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Minireview

Plasmid addiction systems: perspectives and applications in biotechnology

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Summary

Biotechnical production processes often operate with plasmid-based expression systems in well-established prokaryotic and eukaryotic hosts such as *Escherichia coli* or *Saccharomyces cerevisiae*, respectively. Genetically engineered organisms produce important chemicals, biopolymers, biofuels and high-value proteins like insulin. In those bioprocesses plasmids in recombinant hosts have an essential impact on productivity. Plasmid-free cells lead to losses in the entire product recovery and decrease the profitability of the whole process. Use of antibiotics in industrial fermentations is not an applicable option to maintain plasmid stability. Especially in pharmaceutical or GMP-based fermentation processes, deployed antibiotics must be inactivated and removed. Several plasmid addiction systems (PAS) were described in the literature. However, not every system has reached a full applicable state. This review compares most known addiction systems and is focusing on biotechnical applications.

Introduction

Fermentations of microorganisms in biotechnological production processes often depend on foreign genetic information located on plasmids, e.g. the production of human insulin (Johnson, 1983). Further industrial relevant plasmid-based processes for the production of, for example, amylases, lipases, proteases, vitamins or antibiotics were described (Vary *et al.*, 2007). Plasmids are separate genetic elements and autonomously replicated from the chromosomes. Furthermore, several fields of research utilize plasmids as important and essential tools. The success of plasmid-based microbial production systems significantly depends on plasmid stability, copy number and choice of suitable promotors. Plasmids are frequently used to introduce the formation of additional proteins or even for the establishment of complete metabolic pathways. Actual experiments in the fields of gene therapy and genetic vaccination using naked plasmid DNA as therapeutic vectors were successful. For these reasons plasmids became an important medical and economical product (Friehs, 2004). The easy transfer into a new host and the occurrence in multiple copies in the cells provides advantages. Frequently used cloning and expression plasmids harbour antibiotic resistance genes and require the addition of antibiotics to the cultivation medium for plasmid maintenance. Whereas this procedure is feasible at the laboratory scale, it is not applicable at large-scale cultivations in industry due to the high costs and due to ecological constraints. Moreover, plasmid instability can even occur despite the addition of antibiotic during cultivation (Zabriskie and Arcuri, 1986). Chromosomal gene integration is another strategy to stabilize foreign genes; it is mostly associated with single-copy insertions which often cause lower levels of recombinant protein or negative polar effects in contrast to the use of (multi-copy) plasmids.

Most naturally occurring plasmids as well as most constructed plasmids do not encode information required for viability or survival of the host cell. They often provide advantages to the host under specific environmental conditions such as utilizing certain chemical compounds or providing resistances towards adverse substances (Nordstrom and Austin, 1989). Plasmids may exert a metabolic burden to the host in comparison to plasmid-free cells (Zielenkiewicz and Cegłowski, 2001). Diverse mechanisms have been evolved to ensure stable maintenance of plasmids in cells (Nordstrom and Austin, 1989): (i) site-specific recombination systems function as plasmid maintenance systems for most high-copy plasmids (Alonso *et al.*, 1996; Grindley *et al.*, 2006), (ii) active partition systems, essential for distribution of plasmids with moderate or low copy number, consisting of two

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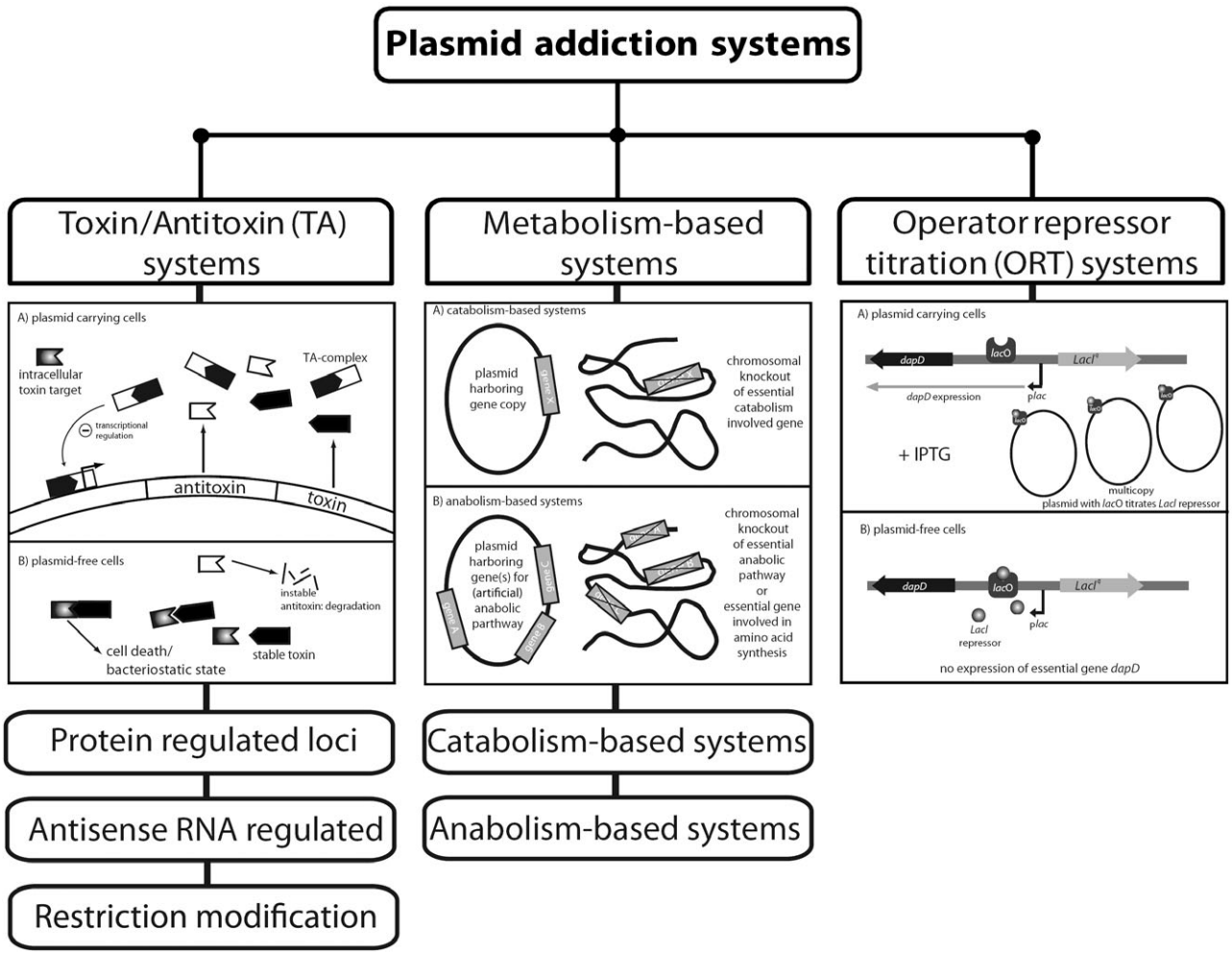

Fig. 1. Overview and classification of the most common plasmid addiction systems (PAS) depending on their functionality and principle of mechanism. Toxin/antitoxin (TA) systems contain three separate subgroups with protein regulated loci, antisense RNA-regulated mechanism or specific restriction modification. The general function of this group is illustrated above by the mechanism of TA systems. Metabolism-based systems are divided into catabolism and anabolism-based systems depending on their target pathway and complementation principle. The operator repressor titration (ORT) system is emphasized as a stand-alone group.

proteins and a centromere-like site actively segregating plasmids to progeny cells during cell division (Gerdes *et al*., 2000; Funnell and Slavcev, 2004), and (iii) plasmid addiction systems (PAS) preventing the survival of plasmid-free cells due to selective killing (Zielenkiewicz and Cegłowski, 2001; Gerdes *et al*., 2005).

The present review is divided into three major parts. The first section 'Variants of PAS and their functioning' is a synopsis of current PAS. It depicts several examples and classifies the PAS related to their functionality. The following section 'PAS and their applications' focuses on applied PAS with biotechnical background. Here a couple of approved examples are displayed and discussed in more detail. Finally, the section 'Conclusion and outlook: applications and perspectives of PAS in biotechnology' summarizes the entire topic and provides comments on possible future developments of PAS.

Variants of PAS and their functioning

This section provides an overview of three major groups of PAS according to their principle of function: (i) toxin/antitoxin (TA)-based systems, (ii) metabolism-based systems and (iii) operator repressor titration (ORT) systems (Fig. 1). It must be emphasized that most of the presented PAS occur naturally (see the host organisms in Tables 1–3) and were modified for special applications or adapted to extend the range of hosts. Exceptions occur in the metabolism-based and ORT systems which are completely synthetic engineered PAS.

TA-based systems

The principle of TA systems is the counteraction of two proteins from which one is a stable toxin and the other is an unstable antitoxin. Mainly, three different groups of TA

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Table 1. Overview of toxin/antitoxin-based addiction systems that were described in literature.

Name of locus	Toxin/antitoxin	Organism	References
hok/sok	Hok/Sok	*E. coli*	Gerdes *et al.* (1986a,b); Gerdes (1988); Thisted and Gerdes (1992); Pedersen and Gerdes (1999); Kobayashi (2004)
par	RNA I/RNA II	*E. faecalis*	Weaver and Tritle (1994); Weaver *et al.* (1996; 2004); Greenfield *et al.* (2000)
ldrD–rdlD	LdrD/RdlD	*E. coli*	Kawano *et al.* (2002)
ratA–txpA	RatA/TxpA	*B. subtilis*	Silvaggi *et al.* (2005)
symER	SymR/SymE	*E. coli*	Kawano *et al.* (2007)

systems can be distinguished: (i) antisense RNA-regulated loci, (ii) protein-regulated loci and (iii) restriction modification systems (Fig. 1).

