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Author
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Publication Date
2007

Peer reviewed|Thesis/dissertation
DNA repair is the target of novel antibiotics

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

Carl Wayne Gunderson

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University of California, San Diego

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San Diego State University

Professor Anca Segall, Chair
Professor Stanley Maloy
Professor Forest Rohwer

2007
The Dissertation of Carl Wayne Gunderson is approved, and it is acceptable in quality and form for publication on microfilm:

____________________________________________________________________

Chair

University of California, San Diego
San Diego State University
2007
This dissertation is dedicated to my family. Thank you for all you’ve done to help me get through. I couldn’t have done it without you. Love you all, always.

Mom and Dad, thank you for encouragement.

Felizza, thank you for love.

Valerie, thank you for taking the pressure off.

I would also like to dedicate this work to Curtis Gunderson.
The more I learn, the less I know.
Mostly because I think my brain is leaking.

Tequila drinking, oh, our minds will wander to wondrous places.
Dave Matthews
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<tr>
<th>Symbol</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>φ</td>
<td>bacteriophage (phage)</td>
<td></td>
</tr>
<tr>
<td>λ</td>
<td>bacteriophage lambda</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>dsb</td>
<td>double-strand DNA break</td>
<td></td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
<td></td>
</tr>
<tr>
<td>HJ</td>
<td>Holliday junction</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>homologous recombination</td>
<td></td>
</tr>
<tr>
<td>IC₅₀</td>
<td>inhibitory concentration 50 (concentration at which an inhibitor achieves 50% inhibition)</td>
<td></td>
</tr>
<tr>
<td>Int</td>
<td>phage lambda Integrase</td>
<td></td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani media</td>
<td></td>
</tr>
<tr>
<td>MHB</td>
<td>Meuller-Hinton Broth media</td>
<td></td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
<td></td>
</tr>
<tr>
<td>PFGE</td>
<td>pulsed field gel electrophoresis</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>SEI</td>
<td>strand exchange intermediate</td>
<td></td>
</tr>
<tr>
<td>SSB</td>
<td>single-strand binding protein</td>
<td></td>
</tr>
<tr>
<td>SSR</td>
<td>site-specific recombination</td>
<td></td>
</tr>
<tr>
<td>STm</td>
<td><em>Salmonella enterica</em> serovar Typhimurium</td>
<td></td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling</td>
<td></td>
</tr>
<tr>
<td>wrwycr</td>
<td>peptide composed of d-form amino acids</td>
<td></td>
</tr>
<tr>
<td>WRWYCR</td>
<td>peptide composed of L-form amino acids</td>
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I would like to acknowledge Professor Anca Segall for guidance and direction throughout the course of this project.

Though there are too many to name here, thanks to all the members of the Segall lab for scientific help, laughs, and the occasional distraction.


Portions of Chapter 3 are in preparation to be published as: Gunderson, Carl and Segall, Anca, 2007. An antimicrobial hexapeptide acts by binding recombinative DNA repair intermediates and influencing their resolution.
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In preparation. Gunderson, CW and Segall AM. An antimicrobial hexapeptide acts by binding recombinative DNA repair intermediates and inhibiting their resolution.

Presentations

01.13.07 CSU Annual Biotechnology Symposium, Los Angeles, Ca. "Antimicrobial Peptides that Bind Holliday Junctions and Branched DNAs Cause DNA Fragmentation and Inhibit DNA Repair"

08.11.06 2006 Salk/Caltech Meeting on DNA Replication and Genome Integrity, La Jolla, Ca. "Antimicrobial Peptides that Bind Holliday Junctions and Branched DNAs Cause DNA Fragmentation and Interfere with DNA Repair"

10.13.05 The Seventh Analytical Genetics Meeting, San Diego, Ca. "DNA Repair of antimicrobial peptides that trap Holliday Junctions"

03.08.05 Gordon Research Conference on Antimicrobial Peptides, Ventura, Ca. "Peptides that trap Holliday junctions in vitro cause DNA damage and have antibiotic activity in vivo"

02.09.04 Keystone Symposia on Bacterial Chromosomes, Santa Fe, NM. "Peptides that trap Holliday junctions in vitro cause DNA damage and have antibiotic activity in vivo"

08.24.02 Molecular Genetics of Bacteria & Phages, Cold Spring Harbor Laboratories, NY. "Novel Antibiotics: Peptides that block Holliday junction resolution in vitro block chromosome segregation in vivo"

Awards and Honors

2007 Don Eden Graduate Student Award Finalist. Annual CSUPERB CSU Biotechnology Symposium, January, 2007
DNA repair is the target of novel antibiotics

by

Carl Wayne Gunderson

Doctor of Philosophy in Biology

University of California, San Diego, 2007
San Diego State University, 2007

Professor Anca Segall, Chair

DNA repair is an important cellular process that is conserved in Bacteria, Archaea, and Eukaryotes. The process of DNA repair involves many different proteins that function to create and process branched DNA intermediates. The branched intermediates include 3-way branches (such as replication forks) and 4-way branches (Holliday junctions). Previously, our laboratory isolated peptides based on their ability to inhibit site-specific recombination. The biochemical activity of the most potent peptides was found to be a result of their specific binding to Holliday junctions and branched DNA intermediates. We hypothesized that if the peptides bind to these 3-
and 4-armed intermediates and inhibit their resolution, we would expect them to have antimicrobial activity. We have found that some of these hexapeptides are potent antimicrobials, bactericidal against both Gram+ and Gram- Bacteria. The hexapeptides cause DNA segregation abnormalities, filamentation, and DNA damage. Using epifluorescence microscopy and flow cytometry, we have extensively characterized the physiology of bacterial cells treated with these peptides. Our model proposes that the peptides kill bacterial cells by trapping the intermediates in the repair of endogenous or exogenous DNA damage and preventing its completion. We show that, in agreement with our predictions, our most potent peptide inhibitor causes double strand breaks and DNA fragmentation in Salmonella enterica Typhimurium as shown by pulsed field gel electrophoresis (PFGE). We have compared the extent of peptide-dependent DNA fragmentation in wild-type cells and in mutants with defects in DNA repair. The data indicate that peptide treatment creates substrates for processing by the RecBCD double strand break (DSB) repair pathway and the RecFOR single strand break (SSB) repair pathway. We propose that the peptides influence the processing of DNA repair intermediates by increasing the time that it takes cellular machinery to process of Holliday junctions and branched intermediates. The data support our previous hypothesis that peptide d-wrwycr inhibits bacterial growth by exacerbating DNA damage and interfering with DNA repair.
Chapter 1: Introduction

Section 1.1: An abbreviated history of hexapeptides that inhibit lambda Integrase

Bacteriophage lambda is a well characterized bacterial virus that infects *Escherichia coli*. Upon lambda infection a genetic decision between lysis and lysogeny is made based on the environment within the host. Dictated by the conditions of the host bacterial cell, the phage’s choice to lyse the cell is made if the cells are rapidly metabolizing in their environment. Conversely, if the cell is under starved conditions, the phage enters lysogeny and elects to integrate its chromosome into that of the host (Ptashne, 1992). Integration of the phage’s genome is reversible, and as conditions of the host cell improve, the phage can excise and enter the lytic phase of its life cycle. Residing at the center of the integration and excision process is the 4-armed DNA molecule known as the Holliday Junction (Figure 1.1).

Integrase (Int), a phage-encoded protein, catalyzes the DNA exchange that integrates and excises lambda’s genome from the host chromosome. Integrase catalyzes the site-specific recombination reaction by first forming a Holliday junction, and then resolving the intermediate to create the contiguous integrated product (Figure 1.1C). When the time comes for lambda to produce new particles and burst the cell, the excision reaction proceeds through the same intermediate. The biochemistry of the mechanism of lambda Int had been well studied except for the Holliday junction itself. The Holliday junction is a very transient intermediate in the
**Figure 1.1**: Mechanism of bacteriophage lambda integration. A) Cartoon representing adsorption of phage lambda (red ‘lunar lander’) to the *E. coli* cell (blue blob) surface. B) After the phage injects its DNA into the cell, and the decision is made to lysogenize the host cell, the attachment (*att*) sites are brought together by the phage encoded Int protein. The *attP* site is located on the phage genome, and the *attB* site on that of the bacteria. C) The synaptic complex that forms between the *att* sites and four Int monomers (green ovals) rapidly undergoes one set of cleavage and strand exchange reactions, catalyzed by Int monomers on opposite strands, and forms the Holliday junction (HJ) intermediate. Isomerization of the symmetric HJ intermediate directs which monomers of Int become active, and upon the switch, a second set of cleavage and strand exchange reactions resolves the Holliday junction. The resultant product is the integration of the phage genome into that of the host. Here, the phage can lie in wait for appropriate conditions to excise (the exact opposite reaction of integration) from the host and replicate.
pathway, and forms from substrates and is resolved to products very rapidly. The Segall lab set out to dissect the role of the Holliday junction in the mechanism of site specific recombination catalyzed by Int.

Due to its transient nature, direct study of the Holliday junction had been elusive. Integrase does not require ATP or any other high-energy cofactors for its activity, thus it was necessary to develop a tool to study its biochemistry. Combinatorial libraries of hexameric peptides were screened for the ability to inhibit site-specific recombination by lambda Integrase \textit{in vitro}. The combinatorial pools were assayed against different pathways of lambda Integrase, bent-L ($\text{attL} \text{ tenP}^*1 \times \text{attL}$) and excision $\text{attL} \times \text{attR}$). Deconvolution of the peptide libraries using the bent-L reaction assay, yielded the peptides KWWCRW and WKHYNY (Klemm \textit{et al.}, 2000, Cassell \textit{et al.}, 2000). A different peptide, WRWYCR, was found when the pools were screened using the excision reaction as the assay (Boldt \textit{et al.}, 2004).

The biochemical activity of the peptides has been well characterized by the lab. The composition and functionality of the peptides’ side chains is well understood, and supports a model where peptide inhibition is a result of peptide binding at the open center of the Holliday junction (Ghosh \textit{et al.}, 2005, Boldt, Kepple, Rideout, and Segall, unpublished). The peptides are rich in the hydrophobic amino acids tryptophan and tyrosine, which are important for the formation of base stacking interactions with exposed bases of the DNA at the center of the junction (Kepple, Patel, and Segall, in preparation). Positively charged residues in the peptides, such as lysine and histidine, allow the peptide to make charge interactions with the DNA, stabilizing the binding of the peptide to the junction (Kepple, Patel, and Segall, in
preparation). In addition, two of the peptides feature cysteines, allowing dimerization through the formation of disulfide bridges. Dimerization of the peptides through the cysteines is essential for full inhibitory function of the peptides \textit{in vitro} (Boldt et al., 2004).

Of the three more potent peptides (KWWCRW, WKHYNY, and WRWYCR), peptide WRWYCR is the most potent inhibitor of the excision, integration, and bent-L reactions of lambda Int. WRWYCR is 10 to 100-fold more potent than either of the other peptides at inhibiting these pathways, and KWWCRW is 10-fold more potent than WKHYNY (Klemm et al., 2000). One distinct difference between the more potent peptides WRWYCR and KWWCRW and the less potent peptide WKHYNY is their ability to bind dsDNA. Only WKHYNY is unable to bind dsDNA at high concentrations. The dsDNA binding activity exhibited by WRWYCR is a non-specific activity of the peptide, as the peptide inhibits Integrase in the range of IC\textsubscript{50} concentrations (concentration that inhibits 50% of enzyme activity) between 0.005 to 0.021 \( \mu \text{M} \) but fails to bind dsDNA at 0.5 \( \mu \text{M} \). WRWYCR binding to dsDNA occurs at 5 \( \mu \text{M} \) (Boldt et al., 2004), thus the inhibitory activity of the peptides is specific to lambda Integrase.

Evidence, in the form a crystal structure, suggested that the peptides bind to the center of the Holliday junction and alters its geometry, preventing Int from acting on the junction (Ghosh \textit{et al.}, 2005). Subsequent studies have shown that the peptides are specific for the Holliday junction DNA itself, binding with strong specificity to junction DNA in the absence of protein (Kepple \textit{et al.}, 2005). Peptide WRWYCR binds HJ at 0.05 \( \mu \text{M} \), and cannot be competed away by 240-fold excess of non-specific, double-stranded DNA. This specificity of the peptide for the HJ structure,
and not the HJ/protein complex, led to the hypothesis that the peptides would inhibit other proteins that act on HJ, based on target specificity.

The RecG helicase is able to branch migrate several different branched DNA structures, including replication forks and Holliday junctions. Peptide WRWYCR can inhibit RecG unwinding at IC\textsubscript{50} concentrations ranging from 0.025 to 0.2 \(\mu\)M, depending on the structure of the substrate DNA (Kepple et al., 2005). The peptide is most potent inhibiting RecG action on a substrate that mimics a replication fork with a lagging strand, but no leading strand. The peptide has the least effect on the opposite substrate, a fork with a leading strand but no lagging strand. The proteins RuvA and RuvB form a complex that also branch migrates Holliday junctions. The difference with this complex is that, when it finds a recognition sequence in the DNA, the RuvC protein binds to the complex and cleaves two strands of the HJ. The peptide is able to inhibit RuvABC cleavage activity, with an IC\textsubscript{50} value of 0.064 \(\mu\)M. Peptide WRWYCR is much less potent in its inhibition of RuvAB HJ migration (IC\textsubscript{50}= 20 \(\mu\)M).

The peptides are specific for Holliday junctions, inhibiting the proteins that act on them, but do not inhibit every protein that acts on DNA. The peptides only non-specifically inhibit restriction endonucleases and topoisomerases (Boldt et al., 2004, Cassell et al., 2000, Klemm et al., 2000). Initial experiments indicate that the peptides are capable of specifically inhibiting RecBCD catalysis on a substrate with chi located near the junction center (Boldt and Segall, unpublished data), but the mechanism of this inhibition is currently not known. The inhibitory effect of the peptide on a protein is exquisitely linked to the peptide’s affinity to the proteins’ substrate. That is, the more closely a DNA substrate resembles a Holliday junction, the more effective the peptide
will be at inhibiting a given proteins’ catalysis on that substrate. Numerous peptide targets (3- and 4-armed DNAs) are formed and resolved in the pathways of homologous recombination and recombinative DNA repair (Figure 1.2).

Section 1.2: Pathways of DNA repair and recombination

Homologous recombination dependent repair of DNA breaks occurs through two major pathways. Double-strand breaks are processed by the RecBCD system and single-strand breaks are processed by the RecFOR system (reviewed in Rocha et al., 2005). These systems differ in the initial substrates on which they act, but yield a similar result, a length of single-stranded DNA coated with RecA proteins. The RecA/DNA nucleoprotein filament facilitates strand invasion of the single strand into a homologous sister duplex. This strand invasion event allows for the precise repair of any discontinuities in the sequence. Holliday junctions are formed as a part of both of these pathways and they must be resolved to complete repair.

When a double-strand break in the DNA has occurred, RecBCD, a 330 kD heterotrimeric protein complex, recognizes and forms on the end of the broken DNA strand. The RecB subunit is a 3′-5′ helicase and RecD, a 5′-3′ helicase (Singleton et al., 2004). RecC provides the pin that separates the DNA duplex as well as the chi scanning sites. Crossover hotspot instigator, chi, is an 8 base pair sequence that switches the nuclease activity of RecBCD (Lam et al., 1974, Singleton et al., 2004). When the RecBCD complex binds a double strand break, RecB degrades both strands of the duplex by switching activity from the 3′ tail to the 5′ tail. This ‘double-
**Figure 1.2:** Models of homologous recombination and fork regression. A) RecBCD processing of a double strand break and the resulting RecA-mediated crossover. B) A single-strand break resulting in a gap can be widened by RecJ, with the help of a helicase such as RecQ. The ends of the gap are stabilized by the RecFOR complex, which loads RecA on the single strand region. RecA mediated crossover in this case results in a double Holliday junction intermediate. C) Model of fork regression that can be catalyzed by RecG of RecA. The blue circles in all panels represent potential peptide binding targets. Please refer to chapters 1 and 3 text for full discussion of these pathways. Adapted from Rocha, Cornet, and Michel, PLoS Genetics 1:2, 2005
strand' nuclease activity continues until RecC recognizes the chi site. Enhanced binding of RecC to the chi site changes the nuclease activity of RecB, which then preferentially degrades the 5’-3’ strand. A free single strand with a 3’ hydroxyl end is created, and RecB facilitates loading of RecA monomers onto the single-stranded region. The resulting nucleoprotein filament is now a substrate for strand invasion of a homologous duplex. The strand invasion event yields a single crossover, and formation of a Holliday junction (Figure 1.2A).

The mechanism of loading RecA on single-strand DNA (ssDNA) gaps in the DNA is not dependent on RecBCD, but on the RecFOR complex. The RecFOR complex binds the 5’ terminated end of a SSB coated ssDNA gap, and helps displace the SSB proteins and load RecA onto the single-strand region in its place (Morimatsu and Kowalczykowski, Molecular Cell, 2003). The RecFOR complex also helps stabilize the RecA nucleoprotein filament. Prior to RecFOR binding, nicks and gaps in the DNA can be widened by the combined action of a nuclease, such as RecJ, and a helicase (Han et al., 2006). RecJ is a 5’-3’ ssDNA exonuclease that degrades the 5’ side of the gap. Again, the RecFOR complex is able to bind the edges of the gaps, and load RecA on the gap. The strand invasion of the gap into a homologous duplex results in the formation of a double crossover structure, containing two Holliday junctions (Figure 1.2).

RecA protein does not simply function as a mediator of strand exchange, but also as a signal for the activation of the SOS response. The SOS response is the coordinated response of a cell to DNA damage (Friedberg, 1995). The SOS
Figure 1.3: Model of SOS induction. The rounded rectangular lines represent replicating chromosomes. Representative genes of the SOS regulon are depicted for illustration of the system's repression (top) and de-repression (bottom). Please refer to text for discussion of the role of LexA and RecA in SOS induction. The sulA gene product is an inhibitor of cell division that allows for DNA to be repaired prior to cell division.

CPD = cyclopyrimidine dimer. Adapted from Friedberg et al. DNA Repair and Mutagenesis, 2nd edition, 2006
response functions to de-repress a regulon of over 30 genes. The RecA-ssDNA nucleoprotein filament that forms, either through the repair of a double-strand or single-strand DNA break, causes RecA’s co-protease function to become active (Figure 1.3). In the active state, RecA activates the autoproteolytic activity of LexA. LexA is the repressor that binds to SOS boxes in order to repress gene expression. So, when damage occurs, and RecA is loaded on to ssDNA, causing LexA to self-cleave, and thus de-represses the regulon. The SOS regulon consists of genes involved in processes that repair and/or increase tolerance of DNA damage, such as nucleotide excision repair and lesion bypass synthesis by error-prone polymerases. The genes of the regulon all differ both in the extent in which they are expressed when in the repressed state, and the fold increase in expression that occurs when the system become de-repressed. The \textit{recA} gene itself is under the control of the LexA repressor, and its expression increases 10-fold when the repressor cleaves (Friedberg, 1995), but the gene is also expressed when the repressor is bound (~7,200 molecules/cell, uninduced). The \textit{sulA} gene, which encodes an inhibitor of septum ring formation, is the gene which shows the largest increase in induction when the system is de-repressed, increases nearly 100-fold (Friedberg, 1995). Two genes that are moderately upregulated by SOS induction code for proteins that are involved in Holliday Junction resolution, \textit{ruvA} and \textit{ruvB} (Friedberg, 1995).

