Investigating Maintenance Of The Yeast 2-Micron Family Of Plasmids

by

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Abstract

The 2µm plasmid of *Saccharomyces cerevisiae* is the best-studied member of a family of multi-copy plasmids found only in Saccharomycetaceae budding yeast. All members of the family are small circular double-stranded DNAs that share structural and functional similarities but lack significant nucleotide sequence identity. These plasmids use yeast replication and segregation machineries for their own maintenance but do not seem to benefit or pose a detriment to the host. For the 2µm plasmid, association of the plasmid-encoded Rep1 and Rep2 proteins with the *cis*-acting *STB* partitioning locus has been shown to be required for equal partitioning of plasmid copies at host cell division. Other members of the family, the 2µm-like plasmids, remain mainly uncharacterized. The goal of this research was to experimentally analyze 2µm-like plasmids pSR1 and pSB3 from *Zygosaccharomyces rouxii* and pKW1 from *Lachancea waltii* to gain a better understanding of the components and features required for plasmid maintenance, as well as to investigate the contribution of the 2µm Rep proteins to the plasmid partitioning mechanism.

Plasmid inheritance assays were used to identify the 2µm-like pSB3 plasmid partitioning locus (*PAR*) and show that the pSB3 plasmid could efficiently partition in a heterologous *S. cerevisiae* host. Despite this, the partitioning loci of pSR1, pSB3 and pKW1 could not functionally substitute for 2µm *STB* and increase the inheritance of a 2µm-based plasmid in *S. cerevisiae*. These results suggest that partitioning locus recognition by the plasmid proteins is plasmid-specific and sequence differences between the loci might determine the specificity of the *cis*-acting sequence. Consistent with this, the pSB3 partitioning proteins Rep1 and C recognized the pSB3 *PAR* sequence *in vivo* but could not associate with the 2µm *STB* DNA sequence.

The pSR1 and pSB3 partitioning proteins associated with their partners *in vivo*, with pSB3 Rep1 and C interacting through their amino-terminal regions, as previously observed for 2µm Rep1 and Rep2. Additionally, the C-terminal domain of pSB3 Rep1 was required for the efficient association of the pSB3 C protein with the pSB3 *PAR* locus and plasmid gene promoters. Taken together, these results suggest that the mechanism of plasmid partitioning between pSB3 and 2µm plasmids is conserved as well as the transcriptional regulation of plasmid genes by the partitioning proteins.

Artificial tethering of proteins to a non-partitioning plasmid was used to characterize a novel Rep1-independent role of 2µm Rep2 in 2µm plasmid partitioning and to show that motifs that enable this function can now be assessed by mutational substitutions. Furthermore, the C-terminal 65 amino acids of 2µm Rep2, required for this Rep1-independent function, could be functionally substituted by the corresponding C-terminal region of the pKW1 C partitioning protein.

This research represents the first experimental analysis of partitioning proteins encoded by other members of the 2µm-like family of plasmids and has provided insight into a contribution of 2µm Rep2 to plasmid partitioning that is independent of its interactions with the Rep1 partitioning protein. Additionally, results obtained in this research suggest that all members of the family function in the same way which may reflect their evolution from a common ancestor. Furthermore, molecular tools developed in this study will now allow further investigation of the partitioning mechanism by which the 2µm-like plasmid family is stably maintained.

List of Abbreviations Used

AD	transcriptional activation domain
ARS	autonomously replicating sequence
BD	DNA-binding domain
CEN	centromere
DNA	deoxyribonucleic acid
$Gal4_{AD}$	Gal4 transcriptional activation domain
IgG	immunoglobulin
IR	inverted repeat
$LexA_{BD}$	LexA DNA-binding domain
NLS	nuclear localization sequence
ORF	open reading frame
ORI	origin of replication
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
SC	synthetic complete
SD	synthetic defined
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
WT	wild-type
YPAD	yeast extract peptone adenine dextrose

Yeast nomenclature

AAA	wild-type gene
aaa	mutant gene
*aaa*Δ	gene deletion
Aaa	wild-type protein
Aaa_{XX}	mutant protein
$aaa^{—}$	absent protein

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Chapter 1. Introduction

The 2µm plasmid of *Saccharomyces cerevisiae* is the best studied member of a family of high-copy extrachromosomal plasmids found only in the Saccharomycetaceae lineage of budding yeast (Strope *et al.*, 2015; Volkert *et al.*, 1989) (Figure 1). Faithful maintenance of these plasmids poses no obvious detriment or benefit to the host cells, but plasmids manage to be maintained by utilizing host replication and chromosome segregation machineries (Reviewed in Rizvi *et al.*, 2018). In this thesis I investigate key elements required for the faithful partitioning of the 2µm plasmid and have extended this analysis to 2µm-like plasmids found in other budding yeast species. My research aimed to identify what 2µm and 2µm-like plasmid features determine their host specificity and partitioning function.

1.1 Overview of 2µm Plasmid Organization and Maintenance

The 2µm plasmid is a small, circular double-stranded DNA, about 6.3-kbp in size, found in the nucleus of most strains of the baker's yeast, *S. cerevisiae* (Rizvi *et al*., 2018; Strope *et al.*, 2015) . The 2µm plasmid is organized into two unique regions separated by a pair of inverted repeats (*IR*s), and four protein-encoding genes (*FLP*, *REP1*, *REP2*, *RAF*) (Figure 2). Replication of the 2µm plasmid is initiated at a single origin of replication (*ARS*) and uses the host replication machinery (Brewer & Fangman, 1987). The plasmid exists at ~ 60 copies per cell in two isomeric forms (A and B) that are present in equal amounts in the cell. Switching between the isoforms is catalyzed by the plasmid-encoded site-specific recombinase Flp, that recognizes the specific target sites (*FRT*) in the *IR*s (Volkert & Broach, 1986; Futcher, 1986; reviewed in Murray, 1987). The plasmid also contains a second *cis*-acting sequence, the *STB* partitioning locus, which

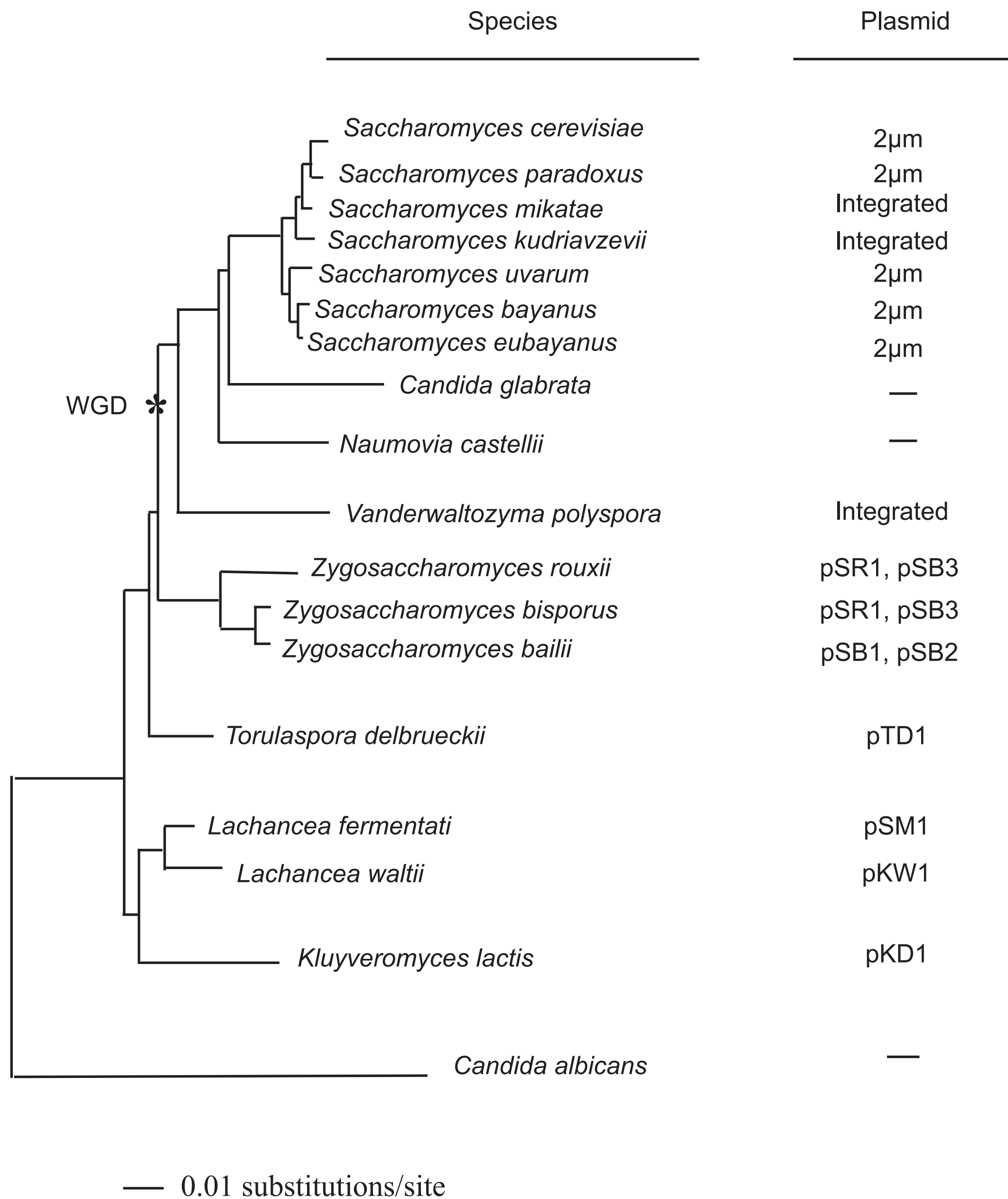


Figure 1. Occurance of 2µm and 2µm-like plamids in Saccharomycetaceae budding yeast species. A phylogenetic tree shows the relationship of yeast species that contain or lack (-) 2µm-like nuclear plasmids or where remnants of the plasmid genes have been identified integrated in the genome (Integrated). An asterisk indicates the whole genome duplication (WGD) event in yeast. Figure adapted from Hedtke *et al*. (2006); OhEigeartaigh *et al*. (2011); Scannell *et al*. (2007); Frank and Wolfe (2009) and Strope *et al*. (2015).

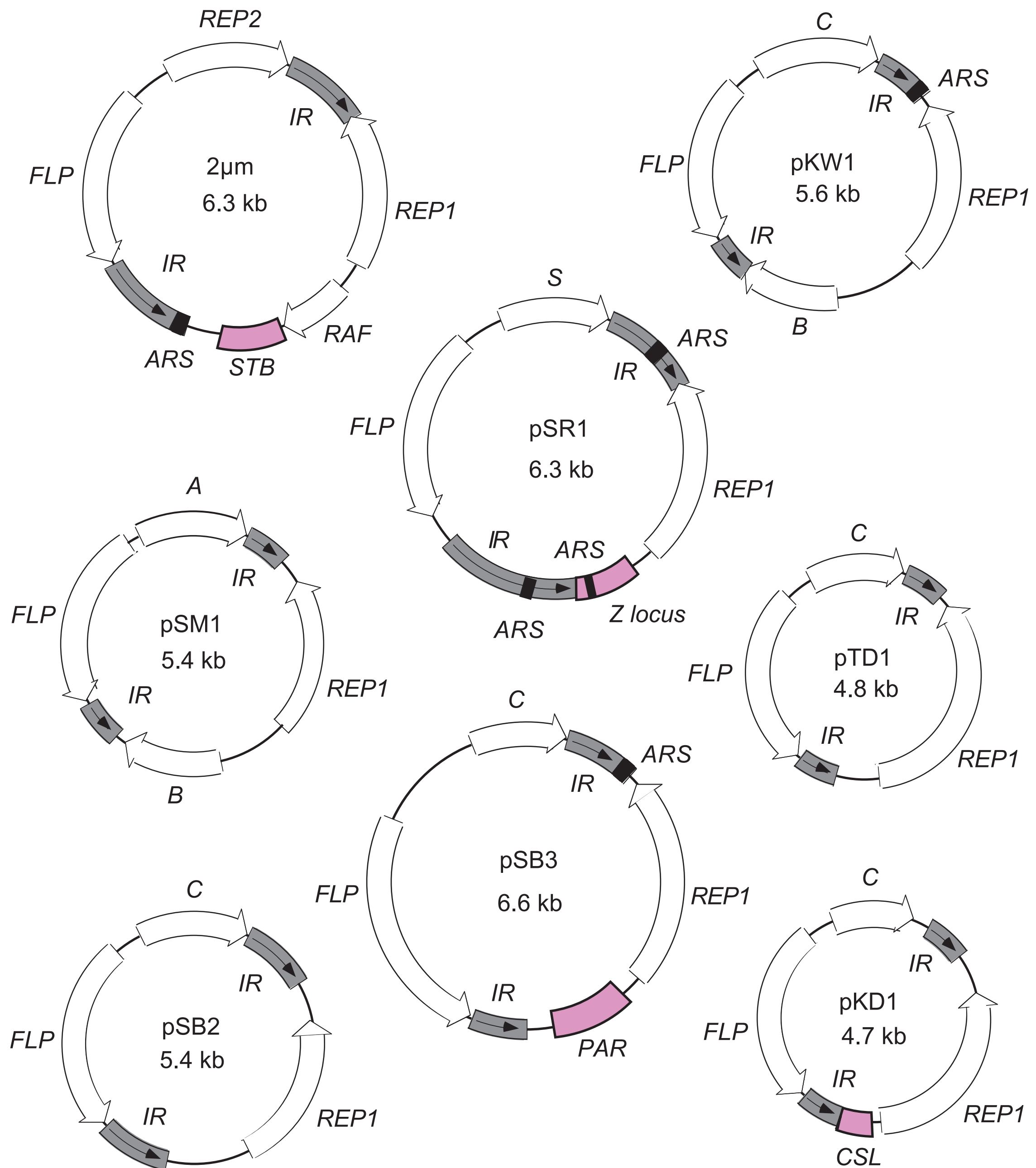


Figure 2. Maps of 2µm and 2µm-like plasmids. Plasmids are *Saccharomyces cerevisiae* 2µm, *Zygosaccharomyces rouxii* pSR1 and pSB3, *Lachancea waltii* pKW1, *Zygosaccharomyces bailii* pSB2, *Zygosaccharomyces fermentati* pSM1, *Kluyveromyces lactis* pKD1, and *Torulaspora delbrueckii* pTD1. Origin of replications (*ARS*) are indicated by black boxes, inverted repeats (*IR*) as grey boxes, plasmid open reading frames are indicated by white arrows, and *cis*-acting stability loci (*STB*, *Z*, *PAR* and *CSL*) are indicated by pink boxes. Plasmid A forms are shown.

is required for plasmid inheritance (Jayaram *et al*., 1983; Kikuchi, 1983; McQuaid *et al.*, 2019).

1.1.1 Amplification of the 2µm Plasmid

Amplification of plasmid copy number is dependent on Flp-mediated recombination. Due to the proximity of the *ARS* to one of the *IR*s, a recombination event during replication is likely to invert only one of the two bidirectional forks resulting in a rolling-circle mode of replication and leading to the amplification of multiple plasmid sequences that are linked end to end (Volkert & Broach, 1986; Futcher, 1986; reviewed in Murray, 1987). A second Flp recombination event restores the direction of the replication fork, allowing the two forks to meet and terminate plasmid amplification. The resulting 2µm plasmid polymer can be converted to multiple monomers by either Flp or host recombination.

1.1.2 Partitioning of the 2µm Plasmid

During host cell division 2µm plasmid copies are equally partitioned between mother and daughter cells. This equal partitioning requires the association of the plasmid-encoded Rep1 and Rep2 proteins with a repeated DNA sequence at the plasmid partitioning locus (*STB*) (Figure 2) (Yang *et al.*, 2004). In the absence of either one of the Rep proteins or *STB*, plasmids fail to be equally partitioned and show a strong maternal segregation bias, with few daughter cells receiving plasmids when they bud from the mother cell (Jayaram *et al*., 1985; Murray & Szostak, 1983).

Rep1 and Rep2 protein association with *STB* triggers the recruitment of host factors, including the nuclear motor protein Kip1 (Cui *et al*., 2009), the centromere-specific histone H3 (Cse4 in yeast), the RCS2 chromatin remodeling complex (Harja *et al*., 2006) and cohesin (Mehta *et al*., 2002). Kinetochore proteins are not recruited to *STB*

and the plasmids are not captured by the plus end of microtubules (Mehta *et al.,* 2002; Mehta *et al.,* 2005) making it unclear how the 2μm copies are equally partitioned during host cell division.

1.1.3 2μm Proteins

Although Rep protein association with *STB* is crucial for the faithful maintenance of the 2μm plasmid, the mechanism is not completely understood and it is unclear whether higher-order complexes of the proteins are involved. The 2μm Rep1 and Rep2 proteins have been shown to self-associate, interact with each other and with a third 2μm plasmid protein, Raf (Ahn *et al.*, 1997; Scott-Drew *et al.,* 2002; Mcquaid *et al*., 2017). The Rep2 protein is required for Rep1 post-translational stability while Rep1 is needed for the efficient recognition of *STB* by 2μm Rep2, suggesting that the Rep1 protein is the key player in *STB* association (Pinder *et al.*, 2013). Consistent with this role, amino acid substitutions that abolished Rep1 interaction with either Rep2 or *STB* led to the failure of plasmid partitioning (Yang *et al*., 2004). However, purified Rep1 does not display DNA-binding activity *in vitro* whereas Rep2 does and can preferentially bind *STB* DNA (Sengupta *et al*., 2001). In plasmon resonance assays purified Rep1 and Rep2 interact with *STB* DNA but only if yeast protein extract is supplied, suggesting that host factors may be required for Rep protein association with *STB in vivo* (Hadfield *et al*., 1995).

1.1.4 2μm Control of Gene Expression

The 2μm Rep1 and Rep2 proteins in addition to their partitioning function also act as transcriptional regulators, repressing transcription of plasmid genes directed by two divergent promoter regions on the 2μm plasmid, *FLP/REP2p* and *REP1/RAFp* (Murray *et al*., 1987; Reynolds *et al*., 1987; Som *et al*., 1988; Veit and Fangman, 1988). Repression of the *FLP* gene provides the feedback mechanism by which 2μm Rep1 and Rep2 control

plasmid amplification. This Rep protein-mediated transcriptional silencing can be alleviated by 2µm Raf (Murray *et al*., 1987), which competes with Rep2 for Rep1 association, disrupting the Rep1/Rep2 repressor complex (McQuaid, *et al*., 2017).

1.1.5 2µm *STB* Stability Locus

Despite the known importance of Rep1 and Rep2 association with *STB* and some identified host factors that are required for the active partitioning of the 2µm plasmid, little is understood about the features of the repeated DNA sequence at 2µm *STB* that contribute to the faithful maintenance of the 2µm copies. The *STB* locus consists of a region lying between the 2µm PstI and AvaI restriction sites and confers equal partitioning when present on non-2µm plasmids, provided that Rep proteins are expressed *in trans* (Kikuchi, 1983). 2µm *STB* can be divided into two regions, *STB*-proximal and *STB*-distal (names indicate relative proximity of each to the 2µm *ARS*) (Kikuchi, 1983; Murray & Cesareni, 1986). *STB*-proximal (here after referred to as *STB* for the purposes of this thesis) consists of five and a half tandem direct repeats of 62-63 bps with sequence identities ranging between 75 to 90% (Jayaram *et al.*, 1985). *STB* was found to be sufficient to confer partitioning if 2µm Rep proteins were present *in trans* (Jayaram *et al*., 1985; Murray & Cesareni, 1986).