In TA systems the antitoxin is usually encoded upstream of the toxin gene (Fig. 1). By binding to each other, the antidote neutralizes the toxin and prevents the organism from cell death. In the case of plasmid loss, the durable toxin is still active, whereas the unstable antidote is degraded rapidly. Thus, the persistent toxin exerts its toxic effect and leads to cell death of the segregant. This mechanism favours stable maintenance of plasmids. The TA systems of antisense RNA-regulated loci have a toxin and antitoxin as antagonistic elements partially on the RNA level. Those mRNA-sequences bind to their target and induce cell death. The antidote is either RNA or a protein, and it inhibits the translation of the toxin RNA and

Table 2. Overview of addiction systems with protein-regulated loci displaying the host origin, involved toxin/antitoxin as described in literature.

Name of locus	Toxin/antitoxin	Organism	References
ccd	CcdB/CcdA	*E. coli*	Ogura and Hiraga (1983); Miki *et al.* (1984a,b); Jaffé *et al.* (1985); Tam and Kline (1989b); Engelberg-Kulka and Glaser (1999); Afif *et al.* (2001); Buts *et al.* (2005); Gerdes *et al.* (2005); Pandey and Gerdes (2005)
hicAB	HicB/HicA	*Haemophilus influenzae*	Mhlanga-Mutangadura *et al.* (1998); Mittenhuber (1999); Pandey and Gerdes (2005); Makarova *et al.* (2006)
higBA	HigB/HigA	*Proteus vulgaris*	Tian *et al.* (1996); Pandey and Gerdes (2005); Christensen-Dalsgaard and Gerdes (2006); Budde *et al.* (2007)
hipBA	HipA/HipB	*E. coli*	Moyed and Bertrand (1983); Moyed and Broderick (1986); Black *et al.* (1991; 1994); Falla and Chopra (1998; 1999); Korch *et al.* (2003)
parDE	ParE/ParD	*Pseudomonas* sp., *E. coli*	Gerlitz *et al.* (1990); Davis *et al.* (1992); Eberl *et al.* (1992); Roberts and Helinski (1992); Roberts *et al.* (1993); Johnson *et al.* (1996); Sobecky *et al.* (1996); Engelberg-Kulka and Glaser (1999); Jiang *et al.* (2002); Anantharaman and Aravind (2003); Gerdes *et al.* (2005); Pandey and Gerdes (2005)
relBE	RelE/RelB	*E. coli*	Bech *et al.* (1985); Gotfredsen and Gerdes (1998); Smith and Rawlings (1998); Pandey and Gerdes (2005); Christensen *et al.* (2001); Anantharaman and Aravind (2003); Grady and Hayes (2003); Gerdes *et al.* (2005)
tasA–tasB	TasB/TasA	*Bacillus thuringiensis*	Fico and Mahillon (2006)
vapBC	VapC/VapB	*Salmonella dublin*	Katz *et al.* (1992); Pullinger and Lax (1992); Sayeed *et al.* (2000); Oláh *et al.* (2001); Pandey and Gerdes (2005); Bodogai *et al.* (2006); Daines *et al.* (2007)
ω-ε-ζ	Zeta/Epsilon	*Streptococci*	Cegłowski *et al.* (1993a,b); de la Hoz *et al.* (2000; 2004); Camacho *et al.* (2002); Meinhart *et al.* (2003); Zielenkiewicz and Cegłowski (2005); Lioy *et al.* (2006); Khoo *et al.* (2007); O'Connor *et al.* (2007)
phd–doc	Doc/Phd	*Bacteriophage P1*	Lehnherr *et al.* (1993); Jensen and Gerdes (1995); Lehnherr and Yarmolinsky (1995); Magnuson *et al.* (1996); Magnuson and Yarmolinsky (1998); Gazit and Sauer (1999a,b); Hazan *et al.* (2001); Pomerantsev *et al.* (2001); Gerdes *et al.* (2005); LoVullo *et al.* (2006)
mazEF	MazF/MazE	*E. coli*	Bravo *et al.* (1987); Metzger *et al.* (1988); Tsuchimoto *et al.* (1988; 1992); Masuda *et al.* (1993); Tsuchimoto and Ohtsubo (1993); Jensen and Gerdes (1995); Aizenman *et al.* (1996); Pedersen *et al.* (2002); Christensen *et al.* (2003); Zhang *et al.* (2003); Gerdes *et al.* (2005); Sørvig *et al.* (2005); Gross *et al.* (2006); Kolodkin-Gal and Engelberg-Kulka (2006); Agarwal *et al.* (2007); Kolodkin-Gal *et al.* (2007); Schmidt *et al.* (2007); Nariya and Inouye (2008); Zhao and Zhang (2008)
fitAB	FitB/FitA	*Nesseira gonorrhoeae*	Clissold and Ponting (2000); Hopper *et al.* (2000); Mattison *et al.* (2006)
mqsRA	MqsR/MqsA	*E. coli*	Zhang *et al.* (2008); Brown *et al.* (2009); Kim and Wood (2009); Kim *et al.* (2009); Yamaguchi *et al.* (2009)
chbB	ChpB/ChpS	*E. coli*	Masuda *et al.* (1993)
yefM–yoeB	YoeB/YefM	*E. coli/Streptococcus pneumoniae*	Cherny and Gazit (2004); Nieto *et al.* (2007)
dinJ–yafQ	YafQ/DinJ	*E. coli*	Motiejūnaite *et al.* (2007)

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Table 3. Overview of restriction–modification (RM) systems.

Name of locus	Toxin/antitoxin	Organism	References
ecoRI	EcoRI/M.EcoRI	*E. coli*	Hedgpeth *et al.* (1972); Kulakauskas *et al.* (1995)
ecoRII	EcoRII/M.EcoRII	*E. coli*	Som *et al.* (1987); Som and Friedman (1993); Karyagina *et al.* (1997); Takahashi *et al.* (2002); Ohno *et al.* (2008)
ecoRV	EcoRV/M.EcoRV	*E. coli*	Nakayama and Kobayashi (1998)
ssoII	SsoII/M.SsoII	*Shigella sonnei*	Karyagina *et al.* (1997); Kobayashi (2004)
paeR71	PaeR71/M.PaeR71	*P. aeroginosa*	Wilson and Murray (1991); Kusano *et al.* (1995); Kobayashi (2004)
pvuII	PvuII/M.PvuII	*P. vulgaris*	Tao *et al.* (1991); Cheng *et al.* (1994); Calvin-Koons and Blumenthal (1995); Gong *et al.* (1997)
bsp6I	Bsp6I/M.Bsp6I	*Bacillus* sp. RFL6I	Kulakauskas *et al.* (1995); Lubys and Janulaitis (1995)
haeII	HaeII/M.HaeII	*Haemophilus aegyptius*	Tu *et al.* (1976)
dcm–vsr	None/Dcm	*E. coli*	Lieb (1991); Palmer and Marinus (1994); Takahashi *et al.* (2002); Bunting *et al.* (2003)

Table displays the host origin, involved toxin/antitoxin as described in literature.

therefore neutralizes toxic effects (Jensen and Gerdes, 1995). In the subgroup of TA systems based on protein-regulated loci, the two antagonists are proteins. The group of so-called restriction modification systems differs from the two other groups in that the toxin is a restriction enzyme which cleaves the host DNA at special nucleotide sequences and thus kills the cell. The corresponding antitoxin is a modification enzyme that methylates the target sequence thereby protecting the DNA from cleavage (Kobayashi, 2004).

In 2007, a web-based tool was developed, 'Rapid Automated Scan for Toxins and Antitoxins in Bacteria' ('RASTA-Bacteria', accessible at http://genoweb.univ-rennes1.fr/duals/RASTA-Bacteria), to improve and relieve the identification of putative TA loci in prokaryotic genomes (Sevin and Barloy-Hubler, 2007). The software 'RASTA-Bacteria' annotates toxin and antitoxin genes based on general characteristics: (i) TA systems consist of, at least, two genes, (ii) with a size of a few hundreds nucleotides each and (iii) 1–20 overlapping nucleotides in general, (iv) with the antitoxin usually being located upstream of the toxin and (v) with the antitoxin gene shorter than the toxin gene. Although restriction–modification systems belong also to TA systems, they were excluded from the study as well as the ω-ε-ζ TA family with its three components due to their specific characteristics.

'RASTA-Bacteria' confirmed all, except two, TA loci that were previously predicted by Pandey and Gerdes (2005), whereby the exceptions were not detected because of their low homology or low confidence score. 'RASTA-Bacteria' was able to identify new loci in all studied genomes except in the obligate intracellular organisms without TA systems and in *Bacillus* spp., which harbour a single TA locus that was known before. Among the 532 TA loci predicted in this study, the major family was *vapBC* comprising 37% of the members, whereas five other families (*mazEF*, *relBE*, *higBA*, *hipBA*, *parDE*) each comprised only about 10% of the loci. In contrast, *phd/doc* and *ccdAB* were less frequently detected (3% and 0.009%, respectively); the other TA loci remained unclassified. 'RASTA-Bacteria' was also able to analyse eukaryotic genomes, although it was not designed for that task. However, further improvements are necessary, since not all TA families have been included, yet, and the predicted TA loci have to be validated in detailed characterization studies. The currently known TA systems are summarized in Table 1.

Antisense RNA-regulated systems. These systems consist of a toxin and antitoxin; both are in most cases RNA molecules. The toxin is encoded by a stable mRNA which upon expression forms a protein that may kill the cell if it is present in high concentrations. In antisense RNA-regulated systems, a small, unstable transcript that functions as antidote binds to the toxin-encoding RNA and inhibits its translation (Gerdes *et al.*, 1997).

The most intensively studied example is the *hok/sok* system from *Escherichia coli* (Kobayashi, 2004). This system stabilizes plasmid R1 in *E. coli* and is encoded in the *parB* region. The *hok/sok* locus of plasmid R1 from *E. coli* consists of three genes and is one of the best-characterized antisense RNA-regulated TA loci (Gerdes *et al.*, 1997). The *hok* gene encodes a toxic protein that leads to an irreversible damage of the cell membrane potential (Gerdes *et al.*, 1986a,b). As a result the cell is rapidly killed and shows a characteristic 'ghost morphology'. Expression and translation of *hok* requires the *mok* gene; the translation of the latter is blocked by the *sok* reading frame encoding an unstable antisense RNA (Thisted and Gerdes, 1992). Sok-RNA binds to *hok* mRNA, and the resulting *hok* mRNA:Sok-RNA duplex is cleaved by RNase III (Gerdes *et al.*, 1992).

