

MicroReview

Paradigms of plasmid organization

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Summary

Plasmids are extrachromosomal elements built from a selection of generally quite well understood survival and propagation functions, including replication, partitioning, multimer resolution, post-segregational killing and conjugative transfer. Evolution has favoured clustering of these modules to form plasmid cores or backbones. Co-regulation of these core genes can also provide advantages that favour retention of the backbone organization. Tumour-inducing and symbiosis-determining plasmids appear to co-regulate replication and transfer in response to cell density, both being stimulated at high density. Broad-host-range plasmids of the IncP-1 group, on the other hand, have autogenous control circuits, which allow a burst of expression during establishment in a new host, but a minimum of expression during maintenance. The lessons that plasmids have for clustering and co-regulation may explain the logic and organization of many small bacterial genomes currently being investigated.

Introduction

Plasmids are key players in the team of mobile genetic elements that fuel bacterial adaptability and diversity. This can be a problem when it results in the rapid spread of antibiotic resistance, but a benefit when it promotes the appearance of the ability to degrade a novel pollutant. The potential of plasmids to promote the escape of genetically modified DNA is also of major public concern.

Plasmids promote adaptability in a number of ways. First, in many cases, when a gene moves onto a plasmid, its copy number per bacterium rises, so that the pool of molecules is enlarged and, thus, the overall mutation rate is increased. Secondly, they are often self-transmissible or mobilizable, so that they increase the chance of the gene moving between bacteria. Thirdly, they remove the

need for the gene to integrate into the bacterial chromosome in order to become established in a new bacterium. Most plasmids can replicate within the species of at least one genus, so they can easily spread between species of that genus. However, a significant number can replicate within the species of many genera – broad-host-range plasmids. Self-transmissible, broad-host-range plasmids are thus potentially the most active vehicles for a pool of genes that are available to many bacteria, of many species, genera and even families – the horizontal gene pool (Thomas, 2000).

To understand this dimension of the bacterial genome, we need a detailed knowledge of the component parts from which these vehicles are constructed – the replication, stability and transfer systems. But we also need to think of the properties of the different genetic machines that emerge from the different permutations and combinations of component parts. The resultant knowledge should ultimately lead to a greater ability to predict the properties of this sector of the genome, when a new organism or plasmid is encountered, as well as a greater knowledge of the gene flow occurring across the bacterial kingdom. The accompanying reviews focus on various individual aspects of plasmid replication and stable inheritance. In contrast, what I do here is to give a broader perspective and consider how juxtaposition and co-ordinate regulation of related survival functions can provide a higher level of organization that represents a stabilizing force, explaining the similarities in the genomic maps of many different bacterial plasmids.

The plasmid survival kit

By definition, a plasmid is a unit of extrachromosomal genetic inheritance. Thus, for a plasmid to come into existence, a new segment of DNA must acquire the ability to self-replicate – a new replicon must be born, or an existing plasmid must change sufficiently so that it can take on an existence distinct from its parent (Fig. 1). There appear to be a limited number of replication strategies. One strategy is to use either a primary transcript or a set of repeated binding sites (iterons) for a Rep protein to unwind the region that becomes the replication origin. In the case of the iteron system, the Rep protein leads in helicase, which unwinds the origin and

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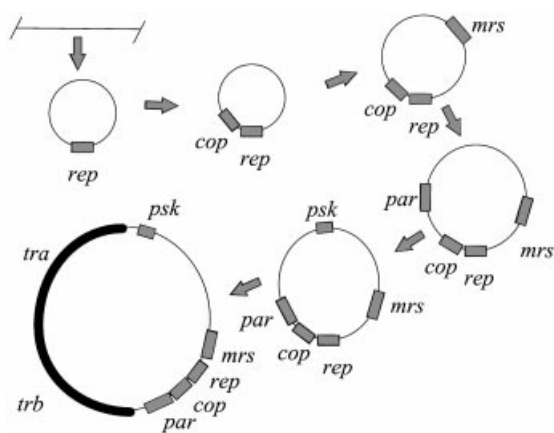