Our lab has shown that Rep1 can associate with a single 63-bp *STB* repeat *in vivo*, while direct repeats of this sequence could functionally substitute for native *STB* (McQuaid *et al*., 2019a). Mutations of two TGCA motifs in the *STB* repeat abolished Rep protein association with *STB in vivo*, while plasmid partitioning was impaired when either TGCA or the adjacent T-tract were altered (McQuaid *et al*., 2019a). Although the TGCA motifs and the adjacent T-tract are important for *STB* partitioning function, it remains to be established whether these DNA elements are recognized by Rep1 or by a host factor

protein that recruits Rep1 to the locus.

1.2 2µm-like Plasmids in Other Budding Yeast

Small DNA plasmids resembling the 2µm plasmid of *S. cerevisiae* have been identified in other closely-related budding yeast species: pSR1 in *Zygosaccharomyces rouxii* (Araki *et al*., 1985), pSB3 in *Zygosaccharomyces bisporus* (Toh-e *et al*., 1984), pSB1 and pSB2 in *Zygosaccharomyces bailii* (Toh-e *et al*., 1984), pTD1 in *Torulaspora delbrueckii* (Blaisonneau *et al*., 1997) , pSM1 in *Lachancea fermentati* (Utatsu *et al*., 1987), pKW1 in *Lachancea waltii* (Chen *et al*., 1992), and pKD1 in *Kluyveromyces lactis* (Chen *et al*., 1986) (Figure 1). These 2µm-like plasmids share structural and functional similarities with the 2µm plasmid and range in size from 4.7- to 6.6-kbp (Figure 2). All plasmids have an *ARS* sequence, a pair of *IR*s, and three or four open reading frames (ORFs) (Figure 2). Just like the 2µm plasmid, other members of the family exist at high copy-number in two isomeric forms and are stably maintained in their respective hosts (Araki *et al*., 1985; Chen *et al*., 1986; Toh-e *et al*., 1984; Toh-e & Utatsu, 1985; Utatsu *et al*., 1987). These 2µm-like plasmids, however, show no significant nucleotide sequence similarity with 2µm (Murray *et al*., 1988) although, based on the predicted amino acid sequence, each encodes a recognizable Flp recombinase and a Rep1 homolog. Functional analysis of 2µm-like plasmids has been limited.

Mutational analysis of pSB3, pSR1 and pKW1 has shown that deletion or disruption of either the *REP1* gene or the *REP2*-positioned ORF (C, S, and C, respectively) impairs plasmid inheritance, suggesting the second protein is functioning as a Rep2-equivalent (Chen *et al*., 1992; Jearnpipatkul *et al*., 1987a; Toh-e *et al*., 1984; Toh-e & Utatsu, 1985). Based on the plasmid nucleotide sequences, the 2µm-like plasmids lack a recognizable *STB* analog. However, *cis*-acting loci required for the plasmid

partitioning have been identified for the pSR1, pKD1 and pKW1 plasmids (*Z*, *CSL*, and *RB* (the *REP1-B* intergenic region), respectively) (Jearnpipatkul *et al*., 1987b; Bianchi *et al*., 1991; Mereshchuk, Dalhousie University, honours thesis).

1.3 Overview

Despite recent discoveries related to the maintenance of the *S. cerevisiae* 2µm plasmid, the mechanism of the 2µm and 2µm-like plasmid partitioning is still not understood. Contributions of the plasmid proteins and host factor requirements as well as the specificity and mode of protein interactions remain to be clarified. In this thesis bioinformatic tools and *in vivo* assays have been used to identify protein domains and features required for the partitioning of the 2µm and 2µm-like plasmids. I show that the pSR1 and pSB3 partitioning proteins can associate with their native partners in *S. cerevisiae*. When co-expressed in *S. cerevisiae,* pSB3 Rep1 and C could recognize the pSB3 *PAR* sequence, identified as the pSB3 partitioning locus, and the pSB3 gene promoters. I further show that amino-terminal domains of the pSB3 Rep1 and C proteins are required and sufficient for their interaction, similar to the domains required for 2µm Rep1 and Rep2 association. Additionally, I identify a function for the C-terminal domain of 2µm Rep2 that can mediate Rep1-independent plasmid partitioning *in vivo* with this function being conserved in the corresponding domain of the C protein encoded by the *REP2*-positioned ORF of the 2µm-like plasmid pKW1. Efficient partitioning of the *Zygosaccharomyces* pSB3 plasmid in *S. cerevisiae* was also demonstrated, giving the first experimental evidence that host factors required for the faithful maintenance of these plasmids might be conserved between some budding yeast species.

Chapter 2. Methods

2.1 Strains and Media

Yeast strains used is this study are listed in Table 1. Yeast were cultured at 28°C in YPAD (1% yeast extract, 2% Bacto Peptone, 0.003% adenine, 2% glucose), in synthetic complete (SC) (0.67% Difco yeast nitrogen base without amino acids, 2% glucose, 1% Difco casamino acids, 0.003% adenine, 0.002% uracil, 0.002% tryptophan), or in synthetic defined (SD) medium (0.67% Difco yeast nitrogen base without amino acids, 2% glucose, 0.003% adenine, 0.002% uracil, and all required amino acids) (Burke, Dawson, & Stearns, 2000). To select for the presence of plasmids with specific nutritional marker genes, SC or SD medium lacking specific bases or amino acids was used. To select for the presence of *kanMX4*-tagged plasmids, YPAD supplemented with 200 mg/L geneticin (G418, Sigma) was used. Solid media were prepared by addition of 2% agar. Yeast were transformed using the LiAc/SS-DNA/PEG method (Gietz *et al*., 1995).

Escherichia coli strain DH5α was used for propagation of plasmids. *E. coli* were cultured and manipulated by standard protocols (Sambrook *et al*., 1989).

2.2 Plasmids

Plasmids used in this study are listed in Table 2. Oligonucleotides used in this study are listed in Table 3. Polymerase Chain Reaction (PCR) was performed using Phusion polymerase according to recommendations from the supplier (Thermo Scientific). PCR products were purified using a Nucleospin Kit (Macherey-Nagel) and Topo-TA cloned into pCR2.1-TOPO (Invitrogen). Generated plasmids were confirmed by sequencing (Robarts).

Table 1. Yeast strains used in the study

S. cerevisiae Strain	Genotype	Source
MD83/1c	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 [cir^{0}]*	(Dobson *et al.*, 2005)
CT/MD/3a	*MATa his3 trp1 leu2-3,112 ade2-1 ura3 met URA3:(lexA$_{(op8)}$)-lacZ [cir^{0}]*	(Pinder *et.al.*, 2013)
AG8/5	*MATa ade2Δ ::URA3 his3-11,15 leu2-3, 112 trp1-1 ura3-1 [cir^{0}]*	W303 a/α [cir^{0}]
JP48/2b	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 [cir+]*	W303 a/α [cir^{+}]
JP49/6b	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 [cir^{0}]*	W303 a/α [cir^{0}]
EP7	*MATα ade2 his3-11,15 trp1-1 ura3-1 leu2-3, -112 URA3:: p-lacZ [cir^{0}]*	(McQuaid e*t al.*, 2019)
EP8-STB(F)	*MATα ade2 his3-11,15 trp1-1 ura3-1 leu2-3, -112 URA3:: STB(F)-p-lacZ [cir^{0}]*	(McQuaid *et al.*, 2019)
JP49/6b-pKW1-RB	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::pKW1 RB-p-lacZ [cir^{0}]*	JP49/6b
JP49/6b-pSR1-Z	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::pSR1 Z-p-lacZ [cir^{0}]*	JP49/6b
JP49/6b-pSB3-PAR	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::pSB3 PAR-p-lacZ [cir^{0}]*	JP49/6b
JP49/6b-pSB3-par$_{528}$	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::pSB3 par$_{528}$-p-lacZ [cir^{0}]*	JP49/6b
JP49/6b-pSB3-FLPp	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::pSB3 FLPp-p-lacZ [cir^{0}]*	JP49/6b
Strain Name	Species and Features	Source
Z. rouxii	Wild type *Z. rouxii [pSR1^{+}, pSB3^{+}]*	ATCC 56076

Table 2. Plasmids used in this study

Plasmid Name	Features	Source
pJL638	URA3 (no UASp)-lacZ	(Li & Herskowitz, 1993)
pJL638-pKW-RB	URA3 pKW RB-lacZ	A. Mereshchuk*
pJL638-pSR1-Z	URA3 pSR1 Z-lacZ	This study
pJL638-pSB3-PAR	URA3 pSB3 PAR-lacZ	This study
pJL638-pSB3-FLPp	URA3 pSB3 FLPp-lacZ	This study
pJL638ΔSB-pSB3-PAR	URA3 pSB3 PAR-lacZΔSmaI/BglII	This study
pJL638ΔSB-pSB3-par_{528}	URA3 pSB3 par_{528}-lacZΔSmaI/SpeI	This study
pGAD424	LEU2 2µm ADH1p-$GAL4_{AD}$	Clonetech
pGADREP1	LEU2 2µm ADH1p-$GAL4_{AD}$-REP1	(Sengupta et al., 2001)
pGADREP2	LEU2 2µm ADH1p-$GAL4_{AD}$-REP2	(Sengupta et al., 2001)
pGADRAF	LEU2 2µm ADH1p-$GAL4_{AD}$-RAF	(McQuaid et al., 2017)
pGADKW-REP1	LEU2 2µm ADH1p- $GAL4_{AD}$ -pKW REP1	A. Mereshchuk*
pGADKW-C	LEU2 2µm ADH1p- $GAL4_{AD}$ -pKW C	A. Mereshchuk*
pGADKW-B	LEU2 2µm ADH1p- $GAL4_{AD}$ -pKW B	A. Mereshchuk*
pGADSR1-REP1	LEU2 2µm ADH1p- $GAL4_{AD}$ -pSR1 REP1	This study
pGADSR1-S	LEU2 2µm ADH1p- $GAL4_{AD}$ -pSR1 S	This study
pGADSB3-REP1	LEU2 2µm ADH1p- $GAL4_{AD}$ -pSB3 REP1	This study
pGADSB3-C	LEU2 2µm ADH1p- $GAL4_{AD}$ -pSB3 C	This study
pGADSB3-C_{1-59}	LEU2 2µm ADH1p- $GAL4_{AD}$ -pSB3 C_{1-59}	This study
pGADSB3-C_{1-174}	LEU2 2µm ADH1p- $GAL4_{AD}$ -pSB3 C_{1-174}	This study
pGADSB3-C_{60-213}	LEU2 2µm ADH1p- $GAL4_{AD}$ -pSB3 C_{60-213}	This study
pSH2-1	HIS3 2µ m ADH1p-$LexA_{BD}$	(Lech et al., 1988)
pSHREP1	HIS3 2µm ADH1p- $LexA_{BD}$ -REP1	(Sengupta et al., 2001)
pSHRep1_{1-224}	HIS3 2µm ADH1p- $LexA_{BD}$ -$REP1_{1-224}$	This study
pSHREP2	HIS3 2µm ADH1p- $LexA_{BD}$ -REP2	(Sengupta et al., 2001)
pSHRAF	HIS3 2µm ADH1p- $LexA_{BD}$ -RAF	(McQuaid et al., 2017)
pSHKW-REP1	HIS3 2µm ADH1p- $LexA_{BD}$ -pKW REP1	A. Mereshchuk*
pSHKW-C	HIS3 2µm ADH1p- $LexA_{BD}$ -pKW C	A. Mereshchuk*
pSHKW-B	HIS3 2µm ADH1p- $LexA_{BD}$ -pKW B	A. Mereshchuk*
pSHSR1-REP1	HIS3 2µm ADH1p- $LexA_{BD}$ -pSR1 REP1	This study
pSHSR1-S	HIS3 2µm ADH1p- $LexA_{BD}$ -pSR1 S	This study
pSHSB3-REP1	HIS3 2µm ADH1p- $LexA_{BD}$ -pSB3 REP1	This study

Plasmid Name	Features	Source
pSHSB3-Rep1$_{1-254/378-517}$	*HIS3 2µm ADH1p- LexA$_{BD}$ - pSB3 REP1$_{1-254/378-517}$*	This study
pSHSB3-Rep1$_{1-143}$	*HIS3 2µm ADH1p- LexA$_{BD}$ -pSB3 REP1$_{1-143}$*	This study
pSHSB3-Rep1$_{1-254}$	*HIS3 2µm ADH1p- LexA$_{BD}$ -pSB3 REP1$_{1-254}$*	This study
pSHSB3-Rep1$_{70-517}$	*HIS3 2µm ADH1p- LexA$_{BD}$ -pSB3 REP1$_{70-517}$*	This study
pSHSB3-Rep1$_{70-254/378-517}$	*HIS3 2µm ADH1p- LexA$_{BD}$ -pSB3 REP1$_{70-254/378-517}$*	This study
pSHSB3-C	*HIS3 2µm ADH1p- LexA$_{BD}$ -pSB3 C*	This study
pKan	*kanMX4 2µm flp- REP1 REP2 RAF STB*	(Pinder *et al*., 2013)
pKanΔBg	*kanMX4 2µm flp- REP1 REP2 RAF*	(McQuaid *et al*., 2019)
pKan-pKW RB	*kanMX4 2µm flp- REP1 REP2 RAF pKW1 RB*	A. Mereshchuk*
pKan-pSR1 Z	*kanMX4 2µm flp- REP1 REP2 RAF pSR1 Z*	This study
pKan-pSB3 PAR	*kanMX4 2µm flp- REP1 REP2 RAF pSB3 PAR*	This study
pAM1	*kanMX4 pKW1 ARS*	A. Mereshchuk*
pAM1-pSR1-Z	*kanMX4 pKW1 ARS pSR1 Z*	This study
pAM1-pSB3-PAR	*kanMX4 pKW1 ARS pSB3 PAR*	This study
pTZ18R-pSB3-A1	*pSB3 FLP REP1 C PAR*	This study
pTZ18R-pSB3-A2	*pSB3 FLP REP1 C PAR*	This study
pKan-pSB3-FLP	*kanMX4 pSB3 FLP PAR*	This study
pKan-pSB3-FLP-Rep1	*kanMX4 pSB3 FLP REP1 PAR*	This study
pKan-pSB3	*kanMX4 pSB3 FLP REP1 C PAR*	This study
pKan-pSB3ΔAS	*kanMX4 pSB3 flp- REP1 C PAR*	This study
pCD1	*TRP1 ARS*	C. Desroches Altamirano*
pCD1-lexA$_{(op8)}$	*TRP1 ARS lexA$_{op8}$*	M. Dobson*
pMM7a	*HIS3 ARS/CEN 2µm GAL1p-LexA$_{BD}$*	M. McQuaid*
pMM7a-REP1	*HIS3 ARS/CEN 2µm GAL1p-LexA$_{BD}$-REP1*	M. Dobson*
pMM7a-REP2	*HIS3 ARS/CEN 2µm GAL1p-LexA$_{BD}$-REP2*	M. Dobson*
pMM7a-KW C	*HIS3 ARS/CEN 2µm GAL1p-LexA$_{BD}$-pKW C*	This study
pMM7a-Rep2$_{1-231}$-SV40	*HIS3 ARS/CEN 2µm GAL1p-LexA$_{BD}$-REP2$_{1-231}$-SV40*	This study
pMM7a-Rep2$_{1-231}$-C$_{WT}$	*HIS3 ARS/CEN 2µm GAL1p-LexA$_{BD}$-REP2$_{1-231}$-C$_{WT}$*	This study
pMM7a-Rep2$_{1-231}$-C$_{AAA}$	*HIS3 ARS/CEN 2µm GAL1p-LexA$_{BD}$-REP2$_{1-231}$-C$_{AAA}$*	This study
pMM7a-Rep2$_{1-231}$-C$_{GGP}$	*HIS3 ARS/CEN 2µm GAL1p-LexA$_{BD}$-REP2$_{1-231}$-C$_{GGP}$*	This study

Plasmid Name	Features	Source
pAS27	*ADE2 2μm flp- REP1 REP2 RAF STB*	J. Pinder*
pAS27ΔStuI	*ade2*, *2μm flp- REP11*$_{130-373}$ *REP2 raf-*	This study
pAS27ΔStuI-pKW1 C	*ade2*, *2μm flp- REP1*$_{130-373}$ *pKW1 C raf-*	This study
pAS27ΔStuI-Rep2$_{1-231}$-SV40	*ade2*, *2μm flp- REP1*$_{130-373}$ *REP2*$_{1-231}$*-SV40 raf-*	This study
pAS27ΔStuI-Rep2$_{1-231}$-C$_{WT}$	*ade2*, *2μm flp- REP1*$_{130-373}$ *REP2*$_{1-231}$*-C*$_{WT}$ *raf-*	This study
pAS27ΔStuI-Rep2$_{1-231}$-C$_{AAA}$	*ade2*, *2μm flp- REP1*$_{130-373}$ *REP2*$_{1-231}$*-C*$_{AAA}$ *raf-*	This study
pAS27ΔStuI-Rep2$_{1-231}$-C$_{GGP}$	*ade2*, *2μm flp- REP1*$_{130-373}$ *REP2*$_{1-231}$*-C*$_{GGP}$ *raf-*	This study
pAS27-pKW1 C	*ADE2 2μm flp- REP1 pKW1 C RAF STB*	This study
pAS27-Rep2$_{1-231}$-SV40	*ADE2 2μm flp- REP1 REP2*$_{1-231}$*-SV40 RAF STB*	This study
pAS27-Rep2$_{1-231}$-C$_{WT}$	*ADE2 2μm flp- REP1 REP2*$_{1-231}$*-C*$_{WT}$ *RAF STB*	This study
pAS27-Rep2$_{1-231}$-C$_{AAA}$	*ADE2 2μm flp- REP1 REP2*$_{1-231}$*-C*$_{AAA}$ *RAF STB*	This study
pAS27-Rep2$_{1-231}$-C$_{GGP}$	*ADE2 2μm flp- REP1 REP2*$_{1-231}$*-C*$_{GGP}$ *RAF STB*	This study

