Antisense and yet sensitive: Copy number control of rolling circle-replicating plasmids by small RNAs[§]

Advanced Review

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[§]Dedicated to Prof. Ramón Díaz-Orejas on occasion of his retirement

ABSTRACT

Bacterial plasmids constitute a wealth of shared DNA amounting to about 20% of the total prokaryotic pangenome. Plasmids replicate autonomously and control their replication by maintaining a fairly constant number of copies within a given host. Plasmids should acquire a good fitness to their hosts so that they do not constitute a genetic load. Here we review some basic concepts in Plasmid Biology, pertaining to the control of replication and distribution of plasmid copies among daughter cells. A particular class of plasmids is constituted by those that replicate by the rolling circle mode (RCR-plasmids). They are small double-stranded DNA molecules, with a rather high number of copies in the original host. RCR-plasmids control their replication by means of a small short-lived antisense RNA, alone or in combination with a plasmid-encoded transcriptional repressor protein. Two plasmid prototypes have been studied in depth, namely the staphylococcal plasmid pT181 and the streptococcal plasmid pMV158, each corresponding to the two types of replication control circuits, respectively. We further discuss possible applications of the plasmid-encoded antisense RNAs and address some future directions that, in our opinion, should be pursued in the study of these small molecules.

(191 words)

Key words: Bacterial plasmids / Copy number / Control of replication / Countertranscribed RNA / Hairpins / Kissing complex / Rolling-circle replication / Transcriptional repression

INTRODUCTION

Flourishing of the RNA world could not have been possible without the pioneer work radiated from the Tomizawa laboratory more than 30 years ago, when they published the first reports on antisense RNAs (asRNA) and their role in the control of replication of plasmid ColE1^{1,2}. These findings opened the ways to the discovery and use of small non-coding asRNAs, to the strategy of RNA interference in gene therapy³, and to the development of transcription-activating small RNAs⁴. Nowadays, to achieve a proper perspective of the field, it is important to review basic concepts on replication of bacterial plasmids, its control, and their influence on the bacterium/plasmid lifestyles. By doing so, we can obtain a broad view of a field that guided the research on small RNAs for a number of years, and that is still useful in constructing tools for System Biology-based elements to control gene expression by RNAs⁵. Bacterial plasmids could be considered as selfish DNA molecules that, in some cases, have become beneficial or symbionts to the hosts⁶. Plasmids have a size that varies from ~1 to several hundreds of kilobase pairs (kb). In the first case, the plasmid will carry only the functions involved in replication and its control^{7,8}, whereas large plasmids incorporate into their backbones one or several operons that may be involved in degradative pathways of xenobiotic compounds⁹, symbiosis for nitrogen fixation by plants, as in the rhizobia family of bacteria¹⁰, antibiotic-resistance traits¹¹, etc. Plasmids replicate in autonomous and controlled manner, although they use different mechanisms to do so¹². We will review examples of replication control circuits in small plasmids replicating by the asymmetric rolling circle mechanism, termed RCR-plasmids. Whereas these plasmids have in common their replicative mechanisms, they control their replication by, at least, two ways: i) a single antisense (as)RNA, and ii) a combination of an asRNA and a transcriptional repressor protein¹³. These regulatory processes are, by no means, exclusive of RCR-plasmids, since they are shared with plasmids replicating by the theta and by the strand-displacement modes¹²⁻¹⁴. Further, involvement of a transcriptional repressor protein in conjunction with an asRNA, has been reported for the theta-replicating plasmid R1^{15,16}, and has been reviewed in depth¹⁷. The examples of these copy number control mechanisms present in theta-replicating plasmids will not be addressed here, and will be suggested as further readings.

Bacterial plasmids are supercoiled DNA molecules that replicate autonomously and in a controlled manner. Plasmid replication is random and independent of the replication of the host chromosome, although plasmids use the host replication machinery thoroughly, especially RNA- (RNAP) and DNA- (DNAP) polymerases, single-stranded DNA binding protein (SSB), and DNA gyrase. To control their replication, plasmids encode negatively-acting elements that: i) limit their replication frequency to one round per copy and per cell cycle¹⁸, and ii) correct the fluctuations in their copy numbers that occur along the host cell cycle¹⁹⁻²². Replication control is exerted at the initiation stage²³, and not at later events (elongation or termination) to avoid useless energy expenses and, in the case of plasmids, to control their replication by elements encoded by the replicon and not by delegation of functions to the host (always a risky matter). Because of their extrachromosomal location, their use of the host replicative machinery, and their number of copies (from one to several dozens), plasmids may constitute a genetic cargo to the host that carries them, although selective advantages may eventually exist. Depending on the plasmid, the host, and environmental conditions, harbouring

a plasmid may impose a metabolic burden to the cell carrying them. This would result in an increase in the doubling time of the plasmid-carrying cells compared to the plasmid-free ones. As a consequence, in mixed populations of plasmid-free and plasmid-carrying cells, the former population would overgrow the latter, and the plasmid would be eliminated from the population^{24, 25}. It follows that the genetic load imposed by the plasmid must be compensated by an increase in the fitness of the plasmid to the host. Then, this genetic burden can be considered negligible²⁶. An improper fitness will result in plasmid segregational instability and, ultimately, in plasmid loss²⁷⁻²⁹.

