DNA-Protein Complexes Created by Mutant EcoRII Methyltransferase and Quinolone Antibiotics in *Escherichia coli*

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy in the
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ABSTRACT

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Abstract

Expression of mutant M.EcoRII protein (M.EcoRII-C186A) in *Escherichia coli* leads to tightly bound DNA-protein complexes (TBCs), located sporadically on the chromosome rather than in tandem arrays. The mechanisms behind the lethality induced by such sporadic TBCs are not well studied, nor is it clear whether very tight binding but non-covalent complexes are processed in the same way as covalent DNA-protein crosslinks (DPCs). Using 2D gel electrophoresis, we found that TBCs induced by M.EcoRII-C186A block replication forks *in vivo*. Specific bubble molecules were detected as spots on a 2D gel, only when M.EcoRII-C186A was induced, and a mutation that eliminates a specific EcoRII methylation site led to disappearance of the corresponding spot. We also performed a candidate gene screen for mutants that are hypersensitive to TBCs induced by M.EcoRII-C186A. We found several gene products necessary for protection against these TBCs that are known to also protect against DPCs induced with wild-type M.EcoRII (after aza-C incorporation): RecA, RecBC, RecG, RuvABC, UvrD, FtsK, and SsrA (tmRNA). In contrast, the RecFOR pathway and Rep helicase are needed for protection against TBCs but not DPCs induced by M.EcoRII. We propose that stalled fork protection by RecFOR and RecA promotes release of tightly bound (but non-covalent) blocking proteins, perhaps by inducing Rep helicase-driven dissociation of the blocking M.EcoRII-C186A. Our studies also argued against the involvement of several proteins that might be expected to protect against TBCs.

We took the opportunity to directly compare the sensitivity of all tested mutants to two quinolone antibiotics, which target bacterial type II topoisomerases and induce a unique form of DPC. We uncovered *rep* and *ftsK* as novel quinolone hypersensitive

mutants, and also obtained evidence against the involvement of a number of functions that might be expected to protect against quinolones. Other work with quinolones showed a mild reduction in the SOS response to DNA damage by quinolones in *recQ* mutants. We also determined that the quinolone resistance seen in an *icdA* mutant is not due to the up-regulation of the AcrAB-TolC efflux pump.

Dedication

To my daughter, Evelyn Adair Henderson, who was born March 30, 2014, three days after my defense.

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Abbreviations

aza-C:	5-azacytidine
Cipro:	ciprofloxacin
DPC:	DNA-protein crosslink
DSB:	double-strand break
HR:	homologous recombination
M.EcoRII:	EcoRII methyltransferase
M.EcoRII-C186A:	mutant EcoRII methyltransferase
MMC:	Mitomycin-C
Nal:	nalidixic acid
NER:	nucleotide excision repair
RFB:	replication fork barrier
RNAP:	RNA polymerase
ROS:	reactive oxygen species
ssDNA:	single-stranded DNA
TBC:	tightly bound DNA-protein complex
UV:	ultraviolet light

Acknowledgements

I would like to acknowledge my advisor, Kenneth Kreuzer, for all guidance and encouragement. I would also like to acknowledge current and former committee members, Tao Hsieh, Meta Kuehn, K. V. Rajagopalan, Sue Jinks-Robertson, and Laura Rusche, for thoughtful discussions and advice.

Chapter 1: Background

1.1 Prokaryotic model systems for studying genome instability relevant to cancer

Escherichia coli is a valuable system for studying DNA damage, because DNA replication, repair, and protection mechanisms are highly conserved from bacteria to humans. Bacteria are also exposed to most of the same DNA damaging agents as eukaryotes, have similar protective mechanisms, a short generation time and are easily manipulated, making *E. coli* a great model system.

A relationship between cancer and genomic instability has been well established [1]. One of the best examples of the value of using a prokaryotic system to understand DNA repair and carcinogenesis in humans is mismatch repair (MMR), which was first discovered in *E. coli* from a study of mutants with an elevated mutation frequency (for review, [2]). Mutations in the *E. coli* genes *mutS, mutL, mutH, uvrD* and *dam* [3,4,5,6] lead to an elevated mutation rate. These genes were determined to be involved in the post replicational repair of DNA mismatches referred to as the mismatch repair system. A similar mismatch repair system was found in humans [7]. Mutations in the human homologs of MutS and MutL result in susceptibility to hereditary non-polyposis colorectal cancer (HNPCC), one of the most common cancer predisposition diseases in humans (for review, [8]).

Three other genes in *E. coli* found to yield a high mutation frequency when disrupted are *mutM*, *mutY*, and *mutT* [9]. Studying these genes uncovered how bacteria and human cells repair damage caused by 8-oxoG, created by oxidative damage to a

guanine residue. 8-oxoG is highly mutagenic because it can pair with adenine as well as cytosine, leading to G:C → T:A transversions. MutM removes 8-oxoG residues opposite cytosine residues [10,11] and MutY removes adenine residues across from 8-oxoG [9], allowing for proper pairing with cytosine. Mutations in the corresponding human proteins OGG1 and MYH, respectively, can lead to a susceptibility to colon cancer [12]. MutT, the first mutator mutant described in *E. coli* [13], removes 8-oxoG from the nucleotide pool [14] and has a corresponding human protein, MTH, which performs the same function.

Alkylating mutagens can damage guanine by attaching a methyl group to the oxygen atom, leading to mispairing with thymine rather than cytidine. The repair of O6-methylguanine was defined in *E. coli* mutants defective for two DNA repair methyltransferases [15]. These mutants were found to have a high rate of spontaneous mutation, suggesting they were deficient in a DNA repair or protection mechanism and were therefore studied further. The study of these mutants uncovered the mechanisms for repairing O6-methylguanine lesions. Eukaryotic cells are also subject to O6-methylguanine lesions and repair the lesions in a similar manner as prokaryotes [16].

1.2 SOS response to DNA damage

The SOS response in *E. coli* is a physiological response to DNA damage that involves the regulation of 20-40 genes. The mechanisms for the regulation of the SOS response are well understood (for reviews, see [17,18,19,20]). The genes regulated by the SOS response encode proteins involved in DNA repair, cell-cycle inhibition, and SOS-induced mutagenesis. The expression of genes involved in the SOS response is controlled by RecA and LexA. In uninduced cells, LexA binds to operator sequences

upstream of each SOS gene, repressing transcription. The SOS response is induced when RecA binds regions of single-stranded DNA, created by DNA damage or during the process of repairing the damage. RecA then undergoes a conformational change that leads to activation of its coprotease activity, and then RecA promotes the autocleavage of LexA. Cleavage of LexA removes repression from SOS genes and leads to induction of these genes. When exposed to quinolone antibiotics, the SOS response is induced through the double-strand break (DSB) processing RecBCD pathway [21]. Mitomycin-C (MMC) and UV also induce the SOS response, but do so through the RecFOR pathway, presumably because MMC and UV damage lead to single stranded DNA (ssDNA) and blocked forks rather than DSBs [21].

1.3 Introduction to E. coli HR pathways

Homologous recombination (HR) is a method to repair damaged DNA by exchanging nucleotide sequences between two DNA molecules. RecA, which catalyzes the strand exchange reaction, is the crucial protein in all HR reaction. RecBCD assists RecA to repair DSB damage via HR (Figure 1; for reviews, see [22,23,24]). DNA damage, such as a fork blocked by a TBC, can lead to a broken replication fork. Homologous recombination can restart a broken replication fork, which requires both RecA and RecBCD. RecBCD helicase/exonuclease binds to a double-stranded DNA end and unwinds the 5’ DNA end until it reaches the Chi sequence (5’ GCTGGTGG 3’) and nicks the 3’ end. This produces the ssDNA substrate of a 3’ overhang for RecA to bind. RecBCD loads RecA onto the DNA. RecA performs strand invasion with a homologous region of DNA. Stand invasion produces a D-loop which serves as a site for

PriA to initiate assembly of the replisome. The recombination reaction yields a Holliday junction, which is resolved by either the RuvABC resolvasome or branch migration by RecG helicase [25,26,27].

The RecFOR pathway is another pathway that assists in RecA-mediated recombination, but the RecFOR pathway processes single-stranded gaps rather than DNA ends (Figure 2; [28,29]). A gap in the DNA can arise when a damaged DNA template is replicated. RecJ exonuclease binds to the 5' end of a single-strand gap in DNA and along with RecQ helicase, moves along the DNA cleaving the 5' strand creating a 3’ overhang [30]. RecFOR loads RecA onto the 3’ overhang [31] allowing RecA to catalyze HR. RecA performs strand invasion with a homologous region. This forms a Holliday junction which can be branch migrated by RuvABC and possibly RecG. The Holliday junction is resolved by RuvABC.

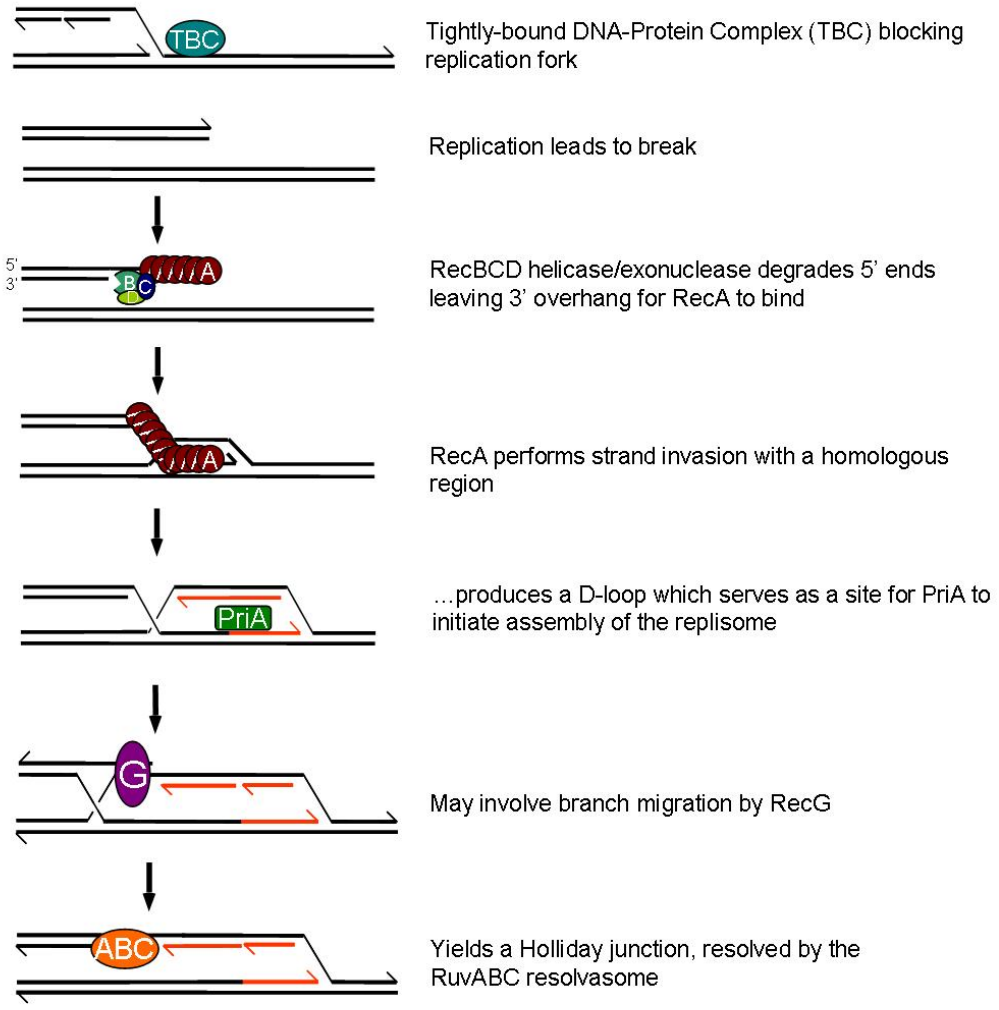

Figure 1: RecBCD assisted RecA-homologous recombination

This pathway involves the response of a fork that is blocked by a TBC. Parental DNA is black, the nascent strand is orange. TBC: tightly bound DNA-protein complex, A: RecA, BCD: RecBCD, PriA: PriA, G: RecG, ABC: RuvABC.

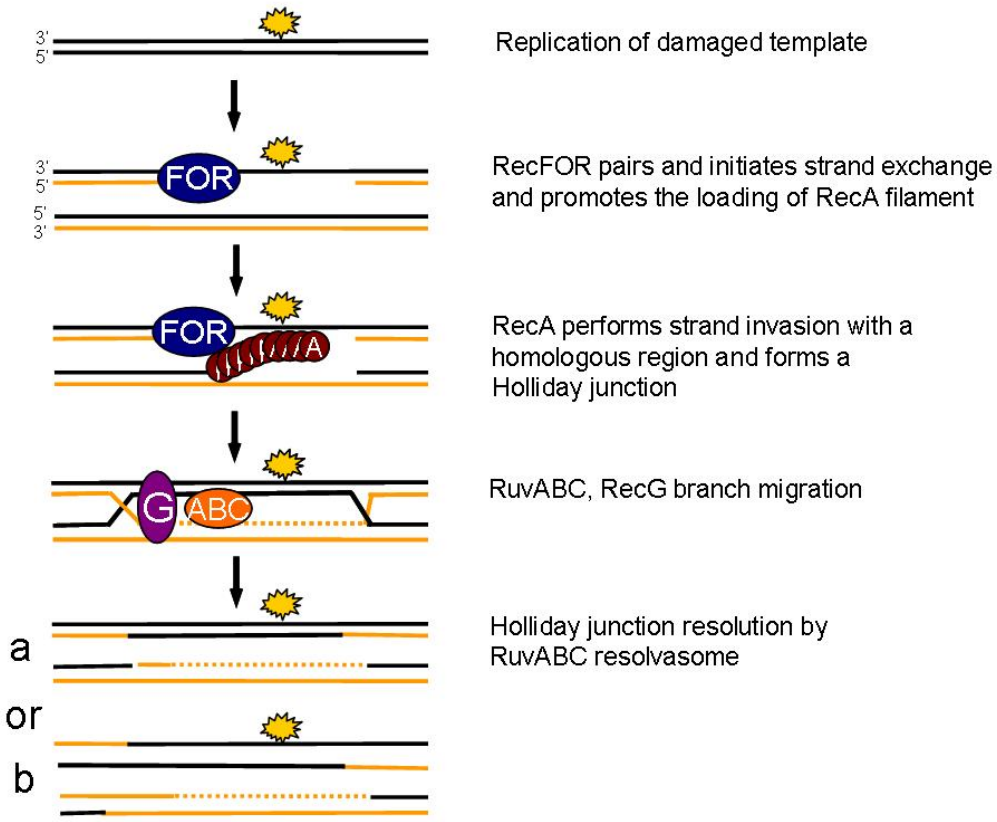

Figure 2: RecFOR assisted RecA-homologous recombination model for daughter strand gap repair

This pathway involves DNA damage that leads to a gap. Parental DNA is black, the nascent strand is orange. Star: template damage, A: RecA, FOR: RecFOR, G: RecG, ABC: RuvABC.

1.4 Replication fork blockage and restart pathways

When the DNA replication machinery encounters DNA damage of various kinds, or unusual situations such as secondary structures, DNA crosslinks and tightly bound proteins, replication fork blockage can occur (for reviews, see [32,33]). Blocked replication forks in turn can induce DNA damage responses, and can also lead to mutation or cell death. Recent work has begun to unravel multiple pathways that cells use to restore active replication after fork blockage; some of these involve damage repair while others simply avoid the damage, leaving it in one of the daughter duplexes (for reviews, see [34,35,36]).

1.4.1 Replication fork direct restart

Direct restart can reactivate a stalled replication fork, if the fork block is quickly removed. Direct restart requires PriA, a component of the primosome. The primosome is a six-protein complex that begins assembly with PriA binding to DNA. Binding of PriB, PriC and DnaT follows. Then DnaC loads DnaB, the replicative DNA helicase. DnaG associates and synthesizes the RNA primer [37,38]. When the block is cleared and the replicative helicase is reloaded, replication can resume. Direct fork restart can also occur from D-loops formed during recombination.

1.4.2 Fork regression pathway for replication fork restart

If the fork block is not quickly removed, fork regression may be necessary for repair (for review, see [39]). If the lesion is not in duplex DNA, fork regression can move it back into a duplex region, allowing for repair from a conventional pathway, such as NER (Figure 3). Another model is strand-switch synthesis, which also starts with fork

regression (Figure 3). Then a lesion in the leading-strand can be bypassed by using the lagging-strand product as a template [40]. The lesion can be repaired before subsequent rounds of replication.

1.4.3 Recombination dependent replication fork restart

Homologous recombination can restart a broken replication fork. RecA and RecBCD are crucial for this pathway. The steps are described in Section 1.3 and Figure 1.

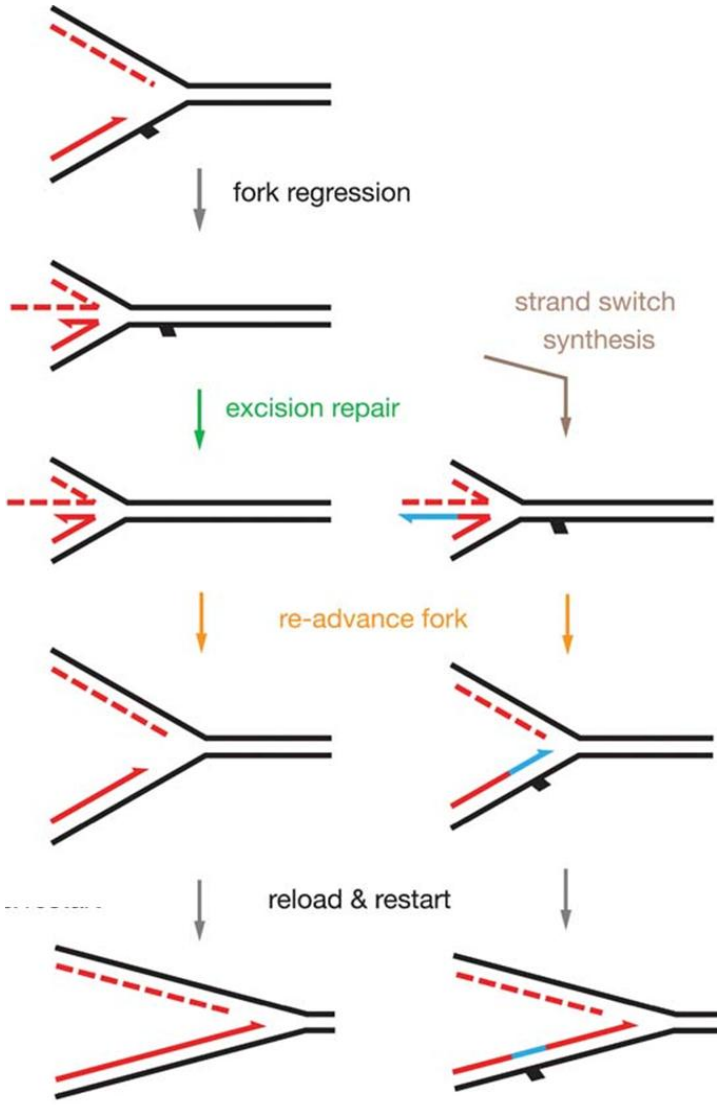

Figure 3: Models of replication fork restart

These pathways involve the response of a fork that is stalled by leading-strand damage; some of these pathways are also relevant for lagging-strand damage. Various steps are indicated by arrows; the color of the arrow corresponds to the color of the text describing a particular step. Parental DNA is black, the nascent leading strand is solid red, and the nascent lagging strand is dashed red. Template DNA damage is indicated by the small black boxes, and the short segment of DNA from strand switch synthesis is blue (from reference [39]).