Another system based on the unique *par* locus, which was found in *Enterococcus faecalis*, is so far the only post-segregational-killing system detected in Gram-positive bacteria. The *par* locus consists of two genes

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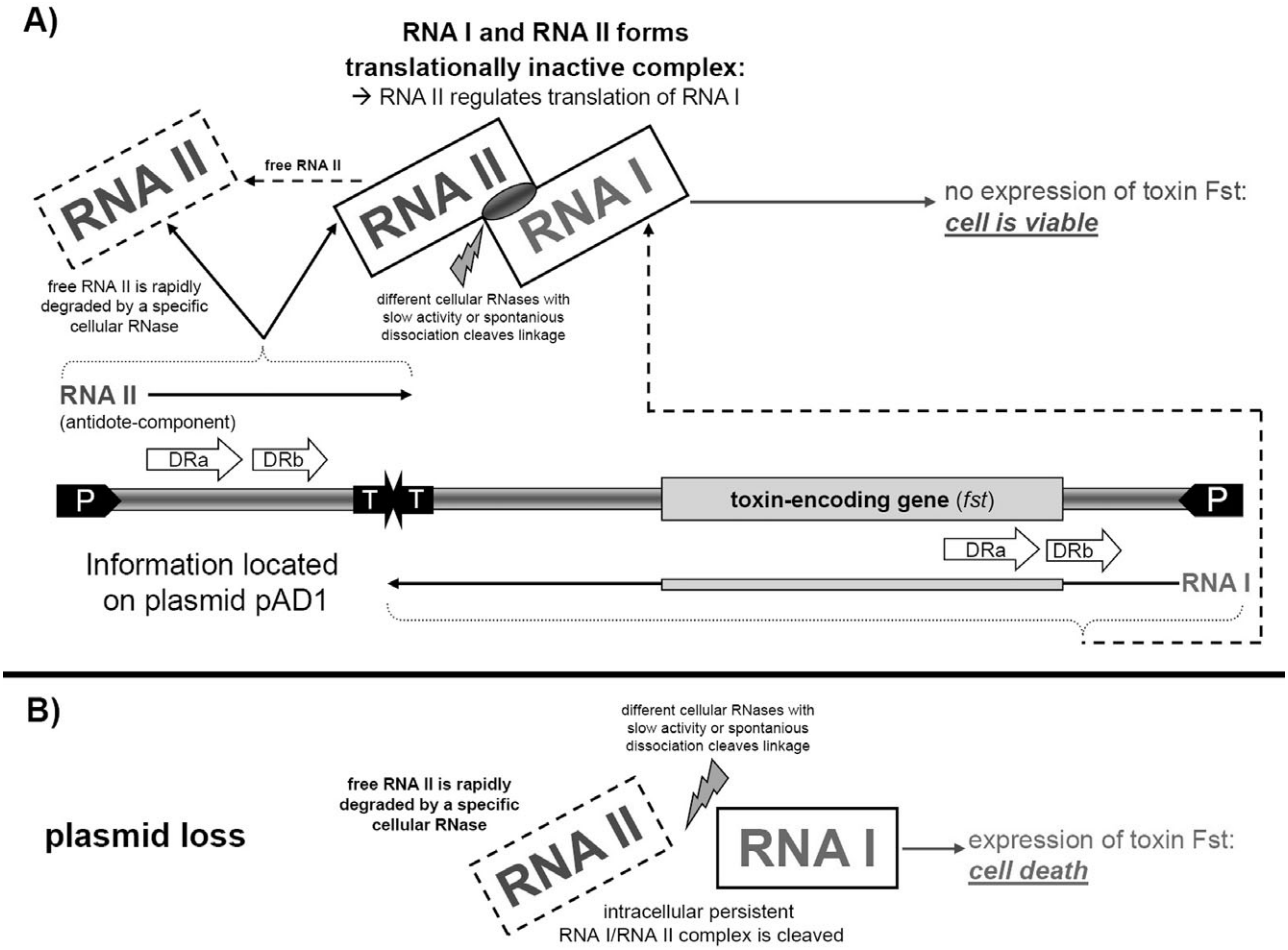

Fig. 2. Principle and scheme of an antisense RNA-regulated toxin–antitoxin plasmid addiction system based on the *par* locus of *Enterococcus faecalis*. The natural occurring *par* locus contains the RNA I and RNA II encoding sequence in series on plasmid pAD1. The complete *in vivo* regulation and function of the native *par* locus is not known in detail, yet. Plasmid loss leads to cell death because of the persistence of the RNA I–RNA II complex and subsequent toxin expression after removal and degradation of the RNA II molecule (Weaver *et al*., 2004).
A. Plasmid carrying cells encode the RNA II fragment which acts as the antitoxin being an antisense RNA that inhibits translation of the toxin message by forming a complex with the chromosomally encoded RNA I. Downstream of the promoter (P) both sequences encoding RNA I and RNA II transcripts contain direct repeats (DRa; DRb) with a bidirectional *rho*-independent transcription terminator (TT). They provide the complementary regions required for interaction of RNA I and RNA II, because transcription occurs in opposite directions across DRa and DRb. While RNA I and RNA II form a translationally inactive complex, the toxic gene product Fst does not appear, and the cells are viable. The RNA I/RNA II complex is cleaved either by an RNase or by spontaneous dissociation of the two components resulting in the release of RNA I. Since there is a counteraction by the acquisition of the RNA I/RNA II complex, cells still hold a viable state.
B. In the case of plasmid loss the open reading frame encoding the toxin on the persistent RNA I is translated by the ribosomes and the toxin Fst leads to cell death by disrupting the cell membrane.

which encode the 210-nucleotide antisense RNA, RNA II and the 63-nucleotide toxin-encoding RNA I (*fst*) (Greenfield *et al*., 2000). Because their sequences are complementary to each other, RNA II is capable of regulating the transcription of RNA I and suppresses *fst* translation (Weaver *et al*., 1996). The *par* toxin, Fst, is activated by removal of RNA II when the plasmid is lost (Weaver *et al*., 2004). Figure 2 describes the *par* system as a representative of the antisense RNA-regulated systems.

The *E. coli ldrD–rdlD* locus in is a third example of an antisense RNA-regulated locus. Here, the *ldrD* gene encodes a peptide that kills the host cell if overexpressed. The antitoxin, RdlD, functions as a *trans*-acting regulator of *ldrD* translation (Kawano *et al*., 2002).

The *ratA–txpA* system occurs in *Bacillus subtilis*. The accumulation of the toxin, a peptide encoded by the *txpA* gene, is blocked by the antitoxin, an antisense RNA encoded by *ratA* gene (Silvaggi *et al*., 2005).

The last example of antisense RNA-regulated addiction systems is the *symER* locus, where the toxin SymR is repressed by the RNA-molecule SymE (Kawano *et al*., 2007). SymE synthesis is not only inhibited at the level of translation, but also at the level of transcription or protein by the LexA repressor and Lon protease. An important

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difference to all other TA systems is that the toxin is not rapidly degraded. One part of the two molecules that might be attacked by the Lon protease is the toxin, and not the antitoxin (Kawano *et al*., 2007). This system has not yet been analysed in more detail.

Protein-regulated systems. In protein-regulated systems (PRS) two proteins are encoded by two adjacent coexpressed genes encoding the stable toxin and the metastable antitoxin. In most PRS the antitoxin gene is located upstream of the toxin gene (Jensen and Gerdes, 1995). Protein-encoded addiction systems are mostly organized in operons and are autoregulated with the antitoxin or the TA complex repressing the promoter of this operon (Tam and Kline, 1989a,b, see Fig. 1). The accumulation of the toxic protein in plasmid-free cells will lead to cell death. There are at present several examples known which all function according to the same principle (see Table 2). Whereas the functions and genetic structures of most PRS are similar, they differ slightly in their sequence. In their location PRS may partially chromosally or completely extrachromosomally located. Further variables are the target of the toxin and the mechanism by which the antitoxin is degraded (Engelberg-Kulka and Glaser, 1999). Whereas the role of plasmid located PRS as plasmid-stabilization systems was demonstrated by several studies, the biological role of chromosomally located TA loci is subject of debate. Hitherto the function of chromosomal located systems is not completely investigated. Magnuson (2007) discussed several possible functions ranging from 'genomic junk' over 'programmed cell arrest' to 'growth control'. Others proposed that chromosomal PRS induce programmed cell death (Engelberg-Kulka *et al*., 2005; Kolodkin-Gal and Engelberg-Kulka, 2006), while Gerdes and others suggested that these PAS only inhibit cell growth (Pedersen *et al*., 2002; Buts *et al*., 2005; Gerdes *et al*., 2005). These contrary views probably reflect differences in the activities of TA-encoded toxins. For example, cell death caused by *mazEF* in *E. coli* has been observed as a response to stress conditions (Sat *et al*., 2001; 2003; Hazan *et al*., 2004; Kolodkin-Gal and Engelberg-Kulka, 2006), whereas induction of *relBE* (and in some cases *mazEF*) did not affect cell viability (Christensen and Gerdes, 2003; Christensen *et al*., 2003). An outstanding example is the MqsR/MqsA system which regulates more than its own locus and is implicated in the role of cell-persister formation (Kim and Wood, 2009; Kim *et al*., 2009). Furthermore, some PRS are associated to biofilm formation (Kim *et al*., 2009; Kolodkin-Gal *et al*., 2009). A comprehensive overview of PRS with continuative literature is given in Table 2.