Fig. 1. Schematic view of the development of survival functions of a plasmid. The birth of the plasmid is shown as the appearance of a self-replicating circle. Subsequently, gene blocks with specific functions are first recruited by the plasmid and then shuffled by recombination, with fitter combinations being constantly selected from the pool. Gene names are: *rep*, replication; *cop*, copy number control; *mrs*, multimer resolution; *par*, partitioning; *psk*, post-segregational killing; *tra*, DNA processing genes for transfer; *trb*, mating-pair formation genes for transfer.

allows primase to initiate leading strand synthesis – a strategy shared with the chromosomal replication system (Chattoraj, 2000). A commonly used alternative strategy is to nick one strand to generate a 3'-OH, which can act as the primer for rolling-circle replication (RCR plasmids; Khan, 2000). Subsequent events that make the rudimentary replicon more efficient and join it to other genetic functions that can promote stable inheritance and propagation will survive. Key stages would be for the efficiency of replication initiation to increase and also for the acquisition of control circuits (del Solar and Espinosa, 2000) that can regulate copy number as well as link plasmid replication to cell growth, so that the plasmid does not become an unacceptable metabolic load on its host.

Although plasmids may not have been built up stepwise from a small self-replicating DNA molecule, imagining them developing in this way is quite helpful when considering the additional functions that they carry. So, from the point at which the plasmid has a moderately efficient, controlled replication system, it can develop in one of two directions.

One direction is to evolve as a small, high-copy-number plasmid, in which case the only additional function that may be needed is to ensure that multimers do not accumulate as a result of recombination between the identical copies of the plasmid (see below). Even this ability does not seem to be essential for RCR plasmids, as their replication strategy disfavors the accumulation of multimers.

Other plasmids have gone in a different direction. Recombination events may have resulted in expansion of

the replicating DNA fragment and acquisition of genes that helped to boost the new unit by improving the phenotype of the host for a character that confers a selective advantage in the environment in which the host–plasmid pair find themselves. Bacteria carrying such DNA may initially have fared better than bacteria lacking the new plasmid because of the altered phenotype but, if the plasmid started as a relatively high-copy-number element, then mutations that led to tighter control, and therefore reduced copy number, could have been favoured by lowering the metabolic burden on the host. However, reductions to below about 5–10 copies per chromosome would create a situation in which acquisition of an active partitioning mechanism would be essential if the plasmid were to be inherited stably over many generations (Gerdes *et al.*, 2000).

Identical copies of a plasmid or chromosome in a bacterial cell will tend to recombine, especially when prompted by DNA damage and, for circular molecules, this is a problem because it can cause dimerization, which prevents separation of the monomers at partitioning. So, for both high- and low-copy-number plasmids, a multimer resolution system (*mrs*) that ensures that each copy of the plasmid genome functions as a separate unit of inheritance would provide the plasmid with a major advantage. Many plasmids have a plasmid-specific *mrs*, often related to the host XerC/XerD-dependent system (Blakely *et al.*, 1993) but, in other cases, the resolvase of a transposon acquired by the plasmid can double up for this purpose and, in at least one recently described case, appears to be more effective than the former (Tomalsky *et al.*, 2000).

A final element in the array of survival mechanisms is the post-segregational killing (or plasmid addiction) system that results in the death of plasmid-free segregants (Jensen and Gerdes, 1995; Gerdes *et al.*, 1997). Such beneficial units will include carriage of restriction-modification enzymes (Kobayashi, 1998) or bacteriocin production and immunity. Possession of this armoury ensures almost completely stable inheritance, so that many naturally occurring plasmids are maintained for years in their natural host in the absence of selection.

A further increase in the ability to survive and multiply would only be achieved by acquiring the ability to spread between bacteria and, thus, conjugative transfer and mobilization systems, even if rudimentary, would have been selected (Zechner *et al.*, 2000), thus completing the basic repertoire of functions that ensure that plasmids replicate and spread efficiently.

