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MINIREVIEW

Active Stable Maintenance Functions in Low Copy-Number Plasmids of Gram-Positive Bacteria
I. Partition Systems

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Abstract

Low copy number plasmids cannot rely on the random segregation during bacterial cell division. To be stably maintained in the population they evolved two types of mechanisms (i) partition systems (PAR) that actively separate replicated plasmid molecules to the daughter cells and (ii) toxin-andidote systems (TA) that act after cell division to kill plasmid-less cells. Our knowledge of partition systems has been based mainly on analysis of plasmids from Gram-negative bacteria. Now, numerous partition systems of plasmids from Gram-positive bacteria have also been characterized and make significant contribution to our understanding of these mechanisms.

Key words: Plasmid stable maintenance, partition, Gram-positive bacteria

Introduction

The primary maintenance function in bacterial plasmids is played by the replication and copy number control systems, two mechanisms strictly correlated. Whereas plasmid replication system delivers sufficient number of plasmid copies, its copy number control mechanism provides the safety measure to minimize the metabolic cost on the host. Low copy number plasmids seem to have an advantage over high copy-number plasmids in exerting less metabolic burden on their hosts. However, the disadvantage of being a low-copy-number replicon is a high probability of loss from the population due to missegregation during cell division. On the basis of random segregation, the probability of plasmid-less cell appearance is 2^{1-n} (where n is the plasmid copy number) (Summers, 1991), what means that a plasmid existing in two copies per cell has a 50% chance of being inherited by the host progeny.

During evolution, plasmids have acquired/developed a number of features ensuring their stable maintenance in bacterial populations, to counteract the rule of copy number related loss-rate. The multimer resolution system (MRS) maximizes the number of plasmid molecules accessible for segregation, therefore is regarded as "passive" stabilization system that supports the random segregation of plasmids to daughter cells. The better than random segregation is ensured by two "active" stabilization systems: partition (PAR) and post-segregational killing systems (PSK) also called toxin-antidote (TA) systems, this review and the accompanying review will focus on, respectively.

Partition systems actively separate and distribute plasmid molecules to the distal parts of the parental cell before it undergoes the division, ensuring that the progeny cells inherit at least one copy of the plasmid. The PSK/TA systems (Dmowski and Jagura-Burdzy, 2013) either induce safety measures when the copy number of the plasmid drastically drops or eliminate bacterial cells that have accidentally lost the plasmid.

On the top of that, the conjugation ability provides the conjugative plasmid with a chance to re-infect those cells, which did not receive the plasmid copy during cell division, hence increasing plasmid "stability" in the population.

Plasmid replication and copy number control

Plasmid replication regulation depends on its mode of action. In general the replication of bacterial plasmids can operate either by σ (sigma) or θ (theta) mechanism, reviewed by del Solar *et al.* (1998). First analysis of bacterial replicons suggested that those from Gram-negative bacteria are mostly θ-type, whereas those from Gram-positive bacteria are of σ-type. This assumption

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had to be verified after discovery of θ – replicating plasmids in Gram-positive species and also σ – replicating plasmids among Gram-negative bacteria (Kleanthous *et al*., 1991; Yasukawa *et al*., 1991; del Solar *et al*., 1993; Bruand *et al*., 1991; Bruand *et al*., 1993; Le Chatelier *et al*., 1993; Benachour *et al*., 1995; Meijer *et al*., 1995).

The σ type replication, also called rolling circle replication (RCR) is unidirectional and asymmetric since the leading and the lagging strand synthesis is uncoupled. The replication initiates at the *dso* (double-stranded origin), where a site-specific nick is introduced by the Rep protein on the plus strand (Fig. 1). The resulting 3'-OH end is used as the priming site for synthesis of the leading strand. This process involves the DNA polymerase III holoenzyme, the single strand binding protein SSB and a helicase. Further single strand elongation stops when the replisome reaches the *dso*. As a result, a dsDNA molecule and a ssDNA molecule are produced. The dsDNA is composed of the parental minus strand and the newly synthesized complementary strand, whereas the ssDNA is the parental plus strand. The strand complementary to the ssDNA is synthesized from the *sso* (single-stranded origin) by host proteins (del Solar *et al*., 1998). The initiation of σ type plasmid replication may be controlled by an antisense RNA (complementary to the *rep*-mRNA) and/or by a repressor protein (Espinosa *et al*., 2002). The conjugative plasmids of Gram-negative bacteria use the σ mode of replication for production of ssDNA for transfer to the recipient cell, where the complementary strand is synthesized (Waters and Guiney, 1993; Llosa *et al*., 2002).

The designation "θ type replication" comes from the observation of forms of replicating plasmid molecules that resemble the letter θ. These forms result from the way that the replication proceeds: the parental DNA strands are melted at the origin of replication (*oriV*) with the help of plasmid-encoded initiator (Rep protein or RNA) and then the host replisome is loaded (Fig. 1). DNA synthesis is continuous on the leading strand but not on the lagging strand (Okazaki fragments). θ type replication can occur uni- or bidirectionally.

The replication and copy number control of θ type plasmids, which encode a Rep protein and contain repetitive Rep binding sites (designated iterons) is controlled by Rep and also intermolecular Rep-iterons complexes ("handcuffing") (Das and Chattoraj, 2004), sometimes additional repressor proteins are involved (RK2 of IncP-1 or RA3 of IncU) (Pansegrau *et al*., 1994, Markowska A. and Jagura-Burdzy G., unpublished). In plasmids which use RNA initiator extended by DNA polymerase I before replisome loading (*ColE1*-type), the copy number control is usually conferred by an antisense RNA, which inhibits initiator RNA interaction with DNA (Espinosa *et al*., 2002).

The variation of θ type replication designated the "strand displacement" proceeds in IncQ plasmids (reviewed by Meyer, 2009). The synthesis of complementary strand is uncoupled in time leading to the formation of dsDNA copies and accumulation of ssDNA molecules corresponding to plus strands.

Plasmid-encoded partition systems

The best studied partition systems originate from plasmids of Gram-negative bacteria. These systems will be briefly described below. Then, attention will be drawn to PAR systems of plasmids from Gram-positive bacteria.

In general plasmid partition systems are composed of a *cis* acting centromere-like site (*parS* sequence), and a bi-cistronic *par* operon encoding: a DNA binding protein (ParB-like protein) which binds to the centromeric site, and an NTPase (ParA-like protein) which is thought to deliver energy and the dynamic scaffold for plasmid molecules to be moved towards cell poles. The two proteins, together with specific DNA sequences form a nucleoprotein complex designated segrosome. So far the PAR systems have been classified into three types (four subgroups) on the basis of NTPase structure, the size of both Par proteins and the location of *parS* sequences (Gerdes *et al.,* 2000, Moller-Jensen and Gerdes, 2007) (Fig. 2).

