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Review

Mechanisms of plasmid stable maintenance with special focus on plasmid addiction systems✪

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The stable inheritance of bacterial plasmids is achieved by a number of different mechanisms. Among them are resolution of plasmid oligomers into monomers, active plasmid partitioning into dividing cells and selective killing of plasmid-free segregants. A special focus is given to the last mechanism. It involves a stable toxin and an unstable antidote. The antidotes neutralize their cognate toxins or prevent their synthesis. The different decay rates of the toxins and the antidotes underlie molecular mechanisms of toxin activation in plasmid-free cells. By eliminating of plasmid-free cells from the population of plasmid-bearing ones the toxin-antidote couples therefore act as plasmid addiction systems.

Plasmids are separate, autonomous genetic elements present in a cell independently of chromosomes. Most plasmids are small: from several to 100 kb, but sometimes they are so large that using the size criteria their distinction from the chromosome is difficult (e.g. in *Vibrio cholerae,* Yamaichi *et al.,* 1999; in *Rhizobium meliloti,* Honeycutt *et al.,* 1993). Different plasmids can constitute even up to 50% of bacterial DNA (e.g. in *Borrelia burgdorferi,* Fraser *et al*., 1997*; Bacillus cereus,* Carlson & Kolstø, 1994). It is commonly accepted that plasmid genes do not encode information indispensable for the functioning of the host cell. However, plasmids specify numerous features advantageous for the host in specific environments, such as resistance to harmful agents, ability to degrade rare compounds, pathogenicity, toxin production, nitrogen assimilation etc.

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Plasmids are very widely distributed among Prokaryota and, in general, are inherited with a high degree of stability. In special environmental conditions some plasmid genes confer a selective advantage. But, in many cases, plasmids are retained over generations without any selective pressure. Thus, there have to exist mechanisms which enable the maintenance of the plasmid during cell growth in nonselective conditions. Systems that contribute to this stability are encoded by DNA cassettes and are, in most cases, independent of one another. A particular plasmid can carry different stabilizing cassettes. Even more, cassettes from different plasmids may be combined to give a stable replicon.

RANDOM- AND BETTER-THAN-RANDOM PLASMID DISTRIBUTION

During the process of cell division plasmid copies are distributed between two descendants. If plasmid distribution within a growing cell is random, in the "ideal replicon", it is the number of plasmid molecules inside the dividing cell that determines the probability (P_0) that one of the daughters will be plasmid free (Nordström & Austin, 1989): $P_0 = 2^{(1-n)}$, where n is the number of plasmid copies. Therefore, a cell having 30 plasmid copies at the time of division would produce in 10^9 cells only two plasmid-free ones, whereas in the case of 5 copies, the chance of giving a plasmid-free cell is as much as 1 per 16.

As long as the plasmid copy number remains high, the subpopulation of plasmid-free cells is extremely limited. For low copy number plasmids obeying the random distribution law would mean that a significant fraction of daughter cells will be plasmid free. This is in contrast to the observed maintenance of plasmids in the host cells. Several mechanisms have been proposed, and shown to operate, to explain this discrepancy (see below). They can be divided into three classes: A, B and C.

A – **site-specific recombination systems** ensuring that plasmid multimers arisen during replication and(or) recombination will be resolved and thus every monomer copy will be independently subjected to random distribution;

B – **active partition process** which precisely distributes plasmid copies to each daughter cell at division;

C – **plasmid addiction systems,** e.g. functions that kill or reduce growth of plasmid-free descendant cells.

While mechanisms described in class A lead to the optimization of random plasmid distribution, class B and C mechanisms ensure better than random plasmid inheritance.

SITE-SPECIFIC RECOMBINATION SYSTEMS

Identical copies of a given plasmid frequently form oligomers. This reduces the number of independent units during segregation. Moreover, since origins of plasmid replication are selected at random (Summers *et al.,* 1993), the probability that plasmid monomers will be selected for replication is, for instance, two-fold lower than for plasmid dimers. Consequently, dimers will out-replicate monomers. The outcome of this 'dimer catastrophe' (Summers *et al.,* 1993) would be a considerable plasmid loss from the host cells. To ensure a plasmid distribution process to be highly efficient, there is a need for every plasmid copy to be accessible for distribution. This is achieved by site-specific recombination systems, also called **mrs** – **m**ultimer **r**esolution **s**ystems. In this process plasmid oligomers naturally formed during replication or recombination are resolved into monomers increasing the number of independent molecules accessible for distribution.

The multimer resolution systems consist of a site-specific recombinase, so called resolvase, and the defined nucleotide sequence *res* located on the plasmid. By specific recombina-

tion between repeated *res* sequences, the recombinase resolves oligomers to monomers. The mrs systems can be encoded entirely by the plasmid (e.g. *loxP-cre* of P1; Austin *et al.,* 1981) or combine the *res* site of the plasmid with a host encoded recombinase (e.g. *cer* of ColE1 and host *xerC, xerD, argR, pepA*; Summers & Sherrat, 1984; Sharpe *et al.,* 1999).

The multimer resolution systems of the *inc*18 plasmid family from Gram-positive bacteria, although encoded by plasmids, require the host factor Hbsu to mediate resolution (Rojo & Alonso, 1994; Alonso *et al.,* 1995; Janniére *et al.,* 1996).

In the majority of high-copy number plasmids, the multimer resolution system is the sole additional mechanism influencing their stable inheritance (for a review see Nordstrom & Austin, 1989; Hiraga, 1992; Summers, 1998). In combination with the copy number and cell division control it ensures a very low frequency of plasmid loss.

ACTIVE PARTITION

Many plasmids, especially those having moderate or low copy number, possess an active (i.e. energy consuming) mechanism for precise distribution prior to cell division of available plasmid copies to prospective daughter cells. This process, named by analogy to chromosome partitioning, as active partitioning, is very efficient (for example: the probability of loss of P1 prophage, which is present as one extrachromosomal unit in the cell, is <1 in 10^5 per generation; Abeles *et al.,* 1985).

Models for the active partitioning process include the pairing of plasmid molecules at *cis*-acting sites and subsequent separation of single plasmid copies to the opposite poles of the cell. The involvement of cellular components in the process of plasmid separation is proposed (Watanabe *et al.,* 1989; Bignell *et al.,* 1999).