1.5 Systems that create deleterious DNA-protein complexes

1.5.1 Aza-C-induced M.EcoRII DNA-Protein crosslinks

DNA-protein crosslinks (DPCs) are particularly difficult to study, therefore poorly understood form of damage that creates blocked replication forks [41,42,43]. A challenge in studying DPCs is that many inducing agents, such as radiation and formaldehyde, create DPCs throughout the chromosomal DNA and the crosslinks involve a wide variety of different DNA-binding proteins [41,42]. A number of DNA damaging agents, including ultraviolet light (UV), ionizing radiation, formaldehyde, certain carcinogens, and some chemotherapeutic drugs, cause proteins to become trapped on DNA in DPCs, which have been shown to block DNA replication.

One system used to avoid the challenges of studying DPCs that are formed with a variety of proteins across the chromosome utilizes the chemotherapeutic drug Azacytidine (aza-C). Aza-C leads to DPCs that are both DNA site-specific and protein-specific. Aza-C is a cytidine analog in which the carbon-5 (C5) of the pyrimidine ring is replaced with nitrogen. M.EcoRII acts on this particular cytosine residue within the EcoRII recognition sequence by transferring the methyl group from S-adenosylmethionine to C5 [44]. However, when the cytidine analog Aza-C is incorporated into the target sequence, an irreversible and covalent DPC is formed between M.EcoRII and its DNA recognition sequence (5'-CCWGG). Several groups have used aza-C-induced M.EcoRII DPCs as a model system to investigate how cells respond to and repair DPCs ([28,43,45,46,47,48,49]; reviewed in [42]; Krasich et al., unpublished data).

Previous work from our lab used 2D agarose gel electrophoresis to visualize the accumulation of replication forks blocked at aza-C-induced DPCs [43]. Sensitivity of mutants to aza-C-induced DPCs has been studied in our lab and others and results are summarized in Chapter 4, Table 4. Mutants of *recA* ([45,46,47,48]; Krasich et al., unpublished data) and *recC* ([48]; Krasich et al., unpublished data) are hypersensitive to aza-C-induced DPCs, arguing that DSBs are formed. RecBCD and RecA may play a direct role in repairing DPCs, or may be involved in repairing downstream DNA damage such as broken replication forks.

As mentioned above, to restart replication after the formation of DSBs, RecBCD assists in RecA-mediated recombination which generates Holliday junctions that can be resolved by RuvABC. Mutations in *ruvABC* also cause hypersensitivity to aza-C ([48]; Krasich et al., unpublished data). Helicases UvrD [48,49] and DinG (Krasich et al., unpublished data) may play a special role in DPC repair, as mutations in both lead to hypersensitivity to aza-C-induced DPCs. An interesting finding in our lab is that *ssrA* mutants are hypersensitive to aza-C-induced DPCs, arguing that tmRNA plays an important role in clearing stalled ribosome-mRNA complexes generated after transcription is blocked by aza-C-induced DPCs [49].

1.5.2 DPCs with topoisomerase

In *E. coli,* quinolone antibiotics target topoisomerase IV [50] and topoisomerase II (gyrase) [51,52] and stabilize a unique form of DPC called the cleavage complex. Quinolone-stabilized DNA gyrase cleavage complexes block replication forks *in vivo* [53]. In eukaryotes, the chemotherapeutic drugs, including doxorubicin and etoposide,

target topoisomerase II and also stabilize the cleavage complex. This topic is discussed further in Chapter 3.

1.5.3 Tandem repressor-operator complexes

Replication can also be blocked by tightly bound but non-covalent complexes [54,55], and it is not clear whether the processing and consequences of TBCs are similar to those of DPCs. One system that has been used to study the consequences of replication fork blocks created by TBCs involves tandem repressor-operator complexes. *E. coli* strains expressing either TetR or LacI repressor, with tandem arrays of 240 copies of the respective binding sites (*tetO* or *lacO*) in the chromosome, demonstrated site-specific and reversible blockage of chromosomal replication at the TBC array [55]. Replication appeared to sometimes proceed a short distance into the array, suggesting some limited success at replication through the blockage. When the TBCs were present for 2 hours and then reversed by addition of the appropriate inducer, replication was shown to rapidly restart in either wild-type or *recA* mutant cells [55]. However, maintaining the chromosomal TBC array for 4 hours in wild-type cells led to a massive loss of viability, and so eventually some toxic event is induced by the long TBC arrays [55].

Shorter TBC arrays (22 or 34 copies) were also found to create replication fork blockage *in vivo*, and arrays even shorter than that blocked replication *in vitro* but not *in vivo* [56]. These results suggested that factors, such as recombination proteins and/or helicases, can prevent or ameliorate fork blockage *in vivo*. Interestingly, RecA, RecBCD, and RecG were all required for cell viability in the presence of the 34-copy TBC arrays,

whereas RecF, Rep, UvrD, and RuvABC were not [56]. These genetic results imply that DSBs are somehow formed in response to the replication blocks. While *rep* and *uvrD* single mutants were viable with the 34-copy TBC arrays, a later study revealed a synthetic phenotype indicating redundant roles of Rep and UvrD in promoting replication through the 34-copy TBC array [57].

1.5.4 Solo, non-arrayed TBCs

Tandem arrays of TBCs present a rather unnatural obstacle to DNA replication, and their processing and consequences might be distinct from single TBCs. Two different tight binding proteins, active site (catalytically inactive) mutants of a restriction nuclease and a restriction methyltransferase, have been used to generate solo, sporadically located (non-arrayed), TBCs. The E111G mutant of EcoRI nuclease binds very tightly to its recognition sequence and causes lethality when expressed at high levels [58], as does the C186A mutant of M.EcoRII [59,60,61]. M.EcoRII-C186A induced TBCs are the subject of Chapter 2 and will be discussed in greater detail. The E111G EcoRI protein blocks *E. coli* replication forks *in vitro*, and either Rep or UvrD helicase (but not RecG, PriA or Mfd) can relieve the replication block [57]. TBCs created with a mutant EcoRI nuclease were also shown to block transcription *in vitro* [62], although a later study showed that additional trailing RNA polymerases can allow the transcription complex to push through the TBC, dislodging the protein in the process [63]. These studies show that solo TBCs are potent replication and transcription blocks *in vitro*, and that they can be cytotoxic *in vivo*. However, they do not illuminate the *in vivo* molecular consequences nor the pathways that cells might use to overcome solo TBCs. The

studies with both tandem and solo TBCs also support an important redundancy in which Rep and UvrD generally assist the replication fork in overcoming protein blockage of various types.

1.5.5 Collisions with RNA polymerase

Several studies argue that the most important and/or common fork blockage in nonstressed cells is caused by collisions with RNA polymerase, not template damage [57,64]. Collisions between replisomes and transcribing RNA polymerases lead to fork blockage in both bacteria and eukaryotes [29,65]. Cells have multiple protective mechanisms to reduce the complications due to these collisions and most collisions lead to pausing, not inactivation of the replisome [64].

The orientation between the replisome and RNAP effects the extent of the damage. Head-on collisions are more inhibitory to replication forks than co-directional collisions [66,67,68,69,70,71]. Bacteria have evolved to minimize head-on collisions, by organizing the majority of highly-transcribed genes in the same orientation [72]. Reducing the frequency of RNAP stalling and backtracking reduces the frequency of collisions. Multiple RNAPs translocating on the same gene reduces the frequency of backtracking. Helicases, Rep and UvrD, are hypothesized to inhibit the frequency of RNAP backtracking [57]. After a collision has occurred, the *E. coli* replisome might be able to displace the stalled RNAP [73]. If the replisome is unable to displace stalled RNAP, Mfd, a dsDNA translocase, may be able to act [74]. Restart of the blocked replication forks can be initiated by PriA.

1.5.6 Tus *Ter* replication fork traps

It is important to note that bacterial cells maintain a specialized and intentional fork-blockage system to assist in the completion of DNA replication. *E. coli* replication forks are paused, in a unidirectional fashion, when the forks encounter the Tus protein bound to *Ter* DNA sites in the terminus region [75,76,77]. When Tus binds to the *Ter* sites, a barrier called a fork trap is formed [78]. The fork trap allows forks to enter the terminus region, but they are not able to exit, creating stalled replication forks at the *Ter* site [79]. The Tus *Ter* system can be used to study the consequences of forks blocked at TBCs. When *Ter* sites are inserted in regions outside the terminus, RecBCD-mediated homologous recombination (HR) and induction of the SOS response are required to tolerate the blocked replication forks [80,81,82]. UvrD is also required for survival in this situation, which was traced to the ability of UvrD to remove the bound Tus from *Ter* sites [83,84].

1.5.7 Fork blockage by UV damage and DnaB^{ts} mutant

Two other fork blockage systems have been studied in some detail and provide an interesting comparison to the systems described above. Following UV exposure, the replication machinery can be blocked by pyrimidine dimers [85,86]. RecA, along with loading factors RecF, RecO and RecR, are needed to stabilize the arrested replication fork after UV damage [87,88,89]. Furthermore, RecA-mediated HR is required for the processing of these blocked forks [27], and Rep helicase, along with primosome proteins PriB and PriA, are required for restarting replication [90]. The second system involves fork blockage after temperature shift of a DnaB^{ts} mutant, which can lead to the formation

of RuvABC-mediated DSBs [91]. In this case, the presence of RecA contributes to DSB formation, presumably by RecA-mediated Holliday junction formation for RuvABC action [92]. The forks stalled following DnaB inactivation are degraded by RecFOR, along with RecJ, RecG, and ExoI (SbcB) [93].

1.5.8 DNA-protein complexes in eukaryotes

In *Saccharomyces cerevisiae*, the Fob-block system consists of Fob1 which binds to the replication fork barrier (RFB) site in ribosomal DNA (rDNA), preventing the replication fork from progressing in the wrong direction [94,95]. This system has been used as a model showing that overcoming DNA-protein complexes is also important in eukaryotic cells [96]. An inducible Fob-block system was used to show that MRX (Mre11, Rad50, and Xrs2) plays a role in maintaining fork integrity at these strong, but non-covalent protein-DNA barriers. In *E. coli,* RecBCD, RecQ, and RecJ play roles similar to the MRX complex.

In *Schizosaccharomyces pombe*, RTS1 is a terminator of replication that controls the direction of replication at the *mat* locus by creating an RFB site [97]. Using a system to express RTS1, it was discovered that homologous recombination is necessary for cell viability [98]. While the recombination is required for cell viability when forks are stalled, the recombination occasionally causes gross chromosomal rearrangements [98].

1.6 Helicases clearing stalled replication forks

Helicases play important roles in restarting stalled replication forks by displacing the blocks to replication. Rep and UvrD are helicases that translocate from 3′ to 5′ [99,100]. Rep helicase allows replication fork progression past a protein-DNA complex

including E111G EcoRI protein blocks and TBC arrays [57]. Rep is also required for restarting replication stalled at UV-damage [90]. UvrD, like Rep, also allows replication fork progression past TBC arrays [57]. UvrD is capable of removing the bound Tus from *Ter* sites [83,84]. Rep, but not UvrD interacts with the replicative helicase to facilitate the replication of DNA with a bound protein [57]. Phage T4 Dda helicase is capable of clearing DNA bound by RNA polymerase (RNAP) *in vitro* [101].

1.7 Major questions to be addressed

The scope of this work is to analyze effects of a solo TBC, using the mutant M.EcoRII protein (C186A) to compare to the consequences of TBCs and DPCs discussed in Chapter 1. We use 2D gel electrophoresis to determine if a single TBC can block replication forks *in vivo*, as was previously shown for covalent DPCs [43]. We compared a colletion of twenty-five mutants' sensitivity to M.EcoRII-C186A TBCs with aza-C-induced DPCs. In addition to studying the effects of M.EcoRII-C186A TBCs, we have performed tests with quinolone antibiotics which stabilize the cleavage complex DPC. The same collection of mutants were tested for quinolone sensitivity.

Table 1: Functions of relevant genes

gene	function
dinD	function unknown; upregulated during SOS response
dinG	helicase
dnaJ	chaperone
dnaQ	epsilon subunit of DNA polymerase
ftsK	essential cell division protein linking cell division and chromosome segregation
helD	helicase IV
hflC	part of HflCK complex that regulate FtsH protease
intQ	predicted defective integrase
lexA	transcription repressor that regulates the SOS response
mcrA	component of restriction system for defending the cell against foreign DNA
mfd	transcription-coupled repair factor
polB	DNA polymerase II; combined polymerase and exonuclease
priA	component of primosome
rarA	conserved ATPase involved in responses to fork stalling
recA	DNA strand exchange and recombination protein with protease and nuclease activity
recB	helicase; component of the RecBCD helicase/nuclease (exonuclease V) complex that is essential for recombination and dsDNA break repair
recC	helicase; component of the RecBCD helicase/nuclease (exonuclease V) complex that is essential for recombination and dsDNA break repair
recD	helicase; component of the RecBCD helicase/nuclease (exonuclease V) complex that is essential for recombination and dsDNA break repair
recF	component of the RecFOR complex which functions in RecA-mediated recombination
recG	helicase; catalyzes branch migration on forked DNA structures
recJ	Exonuclease; functions with RecQ & RecFOR
recO	component of the RecFOR complex which functions in RecA-mediated recombination
recQ	Helicase; functions with RecJ & RecFOR
recR	component of the RecFOR complex which functions in RecA-mediated recombination
rep	helicase; removes proteins from DNA ahead of replication fork
ruvA	helicase; Holiday junction resolvasome
ruvC	endonuclease; Holiday junction resolvasome
sbcCD	dsDNA exonuclease and ssDNA endonuclease
ssrA	specialized tmRNA that releases stalled ribosomes and tags the truncated peptides for degradation
uvrA	subunit of the UvrABC NER generalized DNA repair process
uvrD	helicase II; also functions with NER
xseA	exonuclease VII

Chapter 2: Functions that protect from tightly bound DNA-protein complexes created by mutant EcoRII methyltransferase

2.1 Introduction

2.1.1 M.EcoRII-C186A tightly-bound DNA-protein complexes

As mentioned in chapter one, the mechanisms behind the lethality induced by solo TBCs have not been well studied, nor is it clear whether very tight binding but noncovalent complexes are processed in the same way as covalent DPCs. We used a system to express tight-binding mutant M.EcoRII protein (C186A), which creates solo, site-specific, and protein-specific TBCs, to analyze the *in vivo* consequences and protein requirements for survival. A powerful aspect of this system is that previous studies, from our lab and others, have already identified a variety of mutants that are hypersensitive to aza-C-induced DPCs that involve the same M.EcoRII protein (except that the DPCs utilize the wild-type version of the protein) [28,43,45,46,47,48,49] (reviewed in [42]; Krasich et al., unpublished data). The active site cysteine residue (C186) of M.EcoRII acts on a cytosine residue in the recognition sequence 5'-CCWGG by transferring the methyl group from S-adenosylmethionine [44]. When the active site cysteine residue is substituted with alanine, a TBC is formed between the mutant M.EcoRII and the EcoRII site containing the target cytosine [60]. Covalently trapped DPCs are formed at the same sites with the wild-type M.EcoRII when the cytidine analog, aza-C, has replaced the target cytosine [60,102,103].

We performed an extensive candidate gene screen for mutants that are hypersensitive to the M.EcoRII-induced solo TBCs, using an arabinose expression

system that allows carefully titrated expression of M.EcoRII. We found several gene products necessary for protection against these TBCs that are known to also protect against DPCs induced with wild-type M.EcoRII (after aza-C incorporation): RecA, RecBC, RecG, RuvABC, UvrD, FtsK, and SsrA (tmRNA). In contrast, a functional RecFOR pathway and Rep helicase are needed for protection against TBCs but not DPCs induced by M.EcoRII-C186A. Overall, our results support a model in which RecFOR/RecA-dependent fork stabilization promotes dissociation of a TBC, and also reveal that the tmRNA system helps protect against TBCs, presumably helping to overcome TBC-mediated blockage of coupled transcription-translation complexes. Interestingly, different but overlapping functions are involved in surviving TBCs versus DPCs, both involving the M.EcoRII protein.

2.1.2 Replication intermediates on 2D gel electrophoresis

2D gel electrophoresis allows for the separation of DNA intermediates based on size and shape [104]. Previous work from our lab used 2D agarose gel electrophoresis to visualize the accumulation of replication forks blocked at aza-C-induced DPCs involving the wild-type M.EcoRII protein [43]. The site-specific DPCs between M.EcoRII and its recognition sequence create specific bubble molecules detected as spots on the 2D gel. A mutation that eliminates an EcoRII methylation site leads to disappearance of the corresponding spot. 2D gel electrophoresis has been used to visualize fork-blockage following UV-damage, but because the damage is not site-specific, the pattern that can be seen is less distinct, involving a cone-shaped region [105]. We replicated the 2D gel electrophoresis technique used for aza-C-induced DPCs [43] and determined that a

single, tightly bound (but non-covalent) M.EcoRII complex could also block replication forks *in vivo.* Interestingly, based on the molecular forms observed, the downstream consequences of fork blockage are different depending on whether M.EcoRII is in a covalent or non-covalent complex on the DNA.

2.2 Results

2.2.1 Fork blockage at TBCs created by M.EcoRII-C186A

2D gel electrophoresis was used to visualize DNA replication intermediates to determine whether a single tightly bound (but non-covalent) M.EcoRII complex could block replication forks (Figure 5). We found an accumulation of bubble molecules at locations consistent with the EcoRII methylation sites at 1,443 and 1,060 bp (and less convincingly at 131 bp) (Figure 5). The spot corresponding to the 1,443-bp recognition site was consistently stronger than the other two, suggesting that M.EcoRII sites may be saturated with the mutant M.EcoRII protein, leading to more frequent fork blockage at sites that are encountered earlier in replication of the plasmid (i.e. close to the replication origin; note that the M.EcoRII site at position 2,501 is very close to the origin - we do not know whether forks are blocked at that site, since the resulting bubble spot would be difficult or impossible to detect in the vicinity of the very intense monomer spot). The spots on the bubble arc were only detected with the addition of arabinose to induce expression of M.EcoRII-C186A. To directly test whether TBC formation at M.EcoRII sites was leading to the blocked replication forks, we analyzed a mutated pBR322-C1060A plasmid, which lacks the methylation site at 1,060 bp. Indeed, the bubble spot corresponding to the 1,060-bp site disappeared while the spot corresponding to the

1,443-bp site remained, confirming that the accumulation of bubble molecules is due to blockage at *Eco*RII methylation sites. We conclude that solo TBCs involving the mutant M.EcoRII protein are capable of blocking replication forks *in vivo*, as do DPCs formed after aza-C treatment with wild-type M.EcoRII.

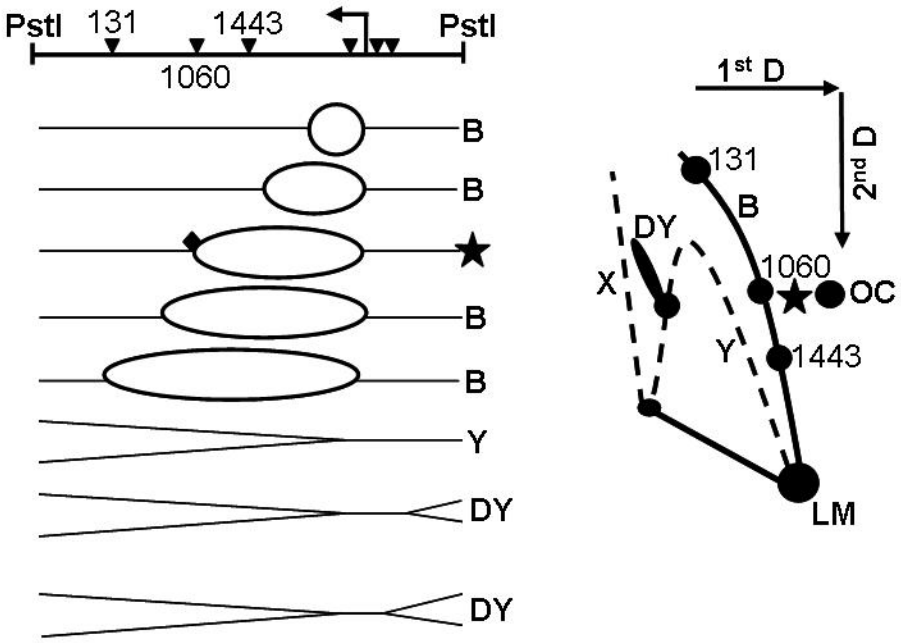

Figure 4: Predicted replication intermediates and two-dimensional gel pattern of linearized pBR322.

