

Mechanisms of Theta Plasmid Replication in Enterobacteria and Implications for Adaptation to Its Host

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ABSTRACT Plasmids are autonomously replicating sequences that help cells adapt to diverse stresses. Theta plasmids are the most frequent plasmid class in enterobacteria. They co-opt two host replication mechanisms: replication at *oriC*, a DnaA-dependent pathway leading to replisome assembly (theta class A), and replication fork restart, a PriA-dependent pathway leading to primosome assembly through primer extension and D-loop formation (theta classes B, C, and D). To ensure autonomy from the host's replication and to facilitate copy number regulation, theta plasmids have unique mechanisms of replication initiation at the plasmid origin of replication (*ori*). Tight plasmid copy number regulation is essential because of the major and direct impact plasmid gene dosage has on gene expression. The timing of plasmid replication and segregation are also critical for optimizing plasmid gene expression. Therefore, we propose that plasmid replication needs to be understood in its biological context, where complex origins of replication (redundant origins, mosaic and cointegrated replicons), plasmid segregation, and toxin-antitoxin systems are often present. Highlighting their tight functional integration with *ori* function, we show that both partition and toxin-antitoxin systems tend to be encoded in close physical proximity to the *ori* in a large collection of *Escherichia coli* plasmids. We also propose that adaptation of plasmids to their host optimizes their contribution to the host's fitness while restricting access to broad genetic diversity, and we argue that this trade-off between adaptation to host and access to genetic diversity is likely a determinant factor shaping the distribution of replicons in populations of enterobacteria.

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INTRODUCTION

Plasmids are autonomously replicating sequences. They are found across all kingdoms of life but are particularly abundant in prokaryotes (1). They typically carry nonessential genes that have adaptive value under specific stresses such as heavy metal exposure, antibiotics, or adapting to a host (virulence genes) (2, 3).

Here, we review the mechanism of plasmid replication known as theta, which is by far the predominant type in *Enterobacteriaceae*. This prokaryotic family groups a number of related genera (*Escherichia*, *Shigella*, *Salmonella*, *Yersinia*) that share the intestinal tract as their central ecological niche and that have been extensively studied due to their clinical relevance (4, 5).

Enterobacterial plasmids share similar features. Nearly all of them can be transferred between strains in one way or another, a process known as horizontal gene transfer (HGT). This allows plasmids to serve as platforms for the exchange of genetic material, for capturing new adaptive genes from the environmental microbial gene pool, and for shuffling and spreading these genes among pathogens (3, 6, 7). There are three main mechanisms of HGT, namely, cell-to-cell contact and formation of a bridge (conjugation), direct uptake from the environment (transformation), and through bacteriophages (transduction) (8). Conjugation is the predominant mechanism in Gram-negative bacteria. It involves copying the plasmid DNA, relaxing it, and moving the DNA through a type IV secretion system to a recipient cell in an ATP-dependent fashion (reviewed in references 3, 7, and 9). The majority of enterobacterial plasmids carry sets of conjugation genes, although these are not always active or complete (10). Some virulence plasmids do not have a conjugation machinery but can be transferred along with a conjugative plasmid, which acts as a helper; this process is known as plasmid mobilization (10). Transduction is also frequent in enterobacteria (11). It occurs when a bacteriophage packages bacterial genetic material along with its own genetic material and infects another bacterium (generalized transduction) or when a phage that has integrated into the bacterial chromosome is improperly excised and packages bacterial DNA (specialized transduction) (12).

Here, we present our current mechanistic understanding of theta plasmid replication and its regulation. We also present concepts that are emerging from genomic studies, which help place the replication of these plasmids in its broader biological context. Given that plasmids isolated from a variety of *Enterobacteriaceae* share similar features (5), we use virulence plasmids in extraintestinal pathogenic *Escherichia coli* (ExPEC) as representative of enterobacteria.

PLASMID REPLICATION INITIATION AT ORIGINS OF REPLICATION

Plasmid replication starts at a specific location known as the plasmid origin of replication, or *ori*. The origin of replication contains nucleotide sequence motifs that are recognized in *trans* by the replication machinery. This machinery typically involves a combination of plasmid and host elements. Since borrowing genes from the host minimizes the metabolic cost of plasmid maintenance, plasmid-encoded replicative elements are typically

restricted to replication initiation, with downstream steps frequently relying exclusively on host proteins. Having plasmid-specific mechanisms of replication initiation ensures autonomy from the host and facilitates the regulation of plasmid copy number by providing specific targets for regulation. Plasmid replication initiation involves plasmid-encoded replication proteins (Reps) and/or plasmid-specific primers. Even these plasmid replication initiation factors are often assisted by host proteins (see below).

Host proteins participating in plasmid DNA synthesis are part of the machinery that replicates the chromosome at *oriC* (recently reviewed in references 8 and 13–15) and/or that restart replication following replication fork collapse (reviewed in references 16–19). These include proteins facilitating the melting of the DNA duplex (DnaA), helicases (PriA, DnaB), proteins that catalyze primer synthesis (DnaG) and primer extension (Pol I, Pol III holoenzyme), proteins that process primers and gaps (Pol I, ligase), proteins that control the local supercoiling status of DNA (topoisomerases), and proteins that bind and stabilize single-stranded DNA (ssDNA) (SSB). Note that replication proceeds in the 5' to 3' direction. The synchronous replication of leading and lagging strands involves two core Pol III enzymes, with the enzyme replicating the leading strand doing so continuously and the enzyme replicating the lagging strand doing so discontinuously, i.e., in segments. The Pol III core enzymes in the Pol III holoenzyme are joined by two τ (tau) subunits and assisted by β units that act as mobile sliding clamps, which ensures the coordination and processivity of the replication in the two strands.

MECHANISMS OF PLASMID REPLICATION

Replication initiation determines the mechanics of plasmid replication. Within theta plasmids, we can distinguish two broad mechanisms of replication initiation: *oriC*-like (which parallels replication initiation at *oriC*) and PriA-mediated (which is similar to replication restart after replication fork collapse). The mechanisms involved in these two categories of plasmid replication (as well as those of nontheta plasmids) are systematically compared in [Table 1](#) and discussed in detail below.

oriC-like plasmids correspond to class A theta plasmid replication. PriA-mediated plasmids correspond to theta classes B, C, and D. Both categories converge at the DnaB loading step and rely on the hosts' Pol III holoenzyme to

Table 1 Comparison of the different modes of plasmid replication^a

Characteristic	Theta A	Theta B ^b	Theta C	Theta D	Strand displacement	Rolling-circle
Directionality of replication (unidirectional vs bi-directional)	Bi/uni	Uni	Uni	Uni	Bi	Uni
Coupling of leading- and lagging-strand synthesis	Coupled (replisome)	Coupled (replisome)	Coupled (replisome)	Coupled (replisome)	Uncoupled	Uncoupled
Simultaneous leading- and lagging-strand synthesis	Yes	Yes	Yes	Yes	Yes	No, with generation of single-stranded intermediate
Leading strand synthesis	Continuous	Continuous	Continuous	Continuous	Continuous	Continuous
Initiation factor	Rep melts duplex and recruits DnaB helicase, DnaA and by NAPs	NA	RepA (also primase activity)	Rep E melts duplex only and has active role in primer generation	Rep C (DnaA-like)	Rep (generates initial nick and seals it)
Initiation factor binding site	Multiple iterons with induced bending	NA	Iteron with two functional subregions: (I) binding and (II) initiation of DNA replication	No iterons, no bending	<i>ssi</i> sites (hairpins)	<i>Dso</i> (hairpin cruciform structure with binding domain and nicking domain)
Leading-strand primer	DnaG primase (host)	Transcript processed by RNaseHI	ppApGpA produced by RepA	RepE transcript, possibly processed by RepE itself	RepB' (interacting at the base of the hairpin)	Rep C-generated nick produces a 3'-OH
Helicase	DnaB ^b	PriA + DnaB ^b	PriA + DnaB ^b	PriA + DnaB ^b	RepA	DnaB ^b
Lagging-strand synthesis	Discontinuous	Discontinuous	Discontinuous	Discontinuous	Continuous	Continuous
Continuous vs discontinuous	Discontinuous	Discontinuous	Discontinuous	Discontinuous	Continuous	Continuous
Lagging-strand primer	DnaG	DnaG	DnaG	DnaG	RepB'	DnaG
Helicase	PriA+ DnaB ^b	PriA+ DnaB ^b	PriA+ DnaB ^b	PriA+ DnaB ^b	RepA in coordination with Pol III	Not needed (it is ssDNA)
Additional comments	Predominant type in Gram-negative bacteria	Limits size of the plasmid to ~40kb	Minimal size of <i>cis</i> -acting <i>ori</i>	2 sites of replisome arrest: for Top I-like enzyme and for resolvase	More independent of host factors Limits the size of the plasmid to ~15 kb	Predominant type in Gram-positive bacteria, also associated with conjugation

^aBased on references 66, 78, and 79.^bPcrA in Gram-positive bacteria.

complete the replication of both strands. Thus, theta plasmids are characterized by coordinated leading- and lagging-strand replication, where lagging-strand replication is discontinuous. This leads to the generation of bubbles in the early stages of replication that resemble the Greek letter θ (theta) under the electron microscope, which gives the name to this type of plasmid replication.

oriC Chromosomal Replication Initiation

DnaA is an AAA+-family ATPase that is central for replication initiation in the chromosome. In this capacity, DnaA has three roles: melting duplex DNA at *oriC*, recruiting the replisome through DnaB helicase, and controlling the timing of replication initiation.

