The role of the ParF protein in governing TP228 plasmid segregation: mutational analysis

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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<td>A (Ala)</td>
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<td>α</td>
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<td>ADP</td>
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<td>AM&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Ampicillin resistance</td>
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NaOH  sodium hydroxide
ng  nanogram
nm  nanometer
NTPase  nucleoside triphosphatase
OD  optical density
OE-PCR  overlap extension polymerase chain reaction
O_F  operator of par complex of TP228 plasmid
oriC  origin of replication
oriT  origin of transfer
P (Pro)  proline
P1  external non-mutagenic forward primer
P2  internal mutagenic reverse primer
P3  internal mutagenic forward primer
P4  external non-mutagenic reverse primer
p_lac  promoter of lac operon
Par  partition loci
par  partition gene
PCR  polymerase chain reaction
PSK  post-segregational killing
PSM  pentapeptide scanning mutagenesis
Q (Gln)  glutamine
R (Arg)  arginine
r.p.m.  revolutions per minute
RHH  ribbon-helix-helix
S (Ser)  serine
T (Thr)  threonine
TAE  tris acid-EDTA
Tris-HCl  tris (hydroxymethyl) methylamine hydrochloride salt
UV  ultraviolet
v  volume
V (Val)  valine
W (Trp)  tryptophan
WT  wild-type
Y (Tyr)  tyrosine
Abstract of thesis submitted by Beriwan Ali for the Degree of Ph.D entitled The role of the ParF protein in governing TP228 plasmid segregation: mutational analysis Month and year of submission September 2016.

TP228 is a low-copy number, multidrug resistant plasmid that replicates in Escherichia coli and which is stabilized by an active partitioning system. The TP228 partition complex consists of three main components: the ParF and ParG proteins that assemble on the centromere site parH to form the segrosome complex. ParF (206 amino acids) is a polymerizing ATPase that plays an essential role in directing the faithful movement of plasmids to daughter cells at cell division. In this study, ParF was subjected to pentapeptide scanning mutagenesis which generated nine unique random pentapeptide insertions in the protein. Insertions at different positions in ParF identified regions in the protein that are crucial for segregation, for ParF self-association, and for the ParF-ParG interaction in vivo. Two of the mutant ParF proteins were entirely impaired in plasmid partitioning, ParF self-association, and interaction with ParG. Three mutants conferred severe reductions in, but did not entirely abolish, plasmid segregation, whereas the interactions with wild-type ParF and ParG were abrogated in these ParF mutants. A pentapeptide insertion between residues Lys151 and Glu152 did not affect ParF self-association but did impair both the ParF-ParG interaction and plasmid segregation which is a novel observation. Longer oligopeptide insertions between these positions exerted similar effects to those observed with the pentapeptide insertion: insertions up to 33 amino acids did not perturb the ParF-ParG interaction indicating that this region of the protein is tolerant of insertions with respect to ParF self-association. Substitution and deletion analysis of residues Lys151 and Glu152 were examined to assess further the importance in ParF-ParG interaction and plasmid partitioning of the α6 helix structural element that contains these residues. The impacts of K151A substitution and Δ151 deletion were similar to the effects produced by the pentapeptide insertion between Lys151 and Glu152. In contrast, the ParF-E152A protein conferred wild-type activity in ParF self-association and ParG interaction assays and as well as in partition assays. The ParFΔ152 mutant was impaired in plasmid segregation. In addition, pentapeptide insertions at positions 160 in the β7 β-strand element, 185 in the turn before the α8 helix, and 205 at the C-terminus of ParF did not produce any detectable change in protein function. However, insertions longer than five amino acids between positions Lys160-Ala161 and Ser185-Ser186 dramatically reduced plasmid partitioning activity. The pentapeptide mutant ParF205GVPLF was unaffected in partition activity and also self-associated and interacted with ParG. The role of the protein C-terminus in ParF-mediated plasmid segregation was assessed further by truncation mutations. ParFΔ206 that lacks the final residue in ParF and ParFΔ205-206 in which two amino acids were deleted did not perturb plasmid segregation, whereas deletion of three amino acids from the ParF C-terminus abolished accurate plasmid partitioning. Nevertheless, the ParFΔ204-206 protein was not perturbed in ParF self-association or in the ParF-ParG interaction indicating the involvement of the C-terminus of ParF in an unknown aspect of plasmid partitioning. Overall, pentapeptide scanning mutagenesis was a powerful mutagenesis strategy to dissect the organization of the TP228 segregation complex by identifying regions in ParF that are tolerant to insertions, defective in known functions of the protein, and disrupted for the ParF-ParG interaction. The data provide new insights into the structure and function of ParF which is a potential target for new antibacterial compounds that disrupt the segregation of multi-resistance plasmids.
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Chapter 1: Introduction
1.1 Plasmids

Bacterial plasmids are extrachromosomal DNA molecules that are of importance in medical microbiology, as they frequently accommodate the genes responsible for antibiotic resistance and pathogenicity. The acquisition of resistance genes, specially by horizontal transfer of plasmids and other mobile elements that harbour these genes, has been crucial to the rapid development of the current antibiotic resistance crisis. This phenomenon may be attributed to a range of biochemical and physiological mechanisms produce by bacteria. The autonomous replication and segregation of plasmids ensure their stable maintenance generation after generation. Understanding the molecular mechanisms by which plasmids are segregated at cell division is important in order to clarify the potential of the segregation apparatus as a novel antibacterial target (Gordon et al., 1997; Lederberg, 1952). The main structure and function of a bacterial cell are encoded by chromosomial genes and bacteria typically do not rely on plasmids for fundamental properties. However, plasmids contribute to genetic diversity and evolution by horizontal gene transfer (Hayes, 2003a; Thomas, 2000). Plasmids generally vary in size from a few kilobases to hundreds of kilobases and their geometry is commonly circular or linear. Plasmids may encode traits including resistance to antibiotics, production of bacteriocins and also resistance to heavy metals, ultraviolet light as well as many other metabolic functions. However, a major clinical concern is related to plasmids that possess one or a number of genes encoding antibiotic resistances (Clewell et al., 2014; Hayes, 2010; Thomas, 2000).

The number of plasmid copies per chromosome is maintained by replication control circuits for every replication event per cell cycle. Consequently plasmid persistence is associated to the virulence, pathogenicity and disease transmission (del Solar and Espinosa, 2000). The numbers of plasmid copies are varied. High copy number plasmids usually are small in size and replicate randomly during the cell cycle and depend on passive diffusion through the cytoplasm for distribution at cell division. In contrast, low copy number plasmids do not rely on random distribution but instead ensure their accurate distribution by possessing partition (par) loci as discussed further below (Ebersbach and Gerdes, 2005; Hayes and Barillà, 2006a; Salje, 2010). In addition, other mechanisms exist by which both high and low copy number plasmids increase their stability and guarantee their distribution into the new progeny at cell division (Hayes and Barillà, 2010). The first mechanism involves multimer resolution systems. Due to replication and homologous recombination between sister plasmids, the formation of plasmid dimers and multimers will prevent their accurate
segregation. However, by the action of site specific recombinase enzymes these dimers and multimers are resolved to monomers (Sengupta and Austin, 2011; Summers, 1998; Hayes, 2003a). For example, the multimer resolution system of the P1 low copy number plasmid assists the stable maintenance of this plasmid (Austin et al., 1981).

The second mechanism involves toxin-antitoxin (TA) systems in which any plasmid-free cells that emerge are eliminated by a post-segregational killing (PSK) process (Gerdes et al., 1986). This event requires two factors that are encoded by the plasmid, a toxin and a neutralizing antitoxin which form a complex. Plasmid-free cells inherit this complex, however, the antitoxin is less stable than the toxin so the latter is liberated and either kills or stops growth of plasmid-free cells (Bukowski et al., 2011; Hayes, 2003b; Hayes and Van Melderen, 2011). PSK mechanisms have been categorized into six types based on the mechanisms of toxin inhibition and toxin-antitoxin interaction (Hayes and Kędzierska, 2014; Kędzierska and Hayes, 2016). Among the best characterized TA mechanisms is type I hok-sok complex encoded by plasmid R1 in which the toxin mRNA is inhibited by an antitoxin mRNA (Gerdes et al., 1990) and a new type has been proposed in which the bacterial toxin inhibits DNA replication elongation (Aakre et al., 2013). Generally TA systems, including those encoded by bacterial chromosomes, are involved in bacterial pathogenicity, virulence promotion and biofilm formation. Therefore these systems are considered to be potential targets for novel antibiotics (Kędzierska and Hayes, 2016).

The main mechanism by which low-copy number plasmids are stabilized is characterized by active partitioning systems (Thomas, 2000). These mechanisms ensure the flow of plasmids into the next generation by directing the replicated plasmids to either side of the cell prior to cell division. Partitioning genes are present on various types of low copy number plasmids (Schumacher, 2012). The partitioning system of multidrug resistance TP228 plasmid that is under study in this project is described further below. Partitioning, multimer resolution and TA mechanisms act together in plasmid maintenance and persistence to ensure plasmid retention in the population.

1.2 Multidrug resistance plasmids

Over recent decades an alarming rate of multidrug resistant bacteria has emerged and generation of new antibiotics is required (Frieri et al., 2016). The segregation stability of
low-copy number plasmids associated with antibiotic resistance is achieved by partitioning proteins. Thus, segregation mechanisms are potential targets for new antimicrobial compounds that can proficiently fight bacterial infections. One or more resistance determinants often are found on many plasmids, whereas other plasmid genes may contribute to bacterial virulence and pathogenicity (Clewell, 2014; Johnson and Nolan, 2009; Saeed et al., 2015). For example enterococcal plasmids often encode resistance to one or more antibiotics (Clewell et al., 2014). As another example, certain serotypes of Salmonella enterica, including serotypes Infantis and Typhimurium, possess plasmid-located resistance genes and are major concerns for public health worldwide (Aviv et al., 2016).

Plasmid encoded fosA3 gene is mediated fosfomycin resistance in Enteobacteriaceae (Yao et al., 2016).

The major form of horizontal gene transfer in bacteria is conjugation which is a transfer of DNA molecules from a donor cell into a recipient through a conjugation apparatus (Clewell, 2014; Thomas, 2000). Conjugative transfer of genetic material is considered to be the most important method of transfer antibiotic resistance. Conjugative plasmids are the vehicle of horizontal gene transfer and harbour an origin of transfer (oriT) region as well as accessory genes that encode the proteins for mating pair formation. One or more plasmid-located transfer operons can form the DNA transport machinery (Goessweiner-Mohr et al., 2014).

The modes of action of antibiotics within bacteria generally include interacting with specific targets such as inhibition of protein synthesis, targeting DNA replication and distribution as well as inhibition of bacterial cell wall synthesis. For example, both penicillin and cephalosporin interfere with bacterial cell wall biosynthesis (Neu, 1992). Resistance genes target antibiotics by specific biochemical resistance mechanisms which may involve inactivation of the enzymatic activity of the drug or changes in the antibiotic target site. Resistance mechanism of β-lactamase family of enzymes includes hydrolyzing of β-lactam ring of penicillins, cephalosporins and carbapenems. Prevention of antibiotic access to the target site is another mechanism of antibiotic resistance which may attribute to the permeability barrier or presence of an efflux pump mechanism (Spratt, 1994). In addition, chromosomal loci may contribute to antibiotic resistance as does transposon acquisition (Clewell, 2014).
1.3 Plasmid Partition

Understanding of plasmid segregation mechanisms has come from analysing the biochemistry, genetics, protein structures and cell biology of partition systems. Low copy number plasmids are specially informative models for studying segregation. Moreover, the apparent simplicity of the segregation of these types of plasmids permits dissection of the basic molecular events involved in their mechanisms (Hayes and Barillà, 2006a; Hayes and Barillà, 2006b; Hayes and Barillà, 2010; Schumacher, 2012). The partition systems of low copy number plasmid consist of three main components: a nucleotide tri-phosphatase (NTPase), a centromere site and a centromere binding protein (CBP) (Figure 1.1) (Abeles et al., 1985; Hayes and Barillà, 2010; Schumacher, 2012). The CBP recognizes the specific centromere sequence of the par module for commencing the partition events. The CBP also acts as the main regulatory protein or as a co-regulator for transcription of the partition genes. The CBP recruits the NTPase to bind which results in the formation of a nucleoprotein partition complex known as the segrosome. This complex can be categorized into subtypes based on its molecular components (Figure 1.1) (Schumacher, 2008).

1.4 Partition systems: different models perform similar functions

Considering the organization of genes that encode partition complexes and the evolutionary relationships between the proteins, partition cassettes have been categorized into four different types (Figure 1.1) (Ebersbach and Gerdes, 2005; Hayes and Barillà, 2006b; Salje et al., 2010). The type I system contains an ATPase with ATP binding folds (ParA) and CBPs termed ParB. In addition, depending on the size of the two proteins as well as sequence homology, type I complexes can be sub-divided into type Ia and type Ib. Type Ia includes ParA proteins (Abeles et al., 1985; Walker et al., 1982) of approximately 320-420 residues and ParB proteins of approximately 310-340 residues (Hwang et al., 2013; Schumacher and Funnell, 2005; Surtees and Funnell, 2001; Vecchiarelli et al., 2013; Vecchiarelli and Funnell, 2013). Protein of the type Ib system include shorter ParA homologues of approximately 190-310 amino acids. The CBP in type Ib complexes are unrelated evolutionary to ParB and are smaller proteins of approximately 45-130 residues (Golovanov et al., 2003; Schumacher, 2007). These CBPs commonly possess ribbon-helix-helix (RHH) DNA binding folds (Hayes and Barillà, 2010).

The second type of partition system is the Type II complex (Figure 1.1). Here, the NTPase is known as ParM (320 residues) and possesses an actin-like folds (Bharat et al., 2015; Bork et al., 1992; Gayathri et al., 2012; Gerdes et al., 1986; Orlova et al., 2007; Salje et al., 2010).
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The CBP is often denoted ParR (~109 residues) and, as in the type Ib complex, is a RHH protein (Campbell and Mullins, 2007; Choi et al., 2008; Garner et al., 2007; Møller-Jensen et al., 2003; Møller-Jensen et al., 2007; Popp et al., 2007; Popp et al., 2008; Salje and Löwe, 2008; Schumacher et al., 2007a).

The type III par complex comprises an NTPase and CBP known as TubZ and TubR, respectively (Akhtar et al., 2009; Aylett and Löwe, 2012; Berry et al., 2002; Larsen et al., 2007; Tang et al., 2006; Tinsley and Khan, 2006). The TubZ protein (~390 residues) is a tubulin homologue, binds to the TubR factor (~421 amino acids) forming the partition complex (Aylett et al., 2010; Hoshino and Hayashi, 2012; Ni et al., 2010). The type IV partition cassette has been found on the pSK1 plasmid of *Staphylococcus aureus* and encodes just one protein that has not been well-characterized (Liu et al., 2013; Simpson et al., 2003). The CBP recognizes specific sequence within the par operon for commencing the partition events and/or to act as a regulatory protein for the same operon. When the partition process starts, the CBP at the centromere recruits the NTPase to form the segrosome nucleoprotein complex. This complex can be categorized into subtypes based on their molecular components (Schumacher, 2008). Different possibilities have been suggested for how the segregation complex achieves segregation. 1. Bidirectional pushing of plasmids as for type II systems. 2. Bidirectional pulling mechanism. 3. A tram mechanism as for type III systems (Barillà et al., 2005; Hayes and Barillà, 2006a; Larsen et al., 2007; Ringard et al., 2009; Schumacher, 2012).

1.4.1 Type I partition system

The type I par system is the most widely distributed module among the partition systems studied to date (Hayes and Barillà, 2010). The plasmid-encoded proteins that participate in this partition machinery are generally termed ParA and ParB (Figure 1.1). These proteins assemble on a specific, cis-acting centromere region that is often termed parS. ParB binds to the centromere region, while ParA interacts with ParB producing the segrosome complex that directs plasmid copies to new cells before cytokinesis (Barillà et al., 2007; Hayes and Barillà et al., 2006a; Surtees and Funnell, 2001; Vecchiarelli et al., 2012). Type I systems have been found in different types of bacterial plasmids as well as in archaea. The ParA protein contains conserved sequence motifs (Walker boxes) which are involved in nucleotide recognition and hydrolysis in a larger group of diverse ATPases (Walker et al., 1982). ParA superfamily proteins have size ranges from ~200 to ~450 amino acids (Hayes et al., 2003; Møller-Jensen et al., 2003; Møller-Jensen et al., 2007; Popp et al., 2007; Popp et al., 2008; Salje and Löwe, 2008; Schumacher et al., 2007a).
and Barillà, 2010; Koonin, 1993; Motallebi-Veshareh et al., 1990). However the sequences and structures of the CBPs are different in type Ia and Ib complexes (Schumacher, 2008).

**Figure 1.1. Genetic organization types I-III partition systems in low copy number plasmids.** The orange and yellow boxes represent the CBP and the NTPase, respectively. The centromere sites are marked by red boxes. Orange arrows represent centromere binding by CBP and yellow arrows illustrate the recruitment of NTPase by the CBP to form the partition complex. The purple arrows show transcriptional repression.
CBPs in type Ib complexes contain a RHH fold which is a DNA binding motif involved in centromere recognition (Zampini et al., 2009). By contrast ParB proteins in type Ia complexes have a helix-turn-helix (HTH) motif and a six-stranded β-sheet coiled-coil that contact the centromere site (Schumacher and Funnell, 2005). Moreover, the CBP in both type Ia and Ib systems stimulates ATP hydrolysis by the ParA protein. This stimulation may involve an arginine finger like motif (Barillà et al., 2007). This stimulation likely plays a conserved role in the ATPase regulation of all members of ParA superfamily with their partner proteins that participate in plasmid segregation (Hayes and Barillà, 2010).

1.4.2 Type Ia partition system
The par system of the P1 plasmid in Escherichia coli is a well-described model for studying the type Ia partition cassette (Abeles et al., 1985; Hayes and Barillà, 2006b; Li and Austin, 2002). The parABS operon of P1 encodes the ParA Walker type ATPase and the ParB CBP that assemble on the parS centromere site (Figure 1.1). The structure of ParB is distinct from those in type Ib complexes (described below). The ParB protein consists of three domains including domains for binding to ParA (N-terminal domain), a central HTH region and a C-terminal dimerization domain bind to the A-boxes and B-boxes of parS, respectively, (Schumacher et al., 2007b; Schumacher, 2012). Due to this subdomain composition ParB possesses a multi-bridging capability which bridges between parS centromere site boxes located on different plasmids (Schumacher and Funnell, 2005; Schumacher, 2007; Schumacher, 2008). The ParA protein performs two main functions: ATP binding and hydrolysis (Bouet and Funnell, 1999) and repression of the parAB operon through the binding of ParA to an upstream promoter. This autoregulation is enhanced by ParB binding to ParA (Davis et al., 1992; Hayes et al., 1994). The parS site is located directly downstream of the parAB genes. The site consists of ~80 bp that comprises two arms that have hexamer B box and heptamer A box motifs that are recognised by ParB (Surtees and Funnell, 2001). The arms are separated by a central binding site for integration host factor (IHF) (Bouet et al., 2000). This factor has an architectural role and bends the parS arms for assembling and spreading of ParB around the arms and adjacent sequences (Funnell, 1991; Hayes and Austin, 1994; Rodionov and Yarmolinsky, 2004).

ParA is a motor protein that mediates the segregation of replicated plasmid by interaction with ParB assembled at parS. There is no direct contact between ParA and the centromere, but ParA instead is recruited by ParB into the segrosome that drives the intracellular movement of plasmids (Bouet and Funnell, 1999; Erdmann et al., 1999; Li et al., 2004;
ATP binding and/or hydrolysis by ParA is the motive force for driving plasmid segregation. In contrast, the binding of ADP stimulates the interaction of ParA with the operator during transcriptional repression (Davey and Funnell, 1997; Davis et al., 1992; Hayes and Austin, 1994; Vecchiarelli et al., 2010).

Certain important properties have been described recently for the P1 ParA protein. First, the interaction of ParA with non-specific DNA, including the bacterial nucleoid has been shown (Davis et al., 1992; Davey and Funnell, 1994, 1997; Havey et al., 2012; Vecchiarelli et al., 2012). ParA positioned on the nucleoid surface may lead to a dynamic oscillation pattern (Sengupta et al., 2010). The ParB is affected by the stimulatory activity of the ParA proteins and the ParB-centromere interaction is abolished in the presence of high concentration of ATPase activity (Barillà and Hayes, 2003; Bouet and Funnell, 1999). In addition, mutation of specific residues in the Walker A motif of the ATP binding site affected the ability of ParB to stimulate ATPase activity as well as the repressor activity (Vecchiarelli et al., 2013).

The complex formation of centromere binding factor ParB and the centromere site has a concentration-dependant manner for DNA binding, polymerization and ATP hydrolysis (Salje, 2010). Moreover, the dynamic oscillation is not observed in the absence of ParB but the ParA remains in contact with the nucleoid and the movement of two replicated plasmids by ParA proteins is present within the nucleoid region (Derman et al., 2008; Ebersbach and Gerdes, 2005; Erdmann et al., 1999; Havey et al., 2012; Hatano and Niki, 2010; Ringgaard et al., 2009; Vecchiarelli et al., 2012). This understanding has developed into a model in which ParA coats the bacterial nucleoid by non-specific binding. The interaction of the ParA-ParB-parS complex with the nucleoid and the conversion of ParA from the ATP bound form to the ADP bound form cause the disassociation of ParA from the nucleoid. Thus, a gradient of ParA concentration is set up across the nucleoid which is ‘chased’ by the ParA-ParB-parS complex leading to the directional movement of the P1 plasmid (Hwang et al., 2013).

P1 plasmid segregation cassette is a relatively well-defined system. The mechanism of transcriptional auto-regulation of the parAB operon also has been well-studied (Bouet and Funnell, 1999; Davis et al., 1996; Hayes et al., 1994). ParA bound to ADP is involved particularly in this regulation. When bound to ADP, ParA adopts a specific dimer state which is required for operator binding and repression of parAB operon expression (Dunham
et al., 2009). The major role of ParA in transcriptional repression is dissimilar to the short ParA proteins in type Ib complexes. These ParAs lack specific motifs for DNA-binding. Instead the CBFs in type Ib complexes are the principal transcriptional repressors of their operons (Carmelo et al., 2005; Wu et al., 2011; Zampini et al., 2009).

1.4.3 Type II segregation: an insertional polymerization mechanism

The best understood type of active plasmid partition system is the type II complex. This type is represented by the par locus of multiple-antibiotic-resistance plasmid R1 which replicates in E. coli. The parMRC locus encodes the proteins that participate in plasmid segregation of R1 plasmid. These proteins comprise ParM, a member of the actin superfamily of ATPases, that forms polar, left-handed, double helical filaments (Bork et al., 1992; Jensen and Gerdes, 1997; van den Ent et al., 2002), and ParR which is a CBP that binds to the parC centromere (Gerdes et al., 1986, 2010). The operon is autoregulated by binding of ParR to an operator (39 bp) which is located within the parC centromere site (Breuner et al., 1996; Dam and Gerdes 1994; Jensen et al., 1994; Salje et al., 2010). The molecular structures of the ParM and ParR proteins and their interaction have been studied comprehensively. ParR (13 kDa) is a RHH dimer (Møller-Jensen et al., 2007; Schumacher et al., 2007) that binds to the multiple repeats of parC (ten 11-bp motifs) (Dam and Gerdes, 1994; Jensen et al., 1998). The crystal structure of ParR-parC revealed the formation of a partition complex with a ring-like (helical) structure. This complex ranged between 15 and 20 nm in diameter around which the DNA was externally wrapped (Møller-Jensen et al., 2007).

ParM forms polar, left-handed, short-lived double stranded filaments in response to ATP binding (van den Ent et al., 2002). These polymers are unstable at both ends but they stabilize when two ParM filaments align antiparallely forming a bipolar spindle (Fink et al., 2016). The growth of ParM filaments is accelerated by ParRC complex at one end of the polar filaments (Gayathri et al., 2012). Only the filaments that are bound to the ParR-parC complex will be available to extend and elongate (Campbell and Mullins, 2007). Such elongation (bidirectional growth of ParM polymers) restricts the movement of two plasmids to the opposite cell poles. Thus, the ParR-parC plasmid-bound nucleoprotein complex has a key role in covering and stabilizing of ParM filament ends by binding to them (Choi et al., 2008; Garner et al., 2007; Orlova et al., 2007; Salje and Löwe, 2008).
Consequently, the bundled filaments of the ParM actin homologue drive DNA segregation by a process called insertional polymerization (Møller-Jensen et al., 2002, 2003). This mechanism is considered to be crucial feature of the type II par system. Different studies have been performed, including electron microscopy studies, to investigate the exact structure and function of ParM filaments. These studies confirmed in vivo and in vitro observations that ParM proteins produce dynamic polymeric structures that form double stranded filaments (Garner et al., 2007). A new asynchronous model has been suggested for plasmid segregation in contrast to the ideas of ParMRC locus of R1 plasmid segregation (Salji et al., 2009). In the proposed model ParM doublets formed antiparallel spindles (Figure 1.2) which assembles to produce mitotic machinery (Bharat et al., 2015).

The dynamic structure of the ParM polymer allows binding to pairs of ParR-parC nucleoprotein complexes (Chio et al., 2008; Salje and Löwe, 2008). Through this stabilization the ParM polymer polymerizes continuously by adding new ParM subunits at each end of the ParR-parC cap (Campbell and Mullins, 2007; Garner et al., 2004, 2007). This bidirectional polymerization of ParM-ATP filaments was observed in vitro using total internal reflection fluorescence-microscopy (Garner et al., 2004; Popp et al., 2007). This insertional mechanism pushes plasmids towards opposite cell poles and beyond the nucleoid (Campbell and Mullins, 2007; Garner et al., 2007; Møller-Jensen et al., 2003; Orlova et al., 2007; Popp et al., 2008). It is unknown if any host factor is required to aid the plasmids in reaching the cell poles (Dobruk-Serkowska et al., 2012; Salje et al., 2010; Schumacher et al., 2012). Although ATP induces the polymerization of ParM protein in vitro (Møller-Jensen et al., 2002), the protein also is an effective GTPase and this activity is more efficient than ATPase hydrolysis (Popp et al., 2008). Whether ATP or GTP is the actual promoter of ParM polymerization in vivo is uncertain.

Interestingly, many ParM filamentation/disassociation rounds combined with plasmid motion have been observed during one cell cycle. This fact suggests that this par system is separated from the cell cycle and that the participation of a host factor(s) is unexpected (Campbell and Mullins, 2007; Garner et al., 2004). It is thought that the induction of structural change in subunits at the tips of ParM polymers may reflect the release of nucleotide from the ends of the polymers which causes the dissociation of end subunits (Hayes and Barillà, 2010; Popp et al., 2008).
Figure 1.2. Cryo-EM microscopy image and atomic model of ParM doublets. In the atomic model the interacting residues are shown in red and orange in each interacting filament (from Bharat et al., 2015)

1.4.4 Type III partitioning system utilizes a tubulin homologue

The type III partition system has been identified on two different plasmids which are pBtoxis from Bacillus thuringiensis and pXO1 from Bacillus anthracis (Berry et al., 2002; Hoshino and Hayashi, 2012; Larsen et al., 2007; Oliva et al., 2012; Tang et al., 2006; Tinsley and Khan, 2006). The former is the best understood type III complex (Larsen et al., 2007; Tang et al., 2006). The type III cassette consists of an upstream gene termed tubR and a downstream gene tubZ (Figure 1.1) that encode TubR and TubZ proteins, respectively (Larsen et al., 2007). The tubC centromere site was first described as four direct repeats of 12-bp (Tang et al., 2006). However, recent findings showed that this site in pBtoxis contains two clusters of binding sites. Sites 1-3 and 4-7 comprise the first and second clusters, respectively (Aylett and Löwe, 2012). The TubR transcriptional repressor and CBP form an intertwined dimer containing a winged helix-turn helix (HTH) domain in which the DNA recognition helices are used principally for dimerization instead of DNA binding (Figure 1.3 A) (Ni et al., 2010). Thus, TubR exhibits a new structure for DNA binding (Schumacher, 2012). TubR binds specifically to the centromere site utilizing residues in the wing regions for interaction with the DNA minor groove and the N-termini of the recognition helices to insert into a single major groove (Ni et al., 2010). In this way TubR coats tubC (Figure 1.3 B) (Aylett and Löwe, 2012).

The motor protein (TubZ) is a member of the tubulin/FtsZ GTPase superfamily: TubZ contains a tubulin/FtsZ fold and a flexible C-terminal tail. TubZ polymerizes in response to
GTP binding forming dynamic double stranded filaments. These polymers perform a directional polymerization \textit{in vivo} and \textit{in vitro} that is required for plasmid stability (Aylett \textit{et al}., 2010; Larsen \textit{et al}., 2007). The presence of GTP that caps the termini of TubZ polymers is proposed to stabilize the hydrolysable subunits within the filaments to protect their disassembly. At least four types of TubZ-like sequence that are encoded by different plasmids in \textit{Bacillus} species have been defined (Chen and Erickson, 2008). This fact supports the presence of many type III \textit{par} systems (Anand \textit{et al}., 2008; Chen and Erickson, 2008; Ni \textit{et al}., 2010; Oliva \textit{et al}., 2012; Tinsley and Khan, 2006).

The structures of TubR and TubZ have been reported by Ni \textit{et al}., (2010). The interaction between the two proteins is performed by the C-terminal regions of TubZ located at the tip of the filaments which is the optimal interaction region with TubR, although the co-crystal structure has not yet resolved (Aylett \textit{et al}., 2010). In addition, \textit{in vitro} analysis suggests that TubZ filament consists of two-strands pre-hydrolysis with intermediates post-hydrolysis forming a four-stranded filament (Montabana and Agard, 2014). Interestingly, the formation of dynamic filaments by TubZ involves a distinctive action called ‘treadmilling’ (Chen and Erickson, 2008; Larsen \textit{et al}., 2007; Ni \textit{et al}., 2010). Furthermore, TubZ treadmilling does not need any known host factor (Larsen \textit{et al}., 2007). Consequently, a tram-like mechanism is suggested for type III partitioning that differs from pulling, pushing or the gradient mechanisms of types I and II systems (Aylett \textit{et al}., 2010; Chen and Erickson, 2008; Ni \textit{et al}., 2010).

In the tram model, the TubRC complex recruits TubZ (Ni \textit{et al}., 2010; Tang \textit{et al}., 2007). The complex forms a ring-like structure which increases the polymerization of TubZ (Aylett and Löwe, 2012; Oliva \textit{et al}., 2012). TubR attaches to TubZ filaments through its exterior C-terminal region. The movement of TubZ filaments along one side of the cell is mediated by following the membrane edge at the end poles. Then the TubZ filaments return in the direction of opposite side of the cell (Schumacher, 2012). The treadmilling action is achieved by hydrolysis of GTP within the TubZ polymer, removal of TubZ-GDP subunits from one end, and attachment of TubZ-GTP subunits at the opposite end. Thus, elongation at one end and retraction at the other end of these dynamic TubZ filaments result in translocation of the plasmid/TubR complex. The complex is presumed to move with the growing filaments (Larsen \textit{et al}., 2007; Sengupta \textit{et al}., 2010). The mechanism of final disassembly of TubR-plasmid partition complexes is unknown (Schumacher, 2012; Salje, 2010). Interestingly, as formation and dynamics of TubZ filaments are important for
plasmid stability (Larsen et al., 2007), a self-assembly of TubZ filaments into a prokaryotic minus end-tracking system has been observed recently suggesting direct DNA transport through pulling forces (Fink and Löwe, 2015).

**Figure 1.3.** The TubR-centromere structure of pBtoxis plasmid of *Bacillus thuringiensis*. A, Crystal structure of TubR-tubC from (Aylett and Löwe, 2012). B, Ribbon diagram of the TubR-DNA model with the recognition helices coloured yellow (Ni et al., 2010).

### 1.5 Segregation of the TP228 plasmid

TP228 is a conjugative multidrug resistant plasmid (72 kb) originating from *Salmonella enterica* serotype Newport. The plasmid exhibits a wide range of antibiotic resistance including to kanamycin, neomycin, spectinomycin, streptomycin, sulphonamides and tetracycline, and some metal ions. TP228 plasmid replicates at low copy number in *E. coli* and is segregationally stable when grown without selective pressure. The plasmid was
screened for the occurrence of a partition system which revealed a segregation cassette that includes a member of the ParA superfamily (Hayes, 2000). The components of the TP228 partition system consist of ParF (22 kDa) a which is ParA-type protein, and ParG which assemble on the centromere site parH (Figure 1.1). The O_F operator site that is involved in transcriptional repression of parF-parG is located between the parH centromere and parF. Studies showed that parH comprises a series of degenerate 5’-ACTC-3’ boxes flanked by AT-rich separators. Similarly, the O_F locus consists of eight degenerate tetramer boxes orientated in a direct and inverted pattern (Wu et al., 2011; Zampini et al., 2009). ParG is a sequence-specific binding protein (Barillà and Hayes, 2003) at parH and O_F. When ParG bound at parH, the ParF protein is recruited to produce the segrosome (Schumacher et al., 2012).

