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Authors

Lilly, Joshua

Camps, Manel

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Mechanisms of Theta Plasmid Replication

JOSHUA LILLY[1] and MANEL CAMPS[1]

[1]Department of Microbiology and Environmental Toxicology, University of California Santa Cruz, Santa Cruz, CA 95064

ABSTRACT Plasmids are autonomously replicating pieces of DNA. This article discusses theta plasmid replication, which is a class of circular plasmid replication that includes ColE1-like origins of replication popular with expression vectors. All modalities of theta plasmid replication initiate synthesis with the leading strand at a predetermined site and complete replication through recruitment of the host's replisome, which extends the leading strand continuously while synthesizing the lagging strand discontinuously. There are clear differences between different modalities of theta plasmid replication in mechanisms of DNA duplex melting and in priming of leading- and lagging-strand synthesis. In some replicons duplex melting depends on transcription, while other replicons rely on plasmid-encoded *trans*-acting proteins (Reps); primers for leading-strand synthesis can be generated through processing of a transcript or in other replicons by the action of host- or plasmid-encoded primases. None of these processes require DNA breaks. The frequency of replication initiation is tightly regulated to facilitate establishment in permissive hosts and to achieve a steady state. The last section of the article reviews how plasmid copy number is sensed and how this feedback modulates the frequency of replication.

INTRODUCTION

Plasmids have been used as convenient models for the study of molecular mechanisms of replication and DNA repair due to their small size, dispensability to the host, and easy manipulation. In addition, plasmids are key facilitators for the evolution and dissemination of drug resistance and for the evolution of complex interactions with animal or plant hosts. Understanding plasmid replication and maintenance therefore has significant practical implications for the clinic and for bioremediation.

Circular plasmids use a variety of replication strategies depending on the mechanism of initiation of DNA replication and depending on whether leading- and lagging-strand synthesis are coupled or uncoupled. This article focuses on replication of circular plasmids whose lagging strand is synthesized discontinuously, a mechanism known as theta replication because replication intermediates have the shape of the Greek letter θ (theta). Our discussion will focus on replication initiation, which informs different biological properties of plasmids (size, host range, plasmid copy number, etc.), and on how initiation is regulated in these plasmids. To highlight unique aspects of theta plasmid replication, this mode of replication will also be compared with another mode of circular plasmid replication, strand-displacement.

REPLICATION INITIATION

General Structure of Plasmid Origins of Replication

Replication initiation depends on a section of sequence known as the plasmid origin of replication (*ori*). *Basic replicon* refers to the minimal sequence that supports replication, preserving the regulatory circuitry. *Minimal replicon* refers to the minimal portion of sequence supporting plasmid replication even though replication may not be properly regulated, as seen in alterations in plasmid copy number or in the compatibility properties of

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Correspondence: Manel Camps, mcamps@ucsc.edu

the plasmids. Finally, there is an even narrower definition of *ori*, which refers to the portion of sequence that is targeted by replication initiation factors *in trans* to initiate replication. In this article we will use the term *origin of replication*, or *ori*, to refer to the *cis-ori*, and *replicon* to refer to basic or minimal replicons.

Rep proteins are plasmid-encoded initiators of replication, although some theta plasmids rely exclusively on host initiation factors for replication. Rep recognition sites typically consist of direct repeats or *iterons*, whose specific sequence and spacing are important for initiator recognition. Spacing is critically relevant so that the distance matches the helical periodicity of the DNA double helix, allowing recognition of specific DNA sequences (1). Iterons are intrinsically bent, and iteron curvature is enhanced by Rep binding.

Rep proteins are essential and rate-limiting for plasmid replication initiation. Controlled expression of two Rep proteins (π of R6K and RepA of ColE2) can produce a wide range of plasmid copy numbers per cell (between 1 and 250 copies), providing a convenient system for gene dosage optimization of recombinant proteins (2).

Plasmid replicons have a modular structure. Replicons often have motifs that are recognized by plasmid-encoded Reps, A+T-rich areas, G+C-rich areas, methylation sites, and binding sites for host initiation and/or remodeling factors. *Rep* loci, when present, are typically upstream of the plasmid *ori*, immediately adjacent or in close proximity to it.

Replication Initiation: Duplex Melting and Replisome Assembly

Depending on the replicon, duplex melting can be either dependent on transcription or mediated by plasmid-encoded *trans*-acting proteins (Reps). Rep binding of *ori* iterons generally leads to the formation of a nucleoprotein complex that opens up the DNA duplex at the A+T-rich segment.

Opening of the DNA duplex is necessary for replisome assembly, which in theta-type plasmids can be DnaA-dependent or PriA-dependent. DnaA-dependent assembly closely resembles replication initiation at *oriC*, the site initiating chromosomal replication. By contrast, PriA-dependent assembly parallels replication restart following replication fork arrest, which depends on D-loop formation, with the extra DNA strand supplied by homologous recombination (3–5).