Plasmid building blocks and architecture

The availability of DNA sequences for many plasmids has revealed that, for all the survival systems described above, we can identify families of phylogenetically related

functions distributed across a range of disparate plasmids (for example, see del Solar *et al.*, 1998; Gerdes *et al.*, 2000). One may therefore ask whether we already know all the survival functions that plasmids use: do plasmids that we have not yet characterized, perhaps from little-studied sectors of the bacterial kingdom and from unexplored environments, use functions unrelated to those that have already been studied? The answer to this often appears to be yes on the basis of trying to classify new plasmids by hybridization to probes of standard plasmid collections, but sequencing of new plasmids frequently reveals similarities to a range of known plasmid survival genes, albeit at a level that would not easily give cross-hybridization or yield a polymerase chain reaction (PCR) product with gene-specific primers. For example, many of the plasmids of the Gram-negative anaerobe *Fusobacterium nucleatum* are related, and recent sequence analysis shows that they contain a ubiquitous RCR replicon (Haake *et al.*, 2000). Similarly, the low-copy-number linear plasmid pCLP from the slow-growing mycobacterial species *Mycobacterium celatum* contains a replicon related to previously characterized replication functions in mycobacteria, as well as a gene related to the general *parABS* system of P1 (Picardieu *et al.*, 2000). A *parA* homologue involved in active partitioning has also been discovered recently on a 60 kb lactococcal plasmid (Kearney *et al.*, 2000), so it appears that such systems are not as rare in Gram-positive bacteria as it once appeared. I would argue that these studies are indicating that we know a good proportion of the existing families of plasmid survival functions, and that we can expect to find that it is these blocks from which the survival machinery of most plasmids is built.

Also, from these sequences, it is clear that plasmids, like bacterial chromosomes and viruses, have complex recombinational histories. These recombinational events can occur by homologous exchanges, by transposition events and by processes that we still do not understand. Some survival functions depend on just one genetic locus, but most depend on at least two – even replication systems dependent on a single plasmid-encoded protein also need a *cis*-acting replication origin as a second locus. Homologous recombination can occur within survival genes to create mosaics, but non-homologous events can lead to reassortment of the survival building blocks. The chance of survival loci being dispersed by recombination will be reduced if they are close to each other: clusters of survival functions will persist longer than well-separated modules. The first sorts of modules to emerge would probably have consisted of interdependent functions, for example a pair of partitioning genes and their associated centromere-like sequence, or the series of functions needed to promote mating-pair formation during conjugation. In parallel, one can then imagine independent

but mutually beneficial functions becoming clustered. Thus, for plasmids where molecular details are available, one often finds replication functions closely associated with other survival functions. For example, the primary maintenance system of F consists of a complex *rep* module, a *par* module, a *pas* (*psk*) module and an *mrs* module (Fig. 2). Events that disrupt this combination of genes will be less efficient at avoiding plasmid loss and will not survive: thus, the plasmid organization will continually improve until the potential of the system is exhausted. The specific arrangement of survival functions that persists in an evolving family is called the backbone of that particular plasmid family.

The converse of this is that one will tend to find that most of the expansion of a plasmid that occurs by transposition into the plasmid will occur in the same sector. The region that already harbours a transposable element will mark a place at which the backbone can be interrupted. Thus, in the classic studies of the F-related plasmids, there is a sector containing all the insertion sequence (IS) and transposon (Tn) elements, and these can dissociate and reassociate. The pSYM plasmids of *Rhizobium*-like pNGR234a (Freiburg *et al.*, 1997) obey the same rule, with replication, partitioning and transfer functions clustered and multiple copies of IS elements that can mediate recombination and rearrangements. Although in a plasmid as large as pNGR234a (536 165 bp),