Although the tertiary structures of ParF and ParG have been determined, the structure of the intact segrosome is unknown (Schumacher, 2012). In fact, the complete segrosome structure has not been determined for any type I, II or III complexes. Like other ParA proteins, ParF is an ATPase (Barillà and Hayes, 2003) with Walker-type nucleotide binding motifs typical of ParA superfamily members (Hayes, 2000). This ATP binding may confer a kinetic force for segregation in the ParF-ParG system. ParF-ATP polymers may be destabilized by conversion of ATP to ADP which may cause the pulling of the attached plasmids to opposite cell poles. Alternatively, the binding of ATP to ParF in polymerization may induce a pushing mechanism (Schumacher et al., 2012).

The ParF protein is a vital component for the formation of the partition complex that drives plasmids towards opposite bacterial cell poles (Hayes and Barillà, 2006a). There is no direct interaction between ParF and parH. Instead ParF assembles into the segregation complex by means of interaction with the ParG protein. This interaction in turn remodels ParF (Barillà and Hayes, 2003; Wu et al., 2011). The ratio of the two partition proteins in vivo is likely to be a determining factor in the plasmid segregation process (Barillà and Hayes, 2003; Barillà et al., 2005, 2007). Following segrosome formation, the next step generally in plasmid partition is thought to be plasmid pairing (Figure 1.4). Each plasmid from the pair can then be segregated in opposite directions. Although plasmid pairing by the ParF-ParG complex has not been tested, pairing has been demonstrated in other cases (Edgar et al., 2001).
Figure 1.4. Models show the possible molecular events during plasmid segregation mediated by the ParF-ParG complex. A pair of partition complexes is positioned centrally in the cell in both models. Each complex is formed from ParF (maroon rectangle) and dimeric ParG (yellow circle) proteins assembled on the partition site parH (green diamond) of the replicated plasmids. The binding of ATP to ParF causes a structural change in the protein (shown as horizontal rectangle changed to diagonal rectangle). Bipolar filamentation of ParF occurs in response to ATP binding. In bidirectional pushing (A), the polymerization of ParF drives the two plasmids towards the cell poles. In bidirectional pulling (B), the ParF filaments are stabilized at opposite cell poles by a hypothetical host structure (blue rectangle). As a result of depolymerization of ParF due to ATP hydrolysis, the plasmids are drawn away from the mid cell (Barillà et al., 2005).

1.5.1 ParF protein

ParF and closely-related proteins form a distinct subgroup of the ParA superfamily of ATPases involved in the partition machinery of low copy number plasmids (Hayes, 2000). ATPase proteins have different functions, but generally utilize ATP for providing a source of chemical energy for dynamic movement of the protein or to elicit changes in the protein conformation (Matte and Delbaere, 2010). The membership of ParF in the ParA superfamily is ascribed particularly to the conserved ATP-binding and hydrolysis motifs (Koonin, 1993; Leipe et al., 2002; Motallebi-Veshareh et al., 1990; Wendler et al., 2012). The ParF protein
was first identified when the partition system of multidrug resistant plasmid TP228 was isolated which was accompanied by the identification of ParF homologues from other plasmids (Hayes, 2000).

1.5.2 ParF: an ATPase of the Walker superfamily

ParF harbours conserved ATP-binding motifs that identify the protein as a member of the ParA superfamily of ATPases that have a deviant Walker A box motif GKGGHGK(S/T). The Walker A motif is located at the N-terminus of an α-helix in ParA proteins and is involved in interactions with the bound ATP molecule. This motif is characterized by the second lysine residue adjacent to its N-terminal end (Hayes, 2000; Lutkenhaus and Sundaramoorthy, 2003; Motallebi-Veshareh et al. 1990; Wendler et al., 2012). Proteins that possess this deviant Walker A motif have different functions. For example, MinD protein is a ParA Walker type ATPase that is involved in septum site placement in *E. coli* which regulates cell division. ParF is more related to MinD than to well-characterized ParA proteins such as those encoded by the P1 and F plasmids. ParA ATPases also possess a second motif, the B box, which is located at residues 73-83 in ParF. This box is characterized by residues with negative charge (Glu or Asp) and functions in magnesium binding and ATP hydrolysis (Schumacher et al., 2012). Similar to other ParA superfamily members, ParF and other ParA proteins possess a weak ATPase activity which is necessary during DNA segregation (Barillà et al., 2005; Davis et al., 1996; Fung et al., 2001). This activity in ParF and close homologues has been demonstrated using thin layer chromatography (Barillà et al., 2005, 2007; Dobruk-Serkowska et al., 2012; Machón et al., 2007).

1.5.3 The molecular structure of ParF

ParF is a polymeric protein in its ATP-bound form and monomeric in the ADP-bound state (Barillà and Hayes, 2003; Schumacher et al., 2012). The ParF structure constitutes a single domain that consists of central seven-stranded twisted β-sheets flanked on each direction by four α-helices. The topology of ParF is: β1(residues 1–8)-α1 (residues 14–29)-β2 (residues 32–39)-α2 (residues 43–49)-β3 (residues 55–60)-α3 (residues 64–73)-β4 (residues 77–85)-α4 (residues 89–98)-β5 (residues 99-106)-α5 (residues 111–123)-β6 (residues 131–139)-α6 (residues 145–157)-β7 (residues 159–163)-β7 (residues 171–178)-310 (residues 182–184)-α8 (residues 189-205) (Figure 1.5A) (Schumacher et al., 2012). The Walker A motif that includes Lys10 of ParF interacts with ATP across the interface to form a dimer (Figure 1.5B).
Figure 1.5. Molecular structure of ParF. A, ribbon diagram of the monomer ParF-ADP complex. The secondary structural elements are labelled. Helices are red, strands are yellow, and loops are green. The ADP molecule is shown as sticks. B, ParF dimer, with the non-hydrolysable ATP analogue, AMPPCP (Schumacher et al., 2012).

1.5.4 ParG: a DNA binding ribbon-helix-helix protein

The second protein in the segregation complex of plasmid TP228 is the ParG CBP that has a monomeric molecular mass of 8.6 kDa (Barillà and Hayes, 2003; Hayes, 2000). ParG is not related to large ParB proteins such as that encoded by the P1 plasmid. ParG structurally is a symmetric dimer, consisting of a folded domain (76 residues) that has a RHH architecture. The folded structure in ParG comprises two intertwined C-terminal parts (residues 33-76) containing double-stranded β-sheets and two mobile N-terminal regions (residues 1-32) (Figure 1.6) (Golovanov et al., 2003). These N-terminal tails are unstructured in solution, so that they take on a random conformation. The RHH folded domain is crucial for DNA binding by ParG (Golovanov et al., 2003; Zampini and Hayes, 2012). Like other RHH proteins (Schreiter and Drennan, 2007), the insertion of the double-stranded β-sheets into the major groove is thought to mediate binding of ParG at both parH and O_F, although a ParG-DNA co-structure is not yet available. The tail of ParG is required for transcriptional repression of the parFG cassette (Carmelo et al., 2005; Zampini et al., 2009).

The ParG protein stimulates the ATPase activity of ParF. This process is achieved by the ParG N-terminal tail which also remodels the structure of ParF (Barillà et al., 2007). In addition, the ParG N-termini promote ParF polymerization (described below). Moreover,
the deletion of the N-terminal region affected the binding function of ParG to the \textit{parH} centromere site (Barillà and Hayes, 2003; Barillà \textit{et al.}, 2007; Carmelo \textit{et al.}, 2005; Machón \textit{et al.}, 2007; Wu \textit{et al.}, 2011). Collectively the ParG C-terminal domain is involved in self-association/dimerization, the ParF interaction, and binding at the centromere and \textit{O} \textit{F} site. Notably, alanine scanning mutagenesis characterized the crucial role of residues Phe49, Trp71 and Leu72 in the RHH domain of ParG in dimerization and consequently in segregation activity (Saeed \textit{et al.}, 2015).

![Figure 1.6. Schematic diagram of the ParG CBP showing the structure of domains that participate in variety of functions upon plasmid partitioning (from Schumacher, 2008).](image)

### 1.5.5 Polymerization of ParF and plasmid segregation

ParF assembles \textit{in vitro} to form highly distinctive polymers. ParF polymerization is increased dramatically by ATP binding (Barillà \textit{et al.}, 2005). ADP has an opposite effect of polymerization compared to ATP, i.e, polymerization is suppressed by ADP. Interestingly, the destabilization of ParF-ATP polymers by means of conversion of ATP to ADP may cause the pulling of the attached plasmid to opposite cell poles whereas the binding of ATP to ParF (polymerization) may induce the pushing mechanism by growing filaments (Figure 1.4) (Barillà \textit{et al.}, 2005; Schumacher \textit{et al.}, 2012). The pre-filamentous structures formed in the absence of ATP might act as nucleation sites for more extensive polymerization. The ultrastructure of ParF polymers has been investigated using negative-stain electron microscopy. When incubated with ATP, ParF forms extensive multistranded filament bundles. These polymers possess one irregular end and one end with a compact structure (Figure 1.7A). This suggested that the irregular end of ParF polymers might act as an active
site for extension of the polymerization (Barillà et al., 2005). Based on the crystal structure, the linear polymer of ParF showed that the building blocks consist of ‘dimer of dimer’ units which possess two complementary types of surfaces (Figure 1.7 B). The complementary interactions between the tips and the elongated surfaces of the dimer units produce an irregular polymer. Two types of interface are formed during ParF polymerization: the contact of residues 61-71 with two separate groups (87-98 and 117-129 residues) creates the extensive interface 1. In addition, interface 2 is produced by the contact of residues 49-60 and 168-192 (Schumacher et al., 2012).

The effects of mutations in the ATP binding motifs and in other regions of ParF have been examined to further investigate polymerization of the protein. Mutation of residues K15Q and G11V in the Walker A motif perturbed ATPase activity of ParF and also abrogated the protein’s polymerization kinetics. In agreement, the mutations also reduced plasmid segregational stability (Barillà et al., 2005). Moreover, mutations at the ParF-ParF polymer interface also inhibited polymerization and decreased partition activity (Schumacher et al., 2012). Interestingly, mutation of certain conserved residues in ParF increased the protein’s ATPase activity. These residues are situated outside of the conserved Walker box motifs. The polymerization properties of proteins bearing these mutations were altered and partition activity was reduced dramatically. These combined mutational studies highlight that a balance between ATP-induced polymerization of ParF and ATP hydrolysis by the protein is crucial for correct segregation activity. Moreover, preserving the structure of the nucleotide-binding site of ParF is necessary for ATPase activity and the polymerization mechanism of plasmid segregation (Dobruk-Serkowska et al., 2012).

1.5.6 Modulation of ParF ATPase activity and polymerization by ParG

ParG has a crucial role both in stimulating ATP hydrolysis by ParF and in the dynamics of ParF polymerization. An arginine finger-like motif was identified in the ParG flexible N-terminal tail (Barillà et al., 2007). Commonly, P-loop NTPases (nucleotide phosphate-binding motifs) use arginine fingers either in cis, when the residue is from the same protein, or in trans, where it is located on a partner protein, as the case of ParG and ParF (Scheffzek et al., 1998). Typically, this arginine finger is present in mobile loop regions of the binding protein. Therefore, stimulation of nucleotide hydrolysis is performed by the insertion of an arginine side chain into a suitable active site position of the partner protein (Ahmadian et al., 1997). In this way, ParG stimulates ATP hydrolysis by ParF ~30-fold in vitro (Barillà et al., 2005).
Separately from its role in stimulating ATP hydrolysis by ParF, ParG influences ParF polymerization in the presence of ATP, but also promotes polymerization independently of ATP. Moreover, ParF fibres formed in the presence of ParG are thicker and longer compared to in the absence of ParG. Thus, ParG promotes bundling and remodelling of ParF filaments (Figure 1.7 C). The N-terminal tail of ParG is important for this interaction (Barillà et al., 2005, 2007). Therefore, ParG has a separable effect on ATP hydrolysis and polymerization of ParF and the flexible N-terminal tail is important in both processes. Consequently the ParG stimulatory action on the ATPase activity of ParF is an important factor in the control of ParF polymerization and depolymerisation mechanisms (Barillà et al., 2005, 2007).

In conclusion, ParF and other ParA proteins are crucial for segrosome assembly and function. Polymerization of these factors appears to be essential to ensure the stable maintenance of low copy number plasmids. Other models that involve the interaction of ParA with the nucleoid also have been proposed. Blocking the activity of ParA proteins using new antibacterial compounds may disrupt the segrosome and thereby interfere with
the accurate segregation of antibiotic resistance plasmids. The characterization of the specific interfaces in ParA that are involved in polymerization and interaction with the partner CBP is necessary to allow the rational design of new compounds that target those interfaces. Here, we perform structure-function studies of the ParF protein whose tertiary structure was described as this project began.

1.6. The aims of the study

The ParF protein plays a crucial role in the partition of multidrug resistance plasmid TP228 in *E. coli*. The crystal structure of the protein has been determined recently. The main aims of this study are:

- To undertake mutational studies of ParF and to correlate the results with the newly determined structure.
- To apply pentapeptide scanning mutagenesis (PSM) to generate a library of mutant ParF protein this will provide new insight into ParF activity.
- To use PSM to identify which regions in ParF are crucial for its function.
- To identify which regions in ParF are insensitive to the effects of pentapeptide insertions.
- To examine the effect of pentapeptide insertions on self-association of ParF by two hybrid analysis and to correlate these data with previous studies on ParF self-association.
- To examine the effect of pentapeptide insertions in ParF on plasmid segregation.
- To examine the effect of pentapeptide insertions in ParF on association with the partner ParG protein by two hybrid analysis.
- To identify the region(s) in ParF that interacts with ParG by PSM.
- To characterize further the ParF interface with ParG by site-directed mutagenesis and functional studies, including segregation assays and two hybrid analysis.
- To test the contribution of the C-terminus of ParF in protein function.
- To identify regions in ParF that may be novel targets for new antibacterial agents that disrupt segrosome assembly.
Chapter 2:
Materials and Methods
2.1 Bacterial strains and growth conditions

*Escherichia coli* DH5α is one of the *E. coli* K12 strain derivatives (endA1/hsdR17(rk’ mk+) supE44 thi-1 recA1 gyrA96 (Nal’ relA1Δ(lacZYA-argF)U169 deoR (Φ80lacΔ(lacZ)M15)) (Woodcock *et al*., 1989) was used for plasmid propagation and cloning. Streptomycin-resistant strain DS941 (thr1 leuB6 hisG4 thi1 ara14Δ (gpt-proA)62argE3galK2supE44 xyl5 mtl1 tsx33 lacY1 rpsL31 recF143 lacZ ΔM15lacIq) (Summers & Sherratt, 1988), was used as the recipient in conjugation experiments. *E. coli* FH395 contains transposon Tn4430 on a derivative of the F plasmid that lacks other transposable elements (pOX38:Tn4430) (Hayes, 2000). Target plasmids for pentapeptide scanning mutagenesis (PSM) were cotransformed into FH395 and used as a donor in mating tests. *E. coli* SP850 (Hfr (PO1) λ e14’ relA spoT1 Δ(cyaA1400::kan) thiE1) (Shah and Peterkofsky, 1991) was used in bacterial two-hybrid assays. A polA mutant host (BR825) was used in plasmid segregation assay (Ludtke *et al*., 1989). For methylation-sensitive restriction enzymes, *E. coli* SCS110 (rpsL thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44 Δ(lac-proAB)F’traD36 proAB lacIqZ ΔM15) was used to prepare DNA plasmid free of Dam and Dcm methylation for selected clonings (Hanahan, 1983). Bacterial cultures were grown as required on Luria-Bertani (LB) broth medium (Fisher Scientific) or on LB agar at 37°C. MacConkey agar (Thermo-Scientific) plates containing 1% (w/v) maltose were used for qualitative bacterial two-hybrid assays at 30°C. These media were supplemented with appropriate antibiotics with incubation periods of 16-36 hours.

2.2 Antibiotics

Antibiotics (Sigma-Aldrich) were added to growth media at the following final concentrations (μg/ml) when required: ampicillin, 100; chloramphenicol, 10; kanamycin, 50; and streptomycin, 50.

2.3 Extraction kits, DNA modifying enzymes and chemicals

The ISOLATE II Plasmid Mini Kit and Gel and PCR Purification Kits were purchased from Bioline and used according to the manufacturer’s instructions. Restriction enzymes (BamHI, BspDI (ClaI), EcoRI, EcoRV, HindIII, HpaI, KpnI, SalI, SexAI, SmaI, SnaBI, StuI, XhoI), Phusion High Fidelity DNA polymerase, T4 DNA ligase, calf intestinal alkaline phosphatase (CIAP) and T4 polynucleotide kinase were purchased from New England Biolabs, as were adenosine-5’-triphosphate (ATP), and 1 kb and 100-bp DNA ladders. Molecular biology grade chemicals were from Sigma-Aldrich or Fisher Scientific.
2.4 Plasmids

Plasmids used in this study are listed in Table 2.1. Some plasmids were from laboratory stocks and others were constructed during this project. Plasmids were constructed by pentapeptide scanning mutagenesis (Section 2.19), PCR (Section 2.11) or OE-PCR (Section 2.14) and restriction enzyme cloning.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Descriptiona (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFH450</td>
<td>Partition assay vector that possesses the P1 and Co1E1 replicons; Cm' (Hayes, 2000)</td>
</tr>
<tr>
<td>pFH547</td>
<td>Plasmid pFH450 harbouring the parFGH partition cassette; Cm' (Hayes, 2000)</td>
</tr>
<tr>
<td>pFH547-16039</td>
<td>pFH547-160 derivative in which 18-bp were deleted from the 57-bp insertion at position 160 in parF by digesting with SmaI and SnaBI and religating; Cm' (this study)</td>
</tr>
<tr>
<td>pFH547-16045</td>
<td>pFH547-160 derivatives in which 12-bp were deleted from the 57-bp insertion at position 160 in parF by digesting with SmaI and EcoRV and religating; Cm' (this study)</td>
</tr>
<tr>
<td>pFH547-16051</td>
<td>pFH547-160 derivative in which 6-bp were deleted from the 57-bp insertion at position 160 in parF by digesting with SnaBI and EcoRV and religating; Cm' (this study)</td>
</tr>
<tr>
<td>pFH547-16057</td>
<td>pFH547ParF160GVPLK derivative in which double-stranded oligonucleotide 123/124 (42-bp; Section 2.21) was inserted into the KpnI site thereby increasing the insert size from 15-bp to 57-bp; Cm' (this study).</td>
</tr>
<tr>
<td>pFH547-18539</td>
<td>pFH547-185 derivative in which 18-bp were deleted from the 57-bp insertion at position 185 in parF by digesting with SmaI and SnaBI; Cm' (this study)</td>
</tr>
<tr>
<td>pFH547-18545</td>
<td>pFH547-185 derivative in which 12-bp were deleted from the 57-bp insertion at position 185 in parF by digesting with SmaI and EcoRV and religating; Cm' (this study)</td>
</tr>
<tr>
<td>pFH547-18557</td>
<td>pFH547ParF185RGTPS derivative in which double-stranded oligonucleotides 123/124 (42-bp; Section 2.21)</td>
</tr>
<tr>
<td>pFH547E152A</td>
<td>was inserted into the KpnI site thereby increasing the insert size from 15-bp to 57-bp; Cm’ (this study).</td>
</tr>
<tr>
<td>----------------</td>
<td>------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>pFH547K151A</td>
<td>parFK151A cloned into pFH547 using restriction enzyme digestion; pT18ParF*K151A was digested with SexAI and ClaI and 229-bp gel extracted fragment was inserted between the equivalent sites in pFH547; Cm’ (this study)</td>
</tr>
<tr>
<td>pFH547ParF151GVPLK</td>
<td>pFH547 derivative in which 15-bp were inserted 3’ of codon 151 in parF by PSM; Cm’ (this study)</td>
</tr>
<tr>
<td>pFH547ParF160GVPLK</td>
<td>pFH547 derivative in which a 229-bp fragment harbouring the 160GVPLK insertion from pT18ParF*160GVPLK was obtained by digestion with SexAI and ClaI and inserted between the equivalent sites in pFH547; Cm’ (this study)</td>
</tr>
<tr>
<td>pFH547ParF164GVPRT</td>
<td>pFH547 derivative in which a 229-bp fragment harbouring the 164GVPRT insertion from pT18ParF*164GVPRT was obtained by digestion with SexAI and ClaI and inserted between the equivalent sites in pFH547; Cm’ (this study)</td>
</tr>
<tr>
<td>pFH547ParF165RGTPA</td>
<td>pFH547 derivative in which 15-bp were inserted 3’ of codon 165 in parF by PSM; Cm’ (this study)</td>
</tr>
<tr>
<td>pFH547ParF166RGTPA</td>
<td>pFH547 derivative in which 15-bp were inserted 3’ of codon 166 in parF by PSM; Cm’ (this study)</td>
</tr>
<tr>
<td>pFH547ParF185RGTPS</td>
<td>pFH547 derivative in which 15-bp were inserted 3’ of codon 185 in parF by PSM; Cm’ (this study)</td>
</tr>
<tr>
<td>pFH547ParF205GVPLF</td>
<td>pFH547 derivative in which a 354-bp OE-PCR fragment harbouring a 15-bp insertion 3’ of codon 205 in parF was digested with Clal and Hpal and inserted between the equivalent sites in pFH547; Cm’ (this study)</td>
</tr>
<tr>
<td>pFH547ParF67GVPLY</td>
<td>pFH547 derivative in which 15-bp were inserted 3’ of codon 67 in parF by PSM; Cm’ (this study)</td>
</tr>
<tr>
<td>pFH547ParF71GVPRK</td>
<td>pFH547 derivative in which 15-bp were inserted 3’ of codon 71 in parF by PSM; Cm’ (F. Hayes personal)</td>
</tr>
<tr>
<td></td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>pFH547Δ151</td>
<td>pFH547 derivative in which codon 151 in <em>parF</em> was deleted by OE-PCR; PCR product was digested with SexAI and ClaI and inserted between the equivalent sites in pFH547; Cm&lt;sup&gt;r&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pFH547Δ152</td>
<td>pFH547 derivative in which codon 152 in <em>parF</em> was deleted by OE-PCR; PCR product was digested with SexAI and ClaI and inserted between the equivalent sites in pFH547; Cm&lt;sup&gt;r&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pFH547Δ204-206</td>
<td>pFH547 derivative in which codons 204, 205 and 206 in <em>parF</em> were deleted by OE-PCR; PCR product was digested with ClaI and HpaI and inserted between the equivalent sites in pFH547; Cm&lt;sup&gt;r&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pFH547Δ205-206</td>
<td>pFH547 derivative in which codons 205 and 206 in <em>parF</em> were deleted by OE-PCR; PCR product was digested with ClaI and HpaI and inserted between the equivalent sites in pFH547; Cm&lt;sup&gt;r&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pFH547Δ206</td>
<td>pFH547 derivative in which codon 206 in <em>parF</em> was deleted by OE-PCR; PCR product was digested with ClaI and HpaI and inserted between the equivalent sites in pFH547; Cm&lt;sup&gt;r&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pFH554</td>
<td>Derivative of pFH547 deleted of the Co1E1 replicon and that replicates solely under the control of P1 replicon; Cm&lt;sup&gt;r&lt;/sup&gt; (Hayes, 2000)</td>
</tr>
<tr>
<td>pOX38::Tn4430</td>
<td>F plasmid derivative used as a source of Tn4430 for PSM; Km&lt;sup&gt;r&lt;/sup&gt; (Mahillon and Lereclus, 1988)</td>
</tr>
<tr>
<td>pT18</td>
<td>Two-hybrid vector; Ap&lt;sup&gt;r&lt;/sup&gt; (Karimova <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td>pT18ParF</td>
<td><em>parF</em> cloned into pT18 vector; Ap&lt;sup&gt;r&lt;/sup&gt; (Barillà and Hayes, 2003)</td>
</tr>
<tr>
<td>pT18ParF*</td>
<td>KpnI sequence removed and BamHI sequence inserted in pT18ParF; Ap&lt;sup&gt;r&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pT18ParF*151&lt;sup&gt;33&lt;/sup&gt;</td>
<td>pT18ParF*151&lt;sup&gt;37&lt;/sup&gt; derivative in which 24-bp were deleted from the 57-bp insertion at position 151 in <em>parF</em> by digestion with SmaI and SstI and religation; Ap&lt;sup&gt;r&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pT18ParF*151&lt;sup&gt;39&lt;/sup&gt;</td>
<td>pT18ParF*151&lt;sup&gt;39&lt;/sup&gt; derivative in which 18-bp were deleted from the 57-bp insertion at position 151 in <em>parF</em> by digestion with Smal and SnaBI and religation; Ap&lt;sup&gt;+&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>pT18ParF*151&lt;sup&gt;51&lt;/sup&gt;</td>
<td>pT18ParF*151&lt;sup&gt;57&lt;/sup&gt; derivative in which 6-bp were deleted from the 57-bp insertion at position 151 in <em>parF</em> by digestion with SnaBI and StuI and religation; Ap&lt;sup&gt;+&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pT18ParF*151&lt;sup&gt;57&lt;/sup&gt;</td>
<td>pT18ParF*151&lt;sup&gt;57&lt;/sup&gt; derivative in which double-stranded oligonucleotide 123/124 (42-bp; Section 2.21) was inserted into the KpnI site thereby increasing the insert size from 15-bp to 57-bp; Ap&lt;sup&gt;+&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pT18ParF*151&lt;sup&gt;99&lt;/sup&gt;</td>
<td>pT18ParF*151&lt;sup&gt;99&lt;/sup&gt; derivative in which two copies of double-stranded oligonucleotide 123/124 (42-bp; Section 2.21) were inserted into the KpnI site thereby the size of insertion increased from 15-bp to 97-bp; Ap&lt;sup&gt;+&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pT18ParF*151GVPLK</td>
<td>pT18ParF* derivative in which 15-bp were inserted 3’ of codon 151 in <em>parF</em> by PSM; Ap&lt;sup&gt;+&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pT18ParF*160GVPLK</td>
<td>pT18ParF* derivative in which 15-bp were inserted 3’ of codon 160 in <em>parF</em> by PSM; Ap&lt;sup&gt;+&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pT18ParF*164GVPRT</td>
<td>pT18ParF* derivative in which 15-bp were inserted 3’ of codon 164 in <em>parF</em> by PSM; Ap&lt;sup&gt;+&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pT18ParF*165RGTPA</td>
<td>pT18ParF* derivative in which 15-bp were inserted 3’ of codon 165 in <em>parF</em> by PSM; Ap&lt;sup&gt;+&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pT18ParF*166RGTP</td>
<td>pT18ParF* derivative in which 15-bp were inserted 3’ of codon 166 in <em>parF</em> by PSM; Ap&lt;sup&gt;+&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pT18ParF*205GVPLF</td>
<td>pT18ParF* derivative in which 15-bp were inserted 3’ of codon 205 in <em>parF</em> by PSM; Ap&lt;sup&gt;+&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pT18ParF*67GVPLY</td>
<td>pT18ParF* derivative in which 15-bp were inserted 3’ of codon 67 in <em>parF</em> by PSM; Ap&lt;sup&gt;+&lt;/sup&gt; (this study)</td>
</tr>
<tr>
<td>pT18ParF*E152A</td>
<td>pT18ParF* derivative in which codon 152 in <em>parF</em> was altered by OE-PCR to encode Ala instead of Glu; PCR product was digested with SexAI and ClaI and inserted between the equivalent sites in pT18ParF*; Ap&lt;sup&gt;+&lt;/sup&gt; (this study)</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT18ParF*K151A</td>
<td>pT18ParF* derivative in which codon 151 in parF was altered by OE-PCR to encode Ala instead of Lys; PCR product was digested with SexAI and ClaI and inserted between the equivalent sites in pT18ParF*; Ap(^r) (this study)</td>
</tr>
<tr>
<td>pT18ParF185RGTPS</td>
<td>pT18 derivative in which parF185RGTPS gene was PCR amplified from pFH547ParF185RGTPS and inserted between XhoI and HindIII restriction sites; Ap(^r) (this study)</td>
</tr>
<tr>
<td>pT18ParF71GVPRK</td>
<td>pT18 derivative in which parF71GVPRK gene was PCR amplified from pFH547ParF71GVPRK and inserted between XhoI and HindIII restriction sites; Ap(^r) (this study)</td>
</tr>
<tr>
<td>pT18ParFΔ151</td>
<td>parFΔ151 gene cloned into pT18ParF by digestion of pFH547Δ151 with SexAI and ClaI and insertion of a 229-bp gel extracted fragment between the equivalent sites in pT18ParF; Ap(^r) (this study)</td>
</tr>
<tr>
<td>pT18ParFΔ204-206</td>
<td>pT18 derivative in which the parFΔ204-206 gene was PCR amplified from pFH547Δ204-206 and inserted between XhoI and HindIII restriction sites of pT18 vector; Ap(^r) (this study)</td>
</tr>
<tr>
<td>pT25ParF</td>
<td>parF cloned into pT25 vector; Cm(^r) (Barillà and Hayes, 2003)</td>
</tr>
<tr>
<td>pT25ParG</td>
<td>parG cloned into pT25 vector; Cm(^r) (Barillà and Hayes, 2003)</td>
</tr>
</tbody>
</table>

\(^a\) Ap\(^r\), ampicillin-resistance; Cm\(^r\), chloramphenicol-resistance; Km\(^r\), kanamycin-resistance.

#### 2.5 Plasmid DNA extraction

Plasmid DNA was prepared from *E. coli* DH5α cultures grown for ~16 hours in LB broth with aeration at 37°C. Plasmid DNA was extracted using an ISOLATE II Plasmid Mini Kit as follows. The stationary phase culture was harvested by centrifugation of 1.5 ml at 13,000 r.p.m. for five minutes using an Eppendorf Centrifuge 5417R. The cell pellet was resuspended in 250 µl Resuspension Buffer by thorough vortexing. 250 µl Lysis Buffer was added and the sample was inverted four to six times carefully in order to lyse the cells followed by incubation for two to three minutes at room temperature. 350 µl Neutralization Buffer was added, the microtube was inverted gently five to six times followed by 10 minutes centrifugation at 13,000 r.p.m. for precipitation of cellular debris. The supernatant (~800 µl) was applied to a silica membrane spin column which was centrifuged at 13,000
r.p.m. for one minute. The filtrate was discarded from the collection tube into which the spin column was reinserted. The membrane was washed twice by adding 500 µl and 700 µl from Wash Buffers AP and BP, respectively. The column was centrifuged at 13,000 r.p.m. for one minute to remove any buffer. The centrifugation step was repeated once more to remove all traces of ethanol. The collection tube was discarded and the column was placed into a sterile 1.5 ml microcentrifuge tube. 50 to 70 µl of sterile dH₂O were added with incubation at room temperature for one minute. Then, a final centrifugation was performed for one minute at 13,000 r.p.m. in order to elute the DNA which was stored at -20°C.

2.6 Restriction enzyme digestion of DNA and agarose gel electrophoresis
Restriction digests of plasmid DNA and PCR products were performed in final volumes of 30-50 µl. 10 to 50 µl (~100 ng/µl) DNA was mixed in the presence of appropriate 10X Restriction Buffer with 5 to 10 units of restriction enzyme(s). The reaction was incubated for one hour at 37°C followed by agarose gel analysis after addition of an appropriate volume of loading dye. Sample DNA was electrophoresed on 1.8% (w/v) agarose gels which were prepared in 1X TAE buffer (40 mM Tris-Acetate, pH 8.0, 1 mM EDTA) using a mould and appropriate comb to hold the gel and to create desired wells for the sample. An electrophoresis tank was filled with 1X-TAE buffer, the samples were loaded in the wells and electrophoresed at 11 volts/cm for the required time (40 minutes to two hours) depending on the sizes of the fragments being separated. Gels were stained with ethidium bromide (0.5 µg/ml) for 15 minutes. The gels were destained in dH₂O and then visualized by UV light (264-366 nm). Gel images were printed or saved using a gel documentation system. Depending on the purpose of the restriction digestion, excision of appropriate bands was performed when required using a scalpel. Gel fragments were collected and placed in a microcentrifuge tube. PCR and Gel Purification Kit was use to recover DNA from gel slices (Section 2.7).

2.7 DNA extraction from agarose gels
Digested DNA fragments (plasmid vectors and PCR fragments) were separated on a 0.8-1.2% (w/v) agarose gel using 1X TAE buffer (40 mM Tris-Acetate, pH 8.0, 1 mM EDTA). The desire fragment was excised on a UV transilluminator and placed in a 1.5 ml tube. The DNA was eluted from the gel slice using a PCR and Gel Purification Kit as follows. Gel Solubilizer (600 µl) was added to the gel slice (~0.3 gm) and incubated at 60°C for 10-15 minutes for agarose gel solubilisation. 200 µl of DNA Binding Optimizer Buffer A was added and vortexed vigorously. The sample was transferred to a binding column and
centrifuged at 13,000 r.p.m. for 30 seconds immediately. The column was washed with Washing Buffer 2 to which ethanol (80%; v/v) was added and centrifuged at 13,000 r.p.m. for one minute to remove impurities in the sample. The sample was centrifuged again and the column transferred to a fresh 1.5 ml microcentrifuge tube. The DNA was eluted from the membrane by adding 30 µl dH₂O to the column with one minute incubation at room temperature. The tube was centrifuged at 13,000 r.p.m. for two minutes. The DNA sample was stored for further use at -20°C.