The proteins RuvA and RuvB form a heterotrimeric complex with a third protein, RuvC. The RuvABC complex acts to branch migrate Holliday junctions, scanning the DNA for a consensus sequence that triggers site-directed cleavage of the junction to resolved products (dsDNA’s). RuvA, as an octamer, first recognizes
and binds the Holliday junction, holding the junction in an open conformation (Parsons et al., 1995, Parsons et al., 1992, Yamada et al., 2002). The RuvB protein forms a pair of hexamer “doughnuts”, surrounding two opposite arms of the junction, providing the motor that drives the migration of the HJ (Yamada et al., 2002). Lastly, the RecB protein, when it recognizes the ruvC cleavage consensus sequence, directs a RuvC dimer to cleave, resolving the junction (Iwasaki et al., 1991). A recent study has indicated that the RuvABC complex is essential to repair collapsed replication forks, once recombination repair has been initiated (Mahdi et al., 2006).

The RecG protein also acts to migrate Holliday junctions, but unlike RuvABC, has no HJ cleavage activity. In some situations, where the junction can encounter a dsDNA break, branch migration may be enough to resolve recombination substrates to linear products. The RecG protein is one of the primary mechanisms of fork regression, regressing stalled forks and forming ‘chicken foot’ structures that are structurally equivalent to HJ’s (Figure 1.2C). The RecG protein has a wedge domain that situates inside the cleft of the Holliday junction and facilitates separation of the DNA strands as the motor domain translocates the junction (Singleton et al., 2004). It has been suggested that RecA is also able to regress stalled forks, but is likely that RecA and RecG function in different situations, as their effect on fork substrates in vitro is antagonistic (Robu et al., 2004).
Bacteria are under constant stress from DNA damaging agents of intracellular and extracellular origins. Frequently, the replication machinery falls apart because it is unable to circumvent lesions caused by DNA damaging agents. In these cases, the lesion must be repaired or bypassed, and the replication fork needs to restart. This process may or may not necessitate the reversal of the replication fork into a “chicken foot” structure (Figure 1.2).

Any collapsed fork is going to require reloading of DnaB, the replicative helicase, in order to resume replication. There are several mechanisms in place to do this and their use is dictated by the architecture of the strands at the fork. One pathway of restart is dependent on the 3’-5’ helicase PriA, which prefers the presence of a leading strand at the fork center. PriA can also work on D-loop structures that arise as a result of recombination, as long as the leading strand is present (Heller & Marians, 2006b, Heller & Marians, 2005a). Another protein, Rep, has the same helicase activity as PriA, and may be able to substitute (Heller & Marians, 2005a, Mahdi et al., 2006). PriC mediates an alternative pathway of fork restart, but preferring a single-strand gap on the leading strand, loading DnaB to the lagging strand template (Heller & Marians, 2005a). Gaps at the site of the fork occur when the replicative polymerase becomes decoupled, and continues to synthesize one of the strands after stalling on the other (Pages & Fuchs, 2003).

The alternative to the “spontaneous” or direct restart of stalled forks, catalyzed through the PriA and PriC pathways, is the RecG or RecA-mediated regression of the
fork (Robu et al., 2004). After fork regression, and assuming the upstream lesion has been repaired, there are several options for resolution of the now collapsed fork (discussed in detail in later chapters). The first option is fork progression, reforming of the fork from the Holliday junction substrate (Higgins et al., 1976, Robu et al., 2004). The chicken foot structure has a free double strand end, a substrate for RecBCD processing, which can be degraded to yield a fork (Figure 1.2C). Given a gap on the lagging strand, the RecFOR system can bind the 5′ end of the gap, and through strand invasion displace the leading strand, which would be subject to nuclease attack, restoring the fork (discussed in chapter three). Alternatively, RuvAB can stabilize the regressed junction, facilitating branch migration of the junction, resulting in the RuvC-dependent cleavage of the junction to create a double strand break (Flores et al., 2001, McGlynn & Lloyd, 2001).

Once Ruv has acted upon a junction, RecBCD are required for restoration of the fork and resumption of replication (Cox, 2001, McGlynn & Lloyd, 2001, Mahdi et al., 2006, Michel et al., 2004). The RecBCD complex processes the double strand break in a recombination dependent manner. This process proceeds through a Holliday junction intermediate, which must be resolved, likely by a second round of action by Ruv. After the replication fork has been restored, the replication machinery can be reassembled via restart mediated by the PriA, PriC, and Rep proteins.
Section 1.4: Specific aims

When I began in the Segall laboratory, the goal of my project was to discover if the peptides were able to inhibit recombination in vivo. At the time, there were many people in the lab studying the biochemistry of the peptides, and how they inhibit lambda Integrase, but no one had studied their effects on bacterial cells. We hypothesized that the peptides could be used as tools to study different aspects of bacterial chromosome maintenance. The specific assays we were interested in were going to study homologous recombination between sister chromatids, and then use that information to asks questions about temporal and spatial organization of the chromosome. My early experiment focused on the cloning of a mini-gene that would express the peptides under inducible control on a plasmid. Although I was able to clone the genes and make the plasmids, I was never able to detect peptide activity either in the cells, or in whole cell extracts. After some time we decided to try applying the peptides exogenously. At first, using disk diffusion assays to look at peptide effects, things did not seem promising. However, once I attempted to treat cells in solution, my project took off in whole new directions.

We discovered that the peptides were capable of inhibiting bacterial growth in rich media culture. This was not an unexpected result, as we knew that the peptides inhibit proteins involved in DNA repair. After a few simple experiments looking at the growth kinetics of peptide-treated cells, it became clear that one focus of the project was to characterize the antimicrobial effects of the peptide. I used a number of diverse assays to address this specific aim: growth curves, minimum inhibitory concentration (MIC), viability, microscopy, and TUNEL (including flow cytometry). The
results of this study led to the formation of a model where peptide treatment caused DNA damage and inhibited the repair of stalled replication forks. Out of these results came the second specific aim of this study, which was to determine, genetically, the targets of peptide antimicrobial activity, and their role in the inhibition of DNA repair. I used pulsed field gel electrophoresis and viability assays to discern the peptide’s effects on *Salmonella* DNA repair. From this data, which corroborated our previous results, we have built a model that explains the peptides’ antimicrobial activity by altering the pathways that repair stalled replication forks.
Chapter 2: Analysis of peptides’ effects on bacterial cells

Section 2.1: Introduction

Holliday Junctions (HJ) are central intermediates in many essential prokaryotic and eukaryotic cellular processes. Homologous recombination (HR) and site-specific recombination (SSR) performed by tyrosine recombinases are the canonical pathways for the formation of HJ (Kreuzer, 2005, Van Duyne, 2002, Walker, 1996). In eukaryotes, HR is mandatory for meiosis. In both prokaryotes and eukaryotes, recombination repair of DNA lesions and collapsed replication forks proceed through HJ intermediates. HR is thought to occur at least once during each division cycle (Sherratt et al., 2004, Steiner & Kuempel, 1998, Lesterlin et al., 2004). In bacteria with circular chromosomes, an odd number of HR events between sister chromosomes results in formation of a dimeric chromosome which must be resolved via site-specific recombination prior to cell division (Lesterlin et al., 2004, Sherratt et al., 2004). HR is also important in pathways in the repair of DNA damage (Lesterlin et al., 2004, McGlynn & Lloyd, 2002, Michel et al., 2004), and failure to resolve HJ intermediates is detrimental to cell viability (Kreuzer, 2005, Michel et al., 2004).

Our laboratory previously reported the isolation and characterization of hexapeptides that bind HJ structures as dimers, and prevent their resolution to products. The peptides were identified as potent inhibitors of lambda Integrase (Int) \textit{in vitro} (Boldt et al., 2004, Cassell et al., 2000). The phage lambda site-specific tyrosine recombinase, Int, is mechanistically and structurally closely related to the bacterial proteins XerC and XerD. These proteins together catalyze site-specific recombination
between pairs of *dif* sites on dimerized chromosomes in order to regenerate monomer chromosomes or between a plasmid and the chromosome in order to integrate the plasmid (Lesterlin et al., 2004, Sherratt *et al.*, 1995, Sherratt *et al.*, 2004). Thus, the XerC and XerD proteins play a critical role in the faithful replication and inheritance of genetic material.

Two of the most potent peptides (see below) also inhibit branch migration by the RecG helicase and cleavage by the RuvABC HJ resolvase complex (Kepple *et al.*, 2005). RecG and the RuvABC complex play important roles in DNA lesion repair and the repair of collapsed replication forks (Flores *et al.*, 2001, Marians, 2004, McGlynn & Lloyd, 2002, Meddows *et al.*, 2004, Seigneur *et al.*, 1998, Michel *et al.*, 2004). Both RecG and RuvABC process HJ, but are mechanistically unrelated to each other, or to the tyrosine recombinases. The peptides’ activity on this diverse group of enzymes is explained by their structure-specific binding to protein-free HJ and some branched DNA molecules that resemble replication forks (Kepple *et al.*, 2005).

Our best characterized HJ-trapping peptides are WRWYCR, KWWCRW, and WKHYNY (by definition, peptides consisting of L amino acids will be designated with capital letters, and d amino acid peptides will be designated by lower case letters). These peptides fall into two classes based on their potency and DNA binding properties. WRWYCR and KWWCRW are 10-20 fold more potent inhibitors of Int recombination, bind to free HJs, and inhibit RecG and RuvABC activity, but show some non-specific DNA binding at higher concentrations (Table 2.1 also Cassell *et al.*, 2000, Klemm *et al.*, 2000, Boldt *et al.*, 2004, Kepple *et al.*, 2005). All the *in vitro* activities of these peptides are strongly dependent on the formation of disulfide
bridges between the cysteines present in both (Boldt et al., 2004, Kepple et al., 2005). Although WKHYNY is less potent than WRWYCR or KWWCRW in inhibiting Int and does not inhibit RecG, it is the most specific for the HJ structure and does not display any nonspecific DNA binding (Klemm et al., 2000). The in vitro activities of these peptides suggested that they may possess antibacterial activity.

Antimicrobial peptides are widespread in nature as part of the innate immune response, and are extremely diverse in structure and sequence. The HJ-binding, recombination-inhibiting peptides are cationic and hydrophobic, and somewhat similar to the lactoferricin family of peptides. However, the HJ-binding peptides are significantly shorter than the naturally-occurring lactoferricin-derived peptides, which vary in length from 20 to 60 amino acids and adopt specific twisted beta-sheet folds (Epand & Vogel, 1999). Despite the shared cationic and aromatic character of our peptides with some of the natural antimicrobial peptides or their derivatives (for a recent review, see (Brogden, 2005), the exact composition of our peptides is distinct and not present in any sequence currently in GenBank. Herein, we have assessed the antimicrobial activity of the HJ-binding peptides, and show that these peptides kill bacteria by interfering with the activity of intracellular targets. We show that the peptides cause accumulation of DNA breaks that leads to filamentation, faulty DNA partitioning, and anucleate cells. Examining the effects of the peptides on cells experiencing different DNA-related stresses revealed that the HJ-binding peptides kill bacterial cells in a SOS- and RecA-independent manner.
Table 2.1: Peptide potency in the inhibition of HJ processing enzymes (IC\textsubscript{50}) and DNA binding activity (all values in \(\mu\)M).

<table>
<thead>
<tr>
<th>Protein/substrate</th>
<th>WRWYCR</th>
<th>KWWCRW</th>
<th>WKHYNY</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\lambda) Int\textsuperscript{a}</td>
<td>0.005-0.02</td>
<td>0.02-1.1</td>
<td>0.2-20</td>
</tr>
<tr>
<td>HJ binding\textsuperscript{b}</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Ns DNA binding\textsuperscript{b}</td>
<td>Y/N</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>XerCD\textsuperscript{c}</td>
<td>0.05</td>
<td>0.05</td>
<td>nd\textsuperscript{d}</td>
</tr>
<tr>
<td>RecG\textsuperscript{e}</td>
<td>0.025-0.2</td>
<td>nd</td>
<td>NE\textsuperscript{d}</td>
</tr>
<tr>
<td>RuvABC\textsuperscript{e}</td>
<td>0.064</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

\textsuperscript{a} IC\textsubscript{50} values vary depending on the recombination pathway mediated by phage lambda Int (data from Boldt et al., 2004, Cassell et al., 2000, Cassell & Segall, 2003, Klemm et al., 2000).

\textsuperscript{b} Binding activity: HJ, Holliday junctions; nsDNA, nonspecific double stranded DNA; Y, significant dsDNA binding; Y/N, moderate to slight dsDNA; N, no DNA binding (data from Boldt et al., 2004, Cassell & Segall, 2003, Kepple et al., 2005, Klemm et al., 2000).

\textsuperscript{c} XerCD-mediated recombination was tested between two psi sites in the presence of ArgR and PepA proteins. Reactions were assembled and performed as described in Bregu et al. (Bregu et al., 2002). These experiments were performed by A. Segall in David Sherratt’s lab with the gracious help of Lidia Arciszewska, Miguena Bregu, Ivailo Zhekov, Rachel Baker, and David Sherratt.

\textsuperscript{d} nd, not done; NE, peptide has no effect in the reaction.

\textsuperscript{e} IC\textsubscript{50} values vary depending on the reaction substrate (data from Kepple et al., 2005).
Section 2.2: Results

**Peptide-mediated inhibition of bacterial growth.** Because HJs are intermediates in both recombination-dependent DNA repair and chromosome dimer resolution by XerC and XerD, we reasoned that peptides which interfere with these processes may inhibit bacterial growth. We investigated the antimicrobial activity of the HJ-trapping peptides by performing standard minimum inhibitory concentration (MIC) assays in microtiter plates. The Gram-positive (Gm+) organisms *Bacillus subtilis*, *Staphylococcus aureus* (as well as a methicillin-resistant MRSA hospital isolate), and *Lactococcus* (*Streptococcus*) *lactis* (Table 2 and data not shown) were more sensitive to peptide treatment than the Gram-negative (Gm-) organisms *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (STm). The STm *galE rfa* strain (also known as deep rough), which has short LPS chains (Ames et al., 1973; Gabriel, 1987; Nikaido, 1996) and is more permeable to a variety of exogenous agents (Ames et al., 1973), showed sensitivity to peptide treatment similar to the sensitivity of the Gm+ bacteria. This suggests that the LPS of the Gm- is a barrier to the peptides. Mutations in either *galE* alone or in the *rfb* locus (resulting in cells with intermediate length LPS chains) reduced the MIC partially but not as much as the combined *galE rfa* mutations. Because the deletion which removes *galE* and *rfa* also removes the *uvrB* locus, we tested and found that the *uvrB* mutation (in the absence of UV treatment) did not render the strain hypersusceptible to peptide (Table 2.2). In addition to the organisms shown in Table 2.2, two other virulent organisms were tested: the Gm- *Salmonella enterica* 14028S had an MIC of 64 μg ml⁻¹ and the Gm+ *Listeria monocytogenes* had an MIC of 12.5 μg ml⁻¹ for peptide WRWYCR and its d-
amino acid isomer, wrwycr (L. Su and A. Segall, unpublished). In summary, of the peptides tested, WRWYCR and wrwycr were the most potent inhibitors of bacterial growth with peptide KWWCRW following close behind, while peptide WKHYNY had no detectable effect on the growth of bacteria. The inhibitory potency of the different peptides on bacterial growth correlated with their potency \textit{in vitro}, suggesting that the inhibition of growth could be due to inhibition of recombination or preventing processing of repair intermediates (see below for more discussion).

\textit{In vitro}, both L- and d-form peptides bind to junctions and inhibit Int-mediated recombination and junction-resolution by RecG and RuvABC \textit{in vitro} (Boldt et al., 2004, Kepple et al., 2005, K. Kepple and A. Segall, unpublished) the L- and d-form peptides can bind to HJs with nearly equivalent affinity but may trap different isomer forms of the junction (J.L. Boldt and A.M. Segall, unpublished results). Since d-amino acid peptides are expected to resist degradation by intracellular peptidases, we tested the d-peptides in MIC assays and growth curve experiments. Bacterial growth rates in LB medium in the presence of peptides largely correlated with the MIC endpoint assays. \textit{B. subtilis} was completely inhibited for the first 8 to 10 hours by 8 \(\mu\)M WRWYCR and 5 \(\mu\)M wrwycr, respectively (Figure 2.1C). \textit{E. coli} was less sensitive to peptides in growth curve experiments, even at concentrations as high as 100 \(\mu\)M (Figure 2.1A), which is above its MIC value of 64 \(\mu\)M. The major difference between the MIC and growth curve experiments is the media in which they were performed – Mueller-Hinton (MH) broth for the MICs (as specified by the NCCLS protocol) versus LB for the growth curves. When the growth curves were performed in MH broth, we
Table 2.2: Minimum inhibitory concentration (MIC) values, expressed in μg ml⁻¹ of hexapeptide.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>WRWYCR</th>
<th>wrwycr</th>
<th>KWWCRW</th>
<th>kwcrw</th>
<th>WKHYNY</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli W3110</td>
<td>W3110</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>NT</td>
<td>&gt;128</td>
</tr>
<tr>
<td>E. coli W3110</td>
<td>xerC</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>NT</td>
<td>&gt;128</td>
</tr>
<tr>
<td>E. coli W3110</td>
<td>xerD</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>NT</td>
<td>&gt;128</td>
</tr>
<tr>
<td>E. coli W3110</td>
<td>xerC xerD</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>NT</td>
<td>&gt;128</td>
</tr>
<tr>
<td>E. coli W3110</td>
<td>uvrA::Tn10</td>
<td>64</td>
<td>32-64</td>
<td>NT</td>
<td>NT</td>
<td>&gt;128</td>
</tr>
<tr>
<td>E. coli MG1655</td>
<td>MG1655</td>
<td>64</td>
<td>32</td>
<td>64</td>
<td>NT</td>
<td>&gt;128</td>
</tr>
<tr>
<td>E. coli MG1655</td>
<td>sbcC</td>
<td>32</td>
<td>32</td>
<td>64</td>
<td>NT</td>
<td>&gt;64</td>
</tr>
<tr>
<td>S. enterica Tm</td>
<td>LT2</td>
<td>64</td>
<td>64</td>
<td>&gt;128</td>
<td>NT</td>
<td>&gt;128</td>
</tr>
<tr>
<td>S. enterica Tm</td>
<td>Δ(galE- rfa- uvrB)</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>32</td>
<td>&gt;128</td>
</tr>
<tr>
<td>S. enterica Tm</td>
<td>galE::Tn10d(attR,)</td>
<td>32-64</td>
<td>32</td>
<td>NT</td>
<td>NT</td>
<td>&gt;128</td>
</tr>
<tr>
<td>S. enterica Tm</td>
<td>rfb</td>
<td>32</td>
<td>32</td>
<td>NT</td>
<td>NT</td>
<td>&gt;128</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>JH642</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>32</td>
<td>&gt;128</td>
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<tr>
<td>B. subtilis</td>
<td>recA260</td>
<td>8</td>
<td>8</td>
<td>NT</td>
<td>NT</td>
<td>&gt;128</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>recA::Kn⁺</td>
<td>4</td>
<td>4</td>
<td>NT</td>
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<td>&gt;128</td>
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<td>S. aureus</td>
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<td>&gt;128</td>
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<td>S. aureus 33591</td>
<td>MRSA</td>
<td>32</td>
<td>32</td>
<td>64</td>
<td>NT</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

NT = not tested; MRSA = methicillin resistant *Staphylococcus aureus*
saw increased sensitivity of *E. coli* to peptide (Figure 2.1B). One of the major differences between the formula of MH and that of LB is the NaCl content (0.5%) of LB. Indeed, when we added 0.5% NaCl to MH broth, we saw increased resistance to peptide (Figure 2.1B). Similar salt-dependent reduction in peptide potency has also been observed for some classes of naturally occurring antimicrobial peptides, and it is thought that the peptides’ interaction with the bacterial membrane is salt-dependent (Goldman *et al.*, 1997, Tam *et al.*, 2002).