DnaA binds DnaA boxes, 9-nucleotide (nt) motifs found in the vicinity of *oriC* that have high affinity for DnaA. Lower-affinity motifs (with small changes to the consensus sequence) are also present, and these are only recognized by DnaA-ATP. Cooperative oligomerization of DnaA occurs when both low- and high-affinity DnaA motifs are bound and leads to the formation of a nucleoprotein helical structure, which generates torsional forces that open up the DNA in an adjacent, ATP-rich area called the DUE, for DNA unwinding element (20, 21). The opening of replication origins by DnaA-ATP occurs by a direct stretching mechanism, similar to other nucleic acid-dependent AAA+ systems (22). The continued DnaA oligomerization in the top strand of the exposed ssDNA and SSB binding to the bottom strand keeps the DNA open (23, 24). DnaA filaments on ssDNA are stabilized by an origin element composed of repeated trinucleotide motifs (25). DnaB is loaded on the bottom/lower strand first, possibly by direct interaction with DnaA; DnaC loads DnaB on the top/upper strand invaded by DnaA molecules (26, 27). The order of helicase loading is important, providing the necessary orientation for the head-to-head loading of the helicase (28). DnaB, in turn, recruits DnaG, which is the primase. Primase activity leads to the release of DnaC and to the recruitment of the β -clamp loader and β -clamp and of the Pol III core (one per each strand). Once the replisome has been loaded, replication proceeds bidirectionally until the two forks meet and resolve. The terminus region where the two forks generally meet, which is antipodal to *oriC*, is flanked by a series of 20-nt-long replication termination (*Ter*) signals that act as direction-dependent arrest sites for fork progression, where forks are allowed to enter but not leave (29, 30). This direction-dependent arrest ensures that if one of the replication forks is

delayed, the two forks still meet and resolve in the terminus region of the chromosome. The mechanism conferring directionality to the fork arrest has not been completely elucidated but involves DnaB-induced strand separation and asymmetric contacts between a specific terminator protein (Tus) and its cognate *Ter* sequence, leading to a highly stable “locked” complex (31).

The specificity of DnaA-ATP for low-affinity DnaA sites acts as a mechanism to control the timing of replication initiation (20, 32). Following replication, DnaA-ATP moves from *oriC* to chromosomal titration sites such as *datA* (preference for DnaA-ATP) until the intrinsic ATPase activity of DnaA is stimulated by a component of the replisome (Hda) (33–35). DnaA is then recharged to DnaA-ATP through new DnaA synthesis, by relocation to DnaA reactivation sequences, or through contact with acidic membrane phospholipids (35, 36). Two nucleoid-associated proteins (NAPs), FIS and IHF, and Dam methylation also help coordinate the timing of replication initiation because DNA bending by FIS and IHF and binding of hemimethylated DNA by SeqA prevent extension of the DNA filament until the cell is ready for a new round of replication (reviewed in reference 36).

oriC-Like Plasmid Replication Initiation

Similar to chromosomal replication at *oriC*, *oriC*-like plasmid replication initiation involves recognition of a sequence motif by a replication initiation protein. In this case, however, the replication initiation protein (Rep) is plasmid-encoded. Reps are generally named with letters of the alphabet (e.g., RepA, RepB, RepE, etc.), but these names are arbitrary, i.e., two Reps with the same name being in different plasmids does not generally mean that the two Reps are phylogenetically related. Methods for typing plasmids based on sequence homology are discussed further below.

The role of Reps in class A theta replication initiation is summarized in Fig. 1. Class A theta replicons typically include a replication initiator, DnaA boxes upstream or downstream of the Rep, iterons, and an AT-rich DUE. As an example, Fig. 1 shows the arrangement found in plasmid R2K, which belongs to the IncP compatibility group. Sites for binding of NAPs (such as IHF) or for methylation by Dam methylase are frequently present as well. Reps cooperatively bind iterons until those iterons are saturated. Next, the DnaB helicase is recruited through Rep, with a variable involvement of DnaA binding to adjacent DnaA boxes, leading to open complex formation.

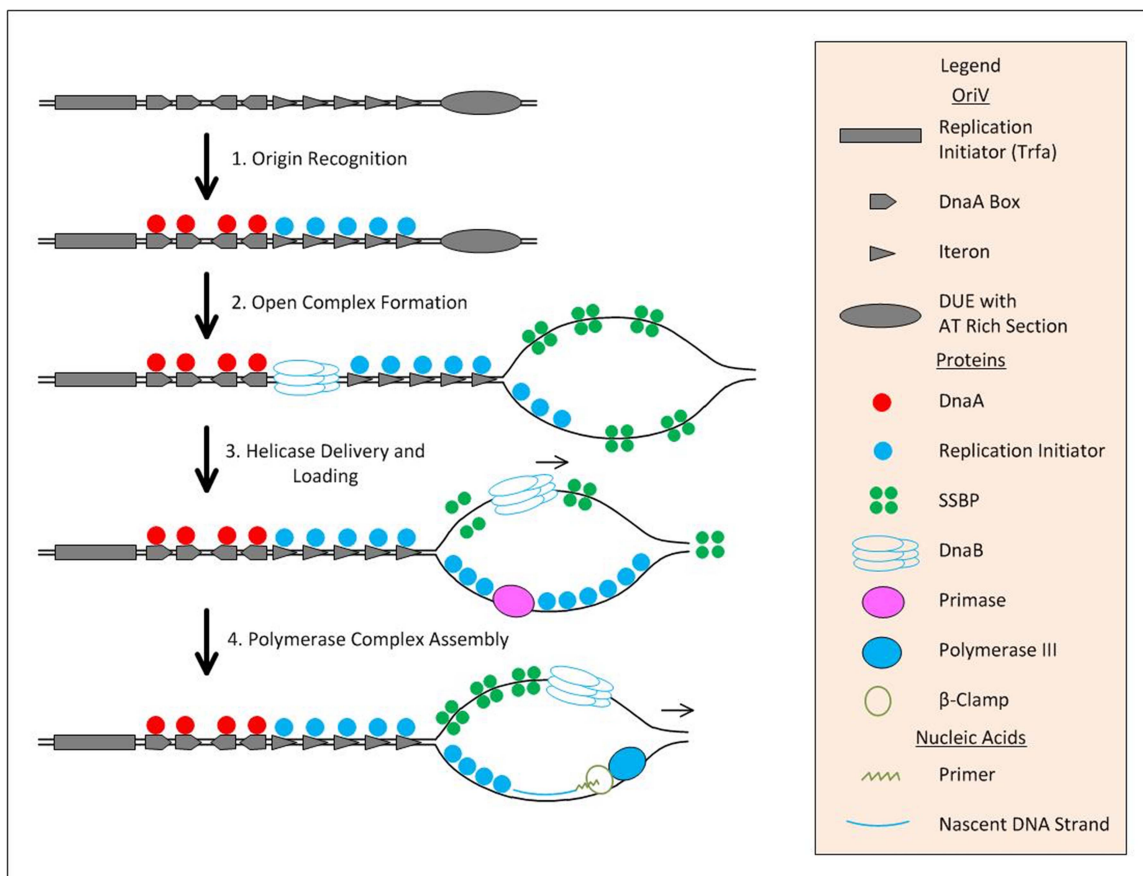


Figure 1 Role of replication initiation proteins in initiation in class A theta plasmid replication (based on references 43 and 97). Class A theta replicons typically include a replication initiator gene: *rep* (rectangle), DnaA boxes upstream or downstream of the Rep (square), iterons (arrow heads), and an AT-rich DNA unwinding element (DUE, oval). Sites for binding of nucleoid associated-proteins (such as IHF) or for methylation by Dam methylase are also frequently present as well but not shown. *oriV* of the RK2 plasmid (which belongs to the IncP compatibility group) is shown as an example. RK2's replication initiator is TrfA. The *trfA* gene is under the control of a strong promoter. Transcription produces two products (a longer and a shorter one) that in this figure are considered largely redundant. During plasmid origin recognition (phase 1), replication initiation proteins (in their monomeric forms) cooperatively bind iterons until those iterons are saturated. The formation of an open complex (phase 2) involves unwinding of the DUE and continued binding of the replication initiator into the bottom strand of ssDNA; the rest of the bottom strand of ssDNA and the top strand are bound by SSB, which is a tetramer. In the case of RK2, the plasmid encodes its own SSB, which is under the control of the same promoter as the replication initiator gene (*trfA*). DnaA enhances/stabilizes the formation of the TrfA-mediated open complex and assists with the recruitment of DnaBC. Finally, the longer form of TrfA also assists in strand-specific replisome assembly in a DnaA-independent manner via direct interaction with the β clamp and through a sequence-specific interaction with one strand of the plasmid origin DUE (8, 136).