The type I partition systems that encode ParA-like proteins being Walker type ATPases may be further divided into two subgroups Ia and Ib (Gerdes *et al.,* 2000). ParA-like proteins of type Ia systems are longer (251–420 amino acids) than those from type Ib (208–227) (Bignell and Thomas, 2001). Some of ParA-like type Ia proteins are DNA binding proteins and contain a helix-turn-helix motif (HTH) in their N-termini. These ParAs are usually involved in autoregulation of partition genes expression (exemplified by P1 prophage system). However, there are type Ia partition systems (IncP-1 plasmids) that encode two forms of ATPases by alternate translation initiation starts, none containing a DNA-binding motif in the N-terminus (IncC1/IncC2 of RK2), others *e.g.* RA3 of IncU (Kulinska *et al.,* 2008), PromA plasmids (Van der Auwera *et al.*, 2009) or chromosomal *parAB* systems (Quisel and Grossman, 2000; Leonard *et al*., 2005; Lasocki *et al*., 2007) encode only a short form of ParA. Although these ParAs do not recognize specific DNA sequences and are not the main autorepressors they may enhance the repression exerted by ParBs and they retain ability to bind DNA in unspecific manner. Since in some Ia systems, the *par* operon regulatory function may be fulfilled by the ATPase ParA as in P1 (Friedman and Austin, 1988) or additional repressor encoded in the partition operon *e.g.* KorA of

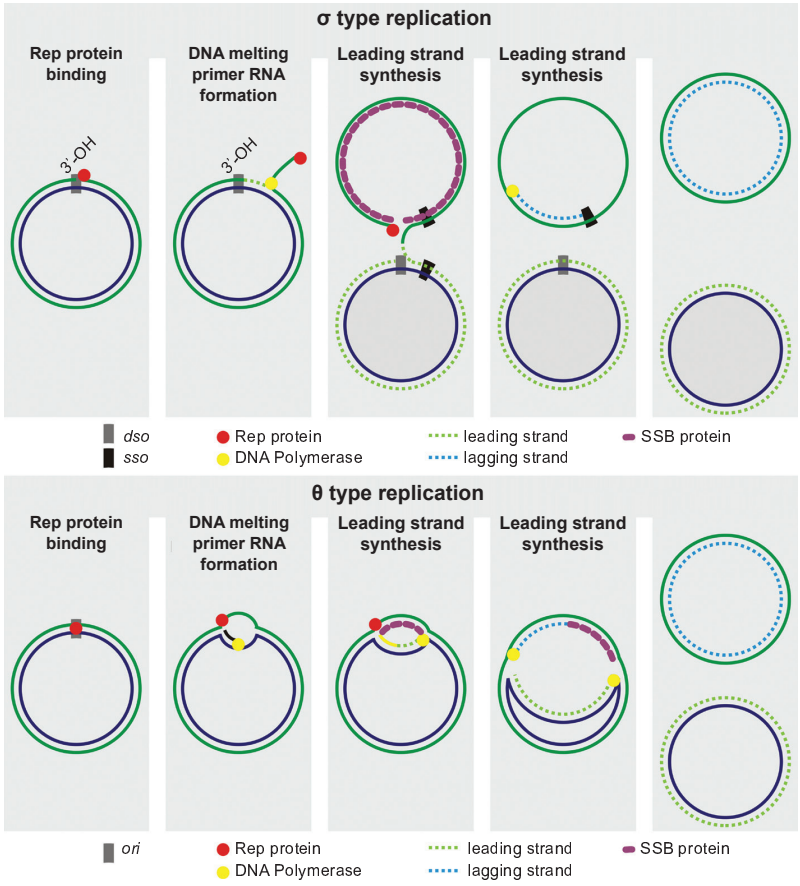

Fig. 1. Two main modes of plasmid replication. A. Scheme of σ type replication. B. Scheme of θ type replication. Details of replication processes are given in the text.

RK2 or KorA of RA3 (Kostelidou *et al.*, 1999; Kulinska *et al.*, 2011), the ParB-recognized centromeric sequence are usually located downstream the *par* operon.

In the type Ib partition systems, ParA-like proteins generally do not act as the transcriptional regulators and do not contain specific DNA binding motifs. In these systems the centromere-like site is located upstream the *par* operon in its promoter region and the expression of the operon is regulated by the ParB-like protein *e.g.* pSM19035, TP228 (de la Hoz *et al.*, 2000; Fothergill *et al.*, 2005).

In type II systems, motor proteins are actin-like ATPases (plasmid R1, Jensen and Gerdes, 1997), whereas in the type III this function is played by tubulin-like GTPases (plasmid pBtoxis, Larsen *et al.*, 2007). These two types of systems are organized similarly to type Ib with the centromere located in the promoter region of the operon.

Despite its role in segrosome formation, the DNA-binding component of *par* systems, ParB-like protein may act as the transcriptional regulator of its own expression or regulate other plasmid operons (Fig. 2). The ParB-like proteins of type Ia are larger (182–336 amino acids) than ParBs encoded by Ib, II and III systems (46–113 amino acids). The large ParB-like proteins contain the DNA-binding Helix-Turn-Helix motif (HTH) and are highly conserved forming so-called ParB family. The small ParB-like proteins analyzed so far, are much more variable in the primary amino acids sequence although structurally they are Ribbon-Helix-Helix (RHH) type proteins, with the β-strands from two monomers contacting the DNA major groove (reviewed by Schreiter and Drennan, 2007). A common feature of ParB-like proteins is the ability to dimerize (oligomerize), form segrosomes, large nucleoprotein complexes on DNA around centromere-like sites *parS*, and to interact with the partner, the ParA-like protein.