2.8 Alkaline phosphatase treatment of DNA
The phosphate groups of digested plasmid DNA were removed from 5’ termini to prevent self-ligation of vector plasmid DNA. 50 µl of digested vector were treated with 10 U (1 µl) of CIAP. The reaction was incubated at 37°C for 20 minutes, and then the enzyme was inactivated by adding 0.1 volume of 200 mM ethylene glycol tetraacetic acid (EGTA) pH 8.0 with 10 minutes incubation at 65°C. DNA was recovered with a PCR and Gel Purification Kit (Section 2.9).

2.9 Plasmid vector and PCR product purification from enzymatic reactions
Digested plasmid vector, amplified PCR products and digested PCR fragments were purified from enzymatic reactions using a PCR and Gel Purification Kit. 500 µl Binding Buffer A (5 M guanidine hydrochloride, 30% (v/v) isopropanol) were added to a spin column with an attached collection tube. The treated DNA (~50 µl) was added to the buffer and the solution was mixed gently by pipetting. The column was centrifuged at 13,000 r.p.m. for two minutes. The flow-through was discarded and 500 µl of Wash Buffer 2 containing 80% (v/v) ethanol were used for washing the column, followed by centrifuged at 13,000 r.p.m. for one minute and the washing step was repeated. The collection tube was discarded and the spin column was placed in a sterile 1.5 ml microcentrifuge tube. The DNA was eluted from the column by adding 30-70 µl of dH₂O directly to the spin column membrane followed by incubation at room temperature for one minute. Finally, the sample was centrifuged at 13,000 r.p.m. for two minutes. The eluted DNA was quantified using a NanoDrop spectrophotometer (Thermo-Scientific) and the samples retained for further use.

2.10 Ligation reactions
Ligation reactions were set up using 3 µl of 5X ligation buffer (250 mM Tris (pH 7.5), 50 mM MgCl₂, 50 mM ATP), 1 µl (1-5 units) T4 DNA ligase and appropriate volumes of digested insert and vector in a 1:2 ratio. Vectors were either digested dephosphorylated
plasmids or digested plasmids recovered from agarose gels. Annealed and phosphorylated double stranded oligonucleotides, purified PCR products or purified restriction fragment were used as inserts in the reactions. The final volume was adjusted to 20 μl with dH₂O. Controls were prepared by setting up the same reactions without insert DNA to determine the effectiveness of dephosphorylation and ligation reactions. All reactions were incubated overnight at 16°C. The entire volume of the reactions was transformed into E. coli (Section 2.17).

2.11 Polymerase chain reaction (PCR)
Mutagenesis of the parF gene in vitro (substitutions, deletions and insertions) and amplification of mutated parF fragments for cloning were made by PCR. PCR mixtures contained Phusion High Fidelity DNA polymerase (1 unit), designed primers which were purchased from Sigma-Aldrich (Table 2.2), template DNA and 100 mM deoxynucleotide triphosphates (dNTPs) (Table 2.3). Primers were resuspended in dH₂O to a concentration of 100 μM. After mixing of all components, the reactions were subjected to thermal cycling (Table 2.4) using an Eppendorf Master Cycler instrument with the instrument lid set to 102°C. An aliquot (3-7 μl) of every completed reaction was checked on an agarose gel.

Table 2.2: Oligonucleotides used in PCR reactions

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5’-3’</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT18XhoI-Forward</td>
<td>TCTCTCCTCGAGTATGAAAGT GATCTCATTT</td>
<td>Used with pT18HindIII'-Reverse for amplifying genes encoding ParF71GVPRK, ParF185RGTPS and ParFΔ204-206 for cloning into pT18; restriction site (XhoI) is underlined.</td>
</tr>
<tr>
<td>pT18HindIII'-Reverse</td>
<td>TCTCTCAAGCTTTCCCTCAAATTCTAAC</td>
<td>Used with pT18XhoI-Forward for amplifying genes encoding ParF71GVPRK and</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT18HindIII²-Reverse</td>
<td>TCTCTCAAGCTTTCTCTAACT ATCTCTTT</td>
<td>Used with pT18XhoI-Forward for amplifying gene encoding ParFΔ204-206 for cloning into pT18; restriction site (HindIII) is underlined. This primer differs from pT18HindIII¹-Reverse in lacking the last three codons of ParF.</td>
</tr>
<tr>
<td>ParF-P1-Forward</td>
<td>GGTTCACTCTCAGTAATCACA</td>
<td>External non-mutagenic primer for substitution of codon 151 and deletion of 151 and 152 codons in parF.</td>
</tr>
<tr>
<td>ParFP2-151-Reverse</td>
<td>TTTGATACTTTCTGCAAGCAC</td>
<td>Internal mutagenic primers for K151A substitution (underlined) in parF.</td>
</tr>
<tr>
<td>ParFP3-151-Forward</td>
<td>GTGCTTGCAGAAAGTATCAA</td>
<td></td>
</tr>
<tr>
<td>ParFP4-Reverse</td>
<td>AAACACGCTGTCGCCATCAAG</td>
<td></td>
</tr>
<tr>
<td>ParF-P2-152-Reverse</td>
<td>TTTGATACTTCTTTAAAGCAC</td>
<td>Internal mutagenic primers for E152A substitution (underlined) in parF.</td>
</tr>
<tr>
<td>ParF-P3-152-Forward</td>
<td>GTGCTTAAGCAAGTATCAA</td>
<td></td>
</tr>
<tr>
<td>ParF-P2-Δ151-</td>
<td>GATACTTTCAAGCACATTTCA</td>
<td>Internal mutagenic</td>
</tr>
<tr>
<td>Reverse</td>
<td>Forward</td>
<td>Primers for Δ151 mutation in parF.</td>
</tr>
<tr>
<td>-------------</td>
<td>----------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>ParF-P3-Δ151-Forward</td>
<td>TTGAATGTGCTTGAAAGTATC</td>
<td></td>
</tr>
<tr>
<td>ParF-P2-Δ152-Reverse</td>
<td>GATACTTTTAAGCACATTTCAA</td>
<td>Internal mutagenic primers for Δ152 mutation in parF.</td>
</tr>
<tr>
<td>ParF-P3-Δ152-Forward</td>
<td>TTGAATGTGCTTTAAAAGTATC</td>
<td></td>
</tr>
<tr>
<td>ParF-A&lt;sub&gt;p&lt;/sub&gt;-Forward</td>
<td>GCTATTACACAACGTCAGGTT</td>
<td>External non mutagenic primer used with ParF-B&lt;sub&gt;p&lt;/sub&gt;-Reverse, ParF-B&lt;sub&gt;p&lt;/sub&gt;&lt;sup&gt;1&lt;/sup&gt;-Reverse, ParF-B&lt;sub&gt;p&lt;/sub&gt;&lt;sup&gt;2&lt;/sup&gt;-Reverse and ParF-B&lt;sub&gt;p&lt;/sub&gt;&lt;sup&gt;3&lt;/sup&gt;-Reverse for amplifying PCR1/PCR3 products in cloning of gene encoding ParF&lt;sub&gt;Δ&lt;/sub&gt;206 into pFH547, PCR1/PCR3 products in cloning of gene encoding ParF&lt;sub&gt;Δ&lt;/sub&gt;204/205 into pFH547 and PCR1/PCR3 products in cloning of gene encoding ParF&lt;sub&gt;Δ&lt;/sub&gt;204-206 into pFH547, respectively.</td>
</tr>
<tr>
<td>ParF-B&lt;sub&gt;p&lt;/sub&gt;-Reverse</td>
<td>GGGGTTACCCTATTTGAGTAAT</td>
<td>Internal mutagenic primers used in cloning of ParF&lt;sub&gt;Δ&lt;/sub&gt;204-206 into</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ParF-D&lt;sub&gt;p&lt;/sub&gt;-Reverse</td>
<td>TACACATTTACTCA</td>
<td>pT18. 15-bp insertion is in bold.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>External non-mutagenic primer used with ParF-C&lt;sub&gt;p&lt;/sub&gt;-Forward, ParF-C&lt;sub&gt;p&lt;/sub&gt;&lt;sup&gt;1&lt;/sup&gt;-Forward, ParF-C&lt;sub&gt;p&lt;/sub&gt;&lt;sup&gt;2&lt;/sup&gt;-Forward, ParF-C&lt;sub&gt;p&lt;/sub&gt;&lt;sup&gt;3&lt;/sup&gt;-Forward for amplifying PCR2/PCR3 products in cloning of gene encoding ParF&lt;sub&gt;205GVPLF&lt;/sub&gt; into pT18, PCR2/PCR3 products in cloning of gene encoding ParF&lt;sub&gt;Δ206&lt;/sub&gt; into pFH547, PCR2/PCR3 products in cloning of gene encoding ParF&lt;sub&gt;Δ204-205&lt;/sub&gt; into pFH547 and PCR2/PCR3 products in cloning of gene encoding ParF&lt;sub&gt;Δ204-206&lt;/sub&gt; into pFH547, respectively.</td>
</tr>
<tr>
<td>ParF-B&lt;sub&gt;p&lt;/sub&gt;&lt;sup&gt;1&lt;/sup&gt;-Reverse</td>
<td>GTGTAATTTAATTTTCTAC</td>
<td>Internal mutagenic primer used in deletion of codon 206 in parF.</td>
</tr>
<tr>
<td>ParF-C&lt;sub&gt;p&lt;/sub&gt;&lt;sup&gt;1&lt;/sup&gt;-Forward</td>
<td>GTTAGAATTTTTTAATTACAC</td>
<td>Internal mutagenic primers used in deletion of codons 205/206 in parF.</td>
</tr>
<tr>
<td>ParF- B&lt;sub&gt;p&lt;/sub&gt;&lt;sup&gt;2&lt;/sup&gt;-Reverse</td>
<td>GTGTAATTATTCTAACTAT</td>
<td></td>
</tr>
<tr>
<td>ParF-C&lt;sub&gt;p&lt;/sub&gt;&lt;sup&gt;2&lt;/sup&gt;-Forward</td>
<td>ATAGTTGAAATTATAATTACAC</td>
<td></td>
</tr>
<tr>
<td>ParF-B&lt;sub&gt;p&lt;/sub&gt;&lt;sup&gt;3&lt;/sup&gt;-Reverse</td>
<td>GTGTAATTTACTATCTACTTC</td>
<td></td>
</tr>
<tr>
<td>Component</td>
<td>Volume (µl)</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>DNA template (~20 ng/µl)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Forward Primer (5 pmol/µl)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Reverse Primer (5 pmol/µl)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>dNTPs (5 mM each dATP, dCTP, dGTP, dTTP)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>10X reaction buffer</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>dH₂O</td>
<td>added to 60 µl</td>
<td></td>
</tr>
<tr>
<td>Phusion High Fidelity DNA polymerase (1U/µl)</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.4: Thermal cycle programme of PCR reaction**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (ºC)</th>
<th>Time (minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial denaturation</td>
<td>93</td>
<td>2</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>98</td>
<td>1</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>4. Elongation [(Kb)x2]</td>
<td>72</td>
<td>2</td>
</tr>
<tr>
<td>5. Going to step 2 and repeating for 29 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Final elongation</td>
<td>72</td>
<td>6</td>
</tr>
<tr>
<td>7. Holding and cooling</td>
<td>4</td>
<td>Overnight</td>
</tr>
</tbody>
</table>
2.12 Phosphorylation reaction

Commercial sources supply oligonucleotides in unphosphorylated form. For providing 5’ phosphate groups, phosphorylation was performed using 2 µl of DNA, 1 µl from 100mM ATP, 4 µl 10 X phosphorylation buffer and 1 µl T4 polynucleotide kinase then dH₂O was added to 40 µl as a total volume of reaction sample. The 10 X phosphorylation buffer consists of 0.5 M Tris-HCl (pH 7.6), 0.1 M MgCl₂, 50 mM DTT, 1 mM Na₂EDTA and 50% (v/v) glycerol. Reactions were incubated at 37°C for 45 minutes, and then an extra 1 µl of T4 polynucleotide kinase was added and incubated at 37°C for a further 45 minutes.

2.13 Alcohol precipitation of DNA

The phosphorylated oligonucleotide (Section 2.12) was precipitated with 0.1 volume of 3 M sodium acetate, pH 5.3, and two volumes of ethanol and incubated at -20°C for approx. two hours. The oligonucleotide was harvested at 13,000 r.p.m. for 10 minutes in an Eppendorf MiniSpin centrifuge. The supernatant was discarded and the DNA pellet was dried and resuspended in 50 µl dH₂O.

2.14 Site-directed mutagenesis by Overlap Extension-Polymerase Chain Reaction (OE-PCR)

Residues in α6 and the C-terminus of ParF were subjected to site-directed mutagenesis. Deletion and substitution of individual amino acids were performed by OE-PCR (Figure 2.1) (Ho et al., 1989). In addition, construction of the mutant gene encoding ParF205GVPLF in the partition vector pFH547 was carried out by the same technique (Lee et al., 2010). OE-PCR involves designing four primers and performing two rounds of PCR amplification which generate DNA fragments (PCR1 and PCR2) harbouring overlapping ends (Figure 2.1). In the subsequent amplification (PCR3) the overlapping ends anneal and further amplification of the fusion product occurs. For point mutations, two non-mutagenic external forward (Aₚ) and reverse (Dₚ) primers with upstream and downstream sequences that flank restriction sites on the parF gene were designed. Two internal mutagenic forward (Cₚ) and reverse (Bₚ) primers were designed containing the required mutation of a specific codon in parF (Figure 2.1). In case of insertion mutagenesis the mutagenic primers contained the additional in-frame mutation. The primers for all mutations made by OE-PCR are listed in Table 2.2. PCR1 and PCR2 reactions were performed as described in Table 2.3. The components of PCR1 reaction were the same of as PCR2 except that primers Aₚ/Bₚ were used in the former and primers Cₚ and Dₚ were used in the latter. Both PCR1 and
PCR2 reactions were purified as outlined in Section 2.9 and the sizes of the products were verified on 1.8% (w/v) agarose gels using 1X TAE buffer (40 mM Tris-Acetate, pH 8.0, 1 mM EDTA). The final round of PCR amplification (PCR3) was set up using purified PCR1 and PCR2 products with addition of 5 µl (5 pmol/µl) A_p and D_p primers (Table 2.5). A standard thermal cycling reaction was set up for all the cycles (PCR1, PCR2 and PCR3) as per Table 2.4.

The final products of PCR3 were purified (Section 2.9) and approximately 7 µl were analysed on a 1.8% (w/v) agarose gel using 1X TAE buffer (40 mM Tris-Acetate, pH 8.0, 1 mM EDTA). The correct size of the amplified DNA fragments were confirmed and appropriate restriction digestions were performed (Section 2.6). The PCR products were recovered from the gel and purified (Section 2.8). The digested and purified PCR3 products were cloned into the purified digested plasmid vector.

### Table 2.5: PCR3 reaction components

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR1 (~20 ng/µl)</td>
<td>1</td>
</tr>
<tr>
<td>PCR2 (~20 ng/µl)</td>
<td>1</td>
</tr>
<tr>
<td>Primer A_p Forward (external non-mutagenic) (5 pmol/µl)</td>
<td>5</td>
</tr>
<tr>
<td>Primer D_p Reverse (external non-mutagenic) (5 pmol/µl)</td>
<td>5</td>
</tr>
<tr>
<td>dNTPs (5 mM each dATP, dCTP, dGTP, dTTP)</td>
<td>10</td>
</tr>
<tr>
<td>10X reaction buffer</td>
<td>6</td>
</tr>
<tr>
<td>dH_2O</td>
<td>added to 60 µl</td>
</tr>
<tr>
<td>Phusion DNA polymerase (1U/µl)</td>
<td>1</td>
</tr>
</tbody>
</table>

### Figure 2.1 (overleaf). Schematic diagram of OE-PCR. Three rounds of PCR amplification are shown. Four primers (two external non-mutagenic and two internal mutagenic) participate in this process. 1. Point mutation and deletion of specific amino acids in the *parF* sequence are produced by using primer B_p and C_p which have complementary single base pair mutations or deletions of one
Two overlap products are generated as a result of PCR1 (using primers $A_p$ and $B_p$) and PCR2 (using primers $C_p$ and $D_p$), whereas in the third round of amplification (PCR3), products from PCR1 and PCR2 anneal and are amplified using $A_p$ and $D_p$ primers. Insertional mutation by OE-PCR for construction of a $parF$ gene encoding ParF205GVPLF in the pFH547 partition vector. The main difference from panel 1 is insertion of the specified 15-bp sequence in primers $C_p$ and $B_p$ which are complementary to each other in their 5’ ends.
2.15 Cloning of PSM mutants in the pT18 vector
Genes encoding ParF71GVPRK and ParF185RGTPS were generated directly in pFH547 instead of in pT18ParF*. In order to study the effect of these insertions in parF in the two-hybrid system, the mutated genes were subcloned into the pT18 vector using XhoI and HindIII restriction sites. For this purpose two primers (pT18XhoI-Forward and pT18HindIII1-Reverse) were designed (Table 2.2) for amplification of the mutated parF. The same forward primer (pT18XhoI-Forward) and a different reverse primer (pT18HindIII2-Reverse) were used similarly to amplify the gene encoding ParFΔ204-206 for cloning into pT18 to test the effect of this mutation in two-hybrid assays.

2.16 DNA sequencing and primers
Samples were sent for sequencing to GATC-Biotech (Germany). Primers used for the sequencing of parF genes cloned in pT18 and pFH450-based plasmids were 5’-GATAACAATTTCACACAG-3’ for pT18; and 5’-CCCAAATGCACCAGAAAGTA-3’ and 5’-AATCCGAAAGGGGGGTTCAGGT-3’ for pFH450. Sequences were downloaded and edited with SnapGene software. ABI sequencing chromatograms were visualised using Finch TV software.

2.17 Recombinant plasmid analysis
Colonies from transformation plates were inoculated in 5 ml LB broth, and incubated overnight growth at 37°C with appropriate antibiotics. Plasmid DNA was extracted and digested with appropriate restriction enzymes as described in Sections 2.5 and 2.6. The correct size of the digested DNA was verified by agarose gel electrophoresis and plasmids containing the insert of the right size were sent for DNA sequencing (Section 2.16).

2.18 Competent cell preparation and transformation of E. coli
E. coli DH5α competent cells were prepared as follows. A loop of bacterial culture from a -80°C stock was streaked on LB agar supplemented with appropriate antibiotic. Single colonies were inoculated in 5 ml LB broth with overnight incubation at 37°C. One ml sample from this overnight culture was inoculated in 100 ml LB broth and incubated at 37°C in a shaker incubator for ~2 h in order to obtain cultures in mid-log phase (OD600 ~0.4). Cells were harvested in 50 ml volumes at 10,000 r.p.m. for 10 minutes at 4°C. The supernatant was discarded, the pellet was resuspended in 5 ml ice cold 50 mM CaCl2, and incubated on ice for 30 minutes. Cells were again harvested and resuspended in 5 ml ice
cold CaCl2. Finally, 2.5 ml of 50% (v/v) ice cold glycerol were added to the cell suspension to provide a final glycerol concentration of ~15% (w/v). Competent cells were frozen at -80°C in 500 µl aliquots. Competent cells were thawed on ice and 100 µl of cells were mixed with 20 µl of plasmid DNA. The mixture was incubated on ice for 30 minutes, and a heat-shock at 42°C for 90 seconds precisely was applied. Cells were returned to ice and 900 µl of LB broth were added to each sample followed by incubation for one hour at 37°C in a shaker incubator. 100-200 µl of the cells from the transformation mixture were spread on LB agar plates with appropriate antibiotic(s) and plates were incubated overnight at 37°C. A control for each transformation also was prepared without DNA plasmid.

### 2.19 Bacterial Two-Hybrid Assay

The bacterial two-hybrid system is a simple qualitative assay developed by Karimova et al. 1998 that depends on specific protein-protein interactions in *E. coli*. The T18 and T25 fragments constitute the catalytic domain of adenylate cyclase (CyaA) in *Bordetella pertussis*. When T18 and T25 are produced from separate plasmids, CyaA activity is not reconstituted in *E. coli* that possesses a mutated cya gene, e.g., strain SP850. However, when the genes for the two fragments are fused to genes whose products interact, the T18 and T25 polypeptides are brought in close proximity, the CyaA enzyme is reconstituted, and cAMP production is restored in *E. coli* SP850. Cyclic AMP is a transcriptional activator of several catabolic operons including the maltose operon used here (Figure 2.2) (Karimova et al., 1998). The sequence encoding ParF which is under investigation in this study previously was fused with the genes specifying both the T18 and T25 fragments (Figure 2.3). When the ParF fusion proteins self-associate in this system, the CyaA fragments are brought sufficiently close together that cAMP production is regenerated in an *E. coli* cya mutant (Barilla and Hayes, 2003). This can be observed as activation of the maltose operon which can be detected as red colony colour of SP850 on MacConkey-maltose plates compared to white colony colour when cAMP production is not restored.

### 2.20 pT18 vector sequencing

The pT18ParF* and pFH547 plasmids were used for PSM of ParF (Section 2.21). The sequence of the latter was known and DNA manipulation was facile. In contrast, the sequence of the bacterial two-hybrid vector pT18 was not available in database and we initially depended on restriction endonuclease analysis to determine the sizes of the fragments for the PSM target plasmid pT18ParF* (Karimova et al., 1998).
Figure 2.2. Functional complementation of CyaA fragments in an E. coli two-hybrid assay. A. The T18 and T25 fragments of Bordetella pertussis CyaA are represented by red and purple shapes, respectively. The intact CyaA protein produces cAMP from ATP. cAMP binds to the catabolite activator protein (CAP) which binds to DNA regulatory sites and activates the transcription of reporter genes, e.g., lacZ, mal and ara. B. When the T18 and T25 fragments are expressed as independent polypeptides they do not interact and no cAMP synthesis occurs. C. The association of interacting proteins, X and Y, which are fused to the T18 and T25 fragments brings the latter into close proximity and reconstitutes CyaA enzymatic activity (Karimova et al., 2000).
Figure 2.3. Schematic representation of cloning vectors pT25 and pT18 were used in two-hybrid analysis. The open reading frames of T25 and T18 fragments are represented by green arrows as well as chloramphenicol (cat) and ampicillin (bla) resistance genes (brown arrows). The multi-cloning sequences (MCS) are shown with the corresponding open reading frames. The MCS in both plasmids allows insertion of foreign genes (Karimova et al., 2000).

For this purpose the primer 5’-GATAACAATTTCACAG-3’ which anneals upstream of the multiple cloning site in pT18 was used to initiate sequence determination of pT18. Based on the result with this primer the rest of pT18 was sequenced by primer walking which involved designing four additional primers (5’-CCCGGCAAGTCTTGCACGCCG-3’, 5’-GGCATTTTGCCTTCCTGTTTT-5’, 5’-TCTAGGTGAAGATCCTTTTG-3’, and 5’-GTATGTTGTGTGGAATTGTGA-3’) that anneal at approximately 600-800 bp intervals
on the plasmid. Therefore, the entire sequence of pT18 was determined here to facilitate the sub-cloning of \textit{parF} mutants by assembling the overlapping reads from these reactions. SnapGene® software was used to display the nucleotide sequence and the circular plasmid (Appendix A and B).

### 2.21 Pentapeptide scanning mutagenesis

Pentapeptide scanning mutagenesis (PSM) is a transposon-based approach in which 15-bp are introduced randomly into a target gene thereby producing random five amino acid insertions in a target protein (Figure 2.4) (Hallet \textit{et al.}, 1997). The mechanism involves insertion and liberation of the transposon Tn4430 (~4-kb) \textit{in vivo} and \textit{in vitro}, respectively. Tn4430 from \textit{Bacillus thuringiensis} can transpose efficiently in \textit{E. coli} DH5α. This transposon contains \textit{KpnI} sites 5-bp from the ends of its terminal inverted repeats. The transposon duplicates 5-bp of target site DNA during transposition (Mahillon and Lereclus \textit{et al.}, 1988; Hallet \textit{et al.}, 1997). Thus, removal of the bulk of Tn4430 by \textit{KpnI} digestions and religation leaves a 15-bp fingerprint in the target gene which produces a pentapeptide insertion in the target protein (Figure 2.4 A). Plasmid pOX38:Tn4430 is a conjugative, kanamycin-resistant F derivative carrying the transposon but devoid of any other mobile genetic elements (Hayes, 2000).

Two different target plasmids (pT18ParF and pFH547) were chosen for PSM. Plasmid pT18 (Table 2.1) used in two-hybrid assays (Section 2.19) expresses the T18 fragment of \textit{Bordetella pertussis} adenylate cyclase (Karimova \textit{et al.}, 1998). A two-hybrid plasmid was used in order to test the ParF mutants generated by PSM for self-association and interaction with the partner protein ParG. Plasmid pT18ParF comprises the \textit{parF} gene cloned between the \textit{KpnI} and \textit{HindIII} sites of pT18 (Barillà and Hayes, 2003). As the target plasmid for PSM must lack \textit{KpnI} sites, pT18ParF* was designed (Section 2.22). Plasmid pFH547 (Table 2.1) contains the \textit{parFGH} partition cassette of the TP228 plasmid (Hayes, 2000) and was used as a second target plasmid in PSM to allow testing of the effects on plasmid partition of pentapeptide insertion in ParF (Section 2.25).

Target plasmids (pT18ParF* and pFH547) were transformed into \textit{E. coli} FH395 (pOX38:Tn4430) with selection for kanamycin and ampicillin or kanamycin and chloramphenicol to guarantee the presence of both plasmids (Figure 2.2 A) throughout different trials. Following overnight incubation, colonies were collected \textit{en masse} from
multiple transformation plates and retained as a donor cell suspension for conjugation experiments. Transposition of Tn4430 involves formation of a cointegrate with the target molecule (Figure 2.4 B). Thus, Tn4430 insertions in the target plasmid can be separated from chromosomal insertions by conjugal transfer of the cointegrate to a plasmid-free recipient. Colonies of the DS941 recipient were resuspended in 500 μl LB broth. Ten-fold dilutions of the donor suspension (100 μl) and resuspended recipient cells (100 μl) were co-plated on agar plates without antibiotic. Plates were incubated for three hours at 37°C to allow conjugal transfer of pOX38:Tn4430::pT18ParF* or pOX38:Tn4430::pFH547 cointegrate plasmids into DS941. The lawn of cells was washed from conjugation plates with LB broth (500 μl) and plated on LB agar supplemented with ampicillin and streptomycin at 37°C overnight. The former selects against DS941 recipient cells that have not acquired the pOX38:Tn4430::pT18ParF* or pOX38:Tn4430::pFH547 cointegrate plasmid, whereas streptomycin eliminates donor cells. Thus, this antibiotic combination selects specifically for DS941 transconjugants into which the pOX38:Tn4430::pT18ParF* or pOX38:Tn4430::pFH547 cointegrate has transferred by conjugation. A pool of many thousands of transconjugants was collected by washing each plate with LB broth (~3 ml).

The cointegrate plasmid resolves rapidly in transconjugants to regenerate pOX38:Tn4430 and the target plasmid bearing a copy of the transposon (Figure 2.4 C). As pOX38:Tn4430 is large and low copy number, plasmid DNA isolated from the pooled cells effectively consists of a library of pT18ParF*::Tn4430 or pFH547::Tn4430 derivatives. To discriminate between Tn4430 insertions in the parF gene and in the target plasmid backbone (Figure 2.4 C), two different strategies were applied. First, candidates from the library of pT18ParF*::Tn4430 transconjugants were screened in a two-hybrid assay as described in Section 2.19. Most Tn4430 insertions in parF were expected to abolish the self-association of ParF that is detectable in this assay. Therefore, Tn4430 insertions in the parF gene in pT18ParF*::Tn4430 plasmids should produce white colonies when tested with pT25ParF on MacConkey-maltose agar plates. In contrast, insertions in the vector backbone will not affect ParF self-association and generate red colonies. Second, a pooled plasmid preparation was made in the case of pFH547::Tn4430. This preparation was digested with restriction enzymes SalI and Hpal that liberate the parF::Tn4430 fragment.
Figure 2.4. Pentapeptide scanning mutagenesis. A. The target plasmid (pT18ParF* or pFH547) (red) is introduced into *E. coli* FH395 (pOX38::Tn4430) (blue). The target plasmid includes the parF gene (yellow). Plasmid pOX38::Tn4430 is a conjugative F plasmid derivative onto which Tn4430 (black) has been inserted *in vivo*. B. Transposition of Tn4430 onto the target plasmid involves formation of a cointegrate intermediate that can be transferred by conjugation to a plasmid-free recipient, DS941. C. The cointegrate resolves rapidly to regenerate pOX38::Tn4430 along with the target plasmid bearing a copy of the transposon which may either be in parF or in the vector backbone. Strategies to discriminate between these possibilities are described in the text. D. Deletion of the bulk of the transposon by digestion of target plasmid::Tn4430 with KpnI and religation produces pT18ParF* and pFH547 derivatives that possess 15-bp insertions in parF. E. The structure
of Tn4430 transposon. Two KpnI sites are underlined (Hayes and Hallet, 2000). Features on the map are not drawn to scale for clarity.

This fragment that consists of parF with Tn4430 insertions at different locations was purified from an agarose gel (1X TAE buffer) and cloned into pFH547 (Table 2.1) that was digested with the same restriction enzymes and recovered from a gel as described in Section 2.7. Thus, the wild-type parF gene in pFH547 was replaced by parF::Tn4430 versions. In either case above the bulk of Tn4430 was deleted subsequently from selected plasmids by KpnI digestion and religation to generate parF derivatives with 15-bp insertions (Figure 2.4 D).

2.22 Construction of the pT18ParF* target plasmid

PSM requires a target plasmid without a KpnI site as deletion of the bulk of Tn4430 involves digestion with KpnI and religation (Figure 2.2 D). The presence of additional KpnI sites on the target plasmid would complicate this process. The pFH547 target plasmid is empty of KpnI sites but the two-hybrid plasmid pT18ParF contains a KpnI site in the vector backbone. A double-stranded oligonucleotide with KpnI overhangs (5’-TGGATCCTGTAC-3’/3’-CATGACCTAGGA-5’) was used in removing this KpnI site.

2.23 Synthetic oligonucleotides insertion mutagenesis

PSM generated a 15-bp insertion at codon 151 of the parF gene. The insertion contains a unique-KpnI site that was used to increase the insertion size at this position. Increasing the insertion size from 15-bp to 57-bp in pT18ParF*151GVPLK was performed using synthetic oligonucleotide. The resulting proteins were tested to examine the effects of these larger insertions on self-association of ParF and interaction with ParG in two-hybrid assays. In addition, the same strategy was applied with pFH547160GVPLK and pFH547185RGTPS to test the partition efficiency of parFGH cassette. An in-frame double stranded synthetic oligonucleotide was used for increasing the insertion size at codon 151 of parF. 123/124 (Hayes et al., 1997) was used to increase the insertion size from 15-bp to 57-bp, thereby generating a ParF derivative with a 19 amino acid insertion. This oligonucleotide contained a set of restriction sites which all generate blunt ends (Figure 2.5). Digestion and religation of appropriate combinations of these enzymes delete the intervening sequence and consequently in-frame insertions shorter than 57-bp are produced.
Figure 2.5. 123/124 oligonucleotide used to increase the insertion size in selected *parF* genes that contain 15-bp fingerprints generated by PSM. Note that *KpnI* sites at the ends of the oligonucleotides have sticky ends (cohesive ends) which consist of unpaired oligonucleotides (overhangs).

