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MINIREVIEW

Active Stable Maintenance Functions in Low Copy-Number Plasmids of Gram-positive Bacteria II. Post-segregational Killing Systems

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Abstract

Active support is needed for low copy-number plasmids to be stably maintained in bacterial cells. The mechanisms that fulfill this role are (i) partition systems (PAR) acting to separate plasmid molecules to daughter cells and (ii) toxin-andidote (TA) (post-segregational killing-PSK) systems which arrest cell growth until the plasmid reaches the correct copy-number or kill the cells that have not inherited the plasmid. Our knowledge of toxin-antidote systems comes mainly from studies on Gram-negative bacteria. However, some addiction systems of Gram-positive bacteria have been characterized in detail or recently identified. Altogether, they bring new interesting data on toxin-antidote functioning in bacteria.

Key words: Plasmid, stable maintenance, post-segregational killing, Gram-positive bacteria

Introduction

Low copy-number plasmids evolved mechanisms that ensure their maintenance in the bacterial population. The issues that arise for a plasmid in a bacterial population and the way plasmids deal with these problems (copy-number control, multimer resolution systems – MRS, partition systems – PAR and post-segregational killing systems – PSK) are shortly presented in the introduction of the accompanying review of partition systems of Gram-positive bacteria (Dmowski and Jagura-Burdzy, 2013). Here, the best known post-segregational killing systems that come from plasmids of Gram-negative bacteria will be briefly described. Then, PSK (TA) systems of plasmids from Gram-positive bacteria will be presented.

Toxin-antidote or post-segregational killing systems

Stabilization of low-copy number plasmids in the bacterial population can be achieved by the action of post-segregational killing systems (PSK) also designated toxin-antidote systems (TA) or addiction systems, for a review see Hayes and Van Melderen (2011). Their action results either in enhancing plasmid replication to compensate the dangerous drop in the copy number – the *copy number rescue* effect proposed by de la Cueva-Mendez and Pimentel (2007) or elimination of plasmid-free segregants from the bacterial population (Zielenkiewicz and Ceglowski, 2001; Hayes 2003). A bacterial cell, which after division has not inherited the plasmid molecule with TA system encoded, is exposed to the toxin remaining in the cytoplasm, whereas the labile antidote is quickly degraded. As the toxin is no longer neutralized, it mediates cell death or a reversible growth arrest (Fig. 1). Another benefit of the plasmidic toxin-antidote systems action was proposed to be the exclusion of competing plasmids (Cooper and Heinemann, 2000).

The PSK systems have been divided into two groups according to the type of the antidote. In the type I systems the antidote is an antisense RNA complementary to the mRNA of the toxin gene. In the presence of the antisense RNA, the toxin mRNA cannot be translated or is prone to degradation and the toxin is not produced (Fig. 1). The best studied PSK system of this type is the *hok-sok* from plasmid R1 (Gerdes *et al.*, 1997). The type II encompasses proteic plasmid addiction systems (PPAS). Here, the antidote as well as the toxin are proteins. In plasmid carrying cells, the two proteins form an inactive complex, what stops the toxin from killing the cell. However, in a daughter cell that has lost the plasmid, the stable toxin protein is no longer neutralized

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(Fig. 1). The well understood proteic systems have been described for plasmids F (*ccdA-ccdB*), P1 (*phd-doc*) and R1 (*kid-kis*); for details see Hayes and Van Melderen, (2011) and references therein.

Multiple toxin-antidote systems were also identified in bacterial chromosomes *e.g. relE-relB* (Cherny *et al*., 2007; Takagi *et al*., 2005) and *mazE-mazF* in *Escherichia coli* (Aizenman *et al*., 1996; Engelberg-Kulka *et al.*, 2005; Kolodkin-Gal *et al*., 2007). The chromosomal TA systems are implicated in the growth control, programmed cell arrest and preservation of the commons, as well, as antiphage action (Magnuson, 2007). Tsilibaris *et al*. (2007) have also suggested the role of chromosomally encoded TA systems in long-term evolution, rather than stress response. These functions were also reviewed by Van Melderen and De Bast (2009).