The partition process is carried out by two proteins usually designated as A and B. The genetic organization of various partition systems is similar, with the gene for the A protein preceding the protein B-encoding gene and an adjacent centromere-like DNA sequence. As an example, the structure of the partition operon of P1 plasmid is shown (Fig. 1).

The partition operons are negatively autoregulated by protein A (i.e. plasmids P1, F) or protein B (i.e. plasmids NR1, R1, pTAR). The repressor activity of one protein can be enhanced by the other. The tight regulation of *par* operons expression is very important since an excess of protein B leads to plasmid destabilization (i.e. SopB of F plasmid; Kusukawa *et al.,* 1987; ParB of P1; Łobocka & Yarmolinsky, 1996). Protein B excess can also silence genes flanking the *cis*-acting sites (up to several kilobases away) by polymerization along the DNA starting from a certain point,

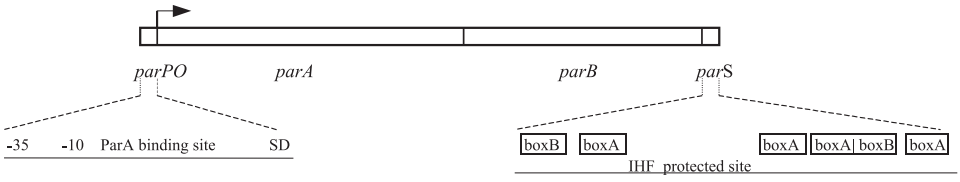

Figure 1. Schematic representation of the *par* operon organization of P1 plasmid.

The promoter–operator *parPO* and *parS* regions are blown up. Binding sites for ParB protein are boxed. Broken arrow indicates the promoter.

as has been shown for SopB (Lynch & Wang, 1995) and ParB (Rodionov *et al.,* 1999).

All three components, partition proteins A, B and *par* site, are required for partition. The ParB proteins bind specifically to their cognate *cis*-acting DNA sequences termed centromere-like regions (Mori *et al.,* 1989; Watanabe *et al.,* 1989). These *cis*-acting sites are located downstream (plasmids P1, F, RK2) or upstream (NR1, R1, pTAR) from *par* genes. Their structure is unique for different plasmids, but always contains a characteristic number of iterated repeats which are the sites for specific binding of protein B.

A motif characteristic for proteins showing ATPase activity is found in partition A proteins suggesting their role in providing energy necessary for the process (Motallebi-Veshareh *et al.,* 1990; Davies *et al.,* 1991). On the basis of sequence similarity ParA proteins from different plasmids can be grouped into two quite distinct families: those with conserved Walker motif of ATPases and those showing homology to actin-like ATPases (Gerdes *et al.,* 2000). Based on sequence analysis of proteins related to the ParF protein from the multidrug resistance plasmid TP228, Hayes proposed recently (Hayes, 2000) the existence of a third distinct subgroup of the ParA superfamily, evolutionarily related to the MinD subgroup of cell division proteins. Proteins of the ParB group are significantly more diverse and difficult to classify.

Recent experiments with fluorescence microscopy have visualized *bona fide* plasmid positioning during the time of cell growth at one- and three-quarter distance to cell poles, (plasmids F, Kim & Wang, 1998; P1, Erdmann *et al.,* 1999; and RK2, Bignell *et al*., 1999) or at the mid-cell with subsequent movement to opposite cell poles (plasmid R1, Jensen & Gerdes, 1999), correlated with the presence of ParB proteins. The factors involved in the process of plasmid DNA movement are still unknown.

Chromosomal homologues of plasmid partition proteins have been identified in many bacteria, including *Bacillus subtilis*, *Caulobacter crescentus* and *Pseudomonas putida*. Their role in chromosome segregation has been shown for example in *B. subtilis* (*locus soj-spo0J*; Sharpe & Errington, 1996; 1998) or *C. crescentus* (genes *parA, parB* and *parS*; Mohl & Gober, 1997). In spite of a functional similarity between chromosome and plasmid partitioning these two processes proceed distinctly (Gordon *et al.,* 1997).

PLASMID ADDICTION SYSTEMS

Independently of the processes that increase the probability of receiving a plasmid by a dividing cell there are very special strategies adopted by plasmids preventing plasmid-free segregants from surviving.

The idea of an "addiction" mechanism leading to very efficient plasmid maintenance comes from Koyama (Koyama *et al.,* 1975). In his considerations he pointed out that if cells that lose an established plasmid die, the population would never contain viable cured cells. Terms like ***killer system***, ***killing-anti-killing, post-segregational killing, toxin-antitoxin, poison-antidote, plasmid addiction system*** or ***programmed cell death*** are all used to describe the situation when the host cell is selectively killed if it has not received any copy of the plasmid.

The molecular basis of this killing requires the existence of at least two plasmid genes: one specifying a stable toxic agent and another coding for an unstable factor which prevents the lethal action of the toxin. While the toxin is always a protein, the antidote is either antisense RNA (which inhibits translation of toxin mRNA) or a protein (Jensen & Gerdes, 1985).

Antisense RNA regulated plasmid addiction systems

Plasmid stabilization systems regulated by antisense RNA constitute a well conserved

group called the ***hok-sok*** family (the name reflecting functions of the ***ho**st* ***k**illing* and ***s**uppression* ***o**f* ***k**illing* genes from plasmid R1) extensively studied and described in detail by Gerdes (Gerdes *et al.*, 1997). This family has been found only in Gram-negative bacteria (see Table 1).

Sok-RNA is very labile (half-life about 30 sec), *hok* mRNA is stable for hours (Gerdes *et al.*, 1988).

The *hok* mRNA exists in a plasmid-carrying cell in two forms: the inert full-length and a shorter active one. In full-length *hok* mRNA the "*f*old-*b*ack-*i*nhibition" element (*fbi*) present

Table 1. Plasmid- and chromosome-encoded *hok* homologues

Locus	Replicon	References
hok/sok	R1	Gerdes *et al.*, 1990
flm	F	Loh *et al.*, 1988
*srn*B	F	Ono *et al.*, 1986
pnd	R483	Ono *et al.*, 1987; Gerdes *et al.*, 1992
pnd	R16	Sakikawa *et al.*, 1989; Gerdes *et al.*, 1992
pnd	R64	Furuya & Komano, 1996
*hok*A	*E. coli* C	Pedersen *et al.*, 1997
*hok*X	*E. coli* B	Pedersen & Gerdes, 1999
*hok*B	*E. coli* K12	Gerdes *et al.*, 1997
*hok*C (*gef*)	*E. coli* ECOR24	Poulsen *et al.*, 1989
*hok*D (*rel*F)	*E. coli* K12	Bech *et al.*, 1985; Gerdes *et al.*, 1986
*hok*E	*E. coli* K12	Gerdes *et al.*, 1997
*hok*H	*H. alvei*	Fecker *et al.*, 1997 Gerdes *et al.*, 1997

A model of the genetic organization of the *hok-sok* system is presented in Fig. 2.