* Dalhousie University

Table 3. Oligonucleotides used as PCR primers in the study

Name	Sequence (5' – 3')	Description
pSR1-REP1-F pSR1-REP1-R	cagaattcAATATGTTTACTAGTCAGG gagtcgACACGGTCTCAATAGTC	Generate pSR1 *REP1* ORF with EcoRI/SalI overhangs
pSR1-ORFS-F pSR1-ORFS-R	caggatCCAGATGCAAATACAAAAC gagtcgaCAATCCTAATCTATTAGCC	Generate pSR1 *S* ORF with BamHI/SalI overhangs
pSB3-REP1-F pSB3-REP1-R	cagaattcGGTATGAACTTTGATCTTGATAG gagtcgACTATACACTATGCGTCAG	Generate pSB3 *REP1* ORF with EcoRI/SalI overhangs
pSB3-ORFC-F pSB3-ORFC-R	caggatccaaATGCCTCCTAGACGTAC gagtcgacAACAAAGCAGTTTGACAAC	Generate pSB3 *C* ORF with BamHI/SalI overhangs
pSB3-STB-Bg pSB3-STB-Bm	caGATCTATCAAGATCAAAGTTC caggatCCACGCACAAACAAGTAC	Amplify pSB3 *PAR* locus with BglII/BamHI overhangs
pSB3-FlpP-Bg pSB3-FlpP-Bm	cagatctCATTTTAATTCGGTTATCTTG caggatccCATACTTTTTATGTGTGATG	Amplify pSB3 *FlPp* region with BglII/BamHI overhangs
pSR1-Z-Bm pSR1-Z-Bm2	caggatccGATGATTGTTTATGGCTCC caggatccATAGTCGGTACCCGTAG	Amplify pSR1 *Z* locus with BamHI overhangs
REP1-12 REP1-34	CGGAATTCATGAATGGCGAGAGACTG caggatcCCTCTTCAGGCGGTAG	Generate 2µm $REP1_{1\text{-}224}$ with EcoRI/BamHI overhangs
pSB3-REP1-254-R	cttcggatCCGCTTTCATTTTCGCTTC	Generate pSB3 $REP1_{1\text{-}254}$ with EcoRI/BamHI overhangs
pSB3-REP1-378-F	gaaagcggatccGAAGCTGAAACTGCAGAAG	Generate pSB3 $REP1_{378\text{-}517}$ with BamHI/SalI overhangs

Name	Sequence (5' – 3')	Description
pSB3REP1-R143	cagtcgACTGCCGCGGAACGCC	Generate pSB3 $REP1_{1-143}$ with EcoRI/SalI overhangs
pSB3REP1-F70	gagaattcTCTAATGTACGTGGCATG	Generate pSB3 $REP1_{70-517}$ with EcoRI/SalI overhangs
pSB3-C60-R	caGTCGACtctctagtagggagatatttc	Generate pSB3 C_{1-59} with BamHI/SalI overhangs
pSB3-C61-F	ctggatccgaaccggtGAGACTTTGCAGGAGATG	Generate pSB3 C_{60-213} with BamHI/SalI overhangs
REP2-10	CGGAATTCATGGACGACATTGAAACAG	Amplify chimeric 2µm *REP2*-pKW1 *C* ORF with EcoRI/BamHI overhangs
REP2-228F	CCAGTCTTGAATCTCACTCTTTTTTTGCTG	
LW-ORFC-238F	GTGAGATTCAAGACTGGCCTC	
pSHrev2	AAAGCAACCTGACCTACA	
REP2-30	CAGCGGCCGCCgacctttctcttctttttggaggctcgagtTCACTCTTTTTTTGCTGTAAAC	Generate chimeric 2µm *REP2*-pKW1 *C* ORF with XhoI site at the junction
LW-ORFC-X	gtctcgagGACTGGCCTCAAGTAAAAG	
pKW1-C-NotI	cagcggccgcTCATGTTGTTCTCAATC	
AT1-F	CAAGTAAAAGAGGCCGAGCGGCTGCAAGAAGAAGTGTTACAG	Mutate pKW1 *C* GRP motif to AAA
AT-R	CTCGGCCTCTTTTACTTG	
AT2-F	CAAGTAAAAGAGGCCGAGGTGGCCCAAGAAGAAGTGTTACAG	Use with AT-R to mutate pKW1 *C* GRP motif to GGP

2.2.1 Reporter Plasmids for One Hybrid Assays

Plasmids encoding a DNA sequence of interest upstream of a *lacZ* reporter gene were used to create one-hybrid yeast reporter strains to test partitioning protein recognition of partitioning DNA sequences *in vivo*. The partitioning loci from pSR1 (*Z* locus), pSB3 (*PAR* locus) pKW1 (*RB* locus), and the *FLP* gene promoter region from pSB3 (*FLPp*) were amplified from the native plasmids flanked by BglII and BamHI compatible restriction sites. The resulting fragments were cloned at the BglII site in the *URA3* gene-based one-hybrid vector pJL638 (Li & Herskowitz, 1993) upstream of the *lacZ* reporter gene, creating the pJL638 plasmid series, pJL638-pSR1-Z, pJL638-pSB3-PAR, pJL638-pKW1-RB, pJL638-pSB3-FLPp, respectively (Table 2). To test sub-portions of the pSB3 *PAR* sequence for pSB3 partitioning protein recognition, pJL638-pSB3-PAR was digested with SmaI and SpeI to remove the 317-bp distal to the *REP1* promoter, overhangs filled-in and the plasmid circularized, creating pJL638ΔSB-pSB3-par_{528}. As a control, pJL638-pSB3-PAR was digested with BglII and SpeI, overhangs filled-in, the plasmid circularized, to create pJL638ΔSB-pSB3-PAR. To integrate the reporter genes into the yeast genome, pJL638 plasmids were linearized with StuI, which cuts within the *URA3* gene in the pJL638 backbone, and used to transform *S. cerevisiae* strain JP49/6b (lacks 2µm; *ura3-1*) to uracil prototrophy.

2.2.2 Yeast One- and Two-Hybrid Assay Plasmids

Plasmids encoding 2µm Rep1, Rep2, Raf and pKW1 Rep1, C, B and fused to the transcriptional activation domain (amino acids 768-881) of Gal4 ($Gal4_{AD}$; pGADREP1, pGADREP2, pGADRAF, pGADLWREP1, pGADLWORFC, pGADLWORFB) or to the DNA-binding domain of the bacterial repressor protein LexA (amino acids 1-87) ($LexA_{BD}$; pSHREP1, pSHREP2, pSHRAF, pSHLWREP1, pSHLWORFC,

pSHLWORFB) were previously described (Sengupta *et al*., 2001; McQuaid *et al*., 2017; Mereshchuk, Honours thesis). Plasmids expressing pSR1 Rep1, S; pSB3 Rep1, C fused to $Gal4_{AD}$ or to $LexA_{BD}$ were created by PCR amplification of the pSR1 and pSB3 *REP1* open reading frames (ORFs) flanked by EcoRI and SalI restriction sites and pSR1 *S* and pSB3 *C* ORFs flanked by BamHI and SalI restriction sites that allowed them to be cloned at the appropriate restriction sites and in frame with $Gal4_{AD}$ or $LexA_{BD}$ in the pGAD424 and pSH2-1 vectors, respectively, creating pGADSR1REP1, pGADSR1ORFS, pGADSB3REP1, pGADSB3ORFC, pSHSR1REP1, pSHSR1ORFS, pSHSB3REP1, and pSHSB3ORFC plasmids.

A plasmid to express a truncated 2μm Rep1 protein ($Rep1_{1-224}$) fused to $LexA_{BD}$ was created by PCR amplification of the first 224 codons of the 2μm *REP1* ORF flanked by EcoRI and BamHI restriction sites and then cloned into EcoRI/BamHI-digested pSH2-1, creating the plasmid pSH-$Rep1_{1-224}$. Plasmids encoding pSB3 truncated Rep1 proteins fused to $LexA_{BD}$ were created by PCR amplification of the pSB3 *REP1* codons 1-143 or 70-517 ($Rep1_{1-143}$; $Rep1_{70-517}$) with flanking EcoRI and SalI sites which were cloned into EcoRI/SalI-digested pSH2-1, creating the plasmids pSHSB3-$Rep1_{1-143}$ and pSHSB3-$Rep1_{70-517}$. A plasmid encoding truncated pSB3 Rep1 protein ($Rep1_{1-254}$) fused to $LexA_{BD}$ was created by PCR amplification of the first 254 codons of pSB3 *REP1* ORF flanked by EcoRI and BamHI which was cloned into EcoRI/BamHI-digested pSH2-1, creating the plasmid pSHSB3-$Rep1_{1-254}$.

To express an internally-deleted version of pSB3 Rep1 that lacked 124 amino acids between positions 254 and 378 ($Rep1_{1-254/378-517}$) fused to $LexA_{BD}$, pSB3-REP1-378-F and pSB3-REP1-R primers were used to amplify pSB3 Rep1 codons 378-517 ($Rep1_{378-517}$) flanked by BamHI and SalI restriction sites which was then cloned into

BamHI/SalI-digested pSHSB3-Rep$1_{1\text{-}254}$, generating the plasmid pSHSB3-Rep$1_{1\text{-}254/378\text{-}517}$. To express an amino-terminal truncation of the pSB3 Rep$1_{1\text{-}254/378\text{-}517}$ protein lacking the first 69 amino acids (Rep$1_{70\text{-}254/378\text{-}517}$) fused to $LexA_{BD}$, pSB3REP1-F70 and pSB3-REP1-R primers were used to amplify pSB3 Rep$1_{70\text{-}254/378\text{-}517}$ from the pSB3 Rep$1_{1\text{-}254/378\text{-}517}$ template flanked by EcoRI and SalI sites. The fragment was then cloned into EcoRI/SalI-digested pSH2-1 creating the plasmid pSHSB3- Rep$1_{70\text{-}254/378\text{-}517}$.

pGAD424 plasmids encoding truncated versions of the pSB3 C protein, comprising amino acids 1-59, 1-174, or 60-213 ($C_{1\text{-}59}$; $C_{1\text{-}174}$; $C_{60\text{-}213}$) fused to $Gal4_{AD}$ were created by PCR amplification of the relevant portions of the pSB3 *C* ORF with flanking BamHI and SalI restriction sites. The resulting fragments were cloned into BamHI/SalI-digested pGAD424, generating the plasmids pGADSB3-$C_{1\text{-}59}$, pGADSB3-$C_{1\text{-}174}$, and pGADSB3-$C_{60\text{-}213}$.

2.2.3 2µm-Based Plasmids

A *KanMX4*-tagged 2µm plasmid either with the native *STB* locus (pKan) or with *STB* deleted (pKanΔBg) has been previously described (Pinder *et al.*, 2013; McQuaid *et al.*, 2019). To create a pKan-based plasmid that had pSR1 or pSB3 partitioning loci in the place of the native 2µm *STB*, the pSR1 *Z* locus with flanking BamHI sites and the pSB3 *PAR* with flanking BglII/BamHI sites were amplified by PCR and cloned at the BglII site in the pKanΔBg vector generating plasmids pKan-SR1Z and pKan-SB3PAR. Creation of the pKanΔBg-based plasmid with the pKW1 *RB* locus in the place of 2µm *STB* (pKan-LWRB) has been previously described (Mereshchuk, Honours thesis).

2.2.4. Non-2µm-Based Plasmids for 2µm-like Plasmid Partitioning Loci Identification

A *KanMX4*-tagged plasmid with the replication origin (*ARS*) from *L.waltii*

pKW1(pAM1) has been previously described (Mereshchuk, Honours thesis). The partitioning loci from pSR1 and pSB3 2µm-like plasmids, that had been amplified with BamHI overhangs for pSR1 *Z* and with BglII/BamHI overhangs for pSB3 *PAR* were cloned at the BamHI site in the pAM1 vector, creating the plasmids pAM1-pSR1-Z and pAM1-pSB3-PAR.

2.2.5. *KanMX4*-tagged pSB3 plasmids

Genomic DNA was extracted from *Z. rouxii* yeast (ATCC 56076) (Cryer *et al*., 1975), digested with the restriction enzyme XhoI, resolved by agarose gel electrophoresis, and a 6.6-kbp fragment corresponding to the full-length pSB3 2µm-like plasmid was purified using a Nucleospin Kit (Macherey-Nagel) and cloned in SalI-digested pTZ18R (Pharmacia) to yield pTZ-pSB3-A1 and plasmid pTZ-pSB3-A2 plasmids in which the amp^R gene in the pTZ18R bacterial backbone is closer to either the pSB3 *REP1* or *FLP* ORF, respectively. A *KanMX4*-tagged plasmid containing the pSB3 *FLP* gene and *PAR* (pKan-pSB3-FLP) was created by ligating a 3.2-kbp EcoRI/MluI DNA fragment from pAM1 with a 2.9-kbp EcoRI/MluI fragment from pTZ18R-pSB3-A2. A 6-kbp EcoRI (filled in)/BamHI fragment from pKan-pSB3-FLP was ligated with a 2.4kbp MluI (filled in)/BamHI fragment from pTZ18R-pSB3-A1, generating a *KanMX4*-tagged plasmid containing pSB3 *FLP* and *REP1* ORFs, and pSB3 *PAR* (pKan-pSB3-FLP-REP1). To create a *KanMX4*-tagged plasmid that had all pSB3 sequences with no disrupted ORFs (pKan-pSB3), a 1.4-kbp MluI fragment from pTZ18R-pSB3-A1 was cloned into MluI-digested pKan-pSB3-FLP-REP1. To create a pKan-pSB3 version that lacked 691-bp in the pSB3 *FLP* ORF (pKan-pSB3ΔFLP), pKan-pSB3 was digested with AgeI/StuI, the overhangs filled in, and the plasmid circularized by ligation.

2.2.6. Plasmids for Assessing Rep1-Independent Partitioning Function of 2μm Rep2

A *TRP1*-tagged *S. cerevisiae ARS*-containing plasmid (pCD1) has been described previously (McQuaid *et al*., 2019a). To create a pCD1-based plasmid that contained 8 copies of the LexA operator sequence ($lexA_{(op8)}$) (pCD1-$lexA_{(op8)}$), a BamHI/NruI fragment encoding $lexA_{(op8)}$ was PCR-amplified from the reporter construct integrated in the genome of the yeast two-hybrid strain CTY10/5d (Bartel *et al*., 1993) and cloned in BamHI/NruI-digested pCD1 (M. Dobson, unpublished).

A *HIS3*-tagged single-copy yeast plasmid containing an *S. cerevisiae ARS* and centromere (*CEN*) sequences and expressing $LexA_{BD}$ under the control of the *ADH1* promoter (pMM7a) was created by Dr. Mary McQuaid (unpublished results). To express 2μm Rep2 and pKW1 C proteins as $LexA_{BD}$ fusions in yeast, the ORF sequences were amplified by PCR and cloned into pMM7a creating, the pMM7a-REP2 and pMM7a-KW-C plasmids. To generate a plasmid that encoded 2μm Rep2 (codons 1-231; $Rep2_{1-231}$) fused to $LexA_{BD}$ and with a C-terminal SV40 nuclear localization signal, REP2-10 and REP2-30 primers were used to PCR-amplify a $Rep2_{1-231}$-SV40 fused coding sequence flanked by EcoRI sites and with a NotI site immediately downstream of the nuclear localization sequence. The DNA was then digested with EcoRI and cloned into EcoRI-digested pMM7a, creating the pMM7a-$Rep2_{1-231}$-SV40 plasmid. To create a plasmid that encoded a chimeric 2μm Rep2 (codons 1-231; $Rep2_{1-231}$) – pKW1 C (codons 238-306; $C_{238-306}$) coding sequence fused to $LexA_{BD}$ with a unique XhoI at the *REP2-C* junction, DNAs encoding $Rep2_{1-231}$ and pKW1 $C_{238-306}$ were PCR-amplified with flanking EcoRI/XhoI and XhoI/NotI sites, respectively. The generated fragments were digested with XhoI and ligated, yielding an EcoRI/NotI fragment encoding codons 1-231 of 2μm *REP2* fused to residues 238 to 306 of pKW1 C, the domain containing an AT-hook motif

(McQuaid *et al*., 2017). This fragment was cloned into pMM7a to generate pMM7a-Rep2$_{1-231}$-C$_{WT}$. Site-directed mutagenesis was carried out by overlap-PCR to introduce AAA and GGP mutations in the AT-hook GRP motif of pKW1 C$_{238-306}$. AT1-F, AT-R, LW-ORFC-X and pKW1-C-NotI primers were used in PCR amplification to generate pKW C$_{238-306}$ with the GRP motif changed to AAA and flanked with XhoI and NotI sites (C$_{AAA}$). AT2-F, AT-R, LW-ORFC-X and pKW1-C-NotI primers were used in PCR amplification to generate pKW1 C$_{238-306}$ with the GRP motif changed to GGP and flanked with XhoI and NotI sites (C$_{GGP}$). C$_{AAA}$ and C$_{GGP}$ were ligated with XhoI-digested Rep2$_{1-231}$-SV40 PCR product yielding *REP2*$_{1-231}$-*C*$_{AAA}$ and *REP2*$_{1-231}$-*C*$_{GGP}$ with EcoRI/NotI overhangs. NotI (filled-in)/EcoRI fragments were cloned into BamHI (filled-in)/EcoRI-digested pMM7a, creating the pMM7a-Rep2$_{1-231}$-C$_{AAA}$ and pMM7a-Rep2$_{1-231}$-C$_{GGP}$ plasmids.

An *ADE2*-tagged *flp*$^-$ 2µm plasmid that has a unique in-frame NotI site immediately after the 2µm *REP2* stop codon and that partitions in *S. cerevisiae* as efficiently as a native 2µm plasmid (pAS27) (J. Pinder, unpublished) was used as a basis for plasmids to assess function of *REP2* chimeric ORFs in *S. cerevisiae*. To create pAS27-based plasmids that had either pKW1 *C* or truncated or mutated versions of 2µm *REP2* in the place of the native *REP2* gene, pAS27 was first digested with StuI and self-ligated to generate plasmid pAS27ΔStuI to remove regions containing restriction sites that would interfere with *REP2* gene replacement. PCR-generated pKW1 *C*, Rep2$_{1-231}$-SV40, Rep2$_{1-231}$-C$_{WT}$, Rep2$_{1-231}$-C$_{AAA}$ and Rep2$_{1-231}$-C$_{GGP}$ with EcoRI/BamHI (filled-in) or EcoRI/NotI overhangs were cloned in either EcoRI/NotI (filled-in) or EcoRI/NotI-digested pAS27ΔStuI creating pAS27ΔStuI-pKW1 C, pAS27ΔStuI -Rep2$_{1-231}$-SV40, pAS27ΔStuI -Rep2$_{1-231}$- C$_{WT}$, pAS27ΔStuI -Rep2$_{1-231}$-C$_{AAA}$, pAS27ΔStuI -Rep2$_{1-231}$-

C_{GGP}. To restore the rest of the pAS27 sequence, 1.5-kbp SphI fragments from pAS27ΔStuI plasmids were ligated with 11-kbp SphI-digested pAS27 backbone creating pAS27 plasmids in which native 2µm *REP2* gene had been replaced by the variant coding regions.

2.3 Sequence Alignment

Sequences of pSB3-encoded proteins were obtained from overlapping reads of the cloned plasmid sequences (Dobson, unpublished results). The 2µm and pSR1 plasmid sequences were obtained from the National Centre for Biotechnology Information (NCBI) (Gene-bank accession numbers: J01347.1 and X02398.1, respectively). DNA and predicted protein sequences were aligned using Clustal Omega with default settings. Alignments generated by Clustal Omega were shaded using Boxshade 3.21 with default settings.

2.4 One-Hybrid and Two-Hybrid Assays

To test for protein-protein interactions *in vivo*, an *S. cerevisiae* two-hybrid reporter strain (CT/MD/3a) that lacked 2µm plasmid (*cir^0*) was co-transformed with two plasmids, one expressing one of the proteins fused to the Gal4 activation domain ($Gal4_{AD}$) (pGAD424-based plasmids) and the other expressing the second protein fused to the LexA DNA binding domain ($LexA_{BD}$) (pSH2-1-based plasmids) (Bartel *et al*., 1993; Pinder *et al*., 2013). The reporter strain contains 8 copies of the LexA operator sequence upstream of a *lacZ* reporter gene integrated in the genome.