The diagram on the left depicts plasmid pBR322 linearized by PstI at 3,607 bp; leftward arrows, replication start site (2,535 bp). Inverted triangles, EcoRII methylation sites (at positions 131, 1,060, 1,443, 2,501, 2,622, and 2,635 bp from left to right); diamond, tightly bound MTase at a blocked fork. The diagram on the right depicts B, bubble; DY, double Y; X, X structures; OC, open circle; LM, linear monomer; star, replication intermediate blocked at the 1,060 methylation site and the resulting accumulation on the two-dimensional gel. This figure is modified from Kuo et al. [43].

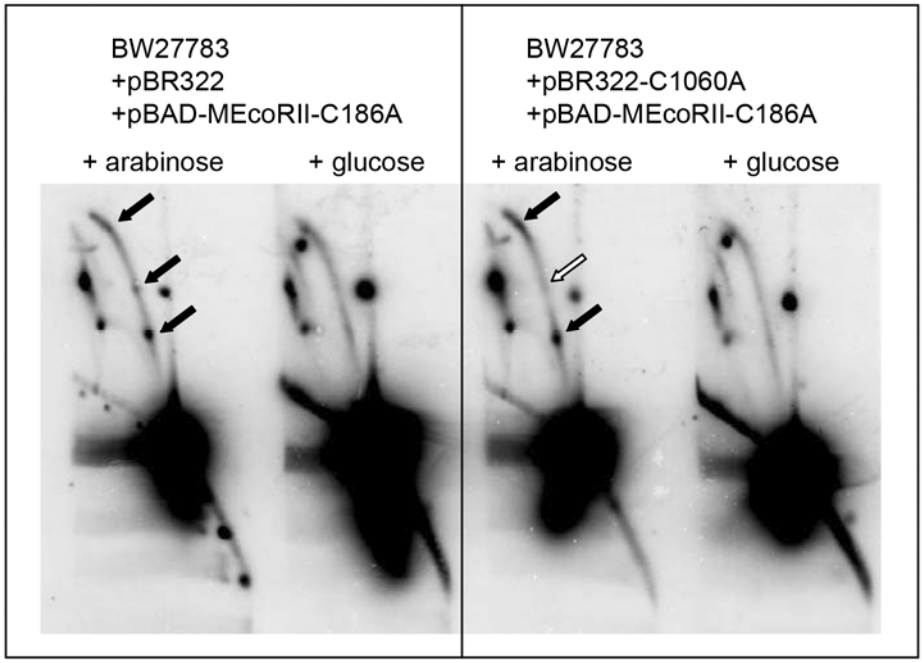

Figure 5: DNA replication is blocked in cells expressing mutant EcoRII C186A methyltransferase.

DNA was digested with Pst1-HF, run on two-dimensional gel electrophoresis and visualized with Southern hybridization. *Closed arrows* show accumulation of bubble molecules at locations consistent with the *Eco*RII methylation sites at 131, 1,060, and 1443 bp. *Open arrow* shows disappearance of the 1,060 bp spot with the mutated pBR322-C1060A plasmid.

2.2.2 Functions that protect from TBCs created by M.EcoRII-C186A

To determine which proteins play a role in protecting from the damage created by TBCs induced by M.EcoRII-C186A, a collection of knockout mutants was created in a genetic background that allows carefully titrated expression from an arabinose promoter. We then compared sensitivities by examining growth (or lack thereof) in the presence of increasing concentrations of arabinose. The collection was largely chosen based on known roles of the corresponding proteins in protection from DPCs or other damage that leads to stalled replication forks. The proteins could be involved in directly reversing the TBC, protecting from the immediate consequences of the TBC (e.g. restarting replication forks or dealing with stalled transcription/translation), or repairing downstream damage from the TBCs (e.g. broken replication forks).

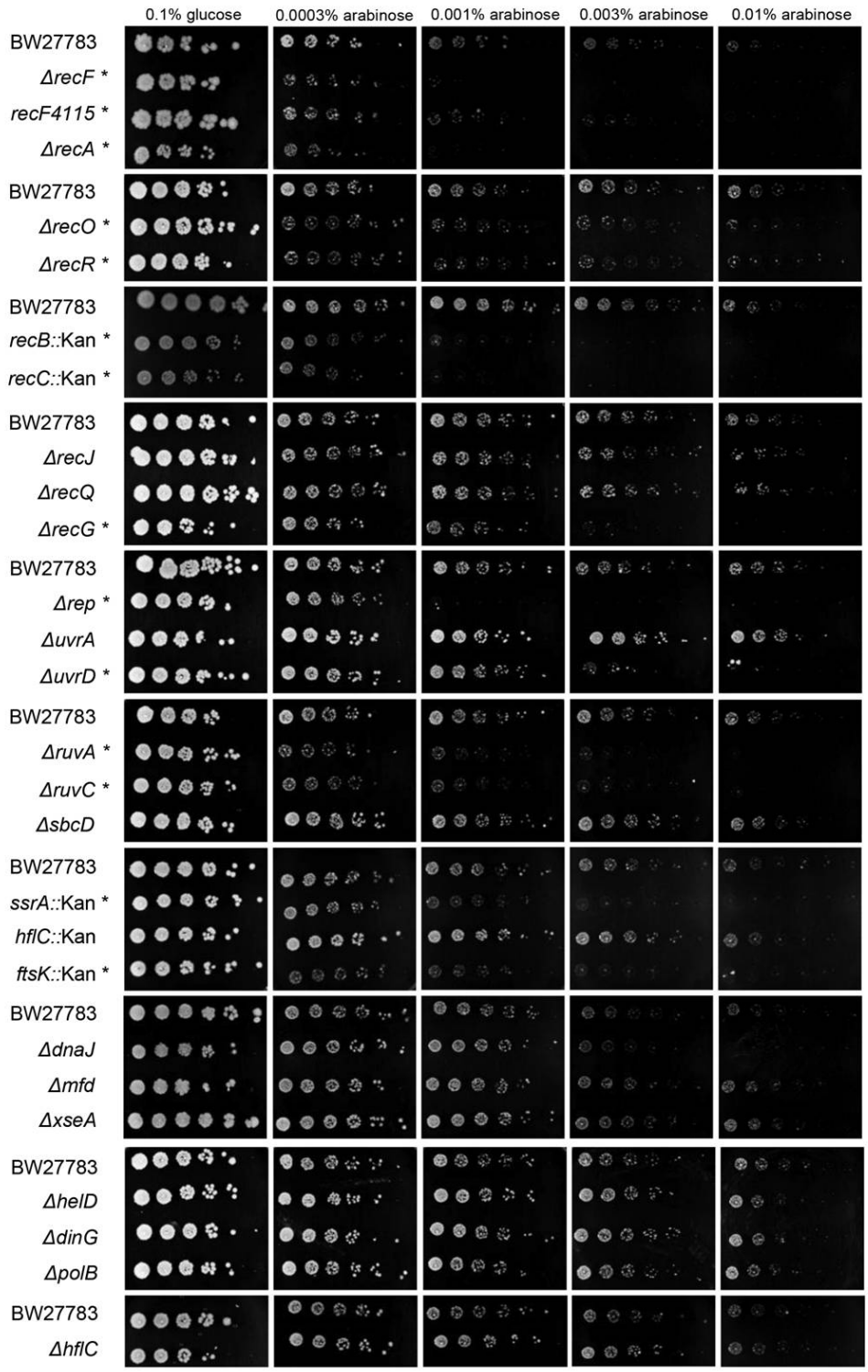
0.1% glucose
0.0003% arabinose
0.001% arabinose
0.003% arabinose
0.01% arabinose
BW27783
ΔrecF *
recF4115 *
ΔrecA *
BW27783
ΔrecO *
ΔrecR *
BW27783
recB::Kan *
recC::Kan *
BW27783
ΔrecJ
ΔrecQ
ΔrecG *
BW27783
Δrep *
ΔuvrA
ΔuvrD *
BW27783
ΔruvA *
ΔruvC *
ΔsbcD
BW27783
ssrA::Kan *
hflC::Kan
ftsK::Kan *
BW27783
ΔdnaJ
Δmfd
ΔxseA
BW27783
ΔhelD
ΔdinG
ΔpolB
BW27783
ΔhflC

Figure 6: Sensitivity to M.EcoRII-C186A

Overnight cultures of BW27783 (wild-type) and the indicated BW27783 derivatives, containing plasmid pBAD-MEcoRII-C186A, were serial diluted five-fold and spotted onto LB plates containing chloramphenicol with either glucose (0.01%) or arabinose (0.0003%, 0.001%, 0.003%, or 0.01%) and incubated at 37° overnight. Those mutants deemed to be hypersensitive are indicated with an asterisk.

2.2.3 Recombination proteins and sensitivity to TBCs

Recombination and repair proteins play important roles in survival from damage due to DPCs and topoisomerase cleavage complexes. For example, RecA, which catalyzes strand exchange, and RecBCD, which prepares broken ends for HR, greatly improve survival from aza-C-induced DPCs [23,55,56,58,63] (Krasich et al., unpublished data) and quinolone-induced cleavage complexes [106,107] (also see Chapter 3). HR by RecA is also required for the processing of replication forks blocked at UV-induced pyrimidine dimers [27]. We found that *recA*, *recB* and *recC* mutants were all markedly sensitive to TBCs generated by expression of M.EcoRII-C186A (Figure 6). Therefore, DSBs are apparently generated after formation of both DPCs and TBCs. RecA and RecBCD may play a direct role in repairing DNA-protein complexes (presumably with DSB formation), or alternatively, these proteins may be involved in repairing downstream DNA damage such as replication forks that are broken after fork blockage (Figure 1).

Like RecBCD, RecFOR assists in RecA-mediated recombination, but RecFOR proteins target RecA to single-stranded gaps rather than DNA ends [48,66]. The action of RecF, RecO and RecR stabilize and maintain replication fork arrest at sites of UV damage, while RecJ exonuclease and RecQ helicase can degrade nascent lagging strand DNA at the blocked fork [87,88,89,105,108]. We found that *recF*, *recO* and *recR* mutants are hypersensitive to TBCs induced by M.*Eco*RII-C186A (Figure 6). Notably, past studies showed that *recFOR* mutants are not sensitive to aza-C-induced DPCs [47] (Krasich et al., unpublished data), nor are they sensitive to quinolones [107]; also see

below). Perhaps fork stabilization by RecFOR is particularly beneficial in the case of non-covalently attached proteins (see Further Discussion; Figure 7). Mutants without RecJ or RecQ showed little or no sensitivity to M.EcoRII-C186A-induced TBCs, indicating that erosion of the nascent lagging strand is not relevant to the processing pathway (Figure 6; the *recJ* and *recQ* mutants sometimes appeared to be very slightly sensitive, but not in a convincing and reproducible manner).

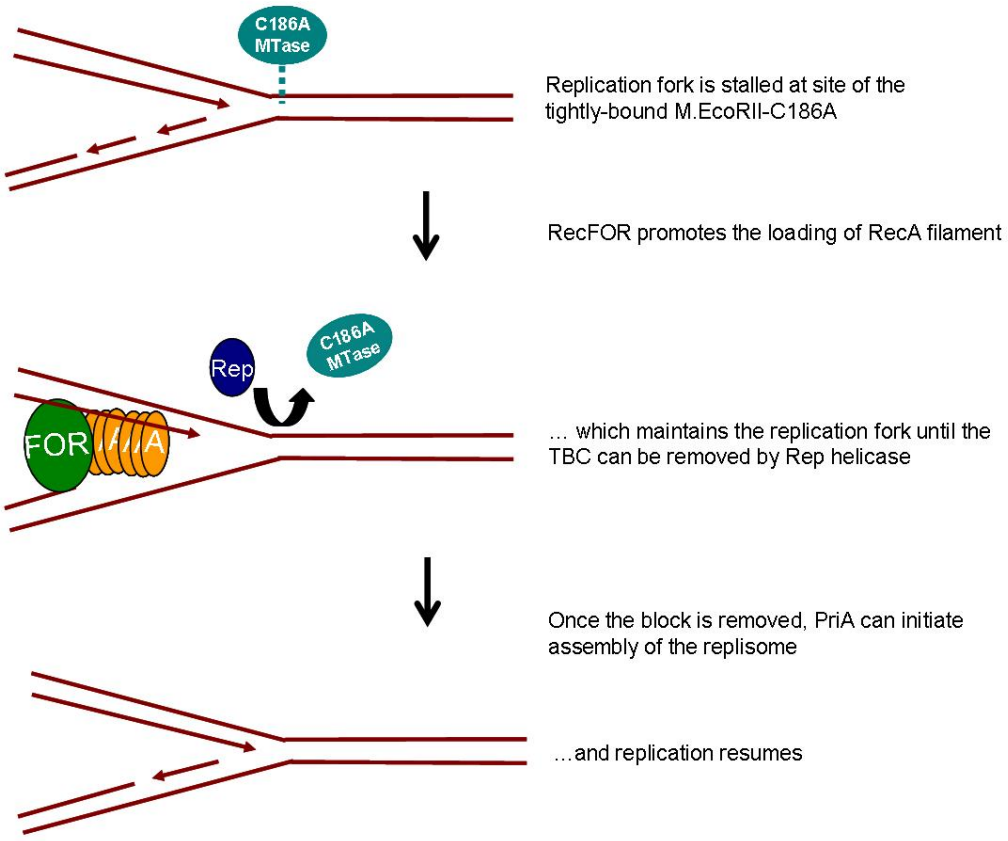

Figure 7: Model of involvement of RecFOR, RecA, and Rep in TBC repair

This pathway involves the response to a replication fork that is blocked by a TBC. C186A Mtase: M.EcoRII-induced TBC, A: RecA, FOR: RecFOR, Rep: Rep

RuvA and RuvC are subunits of the Resolvasome, which resolves Holliday junctions and contributes to rescue of blocked DNA replication forks through replication fork reversal [52,69]. RuvA and RuvC knockout mutants are hypersensitive to aza-C-induced DPCs [48] (Krasich et al, unpublished data) and to quinolone-induced cleavage complexes [65,70]. RuvAB-mediated fork regression does not appear to be involved in rescuing forks blocked by UV damage [109], however *ruvAB* and *ruvC* mutants accumulate unresolved Holliday junctions after UV treatment, leading to decreased cell viability [110]. We found that *ruvA* and *ruvC* mutants are hypersensitive to TBCs induced by M.EcoRII-C186A (Figure 6). Together with the evidence for TBC-induced fork blockage and hypersensitivity of *recBC* mutants (see above), we speculate that TBC-blocked replication forks are sometimes cleaved by a nuclease to generate broken forks, which need to be repaired by RecBCD- and RuvABC-dependent HR. In this view, the hypersensitivity of *ruvA* and *ruvC* mutants suggests that some other nuclease is responsible for fork cleavage. An alternative explanation is that RuvABC induces fork cleavage (see [92]), with subsequent RecBCD-dependent HR, and that this fork cleavage/repair pathway is somehow important for overcoming the blocking TBC (e.g. by promoting removal of the tight-binding protein).

2D gel electrophoresis was used to visualize the replication intermediates of a *ruvC* mutant. We found an accumulation of bubble molecules at locations consistent with the EcoRII methylation sites at 1,443 bp and less convincingly at 1,060 bp (Figure 8). The spot corresponding to the 131-bp recognition site was not visible and is possibly covered by another spot, unrelated to M.EcoRII-C186A expression. When the pBR322-C1060A plasmid, which lacks the methylation site at 1,060 bp, was used, the bubble

spot corresponding to the faint 1,060-bp site disappeared while the spot corresponding to the 1,443-bp site remained, confirming that the accumulation of bubble molecules is due to blockage at *Eco*RII methylation sites. In all conditions, there is an accumulation of X structures, consistent with unresolved Holliday junctions expected in a *ruvC* mutant. The X structures are not dependent on M.EcoRII-C186A expression, and there is no obvious increase in its intensity with TBC formation. Due to the M.EcoRII hypersensitivity of the *ruvC* mutant, we expected to see an increase in X structures with arabinose addition. Perhaps the number of blocked and then cleaved forks created by TBCs are too few to cause a detectable increase in X structures.

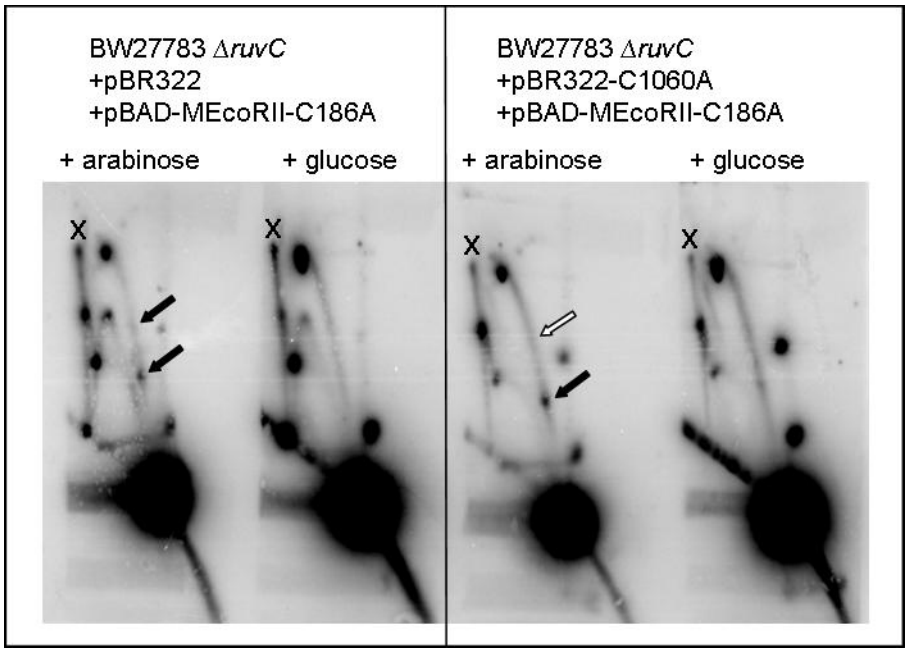

Figure 8: Accumulation of unresolved Holliday junctions in *ruvC* mutant.

DNA was digested with Pst1-HF, run on two-dimensional gel electrophoresis and visualized with Southern hybridization. *Closed arrows* show accumulation of bubble molecules at locations consistent with the *Eco*RII methylation sites at 1,443, and 1,060. *Open arrow* shows disappearance of the 1,060 bp spot with the mutated pBR322-C1060A plasmid. *X* shows an accumulation of X structures.

One candidate for a nuclease that might cleave blocked forks, or even DNA near the tightly bound protein, is SbcCD. This protein has double-strand DNA exonuclease activity as well as single-strand DNA endonuclease activity [111], and has been shown to cleave palindromic branched DNA [112] as well as DNA near a tightly bound protein (streptavidin bound at biotin-tagged DNA end) [113]. We found that *sbcD* mutants are not hypersensitive to TBCs induced by M.EcoRII-C186A (Figure 6). Knockouts of SbcCD also display wild-type sensitivity to aza-C-induced DPCs (Krasich et al., unpublished data).