The locus consists of three juxtaposed genes: *hok* specifying a toxin, ***mok*** ***(m**odulation* ***o**f* ***k**illing)* required for expression and regulation of *hok* translation and *sok* coding for antisense RNA complementary to the *hok* mRNA leader region. Whereas

Figure 2. Schematic representation of the *hok-sok* system organization.

The shadowed box represents Hok toxin; open box, the *mok* regulatory gene; Sok-RNA, antisense RNA, and *sokT*, Sok-RNA target region. The thick, thin and broken arrows represent *hok* mRNAs, *sok* antisense RNA and promoter, respectively. In the full length mRNA the presence of *fbi*, the *f*old-*b*ack-*i*nhibition element, is indicated.

at 3′ end pairs with the 5′ end giving an RNA structure inactive both in translation and antisense RNA binding (Thisted *et al.*, 1994a). In this form *hok* mRNA is accumulated inside the cell. The full-length stable form is slowly processed at the 3′ end (Thisted *et al.*, 1994b). After removal of the *fbi* element, a refolded structure of *hok* mRNA is accessible for antisense RNA binding and for translation. The constitutively expressed 67nt Sok-RNA binds to the leader sequence of *hok* mRNA. This complex is immediately cleaved by RNase III (Gerdes *et al.*, 1992), thus impeding translation of *hok* mRNA. In a plasmid-free cell, the absence of Sok-RNA (degraded by RNaseE) enables the translation of *hok* mRNA.

Hok-like proteins are small (about 50aa), membrane-associated polypeptides. Overexpression of Hok leads to a decrease of cell membrane potential, arrest of respiration and efflux of small molecules, resulting in cell

death (giving rise to characteristic "ghost" in phase-contrast microscopy).

The *hok-sok* systems may be very efficient. The stabilization of unrelated replicons ranges between 50 (for high copy number plasmid) and 10000 fold (for low copy number plasmids), as compared to random distribution (Wu & Wood, 1994).

A number of homologues of *hok-sok* genes from R1 plasmid system have been identified on other plasmids (see Table 1). If tested, all of them were able to post-segregational killing in a manner similar to R1. In contrast, homologues of this family found on bacterial chromosomes are not functioning as post-segregational killing agents. However, when placed on a plasmid they clearly showed all characteristics of the *hok-sok* genes products including the active toxic transmembrane protein (e.g. *hocC* (=*gef*) (Poulsen *et al*., 1989) and *hocD* (=*relF*) (Gerdes *et al*., 1986). The origin and possible functions of these genes, widely distributed in bacterial chromosomes, remain mysterious.

The antisense RNA-regulated stability determinant has been also found in Gram-positive bacteria. This determinant, designated *par*, stabilizes the enteroccocal plasmid pAD1 (Weaver *et al*., 1996) and has no homology to the *hok-sok* family. The 400nt long *par* region encodes two small, convergently transcribed RNAs (210nt long RNA I and 65nt long RNA II), with 33 codons for the ***fst*** (***f****aecalis* plasmid-***s****tabilizng* ***t****oxin*) peptide inside the longer RNA I. The smaller RNA II shows high degree of complementarity to RNA I. Complex formation between RNA I and the antisense RNA II was recently demonstrated *in vitro* (Greenfield & Weaver, 2000). In this complex RNA II inhibits *fst* translation by preventing ribosome binding to the SD sequence sequestered between complementary direct repeats.

The half-life of RNA II (about 10 min) is substantially shorter then that of RNA I (>1 h) (Greenfield *et al.,* 2000) as expected for antisense-regulated stabilizing systems. Overproduction of RNA I (or Fst peptide) causes host cell death (Weaver & Clawell, 1989). The lethal action of Fst can be prevented by supplying RNA II in excess (Weaver *et al.,* 1996).

PROTEIC PLASMID ADDICTION SYSTEMS (PPAS)

Common features of the proteic plasmid addiction systems

As mentioned above, in PPAS both the toxin and its antagonist (preventing agent) are proteins. Its functioning as the post-segregational killing system is based on different decay rates of two proteins involved: the toxin is much more stable than the antidote. This can be shown by provoking synchronous plasmid curing by inhibiting plasmid replication without affecting cell growth and division (Jensen *et al.,* 1995).

The antidote prevents the lethal action of its cognate toxin by forming a tight complex with it. So, as long as the antidote protein remains in excess relative to the toxin, the activity of the toxin is blocked. In a cured cell, when *de novo* synthesis of plasmid encoded proteins is impossible, the concentration of antidote decreases quickly due to degradation by cellular proteases. The toxin, now released from the complex, executes its toxicity causing the cell death. Thus, in a population only cells carrying the plasmid will exist (Fig. 3).

All PPAS have similar genetic organization, shown schematically in Fig. 4.

In all described cases the genes constituting proteic killer systems are organized in operons. With one exception of pRts1 (Tian *et al.,* 1996), the first cistron encodes the antidote and the second the toxin.

An important characteristic of these operons is their autoregulation at the transcriptional level. The antidote protein itself or in a complex with the toxin is a repressor of the promoter in question. In the majority of cases, toxins enhance the promoter repression by the antidote (e.g.: plasmid P1,

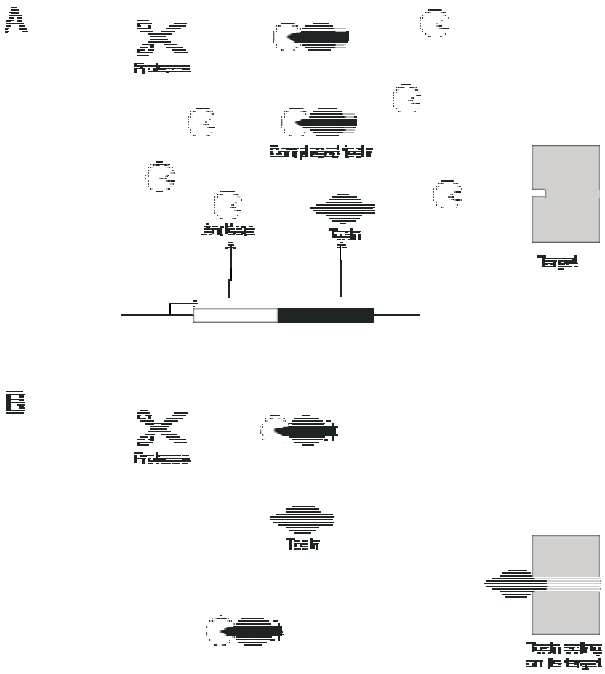

Figure 3. The principle of PPAS functioning.

