The ABCs of plasmid replication and segregation

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Abstract | To ensure faithful transmission of low-copy plasmids to daughter cells, these plasmids must replicate once per cell cycle and distribute the replicated DNA to the nascent daughter cells. RepABC family plasmids are found exclusively in alphaproteobacteria and carry a combined replication and partitioning locus, the *repABC* cassette, which is also found on secondary chromosomes in this group. RepC and a replication origin are essential for plasmid replication, and RepA, RepB and the partitioning sites distribute the replicons to predivisional cells. Here, we review our current understanding of the transcriptional and post-transcriptional regulation of the Rep proteins and of their functions in plasmid replication and partitioning.

Alphaproteobacteria
A group of Gram-negative, generally flagellated bacteria.

Plasmids
Semi-autonomous DNA sequences that are dispensable for bacterial growth but often confer useful new survival and colonization strategies on their bacterial hosts.

Partitioning
The distribution (or segregation) of newly replicated daughter plasmids to each of two nascent daughter cells.

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The class Alphaproteobacteria contains a fascinatingly diverse group of bacteria, including the human and animal pathogens *Brucella* spp., *Bartonella* spp. and *Rickettsia* spp., the plant-pathogenic *Agrobacterium* spp., the nitrogen-fixing plant symbionts *Rhizobium* spp., the nitrate-consuming phototroph *Rhodobacter sphaeroides*, the prosthecate bacterium *Caulobacter crescentus*, insect endosymbionts of the genus *Wolbachia* and many others. In many cases, the survival strategies of these bacteria require genes found on extrachromosomal genetic elements called plasmids. For example, *Agrobacterium tumefaciens* requires plasmid-encoded genes to cause crown gall tumours on host plants. Similarly, *Rhizobium* spp. require plasmid-encoded genes to form and colonize the host legume root nodules, where they reduce atmospheric nitrogen. The majority of alphaproteobacterial plasmids, particularly the larger ones, encode at least one *repABC* cassette, the encoded products of which direct plasmid replication and partitioning to daughter cells in a process analogous to mitosis.

Alphaproteobacteria challenge the conventional distinctions between chromosomes and plasmids. Plasmids are generally thought of as being much smaller than chromosomes and not essential for viability. However, some alphaproteobacterial plasmids are comparable in size to the main chromosome, and in most cases dispensability has not been tested. In the current genomics era, replicons that have been sequenced are designated plasmids if they seem to lack genes which are essential for core functions such as prototrophy or protein synthesis[1]. Many alphaproteobacteria have replicons that are referred to as secondary chromosomes or, more recently, as chromids[2,3]. These replicons have one or more genes that are essential for core physiology, as well as a GC content similar to that of the primary chromosome, but possess plasmid-like replication and partitioning systems. Secondary chromosomes also tend to share synteny only within the same genus. Although we support the concept of chromids as elements that are intermediate between chromosomes and plasmids, in this Review we follow the more standard plasmid nomenclature that has been used in the genome annotations.

To paraphrase a popular aphorism, the goal of every plasmid is to become plasmids. However, if plasmids are dispensable for survival of the host in nature, one threat to their long-term survival is the birth of 'cured' (that is, plasmid-free) daughter cells during cell division. The long-term survival of unit-copy (that is, single-copy) and low-copy plasmids requires faithful vertical transmission from the mother cell to daughter cells, and most such plasmids optimize vertical transmission through a variety of mechanisms (FIG. 1). First, replication must occur frequently enough to ensure that sufficient copies exist to populate daughter cells. Second, low-copy plasmids rely on partitioning systems to physically distribute newly replicated plasmids into the two nascent daughter cells (high-copy plasmids, by contrast, tend to be distributed stochastically to daughter cells)[4]. Third, multimer resolution systems convert plasmid multimers into monomers through site-specific DNA recombination, to facilitate distribution[5,6]. Fourth, post-segregational killing of host cells causes plasmid-free daughter cells to lose viability owing to the action of plasmid-encoded toxin molecules, whereas plasmid-containing cells

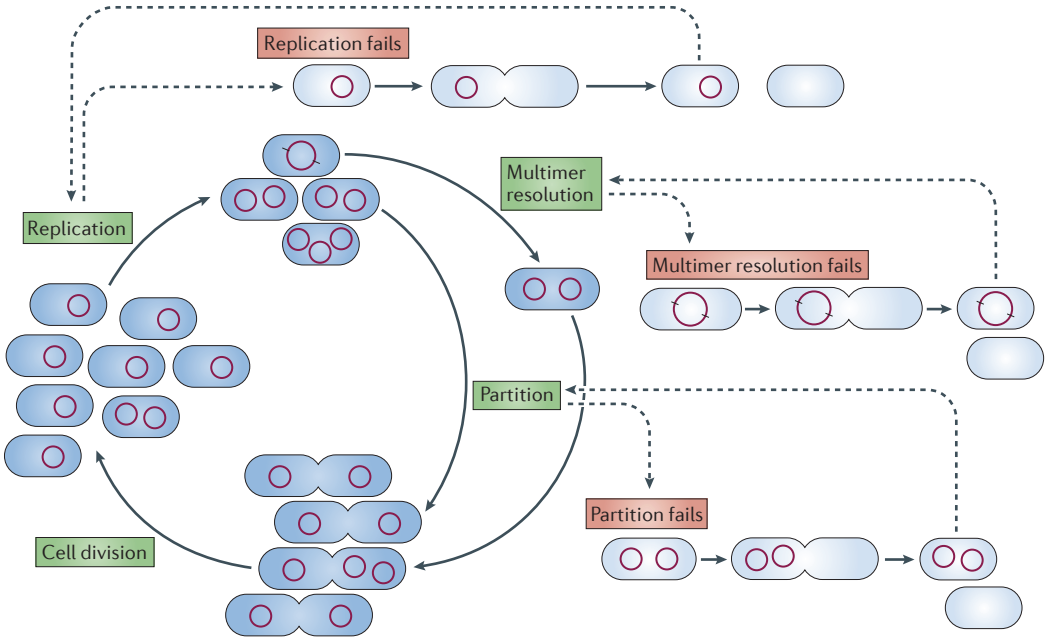


Figure 1 | **Replication and partitioning of low-copy plasmids.** Efficient transmission of low- or single-copy plasmids into daughter cells requires replication once per cell cycle and accurate partitioning of the two plasmids to daughter cells. Plasmids that undergo recombination-mediated multimerization must be converted back to monomers. A failure in any of these functions can lead to plasmid-free daughter cells. Thus, many plasmids contain post-segregational killing systems to eliminate plasmid-free cells. Figure is modified, with permission, from REF. 7 © (2011) American Society for Microbiology.