Despite clear mechanistic similarities with *oriC*, Rep function in these plasmids appears to be only partially redundant with that of DnaA. Some Reps have been shown to bind DnaA (37–39), and DnaA boxes are frequently found in plasmid origins of replication (40–42), suggesting some level of cooperation between the plasmid and the chromosome replication machineries. Compared to DnaA, plasmid Rep proteins in IncP plasmids (which are the ones that have been most thoroughly studied) also have important functional differences; for a side-by-side comparison, see Table 2. Both IncP Reps and DnaA cooperatively oligomerize upon binding specific

repeats, which creates torsional forces leading to the melting of the DNA duplex nearby and to the formation of an open complex. However, while IncP plasmid Rep monomers bind DNA through winged helix domains, DnaA does so through a different domain (helix-turn-helix [HTH]). The cognate binding sites for Reps are unrelated to DnaA boxes: they are direct repeats of about 20 nt in length known as iterons (43–45). Iterons are intrinsically bent (8, 46), and their spacing matches the helical periodicity of the DNA double helix (47), consistent with the formation of a helical oligonucleoprotein structure, but the existence of such a structure has not

Table 2 Comparison between DnaA and theta A Rep-mediated replication initiation^a

Feature	DnaA	Rep	References
Structural domains	HTH domain	Winged helix domain	143 , 144
DNA cognate site	DnaA box	Iterons	8 , 43
Number of nucleotides bound	3	3	22 , 144 , 145
DNA melting	AT-rich DUE	AT-rich DUE	14 , 20 , 21 , 146
Can bind within single-stranded region of melted DUE	Yes	Yes	22 , 145 , 147
ATP binding to replication initiator required for DNA melting	Yes, used to regulate the timing	No, instead timing regulated by transcriptional autoregulation, monomer concentration and activation, and handcuffing	48 , 49 , 148
Presence of high- and low-affinity binding motifs in the <i>ori</i>	Yes, role in controlling oligomerization	No	20 , 32
Melting assisted by NAPS; DnaA binding to origin of replication enhanced by the presence of NAPS IHF and HU	Yes	Sometimes	46 , 149–151
Role in recruitment of replisome	Yes	Yes	26 , 28 , 49 , 103 , 136 , 144 , 152
Role in transcriptional autoregulation	No	Sometimes	44 , 153
Formation of dimers in solution	No	Yes ^b	44 , 153 , 154

^aLargely based on reference [8](#).

^bpAMbeta1 is the exception ([72](#)).

been demonstrated for Reps ([8](#)). Another important difference is that Rep binding of iterons does not require ATP ([48](#), [49](#)); this is unlike DnaA, which has to be in the ATP-bound state to bind high-affinity sites. Finally, while DnaA is monomeric, IncP Reps can form dimers, a mechanism that is critical for the regulation of plasmid copy numbers (see below).

PriA-Mediated Replication Fork Restart

Primosome (PriA-mediated replisome) assembly is a pathway specializing in replication fork restart ([19](#)). PriA is recruited by free 3' ends representing the nascent leading-strand DNA close to the branch junction, which is the landmark of stalled DNA replication forks ([16](#), [17](#), [19](#)). Three-way junctions can also be found when a third strand pairs to one of the main strands in the melted double helix, displacing the other complementary main strand. If the third strand is RNA, this structure is called an *R-loop*; if it is DNA (for example, as a result of strand invasion during recombination or as a result of primer extension), the structure is called a D-loop. A stalled fork can also produce a large single-stranded DNA gap, in which lagging-strand DNA synthesis has proceeded past the nascent leading strand. These large ssDNA gaps appear to be the preferred substrate of a PriA homologue, PriC ([50](#)). PriA recognition is mediated by a small

binding pocket at the N-terminal domain. This directs the proper positioning of the helicase domain of PriA on the double-stranded DNA ([51](#)). Primosome assembly involves two additional proteins (PriB and DnaT) that form a PriA-PriB-DnaT-DNA quaternary complex that loads the DnaB/C complex onto the lagging strand.

The PriA pathway of replication initiation was originally identified in DnaA-deficient mutants and described as stable DNA replication (SDR) ([52](#)). SDR can be achieved in at least three different ways: (i) increased R-loop formation (the processing of R-loops by RNaseE or by RNaseH leads to the formation of a free 3'-OH that can be used for replication restart, leading to constitutive SDR [cSDR] [[53–55](#)]), (ii) increased recombination, a phenomenon known as inducible SDR (iSDR) (in this case, the generation of a free 3'-OH involves exonuclease processing of the invading strand), and (iii) formation of 3' flaps after replication fork collision in replication termination (*Ter*) sites [RecG SDR] [reviewed in references [30](#) and [53](#)].

R-loops have become the focus of increased attention due to their role in generating genetic instability in cancer and in neurodegenerative disease as well as their role producing genetic variation during somatic hypermutation ([56–58](#)). In prokaryotes, unscheduled R-loop formation can be highly deleterious, causing transcription blocks and

inhibiting elongation (59–61). During transcription, the DNA duplex is transiently opened to allow access of the RNA polymerase (RNAP) to the template, and a short 8- to 10-bp-long RNA:DNA hybrid is generated upon initiation of RNA synthesis (62). As the RNAP moves forward, the formation of R-loops is prevented because the size of this RNA loop is maintained by the displacement of the nascent RNA via an RNA exit channel and by the re-annealing of the upstream DNA strands (53). Coupling transcription to translation and factor-dependent transcription termination are additional mechanisms preventing the hybridization of nascent transcripts with DNA in *E. coli* (62–64). However, increased R-loop formation can be observed in genetic backgrounds favoring negative supercoiling due to decreased topoisomerase I activity or increased gyrase activity, in strains that are deficient in RNaseH, or in strains harboring point mutations in RNAP that favor R-loop formation (reviewed in reference 53). Unscheduled R-loops may also form when the RNA polymerase falls off (a process that can be facilitated by DksA [65]) or as a result of RNaseE-mediated cleavage and facilitated rotation of the transcript (63).

Three classes of theta plasmid replication depend on PriA-dependent replisome assembly: class B, which corresponds to ColE1-like plasmids, class C, which corresponds to ColE2 and ColE3 plasmids, and class D, which corresponds to pAM β 1 and other large, low-copy streptococcal plasmids (reviewed in reference 66). All three classes recruit PriA via R-loop formation, leading to replicon assembly on the lagging strand. Despite assembling a potentially bidirectional replication fork, replication is unidirectional because these replicons have replication blocks on the 5' end (67, 68).

Class B theta plasmid replication is illustrated in Fig. 2. These origins of replication encode an RNA transcript (RNAII) that acts as the third strand in a melted duplex DNA, generating an R-loop. The formation of this R-loop is guided by a G-rich sequence in the *ori* DNA that pairs with a C-rich stretch in the transcript. Two hairpin structures further downstream in the preprimer are also important functional elements for R-loop formation, preprimer processing, and extension (reviewed in reference 66). Following its complete transcription, the preprimer RNA in this R-loop is processed by RNaseH to generate a free 3'-OH terminus that is extended by DNA polymerase I (Pol I). RNaseH processing requires the transcript to be in the right conformation and orientation. Pol I extension of the RNA primer further opens the

duplex and reveals a hairpin structure in the lagging strand that can serve as a primosome assembly signal (*pas*). Primosome assembly initiates the coordinated replication of both strands by Pol III, although there is some evidence that Pol I can functionally replace Pol III (69). The mutation footprint produced by error-prone Pol replication also suggests that when the replisome finishes replicating the leading strand, it leaves a gap of approximately 500 nucleotides on the lagging strand that is filled in by Pol I (69). The formation of the R-loop is the only essential step in this process. In the absence of both RNaseH and Pol I, replication initiation is still possible in class B theta plasmids, albeit at reduced efficiency (70).

Class C (ColE2 and ColE3) plasmids encode a Rep (RepA) protein that has a dual function as replication initiation factor and as a primase. The primase produces a unique primer: ppApGpA (71). This primer is extended by Pol I, recruiting the replisome through the primosome assembly pathway, as seen in ColE1. Given that, in this case, the primer is produced by a plasmid-encoded Rep, the *cis ori* sequence required for replication initiation is minimal, only 32 to 33 nt (68).

In class D theta plasmids, replication is initiated by processing of a long transcript, but in this case, the transcript is at the same time the mRNA encoding for a replication initiator factor (RepE in the case of pAM β 1). RepE assists with duplex melting (72), but it does not appear to be involved with the recruitment of the replisome, because (similar to ColE1) replisome recruitment involves extension by Pol I of a transcript, leading to D-loop formation, and uncovering of a cryptic *pas* site, leading to primosome assembly (73). Two unique features of type D theta plasmid replication are that the Rep (RepE) has an active role in primer generation, either by pausing transcription or by processing the transcript (74) and that Pol I progression is arrested at two sites: ~190 nt and ~230 bp downstream of the RNA-DNA switch, respectively. The arrest likely assists with the switch from Pol I to Pol III and appears to be mediated by a plasmid-encoded topoisomerase I-like enzyme (first site) (75) and by a collision with the plasmid-encoded site-specific resolvase, Res β , bound to its resolution site (second site) (76).