In theta-type plasmids, Rep-mediated duplex melting leads to loading of DnaB on the replication fork, often with DnaA assistance. In plasmids that instead rely on transcription for duplex melting, the transcript itself can be processed and becomes the primer for extension. Continuous extension of this primer initiates leading-strand synthesis, facilitating the formation of a displacement loop, or D-loop, as the nascent single-stranded DNA (ssDNA) strand separates the two strands of the DNA duplex and hybridizes with one of them. In this case, PriA (initiator of primosome assembly) can be recruited to the forked structure of the D-loop; alternatively, PriA can be recruited to a hairpin structure that forms when the double-stranded DNA opens (6). PriA promotes both the unwinding of the lagging-strand arm and assembly of two additional proteins (PriB and DnaT) to load DnaB onto the lagging strand template. Thus, in this case loading of DnaB is independent of DnaA.

After loading of DnaB, both DnaA-dependent and -independent modes of replication converge. In both cases, replisome assembly involves the following additional players: SSB (single-stranded binding protein), DnaB (helicase), DnaC (loading factor), the DnaG (primase), and the DNA polymerase III (Pol III) holoenzyme. SSB is recruited to exposed areas of ssDNA, stabilizing them. DnaB is loaded onto the replication fork in the form of a complex with DnaC and recruits DnaG (the primase), which distributively synthesizes RNA primers for lagging-strand synthesis (7). Replisome assembly is completed by loading of the Pol III holoenzyme (8). This holoenzyme contains a core (with α, a catalytic, and ε, a 3′→5′, catalytic subunit), a β_2 processivity factor, and a DnaX complex ATPase that loads β_2 onto DNA and recruits the Pol III core to the newly loaded β_2 (9). DnaB helicase activity is stimulated through its interaction with Pol III and modulated through its interaction with DnaG, facilitating the coordination of leading-strand synthesis with that of lagging-strand synthesis during slow primer synthesis on the lagging strand (10).

Unlike Gram-negative bacteria, which have a single replicative polymerase (Pol III), Gram-positive bacteria have two replicative polymerases: PolC and DnaE. PolC is a processive polymerase responsible for leading-strand synthesis, while DnaE extends DnaG-synthesized primers before handoff to PolC at the lagging strand (11, 12).

In theta plasmids, lagging-strand synthesis is discontinuous and coordinated with leading-strand synthesis. The replicase extends a free 3′-OH of an RNA primer, which can be generated by DnaG primase (in Gram-negative bacteria), by the concerted action of DnaE and DnaG primase (in Gram-positive bacteria), or by alternative plasmid-encoded primases. Discontinuous lagging-strand synthesis involves repeated priming and elongation of Okazaki fragments and is comparable in plasmids

and chromosomes, although Okazaki fragments were found to be smaller in a ColE1-like plasmid, approximately one-third the length of Okazaki fragments in the chromosome (13).

DNA polymerase I (Pol I) contributes to plasmid replication in several ways. In ColE1 and ColE1-like plasmids, Pol I can extend a primer to initiate leading-strand synthesis and open the DNA duplex; this process can expose a hairpin structure in the lagging strand, known as a single-strand initiation (*ssi*) site or primosome assembly (*pas*) site, and/or generate a D-loop. Both hairpins and forked structures recruit PriA, which is the first step in the replisome initiation complex. Following replisome assembly, Pol I plays a critical role in discontinuous lagging-strand synthesis, removing RNA primers through its 5′→3′ exonuclease activity and filling in the remaining gap through its polymerase activity (14). In addition, two lines of evidence suggest that Pol I can functionally replace Pol III in *Escherichia coli*: (i) Pol I is essential for *polC* (Poll III-minus) strain viability, showing that both polymerases are functionally redundant (15). (ii) Mutations generated through error-prone Pol I replication of a ColE1-like plasmid *in vivo* strongly suggest that Pol I replicates both plasmid strands with similar frequency beyond the point where the switch to Pol III is expected, again suggesting that Pol I can be redundant with the Pol III replisome (16).

THETA PLASMID REPLICATION

Three modes of replication can be distinguished for circular plasmid replication: theta, strand-displacement, and rolling circle. This review focuses on theta. This mode of replication is similar to chromosomal replication in that the leading and lagging strands are replicated coordinately, with discontinuous lagging-strand synthesis. No DNA breaks are required for this mode of replication. Coordinated replication of both strands leads to the formation of bubbles in the early stages of replication, seen as the Greek letter θ under electron microscopy. Four classes of theta-type plasmids can be distinguished based on their mode of replication initiation, although the last two categories show hybrid features of the first two and will be discussed together (see theta replication section in Table 1).