Replicons
Autonomously replicating DNA molecules.

Chromids
Replicons with both plasmid- and chromosome-like features: chromids have similar GC contents to cognate primary chromosomes and carry genes that are essential for core physiology, but they use plasmid-like partitioning and replication systems. The term chromid is largely synonymous with the term secondary chromosome.

Conjugative transfer
A form of interbacterial plasmid transfer that requires contact between a donor cell and a recipient cell.

Origin of replication
The minimal DNA region that supports autonomous replication. In plasmids, this is called *oriV*.

ParA and ParB
Proteins that mediate the partitioning of plasmids, prophages or chromosomes to nascent daughter cells.

dnaA
A gene encoding a protein that binds to bacterial replication origins and recruits other components of the replication machinery.

are protected by a plasmid-encoded antitoxin[7,8]. Fifth, conjugative transfer can provide a mechanism for some plasmids to recolonize cured cells[9].

As described above, most of the larger alphaproteobacterial plasmids, as well as all secondary chromosomes, replicate their DNA and distribute it to daughter cells via proteins encoded by *repA*, *repB* and *repC*, which seem to be expressed as an operon. RepC is sufficient for replication, whereas RepA and RepB direct the partitioning of daughter plasmids. The origin of replication and partitioning (*par*) sites are also localized within or near the *repABC* operon. RepA and RepB resemble members of a large family of partitioning system proteins found throughout bacteria, whereas RepC does not resemble proteins from any other family. The fact that all three proteins are encoded in one operon is highly unusual among plasmid maintenance systems and might point to there being special challenges in ensuring appropriate levels of expression. Over the past decade, a great deal has been learned about these replication cassettes, although much work remains to be done. Here, we review recent insights into the expression of *rep* genes and the functions of the encoded proteins.

Distribution of RepABC-type systems

In 1989, a genetic locus bearing three genes, *repA*, *repB* and *repC*, was reported to be required for replication and partitioning of the octopine-type tumour-inducing (Ti) plasmids of *A. tumefaciens*[10]. RepC was found to be essential for plasmid replication, and RepA and RepB were shown to be dispensable for replication but required for efficient vertical plasmid transmission. Since that report, homologous genes have been identified in hundreds of alphaproteobacterial isolates, almost always found to be linked in an apparent operon and in the same gene order[11–14] (see Supplementary information S1 (table)). More than 600 RepC-type proteins were identified in a BLAST search of the Genbank protein database (as of November 2011), all of which are found in alphaproteobacteria; RepA and RepB homologues are found in a much wider range of bacterial plasmids, episomal prophages and chromosomes (see below).

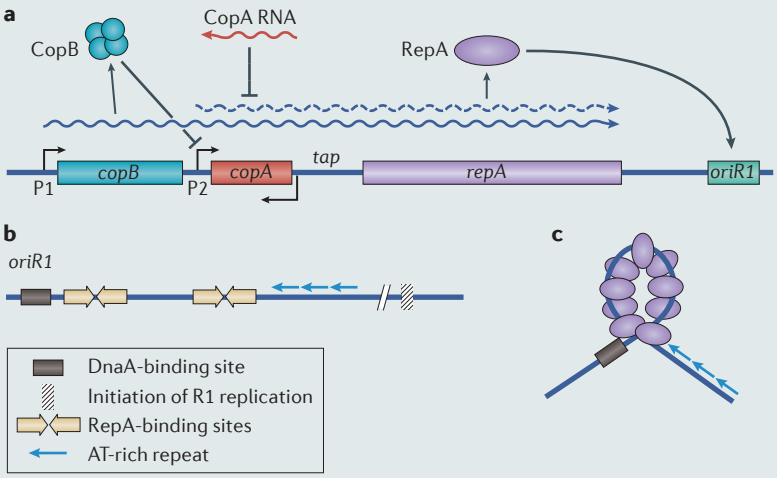
More than one-third of the alphaproteobacterial genome sequences deposited in Genbank fall within the order Rhizobiales, and the distribution of *repABC* operons in this group has been recently described[11,12,15]. Of the 56 finished Rhizobiales genomes found in Genbank in late 2011, 43 are annotated as having just one chromosome, 12 have two chromosomes and one has three chromosomes. The 56 genomes also collectively contain 93 plasmids ranging in size from 4.1 Kb to 2.4 Mb (see Supplementary information S1 (table)).

Almost all of these 163 Rhizobiales replicons have partitioning loci that resemble those encoding ParA and ParB (see Supplementary information S1 (table)). These genes fall into two well-separated clades: those found only on primary chromosomes, and those found only on secondary chromosomes and plasmids[11]. Genes in the primary-chromosome clade are designated *parA* and *parB*, whereas genes in the plasmid clade are designated *repA* and *repB*. Primary chromosomes invariably contain one *dnaA* gene, which encodes a replication initiator (see below). In all cases, *dnaA* is unlinked to the *parAB* locus. None of the primary chromosomes contains *repC*.

Box 1 | **Replication of enteric plasmid R1**

Plasmid R1 provides a well-studied model for replication systems of enteric plasmids[81–89]. In this plasmid, the replication initiator RepA binds to the origin site, *oriR1*, which lies downstream of *repA* (see the figure, part **a**). This *oriR1* site contains binding sites for RepA flanked by a DnaA box at one end and three AT-rich repeats at the other (see the figure, part **b**). DnaA is not essential for replication of this plasmid, but seems to have an accessory role. DNA loop formation, mediated by RepA (see the figure, part **c**), is thought to drive DNA melting at the AT-rich region, which allows DnaC to load the replicative DNA helicase, DnaB. Replication initiates 400 nucleotides downstream of this site.

The *repA* gene is expressed from two promoters and is subjected to two levels of negative regulation (see the figure, part **a**). First, *copB* is co-transcribed with *repA* from promoter P1, and the protein encoded by *copB* represses initiation of *repA* transcription from promoter P2, which remains silent under steady-state conditions (indicated by the dashed line for the transcript). Second, a counter-transcribed RNA, designated CopA, acts at a post-transcriptional level to block RepA synthesis by preventing *tap* translation and, therefore, *repA* translation. *tap* encodes a small leader peptide that is translationally coupled to RepA, and thus translation of *tap* is required for RepA synthesis. With the exception of iteron-type plasmids, most other plasmids use a counter-transcribed RNA to limit replication. Figure is reproduced from REF. 89 © (2008) Henry Stewart Talks Ltd.