The growth curve experiments also show recovery of growth after extended exposure to peptide. This recovery is probably not due to a heritable genetic change, since we have not been successful in isolating spontaneous peptide-resistant mutants from those cultures (J. Patelzick, C. Gunderson and A. Segall, unpublished). Like other antibiotic compounds, the peptides may be subject to efflux from the cell, since *acrAB*− mutants of *E. coli* or *Salmonella*, defective in the major efflux pump (Nikaido & Zgurskaya, 2001), show lesser recovery in similar experiments (C. Gunderson, J. Johnson, N. Radosevich, and A. Segall, unpublished). Again, the increased potency of the d-peptides is evident for both *E. coli* (Figure 2.1A - C filled symbols) and *Salmonella* (data not shown); we assume that resistance of the d-peptides to peptidases accounts for at least part of this response.

We also tested whether the peptides halted bacterial growth when added to growing cultures (flattening of growth curves), or if the peptides caused cell lysis (seen as a decrease in the optical density of the culture), to gain further insight into the peptides’ mechanism of antimicrobial action. The peptides were added to bacterial cells 1 hour or 3 hours after subinoculation of cultures into fresh LB media.
Figure 2.1: Peptides WRWYCR and wrwycr inhibit the growth of *E. coli* MG1655 and *B. subtilis* JH642. A) Growth of *E. coli* MG1655 in LB broth without (closed circles) and with 100 µM WKHYNY (open circles). WRWYCR (open symbols) and wrwycr (closed symbols) were tested at 50 µM (diamonds) and 100 µM (squares). B) Growth of *E. coli* MG1655 in MH broth with (red symbols and lines) and without (black symbols and lines) added sodium chloride. Control growth without peptide is shown by filled circles. Cultures treated with 25 µM WKHYNY are represented by open circles. Cultures treated with 25 µM WRWYCR and 10 µM wrwycr are shown by open squares and filled triangles, respectively. C) Growth of *B. subtilis* JH642 without (filled circles) and in the presence of 8 µM WKHYNY (open circles). WRWYCR (open symbols) and wrwycr (filled symbols) were added to cultures at concentrations of 2, 5, and 8 µM (triangles, diamonds, and squares, respectively). D) Growth of *E. coli* (black lines) and *B. subtilis* (red lines) in the absence of peptide (filled circles), or with peptide added at 1 hour (open diamonds) or 3 hours (open triangles) after subculture (t = 0); 100 µM peptide was added to the *E. coli* cultures, and 25 µM to the *B. subtilis* cultures. Error bars in A-C represent the standard deviation from the mean among 3 independent cultures.
Peptides WRWYCR and wrwycr prevented or slowed further growth without lysing cells, seen as unchanged or increased OD after peptide addition (Figure 2.1D). Also, we did not detect any cell debris as evidence of lysis via microscopy (Figures 2.3, 2.4, 2.6, 2.9, and data not shown). However, while the peptides retained full effectiveness against *B. subtilis*, they lost some of their effectiveness against *E. coli* as the culture grew to higher density, further illustrating the higher sensitivity of Gram-positive bacteria.

The block to further growth without lysis does not distinguish whether the peptides are bactericidal or bacteriostatic. To test this, dilutions of cultures treated with concentrations of peptide representing 0.5X, 1X, and 2X MIC were plated on nonselective LB agar and viable counts were determined. The common definition of bactericidal action is that viability of the cultures is reduced 3 logs (i.e., 99.9% of the bacteria are killed). By this definition, both peptides WRWYCR and wrwycr are bactericidal to the methicillin-resistant *S. aureus* strain 33591, to *E. coli* MG1655, and to *Salmonella* LT2 at 1X MIC (Table 2.3). Peptide wrwycr is bactericidal at 1X MIC to *S. aureus* 25923 and to *B. subtilis*, while peptide WRWYCR is bactericidal to these two bacterial strains only at 2X MIC. Peptide KWWCRW shows less consistent killing at 1X MIC – it is only bactericidal to the gram positive bacteria tested, although it does reduce the viability of *E. coli* by 2 logs (99%) (Table 2.3). As expected, peptide WKHYNY has no significant effect on the viability of any of the bacteria tested.

**Effects of peptides on DNA and RNA synthesis.** We tested the effect of the hexapeptides on the synthesis of DNA and RNA by measuring their effect on the
Table 2.3: Bacterial viable counts. Expressed as the surviving fraction (S/S₀) remaining after 18h peptide treatment.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Peptide: None</th>
<th>WRWYCR</th>
<th>wrwycr</th>
<th>KWWCR</th>
<th>WKHYNY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose:</td>
<td>0.5X MIC</td>
<td>1X MIC</td>
<td>2X MIC</td>
<td>0.5X MIC</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>-</td>
<td>0.261</td>
<td>0.652</td>
<td>&lt;0.0004</td>
<td>&lt;0.0004</td>
</tr>
<tr>
<td>JH642</td>
<td>2.3 x 10⁷</td>
<td>0.056</td>
<td>&lt;0.0001</td>
<td>0.100</td>
<td>0.007</td>
</tr>
<tr>
<td>S. aureus</td>
<td>1.0 x 10⁸</td>
<td>1.500</td>
<td>&lt;0.0003</td>
<td>1.667</td>
<td>&lt;0.0003</td>
</tr>
<tr>
<td>25923</td>
<td>3.0 x 10⁷</td>
<td>0.151</td>
<td>&lt;0.00003</td>
<td>0.091</td>
<td>&lt;0.00003</td>
</tr>
<tr>
<td>S. aureus</td>
<td>3.5 x 10⁸</td>
<td>0.857</td>
<td>&lt;0.00003</td>
<td>&lt;0.00003</td>
<td>0.571</td>
</tr>
<tr>
<td>33591</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>0.151</td>
<td>&lt;0.00003</td>
<td>0.091</td>
<td>&lt;0.00003</td>
</tr>
<tr>
<td>MG1655</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. Tm LT2</td>
<td>3.5 x 10⁸</td>
<td>0.857</td>
<td>&lt;0.00003</td>
<td>&lt;0.00003</td>
<td>0.571</td>
</tr>
</tbody>
</table>

a Average values for the surviving fraction of colonies from two independent peptide-treated cultures. Cultures were set up as described for MIC determination in Mueller-Hinton broth. After 18 hours incubation at 37°C, samples were taken from wells of the 96-well plate, diluted appropriately to obtain countable numbers of colonies in 1 mM Tris-10 mM magnesium chloride (pH 7.4), and plated on non-selective LB agar plates. Plates were incubated no longer than 20 hours and counted.

b For comparison, average values of the final CFU ml⁻¹ of DMSO-treated (control) cultures are given. Values are averages of triplicate cultures.

c MIC values differ with the specific bacterium; In the case of B. subtilis treated with peptide WRWYCR or wrwycr, 0.5X MIC = 4 μM, 1X MIC = 8 μM, and 2X = 16 μM; with peptide KWWCRW, the MIC = 8 μM. In the case of both S. aureus strains treated with peptide WRWYCR or wrwycr, 0.5X MIC = 16 μM, 1X MIC = 32 μM, and 2X = 64 μM; with peptide KWWCRW, the MIC = 64 μM. In the case of E. coli and STm treated with peptide WRWYCR or wrwycr, 0.5X MIC = 32 μM, 1X MIC = 64 μM, and 2X = 128 μM; with peptide KWWCRW, the MIC is 64 μM.
Figure 2.2: Peptide wrwycr partially inhibits the uptake and incorporation of \(^{3}H\)-thymidine (A) and \(^{3}H\)-uridine (B). A) Incorporation of \(^{3}H\)-thymidine by *E. coli* MG1655 was moderately inhibited by 32 and 64 \(\mu M\) wrwycr (open triangles and diamonds) as compared to nalidixic acid (200 \(\mu g\) ml\(^{-1}\)), which inhibited the uptake of label characteristic of the gyrase inhibitors (open squares). B) Untreated *E. coli* MG1655 (filled squares) incorporated \(^{3}H\)-uridine with greater efficiency than *E. coli* treated with 32 and 64 \(\mu M\) wrwycr (open triangles and diamonds). Inhibition by wrwycr was not as great as that demonstrated by rifampicin at a concentration of 200 \(\mu g\) ml\(^{-1}\) (open squares).
incorporation of radiolabeled precursors \textit{in vivo}. Both $^3$H-thymidine and $^3$H-uridine incorporation were inhibited by the addition of peptide at the beginning of a 2 hour time-course. The peptides inhibit DNA synthesis somewhat less than nalidixic acid and inhibit RNA synthesis much less than rifampicin (Figure 2.2). We do not yet know whether inhibition of DNA synthesis or RNA synthesis is direct or indirect. However, the peptides do not inhibit the replication of an OriC-dependent plasmid \textit{in vitro} (J. Kaguni, personal communication).

\textbf{Hexapeptides interfere with DNA segregation and septation.} DNA damage in \textit{E. coli} is often manifested by filamentation due to induction of the SOS response and the consequent SulA-induced block to cell division (Hill \textit{et al.}, 1997, Huisman \textit{et al.}, 1984, Walker, 1996). Moreover, if XerC and XerD were targets of the peptide (see below) we would have expected some peptide-dependent defects in chromosomal segregation (Blakely \textit{et al.}, 1993, Sherratt \textit{et al.}, 2004). \textit{E. coli} MG1655 was grown for 90 minutes in LB at 37°C, followed by addition of peptide and continued incubation for 90 minutes. After treatment, cells were stained with DAPI and visualized by microscopy (Figure 2.3). Peptides WRWYCR and wrwycr had similar effects on cell morphology, including filamentation and DNA segregation defects. The number and length of filamentous cells increased as the dose of peptide was increased from 50 $\mu$M (Figure 2.3 panels B, C, G, H) to 100 $\mu$M (Figure 2.3 panels D-F and I-K) compared to DMSO (mock)-treated cells (Figure 2.3A). Similar effects were also observed in peptide-treated \textit{Salmonella} (data not shown), and \textit{B. subtilis} cultures treated with 25 $\mu$M peptide \textit{d-wrwycr} (Figure 2.3 M-N). Defects in
Figure 2.3: Treatment of *E. coli* with d and L forms of WRWYCR leads to filamentation, DNA segregation defects, and anucleate cells. A) MG1655, untreated; B-C) MG1655, 50 µM WRWYCR; D-F) MG1655, 100 µM WRWYCR; G-H) MG1655, 50 µM wrwycr; I-K) MG1655, 100 µM wrwycr. L) *B. subtilis* JH642, untreated; M-N) *B. subtilis*, 25 µM wrwycr.
septum formation or cell morphology of peptide-treated *S. aureus* were not detected either by fluorescence microscopy or by transmission electron microscopy (data not shown).

Several hydrophobic amino acid residues are contained in the composition of peptide wrwycr. To address the possibility that the peptide may destabilize the membranes of bacterial cells, *E. coli* was treated with peptide or EDTA, and stained with propidium iodide. At high concentrations EDTA is known to destabilize the outer membrane of gram negative bacteria (Leive, 1974, Nikaido, 1996). The fluorescent DNA strain, propidium iodide (PI), is cell impermeant, unless the cells have become compromised. *E. coli* treated with 0.2 mM EDTA or 1 µg ml⁻¹ peptide do not allow PI staining of the DNA (Figure 2.4). Treatment with either 2.0 µM EDTA or 100 µg ml⁻¹ of peptide does allow entry and staining by propidium iodide to occur (Figure 2.4).

During the microscopy analysis, a higher fraction of anucleate cells appeared in peptide-treated cultures compared to the untreated controls. To obtain more quantitative data on larger numbers of cells than could be easily obtained with microscopy, we used flow cytometry. *E. coli* cultures were grown in LB as above, treated with peptide, and stained with PicoGreen, which fluoresces green when bound to DNA, and counterstained with the membrane-specific dye FM 4-64, which fluoresces red when in a lipophillic environment. During flow cytometry analysis, anucleate cells would be detected in the red channel only, while normal cells would
Figure 2.4: Effects of wrwycr treatment on the permeability of *E. coli* W3110 membranes. A) DMSO (mock) treated *E. coli* cells stained with propidium iodide (PI). The membranes are intact, so PI cannot enter the cell and stain the DNA. B) W3110 cells treated with 0.2 mM EDTA and PI. This treatment does not allow staining of the DNA. C) W3110 treated with 2.0 mM EDTA and PI. Cells stain red, suggesting that the membranes of the cells have become destabilized, and allowed PI to enter. D) Treatment with 1 µg ml⁻¹ peptide does not allow PI to stain, but 100 µg ml⁻¹ (E) does.
fluoresce in both red and green channels. Dot plots of representative data from one experiment are shown in figure 2.5. The entire population of cells, P1, was subdivided into several subpopulations using control reactions in which cells were not stained at all or were stained only with one or the other dye, in order to set the fluorescence detection “gates”. The minority P2 population represents background signal and unstained particles, probably cell fragments or other non-bacterial particles. The P3 and P4 populations represent cells stained only with PicoGreen or only with FM4-64, respectively, while the P5 population represents cells stained with both PicoGreen and FM4-64. The P4 population represents anucleate cells, while the P5 population represents normal cells. The P3 population is very small (<0.3% of events) and probably represents PicoGreen-stained aggregates in the media or, less likely, DNA aggregates from a few lysed cells. We observed an eight-fold increase in anucleate cell formation when MG1655 was treated with 50 μM wrwycr, a sub-lethal dose of peptide (quantitated in Figure 2.5M, data shown in Figure 2.5H versus 2.5F). No increase in anucleate cells was seen with 100 μM peptide WKHYNY, which has no effect on bacterial growth (Figure 2.5G). In addition, an increase in cells with apparent sub-normal amounts of DNA (PicoGreen signal) was observed in the wrwycr-treated cells but not in the WKHYNY-treated cells (Figure 2.5M, open bars). The P5 subpopulation was further subdivided into two subpopulations, P6 and P7. The P6 subpopulation presumably consists of cells with wild type amounts of DNA. The P7 subpopulation increased significantly in the wrwycr-treated cells (Figure 2.5L versus 2.5K or 2.5J), and may represent sub-unit length DNA fragments in cells that have suffered mis-segregated chromosomes. This increase was dose-dependent – the fraction of P7 events almost doubles, from 4.9% to 9.7%, with a doubling of wrwycr
**Figure 2.5:** Peptides increase the fraction of anucleate *E. coli* MG1655.

MG1655 cells in the populations labeled in panels A-L represent the following:
P1) all events; P2) unstained events; P3) PicoGreen-positive events; P4) FM4-64-positive events; P5) double-stained events; P6) high intensity PicoGreen staining; P7) low intensity PicoGreen staining. FSC vs. SSC plots:
A) Untreated (DMSO); C) 100 µM WKHYNY; D) 50 µM wrwycr. FM4-64 vs. PicoGreen plots: B) PicoGreen-stained cells; E) FM4-64-stained cells; F) double-stained cells; I) PicoGreen-stained cells, and double stained cells (J). 100 µM WKHYNY (C, G, K) and 50 µM wrwycr (D, H, L) treated plots: C, D) FSC vs. SSC of double stained cells; G, H) FM4-64 vs. PicoGreen dot plot of double stained cells; K, L) FSC vs. PicoGreen of double stained cells. M) Percentage of MG1655 anucleate cells (filled bars) and cells with fragmented DNA (open bars) after treatment with peptides wrwycr (50 µM and 100µM) and WKHYNY (100 µM). Anucleate cells = (P4)/(P4+P5). Error bars show the standard deviation from the mean among 3 independent cultures.
dose from 50 to 100 μM (Figure 2.5J-M). Untreated cells and WKHYNY-treated cells had only 2.7% and 2.8% P7-type events, respectively. Thus, the chromosome segregation defects induced by peptide wrwycr resulted in a larger fraction of anucleate cells and increased peptide-dependent DNA fragmentation.

One of the expected cellular targets of the peptides is the XerC/D site-specific recombinase, which mediates site-specific resolution of chromosome dimers at the terminus-proximal dif site and is mechanistically and structurally related to Int (Lesterlin et al., 2004, Sherratt et al., 2004). Peptide-induced segregation defects and higher numbers of anucleate cells would be consistent with the peptide inhibiting XerC/D-mediated recombination. In vitro, peptides WRWYCR and KWWCRW inhibited XerC/D recombination between two plasmid psi sites (similar to the chromosomal dif site) in the presence of the required accessory proteins ArgR and PepA (Summers, 1998) with an IC₅₀ of 50-100 nM (Table 2.1). If Xer-mediated recombination was the only, or the major, generator of targets for the peptides in vivo, we would expect that xerC and/or xerD mutations would render cells (more) resistant to peptide killing. In E. coli, neither single nor double Xer⁻ mutants offered any protection from peptide treatments, either when tested by comparing growth curves of peptide-treated mutant versus wild type cells (data not shown), comparing MIC values (Table 2.2), or by microscopy (Figure 2.6). The Salmonella xerD mutant behaved like the E. coli xerD mutant (data not shown). A mutation in recA would make the XerC/D system less necessary, because the crossovers that would lead to chromosome dimerization would not occur. The sensitivity of a Salmonella recA xerD double
Figure 2.6: Peptide treatment is epistatic to xer mutant filamentation phenotypes. E. coli W3110 xer mutants were stained with DAPI (panels A-H) or DAPI and FM4-64 (panels I-N) and viewed using epifluorescence microscopy. Untreated cultures: A & I) W3110; B & J) W3110 xerC; C & K) W3110 xerD; and D) W3110 xerC xerD. Cultures treated with 100 µM wrycr: E & L) W3110; F & M) W3110 xerC; G & N) W3110 xerD; and H) W3110 xerC xerD. A Salmonella xerD strain showed the same phenotype in the presence or absence of peptide as the E. coli W3110 strain shown.
Figure 2.7: Growth of *Salmonella* recA and xerD mutants in the presence of d-wrwycr. A) Strains were grown in MH broth, subcultured into peptide containing media at the time=0. *Salmonella* LT2, black curves; LT2 recA, red curves; LT2 xerD, blue curves; LT2 recA xerD, green curves. Two concentrations of peptide d-wrwycr were used: squares, mock treated (DMSO) control, triangles, 16 µM peptide; circles, 32 µM peptide. Error bars represent the standard deviation from the mean of three independent cultures. B) Photographs of LB plates used for dilution series have dilutions labeled across the top, and treatment conditions labeled down the side. Photographs represent data from triplicate independent cultures from at least 2 different days (n of at least 6)
mutant was tested by a growth curve experiment (Figure 2.7). All three of the strains tested show a similar mild enhancement in sensitivity to peptide treatment, none afford protection. The morphology of xerC or xerD bacteria, observed by microscopy, shows that the mutations are epistatic to peptide treatment because the effect of peptide treatment results in cell morphologies very similar to the untreated (Table 2.2, Figure 2.6, 2.7). These data do not rule out that XerC and XerD are inhibited by the peptides; rather, they indicate that if XerC and XerD are inhibited, they are not the sole targets of the peptides in vivo, consistent with their inhibition of RecG and RuvABC complexes (Kepple et al., 2005).

