization of antibiotic resistance genes (*aac(6')-Ib, aadA, and oxa 9*) in the multiresistance transposon Tn*1331*. *Plasmid* 29, 31-40 (1993)

15. Tolmasky, M.E., L.A. Actis, & J.H. Crosa: Plasmids. In: Encyclopedia of Microbiology. Ed: Lederberg J , Academic Press, San Diego, CA 431-442 (1992)

16. Abeles, A.L., K.M. Snyder, & D.K. Chattoraj: P1 plasmid replication: replicon structure. *J Mol Biol* 173, 307-324 (1984)

17. Hayakawa, T., T. Tanaka, K. Sakaguchi, N. Otake, & H. Yonehara: A linear plasmid-like DNA in *Streptomyces* sp. producing lankacidin group antibiotics. *J Gen Appl Microbiol* 25, 255-260 (1979)

18. Hinnebusch, J., & H. Tilly: Linear plasmids and chromosomes in bacteria. *Mol Microbiol* 10, 917-922 (1993)

19. Margolis, N., D. Hogan, K. Tilly, & P.A. Rosa: Plasmid location of *Borrelia* purine biosynthesis gene homologs. *J Bacteriol* 176, 6427-6432 (1994)

20. Barbour, A.G., & C.F. Garon: Linear plasmids of the bacterium *Borrelia burgdorferi* have covalently closed ends. *Science* 237, 409-411 (1987)

21. Barbour, A.G.: Linear DNA of *Borrelia* species and antigenic variation. *Trends Microbiol* 1, 236-239 (1993)

22. Kitten, T., & A.G. Barbour: Juxtaposition of expressed variable antigen genes with a conserved telomere in the bacterium *Borrelia hermsii. Proc Natl Acad Sci USA* 87, 6077-6081 (1990)

23. Plasterk, R.H., M.I. Simon, & A.G. Barbour: Transposition of structural genes to an expression sequence on a linear plasmid causes antigenic variation in the bacterium *Borrelia hermsii. Nature (London)* 318, 257-263 (1985)

24. Ferdows, M.S., P. Serwer, G.A. Griess, S. J. Norris, & A.G. Barbour: Conversion of a linear to circular plasmid in the relapsing fever agent *Borrelia hermsii. J Bacteriol* 178, 793-800 (1996)

25. Hirochika, H., K. Nakamura, & K. Sakaguchi: A linear plasmid from *Streptomyces rochei* with an inverted terminal repetition of 614 base pairs. *EMBO J* 3, 761-766 (1984)

26. Sakaguchi, K., H. Hirochika, & N. Gunge: Linear plasmids with terminal inverted repeats obtained from *Streptomyces rochei* and *Kluyveromyces lactis*. In: Plasmids in bacteria. Eds: Helinski DR, Clewell DB, Jackson DA , & Hollaender A, Plenum Press: New York & London 433-451 (1985)

27. Hinnebusch, J., S. Bergstrom, & A.G. Barbour: Cloning and sequence analysis of linear plasmid telomeres of the bacterium *Borrelia burgdorferi*. *Mol Microbiol* 4, 811-820 (1990)

28. Hinnebusch, J., & A.G. Barbour: Linear plasmids of *Borrelia burgdorferi* have a telomeric structure and sequence similar to those of a eukaryotic virus. *J Bacteriol* 173, 7233-7239 (1991)

29. Baroudy, B.M., S. Venkatesan, & B. Moss: Incompletely base-paired flip-flop terminal loops link the two DNA strands of the Vaccinia virus genome into one uninterrupted polynucleotide chain. *Cell* 28, 315-324 (1982)

30. Gonzalez, A., A. Talavera, J.M. Almendral, & E. Vinuela: Hairpin loop structure of African swine fever DNA. *Nucleic Acid Res* 14, 6835-6844 (1986)

31. Dinouel, N., R. Drissi, I. Miyakawa, F. Sor, S. Rousset, & H. Fukuhara: Linear mitochondrial DNAs of yeasts: closed-loop structure of the termini and possible linear-circular conversion mechanisms. *Mol Cell Biol* 13, 2315-2323 (1993)

32. Austin, S.T.: Plasmid partition. *Plasmid* 20, 1-9 (1988)

33. Hiraga, S.: Chromosome and plasmid partition in *Escherichia coli. Annu Rev Biochem* 61, 283-306 (1992)

34. Williams, D.R. & C.M. Thomas: Active partitioning of bacterial plasmids. *J Gen Microbiol* 138, 1-16 (1992)

35. Firshein, W. & P. Kimm: Plasmid replication and partition in *Escherichia coli*: is the cell membrane the key? *Mol Microbiol* 23, 1-10 (1997)

36. Kim, S.-K. & J.C. Wang: Localization of F plasmid SopB protein to positions near the poles of *Escherichia coli* cells. *Proc Natl Acad Sci USA* 95, 1523-1527 (1998)

37. B Kittell, & D. Helinski: Plasmid incompatibility and replication. In: Bacterial Conjugation. Ed: Clewel D. Plenum Press (1993)

38. Novick, R.: Plasmid incompatibility. *Microbiol Rev* 51, 381-395 (1987)

39. Couturier, M., F. Bex, P.L. Bergquist, & W.K. Maas: Identification and classification of bacterial plasmids. *Microbiol Rev* 52, 375-395 (1988)