Nontheta Plasmid Replication

Two nontheta types of plasmid replication initiation rely on signals in the plasmid *ori* sequence forming higher-order secondary structures that are recognized by Reps. The two types are strand displacement and rolling-circle

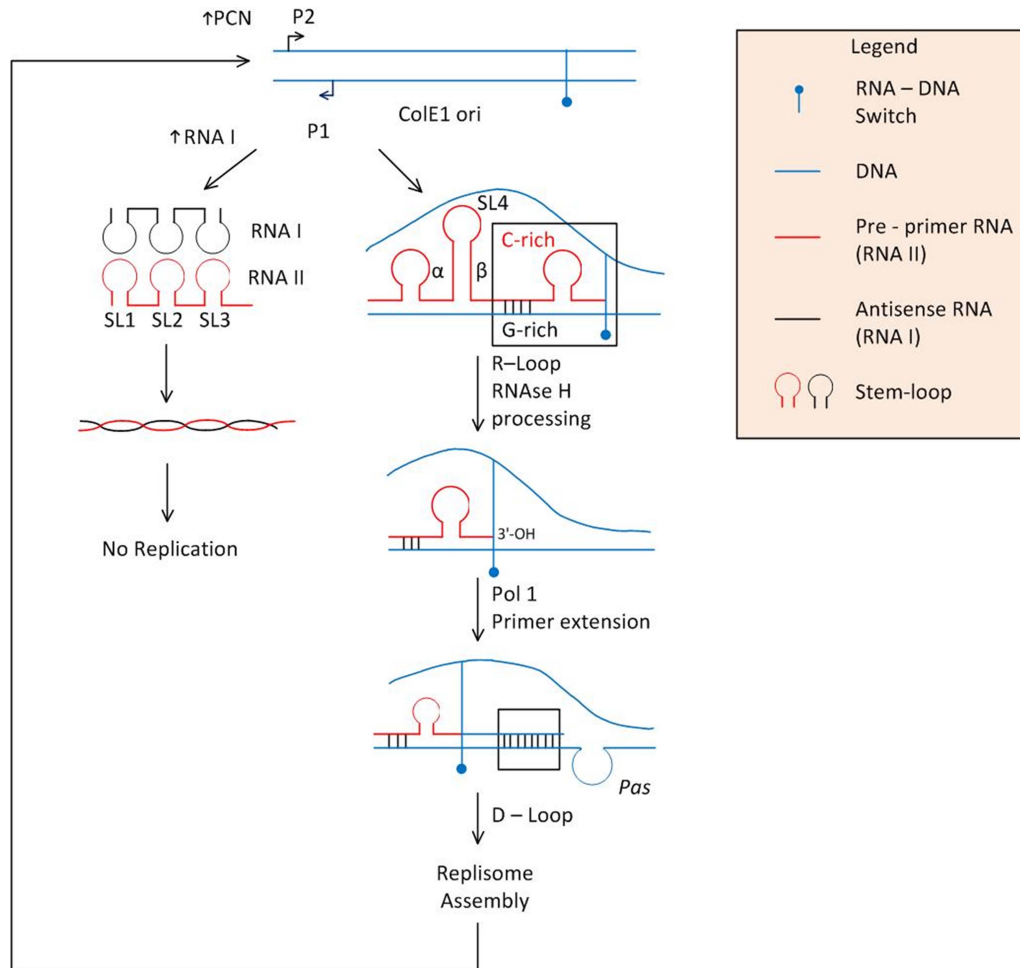


Figure 2 For replication initiation, the *ori* transcript RNAII acts as the third strand in a melted duplex DNA, generating an R-loop, guided by G-rich sequences in the *ori* DNA that pair with C-rich sequences in the transcript; other hairpin structures further downstream in the preprimer are also important for R-loop formation, preprimer processing, and extension (reviewed in reference 66). Processing of the R-loop terminus by RNaseH generates a free 3'-OH terminus. RNAII needs to be in the right conformation and orientation for processing by RNaseH. The 3'-OH terminus generated by RNaseH processing is extended by DNA polymerase I (Pol I), further opening the duplex and revealing a hairpin structure in the lagging strand that can serve as a primosome assembly signal (*pas*). PriA-mediated replisome assembly starts the coordinated replication of both strands by Pol III, although there is some evidence that Pol I can functionally replace Pol III (69). For antisense RNA regulation, as it is being transcribed from promoter P2 in the sense direction, the preprimer (RNAII) forms three symmetrical stem-loop structures (stem-loops 1, 2, and 3 [SL1, SL2, and SL3]). A small and short-lived antisense transcript (RNAI) that is transcribed from a promoter going in the opposite direction also forms these three stem-loops. The RNAII nascent transcript and the antisense RNAI contact each other through the 6- to 7-nt loop portion of their respective stem-loops. This pairing makes the preprimer incompetent for R-loop formation, thus blocking replication initiation (reviewed in references 66, 92, and 93). The half-life of RNAI is short because it contains RNase E recognition sites. Preprimer transcripts larger than 200 nt long are refractory of RNAI-induced inhibition because they form an alternate stem-loop (SL4) by pairing two sequence areas of the transcript (α and β), further reducing the effective half-life of RNAI. This short half-life ensures that RNAI-mediated suppression of replication initiation is reflective of plasmid copy number.

replication. These two alternative types of plasmid replication differ from theta plasmid replication in two additional key aspects: (i) lagging-strand synthesis is decoupled from leading-strand synthesis, and (ii) lagging-strand synthesis is continuous.

In the case of strand displacement, replication initiation is mediated by RepC, which (similar to class A theta

plasmids) binds cognate iteron sequences and melts an adjacent DUE. In this case, though, DUE melting is assisted by a plasmid-encoded helicase, RepA, and exposes two initiation signals known as single-strand initiation (*ssi*) sites. There is one site on each strand. Each *ssi* forms a hairpin. The base of this hairpin is recognized by a plasmid-encoded primase, RepB, which generates a primer. Note that RepB is the only primase used here;

this is unlike theta C plasmids, which have a primase that initiates replication and then switch to DnaG. This primer is extended by Pol III, forming a D-loop (reviewed in references [77](#) and [78](#)). Strand-displacement plasmids are unique in the number of replication enzymes they encode in addition to replication initiation, which allows these plasmids to function in a wide range of hosts (see “Broader Biological Context: Host Range” below).

In the case of rolling-circle replication (reviewed in reference [79](#)), replication of the two strands is completely asynchronous. Leading-strand replication starts at the double-strand origin (*dso*), which has a hairpin cruciform structure. The *dso* is recognized by a plasmid initiation factor (RepC), which nicks this structure, generating a 3'-OH terminus at the nick site. This 3'-OH is extended by Pol III. RepC also catalyzes the final transesterification step that joins the 5' end generated by replication and the 3' end generated in the cleavage site, sealing the replicated leading strand and producing a single-strand intermediate. The presence of a circular single-strand intermediate is a hallmark of this class of plasmid. Lagging-strand synthesis starts at a single-strand origin (*sso*) with the generation of a primer by the host primase DnaG. This primer is extended by Pol III, apparently without the need for a helicase because the template is single-stranded.

SEQUENCE-BASED CLASSIFICATION OF PLASMID REPLICONS

Replicons can also be classified according to sequence homology. The first sequence-related classification method grouped replicons based on incompatibility, i.e., on the observation that when two low-copy-number plasmids cannot be distinguished by one or more maintenance systems, the two plasmids are randomly partitioned into daughter cells, leading to the loss of one of them ([80](#)). Five compatibility groups were initially defined in *E. coli* according to conjugation experiments in *E. coli* ([81](#)). These 5 compatibility groups were later expanded to 23.

Detection of sequence homology based on primers for regions that are unique to each plasmid group was later developed; these methods are collectively known as PCR-based replicon typing, or PBRT ([82](#)). The target for the diagnostic primers in enterobacteria is listed in [Table 3](#) (column 3). PBRT was consistent with the original conjugation-based scheme and has become the most commonly used technique for plasmid typing in

Enterobacteriaceae (28 incompatibility [Inc] groups). More recently, PBRT has been extended to *Pseudomonas* (14 Inc groups) and *Staphylococcus* (18 Inc groups). [Table 3](#) summarizes the Inc groups found in *Enterobacteriaceae*, listing their shared associated biological traits and some specific plasmids belonging to each of these groups. Note that there is a direct correspondence between some *Pseudomonas* and enterobacterium Inc groups (noted in column 1).

A set of mobility (MOB) genes, which are relaxases involved in conjugation, have been used as an alternative for replicon typing ([83](#)). Even though this method excludes nonconjugative plasmids, it produces higher granularity and is consistent with the PBRT method, as each Inc type corresponds to relaxases of a single MOB superfamily. For the remainder of the review, we are going to refer exclusively to the PBRT classification.