Another possibility we considered is that the peptides stabilize normally transient cruciforms extruded from otherwise double-stranded DNA, even relatively short ones, as a consequence of their junction-binding activity. Such structures might then become substrates for cleavage by the SbcC/D endonuclease (Connelly et al., 1999, Connelly et al., 1998), and cause an increase in double strand breaks. If such substrates were indeed the major targets of the peptides, we would have expected sbcC or sbcD mutants to be more resistant to peptide treatment than wild type, but this was not observed (Table 2.2). Again, two possibilities exist: either such structures are not targets for the peptides or alternatively, while the peptides stabilize such extruded cruciforms, they also inhibit cleavage by the SbcC/D nuclease.

Peptide treatment leads to accumulation of DNA breaks. Interference by the peptides with DNA repair processes was expected to cause an increase in strand breaks, the half-life of which would be longer in the presence of peptide. To obtain direct measurements of such damage we used the TUNEL (terminal
Figure 2.8: Peptide treatment causes the accumulation of 3’OH DNA ends, as detected using the TUNEL assay and flow cytometry. (These examples of dot plots correspond to the data in Table 2.4.) In all parts, populations are labeled: P1, all cells; P2, unstained events; P3, PI positive events; P4, TUNEL positive events; P5, double positive events. A) Untreated *E. coli* MG1655 FSC vs SSC. B-D) Untreated MG1655 FITC (TUNEL positive) vs. PI dot plots; B) unstained cells; C) cells stained with PI only; D) FITC-(TUNEL positive) cells only; E-F) MG1655 treated with 50 µM wrwycr; G-H) *B. subtilis* JH642 treated with 10 µM wrwycr; I-J) *Salmonella* LT2 treated with 50 µM wrwycr; K-L) *S. aureus* 25923 treated with 25 µM wrwycr.
**Table 2.4. Flow cytometry analysis of TUNEL assay.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment (medium)</th>
<th>PI</th>
<th>TUNEL</th>
<th>% Labeled (SD)</th>
<th>Fold difference +pep/-pep</th>
</tr>
</thead>
<tbody>
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<td><em>E. coli</em> MG1655</td>
<td>DMSO (LB)</td>
<td>37988</td>
<td>348</td>
<td>1.2 (0.4)</td>
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</tr>
<tr>
<td></td>
<td>50 μM wrwycr (LB)</td>
<td>27541</td>
<td>2364</td>
<td>8.5 (1.1)</td>
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<tr>
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<td>4446</td>
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</tr>
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<tr>
<td></td>
<td>64 μM wrwycr (MH)</td>
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<td>2002</td>
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<tr>
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<td>3382</td>
<td>26.2 (3.4)</td>
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</tr>
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<td></td>
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<tr>
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<td>4.0 (0.8)</td>
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<td><em>S. aureus</em> 25923</td>
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<td>124</td>
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<td>2367</td>
<td>340</td>
<td>16.3 (2.8)</td>
<td>5.8</td>
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</table>

a The standard deviation from the mean of 3 independent cultures is shown in parentheses. Representative dot plots are shown in Figure 6.
deoxyribonucleotide transferase-mediated dUTP nick end labeling) assay, adapted to bacteria, to label free 3’ hydroxyl (3’OH) DNA ends inside cells with a fluorescein-conjugated nucleotide triphosphate (Rohwer & Azam, 2000, see Materials and Methods). Free 3’OH ends accumulate subsequent to many types of DNA damage such as nicks or gaps, double strand breaks, and stalled, collapsed or regressed replication forks (McGlynn & Lloyd, 2002, Michel et al., 2004, Rohwer & Azam, 2000). Thus, cells containing DNA damage will be labeled with fluorescein and can be detected either by epifluorescence microscopy or by flow cytometry. The TUNEL assay provides a simple, specific, and direct means of detecting DNA damage (Gorczyca et al., 1993, Rohwer & Azam, 2000, Roychoudhury et al., 1976). Peptide wrwycr-induced DNA breaks were measured via the TUNEL assay. Cells were counterstained for DNA content using propidium iodide and analyzed by flow cytometry (Figure 2.8 and Table 2.4). Forward scatter (FSC; measure of cell size) versus side scatter (SSC; measure of granularity, see Materials and Methods, Chapter 7) plots indicate all the bacterial cells (P1). The fluorescence dot plots show FITC fluorescence intensity versus PI fluorescence intensity (Figure 2.8B, 2.8C, 2.8D, 2.8F, 2.8H, 2.8J, and 2.8L) of the events within population P1. Four subpopulations within population P1 were identifiable: unstained cells or other particles (P2); cells stained with propidium iodide (PI) only, representing cells with DNA but without appreciable DNA damage (P3); cells stained with FITC only (P4); and cells that sustained DNA damage and were stained with both PI and FITC (population P5). We interpret the events not stained with PI as bacteria lacking DNA. Note that we have not yet attempted to quantitate TUNEL signal on a per cell basis – we have simply counted the number of cells which are or are not labeled.
Figure 2.9: TUNEL damage accumulates rapidly after peptide treatment. *B. subtilis* JH642 and *B. subtilis recA::Kn* cells were treated with peptide wrwycr for 5, 15, 30, 60 minutes or left untreated for 60 minutes, and assayed using the TUNEL procedure. A) top row: untreated *B. subtilis* JH642 cells sampled at 5, 15, 30 or 60 minutes (left to right, respectively). A) bottom row: 100 µM wrwycr treated *B. subtilis* JH642 cells sampled at 5, 15, 30 or 60 minutes (left to right, respectively). Cells were methanol-fixed on slides prior to viewing under the microscope. Images shown are superimposed phase contrast and epifluorescence pictures of representative fields. B) Using the micrographs, a minimum of 500 cells were counted for each treatment and time-point and the percentage of labeled cells was calculated. Error bars represent the standard deviation from the mean from two independent experiments on two different days.
Increased variability is seen in the FSC versus SSC (Figure 2.8A, 2.8E, 2.8G, 2.8I, and 2.8K) populations as peptide concentration increased (note scattered events outside the main population in the upper right region of Figure 6E that are not present in Figure 2.8A). This variability correlates with the changes in morphology (filamentation) caused by peptide treatment. The accumulation of TUNEL-labeled cells is peptide- and dose-dependent and the potency of the peptide as determined by MIC correlates with the number of cells containing DNA damage. *B. subtilis*, which has a lower MIC for wrwycr than *E. coli*, shows greater TUNEL labeling in response to peptide treatment (Table 2.4). Initially, these experiments were done in LB broth at sub-lethal concentrations of peptide in the case of *E. coli*, STm and *S. aureus*, and just above the MIC in the case of *B. subtilis*. In these conditions, STm and *S. aureus* showed fewer strand breaks than *E. coli* and *B. subtilis*. The experiment was repeated, growing *E. coli*, STm, and *S. aureus* in MH broth containing 0.5x, 1x, and 2x MIC of peptide wrwycr to illustrate the dose-dependent increase in breaks (Table 2.4). In the case of STm, the differences in membrane structure with respect to *E. coli* may account for its greater resistance at sub-MIC concentrations (Table 2). This may also be the case for *S. aureus*.

To determine the kinetics of DNA damage accumulation, *B. subtilis* was treated with peptide for increasing periods of time, then assayed using TUNEL (Figure 2.9). *B. subtilis* cultures were treated (or not) with 100 μM hexapeptide wrwycr in LB broth for 5, 15, 30, or 60-minute intervals. An untreated culture showed a constant level of labeling at all time points (Figure 2.9A, top four micrographs). The peptide-treated culture showed a time-dependent increase in TUNEL labeling (Figure
**Table 2.5:** Peptide wrwycr induces the SOS response (fold induction measured by ELISA).

<table>
<thead>
<tr>
<th>Strain</th>
<th>MMC (1 μg ml⁻¹)</th>
<th>wrwycr (50 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli dinD1::MudI (lacZ, Ap&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>2.8</td>
<td>4.0</td>
</tr>
<tr>
<td>STm LT2 recN557::MudJ(lacZ, Km&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>6.4</td>
<td>6.4</td>
</tr>
</tbody>
</table>
2.9A, bottom four micrographs). Clearly peptide-treated cells accumulated increasing DNA damage over time. If the accumulation of DNA damage depended on either recombination or induction of the SOS response via activation of RecA, a recA mutant should not accumulate TUNEL signal above background. On the other hand, if the damage is caused directly by peptide treatment, TUNEL signal accumulation should occur with similar kinetics in both wild type and recA strains. We tested the effect of a B. subtilis recA mutation on the results of the TUNEL assay in parallel (Figure 2.9B). Not only was damage not dependent on the presence of RecA protein, but it accumulated faster in the recA mutant, suggesting that RecA is involved in the repair of peptide-induced DNA damage. This is also the case in E. coli (see Figure 2.11, below).

**Hexapeptides create SOS-independent DNA damage.** If the peptides directly cause the accumulation of DNA damage, then they would be expected to induce the SOS response. An E. coli dinD1::MudA(lacZ) strain and a Salmonella recN557::MudJ(lacZ) strain were used to determine if the peptides induce the SOS response. Both strains produce β-galactosidase from DNA damage-inducible (din) promoters in response to cleavage of the LexA repressor by RecA, which is activated by higher levels of DNA damage within the cell (Walker, 1996). Peptide treatment prevented measurement of β-galactosidase activity because it interfered with the colorimetric output of the activity assay (data not shown). Therefore, the level of β-galactosidase itself was determined using a double-capture ELISA assay (Sambrook, 1989). Peptide wrwycr induced the SOS response similarly to the bifunctional cross-linking agent mitomycin C (MMC) in both E. coli and Salmonella
Figure 2.10: Peptide d-wrwycr damage is independent of the SOS-response.
A) Growth of *Salmonella enterica* Typhimurium strains: LT2 (squares); LT2 *recA* (diamonds); LT2 *lexA3* (Ind-) (triangles); and LT2 *sulA* (circles) in the absence (filled symbols) or presence of 50 µM wrwycr (open symbols). B) Surviving fraction (S/ S₀) after 5 and 8 hours of wrwycr treatment. LT2 (squares), LT2 *recA* (diamonds), LT2 *lexA3* (Ind-) (triangles), and LT2 *sulA* (circles) were treated with 50 µM wrwycr. C) Accumulation of DNA damage, as determined by TUNEL assay and flow cytometry, by wrwycr treatment of LT2 (white bars); LT2 *recA* (light grey bars); LT2 *lexA3* (Ind-) (medium grey bars); and LT2 *sulA* (dark grey bars). Error bars represent one standard deviation from the mean of three independent cultures. Data is representative of one experiment out of three on successive days.
(Table 2.5). The relatively low induction level of β-galactosidase is probably a consequence of having to detect induction by ELISA compared to the activity assays performed in other studies, either due to lower sensitivity and/or due to the lack of the amplification usually seen in an activity assay.

We determined if the peptide induced DNA damage directly or indirectly in Gram negative bacteria by testing the peptide sensitivity of recA, lexA3(Ind-), and sulA mutant Salmonella strains. The recA strain is deficient both in homologous recombination and in inducing the SOS response. The lexA3(Ind-) mutant allele produces a non-cleavable version of the repressor; this mutant is capable of homologous recombination using basal levels of RecA but is unable to activate the SOS response (Lin & Little, 1988, Mount et al., 1972, Walker, 1996). This allele was introduced into Salmonella by Kim Bunny (Bunny et al., 2002). SulA, an SOS-inducible protein, inhibits cell division upon a rise in DNA damage and is presumed to allow DNA repair between sister chromosomes before cells divide (Hill, 1997 #33; Huisman, 1984 #34; Walker, 1996 #110). The sulA strain has all of the SOS and homologous recombination activities of the wild type, but cannot inhibit cell division in response to DNA damage. All three strains were grown in the presence of 50 μM wrwycr, a sub-lethal peptide concentration, and the cultures were monitored by optical density over time. All mutant strains except recA grew comparably to the wild type LT2 in the absence of peptide (Figure 2.10A). Upon treatment with 50 μM of wrwycr, the wild type culture and the lexA3(Ind-) cultures showed the same level of inhibition, approximately 50% over most of the timecourse. The sulA strain showed significant inhibition early but began to recover. The growth of the recA strain was almost completely inhibited over the time tested.
In parallel, we tested the viability of the same strains after wrwycr treatment. Samples were taken from the cultures above and were diluted and plated to determine viable counts. The relative viability (CFU ml\(^{-1}\) treated/CFU ml\(^{-1}\) untreated) of the strains after 5 and 8 hours of 50 \(\mu\)M wrwycr treatment is shown in Figure 8B (again, a sub-MIC concentration for Salmonella was used). The wild type, lexA3(Ind-), and sulA strains showed a log decrease in viability at the 5 hour timepoint, and recovered as the timecourse progressed. The recA strain showed almost a two-log decrease in viability after 5 hours of treatment and greater than a two-log decrease at 8 hours, but also eventually recovered at 24 hours (not shown). If the DNA damage induced by wrwycr were SOS-dependent, we would have expected the lexA3(Ind-) strain to be less susceptible to peptide treatment than the wild type. We also used the TUNEL assay and flow cytometry to quantify DNA breaks in these strains after peptide treatment (Figure 2.10C). With wrwycr treatment, the recA strain shows consistently higher TUNEL labeling than the wild type, which is also true in B. subtilis and E. coli (Figures 2.9 and 2.11, respectively). In contrast, the wrwycr-treated lexA3(Ind-) strain accumulates fewer breaks compared to the recA strain, although more than the wild type, indicating that SOS-controlled enzymes do contribute significantly to peptide wrwycr-linked DNA damage.

**Peptide treatment is synergistic with some DNA damaging agents.** If the peptides are capable of trapping Holliday junction or branched intermediates in the repair of DNA damage, they should be synergistic with DNA damaging agents that require recombination for repair. Wild type and recA strains of E. coli MG1655 cultures were treated with peptide wrwycr or with a combination of wrwycr and methylmethane sulfonate (MMS) or UV. In bacteria, the alkylation damage caused by
**Figure 2.11:** Peptide wrwycr is synergetic with UV-induced DNA damage but not MMS. A) Examples of dot plots obtained in flow cytometric analysis of peptide wrwycr and MMS treated and TUNEL assayed cultures. Upper row, FSC vs. SSC dot plots, lower row FITC (TUNEL) vs. PI dot plots. Left to right: untreated; 0.1% MMS; 50 µM d-wrwycr; and MMS with 50 µM wrwycr. B) Graphical representation of the flow cytometric data. Open bars represent MG1655 and filled bars, MG1655 recA. Treatments are indicated below each bar in the figure. C-D) Same as in parts A and B, except cells were treated with 2 mJ UV and/ or 25 µM wrwycr. In the flow cytometry analyses, the populations (P1-P5) are labeled exactly as in Figure 2.8. Error bars represent the standard deviation from the mean among 3 independent cultures.
the monofunctional alkylating agent MMS is repaired by recognition and excision of the modified bases by specific glycosylases as part of the adaptive response, resulting in the creation of apurinic or apyrimidinic (AP) sites (Friedberg, 1995, Volkert & Landini, 2001) or by direct reversal of the damage via alkB activity (Delaney & Essigmann, 2004, Falnes et al., 2002, Trewick et al., 2002). Subsequent repair of abasic sites involves endonuclease and polymerase activity. However, recA may also be required in the repair of these damages, as subsequent processing of the AP sites by the base excision repair (BER) machinery requires recombination repair (Friedberg, 1995, Spek et al., 2002, Spek et al., 2001). Presumably, recA is most important in cases where replication forks encounter the excision site and create a double-strand break. Pyrimidine dimers generated by UV exposure are normally excised by the UvrABC excision repair system, but they can block passing replication forks before they are repaired. The collapsed forks may regress to form HJ and be repaired either by RecG and/or RuvABC (McGlynn & Lloyd, 2002) or by a yet-unknown mechanism (Michel et al., 2004). In either case, peptide binding to HJ or collapsed replication forks would stabilize them and increase the concentration of free 3'OH ends. To test these possibilities, the cultures were assayed by TUNEL and analyzed by flow cytometry (Figure 2.11). In both wild type and recA strains, similar numbers of DNA breaks are seen in the MMS-treated culture as in the culture treated with both MMS and wrwycr, suggesting that the effects of the peptide and MMS are not additive. In contrast, we see a synergistic relationship between peptide- and UV-dependent damage in MG1655, an effect that is enhanced in the recA background.

The difference in DNA breaks between MMC-peptide co-treatment and MMS-peptide cotreatment was somewhat puzzling. Because of this, we also tested the
Figure 2.12: Peptide wrwycr and mitomycin C show synergy. A) Growth of *E. coli* MG1655 in the presence of 25 µM wrwycr (open squares) or in DMSO (filled squares). *E. coli* was also treated with MMS (diamonds) and MMC (triangles) alone (filled symbols) or in combination with peptide (open symbols). B) Fraction of *E. coli* MG1655 colony forming units surviving (after the 22 hour treatment shown in panel A) with peptide alone, or in combination with MMS or MMC at the indicated concentrations (relative viability = CFU of treated cells / CFU of untreated cells).
effect of co-treatment with MMS or MMC with wrwycr on the viability and growth rates of wild-type *E. coli* (Figure 2.12). Growth was monitored for 22 hours by optical density (Figure 2.12A). Both MMC and MMS by themselves significantly slowed further growth after an apparent threshold of damage had accumulated. Combined, MMC and wrwycr inhibited growth more severely than MMS and wrwycr, and both combined treatments had effects of larger magnitude than any individual treatment. The viability of the treated cells confirmed the growth rate results: co-treatment with MMC and peptide exerted strongly synergistic effects on cell viability, while co-treatment with MMS and peptide exerted a moderately additive effect. Synergistic results are also seen between peptide and H$_2$O$_2$ treatments (data not shown), a known causative agent of DNA breaks (Imlay & Linn, 1987). Thus, peptide wrwycr is synergistic in causing DNA damage with some mutagens (MMC and UV) but not others (MMS). These differences might reflect the speed and/or efficiency with which different lesions are repaired, and thus the chance that a replication fork will run into the lesion and collapse.

Section 2.3: Discussion

In the present study, we have characterized the effects on bacteria of hexapeptides WRWYCR, KWWCRW, and WKHYNY, which were isolated as inhibitors of phage lambda Integrase *in vitro* (Boldt et al., 2004, Cassell et al., 2000). Peptides WRWYCR and KWWCRW also bind to free Holliday junctions, and inhibit the Holliday junction RecG helicase and the RuvABC resolvase (Kepple et al., 2005). In comparison, peptide WKHYNY is less potent than the other two at inhibiting Int-
mediated recombination, does not bind protein-free Holliday junctions (Ghosh et al., 2005, Kepple et al., 2005), and does not inhibit RecG or RuvABC activity (Kepple et al., 2005). We now show that the two most potent peptides have broad-spectrum antimicrobial activity and are bactericidal, while peptide WKHYNY does not inhibit bacterial growth. Both L- and d-is forms of peptides WRWYCR and KWWCRW are effective, but wrwycr is somewhat more potent inhibitor of bacterial growth. While the d-peptides are slightly less potent in the inhibition of Int-mediated recombination than the L-peptides (Boldt et al., 2004), the d-isoforms are expected to resist degradation by cellular peptidases. In most situations, lack of stereospecificity in inhibition would signal nonspecific activity; in the case of these peptides, however, the 2-fold symmetry of the HJ and the fact that it itself can be found in two isomeric forms most likely accounts for the nearly equivalent inhibition of recombination.