40. Kues, U. & U. Stahl: Replication of plasmids in gram negative bacteria. *Microbiol Rev* 53, 491-516 (1989)

41. Novick, R.: Staphylococcal plasmids and their replication. *Annu Rev Microbiol* 43, 537-565 (1989)

42. Crosa, J.H.: Three origins of replication are active *in vivo* in the R-plasmid RSF1040. *J Biol Chem* 255, 11075-11077 (1980)

43. Inuzuka, N., M. Inuzuka, & D.R. Helinski: Activity *in vitro* of three replication origins of the antibiotic resistance plasmid RSF1040. *J Biol Chem* 255, 11071-11074 (1980)

44. Bramhill, D., & A. Kornberg: A model for initiation at origins of DNA replication. *Cell* 54, 915-918 (1988)

45. Scott, J.R.: Regulation of plasmid replication. *Microbiol Rev* 48, 1-23 (1984)

46. Itoh, T. & J. Tomizawa: Initiation of replication of plasmid ColE1 DNA by RNA polymerase, ribonuclease H, and polymerase I. *Cold Spring Harbor Symp Quant Biol* 43, 409-418 (1978)

47. Itoh, T. & J. Tomizawa: Formation of an RNA primer for initiation of replication of ColE1 DNA by ribonuclease H. *Proc Natl Acad Sci USA* 77, 2450-2454 (1980)

48. Itoh, T. & J. Tomizawa: Purification of ribonuclease H as a factor required for *in vitro* ColE1 DNA replication. *Nucleic Acids Res* 10, 5949-5965 (1982)

49. Morotsu, T., K, Matsubara, H. Sugusaki, & M. Takanami: Nine unique repeating sequences in a region essential for replication and incompatibility of the mini-F plasmid. *Gene* 15, 257-271 (1981)

50. Vocke, C., & D. Bastia: Primary structure of the essential replicon of the plasmid pSC101. *Proc Natl Acad Sci USA* 80, 6557-6561 (1983)

51. Bidinost, C., J.H. Crosa, & L.A. Actis: Localization of the replication region of the pMJ101 plasmid from *Vibrio ordalii. Plasmid* 31, 242-250 (1994)

52. Kamio, Y., Y. Itoh, & Y. Terawaki: Purification of Rts1 RepA protein and binding of the protein to mini-Rts1 DNA. *J Bacteriol* 170, 4411-4414 (1988)

53. Gammie, A.E., J.H. Crosa: Co-operative autoregulation of a replication protein gene. *Mol Microbiol* 5, 3015-3023 (1991)

54. Kittell, B.L., & D.R. Helinski: Iteron inhibition of plasmid RK2 replication *in vitro*: Evidence for intermolecular coupling of replication origins as a mechanism for RK2 replication control. *Proc Natl Acad Sci USA* 88, 1389-1393 (1991)

55. Datta, N., & P. Kontomichalou: Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. *Nature (London)* 208, 239-241 (1965)

56. Kontomichalou, P., M. Mitani, & R.C. Clowes: Circular R-factor molecules controlling penicillinase synthesis, replicating under either relaxed or stringent control. *J Bacteriol* 104, 34-44 (1970)

57. Germino, J., & D. Bastia: The replication initiator protein of plasmid R6K tagged with beta-galactosidase shows sequence-specific DNA-binding. *Cell* 32, 131-140 (1983)

58. Germino, J., J.G. Gray, H. Charbonneau, T. Vanaman, & D. Bastia: Use of gene fusions and protein-protein interaction in the isolation of a biologically active regulatory protein: The replication initiator protein of plasmid R6K. *Proc Natl Acad Sci USA* 80, 6848-6852 (1983)

59. Germino, J., & D. Bastia: Rapid purification of a cloned gene product by genetic fusion and site-specific proteolysis. *Proc Natl Acad Sci USA* 81, 4692-4696 (1984)

60. de Lorenzo, V., & K.N. Timmis: Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. *Methods Enzymol* 235, 386-405 (1994)

61. Miller, V.L., & J.J. Mekalanos: A novel suicide vector and use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* 170, 2575-2583 (1988)

62. Patel, I., & D. Bastia: A replication origin is turned off by an origin-"silencer" sequence. *Cell* 47, 785-792 (1986)

63. Germino, J., & D. Bastia: Primary structure of the replication initiation protein of plasmid R6K. *Proc Natl Acad Sci USA* 79, 5475-5479 (1982)

64. Inuzuka, M., & D. R. Helinski: Replication of antibiotic resistance plasmid R6K DNA *in vitro*. *Biochem* 17, 2567-2573 (1978)

65. Kolter, R., M. Inuzuka, & D.R. Helinski: Trans-complementation-dependent replication of a low molecular weight origin fragment from plasmid R6K. *Cell* 15, 1199-1208 (1978)

66. Shafferman, A., R. Kolter, D.M. Stalker, & D.R. Helinski: Plasmid R6K replication. III. Regulatory properties of the Pi initiation protein. *J Mol Biol* 161, 57-76 (1982)

67. Stalker, D.M., R. Kolter, & D.R. Helinski: Plasmid R6K replication. I. Complete nucleotide sequence of an autonomously replicating segment. *J Mol Biol* 161, 33-43 (1982)

68. Filutowicz, M., E. Uhlenhopp, & D.R. Helinski: Binding of purified wild-type and mutant Pi initiation proteins to a replication origin region of plasmid R6K. *J Mol Biol* 187, 225-239 (1985)

69. Germino, J., & D. Bastia: Interaction of the plasmid R6K-encoded replication initiator protein with its binding sites on DNA. *Cell* 34, 125-134 (1983)