The general mode of action of the partition machinery seems to be similar regardless of the type of the system (Ia, Ib, II or III). Advances in this field were subsequently reviewed by Gerdes *et al.* (2004); Hayes and Barilla (2006); Shih and Rothfield (2006), Schumacher (2007), (2012), Salje (2010), Salje *et al.* (2010), Gerdes *et al.*, (2010). The crucial event in the partition process is the assembly of the segrosome which enables plasmid pairing and then separation. This nucleoprotein complex is formed on the *cis*-acting centromeric region, which is usually composed of repeated (in direct

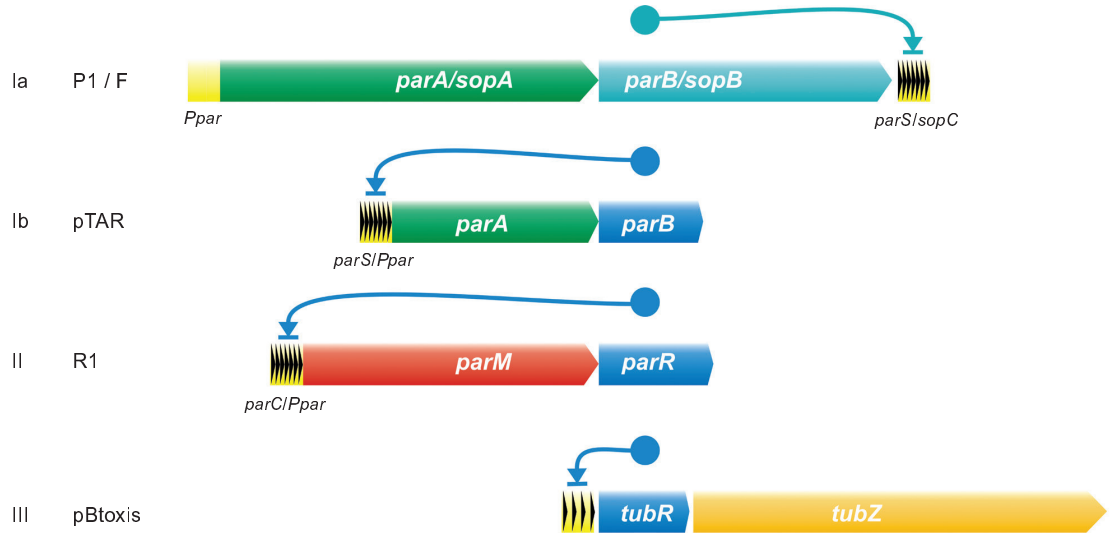

Fig. 2. Schemes of organization of type Ia, Ib, II and III partition systems. The Walker-type ATPases are shown in green, the actin-like ATPase is shown in red, the tubulin-like GTPase is shown in yellow. The centromere binding proteins are shown in blue and centromeric sequences as multiple black arrowheads.

or inverted orientation) sequences. The single repeat length may vary from several to dozens of nucleotides repeated over tenfold. Hence, each partition system has a specific organization of the centromeric region recognized and bound only by the cognate ParB-like protein (Fothergill *et al.*, 2005). In some plasmids multiple ParB binding sites are scattered through the genomes despite the fact that the single site seems to be sufficient for fulfilling its partition role as demonstrated for RK2, N15 and RA3 plasmids (Williams *et al.*, 1998; Dorokhov *et al.*, 2010; Kulińska *et al.*, 2011). All motor proteins (Walker type, actin-like, tubulin-like NTP-ases) can polymerize *in vitro* to form dynamic filaments in a process regulated by NTP binding and hydrolysis and stimulated by ParB-like proteins. The filaments form a scaffold enabling directed movement of plasmid molecules through interactions of NTPase molecules with the segrosomes. The processes of polymerization and depolymerization of actin-type and tubulin-type filaments mediate separation of plasmid molecules by either pushing or tramming (see Gerdes *et al.*, 2010 and references therein) (Fig. 3). There is a controversy over the mechanism the most commonly occurring Walker-type ATPases use to distribute plasmids equidistant from one another (Howard and Gerdes, 2010): the pulling mechanism by retracting filaments of ParA-like proteins has been proposed by Ringgaard *et al.* (2009) and the diffusion-ratchet mechanism that relies on ATP-bound ParA association with the nucleoid and dynamic re-distribution of ParA "cloud" within the cell tracked by the segrosome was recently proposed by Vecchiarelli *et al.* (2010) (Fig. 3).

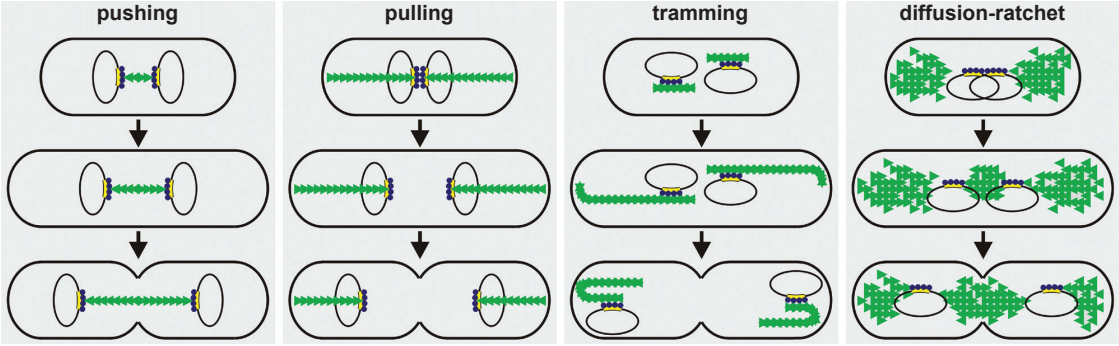

Fig. 3. Schematic representation of plasmid molecules separation during partition. The ParB-like proteins (blue circles) bind to the centromeric sequences (shown in yellow) on replicated plasmids. The polymerizing ParA-like protein (green triangles) interact with the ParB-DNA complex move the sister molecules toward bacterial cell poles.

Discoveries of new plasmids and analysis of their maintenance functions point out the prevalence of mechanisms described above although the list of partition systems may be far from exhaustion. The single partition protein has been identified as product of pSK1 plasmid gene *par* (described below). Recently, another stabilization system has been identified on plasmid R388 (Guynet *et al.*, 2011). It links localization and equipartition of plasmid molecules with functioning of conjugal transfer system.

Partition systems of Gram-positive bacteria

Type I systems with Walker-type ATPases

Plasmid pSM19035. The pSM19035 plasmid of inc18 family is one of the best studied plasmids that can replicate in a wide spectrum of Gram-positive bacteria. Its genome of 29 kb has large sections of plasmid backbone functions duplicated forming two reversely oriented arms separated by erythromycin resistance cassette and transfer genes. It had been shown that its derivative pBT233, containing a single arm (copy number ~7 per cell) is stably maintained in *B. subtilis* bacterial populations even in the absence of selection pressure (Ceglowski *et al.*, 1993). Later, it was demonstrated that its high stability is ensured by the partition system built of genes δ and ω (Dmowski *et al.*, 2006) and the post-segregational killing system – genes ε and ζ (Zielenkiewicz and Ceglowski, 2005) that are present in all plasmids from inc18 family.