2.2.4 Helicases and sensitivity to TBCs

Helicases function in diverse cellular processes, and multiple studies implicate various helicases in responding to DNA-bound proteins. For example, Rep helicase allows replication fork progression past a protein-DNA complex [57,114,115], and also prevents the formation of DSBs following replication forks arrest [116]. UvrD helicase functions in NER and MMR and can remove the replication terminator protein, Tus, bound at *Ter* sites or RecA protein from filaments [83,84,117]. We found that strains lacking either Rep or UvrD helicase are hypersensitive to TBCs induced by M.EcoRII-C186A, with the *rep* mutant being the more hypersensitive of the two (Figure 6).

Notably, the response to aza-C-induced DPCs involving M.EcoRII was distinct, with *uvrD* mutants being hypersensitive, but not *rep* mutants [48] (Krasich et al., unpublished data). Given Rep's well-studied ability to remove proteins bound to DNA, it seems likely that Rep removes the mutant M.EcoRII from TBCs but is unable to remove M.EcoRII from aza-C-induced DPCs, due to the covalent linkage (see Discussion). A

knockout of UvrA, which functions in nucleotide excision repair, is not sensitive to TBCs induced by M.EcoRII-C186A, demonstrating that some UvrD function outside of NER is important. Likewise, a UvrA knockout is not hypersensitive to aza-C-induced DPCs, excluding NER as an important component of DPC repair with these lesions [45,46,48,118] (Krasich et al., unpublished data).

RecG helicase functions in DSB repair and can catalyze branch migration of forked DNA structures and Holliday junctions [26,27]. RecG mutants are hypersensitive to both M.EcoRII-C186A-induced TBCs (Figure 6) and aza-C-induced DPCs [48] (Krasich et al., unpublished data). The role of RecG could involve branch migration during RecBCD-mediated DSB repair, and/or modulation of blocked replication forks.

HelD is a helicase that functions in the RecF pathway of HR [119]. Even though the RecF pathway appears to be important in surviving TBCs induced by M.EcoRII-C186A, a *helD* knockout was not hypersensitive (Figure 6). Finally, DinG helicase has a poorly understood role in DNA repair and replication [120]. We found that a *dinG* knockout is not hypersensitive to TBCs induced by M.EcoRII-C186A (Figure 6), but we recently showed that it is hypersensitive to aza-C-induced DPCs (Krasich et al., unpublished data), consistent with a special role in repairing DNA-protein crosslinks.

Mfd, the transcription-coupled repair factor in bacteria, removes RNA polymerase stalled at DNA damage, such as UV-induced pyrimidine dimers, and recruits the nucleotide excision repair machinery [121]. Mfd is unable to promote fork movement through the TBC generated by a tight-binding restriction nuclease mutant (EcoRI-E111G) *in vitro* [57], but it does somehow promote the rapid recovery of gene expression following UV-induced DNA damage [122]. An Mfd knockout mutant is not

hypersensitive to TBCs induced by M.EcoRII-C186A (Figure 6). We previously found that inactivation of Mfd also does not cause hypersensitivity to aza-C-induced DPCs, arguing that it plays no unique role in releasing RNA polymerase stalled at DPCs [48,49].

2.2.5 FtsK and SsrA (tmRNA) protect against TBCs

We tested a number of other knockout mutants, involved in diverse cellular functions that could conceivably play a role in protecting cells from TBCs. FtsK is an essential cell division protein linking cell division and chromosome segregation. We previously isolated an *ftsK* insertion mutant in a transposon mutagenesis screen for mutants hypersensitive to aza-C-induced DPCs (Krasich et al., unpublished data). The location of *ftsK* in *E. coli* K12 is nucleotide 933224 through 937213. The last nucleotide in the transposon mutants is 937143 (2% disrupted) and 934308 (75% disrupted). The essential N-terminal 210 amino acids are contained in both mutants. These mutants are expected to be viable, but unable to stimulate XerCD recombination [123]. Here, we find that the *ftsK* insertion mutant is also hypersensitive to TBCs induced by M.EcoRII-C186A (Figure 6). Hypersensitivity could reflect some important role in segregation of chromosomes after damage, for example assisting XerCD in chromosome dimer resolution following HR induced by DPCs or TBCs (Figure 9). Alternatively, FtsK is capable of stripping proteins off DNA *in vitro* [124] and perhaps has a function in removing the tightly-bound M.EcoRII-C186A protein (Figure 9).

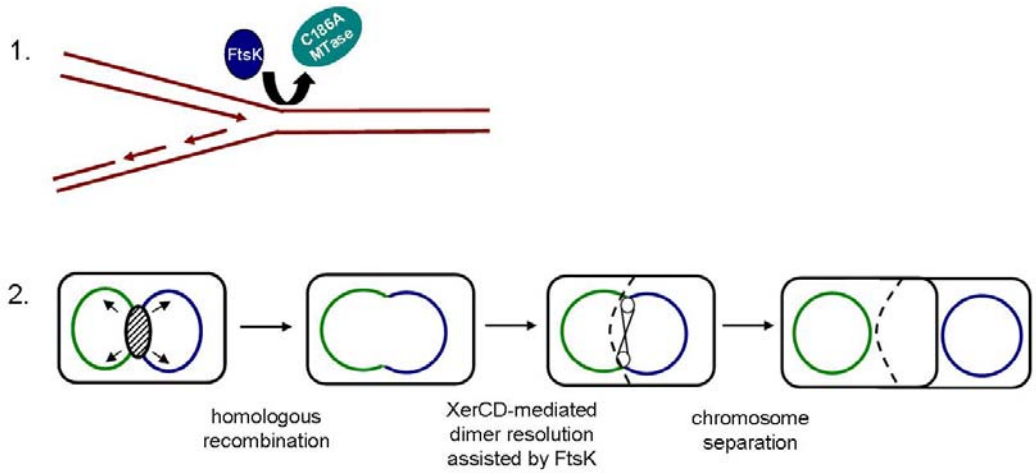

Figure 9: Two models of involvement of FtsK in TBC repair

1. Removal of M.EcoRII-C186A-induced TBC. 2. Chromosome dimmer resolution with XerCD (modified from [125])

SsrA (tmRNA) releases stalled ribosomes from the end of an mRNA lacking a stop codon. We showed previously that *ssrA* mutants are hypersensitive to aza-C-induced DPCs, arguing that tmRNA plays an important role in clearing stalled ribosome-mRNA complexes generated after transcription is blocked by aza-C-induced DPCs [49]. Likewise, we find that an *ssrA* knockout mutant is hypersensitive to TBCs induced by M.EcoRII-C186A (Figure 6). We therefore propose that M.EcoRII-C186A, bound to its recognition sites, can block RNA polymerase and the coupled translation machinery in much the same way as covalently attached M.EcoRII (Figure 10).

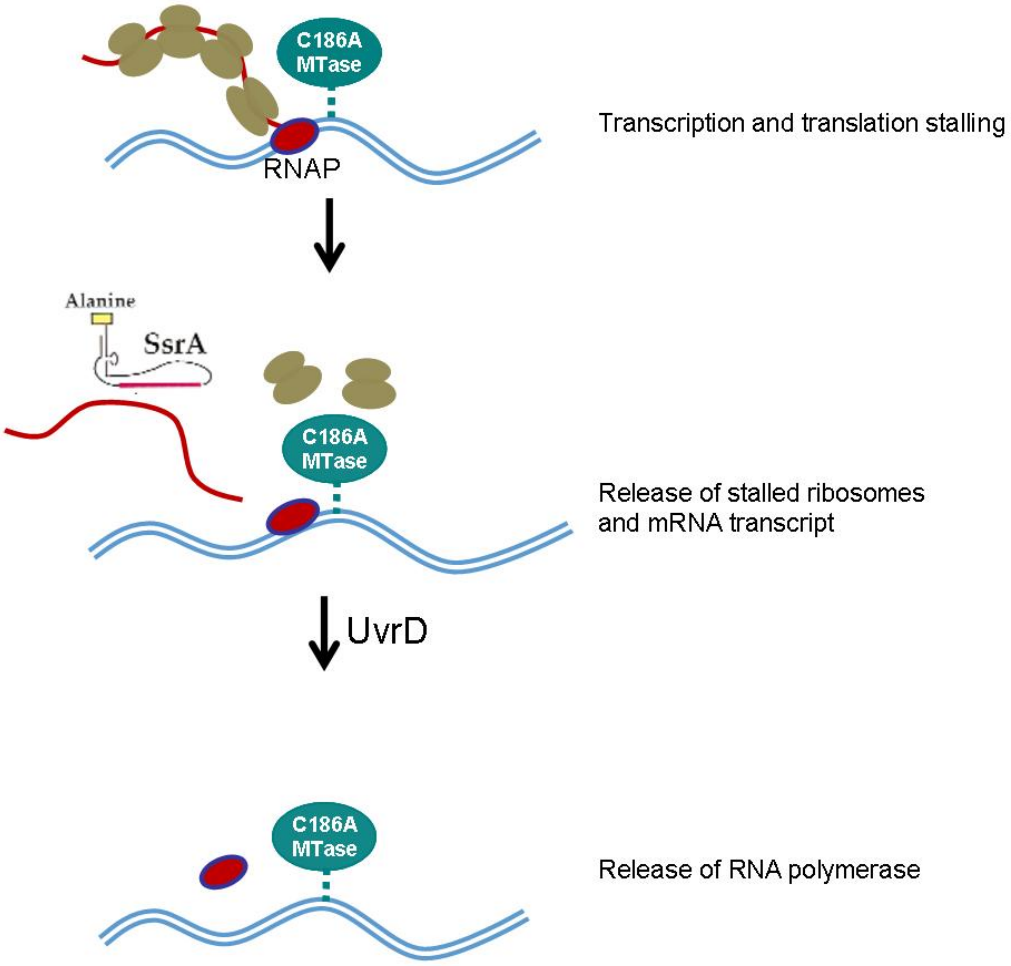

Figure 10: "Chain-reaction" model for clearing blocked elongation complexes

SsrA (tmRNA) clearing stalled ribosome-mRNA complexes generated after transcription is blocked by M.EcoRII-C186A-induced TBC. C186A Mtase: M.EcoRII-induced TBC, RNAP: RNA Polymerase

Both *dnaJ* and *hflC* mutants are hypersensitive to aza-C-induced DPCs [49]. Either or both of these proteins could potentially function in processing the protein within DPCs, since DnaJ is a chaperone that assists in protein folding, while HflC is part of the HflK-HflC complex which interacts with FtsH to regulate the degradation of various proteins [126,127]. We found that neither *dnaJ* nor *hflC* knockout mutants are hypersensitive to M.EcoRII-C186A (Figure 6), suggesting that DnaJ and HflC might play a special role in degrading covalently bound proteins that cannot be dislodged by a helicase (see Discussion).

XseA is the large subunit of Exonuclease VII (ExoVII) [128], and ExoVII-deficient mutants are sensitive to both UV irradiation [129] and nalidixic acid [130], for unknown reasons. We found that an *xseA* mutant shows wild-type levels of sensitivity to the TBCs induced by M.EcoRII-C186A (Figure 5). Finally, DNA polymerase II (Pol II; encoded by *polB*) functions as a polymerase and exonuclease with roles in replication restart following UV exposure, translesion synthesis and nucleotide excision repair (NER) [131,132,133,134,135]. Knockout mutants in *polB* are not hypersensitive to TBCs induced by M.EcoRII-C186A (Figure 6). Pol II also does not contribute to survival, mutagenesis, or restoration of DNA synthesis following UV irradiation [136] or survival from aza-C-induced DPCs [48].

2.2.6 Conflicting results between two *rarA* knockouts

RarA is a highly conserved DNA-dependent ATPase, with orthologs Mgs1 (yeast) and Wrnip1 (mammal) which are involved in replication fork stability [137]. They are

classified in the clade of "clamp loader" AAA+ proteins based on DNA sequence and structure [138,139,140]. Studies suggest that RarA and RecA are functionally redundant in rescuing stalled replication forks [141]. RarA colocalizes with SeqA and RecQ at the replication fork, possibly rescuing stalled replication forks [142]. Previously, the only phenotypes observed in a *rarA* knockout also require *xer* or *dif* mutations. We have discovered that the BW27784 Δ*rarA* knockout, created by P1 transducing a *rarA::*Kan construction created in the Mike Cox lab, is hypersensitive to M.EcoRII-C186A induced TBCs (Figure 11). However, the Keio collection *rarA* knockout was not sensitive to the M.EcoRII-C186A induced TBCs (Figure 11).

We tried to clarify these contradictory results using a complementation test. Plasmid pEAW354, a *rarA* overproduction vector, was transformed into the BW27784 Δ*rarA* Cox lab knockout. Based on plasmid pET-21a, pEAW354 has a T7 promoter and requires T7 RNA polymerase for full expression. We attempted complementation, relying only on leaky expression, in the absence of T7 RNA polymerase. The negative result we obtained, of the plasmid failing to compliment the *rarA* knockout, is unfortunately not conclusive (Figure 11). Surprisingly, the Keio *rarA* knockout actually seemed to be resistant to arabinose treatment. Further studies are needed to investigate the possible role of RarA in response to TBCs.

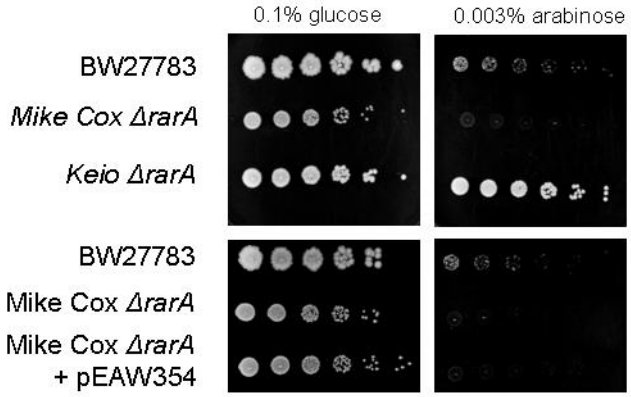

Figure 11: Sensitivity of ∆*rarA* mutants to M.EcoRII-C186A

Overnight cultures of BW27783 (wild-type) and the indicated BW27783 derivatives, all containing plasmid pBAD-MEcoRII-C186A, and plasmid pEAW354 when indicated, were serial diluted five-fold and spotted onto LB plates containing chloramphenicol with either glucose (0.01%) or arabinose (0.003%) and incubated at 37° overnight.

2.3 Discussion

In this work, we analyzed fork-blockage by M.EcoRII-C186A-induced TBCs, and the sensitivities of candidate mutants. A powerful aspect of our system is the ability to compare aza-C-induced DPCs involving wild-type M. EcoRII with M.EcoRII-C186A-induced TBCs, formed at the same M.EcoRII DNA recognition sequence. Using the same 2D gel electrophoresis technique employed for the aza-C-induced DPCs [43], we can directly compare the replication intermediates formed in the presence of DPCs and TBCs. We determined that an M.EcoRII-C186A-induced TBC could also block replication forks *in vivo* (Figure 5).

Strikingly, the 2D gel patterns after aza-C-induced DPC formation with wild-type M.EcoRII [43] showed two prominent features that were not seen here after TBC formation with M.EcoRII-C186A. First, aza-C-induced DPCs caused an accumulation of RecA-dependent X structures, presumably due to RecA-dependent recombination or replication fork regression. Second, a prominent Y-arc with spots was seen after DPC formation, and attributed to the induction of rolling-circle replication (confirmed by EM analysis; [43]). These results indicate that DPCs lead to more frequent fork breakage than TBCs.

Table 2: Proposed models for involvement in repair of M.EcoRII-C186A-induced TBCs, quinolones, and aza-C-induced DPCs

This table summarizes the proposed models for involvement in repair of M.EcoRII-C186A-induced TBCs, quinolones, and aza-C-induced DPCs. X indicates that proposed model applies.

	M.EcoRII-C186A-induced TBCs	Quinolones	aza-C-induced M.EcoRII DPCs	Proposed model for involvement
DinG			X	prevent replication/transcription collisions
DnaJ			X	processing the protein within DPCs; chaperone that assists in protein folding
FtsK	X	X	X	stripping a protein from DNA or role in assisting XerCD in chromosome dimer resolution
HflC			X	processing the protein within DPCs; involved in regulation of protein degradation
RecA	X	X	X	homologous recombination
RecBCD	X	X	X	RecBCD assisted RecA-HR may play a direct role in repairing DNA-protein complexes or repairing downstream DNA damage such as replication forks that are broken after fork blockage
RecFOR	X			RecFOR assisted RecA-HR or stabilization of stalled replication fork
RecG	X	X	X	branch migration following RecA-HR or modulation of blocked replication forks
Rep	X	X		removal of protein
RuvABC	X	X	X	Holliday junction resolution following RecA-HR
SsrA	X		X	clearing blocked elongation complexes
UvrD	X	X	X	removal of RNAP following SsrA clearing blocked elongation complexes

Based on this study of TBCs induced by M.EcoRII-C186A and past studies of aza-C-induced DPCs induced by wild-type M.EcoRII, we now have a direct comparison of cellular functions that protect against the same tightly bound protein in either non-covalent or covalent form (summarized in Table 4, Chapter 4). Several gene products are needed for protection against both kinds of complexes, notably RecA, RecBC, RecG, RuvABC, UvrD, FtsK, and SsrA (tmRNA). The first group of functions could play key roles in processing blocked replication forks or downstream DNA breaks resulting from blocked forks, given that both kinds of M.EcoRII complex block replication forks (Figure 5; [43]). Involvement of the tmRNA pathway indicates that both kinds of complex also block the coupled transcription-translation machinery (see [49]). We have speculated on the possible roles of FtsK above.

A very notable difference was observed between the responses to the two kinds of M.EcoRII complexes: the RecFOR pathway is important in the response to TBCs induced by M.EcoRII-C186A but not to DPCs induced by aza-C with the wild-type protein. By analogy with the role of RecFOR in response to UV damage, we infer that a pathway of fork protection (by RecFOR and RecA) after blockage by the TBC sometimes allows a productive replication restart event – perhaps by inducing helicase-driven dissociation of the blocking M.EcoRII-C186A (presumably by Rep, possibly with assistance from UvrD). Note that the *rep* knockout mutant was uniquely sensitive to TBCs induced by M.EcoRII-C186A, and that Rep helicase has previously been shown to play roles in dissociation of proteins from DNA (see Introduction).

These genetic results are consistent with the physical analyses of replication intermediates resulting from the two kinds of complexes. While both TBCs and DPCs

lead to fork blockage (accumulation of bubble molecules), only the DPCs led to Y forms and X structures (Figure 5; [43]). Therefore, the ability of the RecFOR pathway to stabilize the forks stalled by TBCs may minimize fork breakage and permit Rep to dissociate the TBC.

Replication forks are blocked by both a single TBC induced by M.EcoRII-C186A and arrays of TBCs created with tandem repressor-operator complexes (Figure 5; [55]) . Inactivation of RecA, RecBCD, or RecG causes sensitivity to both types of TBC, implying that DSBs are formed in both systems following replication blocks (Figure 6; [56]). It is also interesting to note that Rep and UvrD play redundant roles in surviving the 34-copy TBC arrays consisting of transcription factors, with only the double knockout mutant showing a phenotype [57], while each single mutant is sensitive to TBCs induced by M.EcoRII-C186A. This is consistent with the possibility that UvrD plays some other important role in survival after formation of TBCs induced by M.EcoRII-C186A. It is very interesting that RecFOR and RuvABC protect from TBCs induced by M.EcoRII-C186A, but not from arrays of TBCs created with tandem repressor-operator complexes (Figure 6; [56]). This suggests that fork stabilization by RecFOR is not important in rescuing forks blocked by tandem repressor-operator complexes. Further studies are needed to deduce whether these differing protein requirements relate to the tandem nature of the arrayed TBCs or perhaps to the different binding strength of the two kinds of protein (very tight binding mutant M.EcoRII versus weaker binding transcription factors).