RCCR-PLASMIDS

The RCCR-plasmid family is very abundant (hundreds of them sequenced so far); many of them carry a mobilization module, so they can be horizontally transferred among several hosts by the functions provided by bigger, 'auxiliary' plasmids³⁰. Thus, these RCCR-plasmids constitute a large pool of DNA that is shared among bacteria³¹. RCCR-plasmids have a small size that, in general, varies from ~1 to less than 10 kb, although plasmids of ~30 kb have been reported³². This limitation of maximum size is related to their mode of replication because it generates single-stranded DNA intermediates (Figure 1) that are recombinogenic and deleterious for the harbouring cells³³⁻³⁵. RCCR-plasmids are constructed by combination of different gene cassettes, so that they usually harbour up to three main modules involved in: i) replication of the leading strand and copy number control; this is the only essential module and it is the one that constitutes the plasmid signature and defines the RCCR-plasmid families; ii) antibiotic-resistance determinant, and iii) mobilization, since many of them are mobilized by functions provided by auxiliary replicons^{12, 30, 36}. Further, some open reading frames related to metabolic or to unknown functions ('cryptic' plasmids) have been reported³⁷. The initiator of replication protein (generically termed Rep) and the relaxase that initiates mobilization (Mob) have both DNA nicking and closing activities on supercoiled DNA, and they both induce hairpin extrusion at their cognate origins of replication (*dso*) and of transfer (*oriT*)^{38, 39}. Nicking of a supercoiled molecule by the cognate Rep protein would result in relaxed plasmid forms that would be unproductive for transfer, and vice versa^{35, 36}. In addition, RCCR-plasmids contain one or two origins of lagging-strand replication, termed *sso*; they are specific noncoding regions that have the potential to generate imperfect hairpin structures on single-stranded (ss) DNA. The *sso* regions are functional in an orientation-dependent manner⁴⁰ and require unpaired sequences within this region^{36, 41, 42}. Based on the replication and control module, RCCR-plasmids have been grouped into three main families: i) Rep_trans, represented by plasmids pT181 and pC221; ii) Rep_1, represented by plasmids pUB110 and pC194, and iii) Rep_2, the representative being plasmids pMV158 and pE194⁴³. Plasmids belonging to the different families seem to control their replication by different mechanisms (see below).

FIGURE 1 Simplified model of plasmid replication by the rolling circle mechanism. The initiator of replication (Rep) dimeric protein (red ellipses) recognizes, binds to it, and cleaves the phosphodiester bond of a specific di-nucleotide (within a stem-loop structure, represented by a grey circle) at the origin of replication of the leading strand (*dso*) on supercoiled DNA. This reaction leaves a 3'-OH end that will be used as primer to assemble the replisome. The various stages of the process (A-F) are detailed in the text. The single-stranded origin of replication, *sso* (filled circle in blue), is generated in the ssDNA intermediate by intrastrand pairing. More detailed representations of the mechanism of replication by the RC-mode have been published^{35, 36, 44, 45}.

Being small, RCR-plasmids respond to the philosophy of 'travel light', that is: i) to encode only the essential genes required for their vertical propagation and, in several instances, horizontal transfer, and ii) to use host proteins that are abundant and present in all hosts, like DNAP, RNAP, SSB, DNA gyrase, and DNA helicase^{46, 47}; this latter feature is not exclusive of RCR-plasmids since several other replicons also use the same kind of proteins, with some differences, like those found the IncQ plasmids that encode their own DNA primase⁴⁸, or in the IncW plasmid family that encode a relaxase with DNA helicase activity⁴⁹. Replication by the RC mode can be succinctly schematized (Figure 1): the Rep initiator protein recognizes its cognate *dso* on the leading strand and induces hairpin extrusion on a curved region of the supercoiled DNA substrate^{39, 50} (Figure 1A). The Rep initiator has site-specific endonuclease nicking-closing activities, and cleaves the phosphodiester bond of a definite dinucleotide, leaving a 3'-OH end while the protein remains bound to the 5'-end of the cleaved strand (Figure 1B). The nick generated by the Rep initiator is elongated by host-encoded proteins (DNAP, a helicase, and SSB). As the replisome progresses, it displaces the parental leading strand that is replaced by the newly synthesized strand. The end products of this stage (Figure 1C) are a double stranded (ds) DNA molecule that has one parental and one newly synthesized strand and a ssDNA intermediate^{40, 51, 52}. Further, the Rep protein is released as an inactive heterodimer (Rep::Rep::DNA) composed of one intact Rep molecule and one modified subunit which has a short oligonucleotide covalently bound to it⁵³. The half replicated molecule would be supercoiled by the host DNA gyrase, leaving a plasmid

copy ready for the next round of replication (Figure 1C). In addition, the single-stranded DNA (ssDNA) intermediate (that would be coated by SSB) is converted to a dsDNA molecule by lagging strand synthesis (Figure 1D). This starts from the sso that, upon hairpin generation on ssDNA, would generate a so-called 'ssDNA promoter'⁵⁴. From this promoter, the host RNAP synthesizes a short RNA primer that is elongated by the DNAP I, to be most likely replaced later by DNAP III⁴¹ (Figure 1E). Thus, the end-product of the lagging strand synthesis is a hemireplicated dsDNA molecule that would be supercoiled by the host DNA gyrase (Figure 1F). In-depth reviews on replication by the RC mechanism have been published along the years^{12, 35, 36, 45, 55-57}.

