Use of two-replisome plasmids to characterize how chromosome replication completes.

by
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Abstract

All living organisms need to accurately replicate their genome to survive. Genomic replication occurs in three phases; initiation, elongation, and completion. While initiation and elongation have been extensively characterized, less is known about how replication completes. In *Escherichia coli* completion occurs at sites where two replication forks converge and is proposed to involve the transiently bypass of the forks, before the overlapping sequences are resected and joined. The reaction requires RecBCD, and involves several other gene products including RecG, ExoI, and SbcDC but can occur independent of recombination or RecA. While several proteins are known to be involved, how they promote this reaction and the intermediates that arise remain uncharacterized.

In the first part of this work, I describe the construction of plasmid "mini-chromosomes" containing a bidirectional origin of replication that can be used to examine the intermediates and factors required for the completion reaction. I verify that these substrates can be used to study the completion reaction by demonstrating that these plasmids require completion enzymes to propagate in cells. The completion enzymes are required for plasmids containing two-replisomes, but not one replisome, indicating that the substrate these enzymes act upon *in vivo* is specifically created when two replication forks converge.

Completion events in *E. coli* are localized to one of the six termination (*ter*) sequences within the 400-kb terminus region due to the autoregulated action of Tus, which binds to *ter* and inhibits replication fork progression in an orientation-dependent manner. In the second part of this work, I examine how the presence of *ter* sequences affect completion on the 2-replisome plasmid. I show that addition of *ter* sequences modestly decreases the stability of the two-replisome plasmid and that this correlates with higher

levels of abnormal, amplified molecules. The results support the idea that *ter* sites are not essential to completion of DNA replication; similar to what is seen on the chromosome.

Rec-B-C-D forms a helicase-nuclease complex that, in addition to completion, is also required for double-strand break repair in *E. coli*. RecBCD activity is altered upon encountering specific DNA sequences, termed *chi*, in a manner that promotes crossovers during recombinational processes. In the third part of this work, I demonstrate that the presence of *chi* in a bidirectional plasmid model promotes the appearance of over-replicated linear molecules and that these products correlate with a reduced stability of the plasmid. The effect appears specific to plasmids containing two replisomes, as *chi* on the leading or lagging strand of plasmids containing one replisome had no-effect. The observation implies *chi* promotes a reaction that may encourage further synthesis during the completion reaction, and that at least on the mini-chromosomes substrates, this appears to be a destabilizing force.

Dedication

I would like to thank my adviser, Dr. Justin Courcelle, for never giving up on my continuous progress and providing me the resources and much needed advice and expertise, along with a ton of patience, that I required to better myself in my graduate tenure. Secondly, I would like to thank my other committee members Dr. Rahul Raghavan and Dr. Jeffrey Singer for their patience, and assistant with my various learning endeavors including classes and facilitating seminars. In equal addition I would like to thank Dr. Charmain Courcelle for the limitless hands-on training and continuous support with countless "upping my game" pep talks which was necessary to overcome many of my self-made obstacles; additionally, helping engineer some rather difficult plasmids. I would also like to thank my lab mates, Dr. Brian Wendel (who first synthesized the two bidirectional plasmids) and Jessica Cole, who have made the hardest challenge of my life achievable and have installed a new and intense appreciation for scientific research and comradery. Lastly, I want to thank my husband, Jeremy Hamilton, who has spent countless hours encouraging and supporting me through the rough days, celebrating my triumphs, and overall being a pillar of figurative and actual physical strength for me; I could not have done this without him, everyone mentioned above, and of course the unconditional love and support from family and friends not mentioned. Thank you all!

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Research purposes

Introduction

The DNA of a genome must be accurately replicated and passed on to daughter cells each generation. In addition to the challenge of accomplishing this task, DNA is also constantly bombarded by chemicals and radiation that compromise its integrity. These include chemicals found in tobacco smoke that oxidize bases or induce single strand breaks, UV-C radiation that induces pyrimidine adducts, deamination by hydrolysis in the presence of acid or heat, and harmful chemical metabolites that can react with the DNA to form adducts (Sachs et al., 1992; Friedberg 1995; Leanderson and Tagesson, 1992; reviewed in Sinha et al., 2002; reviewed in Zeman et al., 2014; Gorden et al., 2018). Therefore, cells contain numerous enzymatic systems that ensure the DNA is faithfully copied and can be repaired when damaged. Some of these regulatory mechanisms include proofreading during and after replication, nucleotide excision repair, mismatch repair mechanisms, and global stress responses such as the SOS and SoxR/OxyR response during DNA damage (Hopefield et al., 1976; Wang and Smith 1986; Gonzalez et al., 1998; reviewed in Crowley and Courcelle, 2002; reviewed in Hanawalt et al., 2003; Heyer et al., 2010; reviewed in O'Donnell et al., 2013; Lee et al., 2015). An additional process that presents challenges to maintaining genomic stability is the completion of DNA replication (Wendel et al., 2014). The mechanism by which completion occurs has only recently been recognized and far less is known about this process than the associated steps of initiation or elongation. However, these events must occur thousands of times per division in human cells; implying that it must occur with high efficiency (Hopefield 1974; Errico and Constanzo, 2012; reviewed in Costa et al., 2013; Wendel et al., 2014). Each singular

convergence must somehow recognize replicated regions, resolve torsional complexities created by the supercoiling of two convergent replisomes, ensure that any overlapping or redundant sequences are resected or degraded, and finally joining the nascent strands at the precise point where all sequences have doubled (reviewed in Courcelle et al., 2015).

Our lab has recently identified several enzymes that are required for this reaction to occur in *Escherichia coli*. These include RecBCD, RecG, ExoI, and SbcDC (Wendel et al., 2014; Wendel et al., 2018). Of these enzymes RecBCD appears to play a critically central role, as cells lacking RecBCD have severely reduced viability and growth rates, and fail to maintain the region of the chromosome where replication completes (Wendel et al., 2014). Interestingly, RecBCD also has a long-established role in double strand break repair and homologous recombination (Klein and Kreuzer, 2002; reviewed in Smith 2012; Wendel et al., 2014). The enzyme complex has a number of remarkable activities, including two helicases with differing polarities, as well as both exonucleolytic, and endonucleolytic activities. Despite decades of characterization, both *in vivo* and *in vitro*, many of the molecular aspects and intermediates for which RecBCD catalyzes recombination remain uncharacterized. Considering RecBCD's newly identified and critical role in completion of replication, it seems likely that its functional role in both processes will be similar if not identical. Thus, characterization of the completion reaction presents a real opportunity to learn more about the cellular role and function of this complex enzymatic machine.

In this work, I examine two DNA sequence motifs that are known to affect RecBCD function in recombination or completion. *Chi* sequences are nonpalindromic G-rich octamers that are heavily enriched and over-represented in the leading strand template (Blattner et al., 1997). They alter RecBCD activities, and during recombination processes, determine where cross-over events are joined between two parental molecules (Lam et al.,

1974; Henderson and Weil, 1975; Stahl and Stahl, 1977). They are hotspots for spontaneous recombination events mediated by RecBCD (Kuempel et al., 1977; Horiuchi et al., 1994). The other sequence motif that was examined is the *ter* sequence. These are 23-bp sequences that bind Tus protein and block replication forks in the terminus region of the chromosome in a polar manner and have been identified throughout the chromosome as can be seen in Figure 1.1A. To further characterize the completion event and the cellular role of RecBCD, I engineered these sequences into "mini-chromosomes", or plasmids, that we adapted in our lab to examine completion events on defined substrates. While the completion reaction is independent of recombination (Courcelle et al., 2015), the two processes share many of the same enzymes, suggesting they are likely to function on similar substrates or intermediates, even though the end products of these reactions are quite distinct. While little is known about completion, recombination has been extensively studied over the last decades, and I briefly review aspects of this process in *E. coli* below.

Basics of Homologous Recombination

Homologous recombination is a highly conserved process found across all domains of life. It plays a critical role in the production of genetic diversity during meiosis and sexual cycles in eukaryotes and prokaryotes, and the gene products are important for maintaining genomic integrity and survival in the presence of DNA damage (Boyce and Howard-Flanders, 1964; reviewed in Bianco 1998; Sung and Klein 2006; reviewed in San et al., 2008; Amunugama and Fishel, 2012). Common to all homologous recombination systems is a core recombinase capable of searching DNA for homologous regions, performing DNA strand pairing and exchange (reviewed in Bianco et al., 1998; Meselson and Weigle, 1961). Fascinatingly, this conserved archetype is found in nearly all organisms; eukaryotes depend on Rad51 and Dmc1, archaea rely on RadA, viruses

like B=bacteriophage T4 need UvsX, and RecA is associated with the bacterium *Escherichia coli* (Lee et al., 2015; reviewed in Bianco et al., 1998; Qi et al., 2015; Seitz et al., 1998). A physical and biochemical comparison of a few of these recombinases suggests highly conserved functionality; implying that studying one will give insight into the others (Reviewed in Bianco 1998).

recA was originally identified as a mutation in a screen for recombination deficient strains of *E. coli* K-12 as monitored by conjugation of a mutagenized F- strain with a Hfr strain (Clark and Margulies, 1965). In control experiments, the authors demonstrated that the donor DNA was taken up by the recipient cells, leading the authors to infer that the defect in *recA* mutants was related to a failure in their ability to exchange or recombine DNA strands (Clark and Margulies, 1965; Howard-Flanders and Theriot, 1966B).

Purified RecA has the ability to form filaments. The monomeric form of RecA has two binding sites, one capable of binding to another RecA monomer and the other capable of binding to either ssDNA or a ssDNA-dsDNA complex. These properties are thought to allow RecA to form extended filaments that are able to survey and identify homologous sequences and pair them together with single stranded regions (Chen et al., 2008; Savir et al., 2010; De Vlaminck et al., 2012; Lesterlin et al., 2014). Once the homology is found the DNA strand exchange occurs through a process in which the RecA filament displaces single-stranded DNA binding protein (SSB) (Mackay et al., 1974) creating a three-stranded D-loop intermediate (Cox and Lehman, 1982; Stasiak et al., 1984). DNA binding, exchange and release are regulated through ATP hydrolysis via creating DNA duplexes where base pairing is not only subject to Watson-Crick strategies but reliant on the intact strand, which plays an important role in differentiating between homologous and non-homologous sequences (Chen et al., 2008).

RecBCD creates 3' ssDNA substrates for RecA in DNA repair

In *E. coli* three different homologous recombination 'pathways' have been characterized, which act on different substrates or conditions to promote RecA-mediated recombination: RecBCD, RecET, and RecFOR (Smith 1989; Kiem and Lark, 1990; Clark 1991; Shiraishi et al., 2006).

Early genetic screens identified mutations in each of these three pathways. *recBC* mutations reduced conjugation or transductional recombination by more than three orders of magnitude (reviewed in Anderson 1997A). In the absence of *recBC* the remaining 0.1% of recombination was dependent on *recFOR* or *recE* genes (Birge and Low, 1974). The recombination defects in *recBC* mutants could also be suppressed by mutations in *sbcDC or xonA* (Allgood and Silhavy, 1991). Several early studies suggested that these mutations activated the RecF or RecE pathways since the recombination remained dependent on these proteins (Karu and Belk, 1982; Lloyd and Thomas, 1983; Clark et al., 1993). RecE and T were subsequently found to be prophage genes that were absent in many of the strains used (Clark et al., 1993; Handa and Kobayashi, 2005A; Shiraishi et al., 2006). Genes that were placed into the RecF pathway were often suggested to be responsible for repairing single-strand gaps or plasmid recombination (Kushner et al., 1971; Stahl et al., 1977; reviewed in Smith 1989; Keim 1990). However, more recent studies have shown that the RecF pathway genes are intimately associated with replication (Stahl et al., 1972) and much of the recombination associated with these genes appears to occur through the initiation of replication when single strand 3' ends are paired with homologous duplex (Courcelle et al., 1997). In the presence of DNA damage that blocks DNA polymerase, RecF pathway genes are associated with processing and maintaining the arrested replication fork structure in a manner that allows the blocking lesions to be repaired so that replication may resume

(Courcelle et al., 1997; Courcelle et al., 1999; Courcelle and Hanawalt, 1999; Courcelle et al., 2001; Courcelle et al., 2003; Chow and Courcelle, 2004; Courcelle et al., 2006).

RecBCD, the enzyme pathway of interest, is essential to maintain the chromosome during the completion reaction, forms an ATP-dependent helicase-nuclease heterotrimeric complex that contains a slow 3'-5' helicase and nuclease on the RecB subunit (Yu et al., 1998; Amundsen et al., 1990; Taylor et al., 2003), a fast 5'-3' helicase on the RecD subunit (Amundsen et al., 1986; Taylor et al., 2003) and a sequence-dependent recognition site for a unique octamer called Crossover hotspot instigator (*chi*) in the RecC subunit (Lam et al., 1974, Amundsen et al., 1990; Taylor et al., 2016; Amundsen et al., 2016), and an exonucleolytic and endonucleolytic activity in the RecB (Yu et al., 1998).

Biochemically, the enzyme complex was initially thought to be made up of two subunits, RecB and RecC (Amundsen et al., 1986). RecD was subsequently identified as a 58-kDa polypeptide that dissociated at higher salt concentrations during purification (Amundsen et al., 1986). Over the years, RecBCD's helicase and nuclease activities have been dissected through both genetic and biochemical characterization of point mutants.

It was observed that in the absence of the RecD subunit, no nuclease activity was detected, *in vitro* or *in vivo*, leading early work to infer that RecD likely contained the nuclease (Biek and Cohen, 1986). However, a point mutation in $RecB_{D1080A}$ was also seen to attenuate nuclease activity (Anderson et al., 1999). Subsequent work demonstrated that RecB contained the nuclease, which was activated by the presence of RecD (Anderson et al., 1999). This was later confirmed from X-ray crystallographic imaging of the subunit revealing components necessary for nuclease activity similar to other nucleases (Singleton et al., 2004).

In vivo and *in vitro*, the nuclease activity of RecBC is also attenuated upon encountering a *chi* site (Dabert et al., 1992). This led to some to suggest that RecD dissociated from the complex at these sites (Stahl et al., 1990). However, *in vitro* comparisons of RecBC(D-) purified enzyme do not entirely mimic those of the RecBCD following *chi*, leading to the idea that the subunit may remain associated in an altered conformation (Thaler et al., 1989; Anderson et al., 1997B). This view was additionally supported in single molecule studies using fluorescent tagged RecBCD molecules demonstrating that RecD remained associated as it approached and passed *chi* (Handa et al., 2005B).

How the enzyme complex degrades DNA as it unwinds has also been debated. Some suggest RecBCD degrades both strands of DNA up to encountering a *chi* site, at which point only 3' strand is degraded (Dixon and Kowalczykowski, 1993). A second model purposes that the enzyme primarily unwinds the DNA, and cuts at *chi* (Singleton et al., 2004). Support for both models can be observed *in vitro* and appears to be primarily depend on the Mg^{+2} and ATP concentration used in the reaction (Taylor and Smith, 1995; Fan and Li, 2009).

RecBCD change linked by Crossover Hotspot Instigator (*chi*)

The conformational changes and altered activities of RecBCD are all triggered by the ssDNA recognition of a non-palindromic, *chi* sequence 5'-GCTGGTGG-3' (Smith et al., 1981; Stahl et al., 1990; Taylor and Smith, 1992; Dixon and Kowalcyzkowski, 1993; Bianco et al., 1997; Kulkarni and Julin, 2004; Amundsen et al., 2007A; Handa et al., 2012; Taylor et al., 2016). The RecC subunit recognizes *chi*, inducing a pause in the processive unwinding (reviewed in Bianco et al., 1997; Dohoney and Gelles, 2001; Handa et al., 2012). A conformational shift in RecD subunit (Anderson et al., 1997B; Handa et al.,

2005B; Yang et al., 2012) alters the enzyme's activity effectively slowing down the processivity by inactivating the fast 5'-3' helicase RecD activity and shifting the leading translocation motor to that of the slower helicase in the RecB subunit (Anderson et al., 1997B, Spies et al., 2003; Handa et al., 2005B; Spies et al., 2007; Yang et al., 20012). Additionally, this conformational change modifies the location of the nuclease activity in RecB such that DNA is incised a few nucleotides before *chi* on the opposite strand (Spies et al., 2005; Cheng et al., 1987; Dixon and Kowalczykowski, 1993; Anderson et al., 1997B). RecB's slow helicase is seemingly the only activity unaffected by the *chi* (Cho et al., 2018). The net result of the single helicase action is the creation of a 3' loop ssDNA, which is thought to produce a substrate for RecA loading (Wong et al., 2006).

chi was first identified as a mutation arising on lambda (λ) phage genome that increased the plaque size during infection (Lam et al., 1974). The molecular process by which *chi* affected this is through the protection of the λ-phage DNA by inactivation of the RecBCD nuclease, allowing λ to persist and initiate lytic rolling circle replication (Chattoraj et al., 1979; Stahl 1979; Murphy 1991; Dabert et al., 1992; Köppen et al., 1995; Kuzminov et al., 1994).

Although extensively characterized, aspects of *chi's* functional role in the *E. coli* life cycle remain unaddressed. The non-palindromic sequence means that *chi* affects RecBCD in an orientation specific manner and is found several times more frequently on the *E. coli* chromosome than would be expected to occur (Malone et al., 1978; Stahl and Stahl 1977; Kobayashi et al., 1982; Blattner et al., 1997). Additionally, these sequences are heavily over-represented specifically on the leading strand template of the *E. coli* genome (Blattner et al., 1997). The reason for this orientation specific effect and strand bias is difficult to explain based on current models of recombination.