REGULATION OF PLASMID REPLICATION

Plasmid replication initiation is subject to tight regulation because plasmid copy number determines the plasmid gene dosage present at any given time in the cell, which in turn has a direct impact on the level of expression of all the genes encoded in the plasmid. Indeed, unlike transcription, which allows for gene- or operon-specific regulation through individual promoters, gene dosage consistently impacts the expression of all the genes encoded in the plasmid ([84](#)). Thus, tight control of steady-state plasmid copy number is critical, particularly with plasmids expressing a large number of adaptive genes. The timing of plasmid replication and plasmid segregation should also be critical for optimizing a plasmid's adaptive value due to their impact on plasmid gene dosage.

Plasmid copy number is mainly regulated through control of replication initiation by mechanisms that are sensitive to the abundance of plasmid copy number relative to cell volume ([43](#), [44](#), [85](#)). Having multiple origins of replication and/or multiple replicons can provide additional versatility in the control of plasmid copy number (see below).

The regulation of replication initiation in class A theta plasmids is illustrated in [Fig. 3](#). It is based on several factors: (i) Rep proteins are limiting and can be targeted by proteases, (ii) Rep proteins may need to be in an activated monomeric form to bind their target iteron sequences, a process that can be facilitated by chaperones,

Table 3 Inc plasmid classification and associated biological traits

PBRT group (corresponding <i>Pseudomonas</i> PBRT in parenthesis)	Examples ^a	Targets for PCR-based replicon typing (82)	Plasmid copy number (10)	Size range (kb) (10)	Host range (5, 10)	HGT (10)	Additional notes
IncP (IncP-1)	RK2 (1α) R751 (1β) pJP4 pQK54 pKJK5 RP1 RP4 R68 R18	Iterons	5–7	70–275	Broad	Conjugative	Environmental: found in manure, soils, water treatment plants Extremely stable
IncHI1	R27		Low	75–400	Broad	Conjugative	HI1A/B multireplicon, frequently also IncFIA/B, occasionally also IncX1
IncHI2	R478		Low	75–400	Broad	Conjugative	Usually IncF-compatible
IncFIA	F	Iterons	Low	45–200	<i>Enterobacteriaceae</i>	Conjugative	Multireplicon plasmid with combinations of FIA/B, FII, and FrepB Most frequent in human (North and South America) and animal (Asia) sources
IncFIB		<i>repA</i>					
IncFII	R1 NR-1 R6-5	<i>repA2</i>					
IncFrepB		<i>RNAI/repA</i>					
IncN	N3 pCU1 R46	<i>repA</i>	Low	30–70	Broad (relative)	Conjugative	Animal-associated environments Often colocalized with IncF
IncA/C (IncP-3)	RA1, pRMH760	<i>repA</i>	Low	18–230	Narrow	Conjugative	
IncL	pK01-34 R471 pEL60	<i>repA, repB, repC</i>	Low	60–90	Broad	Conjugative	Highly conserved, sharing the majority of their genes. Important role in dissemination of β-lactamases
IncR	pKP1780	<i>repB</i>		40–160	Broad	Mobilizable	
IncY	P1 pMCR-1- P3	<i>repA</i>	Low	90–140			Prophage
IncQ (IncP-4)	RSF1010 IncP-4 R1162 R300B		Medium (4–12 copies/cell)	8–14	Broad	Mobilizable	Lacks partitioning or plasmid stability system
IncW	R388 R7K pSa pVX2	<i>repA</i>	Low	<40	Broad	Conjugative Smallest conjugative plasmid; can act as helper for ColE and IncQ	
ColE		ColE-like <i>ori</i>	Variable (1–20 copies/cell)	6–40	Narrow	Mobilizable	Produce colicins, proteins produced by certain strains of <i>E. coli</i> lethal for related strains Associated with the spread of qnrS1, qnrB19

^aPartially based on reference 97.

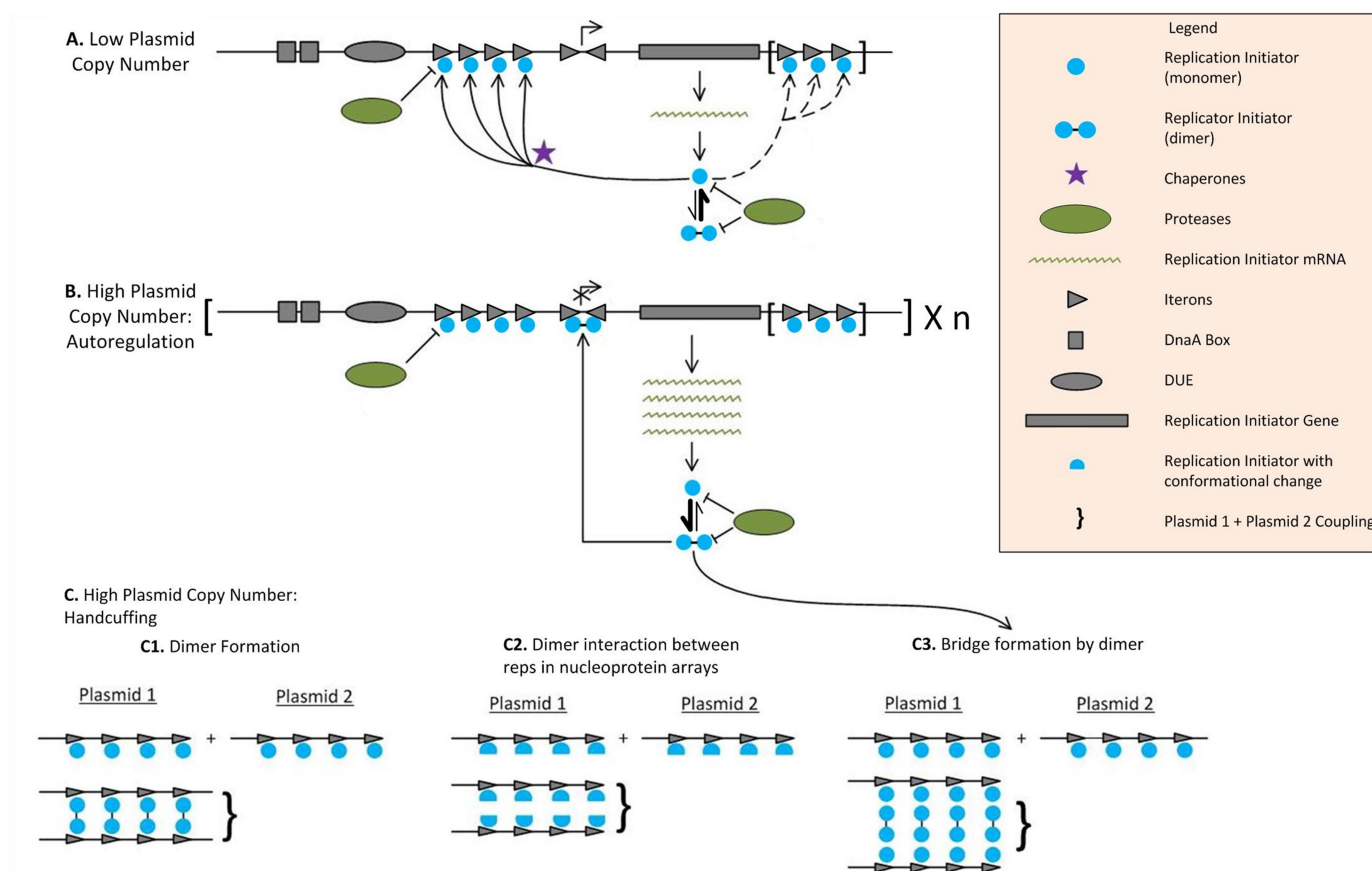


Figure 3 Replication initiator proteins as negative regulators of replication. The rate of replication is determined by the concentration of free (non-Rep-bound) iterons, which is determined by both plasmid copy number and cell volume. Given the cooperative interactions involved in saturating iterons, this regulatory mechanism is ultrasensitive to plasmid copy number and switch-like. Generic class A theta replicon structure: *rep* gene (rectangle), DnaA boxes, which can be found upstream or downstream of the Rep (squares), iterons (arrowheads), and an AT-rich DNA unwinding element (DUE, oval). (A) Replication initiator proteins (Reps) when the plasmid copy number is low. Reps (blue circles) are expressed from strong promoters and tend to be in their monomeric (active) form; in some cases, the activation of these monomers needs to be facilitated by chaperones (purple stars). Reps are also sensitive to protease activity (green ovals). Active monomers bind iterons until saturation; in some cases, additional clusters of iterons are present to decrease the level of Rep available, further tightening plasmid copy number control (86). (B) Replication initiator proteins when plasmid copy number is high. High Rep protein expression resulting from a high plasmid copy number favors the dimeric form of Rep. The symmetrical conformation of the dimeric form matches inverted iterons found in the promoter of *rep* genes. This results in a binding affinity for the promoter that is higher than that of the RNA polymerase, blocking *rep* transcription (137). Transcriptional autorepression by Reps is seen in IncFIA, IncN, and IncP plasmids and is also consistent with the structure of replicons from other groups, such as IncHI1 and IncY, although in the case of IncN and IncY, the iterons that overlap with the Rep promoter are not inverted (43, 138). Rep dimers can also bridge Rep-bound iteron arrays, one of the proposed mechanisms of handcuffing (see panel C3). (C) Different handcuffing mechanisms. Once iteron-bound Rep arrays form, they can couple two different plasmids, a reaction *in trans* known as plasmid handcuffing that blocks *ori* melting by steric hindrance (139). Three mechanisms that pair different plasmids through iteron-bound replication initiation proteins have been proposed (43). From left to right: direct dimerization of the iteron-bound initiators (proposed for the plasmid RK6 [140]), direct interaction between arrays of iteron-bound monomers, associated with a Rep conformational change induced by iteron binding (proposed for the plasmid pPS10 [141]), and bridging via dimer formation (proposed for RK2 [139]). Chaperones counteract handcuffing by facilitating the dissociation of dimers to monomers or by increasing monomer-to-dimer ratios (139, 142).