The cellular targets of known toxins of PSK/TA systems have been characterized only for certain, best studied systems. The CcdB and ParE toxins from F and RK2 plasmid, respectively, affect the DNA gyrase action: ParE inhibits the gyrase activity and promotes conversion of supercoiled DNA into a linear form (Jiang *et al*., 2002), whereas the CcdB toxin of plasmid F induces double strand breaks in DNA upon interaction with the gyrase. The CcdB-gyrase-DNA complex also inhibits replication (Van Melderen, 2002). Another toxin, the Kid toxin of plasmid R1, was suggested to block the replication complex assembly or replication initiation by interfering with the DnaB protein (Ruiz-Echevarria *et al*., 1995; Potrykus *et al*., 2002). Finally it has been demonstrated that Kid of R1 acts as an RNase cleaving the host mRNA at 5'-UUACU-3' sites, which shuts off host gene expression and thus stops cell growth (Pimentel *et al*., 2005, de la Cueva-Mendez and Pimentel, 2007). It also degrades specific plasmidic copy-control element *copB-repA* mRNA what causes derepression of the *repA* gene and rescues the plasmid copy number. The pre-segregational function of Kid results in stabilization of the plasmid R1. Chromosomally encoded RelE and MazF toxins inhibit translation and act bacteriostatically (Christensen *et al*., 2001; Pedersen *et al*., 2002; Amitai *et al*., 2004).

Post-segregational killing systems of Gram-positive bacteria

Plasmid pAD1. The pAD1 plasmid was the source of the first described post-segregational killing system originating from Gram-positive bacteria, misleadingly designated *par* (Weaver and Tritle, 1994). A putative similar TA system was also identified on the staphylococcal plasmid pSK1 (Jensen *et al.*, 2010a). The *par* region of pAD1 encodes two convergently transcribed RNAs: RNAI (210 nt) and RNAII (65 nt) (Fig. 2) (Weaver *et al*., 1996). The RNAI encodes an open reading frame *fst* (*faecalis* plasmid-*stabilizing toxin*) translated into 33 amino acids long peptide (Greenfield *et al*., 2000). This peptide acts as the toxin of the *par* system. In *E. faecalis* the Fst toxin affects the integrity of the bacterial cell membrane, by an unknown mechanism. It also inhibits macromolecular synthesis and affects chromosomal segregation and cell division causing uneven distribution of DNA to daughter cells (Weaver *et al*., 2003, Patel and Weaver, 2006). In *B. subtilis* cells the Fst toxin acts mainly on the nucleoid structure and has minor effects on peptidoglycan synthesis (Patel and Weaver, 2006); its toxicity was also demonstrated in *S. aureus* (Weaver *et al*., 2009). In the same work, authors have found the *fst* gene in the chromosomes of *E. faecalis, Lactobacillus casei* and *Staphylococcus saprophyticus*, on plasmids from *E. faeacali*s, *Lactobacillus curvatus* and *S. aureus* and on a *Lactobacillus gasseri* phage. Peptides encoded by these genes contain a conserved hydrophobic domain which predicts their membrane localization.

The RNAII transcript of pAD1 acts as the antidote since it interacts posttranscriptionally with RNAI: the 5' terminal part of RNAII encompasses direct repeats DRa and DRb complementary to DRa and DRb repeats flanking the translation initiation region of *fst* at the 5' end of RNAI (Fig. 2) (Greenfield and Weaver, 2000). The RNAI-RNAII interaction inhibits ribosome binding and in consequence, inhibits the toxin production. Complementary regions also correspond to the transcriptional terminator stem-loops at RNAI and RNAII 3' ends (Fig. 2). The RNAI-RNAII interaction is a two steps process. First, the RNAI translation is limited by the stem-loop (SL) formation at 5' end presumably until RNAII is ready to interact with RNAI; disruption of the SL results in increased *fst* translation *in vitro* (Shokeen *et al.*, 2008). Then, an initial kissing complex is created between stem-loops formed by transcriptional terminators, followed by pairing of complementary direct repeats DRa and DRb what prevents ribosome binding and *fst* translation (Greenfield *et al*., 2000). RNAI and RNAII form a relatively stable complex (half life of at least 15 minutes), what results in accumulation of a pool of paired RNAs. When the complex dissociates, RNAII is rapidly degraded, but unless the plasmid is lost, the new transcripts can replace those degraded. Since the half-life of RNAI (45 minutes) exceeds 10-fold the half-life of RNAII, in the plasmid-free cell, in the absence of RNAII, the stable RNAI can be translated and the cell dies (Weaver *et al*., 2004). The high level of RNAI stability is ensured by its intramolecular structure, formed by the 5' UH (upstream helix) and 3'UH sequences. Disruption of this structure made RNAI sensitive to degradation by the J2 RNase in *B. subtilis* (Shokeen *et al*., 2009).