Class A Theta Replication

Class A theta plasmids include R1, RK2, R6K, pSC101, pPS10, F, and P. All these plasmids depend on Rep proteins for replication initiation: RepA for R1, pSC101, pPS10, and P1; Trf1 for RK1; and π for R6K. Note that the name of these Reps is incidental, so sharing a name is not an indication of related structure or mode of action. Rep proteins bind direct repeats (iterons) in the plasmid *ori*. In class A, these iterons are rarely identical, although they frequently conform to a consensus motif. In plasmid P1, RepA monomers contact each iteron through two consecutive turns of the helix, leading to in-phase bending of the DNA, which wraps around RepA (17). Similarly, in R6K plasmids, π binding of its cognate iterons bends the DNA and generates a wrapped nucleoprotein structure (18).

There are two prominent exceptions to the presence of multiple iterons in class A theta plasmid *ori*s:

TABLE 1 Comparison of the three basic modes of plasmid replication initiation in circular plasmids

Type of replication	Leading-strand synthesis		Lagging-strand synthesis		
	Plasmid initiation factors	Host factors	Coupling with leading strand	Plasmid factors	Host factors
Theta class A	Rep (duplex melting)	DnaA-replisome	Yes	No	Replisome
Theta class B	None	RNAP Pol I RNase H PriA-replisome	Yes	No	Replisome
Theta class C	Rep (duplex melting, primase)	Replisome	Yes	No	Replisome
Theta class D	Rep (duplex melting, RNA processing?)	RNAP PriA-replisome	Yes	No	Replisome
Strand-displacement	Rep A (helicase) Rep B (primase) Rep C (initiator)	Replisome (recruited by RepA)	No (simultaneous)	Rep A (helicase) Rep B (primase) Rep C (initiator)	None

(i) Plasmid R1, which features two partial palindromic sequences instead of iterons; however, similar to other plasmids of this class, R1 palindromic sequences are recognized by RepA. (ii) The R6K plasmid, which has three *ori*s, only one of which has multiple iterons: γ (with seven iterons), a second origin (α) with a single iteron, and a third origin (β) with only half an iteron. It appears that the γ *ori* is an establishment origin, allowing replication initiation immediately following mobilization, when levels of π protein are low, whereas α and β *ori*s would be maintenance origins in cells inheriting the plasmid by vertical transmission (19). In any case, γ ori acts as an enhancer, favoring the long-range activation of α and β oris by transfer of π. Thus, α and β *ori*s are still dependent on the multiple iterons present in *ori* γ.

Rep binding of a cognate sequence in the plasmid *ori* mediates the earliest step in replication initiation: duplex DNA melting. A Rep-DnaA interaction is frequently involved, although the importance of this interaction varies between individual *ori*s. In plasmid pSC101, RepA serves to stabilize DnaA binding to distant *dnaA* boxes, leading to strand melting (20). Plasmid P1's *ori* has two sets of tandem *dnaA* boxes at each end; DnaA binding loops up the DNA, leading to preferential loading of DnaB to one of the strands (21). By contrast, RK2's TrfA was shown to mediate open complex formation and DnaB helicase loading in the absence of *dnaA* boxes, although the presence of DnaA protein was still required (22).

As mentioned above, the double strand melts in response to iteron binding by Rep protein. Melting occurs at an AT-rich region. Similar to chromosomal *oriC*, AT-rich segments of sequence frequently have sites for host factors playing an architectural role such as histone-like protein, integration host factor, and factor for inversion stimulation. These host factors help with DNA melting and with the structural organization of the initiation complex (1, 23, 24).

Class B Theta Replication

Class B theta plasmids include ColE1 and ColE1-like plasmids, which are frequently used for recombinant gene expression. Unlike class A, class B plasmids rely exclusively on host factors for both double-strand melting and primer synthesis. The DNA duplex is opened in this case by transcription of a long (~600 bp) preprimer called RNA II, which is transcribed from a constitutive promoter P2. Constitutive expression from this promoter is enhanced by a 9-bp motif 5′-AAGATCTTC, which is located immediately upstream of the -35 box (25). The 3′ end of the preprimer RNA forms a stable hybrid with the 5′ end of the lagging-strand DNA template of *ori*. This stable RNA-DNA hybridization (R-loop formation) is facilitated by the pairing of a stretch of G-rich sequence on the transcript with a C-rich stretch on the lagging-strand DNA template and by a hairpin structure located between the G- and C-rich stretches (26). Following R-loop formation, the RNA preprimer is processed by RNase H (which recognizes the AAAAA motif in RNAII), producing a free 3′-OH end. Extension of this RNA primer by Pol I initiates leading-strand synthesis. The point where the RNA primer is extended (known as RNA/DNA switch) is considered the replication start point (reviewed in references 27–29).

As mentioned above, the nascent leading strand separates the two strands of the DNA duplex and can hybridize with the leading-strand template, forming a D-loop. PriA is recruited to the forked structure of the D-loop; alternatively, PriA can be recruited to hairpin structures forming on the lagging-strand template when the duplex opens. Indeed, *priA* strains do not support ColE1 plasmid replication, and hypomorphic mutations in *priA priB* result in a reduced ColE1 plasmid copy number (30–32).