The transcriptional organization of stability systems of the pSM19035 plasmid is unique. First, it was directly shown by RT-PCR (Dmowski *et al.*, 2006) that the *parB*-like ω gene is transcribed independently from the *parA*-like gene δ. Despite the lack of the common transcript for partition genes, the proper balance of expression is achieved at the level of transcription that is controlled by a common regulator. Both promoters *P*δ and *P*ω are repressed by the ParB-like Omega protein, that also regulates the pSM19035 plasmid copy number by repressing the *copS* gene expression (Fig. 4) (de la Hoz *et al.*,

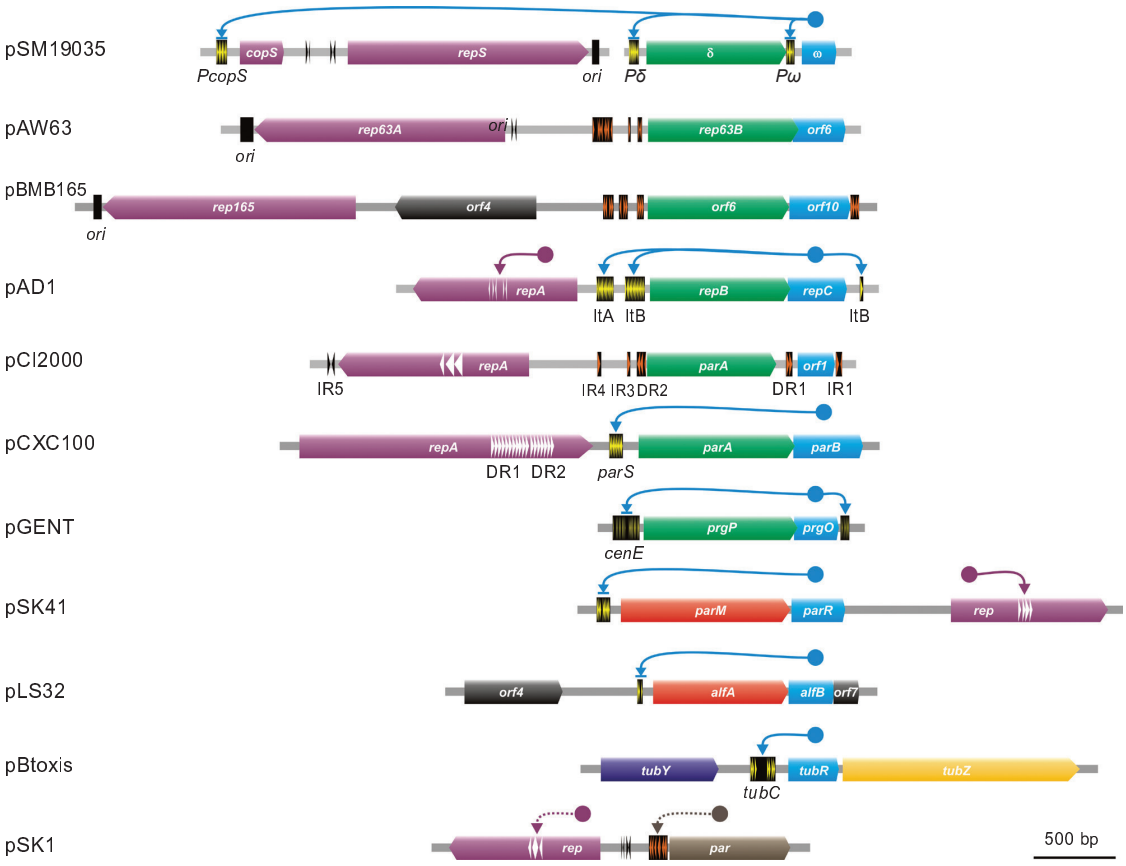

Fig. 4. Graphic representation of the genetic organization of partition modules from plasmids of Gram-positive bacteria. The Walker-type ATPases are shown in green, the actin-like ATPase are shown in red, the tubulin-like GTPase is shown in yellow. The centromere binding proteins are shown in blue. Genes encoding replication proteins are shown purple. The centromeric sequences are presented as multiple yellow or orange (for putative) arrowheads.

2000). Therefore, the notions of both co-regulation and auto-regulation of partition genes are addressed. Second, the ω gene is transcribed together with the ε gene for the antidote (Dmowski *et al*., 2006) and the ζ gene encoding the toxin (Ceglowski *et al*., 1993). The presence of the partition gene ω in one transcriptional unit with the post-segregational killing system is unique.

The δ and ω genes together with their promoter regions constitute the partition system and can stabilize unstable replicons in *B. subtilis* cells regardless of the type of replicon (θ or σ) under slow growth conditions (Dmowski *et al*., 2006). The δ–ω partition system can also stabilize an unstable replicon in *E. coli* cells although with lower efficiency (M. Dmowski, unpublished data).

The Delta and Omega proteins of pSM19035 have been thoroughly characterized. The crystal structures of both proteins are being solved (Murayama *et al*., 2001, Pratto *et al*., 2008) and protein-protein interactions analyzed both *in vivo* and *in vitro* (Dmowski and Jagura-Burdzy, 2011). Omega is a small, 71 amino acids protein (~8 kDa) whose 3D structure has shown similarity to the Arc/MetJ family of repressors. This group of proteins has the ability to bind DNA using a RHH (Ribbon-Helix-Helix) motif (reviewed by Schreiter and Drennan, 2007). The crystal structure of Omega demonstrated that it forms dimers *in vitro* through hydrophobic residues in the C-terminal helices α1 and α2 (Murayama *et al*., 2001). The *in vivo* analysis of Omega dimerization using the bacterial two-hybrid system (Dmowski and Jagura-Burdzy, 2011) not only has confirmed predictions made by Murayama *et al*. (2001) about the role of both α-helices but also demonstrated the role of the β structure in Omega-Omega interactions. Although RHH proteins do not share high sequence similarity, the hydrophobic residues involved in dimerization are conserved (Schreiter and Drennan, 2007; Dmowski and Jagura-Burdzy 2011). The N-terminus of Omega does not play any role in dimerization but is crucial for its interaction with Delta as demonstrated both *in vivo* and *in vitro* (Dmowski and Jagura-Burdzy 2011). Delta needs to form dimers for interaction with Omega, however, in contrast to what has been observed for other partition proteins, Omega protein impaired in dimerization can still interact with Delta.