RecBCD's new found role in completion of replication

Although all previous work has focused on RecBCD's role in double strand break repair via homologous recombination, this multicomponent enzyme has recently also been characterized to be essential to complete DNA replication by accurately resecting and rejoining over-replicated replication forks (Wendel et al., 2014). This new role was initially inferred from observations that the growth and viability of *recBCD* mutants was severely impaired and plasmids were less stable in the mutants of *recD* (Wendel et al., 2014). These phenotypes are not seen in *recA* mutants, meaning that some functions of RecBCD appear to be independent of double strand break repair or RecA. As see in Figure 1.1B, using high-throughput sequencing to compare the copy number of the sequences around the chromosome of *E. coli* showed that *recBC* mutants were unable to maintain the region of the chromosome where replication forks converge (Wendel et al., 2014). Importantly, no defects are observed in *recA* mutants, arguing that the inability to maintain this region is not associated with double strand breaks (Wendel et al., 2014). Other mutants, including *recD, xonA sbcDC,* and *recG*, exhibit an over-replication of this region on the chromosome (Wendel et al., 2014; Wendel et al., 2018). However, much is yet to be determined; including the biochemical mechanisms by which RecBCD and these other enzymes catalyze this reaction (Fig 1.1C).

Could *chi* be involved in completion as well?

Considering RecBCD's new-found role in completion, and its known interactions with *chi*, it seems reasonable that *chi* may affect the completion reaction (Stahl 1979; Kobayashi et al., 1982; Wendel et al., 2014).

In order to examine this question, in this work I describe the construction of plasmid "mini-chromosomes" that contain a bidirectional origin of replication that can be used to

examine the intermediates and factors required for the completion reaction. I initially verify that these substrates can be used to study the completion reaction by demonstrating that these plasmids require completion enzymes to propagate in cells. The completion enzymes are required for plasmids containing two-replisomes, but not one replisome, indicating that the substrate these enzymes act upon *in vivo* is specifically created when two replication forks converge. I then utilize these plasmids to examine how *chi* and or *ter* sequences affect the ability to complete replication in the presence and absence of the various genes required to complete replication on the chromosome.

Figures

Figure 1.1A)

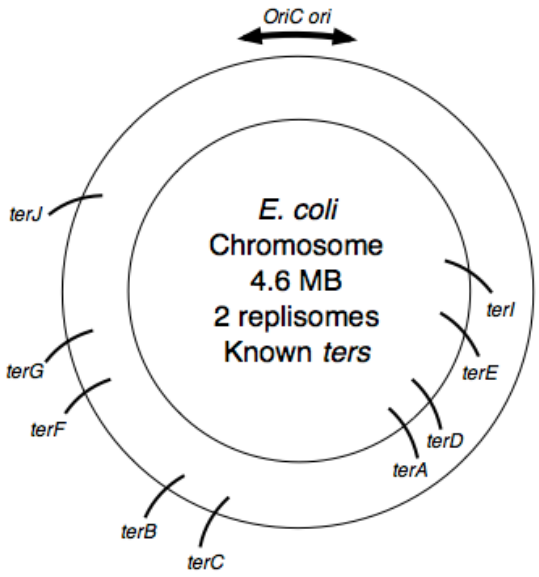


Figure 1.1B)

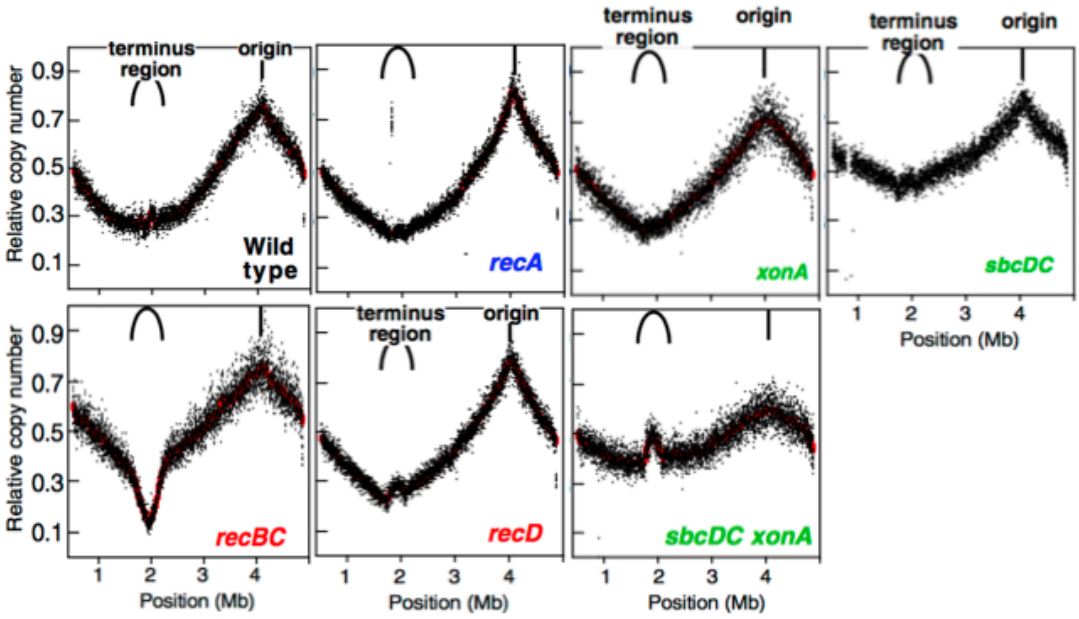


Figure 1.1C)

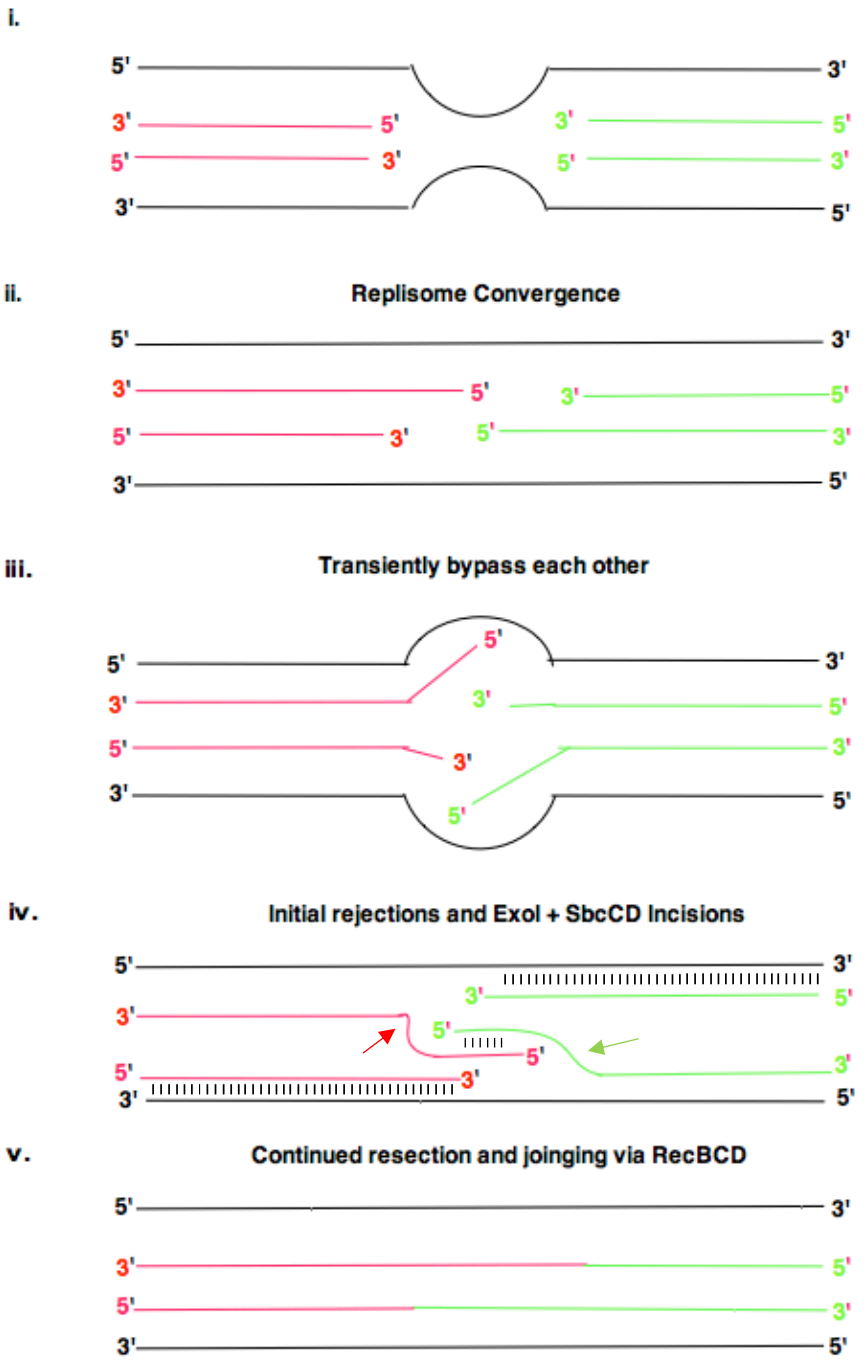

i.
ii.
Replisome Convergence
iii.
Transiently bypass each other
iv.
Initial rejections and ExoI + SbcCD Incisions
v.
Continued resection and joinging via RecBCD

Figure 1.1: The current model for the accurate completion of DNA replication involves RecBCD, ExoI (encoded by *xonA*), and SbcDC in *E. coli*. (**A**) Schematic of the *Escherichia coli* circular chromosome containing a single bidirectional origin of replication and *ter* sequences which block replication progression in an orientation-specific manner. (**B**) Replication profiling reveals that *recBC* is required to maintain the region where replication forks converge on the chromosome, whereas *recD*, or s*bcDC* and *xonA* are required to resect over-replicated DNA that arises in this region. Genomic DNA from replicating cultures was purified, fragmented, and profiled using high-throughput sequencing. Sequence read frequencies, normalized to stationary-phase cells, are plotted relative to their position on the genome (Wendel et al., 2014). (**C**) Model of completion of replication. (**i-iii**) Replication forks converge, bypass each other in the terminus region creating over-replicated regions of the genome. (**iv**) ExoI and SbcDC incise a structure created by these over-replicated regions to initiate resection. (**v**) RecBCD is required to complete resection and promotes joining of the convergent nascent strands.

Chapter II

Methods and Materials

Bacterial strains and Plasmids

The strains used in this study were all derivatives of W3110; SR108 is a strain incapable of synthesizing its own thymine; *thyA36 deoC2* (Mellon and Hanawalt, 1989). All other mutants are derived from this parent and described in Table 2.

Plasmid constructions were performed according to published protocols for *in vivo* recombineering (Sawitzke et al., 2012), construction by amplification (Casini et al., 2014), and Gibson assembly (Gibson et al., 2009). Plasmid and their features used in this study are listed in Table 2.2. All plasmids containing unidirectional origins of replication were derived from pBR322 (Ampicillin and Tetracycline resistance, pMB1 origin), which has been described previously (Bolivar et al., 1977). pCL07 contains a *chi* sequence engineered into leading strand of parent pBR322. pCL08 contains a *chi* sequence engineered into lagging strand of parent pBR322. To accomplish this, primer pairs 5'-catgcccggttactggaacggctggtggttgtgagggtaaacaactgg-3' + 5'-cgccgcatacactattctca-3' and 5'-ccagttgtttaccctcacaaccaccagccgttccagtaaccgggcatg-3' + 5'-tgagaatagtgtatgcggcg-3' were used for pCL07 and pCL08 respectively, with pBR322 as a template and amplified for 25 cycles using Pfu Turbo Polymerase (Agilent). PCR products were examined and purified by agarose gel electrophoresis. The amplified fragments were combined with DpnI digested pBR322, and the fragments were then joined and transformed using Gibson assembly (New England Biolabs) to generate pCL07 and pCL08. Plasmids were sequenced to verify sequence changes.

All plasmids containing bidirectional origins of replication are derived from pCB104 (Potrykus et al., 2002) and contain an ampicillin-resistant cassette from pBR322.

pCL03 was constructed using primer pairs 5’-gtcggttcagggcagggtcgtgga-cggtctgacagttaccaatgc-3’ and 5’-ggcggtttgcgtattgggcgcggtctgacagttaccaatgc-3’ to amplify the *ampR* gene from pBR322. 0.2 μg gel purified PCR product was then combined with 0.5 μg BamHI-digested pCB104 and amplified for 25 cycles using Pfu Turbo Polymerase (Agilent). PCR products were examined by agarose gel electrophoresis and products running larger than 5kb were gel purified and transformed into recombineering strain DY329 (Yu et al., 2000) to generate the ampicillin resistant plasmid, pCL03. pCL01 was made by removing a *chi* sequence proximal to the origin using primer sets 5’-attgctgataaatctgga-3’ + 5’-ctttggaatccagtccctcttcctcctgctgatctgcgacttatcaac-3’ and 5’-tccagatttatcagcaat-3’ + 5’-gttgataagtcgcagatcagcaggaggaagagggactggattccaaag-3’ to amplify overlapping fragments of the plasmid template using Pfu Turbo Polymerase (Agilent). The fragments were then joined and transformed using Gibson assembly (New England Biolabs) to generate pCL01. pCL05 was constructed by inserting a *chi* sequence into the terminus region of plasmid pCL03, using plasmid pairs 5'-ctgcgctcggcccttccg-gctgccaccagcattgctgataaatctgga-3' + 5'-tccagatttatcagcaatgctggtggcagcggaagggccgag--cgcag-3' and 5'-gttgataagtcgcagatcagcaggaggagaagagggactggattcc-aaag-3' + 5'-ctttggaatccagtccctcttcctcctgctgatctgcgacttatcaac-3' to amplify overlapping fragments which were joined and transformed using Gibson assembly (New England Biolabs) to generate pCL05; mutation to inactive *chi* near λ bacteriophage origin of parental plasmid pCL03 as well as mutation to active *chi* inside the ampicillin cassette. pCL02, pCL04, and pCL06 are identical to pCL01, pCL03, and pCL05 but contain *terB* and *terC* sequences inserted flanking the *ampR* in the terminus region. pCL04 was constructed using primer pairs 5’-gtcggttcagggcagggtcgtggatccactttagttacaacatacttattcgcggaacccctatttgttt-3’and 5’-ggcggtttgcgtattgggcgcatattagttacaacatcctatatggtctgacagttaccaatgc-3’to amplify the *ampR*

gene from pBR322. 0.2 µg gel purified PCR product was then combined with 0.5 µg BamHI-digested pCB104 and amplified for 25 cycles using Pfu Turbo Polymerase (Agilent). PCR products were examined by agarose gel electrophoresis and products running larger than 5kb were gel purified and transformed into recombineering strain DY329 (Yu et al., 2000) to generate the ampicillin resistant plasmid, pCL04. Primer pairs 5'-ctgcgctcggcccttccggctgccaccagcattgctgataaatctgga-3' +5'-tccagatttatcagcaatgct--ggtggcagcggaagggccgagcgcag-3' and 5'-gttgataagtcgcagatcagcaggaggagaagagggact--ggattccaaag-3' + 5'-ctttggaatccagtccctcttcctcctgctgatctgcgacttatcaac-3' were used to amplify overlapping fragments of each plasmid. Fragments were joined with pCL04 as a template and transformed using Gibson assembly (New England Biolabs) to generate pCL02 and pCL06.

Transformation efficiency assay

Electro-competent cells were prepared by growing a 100-fold dilution of a fresh overnight culture in 10 mL LB with thymine (LBthy) to an OD 600 of 0.4. Cells were then pelleted, and serially washed with 30 mL water, 30 mL 10% glycerol, and then resuspended in 200 µL of 10% glycerol and stored at -80°C. 40 µL of competent cells were mixed with 50 ng of plasmid and electroporated at 1.8 kV 25 µFD 200 Ohms and allowed to recover at 37 °C for 30-60 minutes in 1 mL SOC media. The transformation reactions were then diluted and aliquots were spread on LB thy plates with and without 50 ug/mL ampicillin to determine the number of transformants and viable cells, respectively. Colonies were counted following overnight incubation at 37 °C. The same preparations of competent cells and plasmid preparations were used for comparisons between strains and plasmids. The relative transformation efficiency of each strain was calculated as the ration of the

transformants per viable cells in the mutant cultures to the transformants per viable cells in wild-type cultures.

Plasmid stability assay

Cells from overnight cultures of strains containing the plasmid grown in LBthy medium with 50 μg/ml ampicillin were pelleted and used to inoculate 10ml cultures of LBthy medium at 1:1000 dilution. Cultures were grown without ampicillin selection at 37 °C with aeration overnight. The resulting cultures were then sampled to determine the ratio of cells retaining the plasmid and used to reinoculated 10ml LBthy medium at 1:1000 dilution. This was repeated for three iterations. To determine plasmid retention, 10-μl aliquots of serial 10-fold dilutions were spotted on LBthy plates in the presence or absence of 50 μg/ml ampicillin. Colonies were counted following overnight incubation at 37 °C to determine the percent of plasmid-containing cells (Wendel et al., 2014).

Total genomic DNA extraction

750 μl of cultures was mixed with 750 μl of cold 2x NET (100 mM NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA). Cells were pelleted and frozen at -80 °C. Samples were resuspended in 140 μl of lysozyme (1 mg/mL) and RNaseA (0.2 mg/mL) in TE (10 mM Tris, pH 8.0, 1 mM EDTA) and lysed for 30 minutes at 37 °C. Then Sarkosyl (10 μl of 20% [wt/wt]) and Proteinase K (10 ul of 10 mg/mL) was added and incubation continued for 60 minutes. Samples were then serially extracted with 4 volumes phenol/chloroform (1/1) and 4 volumes chloroform followed by dialysis for 1 hour on 47 mm Whatman 0.05-um pore disks (Whatman #VMWP04700) which were floated on a 250-mL beaker of TE (1 mM Tris, pH 8.0, 1 mM EDTA).