When the Pol III holoenzyme is loaded (27, 28) this polymerase continues leading-strand synthesis and initiates lagging-strand synthesis. Pol III replication of the lagging strand toward the RNA II sequence is arrested 17 bp upstream of the DNA/RNA switch, at a site known at *terH*, ensuring unidirectional replication (33). Lagging-strand replication by Pol III appears to end a few hundred nucleotides upstream of the *terH* site (33), leaving a gap that is filled by Pol I (16).

The only step that is essential in this process of replication initiation is R-loop formation; deficits in RNase H and/or Pol I do not prevent initiation, although they have a substantial impact on the efficiency of replication initiation. In the absence of RNase H, unprocessed transcripts can still be extended with some frequency, and in the absence of Pol I, the Pol III replisome can still be loaded on an R-loop formed by the transcript and lagging-strand template (28).

R-loop formation can happen as a result of local supercoiling in the trail of the advancing RNA polymerase during transcription and is highly deleterious because R-loops block transcription and the elongation step during translation (34). Therefore, cells have mechanisms to suppress unscheduled R-loop formation. The most important ones are relaxation of the DNA template by type I topoisomerase activity, RNA degradation by RNase H, RecG dissociation of R-loops by branch

migration, factor-dependent transcriptional termination, and coupling transcription to translation (reviewed in reference 35). Accordingly, titration of R-loop-suppressing factors through uncoupling transcription from translation (by starvation, temperature shift, or chloramphenicol treatment) results in increased ColE1 plasmid copy number (36), whereas RecG overexpression dramatically suppresses replication initiation (37). However, loss of topoisomerase I and RNase H activity do not increase plasmid copy number despite inducing increased R-loop formation because these activities are also required for plasmid replication initiation (particularly RNase H).

Hybrid Classes of Theta Replication (Classes C and D)

Classes C and D have specialized priming mechanisms combined with elements of class A and class B replication. Like class A plasmids, class C and D plasmids have Rep proteins, located immediately upstream of *ori*. Like class B plasmids, however, both initiate leading-strand synthesis by Pol I extension of a free 3′-OH. Class C and D plasmids both have termination signals in the 3′ direction of lagging-strand synthesis, making replication of these plasmids unidirectional.

Class C and D theta plasmid replication is based on the evolution of more efficient ways to prime replication initiation. The evolution of plasmid-specific primases exploits the specificity provided by Rep interaction with *ori* to minimize the size of the *cis-ori* sequence. Such specificity is not possible when multiple primers are needed, as in the case of lagging-strand synthesis in the chromosome. Also, the evolution of specialized priming mechanisms broadens the host range of these plasmids by reducing dependence on host factors (38).

Class C includes ColE2 and ColE3 plasmids. The *ori*s for these two plasmids are the smallest described so far (32 bp for ColE2 and 33 bp for ColE3); these two *ori*s differ only at two positions, one of which determines plasmid specificity (39). ColE2 and ColE3 *oris* have two iterons and show two discrete functional subregions: one specializing in stable binding of the Rep protein (region I) and the other specializing in initiation of DNA replication (region III), with an area of overlap in between (region II) (40). Unlike class A initiator Rep proteins, the Rep protein in class C plasmids has primase activity, synthesizing a unique primer RNA (ppApGpA) that is extended by Pol I at a fixed site in the origin region (41). Class C replication is unidirectional, as the 3′ end of the lagging-strand DNA fragment was mapped to a specific site at the end of the *ori* region. The Rep protein may stay bound to the *ori* after initiation of replication, blocking progression of the replisome synthesizing the lagging strand (42).

Class D includes large, low-copy streptococcal plasmids that replicate in a broad range of Gram-positive bacteria. Examples include pAMβ1 from *Enterococcus faecalis*, pIP501 from *Streptococcus agalactiae*, and pSM19035 from *Streptococcus pyogenes*. In these plasmids, replication shares some features with class B theta replication, specifically a requirement for transcription across the *ori* sequence, Pol I extension and PriA-dependent replisome assembly (43). In this case, the transcript is generated from a promoter controlling expression of rep, which is immediately upstream of the *ori* (43). The replication process has been studied in detail for pAMβ1, although the Rep proteins (RepE for pAMβ1, RepF for pIP501, and RepS for pSM19035) are 97% identical for all three plasmids, and the three plasmids share a replisome structure, suggesting that they share mechanisms for replication initiation and termination. Replication depends on transcription through the origin. Rep binds specifically and rapidly to a unique site immediately upstream of the replication initiation site. This binding denatures an AT-rich sequence immediately downstream of the binding site to form an open complex (44). Compared to class A, this open complex is atypical on several counts: (i) the cognate sequence does not have multiple iterons, (ii) binding does not induce strong bending of the origin, and (iii) melting does not require additional host factors. In addition to opening of the double strand, RepE appears to have an active role in primer processing, as melting increases RepE binding and RepE can cleave transcripts from the repE operon in close proximity to the RNA/DNA switch (45).