There are three sets of Omega binding sites (de la Hoz *et al*., 2000), overlapping the promoter regions of copy number control gene *copS* and partition genes δ and ω comprising 9, 7 and 10 contiguous heptads of sequence 5'-$^{A}/_{T}$ATCAC$^{A}/_{T}$-3', respectively, in direct or inverse orientation (Fig. 4). Omega binds as a dimer to at least two heptads (de la Hoz *et al*., 2004)

The Delta protein is a weak ATPase stimulated in its activity by the N-terminal region of Omega and the Omega recognition sequence (heptamers). When activated by Omega, Delta polymerizes, forms spiral-like structures and oscillates (Pratto *et al*., 2008). The mutational analysis of Delta has demonstrated that substitution of the conserved lysine residue in Walker A motif (K36A or K36E) had no effect on Delta dimerization *in vivo* (Dmowski and Jagura-Burdzy, 2011), consistent with crystal structure observation by Pratto *et al*. (2008) but in contrast to what was observed for equivalent substitutions in other ParA-like proteins. However, the same Delta variants were impaired in interaction with Omega. On the other hand, amino acid substitution in the Walker A' motif (for ATP hydrolysis) had no effect on interaction with Omega, indicating that ATP binding but not hydrolysis is required for Delta association with Omega (Dmowski and Jagura-Burdzy, 2011). The *in vitro* complexes between this Delta variant and Omega bound to the centromeric sequence were defected in disassembly, when compared with native Delta (Pratto *et al*., 2009). The C-terminus deprived Delta variant can dimerize but cannot associate with Omega. The region between A' and B motifs, conserved in ParA-like proteins is essential for Delta protein ability to both dimerize and interact with Omega (Dmowski and Jagura-Burdzy, 2011).

Gerdes and co-workers have intuitively classified the Delta protein as an element of type Ib partition system (Gerdes *et al*., 2000), suggesting that the centromeric sequence of this system may be located in the promoter region of δ gene (*P*δ). However, experimental data indicated that two other Omega binding regions (*PcopS* and *P*ω) could successfully replace the *P*δ region in the centromere function. The partition mediated incompatibility assay (Austin and Nordström, 1990) has demonstrated that any of the three Omega binding regions (*P*δ, *PcopS* and *P*ω) constitutes the incompatibility determinant (Dmowski *et al*., 2006). The hypothesis of multiple centromeres is supported by data provided by de la Hoz and co-workers (2000, 2004) showing no differences in Omega binding to the analyzed regions as long as they contain at least four heptamers.

Recently, Soberón and co-workers (2011) have analyzed the complexes formed on DNA under different molar Omega:Delta ratios and proposed the mechanism of pSM19035 partition. First Omega binds the centromeric sequence and Delta binds to unspecific DNA. At different Omega:Delta ratios different complexes are formed, plasmids molecules are paired and pairs disassembled, Delta polymerizes and depolymerizes and plasmid molecules move towards the cell poles.

Plasmids pAW63, pBMB165 and pXO2. The pAW63 is a large (70 kb) broad-host range conjugative plasmid (Wilcks *et al*., 1998). Its Rep63B protein contains ATPase motifs characteristic for type I ParA-like proteins. Wilcks *et al*. (1999) have also identified sets of 10 octameric direct repeats and 6 octameric inverted

repeats upstream of *rep63A* (encoding the replication protein) as well as repeated sequences upstream of *rep63B* (Fig. 4). The pAW63 derivative carrying *rep63A, rep63B* (with repeated sequences located upstream) and *orf6* was shown to be stable in *B. subtilis* cells grown at 37°C but not at 42°C. The same plasmid, when tested in *B. thuringensis* was stable at 30°C but became unstable at 37°C (Wilcks *et al*., 1999). The effect of temperature on plasmid stability (host growth rate, thermo sensitivity of proteins involved, plasmid copy number) has not been explained so far.

An analysis of the replicon of the large (about 82 kb) plasmid pBMB165 from *Bacillus thuringensis* subsp. *thenebrosis* demonstrated that it replicates *via* θ type mechanism (Bruand *et al*., 1993). Its replication protein Rep165 shows similarity to Rep proteins from pAW63 and pXO2. The *ori* sequence has also been located downstream of *rep165* gene (Huang *et al*., 2006). Another DNA sequence necessary for pBMB165 replication and thought to constitute the binding site for the replication protein has been found upstream of the *rep165* and the oppositely transcribed *orf6* gene (Fig. 4). It contains seven direct and three inverted repeats of [AT[A]GTGTAA] sequence. Similar three repeated sequences are present downstream of *orf10* adjacent to *orf6*. Huang *et al*. (2006) have proposed that the repeated sequences upstream of *rep165* are the Rep165 binding site. Similar regions containing repeated sequences were found in pAW63 and pXO2 plasmids (Wilcks *et al*., 1999).

The *orf6* of pBMB165 encodes a protein designated ORF6 containing Walker type motifs characteristic for partition ATPases. Experiments performed by Huang *et al*. (2006) have proved that the *orf6*, together with the *orf10* located immediately downstream are necessary for stability of pBMB165 plasmid derivatives in *B. thuringensis*. Hence it is highly probable that *orf6*, *orf10* and the repeated sequences upstream of the former constitute a plasmid partition system and the replication and partition systems are intertwined.

Interestingly, genes involved in plasmid replication (*rep165*) and stability control (*orf6* and *orf10*) are separated from each other by IS*bth165* (*orf4*), which has no influence on plasmid function and, as it was demonstrated by Huang and co-workers (Huang *et al*., 2006) is not necessary neither for pBMB165 replication nor stabilization.

Plasmid pAD1. The low copy number, 60kb in size, conjugative plasmid pAD1 was isolated from *Enterococcus faecalis* (Clewell *et al*., 1982, Clewell, 2007). The conjugative system of this plasmid is inducible by a peptide sex pheromone secreted by potential recipient cells (Clewel, 1993). Studies by Weaver and co-workers (Weaver *et al*., 1993) have defined the functions of *repA*, *repB* and *repC* in plasmid pAD1 biology (Fig. 4), as encoding the replication protein RepA, the copy number control protein RepB and the protein RepC involved in the stable maintenance. These genes are also present on the pTEF1 plasmid from *E. faecalis*.

The RepA protein binds to repeated sequences in *oriV*, located in its coding sequence so *repA* gene on its own is sufficient for replication in *E. faecalis* (Francia *et al*., 2004). The *repB* and *repC* genes were shown years later to constitute a plasmid partition system (Francia *et al*., 2007) with RepB being the Walker-type ATPase and RepC – the DNA-binding protein. Moreover, three sets of repeated sequences [TAGTARRR] were identified between divergently transcribed *repA* and *repB* genes as well as downstream of *repC* gene (Fig. 4).

The two repetitive sequences from the intergenic *repA-repB* region (ItA – 13 repetitions and ItB – 12 repetitions), together or separately caused significant stabilization of an otherwise unstable plasmid in *E. faecalis*, when *repB* and *repC* genes were provided *in trans*. The third region, from downstream of *repC* gene (ItC – 3 repeated sequences) had no stabilizing effect in the presence of RepB and RepC (Francia *et al*., 2007). Interestingly plasmids carrying repeated sequences, in the absence of RepB and RepC genes were less stable than their parental vector (a pAD1 replicon). The authors suggest that this effect may be due to the interaction of RepA protein with studied repeated sequences (Francia *et al*., 2007). Moreover, the presence of *repC* alone but not *repB* resulted in neutralization of putative repeated sequences-induced destabilization (without causing increased stability). This could be explained by hypothetical interactions between RepA and RepC.