(iii) Rep proteins often bind iterons in their own promoter, sterically hindering transcription by the RNA polymerase, and (iv) once bound to their target iterons, Rep proteins can mediate the coupling of plasmid origins from different plasmids, blocking replication initiation (a process known as handcuffing).

A variation on Rep autoregulation as a mechanism for control of plasmid copy number has been described for IncP plasmid RK2. The promoter for its *rep* protein (TrfA) is controlled by two plasmid-encoded regulators, KorA and KorB, which bind to sites that overlap with the *trfA* promoter sequence. Cooperatively, they suppress

TrfA expression about 1,000-fold (86). So, in this case, instead of the rep itself, regulation is mediated by two other plasmid-encoded proteins.

Dimerization, illustrated in Fig. 3B, has been best studied in IncP (R2K, R6K, PS10) plasmids. The increased synthesis of Rep associated with high plasmid copy number shifts the equilibrium between the monomer and dimer Rep form toward the dimeric form, which is not competent for iteron binding, thus suppressing replication initiation (43). Chaperones such as ClpB or DnaK/DnaJ/GroE control the conversion from dimer to monomer and also the activation of monomers (44). Proteases such as ClpAP control the level of active monomers as well (87). In RK2, the activity of specific proteases against TrfA is modulated by binding to iterons, and the effects can go in either direction; i.e., iteron binding can increase or decrease initiator sensitivity to proteases depending on the specific protease (88).

High plasmid copy number can also lead to handcuffing, i.e., to the formation of bridges coupling plasmid origins from different plasmids via iteron-bound Reps. This bridge formation blocks replication initiation. In this case, replication rate is more directly linked to iteron abundance than to levels of Rep expression. Three plasmid-coupling mechanisms have been proposed and are illustrated in Fig. 3C. These are: dimerization of iteron-bound initiators (RK6), direct interaction between arrays of iteron-bound monomers (pPS10), and dimer formation bridging iteron-bound monomers (RK2) (43).

Another group of plasmids use RNAI (short and highly structured antisense transcripts) for regulation. The pairing of these antisense RNAs allows the precise targeting of specific areas of the transcript to block translation or to induce conformational changes in the transcript. These antisense transcripts have a short half-life, which is critical to enable tight regulation of replication initiation, due to the presence of RNaseE cleavage sites (89). Antisense plasmid copy number appears to be an example of parallel evolution because it is observed in three groups of plasmids with different replication mechanisms and because in each case the suppression of replication initiation occurs by different mechanisms as well. We explain them in greater detail below.

Several class A theta plasmids, including I-complex (IncI, IncK, IncBO) and IncL plasmids, have a Rep whose start codon overlaps with the stop codon of a short leader

peptide immediately upstream. Translation and correct termination of the leader peptide facilitate the formation of a pseudoknot between two short (8-nt) complementary sequences of leader sequence that is necessary for Rep translation. RNAI blocks the translation of the leader peptide by sterically hindering ribosomes attempting to initiate translation of the leader peptide, therefore suppressing the translation of the Rep as well (90, 91).

In class B (ColE1-like) theta plasmids, a short-lived antisense RNAI and its complementary RNAII preprimer target sequence form three symmetrical stem-loop structures that contact each other through the 6- to 7-nt loop portion of their respective stem-loops (Fig. 2). The 5' end of RNAI (known as antitail) nucleates the hybridization between the two RNAs to form an RNA duplex. This pairing induces conformational changes in the preprimer that make the preprimer incompetent for R-loop formation, thus blocking replication initiation (reviewed in references 66, 92, and 93). Preprimer transcripts larger than 200 nt are refractory of RNAI-induced inhibition, further reducing the effective half-life of RNAI.

In class C (ColE2) theta plasmids, RNAI targets the 5' untranslated region of the *rep* transcript, possibly inducing conformational changes in the preprimer that block its translation (94).

BROADER BIOLOGICAL CONTEXT: HOST RANGE

The vast majority of plasmids found in *Enterobacteriaceae* are conjugative or at least mobilizable (Table 3, column 7). This means that the ability of a plasmid to replicate in a range of phylogenetically distant organisms (exhibiting broad host range, or BHR) can be beneficial in the long term because it allows a wider access to the available gene pool.

Factors conferring BHR properties have thus been the object of increased scrutiny (1, 95, 96). It turns out that plasmids have to meet a surprisingly large number of requirements to be able to replicate in different hosts. These requirements include the compatibility between plasmids and a variety of host replication factors, as well as the compatibility of plasmid replication initiation and transcriptional regulatory circuitry across hosts (84, 97).

BHR appears to be clearly advantageous under some conditions, as some plasmid replication strategies appear to have evolved to maximize host range. These include the

evolution of DnaA-independent replication-initiation factors (some Reps, RNA primers, and nicks) and of plasmid-encoded replication factors (primases, helicases, topoisomerases, polymerases, resolvases), which decreases the dependency of these plasmids on adequate interaction with host-specific factors. The acquisition/evolution of plasmid-encoded replication factors is clearly the strategy adopted by IncQ plasmids, which carry their own primase and helicase (77, 78) and have an extraordinarily broad host range that includes *Alpha*-, *Beta*-, *Gamma*-, and *Deltaproteobacteria* and *Cyanobacteria*.

Another strategy is versatility, i.e., the availability of redundant mechanisms of replication that allow functionality in different hosts. This includes the presence of multiple origins of replication and of multiple replication initiators and decreases the plasmid's dependence on host factor compatibility. IncN plasmids, for example, have three origins of replication, *oriB*, *oriS*, and *oriV*. *oriB* functions as the main origin of replication because it has the iterons that bind RepA; however, in the absence of these iterons, Pol I can still initiate replication at *oriV* (98). In the absence of *oriV*, *oriS* can still start replication (99). Another example is the IncP compatibility group R6K plasmid, which also has three origins of replication: α , β , and γ . γ appears to be the post-mobilization *ori* (which requires rapid amplification), while α and β primarily support vegetative replication, with a lower replication rate (100). An example of re-

dundancy in replication initiation factors is TrfA (the Rep protein in IncP plasmids), which is expressed in two different versions resulting from the alternate translation of a single transcript using separate in-frame translation start sites. In *E. coli*, both forms of TrfA can initiate plasmid replication, whereas in *Pseudomonas*, only the longer form is active because it is not dependent on DnaA (101, 102). More recently, mutants adapted to a nonnative species were shown to accumulate mutations at the N terminus of the long form of TrfA, again pointing to this form of TrfA as critical for extending host range (103).

On the other hand, the complex mechanisms of regulation of plasmid maintenance and the strong genetic evidence of plasmid adaptation to its host point to strong selective pressures restricting plasmid host range. The latter include analysis of G+C content and codon usage and even evidence of host-specific evolution of single-strand origin motifs (1, 95, 104). Thus, it seems that adaptation of plasmids to their host is generally necessary to optimize their contribution to the host's fitness but that this adaptation restricts access to broad genetic diversity.

Figure 4 shows the distribution of plasmid replicons in 233 random ExPEC samples collected at the University of Washington hospital between 2009 and 2012 (105). We found that the three most abundant replicons (IncF, IncI-complex, and ColE) correspond to narrow-host-range (NHR) plasmids, with observed frequencies of 66%,