70. Crosa, J.H., L.K. Luttropp, & S Falkow: Molecular cloning of replication and incompatibility regions from the R-plasmid R6K. *J Mol Biol* 124, 443-468 (1978)

71. Kelly, W.L., I. Patel, & D. Bastia: Structural and functional analysis of a replication enhancer: Separation of the enhancer activity from origin function by mutational dissection of the replication origin gamma of plasmid R6K. *Proc Natl Acad Sci USA* 89, 5078-5082 (1992)

72. Shafferman, A., & D.R. Helinski: Structural properties of the beta-origin of replication of plasmid R6K. *J Biol Chem* 258, 4083-4090 (1983)

73. Miron, A., S. Mukherjee, & D. Bastia: Activation of distant replication origins *in vivo* by DNA looping as revealed by a novel mutant form of an initiator protein defective in cooperativity at a distance. *Embo J* 11, 1205-1216 (1992)

74. Wu, F., I. Goldberg, & M. Filutowicz: Roles of a 106-bp origin enhancer and *Escherichia coli* DnaA protein in replication of plasmid R6K. *Nucleic Acids Res* 20, 811-817 (1992)

75. Wu, F., I. Levchenko, & M. Filutowicz: A DNA segment conferring stable maintenance on R6K gamma-origin core replicons. *J Bacteriol* 177, 6338-6345 (1995)

76. Jemilohun, P.F., C.W. Clark, & E.R. Archibold: Characterization of bacterial cell membrane attachment sites of plasmid R6K. *Biochem Biophys Res Commun* 221, 186-192 (1996)

77. Wu, F., J. Wu, J. Ehley, & M. Filutowics: Preponderance of Fis-binding sites in the R6K gamma origin and the curious effect of the penicillin resistance marker on the replication of this origin in the absence of Fis. *J Bacteriol* 178, 4965-4974 (1996)

78. Shafferman, A., Y. Flashner, I. Hertman, & M. Lion: Identification and characterization of the functional alpha origin of DNA replication of the R6K plasmid and its relatedness to the R6K, beta and gamma origins. *Mol Gen Genet* 208, 263-270 (1987)

79. Ratnakar, P.V.A.L., B.K. Mohanty, M. Lobert, & D. Bastia: The replication initiator protein Pi of the plasmid R6K specifically interacts with the host-encoded helicase DnaB. *Proc Natl Acad Sci USA* 93, 5522-5526 (1996)

80. Crosa, J.H., L.K. Luttropp, & S. Falkow: Use of autonomously replicating mini-R6K plasmids in the analysis of the replication regions of the R-plasmid R6K. *Cold Spring Harbor Symp Quant Biol* 43, 111-120 (1979)

81. Mukhopadhyay, P., M. Filutowicz, & D.R. Helinski: Replication from one of the three origins of the plasmid R6K requires coupled expression of two plasmid-encoded proteins. *J Biol Chem* 261, 9534-9539 (1986)

82. Shon, M., J. Germino, & D. Bastia: The nucleotide sequence of the replication origin beta of the plasmid R6K. *J Biol Chem* 257, 13823-13827 (1982)

83. Kelly, W., & D. Bastia: Replication initiator protein of plasmid R6K autoregulates its own synthesis at the

transcriptional level. *Proc Natl Acad Sci USA* 82, 2574-2578 (1985)

84. Mukherjee, S., H. Ericson, & D. Bastia: Enhancer-origin interaction in plasmid R6K involves a DNA loop mediated by initiator protein. *Cell* 52, 375-383 (1988)

85. Kolter, R., & D.R. Helinski: Plasmid R6K DNA replication. II. Direct nucleotide sequence repeats are required for an active gamma-origin. *J Mol Biol* 161, 45-56 (1982)

86. Filutowicz, M., M.J. McEachern, & D.R. Helinski: Positive and negative roles of an initiator protein at an origin of replication. *Proc Natl Acad Sci USA* 83, 9645-9649 (1986)

87. Mukherjee, S., I. Patel, & D. Bastia: Conformational changes in a replication origin induced by an initiator protein. *Cell* 43, 189-197 (1985)

88. Flashner, Y., J. Shlomai, & A. Shafferman: Three novel plasmid R6K proteins act in concert to distort DNA within the alpha and beta origins of DNA replication. *Mol Microbiol* 19, 986-996 (1996)

89. Astill, D., P. Manning, & M. Heuzenroeder: Characterization of the small cryptic plasmid, pIMVS1, of *Salmonella enterica* ser. *thyphimurium*. *Plasmid* 30, 258-267 (1993)

90. Bagdasarian, M. & M. Bagdasarian: Gene cloning and expression. In: Methods for General and Molecular Bacteriology. Ed: Gerhardt, P. American Society for Microbiology Press, Washington, DC. 406-417 (1993)

91. Dery, K., R. Chavideh, V. Waters, R. Chamorro, L. Tolmasky, & M.E. Tolmasky: Characterization of the replication and mobilization regions of the multiresistance *Klebsiella pneumoniae* plasmid pJHCMW1. *Plasmid* 38, 95-105 (1997)

92. Fu, J., H. Chang, Y. Chen, Y. Chang, & S. Liu: Sequence analysis of an *Erwinia stewartii* plasmid pSW100. *Plasmid* 34, 75-84 (1995)

93. Nomura, N. & Y. Murooka: Characterization and sequencing of the region required for replication of a non-selftransmissible plasmid pEC3 isolated from *Erwinia carotovora* subsp. *carotovora*. *J Ferment Bioeng* 78, 250-254 (1994)