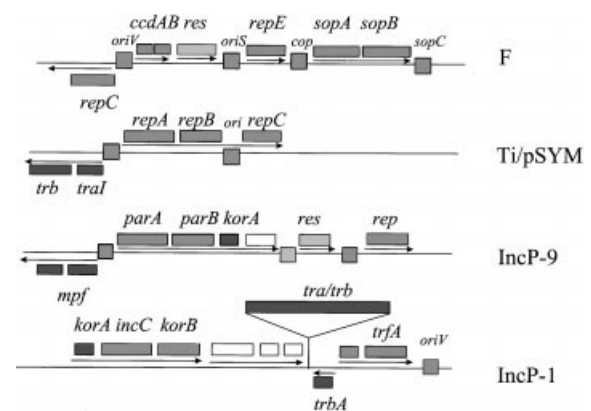


Fig. 2. Clusters of survival functions from selected plasmids. Blocks above the line indicate open reading frames (ORFs) running left to right; blocks below the line indicate ORFs running right to left; blocks on the line indicate *cis*-acting sites; arrows indicate known or probable transcriptional units. Genes are as follows. F: *repC*, activator of *oriV*; *repE*, activator of *oriS*, a secondary origin that is activated when *oriV* is deleted; *ccdAB*, proteic post-segregational killing system; *res*, multimer resolution system; *sopABC*, active partitioning system. Ti/pSYM: *repAB*, partitioning; *repC*, replication; *ori*, replication origin; *traI*, homoserine lactone (agrobacterium autoinducer) synthase; *trb*, mating formation genes. IncP-9 (Greated *et al.*, 2000): *rep*, replication; *res*, possible multimer resolution functions; *parAB*; putative active partitioning; *korA*, putative repressor; *mpf*, equivalent of *trb* in Ti above. IncP-1: *korA*, transcriptional repressor; *incC*, active partitioning; *korB*, transcriptional repressor and active partitioning; *tra/trb*, conjugative transfer functions; *trbA*, transcriptional repressor; *trfA*, activator of *oriV*.

the plasmid survival sector may look insignificant, I will concentrate on these functions, as they are the common element of all plasmids.

Systems with similar organization of survival functions must have arisen many times, because the specific modules combined in a particular way is not constant. For example, the commonest cluster of survival functions appears to be the association between *par* functions belonging to the *sop/par* family and a variety of *rep* genes (Fig. 2). Conversely, essentially the same set of functions can have been assembled by chance in different orders, for example the mating-pair systems of many conjugative plasmids of Gram-negative bacteria have variations in the basic order (see Zechner *et al.*, 2000). But this shuffling not only creates clusters, it also allows co-transcription in the form of operons (Fig. 2). At first, this integration of expression appears to have happened with interdependent genes. For example, the *par* units of P1 and F (Gerdes *et al.*, 2000) have become a single, bicistronic operon, which seems to be able to reassort as a module that is combined with different *rep* genes. These genes are also found in many other plasmids (Gerdes *et al.*, 2000), in some cases combined with extra genes, as in the case of the IncP-1 and IncP-9 plasmids (Fig. 2). Although in the case of the last two examples, it is not clear whether the co-transcribed cistrons add or contribute to another survival function, there are well-characterized systems in which physical juxtaposition has allowed *par* and *rep* genes to become part of the same operon, as in the case of the *repABC* systems of pSYM plasmids of *Rhizobium leguminosarum* (Turner *et al.*, 1996), Ti of *Agrobacterium tumefaciens* (Li and Farrand, 2000) and pTAV1 of *Paracoccus versutus* (Bartosik *et al.*, 1998). Here, *repA* and *repB* belong to the *parA* and *parB* families.

The advantages provided by the organizational structure are reasonably strong, as this combination is widespread and dominant, certainly among the Rhizobiaceae. Indeed, these replicons appear to dominate in many strains of *R. leguminosarum* (Rigottier-Gois *et al.*, 1998), to the extent that they are found on multiple plasmids in the same bacterium and therefore must have undergone sufficient divergence to enter a new incompatibility group and not compete directly with each other.

Co-ordination of backbone functions – an additional stabilizing force

Physical juxtaposition, which allowed genes to be co-transcribed, could in itself provide a balance of the expression of the different genes, but it could also allow control circuits to evolve, providing a variety of advantages. For example, a feedback loop, which provided repression of expression once the products of an operon

had accumulated to a specific level, might allow mutations to increase the strength of key promoters, ensuring a burst of expression of replication and establishment genes on entry into a new host cell. Such changes, under the control of new regulatory circuits, could result in lethal effects on their host if the controls were disrupted. This would create a situation in which rearrangements or deletions/insertions within the plasmid that altered the control circuits would be disfavoured. The evolution of these circuits would therefore add to the inertia of the newly integrated system to further changes.