A direct comparison of sensitivity to M.EcoRII protein within DPCs versus TBCs also revealed a novel role for both DinG helicase and HflC chaperone in survival after DPC formation. These two proteins apparently play some special role that is dictated by

the existence of the covalent DNA-protein bond. One possibility is that they act directly on the DPC, perhaps with DinG playing a role in DPC recognition along the DNA and HflC acting on the covalently bound protein after recognition. A different interpretation is that one or both play some unique role in rescuing the more frequent fork breakage events that appear to occur with the DPC.

2.4 Materials and Methods

2.4.1 Materials

Restriction enzymes were from New England Biolabs; Nytran membranes from Schleicher & Schuell; QuickChange Mutagenesis Kit from Stratagene; Random Primed DNA Labeling Kit from Roche Applied Science; and radiolabeled nucleotides from Perkin-Elmer Life Science. LB (Luria broth) contained bacto tryptone (10 g/L), yeast extract (5 g/L), and sodium chloride (10 g/L).

2.4.2 Plasmids

Plasmid pBAD-MEcoRII contains the wild-type M.EcoRII coding sequence between the KpnI and SphI sites in the multiple cloning region of pBad33, downstream of the *araBAD* promoter [49]. Plasmid pBAD-MEcoRII contains a chloramphenicol resistance gene and is based on the pACYC184 replicon. Plasmid pBAD-MEcoRII-C186A, containing the active site mutant version of the M.EcoRII coding sequence, was created by mutating the active site cysteine codon of M.EcoRII within plasmid pBAD-MEcoRII using Stratagene QuickChange Mutagenesis Kit [60]. Plasmid pBR322-C1060A is a derivative of pBR322 with a cytosine to adenine mutation at location 1060, destroying one M.EcoRII recognition site in the plasmid [43]. Plasmid pEAW354, which

contains *rarA* inserted between the NdeI and BamHI sites of vector pET21A (Novagen) along with ampicillin resistance gene, was obtained from the Mike Cox lab (University of Wisconsin, Madison).

2.4.3 *E. coli* strains

Strain BW27783 [F-, *Δ(araD-araB)567*, *ΔlacZ4787(::rrnB-3)*, λ-, *Δ(araH-araF)570(::FRT)*, *ΔaraEp-532::FRT*, φ*Pcp8araE535*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*)] allows for homogenous and titratable expression from pBAD vectors [143] and was obtained from the lab of HP Erickson (Duke University). *E. coli* knockout mutants from the Keio collection [144] contain kanamycin resistance gene inserts, and are identified in this thesis as Δ followed by gene name (e.g. Δ*recA*). *E. coli* transposon mutants (kanamycin resistance gene) were created in a recent genetic screen (Krasich et al., unpublished data; also see [49]) and are identified here as insertions (e.g. *ssrA::Kan*). In each case, the desired mutation was moved into the BW27783 background via P1 transduction [145], selecting for kanamycin resistance, and confirmed by PCR. The kanamycin-resistance cassette was not removed from the tested strains. The *recF4115* missense mutant (K36Q) was moved into BW27783 using P1 transduction, selecting for a linked Tn10 marker (in *tnaA;* [146]); transductants were screened by DNA sequencing for co-transduction of the *recF* mutation. The RarA knockout strain was created by P1 transducing the *rarA* mutation from EAW98 (obtained from the Mike Cox lab; MG1655 Δ*rarA*) into BW2783. The *rarA* gene is replaced from start codon to stop codon with FRT sites and a Kanamycin resistance gene.

2.4.4 2D gels to visualize DNA intermediates

Replication intermediate were visualized by 2D gel electrophoresis using a procedure similar to that of Kuo et al [43]. Overnight cultures of BW27783 containing plasmid pBAD-MEcoRII-C186A and either pBR322 or pBR322-C1060A were grown with and without 0.01% glucose until OD_{560} = 0.3. Arabinose (0.00005%) was then added to the no-glucose culture and 4-mL samples were collected after 60 min at 37°C. Samples were pelleted and frozen at -80°C. Cell pellets were resuspended in 500 µL of Triton lysis butter [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 1% Triton X-100, and lysozyme at 1.8 mg/mL] and incubated at 65°C for 20 min. Proteinase K (0.5 mg/mL) and SDS (0.2%) were added to the samples and incubated at 55° C for 1 h. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and dialyzed against TE [10 mM Tris-HCl (pH 7.8), 1 mM EDTA] at 4°C overnight. The DNA (50 µL) was digested with Pst1-HF for 2 h at 37°C. Digested DNA was separated by size in the first dimension gel (0.4% agarose) run in 0.5X Tris-borate EDTA (TBE) buffer at 1 V/cm for 29 hours. The desired slices were cut from the gel, rotated 90° counterclockwise, and cast within the top of the second-dimension gel (1% agarose with ethidium bromide at 0.3 ug/mL). The second dimension gel was run at 4.5 V/cm for 15 hours at 4° C with recirculated 0.5X TBE containing ethidium bromide (0.3 µg/mL). The gels were analyzed by Southern hybridization with a 1,158-bp gene probe from pBR322 that does not hybridize with plasmid pBAD-MEcoRII-C186A. The probe was generated by PCR amplification from plasmid pBR322 using primers 5'-CGGTATTCGGAATCTTGCAC-3' and 5'-AGCTCGTTGAGTTTCTCCAG-3' and purified using the DNA Clean & Concentrator Kit

(Genesee Scientific). The probe was ^{32}P-labeled using the Random Primed DNA Labeling Kit (Roche Applied Science). Southern blots were visualized by PhosphorImager.

Chapter 3: *E. coli* response to quinolone antibiotics

3.1 Introduction

Topoisomerases are enzymes that regulate topology (underwinding or overwinding) of DNA. To regulate the topology, topoisomerases bind the DNA and cut the phosophodiester backbone. Type II topoisomerases create two staggered cuts covalently attached at their 5' ends to the topoisomerase in a reaction intermediate known as the cleavage complex. The cleavage complex allows topoisomerase to pass another segment of double-stranded DNA through the transient break to correct the DNA topology and once complete, the DNA backbone is resealed (for reviews, see [147]).

In eukaryotes, topoisomerase II can be inhibited by chemotherapeutic drugs, including doxorubicin and etoposide, that stabilize the cleavage complex. There is evidence that this stabilization of the cleavage complex is necessary for the cytotoxicity [148]. In our lab, studies with phage T4 showed that replication forks were blocked at the site where the chemotherapeutic drugs stabilized the cleavage complex [149].

In *E. coli,* topoisomerase IV [50] and topoisomerase II (gyrase) [51,52] are inhibited by the quinolones antibiotics. Quinolones stabilize the cleavage complex, which is a unique form of DPC. Like aza-C-induced DPCs and M.EcoRII-induced TBCs, quinolone-stabilized DNA gyrase cleavage complexes block replication forks *in vivo* [53]. The mechanism(s) by which quinolone-stabilized cleavage complexes lead to DSB formation and ultimately cell death have been extensively studied but are still not well understood at the molecular level. The formation of the cleavage complex is necessary for the cytotoxicity of quinolones (for reviews, see [112,113,114,115]). However,

formation of the cleavage complex is not sufficient to cause cell death. Cytotoxicity likely results from the conversion of cleavage complexes into a DNA break, but the mechanism of break formation is not known [150]. The cleavage complexes are reversible upon drug removal both in vivo and in vitro [111,116]. Blocking protein synthesis with chloramphenicol protects from the cytotoxic action of nalidixic acid [151], implying that production of a protein is necessary for cytotoxicity.

There is much evidence that the cytotoxic lesion from quinolones is a DSB, including that a functional RecBCD helicase/ nuclease complex is necessary for the induction of SOS in response to quinolones [21] and *recBCD* mutants are hypersensitive to quinolones [106]. As discussed in Chapter 1, RecBCD processes DSBs to allow for RecA-mediated repair. Chromosomal breaks have also been seen after nalidixic acid treatment [152], but these breaks are likely not the same break at the cleavage complex, as the cleavage complex alone is not sufficient for cytotoxicity [150]. One model for how the quinolone-stabilized cleavage complex leads to a DSB is that a nuclease such as SbcCD cleaves the DNA adjacent to the DPC [113]. SbcCD has double-strand DNA exonuclease activity as well as single-strand DNA endonuclease activity [111], and has been shown to cleave palindromic branched DNA [112] as well as DNA near a tightly bound protein (streptavidin bound at biotin-tagged DNA end) [113]. Further support for this model is evidence that Mre-Rad50 nuclease complex can cleave DNA next to a DPC created with the meiotic recombination initiator protein Spo11 in *Saccharomyces cerevisiae* [153]. Another model, shown in phage T4 for a different topoisomerase inhibitor, is the "collateral damage model" where recombination nucleases that act on the forks blocked at the cleavage complex create the DNA breaks [154]. Finding novel

mutants with unique quinolone responses would be useful in uncovering more about the molecular mechanisms of quinolone cytotoxicity.

3.1.1 Is replication necessary for the cytotoxicity of quinolones?

There are contradictions in the literature about the necessity of replication for cytoxicity of quinolones. Gudas and Pardee used a temperature-sensitive *dnaA* mutant, unable to initiate new rounds of replication, to show that DNA replication is necessary for the induction of the SOS reponse by nalidixic acid [155]. However, Sassanfar and Roberts used a temperature-sensitive *dnaC* mutant which cannot load the replicative helicase, DnaB, and showed that nalidixic acid can still induce SOS without ongoing DNA replication [156]. Zhao et al. used a temperature-sensitive *dnaB* mutant, and showed that ongoing DNA replication is not required for killing by nalidixic acid [157]. It is plausible that DNA replication is necessary for killing by quinolones, because the quinolone-stabilized DNA gyrase cleavage complexes have been shown to block replication forks *in vivo* [53]. To clarify the conflicts in the literature, a system that blocks replication, without detrimental effects on the cell, would be useful.

3.1.2 Why do mutations in TCA cycle enzyme, isocitrate dehydrogenase, confer resistance to low-levels of quinolones?

For over three decades it has been know that mutations in *icdA*, which encodes for isocitrate dehydrogenase, lead to resistance to low-levels of the quinolone nalidixic acid, but the cause of this resistance has not been established [158]. The Helling lab hypothesized that these mutants accumulate an intermediate, such as citrate, which upregulates the AcrAB-TolC efflux pump, which then pumps out the intermediate along

with the antibiotic. They supported this hypothesis by showing evidence that the resistance seen in an *icdA* mutant is lost in the *acrA icdA* double mutant [159]. However, their conclusion was based on only a single data point, and another key data point was missing.

The Collins lab's model for cell killing by three major classes of antibiotics, quinolones, β-lactams, and aminoglycosides, is that these antibiotics stimulate the production of highly deleterious hydroxyl radicals. They hypothesized that the resistance in the *icdA* mutant is due to the depletion of NADH pools, which decreases levels of oxidative DNA damage [160]. This proposed model is a large shift from current models, and was the subject of a review published shortly after, pointing out several caveats [161]. More recent studies have provided strong experimental evidence against the Collins' model [162,163]. I revisited this issue by creating *icdA* mutants and *acrA icdA* double mutants, to test if knocking out the AcrA-TolC pump did indeed eliminate the quinolone resistance of an *icdA* mutant.

3.2 Results

3.2.1 Is replication necessary for the cytotoxicity of quinolones?

The nucleotide analog and anti-HIV drug, Azidothymidine (AZT), acts as a chain terminator, because the azido group replaces the 3' OH of thymidine (Figure 12; reviewed in [164]). In *E. coli*, treatment with AZT quickly stops replication [134]. We performed experiments using the drug AZT to block replication, and determined if cell killing by the quinolone nalidixic acid was reduced. We learned that AZT is not a good tool for this test, because it also leads to extensive killing (approximately 20-fold in 1

hour; see Figure 13). AZT was also not an ideal system, because it leads to blocked replication forks on its own and it could be difficult to separate the effects of replication blockage and cell killing by quinolones to ensure that the cellular effects seen are due to the toxic effects of the quinolones.

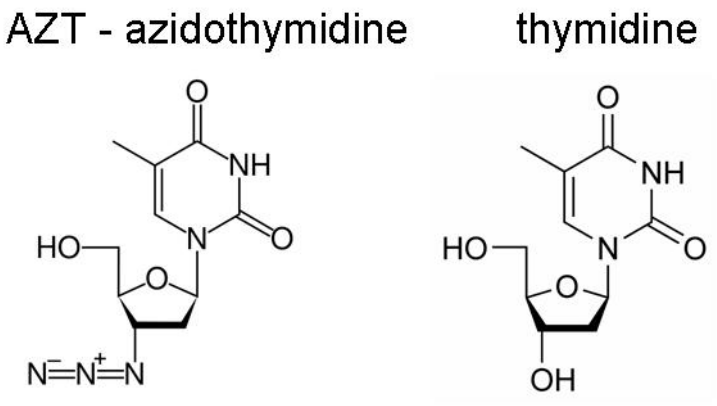

Figure 12: Structure of Azidothymidine (AZT) and thymidine

AZT is a nucleotide analog with an azido group replacing the 3' OH of thymidine.

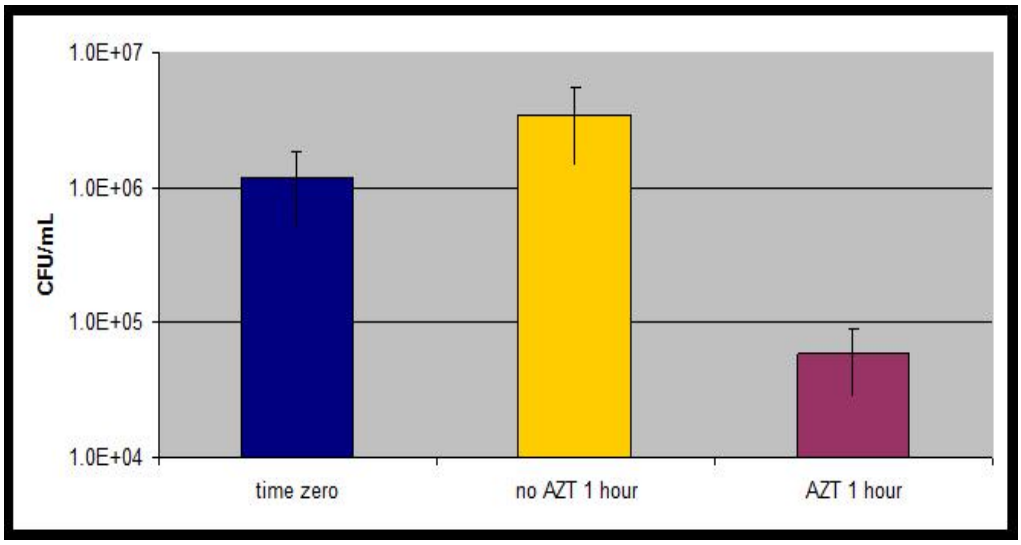

Figure 13: Cells death induced by AZT treatment

Wild-type strain JH39 was grown overnight, diluted 1:100 in LB and incubated for 2 hours at 37° to reach OD_{560}=0.5. The culture was then diluted to measure a pre-drug, time zero cell count (CFU/mL). The culture was split into two parts, receiving either AZT (0.25 µg/mL) or no drug and incubated for 60 minutes at 37°. The culture was then diluted to measure a post-drug cell count (CFU/mL). N=3. Results were plotted on a log scale.

Use of a plasmid expressing coliphage N4 *orf8* looked promising, because it was reported to shut off host DNA replication without killing the cells or inhibiting host transcription and translation [165]. Coliphage N4 *orf*8 inhibits DNA polymerase III holoenzyme *in vitro,* by targeting the clamp loader [165]. When we repeated an assay expressing this plasmid, in four different wild-type strains (MG1655, W3110, W3350 and BW25113), we were surprised to see approximately 500-fold killing, after only 20 minutes of *orf8* expression (Figure 14). We were unable to determine why, in our hands, cells are killed after *orf8* is expressed, while the Rothman-Denes lab saw a bacteriostatic effect.

We hoped that reducing expression of *orf8* would inhibit replication without killing the cells. The *orf8* pBad expression plasmid was therefore moved into the BW27783 strain, for homogenous and titratable expression from the pBAD plasmid [143]. When the arabinose concentration was too low (1.25×10^{-5} %), cell growth was not inhibited completely, but compared to the no arabinose control, we see some inhibition (Figure 15). When arabinose concentration was increased (2.5×10^{-4} % and 5.0×10^{-3} %), cells were killed approximately 30-fold (Figure 15). The killing may be due to the creation of blocked replication forks, which are processed into a toxic intermediate. In summary, neither AZT, nor *orf8* expression, created an applicable system to test the requirement of DNA replication for the cytotoxicity of quinolones. Instead, we found that these two replication inhibitors induce substantial cell killing on their own.

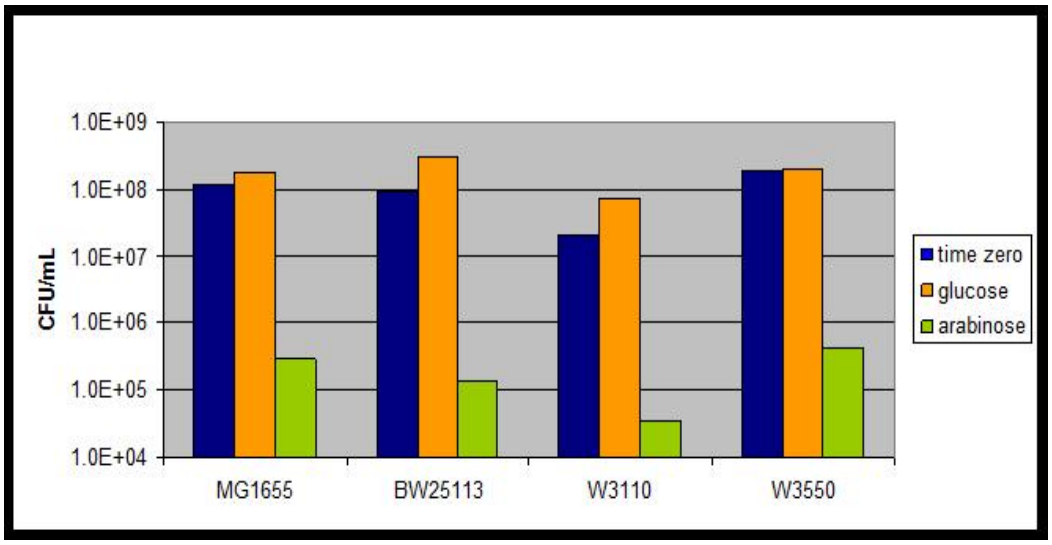

Figure 14: Cell death induced by expressing coliphage N4 *orf8*

Wild-type strains MG1655, BW25113, W3110, and W3350 were grown overnight, diluted 1:100 in LB + 50 µg/mL amp and incubated 1.5 hours at 37° to reach OD_{560}=0.3-0.4. The culture was then diluted to measure a pre-sugar, time zero cell count (CFU/mL). The culture was split into two parts receiving either 0.2% arabinose or 0.2% glucose and incubated 20 minutes at 37°. The culture was then diluted to measure a post-sugar cell count (CFU/mL).