Protein-DNA interactions were assessed *in vivo* using a one-hybrid assay (Li & Herskowitz, 1993). For this assay, proteins of interest fused to $Gal4_{AD}$ were expressed from pGAD424-based vectors in a *S. cerevisiae* yeast reporter strain that contained the DNA sequence of interest integrated in the genome upstream of a *lacZ* reporter gene. To

determine whether the $Gal4_{AD}$ fusion required the presence of another protein to recognize the target DNA, a second protein was expressed in the cell from pSH-based plasmid as a $LexA_{BD}$ fusion. Note that the $LexA_{BD}$ moiety is merely an epitope tag in this context and does not contribute to the interaction as no LexA operator sites are present in the yeast strain.

Activation of the *lacZ* reporter gene was taken as a measure of interaction in the one-hybrid and two-hybrid systems and was monitored using a ß-galactosidase filter assay (Burke *et al*., 2000). Yeast transformants were grown in patches on nitrocellulose membranes overlaid on selective SD solid medium. Cells were lysed by freeze/thaw and the membranes with yeast patches were incubated on a Whatman filter soaked in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM ß-mercaptoethanol) and 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) at 28°C. ß-galactosidase enzyme, the *lacZ* gene product, cleaves X-gal to yield a blue precipitate. For one and two-hybrid assays a minimum of 4 independent transformants were tested and the timing of colour development recorded over 24 hours.

2.5 Plasmid Inheritance Assay

To monitor the inheritance of *KanMX4*-tagged pKan plasmids with 2µm-like plasmid partitioning loci in the place of the native 2µm *STB* or *ADE2*-tagged pAS27 plasmids with mutant or chimeric versions of the 2µm *REP2* gene, a *[cir^0] S. cerevisiae* yeast strain (AG8/5) was used. Inheritance of the *KanMX4*-tagged pAM plasmids containing pSR1 and pSB3 plasmid partitioning loci was tested in *Z. rouxii* yeast. Inheritance of *TRP1*-tagged pCD1-$lexA_{(op8)}$ plasmids was tested by co-transforming *[cir^0] S. cerevisiae* yeast (JP49/6b) with this *ARS*-only plasmid and a *HIS*-tagged pMM7a

CEN/ARS plasmid, expressing either just $LexA_{BD}$ or mutant or chimeric 2µm Rep2 proteins fused to $LexA_{BD}$. In all cases, the fraction of prototrophic or drug-resistant cells was used as a measure of plasmid inheritance. Yeast were grown for ~10 generations in appropriate selective media (16-24 h at 28°C) and appropriate dilutions of the cultures were plated on selective and non-selective solid medium. The fraction of cells carrying the tagged plasmid of interest was determined by comparing the plating efficiency on the two media. Statistical significance was calculated using a two-tailed Student's t-test.

2.6 Western Hybridization Analysis

Proteins were extracted from yeast cultures using alkali lysis, as previously described (Yaffe and Schatz, 1984; Chen *et al*., 2005). Briefly, ~ 1 x 10^8 yeast cells were pelleted, resuspended in 0.2 mL of lysis buffer (1.85M NaOH, 7.4% ß-mercaptoethanol) and incubated on ice for 10 minutes. The lysate was neutralized by addition of 0.2 mL of 50% TCA, incubated on ice for another 10 minutes and subjected to centrifugation for 2 minutes at 16 000 g. Protein pellets were washed with ice-cold acetone, dried and resuspended in equal volumes of urea extraction buffer (8 M urea, 100 mM NaH_2PO_4/Na_2HPO_4, 50 mM Tris) and 2x protein gel loading buffer (125 mM Tris pH6.8, 4.0% sodium dodecyl sulfide (SDS), 20% glycerol, 4.0% ß-mercaptoethanol, 1 M urea, 0.05% bromophenol blue, 0.05% xylene cyanol). Resuspensions of the proteins were heated at 37°C for 5 minutes and subjected to centrifugation as before. The supernatant was resolved by SDS-8% polyacrylamide gel electrophoresis. Proteins of interest were detected using western blotting, as previously described (Sengupta *et al*., 2001). Rabbit polyclonal anti-LexA (Invitrogen), rabbit polyclonal anti-$Gal4_{AD}$ (Sigma) and mouse monoclonal anti-Pgk1 (Abcam) were used as primary antibodies with Horseradish

peroxidase (HRP)-conjugated goat anti-rabbit (Novex) and anti-mouse IgG (Sigma) as secondary antibodies. Chemiluminescent signal was detected with Clarity Western ECL Substrate Kit (BioRad) and captured using a VersaDoc 4000MP imaging system and Quantity One software (BioRad).

To detect proteins on the same western transfers but with different antibodies, blots were washed in phosphate-buffered saline (PBS)-Tween, incubated in 50 mL stripping solution (50 mM Tris pH 6.8, 1% SDS,10 mM 2-mercaptoethanol) at 60°C for 30 min and washed again in PBS-Tween before reuse.

Chapter 3. Results

3.1 Loci Required for the Partitioning of the 2µm-Like Plasmids

Apart from the published sequences and limited functional analysis of some 2µm-like plasmid family members, little is known about the mechanism and features that are required for the partitioning of the 2µm-like plasmid family. Despite a similar organization, 2µm and 2µm-like plasmids do not share any significant nucleotide sequence similarity and their second plasmid-encoded partitioning proteins are not recognized as homologs. Similarly, no sequence similar to the 2µm *STB* partitioning locus can be determined by sequence comparisons and none have a tandemly repeated array resembling that seen for the 2µm *STB* locus. For three members of the 2µm-like plasmid family, plasmids pSR1, pKD1 and pKW1, a DNA sequence that increases plasmid inheritance *in vivo* and acts as a plasmid partitioning locus in the native host has been identified (Jearnpipatkul *et al*., 1987a; Jearnpipatkul *et al*., 1987b; Bianchi *et al*., 1991; Mereshchuk, honours thesis). These 2µm-like partitioning sequences, the *Z, CSL* and *RB* loci respectively, are non-coding and resemble the 2µm *STB* sequence in having A- and T-rich nucleotide stretches. The lack of obvious repeated sequences and sequence similarity with 2µm *STB* suggests that sequence differences between partitioning loci from different plasmids might determine the specificity of protein-DNA interactions and/or indicate that DNA sequence elements required for the plasmid locus recognition differ between the plasmid partitioning proteins.

3.1.1 pSB3 *PAR* Sequence Increases Plasmid Inheritance in *Z. rouxii*

To investigate the nature of the partitioning loci in the 2µm-like family of plasmids, I decided to identify the partitioning sequence for another member of the

family, pSB3. The pSB3 plasmid has been found in some strains of *Z. rouxii* that also contain a second 2µm-like plasmid, pSR1 (Toh-e & Utatsu, 1985). No functional analysis has been performed for the 2µm-like pSB3 plasmid and no sequence required for pSB3 equal partitioning has been identified. Based on the organization of the plasmid elements and plasmid-encoded proteins (Figure 2), I hypothesised that the sequence between the pSB3 *REP1* gene and the plasmid *IR* region was likely to act as the pSB3 partitioning locus (*PAR*). Notably, like the partitioning loci that have been identified in other 2µm-like plasmids and in 2µm, this region of pSB3 is non-coding and has several runs of A and T nucleotides. To determine whether the presence of pSB3 *PAR* could increase the partitioning of an otherwise non-partitioning synthetic plasmid, provided that the pSB3 partitioning proteins, Rep1 and C, were supplied in *trans*, an 840-bp sequence was amplified from *Z. rouxii* and cloned in the vector pAM1 which contains the replication origin from the *L. waltii* 2µm-like plasmid pKW1 and a dominant drug-resistance marker gene (*KanMX4*), creating pAM1-pSB3-PAR. As a control, the previously identified partitioning locus (*Z*) from the *Z. rouxii* 2µm-like plasmid pSR1 was isolated and cloned in pAM1 creating plasmid pAM1-pSR1-Z.

Z. rouxii cells that contained the native pSR1 and pSB3 to supply the plasmid partitioning proteins *in trans* were transformed with each of these plasmids and the inheritance of G418-resistance (conferred by *KanMX4*) was assayed (Figure 3). A relatively small but statistically significant increase in inheritance, compared to pAM1, was observed for both pAM1-pSR1-Z and pAM1-pSB3-PAR. This lower than expected improvement could be due to disruption of the partitioning locus function in this sequence context. Alternatively, the *ARS* sequence on the plasmid which was derived from pKW1 might not function efficiently in this heterologous *Z. rouxii* host. Nonetheless, the results

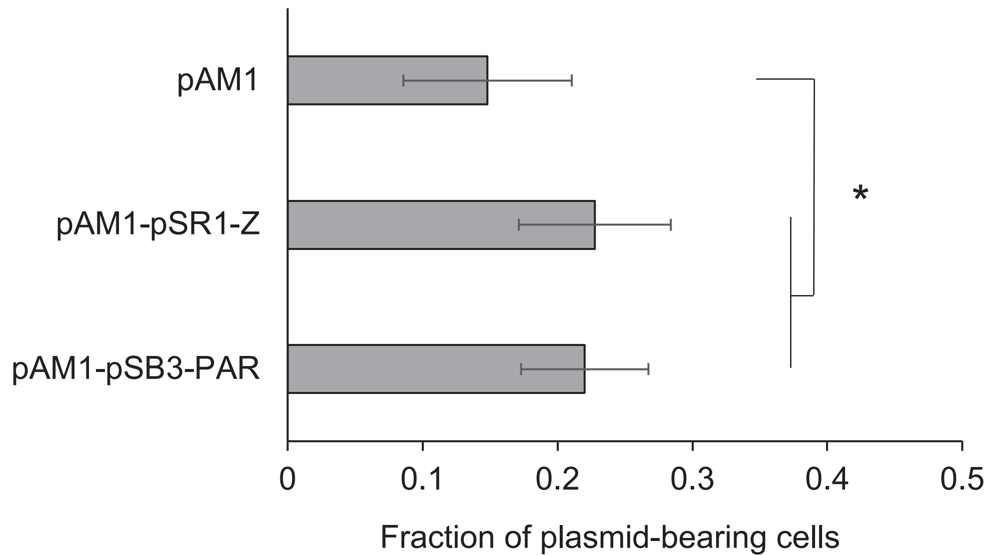


Figure 3. The pSB3 *PAR* sequence increases plasmid inheritance in *Z. rouxii*. A *Z.rouxii* strain containing native pSB3 and pSR1 was transformed with the *KanMX4*-tagged *ARS* plasmid pAM1 or pAM1 containing the *Z* or *PAR* loci from pSR1 and pSB3, respectively. Transformants were cultured overnight in selective medium (YPAD+G418). The fraction of G418-resistant yeast cells indicates the efficiency of plasmid inheritance and was determined by counting the ratio of resistant colonies formed after plating on selective versus non-selective solid medium. Results represent the average (± standard deviation) from assaying six independent transformants for each plasmid. An asterisk indicates a significant difference determined by a two-tailed Student's t-test (*$p \leq 0.05$).

show that the pAM1-based plasmid containing the pSB3 840-bp sequence (pAM1-pSB3-PAR) was inherited with similar efficiency to pAM1-pSR1-Z, suggesting that the hypothesized *PAR* sequence acts as a partitioning locus of the pSB3 plasmid, just as the *Z* locus does for pSR1.

3.1.2 Partitioning Loci from 2µm-Like Plasmids Cannot Functionally Substitute for 2µm *STB* in *S. cerevisiae*

Based on the proposed model of 2µm plasmid partitioning, the association of the 2µm Rep1 and Rep2 partitioning proteins with the *STB* partitioning locus is required for the faithful inheritance of the 2µm plasmid copies (Yang *et al*., 2004). Given that all 2µm-like plasmids share structural similarities with 2µm and have at least one of their partitioning proteins in common (Rep1), I wanted to test whether the partitioning locus from one member of the 2µm-like family could substitute for the *STB* locus and function with 2µm Rep1 and Rep2 to increase the inheritance of a 2µm-based plasmid in *S. cerevisiae*. To test this the 2µm *STB* sequence in a *kanMX4*-tagged 2µm plasmid (pKan) was replaced by the 2µm-like pKW1 *RB*, pSR1 *Z* or pSB3 *PAR* partitioning sequences creating plasmids pKan-pKW1-RB, pKan-pSR1-Z, pKan-pSB3-PAR, respectively, which encode 2µm Rep1 and Rep2 but now have a heterologous partitioning sequence. As a negative control, pKan lacking a native 2µm *STB* sequence (pKanΔSTB) was used. *S. cerevisiae* yeast was transformed to G418-resistance with each of these plasmids and plasmid inheritance was assayed (Figure 4).

2µm-based plasmids bearing either pKW1 *RB*, pSR1 *Z* or pSB3 *PAR* (pKan-RB, pKan-Z, pKan-PAR) were inherited with similar efficiency to the pKanΔSTB plasmid that lacked native 2µm *STB*, demonstrating that none of the 2µm-like partitioning loci could functionally substitute for the 2µm *STB* locus and increase the

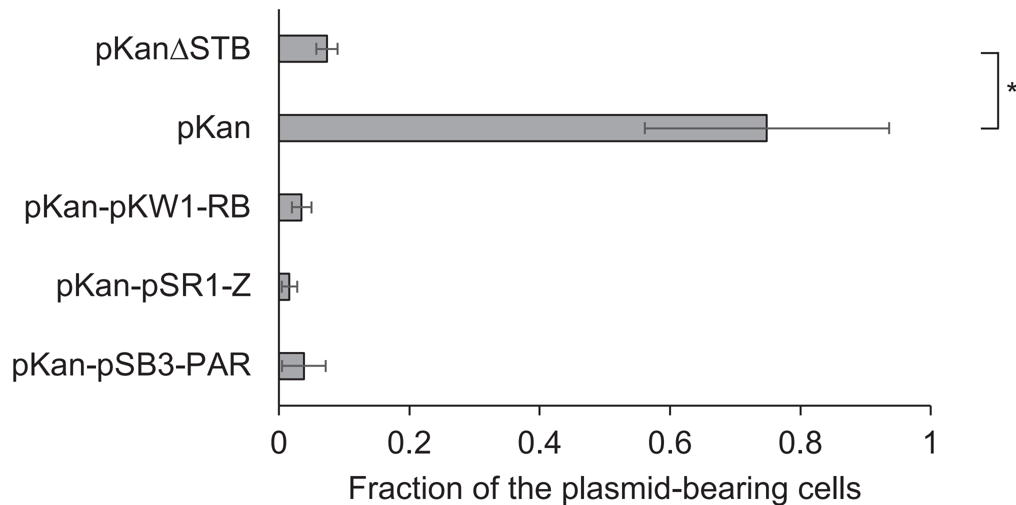


Figure 4. The partitioning loci from 2µm-like plasmids cannot functionally substitute for 2µm *STB*. A *cir⁰* *S. cerevisiae* strain was transformed with 2µm-based pKan plasmids that had no partitioning locus (pKanΔSTB) or contained 2µm (pKan) or 2µm-like partitioning loci in the place of the native 2µm *STB*. Transformants were cultured overnight in selective medium (YPAD+G418). The fraction of G418-resistant yeast cells indicates the efficiency of plasmid inheritance and was determined by counting the ratio of resistant colonies formed after plating on selective versus non-selective solid medium. Results represent the average (± standard deviation) from assaying six independent transformants for each plasmid. An asterisk indicates a significant difference determined by a two-tailed Student's t-test (*p<0.05).

inheritance of the 2µm-based plasmid (Figure 4). These results show that the 2µm Rep1 and Rep2 proteins function only with 2µm *STB*, suggesting the absence of sequence similarity between the 2µm and 2µm-like partitioning loci might reflect the specificity of protein-DNA interactions required for the equal partitioning of the plasmid copies.

3.2 Interactions of the 2µm-Like Plasmid Proteins

No functional analysis has been performed for the 2µm-like plasmid proteins; however, interactions of the 2µm plasmid proteins have been extensively studied. *In vitro* and *in vivo* analyses have shown that 2µm Rep1 and Rep2 can self-associate, interact with each other and with 2µm Raf (Ahn *et al.*, 1997; Scott-Drew *et al.,* 2002; McQuaid *et al*., 2017).

3.2.1 *In vivo* 2µm-Like Plasmid Protein Interaction Analysis

To test whether 2µm-like pKW1, pSR1 and pSB3 plasmid-encoded proteins could associate with their respective partners or cross interact with any other 2µm or 2µm-like plasmid proteins, I performed an *in vivo* two-hybrid protein interaction assay in *S. cerevisiae* (Figure 5). Both pSR1 and pSB3 plasmid proteins interacted with their respective partners, suggesting that for these interactions either host proteins needed for the association are present in *S. cerevisiae* or, like the 2µm Rep1-Rep2 interaction, host proteins are not required. The pSR1 plasmid proteins did not interact with proteins encoded by the other plasmids, however, the pSB3 C protein associated with both 2µm Rep1 and Rep2, suggesting that motifs required for the interaction with 2µm Rep1 and Rep2 may be conserved in pSB3 C (Figure 5). See Section 3.3 for further investigation.