Restriction/modification-based systems. In restriction/modification (RM)-based systems a restriction endonuclease functions as the toxin whereas a modification enzyme serves as the antitoxin. Therefore, it typically consists of two genes. One gene encodes a DNA endonuclease (ENase). The other gene encodes a DNA methyltransferase (MTase) which prevents DNA cleavage by transferring a methyl group specifically to a base and inhibits cleavage at the respective site (Handa and Kobayashi, 1999; Handa *et al*., 2000; Ichige and Kobayashi, 2005). RM addiction systems act as plasmid-stabilization systems (Kulakauskas *et al*., 1995; Nakayama and Kobayashi, 1998) in that way that the MTase-antitoxin protects the cellular target of the ENase-toxin. (Kobayashi, 2004). This variant of addiction system was not investigated in detail, yet. It seems to be different to the two other TA addiction systems described above regarding the regulatory mechanism of post-segregational killing: the toxic effect is conferred by the different stabilities of the two involved protein components in the PAS and in antisense RNA systems, whereas in the RM systems, the dilution of both proteins by cell growth activates the lethal activity of the restriction enzyme (Ichige and Kobayashi, 2005). Upon plasmid loss and further cell growth, the ENase-toxin and MTase-antitoxin are both 'diluted' and are proteolytically degraded in the daughter cells. Furthermore, the amount of the MTase target is simultaneously increasing. The intracellular concentration of the RM enzyme decreases then to a level at which not enough methylase activity remains to modify all DNA sites. Consequently, non-methylated sites appear and DNA cleavage might lead to cell death (Kulakauskas *et al*., 1995). RM systems have been reviewed in detail (Kobayashi, 2004). A scheme and a short overview are given in Fig. 3 and Table 3.

Metabolism-based systems

Metabolism-based addiction systems can be divided into catabolism- and anabolism-based systems.

Catabolism-based systems. Catabolism-based systems (CBS) utilize the necessity of enzymes that degrade an essential carbon and energy source for the microorganism. If the gene for an essential enzyme of the catabolism of such a compound is inactivated, the cells are impaired to use it for growth. However, episomal expression of the respective gene on a plasmid restores the catabolic pathway. *Ralstonia eutropha* H16 is a Gram-negative and facultative chemolithoautotrophic bacterium capable of using gluconate or fructose as carbon and energy sources for growth. Both compounds are catabolized via the Entner–Doudoroff pathway (Gottschalk *et al*., 1964; Blackkolb and Schlegel, 1968). The key intermediate of this pathway 2-keto-3-deoxy-6-phosphogluconate (KDPG) is converted to glyceraldehyde-3-phosphate and

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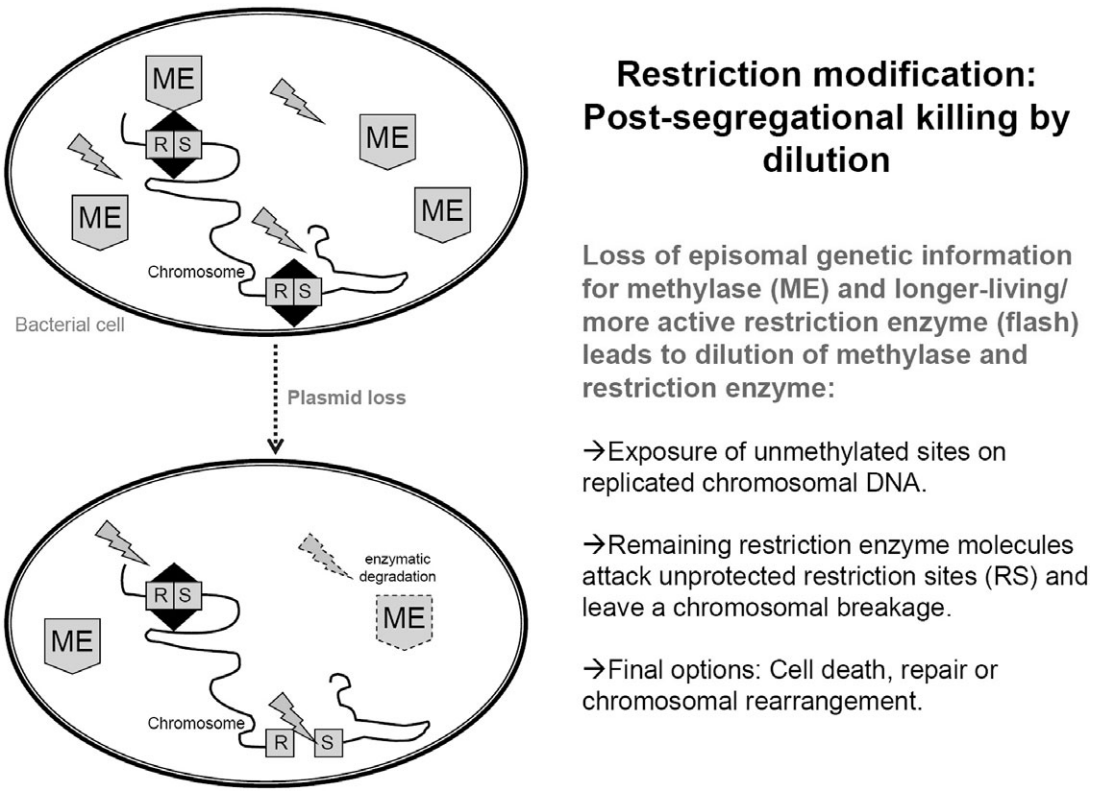

Fig. 3. Scheme of the restriction modification-based post-segregational killing principle by dilution of episomal coded genetic information for methylase and restriction enzyme. Due to the reduced activity or proteolytic degradation of the specific DNA protecting methylase (ME), the persistent or more active restriction enzyme (flash) cleaves the chromosomal DNA at its unmethylated restriction sites (RS), finally leading to extensive chromosomal degradation.

pyruvate by KDPG-aldolase (Kovachevich and Wood, 1955). This key enzyme is encoded by *eda* in *R. eutropha* H16, which is located on chromosome 2 (Pohlmann *et al*., 2006). A plasmid containing *eda* was transferred into the KDPG aldolase-negative mutant *R. eutropha* H16-PHB^{-4}Δ*eda* to restore KDPG-aldolase activity, thereby yielding a first example for a CBS. In addition to *eda*, the plasmid contained the cyanophycin synthetase gene (*cphA*) thereby allowing improved production of cyanophycin (Voß and Steinbüchel, 2006)

Anabolism-based system. Anabolism-based addiction systems (ABS) depend on at least one gene for an essential anabolic pathway. If such a gene is inactivated and if a copy of the intact gene is localized on a plasmid, that is important for a biotechnological process, the production of the respective compound becomes dependant on the presence of the plasmid.

Chromosomally encoded auxotrophic markers like *URA* or *LEU2*, which are complemented by an intact plasmid-encoded gene, are used in the genetics of yeasts and other eukaryotes since many years for selection of plasmids (Strausberg and Strausberg, 2001). One example is the *URA3* gene, encoding orotidine-5′-phosphate decarboxylase. Ura3 represents an essential enzyme in pyrimidine biosynthesis in *Saccharomyces cerevisiae*; its gene is often used on plasmids for complementation of auxotrophic yeast strains (Hensing *et al*., 1995). However, such systems were scarcely employed for production processes in biotechnology. Unsatisfying results were obtained when leucine auxotrophy in a xylitol production strain of *S. cerevisiae* was complemented. Due to the release of leucine from plasmid-containing cells also cells without plasmids containing the *LEU2* auxotrophic complementation selection marker grew (Meinander and Hahn-Hägerdal, 1997). On the other hand, successful complementation of auxotrophy for uracil and proline combined with expression of heterologous proteins was reported in *Pseudomonas fluorescens* (Schneider *et al*., 2005).

Recently, a new anabolic addiction system was developed that relies on the isoprenoid biosynthesis pathway (Kroll *et al*., 2009). In this ABS the native MEP pathway in *E. coli* was disrupted through deletion of the *ispH* gene, thereby disenabling the cells to catalyse the final step of the MEP pathway yielding IPP or DMAPP (Rohdich *et al*., 2002). Deletion of this gene is lethal, due to the absence of essential precursors for isoprenoid biosynthesis

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(McAteer *et al*., 2001). A suppression of this auxotrophic mutation by IPP uptake from the medium is not possible because of the phosphorylated nature of the compound. A synthetic plasmid, which harbours the relevant genes of an artificial IPP-producing mevalonate (MVA) pathway, was transferred to *E. coli* before generating the Δ*ispH* knockout, thereby enabling synthesis of isoprenoids via the foreign pathway in the *ispH* deletion mutant. This system is independent of minimal media and the use of defined carbon sources. The applicability of this addiction system was shown by improved production of cyanophycin in *E. coli* (Kroll *et al*., 2009).

ORT-based systems

To avoid plasmid maintenance through selectable marker genes, Williams and colleagues (1998) developed a novel addiction system depending on plasmid-borne ORT utilizing the *lac* repressor. They integrated a kanamycin resistance gene into the chromosome of *E. coli* and placed it under control of the operator–promoter region of the *lac* operon, resulting in a conditionally essential chromosomal gene under kanamycin selection. The *lac* repressor protein (LacI) binds to the *lac* operator (*lacO*) and therefore represses the expression of the kanamycin resistance gene. When IPTG is available as an inducer, LacI is titrated by *lacO*, and the *E. coli* cells were able to grow in media containing kanamycin due to expression of the corresponding resistance gene. The same result was obtained when high-copy-number plasmids harbouring *lacO* were used for ORT. In this case the plasmid was stably maintained over 72 generations (Williams *et al*., 1998). In a subsequent study, ORT by using high-copy-number plasmids was reported in *E. coli* strains with the essential chromosomal *dapD* gene under control of the *lac* operator–promoter region (Cranenburgh *et al*., 2001; Hanak *et al*., 2003). DapD is involved in lysine biosynthesis and provides diaminopimelate (DAP) for cross-linking of peptidoglycan chains (Schleifer and Kandler, 1972; Richaud *et al*., 1984). Alterations in *lacO* sequences and plasmid copy number were also studied in *E. coli* with LacI-repressed *dapD* gene (Cranenburgh *et al*., 2004). Figure 1 illustrates the previously described ORT system using the regulation of *dapD* expression. The *lacO* of the wild-type *E. coli lac* operon or its palindromic sequence provided sufficient levels of ORT, and lower-copy-number plasmids derived from pBR322, with 39–55 copies per cell (Müller-Hill *et al*., 1968), were shown to be as effective in ORT as high-copy-number plasmids.