Southern analysis of plasmid replication intermediates

Total genomic DNA was digested with *SacII* (New England BioLabs) for strains containing pBR322 derived plasmids or NheI (New England BioLabs) for pCB104 derived plasmids. In both cases, plasmids lack restriction sites for these enzymes. Samples were then extracted with 1 volume of chloroform before equal cell equivalents were loaded on to 0.5% and 1.0% TAE/TBE (220 mM Tris, 180 mM Borate, 5 mM EDTA, pH 8.3) agarose gels and electrophoresed at 1 V/cm. Gels were transferred to Hybond N+ nylon membranes (Amersham GE Healthcare) and probed with either the pBR322 or the pCL01 P^{32}-labelled plasmid. Radioactive labeling was carried out by nick translation kit (PerkinElmer) (Spivak and Hanawalt, 1995). Radioactivity was visualized using a Storm 840 and its associated ImageQuant Software (Molecular Dynamics).

Copy number analysis

Strains containing the plasmids or containing a chromosomal copy of the *ampR* gene (HL946 or CL007) were grown and the genomic DNA purified as described above. Plasmid DNA and chromosomal DNA was digested with EcoRV (New England Biolabs) to linearize plasmid and chromosome species. DNA was then analyzed by standard Southern analysis and quantified as described above.

Strain growth assay

Cultures containing 1 or 2-replisome plasmids, or neither, were grown in LB plus selection over 800 minutes and continuously cataloged in 20-minute intervals for absorbance in OD. The strain of *E. coli* used for this assay was also used to quantify the copy number analysis which has the *ampR* gene (HL946 OR CL007). Growth curves

were graphed showing each cultures ability to replicate until plateauing into stationary phase.

Tables

Table 2.1: *E. coli* strains used in this study		
Courcelle Catalogue	**Genotype**	**Source**
CL001 (SR108)	WT	Mellon & Hanawalt, 1989
CL002	*recA*	Franklin, 1967
CL003	*recBC*	Kushner, 1974
CL004	*recD*	Thaler et al., 1989
CL039	*xonA*	Kushner S et al., 1972
CL2344	*sbcCD*	Gibson et al., 1992
CL2357	*xonA sbcCD*	Jensen 1993
CL008	*recG*	Chua., et al 1993
CL2542	*recBC xonA sbcCD*	Wendel et al., 2018

Table 2.2: Plasmids used in this study				
Courcelle Catalogue	**Construction**	**Replisomes**	**Ter sequence trap**	***chi***
pBR322	(Bolivar et al., 1977)	+		
pCL07	5'-catgcccggttactggaacggctggtg gtt-gtgagggtaaacaactgg-3' +	+		+ Leading strand
	5'-cgccgcatacactattctca-3'			
	5'-ccagttgtttaccctcacaaccaccag-ccgttccagtaacccgggcatg-3'			
	5'-tgagaatagtgtatgcggcg-3'			
pCL08	5'-ccagttgtttaccctcacaagctggtg gc-gttccagtaaccgggcatg-3'	+		+ Lagging strand
	5'-cgccgcatacactattctca-3'			
	5'-catgcccggttactggaacgccaccagctt gtgagggtaaacaactgg-3'			
	5'-tgagaatagtgtatgcggcg-3'			
pCL01	5'-attgctgataaatctgga-3' +	++		
	5'-gttgataagtcgcagatcagca ggaggagaagagggactggattccaaag-3'			
	5'-tccagattatcagcaat-3' +			
	5'-ctttggaatccagtccctcttcctcctgctgatctgcgacttatcaac-3			
pCL02	5'-attgctgataaatctgga-3' +	++	++	
	5'-gttgataagtcgcagatcagcaggaggagaagag-ggactggattccaaag-3'			

	5’-tccagattatcagcaat-3’ +			
	5’-ctttggaatccagtccctcttcctcctgctga-tctgcgacttatcaac-3’			
pCL03	5’-gtcggttcagggcagggtcgtggatcccgcggacccctatttgttt-3’ and 5’-ggcggtttgcgtattgggcg--cggtctgacagttaccaatgc-3’	++		+proximal to origin
pCL04	5’gtcggttcagggcagggtcgtggatccactttagttaca--acatacttattcgcggaacccctatttgttt-3’ and 5’ggcggtttgcgtattgggcgcatattagttacaacatc--ctatatggtctgacagttaccaatgc-3’	++	++	+proximal to origin
pCL05	5’-ctgcgctcggcccttccg-gctgccaccagcatt-gctgataaatctgga-3’ +			terminus region +
	5’-ctttggaatccagtccctcttcctcctgctga-tctgcgacttatcaac-3’			
	5’-tccagatttatcagcaatgctggtggcagc-ggaagggccgagcgcag-3’ +			
	5’-gttgataagtcgcagatcagcaggaggagaag-agggactggattccaaag-3’			
pCL06	5’-ctgcgctcggcccttccggctgccaccagcatt-gctgataaatctgga-3’ +	++	++	terminus region +
	5’-ctttggaatccagtccctcttcctcctgctgatctgcgacttatcaac-3’			
	5’-tccagatttatcagcaatgctggtggcagcgg-aagggccgagcgcag-3’ +			
	5’-gttgataagtcgcagatcagcaggaggaga-agagggactggattccaaag-3’			

+: One-replisome plasmid

++: Two-replisome plasmid

+: Chi inserted

Chapter III

Results

Part I - One-replisome versus two-replisome plasmids during completion of replication

Plasmids containing two-replisomes can be stably transformed, replicated, and grown in cells similar to one-replisome plasmids.

Completion of replication on the chromosome involves an enzymatic process that involves two convergent replisomes resolving with high fidelity (Wendel et al., 2014). The convergent replisomes are thought to transiently bypass each other, before the excess DNA is resected and joined at the point where all DNA has precisely doubled (Wendel et al., 2014). Completion on the chromosome of *E. coli* can be challenging to study because the event occurs once per cell cycle and its location can vary over a 400 kb stretch of the genome (Campbell and Kleckner, 1990). In order to study the process and enzymes involved in this reaction in more detail, it would be useful if it could be studied on plasmids, which contain higher copy numbers and are only a few kilobases in size. However, most plasmids contain unidirectional origins of replication and are replicated by a single replisome, avoiding the event where two replisomes may converge (Reviewed in del Solar et al., 1998). λ phage contains a bidirectional origin of replication that is functionally homologous to that on the *E. coli* chromosome (Furth et al., 1977; Tabata et al., 1983; Meijer et al., 1979), and has previously been shown to replicate as a mini-chromosome when placed on a plasmid (Moore et al., 1977). Dr. Brian Wendel therefore constructed a 5 kb plasmid containing the λ origin to determine if it could be used as a model to study the completion reaction (Fig 3.1A), and initially characterized how well it could transform cells, whether it affected cell growth, and the copy number at which it is propagated in cells.

To examine how the plasmid with two replisomes affected cell growth, we compared the growth of cell cultures containing the two-replisome plasmid, to that of cultures containing the one-replisome plasmid pBR322 utilizing pMB1 (close relative of ColE1) *ori* (Bolivar et al., 1977), or no plasmid at all. Cultures were grown over 800 minutes and continuously cataloged in 20-minute intervals for absorbance. As shown in Figure 3.1B, the growth rate of each culture was similar; indicating that the two-replisome plasmid does not impair growth of the host during replication.

To determine how efficiently the two-replisome plasmid was transformed, 50 ng of plasmid were transformed in 40 ul of wild-type competent cells via electroporation in 2 mm gap cuvettes at 2.5 kilovolts. After a 60-minute recovery period, dilutions of the culture were then plated on LB with 50 ug/mL of ampicillin as well as on LB plates to determine ratio of viable cells that were transformed (Figure 3.1C). Under transformation similar conditions, both the one-replisome and two-replisome plasmid transformed with similar efficiencies, ~200-400 transformants /10^6 viable cells, demonstrating the two-replisome plasmid could enter and initially establish replication similar to other plasmids.

To determine the copy number at which the two-replisome plasmid was maintained, we used Southern analysis in which we used a ^{32}P-labeled ampicillin resistance gene as a probe to compare the radioactive intensity of the signal on the plasmid to the signal from a single copy ampicillin resistance gene integrated into the chromosome. To this end, total genomic DNA was purified from cells containing the plasmid pBR322, pCL01, or without plasmid but having an ampicillin resistance gene on the chromosome. The genomic DNA was then digested with a restriction enzyme that linearized each plasmid and then analyzed by Southern analysis following agarose-gel electrophoresis. A representative gel is shown in Figure 3.1D. Overall, we found through

the radioactive intensity of the signals that the two-replisome plasmid was maintained about 89 copies per chromosome, compared to about 63 copies for pBR322 (Fig. 3.1E). Taken together, the two-replisome plasmid transform, propagate, and are maintained similar to the one-replisome plasmid, pBR322.

Plasmids replicated by two replisomes are less stable than one replisome plasmids and contain more aberrant, multimeric species.

To determine how stably the plasmid is maintained during replication, we monitored the rate of plasmid loss over time in the absence of selection. To this end, wild-type cultures containing either pBR322 or pCL01 were diluted 1/1000 and grown in media without selection overnight, before the process was repeated the next day; similar to the previously published plasmid stability protocol (Wendel et al., 2014). On each passage, dilutions of a passaged sample, to promote proliferation of generations, sample were plated to determine the ratio of cells that maintained the plasmid. Figure 3.2A shows an example of these dilutions for cultures containing both the one-replisome and two-replisome plasmid. We quantified and plotted these ratios over time and the results are shown in Fig 3.2B. Whereas the one-replisome plasmid was stably maintained over the ~30 generation assay, the proportion of cells that maintained the two-replisome plasmid was reduced by ~two orders of magnitude over this same time period.

The results shown in Figure 3.1B-E argue that the instability of the two-replisome plasmid, relative to the one-replisome plasmid is unlikely to be due to detrimental effects on cultures growth rates or comparatively lower copy numbers. Further, neither pBR322 or pCL01 encode any partitioning mechanisms, which would control plasmid segregation

between daughter cells, that could account for the difference in stability (Nordstrom et al., 1980; Austin et al., 1986).

Although the overall copy number between these plasmids were similar, we did observe noticeable differences in the proportion of aberrant non-monomeric, amplified substrates that appeared during the propagation of each plasmid. To examine the form in which the plasmid DNA was maintained in each cell, total genomic DNA was purified from cultures containing each plasmid, and the plasmid DNA was then analyzed by Southern analysis following agarose gel electrophoresis using P^{32}-labeled pBR322 and pCL01 as probe. Representative gels from each are shown in Figure 3.2C and the results are plotted in Figure 3.2D. Overall, the two-replisome plasmid contains elevated amounts of abnormal, multimeric species relative to the one-replisome plasmid.

Similar to the completion of replication on the chromosome, amplifications and genetic instability on two-replisome plasmid are driven by an aberrant RecA-mediated recombination mechanism.

On the chromosome most of the genetic instability and amplifications that arise in the region where replication completes is driven by an aberrant form of RecA-mediated recombination (Wendel et al., 2014; Wendel et al., 2018). To determine if RecA plays a similar role on the plasmid, we examined how its presence or absence affected the stability of the plasmid when grown in the absence of selection, as before. As shown in Figure 3.3A, inactivation of RecA increased the stability of the two-replisome plasmid. The increase in stability brought the two-replisome plasmid to a level that was comparable to the one-replisome plasmid once the ability for homologous recombination was taken away.

We next examined how the form of the plasmid was maintained became affected by the presence of RecA. To this end, total genomic DNA was purified from both wild-type and *recA* mutant cultures containing plasmid and analyzed by Southern analysis as previously described. The increased stability in the absence of RecA correlated with an overall reduction in the amount of amplified, multimeric species (Figure 3.3B and C). A similar reduction in multimeric plasmid species was also seen with the one-replisome plasmid. The observations are consistent with the idea that RecA is driving the instability on two-replisome plasmid and that the instability arises due to amplification or multimeric species generated in its presence (Wendel et al., 2018).

Transformation of plasmids with two replisomes, but not one replisome, depends on the enzymes required to complete replication on the chromosome.

On the chromosome, the completion of replication requires the RecBCD helicase-nuclease (Wendel et al., 2014; reviewed in Courcelle et al., 2015). In its absence, the genomic region where replication forks converge cannot be maintained, is extensively degraded, and growth is severely compromised. Nucleases SbcCD and ExoI are also required to initiate the completion reaction. In the absence of these gene products, maintaining the region where replication forks converge becomes dependent on the aberrant RecA-mediated reaction. In the absence of RecA, growth is severely compromised in these mutants (Wendel et al., 2014; Wendel et al., 2018). In order to determine if these genes are also involved in completing replication in the two-replisome plasmid, we examined the ability of the two-replisome plasmid to transform mutant strains deficient in completion enzymes. As a control, we also examined the ability of the one-replisome plasmid to transform into these mutants. 50 ng of plasmid DNA was

transformed by electroporation into each mutant and the transformation efficiency for each strain was determined, relative to wild-type. In the case of the one-replisome plasmid, transformant samples were obtained for each of the mutants examined, and successful transformation in each case occurred independently of recombination or RecA (Figure 3.4A). However, in plasmid replication involving two replisomes, we observed transformants in most of the mutants examined. Additionally, in *recBC* mutants the transformation efficiency was reduced by greater than two orders of magnitude. In some attempts, a few rare microcolonies could be observed on the selective plates following 3 days incubation (as opposed to the normal overnight incubation). However, in these cases, we were unable to grow the transformants in liquid media beyond a single passage (data not shown). Similarly, in mutants lacking the SbcDC ExoI nucleases, transformation efficiency became dependent on the presence of recombination and RecA (Figure 3.4 A). No other mutants examined depended on RecA for transformation of the two-replisome plasmid. These genetic requirements for transformation of the two-replisome plasmid are required to complete replication on the chromosome and suggest that they are similarly required to complete replication on the two-replisome plasmid. Further, the results would argue that the substrates acted upon by these enzymes, *in vivo*, are specifically created when two replisomes converge, since one-replisome plasmids do not exhibit any requirement for their presence.

Part II: The effect of Replication fork traps and the Completion reaction

Two-replisome plasmids with two additional ter sequences, terB and terC from the Escherichia coli chromosome, are replicated and maintained at similar frequencies as two-replisome plasmids without replication fork trap capabilities.

ter sequences, and their homologs, are found on several bacterial genomes, including *E. coli* and *Bacillus subtillis* (Coskun-Ari et al., 1994) and function as replication fork "traps" which bind to a protein called Tus, and halt replisomes approaching from one side, in a polar manner (Kuempel et al., 1977; MacAllister et al., 1990). Their presence ensures replisome convergence at the terminus area of the chromosome. However, deletion of the *tus* reveals Tus-*ter* mutants have no observable phenotype on growth or viability, arguing that replication fork traps are not essential (Iismaa et al., 1987; Roecklein et al., 1991). Previous experiments have examined *ter* elements in unidirectional plasmids. Perhaps not surprisingly, when *ter* is oriented in a manner that blocks replisomes prior to the point of convergence this causes replication difficulties and induces the SOS response (Hill and Marians., 1990; MacAllister et al., 1990; Hasebe et al., 2018). In order to study how the Tus-*ter* traps affect the completion reaction in *E. coli*, we engineered two *ter* sequences into the two-replisome plasmids in a trapping orientation, similar to that found on the chromosome. The placement of *terB* and *terC* flanking the terminus region, on the two-replisome plasmid is shown in Figure 3.5A. As replication fork "trap" (Tus-*ter*) is dependent on the host derived protein Tus. The *tus* gene is autoregulated and induced in the presence of unbound *ter* sequences (Natarajan et al., 1991; Roecklein et al., 1991). Thus, the presence of additional *ter* sequences in the newly constructed pCL02, would be expected to contain sufficient levels of Tus to ensure that the polar arrest off the replication occurs at these sequences (Figure 3.1E).

To determine how the presence of *ter* traps affects the stability of plasmids containing two replisomes, we monitored the rate of plasmid loss overtime in the absence of selection with similar protocols as described in the previous section. As shown in Figure 3.5B the two-replisome plasmid containing the *ter* trap approximately 10-fold less

stable than the non-*ter* containing plasmid during the ~30 generations of the experiment. In *recA* mutants, which are deficient in homologous recombination, there is an increase in plasmid stability similar to that seen in non-*ter* containing plasmids.

Plasmids replicated by two replisomes with the addition of ter sequences contain more RecA-driven aberrant, multimeric species.

I next examined how the presence of *ter* sequences effect the form of replicating plasmids that contain two replisomes. To this end, plasmids in replicating cultures were purified and examined by Southern analysis as described above. As shown in Figure 3.5C, plasmids containing a *ter* trap contain a larger fraction of multimeric species, however, these species migrate with a pattern that suggests they are intermediates that form unit circles of dimers, trimers, and tetramers. By contrast, most of the multimeric species in the plasmids lacking *ter* sequences migrate as a high linear multimers or branched species. In *recA* mutants, fewer multimeric intermediates were observed irrespective of the presence of *ter* sequences. In Figure 3.5D, I quantified the overall levels of abnormal (non-monomeric) species in each strain. Overall, the results reflect the RecA-catalyzed propagation of abnormal species in both pCL01 vs pCL02. Further, the proportion of abnormal products correlates with the overall level of instability consistent with what is observed on the chromosome; seen more specifically by the overamplified products around the terminus region versus the amplified intermediates of the plasmid substrates.