2.24 Quantitative assessment of two-hybrid interactions by β-galactosidase assay.

Pairs of pT18 and pT25 plasmids were transformed into competent cells of *E. coli* DH5α. Colonies from these plates were incubated overnight at 30°C in LB broth supplemented with ampicillin and chloramphenicol and with 0.2 mM isopropyl-B-D-galactopyranosidase (IPTG). This compound induces expression of the *P*<sub>lac</sub> promoter that drives the cya fusion genes in pT18 and pT25 (Figure 2.3). The cAMP produced by adenylate cyclase induces the *lac* operon including the gene for β-galactosidase (*lacZ*) (Figure 2.2). Overnight cultures were cooled on ice for 20 minutes. The cultures were diluted 1:5 with sterile M63 broth media. OD<sub>600</sub> values were recorded for all samples. 300 μl from each diluted culture was mixed with 700 μl Z buffer (0.06 M Na<sub>2</sub>HPO<sub>4</sub>, 0.04 M NaH<sub>2</sub>PO<sub>4</sub>, 0.01 M KCl, 0.001 M MgSO<sub>4</sub>, pH 7.0). Immediately before use, 54 μl β-mercaptoethanol (50 μM final concentration) was added to 20 ml Z buffer. CHCl<sub>3</sub> (20 μl) and SDS (20 μl of 0.1% solution) were added to the cultures to permeabilize the cells. Samples were vortexed for one minute and incubated at 28°C for 10 minutes. Then 200 μl of nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/ml; 0.13 mM final concentration) were added as a substrate for the reaction. This was recorded as time zero for the assay. When ONPG is hydrolysed by β-galactosidase into galactose and o-nitrophenol, the solution colour turns to yellow. The reaction was stopped by adding 500 μl of 1 M Na<sub>2</sub>CO<sub>3</sub> after a sufficient yellow colour had developed and the time of colour development was recorded (minutes). OD<sub>420</sub> and OD<sub>550</sub> were measured for each sample. Values of β-galactosidase enzyme were determined in Miller units using the formula:

\[
\text{Miller Units (MU)} = 1000 \times \left(\frac{\text{OD}_{420} - (1.75 \times \text{OD}_{550})}{T \times V \times \text{OD}_{600}}\right)
\]

T is reaction time (minutes), V is volume of culture used (ml).
2.25 Plasmid partition assay

The segregational stability conferred by the wild-type and mutated partition cassette of the TP228 plasmid was tested. The partition assay vector pFH450 contains two replicons, the low copy number replicon of P1 and moderate copy number ColE1 (pMB1) origin (Hayes, 1998). The vector uses the Co1E1 replicon in a wild-type host and therefore can be easily isolated and manipulated, whereas in a polA mutant host the plasmid replicates with the P1 replicon. In a polA host the Co1E1 replicon is non-functional because its replication requires DNA polymerase I. Consequently, pFH450 replication comes under the control of the low copy number P1 replicon. This vector lacks accessory stability genes and in the absence of selective pressure after ~25 generations in BR825 shows <2% retention (Hayes, 2000) and was used as a negative control. In contrast, plasmid pFH547 (see Figure 3.18) harbours the wild-type parFGH cassette of TP228 cloned in pFH450 (Hayes, 2000). This plasmid was a positive control for estimating the partition efficiency associated with the parFGH cassette (>65%). The pFH547 derivatives containing mutations in parF generated by PSM and site-directed mutagenesis (Section 2.14) were transformed into E. coli BR825 polA mutant strain with selection on LB agar for chloramphenicol resistance at 37°C for 16 hours. From each transformation eight randomly chosen colonies were streaked on LB agar supplemented with chloramphenicol to ensure that the plasmids were present (Figure 2.6, streak 1). Then LB agar without chloramphenicol was used for streaking of individual colonies from each of the selective streaks which provides non-selective growth of ~25 generations for 16 hours at 37°C (streak 2). Colonies that grow from this streak potentially harbour a mixture of cells that contain the test plasmid and cells from which the plasmid has been lost.

This step was repeated (streak 3) to separate these cell types into different colonies. Eight colonies from each of these eight streaks were stabbed on LB plates with and without chloramphenicol at 37°C for 16 hours. Plasmid retention was determined by counting the number of colonies on both plates and the plasmid retention percentage was calculated. This assay was repeated at least three times with standard deviations <10%.

2.26 Toxicity assay of ParF mutants

Plasmid pFH554 is a low-copy number, chloramphenicol resistance derivative of pFH547 that harbours the parFGH genes and which replicates solely via the P1 replicon (Hayes, 2000) as described above (Section 2.25). pT18ParF* is a moderate-copy-number plasmid
that produces ParF and replicates via the CoE1 (pMB1) replicon and confers ampicillin resistance. A toxicity assay was applied using these plasmids to test the effect of mutated ParF proteins produced from pT18ParF* derivatives on wild-type ParF by measuring the partition activity of pFH554.

For this assay pT18 (negative control), pT18ParF* (positive control) and PSM derivatives of pT18ParF* were introduced into DH5α pretransformed with pFH554 and selected with ampicillin and chloramphenicol. A similar partition assay procedure (Section 2.24) was applied with the selection of ampicillin only in the second and third streaks to ensure the continued presence of pT18 derivatives (Figure 2.6). 64 colonies were stabbed onto LB agar plates which are selective for both pFH554 and pT18 derivatives (ampicillin and chloramphenicol) or for pT18 only (ampicillin). Retention of pFH554 was determined by counting the number of colonies on selective and non-selective plates. Standard deviations of the mean for the assays were approximately 10% of the mean. Thus, the effects of wild-type and mutant versions of ParF produced from pT18-based plasmids on the retention of pFH554 were assessed.
Figure 2.6. Representation of plasmid partition assay. Approximately 25 generation of non-selective growth were provided. The empty partition vector pFH450, pFH547 that consists of the parFGH cassette cloned in pFH450, and derivatives of pFH547 bearing mutated parF genes were transformed separately into E. coli BR825 strain on LB medium supplemented with chloramphenicol. Eight colonies were streaked on LB medium with selective pressure (streak 1). Streaks 2 and 3 were made on LB plates without antibiotic. From streak 3, 64 colonies were stabbed onto LB plate with and without chloramphenicol. Colonies were counted and the retention frequency of the plasmid was determined.
Chapter 3: Results I
Pentapeptide Scanning Mutagenesis of the ParF Protein
3.1 Background

The ParF protein (22 kDa; 206 residues) is an essential constituent of the partition system of multidrug resistant plasmid TP228 (Hayes, 2000). ParF is recruited to the segrosome by the ParG protein which coats the parH centromere site (Golovanov et al., 2003; Wu et al., 2011). This complex drives the attached plasmids during segregation although the molecular mechanisms involved are uncertain. ParF is thought to play a crucial role in segregation through a cycle of nucleotide-dependent polymerization and depolymerization. As a result of this cycle, a polymer pushing or pulling mechanism may mediate plasmid partitioning (Figure 1.4) (Barillà et al., 2005). The solution of crystal structures of ParF in the ADP-bound and AMPPCP-bound states provided a closer insight into the role of the protein in the plasmid segregation mechanism. ParF forms dimers in the ATP-bound state whereas ParF-ADP is monomer that forms dimer-of-dimers building blocks (Schumacher et al., 2012; Barillà and Hayes 2003). The availability of the ParF structure as this project began is complementary to previous structural studies on the partner protein ParG which has a vital role in modulating polymerization and depolymerization of ParF (Barillà et al., 2005; Machón et al., 2007).

Site-specific mutational analysis of ParF has been limited to date. These studies have targeted residues implicated in ATP binding and hydrolysis, certain residues at the polymerization interface, and a triad of residues that are conserved in ParF and closely-related proteins (Figure 3.1). Mutations in conserved residues of the ATP-binding domain of ParF (G11 and K15) indicated their crucial role in DNA segregation (Barillà et al., 2005). In addition, a triple mutant (ParF-K64A-V89Y-M69A) was abrogated in ATP-mediated polymerization and plasmid stability (Schumacher et al., 2012). Site-directed mutations of residues P104, R169 and G179 in ParF showed a crucial role for these residues in ATP binding and hydrolysis and plasmid segregation (Dobruk-Serkowska et al., 2012).

In this study, a more general technique was applied to create a range of random pentapeptide insertion mutations in ParF. Pentapeptide scanning mutagenesis (PSM) depends on insertion and excision of a transposable element (Hallet et al., 1997). The Tn3-related transposon, Tn4430, from Bacillus thuringiensis is a 4,149-kb element (Lereclus et al., 1986). Tn4430 transposes efficiently in E. coli forming a co-integrate structure between donor and target DNA molecules while duplicating 5-bp of the target sequence at the insertion point (Mahillon and Lereclus, 1988; Hallet et al., 1997).
Figure 3.1. Site-specific mutation analysis of ParF. Site-specific mutations that were constructed in ParF prior to this study are shown in the primary (A) and three-dimensional structures (B) (Moreland et al., 2005). Beta-strands, alpha-helices and loops/turns are shown in pink, cyan and yellow, respectively. Asterisks on K64, V89 and M96 indicate a triplet mutant ParF.
The presence of KpnI restriction sites near the outer ends of the transposon terminal inverted repeats gives a particular advantage to the technique of the insertion and deletion. When Tn4430 inserts into a target gene, a 15-bp in-frame fingerprint remains after KpnI digestion and religation. Therefore a five amino acid insertion is introduced into the target protein (Figure 2.4). This technique has been used previously to dissect a variety of proteins. For example, the DspA/E effector of the Erwinia amylovora pathogen is implicated in invasive disease of the Rosaceae family of plants. Certain pentapeptide insertions impaired the function of DspA/E (Siamer et al., 2013). PSM of PdhS which is a histidine kinase regulatory protein that participates in the cell cycle of Brucella abortus produced insertions which had a severe loss-of-function on the protein (Lambert et al., 2010). The Rfc2 protein of fission yeast is involved in ATP binding and chromosome replication. The protein was subjected to PSM and lethal insertions mapped into arginine finger and P-loop regions. In contrast, nonlethal insertions were located to the outer surface of the protein (Gray et al., 2009). In addition, pentapeptide insertions in the β-lactamase enzyme caused substrate specificity changes and provided increased resistance to a third-generation cephalosporin antibiotic comparing with the wild-type protein (Hayes et al., 1997). Thus, PSM is a facile and rapid mutagenesis strategy which can address the influence of insertions on protein structure-function relationships. The above examples of PSM studies prove the advantages of pentapeptide insertions in protein mapping and altering protein activities.

PSM of ParF was undertaken to probe the structure-function relationships in the protein (Figure 3.2) (Hallet et al., 1997). PSM was performed using plasmids pT18ParF (Barilla and Hayes, 2003) (Figure 3.3) and pFH547 (Hayes, 2000) both of which contain the target gene parF. These plasmids were chosen because they show wild-type levels of ParF activity in bacterial two-hybrid and plasmid partition assays, respectively. As a result characterisation of the effects of pentapeptide insertions on ParF protein function was testable directly with these plasmids without sub-cloning in most cases (Table 3.2).
Figure 3.2. Flow chart of the main events of PSM of ParF. Steps in the conjugation process and the target plasmids used in this study are shown. The illustration describes two different mechanisms that were used for identification of pentapeptide mutations in ParF depending on the target plasmid used in each mechanism (pT18ParF* or pFH547).
Figure 3.3. Map of pT18ParF created by SnapGene software. The parF gene (yellow arrow) was cloned between KpnI and HindIII and fused to cyaA (green arrow) (Barillà and Hayes 2003). Three EcoRI sites are shown: in parF, in the multiple cloning site of the pT18 vector and in the vector backbone. Two origins of replication are shown as blue arrows; the brown arrow represents bla (Amp') gene. The lac promoter and operator with CAP binding sites are shown in pink arrow and pink fragments, respectively. Unique restrictions sites (SexAI and Clal) in parF that were used in sub-cloning of certain PSM mutants are marked.

3.2 Construction of pT18ParF* lacking a KpnI site

The first strategy for the identification of Tn4430 insertions in the parF gene utilized a two-hybrid vector plasmid into which parF was cloned (pT18ParF) as described further in Section 3.3. One technical limitation with the PSM procedure is that the target plasmid must entirely lack KpnI sites as this restriction enzyme is used for the step in which the bulk of Tn4430 is deleted (Figures 2.4-D and 3.2). Plasmid pT18ParF harbour a single KpnI site in the vector multiple cloning site (Figure 3.3). Therefore, the first step in applying the PSM procedure to pT18ParF was elimination of this single KpnI site. Site directed mutagenesis was applied for removing the KpnI sequence in pT18ParF. First, pT18ParF was cut with the enzyme and the digested plasmid was verified by gel electrophoresis which showed that the plasmid was linearized as expected as a result of KpnI digestion (Figure 3.4).
To eliminate the KpnI site in pT18ParF, the KpnI digested plasmid was ligated to a 12-bp synthetic double-stranded oligonucleotide 5’-TGGATCCTGTAC-3’/3’ CATGACCTAGGA- 5’ (Section 2.3) and transformed into E. coli DH5α. This linker possessed KpnI compatible sticky ends but the KpnI site was not regenerated following ligation. In addition, the oligonucleotide contained an internal BamHI site for verification of recombinant plasmids. Fourteen colonies from this ligation were grown in LB broth overnight with antibiotic selection. Plasmids from these cultures was extracted and digested with KpnI. The result showed that four of the plasmids were no longer digested by KpnI suggesting that the oligonucleotide had inserted. Six of these 14 digests are presented in Figure 3.5.
As a confirmation that the oligonucleotide was inserted in plasmids that were no longer susceptible to KpnI digestion, selected plasmids were tested with BamHI. The pT18ParF plasmid possesses a single BamHI site (Figure 3.3) and the linker introduces a second site.

Recombinant plasmids that lacked the KpnI site had gained an extra BamHI site as expected (data not shown). Sequence analysis of plasmids in which the KpnI site was abolished and which contained an extra BamHI site confirmed the insertion of the 12-bp oligonucleotide (Figure 3.6). One of the sequenced plasmids was selected for PSM mutagenesis and named pT18ParF*.

![Figure 3.6. Insertion of a 12-bp sequence in the KpnI site of pT18ParF. 1, ABI sequence traces showing the KpnI site in the pT18ParF vector. 2, ABI sequence traces showing pT18ParF*. The 12-bp oligonucleotide that was inserted is shown by blue line. The positions of the KpnI site in pT18ParF and the inserted BamHI site in pT18ParF* are marked with black lines.](image)

**Figure 3.6. Insertion of a 12-bp sequence in the KpnI site of pT18ParF.** 1, ABI sequence traces showing the KpnI site in the pT18ParF vector. 2, ABI sequence traces showing pT18ParF*. The 12-bp oligonucleotide that was inserted is shown by blue line. The positions of the KpnI site in pT18ParF and the inserted BamHI site in pT18ParF* are marked with black lines.

### 3.3 Pentapeptide scanning mutagenesis of the ParF protein

Transposon Tn4430 is available on a derivative of the F-plasmid that lacks other transposable elements (pOX38). As the size of the target plasmid is less than the size of the chromosome of *E. coli*, the majority of Tn4430 transposition events from pOX38:Tn4430 are expected to be into the *E. coli* chromosome and with insertion into pT18ParF* occurring more rarely. However, transposition of Tn4430 is accompanied by cointegrate formation between the donor and target plasmids (Figure 2.4 A and B). Therefore, insertions in pT18ParF* can be isolated by selecting for conjugative transfer of pOX38:Tn4430::pT18ParF* cointegrates to a plasmid-free recipient. The resolution of the co-integrate occurs efficiently in transconjugants producing the target plasmid containing a copy of the transposon (Figure 2.4 C) (Mahillon *et al.*, 1988; Hallet *et al.*, 1997). Insertions in the backbone of pT18ParF* are not expected to disrupt the self-association of ParF produced from this two-hybrid plasmid and from a coresident pT25ParF plasmid.
Therefore, cells bearing this type of Tn4430 insertion will produce red colonies on MacConkey-maltose indicator medium. In contrast, Tn4430 insertions in the parF gene in pT18ParF* are expected generally to abolish the ParF-ParF interaction and generate white-pink colonies on this medium. This provides a screening strategy to differentiate between Tn4430 insertions in parF and those in the pT18ParF* backbone. However, insertions in the lac promoter in the target plasmid which disrupts the transcription of parF also will produce false white-pink colonies as will insertions in the cya gene (Figure 3.2). The false and true white-pink colonies can be determined only by sequencing of mutant samples.

Plasmid pT18ParF* was transformed into E. coli FH395 (pOX38:Tn4430). Transposition of Tn4430 occurs during colony formation by these transformants. A cell suspension from plates of E. coli FH395 (pOX38:Tn4430) transformed with pT18ParF* was diluted and used as donor material in mating experiments with E. coli DS941 (Smr) as recipient. Different dilutions of donor were mixed with the recipient and incubated without selective pressure on LB plates to allow the conjugation process to proceed (Chapter 2.21). The conjugation mixes from five of these plates were recovered and spread separately on selective LB plates containing ampicillin and streptomycin. Ampicillin eliminates recipient cells whereas streptomycin eliminates donor cells. Only transconjugants that contain pOX38:Tn4430::pT18ParF* cointegrate plasmids will grow on both antibiotics. Plasmid DNA was extracted from five pools each of which contained hundreds of selected transconjugants. These pools consist of pOX38:Tn4430::pT18ParF* plasmids that have resolved to pOX38:Tn4430 and pT18ParF*::Tn4430 products. In practice, the large, low copy number pOX38:Tn4430 and unresolved pOX38:Tn4430::pT18ParF* are recovered poorly and the plasmid preparations consist mainly of pT18ParF*::Tn4430 plasmid DNA. The five pools of pT18ParF*::Tn4430 plasmid migrated more slowly in the agarose gel than pT18ParF* suggesting that these pools indeed consisted of target plasmid onto which Tn4430 had transposed (Figure 3.7). Four of these samples were used in screening for parF mutants by two-hybrid analysis.

3.4 PSM of the ParF protein produced by pT18ParF*

A two-hybrid assay was used for screening pools of pT18ParF*::Tn4430 plasmids for mutated parF genes that were disrupted by the insertion of Tn4430. The principle of this assay is described in detail in Chapter 2.19.
Figure 3.7. Agarose gel of pools of pT18ParF*:::Tn4430 plasmid DNA. Lanes: 1, 1-kb ladder; 2, pT18ParF*; 3-7, pT18ParF*:::Tn4430 plasmid pools obtained from matings between an E. coli donor containing pOX38:Tn4430 and pT18ParF* and the DS941 recipient strain. The different plasmid preparations are from separate matings. Selected bands in the 1-kb ladder are indicated.

Briefly, ParF-mediated association of the ParF-T18 and ParF-T25 fusion proteins brings the T18 and T25 fragments sufficiently close that CyaA activity is reconstituted in E. coli SP850, a cya mutant of E. coli DH5α. As a consequence, red colonies are generated on MacConkey-maltose plates (Barillà and Hayes, 2003). In contrast, ParF-T18 alone, for example, produces white-pink colonies (data not shown). To identify pT18ParF* derivatives in which the parF gene was disrupted by insertion of Tn4430, plasmid DNA from the four pT18ParF*:::Tn4430 libraries was cotransformed with pT25ParF into E. coli SP850. Controls involved cotransformation of pT18, pT18ParF, and pT18ParF* with pT25ParF. Double transformants were selected on LB plates containing ampicillin and chloramphenicol at 37°C overnight.

Figure 3.8. MacConkey maltose agar plates showing controls of two-hybrid assay. Each plate harbours co-transformants of SP850 containing: 1, pT25ParF + pT18 (white-pink colonies); 2, pT25ParF + pT18ParF (red colonies); 3, pT25ParF + pT18ParF* (red colonies).
Table 3.1: Summary of screening of pT18ParF*::Tn4430 plasmid pools for two-hybrid interaction with pT25ParF.

<table>
<thead>
<tr>
<th>pT181ParF*:::Tn4430 plasmid pool</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonies tested on MacConkey maltose plates</td>
<td>650</td>
<td>200</td>
<td>450</td>
<td>180</td>
</tr>
<tr>
<td>Red/white-pink colonies</td>
<td>530/120</td>
<td>160/40</td>
<td>390/60</td>
<td>156/24</td>
</tr>
<tr>
<td>Restreaked white-pink colonies that remained white-pink</td>
<td>107</td>
<td>38</td>
<td>45</td>
<td>20</td>
</tr>
<tr>
<td>Sequenced parF genes from white-pink colonies</td>
<td>70</td>
<td>2</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>15-bp insertion mutations identified in parF</td>
<td>One insertion at position 453 and 69 insertions at position 615</td>
<td>16- and 14-bp insertions at positions 59 and 200, respectively</td>
<td>Eight insertions at position 498; single insertions at positions 453, 480, 492 and 495; two insertions at position 201</td>
<td>Six insertions at position 498; three insertions at position 453; two insertions at position 615</td>
</tr>
</tbody>
</table>

Single colonies from *E. coli* SP850 double transformant plates were streaked on MacConkey agar plates containing maltose (1%; w/v) and with ampicillin and chloramphenicol antibiotics. Plates were incubated at 30°C for 36-48 hours until colony colour developed. From the four pools, analysis of 1480 colonies on MacConkey-maltose plates produced 1236 red and 244 white-pink colonies (Table 3.1) (Figure 3.9). 244 white-pink colonies from the four trials were restreaked on MacConkey-maltose agar plates and incubated at 30°C. 210 of these colonies that remained white-pink possess pT18ParF* plasmids in which the *parF* gene is potentially disrupted by Tn4430 (Figure 3.10). Plasmids from these candidates were processed further. 210 white-pink colonies from cotransformation of the pT18ParF*:::Tn4430 libraries with pT25ParF in *E. coli* SP850 were inoculated in broth, grown overnight and plasmid DNA was extracted (Figure 3.11). To separate the pT18ParF*:::Tn4430 plasmids from the pT25ParF plasmid, the two-plasmid preparations were transformed into *E. coli* DH5α with selection for ampicillin only, i.e., for
pT18ParF∗::Tn4430. Single colonies were streaked on separate plates with ampicillin and chloramphenicol and incubated overnight.

Figure 3.9. Example MacConkey maltose agar plates that illustrate screening of pT18ParF∗::Tn4430 plasmid pools in SP850 cotransformed with pT25ParF. Plates harbour co-transformants of E. coli SP850 with: 1, pT25ParF + pT18; 2, pT25ParF + pT18ParF; 3, pT25ParF + pT18ParF∗. All other colonies contain co-transformants generated from four independent pT18ParF∗::Tn4430 plasmid pools to screen for white-pink and red colonies.
Figure 3.10. Restreaked white-pink colonies from pT18ParF*::Tn4430 plasmid pools screened for interaction with pT25ParF in two-hybrid analysis. 1 and 2 represent red and white-pink controls, respectively, on both plates; A, Numbered white-pink colonies indicated mutant samples. Other streaks were falsely identified white-pink colonies in the first screen and were eliminated; B, All samples exhibit white-pink colonies.

Figure 3.11. Example plasmid extracted from cotransformants of pT18ParF*::Tn4430 with pT25ParF from 210 white-pink colonies. Lanes: 1, 1-kb ladder; 2, pT18ParF*. 3-9, Samples harbouring pT18ParF*::Tn4430 and pT25ParF plasmids. Arrows marked A and B indicate pT18ParF*::Tn4430 and pT25ParF plasmids, respectively. The latter comigrates with the pT18ParF* plasmid in lane 2. Selected bands in the 1-kb ladder are indicated.

Most colonies tested were ampicillin-resistant and chloramphenicol-sensitive indicating that the pT18ParF*::Tn4430 plasmids were separated from pT25ParF (Figure 3.12). Plasmids were extracted from the ampicillin-resistant, chloramphenicol-sensitive colonies which harbour the pT18ParF*::Tn4430 plasmids, but not pT25ParF. The presence of pT18ParF*::Tn4430 and the absence of pT25ParF was confirmed in all 210 cases by agarose gel analysis (Figure 3.13).
Chapter 3

Results I

3.5 Restriction and sequence analysis of pT18ParF*::Tn4430 plasmids.

The insertion of Tn4430 in the parF gene in pT18ParF*::Tn4430 plasmids and not in the vector backbone was assessed by EcoRI digests. pT18ParF* (Figure 3.3) possesses three EcoRI sites: in the multiple cloning site, 430-bp from the 5’ end of parF, and in the vector backbone (3.14). Thus, three fragments are produced by EcoRI digestion of pT18ParF*: the first comprises the bulk of the parF gene (430-bp) and the second fragment (711-bp) is from the resident sequence in the plasmid, while the third fragment (~3.1-kb) represents the bulk of the vector backbone (Figure 3.14). Tn4430 does not possess EcoRI sites. Therefore, EcoRI patterns of pT18ParF*::Tn4430 plasmids in which the 430-bp fragment is replaced
by a fragment that is ~4-kb larger identifies clones in which Tn4430 has inserted in this fragment. Analysis of the 210 pT18ParF*:::Tn4430 plasmids described above revealed that the 430-bp EcoRI fragment was replaced clearly by a fragment that was ~4-kb larger in 115 cases. The 430-bp EcoRI fragment was intact in 95 plasmids which were not processed further. Examples EcoRI patterns of the 115 pT18ParF*:::Tn4430 plasmids in which Tn4430 is inserted in the 430-bp EcoRI fragment are presented in Figure 3.15.

**Figure 3.14. Partial map of pT18ParF* used in PSM.** The parF-cyaA’ fusion is shown. Distances between certain restriction sites are shown. B, BamHI; E, EcoRI, K*, KpnI site removed in pT18ParF*.

**Figure 3.15. Restriction analysis of pT18ParF*:4430 plasmids.** Lanes: 1, 1-kb ladder; 2, pT18ParF* digested with EcoRI; 3 and 4 are examples of separate pT18ParF*:::Tn4330 plasmids digested with EcoRI. A, 430-bp fragment into which Tn4430 is inserted. B, bulk of the pT18ParF* backbone; C, 711-bp fragment; D, 430-bp fragment that contains most of the parF gene (Figure 3.14); Selected bands in the 1-kb ladder are indicated.

Tn4430 harbours KpnI restriction enzyme sites 5-bp from both ends of the terminal inverted repeats (Figure 2.4 E). When the bulk of the transposon is liberated from the target gene by KpnI digestion and religation, ten base pairs remain from Tn4430 and five base pairs are a duplication of the target site sequence. Thus, a 15-bp in-frame insertion is left in the target gene (Hallet et al., 1997). The bulk of Tn4430 was deleted from 95 of the 115 pT18ParF*:::Tn4430 plasmids in which the insertion is within the 430-bp EcoRI fragment by
KpnI digestion and religation. Half of each KpnI reaction was analysed by agarose gel electrophoresis to confirm the correct digest pattern (Figure 3.16), and the other half of the reaction was purified (Chapter 2.9) followed by religation and transformation into *E. coli* DH5α with selection for ampicillin resistance. Plasmids were extracted from 95 colonies from each religation procedure and digested with KpnI to demonstrate that the bulk of Tn4430 was deleted. For all 95 pT18ParF*::Tn4430 plasmids, derivatives in which the bulk of Tn4430 was removed were isolated. As a final step before sequencing of the insertions, samples were digested with EcoRI to demonstrate the same pattern as with the parental pT18ParF* plasmid. All 95 plasmids produced the correct pattern (Figure 3.17).

![Figure 3.16. KpnI restriction digests of pT18ParF*::Tn4430 plasmids. Lanes: 1, 1-kb ladder; 2-8, pT18ParF*::Tn4430 plasmids digested with KpnI. L, represents the pT18ParF*:: plasmid with 15-bp derived from Tn4430 and the liberated bulk of the transposon which comigrate to the same position on the gel. Selected bands in the 1-kb ladder are indicated.](image1)

![Figure 3.17. Restriction analysis of pT18ParF*::Tn4430 plasmids from which the bulk of the transposon was deleted. Lanes: 1, 1-kb ladder; 2-9, pT18ParF*::Tn4430 deletion plasmids digested with EcoRI which demonstrated the same pattern as the parental pT18ParF* plasmid (Figure 3.14). A, bulk of pT18ParF*::15-bp backbone; B, 711-bp fragment; C, 445-bp fragment that contains most of the parF gene and with an expected 15-bp generated by PSM. Selected bands in the 1-kb ladder are indicated.](image2)
In total the *parF* gene in 95 candidates of pT18ParF* plasmids that were expected to possess 15-bp insertions were sequenced from the four mutagenesis pools. The results varied between the four trials (Table 3.1). In trial 1, 69 of the 70 insertions surprisingly were located at an identical position 615-bp from the 3’ end of *parF*, whereas one insertion was located at position 453. In trial 2, two insertions were produced. These insertions were at positions 59 and 200 in *parF* and consisted of 16-bp and 14-bp, respectively (Figure 3.18). These insertions were not processed further as protein mistranslation would be a consequence of these out-of-frame insertions. In trial 3, six independent insertions in *parF* gene were generated from 14 sequenced samples. Eight repetitive insertions were located at an identical position 498-bp from the 5’ end of *parF* and two independent insertions were produced at positions 492 and 495 in the same region of *parF*. Another insertion was identical to one of the mutants from trial 1 which was located at position 453-bp. In addition, another insertion in this trial was generated at position 480 in *parF*. Furthermore, two insertions were located at an identical position 201-bp from the 5’ end of *parF*. Trial 4 did not produce any new insertions, as the 11 sequenced samples were identical to those identified in trials 1-3: six insertions were located at position 480, three insertions were inserted at position 453 and two insertions were at position 615 in *parF*. Thus, seven independent insertions in *parF* were produced (see Figure 3.26) from 95 sequenced samples (Table 3.1).

![Figure 3.18. ABI sequence traces showing 14-bp and 16-bp insertions at positions 200 and 59 in parF.](image)

### 3.6 PSM of ParF protein produced by pFH547

Although several PSM trials were carried out using pT18ParF*, the number of unique ParF mutants with pentapeptide insertions was unexpectedly low (seven out of 95 sequenced). Therefore, the partition plasmid pFH547 was selected as a second target for PSM of ParF. The chloramphenicol resistance plasmid possesses the entire segregation cassette from
TP228, including a functional parF gene (Figure 3.19). The process for PSM of pFH547 was similar to that used with pT18ParF* (Section 3.3). Non-conjugative target plasmid pFH547 was transformed into the *E. coli* FH395 strain containing pOX38:Tn4430 from which transposition of Tn4430 occurred. Transformant plates were washed and a cell suspension was prepared and diluted. This suspension was used as donor in matings with the same recipient strain used previously, *E. coli* DS941 (Sm<sup>r</sup>). Different mixtures of the diluted donor and the recipient were incubated on LB plates without antibiotic selection to allow conjugation to proceed (Section 2.20).

![Genetic organization of pFH547 created by SnapGene software](image)

**Figure 3.19. Genetic organization of pFH547 created by SnapGene software.** Two origins of replication are shown in green arrows. Beige arrow represents the *cat* gene encoding chloramphenicol acetyltransferase. Purple fragment is the basis of mobility (bom) region from pBR322 and the violet arrow is the *rop* gene which controls plasmid copy number. The partition cassette of the TP228 plasmid is marked in a brown segment which includes *parH* (red), *O_F* (blue), *parF* (yellow) and *parG* (orange). Restriction sites used in this study are marked.

The plates were washed with LB broth to recover the conjugation mixes and then the mixes were spread on plates containing chloramphenicol and streptomycin. The former antibiotic eliminates donor cells whereas the latter eliminates recipient cells. Thus, only transconjugants that bear pOX38:Tn4430::pFH547 cointegrate plasmids will grow on the
plates containing both antibiotics. Plasmid DNA from these transformants was extracted by preparing pools. Extracted plasmids consist of unresolved pOX38::Tn4430::pFH547, resolved pOX38::Tn4430 but mainly of the second resolution product, pFH547::Tn4430. Agarose gels showed that Tn4430 indeed had transposed onto pFH547 as migration of the pools of pFH547::Tn4430 plasmids was slower than the target plasmid (Figure 3.20). These three pools were collected and concentrated (Chapter 2.9) for identification of parFGH fragments containing Tn4430 using restriction digestion. A direct restriction analysis strategy (Chapter 2.21) was used to isolate Tn4430 insertions in parF of pFH547. The pFH547 plasmid (Figure 3.19) harbours the partition cassette of TP228 cloned in the partition assay vector pFH450 (Hayes, 2000). Two unique restriction sites (SalI and HpaI) within the parFGH cassette were selected for the initial identification of Tn4430.

![Figure 3.20. Agarose gel of pools of pFH547::Tn4430 plasmid DNA. Lanes: 1, 1-kb ladder; 2, pFH547; 3-5, pFH547::Tn4430 plasmid pools obtained from matings between an E. coli FH539 donor containing pOX38::Tn4430 and pFH547 and the E. coli DS941 recipient strain. The different plasmid preparations are from separate matings. Selected bands in the 1-kb ladder are indicated.](image)

Insertions in parF and flanking regions. Digestion of pFH547 with these enzymes produces a ~1.2-kb fragment that includes all of parF along with flanking regions that encompass the parH and O_F sites on one side and the 5' end of parG on the other side (Figure 3.21 and 3.22). A second fragment corresponds to the pFH450 backbone (~7.6-kb). In the case of pools of pFH547::Tn4430 plasmids, two additional fragments are generated by SalI-HpaI digestion. These comprise the ~1.2-kb and ~7.6-kb fragments in which Tn4430 (~4.0-kb) has inserted thereby producing ~5.2-kb and ~11.6-kb fragments. The ~5.2-kb fragment consists of the parF target fragment with Tn4430 inserted randomly. This fragment can be isolated from an agarose gel and cloned back into pFH547 digested with SalI-HpaI to replace the wild-type fragment. This produces a library of pFH547 plasmids in which
Tn4430 is inserted in the ~1.2-kb fragment that includes parF. It is of note that in all pools of pFH547::Tn4430 no vector backbone was detected on agarose gels and the digested pools with SalI and HpaI gave only two fragments which are ~11.6-kb and ~5.2-kb.