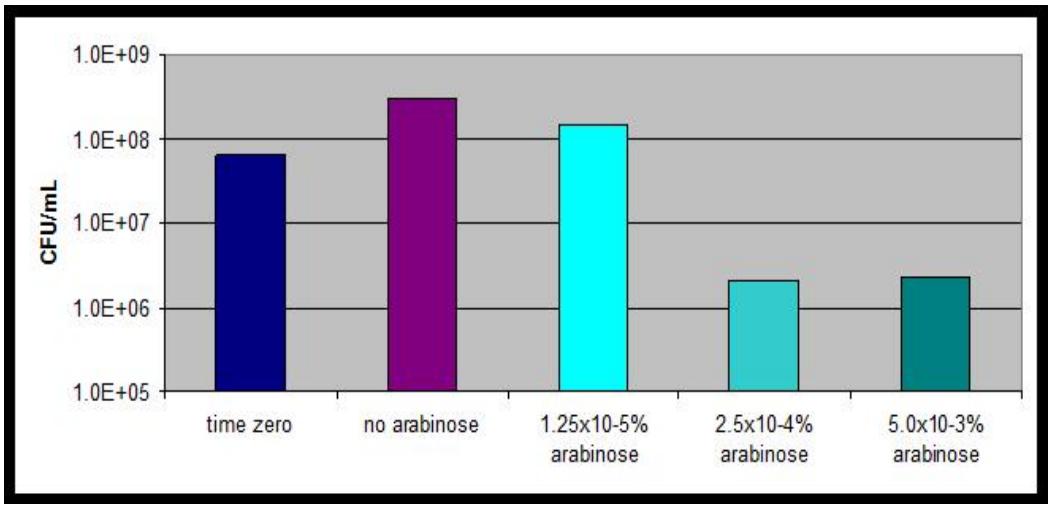

Figure 15: Cell survival with Arabinose titration of *orf8* expression in BW27783

Wild-type strain BW27783 was grown overnight, diluted 1:100 in LB + 50 µg/mL amp and incubated 1.5 hours at 37° to reach OD_{560}=0.3-0.4. The culture was then diluted to measure a pre-arabinose, time zero cell count (CFU/mL). The culture was split into two parts receiving varying arabinose concentrations (0%, 1.25 x 10^{-5} %, 2.5x10^{-4} %, and 5.0 x 10^{-3} %) and incubated for 20 minutes at 37°. The culture was then diluted to measure a post-arabinose cell count (CFU/mL).

3.2.2 Screen for Nalidixic acid bacteriostatic mutants

As a strategy to elucidate the mechanism of cell killing by quinolones, I started a screen to find a transposon insertion mutant that is bacteriostatic in response to Nalidixic acid (nal). Finding a gene knockout that leads to a nal bacteriostatic phenotype could provide insight into the functions involved in killing by quinolones. Previous screens for nalidixic acid resistant mutants looked for mutants that could form colonies on nalidixic acid plates. I screened for mutants with increased survival after 1.5 hours of exposure to 4 µg/mL nalidixic acid and created a collection of potential nalidixic acid bacteriostatic mutants. In a secondary screen, the wild-type control was killed about 25-fold while mutants were killed 4- to 10- fold. Unfortunately, P1 transductions showed that the transposon insertions were not responsible for the phenotype. We decided to suspend further work on this project due to that negative result.

3.2.3 Why do mutations in TCA cycle enzyme, isocitrate dehydrogenase, confer resistance to low-levels of quinolones?

Isocitrate dehydrogenase, *icdA*, mutants are moderately resistant to nalidixic acid [158] and the Helling lab hypothesized that these mutants accumulate an intermediate, such as citrate, which upregulates the AcrAB-TolC efflux pump, which then pumps out the intermediate along with the antibiotic. To test this hypothesis, they compared the growth in the presence of nalidixic acid for wild-type, *icdA*, *acrA,* and *acrA icdA* mutants [159]. However, their conclusion was based on only a single data point, and another key data point was missing. I revisited this issue by creating *icdA*, *acrA,* and *acrA icdA*

mutants, to test if knocking out the AcrA-TolC pump did indeed eliminate the quinolone resistance of an *icdA* mutant. Knocking out the AcrA-TolC pump makes the cells much more sensitive to nalidixic acid, so the data is presented on two graphs with the appropriate scale. We obtained the opposite result of the Helling lab, seeing that the protection in an *icdA* mutant background was still present when the AcrA-TolC pump was knocked out (Figure 16). We conclude that the Helling lab model does not hold up to contradict the Collins lab model. However, recent studies did provide strong experimental evidence against the Collins lab model [162,163]. We suspect the resistance of the *icdA* mutant and other TCA-cycle mutants, is likely due to a slow-growth phenotype.

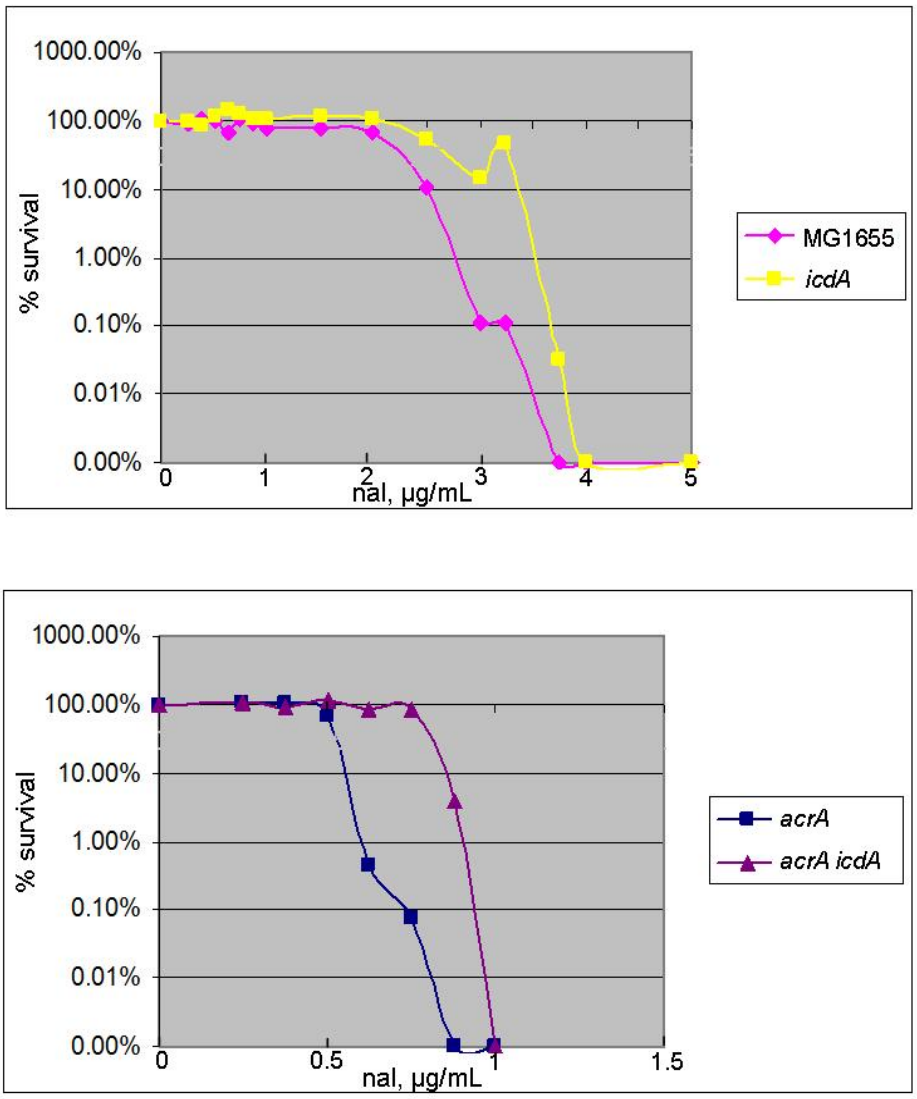

Figure 16: Comparing quinolone resistance of an *icdA* mutant in wild-type and *acrA* strains

MG1655 (wild-type) and indicated MG1655 derivatives were plated to nalidixic acid plates containing Nal at 0.25-1.5 µg/mL. Percent survival was calculated based on CFU on nal plates compared to no-drug control and plotted on a log scale.

3.2.4 Determination of the role of RecQ helicase in the response to quinolones

A strategy our lab has used to learn more about the response to quinolones is to screen for mutants with a defect in inducing the SOS response in the presence of quinolones. We visualize the SOS response using the JH39 strain, which contains a *dinD-lacZ* fusion [166,167]. Upregulation of *dinD*, during the SOS response, can be seen as dark blue on X-gal plates or measured quantitatively by a β-galactosidase assay [168]. Newmark used the X-gal plate method, and performed a transposon mutagenesis screen to find mutants that are defective in inducing the SOS response in the presence of quinolones, but not in response to UV exposure [136]. Mutants with transposon insertions in *recB* and *recC* were the only ones that fit the profile. Mutants with transposon insertions in 11 other genes were found to cause partial defects in SOS induction by one or both pathways.

Mutants that express the SOS response in a partially constitutive manner would have likely been missed by Newmark's screen, as they are always dark blue on X-gal plates, due to constitutive expression of the *dinD-lacZ* reporter. O'Reilly created a collection of these SOS constitutive mutants [169] and tested their SOS profile in response to nalidixic acid and MMC, in the liquid, quantitative assay. Like UV, MMC induces SOS though the RecFOR pathway. A mutant with a transposon insertion in *dnaQ*, which encodes the epsilon subunit of DNA polymerase III, fit the profile of interest. The *dnaQ* knockout showed no increase in SOS induction in response to quinolones, but a normal response to MMC [170].

We suspected that a *recQ* mutant might also fit the profile of interest, with a defect in SOS induction in response to quinolones, because RecQ helicase was shown to be required for SOS induction after blockage of replication forks in a temperature-sensitive *dnaE* mutant (polymerase III alpha subunit) [171]. I therefore created a *recQ* knockout and obtained evidence that *recQ* mutants are partially deficient in SOS induction by nalidixic acid using a quantitative β–Galactoside assay (Figure 17). An independent-samples, 2-tailed, t-test was conducted to compare the SOS response to nalidixic acid for wild-type and the Δ*recQ* mutant (comparing nal - uninduced levels; Table 3). There was a significant difference in the average activity levels for wild-type (A=72.0, SD=22.2) and Δ*recQ* (A=50.2, SD=15.7); n=9, p = 0.029. Although the decrease was modest, these results suggest that the Δ*recQ* mutant does have a statistically significant decreased SOS response to nalidixic acid compared to wild-type.

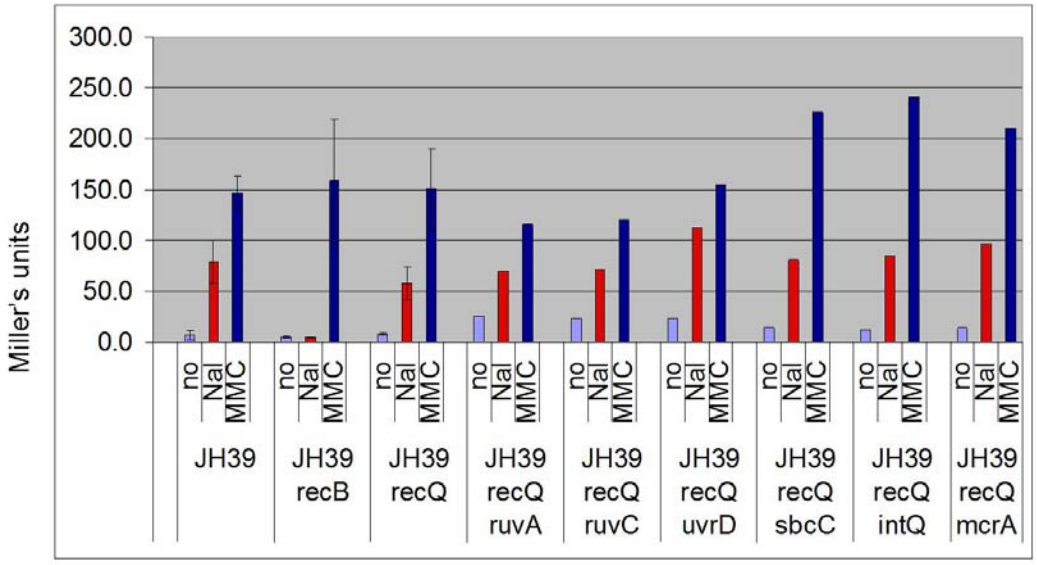

Figure 17: Miller's Assay of SOS induction in response to nalidixic acid in *recQ* double mutants

β-Galactosidase assay was performed on JH39 (wild-type) and the indicated JH39 derivatives. SOS levels were measured in response to nal (10 μg/mL), MMC (1 μg/mL), and a no-drug control. JH39, *recB*, and *recQ;* n=9, double mutants; n=1.

Table 3: Induction of β-Galactosidase by nalidixic acid

The data in this table are the measurements from Figure 17. *n*= number of colonies tested; ± is standard deviation; uninduced numbers are β-Galactosidase units expressed after 2 hours of growth in the absence of nal; + nal numbers are β-Galactosidase units expressed after 2 hours of growth in the presence of nal; nal - uninduced if the difference between β-Galactosidase units in the presence of nal and those in the absence of nal; *p=0.029 is the p-value for the comparison of JH39 *recQ* nal - uninduced to JH39 nal – uninduced.

Strain	*n*	uninduced	+ nal	nal - uninduced
JH39	9	6.9 ± 4.5	78.9 ± 20.7	72.0 ± 22.2
JH39 *recQ*	9	7.6 ± 0.9	57.9 ± 15.9	50.2 ± 15.7 *p=0.029
JH39 *recB*	9	4.9 ± 0.8	4.8 ± 0.6	-0.1 ± 0.6
JH39 *recQ ruvA*	1	24.5	69.9	45.4
JH39 *recQ ruvC*	1	22.6	71.8	49.2
JH39 *recQ uvrD*	1	22.8	112.4	89.6
JH39 *recQ sbcC*	1	14.3	81.2	66.9
JH39 *recQ intQ*	1	12	85.1	73.1
JH39 *recQ mcrA*	1	13.6	96.7	83.1

We were searching for a mutant with a complete loss of SOS induction in response to nalidixic acid, as seen in the *recB* mutant (Figure 17). The modest defect in the *recQ* mutant suggested redundant pathways, and so double mutants with a mutation in *recQ* and either *recJ, helD, uvrD, ruvA, ruvC, sbcC, sbcD, intQ* or *mcrA* were created and tested to see if the SOS response was completely lost. HelD and UvrD were chosen because they are also helicases, and could be performing a function similar to RecQ in response to quinolones. Exonuclease RecJ was chosen, because of its function with RecQ in the RecFOR pathway. Putative integrase IntQ and restriction endonuclease McrA were picked up in the Newmark screen as partially defective in the SOS response upon quinolone exposure [172]. RuvA and RuvC are subunits of the Resolvasome, and were candidates for involvement in the induction of the SOS response, because they can cleave replication forks in certain conditions [91]. SbcCD, a protein that has double-strand DNA exonuclease activity as well as single-strand DNA endonuclease activity [111], was a candidate, because it has been shown to cleave palindromic branched DNA [112] as well as DNA near a tightly bound protein (streptavidin bound at biotin-tagged DNA end) [113].

None of the double mutants were defective in SOS induction as visualized by the *dinD-lacZ* reporter construct on X-gal plates (Figure 18). The very light blue rings seen with the *recB* mutant is the phenotype we hoped to obtain. Viewing color intensity on plates is not clear enough to pick up on gradual differences in SOS induction levels. The indicated mutants were tested using a quantitative liquid assay, to determine if they had a defect in SOS induction specifically to nalidixic acid, but not MMC. SOS constitutive mutants *ruvA, ruvC* and *uvrD*, required testing using a quantitative liquid assay, as they

are always dark-blue on the X-gal plates. Induction levels comparing nal exposure to lack of nal exposure were calculated (Table 3). Some of the double mutants have a greater response to nal (nal - uninduced) than a *recQ* single mutant. This could be due to an increased sensitivity to nal. Also, double mutants were only tested one time and should be repeated at least two more times to look for reproducibility. Unfortunately, none of the double mutants showed a defect in SOS induction (Figure 17).

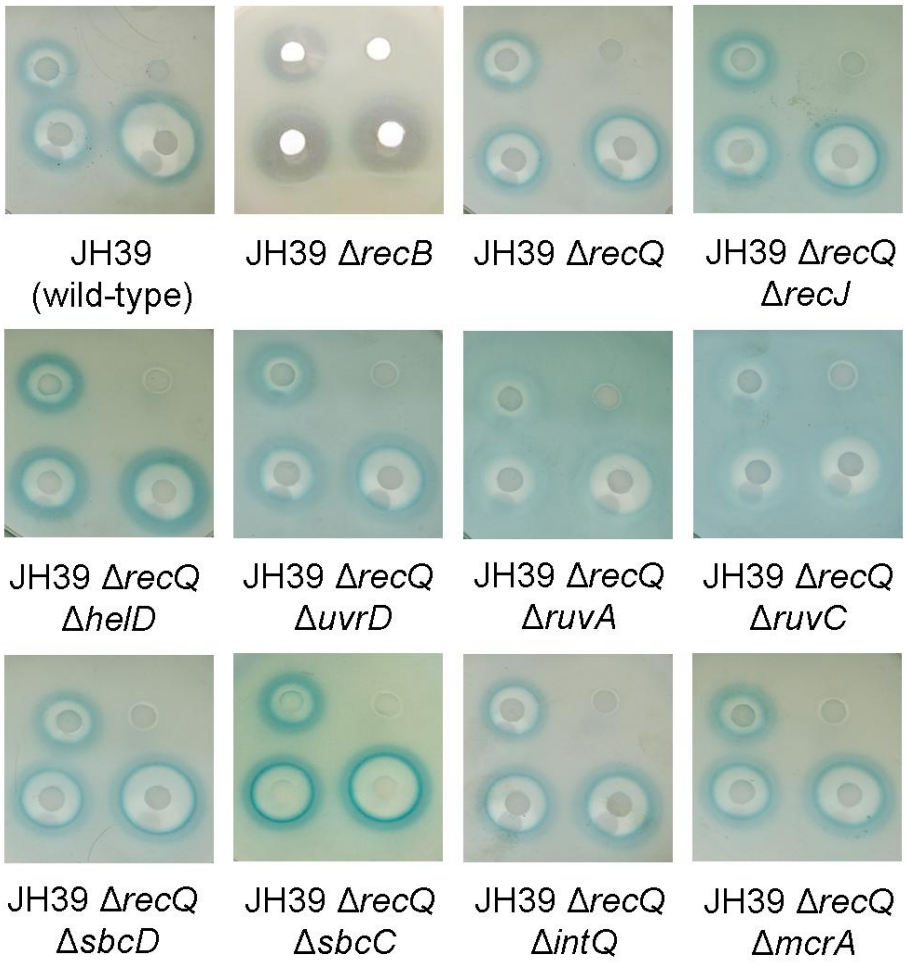

Figure 18: Plate assay of SOS induction in response to nalidixic acid in *recQ* double mutants

Overnight cultures of JH39 (wild-type) and the indicated JH39 derivatives were mixed with LB top agar and poured onto X-gal plates. 0, 10, 20, and 40 µg of nalidixic acid was pipetted onto the filter disk, starting in the top right corner, with concentrations increasing counter-clockwise. Plates were incubated at 37° overnight. The blue color indicates SOS induction, and the lack of color in the *recB* mutant indicates an inability to induce SOS.

One potential model for RecQ helicase's involvement in the response to quinolones is that it regresses a fork stalled at the site of the quinolone-stabilized cleavage complex. This regressed fork, which is also a Holliday junction, could be resolved into a double-strand break by RuvABC or processed with RecG branch migration, leading to cell death unless the break is repaired by RecBCD. I tested this model by creating a *recQ recB* double mutant. If the model was correct, we would have expected the double mutant to be less sensitive to the drug than the hypersensitive *recB* single mutant, because fork regression and break formation should be prevented. The *recQ recB* double mutants were just as sensitive as the *recB* single mutants, not supporting this model (Figure 19). The *recQ* single mutant displayed the same sensitivity as wild-type (Figure 19).