CONTROL AND DISTRIBUTION OF PLASMID COPY NUMBERS

Stable plasmid inheritance can be achieved by several means such as, at least: i) integration into the host chromosome so that the plasmid copy number is identical to that of the chromosome⁵⁸; ii) incorporating into the plasmid backbone some genes that participate in plasmid stable inheritance, like those belonging to the toxin-antitoxin categories⁵⁹, iii) incorporating partitioning systems⁶⁰⁻⁶², and iv) reducing their size and increasing their number of copies so that the probability of plasmid loss is reduced¹³. RCR-plasmids are included in the latter case. These plasmids ensure their stable inheritance because of their elevated number of copies (~20-30 copies per chromosome equivalent in the original host). Further, RCR-plasmids do not seem to carry specialized partitioning systems with the exceptions reported for RCR-plasmids from *Bacillus thuringiensis*³⁷, so that their copy number (N) seems to be distributed randomly between daughter cells at the time of division^{22, 55}. Control of RCR-plasmid replication is exerted at the initiation level and, in the known cases, and it is executed by either asRNAs or by an asRNA and a transcriptional repressor protein^{63, 64}. However, more complex situations involving cross-talks between the mobilization module and copy number control elements have been found since the MobM relaxase of plasmid pMV158 actively participates in the copy number control of the plasmid by hindering transcription of the asRNA that negatively regulates synthesis of the RepB initiator⁶⁵. In the best-known examples of asRNAs involved in copy number control, their coding sequences overlap the sense mRNA encoding the Rep initiator but are transcribed in the opposite direction. Thus, these asRNAs were termed 'counter-transcribed' (ct) RNAs, and they will be referred as such here⁶⁴.

Bacterial populations of cells harbouring plasmids without partition systems, like most RCR-plasmids, distribute their copies between daughter cells in a random mode. Consequently, they can be described as having a Gaussian distribution of the cell numbers versus plasmid copy number (discussed in²⁸). In these plasmids, it could be argued that individual cells may differ in the N values, but from the formal point of view, a Gaussian distribution of copies within the bacterial population is acceptable. It follows that the width of the Gaussian curve may be broad or narrow, indicative of a more or less even distribution of the average copies per cell (N_{av}). When the distribution is too broad, the probability of cells having a reduced N value would be high. In this case, the plasmids located to the left of the Gaussian curve would have $N \ll N_{av}$ before cell division, and the probability of appearance of plasmid-free cells (that usually have a doubling time slightly shorter than that of the plasmid-containing cells) would be higher than in the cases where the width of the

Gaussian distribution is narrow (Figure 2A). However, mechanisms designed to correct fluctuations in the N_{av} do exist and are mediated by the regulatory control elements, like the ctRNAs that participate in the increase or decrease of the rate of replication⁶⁶. We can derive the conclusion that RCR-plasmids, due to their relatively high N_{av} values, should be stably inherited without the need for specialized partitioning systems. This has proved to be correct, except in plasmids in which the lagging strand origin(s) were deleted or were functionally inefficient, showing segregational instability⁶⁷. The experimental rate of plasmid loss per cell and generation (L_{ex}) was determined based on the equation:

$$f_+ = (1-L_{ex})^{n+1}$$

where f_+ is defined as the fraction of plasmid-harboring cells after n generations in the absence of selective pressure, for instance, an antibiotic-resistance determinant^{26, 67, 68}. These parameters can be experimentally determined by growing a culture for several generations in the presence of selective pressure, followed by recollection of the cells by centrifugation, dilution in antibiotic-free medium for several generations more and determination of the total number of cells (plating in antibiotic-free medium) and the number of cells that still harbours plasmids (plating in antibiotic-containing medium). More recently, microscopy-based approaches to screen for plasmid-free cells at short time intervals⁶⁹ have permitted the measurement of plasmid loss rates and to conclude that these rates were lower than those previously determined at least for plasmid R1¹⁷. The mathematical analysis of these and fitness processes of RCR-plasmids have been reported^{26, 29, 70}. When the plasmid is fully stable, the N value remains more or less constant along the generations and, in this case, $N \sim N_{av}$ and $L_{ex} \sim 0$. Plasmids that do not replicate efficiently in a given host may exhibit an increased instability and then $L_{ex} > 0$ and they would be rapidly lost from the population (Figure 2B).