Plasmid DNA from the three pFH547::Tn4430 pools was digested with SalI and HpaI, the fragments were detected on agarose gels, and the ~5.2-kb fragments were recovered (Chapter 2.7) and collected in 1.5 ml tubes. Plasmid pFH547 was digested with the same enzymes to release the wild-type sequence (Figure 3.22) and was then run on agarose gel. The vector was recovered to be ready for ligation reactions with the pFH547::Tn4430 pool fragments. The vector and pool fragments extracted from agarose gels were ligated in a ~1:1 vector:insert ratio. The entire volume of the ligation reaction was transformed into E. coli DH5α and selected on plates supplemented with chloramphenicol. 25 of the colonies that grew on the transformation plate after overnight incubation were inoculated in broth, grown overnight and plasmids were extracted. Recombinant plasmids (pFH547::Tn4430) were verified on agarose gels (Figure 3.23). These plasmids were expected to comprise pFH547 with Tn4430 inserted in the ~1.2-kb SalI-HpaI fragment that includes the parF gene (Figure 3.21).

Figure 3.21. Schematic diagram showing the target fragment of the parFGH cassette in pFH547 used in PSM. Plasmid pFH547 was used as a second target plasmid for PSM following initial tests with pT18ParF*. Tn4430 insertions in parF and flanking regions were isolated from pools of pFH547::Tn4430 plasmids using SalI-HpaI digestion that produces a ~5.2-kb fragment that consists of a ~1.2-kb segment in which the ~4.0-kb transposon Tn44430 has inserted.

Figure 3.22. Agarose gel shows restriction digests of pFH547. Lanes: 1, 1-kb ladder; 2, pFH547 undigested; 3, pFH547 digested with SalI/HpaI. Vector backbone and desired fragment (~1.2-kb) for Tn4430 transposition are marked. Selected bands in the 1-kb ladder are indicated.
The 25 pFH547::Tn4430 plasmids were digested with KpnI to liberate the bulk of the transposon (Figure 3.24). The pFH547::15-bp fragments from which the transposon was eliminated were purified from agarose gels and then religated followed by transformation into E. coli DH5α. Recombinant plasmids from these transformations were digested with SalI and HpaI to verify the same restriction patterns as with the parental pFH547 plasmid. The correct pattern was observed for all 25 plasmids which verified the deletion of the Tn4430 transposon. These plasmids were expected to comprise pFH547 with 15-bp insertions in the ~1.2-kb SalI-HpaI fragment that includes the parF gene (Figure 3.21).

Figure 3.23. Recombinant pFH547::Tn4430 plasmids. Lanes: 1, 1-kb ladder; 2, pFH547; 3-5 examples of pFH547::Tn4430 plasmids. Selected bands in the 1-kb ladder are indicated.

Figure 3.24. Restriction analysis of pFH547::Tn4430 plasmids. Lanes: 1, 1-kb ladder; 2-4 examples of KpnI digested pFH547::Tn4430 plasmids. The pFH547 target plasmid with 15-bp derived from Tn4430 transposition and the liberated bulk of the transposon are marked. Selected bands in the 1-kb ladder are indicated.
Figure 3.25. ABI sequence traces showing nine insertions of 15-bp in the \textit{parF} sequence generated by PSM. In each sequence two codons before and after insertions are showed and marked in black and codons of pentapeptide insertions in red. In ParF205GVPLF sequence, the stop codon is marked with a star in pFH547 plasmid.
Table 3.2: ParF insertions generated by PSM.

<table>
<thead>
<tr>
<th>Position of 15-bp insertions on parF sequence</th>
<th>Target plasmid in which insertion was identified</th>
<th>ParF derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>200-bp</td>
<td>pT18ParF*</td>
<td>ParF67GVPLY</td>
</tr>
<tr>
<td>213-bp</td>
<td>pFH547</td>
<td>ParF71GVPRK</td>
</tr>
<tr>
<td>453-bp</td>
<td>pT18ParF*</td>
<td>ParF151GVPLK</td>
</tr>
<tr>
<td>480-bp</td>
<td>pT18ParF*</td>
<td>ParF160GVPLK</td>
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<tr>
<td>492-bp</td>
<td>pT18ParF*</td>
<td>ParF164GVPRT</td>
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<tr>
<td>495-bp</td>
<td>pT18ParF*</td>
<td>ParF165RGTPA</td>
</tr>
<tr>
<td>498-bp</td>
<td>pT18ParF*</td>
<td>ParF166RGTPS</td>
</tr>
<tr>
<td>556-bp</td>
<td>pFH547</td>
<td>ParF185RGTPS</td>
</tr>
<tr>
<td>615-bp</td>
<td>pT18ParF*</td>
<td>ParF205GVPLF</td>
</tr>
</tbody>
</table>

The parF gene and flanking regions were sequenced in the 25 pFH547::15-bp plasmids. Two insertions were located in the region 5' of parF and were not processed further. Six 15-bp insertions were positioned at 200-bp, another five insertions were located identically at position 453-bp, and six insertions were produced at positions 495-bp. Furthermore, three identical insertions were located at position 498-bp. Insertions at each of the preceding positions were previously identified during PSM of pT18ParF* (Section 3.5). Nevertheless, one insertion at 556-bp is a new insertion identified in mutagenesis of pFH547. It is worth mentioning that insertions at positions 498-bp, 453-bp, 213-bp and 200-bp were constructed previously in parF (F. Hayes, personal communication) using the pFH547 target plasmid. These insertions were produced again in this study except for the mutation at position 213-bp. Accordingly, the nine pentapeptide insertions in ParF obtained from mutagenesis studies with pT18ParF* and pFH547 were named as in Table 3.2. DNA sequence traces of the insertions are shown in Figure 3.25.

3.7 Amplification and cloning of the genes for ParF71GVPRK and ParF185RGTPS into two-hybrid plasmid pT18

As outlined above PSM is a random mutagenesis technique using transposable element Tn4430. The method was carried out here using two different target plasmids (two-hybrid plasmid pT18ParF* and partition plasmid pFH547). Four of the nine insertions were produced in both plasmids independently which allowed for parallel testing of the effects of these insertions on both ParF self-association and plasmid segregation. However, the parF derivatives encoding ParF71GVPRK and ParF185RGTPS were generated in pFH547 but not in the two-hybrid plasmid. Therefore, PCR (Chapter 2.11) was applied using primers pT18XhoI-Forward and pT18HindIII1-Reverse (Table 2.2) to amplify the mutant genes from pFH547 plasmids for cloning in pT18. The PCR products were verified on agarose
gels (Figure 3.26 A) and purified. The pT18 vector and the PCR inserts were digested with XhoI/HindIII to create sticky ends. The digested vector was treated with alkaline phosphatase and purified to prevent religation. Inserts and vector were purified from agarose gels (Figure 3.26 B) to be ready for ligation reactions. The ligation mixtures were transformed into strain E. coli DH5α and ten colonies from each plate were picked and grown in LB broth overnight. Recombinant plasmids were extracted and screened by digesting with XhoI and HindIII (Figure 3.26 C). Relevant samples were sequenced and the clones were verified by the sequencing results. This process produced plasmids pT18ParF71GVPRK and pT18ParF185RGTPS.

Figure 3.26. Agarose gel analysis of transfer of parF genes encoding ParF71GVPRK and ParF185RGTPS into pT18. A, Lanes: 1, 100-bp ladder; 2, control PCR reaction without template DNA; 3 and 4, PCR amplification products (~600-bp) of parF genes encoding ParF71GVPRK and ParF185RGTPS, respectively. B, restriction digested and purified vector and PCR products before ligation reaction. Lanes: 1, 1-kb ladder; 2, pT18 vector (undigested); 3, pT18 digested with XhoI and HindIII; 4 and 5, PCR amplification products of parF genes encoding ParF71GVPRK and ParF185RGTPS, respectively, digested with XhoI and HindIII. C, lanes: 1, 1-kb ladder; 2 and 3, pT18ParF71GVPRK and pT18ParF185RGTPS, respectively, digested with XhoI and HindIII; 4, undigested pT18ParF71GVPRK.

3.8 Amplification and cloning of the genes for ParF160GVPLK and ParF164GVPRT into partition plasmid pFH547

The gene for ParF160GVPLK and ParF164GVPRT were generated in pT18ParF* but not in pFH547 (Table 3.1). To assess the effects on plasmid segregation of these mutations, the genes encoding pT18ParF*160GVPLK and pT18ParF*164GVPRT were cloned in place of the wild-dype parF gene in pFH547 using restriction fragment exchange. SexAI and Clal restriction sites within the parF gene are located on either side of both 15-bp insertions. These sites were selected for replacing the wild-type fragment in pFH547 with the mutated
fragments from pT18ParF* derivatives (Figure 3.27). Note that as the SexAI recognition site is blocked by Dam methylation, the plasmids were transformed into the Dam⁺ E. coli SCS110 strain before manipulation. Two 229-bp fragments were produced from digestion of pT18ParF*160GVPLK and pT18ParF*164GVPRT with SexAI and ClaI. The pFH547 plasmid was digested with the same restriction enzymes to liberate the wild-type sequence. The 229-bp and pFH547 backbone fragments were extracted from an agarose gel (Figure 3.30). Following the preparation of the inserts and pFH547 backbone, samples were ligated and transformed. Plasmid DNA was extracted from selected transformants and verified by KpnI digestion. The pFH547 plasmid lacks a KpnI site whereas the mutated fragments from pT18ParF*160GVPLK and pT18ParF*164GVPRT possess KpnI sites in the 15-bp insertions (Figure 3.28). Sequence analysis verified that the wild-type sequence in pFH547 had been changed to the sequence encoding ParF160GVPLK and ParF164GVPRT.

Figure 3.27. Schematic diagram that illustrates cloning of the genes encoding ParF160GVPLK and ParF164GVPRT from pT18ParF* derivatives into pFH547. A. pFH547; B. pT18ParF*160GVPLK/pT18ParF*164GVPRT; C. pFH547 derivatives in which the SexAI-ClaI fragments from pT18ParF*169GVPLK or pT18ParF*164GVPRT are used to replace the wild-type fragment in pFH547. Yellow, parF; pink, 214-bp wild-type fragment; black, 229-bp fragment harbouring a 15-bp insertion. SexAI and ClaI restriction sites used for fragment exchange are shown.
3.9 Amplification and cloning of the gene for ParF205GVPLF into partition plasmid pFH547

The insertions that produce ParF160GVPLK, ParF164GVPR and ParF205GVPLF were generated in pT18ParF* but not in pFH547. PCR amplification and cloning of the gene encoding ParF205GVPLF was not applicable, as no convenient restriction sites were available for inserting the mutated parF gene in pFH547 in place of the wild-type gene. Therefore, OE-PCR was applied as outlined in Chapter 2.14 to generate the mutation encoding ParF205GVPLF in pFH547. This 15-bp insertion was generated using two sets of external and internal primers (Figure 3.29). Two external primers (ParF-A_F-forward/ParF-D_F-reverse) were used in conjunction with internal primers (ParF-B_F-reverse/ParF-C_F-forward) that contained the appropriate 15-nt sequences as non-annealing tail (Table 2.2). The final PCR fragment was produced using three separate PCR cycles. First, PCR1 (~138-bp) was generated using external primer ParF-A_F-forward and internal primer ParF-B_F-reverse, and PCR2 (~216-bp) was produced using external primer ParF-D_F-reverse and internal primer ParF-C_F-forward. The full-length mutated PCR3 fragment (~354-bp) bearing the 15-bp insertion was generated using PCR1 and PCR2 products as templates and the external primers ParF-A_F-forward/ParF-D_F-reverse (Figure 3.29).

The PCR3 product and pFH547 plasmid were digested with Clal and HpaI and were recovered from an agarose gel. The fragments were ligated and transformed into E. coli DH5α. Twelve colonies were picked from the transformation plate, and plasmid DNA was extracted and verified by restriction digestion with the KpnI enzyme which cuts uniquely...
within the engineered 15-bp insertion (Figure 3.30). Sequencing confirmed that the 15-bp insertion was generated at position 615-bp in \textit{parF}.

Therefore, to summarize the construction of pentapeptide mutations in ParF, nine unique insertions were generated at positions 67, 71, 151, 160, 164, 165, 166, 185 and 205 (Figure 3.31). These insertions were produced using either pT18ParF* or pFH547 target plasmids. In the case of insertions that were available only in one of these plasmids, the corresponding insertions were introduced into the second plasmid by restriction fragment swapping or PCR approaches. Thus, the effects of the nine insertions were testable on plasmid segregation in pFH547-based plasmids and on ParF self-association and interaction with ParG in pT18-based plasmids.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3_29.png}
\caption{Schematic diagram of OE-PCR for producing the gene encoding ParF205GVPLF for cloning into pFH547 in place of the wild-type \textit{parF} gene. Primers (A\textsubscript{p}, B\textsubscript{p}, C\textsubscript{p} and D\textsubscript{p}) and restriction sites are highlighted in red and blue, respectively. The 15-bp insertions are attached to the 5’ of primers B\textsubscript{p} and C\textsubscript{p}. The sequences of \textit{parF} and \textit{parG} are underlined with yellow and orange lines, respectively.}
\end{figure}
Figure 3.30. A, Agarose gel analysis of OE-PCR products for producing the gene encoding ParF205GVPLF for cloning into pFH547 in place of the wild-type parF gene. Lanes: 1, 100-bp ladder; 2, 3 and 4 show PCR1, PCR2 and PCR3 products, respectively. Selected bands in the 100-bp ladder are indicated. B, Restriction enzyme analysis of recombinant pFH547ParF205GVPLF plasmids. Lanes: 1, 1-kb ladder; 2, pFH547 undigested; 3, undigested recombinant plasmid; 4 and 5 examples of pFH547ParF205GVPLF candidate plasmids linearized using KpnI.
Figure 3.31. Pentapeptide insertions in ParF. Insertion positions are mapped to the primary (A) and three-dimensional structures (B) (Moreland et al., 2005). β-strands, α-helices and loops/turns are shown in cyan, pink and yellow, respectively.
3.10 The effects of pentapeptide insertions in ParF on plasmid partitioning

The impact of pentapeptide insertions on ParF protein function was assessed using a partition assay (Chapter 2.25). The assay was performed using pFH547-based plasmids in the *E. coli* BR825 polA mutant strain. The positive control was pFH547 itself that harbours the intact partition cassette of the TP228 plasmid. This plasmid and its derivatives also possess the, CoIE1 moderate copy number and P1 low copy number replicons and a chloramphenicol resistance gene (Figure 3.19). In contrast, pFH450 is the equivalent vector which lacks accessory stability genes and was used as a negative control in partition assay (Hayes, 2000). The principle of the assay is based on transformation of pFH547 plasmids into the *E. coli* BR825 polA strain in which plasmid replication changes to a low copy number under the control of P1 replicon. Plasmid retention after ~25 generations in the absence of selective pressure for positive (pFH547) and negative (pFH450) controls was measured (Chapter 2.25). The plasmids were retained at frequencies of 64 and 2%, respectively, which are similar to values observed previously (Hayes, 2000; Barillà and Hayes, 2003). The ParF160GVPLK, ParF185RGTPS and ParF205GVPLF proteins maintained wild-type levels of plasmid retention (~52-62%) in the assay (Figure 3.32). Thus, the insertions at positions 160, 185, 205 had no detectable effect on the function of the ParF protein in this assay. The effects of elongating the insertions at these positions using synthetic double-stranded oligonucleotides is described in Chapter 5.1.3. The insertion of five amino acids near the C-terminus of ParF did not have any deleterious effect on segregational stability in PaF205GVPLF. Therefore, the C-terminal end was subjected to site-directed mutagenesis to investigate its role *in vivo* (Chapter 5.2).

ParF71GVPRK, ParF164GVPUT and ParF166RGTPPI conferred very low levels of plasmid retention (~11-15%), indicating that the insertions at positions 71, 164 and 166 reduced, but did not abolish, partition efficiency. The remaining pentapeptide insertions (ParF67GVPLY, ParF151GVPLK and ParF165RGTPA) caused severe defects on plasmid partitioning (~3-6% retention) that were very close to the empty vector (pFH450) (Figure 3.32). Therefore, these positions are vital for the function of ParF. To elucidate the impact of pentapeptide insertions *in vivo* in more detail, the ability of the mutant proteins to disrupt the wild-type partition complex (toxicity assays) is described in Section 3.13. In particular the ParF mutants that conferred low levels of plasmid retention were chosen to assess the effect of non-functional ParF variants on plasmid stability.
Figure 3.32. Effects of pentapeptide insertions in ParF on plasmid segregation. Segregation assays were used to determine the efficiency of the parFGH cassette in E. coli BR825 after growth for ~25 generation with non-selective pressure. Plasmid retention was determined as percentages for nine pentapeptide ParF mutants (blue bars). Controls are pFH450 (red bar) which is the empty partition vector that lacks the parFGH cassette (<2%) and pFH547 (green bar) that is pFH450 containing the wild-type partition cassette (~65%). Data represent the means of at least three independent experiments conducted in triplicate with ± standard deviation.

3.11 Effects of pentapeptide insertions on ParF self-association

Monitoring protein-protein interactions via two-hybrid systems is a powerful approach for understanding protein function and association. The bacterial system used here is based on a functional interaction of the two adenylate cyclase fragments (T18 and T25) of Bordetella pertussis which results in synthesis of cAMP. The latter induces the expression of several genes such as involved in lactose or maltose utilization in an E. coli adenylate cyclase-deficient strain. When the proteins of interest that are fused to the T18 and T25 fragments that are able to interact, cAMP will be synthesized as a result of functional complementation between the two fragments. This interaction can be detected on indicator media, e.g., MacConkey-maltose agar plates. Plasmids pT18 and pT25 encode the T18 and T25 fragments, respectively, in vectors in which the lac promoter controls the expression of
fusion proteins. Interaction between the test proteins gives red colonies on MacConkey-maltose agar plate, whereas a white-pink color will be produced when the colonies contain only one of the fusion proteins with T18 or T25, or if the test proteins do not interact (Karimova et al., 1998; Karimova et al., 2000). Investigations based on reconstitution of adenylate cyclase in this two-hybrid system previously have shown ParF self-association and the interaction of ParF with ParG. The interactions were detected using ParF and ParG fused to the T18 and T25 fragments respectively, with ParG (Barillà and Hayes, 2003). In the current study, pT18ParF* was constructed (Chapter 2.2) and used for PSM of ParF. The interactions of the nine ParF derivatives with pentapeptide insertions (Table 3.2) with wild-type ParF were assessed in the two-hybrid assay as described in detail in Chapter 2.19 and 2.21. Briefly, pT18ParF* derivatives and pT25ParF were cotransformed into E. coli SP850. Colony color on MacConkey-maltose plates and β-galactosidase assay were used to examine the effects of the nine insertions on ParF self-association.

Five mutant proteins displayed white-pink colonies on MacConkey-maltose agar plates suggesting that the pentapeptide insertions in ParF67GVPLY, ParF71GVPRK, ParF164GVPRT, ParF165RGTPA and ParF166RGTPI disrupted ParF self-association (Figure 3.33, plate B, streak 3; plate F, streak 1; plate A, streaks 3 and 4; plate J, streak 4). These insertions may affect specifically the ParF-ParF interaction or may have a more general effect on ParF folding and/or stability. Conversely, ParF151GVPLK, ParF160GVPLK, ParF185RGTPS and ParF205GVPLF produced from pT18ParF* resulted in red colonies with wild-type ParF produced from pT25ParF (Figure 3.33, plate K, streak 4; plate J, streak 3; plate B, streak 4; plate G streak 1; plate E, streak 1), indicating that these pentapeptide insertions did not affect the ParF-ParF interaction. The colour intensities produced by these interactions were not visibly different from that generated by self-association of wild-type ParF (Figure 3.33, plate A, streak 1; plate B, streak 1; plate E, streak 2; plate F, streak 2; plate G, streak 2; plate J, streak 1; plate K, streak 5). β-galactosidase activity was measured to assess further the interaction efficiency of the nine ParF mutant proteins with wild-type ParF (Figure 3.34) (Karimova et al., 1998). This test was conducted for assessing the activity of wild-type and mutant ParF and ParG proteins as previously done (Barillà and Hayes, 2003; Barillà et al., 2005; Barillà et al., 2007). In this study, the tests were performed at least three times in three separate experiments for each mutant. pT18ParF* derivatives were co-transformed to the E. coli SP850 (pT25ParF) strain. Wild-type ParF (pT18ParF*) and empty vector pT18 were used as positive and negative controls, respectively. The wild-type
Chapter Three

ParF-ParF interaction produced ~1000 \( \beta \)-galactosidase units and ~80 \( \beta \)-galactosidase units were detectable in the absence of the T18 fragment fused to ParF.

In agreement with the qualitative assays on MacConkey-maltose medium described above, very low \( \beta \)-galactosidase was detectable for wild-type ParF and ParF67GVPLY, ParF71GVPRK, ParF164GVPR, ParF165RGTPA or ParF166RGTPA confirming that the insertion of five amino acids at these positions in the ParF protein abrogated ParF self-association. In contrast, interactions of ParF151GVPLK, ParF160GVPLK, ParF185RGTPS and ParF205GVPLF with wild-type ParF produced ~1200 to 1300 \( \beta \)-galactosidase units. In comparison, self-association of ParF produced ~800 units (Figure 3.34).

The results of \( \beta \)-galactosidase assays for the association of wild-type ParF with mutant proteins coincide with the qualitative colony colour two-hybrid assays. The data support the conclusion that positions 67, 71, 164, 165 and 166 in ParF are crucial for the ParF-ParF interaction or for protein folding/stability in vivo. These insertions also disrupted the plasmid segregation activity of ParF (Figure 3.32). In contrast, positions 160, 185 and 205 are more flexible and insertions of five amino acids at these locations did not affect the interactions with wild-type ParF or plasmid partitioning. Interestingly, ParF151GVPLK interacted strongly with ParF (Figure 3.34) but exerted a pronounced plasmid segregation defect (Figure 3.32), suggesting that this position may be important for an aspect of plasmid partition other than ParF self-association.

Figure 3.33 (overleaf). Association of ParF proteins with pentapeptide insertions with wild-type ParF and with ParG in bacterial two-hybrid assays. Plasmids bearing the relevant pT18ParF* derivatives and either pT25ParF or pT25ParG were cotransformed into *E. coli* SP850. Colonies were streaked on MacConkey-maltose medium and colony colour observed after 36-48 hours incubation at 30°C. A. Examples of wild-type ParF-ParF pentapeptide mutant interaction (white-pink colonies): 1, pT25ParF + pT18ParF*; 2, pT25ParF + pT18; 3 and 4 pT18ParF*164GVPR and pT18ParF*165RGTPA + pT25ParF. B. Wild-type ParF-ParF pentapeptide mutant interactions (white-pink and red colonies) 1, pT25ParF + pT18ParF*; 2, pT25ParF + pT18; 3, pT25ParF + pT18ParF*67GVPL; 4, pT18ParF*160GVPL + pT25ParF. C. Examples of ParG-ParF pentapeptide mutant interactions (white-pink colonies): 1. pT25ParG + pT18ParF*; 2, pT25ParG + pT18; 3 and 4 pT18ParF*67GVPL and pT18ParF*164GVPR + pT25ParG.


I. Wild-type ParG-ParF pentapeptide mutant interaction: 1, pT25ParG + pT18*ParF166RGTPA; 2, pT25ParG + pT18*ParG166RGTPA; 3, pT25ParG + pT18*ParF166RGTPA. J. Wild-type ParF-ParF pentapeptide mutant interaction: 1, pT25ParF + pT18*ParF; 2, pT25ParF + pT18*ParF; 3, pT25ParF + pT18*ParF151GVPLK; 4, pT25ParF + pT18*ParF151GVPLK.

Figure 3.34. Two-hybrid analysis of pentapeptide ParF mutants. Results of two-hybrid analysis using *E. coli* SP850. β-galactosidase assays were used to measure the levels of interactions of ParF mutant proteins with wild-type ParF. Assays were conducted at least three times in three independent experiments for each mutant. Data represent the mean ± standard deviation. Y axis represents β-galactosidase enzymatic activity expressed as Miller units; X axis shows the interactions of ParF mutants with wild-type ParF fused to T25 protein (black bars). Controls are pT25ParF + pT18ParF* (positive control; green bar) and pT25ParF + pT18 (negative control; blue bar).

### 3.12 The effects of pentapeptide insertions in ParF on ParG interaction

Although *in vivo* investigations of mutant ParF derivatives with wild-type ParG are limited, the ParF-ParG interaction can be detected readily using ParF fused to the T18 fragment of adenylate cyclase and ParG fused to the T25 fragment (Barillà and Hayes, 2003). Therefore, the two-hybrid assay was used here to assess the impact of the five amino acid insertions in the ParF protein on the interaction with ParG. The pT18ParF* derivatives and pT25ParG were cotransformed into *E. coli* SP850. Colony colour of the double transformants on MacConkey-maltose plates and β-galactosidase assays were used to examine the effects of the nine insertions in ParF on ParF-ParG interaction.

The interaction between wild-type ParF and ParG was evident as a strong red colony colour in the two-hybrid assay (Figure 3.33, plate C, streak 1; plate D, streak 1; plate E, streak 5;
plate F, streak 5; plate H, streak 2; plate I, streak 1; plate K, streak 2) and as ~1300 β-galactosidase units (Figure 3.35). Five insertions in ParF protein abolished the interaction with ParG in both assays (Figure 3.33, plate C, streaks 3 and 4; plate F, streak 4; plate I, streaks 3 and 4; Figure 3.35). ParF67GVPLY, ParF71GVPRK, ParF164GVPRT, ParF165RGTPA and ParF166RGTP1 displayed white-pink colonies on MacConkey-maltose agar plates revealing that the insertion of five amino acids at these positions disrupted the interaction of the mutant ParF proteins with ParG. The β-galactosidase values obtained with these mutant ParF proteins and ParG were similar to the background levels (Figure 3.35). Thus, two-hybrid assays indicated potential role for these five positions in protein-protein interactions during DNA plasmid segregation. However, as these insertions also affected the association of the mutant ParF proteins with wild-type ParF (Figure 3.34), it is likely that the insertions at positions 67, 71, 164, 165 and 166 instead have a deleterious effect on ParF protein structure and/or stability. In contrast, two-hybrid assays with ParF160GVPLK, ParF185RGTPS and ParF205GVPLF resulted in red colonies with ParG on MacConkey-maltose plates (Figure 3.33, plate D, streak 3; plate E, streak 4; plate H, streak 1), indicating that these pentapeptide insertions did not affect the ParF-ParG interaction. Analogously, β-galactosidase values for the interaction of ParF160GVPLK, ParF185RGTPS and ParF205GVPLF with ParG were similar to those for the wild-type ParF-ParG interactions (Figure 3.35). These observations correlate with the lack of effect that these insertions had no plasmid segregation (Figure 3.32) as segregation requires the ParF-ParG interaction.

Interestingly, the pentapeptide insertion in ParF151GVPLK abolished the interaction with ParG in two-hybrid assays both on MacConkey-maltose plate (Figure 3.33, plate K, streak 1 and plate D, streak 4) and in β-galactosidase assays (Figure 3.35). The defective interaction of ParF151GVPLK protein with ParG but not with wild-type ParF (Figure 3.34) suggests a role for this region of ParF specifically in the ParF-ParG association in vivo. As ParG plays an important role in recruiting the ParF to the segregation processes and also stimulating the ATPase activity of the latter (Barillà et al., 2007). The 151 position may recruit ParG protein to the segregation process.

In summary, five pentapeptide insertions in ParF (positions 67, 71, 164, 165 and 166) negatively affected ParF self-association, the interaction with ParG, and plasmid partitioning (Table 3.3). It is likely that these insertions have disruptive effects on ParF folding and/or stability. In contrast, the insertions at positions 160, 185 and 205 had no
noticeable impact on ParF self-association, the interaction with the partner ParG protein, or on plasmid segregation. These positions appear to be less critical for ParF function. Finally, the insertion at position 151 disrupted both the interaction of ParF with ParG and plasmid segregation activity, but did not impair the self-association of ParF (Table 3.3). These data suggest that this position is of particular importance in the ParF-ParG interaction.

Table 3.3. Summary of effects of pentapeptide insertions on ParF activity

<table>
<thead>
<tr>
<th>ParF Insertion</th>
<th>ParF Self-Association</th>
<th>ParG Interaction</th>
<th>Segregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>67GVPLY</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>71GVPRK</td>
<td>-</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>151GVPLK</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>160GVPLK</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>164GVPRT</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>165RGTPA</td>
<td>-</td>
<td>-</td>
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<tr>
<td>166RGTPi</td>
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<tr>
<td>185RGTPS</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>205GVPLF</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

+, mutation did not affect ParF self-association, ParF-ParG interaction and plasmid segregation; -, mutation affected ParF self-association, ParF-ParG interaction and plasmid segregation; ±, mutation showed medium levels of ParF self-association, ParF-ParG interaction and plasmid segregation.

Analysis of ParF151GVPLK revealed that the insertion of five amino acids at this position impaired DNA segregation (Section 3.10), as well as the interaction with the partner protein ParG, but not the ParF-ParF interaction (Section 3.11 and 3.12). This finding suggested that residues in this region of ParF may participate in the recruitment of the protein by ParG in the segregation process, or may reflect another ParF-ParG interaction. As described in the following chapter: First site-directed mutagenesis of positions 151 and 152 in ParF was applied and the mutants were tested in two-hybrid and partition assays. Second, the effect of increasing the size of the insertion at position 151 from five amino acids to up to 19 residues was assessed. Thus, the data in Chapter 4 clarify the important of residues in the vicinity of amino acid 151 for ParF protein function in plasmid partitioning.
**Figure 3.35. Two-hybrid analysis of pentapeptide ParF mutants interactions.** Results of two-hybrid analysis using *E. coli* SP850. β-galactosidase assays were used to measure the levels of interactions of mutant ParF proteins with wild-type ParG. Assays were conducted at least three times in three independent experiments for each mutant and the data show the mean ± standard deviation. Y axis represents β-galactosidase enzymatic activity expressed as Miller units; X axis shows the interactions of ParF mutants with wild-type ParG fused to T25 protein (black bars). Red bar and blue bar represent positive control (pT25ParG + pT18ParF*) and negative control (pT25ParG + pT18), respectively.

### 3.13 The effects of production of ParF proteins with pentapeptide insertions on the wild-type segregation

Insertions of variable five amino acids in the ParF protein were generated using PSM. The mutagenesis produced nine unique ParF mutant proteins with different effects on the function of ParF in plasmid segregation. Partition and two-hybrid assays revealed that certain pentapeptide insertions abolished segregation function. Other mutations did not affect any of the tested functions. In contrast, the ParF151GVPLK protein was partition-defective, failed to interact with ParG, and guided us to constructing and characterizing more mutations at this position (Chapter 5). In addition, ParF67GVPLY, ParF71GVPRK, ParF151GVPLK, ParF160GVPLK, ParF165RGTPA and ParF185RGTPS were chosen here to investigate the effects of these proteins *in trans* on the wild-type segregation cassette of the TP228 plasmid. The ParF67GVPLY, ParF71GVPRK, ParF165RGTPA proteins confer defects in plasmid partition and in the interactions with both ParF and ParG (Table 3.3). We
speculate that these derivatives may have folding and/or stability deficiencies. ParF151GVPLK also is impaired in plasmid segregation activity and in the interaction with ParG, but associates effectively with wild-type ParF in two-hybrid assays suggesting that it retains at least partial functionality. ParF160GVPLK and ParF185RGTPS appear to be fully functional for plasmid partition as well as in interactions with wild-type ParF and with ParG (Table 3.3).

Plasmid pFH554 contains the functional parFGH cassette and replicates at low copy via the P1 replicon (Hayes, 2000). To assess whether the six ParF insertion mutant proteins with different functionality interested with the wild-type segrosome, the segregational stability of pFH554 in the presence of additional of wild-type ParF or pentapeptide mutant ParF proteins produced in trans from the compatible pT18 plasmid was measured (Figure 3.36). Plasmids pT18, pT18ParF*, pT18ParF*67GVPLY, pT18ParF*71GVPRK, pT18ParF*151GVPLK, pT18ParF*160GVPLK, pT18ParF*165RGTPA and pT18ParF*185RGTPS were co-transformed with pFH554 into DH5α and standard partition assays were performed (Chapter 2.25) except that selection was maintained throughout for the pT18 plasmids. Production of wild-type ParF from pT18ParF* exerted a destabilizing effect on pFH554 which showed <10% retention in the assay. In contrast, pFH554 displayed a normal level of plasmid retention (~65%) in the presence of pT18 without a cloned parF gene (Figure 3.36) thus, the latter showed no destabilizing effects on pFH554 that segregates using the parFGH cassette, whereas the wild-type ParF protein produced from pT18ParF* exerted a poisonous effect toward the partition complex encoded by pFH554 which destabilized the plasmid. This finding agrees with previous observations that excess ParF protein has been shown previously to disrupt the activity of the segrosome (Hayes, 2000).