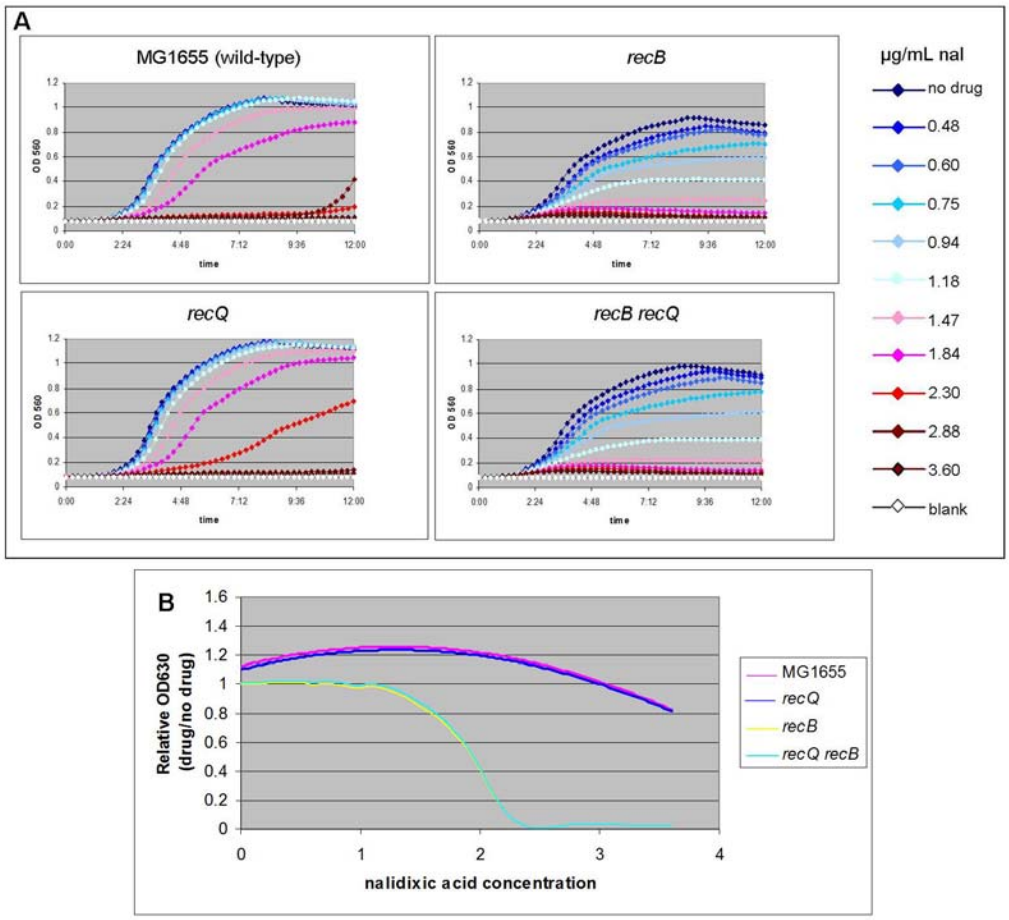

Figure 19: Growth kinetics for nalidixic acid sensitivity of *recQ*, *recB*, and *recQ recB* double mutant

MG1655 (wild-type) and indicated MG1655 derivatives were tested for nal sensitivity using a microtiter plate sensitivity assay. Overnight cultures in LB media at 37°C were diluted 1:200 in LB, and then mixed with an equal volume (75 µl each) of LB containing serial dilutions of nal for a final concentration ranging from 0.48 to 3.6 µg/ml in 96-well plates. The cells were grown in the plate reader at 37°C with constant shaking, and OD630 was measured every 15 minutes for 12 h. Panel A are growth curves for the wild type and mutants. For the comparative titration curves in Panel B, a standardized point in the growth curve was first determined for each cell line, namely the time at which the growth rate of the no-drug culture dropped to 50% of the earlier exponential rate. The OD_{630} value at this time was then taken for each drug concentration and divided by the no-drug control to account for differences in growth rate.

3.2.5 Proteins that protect *E. coli* from quinolone antibiotics

We tested twenty-five knockout mutants for quinolone (nalidixic acid and ciprofloxacin) sensitivity, extending multiple previous studies of quinolone sensitivities [106,107,173,174]. The collection of mutants was selected for TBC studies discussed in Chapter 2. Strains with mutations in *recA, recB, recC, recG, uvrD, ruvA, ruvC, xseA, ftsK,* and *rep* were found to be hypersensitive to exposure to quinolone antibiotics (Figure 21). Knockouts in most of these genes have previously been shown to cause quinolone hypersensitivity (see citations above), but two novel hypersensitive mutants emerged: *rep* and *ftsK*. The latter mutant would not have been identified in past studies (including [107]), because it is an essential gene and was not available as a complete gene knockout. As mentioned in Chapter 2, the transposon mutant used here is viable, because it contains the essential N-terminal 210 amino acids.

We noted an interesting anomaly that should be considered whenever using the *recF* mutant from the Keio collection [144]. The deletion strain was noticeably resistant to quinolones, in contrast to *recR* or *recO* mutants, which behaved like wild type (Figure 21). Inspection of the sequence of the deletion revealed that part of the promoter for the downstream *gyrB* gene is deleted, presumably leading to lower levels of DNA gyrase (and hence resistance to the poisoning effect of quinolones) (Figure 20). To gauge the effect of a simple loss of RecF function, we moved the null *recF4115* point mutant [146] into this background, and found that the mutant displays wild-type sensitivity to quinolones (Figure 21).

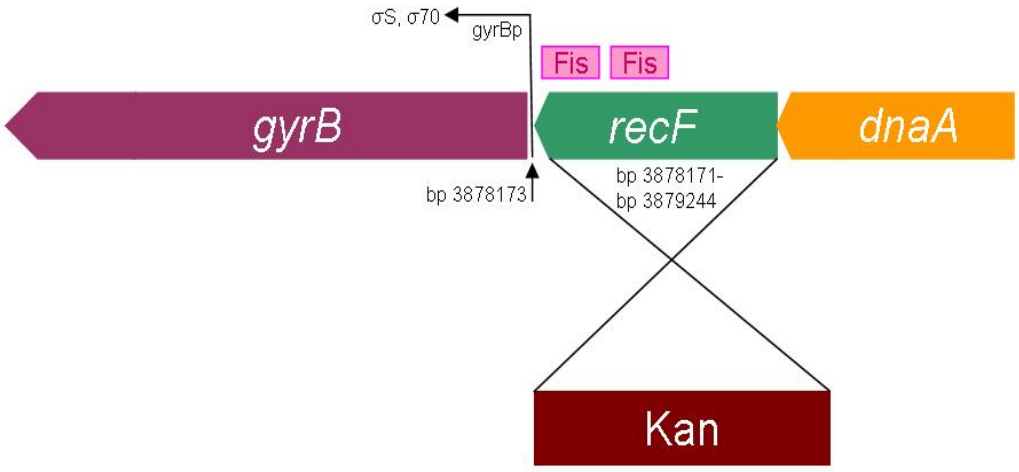

Figure 20: Polar effects on *gyrB* in Δ*recF* mutant

The *recF* gene from start codon (3878171 bp) to stop codon (3879244 bp), is replaced by the Kanamycin resistance cassette (Tn5). The *gyrB* gene and transcription start site (3878173 bp) is intact, but the upstream -35 and -10 promoters and the binding sites of the transcriptional regulator, Fis, are removed in the Keio deletion.

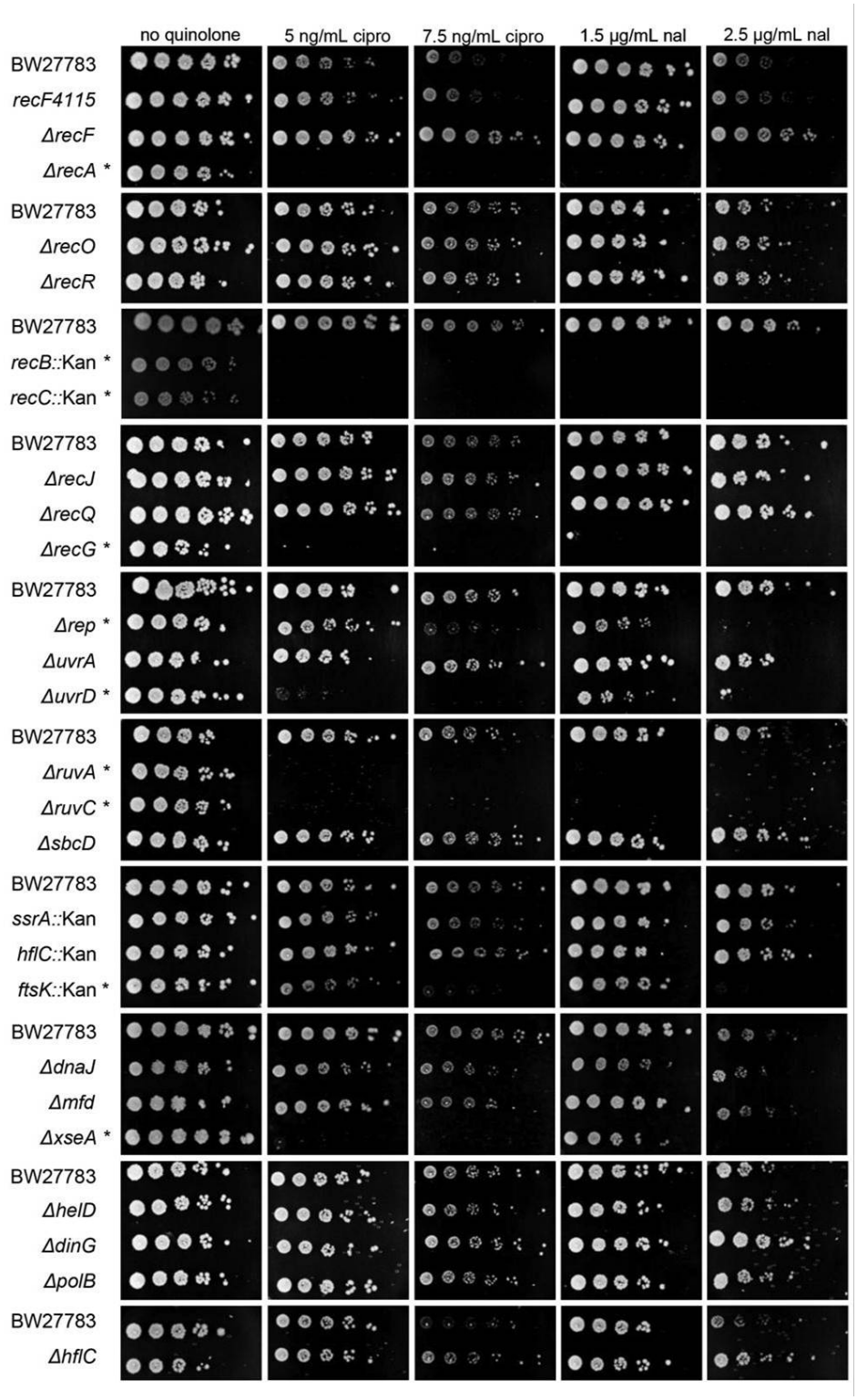
no quinolone
5 ng/mL cipro
7.5 ng/mL cipro
1.5 µg/mL nal
2.5 µg/mL nal
BW27783
recF4115
ΔrecF
ΔrecA *
BW27783
ΔrecO
ΔrecR
BW27783
recB::Kan *
recC::Kan *
BW27783
ΔrecJ
ΔrecQ
ΔrecG *
BW27783
Δrep *
ΔuvrA
ΔuvrD *
BW27783
ΔruvA *
ΔruvC *
ΔsbcD
BW27783
ssrA::Kan
hflC::Kan
ftsK::Kan *
BW27783
ΔdnaJ
Δmfd
ΔxseA *
BW27783
ΔhelD
ΔdinG
ΔpolB
BW27783
ΔhflC

Figure 21: Sensitivity to quinolone antibiotics

Overnight cultures of BW27783 (wild-type) and the indicated BW27783 derivatives were serial diluted five-fold and spotted onto LB plates containing either ciprofloxicin (5 or 7.5 ng/mL) or nalidixic acid (1.5 or 2.5 μg/mL) and incubated at 37° overnight. Those mutants deemed to be hypersensitive are indicated with an asterisk.

3.3 Discussion

I analyzed the quinolone sensitivity of twenty-five knockout mutants, the same mutant collection analyzed for sensitivity to TBCs (Chapter 2), for nalidixic acid and ciprofloxacin. This data allows us to compare survival to the various kinds of DNA-protein complexes in a common genetic background and extend multiple previous studies of quinolone sensitivities [64,65,70,140]. A novel finding was that *rep* and *ftsK* mutants are hypersensitive to quinolones. Both Rep and FtsK have been shown to be capable of stripping proteins from DNA [57,115,124], an activity that could play some role in either resolving topoisomerase cleavage complexes and/or modulating blocked or broken replication forks.

We obtained some useful negative data, namely that mutations in *recF*, *recO*, *recR*, *recJ*, *recQ*, *uvrA*, *sbcD, dinG, dnaJ, helD, hflC, mfd, polB* and *ssrA* cause little or no hypersensitivity to quinolones (Figure 21). Every mutant that had been previously tested for quinolone sensitivity behaved essentially as expected. However, it should be noted that a strain lacking SbcCD was previously shown to be modestly hypersensitive to killing by nalidixic acid (but not ciprofloxacin), while the minimal inhibitory concentration was unaffected [175]. Also, in a spot test comparable to our approach, an SbcCD mutant was not hypersensitive to ciprofloxacin [107].

Attempts to test the necessity of on-going replication for the killing by quinolones were not successful. A difficulty in testing the effects of replication is finding an appropriate way to block replication without deleterious effects. Both the drug AZT and

the expression of coliphage N4 *orf8* led to cell killing, making them unsuitable means of replication blocking for the study.

We discovered that the Helling lab hypothesis of the reason for *icdA* mutants being resistant to low levels of quinolones was not correct. The resistance is not due to the accumulation of an intermediate such as citrate upregulating the AcrAB-TolC efflux pump, and then pumping out the intermediate along with the antibiotic [159]. Repeating their experiments, we saw that the resistance seen in an *icdA* mutant is not lost in an *acrA icdA* double mutant. Additional studies have supported the Collins lab model, that the loss of *icdA* and other TCA cycle genes, decreases NADH pools and has a downstream effect of decreasing the production of cytotoxic superoxide radicals sufficient (for review, see [176]). Recently, two studies provided strong experimental evidence against the Collins' model, by showing that the antibiotics did not lead to increased levels of hydrogen peroxide or elevate levels of free iron. Also, the lethality persisted in anaerobic conditions [162,163]. The resistance of the *icdA* mutant and other TCA-cycle mutants, is likely due to a slow-growth phenotype.

In the continued pursuit of genetic mutants with interesting responses to quinolone exposure, we performed a screen for nalidixic acid bacteriostatic mutants. Unfortunately, the phenotype of the mutants collected was not due to the transposon insertion we created. More comprehensive analysis, like whole-genome sequencing, would be necessary to located the secondary mutations responsible for the phenotype.

We have yet to uncover another mutation that leads to an SOS profile like a *recBC* or a *recQ* mutant, with a failure to induce SOS in response to quinolones, but a regular response to UV or MMC. We did discover *recQ* mutants as partially defective in quinolone induction of SOS. Perhaps, the creation of more *recQ* double mutants would uncover a mutant with the phenotype of interest. The *recB recQ* growth kinetics in the presence of nalidixic acid did not support our hypothesis that RecQ helicase is regressing a fork stalled at the site of the quinolone-stabilized cleavage complex, which can be resolved into a DSB. We hypothesized that this DSB would lead to cell death unless the break is repaired by RecBCD, but the *recQ* mutation did not suppress the extreme hypersensitivity of the *recBC* mutant.

3.4 Materials and Methods

3.4.1 Materials

LB (Luria broth) contained bacto tryptone (10 g/L), yeast extract (5 g/L), and sodium chloride (10 g/L). Kanamycin, nalidixic acid, ciprofloxacin and o-nitrophenyl-β-d-galactopyranoside (ONPG) were purchased from Sigma. 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) was purchased from Gold Biotechnology, Inc.

3.4.2 *E. coli* strains

Strain JH39 [F- *dinD*1::Mu d(Ap^r lac) *lac*Δ*U169 srlD sulA11 thr-1 leuB6(*Am*)* *hisG4*(Oc) *argE3*(Oc) *ilv(ts) galK2(*Oc*) rpsL31*], which has a *dinD-lacZ* fusion allowing for visualization of SOS induction on X-gal plates, was obtained from J. Heitman Lab (Duke University) [166,167]. The damage-inducible gene *dinD* is known to be upregulated during the SOS response, but the function of *dinD* is unknown. The level of

SOS induction in the JH39 strain, as measured by increased expression of the *dinD-lacZ* fusion, can be visualized on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) reporter plates or by a liquid β-galactosidase assay [168]. The JH39 strain also has a mutation in *sulA,* which is a cell-cycle inhibitor induced during the SOS response. This mutation prevents filamentation and is necessary for the survival of many SOS constitutive mutants such as *lexA* [177,178].

Strain BW27783 [F-, *Δ(araD-araB)567*, *ΔlacZ4787(::rrnB-3)*, λ-, *Δ(araH-araF)570(::FRT)*, *ΔaraEp-532::FRT*, φ*Pcp8araE535*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*)] allows for homogenous and titratable expression from pBAD vectors [143] and was obtained from the lab of HP Erickson (Duke University). Strain BW25113- [F-, λ-, *Δ(araD-araB)567*, *ΔlacZ4787(::rrnB-3)*, *rph*-1, *Δ* (*rhaD-rhaB*)568, *hsd*R514] was obtained from Yale CGSC [179]. Strain MG1655- [F^- λ^- *ilvG- rfb*-50 *rph*-1] Strain W3110- [F^- λ^- *rph*-1 INV(*rrnD*, *rrnE*)] Strain W3350- [F^- λ^- *galK*2(Oc) *galT*22 INV(*rrnD-rrnE*)]

E. coli knockout mutants from the Keio collection [144] contain kanamycin resistance gene inserts, and are identified as Δ followed by gene name (e.g. Δ*recA*). In each case, the desired mutation was moved into the wild-type background via P1 transduction [145], selecting for kanamycin resistance, and confirmed by PCR. The kanamycin-resistance cassette was not removed from the tested strains, with the exception of the *recQ* strain used to create the double mutants. The kanamycin-resistance cassette is flanked by FRT sites allowing for removal by expressing Flp recombinase from plasmid pcp20 [180]. Successful removal was confirmed using PCR. The *recF4115* missense mutant (K36Q) was moved into BW27783 using P1

transduction, selecting for a linked Tn10 marker (in *tnaA;* [146]); transductants were screened by DNA sequencing for co-transduction of the *recF* mutation.

3.4.3 Growth kinetics for nalidixic acid sensitivity

Nal sensitivity was measured with continuous growth curves in a temperature-controlled ELx808™ Absorbance Microplate Reader. Overnight cultures in LB media at 37°C were diluted 1:200 in LB, and then mixed with an equal volume (75 µl each) of LB containing serial dilutions of nal (0.48-3.6 µg/ml) in 96-well plates. The cells were grown in the plate reader at 37°C with constant shaking, and OD630 was measured every 15 minutes for 12 h.

3.4.4 Plate assay of SOS induction in response to nalidixic acid

Overnight cultures of JH39 (wild-type) and the JH39 derivatives *recB, recQ*, and *recQ* double mutants were mixed with LB top agar (200 µL culture in 3 mL agar) and poured onto X-gal (90 µg/ml) plates. 0, 10, 20, and 40 µg of nalidixic acid was pipetted onto the filter disk and the plates were incubated at 37° overnight.