On the other hand, when a plasmid colonizes a new plasmid-free host (either by transformation or mobilization), the intracellular concentration of the negative regulators would not exist; thus the incoming plasmid would over-replicate unhindered, reaching a value of $N \gg N_{av}$ (Figure 2C). Then, the control functions would operate to regulate the fluctuations in N until the N_{av} value is reached. This is a typical dose-response fluctuating behaviour that was early studied by System Biology approaches⁷¹. Small fluctuations in the steady-state would be corrected by the participation of the ctRNAs because they have a short half-life⁷². This may not be the case when the host already harbours a resident plasmid: in these cases, the entering plasmid may find difficulties to establish itself if the resident plasmid encodes a transcriptional repressor protein that may interfere with the synthesis of the Rep protein. This has been shown for the RCR-plasmid pMV158 and, although with a different regulatory circuit, for the theta-replicating plasmid R1⁷⁰. Measurements of the N_{av} values are usually referred to a bacterial host genome equivalent. This was early done by extracting total DNA of plasmid-harboring cultures and densitometric scanning of images of stained agarose gels^{73, 74} and by radioactive labelling of exponentially growing cultures of cells-harboring plasmids⁷⁵. More recent approaches include quantitative real-time PCR^{70, 76}, and super-resolved location microscopy⁷⁷.

FIGURE 2 Features of plasmid copy numbers in their bacterial hosts. **A.** The theoretical distribution of plasmid copy numbers at the moment of cell division is provided by a Gaussian curve, in which the N_{av} is indicated by a vertical dotted line. Plasmids having a narrow distribution (red) will have more probabilities to exhibit a stable inheritance than plasmids with a broad distribution (black). **B.** Rate of plasmid loss in cultures grown during n generations in the absence of selective pressure. Stable (red line) and unstable (black line) plasmid inheritance are depicted. **C.** Copy number fluctuations in the N_{av} of a plasmid following colonization of a new bacterial host.

Individual plasmid copies are randomly selected for replication from a pool of molecules that most likely include nonreplicated copies^{22, 55}, because the newly replicated ones may need supercoiling by DNA gyrase before they are ready for a new round of replication (Figure 1). Random selection of the molecules that will replicate, in conjunction with the negative control of replication, leads to the concept that two plasmids sharing the same replicon would be unable to co-exist within the same cell in the absence of selective pressure. This would result in the segregation of the two plasmids within the bacterial population, a well known and important process termed plasmid incompatibility^{13, 21, 78}. Incompatibility was a way to classify plasmids before the studies of the Rep proteins and mechanisms of replication⁷⁹ and has been useful in many experimental approaches to plasmid biology (see the sidebar).

Mechanisms to control the N value in the RCR-plasmid family have been described in-depth for two plasmids, pMV158 and pT181. Other examples, less studied, will be also described below. So far, two main mechanisms appear to exist in

controlling replication of these plasmids: transcriptional attenuation by ctRNAs (Rep_trans; pT181; Figure 3A) and transcriptional repression by a protein plus post-transcriptional repression by a ctRNA (Rep_2; pMV158; Figure 3B).

FIGURE 3 Regulatory circuits of RCR-plasmids pT181 and pMV158. **A.** In the case of pT181, promoter P_{rep} directs synthesis of the RepC initiator (green ellipse), which initiates replication by binding to its cognate *dso*. Replication is controlled by RNAI (red) that is complementary to the untranslated 5'-end of the *repC* mRNA. **B.** For pMV158, the circuit is regulated by the replication initiator protein RepB (green ellipse) that initiates replication by binding to the *dso* and cleaving the phosphodiester bond of a specific dinucleotide, leaving a 3'-OH end that acts as the replicative primer (see also Fig. 1). The negatively-acting elements (red) are the transcriptional repressor protein CopG and the antisense RNAII. The mechanism for controlling synthesis of RepB is provided by both elements: CopG represses synthesis of the initiator by binding to the P_{cr} promoter, whereas binding of RNAII to the *copG-repB* mRNA leads to inhibition of the binding of the ribosomes in the mRNA.

Copy number control by ctRNA

Pioneer work performed in Novick's laboratory demonstrated that copy number control of the staphylococcal RCR-plasmid pT181 was exerted by limiting the synthesis of the initiator RepC protein. Control was shown to be attained at the transcriptional level by two short-lived ctRNAs, termed RNAI and RNAII, which are transcribed from the same promoter; thus, the two ctRNAs share the same 5'-end, but they have different lengths, 85 and 150 nucleotide (nt)-long, respectively⁸⁰⁻⁸². These two ctRNAs are transcribed in opposite direction of the gene encoding the RepC initiator, being complementary to the untranslated 5'-end of the leader *repC*

mRNA (Figure 3A). Thus the *repC* mRNA and the ctRNAs can pair ('kiss') to generate a duplex RNA. Generation of a full duplex does not seem to be needed to hinder the expression of the *repC* mRNA because inhibition of the synthesis of RepC seems to be accomplished faster than generation of a fully stable duplex RNA⁸³. However, determination of the rate constants of formation of the stable RNA duplex were similar to those of the inhibition of *repC* synthesis⁸⁴, leaving open this question on the mechanism of the inhibitory process. Generation of the kissing complex requires the existence of a time window when the sense-antisense RNA can find each other; after this period, the *repC* mRNA adopts a structure, inaccessible to the ctRNAs, that would place the *repC* ribosome-binding site in a position accessible to the translation machinery (Figure 4). However, along the time-frame where the ctRNAs can bind to the *repC* mRNA, pairing would lead to the generation of a secondary structure on the mRNA that would resemble a Rho-independent transcription terminator upstream of the initiation codon of the *repC* gene. As a consequence, premature transcription termination of the essential *repC* mRNA would occur with the concomitant release of non-productive *repC* mRNA molecules (Figure 4)²².