A. In a plasmid-bearing cell: both proteins, the antidote and the toxin, are produced. The antidote (constantly present in excess) neutralizes the toxin by complex formation. **B.** In cell after plasmid loss: the synthesis of both, the antidote and toxin proteins does not occur. The antidote becomes degraded by cellular protease(s) and the toxin remains free. The uncomplexed toxin acts against its cellular target.

Magnuson & Yarmolinsky, 1998; R1, Ruiz-Echevarria *et al.,* 1991b; pTF-FC2; Smith & Rawlings, 1998). In case of F1 plasmid, a complex of both the CcdA and CcdB proteins is needed for the autoregulation of the *ccd* operon (Tam & Kline, 1989). In contrast, the autoregulation of the PPAS encoded by pSM19035 plasmid does not include neither the antidote nor the toxin; it is achieved by a third component that exclusively plays a role of the transcriptional repressor (de la Hoz *et al.,* 2000). Precise autoregulation seems to be a prerequisite for proper functioning of the stabilizing systems.

The antidote proteins are present in excess to the toxins in bacterial cells. Their half-life times *in vivo* are significantly shorter than those of the toxins due to degradation by cellular proteases, as indicated in Table 2.

Proteins of PPAS are generally rather small (about 70aa–130aa), the toxin usually being slightly bigger than the corresponding antitoxin. The only exceptions are the *hig* locus of Rts1 plasmid with a 104aa antidote *versus* a 90aa toxin and a large toxin Zeta protein (287aa) in the ω-ε-ζ system of pSM19035 (see Fig. 4). All PPAS toxins act from within the host cell.

In spite of the structural and functional similarity of various PPAS systems there is no significant sequence identity among their genes. The only weak amino-acid sequence similarity was detected between CcdA of F plasmid and Kis of R1 (Ruiz-Echevarría *et al.,* 1991a) as well as between ParD of RK2 and PasA of pTF-FC2 (Smith & Rawlings, 1997). Instead, it is very common to find highly conserved sequences responsible for plasmid stability on many related plasmids which probably illustrates horizontal transfer of genes between different plasmids and bacteria (Gerdes, 2000; Hayes, 1998).

There is no uniform pattern for the placement of stability cassettes on plasmids, although in a few cases a position close to the origin of replication (e.g. *ccd* of F plasmid and *phd/doc* of P1 plasmid) could be pointed out.

Names given to particular stabilizing loci are not consistent. In the majority of cases they try to describe the observed behaviour of cells when stabilizing genes are activated. As, historically, the function of this type of genes was difficult to distinguish from partitioning, quite often the misleading name ***par*** is used for stability genes. The ***stb*** abbreviation of *stability* becomes frequent in recent literature.

EXAMPLES OF THE BETTER CHARACTERIZED PROTEIC PLASMID ADDICTION SYSTEMS

ccd locus of plasmid F

The *ccd locus* of plasmid F is the best characterized post-segregational proteic killer system. The large, 95 kb conjugative plasmid F

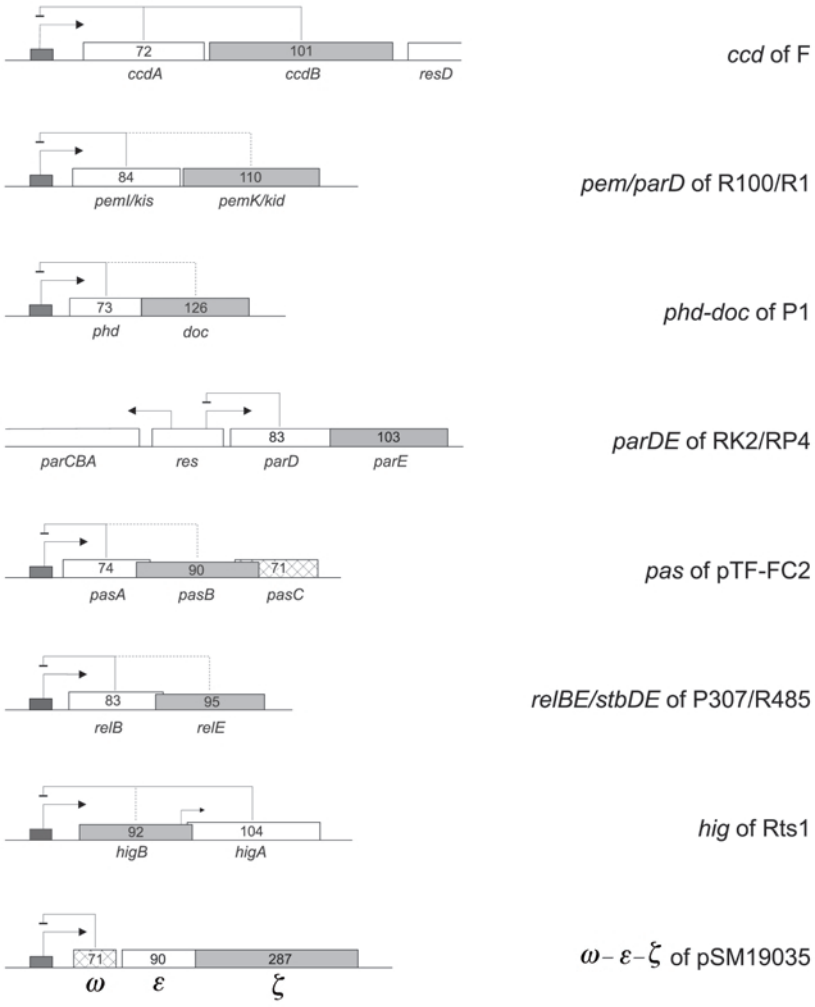

Figure 4. Genetic organization of PPAS operons.