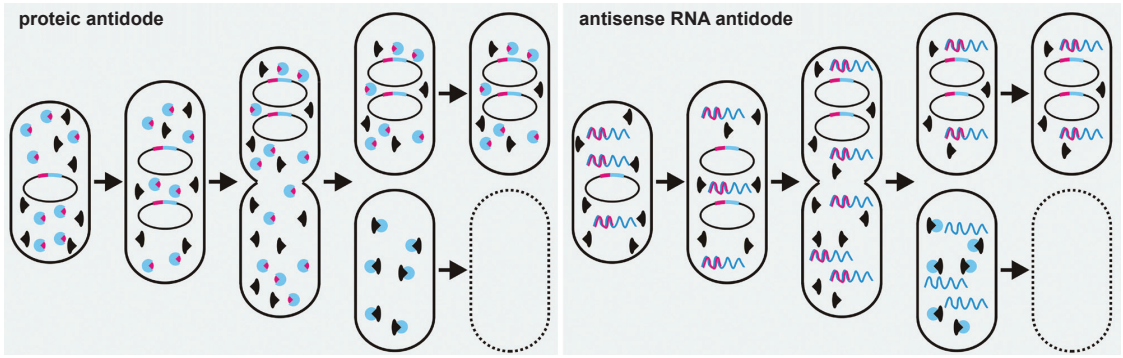

Fig. 1. Schematic representation of PSK/TA systems action. The toxin (light-blue) is neutralized by the labile proteic or antisense RNA antidote (purple). After plasmid loss, the toxin is no longer neutralized and attacks the cellular target (black).

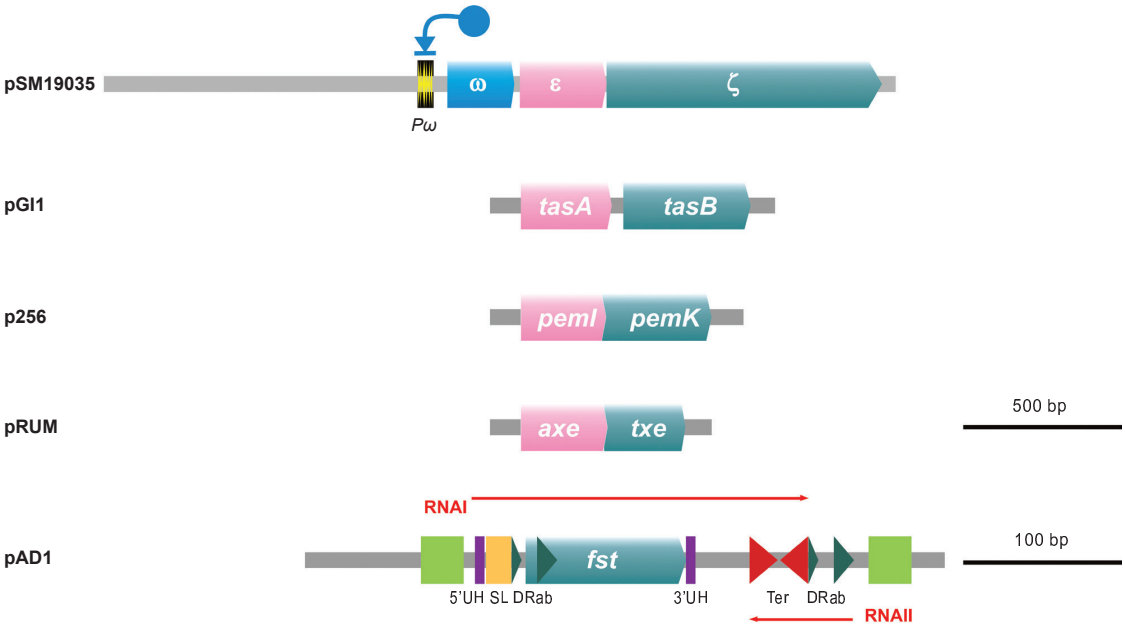

Fig. 2. Post-segregational killing modules from Gram-positive bacteria. The genes coding for toxins are shown in grey-blue, those coding for antidotes are shown in pink.