FIGURE 4 Regulatory circuit of plasmid pT181 indicated by a schematic representation of the interactions between the ctRNA (RNAI, red) and the *repC* mRNA (cyan). The early transcript (~110 nt-long) of the *repC* mRNA would fold as having two main hairpins. In the absence of RNAI, the late transcript would pleat as a complex structure that would place the RBS and the *repC* initiation codon accessible to the translating ribosomes (upper part of the diagram). However, and within a time-window, pairing between the complementary loops of the hairpins generated in the *repC* mRNA and RNAI molecules may take place. In this case, generation of a hybrid RNAI and mRNA would lead to profound conformational changes in the duplex RNA. In this case, the downstream region of the duplex would generate a new hairpin ending in a 5'-UUUUUUUAU-3'

sequence with the configuration of a Rho-independent transcription terminator. The result would be an untranslated truncated RNA duplex (lower part).

Later studies showed that the longer RNAII could be the result of a read-through, and thus a minority by-product of RNAI⁸⁴, but essentially the transcriptional attenuation mechanism for controlling N_{pT181} by RNAI remained unquestioned. A similar regulatory circuit to that of pT181 was early predicted for the staphylococcal plasmid pC221⁸⁵ but, to our knowledge, no experimental data has been published.

In the case of the staphylococcal RCR-plasmids of the Rep₁ family, pC194 and pUB110⁸⁶, two ctRNAs have been proposed to control the synthesis of the initiator proteins, RepA and RepU, respectively. Such control would be exerted at the translational level by binding to the initiation of translation signals of the *rep* mRNA^{87, 88}. A similar situation was proposed to exist for the staphylococcal plasmid pE194⁸⁹. In the case of pUB110, self-regulation of the *repU* gene by the RepU initiator added another level of regulation of N_{pUB110} ⁹⁰. However, detailed knowledge of the mechanisms involved in the copy number control in these plasmids needs further experimentation.

Synthesis of a 115-nt long ctRNA was demonstrated in the case of the 2.9-kb long streptococcal RCR-plasmid pt38⁹¹. The start site of transcription of this ctRNA was shown to be placed at the proper distance from its putative promoter; thus, the ctRNA would overlap the plasmid *rep* mRNA. If this were the case, a mechanism of inhibition of the translation of the Rep initiator protein could be achieved by pairing of the complementary regions of the ctRNA and the *rep* mRNA, thus blocking the access of the ribosomes to their binding site.

The double regulatory circuit of plasmid pMV158

The first instance of a plasmid having a regulatory circuit integrating a transcriptional repressor protein and a ctRNA was that of the RCR-plasmid pMV158^{63, 73}. The copy number control of this plasmid is exerted by the concerted action of the 48 nt-long ctRNA, termed RNAII⁶⁶, and the 45-amino acid transcriptional repressor protein CopG⁹². The genetic organization of the control region (Figure 3B) shows that the genes encoding the *repB* initiator and the repressor *copG* are co-transcribed, although there is no coupled translation of both proteins⁹³. The coding region of gene *copG* is preceded by a near-consensus ribosome binding site (RBS) sequence⁷⁵. Protein CopG binds to a palindromic region that encompasses the single P_{cr} promoter inducing a severe bend in the DNA of $\sim 120^\circ$ ⁹⁴, and thus hindering the binding of the host RNAP to the promoter or even expelling the already bound RNAP⁹⁵.

The coding region of gene *repB* is preceded by a 60 nt-long non-coding region encompassing a translation initiation region (TIR). In late 80s, it was suggested that the following consensus sequence (nucleotides -23 to +3 with respect to the start of the coding region): 5'-AUUUCU...4-5 nt ...UAUA- 9-10 nt ... AUG-3' may be responsible for ribosome binding and therefore the sequence was termed 'atypical' ribosome binding site (ARBS)^{96, 97}, however the molecular details of ARBS binding to the 3' tail of ribosomal 16S RNA (5'-ACCUCCUU-3'; anti Shine-Dalgarno; aSD) or to