Part III: The effect of *chi* sequences on completing replication

chi sequences located at sites where replication forks converge promote a RecA-mediated aberrant replication that correlates with instability

Completion of DNA replication requires RecBCD (Wendel et al., 2014, Courcelle., 2015; Wendel et al., 2018). *chi* sequences, 5'-GCTGGTGG-3' alter the activity of the RecBCD complex and during recombinational processes; they are associated with the locations where cross-over events are joined to form recombinant molecules (Stahl et al., 1975; Stahl and Stahl, 1975; Stahl et al., 1983; Smith et al., 1984; Ponticelli et al., 1985; Taylor et al., 1985). These sequences are highly over-represented in the chromosome and, intriguingly, their presence is heavily biased on the leading strand of the replication (Smith 2012; Courcelle et al., 2015). This bias is difficult to reconcile with its role in the double-strand break repair, since a break would have two double strand ends, whose 3' ends would be both a *chi* enriched strand and a *chi*-depleted strand. Considering RecBCD's role in replication, it is worth considering that *chi* sequences may play a role in the completion reaction. In order to examine the influence *chi* sequences may have on the completion reaction, *chi* sequences were engineered into the two-replisome plasmid pCL01, proximal to the origin, pCL03, and opposite the origin pCL05 (Fig 3.6A) to evaluate influence inside and outside the convergence zone. As controls, I also engineered *chi* sites into both the leading strand and lagging strand of the one-replisome plasmid pBR322, to generate pCL07 and pCL08 respectively (Fig 3.6B).

To determine how *chi* affects the stability of the two-replisome plasmid, each plasmid was monitored over ~15 generations on plates with and without selection to compare viable cells versus cells still containing plasmid, as done previously. As shown in Figure 3.6C, I observed that the presence of *chi* destabilized the plasmid. The destabilizing effect was more prominent when *chi* was located in the terminus region of the plasmid than when it was proximal to the origin.

To determine if the destabilizing effects of *chi* was dependent on the aberrant RecA-mediated form of replication, we also examined the stability in *recA* mutants. I observed that the absence of RecA improved the maintenance of all the plasmids, although it did not eliminate all of the instability observed with one of the *chi* containing plasmids. The results argue that *chi* destabilization is at least partially dependent on RecA, but that *chi* has some effect even in the absence of RecA.

To examine whether the *chi*-destabilization of the plasmids altered its form during the replication *in vivo*, we purified the DNA from replicating cultures and examined it by Southern analysis. As shown in Figure 3.6D and E, in the presence of *chi*, an aberrant high molecular weight intermediate was formed. The intermediate was most prominent when *chi* was present in the terminus region of the chromosome, but could still be observed when the *chi* site was located proximal to the origin of replication. The presence and intensity of the specific aberrant intermediate correlated with the destabilization effect *chi* has on the stable propagation of the plasmid.

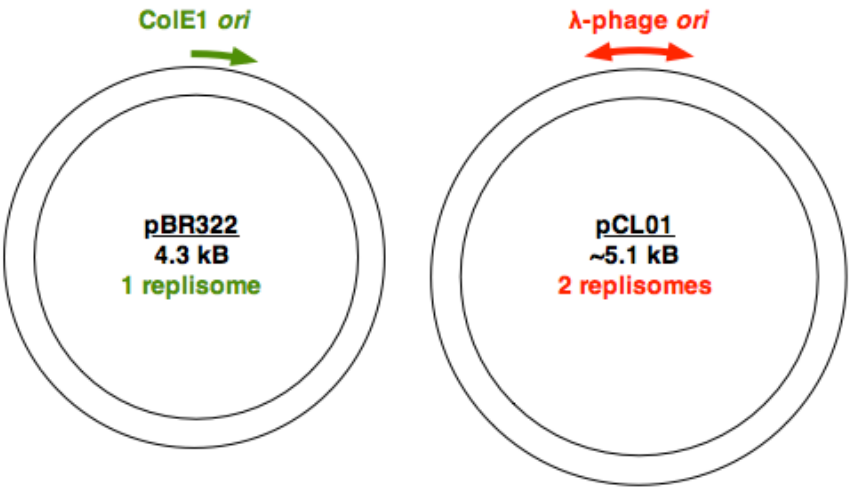
The effect of *chi* appeared to be specific to the plasmid with two-replisomes as no effect on plasmid stability was observed when *chi* sequences were present in either the leading or lagging strand on the one-replisome plasmid (Fig 3.6F, Fig 3.6G and Fig 6.7H). The observation suggests that RecBCD processing during the completion reaction is altered upon encountering a *chi* site in a manner that promotes further replication. At least on the plasmid mini-chromosome substrate, this replication is destabilizing in its effect.

Addition of a terB and terC trap reduces the destabilizing effect of chi by limiting the amount of aberrant replication that can occur

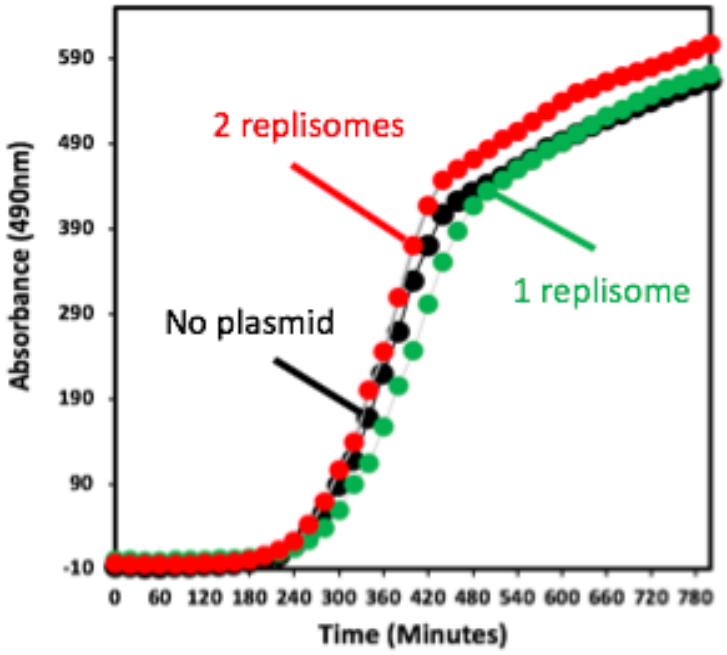
ter sequences are oriented to "trap" replication forks within the region where forks converge. Similarly, the orientation of *chi* sites is heavily biased towards the direction of replication. Considering these orientation biases, we next examined if *ter* may influence the effect *chi* has on the completion reaction. To this end, *terB* and *terC* traps were engineered into the two-replisome plasmids containing *chi* sites (Figure 3.7A and the effect on stability and replication was examined as before. As shown in Figure 3.7B, the addition of the *ter* traps reduced the destabilizing effect of *chi* overall. Further, the presence of the *ter* trap also reduced the destabilizing effect of RecA. When I examined the form of the plasmids in replicating cultures, I found that the *ter* trap similarly reduced or eliminated the presence of the aberrant over-replicated species (Fig 3.7C). The observations suggest that the presence of *ter* reduces the instability of the two-replisome plasmid by preventing or limiting the amount of aberrant RecA-mediated replication that occurs during the completion reaction.

Figures

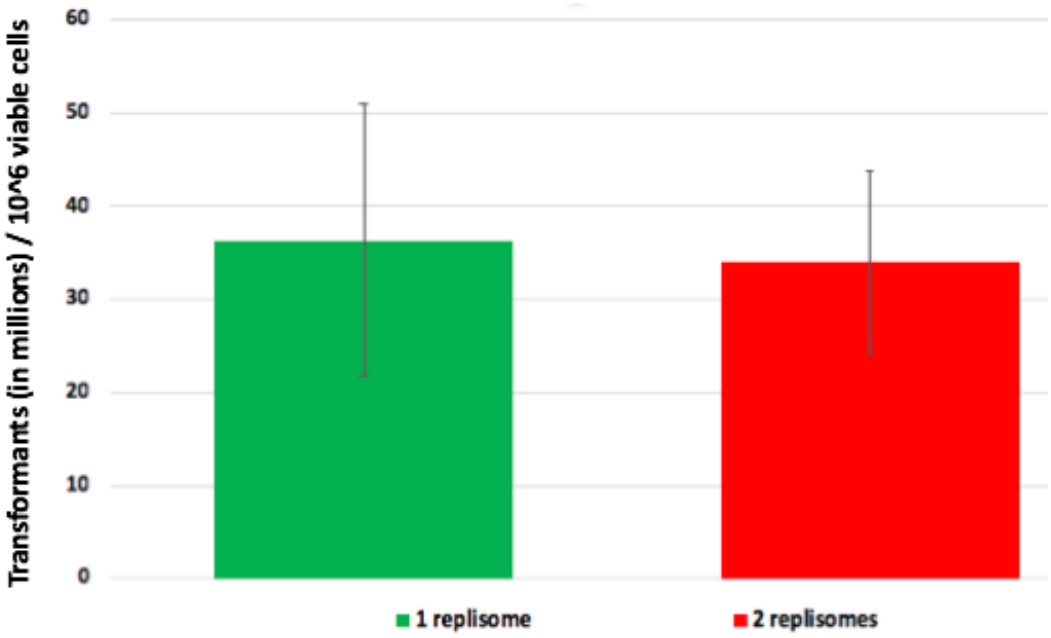
3.1A)



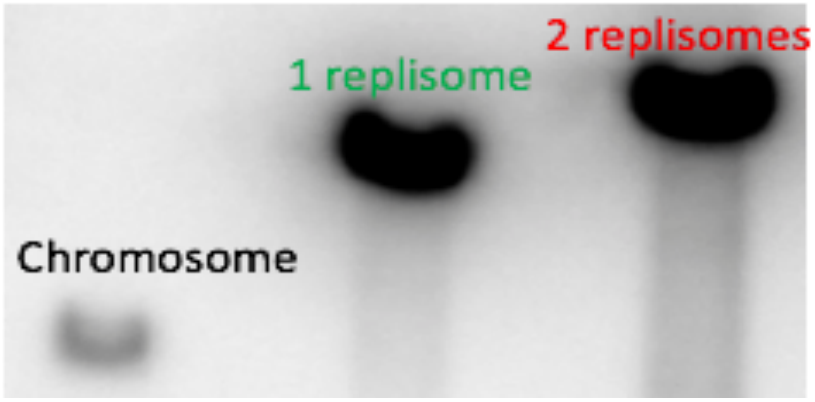
3.1B)



3.1C)


Transformants (in millions) / 10^6 viable cells
60
50
40
30
20
10
0
1 replisome
2 replisomes

3.1D)


2 replisomes
1 replisome
Chromosome

3.1E)

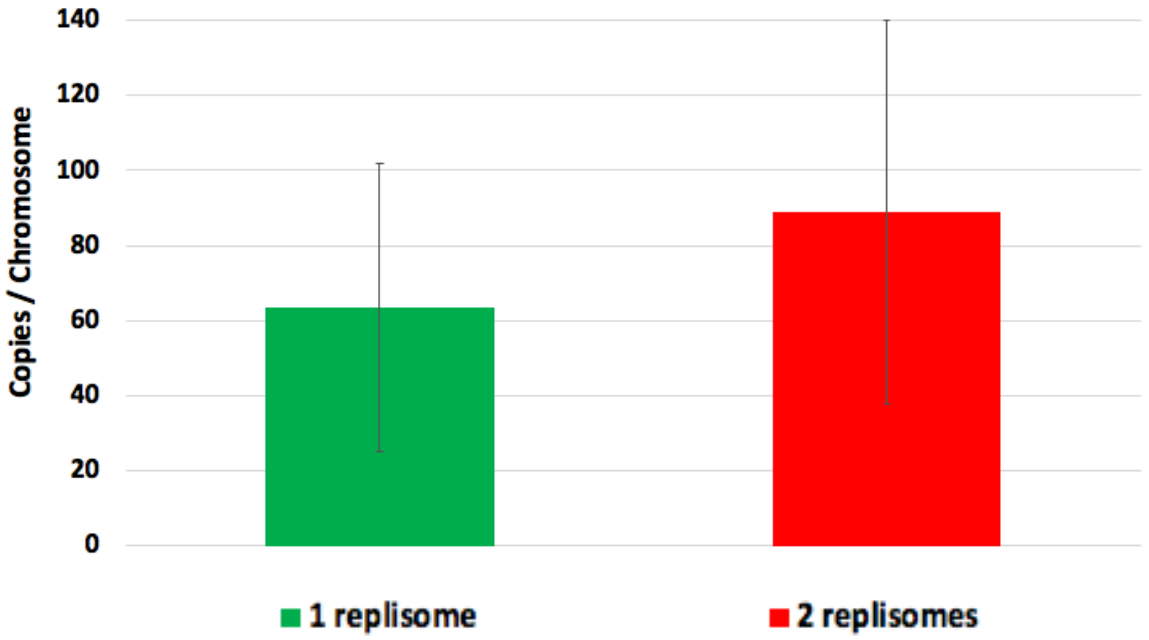
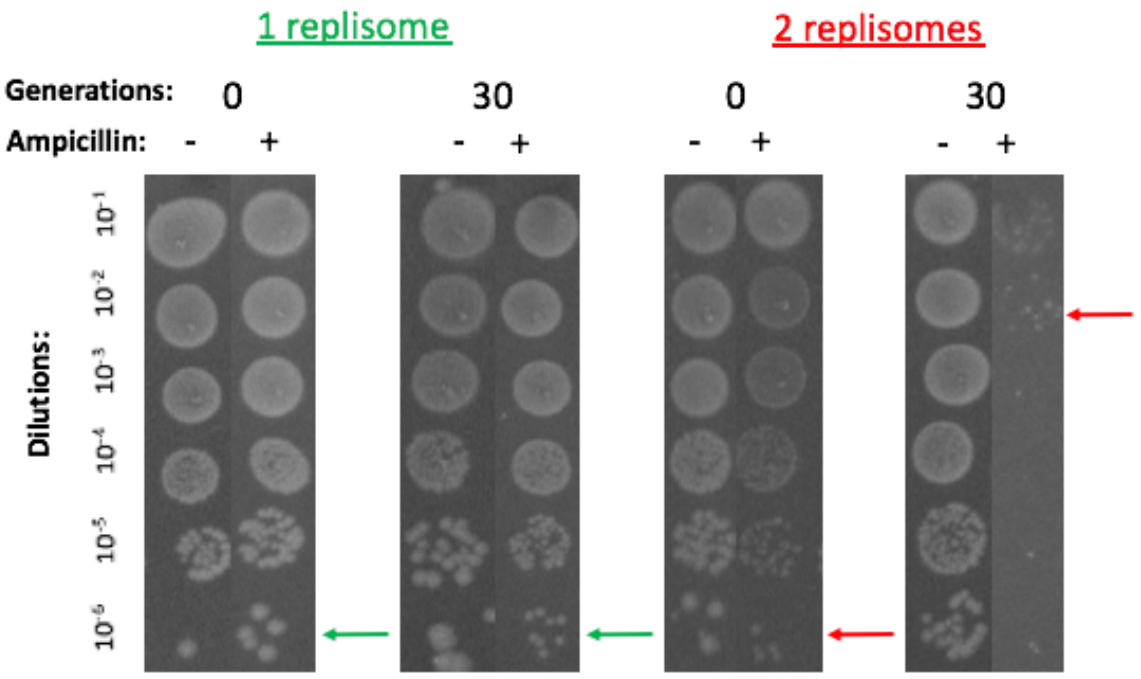
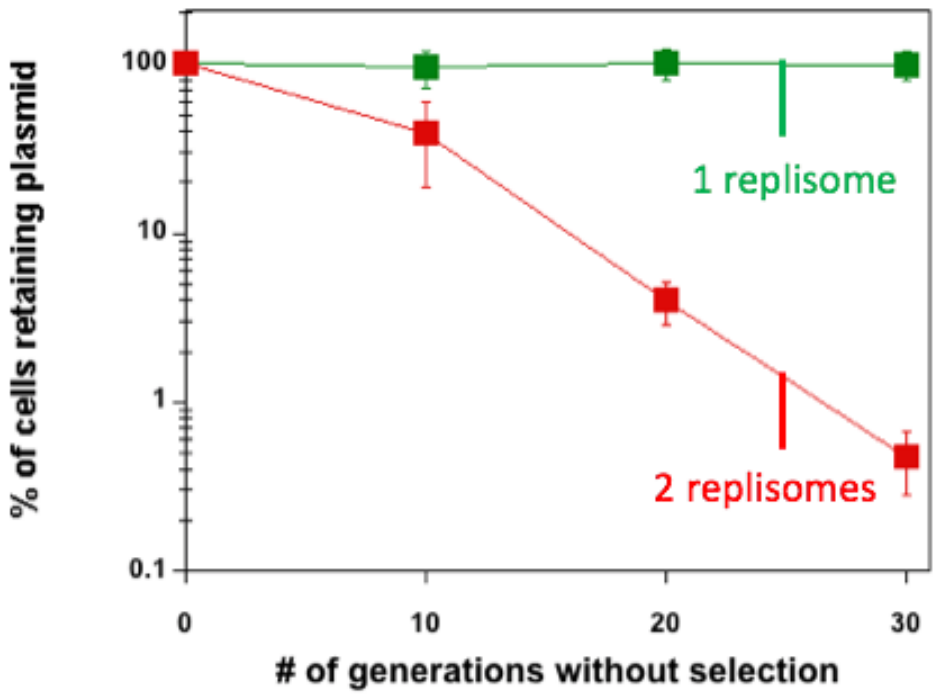


Figure 3.1: The two-replisome plasmid, pCL01, can be stably transformed, replicated, and grown in cells similar to the one-replisome plasmid, pBR322. (**A**) Schematic of pBR322, containing a unidirectional origin of replication, and pCL01 which contains a λ bidirectional origin of replication. (**B**) Strains containing the two-replisome plasmid grow similar to those containing the one-replisome plasmid or no plasmid; *E. coli* strain included ampicillin cassette in this assay (includes data from B Wendel, unpublished). The growth of wild type cultures containing each plasmid as monitored by absorbance at 490nm is plotted over time. (**C**) Plasmids containing two replisomes can be stably transformed at frequencies similar to those with one replisome. The frequency of transformants per colony forming units per mL is plotted following electroporation of 50 ng of each plasmid into WT competent cells. Error bars represent standard error for 3 or more independent experiments. (**D**) The two-replisome plasmid is maintained at a copy number similar to the one replisome plasmid. A representative southern gel of strains containing the ampicillin resistance gene on the chromosome, on the plasmid pBR322, or the plasmid pCL01 is shown. Total genomic DNA was purified from each strain and digested as mentioned in *Southern analysis of plasmid replication intermediates* to linearize the plasmids. Equal number of cell equivalents were then loaded and analyzed by Southern analysis using a P^{32}-labeled ampicillin resistance gene as a probe. (**E**) The copy number of each plasmid, relative to the chromosome is plotted (includes data from B Wendel, unpublished). Error bars represent the standard error for 4 independent experiments.