94. Old, R. & S. Primrose: Principles of gene manipulation. Blackwell Scientific Publications, Oxford (1985)

95. Sambrook, J., E. Fritsch, & T. Maniatis: Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989)

96. Bhagwat, A.S. & S. Person: Structure and properties of the region of homology between plasmids pMB1 and ColE1. *Mol Gen Genet* 182, 505-507 (1981)

97. Balbas, P., X. Soberon, F. Bolivar, & R.L. Rodriguez: The plasmid, pBR322. *Biotechnol* 10, 5-41 (1988)

98. Bolivar, F.: Molecular cloning vectors derived from the CoLE1 type plasmid pMB1. *Life Sci* 25, 807-817 (1979)

99. Covarrubias, L., L. Cervantes, A. Covarrubias, X. Soberon, I. Vichido, A. Blanco, Y.M. Kupersztoch-Portnoy, & F. Bolivar: Construction and characterization of new cloning vehicles. V. Mobilization and coding properties of pBR322 and several deletion derivatives including pBR327 and pBR328. *Gene* 13, 25-35 (1981)

100. Studier, F., A. Rosenberg, J. Dunn, & J. Dubendorff: Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* 185, 60-89 (1990)

101. Tomizawa, J. & T. Itoh: Plasmid ColE1 incompatibility determined by interaction of RNA I with primer transcript. *Proc Natl Acad Sci USA* 78, 6096-6100 (1981)

102. Tomizawa, J.: Control of ColE1 plasmid replication: the process of binding of RNA I to the primer transcript. *Cell* 38, 861-870 (1984)

103. Vieira, J. & J. Messing: The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 19, 259-268 (1982)

104. Ataai, M.M. & M.L. Shuler: Mathematical model for the control of ColE1 type plasmid replication. *Plasmid* 16, 204-212 (1986)

105. Brendel, V. & A.S. Perelson: Quantitative model of ColE1 plasmid copy number control. *J Mol Biol* 229, 860-872 (1993)

106. Brenner, M. & J. Tomizawa: Rom transcript of plasmid ColE1. *Nucleic Acids Res* 17, 4309-4326 (1989)

107. Brenner, M. & J. Tomizawa: Quantitation of ColE1-encoded replication elements. *Proc Natl Acad Sci U S A* 88, 405-409 (1991)

108. Chan, P.T., H. Ohmori, J. Tomizawa, & J. Lebowitz: Nucleotide sequence and gene organization of ColE1 DNA. *J Biol Chem* 260, 8925-8935 (1985)

109. Dasgupta, S., H. Masuakata, & J. Tomizawa: Multiple mechanisms for initiation of ColE1 DNA replication: DNA synthesis in the presence and absence of RNase H. *Cell* 51, 1113-1122 (1987)

110. Eguchi, Y. & J. Tomizawa: Complex formed by complementary RNA stem-loops. Their formation,

structures and interaction with ColE1 Rom protein. *J Mol Biol* 220, 831-842 (1991)

111. Fitzwater, T., Y. Yang, X. Zhang, & B. Polisky: Mutations affecting RNA-DNA hybrid formation of the ColE1 replication primer RNA. *J Mol Biol* 226, 997-1008 (1992)

112. Itoh, T. & J. Tomizawa: Initiation of replication of plasmid ColE1 DNA by RNA polymerase, ribonuclease H, and DNA polymerase I. *Cold Spring Harbor Symp Quant Biol* 43, 409-417 (1979)

113. Masukata, H. & J. Tomizawa: Effects of point mutations on formation and structure of the RNA primer for ColE1 replication. *Cell* 36, 513-522 (1984)

114. Masukata, H. & J. Tomizawa: Control of primer formation for ColE1 plasmid replication: conformational change of the primer transcript. *Cell* 44, 125-136 (1986)

115. Masukata, H., S. DasGupta, & J. Tomizawa: Transcriptional activation of ColE1 DNA synthesis by displacement of nontranscribed strand. *Cell* 51, 1123-1130 (1987)

116. Masukata, H. & J. Tomizawa: A mechanism of formation of a persistent hybrid between elongating RNA and template DNA. *Cell* 62, 331-338 (1990)

117. Merlin, S. & B. Polisky: Analysis of establishment phase replication of the plasmid ColE1. *J Mol Biol* 230, 137-150 (1993)

118. Merlin, S. & B. Polisky: Assessment of quantitative models for plasmid ColE1 copy number control. *J Mol Biol* 248, 211-219 (1995)

119. Nakasu, S. & J. Tomizawa: Structure of the ColE1 DNA molecule before segregation to daughter molecules. *Proc Natl Acad Sci U S A* 89, 10139-10143 (1992)

120. Ohmori, H. & J. Tomizawa: Nucleotide sequence of the region required for maintenance of colicin E1 plasmid. *Mol Gen Genet* 176, 161-170 (1979)

121. Oka, A., N. Nomura, M. Morita, H. Sugisaki, K. Sugimoto, & M. Takanami: Nucleotide sequence of small ColE1 derivatives: structure of the regions essential for autonomous replication and colicin E1 immunity. *Mol Gen Genet* 172, 151-159 (1979)

122. Patnaik, P.K., S. Merlin, & B. Polisky: Effect of altering GATC sequences in the plasmid ColE1 primer promoter. *J Bacteriol* 172, 1762-1768 (1990)

123. Polaczek, P. & Z. Ciesla: Effect of altered efficiency of the RNA I and RNA II promoters on *in vivo* replication of ColE1-like plasmids in *Escherichia coli*. *Mol Gen Genet* 194, 227-231 (1984)