Class D replisome assembly is PriA-dependent. A primosome assembly signal can be found 150 nucleotides (nt) downstream from the *ori* on the lagging-strand template. There is a site for replication arrest induced by Topb, a plasmid-encoded topoisomerase related to topo III, 190 nt downstream for the *ori* (46). A second replication arrest site can be found 230 nt downstream from the plasmid *ori*; in this case arrest is caused by collision with a site-specific resolvase, Resb, which is a plasmid-borne gene responsible for plasmid segregation stability (47). The presence of two independent checkpoints for Pol I progression in pAMβ1 is intriguing; this may be a mechanism that ensures Pol I availability for chromosomal replication and/or that facilitates recruitment of PriA, as PriA is known to be recruited to sites of replication fork arrest. In any case the two replication blocks appear to be largely redundant, as Topb is dispensable for pAMBβ1 replication (46).

COMPARISON OF THE THETA AND STAND-DISPLACEMENT MODES OF PLASMID REPLICATION

Plasmids that replicate using the strand-displacement mode of replication include *E. coli* incompatibility group Q (IncQ) plasmids of γ-proteobacteria such as RSF1010. Strand-displacement replication depends on a specialized primase: RepB. In this case, the function of replication initiator function is provided by a different Rep (RepC). Similar to initiator Rep proteins in class A theta plasmids, Rep C binds cognate iteron sequences, bending the DNA and melting duplex DNA at an adjacent A+T-rich region. An additional plasmid-encoded protein (a helicase, RepA) helps melt the DNA, recruit Pol III, and support continuous replication of one strand. This single-stranded replication produces a daughter ssDNA strand, which separates the two strands of the DNA duplex and allows hybridization with one of them, creating a D-loop (hence the name of this mode of replication).

A model for strand-displacement replication is presented in Fig. 1. After RepC-induced melting of the duplex, RepA monomers assemble around the exposed ssDNA and catalyze bidirectional unwinding of the DNA. This exposes the two different *ssi* sites, which are adjacent and are both palindromic, resulting in inverted repeats on the two DNA strands. When these two sites are exposed in single-stranded configuration, base-pair complementarity favors the formation of two hairpins, one for each strand, (Fig. 1, panel II) (48). Hairpin formation is assisted by a slowdown in RepA progression at a G+C-rich region (reviewed in reference 49). The base of each hairpin contains the start point for DNA synthesis, which is recognized by Rep B, and primer synthesis ensues (50, 51). The Pol III holoenzyme extends off of the synthesized primer (Fig. 1, panels III to V). Initiation can occur at either site independently and is continuous. As replication progresses, facilitated by the RepA helicase, a theta-type intermediate forms (Fig. 1, panels III and IV). Ligation of the two daughter strands produces two double-stranded circles (Fig. 1, panel VI).

Unlike theta-type replication, strand-displacement replication initiation is independent of host factors. This autonomous replication initiation gives these plasmids a very broad range of operation (52). As mentioned above, strand-displacement replication initiation has some similarities to class C theta plasmid replication (with a specialized, plasmid-encoded primase) and similarities to class A theta plasmid replication (with a Rep initiator involved in melting the duplex), but strand displacement presents three major differences relative to theta plasmid replication: (i) no involvement of DnaBC, as RepA is loaded on *ssi* sites exposed in the ssDNA configuration, recruiting the replicase; (ii) priming is carried out by RepB, functionally replacing the host primase DnaG; and (iii) Pol III replicates each strand continuously, initiating at two single-stranded motifs located on opposite strands (*ssiA* and *ssiB*). Note that continuous replication includes the lagging strand, which in this case does not involve synthesis of Okazaki primers (53).

REGULATION OF REPLICATION INITIATION

The frequency of replication initiation is regulated by negative feedback loop mechanisms. These regulatory mechanisms allow for rapid expansion when plasmids colonize a new permissive cell (establishment phase) and later tune the frequency of replication so that, on average, there is one replicative event per plasmid copy number per cell cycle (steady state phase), minimizing fluctuations in copy number (54).

Types of Feedback Regulatory Mechanisms

Plasmid copy number regulation needs mechanisms to monitor the plasmid copy number through a "sensor" and mechanisms to modulate replication initiation in response to feedback through an "effector" (55). The sensor mechanism depends on molecules whose concentration in the cytoplasm is proportional to plasmid copy number. In theta plasmids, inhibition of replication occurs at the initiation step and depends on three types of mechanisms: (i) antisense RNAs that hybridize to a complementary region of an essential RNA (countertranscribed RNAs, or ctRNAs) – dual mechanisms involving ctRNA and an additional protein repressor also occur –; (ii) Rep binding of iterons located in the Rep promoter, suppressing transcription; and (iii) steric hindrance between plasmids by interaction between Rep initiator proteins bound to different plasmids, which "handcuffs" them. Note that in all three cases sensor and effector functions are performed by the same molecule.