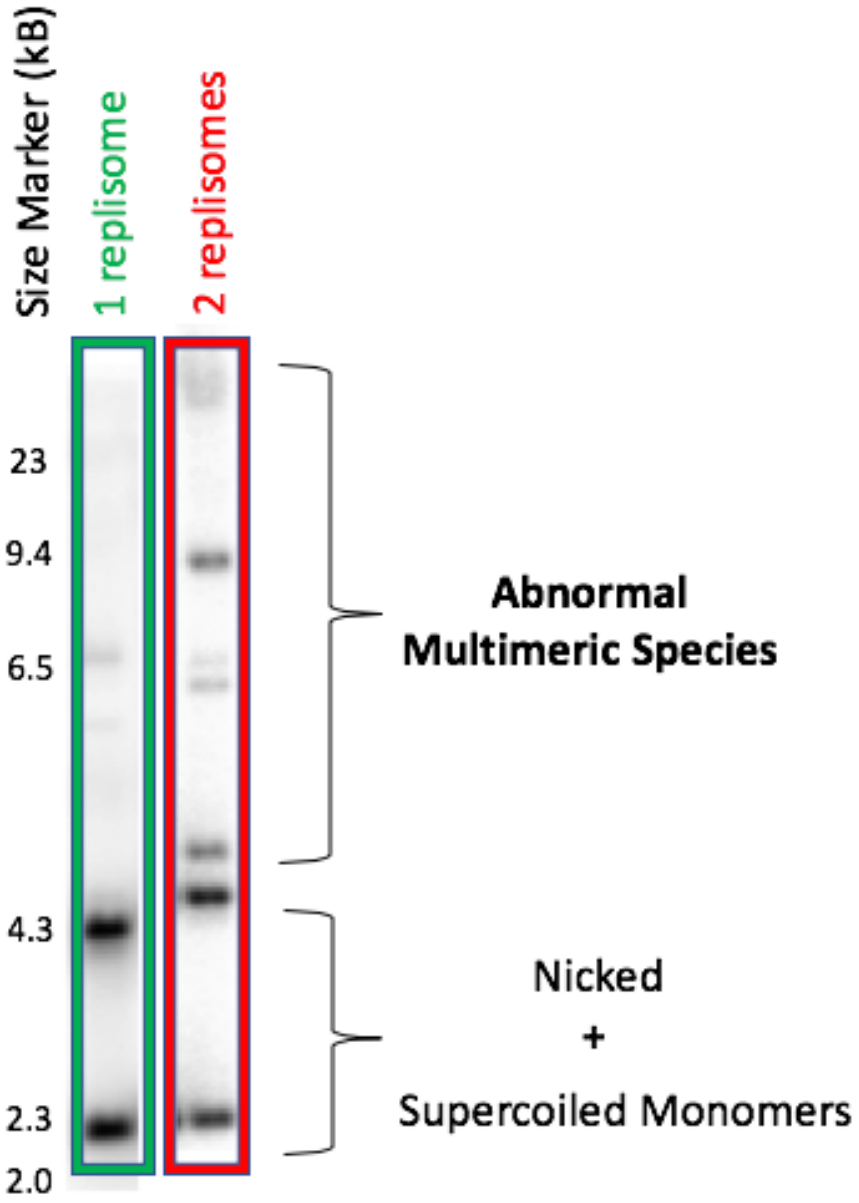
3.2A)


1 replisome
2 replisomes
Generations:
0
30
0
30
Ampicillin:
- + - + - + - +
Dilutions:
10-1
10-2
10-3
10-4
10-5
10-6

3.2B)


% of cells retaining plasmid
100
10
1
0.1
0
10
20
30
# of generations without selection
1 replisome
2 replisomes

3.2C)


Size Marker (kB)
1 replisome
2 replisomes
23
9.4
6.5
4.3
2.3
2.0
Abnormal
Multimeric Species
Nicked
+
Supercoiled Monomers

3.2D)

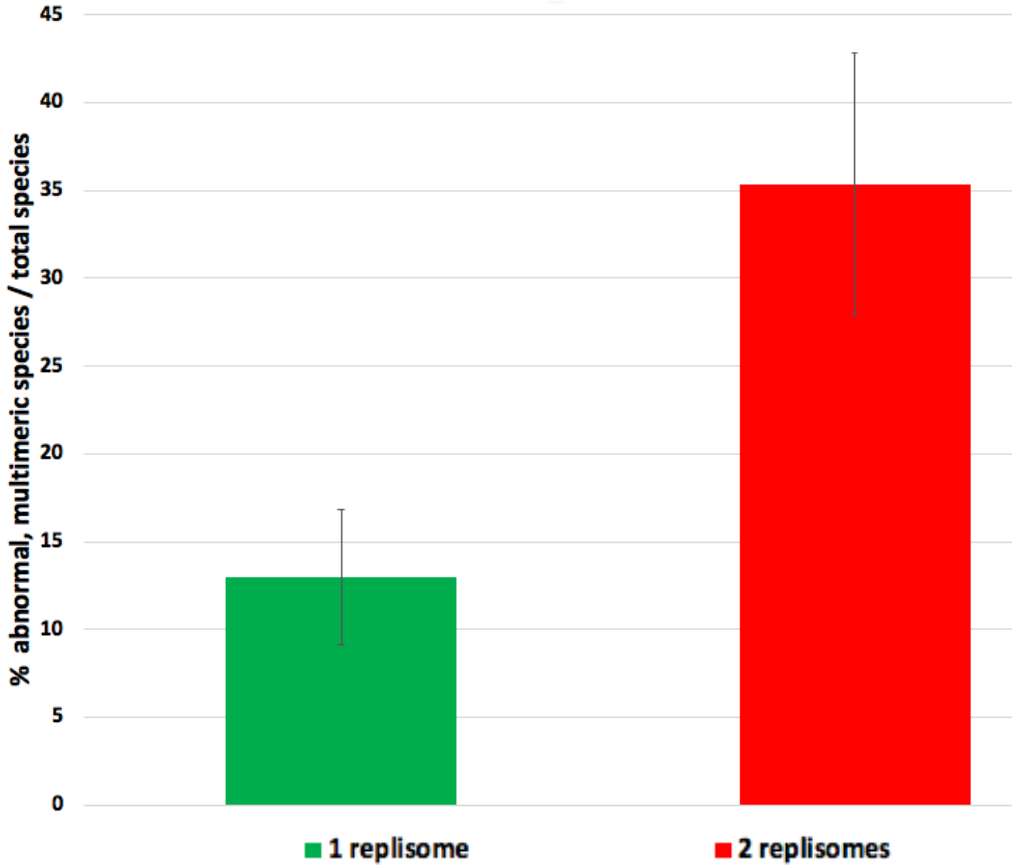
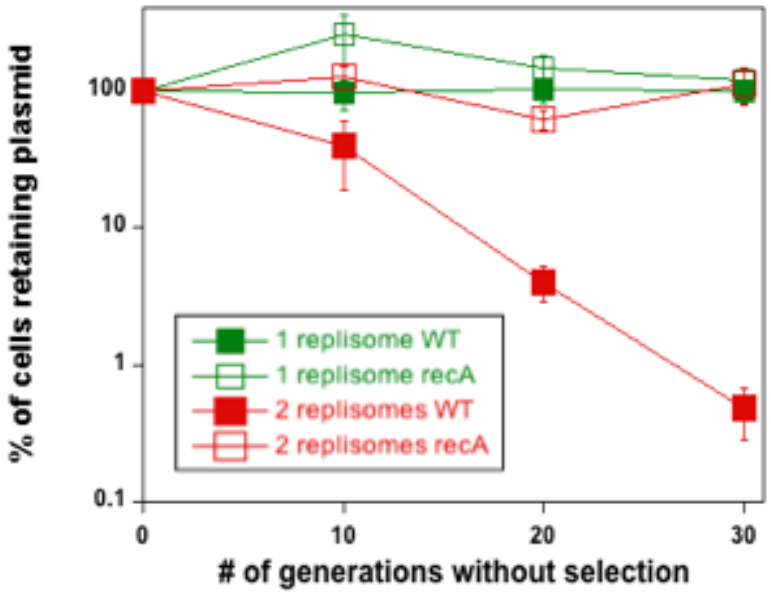
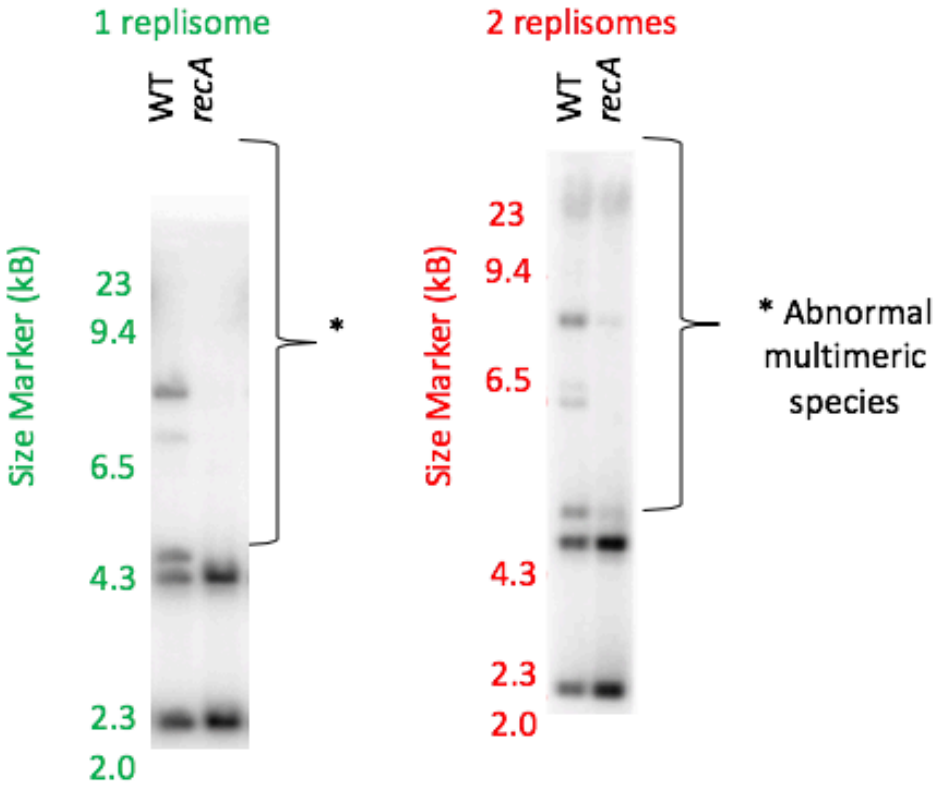


Figure 3.2: Plasmids replicated by two replisomes are less stable than one replisome plasmids and contain more aberrant species. (**A**) In the absence of selection, the two-replisome plasmids are lost more rapidly than the one-replisome plasmid. Cultures containing the one-replisome (pBR322) or two-replisome (pCL01) plasmid were grown for ~30 generations without selection. 10ul drops of 10-fold serial dilutions were plated with and without ampicillin selection to determine the fraction of cells that retain the plasmid. Arrows indicate the highest dilution that was observed to retain the plasmid (**B**) The fraction of cells retaining the one-replisome and two-replisome plasmid is plotted over time. Error bars represent the standard error of 4 or more independent experiments. (**C**) The instability of the two-replisome plasmid, relative to the one-replisome plasmid, correlates with the presence of more abnormal, multimeric species. Total genomic DNA from cells containing the one-replisome or two-replisome plasmid was purified and analyzed by Southern analysis following agarose gel electrophoresis using P^{32}-labeled pBR322 or pCL01 as a probe. (**D**) The fraction of unit length, non-monomeric plasmid in cultures containing the one-replisome and two-replisome plasmid is plotted. Error bars represent the average of four or more independent experiments.

3.3A)


% of cells retaining plasmid
100
10
1
0.1
0
10
20
30
# of generations without selection
1 replisome WT
1 replisome recA
2 replisomes WT
2 replisomes recA

3.3B)


1 replisome
WT
recA
Size Marker (kB)
23
9.4
6.5
4.3
2.3
2.0
*
2 replisomes
WT
recA
Size Marker (kB)
23
9.4
6.5
4.3
2.3
2.0
* Abnormal multimeric species

3.3C)

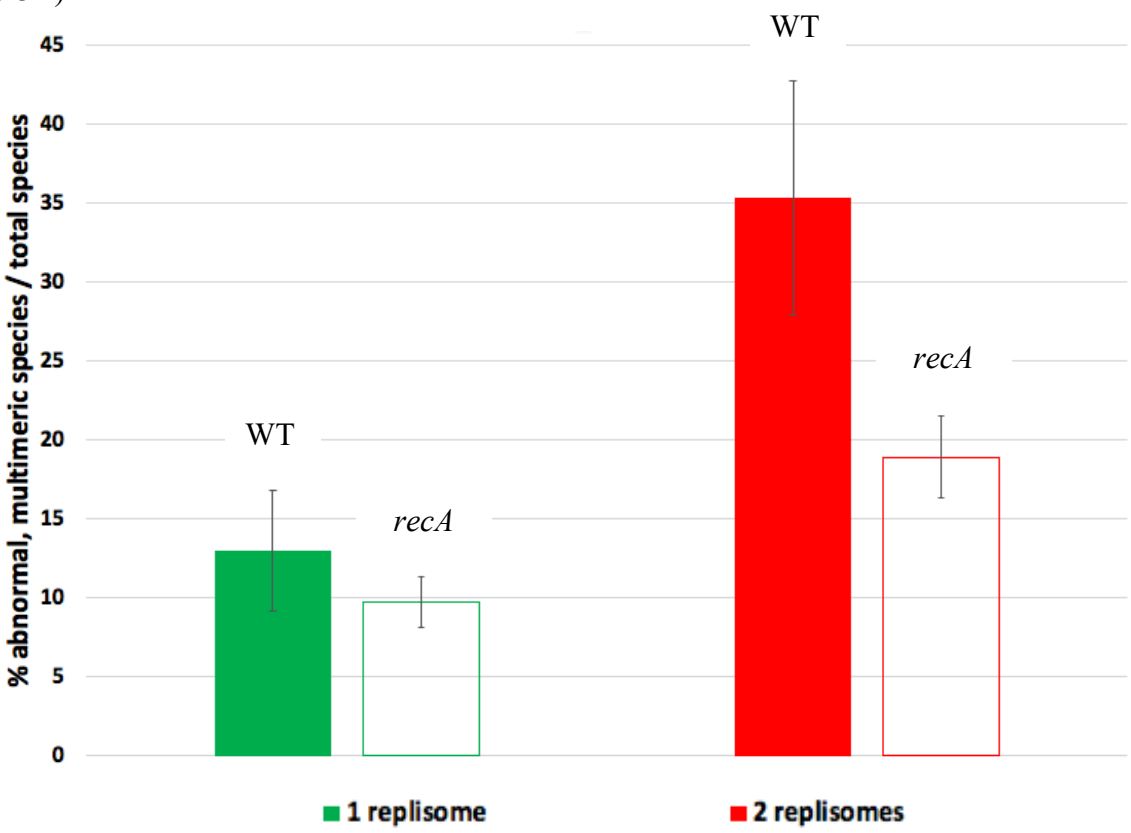


Figure 3.3: Similar to the chromosome, the amplifications and instability on two-replisome plasmids species are driven by the aberrant recombinational mechanism of completing DNA, RecA. (**A**) Inactivation of RecA restores the stability of the two-replisome plasmid to levels that approach that of the one-replisome plasmid. The fraction of cells retaining the one-replisome and two-replisome plasmid in the absence of selection is plotted over time. Error bars represent the standard error of at least 4 independent experiments. (**B**) The increased stability in the absence of the aberrant recombination pathway correlates with an increase in unit-length monomeric plasmids. Total genomic DNA from cells containing the one-replisome or two-replisome plasmid was purified and analyzed by Southern analysis following agarose gel electrophoresis using P^{32}-labeled pBR322 or pCL01 as a probe. (**C**) The fraction of non-monomeric plasmid in each culture is plotted for the one-replisome and two-replisome plasmid in the presence and absence of RecA. Graphs represent the average of at least 4 independent experiments. Error bars represent the standard error.