Similarly, the mutant proteins ParF160GVPLK and ParF185RGTPS that conferred normal levels of partition activity (Figure 3.32) caused poisonous effects on the wild-type partition complex when produced in trans leading to destabilization of pFH554 (~5% retention) (Figure 3.36). In contrast, ParF67GVPLY, ParF71GVPRK, ParF151GVPLK and ParF165RGTPS production in trans conferred mild but reproducible reductions in the stability of pFH554 (~30-50% plasmid retention). These four ParF mutants conferred severe partition defects independently (Figure 3.32). Although ParF67GVPLY, ParF71GVPRK and ParF165RGTPS may have folding and/or stability defects, the proteins appear to retain sufficient activity to disrupt the wild-type ParFGH segrosome and increase the loss of
pFH554, although not as effectively as wild-type ParF or ParF160GVPLK and ParF185RGTPS.

Figure 3.36. Retention of pFH554 containing the *parFGH* cassette when co-cultivated with plasmids which produce ParF proteins containing pentapeptide insertions. Partition assays were used to determine the tolerance of the *parFGH* cassette in the low copy number pFH554 plasmid after growth for ~25 generation with non-selective pressure for pFH554 and continued selection for pT18 plasmids producing wild-ParF (red) or ParF67GVPLY, ParF71GVPRK, ParF151GVPLK, ParF160GVPLK, ParF165RGTPA or ParF185RGTPS (blue). The retention of pFH554 was determined as percentages with presence of pentapeptide ParF mutants (blue). The control is the empty pT18 vector (purple). Data represent the mean ± standard deviations of at least three independent experiments conducted in triplicate.
Chapter 4: Results II

Mutagenesis of ParF151, ParF152 and ParF151GVPLK
(Identification of novel residues involved in the ParF-ParG interaction and plasmid segregation \textit{in vivo})
4.1 Background
Nine ParF mutant proteins were generated using a random pentapeptide insertion mutagenesis approach. Mutants were assessed in two-hybrid assays and partition assays (Chapter 3). Five of the mutants displayed a wild-type phenotype. In contrast the four remaining mutants showed defects in their activity for ParF self-association and/or interaction with the partner protein ParG. Interestingly, the results of both assays confirmed that the insertion of five amino acids between residues 151 and 152 had no effect on ParF self-association whereas the insertion abolished interaction with ParG. To determine the importance of these residues for the structure-function of ParF protein we applied: first, synthetic oligonucleotides were used to increase the size of the insertion in ParF151GVPLK from five to 19 amino acids. The insertion mutagenesis involved cloning of an in-frame double-stranded synthetic oligonucleotide which is 123/124 (Chapter 2.23). Second, restriction digestion and religation of the inserted synthetic oligonucleotides in pT18ParF151GVPLK produced ParF derivatives that contained between five and 19 amino acid insertions. Third, site-directed mutagenesis of residues 151 and 152 which involved substitution of K151 and E152 residues to alanine. In addition residues 151 and 152 were deleted from ParF.

4.2 Increasing the size of the oligopeptide at position 151 in α6 of ParF
4.2.1 Background
PSM generated nine ParF proteins with unique pentapeptide insertions (Chapter 3). The method depends on the insertion of 15-bp in the target gene at random positions using Tn4430 followed by incomplete excision of the transposon by restriction digestion with KpnI and religation. The transposon harbours KpnI cleavage sites close to both its termini and during transposition it duplicates 5-bp of the target sequence. As a result of transposon excision by the KpnI enzyme from the target site, an in-frame 15-bp insertion (10-bp from Tn4430 and 5-bp from target gene duplication) is generated, producing a five amino acid insertion in the target protein (Chapter 2.21) (Hallet et al., 1997). Therefore, all 15-bp insertions generated by PSM in parF contain a unique KpnI site. Results of two-hybrid assays and partition tests indicated that pentapeptide insertions at positions 67, 71, 164, 165 and 166 in ParF abolished ParF self-association and the interaction with the partner protein ParG and also affected ParF activity in faithful plasmid partitioning. Insertions at these positions may have disruptive effects on ParF folding and/or stability. Conversely, the proteins with insertions at positions 160, 185 and 205 displayed wild-type activity in all
assays (Chapter 3.10, 3.11 and 3.12) suggesting that the pentapeptide insertions at these positions had no impact on ParF structure-function relationships. In contrast, the insertion at position 151 in α6 disrupted segregation and the interaction with ParG, but not the ParF-ParF interaction. It is likely that impairment of the ParF-ParG interaction by this pentapeptide insertion had a critical impact on plasmid segregation. Therefore an extended mutagenesis was applied here to address the influence of larger insertions between K151 and E152 in α6 of the ParF protein on protein function, and to test the tolerance of this helix to larger insertions with respect to ParF self-association.

4.2.2 Increasing the oligopeptide length in between K151 and E152 in ParF

The tolerance of α6 to oligopeptide insertions was monitored further by cloning of a double-stranded synthetic oligonucleotide into the unique KpnI site in the 15-bp insertion in the gene that encodes ParF151GVPLK, in order to assess the effect of insertions larger than five amino acids on ParF self-association. Cloning of an 42-bp in-frame, double-stranded synthetic oligonucleotide into the KpnI site of pT18ParF*151GVPLK increased the size of the insertion from 15-bp to 57-bp, thereby producing a ParF derivative with a 19 amino acid insertion instead of a pentapeptide insertion between positions 151 and 152 in α6 of ParF (Chapter 2.23). The 123/124 oligonucleotide has KpnI overhangs to allow for ligation into the KpnI-digested plasmid. The ParF mutant with a 19 amino acid insertions in α6 was constructed and named pT18ParF*151GVPLK. In addition, during the insertion of the 123/124 oligonucleotide into the KpnI site of pT18ParF151GVPLK, another derivative was generated that unexpectedly was produced by ligation of two copies of the oligonucleotide thereby producing a ParF derivative with a 33 amino acid insertion between position 151 and 152 (pT18ParF*151GVPLK).

Plasmid pT18ParF*151GVPLK was linearized with KpnI, dephosphorylated and extracted from an agarose gel to be ready for ligation reactions (Figure 4.1, A). Phosphorylated 123/124 oligonucleotide and the digested plasmid were ligated and transformed into E. coli DH5α. Plasmids were extracted from ten candidate colonies and recombinant plasmids verified with one of the unique restriction site (StuI) introduced as part of the 123/124 oligonucleotide (Figure 4.1, B). The 57-bp in insertion in pT18ParF*151GVPLK was verified by sequencing (Figure 4.2).
Figure 4.1. Increasing the insertion size in pT18ParF*151GVPLK. A. Lanes: 1, 1kb ladder; 2, undigested pT18ParF*151GVPLK; 3, linearized pT18ParF*151GVPLK with KpnI. B. Verification of the pT18ParF*151\textsuperscript{57} recombinant plasmid with one of the unique restriction sites in 123/124 oligonucleotide. Here, StuI was used to verify the insertion of the 123/124. 1, 1-kb ladder; 2, undigested pT18ParF*151GVPLK; 3, undigested pT18ParF*151\textsuperscript{57}; 4, pT18ParF*151\textsuperscript{57} treated with StuI. Selected bands in the 1-kb ladder are indicated.

Figure 4.2. ABI sequence traces showing insertion of the 123/124 oligonucleotide into the KpnI site of the plasmid encoding ParF151GVPLK. A, 15-bp insertion between K151 and E152 in the parF sequence generated by PSM. B, Insertion of 42-bp oligonucleotide into the KpnI site within the 15-bp insertion increases the oligonucleotide size from 15-bp to 57-bp which in turn produces a 19 amino acid insertions instead of pentapeptide insertion. C, Insertion of two copies of oligonucleotide 123/124 into the KpnI site of pT18ParF*151GVPLK increases the size of the insertion from 15-bp to 99-bp. KpnI sites are marked in A, B and C panels with black lines. The 15-bp insertion, 123/124 oligonucleotide insertion and insertion of two copies of oligonucleotide 123/124 into the KpnI site of pT18ParF*151GVPLK in A, B and C are underlined with blue lines,
respectively. The different colours of amino acids in each panel represent the inserted sequences between the ParF residues (black). Amino acids derived from the original 15-bp insertion in all panels are in red and residues derived from the 123/124 oligonucleotide are in blue.

The 123/124 oligonucleotide was designed with a set of restriction sites (Smal, Ecl136, EcoRV, SnaBI, StuI and NruI) which produce compatible blunt ends (Figure 2.5) (Hayes et al., 1997). Digestion of pT18ParF*15157 with combinations of these enzymes and religation to delete the intervening sequence will decrease the insertion size from 57-bp to other in-frame sizes. First pT18ParF*151GVPLK was tested individually with Smal, Ecl136, EcoRV, SnaBI, StuI and NruI to determine the absence or presence of these sites which showed that Smal, StuI and SnaBI sites are not present in the plasmid (Figure 4.3). Thus, these sites in the 123/124 oligonucleotide will be unique in pT18ParF*57. In view of these unique sites in pT18ParF*15157 a set of shorter derivatives that contain 33-, 39- or 51-bp were constructed. Plasmids pT18ParF*15133, pT18ParF*15139 and pT18ParF*15151 were generated from pT18ParF*15157 by deleting 24-bp, 18-bp and 6-bp using digestion with Smal/StuI, Smal/SnaBI and SnaBI/StuI, respectively. The doubly digested samples (Figure 4.4) were extracted and purified from agarose gels, religated and transformed into E. Coli DH5α strain (Figure 4.4). Plasmids from ten samples in each case were extracted and recombinant plasmids verified by digestion of these pT18ParF*15157 derivatives with one of the enzymes within the deleted segment (Figure 4.5). The candidates with the correct digestion patterns were sequenced which verified the lengths of the insertions between codons 151 and 152 in parF (Figure 4.6).

In summary, the insertion and partial deletion of an in-frame, double-stranded synthetic oligonucleotide into the unique KpnI site in pT18ParF*151GVPLK generated five additional ParF mutant proteins with 11, 13, 17, 19 and 33 amino acids inserted between K151 and E152 in α6. The mutants were tested in two-hybrid assays to investigate the effects of these longer insertions on ParF self-association.
Figure 4.3. Agarose gel showing digestion of pT18ParF*151GVPLK with restriction enzymes present in the 123/124 oligonucleotide. Lanes: 1, 1-kb ladder; 2, undigested pT18ParF*151GVPLK; 3 to 8, pT18ParF*151GVPLK digested with SmaI, EcoRV, SnaBI, StuI and NruI restriction enzymes, respectively. Digested and undigested species are marked. The results show that pT18ParF*151GVPLK is not cut with SmaI, StuI or SnaBI. Selected bands in the 1-kb ladder are indicated.

Figure 4.4. Double digestion of pT181ParF*15157 for construction of derivatives with insertions shorter than 57-bp. Lanes: 1, 1-kb ladder; 2, undigested pT181ParF*15157; 3-5 pT181ParF*15157 digested with SmaI/StuI, SmaI/SnaBI and SnaBI/StuI, respectively, to delete 24-bp, 18-bp and 6-bp producing pT18ParF*15133, pT18ParF*15139 and pT18ParF*15151. Selected bands in the 1-kb ladder are indicated.

Figure 4.5. Verification of recombinant pT18ParF*15157 plasmids with insertions shorter than 57-bp. An example is shown of restriction analysis with one of the enzyme that cleaves within the deleted segments of the 123/124 oligonucleotide in pT18ParF*15157. Lanes: 1, 1-kb ladder; 2, undigested pT18ParF*15157; 3, pT18ParF*15157 linearized with StuI as a control; 4-5, candidate recombinant pT18ParF*15133 plasmids treated with StuI that is expected not to digest the plasmids; 6-7, candidate recombinant pT18ParF*15151 plasmids treated with StuI that is expected not to digest the plasmids. In analogous digests, pT18ParF*15139 candidates were treated with SmaI to confirm the deletion of the 12-bp that includes a SmaI site. Selected bands in the 1-kb ladder are indicated.
4.2.3 The effects of increasing the oligopeptide length between K151 and E152 on ParF self-association

The two-hybrid system was used to characterize the effects of increasing the oligopeptide length between K151 and E152 on ParF self-association. The pT18ParF\(^{*151}\), pT18ParF\(^{*151}\), pT18ParF\(^{*151}\), pT18ParF\(^{*151}\) and pT18ParF\(^{*151}\) plasmids and pT25ParF were cotransformed into \textit{E. coli} SP850 and colony colour on MacConkey-maltose plates and \(\beta\)-galactosidase assays were used to determine the effects of insertions between 11 and 33 amino acids on ParF self-association. MacConkey-maltose plates showed that none of the mutant proteins (ParF\(^{151}\), ParF\(^{151}\), ParF \(^{151}\), ParF\(^{151}\) and
ParF151\textsuperscript{33}) affected the interactions with wild-type ParF. The interactions were detectable as strong red colonies for all the derivatives (Figure 4.7 plate A, streak 3; plate B, streaks 3-6) similar to the colour intensities produced by self-association of wild-type ParF (Figure 4.7, plate A, streak 2; plate B, streak 2) on MacConkey-maltose agar plates.

Quantitative β-galactosidase assays were used to examine further the interaction efficiency of the five ParF derivatives with unmutated ParF (Figure 4.8 A). The activity of β-galactosidase was measured by co-transformation of pT18ParF*151\textsuperscript{33}, pT18ParF*151\textsuperscript{39}, pT18ParF*151\textsuperscript{51}, pT18ParF*151\textsuperscript{57} and pT18ParF*151\textsuperscript{99} plasmids with pT25ParF that encodes wild-type ParF into E. coli SP850. Each mutant was tested at least three times in three separate experiments. The pT18ParF* and pT18 plasmids were used as positive and negative controls, respectively. pT18ParF*151GVPLK was used as the second positive control (Chapter 3).

The data for β-galactosidase assays was in accordance with the results of colony colour on MacConkey-maltose plates. Interactions of ParF151\textsuperscript{11}, ParF151\textsuperscript{13}, ParF 151\textsuperscript{17}, ParF151\textsuperscript{19} and ParF151\textsuperscript{33} with wild-type ParF were recorded levels of enzymatic activity that were very similar to self-association of wild-type ParF (~1300 β-galactosidase units) (Figure 4.8A). Altogether, the two-hybrid data showed that increasing the oligopeptide insertion size between K151 and E152 in α6 did not affect the interaction with wild-type ParF protein. The results may reflect a high flexibility of this region in ParF in which insertions as long as 33 amino acids are tolerated and do not alter the association with the wild-type protein. In addition, these results suggest that the α6 element region is not crucial for the stability or proper folding of the ParF protein. Although a pentapeptide insertion between K151 and E152 disrupted partition activity (Chapter 3.10), this defect may be attributable to another function for this region of ParF in plasmid partition. Notably, the ParF151GVPLK protein is defective in the interaction with ParG.
4.2.4 The effects of increasing the oligopeptide length between K151 and E152 in ParF on ParG interaction

A pentapeptide insertion at position 151 abolishes the interaction of ParF with ParG in two-hybrid assays (Chapter 3.12). The two-hybrid system also was used to characterize the effects of increasing the oligopeptide length between K151 and E152 in ParF on the interaction with ParG. Plasmids pT18ParF*8151$^{33}$, pT18ParF*151$^{39}$, pT18ParF*151$^{51}$, pT18ParF*151$^{57}$ and pT18ParF*151$^{99}$ were co-transformed with pT25ParG into E. coli SP850. A white-pink colony colour was observed on MacConkey-maltose plates for all five mutant proteins (Figure 4.16, plate A, streak 6; plate C, streaks 3-6) similar to the colour produced by the empty vector (Figure 4.16, plate C, streak 1). These results were consistent with the data obtained from β-galactosidase assays (Figure 4.17B) in which the mutant proteins produced levels of β-galactosidase similar to that observed with the empty vector. Thus, as noted previously with the pentapeptide insertion between K151 and E152 (Chapter 3.12) (Figure 4.16, plate C, streak 7), increasing the oligopeptide size to 11, 13, 17, 19 or 33 residues at this position in ParF abolished the interaction with ParG. These results imply that α6 in ParF participates in the recruitment of the protein by ParG to the segregation apparatus.

Collectively, the results showed that ParF mutants with oligopeptide insertions up to 33 amino acids still associate with wild-type ParF but are impaired in the interaction with ParG.

Figure 4.7 (overleaf). Association of ParF151$^{11}$, ParF151$^{13}$, ParF151$^{17}$, ParF151$^{19}$ and ParF151$^{33}$ with wild-type ParF and with ParG in two-hybrid assays. Plasmids pT18ParF*151$^{33}$, pT18ParF*151$^{39}$, pT18ParF*151$^{51}$, pT18ParF*151$^{57}$ and pT18ParF*151$^{99}$ were cotransformed into E. coli SP850 with pT25ParF or pT25ParG. MacConkey-maltose medium was used to observe the colony colour after 36-48 hours incubation at 30°C.
Figure 4.8. Two-hybrid analysis of ParF151\textsuperscript{19}, ParF151\textsuperscript{33}, ParF\textsuperscript{11}, ParF151\textsuperscript{13} and ParF151\textsuperscript{17}. $\beta$-galactosidase assays were used to determine the interactions of mutant ParF proteins with wild-type ParF (A) and with ParG (B). Mutant ParF proteins were produced from pT18 and wild-type ParF and ParG were produced from pT25. Black, pT18ParF*151\textsuperscript{57}, pT18ParF*151\textsuperscript{99}, pT18ParF*151\textsuperscript{33}, pT18ParF*151\textsuperscript{13} or pT18ParF*151\textsuperscript{17} with pT25ParF or pT25ParG; green, pT18ParF* with pT25ParF; red, pT18ParF* with pT25ParG; green, blue, pT18 with pT25ParF* or pT25ParG. $\beta$-galactosidase enzymatic activities are expressed as Miller units. For each mutant, assays were performed at least three times in three independent experiments and the data are shown as mean ± standard deviation.
4.3 Site-directed Mutagenesis of ParF151 and ParF152

Results of bacterial two-hybrid assays (Chapter 3) revealed that positions 151/152 in the ParF protein may be crucial for the interaction with ParG in the segregation cassette of the TP228 plasmid. To investigate further the role of residues 151 and 152, OE-PCR was used as outlined in Chapter 2.14 to construct mutations encoding ParFK151A, ParFE152A, ParFΔ151 and ParFΔ152 in the pT18ParF* two-hybrid plasmid and in the pH547 partition assay plasmid. In certain cases the mutations were sub-cloned between these plasmids using restriction fragment exchange. Positions K151 and E152 were substituted to alanine and deleted separately in ParF. The latter was applied to test the effects of the deleted single amino acids on ParF function in vivo. In addition, the effects of these site-directed mutations were compared with those observed with the insertion of five amino acids between positions 151 and 152 in two-hybrid and plasmid partition assays. The conformational change produced by alanine substitution in a protein is very low and is considered the best choice for substitution mutagenesis (Ziolkowska et al., 2006). Plasmid pT18ParF was used initially as a template for all of the site-directed mutation of residues 151 and 152 (Barillà and Hayes, 2003). OE-PCR mutagenesis involves rounds of amplification using two external primers (P1/P4) and two mutagenic primers (P3/P2). The latter were complementary to each other and contained single codon mutations in each case. Figure 4.9 shows an example of primers used to generate the parF gene encoding ParFK151A.

Briefly PCR1 (~200-bp) and PCR2 (~100-bp) were generated using one external and one internal primers in each round of PCR amplification (P1/P2 and P3/P4, respectively). Sizes of amplified PCR products were confirmed on agarose gels (Figure 4.10A) and the two products were used to amplify the full length PCR3 (~300-bp) products using the two external primers P1 and P4 in all mutations (Figure 4.10B). Substitution or deletion of K151 and E152 in ParF required using P1 and P4 primers (ParF-P1-Forward and ParF-P4-Revese) in all cases. In contrast, P2 and P3 were unique for each mutation and corresponded to ParFP2-151-Reverse/ParFP3-151-Forward, ParFP2-152-Reverse/ParFP3-152-Forward, ParF-P2-Δ151-Reverse/ParF-P3-Δ151-Forward and ParF-P2-Δ152-Reverse/ParF-P3-Δ152-Forward (Table 2.2).
Figure 4.9. Schematic diagram of OE-PCR for generating the gene encoding ParFK151A. Primers (P1, P2, P3 and P4) and restriction sites are highlighted in red and blue, respectively. In this figure, primers were designed for producing the gene encoding ParFK151A. P1 and P4 are external primers used with P2 and P3, respectively. In P2 and P3 primers the codon AAA that encodes K151 was changed to GCA that encodes alanine (green). For producing genes encoding ParFE152A, ParFΔ151 and ParFΔ152, the same P1 and P4 primers were used whereas P2 and P3 contained the required mutations (Table 2.2).

Figure 4.10. Examples of agarose gel analysis of OE-PCR products for producing the genes encoding ParFK151A, ParFE152A, ParFΔ151 and ParFΔ152 for cloning into pT18ParF* and pFH547 in place of the wild-type parF gene. A, Lanes: 1, 100-bp ladder; 2 and 3, PCR1 and PCR2 products for the gene encoding ParFK151A, respectively; 4 and 5, PCR1 and PCR2 products for the gene encoding ParFE152A, respectively. B, Lanes: 1, 100-bp ladder; 2-5, PCR3 products for the genes encoding ParFK151A, ParFE152A, ParFΔ151 and ParFΔ152, respectively. Selected bands in the 100-bp ladder are indicated.
The wild-type sequences in pT18ParF* and pFH547 were replaced by PCR3 fragments that contained mutations of codons 151 and 152 in parF. To achieve this, the PCR3 products and pT18ParF* and pFH547 plasmids were digested with SexAI and ClaI (Figure 4.9) and relevant fragments were recovered from agarose gels. The SexAI and ClaI sites within the parF gene are located on either side of the mutation (Figure 4.9). Note that the SexAI recognition site is blocked by Dam methylation, therefor the plasmids were transformed into the E. coli SCS110 Dam- strain before manipulation. The fragments were ligated and transformed into E. coli DH5α. Twelve colonies were picked from transformation plates for each mutagenesis, and plasmid DNA was extracted and verified by restriction digestion with EcoRI which generates three fragments in the case of pT18ParF* (Figure 4.11). These digests confirmed that the general structures of the plasmids were correct, but did not indicate that the required mutations were present. Sequencing of selected candidate plasmids verified that the K151A and E152A mutations had been introduced and that no other mutations were present (data not shown). In contrast, mutated pFH547 plasmids were verified directly by sequencing (Figure 4.13).

Figure 4.11. Verification of recombinant pT18ParF* derivatives encoding ParFK151A and ParFE152A using EcoRI restriction digestion. Lanes: 1, 1-kb ladder; 2, undigested pT18ParF*; 3, EcoRI digested pT18ParF*; 4-7, pT18ParF*K151A candidate plasmids digested with EcoRI; 8-10, pT18ParF*E152A candidate plasmids digested with EcoRI. All candidate plasmids demonstrated the same pattern as the parental pT18ParF* plasmid (Lane 3 and Figure 3.14). A, bulk of pT18ParF*backbone; B, 711-bp fragment; C, 445-bp fragment that contains most of the parF gene bearing single codon mutation generated by OE-PCR. Selected bands in the 1-kb ladder are indicated.

Overlap-PCR was used successfully to construct the genes encoding ParFK151A and ParFE152A in pT18ParF* and ParFΔ151 and ParFΔ152 in pFH547. However, cloning of the genes encoding ParFK151A and ParFE152A in pFH547 and ParFΔ151 and ParFΔ152 in
pT18ParF* was not successful in repeated OE-PCR mutagenesis trial. Therefore restriction fragment exchange was applied using SexAI and ClaI sites to sub-clone genes encoding ParFK151A or ParFE152A and ParFΔ151 or ParFΔ152 from pT18ParF* and pFH547 derivatives, respectively, in place of the wild-type parF gene. Plasmids pT18ParFK151A, pT18ParFE152A, pFH547ParFΔ151 and pFH547Δ152 were digested with SexAI and ClaI to obtain four 230-bp fragments. The pFH547 and pT18ParF* plasmids were digested with the same restriction enzymes to liberate the wild-type sequences. Then, pFH547 and pT18ParF* backbones and the four 230-bp fragments were extracted from an agarose gel (Figure 4.12). The relevant fragments were ligated and transformed into E. coli DH5α. Plasmid DNA was extracted from selected transformants. Sequence analysis verified that the wild-type sequence in pFH547 and pT18ParF* was changed to the sequence encoding ParFK151A and ParFE152A in pFH547 and to ParFΔ151 in pT18ParF* (Figure 4.13). However, repeated attempts to replace the wild-type parF gene in pT18ParF* with a sequence that encodes ParFΔ152 was unsuccessful. The parFΔ152 gene may be unstable or toxic in the context of the two-hybrid vector. In summary, the ParFK151A, ParFE152A, ParFΔ151 and ParFΔ152 mutations were introduced by OE-PCR or by restriction fragment exchange in both the partition assay plasmid pFH547 and the two-hybrid plasmid pT18ParF*, except for the ParFΔ152 change that was not cloned in the latter.

Figure 4.12. Example agarose gel analysis of pT18ParF*K151A, pFH547Δ151 and pFH547Δ152 for exchanging the genes encoding ParFK151A, ParFΔ151 and ParFΔ152 from these plasmids into pFH547 and pT18ParF*, respectively. A. Lanes: 1, 1-kb ladder; 2, undigested pT18ParF*K151A; 3, digested pT18ParF*K151A with SexAI and ClaI. B. Lanes: 1, 1-kb ladder; 2, undigested pFH547Δ151; 3 and 4, digested pFH547Δ151 and pFH547Δ152 with SexAI and ClaI. In A and B, the 230-bp SexAI-ClaI fragments that contain the relevant parF mutations are marked. These fragments were extracted and cloned in place of the wild-type sequences in pFH547 plasmid and pT18ParF*, respectively. Selected bands in the 1-kb ladder are indicated.
Figure 4.13. ABI sequences traces showing sections of the wild-type parF sequence and the equivalent regions with mutations of codons 151 or 152 in pFH547. The 21-bp mutagenic P3 primers for each mutation and the equivalent wild type sequence are underlined. Codons 151 and 152 are marked with black lines above the sequences.

4.3.1 The impact on plasmid partitioning of mutating K151 and E152 in the ParF protein.

The effect of single substitutions and deletions of K151 and E152 in ParF was assessed using the partition assay described in previous chapters (Chapter 2.25). Plasmids pFH547 and pFH450 again were used as positive and negative controls, respectively, in the BR825 polA mutant strain. The level of plasmid retention conferred by wild-type parFGH cassette in pFH547 was ~70%, whereas the pFH450 vector showed ~3% retention (Figure 4.14). These values agree with those published previously (Hayes, 2000) and with those presented in Chapter 3. The K151A substitution in ParF reduced plasmid retention almost to the level of the empty vector (~8%). This result suggests that the K151A substitution either affects a critical function of ParF or alters the conformation of the protein so that it is no longer active. In contrast, the E152A mutation did not affect plasmid segregation suggesting that the ParF protein function and structure are not affected by this change.
Deletion of K151 or E152 residues potentially involves more radical changes in α6-helix of ParF than substitution mutations. The plasmids producing ParFΔ151 or ParFΔ152 showed levels of retention (<10%) similar to those of the empty vector. Indicating that deletion of K151 or E152 strongly reduced plasmid segregation activity by ParF (Figure 4.14). These findings indicate that deletion of K151 or E152 and the K151A substitution had strong effects on impairment of plasmid segregation, whereas the E152A change did not have any deleterious effect. In summary, the data show that K151 or E152 in ParF fulfil a crucial role in plasmid segregation.

**Figure 4.14. Effects of substitution and deletion mutagenesis of K151 or E152 in ParF on plasmid segregation.** Partition assays were performed to assess the efficiency of the wild-type and mutated parFGH cassettes after growth for ~25 generation under non-selective conditions. Plasmid retention was determined as percentages for the ParF mutants (blue bars). The negative (red bar) and positive (green bar) controls showed ~3% and ~70% plasmid retention, respectively. Data represent the means of at least three independent experiments conducted in triplicate ± standard deviations.
4.3.2 Effects of site-direct mutagenesis of K151 and E152 on ParF self-association

Mutant ParF proteins (ParFK151A, ParFE152A and ParFΔ151) were tested in two-hybrid system to observe the effects of substitution and deletion of K151 or E152 in ParF on protein self-association \textit{in vivo}. pT18ParF* and pT18 were used as positive and negative controls, respectively, as described in detail in Chapters 2.19. The assays were performed by co-transformation of pT181ParF* mutant derivatives and pT25ParF into \textit{E. coli} SP850. The effects of the mutations on ParF self-association were investigated using MacConkey-maltose agar plates and a quantitative β-galactosidase assay.

The K151 or E152 substitutions and deletion of the former position did not affect colony colour in the two-hybrid assay compared to the colour detected when wild-type ParF was produced from both vector plasmids (Figure 4.15, plate A, streaks 3 and 4; plate C, streak 4). These qualitative results suggest that ParF self-association is not affected either by substitution changes of K151 or E152 and that even the deletion of K151 did not abrogate the ParF-ParF interaction.

To investigate the effects of the K151 and E152 changes in further detail, the interactions between wild type ParF protein and mutant proteins in the two-hybrid set-up were measured using β-galactosidase assays. ParFK151A, ParFE152A and ParFΔ151 produced \(\sim 1000\) β-galactosidase units with wild-type ParF which was similar to the self-association of wild-type ParF. In contrast, the negative control showed very low values of β-galactosidase activity (\(\sim 80\) units) (Figure 4.16).

In summary, both colony colour assessment on MacConkey maltose agar plates and β-galactosidase measurements showed that the interaction of ParFK151A, ParFE152A and ParFΔ151 proteins with wild-type ParF was unaltered in the two-hybrid system. As the ParFK151A and ParFΔ151 mutations affected plasmid partitioning (see above), the results suggest that K151 and E152 are crucial for ParF-mediated plasmid segregation but that mutation of these residues affects a function other than ParF self-association. Similar results were observed with the pentapeptide insertion between positions 151 and 152 in ParF (Chapter 3).
4.3.3 The effects of substitution and deletion mutagenesis of K151 and E152 in ParF on ParG interaction

*In vivo* interactions between ParF and ParG are detectable in the two-hybrid system (Barillà and Hayes, 2003). This assay was used again to assess the impact of the K151 and E152 changes in ParF on the interaction with ParG. Plasmids pT18ParF*K151A, pT18ParF*E152A and pT18ParF*Δ151 were cotransformed into SP850 with pT25ParG. Screening of colonies on MacConkey-maltose plates and β-galactosidase assays were performed to monitor the effects of the mutations on the interaction with ParG. The positive and negative controls for the interaction between wild-type ParF and ParG were detected as a strong red and white-pink colonies, respectively, on MacConkey-maltose plates (Figure 4.15, plate B, streak 2 and 1; plate C, streak 3 and 2), and as ~1200 and ~35 β-galactosidase units for the ParF-ParG interaction and the negative control, respectively (Figure 4.17). Interestingly, the results with MacConkey-maltose plates showed that the interaction of ParFK151A and ParFΔ151 proteins with wild-type ParG was disrupted in both cases: white-
pink colonies were detected for both mutants with ParG on MacConkey-maltose plates (Figure 4.15, plate B, streak 3; plate C, streak 1). The results were in agreement with the β-galactosidase values for both mutants which were similar to the levels of the negative control (Figure 4.17). In contrast, the E152A substitution in ParF did not abrogate the interaction of the protein with wild type ParG assessed either on MacConkey-maltose medium (Figure 4.15, plate B, streak 4) or by β-galactosidase assays (Figure 4.17).

![Figure 4.17. Two-hybrid analysis of ParG interactions with substitution and deletion variants of residues K151 and E152 in ParF. β-galactosidase assays were used to measure the levels of interaction of mutant ParF proteins with wild-type ParG in strain SP850. The horizontal axis displays the ParF mutants produced from pT18ParF* with ParG fused to the T25 fragment (black bars). The vertical axis represents β-galactosidase enzymatic activity expressed as Miller units. Red and blue bars represent the positive control (pT25ParG + pT18ParF*) and the negative control (pT25ParG + pT18), respectively. Assays were performed at least three times in three independent experiments for each mutant and the data are shown as the mean ± standard deviation.](image)

Overall, the results of site-directed mutagenesis of residues K151 and E152 in ParF pinpointed that mutation of the former impaired both plasmid partitioning and the interaction with ParG. By contrast, the E152A affected neither activity (Table 4.1). Although the deletion of E152 was not tested in the two-hybrid system, partition assays indicated that deletion of this residue in ParF exerted a negative impact on plasmid segregation activity (Section 4.3.1; Figure 4.14). In summary, the data particularly suggest
an important role for residue K151 in the α6 of ParF in the interaction with the ParG partner protein (Table 4.1).

### Table 4.1 Summary of effects of site-directed mutagenesis on ParF activity

<table>
<thead>
<tr>
<th>ParF mutant</th>
<th>ParF Interaction</th>
<th>ParG Interaction</th>
<th>Plasmid Segregation</th>
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<tr>
<td>Wild-type</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ParFK151A</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ParFE152A</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ParFΔ151</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ParFΔ152</td>
<td>NT</td>
<td>NT</td>
<td>-</td>
</tr>
</tbody>
</table>

NT, not tested because the cloning of parFΔ152 into two-hybrid vector was not successful; +, mutation did not affect ParF self-association, ParF-ParG interaction and plasmid segregation; -, mutation affected ParF self-association, ParF-ParG interaction and plasmid segregation; ±, mutation showed medium levels of ParF self-association, ParF-ParG interaction and plasmid segregation.