Conversely, none of the 14 secondary chromosomes or 93 plasmids carry *dnaA*. This pattern is also found in other orders within the class Alphaproteobacteria, although exceptions have been documented[16,17]. All 14 secondary chromosomes and more than two-thirds of the plasmids include at least one *repC* (exceptions are found among the very small plasmids)[11]. Almost all *repA*, *repB* and *repC* genes are arranged in an apparent operon. Of the 93 plasmids, 68 have at least one complete *repABC* locus; five plasmids have two complete operons, and six plasmids have one complete locus and additional incomplete cassettes.

The *repABC* operons of secondary chromosomes do not form a separate clade from the plasmid-encoded operons. Instead, plasmid-encoded and chromosomal *repABC* operons are interspersed on evolutionary dendrograms and virtually impossible to distinguish from each other by sequence[11]. Apparently, there are no major barriers to adapting a plasmid cassette for use in a secondary chromosome or vice versa. In other words, the replication and partitioning systems of the 14 secondary chromosomes are extremely plasmid like. By contrast, the complete absence of horizontal transfer of these genes between primary chromosomes and other replicons is striking. It would seem that *repABC* operons are poorly suited for use in primary chromosomes, and *parAB* and *dnaA* are equally poorly adapted to secondary chromosomes and plasmids.

Unlike other well-studied replication and partitioning systems, found in enterobacteria (BOXES 1,2), *repABC* cassettes are just beginning to be the focus of detailed studies. The best characterized *repABC* cassettes are found on the symbiosis plasmids p42d of *Rhizobium etli* and pSymA from *Sinorhizobium meliloti*, on two different Ti plasmids from *A. tumefaciens* and on plasmid pTAV1 from *Paracoccus versutus*[12,18–24]. All of these model organisms, except for *P. versutus*, are members of the order Rhizobiales. Nonetheless, the genomes of these bacteria are dissimilar. *R. etli* str. CFN42 has one circular chromosome and six circular plasmids ranging in size from 194 Kb to 642.5 Kb; all six plasmids have either one or two *repABC* cassettes (see Supplementary information S1 (table))[25], but p42d has received the most attention, as it carries the *nod* and *nif* genes that are required for root nodulation and nitrogen fixation. *S. meliloti* str. 1021 contains one circular chromosome and two symbiotic plasmids, pSymA (1.35 Mb) and pSymB (1.7 Mb)[26], both of which are essential for root nodulation and replicate using *repABC* cassettes. *A. tumefaciens* str. C58 has a larger circular chromosome, a linear chromosome (2.1 Mb), the Ti plasmid pTiC58 (0.2 Mb) and a second plasmid (0.5 Mb)[27,28]; both plasmids and the linear chromosome replicate using *repABC* cassettes. The genome of *P. versutus* has not been sequenced in its entirety, but it appears to have one circular chromosome and two plasmids, pTAV1 (107 Kb) and pTAV3 (~400 Kb)[29]. Of these, pTAV1 has two replication loci, a complete *repABC* cassette and a second copy of *repC*[24,30], whereas pTAV3 encodes a different type of replication system[31].

Genetic structure of *repABC*-type cassettes

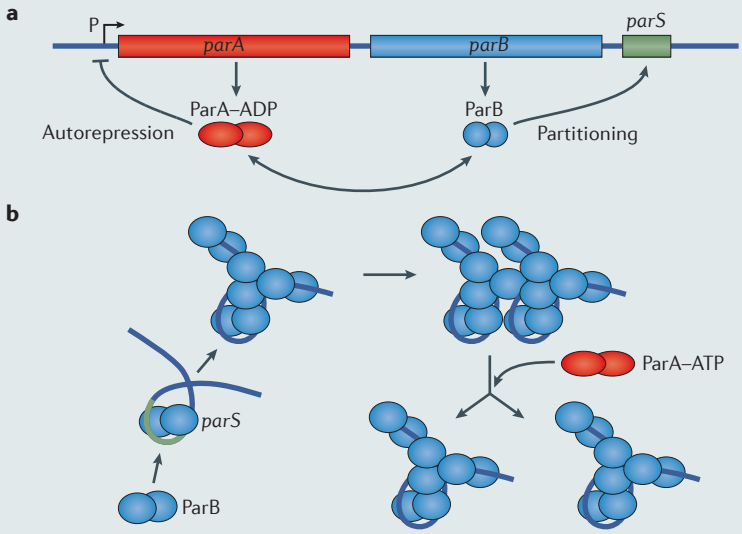
The available data indicate that the *repA*, *repB* and *repC* genes of each plasmid in FIG. 2a are expressed from promoters that lie upstream of *repA*[32,33]. In the *A. tumefaciens* str. R10 plasmid pTiR10 (which is almost identical to other octopine-type Ti plasmids, such as pTiA6, pTiB6, pTi15955 and pTiAch5, found in other strains of *A. tumefaciens*), mutations that block activity of the promoters in this region also block *repC* expression[32,34]. Likewise, in p42d, disruptions of the *repA* promoter block *repC* expression, as do insertions of foreign DNA into *repA* or *repB*, owing to transcriptional polarity[35].

The plasmid pTiR10 encodes a fourth gene, *repD*, which is 228 nucleotides in length and fully spans the gap between *repA* and *repB*[36]. Plasmid pTiC58 has a reading frame that is identical in position and length to *repD* but completely divergent in sequence. This divergence suggests that the expression of the two *repD* genes has no function other than to ensure the expression of downstream genes. Within *repD* of pTiR10 are two RepB-binding *par* sites[36] that are highly conserved in pTiC58 and other members of the family, and are required for plasmid partitioning (see below). By contrast, no gap or small gene is found between *repA* and

Box 2 | **The partitioning system of prophage P1**

Many bacterial plasmids and episomal prophages, and all chromosomes are accurately distributed to predivisional daughter cells in a process referred to as replicon segregation or partitioning. Many of these replicons partition using members of the ParA–ParB family, the best studied members of which are the ParA and ParB proteins of prophage P1 (see the figure), the SopA and SopB proteins of the F plasmid and the Soj and Spo0J proteins encoded by the *Bacillus subtilis* chromosome[90–97]. Partitioning systems also require a site, often referred to as *parS* or *parC*, that is functionally analogous to a eukaryotic centromere and is usually found closely linked to the *parAB* genes[97,98]. Most members of the ParA–ParB family are encoded by bicistronic operons in which *parA* is located near the promoter.