The RepC protein binds cooperatively to the repeated sequences located in each of the three clusters ItA, ItB and ItC. The RepC represses the *repBC* promoter but its involvement in *repA* expression regulation has not been demonstrated yet. It is also possible that there is an autoregulation of *repA* since RepA ability to bind to the *parS*-putative iterons was suggested (Francia *et al*., 2007). The RepB protein binds to the RepC protein in the presence of either ATP or non-hydrolysable ATPγS, demonstrating, that ATP hydrolysis is not necessary for these two proteins association. Moreover, RepB was shown to reduce the cooperativity of RepC binding to It sequences.

Interestingly, the pAD1 octameric sequences from *repABC* region are also involved in switching on/off the expression of genes responsible for the conjugation process of the plasmid (Heath *et al*., 1995). The increase of "iterons" number, from 13 to 17 upstream of *repA* was shown to influence the conjugation efficiency. The stability of a plasmid carrying the *repABC* region with increased number of octamers was slightly reduced, so it was speculated that a potential negative effect of changes in the "iterons" number on the partitioning

process could be compensated by the conjugation process (Francia *et al*., 2007).

The analysis of pAD1 partition system confirms its classification as type Ib as proposed by Gerdes *et al.* (2000): the centromeric sequence is located in the *repBC* promoter region and is regulated by the small RepC (ParB-like) protein. Moreover, RepB (ParA-like protein) does not contain the N-terminal DNA-binding region.

Plasmid pCI2000. Plasmid pCI2000 was isolated from *Lactococcus lactis*. Analysis of the DNA region which is necessary for its replication and stable maintenance has demonstrated that the product of the *repA* gene shows high similarity to the RepA protein from the pAD1 plasmid (Kearney *et al*., 2000). Moreover, two and a half iterons, 54 nucleotides long were identified within the *repA* coding sequence. In contrast to iterons in *repA* of pAD1, iterons located in the *repA* coding sequence of pCI2000 did not constitute the origin of replication (Kearney *et al*., 2000).

Another gene identified in this region encodes a protein, designated ParA, which demonstrates homology to partition Walker type ATPases, (Kearney *et al*., 2000). The *orf1* located downstream of *parA* encodes a small protein which does not show homology to any known proteins (Fig. 4). The presence of these two genes promotes stable maintenance of a pCI2000 derivative, suggesting that they encode an active partition system. It was shown, that the ParA protein can act *in trans* as it can complement a pCI2000 derivative carrying a stop codon in the *parA* coding sequence. So far, the *cis*-acting centromeric sequence has not been identified on plasmid pCI2000 although this role may be played by the repeated elements located upstream of gene *parA* (Kearney *et al*., 2000).

Plasmid pCXC100. Plasmid pCXC100, 51-kb in size, has been isolated from *Leifsonia xyli* subsp. *cynodontis*, a Gram-positive bacterium colonizing without any symptoms the xylem of bermudagrass as well as other crop plants such as oats, maize, rice or sorghum (Evtushenko *et al*., 2000). The RepA protein from pCXC100 shares homology with replication proteins of mycobacterial plasmids from the pLR7 family (Beggs *et al*., 1995), however, some additional regions unrelated to the pLR7 family are present. RepA contains three DNA-binding HTH motifs located in its N-terminal part. Interestingly, the 51 amino acids from the C-terminal part of the RepA protein are not necessary for plasmid replication (Li *et al*., 2004). The coding sequence of *repA* encompasses two regions containing repetitive 21-meres: DR1 with 11 repeats and DR2 with 7 repeats which were suggested to have a regulatory function (Li *et al*., 2004). Other direct repeats (DR3), designated *parS* were also found in the promoter region of *parA* gene encoding the ParA protein, a Walker type ATPase. The DR3 and *parA* together with the *orf4* located downstream of *parA* were suggested to constitute a partition system (Li *et al*., 2004) (Fig. 4).

Sequence analysis of the putative product of *orf4* (now designated *parB*) did not demonstrate sequence homology with ParB-like proteins, however it has been demonstrated that its product of 139 amino acids can cooperatively bind direct repeats located upstream of the *parA* gene (Yin *et al*., 2006). Later, it was shown that ParB is an RHH protein with unstructured N-terminal part (residues 1–65) and the DNA binding C-terminal part. The centromeric sequence is composed of nine contiguous 9-bp direct repeats (Huang *et al*., 2011). A dimer of ParB dimers cooperatively binds DNA composed of at least two nine-nucleotide repeats. Yin and co-workers (2006) have classified the pCXC100 partition system as type Ib.

Plasmid pGENT. The pGENT plasmid from *Enterococcus faecium* ensures high-level of resistance to gentamicin and other aminoglycosides in its host (Simjee *et al*., 1999). A fragment of pGENT, which harbored *prgPO* genes and *cenE,* a set of TATA boxes located upstream (Fig. 4), ensured stable inheritance of plasmids in *E. faecium*. Deletion of *prgP*, *prgO* or *cenE* abolished the stabilizing effect. The *prgPO* genes were found on many plasmids from *E. faecium*, *E. faecalis* and *L. casei* (Hedberg *et al*., 1996; Derome *et al*., 2008).

The product of the *prgP* gene, the protein PrgP belongs to the superfamily of ParA proteins involved in plasmid partition (Derome *et al*., 2008). The PrgO protein forms dimers and binds specifically the *cenE* centromere located upstream of *prgP*. The centromere consists of two clusters designated CESI and CESIII, each composed of seven TATA boxes separated by five nucleotides. The clusters are separated by 26 base pairs sequence designated CESII that PrgO does not bind to. Interestingly, PrgO binds CESI and CESIII independently, with high and low affinity, respectively and PrgO bound to CESI probably does not interact with PrgO bound to CESIII. Moreover a third cluster of six TATA boxes is located downstream of *prgO* and its deletion has no effect on stabilization function (Derome *et al*., 2008). The organization of the intrinsically curved *cenE*, is similar to that of yeast centromeres (Derome *et al*., 2008). Identical sequences, encompassing *prgP* and *prgO* genes are present in plasmids pRE25 and pIP816 from the inc18 family which also contain the PAR and PSK systems of pSM19035.