Countertranscribed RNA Inhibition

These feedback mechanisms share the following elements: two promoters in opposite orientations, one directing the synthesis of an RNA essential for replication and the other directing the synthesis of an inhibitor ctRNA. The ctRNA is complementary to a region near the 5′ end of the essential RNA, is typically strongly expressed, and has a short half-life, whereas its target RNA is expressed at constitutive but low levels. Examples of targets include maturation of a primer required for replication initiation (ColE1 plasmids), inhibition of repA

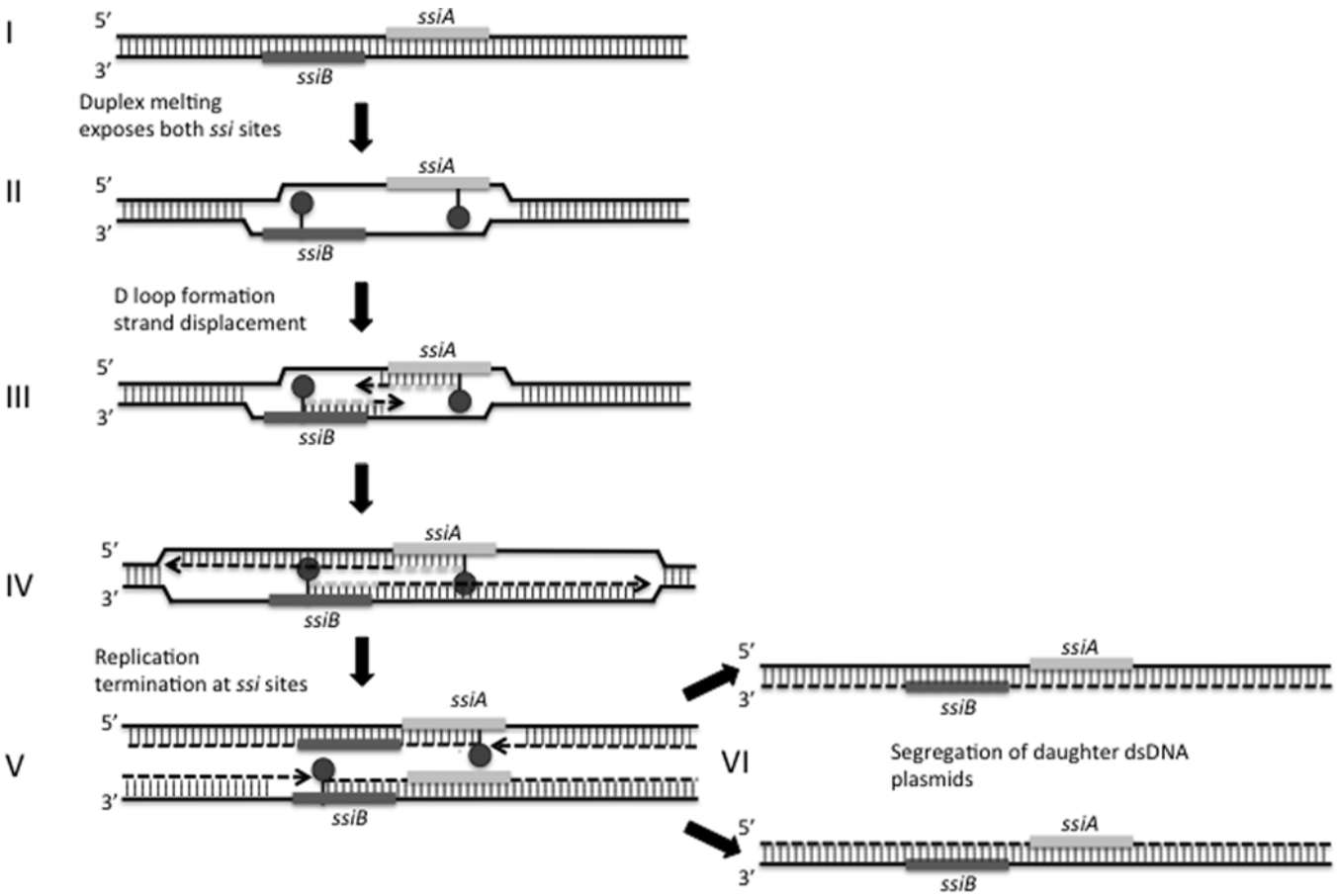

FIGURE 1 Model of plasmid replication by the strand-displacement mechanism. (I) Parental DNA duplex (solid black lines) depicting the two single-stranded replication initiation sites, *ssiA* (light gray box) and *ssiB* (dark gray box). Vertical lines show hybridization between DNA strands. (II) The DNA duplex is melted through binding of RepC (possibly in concert with the RepA helicase), allowing the two *ssi* sites to form hairpins (ball and stick). (III) The base of the hairpin is recognized by RepB′, which initiates the synthesis of an RNA primer (light gray dashed line). Extension of the free 3′-OH of the primer by Pol III (assisted by the RepA helicase) is shown as dashed black arrows. Two D-loops are formed, one for each direction of synthesis, as parental strands are displaced and dissociate from each other, leaving ssDNA intermediates. This is shown as areas where one of the strands has no hydrogen bonding. (IV) Synthesis continues in both directions, extending the area of D-loop formation. (V) Elongation is completed and termination of replication occurs on both strands at the *ssi* sites in which replication began. At this point, the *ssi* sites on the newly synthesized daughter strands are restored. (VI) Segregation: the two daughter strands are ligated, resulting in two DNA duplexes, each containing a parental strand (solid black line) and daughter strand (dashed black line). doi:10.1128/microbiolspec.PLAS-0029-2014.f1

translation (R1), and premature termination of translation of a rep mRNA (class D plasmids). Antisense RNA regulation of plasmid replication has been extensively reviewed elsewhere (55–57).