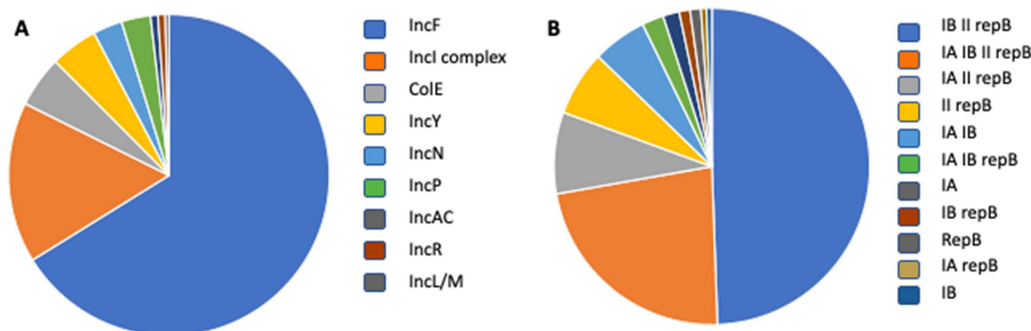


Figure 4 Replicon representation in ExPEC whole genomic shotgun sequences reported by the University of Washington (105). Only 233 of the reported sequences were included in this analysis because of strict quality control standards. (A) Pie chart representation of the replicons identified. The replicons are ordered clockwise from most to least abundant: IncF ($n = 180$), IncI complex ($n = 44$), ColE ($n = 14$), IncY ($n = 13$), IncN ($n = 8$), IncP ($n = 8$), IncAC ($n = 2$), IncR ($n = 2$), and IncL/M ($n = 1$). IncF denotes the presence of at least one of the following replicons: IncFIA, IncFIB, IncFII, or IncFrepB. The Inc-I complex includes IncI1 ($n = 17$), IncK ($n = 17$), and IncBO ($n = 10$). Note that the number of replicons ($n = 272$) is greater than the number of samples included ($n = 233$) because, often, multiple replicons can be found in the same sample. (B) Representation of IncF replicons, which are largely mosaic combinations of IncFIA, IncFIB, IncFII, or IncFrepB. Again, these replicons are ordered clockwise from most to least abundant: IB II repB (49.4%), IA IB II repB (22.8%), IA II repB (8.3%), II repB (6.7%), IA IB (5.6%), IA IB repB (2.2%), IA (1.7%), IB repB (1.1%), repB (1.1%), IA repB (0.6%), and IB (0.6%). Note that these combinations may be found in different plasmids; we are only showing combinations present in the same cell. Note also that we are observing a rapid sequence divergence for IncFII replicons (not shown). Therefore, frequently, IncFII may not have been an exact match for our diagnostic sequence, producing false negatives.

16%, and 5%, respectively. BHR plasmids (IncN, IncP, IncL/M, and IncQ), by contrast, were infrequent: 3%, 3%, 0.5%, and <0.5%, respectively. This snapshot is consistent with previous studies reporting IncF plasmids as one of the most prevalent incompatibility types (10, 106) and IncQ as “rarely detected” in *Enterobacteriaceae* (10). The observed distribution of NHR and BHR plasmids in ExPEC supports the proposed tradeoff between adaptive value for the host and access to genetic diversity. This hypothesis predicts a strong representation of NHR plasmids because they are optimally suited to their hosts and therefore tend to have a stronger positive effect on fitness. This model also predicts the retention of BHR plasmids at low frequency, driven by the benefit of broadening genetic diversity available to the population as a whole.

Figure 4B shows the distribution of IncF replicons in samples that have at least one IncF replicon. Of all IncF samples, only 4% have a single replicon. The replicon mosaicism of IncF plasmids is well established (3, 107). The factors driving this mosaicism are not clear. Part of it appears to be specialization reminiscent of that of R6K, with IncFII likely involved in establishment following conjugation and IncFIA as the primary replicon supporting vegetative growth (reviewed in reference 3). The frequent linkage between IncFIA and IncFrepB may be driven by expanding the compatibility range to allow different IncF plasmids to stably coexist in host cells, as the presence of two compatible replicons overrides the incompatibility between individual replicons (107, 108). As the fraction of genetic diversity carried by one type of NHR plasmid (IncF plasmids in this case) increases, there should be increasing selective pressure for the stable acquisition of multiple variants of these plasmids in the

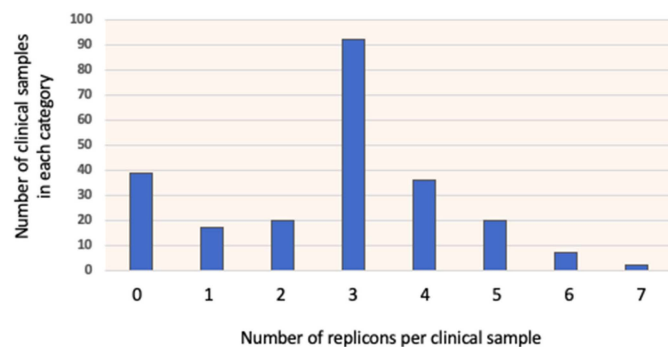


Figure 5 The distribution of replicon number per clinical sample is shown as a bar graph. The average number of replicons for samples that have at least one plasmid is 3.26. If all IncF replicons are considered as a single category, the average number goes down to 1.2.

cell. In this scenario, the availability of expanded compatibility should provide mosaic replicon plasmids such as IncF or IncHI1 with a selective advantage.

Figure 5 shows the distribution of replicons per sample, which for host cells with at least one replicon follows roughly a normal distribution centered around an average of 3.28 per sample. This high average is driven by the abundance of mosaic IncF plasmids; if we lump all IncF replicons in one category, the average goes down to 1.2, highlighting both the strong representation of IncF plasmids in our strains (Fig. 4A) and the mosaic nature of these replicons (Fig. 4B).

BROADER BIOLOGICAL CONTEXT: PLASMID MAINTENANCE

Given that plasmid replication initiation determines the plasmid gene dosage present at any given time in the cell, which in turn has a direct impact on the level of expression of all the genes in the plasmid, controlling plasmid copy number is critical for optimizing a plasmid’s adaptive value for the host. Controlling the timing of replication initiation and plasmid segregation should be important as well, as these processes also have an impact on gene dosage. Therefore, regulation of plasmid replication needs to be understood in the broader context of plasmid maintenance, including control of plasmid segregation through partition (*par*) systems and post-segregational counterselection through toxin-antitoxin (TA) plasmid addiction systems.

The presence of multiple versions of these functional elements adds further complexity. This includes alternate origins of replication within a single replicon as in the cases of IncP and IncN plasmids discussed above (96, 99), mosaic replicons such as IncF and IncHI1 (3, 109), and examples of cointegrated replicons (110–112) and of multiple *par* (113, 114) and/or TA systems (115, 116) coexisting in the same plasmid. We believe that all these systems are tightly interconnected, maintaining the delicate balance between optimizing the adaptive value of the plasmids and access to genetic diversity.

TA systems counterselect plasmid-free cells through post-segregational killing or growth inhibition of plasmid-free cells. According to their mechanism of action and regulation, TA systems can be classified into six types (types I through VI). They typically consist of two elements: a stable protein that targets essential cellular

processes (the toxin) and short-lived protein or an untranslated antisense RNA species inhibiting the toxin activity (the antitoxin). Antitoxins are more labile than toxins because they are degraded more efficiently by host enzymes. Loss of the antitoxin when the plasmid is no longer present allows the toxin to reach or act on its target, causing death or growth restriction in the cell (for comprehensive reviews, see references [117](#) and [118](#)). More recently, additional roles for TA systems have been recognized, including adaptation to intracellular environments, stress response (induction of persistent state and of programmed cell death), and postrecombinational killing ([117](#), [119](#)).

Most TA systems found in plasmids correspond to types II and III, with type II being the most abundant in virulence plasmids ([5](#)). Type II TA systems have been categorized into eight superfamilies: RelBE, MazEF, VapBC, CcdAB, ParDE, HigAB, HipBA, and Phd-Doc ([120](#)). In these TA systems, both the toxin and the antitoxin are proteins. The toxin usually consists of two distinct domains: a DNA-binding domain at the amino-terminus and a toxin-interacting domain at the carboxy-terminus. The antitoxin binds the toxin, forming a protein complex that results in toxin sequestration and inactivation.

Partition (*par*) systems are found in replicons of above 25 kb and ensure that all daughter cells receive at least one plasmid following segregation. *Par* systems may also be needed to counteract plasmid eviction toward nucleoid edges ([121](#)). These systems have three components: a motor protein, a centromere-like specific DNA sequence, and a DNA-binding protein that oligomerizes and serves as an adaptor to connect the motor with the centromere (reviewed in reference [122](#)). The two main types found in plasmids differ in the mode of action of their motor proteins. Type I *par* systems, such as *parABS* and *sopAB*, have Walker-type ATPases as motor proteins that form dynamic gradients in the cell that pull plasmids along. In contrast, type II systems, such as *parMRC* and *stbAB*, have actin-like motor proteins that push plasmids apart as they oligomerize. Most plasmids of above 180 kb carry *ParAB* systems, so type I *par* systems appear to be better suited for large plasmids ([121](#)).

CLUSTERING OF PLASMID MAINTENANCE ELEMENTS

Functionally related elements tend to cluster together as a way to facilitate their stable coregulation/cross-talk ([2](#), [123](#), [124](#)). This is particularly important in plasmids,

which undergo a very high rate of recombination and could then lose elements that are needed ([125](#)). With this in mind, we looked for protein functional domains (using the PFAM database [[126](#)]) that are consistently found within a 10-kb window centered on the PCR amplicon used for PBRT classification in 761 fully assembled *E. coli* genomic sequences available in NCBI as of April 2019. We defined “consistently” as seen in >75% of the isolates for one or more of the Inc groups. The results are shown in [Fig. 6](#), which shows protein family (PFAM) representation compiled using color-coding, with darker colors representing a higher prevalence of protein families within each Inc group. Inc groups were arranged by similarity using a hierarchical clustering algorithm and were grouped into five functional categories: replication initiation, plasmid maintenance, environmental stress protection, conjugation/mobilization, and other factors.