3.4A)

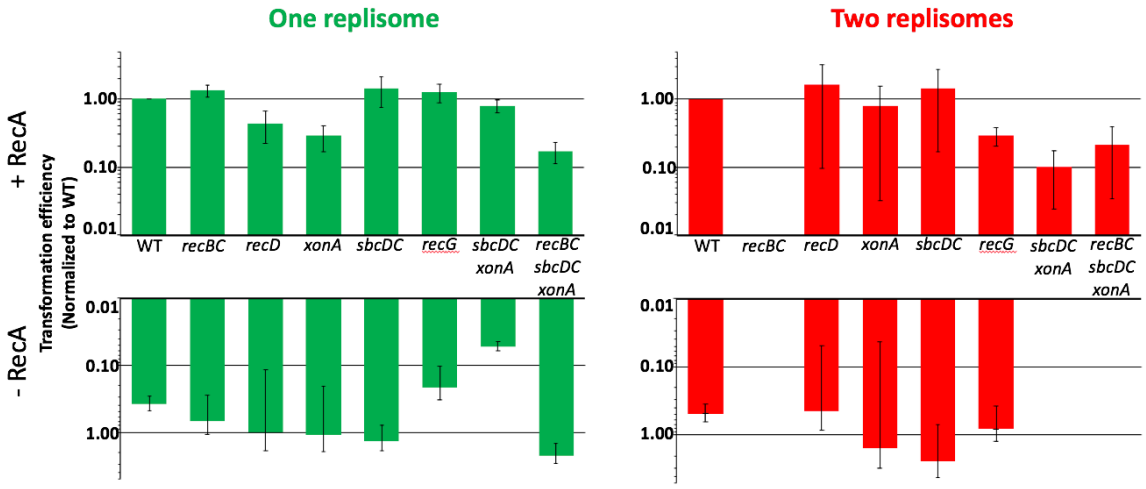
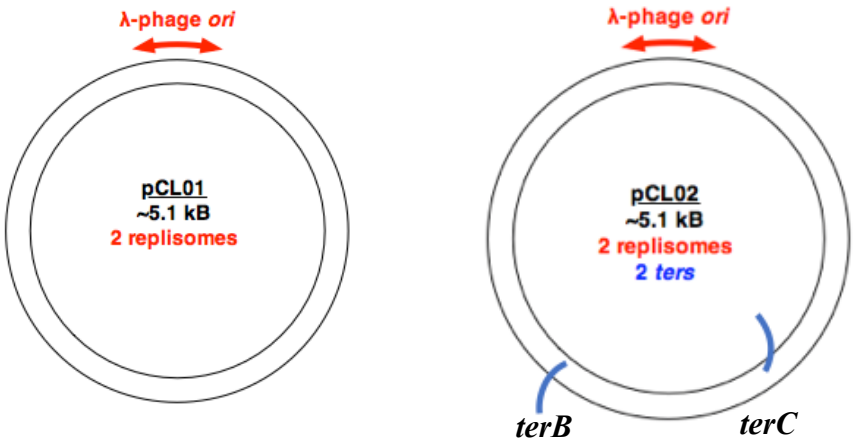


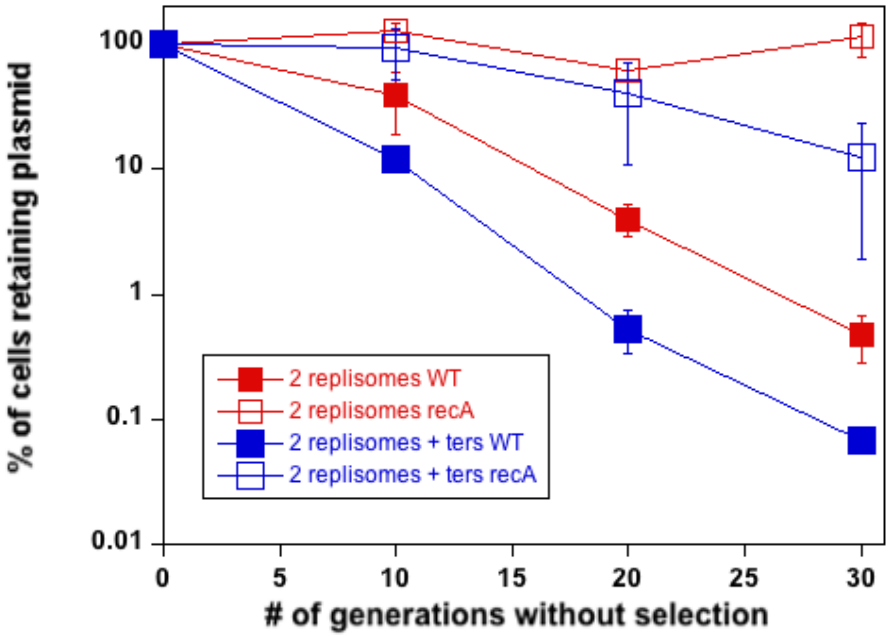
Figure 3.4: Transformation of plasmids with two replisomes, but not one replisome, depends on the enzymes required to complete replication on the chromosome. (**A**) The transformation efficiency of the one-replisome and two-replisome plasmid, relative to wild type cells, is shown for the strains indicated. Error bars represent the standard error of at least two independent experiments (Includes data from B Wendel, unpublished).

PART II: No *ter* vs *ters*

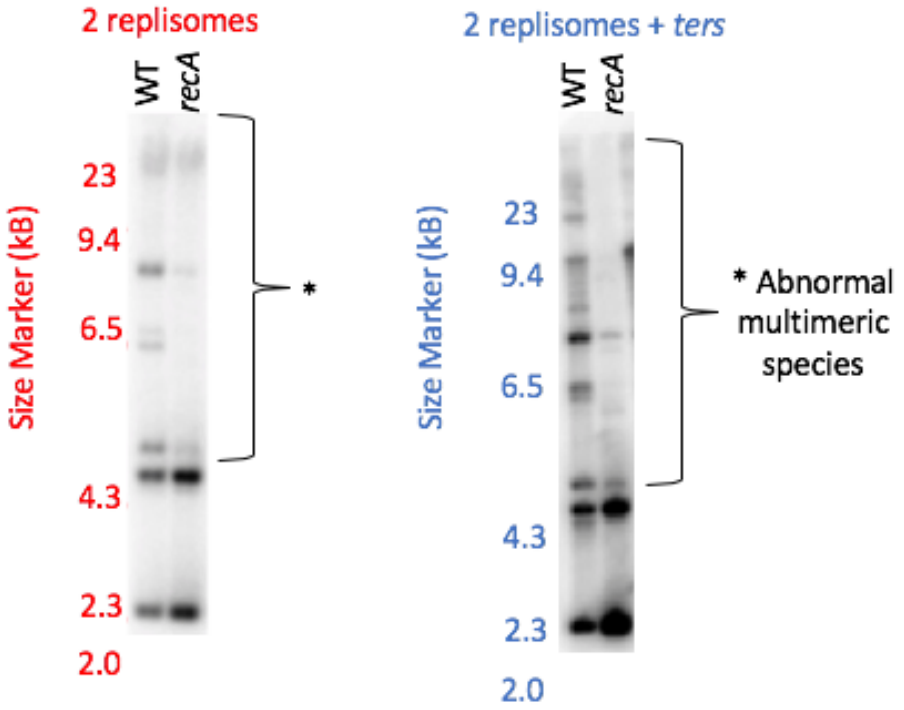
3.5A)



3.5B)



3.5C)


2 replisomes
WT
recA
Size Marker (kB)
23
9.4
6.5
4.3
2.3
2.0
*
2 replisomes + ters
WT
recA
Size Marker (kB)
23
9.4
6.5
4.3
2.3
2.0
* Abnormal multimeric species

3.5D

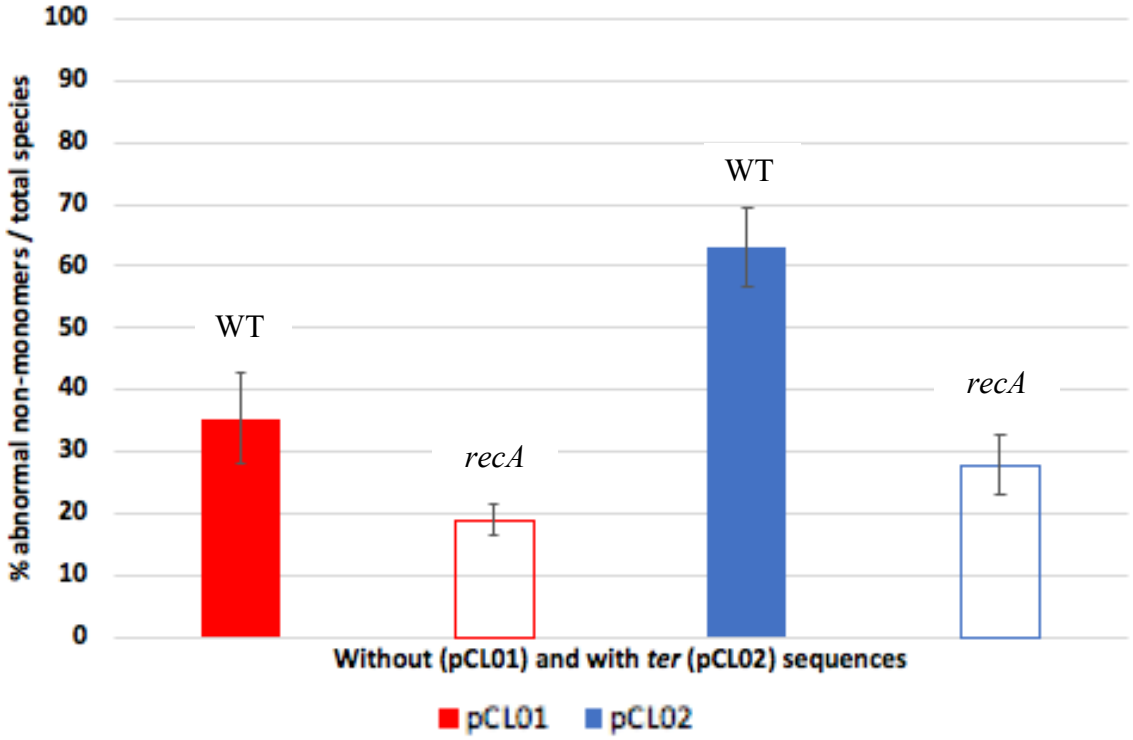
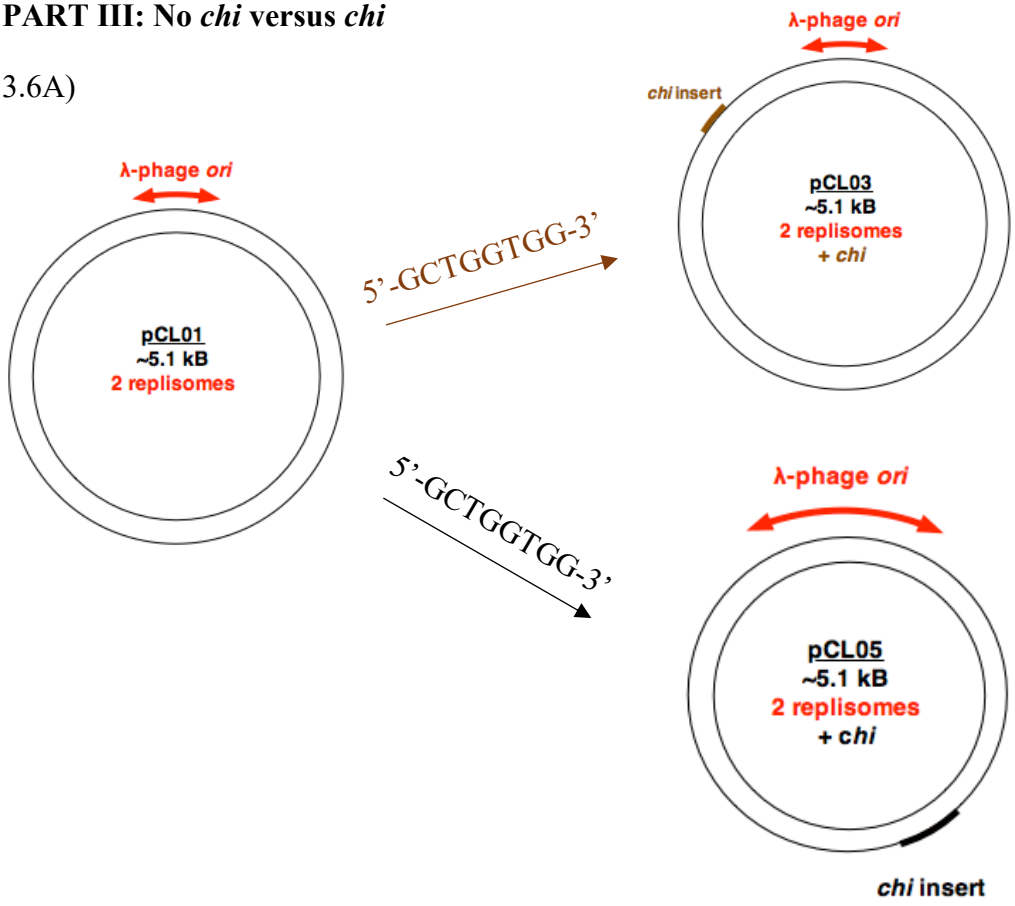

% abnormal non-monomers / total species
100
90
80
70
60
50
40
30
20
10
0
WT
recA
WT
recA
Without (pCL01) and with ter (pCL02) sequences
pCL01
pCL02

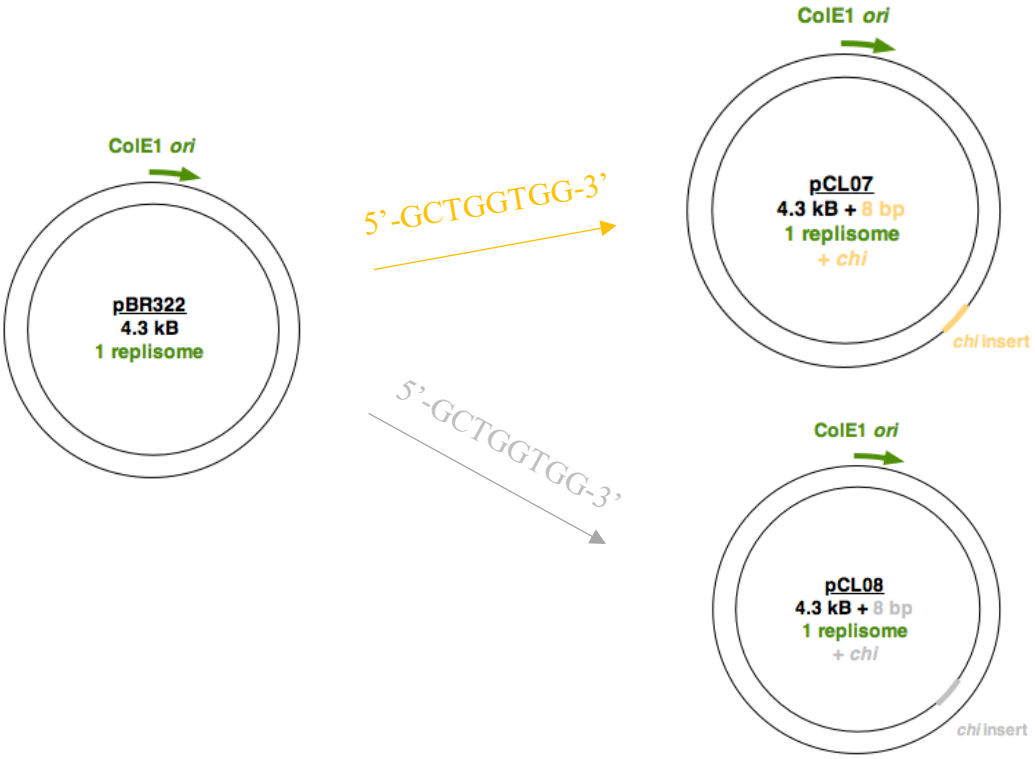
Figure 3.5: *ter* traps engineered onto the two-replisome plasmid only modestly affect the overall stability of the plasmid but appear to increase the amount of abnormal non-monomeric circular plasmid species (A) Diagram of 2-replisome plasmids with and without *ter* traps (*terB* + *terC*). (**B**) The fraction of cells retaining the two-replisome plasmid in the absence of selection is plotted over time. Error bars represent the standard error of at least two independent experiments. (**C**) Increased amounts of RecA-dependent, non-monomeric plasmid species are observed in the presence of *ter* traps. Total genomic DNA from cells containing the indicated plasmid was purified and analyzed by Southern analysis following agarose gel electrophoresis using P^{32}-labeled pCL01 as a probe. (**D**) The fraction of non-monomeric plasmid species in each culture is plotted. Graphs represent the average of at least 4 independent experiments. Error bars represent the standard error.