an alternative ribosomal element remained unclear. In the case of the pneumococcal *dprM* gene the ARBS sequence was reported to be a -10 extended promoter⁹⁸. Further, the *repB* 5'-UTR sequence, and in particular its part proximal to the start codon, was shown to play an important role in the RNAI::*copG-repB* mRNA interactions^{66, 93}. In the absence of toeprinting analyses⁹⁹ that would unambiguously show the ribosome-binding site in the *repB* mRNA, we would like to propose an alternative or complementary, yet unproved, hypothesis that would consider the ribosome-binding site to be a weak version of the Shine-Dalgarno sequence (wSD), while the rest of the conserved sequence of the *repB* 5'-UTR (including the proposed ARBS) would form a structured RNA element that is responsible for the initial mRNA - ribosome interactions. Formation of specific structured motifs upstream the start codon (as predicted for *repB* mRNA) has been shown to be involved in the wSD recognition by the ribosome through S1 protein binding to and unfolding of the structured RNA elements¹⁰⁰. A sequence alignment of the putative rep genes translation initiation region (TIR) of the pMV158 plasmid family indicated the presence of a putative wSD 5'-AGGU-3' (5'-GGGU-3' in pMV158 *repB*) motif, namely: **RN(W)₁₋₂RWUWNW(N)₀₋₅URAGGU(N)₁₋₂AUUUAUANC(W)₀₋₂(U)₀₋₁AUG** (adapted from⁹³; shown are nucleotides with at least 75% conservation; in bold - positions with at least 90% conservation; the sequence of RNAI -10 promotor is shown in italics; the start codon is underlined; N: A/G/C/U; R: A/G; W: A/U). Secondary and tertiary structure prediction of the *repB* mRNA region from nucleotides -60 to +40, strongly suggests the possibility of generation of three hairpins, which could be further engaged in pseudoknot interactions, including kissing-loop pseudoknots. In our proposed structural model of the *repB* 5'-UTR (Figure 6), the wSD 5'-GGGU-3' and the start codon AUG sequences are embedded within a bulged hairpin 2. The hairpin 2 loop is made of a long stretch of conserved bases (5'-AUUAUACUU-3') that form a kissing-loop interaction with hairpin 1 loop (5'-GGCA-3'; Figures 5 and 6). Formation of alternative structures by hairpin 2-loop would be also possible: a pseudoknot with the hairpin 1 - hairpin 2 linker (5'-AAGUCAA-3') or a kissing-loop with the hairpin 3-loop (5'-AAGAUUA-3'; Figure 5). In our proposed hypothesis, the conserved motifs of *repB* mRNA would generate a structure that would interact with the ribosomes through S1 protein. This structure would subsequently be unfolded by the S1 protein leading to *repB* the wSD motif (bases -16 to -13) pairing with the 5' part of the 16S anti-SD sequence (5'-ACCU-3' of 5'-ACCUCCUU-3') and placement of the start AUG codon in the ribosome decoding center. Contribution of SD sequence types (including lack of a SD sequence), structured RNA elements, RBS spacer, or translation enhancers to gene expression regulation in prokaryotic systems is becoming better understood in recent years¹⁰⁰⁻¹⁰². Concerning the mechanism of action (Figure 5), RNAI exerts its inhibitory role at the translational level by pairing, at least partially, with its complementary region in the *copG-repB* mRNA causing its structural switch that occludes the ARBS/wSD sequence from binding to the ribosome^{66, 93}.

Figure 5. Regulatory circuit of plasmid pMV158. Nucleotide positions with reference to the adenine of the *repB* AUG start codon are indicated. **A.** Diagrammatic representation of the secondary structures of *repB* mRNA (cyan), counter-transcript RNAII (red) and their complex (cyan-red; with // indicating a part of the duplex that is not shown). Thin lines in *repB* mRNA structure indicate alternative pseudoknots. Upon mRNA/RNAII duplex formation and structural switch of *repB* mRNA, translating ribosomes would not be able to bind to *repB* translation initiation site anymore. **B.** Sequence and secondary structure of *repB* mRNA. In bold are shown bases with positions conserved at least with 75% (using an extended nucleotide 'alphabet', where R=A/G and W=U/A; alignment from ⁹³). Promoter P_{ctII} (-35 and -10 sequences indicated) directs synthesis of RNAII that starts 17 bases before the start codon of *repB* (highlighted in green) and terminates past the UAA stop codon of *copG* (highlighted in red). Red bases indicate ARBS, and bases highlighted in yellow show wSD (GGGU). Boxed regions highlight mRNA and RNAII mutants that show impaired function ^{78, 93, 103}. The RNAII transcript (-17 to -64) overlaps partially the ARBS/wSD sequences of gene *repB*. In the lower part of the Figure, a dot-bracket notation of *repB* mRNA secondary structure (matching brackets symbolize base pairs and dots show unpaired bases) for mRNA alone (with three alternative pseudoknots) and mRNA upon pairing with RNAII are shown.