RNAI (ColE1, ColE2) and CopA (R1) ctRNA molecules are highly structured. Given that the target preprimer and ctRNA sequence are complementary, higher-order structures for both RNAs are mirror images of each other. The first contact between sense and antisense RNAs occurs by pairing between complementary sequences at the loop portion of stem-loops, a rate-limiting step known as the "kissing complex formation" (58). Point mutations at the loop portion of stem-loops are frequently tolerated, as mutations in the template DNA introduce complementary changes in sense and ctRNA at the same time, preserving base-pairing. These mutations modulate the

affinity of sense RNA–ctRNA interaction, with A-U pairs generally decreasing affinity relative to G-C pairs (for ColE1 plasmids reviewed in reference 27).

Several ctRNAs (ColE1 and ColE2 RNAI and R1 copA) have a short half-life due to the presence of an RNase E cleavage site, which consists of the U-rich sequence and a hairpin structure at the 3′ end. Conditional expression of a hyperactive variant of RNase E has been used for controlled overproduction of ColE1 plasmid DNA (59). RNase E cleavage produces monophosphorylated decay intermediates lacking short portions of the 5′ end. In the case of ColE1 and ColE2, these pRNAI cleavage intermediates are polyadenylated by PAPI, facilitating exonucleotidic digestion by PNPase (60, 61). Deletion of *pcnB*, the gene encoding PAPI, leads to increased cytoplasmic levels of pRNAI cleavage intermediates and to a 5- to 10-fold (ColE1) and 2-fold (ColE2) decrease in plasmid copy number (61, 62). RNase III has also been reported to degrade ColE1 RNAI upstream of RNase E (63). In ColE2, the differential stability between RNAI and its target rep-mRNA is partially due to differential exonuclease recruitment by RNase E (64).

Single mechanisms involving ctRNA inhibition

In ColE1, the ctRNA (RNAI) is transcribed from P1, a promoter located 108 bp downstream from the sense promoter P2. Both preprimer and ctRNA form three stem-loops (SL1-3); the loop portion consists of six to seven unpaired residues. These residues are critical, as their pairing with their complementary counterparts initiates hybridization. Next, the 5′ end of RNAI (antitail) nucleates the hybridization between the two RNAs to form a duplex.

Hybridization between the preprimer and ctRNA leads to conformational changes in the preprimer, blocking R-loop formation further downstream, a phenomenon known as “action at a distance” (reviewed in references 27 and 65). This conformational change is mediated by the interaction of a sequence domain (β) in the preprimer with another sequence domain further downstream (γ), making the preprimer incompetent for R-loop formation. In addition to being short-lived, ColE1 RNA I has a short window of action, because as soon as RNAII is transcribed past position 200 downstream of the RNA/DNA switch, hybridization of the β domain with another sequence domain (α) forms a new loop (SL4), which makes RNAII refractory to RNAI inhibition.

SL1 to SL3 bear a structural resemblance to the cloverleaf structure of tRNAs and even have homology to the anticodon loops of 11 tRNAs (66). Competitive hybridization between tRNA and RNAI or RNAII appears to interfere with RNAI/RNAII hybrid formation (66). In addition, uncharged $tRNA^{ala}$ cleaves RNAI both *in vitro* and *in vivo* (67), and there is evidence suggesting that the 3′-CAA terminus of uncharged tRNAs hybridizes stably with RNAI (68). This functional cross-talk between RNAI and tRNAs may contribute to plasmid copy number deregulation associated with amino-acid starvation in *relA* strains used for recombinant gene expression; one of the key factors is the limiting yield of large-scale recombinant expression (69). Cross-talk between ctRNA and tRNAs may also explain the conservation of the 5′-UUGGCG-3′ sequence at the loop region of many of the antisense RNAs and their targets involved in regulation of replication, suggesting that this sequence is under common and strong selective pressure (70).

In ColE2 plasmids the ctRNA is also known as RNAI and has a complex secondary structure. In this case, RNAI is complementary to the 5′ end of rep mRNA containing an untranslated sequence. Given that the 5′ end portion of RNAI does not cover the initiation codon of Rep or its immediate vicinity, inhibition in this case appears to be caused by structural disruption of secondary or tertiary structures required for translation (70).