Bars represent: open — antidotes, grey — toxins and hatched — additional elements, respectively. Arrows indicate promoters. Autoregulatory circuits are shown for every system. Continuos and discontinuos lines indicate genes directly involved in autoregulation and enhancement of autoregulatory activity, respectively. See text for details.

exists in *E. coli* cells in only one or two copies per chromosome. Yet it is extremely well maintained in a growing population. This high degree of stability plasmid F achieves by combining the effects of multiple control elements: two functional replication systems, several site-specific recombinases, the active partition region *sop* and three post-segregational killing systems (Nordström & Austin, 1989). The ***flm*** (***F l****eading* ***m****aintenance*) locus and ***srnB*** *(****s****table* ***RN****A degradation)* locus are homologous to the *hok/sok* family regulated by antisense RNA (Golub & Panzer, 1988; Ohnishi *et al.,* 1977; Nielsen *et al.,* 1991).

The name of the ***ccd*** locus *(****c****oupled* ***c****ell* ***d****ivision)* originates from the observation (Ogura & Hiraga, 1983; Miki *et al.,* 1984) that cell division is dependent on previous replication of F plasmid. This inadequate description was later deciphered using the same letters as ***c****ontrol of* ***c****ell* ***d****eath* (Van Melderen *et al.,* 1994). Indeed, it was shown (Jaffé *et al.,* 1985) that genes of the *ccd* locus mediate plasmid maintenance primarily by killing plasmid-free segregants.

The *ccd locus* is composed of two genes: ***ccdA*** and ***ccdB*** (also called ***H*** and ***G*** or ***letA*** and ***letB***, respectively) encoding the correspond-

Table 2. Examples of proteic plasmid addiction systems

System	Plasmid	Toxin	Antidote	Protease degrading antidote	Target	Reference
ccd	F	CcdB	CcdA	Lon	gyrase	Jaffé *et al.*, 1985
pem/parD	R100/R1	PemK/Kid	PemI/Kis	Lon	DnaB	Tsuchimoto *et al.*, 1988
phd-doc	P1	Doc	Phd	ClpXP	unknown	Bravo *et al.*, 1987 Lehnherr *et al.*, 1993
parDE	RK2/RP4	ParE	ParD	unknown	unknown	Roberts *et al.*, 1990
pas	TF-FC2	PasB	PasA	Lon	unknown	Smith & Rawlings, 1997
relBE/stbDE	P307/R485	RelE/StbE	RelB/StbD	Lon	unknown	Grønlund & Gerdes, 1999
hig	Rts1	HigB	HigA	unknown	unknown	Hayes, 1998 Tian *et al.*, 1996

ing proteins: the CcdA antidote and the CcdB toxin (Bex *et al.,* 1983; Miki *et al.,* 1984). These genes are organized in an operon together with the resolvase-encoding gene ***resD*** (Lane *et al.,* 1986). Expression of the operon is negatively controlled by a complex of the two Ccd proteins (de Feyter *et al.,* 1989). The domains of both proteins involved in autoregulation are different from those responsible for the killing-antikilling effect (Salmon *et al.,* 1994; Bahassi *et al.,* 1995). In the absence of the CcdA antidote, CcdB causes a reduction in DNA synthesis (Jaffé *et al.,* 1985), activation of the SOS regulon (Karoui *et al.,* 1983; Reece & Maxwell, 1991), cell filamentation (Miki *et al.,* 1984), formation of anucleate cells and, as a consequence, cell death (Jaffé *et al.,* 1985).

Bacterial mutants resistant (Bernard & Couturier, 1992) and tolerant (Miki *et al.,* 1992) to the lethal activity of CcdB were altered in the *gyrA* gene. The product of *gyrA* constitutes a part of an essential enzym – gyrase, responsible for introducing negative supercoils in DNA. During the breaking and rejoining reaction, the 5′-ends of substrate DNA are covalently linked to the A subunit of gyrase. The CcdB toxin traps the cleaved DNA–gyrase complex and impedes the resealing of the nicked DNA (Bernard & Couturier, 1992; Bernard *et al.,* 1993). Formation of double-strand breaks in the DNA, in turn, induces the SOS response.

In addition, the CcdB protein is a direct inhibitor of gyrase (Miki *et al.,* 1992). In the absence of covalently bound DNA CcdB binds to free GyrA subunit making gyrase catalytically inactive. Both CcdB activities are suppressed by the same mutations in GyrA (substitution R462C leading to resistance to CcdB; Bernard *et al.,* 1993) and in CcdB (mutations in the three carboxy-terminal amino acids that annul the lethal activity of CcdB; Bahassi *et al.,* 1995).

The recently determined crystal structures of a large, 59 kDa, fragment of the amino-terminal region of GyrA (Berger *et al.,* 1996) have led to a model for the GyrA–CcdB complex formation (Lorris *et al.,* 1999). The model accommodates the critical C-terminus of the toxin dimer in the central hole of a GyrA dimer during its cycling on DNA, impeding the closing stage and therefore the religation of nicked DNA. The CcdA antidote not only prevents this complex formation, but also releases CcdB from inactive complexes by forming a new tight CcdA–CcdB complex (Bernard *et al.,* 1993; Maki *et al.,* 1996).

The ATP-dependent serine protease Lon is responsible for degradation of CcdA. The half-life of the CcdA protein was estimated to

be about 1 h, whilst CcdB remained stable for over 2 h (Van Melderen *et al.,* 1994).

Among the F plasmid encoded stability cassettes, the *ccd* system is a rather inefficient one: only 10-fold stabilisation is achieved, compared to 100-fold for *flm* or 1000-fold for *sop* (Jensen *et al.,* 1995; Boe *et al.,* 1987). Perhaps, as the CcdB toxin primarily induces the SOS regulon, rather than immediate cell death, part of its role may be to generate genetic diversity in those bacteria which do not have the F plasmid after division (Couturier *et al.,* 1998).

pem/parD locus of plasmids R100/R1

This is the second (and so far the last) PPAS with a known cellular target of lethal action. Loci described as ***pem*** ***(p****lasmid* ***e****mergency* ***m****aintenance)* for plasmid R100 (Tsuchimoto *et al.,* 1988) and as ***parD*** for plasmid R1 (Bravo *et al.,* 1987) encode the antitoxin **PemI** identical to **Kis** ***(ki****lling* ***s****uppressor)* and toxin **PemK** identical to **Kid** ***(ki****lling* ***d****eterminant).*

The *pem/parD* operon possesses the canonical features of PPAS: it encodes two small proteins with the gene for the antitoxin preceding that for the toxin; it is autoregulated at the level of transcription by a complex of the two proteins involved and the decay time for these proteins is different. The degradation of the antidote PemI/Kis is due to the Lon protease (Tsuchimoto *et al.,* 1992).