The peptides are more potent against Gram+ bacteria than Gram- bacteria, except in the case of the Salmonella galE rfa strain. These mutations truncate the lipopolysaccharide (LPS) chains anchored to the outer membrane of the cell, effectively increasing membrane permeability (Leive, 1974; Nikaido, 1996). Thus it appears that peptide potency against Gram- bacteria is diminished by the difficulty in crossing the outer membrane. This model is supported by the observation that 0.02-0.2 mM EDTA increases the sensitivity of the Gram- organisms to peptide treatment (Figure 2.4). EDTA chelates magnesium, which helps anchor the LPS to the outer membrane (Leive, 1974, Nikaido, 1996). The destabilization of the LPS by EDTA changes the polarity of the leaflets of the outer membrane, resulting in an increase in outer membrane permeability (Leive, 1974, Nikaido, 1996). The PhoP/Q system is a two-component regulatory system that regulates genes involved
Figure 2.13: Growth of *Bacillus subtilis* JH642 and *B. subtilis spoOKBCDE* in the presence of d-wrwycr. Growth of wild-type bacillus (black lines) and *spoOKBCDE* mutant (red lines) in the presence of 0.02 and 5 µM peptide wrwycr (x's and diamonds, respectively).
Figure 2.14: Growth of *Salmonella enterica* Typhimurium LT2 and a LT2 *phoP* in the presence of d-wrwycr. Growth of wild-type LT2 (black lines) and *phoP* mutant (red lines) in the presence of 5 and 25 μM peptide wrwycr (triangles and x’s, respectively).
in a variety of cellular mechanisms, including resistance to antimicrobial peptides (Groisman, 2001). A *Salmonella phoP* mutant has increased sensitivity to wrwycr, probably due to the outer membrane remodeling controlled by PhoP/Q (Figure 2.13). A *B. subtilis* strain deficient in one of its peptide transport systems, Δ*spo0KBCDE* (Rudner et al., 1991), retained wild-type sensitivity to peptide, suggesting that this system is not required for entry into or export from *Bacillus subtilis* (Figure 2.14). In this aspect, our peptides resemble natural cationic aromatic antimicrobial peptides like lactoferricins, which do not require active transport to cross the cell wall (Epand and Vogel, 1999). We are currently investigating the effects of the peptides on bacterial membranes in order to better understand the uptake of these molecules into cells.

Peptide-treated cells lose viability, in some cases, even at sub-lethal concentrations (Figure 2.10 and Table 2.3). However, cells can recover after extended treatment with 1x the MIC concentrations of the peptides. One possibility we considered is that the peptides may be effluxed from cells. Indeed, *acrAB* deletions affecting the inner membrane components of the major efflux pump of Gram- cells render *E. coli* and STm sensitive to ~4-fold lower concentrations of peptide and take longer to recover from peptide treatment than wild type strains (J. Johnson, A. Flores, N. Radosevich and A. Segall, unpublished results). Recovery of the cells may also be permitted by the eventual dissociation of the peptides from their targets.

The major phenotypes of bacteria treated with either wrwycr or WRWYCR are filamentation, mis-segregation of chromosomes, and higher fractions of anucleate cells. Filaments greater than 10 times the length of wild type cells are seen with 90
minute treatment at sub-MIC concentrations, even in a sulA mutant strain (data not shown). This indicates that peptide-induced filamentation is independent of SulA mediated inhibition of division. In addition to anucleate cells, peptide treatment caused the appearance of cells with reduced DNA content (Figure 2.5). Flow cytometry proved to be an ideal tool for determining the fraction of anucleate cells, because we could assay large enough cell numbers to obtain a baseline value for these rare events in untreated wild type cultures (typically fewer than 0.5%). Compared to this baseline, wrwycr treatment caused an 8-fold increase in anucleate cells in E. coli.

Several possibilities may account for the peptide-induced mis-segregated and fragmented chromosomes and the formation of anucleate cells. The most likely, given the biochemical activities of the peptides, is that they interfere with the repair of DNA damage, restart of stalled and collapsed replication forks, and with the site-specific resolution of chromosome dimers formed during recombination-dependent repair. The interference of peptides with these processes may cause cells to attempt to divide prior to completing DNA repair, leading to the formation of anucleate cells or preventing division entirely.

Filamentation and DNA segregation abnormalities are common phenotypes of cells suffering DNA damage. Using direct labeling of free 3’OH ends by the TUNEL method, we detected significant increases in DNA breaks in a peptide- and dose-dependent fashion. The TUNEL assay coupled with flow cytometric analysis of large cell populations permitted even relatively rare events to be detected and quantitated reliably. The validity of this assay is also supported by the fact that the fraction of untreated E. coli cells that are TUNEL-labeled agrees extremely well with the fraction
of wild type cells in which SOS is induced, based on the fluorescence seen in cells with a \textit{sulA::gfp} fusion (McCool et al., 2004). DNA breaks observed by TUNEL accumulated rapidly in peptide treated cells (within 5-15 minutes). The time course of DNA break accumulation following peptide wrwycr treatment also suggests that the peptides cause DNA damage directly rather than indirectly via induction of the SOS response. This conclusion was substantiated by demonstrating that accumulation of DNA damage does not depend on the induction of the SOS response, but it does contribute to damage. While SOS-dependent proteins are not necessary for damage accumulation, the peptides do induce the SOS response, as expected of a DNA damage-inducing agent.

The \textit{in vivo} data presented here are fully consistent with our previous \textit{in vitro} observations that the peptides bind to and block the resolution or branch migration of HJ intermediates by proteins involved in DNA repair such as the RecG helicase and the RuvABC junction resolvase (Kepple et al., 2005). If the peptides also bind branched DNAs \textit{in vivo}, then concomitant treatments with DNA damaging agents that are likely to generate a higher concentration of repair intermediates should sensitize cells to peptide treatment. Indeed, we observed synergistic effects between peptide treatment and MMC or UV treatment, but not between peptide and MMS. MMS is an alkylating agent whose damage is repaired in bacteria primarily by the adaptive response, mediated by specific glycosylases which create apurinic/apyrimidinic sites (Friedberg, 1995). This mechanism of repair can proceed without the involvement of HJ intermediates unless replication forks encounter the damage during repair, in which case double strand breaks can be formed. Conversely, UV damage is primarily repaired via UvrABC-dependent excision repair of thymine dimers, which does not
operate through a HJ intermediate. Despite this, UV damage is synergistic with peptide treatment. This may be explained by the fact that replication forks have to regress in order to permit repair and restart after encountering pyrimidine dimers (Courcelle et al., 1999, Courcelle & Hanawalt, 2001, Flores et al., 2001, Keller et al., 2001, McGlynn & Lloyd, 2002, Meddows et al., 2004, Michel et al., 2004). In cases where the UvrABC-directed repair is overwhelmed due to a high number of lesions, the peptides likely show synergy with UV by interfering with the repair of the collapsed replication forks. In contrast, DNA damage caused by the crosslinking agent MMC (Suresh Kumar et al., 1997) frequently requires recombination repair involving HJ intermediates (Keller et al., 2001). Interestingly, the elongation of normal replication forks is not highly sensitive to peptides (Figure 2.2), perhaps because they only gain access to the DNA after the disassembly of the replisome and can compete only with less processive helicases like RecG. Indeed, the peptides have no effect on DNA synthesis in vitro in an ori-C dependent reconstituted replication assay (Jon Kaguni, personal communication).

Peptides WRWYCR or KWWCRW, which are better at trapping HJ complexes and inhibiting HJ-processing enzymes in vitro than peptide WKHYNY, are also much more potent antimicrobials than peptide WKHYNY. These data lead us to conclude that peptides WRWYCR and KWWCRW kill bacteria by trapping HJ and branched DNA intermediates during recombination-dependent repair of collapsed replication forks. In the presence of the toxic peptides, DNA damage accumulates and eventually leads to filamentation, chromosome mis-segregation and DNA loss. These peptide effects are similar in Gram- and Gram+ cells, although they occur at different levels in different bacteria. DNA repair is a frequent event in the bacterial life cycle
Sources of DNA damage range from extrinsic sources (e.g., reactive oxygen species generated by a host’s immune system, ultraviolet and higher energy radiation, and chemical agents) as well as intrinsic sources (e.g., oxygen radicals generated during respiration, trapped protein-DNA complexes, and collapsed or stalled replication forks Park et al., 2005). Interference with DNA repair is a novel mechanism of antibacterial activity. Similar processes may render tumor cells more sensitive to these peptides than relatively insensitive non-tumor cells (D. Fujimoto, C. Vallejo, L. Su, and A. Segall, unpublished). Since HJ and branched DNA substrates are generated by several independent pathways, resistant mutations affecting the target itself should arise very rarely, and many of the resulting mutations may render bacteria more susceptible to killing by the host’s immune system. In fact, we have not been able to identify any spontaneous peptide-resistant mutants (J. Patelzick, C. Gunderson and A. Segall, unpublished). In addition to their use as potential therapeutics, we expect these peptides to be very useful as tools for dissecting recombination and repair pathways in vivo.

Portions of this chapter were published as: Gunderson, Carl and Segall, Anca, 2006. DNA repair, a novel antibacterial target: Holliday junction-trapping peptides induce DNA damage and chromosome segregation defects. Mol. Micro. 59 (4), 1129–1148.
Chapter 3: Genetic analysis of peptide effects on *Salmonella* DNA repair

Section 3.1: Introduction

Chromosomal DNA is constantly under stress from agents that are capable of breaking, bonding to, or otherwise altering its structure and sequence. DNA damage originates from many intrinsic (i.e. protein-DNA covalent complexes and free radical oxygen) and extrinsic (i.e. UV, chemical mutagens) sources (Cox *et al.*, 2000, Kuzminov, 1999, Park *et al.*, 2005, Pennington & Rosenberg, 2007). Faithful and timely repair of DNA lesions is important for all cells, eukaryotic and prokaryotic. Failure to repair these lesions can result in cell death, while failure to repair lesions accurately can cause mutagenesis, leading to cell death or cancer at one extreme and on the other, adaptation. Thus, DNA repair is a frequent cellular process that requires precision and consistency in its operation.

In bacteria, there are two major RecA-dependent pathways of homologous recombination, the RecBCD pathway and the RecJ/RecFOR pathway (Friedberg, 1995, Michel *et al.*, 2004). The fundamental difference between these pathways is the initial substrate on which they act. Double strand DNA breaks (DSB's) are processed by RecBCD, which creates a RecA-coated ssDNA molecule, and facilitates strand invasion of a homologous sister DNA strand (Chaudhury & Smith, 1984, Singleton *et al.*, 2004). Nicks or gaps in the DNA can be widened through the combined action of a helicase and the nuclease RecJ, making the ends of the single-strand binding protein (SSB) coated gap substrates for stabilization by the RecFOR complex.
(Courcelle et al., 2006, Courcelle et al., 1999, Michel et al., 2004, Morimatsu & Kowalczykowski, 2003). The RecFOR complex facilitates RecA loading on the single strand region, displacing SSB and facilitating strand invasion (Morimatsu & Kowalczykowski, 2003). These pathways are not mutually exclusive and in the absence of some activities, others are able to compensate. For example, in the absence of RecBCD activity, it has been suggested that RecQ and RecJ are able to assemble and act on DSB’s (Kowalczykowski et al., 1994, Morimatsu & Kowalczykowski, 2003). Homologous recombination and recombination repair catalyzed by RecA and facilitated by either RecBCD or RecFOR activity always proceed through a Holliday junction intermediate, which must be resolved before the strand exchange is complete.

We have previously shown that a set of peptides kill bacterial cells by causing DNA damage and chromosome partitioning defects (Gunderson & Segall, 2006). The peptides are synergistic with DNA damaging agents, such as mitomycin C, because repair of damage caused by the agents results in formation of targets for peptide binding. The peptides, originally isolated for their ability to inhibit site-specific recombination, were subsequently found to bind preferentially to Holliday junctions, and to a lesser extent, three-way junctions (Boldt et al., 2004, Cassell et al., 2000, Kepple et al., 2005). Peptide binding affinity to three-way junctions varies based upon the architecture of the substrate and the presence of a leading or lagging nascent strand (or both) present in the fork. The peptide d-wrwycr does not bind specifically to a fork that is a flayed duplex (Boldt et al., 2004, Cassell et al., 2000, Kepple et al., 2005, Kepple and Segall, in preparation). Three- and four-armed DNA intermediates are common to all recombination pathways. The inhibitory action of the peptides in
vitro is structure-specific for the branched DNAs rather than being dependent on which protein(s) created the structures, although the proteins (RecG, RuvABC, and tyrosine recombinases) help peptide bind by making a (better) substrate. Thus, the peptides are able to specifically inhibit a diverse number of proteins with different activities on a substrate-dependent level.

Here we present genetic evidence both on the nature of the DNA damage caused by hexapeptide d-wrwycr and on its effects on the repair of that damage. We have used pulsed field gel electrophoresis (PFGE) to analyze DNA damage in peptide-treated *Salmonella enterica* Typhimurium LT2. We have compared the extent of damage caused by the peptide in wild-type cells to that of cells that are recombination and/or repair deficient. We have also determined the viability of the same strains after the same peptide treatments. The PFGE and viability assays allow us to address complementary questions about the activity of the peptides. The PFGE assay, as an endpoint assay, addresses the potential number of targets within the cell. For example, mutations that result in the formation of more branched DNA structures (peptide targets) may exhibit greater fragmentation when treated with the peptide. The viability assays demand that the cells grow on non-selective media after treatment with the peptide, therefore the assay sensitively measures the ability of the cells to faithfully repair the damage caused by the peptides. Data presented herein indicates that peptide treatment creates substrates for processing by both the RecBC double strand break (DSB) repair pathway and the RecJ/RecFOR single strand break (SSB) repair pathway. However, repair of damage caused by the peptide is largely dependent on the RecBCD-dependent recombination machinery. We propose that the peptide is influencing the processing of DNA repair intermediates by increasing
the half-life of branched DNA structures in the cell. The data supports our previous hypothesis that peptide d-wrwycr inhibits bacterial growth by causing DNA damage and interfering with DNA repair.

Section 3.2: Results

**Peptide d-wrwycr causes double strand DNA breaks in wild-type Salmonella.** Previously, we have shown using a TUNEL assay (labeling of free 3’ OH in DNA using a terminal transferase) that peptide wrwycr causes the accumulation of breaks (Gunderson & Segall, 2006). However, the TUNEL assay does not distinguish between SSB and DSB. To test what types of breaks are accumulated due to treatment, we used pulsed field gel electrophoresis (PFGE). Intact bacterial chromosomes are too large to efficiently enter the matrix of the agarose gel during pulsed field gel electrophoresis (PFGE), thus only linear fragments of the chromosome enter the gel. Cultures of *Salmonella enterica* Typhimurium LT2 were treated with peptide d-wrwycr to analyze its effect on the integrity of the chromosome. Treatment with either 32 or 64 μM d-wrwycr causes loss of DNA from the plugs, yielding DNA fragments in the lanes of the gel (Figure 3.1, lanes 5 and 7). There is a synergistic increase in the amount of fragmentation when MMC and peptide treatments are combined (Figure 3.1, lanes 6 and 8). The DNA fragments created by peptide or MMC treatment ranged in size from less than 48 kb to greater than 800 kb, but the DNA fragments created are smaller when the treatments are combined. To show that the DNA in the wells is intact, and not retained in the well for other reasons,
Figure 3.1: Pulsed field gel electrophoresis of *Salmonella enterica* serovar Typhimurium LT2 Chromosomal DNA. Cultures were treated as indicated above the gel. Chromosomal DNA that was not cut after processing is shown on the left portion of the gel. Chromosomal DNA that was digested with I-CeuI is shown on the right half of the gel. Size of standard bands in kilobases are labeled to the left of the gel. Lane 1, yeast chromosome (YC) ladder (preparation of the 16 *Saccharomyces cerevisiae* chromosomes). Lane 2, lambda (λ) ladder (preparation of successively larger phage lambda chromosome concatemers).
we digested chromosomes with the homing endonuclease I-CeuI (Figure 3.1, right portion of gel), which released 7 predicted fragments into the gel (Liu et al., 1993, Liu & Sanderson, 1995). As expected for random chromosome breakage, the largest genome segments seem to be most susceptible to the non-specific fragmentation caused by the peptides. When cells are treated with the MMC, the largest fragment is most susceptible (Figure 3.1, lane 10); this fragment contains all four of the resident prophage in *Salmonella* LT2.

The correlation between the fragmentation seen in the pulse field gels and the viability of the culture after treatment was investigated by plating cells after similar treatment conditions. Cultures of wild-type LT2 were plated for viability after three hours of treatment (Figure 3.2). The wild-type strain showed a similar decrease in viability when treated with either 1 μg ml⁻¹ MMC or 64 μM peptide (Figure 2). When the treatments were combined, the viability dropped below the detection limit of the assay (less than 100 CFU ml⁻¹).

**SOS induction is required to enhance peptide-dependent DNA fragmentation.** The PFG and viability analyses were repeated on a set of *Salmonella* recombination mutants to determine the effects of DNA recombination and repair deficiencies on peptide efficacy. First, the background fragmentation in DMSO (mock) treated cultures of each of the *Salmonella* mutants was determined (Figure 3.2). The mutants fall roughly into two groups: those that show protection over the background level of fragmentation (including *recA*, *recABC*, and *recF*), and those that show greater fragmentation than the wild type (*lexA*(ind-), *recG*, *ruvAB*, and *ruvC*). The intact DNA (well area) of representative pulsed field gels is shown for each strain
Figure 3.2: Viability analysis of Salmonella recA, lexA(ind-), and recD strains. Photographs of LB plates used for dilution series have dilutions labeled across the top, and treatment conditions labeled down the side. From top to bottom and left to right, panels are wild-type LT2, recA, recD, and lexA(ind-). Photographs represent data from triplicate independent cultures from at least 2 different days (n of at least 6, LT2 n=54).
Figure 3.3: Fraction of intact DNA retained in the wells of DMSO (mock) treated cultures. The amount of intact DNA in each of the mutant Salmonella strains (X axis) expressed relative to the amount in the LT2 culture (wild-type, red bar).
Figure 3.4: PFGE analysis of Salmonella recA, lexA(ind-), and recD strains. A) The well portions of representative pulsed field gels are shown, with treatment conditions labeled above. Wild-type LT2 is shown at the top for reference. Gels were stained with Sybr Green I. B) The relative fraction of intact DNA in each lane was calculated by dividing the amount of DNA retained in the well after treatment by the amount of intact DNA in the DMSO control. Each of the mutants was calculated independently. Each bar represents at least 3 independent trials across 2 different days (n of at least 3, LT2 n=33). Error bars represent the standard deviation from the mean.
tested (Figures 3.4, 3.5, 3.7, parts A). The quantity of intact DNA within the each population was measured (staining intensity at the well), and measurements for each treatment were compared to the DMSO (mock treated) control for the same strain (Figures 3.4, 3.5, 3.7, parts B). A *Salmonella* recA mutant culture treated with d-wrwycr alone exhibited a similar amount of fragmentation as the wild-type strain. However, MMC treatment alone yielded more fragmentation in the recA mutant than in the wild-type strain. The amount of fragmentation seen in the recA cultures correlates well with the viability data, as a *Salmonella* recA mutant is more sensitive to MMC than to peptide and, compared to the wild-type strain, is mildly resistant to 64 μM peptide treatment alone (Figure 3.2).