The pKW1 Rep1 partitioning protein associated with 2µm Raf; however, no other pKW1 protein interactions were observed in the two-hybrid assay (Figure 5). Failure to detect interactions in *S. cerevisiae* could be due to the proteins not being expressed in a

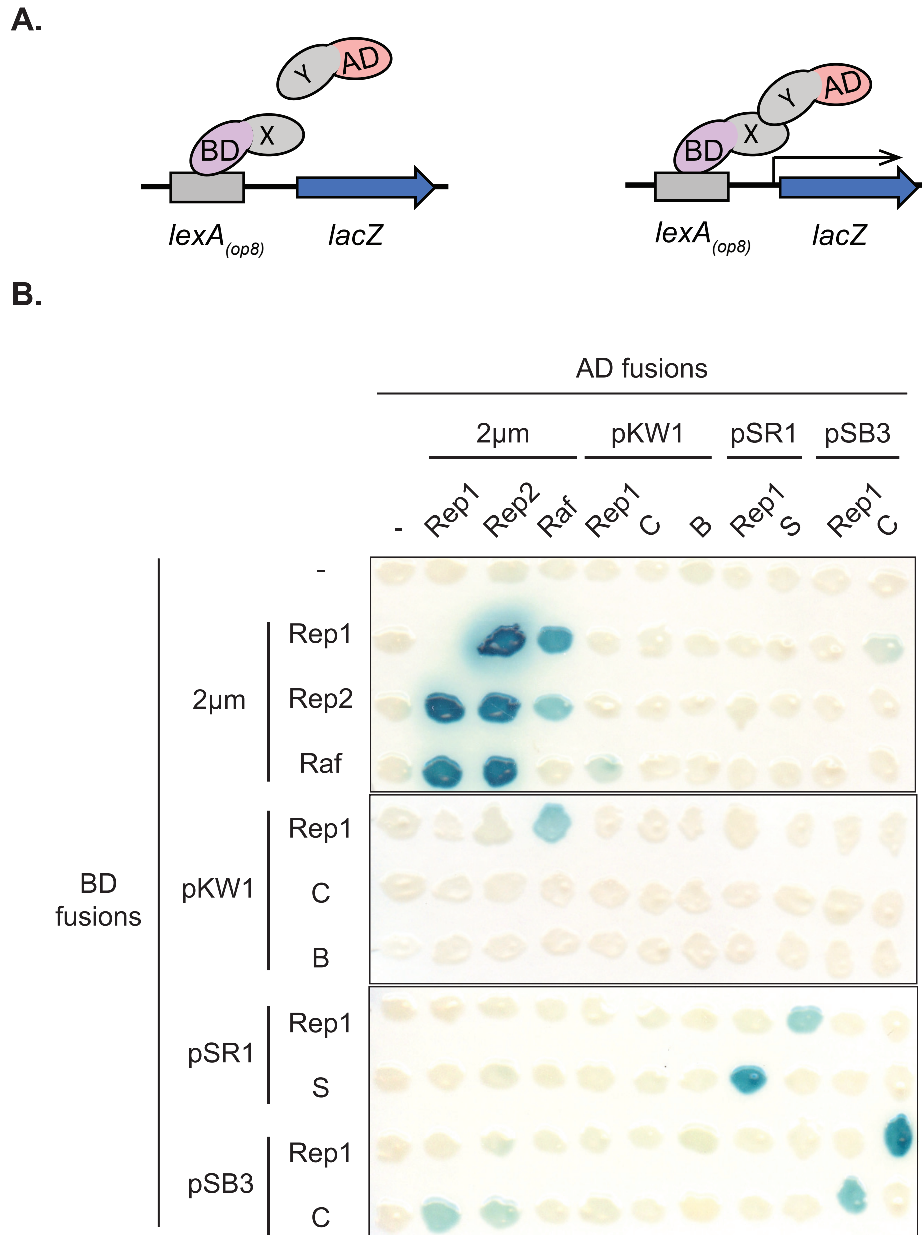


Figure 5. pSR1 and pSB3 proteins interact with their native partners in *S. cerevisiae* in an *in vivo* protein interaction assay, and pSB3 C also interacts with 2 µm Rep1 and Rep2, and pKW1 Rep1 with 2µm Raf. (A) Two proteins were expressed in a *cir⁰* two-hybrid *S. cerevisiae* reporter strain, one fused to the LexA DNA-binding domain (BD), the other to the Gal4 transcriptional activation domain (AD). (B) Co-transformants were grown in patches on a nitrocellulose filter and activation of the *lacZ* reporter gene integrated in the yeast genome downstream of LexA recognition sites ($lexA_{(op8)}$) indicates interaction of the fusion proteins, detected by a filter assay for the *lacZ* gene product, ß-galactosidase, which cleaves X-Gal substrate to produce a blue precipitate.

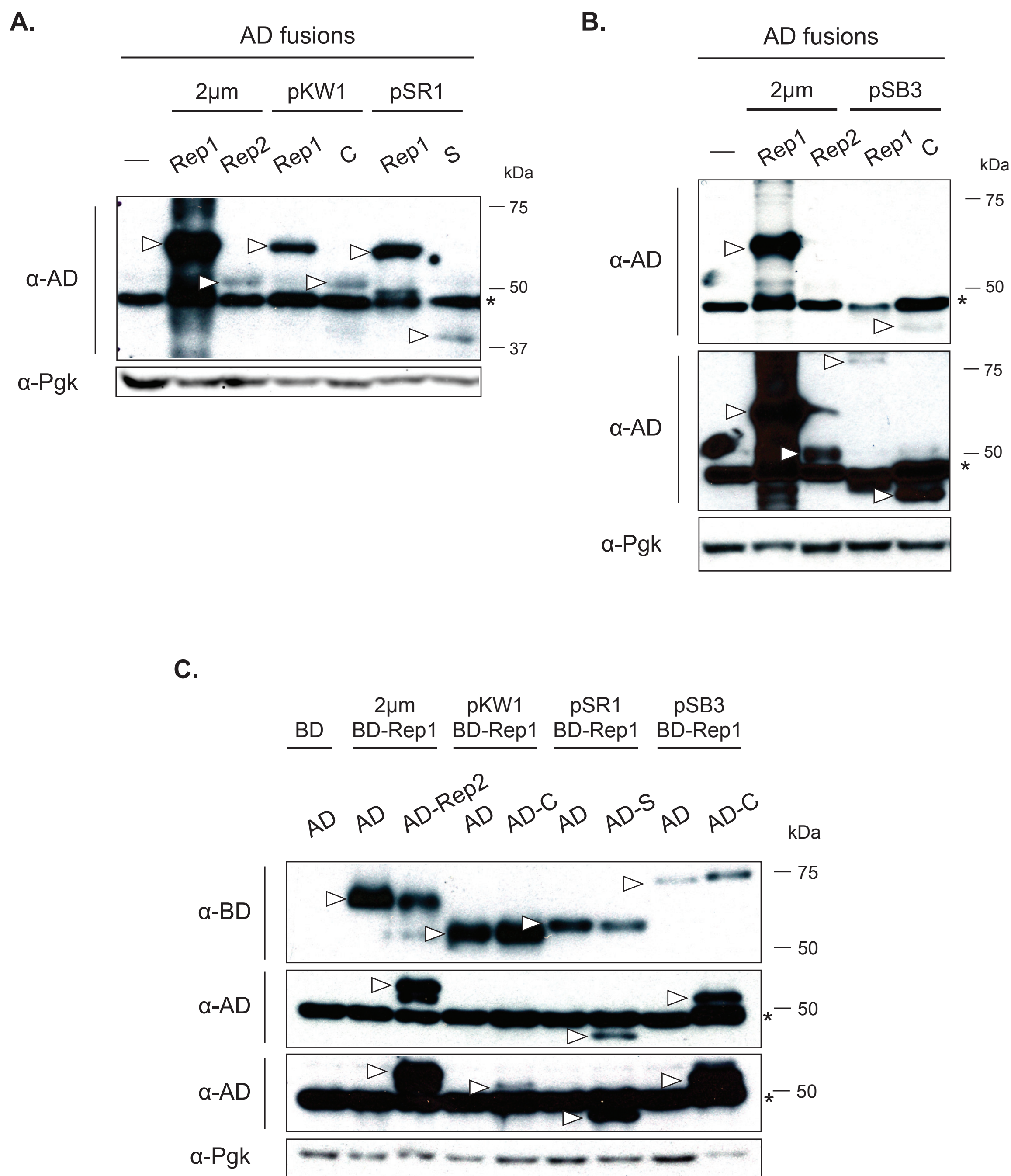


Figure 6. Expression of 2µm-like plasmid partitioning protein fusions in non-native *S. cerevisiae* yeast host. Total protein extracted from yeast transformants in Figure 2 expressing the indicated 2µm and 2µm-like AD and BD protein fusions (A, B) in the absence or (C) the presence of their partner protein and resolved by SDS-PAGE and analyzed by western blotting with anti-$LexA_{BD}$ (α-BD), anti-$Gal4_{AD}$ (α-AD) and anti-Pgk (α-Pgk) antibodies. Species with the mobility expected for each of the protein fusions are indicated with arrowheads. (B, C) A longer exposure of the α-AD western is shown beneath a shorter exposure. A non-target yeast protein detected by the antibody is indicated with an asterisk.

heterologous host. To assess this, western blotting analysis was performed (Figure 6). Some proteins were expressed at much lower levels than 2µm Rep1and Rep2, for example pSB3 Rep1 and pKW1 C. However, the level of the pKW1 C partitioning protein was similar to that of pSB3 Rep1 which did interact, suggesting that the lack of interaction was not due to low levels of the pKW1 fusion proteins in *S. cerevisiae*. Host factors may have interfered with the interaction between the pKW1 partner proteins or they may differ from 2µm, pSR1 and pSB3 partitioning proteins and do not associate.

3.2.2 *In vivo* 2µm-Like Plasmid Protein-DNA Interaction Analysis

Both 2µm Rep1 and Rep2 have been found to associate with *STB in vivo*; however, Rep2 requires the presence of 2µm Rep1 for robust interaction (Pinder *et al*., 2013). Given that the 2µm-like pSR1 and pSB3 plasmid partitioning proteins associated with their respective partners in *S. cerevisiae* (Section 3.2.1), I wanted to test whether the 2µm-like pKW1, pSR1 and pSB3 plasmid proteins could associate with their respective partitioning loci in a manner similar to the 2µm proteins or show any cross-plasmid interactions. Using an *in vivo S. cerevisiae* one-hybrid assay, no protein-DNA interactions, other than the known 2µm Rep1-*STB* association were observed when any of the 2µm-like plasmid partitioning proteins were expressed on their own (Figure 7). However, when the pSB3 Rep1 protein was co-expressed with the pSB3 C partitioning protein fused to a transcriptional activation domain (AD), the pSB3 C fusion was able to interact with the pSB3 *PAR* sequence (Figure 7E). These results suggest pSB3 *PAR* recognition requires the presence of both Rep1 and C partitioning proteins.

Interestingly, when co-expressed with 2µm Rep1, 2µm Rep2 associated weakly with the pSB3 *PAR* sequence (Figure 7B) suggesting that perhaps motifs required for recognition of the locus by the partitioning proteins are conserved between 2µm *STB* and

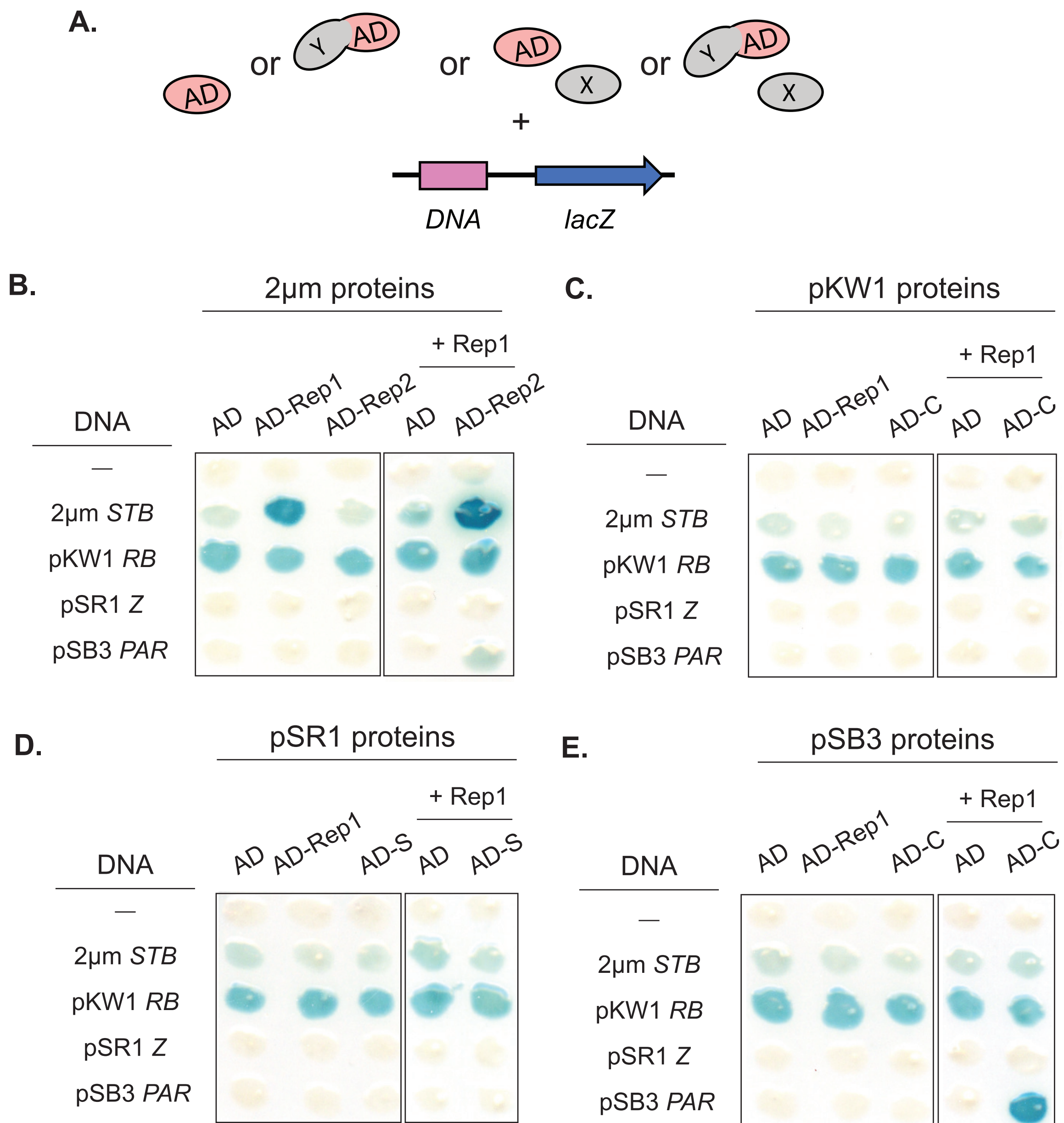


Figure 7. One-hybrid assay for partitioning protein association with plasmid partitioning loci in *S. cerevisiae*. (A) *S. cerevisiae* yeast lacking native 2µm plasmid [cir^{0}] and containing the indicated partitioning sequence or no target sequence (-) integrated in the genome upstream of a *lacZ* reporter gene were transformed with a plasmid expressing AD or the indicated 2µm (B), pKW1 (C), pSR1 (D) and pSB3 (E) partitioning protein fused to AD or co-transformed with two plasmids, one expressing the indicated 2µm and 2µm-like plasmid Rep1 partitioning proteins fused to BD, the other expressing AD or the indicated plasmid partitioning protein fused to AD. Activation of *lacZ* indicates association of the AD fusion proteins with the plasmid partitioning sequence and was assessed by a filter assay for the lacZ gene product, ß-galactosidase, which cleaves X-Gal substrate to produce a blue precipitate. (Note, the pKW1 RB sequence activates expression of the *lacZ* reporter even in the absence of AD expression)

pSB3 *PAR*. Lack of detectable pKW1 protein-*RB* interactions in the one-hybrid assay (Figure 7C) could be due to absence of host factors needed for the pKW1 *RB* locus recognition by the partitioning proteins that differ between the two yeast species or due to the lack of association between pKW1 Rep1 and C in this heterologous host. Notably, I did not detect any pSR1 protein interaction with the pSR1 *Z* partitioning sequence (Figure 7D), despite the observed pSB3 protein-pSB3 *PAR* association. The two 2µm-like plasmids, pSR1 and pSB3, are able to co-exist in *Z. rouxii* cells but perhaps their respective partitioning proteins have co-evolved with their partitioning loci and recognize different sequences. Alternatively, the pSR1 and pSB3 plasmids have co-evolved along with different host factors required for the partitioning locus interaction. Further analysis is needed.

3.3 Domain Mapping of pSB3 Plasmid Partitioning Proteins

The tertiary structures for the 2µm or 2µm-like partitioning proteins have not been identified; however, previously our lab has shown experimentally that amino-terminal domains of both 2µm Rep1 and Rep2 were required and sufficient for protein hetero-interactions (Sengupta *et al*., 2001; McQuaid *et al*., 2017) while the carboxy-terminal domains were required and sufficient for the association with *STB* (McQuaid *et al*., 2017; McQuaid, unpublished data). Despite no significant sequence similarity of the pSB3 C protein and *PAR* partitioning locus with 2µm Rep2 and *STB*, respectively, interactions similar to the 2µm Rep proteins are seen for pSB3 Rep1 and C (as outlined in Section 3.2) suggesting that common protein domains may be required for the protein-protein and protein-DNA interactions and the subsequent partitioning of the plasmids.

3.3.1 Secondary Structure Predictions for 2µm and pSB3 Partitioning Proteins

To screen for potential protein elements that might contribute to the similarities in observed protein interactions between 2µm and pSB3 plasmid partitioning proteins, the predicted amino acid sequences of the 2µm and pSB3 partitioning proteins were used to predict secondary structures of the plasmid partitioning proteins using the secondary structure prediction algorithm JPred4 (Drozdetskiy *et al*., 2015) (Figure 8).

The generated structure predictions did not show any striking similarities between 2µm Rep2 and pSB3 C, consistent with their apparent lack of similarity, but the homologous Rep1 proteins share structural elements, including a notably ß-sheet-rich carboxy-terminus that might be involved in Rep1 recognition of their respective plasmid partitioning loci (Figure 8). One striking difference between the two Rep1 proteins is a putatively unstructured 124-amino acid region in pSB3 Rep1 (between residues 254 and 378) that was absent from 2µm Rep1 and, in fact, from other 2µm-like Rep1 proteins (Dobson, unpublished results).

3.3.2 Amino-Terminal Regions of Both pSB3 Rep1 and C Proteins are Required and Sufficient for pSB3 Protein Interactions in *S. cerevisiae*

Despite dissimilar predicted secondary structures of 2µm Rep2 and pSB3 C partitioning proteins (Figure 8), pSB3 C could associate with its Rep1 partner in *S. cerevisiae* and also with 2µm Rep1 and Rep2, but more weakly (Figure 5). To test whether pSB3 protein domains required for these interactions are similar to those of 2µm Rep1 and Rep2, I designed pSB3 Rep1 and C protein truncations to closely match those of 2µm proteins with break points at positions that did not disrupt any predicted

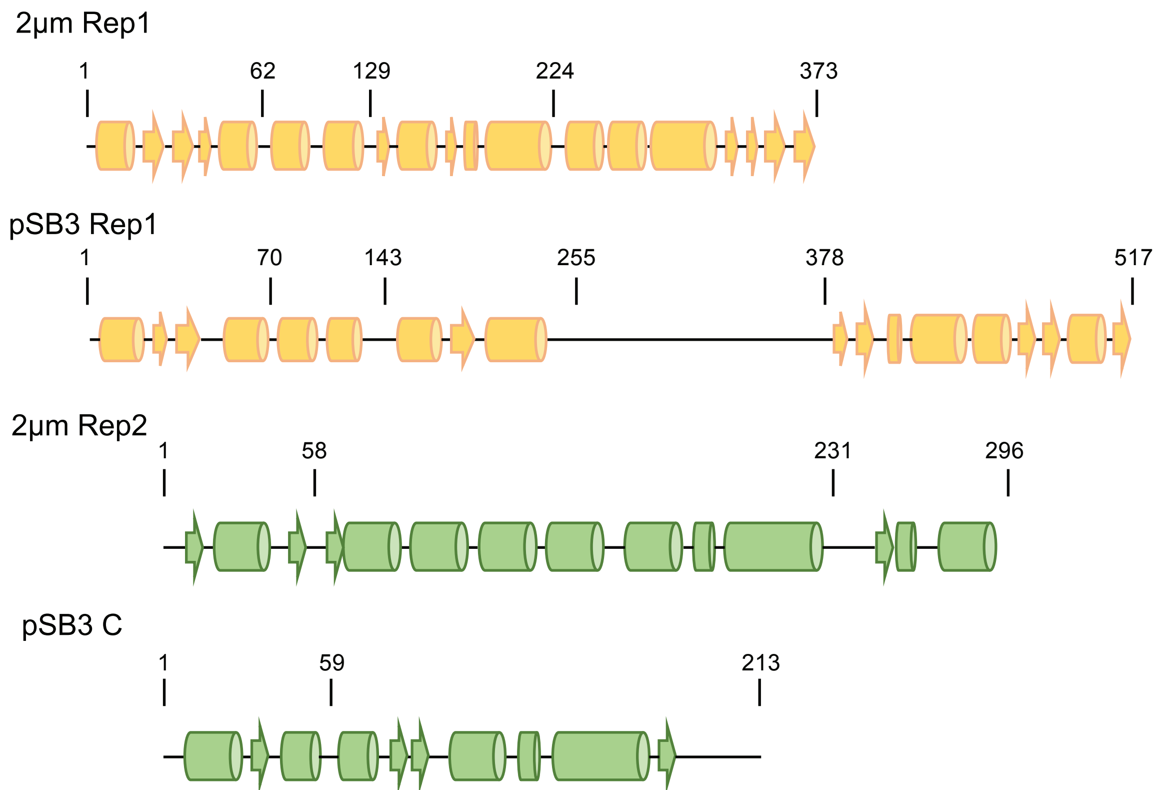


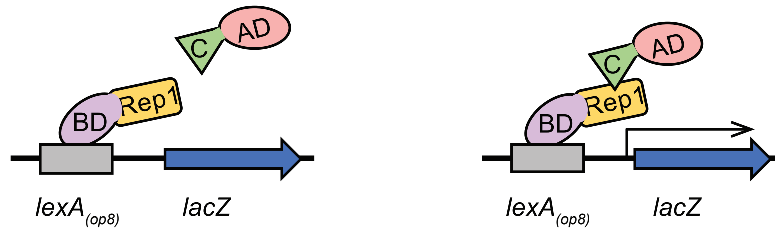
Figure 8. Protein secondary structure prediction. Predicted α-helical (barrels) and ß-sheet (arrows) composition of 2µm Rep1 and Rep2 and 2µm-like pSB3 Rep1 and C partitioning proteins as determined using JPred4 software (Drozdetskiy *et al.*, 2015) and the predicted protein sequences.

secondary structure elements. The resulting protein truncations were tested for their ability to interact with each other and with the full-length partners in the two-hybrid assay (Figure 9).