Plasmid pSM19035. The plasmid addiction system encoded by the pSM19035 plasmid is the most extensively studied proteic TA system from Gram-positive bacteria. Early studies have shown that the DNA fragment encompassing the δ, ε and ζ genes promotes 500–1000 times higher stability of both θ and σ type unstable replicons in *B. subtilis* cells (Ceglowski *et al.*, 1993). Further studies of pSM19035 by Zielenkiewicz and Ceglowski (2005) have demonstrated that the ζ gene encodes a toxin of the post-segregational killing system, whereas ε encodes the antidote. It was shown, that these genes stabilize plasmids in *B. subtilis* cells (Zielenkiewicz and Ceglowski, 2005) and other species of Gram-positive bacteria with low GC content: *S. pyogenes*, *S. aureus*, *Streptococcus agalactiae* and *Lactobacillus plantarum* (Brzozowska *et al.*, 2012). This PSK system can also stabilize unstable replicons in Gram-negative host *E. coli* cells although with lower efficiency (Zielenkiewicz and Ceglowski, 2005). Studies of Moritz and Hergenrother (2007) and Rosvoll and co-workers (2010) demonstrated dissemination of ε-ζ system among vancomycin resistant enterococci. This TA module was associated mainly with the inc18 replicons, *e.g.* pIP501, pSM19035, classified as family 1 by Jensen *et al.*, (2010b) but occurred also with the non-inc18 replicons, classified as family 2, *e.g.* pVEF1, pVEF2. Rosvoll *et al.*, (2010) demonstrated the phylogenetic divergence of ω-ε-ζ operons associated with these replicons and designated them pSM19035-type and pVEF1/2-type, respectively.

Expression of both ε and ζ genes proceeds mainly from the strong *P*ω promoter controlled by the regulatory protein Omega encoded by the ω gene, the first cistron in the ω-ε-ζ operon (Fig. 2). Although transcribed together with the ε and ζ genes, gene ω is not necessary for the activity of the post-segregational killing system (Zielenkiewicz and Ceglowski, 2005). The ε and ζ genes can also be expressed from the weak *P*ε promoter which is not subject to Omega regulation (Sitkiewicz, PhD Thesis, 2002). The activity of the *P*ε promoter may explain the observation that only after more than 90 generations of growth, the absence of ω gene provokes a decrease of ε-ζ mediated stability.

The Zeta toxin is a phosphotransferase. Its N-terminal part (involved in interaction with Epsilon) is responsible for its toxicity. In the work by Nowakowska *et al.* (2005) the analysis of *B. subtilis* survivors of Zeta over-production has shown that alleles coding for non-toxic variants of Zeta accumulated various mutations in the 5' terminus. Moreover, deletion of 12 N-terminal residues, or substitution of the conserved lysine residue in the Walker A motif (K46L) results in loss of Zeta toxicity (Zielenkiewicz *et al*., 2009). The structural characterization of the toxin and the antidote has shown that Epsilon and Zeta proteins associate in a heterotetramer. The N-terminal helix of the Epsilon antitoxin blocks Zeta ATP/GTP binding site, what results in inactivation of its bacteriostatic activity (Meinhart *et al*., 2003). The *in vivo* studies of interactions of Epsilon and Zeta proteins have shown, in agreement with crystal structure data, that Epsilon and Zeta interact through their respective N-terminal parts (Zielenkiewicz *et al*., 2009).

The toxic action of the long lived Zeta protein (half-life more than 60 minutes) is neutralized by the continuous production of Epsilon protein (half-life of 18 minutes) (Camacho *et al*., 2002). The overproduction of Epsilon abolishes the stabilizing effect of the ω-ε-ζ operon on an unstable replicon (Zielenkiewicz and Ceglowski, 2005). Initial studies have shown that the effect of Zeta in *B. subtilis* cells is bacteriocidal; it provoked changes in cell morphology (smaller and finer cells) and has led to cell lysis, whereas the Zeta action in *E. coli* cells is bacteriostatic, cells tend to form filaments, what suggests that this protein may influence cell division (Zielenkiewicz and Ceglowski 2005). Lioy and co-workers (2006) have also shown that Zeta induces stasis of bacterial population, a viable but non-culturable (VBNC) state. Such growth arrest is reversible by the Epsilon antitoxin. The time window during which the toxic effect can be reversed depends on the ζ gene expression level-stronger overproduction needs faster neutralization. Recently Lioy and co-workers (2012) have suggested that the Zeta caused dormancy (through up-regulated expression of *relA* coding for the (p)ppGpp synthase) induces protective responses including repression of membrane biosynthesis. Interestingly although the dormant state is reversible, a fraction of the population cannot either enter or recover from dormancy and dies. Not only in bacteria but also in yeasts Zeta caused growth retardation, the toxic effect was dose-dependent and neutralized in the presence of Epsilon protein (Zielenkiewicz *et al*., 2009).

A TA system homologous to ε-ζ and designated PezAT was found chromosomally encoded in *Streptococcus pneumoniae* (Khoo *et al*., 2007). It possibly constitutes a virulence factor involved in pneumolysin release, as demonstrated in the mouse model (Brown *et al*., 2004). Recently, Mutschler *et al.* (2011) have identified the mode of action of the Zeta/PezT toxin. The PezT kinase impairs cell wall synthesis through phosphorylation of the cell wall precursor UNAG and in consequence, inhibition of MurA, the enzyme involved in peptidoglycan synthesis. The possible application of ε-ζ/PezAT systems in antimicrobial treatment were discussed by Mutschler and Meinhart (2011).