In vitro, it has been shown that the Kis and Kid proteins form a complex. Also, DnaB-dependent initiation of plasmid ColE1 replication was specifically inhibited by the addition of Kid. The replication was restored when the antidote Kis was added. *In vivo,* inhibition of lytic λ prophage induction (whose replication initiation is DnaB dependent) was observed after inactivation of the antagonist protein Kis. Furthermore, overproduction of DnaB suppressed the lethal action of Kid *in vivo* (Ruiz-Echevarría *et al.,* 1995). Taking this into account, it seems well documented that the cellular target of the action of the PemK/Kid protein is DnaB helicase. The primary effect of the *pem/parD* system in a majority of strains is cell division inhibition rather than killing of the plasmid-free segregants (Tsuchimoto & Ohtsubo, 1989; Jensen *et al.,* 1995) thus explaining its modest, less than 10-fold, plasmid stabilization.

phd-doc locus of bacteriophage P1

Bacteriophage P1 lysogenizes *E. coli* cells as an extremaly low-copy number plasmid. Its stable maintenance within bacterial cells is ensured, in part, by an addiction mechanism mediated by two specific proteins: the antidote Phd (***p****revent* ***h****ost* ***d****eath*) and the toxin Doc (***d****eath* ***o****n* ***c****ure*), (Lehnherr *et al.,* 1993). The corresponding genes ***phd*** and ***doc*** are organized in an operon, the transcription of which is negatively autoregulated by Phd (two dimers of the Phd protein bind to the operator, Gazit & Sauer, 1999) and enhanced by the Doc protein (Magnuson & Yarmolinsky, 1998).

Free Phd protein (unbound to the operator or Doc) exists predominantly in an unfolded conformation and is subject to degradation by the ATP-dependent serine protease ClpXP (Lehnherr & Yarmolinsky, 1995). Physical binding between the prophage stabilizing proteins was shown for a nontoxic mutant of Doc *in vitro* (Gazit & Sauer, 1999). The cellular target of the action of Doc remains unknown but under conditions of its action the protein synthesis is inhibited. Recently it was demonstrated that post-segregational killing induced by *phd-doc* addiction genes requires *mazEF* chromosomal system (Hazan *et al.,* 2001). The *phd-doc* system stabilizes mini P1 plasmid about 7-fold (Lehnherr *et al.,* 1993).

parDE locus of plasmids RK2/RP4

The broad-host-range plasmid RK2 (60kb, identical to the plasmid RP4, IncP incompatibility group) is maintained at the relatively low number of 5–8 copies per chromosome

and is very stable in a wide range of Gram-negative bacteria.

The investigation of separate loci involved in the stable maintenance of plasmids from the IncP incompatibility group is extremely difficult because of the interrelated co-ordinated regulation of the vital functions by the plasmid central control region *ccr*. The juxtaposited influence of the regulatory proteins KorA and KorB on transcription of many promoters makes the final effect of the propagation of IncP plasmids particularly dependent on the host strain and conditions of cultivation.

The ***parDE*** operon responsible for post-segregational killing of plasmid-free cells is located inside the ***par*** locus together with the ***parCBA*** operon encoding a site-specific recombination system (Grinter *et al.,* 1989; Roberts *et al.,* 1990; Roberts & Helinski, 1994).

Expression of the ParE protein in the absence of ParD leads to growth inhibition and filamentation of the cells. An uncharacterized region *psa* has been postulated to be responsible for growth arrest not involving filamentation (Jovanovic *et al.,* 1994). The cellular target of the toxin is not known. The effect of the functioning of the *parDE* operon on bacterial growth and morphology is dependent on the host strain and the type of medium in a manner which is not well understood (Roberts *et al.,* 1994).

Both proteins, ParD and ParE, exist as dimers in solution and form tetrameric complexes *in vitro* (Johnson *et al.,* 1996). The antidote ParD negatively regulates transcription of the *parDE* operon (Roberts *et al.,* 1993).

In its natural context, the expression of the *parDE* operon might be subject to global regulation by the KorA and KorB proteins (Pansegrau *et al.,* 1994). Perhaps this is the reason that, unexpected for proteic killer system, when tested in a control system (Jensen *et al.,* 1995) containing the *parDE* operon only, this cassette exerts a 100-fold stabilizing effectiveness. The sole effect of stabilization in the case of conjugal plasmids may be additionally masked by the simultaneous transfer of the plasmid to the plasmid-free cells (Easter *et al.,* 1997).

The RK2 plasmid also carries several potentially host-lethal ***kil*** *loci* ***(kilA, B, C*** and ***E*** also denominated as ***kla***, ***klb***, ***klc*** and ***kle***). *kilB* is involved in conjugal transfer (Motallebi-Veshareh *et al.,* 1992). The function of other *kil* genes is unknown, but their unregulated expression can lead to the death of host *E. coli* cells (Figurski *et al.,* 1982).

An intriguing question whether the *parDE* operon works in concert with the second *par* operon *parCBA* comes from the comparison with the resolution of dimer chromosomes coupled to cell division (Steiner & Kuempel, 1998).

pas locus of plasmid pTF-FC2

An unusual proteic plasmid addiction system has been described for the broad host range plasmid pTF-FC2 from the biomining bacterium *Thiobacillus ferrooxidans*.

The ***pas*** ***(p****lasmid* ***a****ddiction* ***s****ystem)* locus consists of three genes: ***pasA, B*** and ***C***. *PasA* encodes an antitoxin, *pasB* a toxin, and *pasC* a protein which enhances the ability of the PasA antidote to neutralise the toxic effect of PasB. Nevertheless, the pair PasA and PasB is equally efficient in plasmid stabilization in the presence or absence of PasC (Smith & Rawlings, 1997).