The pSB3 Rep1 protein that had the first 143 amino acids ($Rep1_{1-143}$) was designed to be the equivalent to the first 129 amino acids of 2µm Rep1, which has been shown to be required and sufficient for the interaction with Rep2 (Sengupta *et al*., 2001). pSB3 $Rep1_{1-143}$ interacted with pSB3 C in *S. cerevisiae*, demonstrating that amino acids 1 to 143 are sufficient for this interaction and that the protein domain required for the pSB3 Rep1 association with C is similar to that in 2µm Rep1 required for Rep2 association. Similarly, the pSB3 C protein that contained the first 59 amino acids (C_{1-59}) was created to match 2µm $Rep2_{1-58}$, the domain required and sufficient for Rep2 interaction with 2µm Rep1. The pSB3 C_{1-59} truncation interacted equally well with both full-length pSB3 Rep1 and $Rep1_{1-143}$, demonstrating that the amino-terminal domains of both pSB3 partitioning proteins are sufficient for the protein hetero-interactions just as they are for the 2µm Rep proteins (Figure 9).

An amino-terminal truncation of pSB3 Rep1 lacking the first 69 amino acids ($Rep1_{70-517}$) to match 2µm Rep1 with residues 63-373, and one of pSB3 C lacking the first 59 amino acids (C_{60-213}), analogous to 2µm Rep2 with amino acids 58 to 296, were also assessed. Neither pSB3 $Rep1_{70-517}$ nor C_{60-213} associated with either their full-length partitioning partner or any of the analyzed protein truncations (Figure 9), suggesting that N-terminal domains of both pSB3 Rep1 and C are required for the protein hetero-interactions in *S. cerevisiae* just as they are for 2µm Rep protein heterodimerization. Alternatively, the amino-terminal truncations might have failed to interact if levels of the

A.



B.

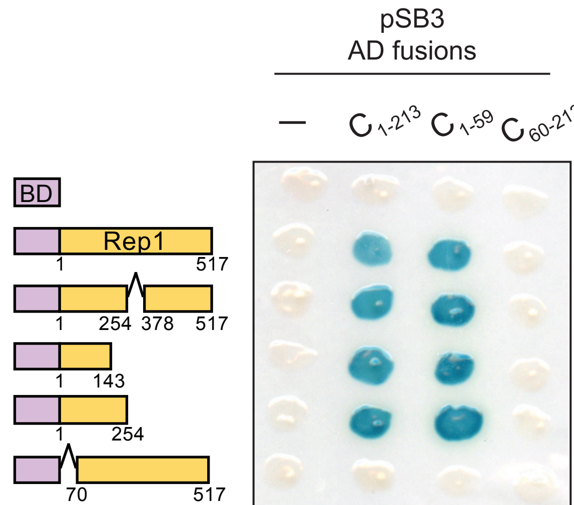


Figure 9. 2µm-like pSB3 Rep1 and C proteins interact through their amino termini. (A) The pSB3 Rep1 and C proteins or the indicated truncations of these were expressed in a *cir*^{0} two-hybrid *S. cerevisiae* reporter strain, with Rep1 fused to the DNA-binding domain of LexA (BD), and C fused to the transcriptional activation domain of Gal4 (AD). (B) Co-transformants were grown in patches on a nitrocellulose filter and activation of the *lacZ* reporter gene integrated in the yeast genome downstream of *lexA*$_{(op8)}$ indicates interaction of the fusion proteins, detected by a filter assay for the *lacZ* gene product, ß-galactosidase, which cleaves X-Gal substrate to produce a blue precipitate.

fusion proteins were too low. Western blotting analysis of pSB3 Rep1 and C protein truncations is needed to assess protein levels.

3.3.3 The C-Terminus of pSB3 Rep1 is Required for Efficient *PAR* recognition

The importance of the interaction between 2µm Rep1 and Rep2 for their association with 2µm *STB* and in mediating the equal partitioning of the 2µm plasmid is not entirely clear. Data from Yang *et al*. (2004) suggest that Rep1-Rep2 interactions are critical for *STB* association whereas McQuaid *et al*. (2017) showed that a Rep2 mutant that could not interact with Rep1 was competent for partitioning provided that 2µm Raf was present to maintain Rep1 and Rep2 levels. As I was able to show that the pSB3 Rep1 and C partitioning proteins associate with each other and with the pSB3 *PAR* locus in *S. cerevisiae*, I wanted to know what protein domains were required for these associations to better understand the nature of the partitioning protein – partitioning sequence associations. To determine the pSB3 protein domains required for *PAR* locus recognition, I tested the pSB3 Rep1 and C protein truncations in the *S. cerevisiae* one-hybrid assay (Figure 10).

As shown previously (Figure 7E), full-length pSB3 C associated with *PAR* as did a truncated version of C containing the first 59 amino acids (C_{1-59}) but only when the pSB3 Rep1 partitioning protein was co-expressed in the same cells (Figure 10). In contrast, C_{1-59} did not associate with *PAR* when co-expressed with pSB3 Rep1 containing only the first 143 or 254 amino acids ($Rep1_{1-143}$ or $Rep1_{1-254}$ respectively) and full-length C only weakly interacted with the locus when co-expressed with either of these Rep1 truncations suggesting that the C-terminal region of the pSB3 Rep1 protein is required for efficient interaction of the Rep1/C complex with the pSB3 partitioning locus (Figure 10). This result is consistent with the 2µm Rep1 association with *STB* that

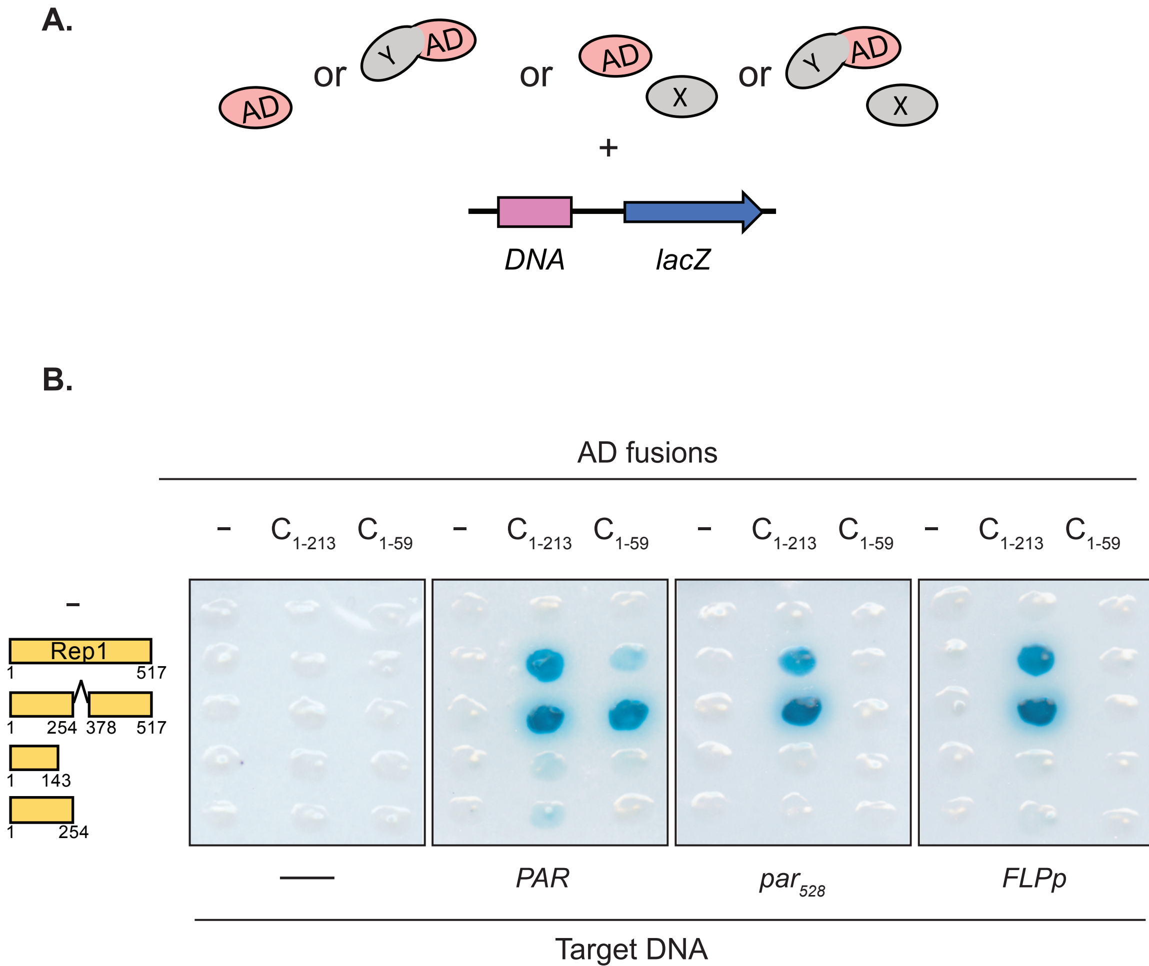


Figure 10. The pSB3 Rep1 and C partitioning proteins associate with the pSB3 *PAR* and plasmid gene promoters in *S. cerevisiae*. (A) *S. cerevisiae* one hybrid strains with pSB3 *PAR* full-length, or the *REP1*-proximal portion (par_{528}) or the *FLP* gene promoter (*FLPp*) integrated in the genome upstream of a *lacZ* reporter gene were co-transformed with plasmids, one expressing full-length pSB3 Rep1 or the indicated Rep1 protein truncations, the other expressing AD or AD fused to full-length pSB3 C or the indicated C truncation. (B) Co-transformants were grown in patches on a nitrocellulose filter and activation of *lacZ* indicates association of the AD fusion protein with the plasmid partitioning sequence and was assessed by a filter assay for the *lacZ* gene product, ß-galactosidase, which cleaves X-Gal substrate to produce a blue precipitate.

requires the presence of amino acids 130-373 of 2μm Rep1 (equivalent to amino acids 144-517 in pSB3 Rep1) for efficient interaction with the 2μm locus (McQuaid, unpublished data). The weaker signal seen for C-terminal truncations of pSB3 Rep1 is unlikely to be due to the lower protein levels of truncated pSB3 Rep1 or C proteins since these same truncations had steady-state levels sufficient to give strong signals in the two-hybrid assay where $Rep1_{1-143}$ and $Rep1_{1-254}$ associated strongly with both pSB3 C and C_{1-59} (Figure 9). However, western blotting analysis is needed to experimentally assess the level of the truncated pSB3 proteins.

3.3.4 A 124-amino Acid Insertion in the pSB3 Rep1 Protein was not Required for Rep1 Association with pSB3 C and with the *PAR* Partitioning Locus

Upon comparison of the secondary structure predictions for the 2μm and pSB3 partitioning proteins, an unusual 124-amino acid region, predicted to be unstructured, was identified for pSB3 Rep1 (Figure 8; outlined in Section 3.3.1). To determine whether this 124 amino acid region of pSB3 Rep1 had any effect on the *in vivo* protein interactions, I deleted the residues from the protein coding region and tested the resulting Rep1 truncation ($Rep1_{1-254/378-517}$) in two-hybrid and one hybrid assays (Figures 9 and 10, respectively). Deletion of the 124 amino acids in pSB3 Rep1 between residues 254 and 378 did not affect protein association with pSB3 C or C_{1-59} in the *S. cerevisiae* two-hybrid assay (Figure 9). The pSB3 $Rep1_{1-254/378-517}$ protein still associated with *PAR*, but only when either C or C_{1-59} were co-expressed in the one-hybrid assay (Figure 10), consistent with previous observations for the full-length pSB3 Rep1. In both cases, the interactions of pSB3 $Rep1_{1-254/378-517}$ were stronger, compared to the full-length pSB3 Rep1, perhaps indicating that the level of the internally-truncated Rep1 protein is higher or the deleted region inhibits interaction with the C protein. Although western blotting and $Rep1_{1-254/378-}$

$_{517}$ partitioning function analysis have yet to be performed, two-hybrid and one-hybrid assay results show that the unusual 124-amino acid region in pSB3 Rep1 does not affect protein interactions and suggest that it may have no functional importance.

3.4 The pSB3 Plasmid Proteins Interact with the Plasmid Gene Promoters *In Vivo*

Apart from partitioning function, the 2μm Rep1 and Rep2 proteins act as transcriptional regulators, repressing transcription of all four plasmid genes. Rep protein association with the two divergent plasmid promoters *in vivo* has been demonstrated by chromatin immunoprecipitation and one-hybrid assays (McQuaid *et al*., 2017), with repression of the 2μm *FLP* gene being required for control of plasmid amplification and copy number (Futcher, 1986; Veit & Fangman, 1988; Volkert & Broach, 1986).

To determine whether the pSB3 Rep1 and C partitioning proteins might similarly regulate pSB3 plasmid amplification, these proteins were assayed for association with the pSB3 *FLP* gene promoter, *FLPp,* and the portion of *PAR* closest to the *REP1* gene, par_{528}, in a one-hybrid assay in *S. cerevisiae* (Figure 10). As seen for interaction with the full *PAR* sequence, full-length pSB3 Rep1 and C interacted with both plasmid promoters, but only when the two proteins were co-expressed. The absence of the carboxy-terminal domain of pSB3 Rep1 virtually abolished the association. In contrast, when the C-terminal region of C was deleted, interaction of C_{1-59} with both *FLPp* and par_{528} was abolished even when the full-length Rep1 partner protein was co-expressed, suggesting that the carboxy-terminal domains of both Rep1 and C are required for their efficient interaction with pSB3 gene promoters (Figure 10). The pSB3 Rep1 protein with 124-amino acids deleted between residues 254 and 378 ($Rep1_{1-254/378-517}$) had similar interactions to the full-length Rep1; however, as with *PAR* association, interaction with *FLPp* and par_{528} was stronger than for the full-length Rep1 (Figure 10). This result may

indicate higher $Rep1_{1-254/378-517}$ protein levels compared to the full-length pSB3 Rep1, which still needs to be assessed by western blotting analysis.

3.5 *Z. rouxii* pSB3 Plasmid Can Partition in *S. cerevisiae*

Prior to my research only limited functional analysis had been performed for some of the 2μm-like plasmids and some of these efforts were flawed due to sequencing errors in the published plasmid sequences that led to mis-identification of coding capacity (Araki *et al*., 1985; Toh-e *et al*., 1984; Jearnpipatkul *et al*., 1987; Dobson, unpublished results). Having shown that the pSB3 Rep1 and C partitioning proteins can interact with each other and associate with the pSB3 *PAR* locus and the pSB3 plasmid promoters in *S. cerevisiae* (Figures 9 and 10) led me to investigate whether pSB3 might be able to partition in a heterologous yeast host.

To determine whether the *Z. rouxii* pSB3 plasmid could be actively maintained in *S. cerevisiae*, a plasmid containing all of pSB3 and tagged with a dominant drug-resistance marker gene (*kanMX4*) was created (pKan-pSB3). The *E. coli* vector and marker gene were inserted downstream of the transcriptional termination sequence for the *C* ORF and adjacent to one of the large inverted repeats where they were least likely to interfere with the normal plasmid partitioning in yeast. As a control, a *KanMX4*-tagged 2μm plasmid (pKan) and pKan lacking the *STB* partitioning locus (pKanΔSTB) were used. All plasmids were then assessed for inheritance in *S. cerevisiae* (Figure 11).

The pKan-pSB3 was inherited with an efficiency similar to that of the 2μm-based pKan plasmid in *S. cerevisiae*, suggesting that *Z. rouxii* pSB3 can partition in a heterologous yeast. To ensure that efficient inheritance of the pKan-pSB3 plasmid in *S. cerevisiae* was not merely due to high plasmid copy number from plasmid amplification overcoming unequal segregation, a pKan-pSB3 plasmid lacking a functional *FLP* gene

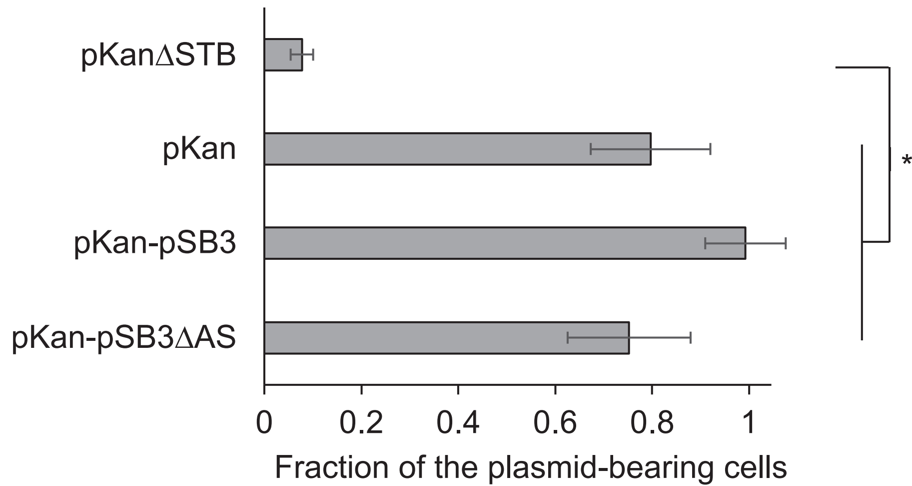


Figure 11. pSB3 2µm-like plasmid is efficiently inherited in heterologous *S. cerevisiae* host. A *cir*^{0} *S. cerevisiae* strain was transformed with *KanMX4*-tagged 2µm plasmids that contained (pKan) or lacked 2µm *STB* (pKanΔSTB) or *KanMX4*-tagged pSB3 plasmids that contained (pKan-pSB3) or lacked (pKan-pSB3ΔAS) a functional pSB3 *FLP* gene. Transformants were cultured overnight in selective medium (YPAD+G418). The fraction of G418-resistant yeast cells indicates the efficiency of plasmid inheritance and was determined by counting the ratio of resistant colonies formed after plating on selective versus non-selective solid medium. Results represent the average (± standard deviation) from assaying six independent transformants for each plasmid. Asterisks indicate a significant difference that was determined by a two-tailed Student's t-test (*p<0.05).

was created (pKan-pSB3ΔAS). This plasmid was also efficiently maintained (Figure 11). This result suggests the pSB3 plasmid partitioning mechanism is fully functional in *S. cerevisiae*, indicating that any host factors required for partitioning of this family of plasmids are conserved between the *Z. rouxii* and *S. cerevisiae* yeast.