One particular organizational motif that occurs repeatedly is the juxtaposition of replication/partitioning and transfer functions (Fig. 2). Although the organization is similar for the examples shown, it seems likely that they have arisen separately, and it is fascinating to speculate on the lineages involved. One striking example is that of the Ti, Ri and pSYM plasmids, in which the same organization is likely to have arisen from a common ancestor but is now present in many different incompatibility groups. So, what purpose does the organization serve? It has been known for some time that the conjugative transfer of Ti plasmids is controlled by both the availability of complex amino acids produced by T DNA-induced plant tumours and the density of the bacterial community (reviewed by Zatyka and Thomas, 1998). The density control has recently been shown to be a key part of the back-to-back arrangement of the *rep/par* and *trb* operons (Li and Farrand, 2000). Basically, there are LuxI and LuxR homologues, termed TraI and TraR, encoded within the transfer region. The *traI* gene is located at the start of the *trb* operon (Fig. 3). The homoserine lactone (HSL) it produces diffuses away from the bacteria but, when cell density is high, enough accumulates in the bacterial environment and re-enters the bacterium in order to associate with a significant level of TraR protein. This converts TraR to a transcriptional activator, which binds to TraR boxes at key points in the transfer region and stimulates transcription. In the absence of HSL-activated TraR, there is no detectable transcription, although a significant signal was found for the *repABC* region (Perret *et al.*, 1999). TraR boxes occur in the gap between *traI*. Although the level of *repABC* transcription does not rise, plasmid replication is stimulated by this protein, perhaps as a result of a conformational change in the DNA (Li and Farrand, 2000). Thus, the physical proximity of *trb* and *rep* allows a co-ordinate stimulation of both replication and transfer.

Co-regulation of the genes of the IncP-1 plasmid backbone

Despite belonging to a single incompatibility group, IncP-1 plasmids of this group are quite diverse. Restriction

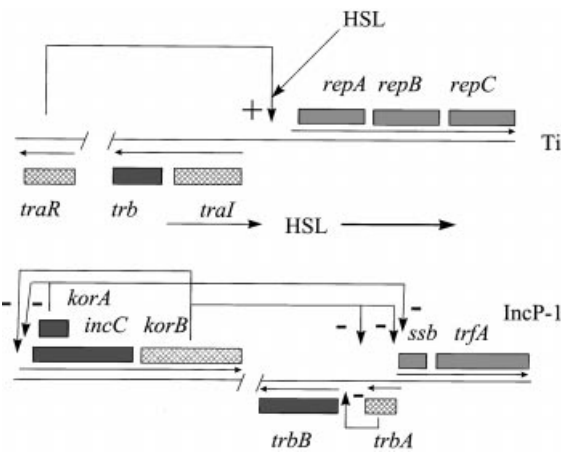


Fig. 3. Co-regulation of replication and transfer genes of Ti and IncP-1 plasmids. Nomenclature is as in the legend to Fig. 2, except that *traR* encodes the activator for which the homoserine lactone (HSL) synthesized by *Tral* is the co-activator. HSL diffuses out into the medium and accumulates. For IncP-1, *ssb* is a single-stranded DNA-binding protein encoded in the same operon as *trfA*.

patterns distinguish two major subfamilies, IncP-1 α and IncP-1 β , and archetypes of the two groups have been completely sequenced (Pansegrau *et al.*, 1994; Thorsted *et al.*, 1998). Despite considerable sequence divergence between members of these subgroups, studies to date have revealed conservation of backbone functions as defined above, in terms of what genes are present and how they are organized. A key feature of this backbone is what is termed 'the central control region', which serves a dual role, providing both active partitioning of the plasmid before cell division and co-regulation of many of the backbone operons. The IncC and KorB proteins, mentioned below, encoded in this region are equivalents of the P1 ParA and ParB proteins described by Gerdes *et al.* (2000). The key regulators from this region are KorA and KorB (Fig. 3): KorA binds to seven places on the RK2 genome, whereas KorB binds to 12. Although all the KorA operators lie in or very close to known promoters, only three of the 12 KorB operators are adjacent to promoters (Kostelidou and Thomas, 2000). Three more are within 80–189 bp of a transcription start point, but the rest are more than 1000 bp away from a promoter. KorB can act as a transcriptional repressor at a distance from where it binds (Jagura-Burdzy *et al.*, 1999a). The IncC protein potentiates the repressor activity exhibited by KorB (Jagura-Burdzy *et al.*, 1999b).