124. Polisky, B., X.Y. Zhang, & T. Fitzwater: Mutations affecting primer RNA interaction with the replication repressor RNA I in plasmid ColE1: potential RNA folding pathway mutants. *Embo J* 9, 295-304 (1990)

125. Selzer, G. & J. Tomizawa: Specific cleavage of the p15A primer precursor by ribonuclease H at the origin of DNA replication. *Proc Natl Acad Sci USA* 79, 7082-7086 (1982)

126. Selzer, G., T. Som, T. Itoh, & J. Tomizawa: The origin of replication of plasmid p15A and comparative studies on the nucleotide sequences around the origin of related plasmids. *Cell* 32, 119-129 (1983)

127. Som, T. & J. Tomizawa: Origin of replication of *Escherichia coli* plasmid RSF 1030. *Mol Gen Genet* 187, 375-383 (1982)

128. Som, T. & J. Tomizawa: Regulatory regions of ColE1 that are involved in determination of plasmid copy number. *Proc Natl Acad Sci U S A* 80, 3232-3236 (1983)

129. Takeya, T., K. Shimada, A. Oka, & Y. Takagi: Location of a ColE1 deoxyribonucleic acid region that affects the plaque-forming ability of lambda-ColE1 hybrid bacteriophage. *J Bacteriol* 140, 294-296 (1979)

130. Tomizawa, J., T. Itoh, G. Selzer, & T. Som: Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. *Proc Natl Acad Sci U S A* 78, 1421-1425 (1981)

131. Tomizawa, J. &T. Som: Control of ColE1 plasmid replication: enhancement of binding of RNA I to the primer transcript by the Rom protein. *Cell* 38, 871-878 (1984)

132. Tomizawa, J.: Control of ColE1 plasmid replication: initial interaction of RNA I and the primer transcript is reversible. *Cell* 40, 527-535 (1985)

133. Tomizawa, J.: Control of ColE1 plasmid replication: binding of RNA I to RNA II and inhibition of primer formation. *Cell* 47, 89-97 (1986)

134. Tomizawa, J. & H. Masukata: Factor-independent termination of transcription in a stretch of deoxyadenosine residues in the template. DNA. *Cell* 51, 623-630. (1987)

135. Tomizawa, J.: Control of ColE1 plasmid replication: intermediates in the binding of RNA I and RNA II. *J Mol Biol* 212, 683-694 (1990)

136. Tomizawa, J.: Control of ColE1 plasmid replication: interaction of Rom protein with an unstable complex formed by RNA I and RNA II. *J Mol Biol* 212, 695-708 (1990)

137. Viguera, E., Hernandez, D. Krimer, A. Boistov, R. Lurz, J. Alonso, & J. Schvartzman: The ColE1 unidirectional origin acts as a polar replication fork pausing site. *J Biol Chem* 271, 22414-22421 (1996)

138. Wrobel, B. & G. Wegrzyn: Replication regulation of ColE1-like plasmids in amino acid-starved *Escherichia coli Plasmid* 39, 48-62 (1998)

139. Yang, Y.L. & B. Polisky: Suppression of ColE1 high-copy-number mutants by mutations in the *polA* gene of *Escherichia coli. J Bacteriol* 175, 428-437 (1993)

140. Inselburg, J.: Replication of colicin E1 plasmid DNA in minicells from a unique replication initiation site. *Proc Natl Acad Sci USA* 71, 2256-2259 (1974)

141. Tomizawa, J., M. Ohmori, & R. Bird: Origin of replication of colicin E1 plasmid. *Proc Natl Acad Sci USA* 74, 1865-1869 (1977)

142. Castagnoli, L., R. Lacatena, & G. Cesareni: Analysis of dominant copy number mutants of the plasmid pMB1. *Nucleic Acids Res* 13, 5353-5367 (1985)

143. Kingsbury, D. & D. Helinski: DNA polymerase as a requirement for the maintenance of the bacterial plasmid colicinogenic factor E1. *Biochem Biophys Res Commun* 41, 1538-1544 (1970)

144. Ohmori, H., Y. Murakami, & T. Nagata: Nucleotide sequences required for a ColE1-type plasmid to replicate in *Escherichia coli* cells with or without RNase H. *J Mol Biol* 198, 223-235 (1987)

145. Naito, S. & H. Uchida: RNase H and replication of ColE1 DNA in *Escherichia coli. J Bacteriol* 166, 143-147 (1986)

146. del Solar, G., M. Moscoso, & M. Espinosa: Rolling circle replicating plasmids from Gram-positive and Gram-negative bacteria: a wall falls. *Mol Microbiol* 8, 789-796 (1993)

147. Espinosa, M., G. del Solar, F. Rojo, & J. Alonso: Plasmid rolling circle replication and its control. *FEMS Microbiol Lett* 130, 111-120 (1995)

148. Galli, D. & D. Leblanc: Transcriptional analysis of rolling circle replicating plasmid pVT736-1: evidence for replication control by antisense RNA. *J Bacteriol* 177, 4474-4480 (1995)

149. Gielow, A., L. Diederich, & W. Messer: Characterization of a phage-plasmid hybrid (phasyl) with two independent origins of replication isolated from *Escherichia coli. J Bacteriol* 173, 73-79 (1991)

150. Gruss, A. & S. Ehrlich: The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. *Microbiol Rev* 53, 231-241 (1989)

151. Kleanthous, H., C. Clayton, & S. Tabaqchali: Characterization of a plasmid from *Helicobacter pylori* encoding a replication protein common to plasmids in Gram-positive bacteria. *Mol Microbiol* 5, 2377-2389 (1991)