PART III: No *chi* versus *chi*

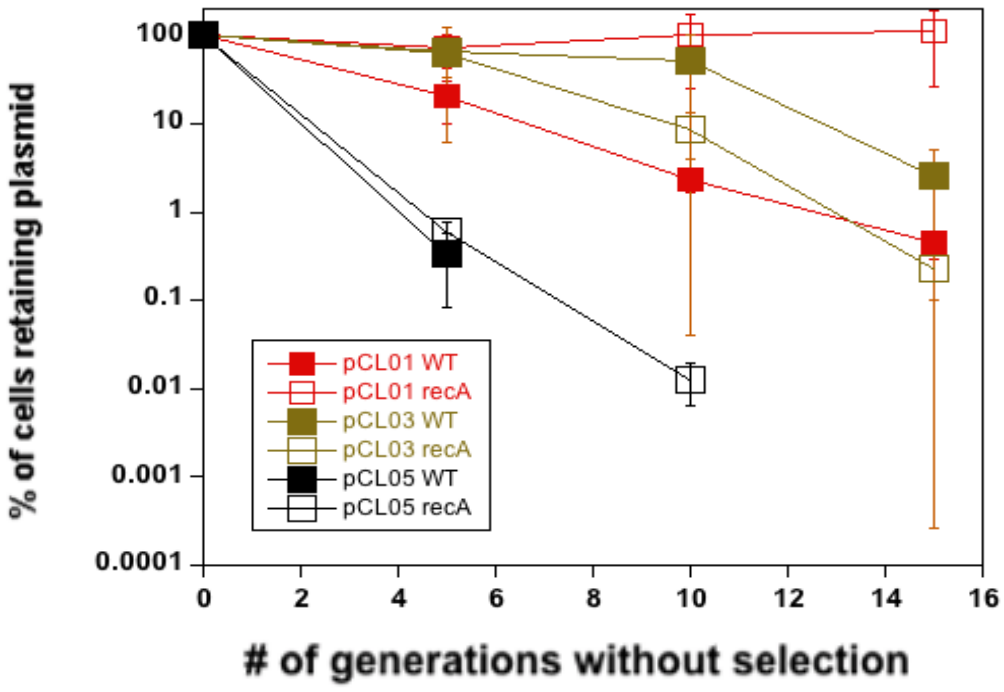
3.6A)



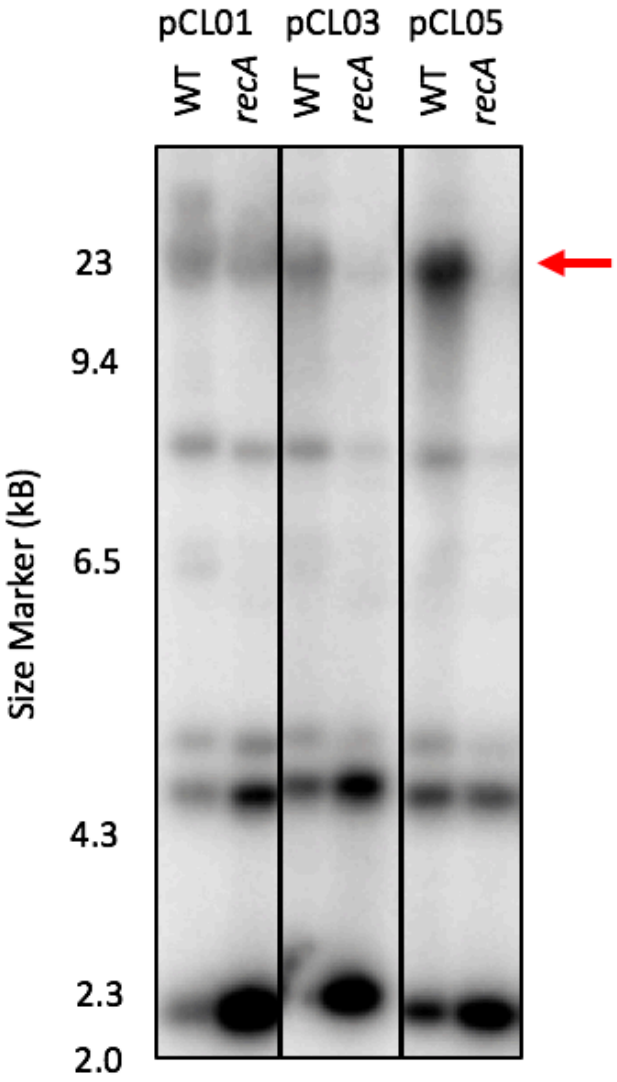
3.6B)


ColE1 ori
pBR322
4.3 kB
1 replisome
5'-GCTGGTGG-3'
ColE1 ori
pCL07
4.3 kB + 8 bp
1 replisome
+ chi
chi insert
5'-GCTGGTGG-3'
ColE1 ori
pCL08
4.3 kB + 8 bp
1 replisome
+ chi
chi insert

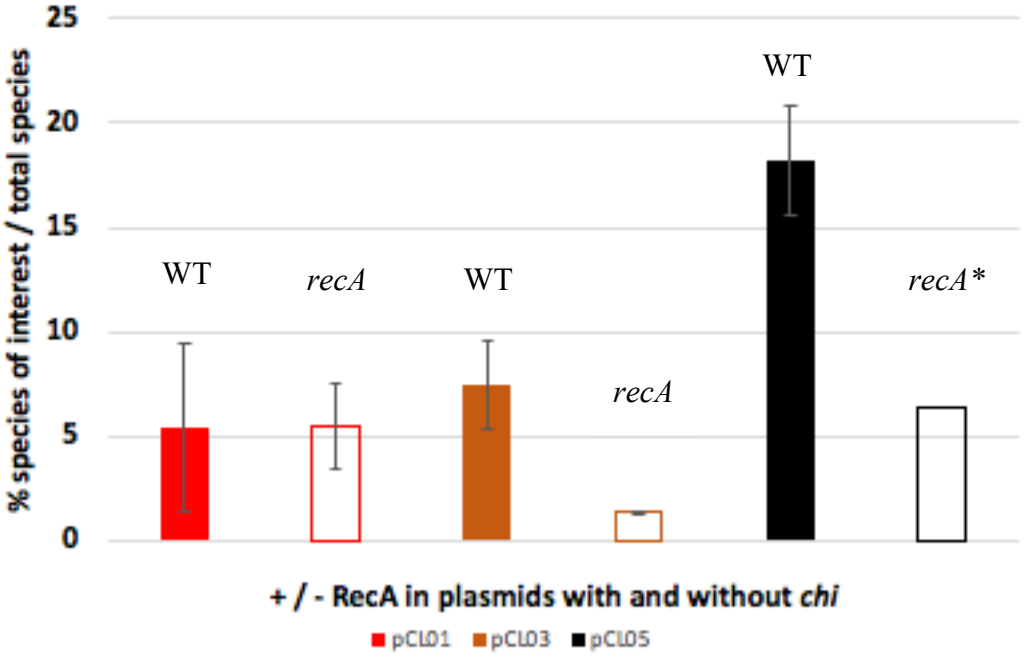
3.6C)


% of cells retaining plasmid
100
10
1
0.1
0.01
0.001
0.0001
0
2
4
6
8
10
12
14
16
# of generations without selection
pCL01 WT
pCL01 recA
pCL03 WT
pCL03 recA
pCL05 WT
pCL05 recA

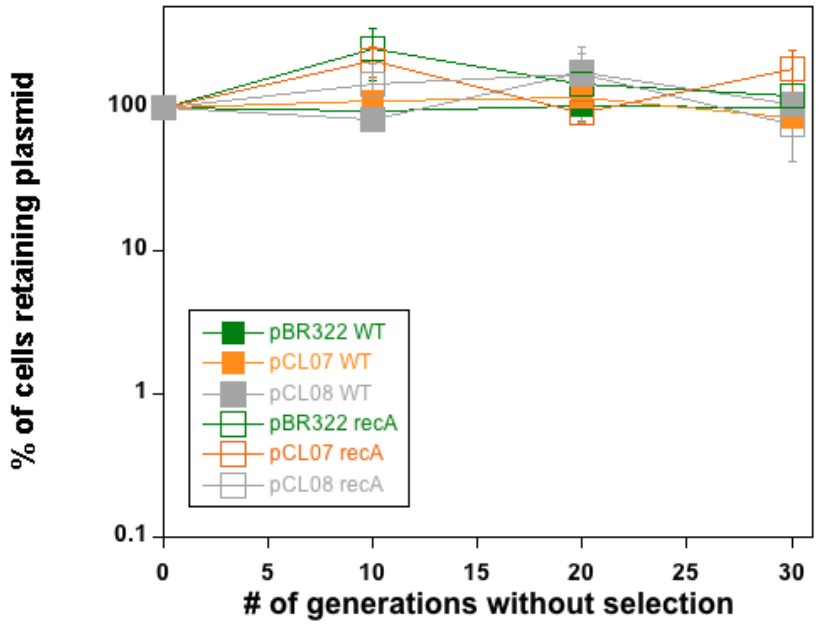
3.6D)


pCL01
pCL03
pCL05
WT
recA
WT
recA
WT
recA
23
9.4
6.5
4.3
2.3
2.0
Size Marker (kB)

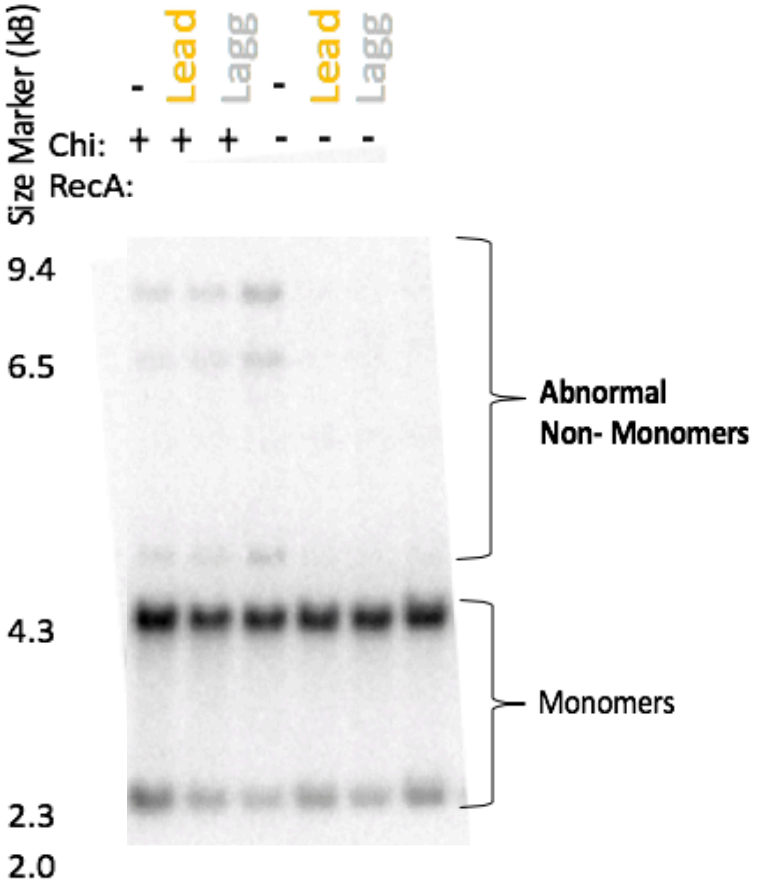
3.6E)


25
20
15
10
5
0
% species of interest / total species
WT
recA
WT
recA
WT
recA*
+ / - RecA in plasmids with and without chi
pCL01
pCL03
pCL05

3.6F)


100
10
1
0.1
% of cells retaining plasmid
pBR322 WT
pCL07 WT
pCL08 WT
pBR322 recA
pCL07 recA
pCL08 recA
0
5
10
15
20
25
30
# of generations without selection

3.6G)


Size Marker (kB)
-
Lead
Lagg
-
Lead
Lagg
Chi: + + + - - -
RecA:
9.4
6.5
4.3
2.3
2.0
Abnormal
Non- Monomers
Monomers

3.6H)

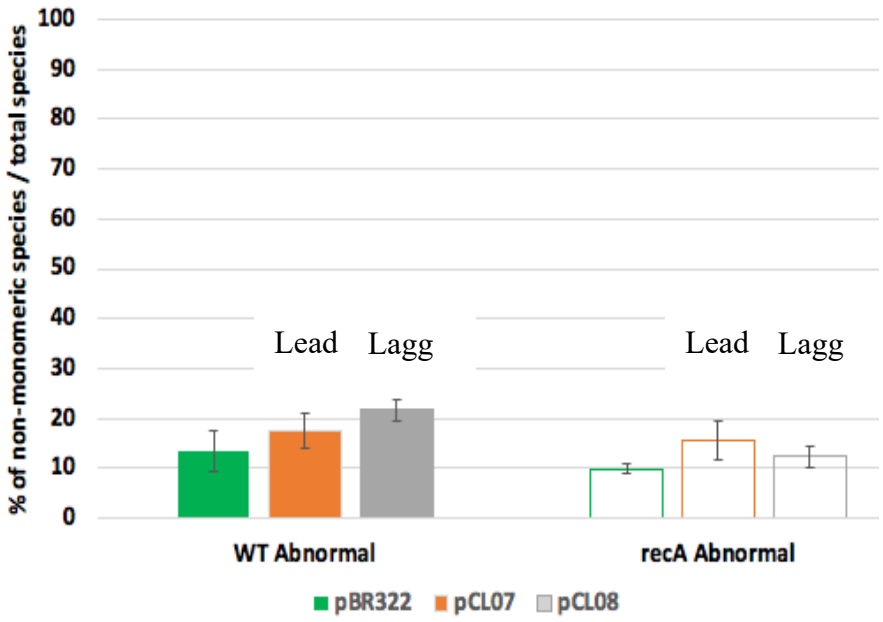
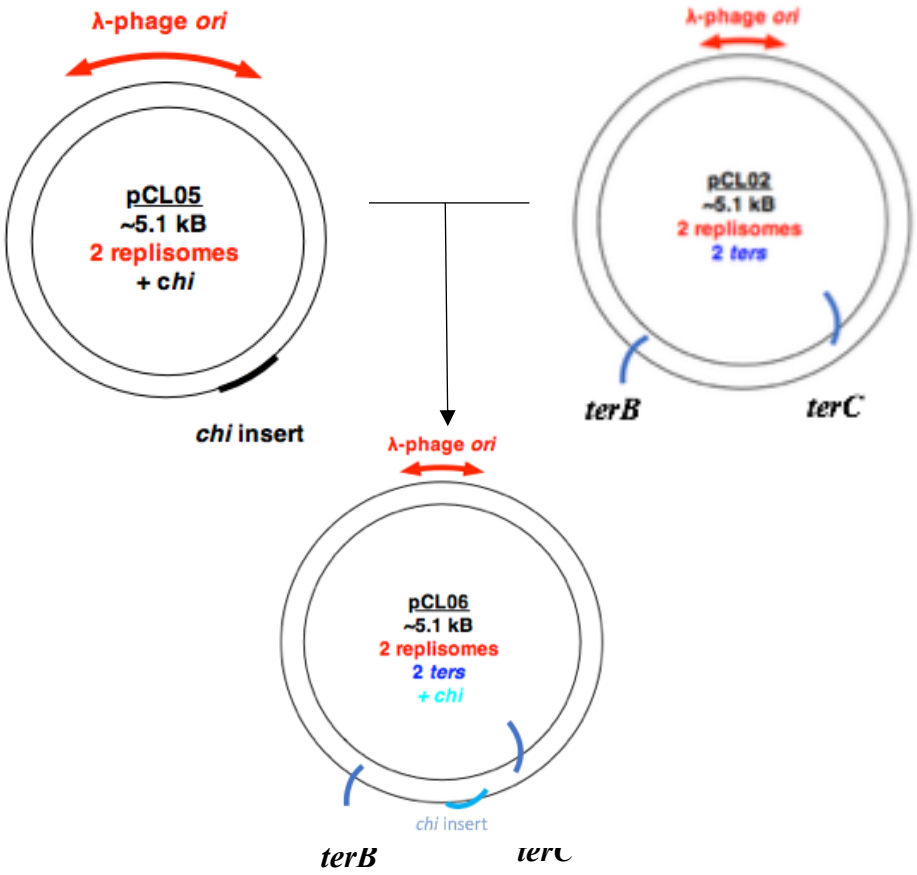

% of non-monomeric species / total species
100
90
80
70
60
50
40
30
20
10
0
Lead
Lagg
Lead
Lagg
WT Abnormal
recA Abnormal
pBR322
pCL07
pCL08

Figure 3.6: Chi decreases the stability of two-replisome, but not one replisome plasmids, in a manner that correlates with the amount of aberrant linear multimeric plasmid that is observed. (A) Diagram of plasmid constructs comparing 2-replisome plasmid, pCL01, no *chi* control, pCL03 where *chi* is mutated near the *ori* site, pCL05 where *chi* is mutated opposite of *ori* site. **(B)** Diagram of one-replisome plasmids containing a *chi* inserted into the leading, pCL07, or lagging strand, pCL08, pBR322 template. **(C)** When present in the terminus region, *chi* destabilized the two-replisome plasmid. The fraction of cells retaining the two-replisome plasmid in the absence of selection is plotted over time. Error bars represent the Standard error of at least two independent experiments. **(D)** When present in the terminus region of the two-replisome plasmid, *chi* induces the formation of an aberrant linear plasmid multimeric species. Total genomic DNA from cells containing the indicated plasmid was purified and analyzed by Southern analysis following agarose gel electrophoresis using P^{32}-labeled pCL01 as a probe. Arrow indicates the position of the linear plasmid multimers. **(E)** The amount of the linear plasmid multimeric species observed is plotted. Graphs represent the average of at least two independent experiments, except for pCL05 *recA* which represents a singular experiment. For all other strains, error bars represent the Standard error. **(F)** The stability of the one-replisome plasmid is not affected by the presence of *chi*. The fraction of cells retaining the two-replisome plasmid in the absence of selection is plotted over time. Error bars represent the Standard error of at least four independent experiments. **(G)** No multimeric species are induced by *chi* on one-replisome plasmids. Total genomic DNA from cells containing the indicated plasmid was purified and analyzed by Southern analysis following agarose gel electrophoresis using P^{32}-labeled pBR322 as a probe. **(H)** The amount of the linear plasmid multimeric species observed is plotted. Graphs represent the average of at least 5 independent experiments. Error bars represent the standard error.