A Salmonella strain that carries the *E. coli* allele for a non-cleavable LexA protein (*lexA3(ind-)*) and is unable to induce an SOS response (Bunny et al., 2002), showed no fragmentation with peptide treatment but enhanced fragmentation when cultures were treated with MMC alone. The *lexA3(ind-)* strain, when treated with both MMC and peptide, had the least intact DNA of the mutants tested in this study (<25%). Although the fragmentation phenotype of the *lexA3(ind-)* mutant is similar to that of the recA strain, its viability differs. Like the recA strain, the *lexA3(ind-)* showed more sensitivity to MMC than the wild-type strain, but not resistance to peptide treatment, most evident at 64 μM (Figure 3.2). A recD mutant’s response to the treatments, assayed via PFGE or viability, is not significantly different from that of the wild-type strain (Figure 3.3, 3.4).

**Single and double strand break repair mutants are protected from peptide-dependent fragmentation.** RecBCD is the major double-strand end
dependent nuclease in *E. coli* (Amundsen & Smith, 2007). Strains defective in double strand DNA break processing, via mutation in *recBC*, showed some protection from fragmentation caused by treatment with a combination of peptide and MMC (Figure 3.5). Each of the agents alone exerted a similar response on the *recBC* mutant as they did on the wild-type strain. Though the strain seemed to be protected from fragmentation, a *recBC* mutant had decreased viability after peptide and MMC treatment (Figure 3.6). A *recABC* mutant strain was more protected from peptide treatment than *recBC* alone, but showed similar fragmentation with MMC treatments (Figure 3.5). However, in the viability assay, the *recABC* mutant was dramatically increased in its sensitivity to all treatments (Figure 3.6).

RecF and RecJ proteins are involved in DNA gap repair, RecJ by widening gaps via its 5' – 3' exonuclease activity, RecF by helping to load RecA onto the single-stranded DNA. *Salmonella recF* mutants were mildly protected from fragmentation by MMC and low peptide concentration treatments (Figure 3.5). The viability of the *recF* mutant, despite showing increased sensitivity to MMC, had wild type sensitivity to peptide (Figure 3.6). In a *recJ* mutant, the combination treatments had similar effects as they did on the wild-type strain, but there was a subtle enhancement of fragmentation with MMC or peptide treatment alone. Despite these results, a *recJ* mutant’s viability did not differ in sensitivity to peptide or MMC compared to the wild-type strain (Figure 3.6). Strains that are *recB recJ* exhibited enhanced fragmentation with peptide treatment, but a *Salmonella recBC recF* mutant’s DNA was protected from fragmentation in all treatments (not shown). A *recB*
Figure 3.5: PFGE analysis of Salmonella recBC, recABC, recF, and recJ strains. A) The well portions of representative pulsed field gels are shown, with treatment conditions labeled above. Wild-type LT2 is shown at the top for reference. Gels were stained with Sybr Green I. B) The relative fraction of intact DNA in each lane was calculated by dividing the amount of DNA retained in the well after treatment by the amount of intact DNA in the DMSO control. Each of the mutants was calculated independently. Each bar represents at least 3 independent trials across 2 different days (n of at least 3, LT2 n=33). Error bars represent the standard deviation from the mean.
**Figure 3.6:** Viability analysis of *Salmonella* recBC, recABC, recF, recJ, recBC recF, and recB recJ strains. Photographs of LB plates used for dilution series have dilutions labeled across the top, and treatment conditions labeled down the side. From top to bottom, left to right, panels are wild-type LT2, recBC, recF, recJ, recA, recABC, recBC recF, and recB recJ. Photographs represent data from triplicate independent cultures from at least 2 different days (n of at least 6, LT2 n=54).
recJ strain was extremely sensitive to both peptide and MMC treatments, failing to show detectable growth in our assay (not shown). Similarly, a recBC recF mutant failed to show growth after any of the treatments (not shown).

**Holliday junction resolution deficiencies sensitize cells to d-wrwy cr treatment.** Strains that are deficient in Holliday junction (HJ) resolution showed the largest increase in peptide-dependent DNA fragmentation (Figure 3.7). Cells deficient in RecG had decreased viability when treated with peptide or MMC compared to wild type (Figure 3.8). These results differ from the pulsed field gel data, which showed a lot of peptide-dependent fragmentation but no MMC-dependent fragmentation of the chromosome in the recG mutant (Figure 3.7). The chromosome of a ruvAB mutant fragmented more than that of a recG mutant when treated with peptide, but not with MMC. A ruvAB mutant showed a larger decrease in viability after the treatments than the recG mutant (Figure 3.8). If recG and ruvAB are deleted in the same strain, the strain’s sensitivity to peptide and MMC-dependent DNA fragmentation resembled more a recG mutant or the wild-type strain than a ruvAB mutant (Figure 3.7). When the ruvC gene is deleted, the strain becomes more sensitive to peptide-dependent fragmentation than to MMC (Figure 3.7). A ruvC mutant was more sensitive than either the recG or ruvAB mutants, and its viability after treatment was similar to that of the double recG ruvAB mutant (Figure 3.8). When the recG and ruvC mutations are combined, the strain is more sensitive to fragmentation caused by a low concentration of the peptide, but not significantly more sensitive to the combination treatments. The resistance to MMC-dependent fragmentation is the same as the recG
**Figure 3.7**: PFGE analysis of *Salmonella* recG, ruvAB, ruvC, recG ruvAB, and recG ruvC strains. A) The well portions of representative pulsed field gels are shown, with treatment conditions labeled above. Wild-type LT2 is shown at the top for reference. Gels were stained with Sybr Green I. B) The relative fraction of intact DNA in each lane was calculated by dividing the amount of DNA retained in the well after treatment by the amount of intact DNA in the DMSO control. Each of the mutants was calculated independently. Each bar represents at least 3 independent trials across 2 different days (n of at least 3, LT2 n=33). Error bars represent the standard deviation from the mean.
Figure 3.8: Viability analysis of *Salmonella* recG, ruvAB, ruvC, recG ruvAB, and recG ruvC strains. Photographs of LB plates used for dilution series have dilutions labeled across the top, and treatment conditions labeled down the side. From top to bottom and left to right, panels are wild-type LT2, ruvAB, ruvC, recG, recG ruvAB, and recG ruvC. Photographs represent data from triplicate independent cultures from at least 2 different days (n of at least 6, LT2 n=54).
or ruvC single mutants. After treatment, the recG ruvC strain is the least viable of this set.

The concentration of the MMC treatments used in the viability assays was chosen to match those used in the PFGE assays. Unfortunately, the concentrations used to give a fragmentation response in the pulsed field assays, killed the strains in the viability assay. To test the synergy between the MMC and peptide, lower concentrations of MMC were used. We can clearly see the synergy between the compounds in recG, ruvC, and recA backgrounds when the MMC concentrations were reduced to concentrations of 0.1 or 0.01 μg ml\(^{-1}\) (Figure 3.9).

A majority of the studies of DNA repair and recombination in bacteria have been performed in Escherichia coli. Salmonella was more amenable to the PFGE assay, so we employed it as the model system in our study. This introduces difficulties in comparing our results to those published in the literature. However, the recombination proteins of E. coli and Salmonella are extremely similar, showing greater than 84% similarity and 90% identity at the amino acid level (Table 3.1). Although the proteins of the recombination systems of these bacteria are very similar, we have identified that there are some differences in the phenotypes of recombination mutants when treated with MMC or peptide d-wrwwyr.

The growth response of Salmonella enterica Typhimurium LT2 recombination mutants to peptide treatment was compared to that of E. coli MG1655. The wild-type Salmonella LT2 strain grows better than E. coli MG1655 in microtiter dish assays (Figure 3.10, panels A and C vs. B and D). This makes the differences between mutants of the two genera difficult to ascertain. The growth kinetics recA, recB and
Figure 3.9: Viability analysis of *Salmonella* recG, ruvAB, ruvC, and recA strains. Photographs of LB plates used for dilution series have dilutions labeled across the top, and treatment conditions labeled down the side. From top to bottom, panels are wild-type LT2, recG, ruvC and recA. Units for all MMC and peptide concentrations are µg/ml and µM, respectively. Photographs represent data from triplicate independent cultures from at least 2 different days (n of at least 6).
Table 3.1: Protein identity and similarity values for the recombination machinery of *Salmonella enterica* Typhimurium LT2 and *Escherichia coli*.

<table>
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<tr>
<th>Protein</th>
<th>% Identity*</th>
<th>% similarity*</th>
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</tr>
<tr>
<td>recC</td>
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</tr>
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</tr>
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</tr>
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<tr>
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*% identity and similarity at the amino acid level.*
Figure 3.10: Comparison of the growth kinetics of *Salmonella* and *E. coli* recombination mutants. A) Growth of wild-type *Salmonella enterica* Typhimurium LT2 and *recA*, *recB* and *recJ* mutants in the absence (black curves) and presence (red curves) of 16 µM d-wrwycr. A) Growth of wild-type *E. coli* MG1655 and *recA*, *recB* and *recJ* mutants in the absence (black curves) and presence (red curves) of 16 µM d-wrwycr. C) Growth of LT2 and *recG*, *ruvAB*, *ruvC*, *recG ruvAB* and *recG ruvC* mutants in the absence (black curves) and presence (red curves) of 8 µM d-wrwycr. D) Growth of MG1655 and *recG*, *ruvAB*, *ruvC*, *recG ruvAB* and *recG ruvC* mutants in the absence (black curves) and presence (red curves) of 8 µM d-wrwycr. Lines represent average of three independent cultures.
*recJ* mutants in the presence of peptide seem comparable in both backgrounds (3.10 A vs. B). *E. coli* strains that are deficient in Holliday junction resolution, such as *recG* and *ruv*, are possibly slightly more sensitive to peptide than their Salmonella counterparts.

The differences in the sensitivity of the recombination mutants to peptide treatment was also determined using the more sensitive TUNEL assay (Table 3.2). We see that *recD* mutants of both *E. coli* and *Salmonella* see dramatic increases in the percentage of cells within the population that are TUNEL labeled. In this case, however, the wild-type W3110 *E. coli* is much more sensitive to peptide than LT2. The number of labeled MG1655 *E. coli* is closer to that of LT2 than the W3110 strain, but MG1655 is still more sensitive to peptide treatment than LT2. Those same differences are borne out in the *recA* and *lexA(ind-)* mutants of MG1655 and LT2 (Table 3.2). However, a *recB* mutant of LT2 is similarly sensitive to a MG1655 *recB* mutant, suggesting that LT2 *recB* has enhanced sensitivity to peptide treatment. Overall, the differences between *Salmonella* and *E. coli* with regard to recombination and repair are subtle. More discussion on this topic as it applies to *recQ* mutants is presented in the discussion of chapter four. Further study would be required to more fully understand the differences between the recombination and repair systems of these bacteria.

The effect of *wrwycr* on the *Salmonella* recombination mutants is also complicated by potential polar effects of the mutations. Complementation tests are necessary to discern if the enhanced resistance or sensitivity to peptide is due do the mutation that disrupts the recombination mutant, or a downstream effect. STm LT2 and a LT2 *recG* mutant were transformed with either a plasmid that expresses RecG
Table 3.2: Comparison of % TUNEL labeled in *E. coli* and *Salmonella* mutants.

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nt – not tested. Numbers are averages of three independent cultures.
under a rhamnose inducible promoter (pRHA recG+), or the empty construct (pRHA empty), and their growth in the presence of rhamnose and wrwycr was monitored by optical density (Figure 3.11). Expression of RecG does enhance the growth of the recG mutant, which is normally sensitive to peptide, unfortunately, even the empty vector enhances the growth of the mutant. A similar effect was seen with RecJ complementation, where the recJ strain is normally resistant to peptide treatment, presence of the pBR plasmid (with or without expression construct) enhances growth of the mutant (data not shown). Although pRHA is a low copy number plasmid, the strains harboring it have extra DNA that they would not normally contain and because of the rhamnose, extra expression will be occurring. Thus one possibility is that increased nucleic acid content of the cell will create more non-specific competition for peptide binding. For this reason, it is not possible to complement the recombination mutants using plasmid expression vectors. It may be necessary to construct chromosomal expression cassettes for these mutants so that the expression of the complementing protein is at wild type levels.
Figure 3.11: Complementation of a *Salmonella enterica* Typhimurium recG mutant. The growth kinetics of STm LT2 and a STm recG mutant (black and green curves, respectively) that contain a either a RecG expressing plasmid (pRHA recG+, triangles) or an empty control plasmid (pRHA empty, diamonds). The plasmid containing strains are compared to the same strains lacking plasmid (squares). All six strains were grown in the presence of 100 µM rhamnose (inducer) and 50 µM wrwycr. Points are averages of three independent cultures (n=3), data is representative of two independent experiments.
Section 3.3: Discussion

Previously, we had shown that peptide d-wrwycr accumulated breaks as visualized by fluorescent labels on free 3’OH (Gunderson and Segall, 2006). The assay is limited because it is unable to distinguish between ssDNA breaks and dsDNA breaks. Using pulse field gel electrophoresis (PFGE) we have shown that the peptides cause DSB’s (Figure 3.1). Since the peptides do not physically break the DNA themselves, we hypothesize that the breaks arise through the collision of nascent DNA replication forks and sites of DNA damage, including previous forks that have stalled at DNA damage (Figure 3.11A). Forks that have been stalled, or have fallen apart at the sites of damage frequently require recombination-based repair to restart replication (Cox et al., 2000, Mahdi et al., 2006, Michel et al., 2004). Based on the \textit{in vitro} activity of the peptide, as well as data presented here, we know that peptide d-wrwycr can stabilize intermediates in repair pathways, delaying repair and subsequent restart of the fork. The stabilization of the repair intermediates allows greater opportunity for forks to collide, resulting in the formation of DSB’s. These new DSB’s will also need to be repaired, increasing the effective number of peptide targets within the cell, making it exponentially more difficult for the cell to successfully replicate and segregate daughter chromosomes to new cells prior to division.

In wild-type \textit{Salmonella} LT2, MMC alone (at 1 \( \mu \text{g ml}^{-1} \)) does not cause a detectable accumulation of breaks in our PFGE assay. However, at elevated concentrations (64 \( \mu \text{M} \)), peptide alone can cause accumulation of damage. MMC and peptide d-wrwycr treatments act synergistically upon the cells to cause fragmentation of the chromosome in \textit{Salmonella}. The synergy we see is consistent with results
previously seen in the TUNEL assay, where peptide and MMC showed synergy as well (Gunderson & Segall, 2006). The background fragmentation we see in the mutant Salmonella strains tested is consistent with the known functions of the genes that are knocked out. We would expect that the strains that undergo less recombination might have fewer peptide targets and thus fewer breaks, whereas strains that are lacking resolution enzymes might have more unresolved break repair intermediates and more peptide targets. The changes in fragmentation we observe in the gel are most likely the result of changes in recombination extents within the population. The increased sensitivity of the recA mutant to MMC treatment in the PFGE assay suggests that RecA-mediated recombination repair prevents fragmentation of the chromosome after MMC treatment. This is not the case for peptide-mediated DNA damage, as peptide treatment of a recA mutant does not cause significantly more, or less, damage than in the wild-type strain. The recA mutant is deficient in both homologous recombination and SOS induction. A lexA3(ind-), recA, and recB mutations render Salmonella most sensitive to MMC-dependent fragmentation, whereas recD, recG, and ruv mutations make
Salmonella more resistant to MMC-dependent breaks (Figure 3.12 A). Mutations in recB also make Salmonella more sensitive to breaks caused by treatment with 64 μM peptide d-wrwycr (Figure 3.12 B). However, unlike the MMC result, the ruvAB and ruvC mutations also sensitize Salmonella to peptide treatment. Interestingly, a recG mutation, alone or in combination with either ruvAB or ruvC, protects from the peptide-mediated fragmentation of the chromosome. Taken together, the data suggests that fork-regression is more detrimental to cells treated with peptide than to those treated with MMC.

In addition to giving insight regarding the number of peptide targets that are formed, the viability assays demand that the cells grow after treatment, which gives us an indication of the strain’s ability to repair peptide damage. The result with the recA mutant, which is resistant to peptide treatment but sensitive to MMC, suggests that RecA makes targets for peptide. Since there is less recombination in the recA strain there will be fewer Holliday junctions and branched intermediates, the peptide’s targets. In this case, the inability of the strain to undergo recombination repair is an advantage, as recombination repair will form more targets for peptide binding. However, resistance to peptide is not seen in the lexA3(ind-), in spite of the sensitivity to MMC being retained, suggesting that the peptide resistance seen in the recA mutant is SOS dependent. The recD mutant has similar sensitivity to peptide and MMC as the wild-type strain, suggesting that a general increase in recombination is not sufficient to increase the sensitivity of the cell to peptide treatment.
Figure 3.12: Model for peptide effects on DNA repair. A) Model for double strand break ‘amplification’ caused by peptide wnwycr. Fork progression stalls at the site of a DNA lesion (lightning bolt). Collision of nascent replication forks with the damage results in a double strand break. Peptide inhibits fork repair, and initiations will continue to occur (backward arrow), causing more breaks. B) A fork with a lagging strand gap at the junction can be targeted by the RecFOR complex, which will load RecA on the gap. A strand invasion event will yield a 3’ OH ssDNA overhang, which will be degraded by exonucleases or serve as template for lagging strand synthesis (see figure 6.2B) C) A fork with leading and lagging strands at the junction can be reversed by RecG or RecA. If the resulting Holliday junction is processed and cleaved by the RuvABC complex, a double strand break will be formed. RecBCD activity on the dsDNA end loads RecA to facilitate strand exchange. RuvABC resolves the substrate to restore the fork.
Figure 3.13: Fraction of intact DNA retained in the wells of 1 µg ml⁻¹ MMC (A), 64 µM d-wwrwy (B), and combined (C) treated cultures. The amount of intact DNA in each of the mutant Salmonella strains (X axis) expressed relative to the amount in the LT2 culture (wild-type, red bar). Note scale change in panel C.
Based on the data presented herein, consider the effect of peptide d-wrwyocr on two scenarios where intrinsic DNA damage will stall a replication fork. In the first case, a lesion blocking synthesis on the lagging strand has caused the polymerase complex to become uncoupled and DNA synthesis has continued on the leading strand (Figure 3.11 B). In the second example, the fork has collapsed and synthesis on both strands has ceased (Figure 3.11 C). These types of damaged fork will be taken in turn in the following discussion, highlighting the effect of the peptide on steps in the repair of each of these stalled forks.

In the case of the single-strand block of the replication fork, there will be a region of ssDNA on the lagging strand of the fork that is coated with SSB (Figure 3.11 B). This substrate will be subject to stabilization by the RecFOR complex, and RecA loading onto the gap will facilitate the strand invasion event that will displace the nascent leading strand, which will be subject to degradation by RecJ or other ssDNA nucleases (Figure 3.11 B). We observed that the peptide effect on recJ or recF mutants is not significantly different than the wild-type strain. This suggests that either the peptide-dependent damage is not repaired in this manner, or that the peptide does not effect this mechanism of fork repair. The latter case is true, as the small amount of growth seen in the peptide treated recB mutant is likely due to the activity of RecJ. That the peptide has little effect on the repair of a decoupled and collapsed replication fork does not mean that the cell will be able to rely on this mechanism for all fork repairs, because forks that are damaged in different ways will require different mechanisms for repair.