Taking together the insertion and site-directed mutagenesis results of K151/E152 in α6, deletion or substitution of K151 abolished the proper function of ParF protein in plasmid segregation, reducing the partition activity to the level of the empty vector (Section 4.3.1) (Table 4.1). A pentapeptide insertion between these residues exerted a similar effect on partitioning (Chapter 3.10). The pentapeptide insertion, between K151 and E152, and substitution and deletion of K151 detectably disrupted the interaction with the partner protein ParG in two-hybrid analysis, but did not affect the association of the mutant proteins with wild-type ParF. Similarly, extending the size of the oligopeptide between K151 and E152 to as much as 33 residues did not impact the interaction with wild-type ParF. In contrast, the E152A mutation had no detectable effect on ParF function (Table 4.1). It is likely that K151 plays an important role in mediating the interaction with ParG in the segosome and that disrupting this interaction has a profound impact on plasmid segregation. Nevertheless, the overall tertiary structure of the ParF protein apparently was not perturbed by the various mutations in α6 as the association with wild-type ParF was maintained even when K151 was deleted or a 33 residue oligopeptide was inserted at the C-terminal side of this amino acid.
Chapter 5: Results III

Testing the effects of oligopeptide length at promiscuous sites in ParF and truncation of the C-terminal end of the protein on ParF function
5.1 Increasing the oligopeptide size in ParF pentapeptide mutants which displayed wild-type partition activity

5.1.1 Background

Insertion of variable five amino acids by PSM produced nine unique pentapeptide insertions in ParF protein. The simple, rapid mutagenesis depended on insertion and excision of Tn4430 into the target gene leaving a unique KpnI site (Hayes and Hallet, 2000). The remaining KpnI site was utilized to generate oligopeptide insertions larger than five amino acids using synthetic oligonucleotide 123/124 (Figure 2.5). Three ParF pentapeptide mutants were proficient in plasmid partitioning to a level similar to that conferred by the wild-type ParF (Chapter 3.10). Two-hybrid analysis showed that these mutants associated effectively with wild-type ParF and also interacted normally with the partner protein ParG (Chapter 3.11 and 3.12). Insertions of pentapeptides at positions 160 in β7 and 185 in the turn before α8 generated ParF160GVPLK and ParF185RGTPS proteins, respectively, which are two of the three partition-proficient mutants. To investigate the impact of longer insertions in ParF at these positions on the stability functions of the partition cassette of the TP228 plasmid, pFH547-160GVPLK and pFH547-185RGTPS were selected for insertional analysis using the synthetic oligonucleotide insertion strategy (Chapter 2.23 and Figure 2.5) at the unique KpnI site generated by PSM. In brief, a strategy similar to that applied for increasing the length of the oligopeptide between K151 and E152 in ParF (Chapter 4.2.2) was used to generate ParF derivatives that contained between five and 19 amino acid insertions between positions 160 and 161 and between 185 and 186. In addition, a pentapeptide insertion was previously identified at position 205 located at the C-terminus of the ParF protein (Chapter 3.5). The mutant showed wild-type activities in association with ParF and in interaction with ParG. Therefore, this position was subjected to site-directed mutagenesis to investigate the influence of this region on ParF function (Section 5.2).

5.1.2 Increasing the oligopeptide length in ParF pentapeptide mutants which displayed positive partition activity

Insertion of five amino acids between either K160 and A161 or S185 and G186 in ParF by PSM did not abrogate plasmid partitioning activity, ParF self-association or interaction with ParG. In contrast a pentapeptide insertion between K151 and E152 disrupted ParF-mediated partition activity and the interaction with ParG, but not the association with ParF (Chapter 3.10, 3.11 and 3.12). We aimed to examine the function of ParF mutant proteins which conferred normal plasmid retention to determine the regions that participate functionally in
plasmid segregation and to identify those regions that are promiscuous for insertions. The 123/124 oligonucleotide (Chapter 2.23) was cloned into the KpnI site of pFH547-60GVPLK and pFH547-185RGTPS plasmids to increase the length of the insertions from five to 19 amino acids. Plasmids pFH547-160GVPLK and pFH547-185RGTPS were linearized with KpnI, extracted from an agarose gel and were prepared for ligation reaction (Figure 5.1, A). Linearized plasmids and the phosphorylated 123/124 oligonucleotide were ligated and transformed into strain E. coli DH5α. Recombinant plasmids were obtained from ten candidate colonies of each mutant and verified with StuI digestion which indicated the insertion of the oligonucleotide (Figure 5.1, B). The final verification of 57-bp insertion was by sequencing results (Figure 5.2). As a result ParF derivatives with 19 amino acids insertions were produced in β7 and the turn before α8. The mutants were named pFH547-160^{57} and pFH547-185^{57}.

**Figure 5.1. Increasing the insert size in pFH547GVPLK and pFH547185RGTPS.** A. Lanes: 1, 1-kb ladder; 2, undigested pFH547-160GVPLK; 3 and 4, pFH547-160GVPLK and pFH547-185RGTPS, respectively, linearized with KpnI. B. Verification of the pFH547-160GVPLK:123/124 and pFH547-185RGTPS recombinant plasmids with one of the unique restriction sites (StuI) in the 123/124 oligonucleotide. 1, 1-kb ladder; 2, undigested pFH547-160^{57}; 3 and 4, pFH547-160^{57} and pFH547-185^{57}, respectively, treated with StuI. Selected bands in the 1-kb ladder are indicated.
Figure 5.2. ABI sequence traces showing insertion of the 123/124 oligonucleotide into the \textit{KpnI} site of the plasmids encoding \textit{ParF160GVPLK} and \textit{ParF185RGTPS}. A and C, 15-bp insertions generated by PSM at K160 and S185 in the \textit{parF} sequence, respectively. B and D, Increasing the insertion size from 15-bp to 57-bp by cloning of a 42-bp oligonucleotide into the \textit{KpnI} site within the 15-bp insertion which results in 19 amino acid insertions instead of pentapeptide insertions between K160 and A161 or S185 and G186, in the \textit{ParF} sequence. \textit{KpnI} sites are marked with black lines in panels A, B, C and D. The 123/124 oligonucleotide insertion into the \textit{KpnI} site of pFH547-160GVPLK and pFH547-185RGTPS in panels B and D are underlined in blue. The different colours of amino acids in each panel represent the inserted sequences of amino acids between the \textit{ParF} residues (black). Amino acids derived from the original 15-bp insertion are in red and residues derived from the 123/124 oligonucleotide are in blue.
A shortening strategy was applied to generate insertions less than 57-bp in pFH547-160\textsuperscript{57} and pFH547-185\textsuperscript{57}. The cloned oligonucleotide contained a set of restriction sites which generate blunt ends (Figure 2.5) (Hayes et al., 1997) as described in detail in Chapter 4.2.2. Briefly, digestion of pFH547-160\textsuperscript{57} and pFH547-185\textsuperscript{57} at combinations of the restriction sites in the 123/124 oligonucleotide and religation was performed to delete the intervening sequence which decreased the insertion size from 57-bp to other in-frame sizes. The absence or presence of the SmaI, EcoRI, SnaBI, StuI and NruI sites in pFH547 was determined by SnapGene software. Data showed that SmaI, SnaBI and EcoRV sites are not present in the plasmid (data not shown). As a result these sites in the inserted 123/124 oligonucleotide were unique. Depending on these data five additional mutants were constructed from pFH547-160\textsuperscript{57} and pFH547-185\textsuperscript{57} with shorter insertions that comprised 39-, 45- or 51-bp and 45- or 51-bp, respectively.

Plasmids pFH547-160\textsuperscript{39}, pFH547-160\textsuperscript{45}, pFH547-160\textsuperscript{51} and pFH547-185\textsuperscript{45} were produced from pFH547-160\textsuperscript{57} by deleting 18-bp, 12-bp and 6-bp using digestion with SmaI/SnaBI, SmaI/EcoRV and EcoRV/SnaBI, respectively. In addition, plasmids pFH547-185\textsuperscript{51} and pFH547-185\textsuperscript{51} were generated from pFH547-185\textsuperscript{57} by deleting 18-bp and 12-bp using digestion with SmaI/SnaBI and SmaI/EcoRV, respectively. Plasmids pFH547-160\textsuperscript{57} and pFH547-185\textsuperscript{57} treated by double digestion with appropriate restriction enzymes (Figure 5.3) were extracted from agarose gels, religated and transformed into strain E. coli DH5α. Plasmids were extracted from ten samples in each case and digestion with one of the enzymes within the deleted sequence was used for verification of the pFH547-160\textsuperscript{57} and pFH547-185\textsuperscript{57} derivatives (Figure 5.4). The samples with the correct digestion patterns were sequenced and the length of the insertions verified by sequencing results (Figure 5.5).

Taking the above results together, an in-frame synthetic oligonucleotide insertion into the unique KpnI site in pFH547-160GVPLK and pFH547-185RGTPS and partial deletion of this inserted oligonucleotide produced an additional seven ParF mutant proteins with 13, 15, 17 and 19 amino acids inserted between K160 and A161 and S185 and D186 in ParF, respectively. The mutants were tested in partition assays to investigate the effects of longer oligopeptide insertions instead of pentapeptide on plasmid segregation activity.
Chapter 5

Results

Figure 5.3. Construction of pFH547-160^{57} and pFH547-185^{57} derivatives with insertions shorter than 57-bp by double digestion with combinations of restriction enzymes with unique sites in the 57-bp sequence and religation (preparative digests used to produce the mutants with shorted inserts). Lanes: 1, 1-kb ladder; 2, undigested pFH547-160^{57}; 3-5, pFH547-160^{57} digested with SmaI/SnaBI, SmaI/EcoRV and EcoRV/SnaBI, respectively, to delete 24-bp, 18-bp and 6-bp producing pFH547-160^{39}, pFH547-160^{45} and pFH547-160^{51}; 6 and 7, pFH547-185^{57} digested with SmaI/SnaBI and Smal/EcoRV, respectively, to delete 18-bp and 12-bp generating pFH547-185^{39} and pFH547-185^{45}. Selected bands in the 1-kb ladder are indicated.

Figure 5.4. Verification of recombinant pFH547-160^{57} and pFH547-185^{57} plasmids with insertions shorter than 57-bp. An example of restriction enzyme analysis with one of the enzymes that cleaves within the deleted segments of the 123/124 oligonucleotide in pFH547-160^{57} and pFH547-185^{57}. Lanes: 1, 1-kb ladder; 2, undigested pFH547-160^{57}; 3-6, candidate recombinant pFH547-160^{39}, pFH547-160^{45}, pFH547-185^{39} and pFH547-185^{45} plasmids treated with SmaI that is expected not to digest the plasmids; 7, candidate recombinant pFH547-160^{51} plasmid digested with EcoRV that is expected not to digest the plasmid. Selected bands in the 1-kb ladder are indicated.
Figure 5.5. ABI traces showing the sequences of the pFH547-16039, pFH547-16045, pFH547-16051, pFH547-18539 and pFH54718545 plasmids. A, 123/124 oligonucleotide sequence cloned in pFH547-16057 and pFH547-18557 with the restriction sites used in shortening the insertion size marked with arrows. B - F, sequence traces of the generated pFH547-16057 and pFH547-18557 derivatives with the restriction sites used in shortening the insertion size marked. The remaining 39, 45, or 51-bp insertions are underlined. KpnI sites in all panels are over lined. Amino acids derived from the original 15-bp insertion in pFH547-16057 and pFH547-18557 are in red and residues derived from the 123/124 oligonucleotide are in blue.
5.1.3 The effects of increasing the oligopeptide length between K160 and A161 and S185 and D186 in ParF on plasmid partitioning

Six pentapeptide ParF mutant proteins produced by PSM impaired plasmid segregation, whereas the three remaining mutants displayed levels similar to wild-type ParF activity (Chapter 3.10). The insertions of five amino acids at positions K160, S185 and F205 did not perturb the function of ParF protein in partition assays. Therefore, further mutagenesis was applied by inserting and deleting sequences of an in-frame synthetic oligonucleotide in the unique KpnI site of ParF160GVPLK and ParF185RGTPS as described above. Seven mutants were generated and tested in partition assays. In addition the C-terminal end of ParF was subjected to site-directed mutagenesis (see Section 5.2) to investigate the role of this region in ParF function.

The impact of insertions longer than five amino acids between positions K160/A161 and S185/S186 were determined using partition assays (Chapter 2.25). Similar to previous tests, the ParF mutants were assessed in the E. coli BR825 polA mutant strain with pFH547 and pFH450 used as positive and negative controls, respectively. Plasmid pFH547 that harbours the wild-type parFGH cassette and the empty vector pFH450 showed ~70% and ~3% levels of plasmid retention, respectively, which are in agreement with those observed previously (Hayes, 2000) and with those mentioned in Chapter 3. In addition, pFH547-160GVPLK and pFH547-185RGTPS were tested again to compare with the results of their derivatives pFH547-160\textsuperscript{39}, pFH547-160\textsuperscript{45}, pFH547-160\textsuperscript{51}, pFH547-160\textsuperscript{57}, pFH547-185\textsuperscript{39}, pFH547185\textsuperscript{45} and pFH547185\textsuperscript{57}.

Insertions of 13, 15, 17 or 19 amino acids between K160 and A161 reduced plasmid segregational stability to very low levels (~10%) (Figure 5.6). These results show that the β7 element in ParF does not tolerate insertions larger than five amino acids. Larger oligopeptide insertions may affect more dramatically the conformation of the β strand that is located in the core of the protein compared to the pentapeptide insertion that is well-tolerated at this position. In contrast, insertion of 13 and 15 amino acids between S185 and S186 in ParF decreased partition function (~45%) only slightly compared to the pentapeptide insertion at this position (~55%) (Figure 5.6). This region of the ParF protein may be more tolerant to oligopeptide insertions as insertions in loops are predicted generally to be less deleterious to protein function than insertions in α-helices or β-sheets (Hayes and Hallet, 2000). However, insertion of 19 amino acids between S185 and S186 in
ParF reduced plasmid retention levels almost to the level of the empty vector (Figure 5.6). This result suggests that the conformational change produced by a 19 amino acid insertion disrupted the function of ParF more dramatically than the 13 and 15 residues insertions.

In summary, these preceding results suggest that the β7 element that includes K160 and A161 and the loop near the C-terminus of the protein that includes S185 and S186 both are required for the function of ParF, although these regions display different tolerances to oligopeptide insertions. Analysis of these mutants in the two-hybrid assay may clarify the effects of longer oligopeptides at these positions on ParF self-association and interaction with ParG.

Figure 5.6. Effects of increasing the oligopeptide sizes between K160 and A161 or S185 and S186 on plasmid segregation. Plasmid segregation assays were used to test the effects of oligopeptide insertions larger than pentapeptide in ParF on the efficiency of plasmid partitioning after growth for ~25 generation under non-selective conditions with the wild-type ParF. Plasmid retention was determined as percentages for the pFH547 derivatives producing wild (green bar) or mutant ParF proteins (blue bars) and the empty vector (red bar). Data represent the means of at least three independent experiments conducted in triplicate ± standard deviations.
5.2 C-terminal truncation of ParF reveals a mutant that is partition-defective but that can interact with wild-type ParF and ParG

5.2.1 Background

PSM analysis of ParF involved constructing a mini-library of mutants for dissecting structure-function relationship in the protein. PSM generated nine unique pentapeptide ParF proteins with a variety of phenotypes in vivo (Chapter 3). The method is based on insertions of five amino acids at random positions in the target protein by transposition and excision of the Tn4430 element (Chapter 2.19). Partition and two-hybrid assays indicated that insertions of five amino acids at certain positions in the ParF protein caused the disruption of protein function. Pentapeptide insertions at V67, K71, T164, A165 and I166 positions exerted severe defects in both tests suggesting that these positions are crucial for the stability and/or folding of the ParF protein. In contrast, insertions at K160, S185 and F205 did not perturb the activity of ParF either in two-hybrid analysis or in partition assays, whereas the insertion at position K151 impaired partition activity and interaction with the partner protein ParG but not ParF self-association.

The length of the oligopeptide was increased at position 151 and substitutions and deletions of K151 and E152 in α6 of ParF also were generated. The partition activity of ParF mutants was impaired by these changes, but the overall structure of the protein apparently was not affected by these different mutations in α6 as ParF self-association was observed with all the mutants in two-hybrid assays (Chapter 4.2.3 and 4.3.2). Instead results revealed that residues in this helix may contribute in the recruitment of ParG to the plasmid partitioning complex which is a novel observation. Interestingly, among the pentapeptide mutant proteins that were unaffected in segregation activity, self-association or the interaction with ParG was a protein with an insertion between the penultimate and the most C-terminal residues in ParF. The sequence in this region was changed from 205-FE-206 to 205-FGVPLFE-211 (Table 3.2).

The contribution of the C-terminus of ParF to segregation function has not been analysed previously. To characterize further the function of the ParF protein, we here subjected the C-terminus to site-directed mutagenesis by truncation of the protein. C-terminal truncation of numerous proteins has resulted in the identification of new phenotypes associated with protein activity (Lambert et al., 2010; Williams et al., 2016).
5.2.2 Elimination of the last three amino acids of the ParF protein

ParF205GVPLF was generated in the two-hybrid vector system by PSM and was constructed in pFH547 by OE-PCR (Chapter 3.9). Insertion of these five amino acids at position F205 did not affect the partition activity of the mutant protein that also was found to self-associate and interact with ParG similarly as wild-type ParF (Chapter 3.10, 3.11 and 3.12). Therefore, this region was subjected to site-directed mutagenesis to elucidate the effects of deletion mutations of this C-terminal region on ParF protein function. Residues 206, 205-206 and 204-206 were deleted separately to test the effects of these deletions on the activity of ParF in performing plasmid segregation in plasmid partition assay (pFH547). Mutations encoding ParFΔ206, ParFΔ205-206 and ParFΔ204-205 were constructed in the pFH547 partition assay plasmid by OE-PCR (Chapter 2.14). The pFH547 plasmid was used as template for all of the mutations. The mutant parF fragments were generated using two sets of external and internal primers (Figure 5.7).

The amplification of mutant fragments included three rounds of PCR using two external primers (A\textsubscript{p}/D\textsubscript{p}) and two mutagenic primers (B\textsubscript{p}/C\textsubscript{p}). The latter were complementary to each other and contained the relevant codon mutations in each case (Table 2.2). Figure 5.7 presents an example of primers used to produce the parF gene encoding ParFΔ206. For all three mutations PCR1 (~138-bp) and PCR2 (~216-bp) were generated using one external and one internal primer in each round of amplification (A\textsubscript{p}/B\textsubscript{p} and C\textsubscript{p}/D\textsubscript{p}, respectively). The correct sizes of amplified PCR products were confirmed on agarose gels and the two PCR products were used to amplify the full length PCR3 (~354-bp) products using the two external primers A\textsubscript{p} and D\textsubscript{p} in all mutations (Figure 5.8). A\textsubscript{p} and D\textsubscript{p} primers (ParF-A\textsubscript{p}-Forward and ParF-D\textsubscript{p}-Reverse) were used in the deletion of E206, F205-E206 and I204-E206. In contrast, B\textsubscript{p} and C\textsubscript{p} were unique for each mutation and corresponded to ParF-B\textsubscript{p}\textsuperscript{1}-Reverse/ParF-C\textsubscript{p}\textsuperscript{1}-Forward, ParF-B\textsubscript{p}\textsuperscript{2}-Reverse/ParF-C\textsubscript{p}\textsuperscript{2}-Forward and ParF-B\textsubscript{p}\textsuperscript{3}-Reverse/ParF-C\textsubscript{p}\textsuperscript{3}-Forward (Table 2.2).

Figure 5.7 (overleaf). Schematic diagram of OE-PCR for generating the gene encoding ParFΔ206. Primers A\textsubscript{p}, B\textsubscript{p}, C\textsubscript{p} and D\textsubscript{p} and relevant restriction sites are highlighted in red and blue, respectively. The primers presented in this figure were designed for generating the gene encoding ParFΔ206.
A_p and D_p are external primers used with B_p and C_p, respectively. The codon GAG that encodes E206 was deleted in primers B_p and C_p. To produce genes encoding ParFΔ205-206 and ParFΔ204-205, the same A_p and D_p primers were used whereas B_p and C_p contained the required mutations (Table 2.2). The sequences of parF and parG are underlined with yellow and orange lines, respectively.

Figure 5.8. Agarose gel analysis of OE-PCR products for producing the gene encoding ParFΔ206 for cloning into pFH547 in place of the wild-type parF gene. Lanes: 1, 100-bp ladder; 2, 3 and 4 PCR1, PCR2 and PCR3 products, respectively. Selected bands in the 100-bp ladder are indicated.

The wild-type parF in pFH547 was replaced by PCR3 fragments which contained the deletion of codons 206, 205-205 and 204-206. Plasmid pFH547 was digested with SexAI and HpaI (Figure 5.7) to liberate the wild-type sequence and the PCR3 products were digested with the same enzymes. The relevant fragments were recovered from agarose gels. The fragments were ligated and transformed into E. coli DH5α. Twelve colonies were
picked from transformation plates for each mutation, and plasmid DNA was extracted and recombinant plasmids were examined by sequencing (Figure 5.9). Sequence analysis verified that the wild-type sequence in pFH547 was changed to the sequence encoding ParFΔ206, ParFΔ205-206 and ParFΔ204-206. The stop (TAA) codon that occupies codon 207 in parF was retained in the mutated genes.

5.2.3 The effects of C-terminal truncations of the ParF protein on plasmid partitioning

Segregation assays were used to test whether the truncated ParF proteins were affected in performing plasmid partitioning. The levels of plasmid retention conferred by ParFΔ206 and ParFΔ205-206 were similar to the levels with wild-type ParF (~70%) (Figure 5.10) and with ParF205GVPLF (Chapter 3.10).

![Figure 5.9](image)

Figure 5.9. ABI sequences traces showing sections of the wild-type parF and equivalent regions with deletions of codons 206, 205-206 and 204-206 in pFH547. The 21-bp mutagenic C primers for each mutation and the equivalent wild type sequence are underlined. Codons 204, 205 and 206 are marked with black lines above the sequences.
In sharp contrast, deletion of the last three amino acids in ParF (ParFΔ204-206) reduced plasmid retention to the level of the empty vector (~2%). This indicates that C-terminal truncation of two residues had no effect on ParF activity in plasmid segregation but that deletion of an extra residue may have affected the structure and/or folding of ParF as removing of the C-terminal end of many proteins resulted in destabilizing their functions (Hamill et al., 1998; Lambert et al., 2010; Williams et al., 2016). Alternatively, the ParF C-terminus may be implicated in a specific function such as self-association or interaction with ParG. To investigate and characterize further the role of the ParF C-terminus in self-association and interaction with ParG, the gene encoding the partition defective ParFΔ204-206 mutant was cloned and analysed in two-hybrid vectors.

Figure 5.10. Effects of C-terminal truncation in ParF on plasmid segregation. Partition assays were used to test the efficiency of the wild-type and mutated parFGH cassettes after growth for ~25 generation under non-selective conditions. Plasmid retention was determined as percentages for the ParF mutants (blue bars). The negative (red bar) and positive (green bar) controls showed ~2% and ~70% plasmid retention, respectively. Data represent the means of at least three independent experiments conducted in triplicate ± standard deviations.

5.2.4 Amplification and cloning of the gene encoding ParFΔ204-206 into two-hybrid plasmid pT18

The results of partition assays confirmed that the deletion of last three amino acids in ParF disrupted the partition activity and that this region is required for wild-type segregation activity (Section 5.2.3). The gene encoding ParFΔ204-205 was amplified from the pFH547-based plasmid with primers pT18Xhol-Forward and pT18HindIII²-Reverse (Table 2.2) for
cloning in pT18. The amplified fragment was verified on an agarose gel and purified (Figure 5.11). The sticky ends were produced for both pT18 vector and PCR fragment by digesting with XhoI/HindIII. Insert and vector were again purified from agarose gels to be ready for ligation reactions. The ligation samples were transformed into *E. coli* DH5α and colonies were picked and plasmid DNA was extracted and screened by digesting with XhoI and HindIII (Figure 5.12). As the cloning was successful and the relevant fragments were seen on agarose gels, samples were sent for sequencing. The clone was verified by the sequencing results (Figure 5.13) thereby producing plasmid pT18ParFΔ204-206.

![Figure 5.11. Agarose gel analysis of the PCR product of the fragment encoding ParFΔ204-206 for cloning into pT18. Lanes: 1, 100-bp ladder; 2, PCR amplification product (~600-bp) of the fragment encoding ParFΔ204-206. Selected bands in the 100-bp ladder are indicated.](image1)

![Figure 5.12. Verification of recombinant pT18ParFΔ204-206 by restriction analysis. Lanes: 1, 1-kb ladder; 2, sample of undigested pT18ParFΔ204-206; 3-8, recombinant plasmid pT18ParFΔ204-206 candidates digested with XhoI and HindIII. Selected bands in the 1-kb ladder and the ~600-bp XhoI-HindIII fragment liberated from plasmid candidates are indicated.](image2)
Figure 5.13. ABI sequences traces showing sections of the wild-type parF and equivalent regions with deletions of codons 204-206 in pT18ParF. The HindIII restriction site used in the cloning is underlined. Codons 204, 205 and 206 codons are marked with black lines above the sequences in wild-type parF.

5.2.5 The effects of C-terminal truncation in ParF on ParF self-association and interaction with ParG

Three ParF deletion proteins, ParFΔ206, ParFΔ205-206 and ParFΔ204-206, were tested above in partition assays. Results revealed that ParFΔ206 and ParFΔ205-206 displayed wild-type plasmid retention. In contrast, ParFΔ204-206 caused a severe defect on plasmid segregation. Therefore, to investigate further the effects of the latter mutation on other functions of the ParF protein, the mutant gene was cloned and tested in a two-hybrid assay plasmid. Plasmid pT18ParFΔ204-206 and pT25ParF or pT25ParG were cotransformed into E. coli SP850 and colony colour on MacConkey-maltose plates and β-galactosidase assays were used to determine the effects of deletion of the C-terminal three amino acids of ParF on the interaction with wild-type ParF and with ParG.

MacConkey-maltose agar plates were used to examine colony colour as an indication of the interaction of the ParFΔ204-206 mutant wild-type ParF and ParG. The plates showed red colonies for the combinations of ParFΔ204-206 with both wild-type ParF and with ParG (Figure 5.14, sectors 3 and 6, respectively). In addition, the interaction efficiency of the ParFΔ204-206 protein with wild-type ParF and ParG was measured using β-galactosidase assays (Figure 5.15). In agreement with the qualitative assay on MacConkey-maltose plates,
ParFΔ204-206/ParF and ParFΔ204-206/ParG interactions produced levels of β-galactosidase similar to those obtained for the ParF/ParF and ParF/ParG combinations, ~1200-1300 Miller units for both interactions (Figure 5.14). The results revealed that deleting the C-terminal three amino acids in ParF did not perturb ParF self-association or interaction with ParG. Thus, truncation of the ParF C-terminus of ParF protein did impair partition activity but not ParF self-association or the interaction with ParG as assessed in two-hybrid assays. These results suggest that the C-terminus of ParF plays a crucial role in the plasmid segregation, but that this role potentially does not involve the interaction with ParG or ParF self-association. Further analysis will clarify the importance of the ParF C-terminus in plasmid partitioning.

**Figure 5.14.** Association of ParFΔ204-206 with wild-type ParF and with ParG in two-hybrid assays. Plasmids pT18ParFΔ204-206 was cotransformed into *E. coli* SP850 with pT25ParF or pT25ParG. MacConkey-maltose medium was used to observe the colony colour after 36-48 hours incubation at 30°C. The strains in the sectors of the plate contain: 1, pT25ParF + pT18; 2, pT25ParF + pT18ParF*; 3, pT25ParF + pT18ParFΔ204-206; 4, pT25ParG + pT18; 5, pT25ParG + pT18ParF*; 6, pT25ParG+ pT18ParFΔ204-206.
5.2.6 The effect of production of ParFΔ204-206 on the wild-type segrosome

Deletion of the C-terminal three amino acids in ParF impaired segregation function but not the interaction with ParF or ParG. The effect of the truncated protein in trans on the wild-type segregation cassette of the TP228 plasmid was investigated to provide further information about the potential role of the ParF C-terminus in the segregation process. As described in Chapter 3.13, pFH554 comprises the parFGH cassette on a constitutively low copy number plasmid that is stabilized by the cassette. The segregational stability of pFH554 in the presence of wild-type ParF or ParFΔ204-206 protein was measured (Figure 5.16). Plasmids pT18, pT18ParF* and pT18ParFΔ204-206 were cotransformed with pFH554 into DH5α and typical partition assays were performed (Chapter 2.25), but with continuous selection for pT18 plasmids and ~25 generations of non-selective growth for pFH554. The controls showed similar levels of plasmid retention to those observed previously (Chapter 3.13). Therefore, production of wild-type ParF from pT18ParF*
displayed a destabilizing effect on pFH554 which showed <10% retention in the assay. In contrast, pFH554 showed a normal level of plasmid retention (~70%) in the presence of pT18 only (Figure 5.16). These controls demonstrated that empty pT18 had no destabilizing effects on the parFGH cassette cloned in pFH554, whereas a poisonous effect was produced by wild-type ParF encoded by pT18ParF* which caused a destabilizing effect on pFH554. These results agree with previous observations (Hayes, 2000) and with data obtained from ParF pentapeptide mutants (Chapter 3.13) and are thought to reflect a negative effect of ParF overproduction that inhibits the partitioning process by a mechanism that is not yet understood.

The partition-defective mutant protein ParFΔ204-206 caused a toxic effect on the wild-type partition complex when produced in trans which destabilized the pFH554 plasmid (~5%) (Figure 5.16). The effect of the C-terminal truncation of ParF on the ParFGH segrosome was as disruptive as that caused by wild-type ParF. The data suggest that ParFΔ204-206 can interact with and perturb the function of the wild-type segrosome encoded by pFH554 which thereby interferes with the maintenance of the latter.

Figure 5.16. Retention of pFH554 containing the parFGH cassette tested with plasmids producing wild-type ParF or ParFΔ204-206 in trans. Partition assays were used to determine the tolerance of the parFGH cassette in the low copy number pFH554 plasmid after growth for ~25 generations with non-selective pressure for pFH554 and continued selection for pT18 plasmids producing wild-type ParF (red) or ParFΔ204-206 (blue). The control is the empty pT18 vector (purple). Data represent the means of at least three independent experiments conducted in triplicate ± standard deviations.
Chapter 6: Discussion
Multidrug resistant bacteria are crucially important in clinical settings and their emergence and spread are global health threats that are associated with increasing morbidity and mortality (Akova, 2016; Rosenthal, 2016; Spellberg et al., 2008). Multidrug resistant Gram-negative bacteria including *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, methicillin resistant *Staphylococcus aureus* and resistant isolates of *Mycobacterium tuberculosis* are a few examples of multidrug resistant strains that are responsible for nosocomial and community acquired infections and which have caused major public health concerns worldwide (Aviv et al., 2016; Schumacher et al., 2014; Yao et al., 2016). In recent decades the development of novel antibiotics has dramatically decreased and the emergence of untreatable infections has risen which guides research activities for discovery of new antimicrobial targets (Frieri et al., 2016). Resistance genes often are located on plasmids (van Hoek et al., 2011). Plasmid pBRG1 of *Enterococcus faecium* (Huycke et al., 1998) and multidrug resistant plasmid pMET1 of *Klebsiella pneumoniae* (Soler Bistue et al., 2008) are few examples of plasmids harbour resistance genes.

TP228 is a conjugative plasmid which was originally identified in *Salmonella newport* and which confers resistance to a range of antibiotics (kanamycin, neomycin, spectinomycin, streptomycin, sulphonamides and tetracycline) and also mercuric ions (Bradley, 1980). Plasmid distribution to daughter cells is ensured by faithful partitioning at cell division although the mechanisms involved are not fully understood. Dissecting and understanding the assembly of proteins that participate in plasmid segregation may be important for finding a new target for antibiotic resistant bacteria. Dissecting and understanding the assembly of proteins that participate in plasmid segregation may be important for finding a new target for antibiotic resistant bacteria. Specifically, by disrupting the segregational stability of resistance plasmids by the use of novel antibacterial agents, the persistence and spread of multiresistance plasmids may be curtailed (Clewell et al., 2014; Saeed et al., 2015).