3.7A)


λ-phage ori
pCL05
~5.1 kB
2 replisomes
+ chi
chi insert
λ-phage ori
pCL02
~5.1 kB
2 replisomes
2 ters
terB
terC
λ-phage ori
pCL06
~5.1 kB
2 replisomes
2 ters
+ chi
chi insert
terB
terC

3.7B)

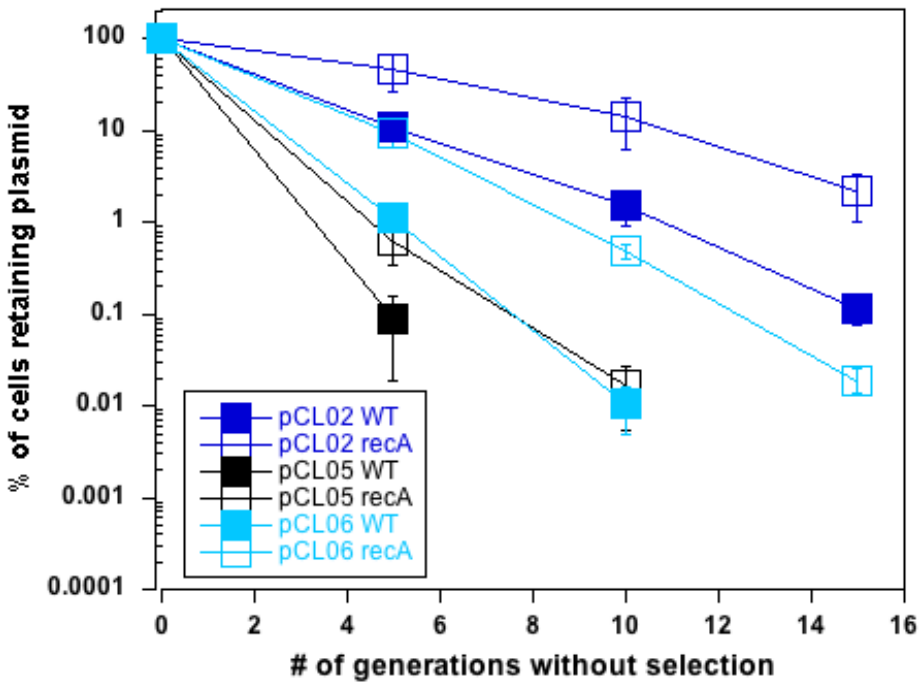

% of cells retaining plasmid
100
10
1
0.1
0.01
0.001
0.0001
0
2
4
6
8
10
12
14
16
# of generations without selection
pCL02 WT
pCL02 recA
pCL05 WT
pCL05 recA
pCL06 WT
pCL06 recA

Figure 3.7C)

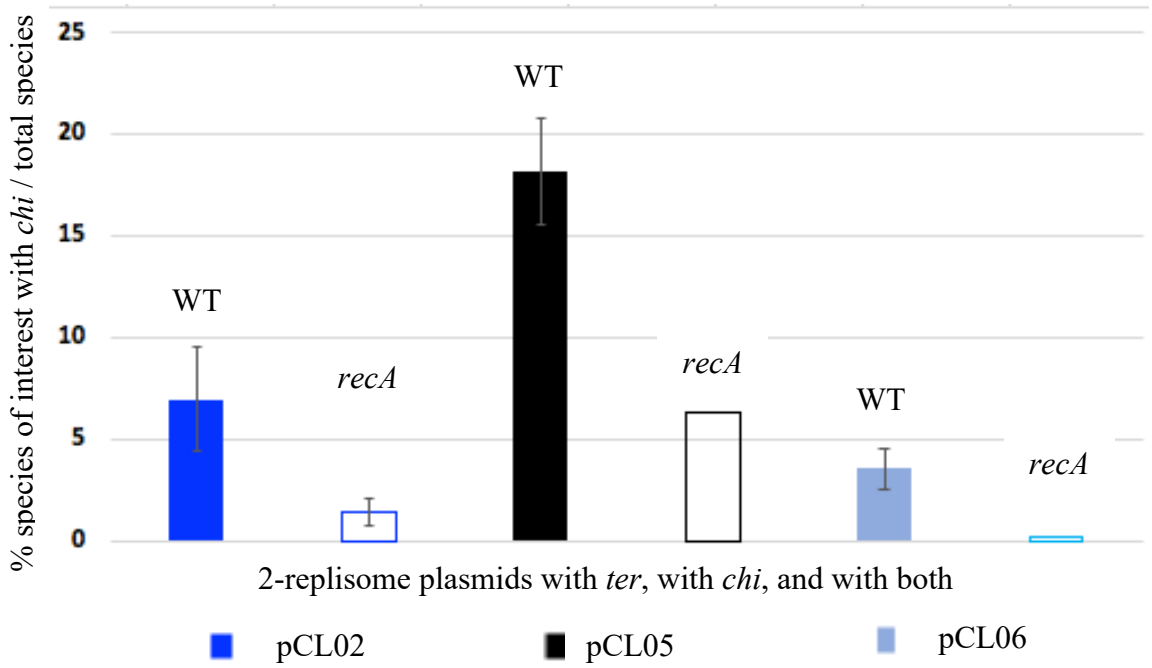


Figure 3.7: The presence of *ter* traps reduces the destabilization by *chi*, and prevents the formation of the RecA-mediated linear plasmid multimeric species (A) Diagram of two-replisome plasmids containing either a *chi* site in the terminus region or *ter* traps or both. **(B)** There is less destabilization by *chi* observed in the presence of a *ter* trap. The fraction of cells retaining the two-replisome plasmid in the absence of selection is plotted over time. Error bars represent the Standard error of at least two independent experiments **(C)** The amount of the linear plasmid multimeric species observed is plotted. Graphs represent the average of at least two independent experiments. Total genomic DNA from cells containing the indicated plasmid was purified and analyzed by Southern analysis following agarose gel electrophoresis using P^{32}-labeled pCL01 as a probe. Except for pCL06 *recA,* which represents only a single experiment. For all other strains, error bars represent the Standard error.

Chapter IV

Discussion

Part I: One-replisome versus two-replisome plasmids during completion of replication

The completion reaction involves two replisomes which converge, resect, resolve, and join the nascent strands of DNA at the point where all sequences have doubled (Wendel et al., 2014). These events occur thousands of times per cell cycle across human chromosomes, meaning the event must occur with remarkable efficiency to maintain cell viability (reviewed in Cvetic and Walter, 2005; Gao and Zhang, 2007; reviewed in Méchali 2010). Prokaryotes, which contain a single bidirectional origin, offer a chance to characterize this event and reaction in a less complex and better-defined system. The use of plasmids can further simplify this analysis and have been successfully used in other works to characterize the molecular mechanisms of replication initiation and elongation (Reviewed in del Solar et al., 1998). However, commonly-used plasmids, such as pBR322 that have origins of replication derived from ColE1, replicate with one-replisome plasmids are unlikely to ever have a completion event where replication forks converge similar to the chromosome (Abe 1980; Wendel et al., 2014). Thus, in this study, I used a plasmid mini-chromosome that contained a bidirectional origin that could be used to assess and examine the completion reaction. I show that maintaining plasmids containing two replisomes depends on the enzymes needed to complete replication on the chromosome. The completion of chromosomal replication requires RecBCD to join the strands of convergent replication forks. In its absence, DNA ends persist, are extensively degraded, and cells fail to maintain these regions of the chromosome (Dimude et al., 2018A; Wendel et al., 204; Courcelle et al., 2015; Wendel et al., 2018). Similarly, I show that transformation of two-replisome plasmids in *recBC* mutants is severely impaired and

the plasmids fail to propagate in cells under selection. On the chromosome, the ExoI SbcDC structure-specific nucleases are required to initiate the faithful completion reaction. In the absence of these proteins, normal completion cannot occur, excess over-replicated regions persist, and the reaction is shunted through a RecA-mediated pathway that is associated with amplifications and genetic instabilities (Indiani et al., 2013; Dimude et al., 2018A; Wendel et al., 2014; Courcelle et al., 2015; Wendel et al., 2018). Here, I show that transformation of two-replisome plasmids in *xonA sbcCD* mutants similarly depends on the RecA-mediated pathway. Thus, both the normal RecBCD-mediated reaction, and the aberrant recombinational process appear to operate on the two-replisome plasmids. Additionally, I show that the requirement for these completion enzymes is specific to plasmids with two replisomes, as transformation was not impaired in the one-replisome plasmid pBR322. The observation implies that the substrate acted upon by ExoI, SbcDC, and RecBCD during the completion reaction is specific to a structure created when two replisomes converge.

Finally, I found that the two-replisome plasmid was less stable than the one-replisome plasmid when propagated in the absence of selection, and showed that this instability was driven by amplifications arising from a RecA-mediated recombination reaction, similar to what is observed on the chromosome at sites where completion occurs (Wendel et al., 2018). Inactivation of RecA increased the stability of the two-replisome plasmid and reduced the proportion of abnormal amplification products that were observed (Fig 3.3A). The high rate of RecA-driven instability on the two-replisome plasmid indicates that the aberrant pathway occurs at relatively high frequency on the plasmid, even when the normal, faithful pathway remains functional. I would speculate that this is likely due to the high copy number of the bidirectional plasmid, and that the

frequency of aberrant completion events on the chromosome is likely to be much less. Although pBR322 demonstrated relatively similar copy number I would not suggest this to have the same effect as the accumulative assays suggest 1-replisome plasmids do not engage or require the same enzymes to complete DNA replication. Under normal conditions in *E. coli*, the completion enzymes are only required to catalyze a single reaction on the chromosome. Whereas the bidirectional plasmid, which is maintained at ~80 copies/chromosome (Fig 3.1E), may exceed the reaction-capacity of the faithful pathway, whose genes are not highly expressed (Eichler and Lehman, 1977; Taylor and Smith, 1980), allowing the aberrant mechanism to operate more frequently than would normally occur.

ExoI and SbcDC nuclease complexes are additive in their effect on the completion reaction and inactivation of the normal completion reaction required deletion of both genes (Wendel et al., 2014; Wendel et al., 2018; Dimude et al., 2018A). On the chromosome, in the absence of both nucleases, the over-replicated regions persist, and the ability to maintain growth and the chromosome region where forks converge becomes entirely dependent on RecA; as the triple mutant *xonA sbcDC recA* shows a complete lack of maintenance in the terminus region instead of over amplification (Wendel et al., 2018). In this report, we show that transformation of two-replisome plasmids similarly becomes dependent on RecA only in the absence of both nucleases (Fig. 3.4B). Mechanistically, ExoI and SbcDC could functionally interact to cooperate and enhance their ability to degrade the substrate(s) created by convergent replication forks. This appears to occur in eukaryotes, where the homologous Mre11-Rad50 interacts with Sae2 and increase its exonuclease activity (Lengsfeld et al., 2007; Deng et al., 2015; Andres and Williams, 2017). Consistent with this, a recent study from Dimude et al. found that

SbcDC alone prevented much of the degradation that occurs in *recBC* mutants (Dimude et al., 2018B), an observation we have confirmed in our lab. *In vitro*, SbcCD has been demonstrated to cleave a palindrome-like substrate similar to that predicted to arise when replication forks bypass each other (Lim et al., 2015; Saathoff et al., 2018). Alternatively, ExoI may act independently of SbcDC to suppress the aberrant recombinational pathway. Early studies suggested a strong physical interaction between ExoI exonuclease and RecA (Bedale et al., 1991; Bedale et al., 1993; Kowalczykowski et al., 1994). Association of this 3' exonuclease with RecA would be expected to strongly degrade recombinogenic 3' ends preventing RecA from initiating recombination at these sites.

In an alternative interpretation of RecBCD function, a recent study and review speculated that chromosome cleavage may frequently occur during septation at cell division, resulting in double strand breaks that require RecBCD for repair (Sinha et al., 2018; Michel et al., 2018). The authors based this argument primarily on the observation that the extensive degradation in *recBC* mutants' centers upon the *dif* locus, where chromosomes ultimately separate as cells divide. However, several observations argue strongly against this possibility. If the defects in *recBC* mutants were due to the presence of double strand breaks, then *recA* mutants, which is essential for repair of double strand breaks, should be similarly, or more severely affected. Yet, in their initial study, the authors failed to examine or address *recA* mutants (Sinha et al., 2018). However, *recA* mutants grow at rates similar to wild type cultures, and unlike, *recBC* mutants, maintain this region of the chromosome normally (Courcelle et al., 2015; Wendel et al., 2018). Further, these authors and previous investigators all note that DNA breaks are not detected on the chromosome of *recA* mutants (Sinha et al., 2018; Wendel et al., 2014; Courcelle et al., 2015; Wendel et al., 2018; Pennington and Rosenberg, 2007). To explain

the absence of DNA breaks in *recA* mutants, a subsequent study from this group proposed that the septation-induced breaks in *recA* genomes are subsequently degraded and therefore undetectable (Sinha et al., 2018). However, based on the viability of *recBC* mutants, ~90% of cells in culture would be experiencing these septation-induced breaks. Synthesis and subsequent degradation of these genomes would be expected to slow culture growth considerably and generate partial genomic degradation intermediates that should be easily detectable, neither of which are observed in *recA* cultures (Sinha et al., 2018; Wendel et al., 2014; Courcelle et al., 2015; Wendel et al., 2018). Further, such models do not consider, and fail to address, how the inactivation of exonucleases SbcDC and ExoI, which are not essential for double strand break repair, would restore *recBCD* mutant growth defects, or why maintaining the terminus region of the chromosome in the absence of these exonucleases would depend on RecA (Templin et al., 1972; Lloyd and Buckman, 1985; Wendel et al., 2018). Finally, as shown here, plasmids containing two replisomes require RecBCD to propagate but do not require RecA and are actually stabilized in its absence. These plasmids lack *dif* sequences, and segregate prior to and independent from cell division, and would therefore not be subject to septation-induced breaks. These observations are all inconsistent with the idea that the requirement for RecBCD is due to double strand breaks caused by cell division, and argue strongly in favor of its role in joining DNA ends of convergent forks, which can occur independently of recombination or RecA. It is also worth considering that although current models for RecBCD in double-strand break repair propose that RecBCD acts prior to RecA, the early *in vivo* studies led several independent labs to conclude that RecBCD acted after RecA, at a later step in completing the recombination reaction (Wilkins 1969; Willets 1975; Hall and Howard-Flanders, 1972; Birge and Low, 1974). Current models placing RecBCD as

an initiator are heavily derived from biochemical studies in which linear double-stranded substrates were used to characterize enzyme binding, helicase, and exonuclease activities (reviewed in (Yeeles and Dillingham, 2010; Dillingham and Kowalczykowski, 2008; Smith 2012)). The initial concept that RecBC acts late in recombination arose from the observation that although *recA* mutants receiving an F' factor were unable to transfer chromosomal genes to another cell, *recBC* mutants could do so at frequencies that approached those of wild-type cells. However, over time (~1 generation), this ability rapidly declined (Hall and Howard-Flanders, 1972; Wilkins 1969; Willetts 1975). The authors inferred that recombination proceeds beyond the point at which the incoming DNA is joined to the chromosome in *recBC* mutants, but that *recA* mutants are blocked prior to this event. In recombinational crosses between Hfr and F- strains carrying non-complementing mutations in lacZ, Birge and Low found that although *recA* mutants were entirely blocked, *recBC* mutants initially produced beta-galactosidase within two-fold of those seen in wild-type cells, indicating that recombination reactions progressed beyond the point where transcribable, mutation-free copies of LacZ+ were produced (Birge and Low, 1974). However, although these recombination intermediates were detectable, the completion of these recombination events was impaired in the absence of RecBC and viable LacZ+ recombinant progeny were reduced 100-1000-fold. The authors inferred that "early steps in recombination can proceed efficiently in RecBC- and RecC-strains, but that late steps, such as the degradation of excess DNA 'tails', might be defective." Using combinations of single and double mutations, Willetts confirmed these previous studies and suggested that recombination proceeded past the first joining reaction of the two DNA molecules, but that RecBCD was required for a second joining needed "to generate a circular unit" that could be inherited (Willetts 1975). These interpretations are

strikingly consistent with RecBCD's apparent role in completing replication on the chromosome, and may suggest that RecBCD function during recombinational events is similar to its role in completion of replication (Wendel et al., 2014; Courcelle et al., 2015).

Replication fork traps and the completion reaction

Although not essential for viability, Tus-*ter* DNA-protein complexes are important to localize the region where converging replication forks meet on the chromosome (Roecklein et al., 1991; Reviewed in Rothstein et al., 2000). By adding *terB* and *terC* sequences to the mini-chromosomes, I observed that their presence altered the form of the replicating plasmid, effectively increasing the proportion of the plasmids that migrated as circular forms. This had a modest but detrimental effect on the overall stability of the plasmid, but could be argued to promote a more 'normal' circular DNA product that can be passed on to the next generation, similar to the circular chromosome of the bacterial host. The modest reduction in stability may be due to the unnaturally small plasmid substrate in which the completion must occur. On the chromosome, this reaction takes place within a 400-kb region. On the plasmid we ask the reaction to occur within 5-kb, which may present some formational impediments for the reaction and provide a rationale for why large circular products are observed. In future work, it would be of interest to create a large plasmid substrate, to determine if this improves the efficiency of the completion reaction. If plasmid stability increased with increasing plasmid size, it would suggest that the completion reaction requires larger substrates to occur. Given that RecBCD degrades and unwinds DNA at rates approaching 1000 bp/sec (Roman et al., 1992) and that the distance between *chi* sites on the chromosome average

several kilobases (Burland et al., 1993), it seems reasonable to suspect that large substrates may be required.

Nevertheless, the increased level of circle DNA products in the presence of *ter* sequences implies that the *ter* sequences are limiting the amount of aberrant runaway replication that occurs, and may provide more time for the completion to occur. I observed that the presence of the *ter* sequences seemed particularly critical or beneficial to the reaction, when *chi* sequences were present, which appear to promote the runaway replication.

Chi sequences and the completion of replication

Chi sequences have been previously shown to determine where RecBCD promoted crossover events that occur during recombination processes; thus, named crossover hotspot instigator by Franklin Stahl (Lam et al., 1974; Henderson and Weil, 1975; Stahl et al., 1977; Stahl 1979). I examined how the presence of *chi* effected RecBCD's ability to catalyze the completion reaction on the bidirectional plasmid. I observed that *chi* sequences increase the aberrant replication on the plasmid, and that this correlates with a reduced stability of the plasmid. This appears to suggest that upon encountering a *chi* site, RecBCD promotes the recruitment of replication. On the plasmid substrates, this appears detrimental. This could be due to the small size or the absence of other critical sequences on the plasmid. On the chromosome, it seems reasonable to consider that this is likely to be a beneficial step. It may be that the *chi* sites induce the RecBCD complex to cease degrading the DNA, perhaps as a 'check' to see if all of the over-replicated regions have been degraded. If it has degraded beyond the over-replicated region, then further synthesis would be needed to complete the replication process. During recombination, *chi* sequences induce RecBCD to promote crossover events at

those locations (Stahl et al., 1977; Stahl 1979). We speculatively suggest that this may similarly reflect joining events during the completion of replication or during the repair of double strand breaks. In both cases, it would be critical that the joining occur at the point where excess tails have been degraded, and we postulate that *chi* is acting to promote this event. This type of activity may also explain why the location of *chi* sequences is heavily biased to the leading strand template of the chromosome (Burland et al., 1993), as the degradation of excess sequences are always orientated away from the origin of replication, allowing more 'checks' to occur during resection in this direction.

Ch V

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