3.4.5 Liquid assay of SOS induction in response to nalidixic acid

Overnight cultures of JH39 (wild-type) and the JH39 derivatives *recB, recQ*, and *recQ* double mutants were diluted 100-fold and grown until they reached OD_{560}=0.4. Each culture was divided into 3 tubes of 2 mL each for exposure to nal (10 µg/mL), MMC (1 µg/mL) or a no-drug control and grown for 2 hours. Cells were then pelleted in a microcentrifuge. β-Galactosidase assays were performed as described by Miller [145]. Cell pellets were resuspended in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 10 mM dithiothreitol). 100 µL of chloroform and 50 µL drop of 0.1%

sodium dodecyl sulfate (SDS) were added to the cell suspension, which was then vortexed vigorously for 10 seconds. Appropriate amounts of the lysate were mixed with Z-buffer to a final volume of 1.0 ml. To start the reactions, 200 µl of o-nitrophenyl-β-d-galactopyranoside (ONPG) (4 mM) was added, and the reaction mixtures were incubated at 30°C until a moderate yellow color was observed. The reactions were stopped with 0.5 ml of 1 M sodium bicarbonate, and the cellular debris was pelleted. The optical density was recorded with an ANTHOS 2001 plate reader with a 405-nm filter. Miller units were calculated as follows: units = 1,000[(OD405/(t × v × OD600)], where OD405 is the optical density at 405 nm of the reaction product, OD600 reflects the cell density at 600 nm, t is the reaction time in minutes, and v is the volume of culture used in the assay.

3.4.6 Spot tests for sensitivity to quinolone antibiotics

Overnight cultures of BW27783 (wild-type) and the indicated BW27783 derivatives, containing plasmid pBAD-MEcoRII-C186A, were diluted to the equivalent of OD_{560} = $5x10^{-4}$ (corresponding to approximately 2 x 10^5 cells per mL). Five-fold serial dilutions were generated across a microtiter plate and 5 µl of each dilution (range of roughly 1,000-0.3 colony forming units for wild-type) was spotted onto LB plates containing chloramphenicol with either glucose (0.01%) or arabinose (0.0003%, 0.001%, 0.003%, or 0.01%). The same dilutions were also spotted onto LB plates containing either ciprofloxicin (5 ng/mL or 7.5 ng/mL) or nalidixic acid (1.5 µg/mL or 2.5 µg/mL). All plates were incubated at 37° overnight. Spot tests were performed on each strain at least three times, and representative examples are shown.

3.4.7 Using AZT to block replication

Wild-type strain JH39 was grown overnight, diluted 1:100 in LB and incubated for 2 hours at 37° to reach OD_{560}=0.5. The culture was then diluted to measure a pre-drug cell count (CFU/mL). The culture was split into two parts, receiving either AZT (0.25 µg/mL) or no drug and incubated 60 minutes at 37°. The culture was then diluted to a range of 1:10-1:10,000 and pipetted onto LB plates to measure a post-drug cell count (CFU/mL) and calculate the percentage killed.

3.4.8 Expressing coliphage N4 *orf8* to block replication

Wild-type strains MG1655, W3110, W3350 and BW25113, were grown overnight, diluted 1:100 in LB + 50 µg/mL amp and incubated 1.5 hours at 37° to reach OD_{560}=0.3-0.4. The culture was then diluted to measure a pre-sugar cell count (CFU/mL). The culture was split into two parts receiving either 0.2% arabinose or 0.2% glucose and incubated 20 minutes at 37°. The culture was then diluted to a range of 1:10-1:10,000 and pipetted onto LB plates to measure a post-sugar cell count (CFU/mL) and calculate the percentage killed. The same protocol was followed for BW27783 wild-type, with the adjustment of arabinose concentrations of 1.25×10^{-5} %, 2.5×10^{-4} %, and 5.0×10^{-3} % used instead of 2%.

3.4.9 Comparing quinolone resistance of an Δ*icdA* mutant in wild-type and Δ*acrA* strains

MG1655 (wild-type) and indicated Keio collection knockouts were grown overnight and diluted to plate approximately 1×10^8 cells onto nalidixic acid plates containing nal at 0.25, 0.375, 0.5, 0.625, 0.75, 0.875, 1 or 1.5 µg/mL and a no-drug

control. Percent survival was calculated based on CFU on nal plates compared to no-drug control and plotted on a log scale.

Chapter 4: Discussion and Future Directions

4.1 Summary of results

In this study, we analyzed the *in vivo* consequences and protein requirements for survival upon expression of the tight-binding mutant M.EcoRII protein (C186A), which creates solo TBCs. As discussed in Chapter 1 and 2, the mechanisms behind the lethality induced by solo TBCs have not been well studied, nor is it clear whether very tight binding but noncovalent complexes are processed in the same way as covalent DPCs. Using the same 2D gel electrophoresis technique performed on aza-C-induced DPCs [43], we visualized that an M.EcoRII-C186A-induced TBC could also block replication forks *in vivo* (Figure 5). A difference in the 2D gel patterns is that the aza-C-induced DPCs caused an accumulation of X structures and a prominent Y-arc [43]. The X structures were not present in a *recA* mutant and are likely created by RecA-dependent recombination or replication fork regression. Electron microscopy was used to determine that aza-C-induced DPCs were causing rolling-circle replication, leading to the prominent Y-arc [43]. Normal theta replication can be converted to rolling-circle replication if the blocked fork is cleaved.

We also performed an extensive candidate gene screen for mutants that are hypersensitive to the M.EcoRII-induced solo TBCs. A powerful aspect of this system is that previous studies, from our lab and others, have already identified a variety of mutants that are hypersensitive to aza-C-induced DPCs that involve the same M.EcoRII protein (except that the DPCs utilize the wild-type version of the protein) [28,43,45,46,47,48,49] (reviewed in [42]; Krasich et al., unpublished data). We now have

a direct comparison of cellular functions that protect against the same tightly bound protein in either non-covalent or covalent form (summarized in Table 4). Several gene products are needed for protection against both kinds of complexes, including RecA, RecBC, RecG, RuvABC, UvrD, FtsK, and SsrA (tmRNA). The recombination proteins likely function in processing blocked replication forks or downstream DNA breaks resulting from blocked forks, given that both kinds of M.EcoRII complex block replication forks ([43]; Figure 5). Involvement of the tmRNA pathway indicates that both kinds of complex also block the coupled transcription-translation machinery (see [49]).

Interestingly, different but overlapping functions are involved in surviving TBCs versus DPCs, both involving the M.EcoRII protein. The most striking difference between the responses to the TBCs versus DPCs is that the RecFOR pathway is important in the response to TBCs induced by M.EcoRII-C186A but not to DPCs induced by aza-C with the wild-type protein. The RecFOR pathway is possibly involved in protecting the replication fork stalled at the TBC from degradation. Rep helicase is another function that is involved in the response to TBCs, but not DPCs. Rep could be involved in dissociating the tightly-bound protein from the DNA. Our model for the repair of M.EcoRII-C186A-induced TBCs is that RecFOR along with RecA stabilize the stalled fork, allowing Rep to remove the TBC. The tmRNA system could help overcome the TBC-mediated blockage of coupled transcription-translation complexes.

In addition to the ability to compare aza-C-induced DPCs involving wild-type M. EcoRII, with M.EcoRII-C186A induced TBCs, formed at the same M.EcoRII DNA recognition sequence, we also have data for the same mutants in response to quinolone-induced DPCs (summarized in Table 4). RecA, RecBC, RecG, and RuvABC

are involved in protection from M.EcoRII-C186A induced TBCs, quinolones, and aza-C-induced DPCs, and due to their roles in replication and repair, this is not a surprising finding. The involvement of these proteins implies that DSBs are formed, as RecBCD is required for processing DSBs to allow for RecA-mediated HR. The recombination reaction yields a Holliday junction, which can be resolved by either the RuvABC resolvasome or branch migration by RecG helicase [25,26,27]. The result of FtsK being hypersensitive to all three is very interesting. Hypersensitivity could be attributed to a role in chromosome segregation after damage or a role in stripping proteins off DNA ([57,115,124]; see below for more discussion). Another novel finding is that in addition to being hypersensitive to M.EcoRII-C186A-induced TBCs, *rep mutants* are also hypersensitive to quinolones. Like FtsK, Rep is also capable stripping proteins from DNA [57,115,124], and could have a role in either resolving topoisomerase cleavage complexes or processing broken replication forks.

Table 4: Sensitivity to M.EcoRII-C186A-induced TBCs, quinolones, and aza-C-induced DPCs

This table summarizes the sensitivity of various *E. coli* mutants to TBCs induced by M.EcoRII (Figure 6; this study), topoisomerase cleavage complexes (Figure 21; this study; also see citations in the text to past studies of quinolone-sensitive mutants), and DPCs induced by wild-type M.EcoRII proteins with aza-C treatment (indicated citations; [#] refers to Krasich, Wu, Kuo and Kreuzer, unpublished data).

	TBCs (Figure 6)	**Quinolones (Figure 21)**	**M.EcoRII DPCs (aza-C-induced)**
dinG	wild-type	wild-type	hypersensitive [#]
dnaJ	wild-type	wild-type	hypersensitive [49]
ftsK	hypersensitive	hypersensitive	hypersensitive [#]
helD	wild-type	wild-type	not reported
hflC	wild-type	wild-type	hypersensitive [49]
mfd	wild-type	wild-type	wild-type [48,49]
polB	wild-type	wild-type	wild-type [48]
recA	hypersensitive	hypersensitive	hypersensitive [45,46,47,48] [#]
recBC	hypersensitive	hypersensitive	hypersensitive [48] [#]
recFOR	hypersensitive	wild-type	wild-type [47] [#]
recG	hypersensitive	hypersensitive	hypersensitive [48] [#]
recJ	wild-type	wild-type	wild-type [48]
recQ	wild-type	wild-type	wild-type [48]
rep	hypersensitive	hypersensitive	wild-type [#]
ruvABC	hypersensitive	hypersensitive	hypersensitive [48] [#]
sbcCD	wild-type	wild-type	wild-type [#]
ssrA	hypersensitive	wild-type	hypersensitive [49]
uvrA	wild-type	wild-type	wild-type [45,46,47,48,49]
uvrD	hypersensitive	hypersensitive	hypersensitive [48,49]
xseA	wild-type	hypersensitive	not reported

4.2 Characterization of hypersensitive mutants and creation of additional mutants

4.2.1 Visualizing replication intermediates to test models for RecFOR, Rep, and UvrD involvement in the processing of M.EcoRII-C186A induced TBCs

Both aza-C-induced DPCs [43] and M.EcoRII-C186A-induced TBCs block replication forks *in vivo* (Figure 5), as seen by an accumulation of bubble molecules on a 2D gel. Only the DPCs led to Y forms and RecA-dependent X structures, which indicates that DPCs lead to more frequent fork breakage than TBCs (Figure 5; [43]; see Chapter 2 discussion). The X structures are likely due to RecBCD-RecA mediated HR or replication fork regression. The ability of the RecFOR pathway to stabilize the forks stalled by TBCs may minimize fork breakage and permit Rep to dissociate the TBC. A future project would be to run 2D gels for the M.EcoRII-C186A hypersensitive mutants. It would be of particular interest to see if Δ*recFOR*, Δ*rep*, and Δ*uvrD* mutants have an accumulation of Y forms and X structures, due to more fork breakage. Seeing an increase in broken forks in Δ*recFOR* and Δ*rep*, but not Δ*uvrD* mutants would support the hypothesis that RecFOR and RecA stabilize forks stalled by TBCs, promoting the release of TBC by Rep. While UvrD can remove transcription factors from arrays (as well as Tus and RecA), we speculate it is not powerful enough to remove a very tight binding M.EcoRII-C186A. We would also expect a decrease in the spots on the bubble arc, in Δ*recFOR* mutants, corresponding to a decrease in stabilized blocked forks. Conversely, we would expect an increase in intensity of spots on the bubble arc in a Δ*rep* mutant when Rep is not clearing TBCs due to less (or more transient) fork blockage.

4.2.2 Models for FtsK involvement in processing DNA-Protein complexes

As mentioned above, we obtained the interesting result that *ftsK* mutants are hypersensitive to M.EcoRII-C186A induced TBCs (Figure 6) and quinolones (Figure 21) while other work also showed *ftsK* sensitivity to aza-C-induced DPCs (Krasich, unpublished data). FtsK is capable of stripping proteins off DNA *in vitro,* including the removal *of* MatP bound to *matS* [124]. MatP binds *matS* in the *ter* region to prevent early segregation of that domain during cell division [181]. FtsK can also displace streptavidin from biotin on DNA (Graham,J.E., Sherratt,D.J., Crozat,E. and Howarth,M. unpublished data cited in [181]). Also, the *Bacillus subtilis* FtsK orthologue, SpoIIIE, is capable of stripping proteins from DNA during spore formation [182]. This supports a model where FtsK has a function in removing the tightly-bound M.EcoRII-C186A protein.

Future work with FtsK can test the hypothesis that is has a function in removing DNA-Protein complexes *in vivo.* A future experiment would be to perform 2D gels, as detailed in Chapter 2, and see if *ftsK* mutants have an increase in blocked forks, as seen by spots on the bubble arc. This would support the model that FtsK is removing the TBC.

The triplex displacement assay used to show that FtsK can remove MatP bound to *matS* [124] could be used to test if FtsK can remove M.EcoRII-C186A-induced TBCs *in vitro*. To perform this assay, a DNA substrate would be engineered containing a triplex binding site downstream of an M.EcoRII recognition site. The substrate would be incubated with M.EcoRII-C186A and radio-labeled Triplex DNA. The displacement of Triplex DNA can be measured using gel electrophoresis. The percentage of M.EcoRII-

C186A displaced by FtsK can be calculated based on the amount of Triplex DNA displaced. The same test could be used for aza-C-induced DPCs.

Alternatively, hypersensitivity could reflect some important role in segregation of chromosomes after damage, for example assisting XerCD in chromosome dimer resolution following HR induced by DPCs or TBCs. XerCD is a site-specific recombinase that resolves chromosome dimers [183]. The DSBs created by the DPCs, TBCs, and quinolones presumably require RecBCD-RecA mediated HR. Depending on how the resulting Holliday junction is resolved, such chromosomal HR can yield two linked copies of the chromosome, which need to be resolved prior to cell division. FtsK assisting XerCD may be required for chromosome dimer resolution before cell division can take place. Mutants of *xerC* and *xerD* would be of interest to test for hypersensitivity to aza-C, quinolones, and M.EcoRII-C186A-induction. This model would be supported if they are also hypersensitive.

4.2.3 Does the primosome have a role in replication fork restart following M.EcoRII-C186A induced TBCs?

PriA and PriB are components for the primosome, which is a complex involved in replication fork restart and the replication of certain phages and plasmids. When forks are blocked at UV damage, PriAB and Rep are required to stabilize the replisome to resume DNA synthesis [90]. It would be of interest to test if *priA* and *priB* mutants are hypersensitive to M.EcoRII-induced TBCs.

Mutants of *priA* have reduced viability and are therefore more difficult to work with. It is important to continuously confirm that additional suppressor mutations have not been acquired. I created a *priA* mutant, but was unable to transform in plasmid

pBAD-MEcoRII-C186A. A functional PriA is required for replication of the pBR322 plasmid [184] and the lack of PriA was potentially the cause of the inability to obtain *priA* pBAD-MEcoRII-C186A transformants. A future direction is to instead create a BW27783 Δ*priB* pBAD-MEcoRII-C186A strain and test its sensitivity to M.EcoRII-induced TBCs.

4.2.4 Model for RuvABC and SbcCD involvement at TBC-blocked forks

RuvA and RuvC are subunits of the Resolvasome, which resolves Holliday junctions and contributes to rescue of blocked DNA replication forks through replication fork reversal [52,69]. RuvA and RuvC knockout mutants are hypersensitive to aza-C-induced DPCs ([48]; Krasich et al, unpublished data), quinolone-induced cleavage complexes [65,70] and TBCs induced by M.EcoRII-C186A (Figure 6). We propose a model that M.EcoRII-C186A-induced TBC-blocked replication forks are sometimes cleaved by a nuclease to generate broken forks, which need to be repaired by RecBCD- and RuvABC-dependent HR. Evidence to support this model is that *recBC* mutants are hypersensitive to M.EcoRII-C186A-induced TBCs and that these TBCs lead to blocked replication forks (see Chapter 2). We presume that a nuclease other than RuvABC is responsible for the fork cleavage as *ruvA* and *ruvC* mutants are hypersensitive to the induction of these TBCs.

One candidate for a nuclease that might cleave the blocked forks, or even DNA near the tightly bound protein, is SbcCD. This protein has double-strand DNA exonuclease activity as well as single-strand DNA endonuclease activity [111], and has been shown to cleave forks and tightly-bound proteins (see Chapter 2). We found that *sbcD* mutants are not hypersensitive to TBCs induced by M.EcoRII-C186A (Figure 6). If

SbcCD is cleaving TBC blocked forks, the broken fork could lead to cell death unless repaired by RecBCD. To test this model, we would create a *sbcD recB* double mutant. If the model was correct, we would expected the double mutant to be less sensitive to M.EcoRII-C186A induced TBCs than the hypersensitive *recB* single mutant, because break formation should be prevented. The same test would be performed with a *sbcD ruvC* double mutant to see if results support an involvement of RuvABC-dependent HR in the repair.

4.2.5 Is the tmRNA system induced when RNA polymerase is blocked by TBCs?

SsrA (tmRNA) releases stalled ribosomes from the end of an mRNA lacking a stop codon. Mutants of *ssrA* are sensitive to both aza-C-induced DPCs [49] and M.EcoRII-induced TBCs (Figure 6). This result argues that the tmRNA system plays an important role in clearing stalled ribosome-mRNA complexes generated after transcription is blocked by both aza-C-induced DPCs and M.EcoRII-induced TBCs. We therefore propose that M.EcoRII-C186A, bound to its recognition sites, can block RNA polymerase and the coupled translation machinery in much the same way as covalently attached M.EcoRII.

To demonstrate that the tmRNA system is involved in surviving aza-C-induced DPCs, aza-C treated cells expressing wild-type M.EcoRII were tested for an increase in SsrA tagged proteins [49]. Performing Western blotting with an antibody against the wild-type SsrA tag, it was shown that aza-C treatment led to a substantial increase in SsrA-tagged proteins in wild-type cells expressing M.EcoRII. The same Western blotting could be performed to determine if M.EcoRII-C186A-induced TBCs also lead to an increase in

SsrA-tagged proteins. We would expect a loss of tagging in the *ssrA* mutant. UvrD and Rep could play a role in removing the coupled transcription-translation machineries blocked by the TBC. Hypersensitive mutants, *uvrD* and *rep,* would also be tested for an increase in SsrA tagging which would support this hypothesis.

4.3 Sensitivity to quinolone-induced DPCs

In addition to the novel finding that *rep* and *ftsK* mutants are hypersensitive to quinolones (discussed above), we uncovered a few additional mutant phenotypes with quinolone studies. We discovered that *recQ* mutants are partially defective in quinolone induction of SOS. However, we did not obtain a double mutant completely defective in quinolone induction of SOS. Double mutants of *recQ rep* and *recQ ftsK* would be of interested to test for SOS response, due to the quinolone hypersensitivity.

We determined that the modest quinolone resistance seen in an *icdA* mutant is not lost in an *acrA icdA* double mutant, invalidating the Helling lab hypothesis that of the reason for *icdA* mutants being resistant to low levels of quinolones is due to upregulation of AcrAB-TolC efflux pump. Two recent studies did provide strong experimental evidence against the Collins lab model that the modest resistance of an *icdA* mutant was due to depletion of NADH pools and therefore a decrease in ROS ([162,163]; see Chapter 4 discussion). We suspect the resistance of the *icdA* mutant is likely due to a slow-growth phenotype. This hypothesis could be tested by measuring the nal resistance of other slow-growing mutants. Measurements of resistance due to growth rate can also be performed by comparing nal resistance in rich media to minimal media and growth at 37°C to 30°C.

4.4 Final remarks

This work expands on the knowledge of how cells tolerate and process TBCs and blocked replication forks. It also allows for a direct comparison to DPCs between the same protein and DNA sequence, as well as DPCs created by quinolone treatment. The M.EcoRII-C186A system has many advantages over existing methods to study TBCs and continued work utilizing this system should prove to be very valuable in the field. Further characterization of the TBC hypersensitive mutants as well as the creation of new mutants, will also greatly expand on our understanding of the mechanisms behind TBC-induced DNA damage.

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