3.6 Rep1-Independendent Partitioning Function of 2µm Rep2

When *S. cerevisiae* cells transformed with a synthetic plasmid containing a functional partitioning system (Rep1-Rep2-*STB*) and a selectable marker are cultured in medium selecting for the plasmid, ~90% of cell contain the plasmid. In the absence of either Rep1 or Rep2, the plasmids show a strong maternal bias in inheritance with only 10-15% of the daughter cells receiving the plasmid at each cell division (Murray & Szostak, 1983). Recently, our lab has discovered that the 2µm Rep2 protein could overcome this bias. Rep2 expressed as a LexA fusion protein was able to increase inheritance of a synthetic plasmid that had LexA-binding site and a yeast origin of replication that lacked any 2µm sequences (Johnston, P., unpublished data). When the LexA Rep2-fusion protein was tethered to the plasmid through LexA-binding sites, 50% of cells cultured in medium selecting for the marker gene on the plasmid contained the plasmid of interest, suggesting that the observed increase in plasmid presence was due to the active segregation of the plasmids. Neither 2µm Rep1 nor Raf, when similarly tested, were able to do so. Further support for the partitioning function of Rep2 being Rep1-independent was the observation that 2µm Rep2 mutants lacking the Rep1 interaction domain ($Rep2_{58\text{-}296}$) or with an amino-terminal substitution that abolished Rep1 interaction ($Rep2_{D22N}$) were still competent for partitioning function. In contrast, a Rep2 mutant that could not self-interact ($Rep2_{AA}$) and one lacking the C-terminal DNA binding domain ($Rep2_{1\text{-}231}$) were impaired for Rep1-independent partitioning function (Johnston,

P., unpublished data).

3.6.1 The C-Terminal Function of 2µm Rep2 is Conserved in the pKW1 C Protein

The failure of the artificially-tethered Rep2 protein lacking the C-terminal 65 amino acid DNA-binding domain (residues 232 to 296) to increase the partitioning of the non-2µm plasmid suggests that the ability of 2µm Rep2 to bind DNA is required for the Rep1-independent partitioning function of Rep2 (McQuaid *et al*., 2017). I wanted to investigate what C-terminal features of Rep2 contribute to the observed ability to mediate plasmid inheritance and whether Rep2 partitioning function is conserved in the other 2µm-like plasmid partitioning proteins encoded by *REP2*-positioned ORFs. To investigate this, I created a plasmid that would express a chimeric Rep2 protein in which the C-terminal residues 232 to 296 were replaced by the corresponding amino acids from *L. waltii* pKW1 C (amino acids 238-306), previously reported to contain a *bona fide* AT-hook DNA-binding motif in this region (McQuaid *et al.*, 2017). This chimeric Rep2-pKW1 C protein ($Rep2_{1\text{-}231}\text{-}C_{WT}$) and the full-length pKW1 C were expressed fused to the DNA binding domain of the bacterial repressor protein LexA ($LexA_{BD}$) and assessed for their ability to promote the inheritance of the non-2µm *TRP1*-tagged plasmid containing an *ARS* and LexA-binding sites (pCD1-$lexA_{(op8)}$). C-terminal truncation might impair Rep2 function by affecting the nuclear localization (Velmurugan *et al*., 1998), so I also created a plasmid that expressed 2µm Rep2 lacking the C-terminal 65 amino acids but replaced with the SV40 nuclear localization signal (NLS) fused to $LexA_{BD}$ ($Rep2_{1\text{-}231}$-SV40).

Plasmid inheritance assays in *S. cerevisiae* showed that when $Rep2_{1\text{-}231}\text{-}C_{WT}$ was artificially tethered to the pCD1-$lexA_{(op8)}$ plasmid DNA, this plasmid was now inherited with an efficiency similar to that seen when full-length Rep2 was tethered (Figure 12).

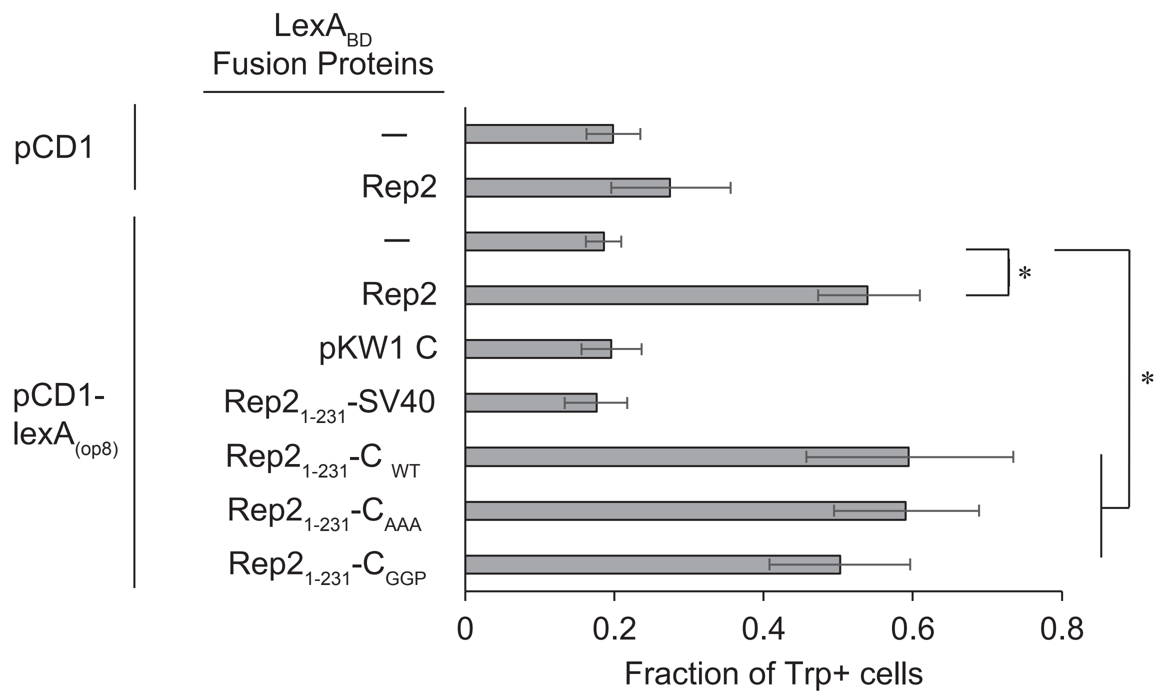


Figure 12. Role of the Rep2 C-terminus in Rep1-independent partitioning can be supplied by the C-terminal AT-hook domain from the pKW1 C protein with AAA and GGP AT-hook mutations in C not impairing this partitioning. A *cir^0 S. cerevisiae* strain was co-transformed with two plasmids, a *TRP1*-tagged, *ARS*-containing plasmid that either lacked (pCD1) or contained (pCD1-lexA$_{(op8)}$) 8 copies of the lexA operator sequence, and a *HIS3*-tagged *CEN/ARS* plasmid that constituatively expressed either LexA$_{BD}$, or LexA$_{BD}$ fused to wild-type 2μm Rep2, pKW1 C, or chimeric 2μm Rep2 proteins that had the first 231 amino acids of Rep2 fused to the SV40 nuclear localization signal (SV40) or to the C-terminal 68 amino acids of pKW1C which was either wild-type, or with the AT-hook GRP motif mutated to AAA or GGP. Co-transformants were cultured overnight in selective medium (SD-his-trp). The fraction of tryptophan-prototrophic cells indicates the efficiency of inheritance of the pCD1 or pCD1-lexA$_{(op8)}$ plasmids and was determined by counting the ratio of tryptophan prototrophic colonies formed after plating on selective (SD-his-trp) versus non-selective (SD-his) solid medium. Results represent the average (± standard deviation) from assaying six independent co-transformants for each. Asterisks indicate a significant difference determined by a two-tailed Student's t-test (*$p<0.05$).

These results suggest that despite the lack of significant sequence similarity between 2μm Rep2 and pKW1 C proteins, their C-terminal function is conserved. pCD1-lexA$_{(op8)}$ plasmid inheritance was not increased by expression of full-length pKW1 C protein fused to LexA$_{BD}$ (Figure 12) suggesting that factors other than the protein C-terminus are required for the Rep1-independent partitioning function of Rep2. Alternatively, the *L. waltii* pKW1 C protein level was too low or its role in plasmid inheritance may differ from that of 2μm Rep2 and cannot be assayed in *S. cerevisiae*, or perhaps some host factors that are required for the Rep1-independent partitioning vary between these yeast species.

To test whether the chimeric Rep2$_{1-231}$-C$_{WT}$ protein could mediate partitioning of the 2μm plasmid, the *REP2* coding region in an *ADE2*-tagged 2μm plasmid (pAS27) was replaced with one encoding Rep2$_{1-231}$-C$_{WT}$ creating pAS27- Rep2$_{1-231}$-C$_{WT}$. As a control, the *REP2* ORF in pAS27 was replaced with the ORF encoding Rep2$_{1-231}$-SV40, creating pAS27- Rep2$_{1-231}$-SV40. The pAS27- Rep2$_{1-231}$-C$_{WT}$ plasmid was inherited with efficiency similar to pAS27, suggesting that the C-terminal part of pKW1 C could fully substitute for that of 2μm Rep2 (Figure 13). The plasmid pAS27-Rep2$_{1-231}$-SV40 was not efficiently inherited (Figure 13) demonstrating that in partitioning of the 2μm plasmid nuclear localization is not the only role of the C-terminal domain of Rep2. These results may also indicate that both 2μm Rep2 and pKW1 C proteins perform similar roles during the partitioning of 2μm and pKW1 plasmids.

3.6.2 AT-Hook Mutations in pKW1 C do not Impair Plasmid Partitioning

The observation that replacement of the C-terminal portion of 2μm Rep2 with the 68 C-terminal residues of the pKW1 C protein was able to restore the Rep1-

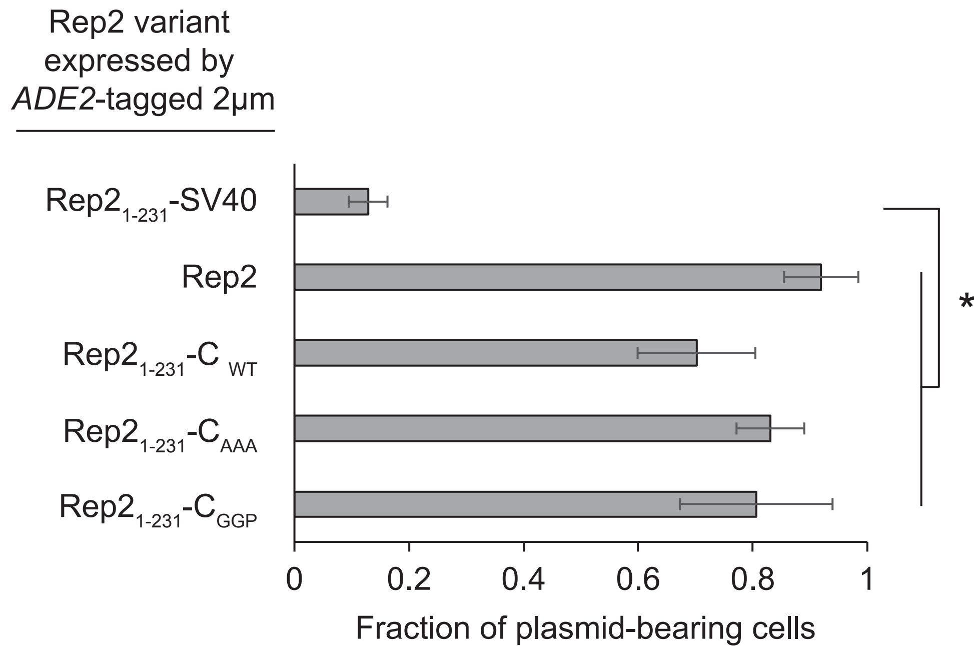


Figure 13. The C-terminal domain of pKW1 protein C can functionally substitute for the corresponding C-terminal part of 2µm Rep2 with AAA and GGP AT-hook mutations having no significant effect on partitioning. A *cir^0 S. cerevisiae* strain was transformed with an *ADE2*-tagged 2µm-based plasmid (pAS27-based) that contained either wild type 2µm *REP2* or an ORF encoding the indicated chimeric version of Rep2 in the place of the native *REP2* ORF. Transformants were cultured overnight in selective medium (SC-ade). The fraction of adenine-prototrophic yeast cells indicates the efficiency of plasmid inheritance and was determined by counting the ratio of adenine prototrophic colonies formed after plating on selective versus non-selective solid medium. Results represent the average (± standard deviation) from assaying six independent transformants for each plasmid. An asterisk indicates a significant difference determined by a two-tailed Student's t-test (*$p<0.05$).

independent partitioning of Rep2 and fully substitute for the Rep2 C-terminus in mediating 2μm plasmid inheritance suggests that the required function is conserved and encoded in the C-terminus of both (outlined in Section 3.6.1). The 2μm Rep2 and pKW1 C proteins have no significant nucleotide or amino acid similarity; however, their C-termini are highly basic with the pKW1 C protein having a recognizable AT-hook (McQuaid *et al*., 2017), a motif that has been shown to bind in the minor groove of AT-rich DNA (Reeves & Nissen, 1990). To assess the contribution of the bona fide AT-hook motif to the chimeric 2μm Rep2-pKW1 C protein ($Rep2_{1-231}$-C_{WT}) partitioning function, I mutated the core GRP motif to either AAA or GGP and tested the resultant mutant chimeras ($Rep2_{1-231}$-C_{AAA} and $Rep2_{1-231}$-C_{GGP}, respectively). When artificially-tethered to a non-2μm plasmid (pCD1-$lexA_{(op8)}$) through their $LexA_{BD}$ moiety, both $Rep2_{1-231}$-C_{AAA} and $Rep2_{1-231}$-C_{GGP} increased inheritance of the plasmid with an efficiency similar to that promoted by the $Rep2_{1-231}$-C_{WT} $LexA_{BD}$ fusion (Figure 12). Similar results were observed for 2μm plasmids (pAS27) in which the coding region for the native Rep2 protein was replaced with ones encoding either $Rep2_{1-231}$-C_{AAA} or $Rep2_{1-231}$-C_{GGP} (pAS27- $Rep2_{1-231}$-C_{AAA} and pAS27-$Rep2_{1-231}$-C_{GGP} plasmids, respectively) (Figure 13). The pAS27 plasmids expressing these versions of the mutant chimeric proteins partitioned efficiently, suggesting that mutations in the AT-hook motif alone are not sufficient to abolish Rep2 partitioning function. Further experiments are needed to assess the importance of the Rep2 C-terminus in Rep1-independent partitioning function and the contributions of the region to the plasmid partitioning mechanism.

Chapter 4. Discussion

4.1 General Overview

The maintenance mechanism of the 2µm-like family of plasmids is not completely understood. However, the equal partitioning of the 2µm plasmid strongly relies on the association of the plasmid-encoded partitioning proteins, Rep1 and Rep2, with the plasmid *STB* partitioning locus resulting in near chromosome-like fidelity of inheritance for the native plasmid. Absence of either partitioning protein or the partitioning locus causes the plasmids to missegregate, showing a strong maternal bias in inheritance.

In this thesis, I identified the sequence that likely functions as the partitioning locus (*PAR*) for the *Zygosaccharomyces rouxii* 2µm-like plasmid pSB3 and showed that partitioning loci from other 2µm-like plasmids cannot functionally substitute for the 2µm *STB* locus. The failure of the 2µm-like partitioning loci to function with the 2µm partitioning proteins suggests that the formation of a functional partitioning complex may depend on sequence-specific recognition of the *PAR* sequence by the proteins encoded by that plasmid.

I have also performed the first experimental analysis of the 2µm-like pKW1, pSR1 and pSB3 proteins, showing that the pSR1 and pSB3 plasmid partitioning proteins can interact with their respective partners, with the pSB3 proteins also recognizing the pSB3 *PAR* sequence. Furthermore, the pSB3 second partitioning protein, C, interacted with both 2µm Rep1 and Rep2, while the pKW1 Rep1 protein associated with 2µm Raf. I was also able to detect weak 2µm Rep1 and Rep2 protein interaction with pSB3 *PAR*.

Analysis of the pSB3 Rep1 and C protein domains further revealed that amino-terminal domains of both Rep1 and C are required and sufficient for their interaction

while the carboxy-terminal domain of pSB3 Rep1 appeared to be required for efficient association with *PAR*. Recognition of the pSB3 *PAR* sequence was also shown to differ from association with the pSB3 *FLP* gene promoter, *FLPp*, or the portion of *PAR* proximal to the *REP1* gene (par_{528}) with the C-termini of both Rep1 and C being required for the association with plasmid promoters.

I have also provided insights into features required for the partitioning of the 2µm-like plasmid family by showing that the *Z. rouxii* pSB3 2µm-like plasmid can partition in a heterologous budding yeast host *S. cerevisiae*. Additionally, I showed that the Rep1-independent partitioning function of 2µm Rep2 requires the C-terminal DNA-binding domain with the function of the protein C-terminus being conserved in the protein encoded by the *REP2*-positioned pKW1 *C* gene.

Overall, this research broadens the understanding of the maintenance mechanism of the 2µm-like family of plasmids and is the first experimental analysis of the interactions of the partitioning proteins of the 2µm-like plasmids. My research supports an active role for the C-terminal DNA-binding domain of the 2µm Rep2 protein in plasmid partitioning and suggests that this function is conserved in other 2µm-like Rep2 protein equivalents despite lack of sequence similarity among the proteins.

4.2 The pSB3 *PAR* Plasmid Partitioning Locus

The *Saccharomyces cerevisiae* 2µm plasmid is the best-studied member of the 2µm-like plasmid family found in Saccharomycetaceae budding yeast. Maintenance of the 2µm plasmid relies on a robust partitioning mechanism and the ability to amplify plasmid copy number if it falls below normal levels due to plasmid missegregation events. The repression of plasmid gene expression by plasmid partitioning proteins prevents this plasmid amplification once copy number achieves normal levels. For equal

partitioning of the 2μm plasmid, the plasmid-encoded Rep1 and Rep2 proteins have to assemble at the plasmid partitioning locus *STB*. The association of the 2μm Rep proteins with the plasmid *STB* sequence has been studied *in vivo* and *in vitro*. However, prior to my investigation, limited functional analysis had been performed for the 2μm-like plasmids and the plasmid partitioning locus had only been identified for three other members of the family, pSR1, pKD1, and pKW1. The *Zygosaccharomyces rouxii* 2μm-like plasmid pSB3 had not been functionally characterized and the plasmid partitioning locus not identified.