The proteins PasA and PasB, but not PasC, are required for the full repression of the *pas* operon (Smith & Rawlings, 1998b). Precise autoregulation of the *pas* operon is very important for its functioning – placing the *pas* genes under a heterologous promoter results in a complete loss of the test plasmid stability (Smith & Rawlings, 1998b).

The efficiency of the pTF-FC2 *pas* poison-antidote stability system in *E. coli* is greatly affected by the host strain: from 100-fold in CSH50 to no increase in JM109. The Lon protease is responsible for degrada-

tion of the antidote PasA (Smith & Rawlings, 1998a).

Although the specific action of the PasB toxin is still unknown and the stabilization efficiency is modest, the *pasABC* system undoubtedly falls into the bactericidal category as shown in *in vivo* experiments (Smith & Rawlings, 1997).

The pTF-FC2 PasA antitoxin is poorly but clearly related (31% aa identity) to the ParD antitoxin of the *parDE* system of RK2 plasmid.

relBE/stbDE locus of plasmids P307/R485

Recently discovered on enteropathogenic plasmid P307 from *E. coli,* the stability system ***relBE*** shares about 50% sequence identity with ***stbDE*** genes on R485 plasmid from *Morganella morganii* (Grønlund & Gerdes, 1999; Hayes, 1998).

Its structural organization is canonical for the toxin-antitoxin genes family with the autoregulatory ***relB*** gene encoding an antidote and the overlapping ***relE*** gene encoding a toxin. When overproduced, the *relE* gene causes immediate decline in viable counts over 5 orders of magnitude, indicating that RelE is a very potent toxin. The killing by RelE can be counteracted by the induction of RelB expression. The protease responsible for RelB degradation is Lon. The *relBE* operon is able to stabilize a mini-P307 replicon.

Homologues of the *relBE/stbDE* operon have been found on Gram-negative bacterial chromosomes. The ***relBEF*** operon of *E. coli* chromosome was found to be affected in stringent control during amino-acid starvation (so called "delayed relaxed response", Lavallé, 1965). *RelB* mutants had an increased level of *relBEF* mRNA and the growth recovered very slowly after the termination of amino acid starvation (Bech *et al.,* 1985). In this context the proposed action for the RelE toxin is inhibition of translation (Gotfredsen & Gerdes, 1998).

The locus *relBE,* at the moment of discovery assigned only to pathogenic bacteria, now seems unexpectedly widely distributed among prokaryotic chromosomes.

hig locus of plasmid Rts1

Compared with other proteic killer genes, the ***hig (host inhibition of growth)*** locus of plasmid Rts1 is unique in that the toxic part ***higB*** lies upstream of the antidote gene ***higA***.

The protein products of the two overlapping genes have been identified: the 92aa killer factor of *higB* and the 104aa antitoxin of *higA*. HigA suppresses the HigB function both in *cis* and in *trans*. Two promoters were shown to be active in the *hig* operon: a stronger one upstream of *higB,* negatively regulated by the HigA antidote, and a weaker but constitutive one upstream of *higA,* within the *higB* coding region (Tian *et al.,* 1996b).

The cellular target of the action of HigB remains unknown.

ω-ε-ζ locus of plasmid pSM19035

The ω-ε-ζ operon of the low-copy-number, broad-host-range plasmid pSM19035 from *Streptococcus pyogenes* encodes the only proteic addiction system discovered in Gram positive bacteria. It consists of three genes: ω, ε and ζ. Two of them, ε and ζ, encode PPAS with Zeta (the product of ζ) acting as a toxin and Epsilon (the product of ε) as an antidote. This system is unique in that neither the antidote nor the antidote–toxin complex are involved in regulation of their own synthesis. The autoregulatory functions are exclusively played here by a third component of the operon, ω gene. It has been shown that ω is also implicated in regulation of expression of two other pSM19035 genes: δ and *copS*. Delta belongs to a family of ATPases involved in active partitioning and CopS regulates plasmid copy number. Hence, Omega-repressor links random (plasmid-copy-number control) and

better-than-random (PPAS, putative partitioning) inheritance of pSM19035 (de la Hoz *et al.,* 2000).

The ω-ε-ζ operon was shown to stabilize heterologous plasmids in Gram-positive bacterium *B. subtilis* and less efficiently in Gram-negative *E. coli*. In both organisms the toxic effects of the excess of protein Zeta were counteracted by the proper expression of the gene ε. In *B. subtilis* the activity of protein Zeta was bactericidal whereas in *E. coli* it was mainly bacteriostatic (Zielenkiewicz *et al.,* 2000). The interaction between Epsilon and Zeta proteins was detected *in vivo* in yeast two-hybrid system (Gerdes *et al.,* 2000). It is important to note the broad spectrum of the toxicity of protein Zeta: it acts against Gram-positive and Gram-negative bacteria as well as eukaryotic *S. cerevisiae* (Zielenkiewicz, unpublished).

The cellular target of the toxin Zeta remains unknown.

CHROMOSOMALLY LOCATED TOXIN-ANTITOXIN GENES

Many of the addiction genes found in plasmids have homologues on bacterial chromosomes. Systematic inspection of data-bases reveals that they are present in every systematic group of Prokaryota, including Archaea. Moreover, several paralogues of toxin-antitoxin genes are frequently repeated on the same chromosome (Gerdes, 2000).

When cloned into plasmids, these genes are able to stabilize an unstable replicon by post-segregational killing. For example, one of the two *E. coli* homologues of the *pem locus,* called ***chpA*** *(*or ***mazEF)*** located in the *rel* operon was tested in detail for its killer properties (Aizenman *et al.,* 1996). After cloning on a plasmid it was clearly demonstrated that these genes possess all of the properties required for an addiction module. MazE is a labile protein degraded by the ClpAP protease and protects the bacterial cell from the toxic effect of the stable MazF protein. Both proteins interact directly. Expression of *mazEF* is regulated by the cellular level of ppGpp, product of the activity of the protein encoded by *relA* gene. As shown in recent papers (Sat *et al.,* 2001; Hazan *et al.,* 2001) inhibition of transcription and/or translation caused by stress conditions (starvation, action of some antibiotics) or the activity of another PPAS, *phd-doc,* triggers *mazEF* lethality by preventing *de novo* synthesis of the short-lived antitoxin.

Irrespective of only partial amino-acid sequence identity, the ChpAI (MazE) or ChpBI (the second homologue of PemI) antitoxin can functionally interact with the PemK/Kid toxin (Santos-Sierra *et al.,* 1997).