In the second case, a fork that has replication stall on both strands, there are two immediate options available to the cell. First is the direct reloading and restart of
the replication fork via the Pri or Rep helicases (Heller & Marians, 2005b, Heller & Marians, 2006a, Mahdi et al., 2006). This mechanism is not likely to be inhibited by the peptides, but the ability of Pri and Rep restart of the forks is dependent on the suitability of the substrates for reloading. If the fork left behind after the collapse is not subject to direct reloading, the fork will be regressed into a chicken foot structure by RecG or RecA (Figure 3.11 C). The RecG protein, whose activity is inhibited by the peptide, has been suggested to be more active during the stationary growth phase, whereas RecA’s branch migration activity, which is likely not inhibited by peptide, occurs during logarithmic growth (Robu et al., 2004). There are several mechanisms that may be able to resolve the collapsed fork. One is direct fork progression catalyzed by RecG, which will be inhibited by peptide. Additionally, exonucleases, including RecBCD, may be able to degrade the free end to restore the fork. This activity would like not be inhibited by peptide, but would again require that the fork structure would be a suitable substrate for restart by Pri or Rep (Figure 3.11 C). However, it has been suggested in the literature that once a fork has been regressed to a chicken foot structure, recombinative repair is essential (Mahdi et al., 2006).

The regressed fork, or chicken foot, is a four-way DNA junction extremely similar in structure to Holliday junctions. For this reason, regressed forks are good substrates for RuvABC. RuvABC action on a regressed fork will result in a double strand break. We know that this activity is inhibited \textit{in vitro} by the peptide (Kepple et al., 2005), however the genetic data presented in this chapter suggests that Ruv action is necessary for bacteria to survive in the presence of the peptides. If the RuvABC complex is able to overcome the peptide to create the DNA dsb, RecBCD activity will be necessary at the break, as RecBCD is required at approximately 99%
of all double strand DNA breaks (Spies & Kowalczykowski, 2006). Unfortunately for the cell, peptide will have little effect on RecBCD processing of the dsb, and the resulting D-loop structure will require a second round of RuvABC activity on a Holliday-junction substrate (Figure 3.11 C). This model explains both the enhanced sensitivity of RecB and Ruv mutants to peptide treatment, suggesting that the regressed fork is the dominant substrate that forms in response to peptide treatment. This pathway of double-strand break repair, it is worthwhile to note, is also applicable to forks that collide with a single-strand nick or gap in the DNA. This would result in a DSB not unlike that described above, and in the presence of d-wrwycr, will again lead to the difficulties described above.

Together, the genetic data suggests that although both RecBCD substrates and RecFOR substrates are being formed after peptide treatment, the RecBCD substrates are either more prevalent or RecBCD is better at repairing (or avoiding) peptide-mediated DNA damage. However, the RecF systems can effect repair of peptide-dependent DNA damage in a RecA-dependent fashion in the absence of RecB. With regard to resolution of the branched intermediates that are formed in response to peptide treatment, the cell can compensate for the absence of recG through other proteins (such as RecA and RuvABC). However, the cell is unable to compensate for the loss of RuvAB, and even less so, RuvC. When both recG and ruvAB are deleted in the same cell, the effect of accumulation of DNA damage in the recG is dominant, and the enhanced sensitivity of ruvAB is lost. Deletions of recG and ruvC additively accumulate DNA fragments when treated with peptide. These results are not in perfect agreement, suggesting that RecG is more involved upstream in the formation of HJ, and that RuvABC acts more to resolve HJ structures.
The hierarchy of the *recG*, *ruvAB*, and *ruvC* mutants’ viability with peptide treatment suggests that cells that have RecG and RuvAB but not RuvC or cells with only RuvC (a *ruvC* mutant and a *recG ruvAB* mutant, respectively) are least capable of repair after treatment. Several possible explanations could explain these results; i) they are less able to repair peptide-dependent damage, ii) the resolution machinery remaining in the mutant cells is more inhibited by the peptide, or iii) wild type cells have several mechanisms to effect resolution of HJ that occur within cells, and as one or more of these resolution pathways are removed, the cell becomes less and less efficient at resolving the intermediate. RecG and RuvC are most necessary for the repair of peptide-mediated damage, or are only moderately inhibited by peptide. Conversely, the RuvABC complex is best at repairing damage or is least inhibited by peptide. *In vitro*, the RuvABC complex is the most sensitive to peptide inhibition, followed closely by RecG, while the individual subunits of RuvABC (RuvAB or RuvC) are less sensitive to peptide inhibition of their enzymatic activity (Kepple et al., 2005). That the *in vitro* data does not exactly correlate with the genetic data suggests that the effects the *recG* and *ruv* mutations have on both the viability and the sensitivity to chromosomal fragmentation of the strains to peptide treatment is more a consequence of which proteins are present to process HJ, as opposed to how efficiently the peptides can inhibit the activity of the particular proteins.

We propose a model where peptide d-wrwycr interferes with DNA damage repair of naturally occurring lesions (Kuzminov, 1999, McCool et al., 2004, Pennington & Rosenberg, 2007). Repair of DNA lesions frequently results in the formation of intermediates which are substrates for d-wrwycr binding, namely complete and partial replication forks and Holliday junctions. Resolution of
recombination normally occurs through multiple pathways, and how the DNA lesion will be repaired will depend on the DNA substrates, the affinity repair proteins have for that substrate, and the availability of the proteins to the substrate. Recently, it has been suggested that RecB may act as a scaffold for repair proteins (Spies & Kowalczykowski, 2006). If so, this would localize repair proteins to the site of damage, creating a bacterial DNA repairosome. If the peptides bind to and stabilize the branched intermediates the repair proteins act upon then it is possible that the life of those substrates will be longer than normal. Disrupting the kinetics of DNA repair will increase the likelihood that newly initiated replication forks will collide with the site of the lesion, assuming that blocks to DNA replication initiation do not occur or will be overcome with time. When the nascent fork collides with the peptide-stabilized fork, double strand breaks will occur. These double strand breaks will also require recombination to be repaired, and in turn will create more peptide targets, increasing the frequency of new fork collision events. This process could amplify double strand breaks that would persist as long as the peptide is present to interfere with DNA repair, or the cell dies.

Data presented here suggest that peptide treatment frequently results in the collapse of replication forks, and their subsequent regression into a chicken foot structure. This structure, which resembles a Holliday junction, can be bound with high affinity by the peptides. Regression of the fork can be carried out by either RecG or RecA (Robu et al., 2004) However, the presence of the peptide probably skews repair pathways toward pathways that do not require recombination to restart stalled replication forks. The direct restart of collapsed forks can be achieved through direct fork progression, possibly by RecG or RecA, and reloading mediated by the Pri or

The model of peptide d-wrwycr action proposed here predicts some mutations which would be expected to confer d-wrwycr resistance. Among these would be mutations that decrease the frequency of HJ formation. Such strains would have decrease recombination frequency. These mutations conferring resistance to peptide d-wrwycr would probably decrease overall fitness of the strain in the absence of peptide. Unlike most modern antibiotics where resistance is allowed to persist in the absence of selection because of suppressors, the peptide resistant strains will be out-competed in the environment. In addition to potential therapeutic applications, we envision that, peptide d-wrwycr will be a valuable tool for dissecting recombination pathways in living bacterial cells.

Portions of this chapter are in preparation to be published as: Gunderson, Carl and Segall, Anca, 2007. An antimicrobial hexapeptide acts by binding recombinative DNA repair intermediates and influencing their resolution.
Chapter 4: Analysis of RecQ mutants in *E. coli* and *Salmonella*

Section 4.1: Introduction

Some proteins that are involved in DNA repair and chromosome maintenance are conserved from bacteria to humans. One example is the *E. coli* protein RecQ, which is the archetypal member of the RecQ family of DNA helicases. RecQ is a 3'-5' DNA helicase that has five homologs in humans, including; WRN, BLM, RecQ1, RecQ4, and RecQ5. The function of the human proteins WRN, BLM, and RecQ4 are well studied, and it is known that loss-of-function mutations in these proteins lead to diseases such as Bloom’s and Werner’s syndromes. These syndromes are characterized by a predisposition to cancer and premature aging, a result of a general loss of genome stability due to the absence of the functional helicase. The roles of RecQ1 and RecQ5 are less well understood but it has been proposed that the function of these homologs might be explained by the function and activity of the RecQ protein from *E. coli* (Magner et al., 2007).

The RecQ protein in *E. coli* has been shown to be able to act in co-ordination with Topoisomerase III to mediate catenation and decatenation of DNA (Harmon et al., 2003). In addition, RecQ can act in an anti-recombinational manner and unwind branched DNA substrates, such as double Holliday junctions (Harmon & Kowalczykowski, 1998). Based on more recent data, the Rosenberg group proposes that the *E. coli* RecQ helicase promotes the formation of recombination intermediates (Magner et al., 2007). They show that a synthetic lethality observed between *ruv* and
uvrD mutations is due to the inability to resolve Holliday junctions, and that viability can be rescued by decreasing recombination frequencies via mutations in recA, recQ, or to some extent a recJ mutation.

The synthetic lethality of the ruv and uvrD mutations is a result of the activity of these proteins in resolving and or removing recombination intermediates. When the activity of these resolving proteins is absent, the cells die from a toxic buildup of strand exchange intermediates. The RuvABC complex is involved in the branch migration and resolution of Holliday junctions in recombination (see Introduction section 1.2 and 1.3). The uvrD gene was originally identified as coding for a gene involved in ultraviolet light resistance. The UvrD protein is a 3' – 5' helicase that removes UV damaged DNA strands after they have been recognized and nicked by the action of the UvrA, UvrB, and UvrC proteins (Friedberg, 1995). It has been know for a long time that uvrD mutants are hyper-recombinogenic. Recently, the hyper-rec phenotype of uvrD mutants appears to be a consequence of the anti-recombinogenic activities of stripping RecA from nucleoprotein filaments (Centore & Sandler, 2007, Flores et al., 2005, Veute et al., 2005), and unwinding of fork structures (Cadman et al., 2006).

The Rosenberg lab proposed a collaboration to use the Holliday junction binding properties of the peptides to help determine the role of E. coli RecQ in chromosome stability. The simple prediction was that, if the peptides inhibit cleavage of Holliday junctions, treatment with the peptide should phenotypic ally mimic a ruvC mutation. Initial work was done to determine the sensitivity of E. coli recQ, ruvC, and uvrD mutants to peptide treatment. Expecting to see resistance to peptide in the recQ mutant strain of E. coli due to a reduction in recombination, and thus peptide targets
we were disappointed after initial testing. However, a *Salmonella recQ* allele did display partial resistance to peptide treatment. With these encouraging findings we continued the study using *Salmonella* strains.

*Salmonella recQ*, *uvrD*, and *ruvC* mutants (as well as double mutants thereof) were treated with Holliday Junction-binding peptide (d-wrwycr) and assayed by pulsed field gel electrophoresis and for viability after treatment. In both assays, cells were treated with peptide alone as well as in combination with mitomycin C (MMC). We have previously shown that there is synergy between the peptide's activity and that of MMC. We hypothesize that synergy between the agents is a result of recombination repair of MMC-dependent DNA damage, which creates targets (HJ and branched DNA intermediates) for peptide binding. The cell physiology of peptide treated bacteria (Chapter 2) and genetic analysis of peptide effects of *Salmonella* recombination mutants (Chapter 3), taken with the known biochemistry of the peptides (Boldt et al., 2004, Kepple et al., 2005), suggest that the peptides' binding to the junctions is their primary mode of antimicrobial activity. These aspects of the peptide's activity, and the response of the *recQ* mutant to peptide treatment, supports the model that RecQ creates substrates for peptide binding, but is not the sole source of strand exchange intermediates (SEI) in the cell.

Section 4.2: Results

The sensitivity of *E. coli* and *Salmonella recQ* mutants to peptide wrwycr was determined by monitoring the growth of the strains by optical density after addition of
the peptide. The *Salmonella recQ* allele was tested in combination both with *uvrD* and *ruvC*. A *Salmonella recQ* mutant is modestly resistant to peptide treatment, and that increased resistance is maintained in an *uvrD*- background (Figure 4.1). An *E. coli recQ* mutant shows no increase or decrease in susceptibility to peptide compared to the wild-type.

Because a number of repair-deficient mutants filament, optical density measurements may not reflect only cell numbers, but also size. Therefore, viability assays were performed to obtain a direct measure of cell numbers using a combination of peptide and MMC treatments. The results of the viability experiments show that *recQ* is somewhat more resistant to peptide treatment than the wild-type cells (seen most clearly at 64 μM peptide), whereas *uvrD* and *ruvC* are more sensitive, seen most clearly at 32 μM peptide (Figure 4.2). The *recQ uvrD* double mutant mimics the wt, thus the *recQ* mutation rescues somewhat the sensitivity of the *uvrD* mutant to peptide treatment. Conversely, the increased sensitivity of the *ruvC* strain is dominant over the *recQ* phenotype, since the *recQ ruvC* double is as sensitive to peptide as *ruvC* alone (Figure 4.2).

DNA damage caused by the peptide (in the presence or absence of MMC) after three hours of treatment was assayed by pulse field gel electrophoresis. In this assay fragmentation of the chromosome is visualized by smears of DNA within the lanes of the gel. The intact DNA remains in the well throughout the run (the well portions of the gels are shown in Figure 4.3, see section 3.2 and Materials and Methods for details on PFGE). The bar graph presented in the bottom portion of the PFGE figure represent the average amount of intact DNA for each strain treatment relative to the mock (DMSO) treated control for that strain (Figure 4.3B).
**Figure 4.1:** Growth kinetics of d-wrwycr-treated *E. coli* 594 and *Salmonella* LT2. A) Growth of *Salmonella* LT2 (black curves), and *recQ* (blue curves), *uvrD* (red curves) and *recQ uvrD* (green curves) mutants in the absence (squares) and presence of 32 and B) 64 µM d-wrwycr (triangles and circles, respectively). C) Growth of *Salmonella* LT2 (black curves), and *recQ* (blue curves), *ruvC* (red curves) and *recQ ruvC* (green curves) mutants in the absence (squares) and presence of 32 µM d-wrwycr (triangles). D) Growth of *E. coli* (black curves), and *recQ* (blue curves), *uvrD* (red curves) and *recQ uvrD* (green curves) mutants in the absence (squares) and presence of 32 µM d-wrwycr (triangles and circles, respectively). Curves are averages of 3 independent cultures. Error bars represent one standard deviation from the mean.
Figure 4.2: Viability analysis of *Salmonella* recQ, *uvrD*, recQ *uvrD*, *ruvC*, and recQ *ruvC* strains. Photographs of LB plates used for dilution series have dilutions labeled across the top, and treatment conditions labeled down the side. From left to right, and top to bottom panels are wild-type LT2, *uvrD*, *ruvC*, recQ, recQ *uvrD*, and recQ *ruvC*. All MMC treatments are 1 μg ml⁻¹. Photographs represent data from triplicate independent cultures from at least 2 different days (n of at least 6, LT2 n=54).
Figure 4.3: PFGE analysis of *Salmonella* recQ, uvrD, recQ uvrD, ruvC, and recQ ruvC strains. A) The well portions of representative pulsed field gels are shown, with treatment conditions labeled above. Wild-type LT2 is shown at the top for reference. Gels were stained with Sybr Green I. B) The relative fraction of intact DNA in each lane was calculated by dividing the amount of DNA retained in the well after treatment by the amount of intact DNA in the DMSO control. Each of the mutants was calculated independently. Each bar represents at least 3 independent trials across 2 different days (n of at least 3, LT2 n=33). Error bars represent the standard deviation from the mean.
The combined treatment of peptide and MMC causes less fragmentation of the chromosome in both a *Salmonella uvrD* mutant and a *recQ* mutant than they do in the wild type strain (Figure 4.3). Although at high concentrations of peptide and MMC, the *uvrD* mutant and the *recQ uvrD* double mutant show similar peptide-dependent fragmentation of chromosomal DNA, the chromosomes of the double mutants are more fragmented when treated with MMC alone (Figure 4.3). A *ruvC* strain is very sensitive both to peptide alone, and in combination with MMC (see also chapter 3). However, the amount of intact DNA in the *recQ ruvC* double mutant after treatment is increased relative to the *ruvC* mutation alone, but is less than that of the *recQ* mutant (Figure 4.3).

Section 4.3: Discussion

As discussed in the previous chapter, the pulse field gels are an endpoint assay with the cells being lysed in agarose blocks after three hours of treatment. The PFGE data thus speaks more to the accumulation of targets within the cell. The viability assays, which demand growth on nonselective media after treatment, address the ability of the cells to repair damage caused by peptide and MMC treatments. The *recQ* mutant, which shows a similar fragmentation response to the wild type, shows only a slight decrease in the number of peptide targets. However, when peptide treatment is combined with MMC, there is protection from fragmentation in the *recQ* mutant. This is consistent with the resistance of the *recQ* mutant to
peptide treatment in the growth assays, suggesting that RecQ is likely involved in formation of peptide targets in *Salmonella*.

With regard to peptide-dependent fragmentation, the *ruvC* mutant treated with 64μM peptide exhibited the greatest decrease in fragmentation (largest increase in targets). These results are consistent with the role of these proteins in reducing the overall number of strand exchange intermediates (SEI) in the cell. The sensitivity of the *ruvC* mutant in the viability assay implies that RuvC is frequently required to resolve recombination intermediates in the repair of damage caused by the peptides. Introducing the *recQ* mutation into the *ruvC* background results in an increase in the amount of intact DNA in cultures treated with high concentrations of peptide, as compared to the *ruvC* mutant alone, suggesting that lacking RecQ-mediated recombination has a protective effect in a *ruvC* null background. However, these cells are no more viable than the *ruvC* mutants, suggesting that RuvC is necessary to resolve the SEIs that accumulate in a RecQ-independent manner.

There is less intact DNA present in the *recQ uvrD* double mutant than either of the single mutants alone, an indication that UvrD prevents the formation of SEI's in both RecQ-dependent and –independent pathways. The viability assays suggest that the antirecombinogenic activity of UvrD affords some protection from the peptides, as the *uvrD* mutant is more sensitive to peptide, but this affect is not cumulative with the loss of RecQ, since the *recQ uvrD* double mutant is more sensitive to peptide treatment than a *recQ* mutant.

There are multiple pathways by which RecQ might be creating targets for peptide d-wrwycr (Harmon & Kowalczykowski, 1998, Magner et al., 2007). If RecQ
and RecJ process the lagging strand of a stalled replication fork prior to fork regression, they will create a fork structure with a gap on the lagging strand (Figure 4.4 A). This fork substrate is similar to one that can form when the replication machinery becomes decoupled, and leading-strand synthesis continues for a time after lagging-strand synthesis has stalled (Figure 4.4 B). The peptide will likely be unable to inhibit the RecQ-dependent formation of this substrate. The RecFOR complex will stabilize the 5’ end of the lagging strand gap, to facilitate RecA loading and strand invasion (Morimatsu & Kowalczykowski, 2003). There are two different models of how this strand invasion event may occur (Figure 4.4 C). In one model, strand invasion will result in the formation of a double Holliday junction structure, a very good substrate for peptide binding (Figure 4.4 C, left). These Holliday junctions would require RuvABC for resolution. The alternative model suggests that the leading strand will be displaced by the strand invasion event, and subject to ssDNA exonuclease activity (Figure 4.4 C, right). The peptide would have less effect on this type of processing because no Holliday junctions are formed. If both of these processes occur, the cell has an alternative to forming the peptide targets. It is possible that the directed use of one of these two pathways is a result of the length of the single strand region, and longer RecA nucleoprotein filaments will favor the double crossover pathway and shorter filaments will displace the leading strand. If this is the case, then the pathway will be dictated by kinetics of RecQ and RecJ binding in competition with the RecFOR binding and stabilization of the 5’ DNA ends. On the other hand, if the assumption that the cell has equal opportunity to use either of these pathways is accepted, then the cell will always have one pathway to resolve
Figure 4.4: Models of RecQ-mediated strand-exchange intermediate formation. A) A stalled replication fork can be processed by RecQ and recJ to create a lagging-strand gap at the junction. B) A similar fork substrate with lagging-strand gap will form if DNA synthesis becomes uncoupled. C) Alternative models for RecFOR and RecA action on the stalled fork. On the left, strand invasion will create a double Holliday junction structure. In the pathway on the right, the longer leading strand is displaced and is subject to exonuclease digestion. D) If there is a nick or gap on a single strand of the DNA, RecJ and RecQ will widen the gap on the 5' side, until the RecFOR complex stabilizes the gap, and subsequent RecA loading lead to formation of a double crossover intermediate. Resolution of the double Holliday junction will be catalyzed by RuvABC. These models are based on the works of Benedicte Michel and Susan Rosenberg.
the intermediates. This would suggest that there is another major pathway of RecQ-mediated formation of peptide targets.