PAS and their applications

Naturally occurring PAS can be considered as regulatory elements in prokaryotic or eukaryotic cells, which control by different mechanisms such as plasmid stability or the adjustment of the transcriptional rate. Hitherto, the precise functions of naturally occurring addiction systems are not fully understood and different hypothesis exist although the main role of these addiction systems is to maintain plasmid stability in daughter cells. Since the discovery of TA systems in the 1980s and due to an increasing interest in antibiotic-independent strategies for stable plasmid maintenance in biotechnology, great efforts have been made to further develop existing TA systems and to increase our knowledge about their characteristics and functionality. The gained knowledge has been used for the implementation of TA systems in a large variety of applications (see Tables 4 and 5). The following section describes the applications of biological containment systems for genetically engineered microorganisms (GEMs) in bioremediation processes, the delivery of antigens to the human immune system and the production of compounds based on enhanced plasmid stability.

Table 4. TA systems used for bacterial containment and in bioremediation.

Application	TA system	Killing induction	References
Model containment system	*hok*	Tryptophan starvation	Molin *et al*. (1987)
	hok	Time-dependent	Molin *et al*. (1987; 1993)
	hok	Lactose or IPTG	Bej *et al*. (1988)
	hok/sok	Phosphate starvation	Schweder *et al*. (1992)
	sacB[a]	Sucrose	Recorbet *et al*. (1993)
	gef	Time-dependent	Klemm *et al*. (1995)
	colE3/immE3, *ecoRI*	Lactose or IPTG	Torres *et al*. (2003)
Bioremediation	*gef*	Absence of degradable compounds	Contreras *et al*. (1991); Jensen *et al*. (1993); Ronchel *et al*. (1995)
	colE3[a]	Lactose or IPTG	Munthali *et al*. (1996a,b)
	stv[a]	Absence of degradable compounds	Szafranski *et al*. (1997); Kaplan *et al*. (1999)
	asd[a], *gef*	Absence of degradable compounds	Ronchel and Ramos (2001)

a. TA-less killing function.
All listed model containment systems have been constructed in *E. coli* and the active containment systems were designed for the use of *P. putida* in bioremediation.

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Table 5. TA systems used in a variety of biotechnical applications.

Application	Organism	TA system	Killing induction	References
Live vector vaccines	*S. typhi*	*hok/sok*	Plasmid loss	Galen *et al.* (1999)
	S. typhimurium	*dapD*[a,b]	Absence of lactose or IPTG	Garmory *et al.* (2005)
Phage therapy	*E. coli*	*gef*, *chpBK*	Lactose or IPTG	Westwater *et al.* (2003)
	E. coli	*bglII*	Lactose or IPTG	Hagens and Bläsi (2003)
	P. aeruginosa	*bglII*	Lactose or IPTG	Hagens *et al.* (2004)
Vectors	*E. coli*	*ccdB*	Insert-less MCS	Bernard *et al.* (1994)
	C. neoformans	*ccdB*	Lactose or IPTG	Mondon *et al.* (2000)
	F. tularensis ssp. *tularensis*	ORF4-ORF5	Plasmid loss	LoVullo *et al.* (2006)
	E. coli	*ccdAB*	Plasmid loss	Wegerer *et al.* (2008)
Anti-antitoxin PNAs	*E. coli*	*hok/sok*	Antitoxin sequestration	Faridani *et al.* (2006)
Gene silencing	*E. coli*	RNA I/RNA II	–	Pfaffenzeller *et al.* (2006a,b)
RASTA-Bacteria[b]	–	–	–	Sevin and Barloy-Hubler (2007)

a. TA-less killing function.
b. Web-based annotation tool.
Bacterial containment systems were listed in Table 4.

Containment systems for GEMs

Pollution of the environment with persistent, toxic and xenobiotic chemicals is of global concern and requires remediation of these sites (Paul *et al.*, 2005). Bioremediation relies on the degradative pathways for various organic substrates as sources of carbon and energy, which have evolved in microorganisms over the time, and which may be more eco-friendly and cost-effective, in comparison with physicochemical processes (Pieper and Reineke, 2000; Cases and de Lorenzo, 2005). For an effective taxonomic affiliation of microorganisms involved in bioremediation processes, polyphasic approaches as a combination of more traditional phenotypic and physiological approaches with modern molecular biology techniques are particularly successful because only a community of microorganisms provides a sufficient metabolic diversity for disposal of these anthropogenic pollutions (Pieper and Reineke, 2000). In addition, physicochemical and also special biological remediation processes are frequently used for the removal of chemicals which resist biodegradation (Dua *et al.*, 2002).

Based on the multitude of sequenced genomes, gene clusters encoding degradative pathways for various aromatic and other hazardous organic compounds were cloned and characterized (Sangodkar *et al.*, 1989; Samanta *et al.*, 2002). Several GEMs with novel or improved degradative properties were constructed, and their potentials as biocatalysts were investigated (Pieper and Reineke, 2000; Furukawa, 2003; Lovley, 2003). Owing to security concerns and fears of non-predictable horizontal gene transfer and uncontrolled proliferation when GEMs would be released to the environment, legal requirements restrict possible applications of GEMs in bioremediation, although they were already successfully investigated under laboratory conditions (Diamand, 1999; Paul *et al.*, 2005). Therefore, bacterial containment systems were developed by combining degradative pathways for organic compounds mainly with TA systems for the use of GEMs in bioremediation (see Table 4). These systems aim at killing the cells by toxin expression, for example by induction through amino acid starvation when they escape the designated environment. TA-absent containment systems using GEMs expressing bacterial haemoglobin to degrade aromatic compounds under hypoxic conditions have been reviewed elsewhere (Urgun-Demirtas *et al.*, 2006). Table 4 summarizes containment systems and gives cross references to the relevant literature.

Model containment systems for controlled killing of bacteria. The first model containment system was designed in *E. coli* with the *hok* gene (see above), encoding the Hok toxin gene in fusion with the promoter of the tryptophan operon (Molin *et al.*, 1987). Under controlled conditions such as in fermentation processes with sufficient supply of tryptophan in the medium, the *hok* gene is not expressed due to an inactivated tryptophan promoter. Inadvertent escape to the environment with tryptophan starvation or genetic rearrangement of the containment system led to expression of the toxin and therefore to cell death. Molin and colleagues suggested that time-dependent toxin induction regardless of growth rate would be more efficient than toxin expression depending on cell division, since the latter is unpredictable in nature (Molin *et al.*, 1987; 1993). Therefore, they placed a *hok* gene under control of the invertible *fimA* promoter periodically expressing type I fimbriae in *E. coli*. The plasmid-located *fim* gene cluster provides antagonistically acting *fimB* and *fimE* gene products which are involved in the regulation of the *trans* activated *fimA* promoter. Because FimB and FimE act as simple activator/repressor proteins, FimB mediates an active promoter configuration resulting in *fimA* transcription (Molin *et al.*, 1987). The investigated mutant *E. coli* MC1000 harboured either one or three additional different compatible plasmids with different

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copy numbers in addition to the above described plasmid carrying the *fim* cluster. In addition, plasmids containing the genes *fimB* alone, *fimB* in combination with *fimE* or only *fimE* were also investigated. Finally, if the positive regulator FimB is present in excess, the cells are killed due to Hok toxin expression. In a slowly growing population such as occurring in the environment, stochastic dying of a cell fraction would lead to a gradual reduction of viable cell counts over time. However, in a fast-growing population such time-dependent killing would be insignificant with regard to the growth rate, thus permitting the controlled containment of bacteria under certain environmental conditions. These results were confirmed in a subsequently performed experiment that used the *gef* gene, a *hok* homologue, under control of the *fimA* promoter in *E. coli* (Klemm *et al*., 1995).

A related suicide vector-based *hok* expressing model containment system with additionally mediated carbenicillin resistance was under control of the *lac* promoter (Bej *et al*., 1988). Cultivation upon induction with IPTG under antibiotic selection led to mutational resistance against *hok* expression, whereas induction under non-selective conditions resulted in a toxin-mediated killing of 90–99% of the cell population. Surviving cells, grown under the latter condition, had developed a resistance against the still active *hok* gene with reduced energy-generating membrane functions and exponential growth. There may be also the following explanations (Bej *et al*., 1988): a mutation in the *lacI* gene occurred, which prevented *hok* induction, or in the cells carrying the suicide vector making them resistant to Hok polypeptide. Thus and due to the results obtained during cultivation in presence of carbenicillin, the authors concluded that their system was not elaborated enough to ensure the fail-safe containment of GEMs.

Additionally, plasmid maintenance by the complete *parB* locus, encoding the *hok/sok* TA system fused to the phosphate-repressed promoter of the alkaline phosphatase gene (P_{phoA}), was investigated in *E. coli* (Schweder *et al*., 1992). This model containment system provided *hok*-mediated cell killing due to phosphate limitation, which often occurs as a growth-limiting factor in the environment. Phosphate is an essential constituent in media, thus it ensures plasmid maintenance under these controlled conditions. Analyses of non-selective and glucose-limited cultivations of strains harbouring a plasmid with *hok/sok* or a P_{phoA}-*hok/sok* construct revealed no plasmid-free cells after 50 generations or only 3% plasmid loss between the 50th and 60th generation. When grown in phosphate-limited medium, cell killing was observed after 4 h of cultivation but about 5% of the population still remained viable after 8 h. Because of the more effective *hok*-based model containment systems Schweder and colleagues suggested that the integration of the complete *parB* locus and resulting *hok* mRNA:Sok-RNA interactions might have been unfavourable for the efficacy of cell killing.

Development of functional bacterial containment systems applicable in bioremediation. Contreras and colleagues (1991) constructed the first active containment system in *Pseudomonas putida* to degrade a variety of substituted benzoates by the use of two plasmid-borne elements. Enhanced substrate range and a 50-fold increased affinity for 3-methylbenzoate as effector were achieved in the substitution mutant XylSR45T. The *gef* gene, a *hok* homologue, was placed under control of a LacI-repressed promoter and the corresponding *lacI* gene under the influence of a XylS-regulated promoter. This provided a suicide function resulting in cell killing through Gef toxin activity in the absence of substituted benzoates as XylS effectors. That means no active form of XylS was formed allowing *lacI* expression and therefore no *gef* (toxin) expression downstream of a P_{tac} promoter. However, as observed in previous studies (Molin *et al*., 1987; Bej *et al*., 1988), few cells became resistant for uninvestigated reasons.

