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A type Ib plasmid segregation machinery of the *Advenella kashmirensis* plasmid pBTK445

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ABSTRACT

pBTK445 is a newly described large (~60 Kb), low-copy number, conjugative plasmid indigenous to the sulfur-chemolithoautotroph *Advenella kashmirensis*. Based on its minimal replication region, a shuttle vector, pBTKS was constructed which can be used for diverse *Alcaligenaceae* members. The construct was found to be stably maintained both in the native host as well as in *Escherichia coli* in the absence of selective pressure which indicated that pBTKS harbors the stabilizing system of pBTK445, that are commonly coded by low-copy-number plasmids. Deletion analyzes of pBTKS confirmed the essentiality of *parA* (encoding a Walker-type ATPase of 214 amino acids) and the downstream located small *parB* (encoding an 85 amino acid protein having no sequence homolog in the database) in the faithful partitioning of pBTK445. A 1075 bp PCR product, containing *parA*, *parB* and an upstream sequence having nine 11 bp direct repeats (*parS* site) was found to comprise the partition functions of pBTK445, stabilizing both low-copy or high-copy number homologous and heterologous replicons in diverse hosts. The incompatibility determinant and the *par* promoter, P_{par} were both found to be present within a 191 bp iterated sequence present upstream of *parA*. ParB was found to regulate the expression of the Par proteins from P_{par}. The presence of a typical Walker-type ATPase motif in ParA, a short phylogenetically unrelated ParB, that acts as a repressor of P_{par}, and location of the iterated *parS* site upstream of *parA*, confirm that the active partition system of pBTK445 belongs to the type Ib.

1. Introduction

While random distribution among daughter cells in high-copy-number plasmids results in their faithful segregation to daughter progeny, low-copy-number plasmids generally encode additional functions to counteract plasmid loss during cell division. These include plasmid multimer resolution systems, copy number control, post-segregational killing, and active partition mechanisms (Gerdes et al., 2000; Hayes, 2000, 2003; Moller-Jensen et al., 2000; Nordstrom and Austin, 1989). Active partition mechanisms of low-copy number plasmids usually require two plasmid-encoded *trans*-acting partition proteins and a *cis*-acting DNA sequence, termed the plasmid partition site or centromere-like site (generically named *parS*, after the well-studied partition system of plasmid P1) (Austin and Abeles, 1983) at which one or possibly both proteins act (Barilla et al., 2005). In all well studied systems, the two partitioning genes are organized in tandem as an operon, and are transcribed from a common transcriptional promoter (Hayes and Barilla, 2006; Kwong et al., 2001). The upstream gene encodes an ATPase called ParA while the one in the downstream encodes a DNA-binding factor called ParB. ParB interacts directly with the *parS* site, normally composed of direct or inverted iterated sequences (Fothergill et al., 2005; Hayes and Barilla, 2006; Surtees and Funnell, 2003;

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Yin et al., 2006) that also serves as the incompatibility determinant in a number of plasmids (Austin and Nordstrom, 1990; Bouet et al., 2007b, 2005; Hayes, 2000; Kalnin et al., 2000; Kwong et al., 2001). The interaction of ParB and ParA to the partition site, results in the formation of a partition complex that helps in the faithful segregation of the daughter plasmids (Barilla et al., 2005; Bouet and Funnell, 1999; Hayes and Barilla, 2006). Depending on the type of ATPase involved, active partitioning systems can broadly be classified as the widespread Type I (Walker-type ATPase) or the infrequent type II (ATPase belonging to the actin/Hsp70 superfamily) systems (Ebersbach and Gerdes, 2005; Gerdes et al., 2000; Hayes, 2000; Koonin, 1993; Motallebi-Veshareh et al., 1990). The type I partition systems are further divided into type Ia and type Ib subgroups based on the size of the Par proteins, localization of the partition site and the mechanism of transcriptional regulation. In contrast to the type Ia system, found in the well studied plasmids P1 and F (Bouet et al., 2007a; Bouet and Funnell, 1999; Bouet and Lane, 2009; Dabrazhynetskaya et al., 2009; Libante et al., 2001; Radnedge et al., 1998; Sengupta et al., 2010; Sergueev et al., 2005; Vecchiarelli et al., 2007), both proteins of the type Ib are much smaller with the phylogenetically unrelated ParB being involved in autoregulation of the Par proteins transcribed from a common transcriptional promoter located upstream of *parA* (Fothergill et al., 2005; Gerdes et al., 2000; Kwong et al., 2001). Recently a type III system has been identified comprising of the TubR (a specific DNA-binding protein) and TubZ (a distant homolog of both tubulin and FtsZ having a strong GTPase activity) proteins encoded by the pBtoxis plasmid from *Bacillus thuringiensis* (Ni et al., 2010). This has opened up unforeseen perspectives on prokaryotic cytoskeletal proteins mediating genome segregation.