ParB of bacteriophage P1 is dimeric and binds specifically to the DNA sequence *parS*, which is located directly downstream of the operon (see the figure, part **a**). A bound ParB dimer serves to nucleate the binding of additional dimers, extending outwards from *parS*[97,99,100] (see the figure, part **b**). The carboxy-terminal half of ParB suffices for *parS* binding[61], whereas the amino-terminal region is required for oligomerization and for binding to ParA[101]. The crystal structure of ParB–*parS* shows that each ParB subunit has two different DNA-binding domains, and a ParB dimer is likely to contact neighbouring plasmids[102,103]. ParA of P1 is also dimeric and contains Walker A and Walker B ATPase motifs. ParA is a weak ATPase, the activity of which is enhanced by binding to ParB. ParA bound to ADP can autorepress the *parAB* promoter (see the figure, part **a**), and purified ParA protects an extensive region surrounding the promoter. ParB increases repression of this promoter through stimulation of the ParA ATPase activity, leading to accumulation of the repressor form, ParA–ADP. The N-terminal domain of ParA is responsible for binding to promoter DNA through a helix–turn–helix motif in a process that requires ADP[96,104]. ParA–ATP dimerizes and associates cooperatively to bind DNA nonspecifically in a dynamic fashion, oscillating over the nucleoid[105], and this is believed to play an important part in partitioning[105,106]. In fact, ParA–ATP bound to the nucleoid attracts the partitioning complex composed of ParB–*parS*, which in turn stimulates ATP hydrolysis and, eventually, the disassembly of the complex. It is proposed that this interaction drives partitioning of the daughter plasmids to the respective daughter cells[107] (see the figure, part **b**). Figure is reproduced from REF. 90 © (2008) Henry Stewart Talks Ltd.



Counter-transcribed RNA
A term used in plasmid biology to describe a type of antisense RNA that is synthesized from the DNA strand which is complementary to its target RNA. Like other antisense RNAs, counter-transcribed RNAs form a duplex with their targets, usually leading to degradation of both strands.

repB of p42d, pSymA or pTAV1. The *par* sites in these plasmids lie directly upstream or downstream of the corresponding *repABC* operons (FIG. 2a).

All *repABC* cassettes contain a gene between *repB* and *repC* that encodes a short, non-translated counter-transcribed RNA that downregulates expression of *repC*[18,20,22], thereby controlling plasmid copy number and incompatibility (see below).

There are two other notable features of these operons (FIG. 2b). First, each has a conspicuous AT-rich region within *repC* that, as described below, seems to contain the origin of plasmid replication. Second, the DNA sequence GANTC is over-represented in the putative replication origin and in the promoter of the counter-transcribed RNA. These sequences are substrates for a DNA methylase that modifies the A residues, and the motifs might play a part in the timing of various events in the cell cycle[37].

RepC and the origin of replication

RepC-type proteins have been found only in alphaproteobacteria[11,13,23,38,39], and they bear no apparent homology to any other replication initiator proteins. Several *repC* genes have been shown to be essential and sufficient for plasmid replication in the hosts from which they originate[18,23,40–43]. The ability of a *repC* coding sequence to confer the ability to replicate on a plasmid indicates that the origin of replication must lie within this gene[12,24,42,43]. Such a location for the origin, although unusual, has precedent in several other types of plasmid and at least one bacteriophage[12,44,45]. The origins of many types of plasmids contain directly repeated DNA sequences called iterons, which serve as binding sites for replication initiators[46–51]. Origins that require RepC for activity are almost unique in their lack of iterons[51]. As mentioned above, all *repC* genes contain an AT-rich sequence of ~150 nucleotides near the middle of the protein-coding sequence; such an AT-rich sequence is another common feature of diverse replication origins. Purified RepC from pTiR10 binds to the AT-rich segment via a highly conserved DNA sequence that includes imperfect dyad symmetry[43]. A second dyad located immediately downstream of the RepC-binding site is even more strongly conserved but does not bind RepC *in vitro*[43], suggesting that it binds some other replication factor.

Secondary-structure predictions and amino acid conservation suggest that RepC has two domains, an amino-terminal domain (NTD) from residues 1 to 265 and a carboxy-terminal domain (CTD) from residues 295 to 439, joined by a 30 amino acid linker that is hydrophilic, unstructured and poorly conserved. A fragment containing just residues 26–158 is sufficient for DNA binding, albeit with strongly reduced sequence specificity[43]. This region of RepC has a structural resemblance to members of the DnaD family of low-GC-content Gram-positive bacteria. DnaD interacts with either DnaA or primosomal protein N′ (PriA) at replication origins or replication restarts, respectively. In low-GC-content Gram-positive bacteria, DnaD, DnaB and DnaI together load the replicative DNA helicase onto the DNA at the origin of replication and at replication restarts[52]. The same region (residues 26–158) of RepC also resembles members of the MarR family of transcription factors, which bind DNA as dimers by means of a winged helix–turn–helix motif[53]. The role of the RepC CTD remains to be revealed; the last 39 amino acids of p42d RepC were found to play a part in plasmid incompatibility[42].

Despite extensive efforts, we have never been able to detect pTiR10 RepC acting in *trans*[43]. The same is true of p42d RepC[42]. Furthermore, overexpression of pTiR10 RepC causes a large increase in the copy number of the