To study factors that limit the killing efficiency of containment systems, plasmids harbouring the *relF* gene, homologous to *hok* and *gef*, fused with the *lac* promoter were used in *E. coli* (Knudsen and Karlström, 1991). The factors were determined to be the mutation frequency of the toxin gene with 10^{-6} per cell per generation, preventing cells from being killed, and a basal toxin expression, which results in a reduced growth under controlled conditions, but provides a selective growth advantage to cells with mutational resistance towards toxin activity. A containment system with a second copy of the *relF*-encoding plasmid led to a reduced mutation rate of $< 5 \times 10^{-9}$ per cell and generation. In a subsequent study, a plasmid harbouring two *relF* genes, which were arranged to prevent inactivation by deletion or recombination and insertional inactivation, resulted in a mutation rate of 10^{-8} per cell and generation (Knudsen *et al*., 1995).

The active containment system of Contreras and colleagues (1991) was altered in a way that a transposaseless transposon bearing the *gef* gene in fusion with a LacI-repressed promoter for random integration into the *P. putida* chromosome, and the XylSR45T mutant with *p*-ethylbenzoate as additional effector molecule were used (Jensen *et al*., 1993). Toxin resistance occurred with a frequency between 10^{-5} and 10^{-6} per cell per generation and 10^{-8} in cells harbouring two copies of the suicide function respectively. A *P. putida* strain with the chromosomally integrated transposon was used for alkylbenzoate degradation and its applicability for bioremediation in alkylbenzoates-containing soils was demonstrated (Ronchel *et al*., 1995).

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Additional containment systems have been reported: a sucrose-sensitive *E. coli* strain harbouring a chromosomally integrated *sacB* gene encoding levansucrase, which converts sucrose to levan that causes cell lysis (Recorbet *et al*., 1993), or the degradation of polychlorinated biphenyls by a *P. putida* strain that is killed due to colicin E3 RNase activity (Munthali *et al*., 1996a,b).

Further improvements were achieved with a novel concept in *P. putida* based on the *stv* gene encoding streptavidin (Szafranski *et al*., 1997; Kaplan *et al*., 1999), which interacts with biotin and thus inhibits biotin-depending enzymes. The constructed *P. putida* strain was used for bioremediation of aromatic hydrocarbons and in the absence of the effector 3-methylbenzoate, a reduction of at least 90% of the population was detected with a mutation rate from 10^{-7} to 10^{-8} per cell and generation.

Ronchel and Ramos (2001) designed a novel bacterial suicide containment system ensuring controlled killing without the risk of mutational inactivation. The P_m promoter was fused to the essential *asd* gene, which is therefore expressed in the presence of substituted benzoates as effector molecules. The *asd* gene encodes the aspartate-semialdehyde dehydrogenase which is essential for biosynthesis of methionine, threonine and isoleucine, lysine and DAP as a constituent of peptidoglycan layers (Truffa-Bachi and Cohen, 1968). The additional integration of a containment system reported previously (Ronchel *et al*., 1995, see above) resulted in an undetectable mutation rate ($< 10^{-9}$ per cell per generation), and the respective GEM population of *P. putida* was eliminated in less than 25 days in soil experiments.

An alternative dual model containment system consists of two TA loci, the colicin E3-immunity E3 system and the EcoRI restriction–modification system, with plasmid-borne toxins and both antitoxins stably integrated into the *E. coli* chromosome (Torres *et al*., 2003). The so-called 'containment efficacy' of this dual containment system was experimentally determined and higher than the ones of single containment systems, but lower than the expected values of theoretical calculations (Knudsen and Karlström, 1991). Mutated clones that escaped the expected killing effect showed deletions or insertions in one or both lethal genes. However, horizontal gene transfer in this dual system was effectively decreased and the presented system provides a valuable strategy to increase containment.

Pandey and colleagues (2005) conceptualized a novel active containment system with improved plasmid maintenance and regulated survivability in the environment. Two different TA systems would be necessary, one with plasmid-borne toxin and chromosomally integrated antitoxin, the other in reverse arrangement. Both toxin genes would be constitutively expressed, and the antitoxins would be under control of promoters of catabolic operons such as P_m. In the absence of effector molecules which induce antitoxin expression, the cells would be killed due to toxin activity. Thus, survival upon escaping from a defined environment would be prevented. Horizontal transfer of the plasmid would result in toxin-mediated cell killing since the respective antitoxin would be lacking in the recipient cell.

Use of TA systems in antimicrobial treatments. Several applications have been developed, which use TA systems for stable plasmid maintenance and killing of progeny cells without plasmids, in antimicrobial treatments. Among these applications are expression plasmids for the delivery of heterologous antigens to the human immune system in attenuated *Salmonella* spp. live vector vaccine strains that were shown to be effective in treatments of *Salmonella* infections. To treat infections of *E. coli* or *Pseudomonas aeruginosa*, other approaches were based on genetically engineered phages, which inject DNA *inter alia* thereby killing the bacteria. Specific peptide nucleic acids (PNAs) were constructed as a novel class of antimicrobial compounds to sequester antitoxin-RNA and to enable toxin activity in *E. coli*. TA system-dependent shuttle vectors have been also designed as genetic tools for the highly pathogenic bacterium *Francisella tularensis* (see below).

Plasmids expressing heterologous antigens for vaccine therapies in Salmonella *spp.*

Expression of immunogenic and protective antigens for delivery to the human immune system from plasmids in attenuated *Salmonella* spp. live vector vaccine strains are one possibility in vaccine development (Galen *et al*., 1999; Garmory *et al*., 2002). Sufficient expression level of antigens and stable plasmid maintenance define the efficacy of live vector vaccine strains. A pre-clinical model of murine intranasal immunization was established for *Salmonella typhi* CVD 908-based live vector vaccines in mice (Galen *et al*., 1997) and various heterologous antigens have been successfully expressed within this strain (Barry *et al*., 1996). Although an addiction system for plasmid maintenance based on an essential gene was designed in attenuated *Salmonella typhimurium* strains (Nakayama *et al*., 1988) and expressed successfully heterologous antigens in mice (Karem *et al*., 1995; Srinivasan *et al*., 1995; Covone *et al*., 1998), stabilization of plasmids with this system in *S. typhi* vaccine strains failed, yet (Tacket *et al*., 1997). Nakayama and colleagues (1988) constructed a *S. typhimurium* mutant lacking the *asd* gene. Mutants without a plasmid-borne *asd* gene or cells grown in absence of DAP were killed.

To alternatively enhance plasmid maintenance and stability in *S. typhi*, two sets of novel non-catalytic mul-

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ticopy expression plasmids were developed that harboured the *hok/sok* antisense RNA-regulated TA locus (see section above) and one or both plasmid partition functions encoded by *par* (Wahle and Kornberg, 1988) and *parA* (Dam and Gerdes, 1994) to avoid random segregation (Galen *et al*., 1999). The sets contained either the *ori*E1 or the *ori*15A origin of replication expected to result in approximately 60 or 15 copies per cell, respectively, and a derivative of the osmolarity-inducible *ompC* promoter cassette (P_{ompC1}) from *E. coli* was used (Norioka *et al*., 1986). The plasmids also harboured the *gfpuv* gene encoding a UV-optimized variant of the green fluorescent protein (GFPuv) as a test antigen. Plasmid maintenance was measured by quantification of GFPuv-mediated fluorescence using flow cytometry in the absence of antibiotics. These experiments revealed that plasmids containing the higher-copy-number *ori*E1 origin of replication were very unstable and even when cultivated in the presence of antibiotic selection, 50–62% of the cell population did not express measurable amounts of GFPuv any longer, depending on the plasmid's maintenance functions. Examinations revealed that non-fluorescing bacteria were viable and sensitive to antibiotic due to plasmid loss.

Consistent with other reports, these results indicate that, in contrast to plasmids containing the *ori*E1 replicon, the *hok/sok* TA system improves the stability of plasmids with *ori*15A origin of replication (Gerdes, 1988), although it does not ensure killing of all plasmid-free cells (Wu and Wood, 1994). To enhance plasmid stability, *hok/sok* was combined with the *parDE* and *pnd* loci; however, long-time cultivation in the absence of an antibiotic did not result in complete plasmid stability (Pecota *et al*., 1997). According to recent studies (Stephens *et al*., 2006), this result gives rise to doubts about strategies using higher-copy-number plasmids to express heterologous antigens in *S. typhi* as the metabolic burden promotes plasmid loss. Galen and colleagues (1999) suggested that the design of *S. typhi* live vector vaccines strains with stabilized low-copy-number plasmids expressing heterologous antigens to be a promising strategy in vaccine development. These plasmids should be able to express high levels of heterologous antigens in response to an environmental signal that occurs *in vivo* at the destination area of immunological induction which the vaccine organism has to reach.

Maintenance of high-copy-number plasmids in the absence of selectable marker genes was achieved by using ORT to successfully express the *Yersinia pestis* F1 antigen in an attenuated *S. typhimurium* strain (Garmory *et al*., 2005). The *lac* repressor gene was chromosomally integrated to ensure effective repression of the essential chromosomal *dapD* gene, which was placed under control of the *lac* operator–promoter region. Despite this, a basal expression of *dapD* in the absence of IPTG was detected; however, growth was only possible in liquid media. Cultivation of *S. typhimurium* for 5 days demonstrated ORT-mediated stable plasmid maintenance without plasmid loss. *In vivo* experiments in mice revealed recombinant bacteria, although significantly lower levels were particularly measured due to the metabolic burden of harbouring high-copy-number plasmids. To provide immunization against plague in mice, the kanamycin resistance gene of a plasmid expressing *Y. pestis* F1 antigen (Titball *et al*., 1997) was exchanged for the palindromic *lac* operator, and no plasmid loss was detected in *S. typhimurium* over 5 days. This was the first time that a single dose of *S. typhimurium*-based vaccine was shown to protect against plague. A comprehensive overview of the mentioned systems for vaccines therapies is provided in Table 3.