The segregation cassette of TP228 consists of ParF and ParG proteins and a centromere-like site *parH*. ParF is a member of the ParA protein superfamily and is more closely related to the MinD cell division protein subgroup than, for example, to ParA of the P1 plasmid (Hayes, 2000; Schumacher et al., 2012). ParF may mediate segregation in bacteria through a polymerization mechanism. Binding of ATP enhances ParF polymerization whereas hydrolysis leads to depolymerization (Barillà et al., 2005, 2007). The partner RHH protein ParG is a centromere binding factor that recruits ParF to the segosome complex and plays a
crucial role in accurate segregation by stimulating both the ATPase and polymerization activities of ParF. The unstructured N-terminus tails in ParG are implicated in these stimulatory activities, and also are required for ParF assembly into the complex (Carmelo et al., 2005; Barillà et al., 2005, 2007; Machón et al., 2007; Wu et al., 2011; Zampini et al., 2009). The partition apparatus of the multidrug resistance TP228 plasmid and other resistance plasmids may provide a novel target for new anti-plasmid compounds (Saeed et al., 2015).

Complete alanine scanning mutagenesis of the RHH domain in ParG characterized the crucial role of residues Phe49, Trp71 and Leu72 in ParG dimerization and consequently in segregation activity (Saeed et al., 2015). Moreover, pentapeptide insertions and deletion mutations of the ParG tails have provided clear insights into the role of the N-terminal tail in ParG function. None of the tail mutations abolished ParG dimerization in vivo and in vitro confirming that the disordered tails are not implicated in ParG dimerization (Carmelo et al., 2005). Nevertheless, segrosome assembly was abolished as a result of removing the flexible tails of ParG (Wu et al., 2011). As mentioned above, mutational analysis has shown that the flexible tails of ParG are required for two separable functions: stimulation of ATP hydrolysis by ParF and promoting ParF polymerization (Barillà et al., 2007).

Previous analysis showed that mutation of the ATP binding motifs perturbs the formation of ParF polymers and also that certain residues around the nucleotide-binding pocket ensure that the conformational structure of the ATP binding niche is maintained (Barillà et al., 2005; Dobruk-Serkowska et al., 2012). In addition, mutational analysis was combined with determination of the ParF tertiary structure to examine the ParF polymer interface (Schumacher et al., 2012). In view of these limited mutational studies to date (Figure 3.1) and their effects on ParF protein function, we applied here a more random in vivo mutagenesis of the protein to understand the role of ParF in plasmid segregation and to investigate the structure-function relationships of the protein. The work benefitted from the determination of the ParF crystal structure as the project began (Schumacher et al., 2012).

6.1 Pentapeptide scanning mutagenesis provides mutant proteins with a range of activities

PSM has been shown to be a valuable approach in analysing structure-function relationships of proteins belonging to different families. This facile strategy is applicable in identification of essential and inessential regions of a protein, specially proteins with known structures.
Mutated proteins with a range of activity levels can be generated using this random mutagenesis technique. Unexpected insights also have been provided in different studies by identifying mutated proteins with novel functions (Hayes and Hallet, 2000). For example, pentapeptide insertions in the β-lactamase enzyme switched substrate specificity and provided increased resistance to a third-generation cephalosporin antibiotic comparing with the wild-type protein. These insertions were located in the vicinity of the catalytic pocket and altered the conformation of the pocket thereby allowing easier access by the cephalosporin (Hayes et al., 1997). Second, PSM was applied to the bacterial XerD site-specific recombinase and insertions were identified that affected the function of the protein during the site-specific recombination reaction (Cao et al., 1997; Hallet et al., 1997). Third, distinct regions were identified in the human tumour suppressor protein BRCA1 which, among other functions, is a transcriptional activator. Moreover, PSM revealed a region in the carboxyl terminus of this protein that is highly tolerant to insertions (Hayes et al., 2000). Fourth, the multiprotein secretion complex of Pseudomonas aeruginosa which transports exoproteins was dissected by PSM and domains required for assembly of the complex were identified (Robert et al., 2002). Fifth, PSM was applied to the ParG centromere binding protein. Pentapeptide insertions in the RHH folded domain of ParG abolished dimerization of the mutant with wild-type ParG whereas insertions in the flexible N-terminus showed that the tail is not required for dimerization \textit{in vivo} or \textit{in vitro} (Carmelo et al., 2005). Sixth, the histidine kinase regulatory protein PdhS of Brucella abortus participates in the cell cycle of this animal pathogen. A number of insertions with specific effects were identified in a domain of this protein required for protein-protein interactions. The PSM and complementary data showed that PdhS acts as an essential regulator for growth and division mechanisms of cell cycle progression in \textit{Brucella abortus} (Van der Henst et al., 2012). Seventh, PSM was applied to a specific pathogenicity effector, DspA/E, of Erwinia amylovora. The pathogen depends on this factor in causing invasive diseases in the Rosaceae family of plants. Thirteen altered proteins with pentapeptide insertions were characterized eight of which impaired the function of DspA/E (Siamer et al., 2013). Finally, the potential of PSM in dissecting protein-protein interactions also has been shown in numerous other studies with a variety of different protein targets (Draghici et al., 2009; Fransen et al., 2005; Gray et al., 2009; Kim et al., 2005; Lambert et al., 2010; Létoffé et al., 2003; Malone et al., 2007; Oliva et al., 2004; Reijns et al., 2005; Sanchez et al., 2009; Sènechal et al., 2010; Zhang et al., 1999, 2008).
In this study, the functional activity and oligomeric state of the ParF protein and the interaction of ParF with the ParG protein were considered. PSM was applied to create a library of mutant ParF proteins. A two-hybrid screen and restriction enzyme digestion strategy were used to identify Tn4430 insertions in the parF gene. As the gene comprises only ~10% of the target plasmid, the frequency of white colonies in the two-hybrid screen, i.e., transposon insertions in parF, was noticeably less than the number of red colonies, i.e., transposon insertions in the pT18ParF* vector backbone. In addition, the frequency of insertions also was low in the case of a second target plasmid, pFH547: only 14 recombinant plasmids were produced from ligation of gel-purified parF::Tn4430 fragments and pFH547 backbone (Figure 3.20). Moreover, contrary to expectations, this study did not yield a large number of unique pentapeptide mutants. From 97 sequences of mutant parF genes only nine unique pentapeptide insertions were produced at different positions in ParF (Figure 3.33). Some insertions were found repeatedly at the same positions in the protein in different PSM trials even when using two different target plasmids and two different insertion strategies (Figure 3.2). Tn4430 belongs to the Tn3 family of transposable elements (Mahillon and Lereclus, 1988). Certain transposons have insertion site specificity and they always insert at the same target sequence. For example, Tn10 insertion is highly sequence-specific, inserting preferentially into GCTNAGC sequences (Halling and Kleckner, 1982), whereas other transposons, including members of the Tn3 family, insert more randomly in DNA (Davies and Hutchison, 1995). Nevertheless, Tn3 has a weak preference for the consensus site TA[A/T]TA (Seringhaus et al., 2006). The Tn3 family member Tn4652 has a similar weak consensus target site (Kivistik et al., 2007).

The insertion site specificity of Tn4430 that was used in the current study has not been examined rigorously. However, the results here show that Tn4430 may not behave entirely as a random transposable element which is consistent with the results of PSM performed by Siamer et al. (2013), who observed that the insertion of Tn4430 into a target plasmid was not random. Among 13 dspA/E mutants that were generated in the study, 12 were located in the first half of the dspA/E sequence with eight insertions positioned in the segment that encodes the β-propeller fold and only one insertion was located in the 3’ end of the gene. In our study, the 15-bp insertions in parF included 71 insertions at position 615, 14 insertions at position 498, four insertions at position 201, two independent insertions at position 492 and 495, two insertions at position 201, and one insertion at position 480. The Results showed that not all the regions of parF were permissive to Tn4430 insertions even with multiple trials of PSM mutagenesis and repetitive insertions were found at the same regions.
in the gene (Table 3.1). The data may reflect that specific regions in the parF sequence are hotspots in which the frequency of Tn4430 insertions occurs more than at other sequences. This could be examined further by analysing the insertion sites from a large number (>100) of independent Tn4430 transposition events to determine whether a consensus sequence exists as performed for other transposons (Brookfield, 1986; Brookfield and Johnson, 2006; Hassan et al., 2016). High-throughput DNA sequencing approaches may be useful in this analysis (Mullany et al., 2012). In addition to the nine unique 15-bp insertions in parF, PSM produced two atypical insertions of 14-bp and 16-bp which were not tested further as they produced frame-shift mutations. This result is consistent with that observed by Gray et al. (2009) who also described a 16-bp insertion derived from Tn4430 transposition.

6.2 Pentapeptide insertions at different positions in ParF produced modest, severe or no defects on plasmid segregation

The effects of pentapeptide insertions on ParF function were examined in vivo using partition assays. The data presented in this study clarify the importance of certain regions in ParF protein function. First, we demonstrated that insertions following Lys71, Thr164 and Ile166 positions reduced, but did not abolish, plasmid segregation activity. Thus, these insertions that caused partial functional impairment of the protein are in positions that can partially tolerate mutagenesis. Second, pentapeptide insertions at positions Tyr67, Lys151 and Ala165 completely impaired plasmid segregation. These insertions are located in α3, α6 and in the loop between β7 and α7 in ParF, respectively. Tyr67, Lys151 and Ala165 are conserved in ParF homologues (Figure 6.1) which supports a vital role for these regions in protein function. Interestingly, the region from residues 61-71 is implicated in one of the interfaces in the ParF polymer (Schumacher et al., 2012). We examined the contribution of these regions to ParF self-association or interaction with ParG using two-hybrid assays to clarify in more detail the disruption of ParF efficiency caused by these insertions. Third, and in contrast to the preceding mutations, pentapeptide insertions at positions Lys160, Ser185 and Phe205 had no apparent effect on plasmid partitioning. These observations suggest the utility of PSM in characterizing the segments in a protein which are tolerant to oligopeptide insertions (Cao et al., 1997; Gray et al., 2009; Hallet et al., 1997; Lambert et al., 2010).
6.3 Effects of pentapeptide insertions in ParF on protein functions \textit{in vivo}

All ParF pentapeptide mutant proteins were assessed in two-hybrid assays to investigate the effects of the insertions on ParF self-association and interaction with the partner protein ParG. The interactions were detected \textit{in vivo} using a two-hybrid system based on reconstitution of adenylate cyclase activity in \textit{E. coli} (Karimova \textit{et al.}, 1998) that has been used previously to dissect the ParF-ParG complex (Barillà and Hayes, 2003; Barillà \textit{et al.}, 2007; Carmelo \textit{et al.}, 2005; Saeed \textit{et al.}, 2015). Pentapeptide insertions at positions Tyr67 and Lys71 in α3 and Thr164, Ala165 and Ile166 in the loop between β7 and α7 (Figure 3.33) disrupted both self-association of ParF and interaction with the partner protein ParG as assessed on MacConkey-maltose agar plates and in β-galactosidase assays. The data suggest that these positions are crucial for ParF protein folding and/or stability as the defects were not specific to a single aspect of ParF function, but affected both self-association and ParG interaction. Sequence alignments of ParF homologues shows that residues Tyr67, Lys71, Thr164, Ala165 and Ile166 are well conserved (Figure 6.1). Among the insertion positions Ala165 is close to the ATP binding pocket that spans residues 166-177 in ParF (Schumacher \textit{et al.}, 2012). In addition, residue Lys71 participates in formation of the larger ParF polymer interface (Barillà \textit{et al.}, 2005; Schumacher \textit{et al.}, 2012). Disruption of protein function due to pentapeptide insertions at these conserved residues may perturb nucleotide binding and/or ParF polymerization. Nevertheless, ParF polymerization is primed by the tertiary structure of the protein which is predicted to be induced by nucleotide binding thereby promoting a conformational change in ParF structure (Shumacher \textit{et al.}, 2012). Western blot analysis using antibodies against ParF would allow estimation of the intracellular stability of the pentapeptide derivatives that apparently disrupt ParF folding and/or the gross structure of the protein. The general structures of the mutant proteins also could be examined \textit{in vitro} by, for example, circular dichroism.
Figure 6.1 (next page). Amino acid sequence alignment of ParF homologues. Accession numbers: 1, plasmid TP228 ParF, AAF-74217.1; 2, chromosome partitioning protein ParA, WP-039587694.1.3, chromosome partitioning protein ParA, WP-000864791.1; 4, chromosome partitioning protein ParA, WP-04424373.1; 5, plasmid partitioning protein ParF, BAS44004.1; 6, plasmid partitioning protein, SAW-39084.1; 7, chromosome partitioning protein ParA, WP-048960560.1; 8, chromosome partitioning protein ParA, WP 058610035.1; 9, partitioning protein ParA, WP-CCP05054.1; 10, chromosome partitioning protein ParA, WP-052177113.1; 11, chromosome partitioning protein ParA, WP-015873814.1. The colour scheme for amino acids is: green, polar negative (D and E); red, polar positive (R, H and K); yellow, polar neutral (N, Q, S and T), blue, non-polar aliphatic (A, I, L, M and V); purple, non-polar aromatic (F, Y and W); orange, special cases (G, P and C). The positions of PSM insertions and site-directed mutants are marked with black arrows.
Remarkably, colony colour on MacConkey-maltose plates and quantitative β-galactosidase assays showed that pentapeptide insertions at positions Lys151 in α6, Lys160 in β7, Ser185 in the loop before α8, and Phe205 in the C-terminus of ParF did not affect ParF self-association. Thus, these regions in the protein are tolerant to pentapeptide insertions for ParF self-association (Figure 3.33) and the changes produced by these insertions did not perturb the conformation of the regions sufficiently to disrupt the interaction between ParF monomers.

The pentapeptide insertion at Lys151 caused a severe defect in plasmid partitioning. However, the mutation did not abolish the interaction with ParF, but did impair the interaction with the partner protein ParG (Table 3.3). Thus, the data indicate a specific role for the region that includes Lys151 in the ParF-ParG interaction. The arginine-finger like motif in the flexible N-terminal tail of ParG interacts with the nucleotide binding pocket in ParF and stimulates ATP hydrolysis by the latter. The unstructured tail in ParG also is implicated in polymerization and remodelling of ParF polymers (Barillà et al., 2005, 2007). The defect caused by the pentapeptide insertion at position 151 in ParF may affect the ability of the N-terminal tail in ParG to stimulate either ATP hydrolysis and/or polymerization of ParF. These possibilities might be investigated further *in vitro* using purified ParF151GVPLK in ATPase stimulation and polymerization assays with ParG. The RHH domain of ParG also interacts with ParF although the molecular basis for this interaction has not been elucidated (Carmelo et al., 2005). The interaction defect with ParG caused by the pentapeptide insertion at position 151 in ParF instead may perturb the binding of ParF with the RHH segment of ParG. Further analysis of this interaction using *in vivo* and/or *in vitro* techniques will clarify the ParG interaction defect in the ParF151GVPLK protein.

In summary, pentapeptide scanning mutagenesis was used to probe the structure of the ParF partition protein (Table 3.3). The impact of the pentapeptide mutations that affected the function of ParF was compared with the previous limited mutational analysis of conserved residues in the protein and was used also to define regions involved in self-association and interaction with ParG. Pentapeptide insertions at positions 67, 71, 164, 165 and 166 in ParF apparently disrupted the overall structure, folding and/or stability of the protein (Figure 3.33). First, the interactions of these mutant proteins with wild-type ParF caused deleterious effects on subunit interactions in two-hybrid assays. Second, the mutant ParF derivatives did not interact with ParG in two-hybrid assays. Third, plasmid partitioning was affected as
a consequence of these five pentapeptide mutants. In contrast, pentapeptide insertions at positions 160, 185 and 205 did not perturb the interaction of the mutant protein with ParF or ParG or plasmid segregation which indicates that these regions in ParF are permissive for insertions. A novel observation came from the pentapeptide insertion at position 151 in α6 (Figure 3.33) which impaired the interaction with ParG and plasmid segregation efficiency, but did not affect ParF self-association. The finding suggests that this region of ParF is vital for the interaction with ParG during segrosome assembly.

6.4 Pentapeptide insertions in ParF exerted a toxic effect on the wild-type segrosome
The effects of the production of ParF pentapeptide mutant proteins on the wild-type protein were tested using the segregation activity of the parFGH cassette. Excess ParF protein disrupts the activity of the segrosome (Hayes, 2000). Surplus ParF may perturb segrosome formation or cause inappropriate ParF polymerization dynamics, or may reflect that ParF needs to be produced at a specific time and location in the plasmid segregation cycle. Here, production of wild-type ParF from pT18ParF* showed a destabilizing effect on the plasmid that contains the wild-type parFGH cassette in pFH554 (<10% retention), whereas pFH554 displayed a normal level of plasmid retention (~65%) in the presence of pT18 without cloned parF. Mutant proteins ParF160GVPLK and ParF185RGTPS that displayed normal levels of partition activity were tested for their overproduction effects towards the wild-type partition complex. The mutants caused poisonous effects which destabilized the activity of pFH554 to similar levels as the wild-type protein. These results confirmed that the insertions at these positions did not inhibit ParF function. In contrast, ParF pentapeptide mutants that conferred severe partition effects only mildly affected the stability of pFH554 (~30-50% plasmid retention). These results support the partition assay data as the mutant proteins which did not show any defect in partition efficiency were poisonous to the wild-type segrosome. In contrast, mutant proteins that conferred severe defects in partition assays reduced the activity of the segrosome. If the gross structure of ParF is disrupted by these insertions, then the mutant proteins may be ineffective in perturbing the wild-type segrosome in trans.

6.5 ParF derivatives with oligopeptide insertions larger than five amino acids between Lys151 and Glu152 maintained self-association
Partition assays and two-hybrid assays confirmed that insertion of five amino acids between residues 151 and 152 did not perturb ParF self-association. In contrast, the insertion
abolished ParF-ParG interaction (Table 3.3). The tolerance of this region of α6 to larger oligopeptide insertions was examined. The insertion size of ParF151GVPLK initially was increased from five to 19 amino acids. Restriction digestion and religation of the resulting recombinant plasmid generated derivatives that encoded insertions less than 19 amino acids as well as a ParF derivative with a 33 residue insertion that was produced fortuitously from the same mutagenesis. The effect of increasing the size of the insertion α6 was examined in two-hybrid assays. None of the mutant ParF proteins (ParF15111, ParF15113, ParF15117, ParF15119, ParF15133) disrupted ParF self-association. These results emphasized the high flexibility of α6 region in ParF in which insertions as long as 33 amino acids did not perturb the interaction with the wild-type protein. It is likely that this region is not crucial for ParF stability or folding or self-association, but does play an important role in interacting with ParG. Thus, the assays showed that the ParF15111, ParF15113, ParF15117 ParF15119 and ParF15133 mutant proteins did not interact with ParG with similar results to that observed with the insertion of five amino acids at the same position. This observation is new among the ParA superfamily of proteins and highlights the use of PSM as a tool for identifying the essential and non-essential regions in proteins. The region in the vicinity of residue 151 in α6 of ParF may be useful for the insertion of synthetic amino acid tags, for example, to allow subcellular localization of the protein. The preceding results guided us to more details investigations by applying site-directed mutagenesis of residues 151 and 152 in ParF.

6.6 Mutagenesis of Lys151 and Glu152 in ParF disrupts plasmid segregation
A pentapeptide insertion and insertions up to 33 residues between positions 151 and 152 did not affect ParF self-association, but did impair the ParF-ParG interaction and plasmid segregation. The ParF-K151A mutant also showed severe reduction in plasmid retention whereas, in contrast, the ParF-E152A mutant was unaffected. The defect produced by substitution of Lys151 with alanine may be attributed to, first, the removal of all side chain atoms of the residue by the alanine substitution. Substitution to alanine often produces protein destabilization experimentally and computationally (Lwin et al., 2007). Second, a conformational change produced by this substitution may render the ParF protein inactive in plasmid segregation. Interestingly, deletion mutations of Lys151 or Glu152 residues are likely to have caused more radical changes in α6 of ParF than the substitution mutations as both deletions caused severe reductions in plasmid segregation. These results with Lys151 and Glu152 can be added to previous substitution mutagenesis studies of ParF including
first, the P104A, R169A and G179A mutations that abolished plasmid segregation and induced ATPase hyperactivity in ParF (Dobruk-Serkowska et al., 2012). In addition, the G11V and K15Q changes impaired plasmid segregation by perturbing nucleotide binding in ParF (Barillà et al., 2005). Finally, the K64A/V89Y/M96A triple mutation that disrupted key residues in formation of one of the ParF polymer interfaces caused a partition defect (Schumacher et al., 2012). In summary, our data show that Lys151 in α6 of ParF fulfils a crucial role in plasmid segregation.

It was reasonable to speculate that Lys151 may be particularly important for the interaction with ParG in view of the properties of the ParF151GVPLK insertion mutant (Table 3.3). In agreement, two-hybrid analysis showed that the K151A or E152A substitutions or deletion of K151 did not abrogate ParF subunit interactions. However, substitution or deletion of Lys151 impaired the interaction with ParG. In contrast, ParF-E152A was unimpaired in the interaction with ParG. ParA proteins, including ParF, and ParB/ParG proteins assemble together on cognate partition sites to form the segrosome (Hayes and Barillà, 2006a). Therefore, it is clear that the interaction between these two partners is mandatory for accurate plasmid partitioning. Several ParB proteins interact with ParA through the N-terminus of the former (Leonard et al., 2005; Ravin et al., 2003). Although ParG is unrelated at the sequence or structural levels to ParB encoded by the P1 plasmid, for example, residues 17-23 in the N-terminal flexible region of ParG are predicted to be vital for interaction with ParF (Golovanove et al., 2003). Deletions of 9, 19 and 30 residues from the N-terminus of ParG affected the interaction with ParF and stimulation of ParF ATPase activity. The flexible N-terminal tails of the ParG might act as tentacles to bundle ParF polymers formed in response to ATP binding (Barillà et al., 2007). Similarly, the interaction of the ParR centromere binding protein of the R1 plasmid stabilizes ParM polymers and is predicted to produce conformational changes in the polymer structure (Gayathri et al., 2012; Møller-Jensen et al., 2007; Salje et al., 2010). The N-terminal flexible tail of ParG and the dimeric RHH DNA-binding fold constitute the interaction surfaces of ParG with ParF (Carmello et al., 2005; Golovanov et al., 2003). Further analysis is required to investigate whether Lys151 in ParF contacts the N-terminal tail or RHH domain in ParG.

6.7 Insertions larger than five amino acids disrupt partition-proficient pentapeptide mutants

Pentapeptide insertions at positions 160 and 185 and at position 205 near the C-terminal did not affect plasmid partitioning, ParF self-association or the interaction of ParF with ParG.
Therefore, we explored the tolerance of positions 160 and 185 to larger oligopeptide insertions and the effects of truncation of the C-terminus of the ParF protein. ParF160GVPLK and ParF185RGTPS were selected for further mutational studies to assess the permissiveness of positions 160 and 185 for oligopeptide insertions. The impact of enlarging the size of insertions from five to 19 amino acids at positions 160 in β7 and 185 in the turn before α8 in ParF was tested. In addition, a shortening strategy was applied and collectively seven mutants were generated that contained 13, 15, 17 and 19 amino acids inserted between Lys160 and Ala161 and between Ser185 and Ser186, respectively. ParF16013, ParF16015, ParF16017, ParF16019, ParF18513, ParF18515 and ParF18519 mutants were tested in partition assays. Insertions of 13, 15 or 17 amino acids between Lys160- Ala161 markedly reduced the levels of plasmid retention which revealed that insertions larger than five amino acids may cause structural disruption that render the β7 strand non-functional or may induce perturbation of ParF stability or folding. In contrast, insertion of 15 amino acids between Ser185 Ser186 affected partition efficiency modestly: plasmid retention was decreased slightly (~45%) compared with the pentapeptide insertion (~55%). Position Ser185 is located in interface 2 in the ParF crystal structure and sequence alignments shows that this residue is among the well conserved residues in this interface (Schumacher et al., 2012 and Figure 6.1). The result shows that oligopeptide insertions in loops often have limited effects on protein function compared to insertions in other protein structural elements (Hayes and Hallet, 2000). However, the 19 amino acid insertion between Ser185 and Ser186 reduced plasmid segregation to the levels of the empty vector. The conformational change produced by 19 amino acids at this position may be more profound than the alterations generated by shorter oligopeptides. Similarly, insertion of more than five amino acids in loop regions was observed to induce great instability in the β-lactamase enzyme (Hayes et al., 1997). In summary, the β7 element that contains Lys160 and Ala161 and the loop near the C-terminus of the protein that includes Ser185 and Ser186 displayed different tolerances to oligopeptide insertions which may reflect different contributions of these regions to ParF protein function.

6.8 The C-terminal end of ParF is required for plasmid segregation

The contribution of the C-terminus of the ParF protein to plasmid partitioning is a significant finding of this study which has not been described previously. Among the three partition-proficient pentapeptide ParF mutant proteins that self-associated and interacted with ParG, the insertion between Phe205 and Glu206, which is the last amino acid in the
protein changed the ParF sequence from 205-FE-206 to 205-FGVPLFE-211. The ParFΔ206 and ParFΔ205-206 deletion proteins conferred levels of plasmid retention similar to wild-type ParF and to that observed with the C-terminal pentapeptide insertion. However, deletion of the last three amino acids (ParFΔ204-206) reduced plasmid retention to the level of the empty vector. Therefore, deletion of the C-terminal two amino acids in ParF does not impair protein function but, in contrast, deletion of an extra residue inhibited segregation activity. To assess the contribution of the ParF C-terminus in a specific function such as self-association or interaction with ParG and for further characterization the role of this element in protein function the gene encoding the partition defective ParFΔ204-206 protein mutants was cloned and analysed in two-hybrid vectors. Deleting the C-terminal three amino acids did not disrupt ParF self-association or interaction with ParG. Moreover, the effect of production of ParFΔ204-206 was disruptive and toxic to the wild-type partition complex by destabilization of the pFH554 plasmid. The results suggest that the C-terminus of ParF fulfils a key role in the plasmid partitioning mechanism that does not involve the interaction with ParG or self-association. The C-terminus of ParF may be required for the interaction with putative host structures which participate in plasmid segregation. Alternatively, the tripeptide deletion may subtly alter the kinetics of protein folding as a result of disruption of a two-state process that involves contact of N- and C-terminal secondary structure elements that play roles in protein folding steps (Krishna and Englander, 2005). Protein C-termini often are crucial for function. For example, predicted phosphorylation sites were lost in a C-terminal mutant of the Hoxa1 protein (Lambert et al., 2010) and the C-terminal end of the Hox protein UBX of Drosophila was reported to contribute to transcription activity (Ronshaugen et al., 2002). In addition, C-terminal truncation mutations of the MECP2 binding protein of post-mitotic neurons in Drosophila caused neuronal apoptosis in vivo which is a novel finding (Williams et al., 2016). Another example is provided by the C-terminal amphipathic helix that mediates membrane binding by the MinD cell division factor (Hu and Lutkenhaus, 2003). Furthermore, removing of the C-terminal tail of TubZ- Bacillus cereus and TubZ- Bacillus thuringiensis tubulin proteins or short truncation impaired polymerization activity of the filaments (Hoshino and Hayashi, 2012; Montabana and Agard, 2014).
6.9 Future work

6.9.1 Improving the pentapeptide scanning mutagenesis strategy

PSM may be a powerful genetic tool for identification of novel targets for development of antibacterial drugs that affect plasmid segregation. The mutational analysis here suggests that insertions generated in ParF have significant use in developing the structure-function analysis of the protein. The results also provide new insights into the segregation machinery of multidrug resistant plasmids. Nine unique insertions were generated at different positions in ParF but the numbers of repetitive samples from multiple trials using different target plasmids were relatively high. The Tn4430 transposon was used as a mutational tool in the insertion process, but the transposon did not behave as randomly in its insertion pattern as in previous studies. Another type of transposable element may generate more diversity in parF insertions. For example, IS21 transposable element produces four or 11 aminoacids in vivo using BglII or SalI cleavage and religate (Hayes, 2003). In addition, direct insertion of Tn4430 in vitro into target gene of a particular DNA fragment may become another strategy to introduce the insertion of five aminoacids to any gene of interest (Hayes and Hallet, 2000).

Pentapeptide insertions at three positions in ParF reduced but did not abolish partition efficiency. The insertions at these positions can be elongated to more than five amino acids and the mutants can be tested in partition assays, although the three mutants are defective in ParF self-association and ParF-ParG interaction. In contrast, pentapeptide mutants ParF67GVPLY and ParF165RGTPS displayed severe defects in partition activity and did not self-associate or interact with ParG. These mutants are excellent candidates for further biochemical tests and kinetic studies, including ATPase and polymerization assays. In addition, pentapeptide mutants ParF160GVPLK and ParF185RGTPS showed wild-type activities in both two-hybrid and partition assays and were subjected to longer insertional mutagenesis and tested for partition activity. It would be interesting to assess whether these proteins with larger insertions interact in two-hybrid assays which may clarify the effects of longer oligopeptides in β7 and in the loop near the C-terminus of ParF on ParF-ParF and ParF-ParG interactions. Thus, all of the ParF pentapeptide mutants might represent interesting tools for further studies.
6.9.2 Dissecting ParF self-association

The insertion at position 151 in ParF impaired plasmid segregation and the interaction with ParG, but did not perturb ParF self-association. Insertions up to 33 amino acids did not affect the latter interaction. It would be valuable to increase the size of the insertion by more than 33 amino acids to test the tolerance of the α6 element to insertions. The resulting proteins could be tested to examine the effects of larger insertions on self-association of ParF in two-hybrid assays as well as in in vitro studies using purified proteins. Structural studies may also reveal the conformation of the α6 element. In addition, site-directed mutagenesis showed that the Δ152 deletion affected plasmid segregation, whereas substitution of this residue did not perturb ParF-ParF or ParF-ParG interactions or plasmid segregation. Deletion mutations generally may have stronger effects than substitution changes. Nevertheless, it would be worthwhile assessing if the ParFΔ152 mutant is active in two-hybrid analysis.

6.9.3 The ParF-ParG interaction

The ParF151GVPLK mutant self-associated but did not interact with ParG. As the mutant was partition defective, the results suggest that ParG interacts with ParF through Lys151 and that the region that includes this residue may have a critical role in ParF-mediated plasmid segregation. The role of 151 was confirmed by site-directed mutations. The separate ParF and ParG structures have been determined (Golovanov et al., 2003; Schumacher et al., 2012). Determination of the ParF-ParG co-structure will reveal the contribution of Lys151 in ParF to formation of the complex. Moreover, ParG stimulates ATP hydrolysis by ParF as well as ParF polymerization (Barillà et al., 2005, 2007) and the proteins interact during segrosome assembly at the parH site (Wu et al., 2011). Biochemical testing of ParF151GVPLK for response to these stimulatory functions of ParG will reveal which aspects of the ParF-ParG interaction are disrupted by the insertion.

6.9.4 The role of ParF protein in the plasmid segregation mechanism

Preliminary studies by electron microscopy demonstrated the organization of ParF polymers (Barillà et al., 2005). Further investigation by microscopy can determine the structure and arrangements of ParF polymer formation in vitro. The dynamics of selected pentapeptide mutants should be established and tested by performing ATPase activity studies and polymerization analyses, potentially including electron microscopy studies. Production of a specific building block (dimer-of-dimer unit) that drives polymer formation is dependent on
ATP binding by ParF which is critical for subsequent ParF polymerization (Schumacher et al., 2012). How are polymer formation and control achieved to mediate DNA segregation? A further study with focus on a broader range of ParF mutants could be therefore suggested.

6.9.5 The contribution of the C-terminal end of the ParF protein to plasmid segregation

Truncation of the C-terminus of ParF revealed that the deletion of the final three residues disrupted plasmid partitioning, but not ParF self-association or the interaction with ParG. This interesting finding can be investigated further to dissect the function of the C-terminus of the protein which will provide further insights on the plasmid partitioning process. In vitro biochemical studies can be used to examine ATP hydrolysis kinetics and polymerization properties of the deletion protein, as well as the capacity of the mutant protein to interact with ParG and to assemble into the segrosome in vitro (Wu et al., 2011). Additional substitution mutations in the C-terminal tripeptide also could be designed and tested. Previous ParF studies and mutational analysis (Barillà and Hayes, 2003; Barillà et al., 2005, 2007; Dobruk-Serkowska et al., 2012; Schumacher et al., 2012) showed ParF-self association, mutant-mutant and mutant-wild type ParF interactions. However, the N-terminus of ParF has not been analysed. Future studies on the N-terminus therefore are recommended, especially systematic truncation of the first ten amino acids to combine with the C-terminus mutants of this study. These studies will reveal whether the N- or C-termini of the protein are involved in the interaction with ParG, for example.

In conclusion, PSM was used successfully to understand structure-function relationships in the ParF plasmid segregation protein. We have generated nine unique insertions which have provided valuable information about ParF organization and activity. A region in ParF which participates in the interaction with ParG was identified. The tolerance of specific regions in the protein was highlighted by pentapeptide and longer oligopeptide insertions. Moreover, the role of the C-terminus of ParF in plasmid segregation was analyzed by truncation of the protein. Future studies that capitalize on the work described here will reveal further aspects of multidrug resistance plasmid segregation.
Appendix A: Genetic organization of the pT18 vector edited by SnapGene software

Appendix B: nucleotide sequence of pT18
References


References


References


References


References


SNAPGENE software from GSL Biotech; available at www.snapgene.com.