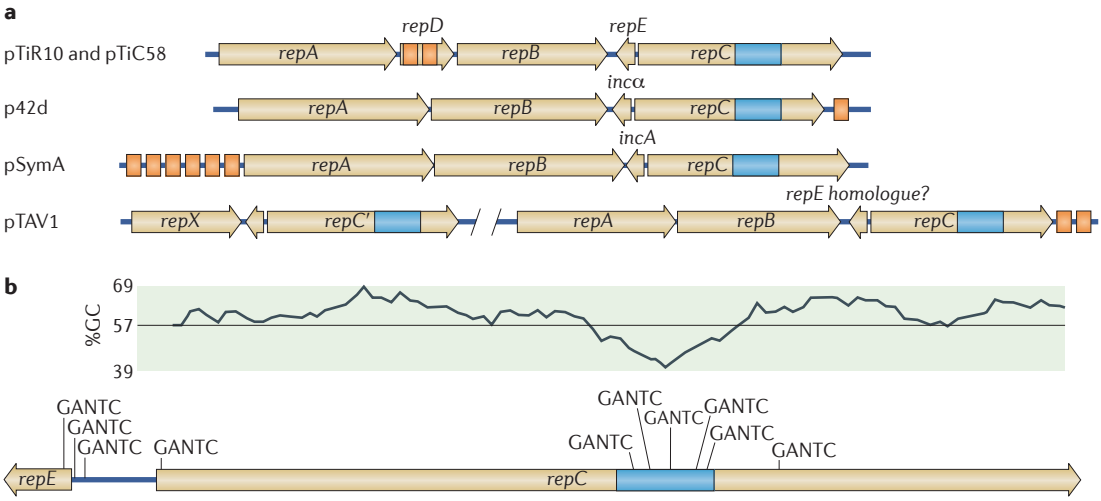


Figure 2 | **Genetic organization of *repABC* systems from representative RepABC plasmids. a** | The replication and partitioning (*repABC*) modules of the *Agrobacterium tumefaciens* tumour-inducing (Ti) plasmids pTiR10 and pTiC58, of the *Rhizobium etli* str. CFN42 plasmid p42d, of the *Sinorhizobium meliloti* plasmid pSymA and of the *Paracoccus versutus* plasmid pTAV1 (see main text for details). The partitioning sites are shown in orange, and the AT-rich regions that are believed to contain the plasmid origin of replication (*oriV*) are shown in blue. Arrows immediately upstream of *repC* represent counter-transcribed RNAs; in the case of pTiR10, this RNA is called *repE*. The *repD* gene of pTiC58 is provisional and based solely on DNA sequence analysis. **b** | The *repE–repC* region of pTiR10, showing the abundance of GANTC sites located near the *repE* promoter and in the AT-rich region. The GANTC sites at these locations are a common feature of *repABC* operons.

plasmid expressing the protein, but has no effect on the copy number of a second RepC-dependent plasmid in the same cell, confirming that RepC functions only in *cis*. Although most other replication initiators function well in *trans*, those of the closely related plasmids R1, NR1 and R100, as well as that of pMU720, function only in *cis*[54–56].

Replication initiator proteins of iteron-type plasmids generally bind to the origin of replication and recruit DnaA, causing melting of the AT-rich region at the origin and thus creating the replication bubble. DNA–DnaA complexes recruit the replicative DNA helicase bound to the loading factor (DnaB and DnaC, respectively, in *Escherichia coli*), and the helicase in turn recruits DNA polymerase. Replication usually proceeds bidirectionally[57]. There are no apparent DnaA-binding motifs matching the consensus sequence for alphaproteobacteria anywhere within *repC*[37]. It nonetheless seems plausible that RepC recruits DnaA, which would then recruit DnaB–DnaC[58]. It is also possible that RepC is able to recruit DnaB–DnaC directly. The resemblance between RepC and DnaD of Gram-positive bacteria suggests a role for RepC in loading the replicative helicase.

It is striking that some bacteria can have as many as six RepABC family replicons (see Supplementary information S1 (table)). One bacterium, *R. etli*, has eight complete cassettes distributed over six replicons. One might wonder how each RepC can function only at its cognate origin rather than at heterologous origins in the same cell. Although most RepC proteins found within a single bacterium tend to be fairly divergent[11], there are several surprising examples to the contrary. One such case is the RepC proteins of the *R. leguminosarum* plasmids pRL9 and pRL12; these proteins are 97% identical. Similar examples are found in plasmids from *Nitrobacter hamburgensis*, *Mezorhizobium loti* str. MAFF303099, *Agrobacterium vitis* str. S4 and *R. leguminosarum* bv. *trifolii* str. WSM1325 (see Supplementary information S1 (table))[42]. Can two almost identical RepC proteins avoid acting at heterologous origins, and if so, how? We hypothesize that all RepC proteins are strictly *cis* acting, as was shown for those of pTiR10 and p42d (see above). If this were true, these proteins would not need to diverge, as they could never act at heterologous origins, no matter how similar the proteins were. We have constructed strains containing two different plasmids with identical *repC* genes and did not notice any incompatibility[43]. However, the opposite was reported for p42d[42], so additional studies are clearly needed.

Plasmid partitioning by RepA and RepB

The RepA and RepB proteins encoded by *repABC* cassettes generally resemble the larger family of ParA and ParB proteins (BOX 2) and are likely to have similar general properties. Insertions, frame-shift mutations or deletions in *repA* or *repB* substantially decrease plasmid stability[10,24,35,59,60]. Sequence homology between RepA and the ParA of prophage P1 is largely restricted to the ATPase domain, which also resembles other ATPase domains in a large range of enzymes. At least two alphaproteobacterial RepA proteins mediate autorepression[33,34]. One of these RepA proteins is that of pTiR10; this RepA autorepresses the P4 promoter (FIG. 3), which contains a 40-nucleotide region of dyad symmetry that lies within

Copy number
The number of copies of a plasmid per bacterial cell; this number is generally held constant by the replication machinery.

Autorepression
The ability of a protein to repress the promoter of the gene encoding that protein.

a 70-nucleotide region protected by purified RepA[34]. Amino acid residues 50–112 of RepA are strongly predicted to have a helix–loop–helix DNA-binding motif similar to that found in residues 43–105 of the crystallized P1 ParA protein[61]. The binding of pTiR10 RepA to its operator is stimulated by ATP (and to a lesser extent by ADP) and by the addition of RepB[34].

The *par* sites of pTAV320, p42d, pSymA, pTiC58 and pTiR10 consist of one or more copies of a 16-nucleotide palindromic sequence with the consensus GTTNNCNGCNGNNAAC. The number and position of *par* sites present in this family of replicons varies widely (FIG. 2a). These sites are essential for plasmid stability, bind RepB and are incompatible with their respective parental plasmid when provided in *trans*[24,36,59,62]. This is presumably due to competition between the two plasmids for the partitioning machinery. Point mutations in the cloned *par* site that reduce RepB binding also eliminate plasmid incompatibility[59]. Purified pTiR10 RepB binds with low affinity to DNA containing the two *par* sites within *repD*, but the affinity of this binding is increased by the addition of RepA[34] (FIG. 3a).

Fluorescence *in situ* hybridization (FISH) has shown that the origins of all *repABC* replicons in *A. tumefaciens* and *S. meliloti* are located at or near the cell pole[63].