Anti-Sok PNA oligomers in E. coli (Faridani *et al., 2006*)

Faridani and colleagues (2006) developed a method to sequester Sok-RNA competitively to inhibit *hok* mRNA:Sok-RNA interactions in *E. coli*. They designed PNA oligomers that were complementary to the 5′ end of Sok-RNA to prevent Sok-mediated degradation of *hok* mRNA; this led to cell killing through synthesis of Hok toxin. To enhance uptake in *E. coli*, the PNA oligomers were attached to the synthetic peptide $(KFF)_3K$ that increases the permeability of the outer membrane (Vaara and Porro, 1996). Already 10 μM of one of the anti-Sok PNAs prevented growth of *E. coli* cells for as long as 20 h, whereas a lower concentration or other PNAs exerted only a transitive growth arrest. Surprisingly, the inhibitory effect of the global transcription inhibitor rifampicin on viable cell counts was significantly lower than that of the PNA oligomers even at a 20-fold higher concentration. Thus, anti-Sok PNAs are more bactericidal to *E. coli* containing a *hok/sok* TA system than rifampicin. After treatment of an *E. coli* culture harbouring *hok/sok* with either anti-Sok PNA or rifampicin, dead 'ghost' cells occurred and mature *hok* mRNA was accumulated. In contrast to antibiotics, which are rapidly exported from cells, there is evidence that PNA oligomers accumulate in *E. coli* (Good *et al*., 2001).

Peptide nucleic acids are potent competitive inhibitors of toxin mRNA:antitoxin-RNA interactions. To enhance their bactericidal activity for clinical applications, their cell influx and toxicity must be further enhanced. Antisense agents targeting essential genes are effective against *E. coli* infections in mice (Geller *et al*., 2005; Tan *et al*., 2005). Faridani and colleagues (2006) proposed that chromosomal antitoxin-RNAs may be sequestered by the cells as a resistance mechanism against PNAs. Mutations that do not inactivate the toxin, could lead to cell death if only the formation of toxin mRNA:antitoxin-RNA

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is prevented. PNA oligomers may therefore present a new class of effective antimicrobials for treatment of bacterial infections.

Novel shuttle vectors for use in F. tularensis *ssp.* tularensis (LoVullo *et al., 2006*)

The Gram-negative bacterium *F. tularensis* is one of the most infectious pathogens known and causes the zoonotic disease tularaemia, which is rarely fatal when treated with appropriate antibiotics, even in its more serious pneumonic and typhoidal forms, but infected persons are often severely debilitated (Titball *et al*., 2007). Biosafety-level three conditions are necessary for manipulation of *F. tularensis* (Dennis *et al*., 2001), and most of the genetic tools such as *E. coli–Francisella* shuttle vectors, derived from the cryptic *Francisella*-plasmid pFNL10 (Norqvist *et al*., 1996), have been developed for the moderate pathogenic subspecies *novicida* and a *holarctica*-derived attenuated live vaccine strain (LVS, see Table 5). Recently, genetic tools including shuttle vectors, transposons and allelic replacement strategies have been exhaustively reviewed (Frank and Zahrt, 2007). A challenge in the development of genetic tools for *F. tularensis* is its resistance to β-lactam antibiotics and the restricted use of other antibiotics that are administered in tularaemia treatments (Baker *et al*., 1985).

In need to develop genetic tools and to test marker genes for the most pathogenic *F. tularensis* ssp. *tularensis*, LoVullo and colleagues (2006) constructed novel *E. coli–Francisella* shuttle vectors, which were derived from the cryptic *Francisella*-plasmid pFNL10 (Pomerantsev *et al*., 2001). In a future perspective, an attenuated *F. tularensis* ssp. *tularensis* vaccine strain might be more effective in providing protective antigens for delivery to the human immune system than the existing vaccine strains derived from other *F. tularensis* subspecies (Conlan and Oyston, 2007). A spontaneous mutant of a specific *F. tularensis* ssp. *tularensis* strain was recently discovered and shown to be both safer and more protective against *F. tularensis* infections in mice than the *holarctica*-derived LVS (Twine *et al*., 2005).

Further applications based on TA systems

Besides the broad applications in bioremediation and antimicrobial treatments, a few other usages of TA systems were reported. Among them are cloning vectors that harbour a toxin gene as a positive selection marker. Plasmids with ColE1-derived origin of replication were successfully shown to silence a chromosomal target gene due to toxin mRNA:antitoxin-RNA interactions. To relieve and improve the identification of novel TA systems, a web-based tool for identifying TA loci in prokaryotes has been developed (Sevin and Barloy-Hubler, 2007).

Incorporation of TA systems into cloning vectors. Two novel positive selection vectors derived from the high-copy-number vectors pUC18/19 have been constructed (Bernard *et al*., 1994). The 3.0 kb vectors harboured both an ampicillin resistance gene and the *ccdB* toxin gene with incorporated MCS differently orientated in both vectors, under control of the *lac* promoter. The cytotoxic active gene *ccdB* was found on the *ccd* (control of cell death) locus on the F plasmid and kills the hosts by interfering with the DNA–gyrase cleavable complex (Bernard, 1995). To avoid killing of vector-harbouring cells, maintenance in DNA gyrase mutants suppressing the toxin activity or hosts expressing *lac* repressor was necessary. By insertion of DNA fragments of at least 150 bp into the MCS, positive selection upon IPTG induction was possible due to disruption of the *ccdB* gene and subsequent occurrence of viable colonies. In a further study, the ampicillin resistance gene was replaced by the kanamycin or chloramphenicol resistance gene (Bernard, 1995).

A novel *ccdB*-based shuttle vector for transformation of the basidiomycetous yeast *Cryptococcus neoformans* was designed that combined positive selection in bacteria with functions for enhanced episomal maintenance in *C. neoformans* (Mondon *et al*., 2000). This approach was used to force plasmid stability with a multitude of positive influences from known elements on plasmid stability in yeasts. Autonomously replicating plasmids have been shown in several yeasts to be lost during mitosis under non-selective conditions. Therefore, the *ccdB* toxin gene, telomere sequences, the STAB fragment enhancing stable plasmid maintenance (Varma and Kwon-Chung, 1998) and the *URA5* gene as a selectable marker on media that contain 5-fluoroorotic acid (Edman and Kwon-Chung, 1990) were assembled to the novel vector. The above mentioned STAB-DNA sequence itself resulted in an enhanced episomal stability in more than 60% of the transformants. Furthermore, plasmid-borne prototrophic stability in these transformants was enhanced up to 80% (up from 0 to 5% regarding to the control experiment). Although vector-mediated CcdB activity was shown to inhibit growth of *E. coli*, no such effect was detected with *C. neoformans*.

Maintenance of ColE1 plasmids due to gene silencing based on RNA interactions. Plasmids derived from vectors with a ColE1-type origin of replication offer the possibility to silence engineered target genes by toxin mRNA:antitoxin-RNA interactions (Pfaffenzeller *et al*., 2006a,b). The replicational regulatory network of these plasmids consists of RNA II that functions as a primer by hybridizing to a region in the origin of replication and RNA I, inhibiting this hybridization by prior binding to RNA II (Brenner and Tomizawa, 1991). Pfaffenzeller and colleagues (2006a) integrated the green fluorescent pro-

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tein (GFP) chromosomally into *E. coli* and obtained sequences of different length complementary to RNA I near to the ribosomal binding site. As a result the use of a ColE1-type plasmid should lead to translation inhibition by interactions of RNA I with the mRNA of the target gene. Investigations on GFP expression in presence and absence of a ColE1-type plasmid showed no difference for the control strain without RNA I-complementary sequence. A decrease in the GFP expression of 36% was observed for the strain with entire RNA I-complementary sequence in the presence of plasmid-encoded RNA II. However, a strain that included only a partially complementary sequence showed a significantly lower GFP expression without ColE1-type plasmid, whereas almost no expression was detected in the presence of the plasmid. These results indicate that not only sequence length but also structural stability were important for the silencing of the target gene. Finally fermentation experiments confirmed the result that a partial complementary sequence to RNA I ensures almost entire gene silencing over 22 h.

Integration of the ccdAB *TA systems into rhamnose-inducible expression vector.* Expression systems for plasmid-encoded heterologous genes need special requirements: (i) they have to ensure high yields of enzymes and proteins (ii) in a cost-effective production (iii) at high plasmid stability (iv) while the expression has to be tightly controllable (Stumpp *et al.*, 2000; Wegerer *et al.*, 2008). A versatile expression vector is pJOE3075, which contains the genes for L-rhamnose uptake and catabolism under control of their native promoter ($rhaP_{BAD}$) as a positive regulator (Stumpp *et al.*, 2000). The completely sequenced expression vector pJOE4056.2, a derivative of pJOE3075, harbours the enhanced green fluorescent protein (eGFP) gene under control of the $rhaP_{BAD}$ promoter (Wegerer *et al.*, 2008). To optimize pJOE4056.2, a stem loop structure was initially inserted into the transcription initiation region of the $rhaP_{BAD}$ promoter resulting in a fourfold increased eGFP production (Wegerer *et al.*, 2008). However, consequences on plasmid maintenance were not detected. Subsequently, combinations of the genetic modules *rop*, *ccdAB* and *cer* with regard to their advantages for the above mentioned qualities of plasmid-encoded expression systems were investigated. The genetic modules have different functions: *rop* is involved in the maintenance of constant but low copy numbers for plasmids (Cesareni *et al.*, 1982), *ccdAB* encodes a TA system, with a toxin activity leading to inhibition of DNA gyrase and therefore to cell killing, and *cer* does not only participate in the resolution of plasmid multimers (Summers and Sherratt, 1984) but also contains a genetic element the transcript of which retards cell division in the presence of plasmid multimers and functions in plasmid maintenance (Patient and Summers, 1993; Balding *et al.*, 2006). Deletion of *rop* led to a more than threefold increase in plasmid DNA without a simultaneous rise in eGFP production due to the high content of eGFP already available. The inadvertent insertion of a *cer* gene tandem had a tremendous stabilizing effect because more than 90% of the cells retained the plasmid under antibiotic selection, whereas over 50% of the population lost a *cer*-less plasmid. Excision of one *cer* gene did not affect plasmid maintenance but increased the yield of eGFP. Surprisingly, the integration of *ccdAB* resulted in an additional plasmid loss; Wegerer and colleagues (2008) ascribed this observation to the fact that two independently positive effects in combination might present a disadvantage. Since expression vectors with $rhaP_{BAD}$ promoter are already efficient, tightly regulated and well balanced, further improvements might be difficult to establish and unfavourable. Therefore, the novel constructed plasmid pWA21 containing the stem loop, a single *cer* gene and the *rop* gene offered both high expression levels and an accurate plasmid stability.