Dual mechanisms involving ctRNA

These mechanisms are plasmid copy number regulatory systems that include two elements: a ctRNA and a transcriptional repressor protein. In these systems, Rep expression is controlled by a strong, repressor-regulated promoter so that there is a high rate of Rep transcription when the repressor does not operate. The two best-studied examples are the R1 plasmid, where the ctRNA is CopA and the repressor is CopB, and pIP501, where the ctRNA is RNAIII and the repressor is CopR. These dual mechanisms may represent an advantage during the establishment phase, particularly for mobilizable plasmids such as class D plasmids.

In R1, repA can be transcribed from an upstream promoter P1 or from an alternative promoter further downstream, P2. Expression of repA is translationally coupled to that of tap, a small leader peptide. CopA inhibits repA expression by inhibiting translation of tap. The second element is a transcriptional repressor of P2, CopB. CopB expression is under the control of P1 but not P2. When levels of CopB are high, tap+repA are transcribed as polycystron copB-tap-RepA RNA from P1 (as P2 is silenced by CopB), but when they are low, the P2 promoter becomes derepressed and tap+repA can

also be expressed from that alternative promoter, leading to a transient increase in tap+repA expression (71).

Class D plasmids have a cop-ctRNA-rep modular structure. In this case the two regulatory elements are RNAIII and a Cop protein. RNAIII is transcribed in the opposite orientation relative to its target DNA (5′ end of rep) from promoter pIII, whereas pI and pII control CopR and Rep expression, respectively, in the sense orientation. In pIP501 plasmids, RNAIII hybridization to its complementary sequence induces folding of RNA into a transcriptional terminator structure that prevents transcription of repR. This mechanism only operates on nascent (<260-nt-long) RNAs, as longer rep transcripts form an alternative secondary structure that is refractory to repR-induced transcriptional attenuation (72). CopR (whose levels reflect plasmid copy number in the cell) inhibits the sense promoter pII. A decreased plasmid copy number leads to pII derepression, resulting in increased RepR expression. In addition, induction of pII (*repR*) transcription results in a substantial decrease in pIII transcription because pIII is supercoiling-sensitive. In pAMBβ1, CopF (the equivalent of CopR), in addition to suppressing RepF transcription, decreases primer formation since CopF transcription generates the primer for replication initiation (see class D in the "Hybrid Classes of Theta Replication" section above).

Transcriptional Regulation by Rep Binding

In some class A theta plasmids, a different mechanism of regulation involves inhibiting Rep transcription by Rep itself. In these plasmids, iterons are located in the promoter of the Rep operon, outside the plasmid *ori*. Rep binding to these cognate sequences inhibits Rep expression and thus acts as an autoregulatory mechanism.

Rep binding of two alternative binding sites (Rep promoter and plasmid *ori*) involves changes in the conformation and oligomerization status of the Rep protein. These changes have been studied in detail in the RepA protein of pPS10 (73). This protein has two winged-helix domains (WH1 and WH2). When Rep A is in dimeric form, it acts as a transcriptional repressor, with the WH1 domain functioning as a dimerization interface. Low concentrations of RepA favor dissociation of Rep dimers into monomers, which are the only form that is active as an initiator. Monomerization involves conversion of the dimerization domain into a second origin-binding sequence and remodeling of the WH1 sequence to bind the opposite iteron end (73). In some cases, monomerization can be assisted by chaperones or by the allosteric effect of binding iterons at the *ori* (74–77).

Steric Hindrance

A different feedback mechanism, known as steric hindrance or handcuffing, was initially proposed for P1 and R6K plasmids (78, 79) but could operate in more iteron-containing plasmids. According to this model, as the number of plasmids in the cell increases, Rep molecules bound to iterons of one origin begin to interact with similar complexes generated in other origins. This pairing (known as handcuffing) produces plasmid pairs linked through Rep-Rep interactions, causing a steric hindrance to both origins that interferes with origin melting (80). Rep molecules are paired through zipping-up DNA-bound RepA monomers (78). A difference between this model and the autoregulation model is that the rate of replication depends on iteron concentration, not Rep expression level. Both mechanisms of autoregulation could be working together for initiators that are limiting (81).

CONCLUDING REMARKS

Plasmids contribute to the adaptation of bacterial hosts to an ever-changing environment through mobilization and amplification of selected genes. Different circular plasmids show differences in duplex melting, leading-strand priming, and lagging-strand synthesis. Learning more about the diversity of the replication mechanisms present in plasmids can help us understand the mechanisms that cells have available to replicate and repair their DNA. Organellar replication and restoration of replication after replication fork arrest are two examples of processes that occur in cells that are mechanistically closely related to plasmid replication. Also, learning more about these mechanisms will improve our understanding of plasmid biology, as mechanisms of replication limit plasmid size, host range, and mobilization capacity. Finally, maintaining a stable plasmid copy number is critical for the host, as loss of the plasmid entails losing the adaptive functions carried in the plasmid sequence, and runaway plasmid replication is lethal. Thus, mechanisms of plasmid replication regulation represent potential targets for antimicrobial intervention.

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