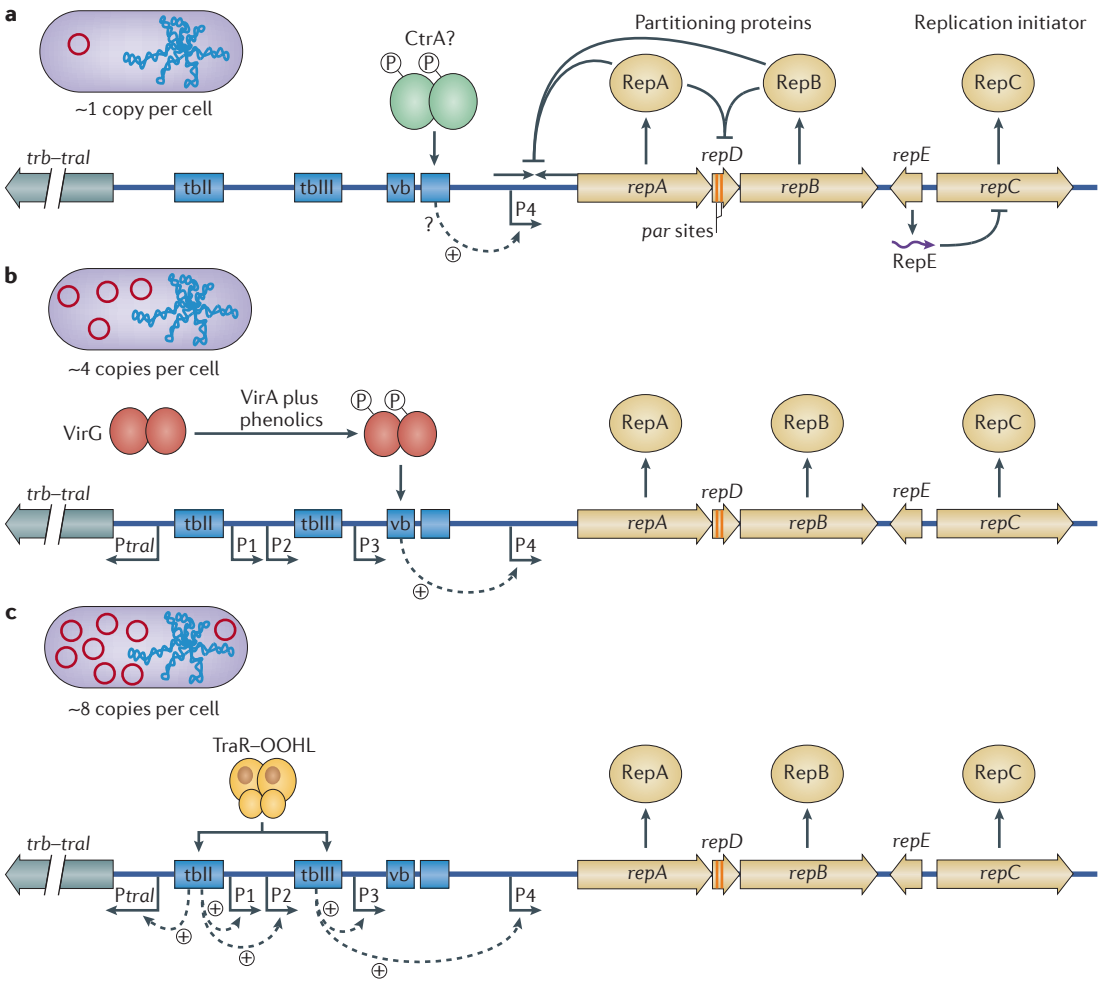


Figure 3 | **Regulation of the *repABC* operon of octopine-type tumour-inducing (Ti) plasmids. a** | Autoregulation. Transcription of the *repABC* operon is inhibited by autorepression mediated by RepA–RepB complexes at the operator region downstream of P4 (a prominent region of dyad symmetry is indicated by inverted arrows) and at the partitioning (*par*) sites located between *repA* and *repB*. Expression of *repC* is inhibited transcriptionally and post-transcriptionally by the counter-transcribed RNA RepE. In the absence of external signals, tumour-inducing (Ti) plasmids are maintained as single copies. Additional regulation may be provided by phosphorylated CtrA. **b,c** | The copy number of Ti plasmids is influenced by at least two diffusible chemical signals. Plant-released phenolic compounds are detected by the VirA–VirG two-component system, triggering VirA-mediated phosphorylation of VirG; phosphorylated VirG binds the *vir* box (vb) to activate transcription from promoter P4 (part **b**). Thus, in the presence of phenolics, which are encountered when bacteria enter the plant stem or root through a wound, the copy number of Ti plasmids increases to about four copies per cell. In addition, TraR–3-oxo-octanoylhomoserine lactone (OOHL) complexes, which are encountered in the environment of crown gall tumours, bind to *tra* box II (tbII) and tbIII, activating the *tral–trb* operon through promoter P*tral* and the *repABC* operon through promoters P1, P2, P3 and P4 (part **c**). In the presence of TraR–OOHL complexes, the copy number of Ti plasmids therefore increases to about eight copies per cell.