IncFIA replicons stand out for the presence of plasmid maintenance systems associated with the *ori*. We find a consistent association between the IncFIA *ori* and genes encoding *ParB*, *ParBc*, and another segregation-related PFAM (centromere-binding protein HTH domain). The association of IncF with plasmid *par* genes is well established ([3](#), [127](#)). IncFIA is also consistently associated with the *CcdA/CcdB* TA (type II) system. F-like plasmids have been reported to carry at least one representative of the various subfamilies of type II TA systems ([3](#)). The location of *ccdAB* genes adjacent to the origin of replication of the F plasmids has been previously reported and thought to enhance IncFIA replicon stability by coupling plasmid replication to host cell division ([128](#)).

Notably, our clustering of consistently represented PFAM groups in [Fig. 6](#) identified three clusters that include Inc replicons that share functional commonalities, highlighting the functional connection between replicons and TA and segregation systems that are found in close physical proximity.

Cluster I includes IncFIA, IncFIB, and IncN. These three replicons share a homologous Rep, although their regulatory mechanisms show considerable divergence ([95](#)). They also share the presence of a phage integrase in the vicinity of *ori*. Y-family polymerase UmuDC is close to the *ori* at some frequency in these three incompatibility types (as well as in IncP and IncHI1). This polymerase may provide increased genetic diversity ([129](#)) and/or protection from DNA damage ([130](#), [131](#)), although how its association with *ori* is relevant to its functionality is not known.

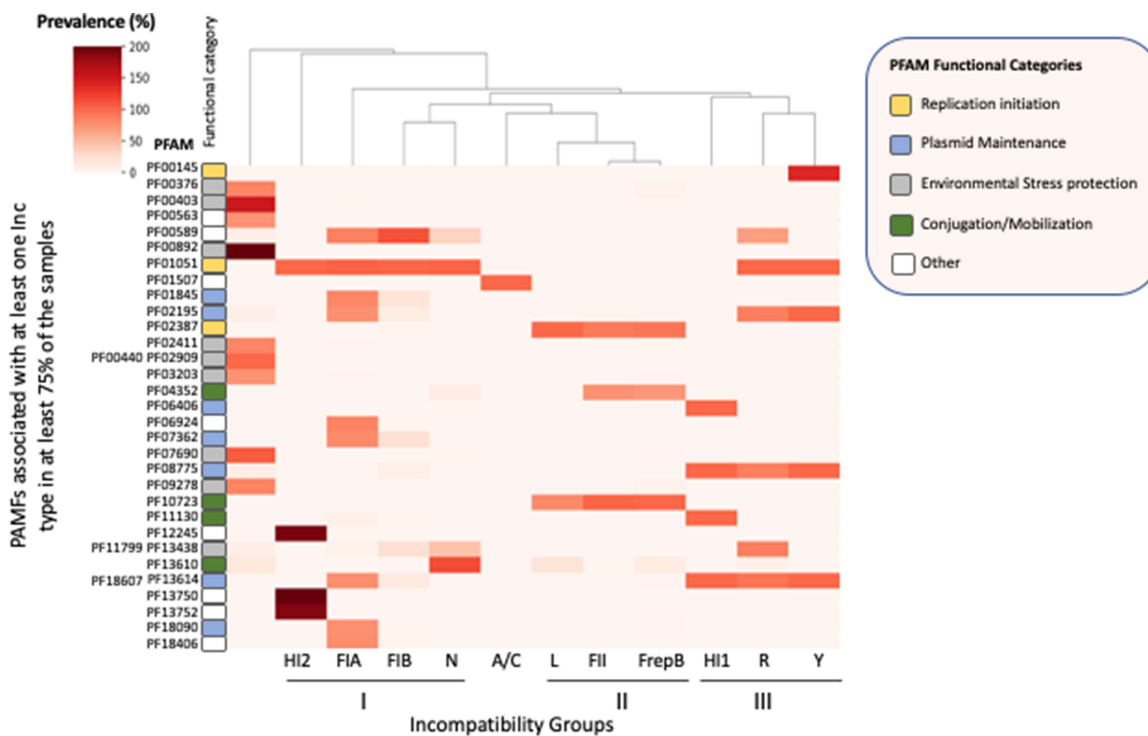


Figure 6 PFAM representation in the vicinity of plasmid replicons. Completely assembled *E. coli* plasmid sequences obtained from NCBI as of April 2019 were grouped by Inc type based on the diagnostic PCR amplicons for the PBRT system (82). Within each incompatibility group, we looked for PFAM matches within a 10-kb window centered around the diagnostic PCR amplicons. PFAM protein families present in at least 75% of the samples in at least one Inc group were identified and mapped to individual open reading frames. These PFAMs were also identified in other Inc groups. These PFAM representation data were compiled into a matrix and plotted using color-coding, with darker colors representing a higher prevalence of protein families within each Inc group. Inc groups are arranged by similarity using a hierarchical clustering algorithm for PFAMs. Note that the percentage can be higher than 100% in the case of duplications or if two domains are found in the same ORF. PFAMs are listed in the y axis, grouped in the following color-coded functional categories. Replication initiation-related PFAMs (orange): m5C methylase (PF00145), initiator replication protein (PF01051), and IncFII RepA protein family (PF02387). Plasmid maintenance PFAMs (light blue): PemK endoribonuclease toxin (PF01845) ParB-like nuclease domain (PF02195), StnA protein (PF06406), post-segregation TA CcdA (PF07362), ParB family (PF08775), ParA AAA+ and HT domains (PF13614 PF18607), centromere-binding protein HTH domain (PF18090). Environmental stress protection PFAMs (light gray): MerR family regulatory protein (PF00376), heavy metal-associated domain (PF00403), EamA-like transporter family (PF00892), mercuric transport protein (PF02411), tetracycline repressor family (PF02909 PF00440), MerC mercury resistance protein (PF03203), major facilitator superfamily (PF07690), MerR (PF09278), impB/mucB/samB Y family polymerase (PF13438, PF11799). Conjugation/mobilization PFAMs (green): phage integrase family (PF00589), ProQ/FINO family (PF04352), replication regulatory protein repB (PF10723), TraC_F_IV F pilus assembly (PF11130), DDE domain found in transposases (PF13610). Other PFAMs (white): EAL domain (PF00563), phosphoadenosine phosphosulfate reductase family (PF01507), DUF1281 (PF06924), bacterial IgE domain (PF12245), bacterial IgE-like domain (group3) (PF13750), DUF4165 (PF13752), ferredoxin-like domain in Api92-like protein (PF18406).

Cluster II comprises IncL, IncFII, and IncFrepB replicons. These replicons are all regulated by RNAI suppression of leader peptide translation, with the presence of a pseudoknot (85, 90, 91). IncFII, IncFrepB, and IncL share rolling-circle replication regulatory protein *repB*, which is likely associated with conjugation.

Cluster III includes IncHI1A, IncR, and incY, which share a homologous Rep (95). ParAB (type I partition system) is consistently associated with the *ori* in these three incompatibility groups. In addition, IncR plasmids have

ParBc close to their plasmid *ori*, and IncHI1A plasmids have a type II *par* gene. ParB-type proteins have recently been recognized as a new class of CTP hydrolases that act as molecular switches. During plasmid segregation, these proteins work in concert with ParA and the prokaryotic SMC/condensin complex, mediating centromere condensation into kinetochore-like structures (132, 133).

In contrast, IncP, IncHI2, and IncA/C have unique PFAM *ori* profiles. IncA/C stands out for the low density of PFAMs consistently found in the vicinity of the *ori*.

IncP is very enriched for genes involved in protection from environmental stress (8 of the 9 consistent *ori*-proximal PFAMs). This is consistent with the association of this plasmid compatibility group with bacteria isolated from manure, soils, and water treatment plants (134, 135).

CONCLUDING REMARKS

The plasmid gene content, level and timing of gene expression, and DNA sequence (G+C content, codon usage, etc.) need to be adjusted to the host for a given plasmid to have maximal adaptive impact. This selective pressure favoring adaptation tends to narrow plasmid host range and conflicts with the need for access to a broader pool of genetic diversity. Here, we argue that the trade-off between these two selective forces likely shapes the distribution of replicons in populations of enterobacteria based on two considerations: (i) a predominance of NHR plasmids in the population of ExPEC, supporting the adaptive value of these plasmids, coupled with a low but consistent prevalence of BHR plasmids, suggesting that expanded access to genetic diversity is important, and (ii) the success of NHR mosaic replicon plasmids such as IncF or IncHI1, which maximizes adaptive value while moderately increasing access to genetic diversity by making these plasmids compatible with other plasmids of the same Inc group.

We also argue that, given its critical importance for modulation of plasmid gene expression, the regulation of plasmid replication needs to be understood broadly. This includes the presence of redundant origins of replication, of mosaic and cointegrated replicons, of plasmid segregation systems, and of TA systems. The reason is that all these elements control not only plasmid copy number at steady state but also variations in plasmid copy number relative to the host's cell cycle, the presence of additional plasmids, and the physiological state of the host cell. Highlighting their tight functional integration with *ori* function, all these elements are frequently found in close proximity to the plasmid *ori*.

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