Synthesis of RNAII is directed by promoter P_{ctII} that is embedded within the coding sequence of *repB* (Figure 5B). The entire nucleotide sequence of gene *rnall* is fully complementary to a region in the *copG-repB* mRNA that is located upstream of the ARBS/wSD and the AUG initiation codon of *repB* ^{66, 78, 103}. This genetic organization is conserved in all plasmids of the pMV158 family ⁹³. Due to the auto-regulation of the *copG-repB* mRNA by CopG, the repressor protein would maintain a fairly constant level of the messenger; on these levels it would operate fluctuating concentrations of RNAII (Figure 3B). Folding of RNAII predicted by the Zuker's algorithm ¹⁰⁴ indicated a simple structure constituted by a single hairpin of 8 paired nt with a 1-nt internal bulge-loop and a 6-nt terminal loop ($\Delta G = -10.5$ kcal/mol). RNAII is constitutively synthesized, has a short half-life (~ 1 min), and being only 48 nt-long is one of the smallest ctRNA described so far ^{13, 93, 103}. The 3'-end of RNAII extends and terminates past the UAA stop codon of gene *copG* (Figure 5B), and contains a

stretch of 14 U residues out of a sequence of 16 nt. This 3'-terminal region of RNAII was shown to be, at least in vitro, a rho-independent transcription terminator with a predicted termination efficiency of 85% and an experimental one of ~87%^{105, 106}. Mapping of the interactions of RNAII with its cognate region in the *copG-repB* mRNA showed an unexpected scenario: instead of the 'classical' hairpin-kissing interactions through unpaired loop regions, the RNA initial duplex started by pairing of the 5'-end of RNAII with its complementary sequence in the mRNA. These interactions have been also predicted computationally when running 3D structure prediction of mRNA-RNAII complex (early complex with partially base paired; Figure 6).

Figure 6 Three-dimensional structural models of pMV158 *copG-repB* mRNA intergenic region (cyan), RNAII (red), and their early and final complex (all models were submitted to RNArchitecture database¹⁰⁷). The models were predicted by SimRNAweb¹⁰⁸ with secondary structure inputs as predicted by RNAstructure¹⁰⁹, and experimentally evaluated (fully for RNAII

and partially for the mRNA intergenic region) by López-Aguilar in G. del Solar laboratory^{78, 103}. Figures were generated using PyMol¹¹⁰.

Interestingly, during progression of the complex formation the mRNA region that includes ARBS 3'-fragment/wSD undergoes structural rearrangement that could be responsible for the inability of the ribosome to bind the mRNA-RNall complex and initiate *repB* translation (final complex; Figure 6). This pairing was helped by generation of kissing hairpins, but progression from the initial duplex to reach the kissing hairpins and, later on, a full duplex molecule, was not required to generate a stable RNall-mRNA complex^{78, 103}. Initial interactions between asRNA and mRNA in several theta-replicating plasmids (like Collb-P9) were shown to be facilitated by a hexanucleotide element that was found to be present in several asRNA¹¹¹. This element termed the 'U-turn' is located at the loop of RNA hairpins and enhances the interactions between sense and asRNAs^{112, 113}. The U-turn motif was not present in the plasmids of the pMV158 family¹⁰³, suggesting an important difference in the initial stages of the asRNA-mRNA interactions between these RCR-plasmids and those using the kissing hairpins pathway⁸³. Further, lack of U-turns could be related to the non-essentiality of an initial kissing complex, at least in plasmids of the pMV158 family¹¹⁴. Nevertheless, and whatever the mechanism is, it is interesting to point out that the rates of asRNA-mRNA association constants were similar in plasmids having or lacking the U-turn¹⁰³.

Generation of mutations in either *copG* or *rnall* genes followed by incompatibility tests showed that the combination of the two elements exhibited an incompatibility greater than that exerted by either element alone. This synergistic effect points to an important role of CopG-mediated repression/derepression of the operon to regulate the control of N_{pMV158} ⁶⁶. When an entering pMV158 encountered high levels of CopG (provided by a compatible plasmid), there was a strong decrease in the frequency of plasmid establishment⁷⁰. Repopulation experiments⁷² in which the N value was determined at different times after plasmid DNA uptake allowed to determine the kinetics of plasmid repopulation⁷⁰. The results showed that the process lasted ~45 min after plasmid transfer (by transformation or conjugation) when CopG was absent from the recipient cells, whereas the presence of intracellular repressor protein dramatically diminished the repopulation of the cells by plasmid pMV158, but not by plasmids lacking the CopG-RNall regulatory circuit⁷⁰. A similar hindrance in plasmid establishment was found in plasmid R1 that has a different regulatory pathway but also encodes a transcriptional repressor protein (CopB) involved in copy number control¹¹⁵.

Summing up, and lacking more detailed experiments, we can advance the following conclusion: out of the three RCR-plasmid families, Rep_trans, Rep_1, and Rep_2, represented by plasmids pT181, pUB110/pC194, and pMV158, respectively^{43, 45, 57}, differences in the mechanisms controlling their number of copies seem to exist: i) Rep_trans and Rep_1 plasmids control their number of copies only by trans-acting ctRNAs, although regulation of the levels of *rep* mRNA by the Rep initiator itself could also participate in the regulatory circuit; and ii) Rep_2 plasmids would integrate two elements within a single circuit: a ctRNA and a CopG-like transcriptional repressor protein. Both elements are active *in trans* and would jointly participate in the regulatory circuit^{13, 45, 114}.