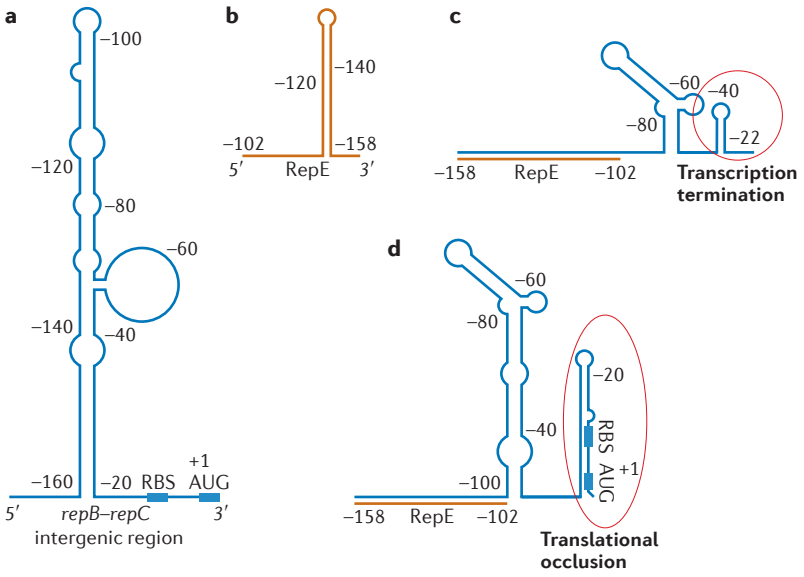


Figure 4 | **Model of the mechanism of action of the counter-transcribed RNA RepE on the control of *repC* expression.** All numbers refer to the position in the mRNA relative to the *repC* translational start site (AUG). **a** | Predicted structure of the mRNA upstream of *repC* in the absence of RepE. Note that the ribosome-binding site (RBS) and the translational start site of *repC* are available for translation initiation. **b** | Predicted structure of RepE. **c** | Binding of RepE to the target mRNA upstream of *repC* could cause the formation of several hairpins, one of which resembles a Rho-independent transcription terminator. **d** | RNA molecules that are not terminated are predicted to have an alternative stem–loop that sequesters the RBS and the translational start site of *repC*.

CtrA
A transcription factor of *Caulobacter crescentus* that is synthesized and phosphorylated during a particular portion of the cell cycle to regulate the expression of various promoters.

VirG
A transcription factor of *Agrobacterium* spp. that is phosphorylated by VirA in response to plant-released phenolic compounds and activates transcription of plasmid tumour-inducing *vir* genes, which direct the transfer of tumorigenic DNA fragments into host cell nuclei.

Quorum sensing
A form of transcriptional regulation in bacteria. Quorum sensing systems consist of a bacterial pheromone (which accumulates at high population density), a pheromone synthase and a pheromone receptor, and they most often function to activate target genes in the presence of the pheromone.

In double-labelling experiments in *A. tumefaciens*, it was shown that the origins of two different replicons rarely colocalize; rather, they occupy close but clearly distinct sites.

Above, we asked how up to eight different RepC proteins can coexist in a single bacterium. The same question arises for RepA and RepB. In a survey of bacteria from the order Rhizobiales, multiple RepA and RepB proteins within the same bacterium were in all cases rather divergent[11,12,15]. In this order, no two RepA proteins in the same bacterium are more than 61% identical, no two RepB proteins are more than 51% identical, and most pairs show lower similarity. This divergence could help to minimize heterologous interactions. It is also noteworthy that the evolutionary dendrograms of RepA and RepB within the order Rhizobiales are congruent, indicating that these proteins have co-evolved without horizontal exchange. A completely different picture emerges when comparing the phylogenies of RepA and RepB with that of RepC: this comparison provides clear evidence of extensive exchange of *repC* genes between heterologous cassettes[11].

Regulation of *repC* by a counter-transcribed RNA

Each *repABC* operon shown in FIG. 2a is thought to have a gene lying between *repB* and *repC*, but in the opposite orientation; this gene encodes a non-translated RNA of approximately 50 nucleotides in length. The RNA includes a predicted stem–loop that might serve as a Rho-independent transcription termination site and might also play a part in contacting complementary mRNA sequences[18,64] (FIG. 4). Similar genes are predicted to be conserved in most or perhaps all members of the *repABC* family. Plasmids that have a cloned copy of *repE* (and that replicate using a different replication system) are incompatible with plasmids that replicate using the cognate *repABC* cassette[18]. The same is true of at least two other homologous systems[20,64]. Point mutations that alter the structure of the RepE RNA or decrease its expression decrease this incompatibility[10,18,20,22,35,64,65].

The counter-transcribed RNA of pTiR10, known as RepE, inhibits both the transcription and translation of *repC*[18]. Downregulation of RepE leads to increased plasmid copy number, and a lack of RepE can lead to lethal runaway replication[18]. RepE is predicted to alter the balance between two alternative stem–loop structures in the mRNA upstream of the *repC* coding sequence. According to this model, in the absence of RepE, the ribosome-binding site of *repC* is available for translation initiation. In the presence of RepE, alternative mRNA folding creates a Rho-independent transcription termination site upstream of the ribosome-binding site. Any mRNA molecules that are not terminated are predicted to make an alternative stem–loop that sequesters the ribosome-binding site (FIG. 4). This regulation, combined with transcriptional repression by RepA and RepB, reduces *repC* expression to almost undetectable levels[34]. The counter-transcribed RNA of plasmid p42d is hypothesized to function in the same way[64].

Transcriptional regulation of *repABC*

Information about transcriptional regulation is somewhat sparse for most *repABC* systems but is most well developed for pTiR10. The *repABC* operon of this plasmid is transcribed from no fewer than four promoters, one of which (P4) provides basal expression of the operon (FIG. 3) and is autorepressed by RepA and RepB (see above). The P4 promoter region also contains a possible binding site for another two-component response regulator, CtrA, which regulates the cell cycle of *Caulobacter crescentus*[37]. A perfect copy of this motif (TTAAN$_7$TTAA) is centred 50-nucleotides upstream of the P4 start site. The role of CtrA in Ti plasmid replication needs to be explored.

Promoter P4 of pTiR10 is also activated by the two-component response regulator VirG, which is phosphorylated by VirA in response to plant pheromones[66] (FIG. 3b). Phosphorylated VirG binds to a *vir* box centred 71-nucleotides upstream of P4, leading to a 3–4-fold increase in Ti plasmid copy number (FIG. 3b). In addition, all four *repABC* promoters are activated by the quorum sensing transcription factor TraR, a Rhizobiaceae family LuxR-type transcription factor that allows cell density-dependent gene expression and which requires the pheromone 3-oxo-octanoylhomoserine lactone (OOHL) for activity[32,34,67]. TraR-mediated activation of these promoters leads to an approximately eightfold increase in Ti plasmid copy number (FIG. 3c) and an increase in tumorigenesis in plants infected with bacteria containing these plasmids[32,65]. Transcriptional activation of *repABC* by TraR–OOHL has been also described for the symbiotic plasmid pRL1JI of *R. leguminosarum*[68]. Two

other rhizobial plasmids, pRL8JI of *R. leguminosarum* and pNGR234a of *Rhizobium* sp. NGR234, have TraR-binding motifs in the same regions, although their roles remain to be tested[69]. Therefore, expression of the octopine-type Ti plasmid *repABC* operon and, ultimately, the collective Ti gene dosage are enhanced both by plant-released chemical signals (leading to phosphorylation of VirG) and quorum sensing pheromones (activating TraR).