Type II systems with actin-like ATPases

Plasmid pSK41. The pSK41 plasmid of 46.4 kb exemplifies large staphylococcal conjugative multiresistant plasmids. It confers resistance to aminoglycosides, gentamycin, tobramycin, kanamycin and neomy-

cin (Byrne *et al*., 1990) and can transfer by conjugation, as well as mediate mobilization of other plasmids (Berg *et al*., 1998). Plasmid pSK41 relies on a θ-type replicon, evolutionary related to the staphylococcal plasmid pSK1 (Firth *et al*., 2000). The pSK41 encoded Rep protein binds to tandem direct repeats located in the *rep* coding sequence (Kwong *et al*., 2004) (Fig. 4). It has been shown that the segregational stability of pSK41 is ensured by the *parMR* operon located upstream of the *rep* gene (Schumacher *et al*., 2007) (Fig. 4).

The partition system of pSK41 plasmid encodes an actin-like ATPase ParM and therefore is classified as type II. The ParR of 109 amino acids belongs to the RHH (ribbon-helix-helix) family of DNA-binding proteins. This protein binds to a series of eight 10-bp DNA repeats located upstream of the *parM* gene (Fig. 4). The RHH fold is located in the N-terminal part of ParR and this region is sufficient for DNA binding of ParR as its short version ParRN (residues 1–53) binds to the specific DNA sequences with identical affinity (Schumacher *et al*., 2007). The minimal DNA region that ParR binds as a dimer of dimers is a pair of 10-bp repeats. Schumacher *et al*., (2007) have analyzed the pSK41 segrosome and concluded that upon DNA binding, ParR dimers form a superstructure which wraps the DNA to form a super-helical structure with six ParR dimers of dimers in one turn. This conclusion was supported by electron microscopy analysis of ParR-DNA complexes where circular structures were observed. DNase I protection assay performed with ParR of pSK41 plasmid has suggested its spreading to DNA sites surrounding the centromere (Schumacher *et al*., 2007). The ability of ParR to interact with ParM (the actin-like protein) was suggested to be ensured by the C-terminal part of the ParR protein. ParR binding stimulates ParM NTPase activity (Popp *et al*., 2010).

The structure of filament-forming ParM protein was determined. Although it belongs to the actin/Hsp70 family, ParM demonstrates strong structural similarity to the actin-like protein from *Thermoplasma acidophilum*. ParM forms polymers in the presence of GTP, ATP and its analogs but not in the presence of ADP or GDP (Popp *et al.,* 2010). Schumacher *et al*., (2007) also proposed that the ParM filament (about 6 nm in diameter), is captured by the circular structure formed by ParR and the centromeric site.

Plasmid pLS32. The pLS32 cryptic plasmid, identified by Tanaka and Koshikawa (1977) in *Bacillus natto* is 70 kb large and maintained at low copy number in bacterial cells. Its 7,2 kb derivative, the pBET131 plasmid can replicate in *B. subtilis* cells at 2–3 copies per chromosome and be stably maintained (Tanaka and Ogura, 1998). Its segregational stability is ensured by the *alfAB* genes. The expression of *alfA* gene, which is organized in one operon with *alfB*, is repressed by the AlfB protein (Fig. 4). AlfB binds specific DNA sequences, designated *parN* and located upstream of *alfA*, that contain three AT-rich octamers; at least two repeats are required for binding (Tanaka, 2010). The AlfB-*parN* binding is prerequisite for plasmid stabilization by *alfAB* genes. Moreover, Tanaka (2010) has shown that AlfA and AlfB proteins form dimers and interact with each other. Since AlfA is an actin-like protein, Tanaka (2010) proposed to classify *parN-alfAB* as type II partition system.

The AlfA protein, distantly related to bacterial actin forms dynamic filaments in the presence of ATP or GTP and at higher critical concentration of ADP or GDP, what differs it from other actin-like proteins (Becker *et al*., 2006; Polka *et al*., 2009). A mutation in *alfA*, that results in D168A substitution in the active centre of ATPase, impairs filament formation and pBET131 stability (Becker *et al*., 2006). The AlfA filaments form mixed-polarity bundles from pole to pole in *B. subtilis* cells. Polka *et al*. (2009) hypothesized that bundles can stabilize interactions with other proteins or that the gathered AlfA polymers could be a template for plasmids movement to cell poles.

Type III systems with tubulin-like GTPases

Plasmids pBtoxis and pXO1. Plasmids pBtoxis and pXO1 were isolated from *B. thuringiensis* subsp. *Israeliensis* and *Bacillus anthracis,* respectively. The pBtoxis encodes six toxins which make *B. thuringiensis* a biological weapon against insects (Berry *et al*., 2002) and pXO1 plasmid encodes the anthrax toxin required for virulence of *B. anthracis* (Guidi-Rontani *et al*., 1999). Both plasmids encode tubulin/FtsZ-like GTPase proteins, TubZ in pBtoxis (Akhtar and Khan, 2012) and RepX in pXO1 (Tinsley and Khan, 2006) involved in replication and segregation. Members of the tubulin family participate in cell division in eukaryotes and prokaryotes.

The *tubR* and *tubZ* genes (Fig. 4) which encode the partition system of pBtoxis are also essential and sufficient for replication (Tang *et al*., 2007). Expression of the *tubRZ* genes is autoregulated by TubR (Larsen *et al*., 2007), a 104 aa protein with a helix-turn-helix (HTH) DNA binding motif (Berry *et al*., 2002). The dimer of TubR binds with high specificity to iterons composed of clusters of three or four 12-bp direct repeated sequences located upstream of *tubR* coding sequence and TubZ binds to the DNA-TubR complex (Tang *et al*., 2007; Ni *et al*., 2010; Aylett, and Löwe, 2012). This 48-bp region plays also the role of the *oriV* sequence as it is sufficient for replication if TubR and TubZ are provided in *trans*.

The GTPase TubZ protein contains a tubulin signature motif (GGGVGTG) involved in GTP binding and hydrolysis (Tang *et al*., 2007). The crystal structure of

TubZ has shown double helical filaments which resemble actin filaments. Presumably, TubZ and actin filaments evolved convergently (Aylett *et al.,* 2010). *In vivo* TubZ forms dynamic polarized bundled polymers with plus and minus ends which translocate in the cell by a treadmilling mechanism (Larsen *et al*., 2007; Aylett *et al.*, 2010). Upon reaching the cell pole, the filament bends and continues growing. TubZ treadmilling has been shown to be essential for pBtoxis plasmid stability (Larsen *et al*., 2007).