152. Yasukawa, H., T. Hase, A. Sakai, & Y. Masamune: Rolling-circle replication of the plasmid pKYM isolated from a gram-negative bacterium. *Proc Natl Acad Sci USA* 88, 10282-10286 (1991)

153. Zhang, N. & J. Brooker: Characterization, sequence, and replication of a small cryptic plasmid from *Selenomonas ruminantium* subspecies *lactilytica. Plasmid* 29, 125-134 (1993)

154. Alonso, J. & M. Espinosa: Plasmids from Gram-positive bacteria. In: Plasmids - A Practical Approach. Ed: Hardy K, IRL Press, N Y 39-63 (1993)

155. Thomas, C., D. Balson, & W. Shaw: *In vitro* studies of the initiation of staphylococcal plasmid replication. *J Biol Chem* 265, 5519-5530 (1990)

156. Moscoso, M., G. del Solar, & M. Espinosa: Specific nicking-closing activity of the initiator of replication protein RepB of plasmid pMV158 on supercoiled or single-stranded DNA. *J Biol Chem* 270, 3772-3779 (1995)

157. Alonso, J.: DNA replication of plasmids from gram-positive bacteria in *Bacillus subtilis*. Plasmid pUB110 as a model system. *Microbiologia* 5, 5-12 (1989)

158. del Solar, G., R. Giraldo, M. Ruiz-Echeverria, M. Espinosa, & R. Diaz-Orejas: Replication of bacterial plasmids. *Microbiol Rev* 62, 434-464 (1998)

159. Baas, P. & H. Jansz: Single-stranded DNA phage origins. *Curr Top Microbiol Immunol* 136, 31-70 (1988)

160. Khan, S.A., S.M. Carleton, & R.P. Novick: Replication of plasmid pT181 DNA *in vitro*: requirement for a plasmid- encoded product. *Proc Natl Acad Sci USA* 78, 4902-4906 (1981)

161. Koepsel, R., R. Murray, W. Rosenblum, & S. Khan: The replication initiator protein of plasmid pT181 has sequence-specific endonuclease and topoisomerase-like activities. *Proc Natl Acad Sci USA* 82, 6845-6849 (1985)

162. Wang, F., S. Projan, V. Herniquez, & R. Novick: Specificity of origin recognition by replication initiator protein in plasmids of the pT181 family is determined by a six amino acid residue element. *J Mol Biol* 223, 145-158 (1992)

163. Gennaro, M. & R. Novick: *cmp*, a cis-acting plasmid locus that increases interaction between replication origin and initiator protein. *J Bacteriol* 168, 160-166 (1986)

164. Henriquez, V., V. Milisavljevic, J. Kahn, & M. Gennaro: Sequence and structure of *cmp*, the replication enhancer of the *Staphylococcus aureus* plasmid pT181. *Gene* 134, 93-98 (1993)

165. Zhang, Q., S. Soares de Oliveira, R. Colangeli, & M. Gennaro: Binding of a novel host factor to the pT181due element. J Mol Biol 223, *145-158 (1992*)

166. Koespel, R. & S. Khan: Static and initiatior protein-enhanced bending of DNA at the replication origin. *Science* 233, 1316-1318 (1986)

167. Iordanescu, S.: The *Staphylococcus aureus* mutation *prcA3* leads to the accumulation of pT181 replication initiation complexes. *J Mol Biol* 221, 1183-1189 (1991)

168. Iordanescu, S.: Plasmid pT181-linked suppresors of the *Staphylococcus aureus prcA3* chromosomal mutation. *J Bacteriol* 175, 3916-3917 (1993)

169. Rasooly, A. & R. Novick: Replication-specific inactivation of the pT181 plasmid initiator protein. *Science* 262, 1048-1050 (1993)

170. Rasooly, A., S. Projan, & R. Novick: Plasmids of the pT181 family show replication-specific initiator protein modification. *J Bacteriol* 176, 2450-2453 (1994)

171. Rasooly, A., P. Wang, & R. Novick: Replication-specific conversion of the *Staphylococcus aureus* pT181 initiator protein from an active homodimer to an inactive heterodimer. *EMBO J* 13, 5245-5251 (1994)

172. Birch, P. & S. Khan: Replication of single-stranded plasmid pT181 DNA *in vitro. Proc Natl Acad Sci USA* 89, 290-294 (1992)

173. Gruss, A., H. Ross, & R. Novick: Functional analysis of a palindromic sequence required for normal replication of several staphylococcal plasmids. *Proc Natl Acad Sci USA* 84, 2165-2169 (1987)

174. Iordanescu, S. & S. Projan: Replication termination for staphylococcal plasmids: plasmids pT181 and pC221 cross react in the termination process. *J Bacteriol* 170, 3427-3434 (1988)

175. Chang, P.-C., & S.N. Cohen: Bi-directional replication from an internal origin in a linear *Streptomyces* plasmid. *Science* 265, 952-954 (1994)

176. Shiffman, D., & S. N. Cohen: Reconstruction of a *Streptomyces* linear replicon from separately cloned DNA fragments: existence of a cryptic origin of circular replication within the linear plasmid. *Proc Natl Acad Sci USA* 89, 6129-6133 (1992)

177. Barbour, A.G., C.J. Crater, V. Bundoc, V., & J. Hinnebusch: The nucleotide sequence of a linear plasmid of *Borrelia burgdorferi* reveals similarities to those of circular plasmids of other prokaryotes. *J Bacteriol* 178, 6635-6639 (1996)