DNA methylation sites in the *repABC* region

DNA methylases have several roles in bacterial physiology[70], perhaps the best known of which is to protect DNA from cognate restriction endonucleases. Another role is thought to be enabling the cell to distinguish parental and newly synthesized daughter DNA. Distinguishing between the two is important for mismatch repair, for initiation of chromosome replication and for the activity of some promoters[71]. Methylation can also decrease the melting temperature of duplex DNA[72–74]. The *E. coli* origin of replication is rich in GATC sites, which are methylated by Dam methylase at the N6 position of A residues. After replication, hemimethylated GATC sites are tightly bound by SeqA, slowing the rate at which these sites are methylated by Dam and sequestering these sites from the replication initiation factor, DnaA. Eventually, Dam succeeds in methylating the sites, and this blocks SeqA binding. Hemimethylated GATC sites therefore block replication early in the cell cycle, and this block is eventually relieved when the sites become fully methylated later in the cell cycle[75].

In the alphaproteobacterium *C. crescentus*, there is an analogous (although not homologous) methylase to Dam called cell cycle-regulated methylase (CcrM), which methylates the N6 position of A residues in the sequence GANTC[76]. The origin of replication in *C. crescentus* has five GANTC sites, and their full methylation is required for replication initiation[77]. GANTC sites are also found in the promoters of several *C. crescentus* genes that have important roles in cell cycle timing[78], and methylation of these promoters influences their expression[78,79]. Alphaproteobacteria do not have a protein homologous to SeqA, but another protein might play an analogous role. There is at least one important difference between Dam and CcrM: Dam is thought to be active throughout the cell cycle, whereas CcrM is synthesized by and is active in predivisional cells only. Therefore, GATC sites of *E. coli* are hemimethylated only transiently after replication, whereas GANTC sites of *C. crescentus* remain hemimethylated for much of the cell cycle[70,78].

The putative origins of replication of *repABC* plasmids are rich in GANTC sites, as are the promoters of the counter-transcribed RNA genes (FIG. 2b). If methylation of these sequences occurs only at a particular point in the cell cycle, this could have important consequences for origin utilization and/or expression of RepC. Methylation does not affect the binding affinity of RepC for DNA *in vitro*[43], but it might influence the binding of some other replication factor or might enhance origin melting[72–74]. Moreover, methylation of the *repE* promoter might influence the production of the counter-transcribed RNA RepE, which downregulates RepC synthesis.

Dam methylase
A DNA methylase that is found in enterobacteria and methylates the A residues of GATC motifs. Cells can recognize newly synthesized DNA by its lack of methylation.

Prospects for future work on RepABC systems

A great deal of work remains to be done on this large family of replication and partitioning systems. Under most conditions, plasmids containing these systems are held at unit copy, and progress has been made in understanding the negative-control checkpoints that confer this property. Furthermore, in the case of *A. tumefaciens*, replication of the four replicons is synchronized, despite the use of different replication initiators for each replicon[80]. The underlying mechanisms for this synchronicity could involve CtrA abundance and phosphorylation (both of which are cell cycle regulated in another alphaproteobacterium[37]), methylation of GANTC sites in or near the replication origins, or both. Methylation could alter the melting temperature of the origin or the binding of replication factors. Future work on synchronized cultures could provide information about the cell cycle dependence of CtrA accumulation and DNA methylation.

As described above, changes in *repABC* transcription can alter plasmid copy number and thereby affect the expression of all plasmid-encoded genes. This can have major consequences for host–bacterium interactions. In the case of *A. tumefaciens*, increasing Ti plasmid copy number results in increased tumorigenesis[15,32]. The discovery that environmental stimuli can affect plasmid copy number might be true of other plasmids that replicate using *repABC* cassettes as well, although this remains to be shown. If it is a common effect, the signals and regulatory systems might differ greatly in different organisms. Artificial overexpression of *repC* causes substantial increases in plasmid copy number[43], and this effect could have profound applications in synthetic biology, including the development of new approaches for constructing transgenic plants and fungi.

Among the Par protein family members, RepA and RepB offer an unprecedented opportunity to understand how up to eight different partitioning protein pairs can function in harmony in a single cell. It will be interesting to examine the possible epistatic relationships between these multiple RepA–RepB pairs, and to determine how segregation is achieved, particularly in cases for which more than one RepA–RepB pair is found in a single replicon. It will also be interesting to learn more about the roles of the many ParA and RepA proteins that lack cognate ParB or RepB partners.

At least some RepA and RepB proteins form heteromultimers that can bind at *par* sites and can also bind *repABC* promoters[34,36]. Can a complex containing RepA and RepB contact a promoter and a *par* site simultaneously, forming a DNA loop? How would loop formation affect the roles of these proteins in partitioning and in autoregulation? It is curious that the positions of the *par* sites can vary so widely (FIG. 2a), and it would be interesting to learn whether moving these sites would affect their function. How much or how little DNA can separate

par sites from *repABC* promoters? One major difference between the RepA–RepB system and the homologous ParA–ParB system is that the autoregulation of RepA and RepB also affects expression of RepC, which in turn regulates replication frequency and copy number. The RepABC system has evolved such that changes in transcription initiation have an impact on *repC* expression, but large fluctuations in RepC expression must be avoided.

RepC appears to be unique to alphaproteobacteria. The NTD of pTiR10 RepC can bind DNA, but with limited sequence specificity. The CTD of this protein is predicted to have very weak structural similarity to bacterial transcription factors, suggesting that this domain has some affinity for DNA independently of the NTD. In preliminary experiments, the CTD does not stably bind DNA in electrophoretic mobility shift assays[43], but this needs to be confirmed using other RepC fragments. The prediction that RepC contains two domains separated by a 30 amino acid linker requires experimental verification, and the two RepC domains are also attractive targets for structural studies.

We know little about the specific consequences of RepC binding to the origin of replication, although in all likelihood this binding recruits the replisome. Most replication initiators recruit DnaA to the origin, which in turn attracts DnaB–DnaC. A few plasmid replication initiators replace either DnaA alone or all three proteins. It will be interesting to determine which host replication proteins are recruited by RepC. RepC does not function at all in *E. coli*; perhaps a library of *A. tumefaciens* DNA could be screened for genes that allow RepC to function in a heterologous bacterial host, and this could help elucidate how RepC functions in its native host. As is the case for all experimental science, every new insight about these genes reveals a dozen or more new questions to be answered, and we now have enough thorny questions to last a lifetime.

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Competing interests statement
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