Plasmids pRUM and pS177. The *axe-txe* system was identified on the pRUM and pS177 plasmids. The pRUM plasmid (24 873 bp), isolated from *E. faecium* confers resistance to chloramphenicol, erythromycin, streptomycin and streptothricin to its host (Grady and Hayes 2003). The recently described 39 kb large plasmid pS177 (Halvorsen *et al.*, 2011) based on the pRUM replicon, encodes resistance to vancomycin (Tn1546), erythromycin, streptomycin, streptothricin and kanamycin (due to the gene cassette from *Staphylococcus intermedius*). The first report on the *axe-txe* system (Grady and Hayes 2003) has shown that *axe* and *txe* genes encode proteic antitoxin and toxin, respectively. The authors have demonstrated that these genes promote segregational stability of otherwise unstable plasmids in *E. faecium* as well as in *B. thuringensis* and evolutionarily distant *E. coli* host. The "broad host range" of this TA system correlates with the fact that homologs of these genes were identified in the genomes of both Gram-positive and Gram-negative bacteria what implies that the mode of action of Axe-Txe system is universal (Grady and Hayes, 2003). Interestingly, amino acid sequence analysis has shown that the Axe antitoxin is related to Phd from P1, whereas the Txe is similar to the RelE superfamily of toxins (Halvorsen *et al.*, 2011; Francuski and Saenger, 2009). It has also been demonstrated that Txe inhibits protein synthesis by cleaving mRNA three bases downstream of the A of the AUG start codon (Halvorsen *et al.*, 2011). The *axe-txe* system has been commonly found in vancomycin-resistant enterococci (Moritz and Hergenrother, 2007) prevalently linked to the pRUM replicon (Rosvoll *et al*., 2010).

Plasmid p256. A toxin-antitoxin-like system has been found on the p256 plasmid (7.2 kb) from *L. plantarum* and characterized by Sørvig *et al.* (2005). This

plasmid replicates *via* a θ mechanism which is Rep-protein independent and is unique for lactic acid bacteria (LAB) plasmids. Its estimated copy number is between five and ten copies. The stability determinants are localized in *orf2* and *orf3* that encode proteins related to PemI and PemK from *E. coli* plasmid R1. The region encompassing studied genes was necessary for p256 derivative stabilization. Transcriptomic analysis of the *orf2* and *orf3* suggests that these two genes are organized in one operon.

Plasmid pGI1. Plasmid pGII from *B. thuringensis* has been shown to encode a specific toxin-antidote system designated *tasA-tasB* (*t*huringensis addiction system). Its analysis by Fico and Mahillon (2006) demonstrated that the toxin TasB is similar to the Doc toxin of P1 *phd-doc* TA system. The antidote TasA has no similarity to Phd but to MazE antitoxin of the *mazEF* system encoded chromosomally in *E. coli*. Hence the TA system from pGI1 may also be considered as a hybrid between two systems (similarly to *axe-txe* system of pRUM). Interestingly, such hybrid loci have been found in a variety of microorganisms, including firmicutes, proteobacteria, chlorobi, cyanobacteria, chlamydiae, one acidobacterium, one bacteroidete and one planctomycete. Fico and Mahillon have also shown that the TasB toxin is functional in *E. coli* although the presence of the *tasA* gene failed to abolish TasB toxicity.

Concluding remarks

Only few PSK/TA systems of Gram-positive bacteria have been identified so far. The best studied are those from pAD1 and pSM19035 plasmid, which are representatives of type I (antisense RNA as antitoxin) and II (proteic antitoxin) of TA systems, respectively.

Numerous plasmids possess both active partition (presented in the accompanying review) and toxin-antidote systems (*e.g.* pSM19035, pAD1). The bacterial cell can inevitably profit from the presence of both stabilization systems (Brendler *et al*., 2004): The PSK system is useful since it eliminates bacterial cells that despite the presence of the partition system did not inherit the plasmid, whereas the partition system minimizes the toxic effect of the PSK system exerted on the bacterial population growth rate and resulting from the death of plasmid-free cells.

The understanding of TA systems functioning can also bring us new antimicrobial treatment possibilities either by inactivating antidotes of TA modules found in various strains of both Gram-positive ant Gram negative bacteria of clinical importance or by direct use of toxins to inhibit bacterial cells proliferation.

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