178. Marconi, R.T., S. Casjens, U.G. Munderloloh, & D.S. Samuels: Analysis of linear plasmid dimers in *Borrelia burgdorferi* sensu lato isolates: implications concerning the potential mechanism of liner plasmid replication. *J Bacteriol* 178, 3357-3361 (1996)

179. Moss, B., E. Winters, & E.V. Jones. In: UCLA Symposium on the mechanics of DNA replication and recombination (1983)

180. de Boer, P.A.J., R.E. Crossley, A.R. Hand, & L.I. Rothfield: The MindD protein is a membrane ATPase required for the correct placement of the *Escherichia coli* division site. *EMBO J* 10, 4371-4380 (1991)

181. Varley, A.W., & G.C. Stewart: The *divIV*B region of the *Bacillus subtilis* chromosome encodes homologs of *Escherichia coli* placement (MinCD) and cell shape (MreBCD) determinants. *J Bacteriol* 174, 6729-6742 (1992)

182. Abeles, A.L., S.A. Friedman, & S.J. Austin: Partition of unit-copy miniplasmids to daughter cells. III. The DNA sequence and functional organization of the P1 partition region. *J Mol Biol* 185, 261-272 (1985)

183. Mori, H., A. Kondo, A. Ohshima, T. Oruga, & S. Hiraga: Structure and function of the F plasmid genes essential for partitioning. *J Mol Biol* 192, 1-15 (1986)

184. Weaver, K.E., D.B. Clewell, & F. An: Identification, characterization, and nucleotide sequence of a region of *Enterococcus faecalis* pheromone-responsive plasmid pAD1 capable of autonomous replication. *J Bacteriol* 175, 1900-1909 (1993)

185. Lopez, J., I. Andres, J.M. Ortiz, & J.C. Rodriguez: Nucleotide sequence and expression of the copy number control gene (cop) of the IncFVII plasmid pSU233. *Nucleic Acid Res* 18, 7177 (1990)

186. Wagner, E. & R. Simons: Antisense RNA control in bacteria, phage and plasmids. *Ann Rev Microbiol* 48, 713-742 (1994)

187. Gerhardt, E., H. Wagner, & R. Simmons: Antisense RNA control in bacteria, phages, and plasmids. *Annu Rev Microbiol* 48, 713-742 (1994)

188. Simons, R.: Natural antisense RNA control in bacteria, phage, and plasmids. In: Antisense nucleic acids and proteins. Eds: Joseph M & van der Krol A. Marcel Dekker Inc, NY (1991)

189. Eguchi, Y., T. Itoh, & J. Tomizawa: Antisense RNA. *Ann Rev Biochem* 60, 631-652 (1991)

190. Masai, H. & K. Arai: *repA* protein and *oriR*-dependent initiation of R1 plasmid replication: identification of a *rho*-dependent transcription terminator required for *cis*-action of RepA protein. *Nucleic Acids Res* 16, 6493-6514 (1988)

191. Dong, X., D. Womble, & R. Rownd: *In vivo* studies on the cis-acting replicator initiator protein of IncFII plasmid NR1. *J Mol Biol* 202, 495-509 (1988)

192. Blomberg, P., H.M. Engdahl, C. Malmgren, P. Romby, & E.G. Wagner: Replication control of plasmid R1: disruption of an inhibitory RNA structure that sequesters the repA ribosome-binding site permits tap-independent RepA synthesis. *Mol Microbiol* 12, 49-60 (1994)

193. Blomberg, P., K. Nordstrom, & E.G. Wagner: Replication control of plasmid R1: RepA synthesis is regulated by CopA RNA through inhibition of leader peptide translation. *Embo J* 11, 2675-2683 (1992)

194. Blomberg, P., E.G. Wagner, & K. Nordstrom: Control of replication of plasmid R1: the duplex between the antisense RNA, CopA, and its target, CopT, is processed specifically *in vivo* and *in vitro* by RNase III. *Embo J* 9, 2331-2340 (1990)

195. Malmgren, C., H.M. Engdahl, P. Romby, & E.G. Wagner: An antisense/target RNA duplex or a strong intramolecular RNA structure 5' of a translation initiation signal blocks ribosome binding: the case of plasmid R1. *RNA* 2, 1022-1032 (1996)

196. Malmgren, C., E.G.H. Wagner, C. Ehresmann, B. Ehresmann, & P. Romby: Antisense RNA control of plasmid R1 replication. The dominant product of the antisense rna-mrna binding is not a full RNA duplex. *J Biol Chem* 272, 12508-12512 (1997)

197. Nordstrom, K., S. Molin, & J. Light: Control of replication of bacterial plasmids: genetics, molecular biology, and physiology of the plasmid R1 system. *Plasmid* 12, 71-90 (1984)

198. Nordstrom, K., E.G. Wagner, C. Persson, P. Blomberg, & M. Ohman: Translational control by antisense RNA in control of plasmid replication. *Gene* 72, 237-240 (1988)

199. Stougaard, P., S. Molin, & K. Nordstrom: RNAs involved in copy-number control and incompatibility of plasmid R1. *Proc Natl Acad Sci USA* 78, 6008-6012 (1981)

200. Wagner, E.G., P. Blomberg, & K. Nordstrom: Replication control in plasmid R1: duplex formation between the antisense RNA, CopA, and its target, CopT, is not required for inhibition of RepA synthesis. *Embo J* 11, 1195-1203 (1992)