Recently, a new widely distributed family of genes, homologous to *E. coli* ***relBE*** has been classified as toxin-antitoxin modules (Gotfredsen & Gerdes, 1998). Again, by use of a plasmid model, it was shown that *relB* specifies an antitoxin that prevents the lethal action of *relE*-encoded toxin; the protein RelB autoregulates the *relBEF* operon at the level of transcription and the genes *relBE* stabilize a mini-R1 test plasmid. The third gene of this operon, ***relF,*** potentially codes for a toxin belonging to the Hok family (Gerdes *et al.,* 1986), although the cistron is not translated. Additionally, the *relBE* genes are clearly homologous to many operons located on different plasmids, some of them with a well documented function of an addiction system (e.g. P307; Grønlund & Gerdes, 1999, R485; Hayes, 1998, pTF-FC2; Smith & Rawlings, 1997).

BACTERIOCINS AND OTHER OUT ACTING AGENTS ENCODED BY PLASMID

Antagonistic interactions between related microorganisms have been noticed since the very beginning of bacteriological studies. In time, substances produced by bacteria that inhibit the growth of other vicinal bacteria from

closely related species received the name bacteriocin.

Production of many bacteriocins is encoded on plasmids together with correspondent gene(s) specifying the immunity to them. The toxic protein is secreted outside the cell, adsorbs to specific outer membrane receptors of the sensitive cell and, consequently, inhibits or kills this cell. The bacteriocin producing cells are resistant to their own toxin thanks to the immunity gene present on a plasmid and expressed at the same time with the gene for the toxin. The detailed information on the function of bacteriocins can be found in recent reviews (Jack *et al.,* 1995; Jack & Jung, 2000).

The mechanism of stabilization achieved by bacteriocin killing is only superficially similar to post-segregational stabilization by addiction systems. Whereas plasmid addiction systems do not permit the progeny without a plasmid to arise, plasmids producing toxins acting from outside protect plasmid-bearing cells from competition by eliminating incoming plasmid-free cells.

To some extent, this is similar to many other phenomena like antibiotic production, pathogenicity, yeast killer viruses and the *Kluyveromyces* killer plasmids.

RESTRICTION-MODIFICATION SYSTEMS (R–M) AS STABILITY DETERMINANTS

A restriction endonuclease (R) recognizes a specific short sequence on duplex DNA and makes double-strand break within or close to it, unless the modification enzyme (M) methylates this sequence protecting it from the cleavage. R–M gene complexes are abundant in Prokaryota, including Archaea, present equally on chromosomes and plasmids. One bacterial cell can contain multiple R–M gene pairs.

R–M systems are believed to have evolved to protect cells from foreign DNA like viruses or plasmids. The incoming DNA is not modified at specific sequences and will be cleaved by the restriction enzyme present in the cell. But, this "cellular defense" hypothesis can hardly explain the exceptional diversity and specificity of recognition sequences. For some "rare cutter" restriction enzymes it is difficult to find foreign DNA containing such sequences.

It is also true that the R–M pairs are not easily lost from their host cell. Those of the descendant cells that lose an RM gene cluster are unable to modify a sufficient number of recognition sequences in their chromosome to protect them from the lethal attack by the remaining restriction enzyme.

When present or moved on a plasmid, R–M gene pairs can increase apparent plasmid stability in a post-segregational host killing manner. Naito *et al.* (1995) have demonstrated that plasmids carrying *Eco*RI or *Pae*R7 R–M gene pairs (r^+m^+) are considerably more stable than the vector alone or plasmids with the modification gene only (r^-m^+). When the replication of r^+m^+ plasmids was temperature arrested, the number of viable cells ceased to increase as well as the number of plasmid carrying cells. The proposed explanation for the cell death after r^+m^+ plasmids loss is the cleavage of the insufficiently methylated host chromosome. It was confirmed by direct observation of chromosomal DNA degradation in cured cells.

It has been experimentally demonstrated that two R–M systems of the same sequence specificity cannot simultaneously force their maintenance on a host (Kusano *et al.,* 1995). That could be the basis of the quick evolutionary diversification of the R–M genes.

From this perspective the restriction enzyme and its cognate methylase are like a toxin-antidote pair. The only difference is that the restriction enzyme is not interacting with the methylase directly. However, as the methylase is protecting the cellular target from the restriction enzyme attack, the latest effect of the R–M pair presence is apparent stabilization of plasmid carrying them.

All these observations allow the R–M genes to be seen as selfish elements, evolved and

fixed as programmed cell death modules, formally similar to plasmid addiction systems (Yarmolinsky, 1995; Kobayashi, 1998).

PPAS EVOLUTION

The origin of potentially lethal genes remains open to speculations. As the addiction cassettes are so frequent in prokaryotic chromosomes and plasmids, the hypothesis of the concentration of multiple and independent smaller replicons to give a new bigger plasmid or even a bacterial chromosome has been proposed. This is supported by an observation that the presence of multiple stability determinants on a given plasmid is often paralleled by multiple replicons (e.g. plasmids F and RK2).

Another hypothesis is proposed basing on sequence comparison among dispersed homologues (Grønlund & Gerdes, 1999) assumed that ubiquity of *relBE* genes is likely due to vertical transmission of an ancestor that have appeared early in evolution before the branching of Bacteria and Archaea.

Toxin-antitoxin cassettes present on chromosomes could have become the source of genes for plasmid addiction systems (Rawlings, 1999). Once captured on a plasmid these genes would be infinitely maintained in population owing the ability to kill plasmid-free cells. At the same time the diversification and horizontal transfer of the toxin-antitoxin genes could have occurred. The opposite direction of moving genes (i.e. from plasmid to the chromosome) cannot be yet ruled out.

The abundance of killing genes on prokaryotic chromosomes also suggests their different that stabilizing functions. Since some of these genes (e.g. *relBE* and *chpA*) are linked to the stringent control and their activation reduces the rate of translation (RelE) or replication (ChpA), it is now proposed that the toxin-antitoxin loci constitute a part of the global stress response (Gerdes, 2000).

The question whether toxin-antitoxin loci, plasmidic or chromosomal, are a kind of DNA self parasites, suicide cassettes for programmed bacterial cell death, elements of stress response or even beneficial in genetic diversification is still to judge.

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