We have recently isolated a large (~60 Kb), low copy number, novel IncP plasmid, named pBTK445, from strains of *Advenella kashmirensis* (Dam et al., 2009; Ghosh et al., 2005; Gibello et al., 2009), and found the same to be highly stable even in the presence of high concentrations of DNA intercalating agents like acridine orange or ethidium bromide that are generally used for plasmid curing. Moreover, although the plasmid lacks any antibiotic selection marker, it was stably maintained in the host. Because of its high stability despite a relatively small size in comparison to the other large low-copy number plasmids; pBTK445 was deemed interesting to be studied for its stability functions. This paper describes the characterization of the segregation machinery of pBTK445 and found it to be a type Ib partition system.

2. Methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1, and their growth conditions are as described earlier (Dam et al., 2009, 2007; Ghosh et al., 2005). *Escherichia coli* TOP10 (Invitrogen) used in arabinose regulator expression study was grown in RM medium (pH 7.4) containing M9 salts (Na_2HPO_4 6 gm, KH_2PO_4 3 gm, NaCl 0.5 gm and NH_4Cl 1 gm per liter water), 2% (w/v) Casamino acid and 1 mM $MgCl_2$. Filter sterilized 0.2% (w/v) arabinose (or glucose) was added when needed. Tetracycline, kanamycin, chloramphenicol or ampicillin when required was added to the media at final concentrations of 20, 50, 60 and 100 μg ml^{-1}, respectively.

2.2. Plasmid construction and expression of recombinant proteins

Plasmid vectors used in this study and their sources are listed in Table 1. All regular DNA manipulations were carried out following standard methods (Sambrook et al., 1989). The construct pBTKE5.7, pBTKS and pBTKSR (Dam et al., 2009) were used to obtain the deletion derivatives for partition assays (Table 1 and Fig. 1). The ParA and ParB overexpression plasmid in pQE30 (pQEPA, and pQEPB) or in pBAD (pBADPB) were constructed by first amplifying the respective genes using primers as described in Table 2 and then cloning them into the *Bam*HI-*Hind*III site of the respective expression vector. The inserted fragment was sequenced in order to eliminate the possibility of any misincorporation of nucleotides. Expressions of the recombinant proteins were checked by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) in pQE30-based constructs in *E. coli* XL1-Blue, or 0.2% (w/v) arabinose in pBAD-based constructs in *E. coli* Top10 (Sambrook et al., 1989). Whenever tight repression from the pBAD-construct was necessary, 0.2% (w/v) glucose was added in place of arabinose. β-Galactosidase assay was performed as described earlier (Dam et al., 2009).

2.3. Plasmid stability assay

Stability of a plasmid within a particular host was determined after growing the cells in absence of selective pressure. Plasmids which are present in low-copy number (pFH450 and its derivatives within BR825; or pBTKS and its derivatives within *A. kashmirensis* SRC1) were grown for approximately 100 generations. Briefly, ten randomly chosen colonies (from plasmid specific antibiotic containing plate) were streaked on non-selective plates and incubated overnight, thereby completing approximately 25 generations of growth. A loop-full cell was then restreaked on fresh plates for three more times, thereby completing approximately 100 generations of non-selective growth. The proportion of cells that retained the plasmid (after every 25 generations) was determined by picking ten isolated colonies from each of these ten streaks (a total of 100 colonies) and stabbing them on plates containing the plasmid specific antibiotic. Plasmids present in high-copy number (pBluescript or pBTKS within XL1-Blue) were treated similarly, except they were grown on non-selective medium for longer period of time, approximately 50 sub-cultures. Assays were performed in triplicate.

2.4. Incompatibility assay

The compatible plasmids pSD5B and pBluescript were used to clone either the complete 1075 bp *par* locus of pBTK445, or the 191 bp iterated sequence present upstream of *parA*. One of the compatible plasmid or its derivative was