In this study I have experimentally identified the sequence that contains the pSB3 *PAR* locus (Figure 3). This sequence was chosen based on its position upstream of the *REP1* gene, the region where the pSR1, pKD1 and pKW1 partitioning loci are positioned (Jearnpipatkul *et al*., 1987; Bianchi *et al*., 1991; Mereshchuk, Dalhousie University, honours thesis). pSB3 *PAR* is one of only two non-protein coding regions on the plasmid other than the large inverted repeats but has no significant nucleotide sequence similarity with *STB*. An assay for plasmid inheritance in *Z. rouxii* showed that the presence of pSB3 *PAR* increased the fraction of cells that maintained the synthetic plasmid to a level similar to that seen for a plasmid containing the previously-identified *Z. rouxii* pSR1 *Z* partitioning locus (Jearnpipatkul *et al*., 1987b).

Although the increase in plasmid inheritance due to the presence of either pSR1 *Z* or the pSB3 *PAR* sequence was significant compared to the *ARS*-only plasmid (Figure 3), the fraction of cells containing the plasmid did not reach the expected 70-80% usually seen for *STB*-containing plasmids in *S. cerevisiae*. One reason for the reduced plasmid inheritance might be poor activity of the *ARS* sequence I had used in making the *ARS*-only vector. In preliminary experiments, I determined that the 2μm *ARS* sequence did not

function efficiently in *Z. rouxii*, so instead, I used the *ARS* from the *L. waltii* 2µm-like plasmid pKW1 to construct the vector used for testing partitioning loci. However, this heterologous *ARS* sequence might also not have been efficiently recognized by the *Z. rouxii* replication machinery which would have led to a reduction in the fraction of cells containing the plasmid despite proper partitioning. Alternatively, either the *ARS* or the pSR1 and pSB3 partitioning sequences cloned in this vector might not have been sufficiently protected from transcription promoted by the bacterial vector sequence in yeast disrupting the function of these sequences and causing the subsequent lower inheritance efficiency. Construction of a better plasmid backbone containing the native *Z. rouxii* pSB3 *ARS* is currently underway which will allow the ability of the presumptive *PAR* sequence to partition a synthetic plasmid in *Z. rouxii* to be determined.

4.3 Functional Substitution of the 2µm *STB* Partitioning Locus

Persistence of the 2µm-like family of plasmids strongly relies on the presence of the *cis*-acting plasmid partitioning locus, however, the mechanism of the plasmid protein association with the locus and sequence-specificity of these interactions are still unclear. Previous studies have shown that mutations of key sequence elements in the 2µm *STB* repeats resulted in a significant decrease in Rep protein association and a reduction in plasmid inheritance (McQuaid *et al.,* 2019a). The sequences of the 2µm and 2µm-like partitioning loci share no significant similarity and key elements such as the TGCATTTTT motif present at *STB* (McQuaid *et al.,* 2019a) are not found in the partitioning loci of all 2µm-like plasmids. The loci do have runs of A and T nucleotides, and these might be required for the recognition by the partitioning proteins and plasmid partitioning *in vivo*. I used *L. waltii* pKW1 *RB*, *Z. rouxii* pSR1 *Z* and *Z. rouxii* pSB3 *PAR* partitioning loci to replace the native *STB* sequence on the 2µm-based plasmid to test

whether, despite a lack of sequence similarity, partitioning function is conserved. Presence of the 2µm-like plasmid partitioning loci failed to increase inheritance of the 2µm-based plasmid (Figure 4), suggesting that the 2µm Rep1 and Rep2 proteins cannot recognize the 2µm-like loci as *cis*-acting DNA sequences. These results also suggest that the association of the plasmid partitioning proteins with the partitioning locus is sequence-specific. Differences among the 2µm and 2µm-like loci might determine the specificity of the protein interactions, thus, allowing the locus to serve as a partitioning sequence for only a specific plasmid.

4.4 Interactions of the 2µm-Like Plasmid Proteins

4.4.1 *In Vivo* Protein-Protein Interactions

Given structural similarities with *S. cerevisiae* 2µm and the overall robust persistence of these 2µm-like plasmids in their respective yeast hosts, I wanted to see whether interactions similar to those reported for the 2µm partitioning proteins could be detected for the corresponding 2µm-like proteins. The *in vivo* two-hybrid assay showed that pSR1 Rep1 and S and pSB3 Rep1 and C proteins resemble 2µm Rep1 and Rep2 in interacting with their respective partners (Figure 5). However, they differ from the 2µm Rep proteins in not displaying self-association. The 2µm-like plasmid proteins were tested for association in *S. cerevisiae*, so the apparent lack of self-association might indicate the inability of the 2µm-like proteins to acquire the levels or conformation needed for the interaction to be detected. Interestingly, the 2µm Rep1 protein needs to be overexpressed for the self-association to be detected (Dobson, unpublished results). Otherwise, in the absence of 2µm Rep2, which normally acts as a chaperone for Rep1, Rep1 protein levels are too low to detect this interaction (Pinder *et al.,* 2013). Similar

experiments have to be performed for the 2µm-like proteins to further test for protein self-interaction in *S. cerevisiae*.

Alternatively, the mechanism for 2µm-like plasmid partitioning may have evolved such that partitioning protein self-association is not required by all or perhaps the 2µm Rep protein self-interactions are a specialized feature of the 2µm partitioning mechanism and represent a plasmid adaptation to compensate for selective pressure and ensure a more robust mechanism of plasmid partitioning. The 2µm-like plasmid proteins might interact but fail to do so in a heterologous host. Specific eukaryotic post-translational modifications are apparently not needed for interaction of the 2µm Rep proteins, as bacterially-expressed versions have been shown to self-associate *in vitro* (Pinder *et al.,* 2013), but the 2µm-like plasmid proteins may be inappropriately modified in *S. cerevisiae*, interfering with their self-association.

The *in vivo* assay also revealed that the pSB3 protein C could interact with both 2µm Rep1 and Rep2, while pKW1 Rep1 interacted with 2µm Raf (Figure 5). These 2µm-like protein interactions with 2µm plasmid proteins suggest protein elements required for these interactions are conserved. However, even if conserved, this does not necessarily indicate that the 2µm-like plasmid proteins can functionally substitute for any of the corresponding 2µm species. In fact, no interactions with other 2µm-like plasmid proteins were seen (Figure 5) suggesting that sequence differences among the plasmid partitioning proteins might determine the specificity of the protein interactions for their specific partner proteins and to their yeast host.

4.4.2 *In Vivo* Protein-DNA Interactions

Prior to my research, no plasmid 2µm-like protein interactions with the plasmid partitioning loci had been reported. In one-hybrid assays, none of the 2µm-like plasmid

partitioning proteins when expressed in the absence of their partner protein were able to interact with either their native plasmid partitioning locus or with any other 2µm-like plasmid partitioning locus (Figure 7). However, when co-expressed, pSB3 Rep1 and C associated with pSB3 *PAR* (Figure 7E). This result suggests that, just like the 2µm partitioning proteins, pSB3 Rep1 and C form a partitioning complex at *PAR*; however, unlike the 2µm plasmid, the pSB3 Rep1 protein did not interact in the absence of pSB3 C. Recognition of the pSB3 partitioning locus by the pSB3 Rep1 and C proteins may require a conformational change induced by the partitioning partner. Alternatively, *PAR* recognition by the pSB3 Rep1 protein may be too weak or dynamic and, when combined with relatively low steady-state levels of the pSB3 Rep1 one-hybrid fusion protein (Figure 6), may not have been detectable in this assay.

Interestingly, I also detected 2µm Rep1 and Rep2 interaction with pSB3 *PAR*, but only if both proteins were present, although the signal was quite weak (Figure 7B). The 2µm protein cross-recognition of the pSB3 partitioning locus suggests that DNA motifs or sequence elements are conserved between 2µm *STB* and pSB3 *PAR*. However, this interaction must not be sufficient to form a functional partitioning complex since the pSB3 *PAR* sequence did not increase the inheritance of the 2µm-based plasmid encoding 2µm Rep proteins when substituted for *STB* (Figure 4).

Lack of detection of *L. waltii* pKW1 protein interactions with the pKW1 *RB* partitioning locus (Figure 7C) could be due to host factor differences between the two yeast species, which are more distantly-related to each other than *S. cerevisiae* is to *Z. rouxii* (Figure 1). However, the failure of *Z. rouxii* pSR1 proteins to associate with pSR1 *Z* (Figure 7D) seems less likely to be explained by variations in the host proteins. The pSB3 and pSR1 plasmids are both found in *Z. rouxii* and can even co-exist in the same

cells. Lack of detectable pSR1 protein interactions with *Z* might instead be due to the accidental blockage of the partitioning sequence elements needed for pSR1 partitioning protein binding by an *S. cerevisiae* protein. Some support for this possibility comes from a previous study that reported the pSR1 *Z* DNA sequence was bound by an unknown *S. cerevisiae* host factor in a gel retardation assay (Araki *et al*., 1993). The pSR1 *Z* locus contains a functional *S. cerevisiae ARS* consensus sequence (Mereshchuk, A. & Mulhall, M., data not shown) and recognition of this sequence in *S. cerevisiae* by the origin of replication complex (ORC) might have prevented binding by the pSR1 partitioning proteins. Alternatively, absence of detectable protein-DNA interaction in *S. cerevisiae* might be due to lack of appropriate post-translational modifications of the 2µm-like plasmid proteins in the heterologous yeast host. Notably, post-translational sumoylation of 2µm Rep1 was required for Rep1 association with *STB* (Pinder *et al*., 2013).

4.5 pSB3 Protein Domains Required for Interactions

Just like 2µm Rep1 and Rep2, the pSB3 partitioning proteins interact with each other and with the plasmid partitioning locus in *in vivo* assays (Figures 5 and 7). I also showed that the protein domains required for these interactions are similarly positioned within the pSB3 and 2µm proteins with their amino-termini being sufficient for the partner protein association (Figure 9). The C-terminal domain of pSB3 Rep1 was required for the efficient association of the pSB3 protein C with the pSB3 *PAR* locus (Figure 10) suggesting that this portion of pSB3 Rep1 contains the DNA-binding domain, similar to that in 2µm Rep1 required for interaction with *STB*.

The contribution of the pSB3 C protein to the interaction with *PAR* is still unclear. Perhaps, the presence of C induces a conformational change in pSB3 Rep1 required for effective interaction with the locus since, when expressed on its own, Rep1 did not

associate with *PAR* in the one-hybrid assay (Figure 7E). Alternatively, the pSB3 C protein might also weakly recognize the *PAR* sequence but require Rep1 for robust association, similar to 2µm Rep2 protein requiring 2µm Rep1 for interaction with *STB* to be detectable in a one-hybrid assay (Pinder *et al.,* 2013). Notably, when co-expressed with C, pSB3 Rep1 protein truncations lacking the protein C-terminal 263 or 374 amino acids interacted with *PAR* weakly but only when the full-length C partner protein was present (Figure 10). This result suggests that either the pSB3 Rep1 amino-terminus is sufficient for weak association with the partitioning locus or the observed one-hybrid interaction is due to the recognition of the *PAR* sequence by the pSB3 C protein. Regardless of the *PAR* locus association mode, the observed similarities with the 2µm plasmid suggest that despite plasmid differences, the 2µm-like family members evolved similarly and are maintained by the same partitioning mechanism. Perhaps variations in plasmid proteins determine the specificity of interactions and act as a cross-species barrier ensuring that they only promote retention of their own plasmids.

4.6 pSB3 Plasmid Protein Association with *PAR* and Plasmid Gene Promoters

The similarity between the 2µm plasmid and pSB3 in their partitioning protein associations with each other and the partitioning locus suggested that the regulation of plasmid gene expression might also be the same. This was tested by determining whether the pSB3 Rep1 and C proteins could associate with the pSB3 *FLP* gene promoter, *FLPp* (Figure 10). As with *PAR* locus recognition, I detected pSB3 Rep1 and C partitioning protein interaction with *FLPp* but only when the full-length versions were co-expressed. This result suggests that like 2µm Rep1 and Rep2, the pSB3 partitioning proteins have the potential to act as transcriptional regulators, repressing the transcription of the pSB3 plasmid genes. The need for the carboxy-termini of both pSB3 Rep1 and C proteins for

this association with *FLPp* and also with the portion of *PAR* that likely acts as the *REP1* gene promoter (*par*$_{528}$) makes this different from the protein interaction with *PAR* where that C-terminus of the C protein was not required for efficient association. These distinct domain requirements for plasmid sequence recognition by the pSB3 partitioning proteins might indicate a different mode of interaction perhaps due to the nature of protein complexes that can be formed at the different loci. These observations together with the previously proposed variations in the protein complexes formed at 2µm *STB* versus plasmid promoters (reviewed in McQuaid *et al*., 2019b) suggest that just like 2µm Rep1 and Rep2, the pSB3 partitioning proteins might perform the dual role of plasmid partitioning and copy number control.

4.7 pSB3 Plasmid Partitioning in *S. cerevisiae*

I showed that the *Z. rouxii* pSB3 plasmid can partition in a heterologous *S. cerevisiae* host (Figure 11). This novel discovery indicates that despite host differences, factors that are required for the partitioning of the 2µm and pSB3 plasmids are conserved between these two yeasts. Maintenance of the 2µm-like plasmid in the heterologous species broadens the understanding of the host contribution to the inheritance mechanism and features that determine the specificity of the 2µm-like plasmids to their respective hosts. Active partitioning of the pSB3 plasmid and the conservation of the pSB3 partitioning protein interactions in *S. cerevisiae* now allow mix and match experiments between 2µm and pSB3 plasmid elements that can help identify the protein motifs, DNA features and host proteins required for the maintenance of the 2µm-like plasmid family.

4.8 The Rep1-Independent Partitioning Function of 2µm Rep2

Despite being the best-studied member of the 2µm-like family of plasmids, little is known about the nature of the 2µm Rep1 and Rep2 protein interactions, more specifically

about the contribution of each plasmid protein to the partitioning mechanism. Our lab has previously found that Rep2 can confer partitioning function in the absence of 2µm Rep1 if artificially tethered to a plasmid DNA. The ability of Rep2 to self-associate and the presence of the C-terminal 65 amino acids were required for this function (Dobson, unpublished data). The role of this C-terminal domain has not been established, however, the region is highly basic and was previously reported to be sufficient for association with *STB* DNA *in vitro* (McQuaid *et al*., 2017). Similarly, the corresponding carboxy-terminal regions of the partitioning proteins encoded by the *REP2*-positioned genes in 2µm-like plasmids are also basic with the pKW1 protein C having a recognizable AT-hook motif (McQuaid *et al*., 2017). Here, I have shown that the role of this carboxy-terminal domain in Rep2-mediated partitioning is conserved in the Rep2-equivalent pKW1 protein C (Figures 12 and 13) suggesting that despite having no significant sequence similarity, the C-terminal domains of the 2µm Rep2 and pKW1 C proteins provide the same function in plasmid partitioning.

Taken together with *in vivo* 2µm Rep2 protein-DNA interaction data (McQuaid, PhD thesis) and the proposed model of chromosomal attachment (Sau *et al*., 2015), I suggest that the observed increase in the non-2µm plasmid inheritance facilitated by the tethered 2µm Rep2 is due to its ability to randomly attach plasmid copies to sister chromatids. The role of Rep1 may be to position Rep2 at *STB*, or perhaps to induce the proper protein conformation needed for Rep2 partitioning function by allowing the correct arrangement of the Rep2 polymers. Alternatively, the role of Rep1 might be to bridge some external host interactions to allow the equal attachment to the sister chromatids and the subsequent chromosome-like partitioning of the 2µm copies.

Mutations in the pKW1 C AT-hook consensus sequence did not abolish the partitioning mediated by the tethered 2µm Rep2 – pKW1 C chimeric protein (Figure 12), suggesting that other protein motifs are sufficient for the observed plasmid inheritance. An AT-hook is a weak DNA-binding domain and is usually found either as an array of AT-hooks or adjacent to other binding motifs (reviewed in Aravind & Landsman, 1998). Previous mutational analysis showed that, in most cases, inactivation of a single AT-hook did not have a significant effect on the protein function and abolishment of multiple domains (Harrer *et al*., 2004; Cattaruzzi *et al*., 2007) or adjacent motifs (Cairns *et al*., 1999) was required to impair the ability of the protein to bind DNA. These observations, taken together with the lack of impact on plasmid inheritance when the pKW1 C AT-hook motif was mutated (Figure 13), suggest that while the C-terminal AT-hook domain in the 2µm Rep2 – pKW1 C protein may contribute to partitioning function, other sequence motifs in the highly basic protein C-terminus have to be investigated for the ability to promote the Rep1-independent partitioning function of 2µm Rep2.

4.9 Concluding Remarks

The 2µm-like plasmid family members use yeast replication and segregation machineries for plasmid maintenance; however, they do not confer any obvious benefit or detriment to the host. All members share structural and functional similarities, but no significant nucleotide sequence identity. Apart from the best-studied 2µm plasmid, limited functional analysis has been performed for the 2µm-like plasmids and the partitioning mechanism by which the 2µm-like family persists in the nuclei of budding yeast is still not completely understood.

My findings are the first experimental analysis of the *Z. rouxii* 2µm-like plasmid pSB3 partitioning proteins and *PAR* locus. Here, I showed that pSB3 Rep1 and C interact

with each other, with the *PAR* locus and with the plasmid gene promoters. These striking similarities to the 2μm Rep1 and Rep2 protein interactions and the ability of the pSB3 plasmid to equally partition in *S. cerevisiae* suggest that 2μm and pSB3 have conserved maintenance mechanisms. However, despite the identification of the cross-species partitioning, the exact mechanism by which these plasmids persist in their respective hosts remains unknown, as well as the contribution of the host factors, plasmid protein structure and the timeline of partitioning complex formation.

I have also shown that the C-terminal function of the 2μm Rep2 protein, required for the Rep1-independent partitioning, is conserved in the Rep2-equivalent pKW1 protein C. This finding suggests that despite having no significant nucleotide or amino acid similarity, these two proteins likely have the same function *in vivo*. This novel 2μm Rep2 role in 2μm partitioning adds at least one new component to the model of chromosomal attachment in which the 2μm plasmids are proposed to evenly attach to paired sister chromatids and co-segregate with these during cell division (Sau *et al*., 2015). Taken together with the observed Rep1-independent partitioning function of Rep2, I hypothesise that the role of 2μm Rep2 is to randomly attach the 2μm plasmid copies to the yeast chromosomes, while the specificity of this attachment is determined either by Rep2 association with Rep1 or with the host proteins. However, my observations still leave questions regarding the required protein motifs and sequence elements as well as involvement of host factors.

Overall, my research broadens the understanding of the 2μm and 2μm-like plasmid maintenance providing novel findings that indicate the conservation of partitioning and self-regulatory mechanisms. Results obtained in my Masters research could be used to further study plasmid and host elements required for the robust

persistence of the 2µm-like family of plasmids. Furthermore, the molecular tools I have created can be used in mix and match experiments to narrow down the protein and partitioning sequence motifs required for proper plasmid maintenance. Understanding gained from this research not only contributes to the field of yeast biology but can also give insights into how these plasmid models could be effectively used as efficient vectors in other yeast species and possibly even in a broader range of hosts.

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