TubR demonstrates structural similarity to transcriptional regulators of the ArsA family and forms dimers through its N-terminal residues (Ni *et al.*, 2010). In contrast to other partition proteins, the TubZ-TubR interaction does not require GTP. The flexible C-terminus of TubZ is not involved in polymerization and may be exposed on the surface of the polymer to interact with TubR (Ni *et al.*, 2010; Aylett *et al.*, 2010). TubR dimers bind repetitive sequences upstream of *tubR* and by lateral coating form a flexible DNA-protein complex that recruits the TubZ polymer. Then, the plasmid, attached to the treadmilling TubZ filament through TubR migrates to the cell pole. Upon reaching the pole, the TubR-bound plasmid is released (Ni *et al.*, 2010; Aylett, and Löwe, 2012).

In the mini pXO1 the FtsZ-like protein RepX (later re-named TubZ) and the inverted repeat of 24 nucleotides downstream of its gene are necessary for replication (Tinsley and Khan, 2006) although Pomerantsev *et al.* (2009) suggested limiting of RepX role only to partitioning. The *repX* gene was also found in other plasmids and megaplasmids among the members of the *B. subtilis* group (Berry *et al*., 2002). RepX exhibits a GTPase activity and polymerizes into filaments in the GTP-dependent manner; a mutant in the functional tubulin motif is defective in both GTPase activity and polymerization *in vitro*. Moreover, GTP hydrolysis probably causes RepX de-polymerization (Anand *et al*., 2008). Nucleotide binding is also necessary for RepX unspecific DNA binding. Therefore, RepX may be a hybrid protein which both binds DNA and polymerizes (Anand *et al*., 2008). *In vivo*, in *B. anthracis*, RepX forms straight or curved helical filaments in a process which depends on protein concentration in the cell (Akhtar *et al*., 2009). RepX filaments were also observed in *E. coli* cells even in the absence of other pXO1 encoded factors.

Interestingly RepX and TubZ which are almost as divergent from each other (21% identity) as they are from FtsZ and tubulin (15–20% identity), have highly similar biochemical properties (Chen and Erickson, 2008). RepX and FtsZ assemble into filaments in the cooperative manner. Upon GTP hydrolysis, the filament subunits disassemble, and reassemble after exchange of GDP to GTP. The capping mechanism of filament formation has been suggested (Chen and Erickson, 2008): the GDP nucleotide dominates in the polymer what makes the polymer unstable; therefore, a small GTP cap must stabilize the filament at the growing end, consistent with the observed growth at one end of the TubZ filament and disassembly at the other (Larsen *et al*., 2007).

The partition systems encoding TubZ-like GTPases are classified as type III systems (Moller-Jensen and Gerdes, 2007). Recently, Oliva and co-workers have identified TubZ-TubR partition system in the *Clostridium botulinum* phage c-st. This linear phage circularizes as a prophage and encodes botulinum toxin crucial to virulence (Oliva *et al*., 2012). They have also found a fourth component, designated *tubY*, located downstream of *tubZ* of c-st or upstream of *tubR* in pXO1 and pBtoxis. TubY contains both, a HTH motif and a C-terminal coiled-coil region involved in TubZ binding. Its hypothetical role is modulating the assembly and reshaping of TubR-TubZ complex with the centromere-like site *tubS*.

Other partition systems

Plasmid pSK1. The pSK1 from *Staphylococcus aureus* is a 28.4 kb plasmid classified to Rep family 15 (Jensen *et al*., 2010a; 2010b) of θ replicons from Gram-positive bacteria (Firth *et al*., 2000). Early studies have demonstrated that the *rep* gene is necessary and sufficient for replication in *S. aureus* and that *orf245* located upstream and transcribed divergently is necessary for plasmid stable maintenance (Firth *et al*., 2000) (Fig. 4). The study by Simpson *et al*. (2003) has confirmed the ability of *orf245*, now designated *par* to stabilize the pSK1 replicon. Interestingly, authors postulate that this partition system is determined by a single gene.

The *par* gene and the intergenic region located upstream have also stabilized an unrelated θ-type replicon – a derivative of the pIP501 plasmid as well as a σ-type replicon – a derivative of the pUB110 plasmid. It was demonstrated that the *par* gene has effect neither on plasmid copy-number nor on host growth rate, the later excluding its involvement in the post-segregational killing. The centromere-like sequence is located upstream of the *par* gene – it contains seven direct repeats and one inverted repeat (Fig. 4). The product of the *par* gene has a half of the HTH motif that confers unspecific binding to DNA. The second half of HTH motif (Brennan *et al*., 1989) is not conserved. Analysis of the Par protein also demonstrated that it lacks any ATP-binding motif (Simpson *et al*., 2003). The Par structure prediction suggested the possibility of coiled-coil formation with high probability score, what implies that Par may be able to form oligomers. Such coiled-coil structures have also been found in transcrip-

tion factors and eukaryotic cytoskeleton proteins. The coiled-coil domain of Par from pSK1 may play the role of molecular switch, usually played by ATP ADP binding (Burkhard *et al*., 2001; Simpson *et al*., 2003).

Concluding remarks

This review of the identified plasmid stabilization systems from Gram-positive bacteria demonstrates how scarce and incomplete the knowledge in this field still is. The picture emerging from the available data on plasmids from Gram-positive hosts points out that the replication and partition systems are not only close by lineage, coordinately regulated as it has been observed in Gram-negative hosts but are tightly interlocked or even overlap in their functions (pBtoxis, pXO1). Moreover, a possible connection between partition and conjugation was proposed for plasmid pAD1.

Par proteins have been initially identified as copy number control elements (*e.g.* pAD1). The *parS* sequences located in the promoter region may act as the transcriptional regulator binding site either for *parB* or *rep* (*e.g.* pAD1, pAW63, pAMB165). Recognition of *parS* by Rep protein (if confirmed) would be unique for Gram-positive plasmids.

The significance of coordination of expression of the diverse systems involved in replication, copy number control, stabilization and transfer has been discussed by Bignell and Thomas (2001). It was proposed that organization in operons might be beneficial in the course of evolution since it promotes co-acquisition. Moreover, coordination of plasmid copy number control with replication and/or partition may enable effective partition of plasmids present in a correct number of copies. The organization of the partition systems of Gram-positive bacteria described above shows that they are similar in genetic organization – their operons are regulated by the centromere binding proteins. These small DNA binding proteins belong mainly to the RHH family. Moreover, partition systems of Gram-positive bacteria are more variable considering the type of motor protein they encode. The majority of them is classified to the group of type Ib with Walker type ATPases (pAW63, pBMB165, pAD1, pCI2000, pCXC100) or to the type II with actin-like NTPases (pSK41, pLS32). However two new systems have been found on plasmids from Gram-positive-bacteria: type III partition systems based on tubulin-like motor protein (pBtoxis, pXO1) and possibly single coiled-coil Par protein combining DNA binding function and scaffold forming "motor" protein (pSK1).

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