Applications in enzyme production

The applicability of complemented *P. fluorescens* mutants with uracil and proline auxotrophy was analysed during production of α-amylase and nitrilase (Schneider *et al.*, 2005). Two plasmids were used: one plasmid encoding the respective enzyme under control of a LacI-repressible promoter and tetracycline resistance, while the other plasmid harboured *lacI* and the kanamycin resistance gene (kam^R). In a first step, the tetracycline resistance gene was replaced by *pyrF*, and subsequently (kam^R) was exchanged by *proC*. Fermentation experiments at the 20 l scale over 75 h showed no difference in enzyme activity and optical density between antibiotic-containing plasmids, those encoding PyrF and kanamycin resistance and plasmids that complemented both auxotrophic mutations. Thus, plasmid loss led to auxotrophy for uracil and proline and plasmid-free progeny cells were killed. Plasmid maintenance was measured with two different methods and revealed that 94–97% of the population retained the plasmids even after 62 generations. The detection of a slower growth rate of auxotrophic cells compared with complemented cells even on medium supplemented with uracil and praline was cited as reason for the small fraction of plasmid-free segregants.

Production of the biopolymer cyanophycin

Cyanophycin (CGP) is a polymer of biotechnological interest (Mooibroek *et al.*, 2007), which consists of a polyaspartic acid backbone linked with arginine moieties (Simon, 1976). Different approaches to increase the production of

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CGP in *R. eutropha* H16 without the addition of antibiotics to stabilize plasmid maintenance were investigated. Strategies based on different plasmid types harbouring the cyanophycin synthetase gene of *Synechocystis* sp. PCC6308 or a chromosomal integration failed and did not enhance the production of CGP in *R. eutropha* H16 yielding only very low CGP contents. Therefore, in an alternative attempt to enhance the CGP content of the cells, the *eda*-dependent CAS was constructed (Voß and Steinbüchel, 2006). Flasks experiments revealed high CGP contents up to 40.0% (w/w) of cell dry weight without CGP precursor substrates. The CGP contents obtained in the absence of plasmid-stabilizing antibiotics were only slightly lower. Fed-batch fermentations at the 30 l and 500 l scale confirmed the results of the flasks experiments. Even in the absence of antibiotics, only 7% of the cells had lost the plasmid because the *eda* gene is essential for growth of *R. eutropha* H16 on gluconate or fructose.

The recently developed novel branch of ABS by Kroll and colleagues in 2009 revealed nearly full plasmid stability and represents a novel PAS without a possible bypass/loophole. This system was used to produce CGP and aims at providing precursors for isoprenoid biosynthesis as the target anabolic pathway. The use of this class of ABS with its independence of using defined carbon sources or cultivation media like mineral media may be very interesting for industrial fermentations. It provides also the possibility to use cheap agricultural commodities such as corn steep liquor or melasse as cultivation media.

Conclusion and outlook: applications and perspectives of PAS in biotechnology

Recombinant microorganisms are frequently used for the production of proteins, enzymes, organic compounds – e.g. amino acids, sugars, vitamins, alkanes and their various derivatives, antibodies and drugs in large-scale fermentation processes. These processes are often based on heterologous expression of plasmid-encoded genes and the systems have to meet the following quality criteria: (i) the respective products must be obtained with high yields, (ii) production must be cost-effective, (iii) plasmid stability must be high, while (iv) expression has to be certainly and tightly controllable (Jana and Deb, 2005; Sørensen and Mortensen, 2005; Wendisch *et al*., 2006; Wegerer *et al*., 2008). The host of choice for production of many recombinant proteins is in particular the Gram-negative bacterium *E. coli* for several reasons: (i) its economical and fast high-density cultivation possibilities (ii) combined with its simplicity, (iii) the extensive biochemical and genetic knowledge, and (iv) the large variety of available biotechnological tools for high-level expression of a multitude of recombinant proteins (Jana and Deb, 2005; Sørensen and Mortensen, 2005). Other organisms such as *Corynebacterium glutamicum* or the yeast *Pichia pastoris* are used for the industrial production of amino acids or therapeutic glycoproteins with fully humanized N-glycosylation structures respectively (Macauley-Patrick *et al*., 2005; Wildt and Gerngross, 2005; Wendisch *et al*., 2006). The importance of biotechnical processes in industrial production is steadily increasing, due to rising oil prices and the demand for ecologically feasible production (Wendisch *et al*., 2006). As most established systems for heterologous productions are plasmid-based, they often require antibiotics for plasmid selection. This is not only cost-intensive but also entails potential risks to the environment from the antibiotics in industrial waste as well as from the spreading of the resistance genes when cells would escape inadvertently (Hägg *et al*., 2004). Furthermore, the use of antibiotics in pharmaceutical production is limited since recombinant therapeutics require a contamination-free product or their use is prohibited for fear of emerging antibiotic resistances when they can be used in antimicrobial treatments (LoVullo *et al*., 2006). Thus, there is a strong need for alternative systems that maintain plasmids highly stable and antibiotic-free.

Toxin/antitoxin systems as the major PAS group can be further divided into three subtypes: (i) antisense RNA-regulated loci, whose antisense RNA-antitoxin binds to toxin mRNA and thereby inhibits toxin translation (Gerdes *et al*., 1997), (ii) restriction modification systems with a restriction endonuclease cleaving foreign DNA and a methyltransferase that prevents host DNA from being cleaved due to methylation (Kobayashi, 2004), and (iii) the classical proteic TA systems, which consists of seven TA gene families (Pandey and Gerdes, 2005), whose toxin and antitoxin are both actively present as proteins (Engelberg-Kulka and Glaser, 1999; Gerdes *et al*., 2005). TA systems were also discovered to be encoded chromosomally (Masuda *et al*., 1993; Aizenman *et al*., 1996; Pedersen and Gerdes, 1999), but their physiological role remains under speculative debate (Magnuson, 2007). The prevalent hypothesis is that they are involved in general environmental stress responses, e.g. to amino acid starvation, by induction of a bacteriostatic state and thus regulating the synthesis of macromolecules, such as DNA or proteins, depending on external nutrient supply (Gerdes *et al*., 2005; Kolodkin-Gal and Engelberg-Kulka, 2006). However, recent studies contradict this hypothesis by demonstrating that chromosomally encoded TA systems did not lead to selective advantages under stressful conditions (Szekeres *et al*., 2007; Tsilibaris *et al*., 2007; Saavedra de Bast *et al*., 2008).

Since the discovery of TA systems as natively occurring PAS in 1983 by Ogura and Hiraga, great efforts have been undertaken to identify additional TA systems and to enhance our knowledge on their characteristics and func-

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tionality (Gerdes *et al*., 2005; Pandey and Gerdes, 2005). The results have been used for implementation of TA systems in a large variety of applications. A major field of application is the construction of containment systems as artificial PAS for GEMs that are used in bioremediation (Lovley, 2003; Paul *et al*., 2005). These GEMs, mostly strains of *E. coli* or *P. putida*, are only viable in a specific environment for the desired task, e.g. the degradation of aromatic, phenolic or other organic compounds (Pieper and Reineke, 2000). Inadvertent escape or horizontal gene transfer is prevented as this induces cell killing mediated by inhibited expression of essential genes. This containment principle can also be employed on GEMs used for biochemical, biotechnological or pharmaceutical processes in industrial production (Molin *et al*., 1987; Schweder *et al*., 1992). The use of TA systems for maintenance of plasmids delivering heterologous antigens to the human immune system enables the necessarily antibiotic-free production and represents a second large area of application. For example, expression plasmids in attenuated *Salmonella* spp. live vector vaccine strains were shown to be effective in treatments of bacterial infections (Galen *et al*., 1999; Garmory *et al*., 2005). Infections of *E. coli* or *P. aeruginosa* were treated with genetically engineered phages, which inject, *inter alia*, DNA-encoded toxins that lead to bacterial cell death (Westwater *et al*., 2003; Hagens *et al*., 2004).

Finally, the following PAS should be mentioned: (i) TA systems as the major group of reported PAS, (ii) metabolism-based PAS including the hitherto only catabolism-based plasmid addiction system (CBS) (Voß and Steinbüchel, 2006), (iii) an outstanding ABS utilizes the biosynthesis pathway of isoprenoids (Kroll *et al*., 2009), (iv) complementation of auxotrophic mutations, e.g. for uracil and proline combined with expression of heterologous proteins in *P. fluorescens*, was reported (Schneider *et al*., 2005), and (v) ORT is based on essential chromosomal genes under control of the *lac* operator–promoter region, resulting in repression by binding of the *lac* repressor protein (Williams *et al*., 1998). Expression is only enabled in presence of plasmids that also harbour a *lac* operator–promoter region to titrate the repressor protein away from the chromosomal gene.

The increasing importance of the biotech industry with its interest in stable and recombinant production of proteins and chemicals especially based on renewable resources will be a key factor to predict PAS an important significance for the future. Nevertheless, not every PAS has reached an industrial applicable status. This might be the problem of an insufficient stabilization during the cultivation process or due to the difficult to integrate the system in existing production processes. Chromosomal integration of genes instead of using plasmid-based systems as a simple solution is not obvious in every case. Important factors like polar effects or limited gene copy numbers are just two disadvantages of this alternative. The development of further applications for addiction systems will be helpful to overcome current problems in biotechnology or pharmaceutical industry. For example, the field of cancer treatment could be supported by the use of special addiction modules. Toxin molecules like RelE or Kid/Kis induce or trigger apoptosis in human cells (Kristoffersen *et al*., 2000; de la Cueva-Mendez *et al*., 2003). As an example, this selective killing mechanism of eukaryotic (cancer) cells could be a future development based on the knowledge of naturally occurred addiction systems.

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