201. Soderbom, F., U. Binnie, M. Masters, & E.G. Wagner: Regulation of plasmid R1 replication: PcnB and RNase E expedite the decay of the antisense RNA, CopA. *Mol Microbiol* 26, 493-504 (1997)

202. Cesareni, G., C. Helmer, & L. Castagnoli: Control of ColE1 plasmid replication by antisense RNA. *Trends Genet* 7, 230-235 (1991)

203. Eguchi, Y., T. Itoh, & J. Tomizawa: Antisense RNA. *Annu Rev Biochem* 60, 631-652 (1991)

204. Polisky, B.: ColE1 replication control circuitry: sense from antisense. *Cell* 55, 929-932 (1988)

205. Lacatena, M., D. Banner, L. Castagnoli, & G. Cesareni: Control of initiation of pMB1 replication: purified Rop protein and RNA I affect primer formation *in vitro*. *Cell* 37, 1009-1014 (1984)

206. Lacatena, R. & G. Cesareni: Base pairing of RNA I with its complementary sequence in the primer precursor inhibits ColE1 replication. *Nature* 294, 623-626 (1981)

207. Eguchi, Y. & J. Tomizawa: Complex formed by complementary RNA stem-loops and its stabilization by a protein: function of ColE1 Rom protein. *Cell* 60, 199-209 (1990)

208. Wong, E. & B. Polisky: Alternative conformations of the ColE1 replication primer modulate its interaction with RNA I. *Cell* 42, 959-966 (1985)

209. He, L., F. Soderbom, E. Wagner, U. Binnie, N. Binns, & M. Masters: PcnB is required for the rapid degradation of RNA I, the antisense RNA that controls the copy number of ColE1-related plasmids. *Mol Microbiol* 9, 1131-1142 (1993)

210. Xu, F., S. Lin-Chao, & S. Cohen: The *Escherichia coli pcnB* gene promotes adenylation of antisense RNA I of ColE1-type plasmids *in vivo* and degradation of RNA I decay intermediates. *Proc Natl Acad Sci USA* 90, 6756-6760 (1993)

211. Tomcsanyi, T. & D. Apirion: Processing enzyme ribonuclease E specifically cleaves RNA I an inhibitor of primer formation in plasmid DNA synthesis. *J Mol Biol* 185, 713-720 (1985)

212. Cesareni, G., M. Cornelissen, M. Lacatena, & L. Castagnoli: Control of pMB1 replication: inhibition of primer formation by Rop requires RNA I. *EMBO J* 3, 1365-1369 (1984)

213. Twigg, A. & D. Sherrat: Trans-complementable copy-number mutants of plasmid ColE1. *Nature* 283, 216-218 (1980)

214. Carleton, I., S. Projan, S. Highlander, & R. Novick: Control of pT181 replication. II. Mutational analysis. *EMBO J* 3, 2407-2414 (1984)

215. Novick, R., G. Adler, S. Projan, S. Carleton, S. Highlander, A. Gruss, S. Khan, & S. Iordanescu: Control of pT181 replication I. The pT181 copy control function acts by inhibiting the synthesis of a replication protein. *EMBO J* 10, 2399-2405 (1984)

216. Novick, R., S. Projan, S. Kumar, C. Carleton, S. Gruss, S. Highlander, & J. Kornblum: Replication control for pT181, an indirectly regulated plasmid. In: Plasmids in bacteria. Eds: Helinski DR, Clewell DB, Jackson DA , & Hollaender A, Plenum Press: New York & London 299-320 (1985)

217. Landick, R., C. Turnbough, & C. Yanofsky: Transcription attenuation. In: *Escherichia coli and Salmonella*. Cellular and Molecular Biology. Ed: Neidhardt F, American Society for Microbiology Press, Washington, DC 1263-1286 (1996)

218. Novick, R., S. Iordanescu, S. Projan, J. Kornblum, & I. Edelman: pT181 plasmid replication is regulated by a countertranscript-driven transcriptional attenuator. *Cell* 59, 395-404 (1989)

219. Pal, S., R.J. Mason, & D.K. Chattoraj: Role of initiator titration in copy number control. *J Mol Biol* 192, 275-285 (1986)

220. Pal, S. & D. Chattoraj: P1 plasmid replication: initiator sequestration is inadequate to explain control by initiator-binding sites. *J Bacteriol* 170, 3554-3560 (1988)

221. Abeles, A.L. & S.J. Austin: Antiparallel plasmid-plasmid pairing may control P1 plasmid replication. *Proc Natl Acad Sci USA* 88, 9011-9015 (1991)

222. Abeles, A.L., L.D. Reaves, B. Youngren-Grimes, & J.S. Austin: Control of P1 plasmid replication by iterons. *Mol Microbiol* 18, 903-912 (1995)

223. McEachern, M.J., M.A. Bott, P.A. Tooker, & D.R. Helinski: Negative control of plasmid R6K replication: Possible role of intermolecular coupling of replication origins. *Proc Natl Acad Sci USA* 86, 7942-7946 (1989)

224. Nordstrom, K.: Control of plasmid replication - How do DNA iterons set the replication frequency. *Cell* 63, 1121-1124 (1990)

225. Filutowicz, M., G. Davis, A. Greener, & D.R. Helinski: Autorepressor properties of the Pi initiation protein encoded by plasmid R6K. *Nucleic Acids Res* 13, 103-114 (1985)

226. Greener A, MS Filutowicz, MJ McEaschern, DR Helinski: N-terminal truncated form of the bifunctional Pi initiation protein express negative activity on plasmid R6K replication. *Mol Gen Genet* 224, 24-32 (1990)

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