There are two other regulators: KorC, which regulates three promoters in two operons; and TrbA, which regulates at least four promoters in three operons (Pansegrau *et al.*, 1994). TrbA is particularly significant, because it is encoded between the divergent *rep* and *trb* operons. This location appears to be a crucial part of the plasmids summarized in Fig. 3, being the most obvious

part of the organization that is conserved across many groups of plasmid. In the IncP-1 plasmids, there is a genetic switch, which promotes expression of replication genes during establishment and delays transfer gene expression until plasmid-encoded repressor KorA starts to accumulate (Jagura-Burdzy and Thomas, 1994). The TrbA protein appears to be a late part of this control. Perhaps significantly, the N-terminal region containing the putative DNA-binding motif of TrbA appears to be derived from a common ancestor, with the repressor protein of temperate phage ϕ 105 of *Bacillus subtilis* controlling the balance between alternative modes of phage propagation, just as replication and transfer are alternative methods of plasmid propagation.

We have discovered recently that KorB shows cooperative interactions with both KorA and TrbA, depending on which pairs of sites are close together (Kostelidou *et al.*, 1999; M. Zatyka and C. Thomas, unpublished). The co-operativity between KorB and TrbA is still exhibited when the operators are separated by more than 160 bp. This raises the possibility that KorB and TrbA interact in regions in which KorB operators are a long distance from either the promoter or the TrbA operator, and thus provide some role for these 'orphan' binding sites. In terms of evolution, it is interesting that the ability of both KorA and TrbA to interact with KorB results from the fact that they possess a highly conserved C-terminal domain, which presumably makes direct contact with KorB. This sharing of a domain is another example of the recombinational shuffling that fuels the appearance of fitter variants with more sophisticated organization and regulation.

Because of the ease with which it was possible to identify KorA, KorB and KorC operators on the basis of their sequence alone, the IncP-1 plasmids were known for a long time to possess operons co-regulated with replication and stable inheritance functions, but with no known function. Many aspects of these co-regulated operons were conserved between the two IncP-1 subfamilies. The implication was that these operons should be part of the array of plasmid survival strategies, but there was little evidence for this. However, we discovered recently that KorB and IncC do not appear to function with full efficiency as active partitioning genes unless accompanied by additional genes encoded within the *kIe* and *kIc* operons whose expression is co-ordinated by KorA, KorB and KorC (Thorsted *et al.*, 1998; Bignell *et al.*, 1999). Although it is not known exactly what role the product of each of the genes plays, it is clear that co-regulation does imply a related or synergistic function. This would lead to the conclusion that, even when the function is not known, co-regulation of plasmid genes and conservation within a divergent family implies an important role in stable inheritance or propagation of the plasmid.

Conclusions and perspectives

DNA sequences are providing us with the basis for cataloguing the combinations and permutations by which plasmids are assembled from the available families of plasmid survival function. The patterns that emerge can be explained by reference to key systems in which the logic of organization has been analysed. There is great potential for sophistication to emerge by superimposing regulatory integration on top of physical clustering. An increasing number of examples are revealing the logic and value of this integration, and this may help to explain the logic of plasmid genomes as whole systems, rather than simply as a sum of the component parts, even if the individual parts can function alone. There are a growing number of examples in which different survival functions seemed to be interlocked in some way, for example the partitioning and replication functions of the Streptococcal plasmid pSM19035 (de la Hoz *et al.*, 2000; del Solar and Espinosa, 2000). Looking for the general features of both organization and regulation may help to define the logic that explains the way these systems have evolved and may provide insights into new systems as they are uncovered.

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