POSSIBLE APPLICATIONS AND FUTURE DIRECTIONS

Ample knowledge has accumulated over the years on the mechanisms employed by asRNAs to control plasmid replication and, hence, to down-regulate gene expression. However, exploitation of this knowledge to develop tools to reduce the expression of genes involved in virulence and/or pathogenicity has been scarce. Reduction in the levels of the staphylococcal alpha-toxin by the use of a regulated asRNA was feasible¹¹⁶, but no follow-up information was reported. Nevertheless, employment of asRNA placed under the control of inducible promoters could be an amenable approach to develop 'killer' or silencing vectors designed to regulate synthesis of an antisense region to any desired essential gene leading to cell growth arrest or even cell death. These vectors could be used as possible 'live' vaccines when provided by non-pathogenic bacteria carrying a plasmid vector with high transfer frequencies and that could be eliminated once they have accomplished their immunogenic role^(117; Espinosa, unpublished). Alternatives to these strategies have been developed, especially by employment of modified asRNAs coupled to oligodeoxynucleotides to silence essential genes at mRNA::DNA levels. Paired DNA::RNA molecules are prone to degradation by intracellular nucleases, and variants of these molecules have been developed especially peptide-nucleic acids¹¹⁸. These compounds, termed PNAs, are analogues of nucleic acids but the sugar-phosphate backbone is substituted by a flexible peptide polymer to which the bases are attached. Thus, PNAs can mimic and pair to DNA or RNA, generating heteroduplexes resistant to degradation by intracellular enzymes. All these approaches have a fundamental drawback that is the delivery of the compound into a clinical format¹¹⁹, although alternatives have been found and clinical trials are in progress as recently reviewed¹²⁰. Importantly, just when this review was being finished, the Journal *Nucleic Acids Research* has launched a special issue gathering selected articles on nucleic acids therapeutics (https://academic.oup.com/nar/pages/nucleic_acids_therapeutics_collection).

Concerning future directions, we envisage at least three, in addition to the above. Firstly, in the drug discovery field, it has been found a very promising approach that consists in developing asRNA molecules to which membrane-penetrating peptides (MPPs) have been coupled. MPPs are short molecules (~40 residues) that have an access to the intracellular space of any living cell. This is because they are highly cationic and rich in arginine and lysine residues, thus being able to carry with them conjugated molecules, nucleic acids among them. Therefore, MPPs are useful molecules to be explored to deliver drugs to the bacterial intracellular *milieu*¹²¹. Phosphorodiamidate-morpholino oligonucleotides mimicking a nucleic acid conjugated to one MPP have been successfully used as an active compound against *Acinetobacter* both in vitro and in vivo¹²². Secondly, there have been important findings of functional microRNA-size RNAs in *Escherichia coli*¹²³, the synthesis of a 20-nt long small microRNA-size encoded by the *E. coli* phage Φ 24B that severely alter the expression of phage- and host-genes¹²⁴, and the existence of a secretable microRNA-size in *Streptococcus sanguinis*¹²⁵. All these novel discoveries on these RNA molecules that are similar to eukaryotic microRNA are yet to be fully explored. And thirdly, we have little information on the cross-talks between chromosomally-encoded proteins and RCR-plasmid regulatory networks. A recent

report has shown that out of the three RNase III-homologs encoded by the cyanobacterium *Synechococcus* sp. strain PCC 7002, one of them is specifically dedicated to cleavage of the ctRNA involved in the control of the number of copies of the indigenous RCR-plasmid pAQ3^{126, 127}, a member of the pMV158 plasmid family. These novel findings open an unexplored avenue of research aimed to unveil the processes of cross-talks between bacteria and their RCR-plasmids, something that we have just started to discover, but not to understand yet.

CONCLUSION

Plasmids constitute an important part (up to 20%) of the bacterial pangenome¹²⁸ and as such participate actively in the response to selective pressures: they play a key role in the spread of antibiotic resistances or in the acquisition of catabolic pathways in response to toxic compounds in soils and waters^{129, 130}. Being shared among different bacterial groups, many plasmids move around posing some 'background noise' in the sequence databases. And furthermore, plasmids have played a fundamental role in the development of the modern Biology, not only as cloning and/or expression vectors but also in the development of tools for Synthetic Biology¹³¹. Now that the basic studies in Plasmid Biology appear to dwindle, it is the time to revisit many of the fundamental studies dealing with the control of plasmid replication by asRNAs. The basic questions being: i) do we know everything that is needed to know? And, if not, ii) what do we need to know? Cross-talks between plasmid modules, plasmid copies, and plasmids and chromosomes come immediately to our minds. Understanding the mechanisms of plasmid horizontal transfer will require further efforts if we want to control the spread of antibiotic-resistances. And, of course, asRNAs can be useful tools in the design of novel targets and strategies to deal with pathogenic bacteria. We should use technologies like single-cell, microfluidics, RNAseq, and global approaches to understand the mechanisms underlying why and how the replicative machinery must be controlled and to fully understand the role of asRNAs in these processes.

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Further readings

We believe that there are, at least, two fundamental books to understand plasmid field:

1. Summers DK. The biology of plasmids, Oxford: Blackwell Science Ltd.; 1996.
2. Thomas CM. The horizontal gene pool, Amsterdam: Harwood Academic Publishers; 2000.

As recommended articles we suggest:

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