The action of RecQ and recJ is not limited to the lagging strand of stalled replication forks. Any gap or nick on a single strand of DNA will be a substrate for RecQ and RecJ processing on the 5’ end (Figure 4.4 D). This pathway, in a RecFOR-dependent manner, requires the double Holliday junction intermediate to form for accurate repair. These Holliday junctions can only be resolved by RuvABC. It could be that these substrates are the dominant source of RecQ-dependent peptide targets.

The data presented here is consistent with the hypothesis that RecQ is involved in one pathway of formation of recombination intermediates that are peptide targets. Peptide-stabilized repair intermediates require recombination for both their formation and their resolution, and ultimately the resolution of the SEIs necessitates the activity of ruvC. This suggests that the intermediates that form cannot be resolved by branch migration either mechanistically, or because the peptide inhibits RecG, and must be cleaved by ruvC. Salmonella recG and ruvAB mutants suffer less peptide-dependent fragmentation than a ruvC mutant (Figure 3.7). The sensitivity of the uvrD mutant to the peptide is consistent with increased recombination levels because RecA nucleoprotein filaments are more stable or more frequent when the UvrD protein is absent, resulting in an increase in potential peptide targets.

The difference between the Salmonella and E. coli recQ mutants may be explained in one of several ways; the RecQ proteins of these bacteria could be different enough that they serve different functions, or that one of the strains, likely E. coli, has a protein that can functionally substitute for RecQ in its absence. The latter
situation is a more likely explanation, considering that *E. coli* and *Salmonella recQ* are 94% identical at the amino acid level. It is also possible that the *recQ* gene is differentially regulated in the two organisms, and that the RecQ protein of *E. coli* is less frequently required to initiate recombination events, and that different pathways of recombination are used at different frequencies in *Salmonella* and *E. coli*. 
As discussed in the first chapter, bacteriophages are capable of integrating their chromosome into that of the host during the lysogenic portion of their life cycle. Bacteriophage lambda utilizes the Integrase enzyme to catalyze the site-specific recombination reaction that integrates and excises its chromosome into and out of the host. Wild-type *Salmonella* has four prophages in its chromosome: Gifsy-1, Gifsy-2, Fels-1 and Fels-2 (Figueroa-Bossi et al., 1997, Gemski et al., 1972, Yamamoto, 1969). Each of these prophages is capable of being induced to replicate and/or excise, in a manner similar to phage lambda. DNA damage is one signal that can lead to activation of prophages, including Gifsy and Fels. In *Salmonella*, null mutations of the *lexA* gene that render cells SOS constitutive are lethal due to activation of the Fels-2 prophage (Bunny et al., 2002).

In the course of performing pulse field gel electrophoretic analysis of the *Salmonella* recombination mutants treated with peptide, we noticed that some strains showed similar fragmentation patterns (see appendix Figure A.10). The smears of DNA within the gel showed tapered bands, ranging from less than 48 kb to greater than 300 kb. These bands are consistent with fragmentation of the chromosome, but occasionally we observed a specific band at that migrated at approximately 50 kb. The bands appeared to be consistent with initiation of prophages replication, and if
the replication occurred while the prophage remained integrated, could explain some of the DNA smearing that we observed.

Since the peptides cause DNA damage and induce the SOS response, it is reasonable that the peptides might also be activating the prophages. However, since the peptides were isolated as inhibitors of site-specific recombination, they might also be capable of inhibiting the excision reaction. To further examine the role of the peptides in prophage excision and replication, we used an *E. coli* phage lambda di-lysogen constructed in the Weisberg lab (Weisberg, 1969). The strain was constructed to test the activity of Integrase mutants, but it also makes it possible to examine the peptides’ effect on the excision step, and distinguish between the *attL* by *attR* and the *attL* by *attL* recombination pathways (Figure 5.1).

The peptides are able to inhibit excision *in vivo* by phage lambda Integrase. The peptides also inhibit excision and/or replication of phage P22 and Gifsy and Fels phage lysogens, as peptide inhibits the production of viable phage after treatment with Mitomycin C (MMC). In addition, the peptide is able to inhibit phage production from the Gifsy and/or Fels prophages. Quantitative PCR showed that the peptides inhibit Gifsy phage excision and Fels-1 replication.

Section 5.2: Results

The peptides were identified as inhibitors of lambda Integrase mediated strand exchange, and subsequently found to have antimicrobial activity. It was important to
Figure 5.1: Assay to analyze peptide d-wrwycr’s effect on phage lambda excision in vivo. A) Diagram of the lambda excision reporter strain. Excision via attL by attL will result in a white temperature sensitive colony. attL by attR excision will result in either a red, temperature resistant colony or a white temperature resistant colony. No excision is indicated by a red, temperature sensitive colony. B) An example of a portion of MacConkey galactose plate showing red, white and sectored colonies.
show that they are capable of acting, mechanistically, within a cell as they do in biochemical reactions. An *E. coli* strain which harbors tandem lambda lysogens allows visualization of phage lambda excision through temperature sensitivity and the ability to utilize galactose. One of the prophages is a lambda cl857 dgal, which is a defective lambda that confers the ability to utilize galactose. The second prophage has a cl857 repressor mutation and can produce virulent phage at a temperature of 42°C. Prior to induction of the prophages by heat pulse at 42°C, the strain is temperature sensitive and will produce red colonies on MacConkey agar with galactose (Mac + Gal). Upon induction, three potential recombination events may occur, and result in the excision of one or both of the prophages (figure 5.1A). An attL by attL (LxL, bent L) recombination event will liberate the λ.dgal phage resulting in white temperature sensitive colonies on Mac + Gal plates. There are two different attL by attR (LxR, excision) recombination events (figure 5.1A), one of which will excise the cl857 virulent prophage (red, temperature resistant colonies) and the other will excise both prophages (white, temperature resistant colonies).

The strain was diluted and plated on Mac + Gal plates at 30°C after induction for five minutes at 42°C in the presence of peptide. After colonies appeared on the plates at 30°C, they were replica printed to fresh Mac +Gal plates, which were incubated at 42°C. By counting colonies we can determine that induction causes nearly 50% excision of the phages, but the presence of peptide d-wrwycr inhibits 18% of the total recombination (Figure 5.2). The LxR recombination event is inhibited 12% by the peptide and LxL recombination is not significantly inhibited (~5%). The peptide inhibits phage lambda's Integrase *in vivo*, but the mechanism of excision by site-
Figure 5.2: Peptide d-wrwycr effect on phage lambda excision in vivo. Bars represent the percentage of colonies, on MacConkey + galactose plates, that have undergone each of the excision reactions labeled below the chart. White bars represent colonies from DMSO (mock) treated culture, and grey bars represent colonies from a culture treated with 10 µM d-wrwycr during the 5 minute induction at 42°C. Bars are the average of three independent determinations, and error bars are the standard deviation from the mean.
specific recombination is not specific to phage lambda, but is shared by many lysogenic phages.

To determine if the peptides are capable of inhibiting the excision of prophages other than lambda, we tested the ability of a phage P22 Salmonella lysogen to produce plaque forming units after treatment with peptide d-wrwycr (Figure 5.3A). After a 1:100 dilution of an overnight culture into fresh MHB, the lysogen strain was grown for two hours at 37°C. The cultures were then treated with MMC, peptide, or both. We see that some phage are produced in the DMSO (mock) treated sample, but mitomycin C (MMC) causes nearly 1000-fold more P22 phages to be produced. A sublethal concentration of the peptide inhibits the production seen in the DMSO 100-fold. Peptide inhibits the number of phage produced by treatment with MMC by about 100-fold. Using this assay, we do not know if the peptides are inhibiting excision or if they inhibit replication of the phage directly. However, since we know that lambda excision is inhibited by the peptides, and that P22 uses a similar mechanism to excise from the chromosome, it is likely that the inhibition we see is due to inhibition of excision rather than inhibition of replication.

Wild-type Salmonella harbors 4 prophages within its chromosome (Gifsy-1, Gifsy-2, Fels-1, and Fels-2), which produce viable phage that will plaque on strains which have had the prophages deleted or deactivated. After treating wild-type LT2 with MMC and peptide and making a lysate of the culture, we can see that plaque-forming units are produced when the culture was treated with MMC, but not when treated with peptide (Figure 5.3B). Again, peptide inhibits the MMC induction of the
Figure 5.3: Peptide d-wrwycr inhibits excision of Salmonella prophage. A) Lysates from wild-type LT2 cultures treated with MMC, peptide, or both (as indicated at right). B) Lysates from wild-type LT2 cultures treated with MMC or peptide (as indicated at right). Lysates were serially diluted 10-fold and plated on a lawn of LT2 cured of its four naturally occurring prophage (Gifsy-1, Gifsy-2, Fels-1, and Fels-2). Lysates were serially diluted 10-fold and 5 µl of each dilution, starting with undiluted, was plated on a lawn of LT2.
phage. Using strains that have been differentially deleted of the different phages, we can see that Fels-1 is the dominant phage being induced under these conditions (data not shown).

Salmonella’s prophages might be induced with the peptide treatments, which caused concern that replication of the Gifsy and/or Fels phages might be interfering with our pulse field gel results because their replication may contribute to the DNA fragments seen. The McClelland lab showed that induction of Fels-1 can cause two different types of replication. In one scenario, the prophage excises and replicates extra-chromosomally. In the other scenario, the phage remains integrated and initiates replication that causes ‘onion-skin’ replication (Fukasawa et al., 1978, Willard & Echols, 1968), which extends beyond the ends of the integrated phage (Frye et al., 2005). These linear replication products can be as long as 900kb. To distinguish between these possibilities, we can use quantitative PCR (qPCR) to measure the relative amounts of attR and attB for each of the prophages (Figure 5.4 A). The attR site will only be present when the prophage is integrated, while the attB site will only be created when the phages excises. By quantifying these two sites, we can elucidate the extent of replication of each of the prophages, and if the replication is taking place within the chromosome, or after excision.

Quantitative real-time PCR was used to determine if excision or replication of the prophages was occurring after the treatments. Wild-type LT2 was subcultured 1:100 from an overnight into fresh MHB, and grown for two hours at 37°C. Peptide and MMC treatments, in addition to combined treatments, were added and the cultures were returned to the 37°C incubator for three hours. The Promega Wizard genomic DNA purification kit was used to isolate genomic DNA from the treated
Figure 5.4: Induction of Gifsy-1 and Gifsy-2 phages after treatment with MMC or peptide d-wrwycr. A) Fold increase (or decrease) in abundance of Gifsy-1 attR (light grey bars) and Gifsy-1 attB (dark grey bars) sites after treatment with 1 µg ml⁻¹ MMC (left pair), 64 µM d-wrwycr (center pair), or both (right pair). B) Fold increase (or decrease) in abundance of Gifsy-2 attR (light grey bars) and Gifsy-2 attB (dark grey bars) sites after treatment with 1 µg ml⁻¹ MMC (left pair), 64 µM d-wrwycr (center pair), or both (right pair). Error bars represent the standard deviation from the mean.
Table 5.1. Primers used to detect Salmonella prophage via quantitative PCR.

<table>
<thead>
<tr>
<th>name</th>
<th>sequence</th>
<th>product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fels1 attL up</td>
<td>5’ – GCAGATTGAGTACACGCAGC – 3’</td>
<td>Fels1 attL</td>
</tr>
<tr>
<td>Fels1 attL down</td>
<td>5’ – TTCTGCGAAAGGTACTATCTGCG – 3’</td>
<td></td>
</tr>
<tr>
<td>Fels1 attR up</td>
<td>5’ – ATTTGCCGGAACCAACCCAG – 3’</td>
<td>Fels1 attR</td>
</tr>
<tr>
<td>Fels1 attR down - 2</td>
<td>5’ – GTGTCAGCTTCACGCTGCG – 3’</td>
<td></td>
</tr>
<tr>
<td>Gifsy1 attL up</td>
<td>5’ – TCCATCGAATCAAGTACCTGAGC – 3’</td>
<td>Gifsy1 attL</td>
</tr>
<tr>
<td>Gifsy1 attL down</td>
<td>5’ – GTG GAA TGT CCA CCG CTG – 3’</td>
<td></td>
</tr>
<tr>
<td>Gifsy1 attR up</td>
<td>5’ – TTGCGGGGTACCTGAGG – 3’</td>
<td>Gifsy1 attR</td>
</tr>
<tr>
<td>Gifsy1 attR down</td>
<td>5’ – GTAGTGTAGATGCCGGCGTTTC – 3’</td>
<td></td>
</tr>
<tr>
<td>Gifsy2 attL up</td>
<td>5’ – CCGCTGGAGTATACCTTGTGAGC – 3’</td>
<td>Gifsy2 attL</td>
</tr>
<tr>
<td>Gifsy2 attL down</td>
<td>5’ – GTGGGATGTCAGAGAGAGCG – 3’</td>
<td></td>
</tr>
<tr>
<td>Gifsy2 attR up</td>
<td>5’ – GGTCTGTGAAATCTGTTTCTG – 3’</td>
<td>Gifsy2 attR</td>
</tr>
<tr>
<td>Gifsy2 attR down</td>
<td>5’ – CAAATCGCGCTACGAGATG – 3’</td>
<td></td>
</tr>
</tbody>
</table>

The attB sites of any of the phages can be amplified using the appropriate attL-up and attR-down primers specific for that phage. Similarly, the attP sites can be amplified by using the attR-up and attL-down primers. The pPCR reaction started with 95°C for 3 minutes, followed by 40 cycles of 95°C, 15 seconds; 56°C, 20 seconds and a plate reading. After the 40 cycles, a melt curve was done to check the specificity of the reactions.
cultures. This genomic DNA was used as the template in the qPCR reactions. The primer sets used in the reactions to detect the attL and attB sites of Fels-1, Gifsy-1, and Gifsy-2 are detailed in Table 5.1. Bio-Rad iQ SYBR Green Supermix was used to provide the polymerase, dNTPs, and detection dye (SYBR Green). Refer to table 5.1 for details of qPCR reaction. We observed that peptide alone has little effect in inducing the prophages of Salmonella. However, when the phages are induced to excise by treatment with Mitomycin C, excision of both Gifsy-1 and Gifsy-2 prophages is inhibited by the peptide. MMC also induces Fels-1 to replicate within the chromosome. The peptide inhibits the Fels-1 replication, but since we know that the peptides have little direct effect on bacterial DNA synthesis machinery, it is likely through an indirect mechanism of peptide action.

Section 5.3: Discussion

One limitation that we have had in the analysis of the peptides’ effect on bacterial cells is that we are not currently able to track or quantify them within the cell. The effects that the peptides have on bacterial cells, including DNA breaks and chromosome fragmentation, and the changes in sensitivity seen with different recombination mutations, have provided indirect evidence that the peptides act on targets within the cell. Here, we have shown that the peptides can enter an E. coli cell and inhibit the site-specific recombination event that results in the excision of lambda and other prophages. We now know that the peptides are able to enter bacterial cells, retaining an active form that is capable of their in vitro activity. Thus, we can be more confident that since the peptides can inhibit site-specific recombination in vivo, they
are also available to inhibit recombination and DNA repair, as discussed in previous chapters.

The effect of the peptide on prophage excision varies slightly from what we observe in biochemical recombination reactions between linear sites. Peptide d-wrwycr is about 4-fold more effective at inhibiting the action of Integrase in bent-L reactions than excision reactions (Boldt et al 2004). Although the peptide inhibits the L x R recombination, we see little effect of the peptide on bent-L excision \textit{in vivo}. One possible explanation for this could be the difference between \textit{in vivo} excision or bent-L recombination on a supercoiled DNA substrate versus those same reactions on linear substrates. Despite these differences, it is clear that the peptides are capable of exerting similar effects on recombination \textit{in vivo} as they do \textit{in vitro}.

The peptides are capable, \textit{in vitro}, of inhibiting more than just lambda Integrase. We have shown that peptide d-wrwycr can inhibit replication of phage P22 lysogens, and Gifsy and/or Fels phages, likely by inhibiting excision. Our data shows that concerns that the excision or replication of the prophages might contribute to DNA fragmentation seen in the PFGE analysis only applies to the MMC treatments, as peptide treatments not only do not induce the phages, but inhibit their excision and/or replication.
Chapter 6: Discussion and future directions

Section 6.1 Discussion

The depth of knowledge in the field of DNA repair and recombination is long, detailed, and diverse. Innumerable genetic and biochemical studies have been, and will be, undertaken in an effort to better understand the mechanisms of genetic stability and chromosomal maintenance. Recombination is an integral part of these processes, and although we currently have a good idea of how a cell’s complement of recombination proteins operate, some aspects of DNA repair remain elusive. The repair of damaged DNA is a balance between the substrate specificity of the repair enzymes and their availability to the site of the damage. A given DNA lesion may be repaired through one of several pathways, catalyzed by different proteins (Figure 6.1 and 6.2). The inherent overlap and promiscuity of DNA repair systems makes them difficult to study. The initial goal of this project was to use the peptide inhibitors of lambda site-specific recombination to probe aspects of chromosome structure and maintenance, including homologous recombination. These processes frequently involve the formation of Holliday junctions, and if the peptides are able to bind and stabilize Holliday junctions in living cells as they do in vitro, the peptides would be valuable tools in their study.

We have shown that peptide d-wrwycr is capable of inhibiting recombination by lambda Integrase in vivo. This showed that the peptide is available within the cell
Figure 6.1: Model of stalled replication fork repair. A) A fork with leading and lagging strands at the junction can be reversed by RecG or RecA. If the resulting Holliday junction is processed and cleaved by the RuvABC complex, a double strand break will be formed. RecBCD will recognize the dsDNA end, and facilitate RecA loading and strand exchange. RuvABC then resolves the substrate, restoring the fork. B) In the case of a fork with a gap on the lagging strand, RecA or RecG can facilitate regression, and polymerases are able to synthesize off the longer lagging strand. Subsequent fork progression can bypass the lesion, restoring the fork downstream of the lesion. In the case of either A or B, PriA will be able to reload DnaB on the fork, the first step of replication fork restart.
Figure 6.2: Model of stalled replication fork repair. A) A fork with a lagging strand gap at the junction can be targeted by the RecFOR complex, which will load RecA on the gap. A strand invasion event will yield a 3' OH overhang, which will be degraded by exonucleases or will serve as template for lagging strand synthesis (see figure 6.2B) B) After RecA or RecG facilitate fork regression, nuclease activity can degrade the free end, restoring the fork. In the case of either A or B, PriA will be able to reload DnaB on the fork, the first step of replication fork restart.
to act on the target it was initially isolated against, and that it has similar activity within the cell as it does *in vitro*. The peptide is also able to inhibit the excision of phage P22 of *Salmonella* as well as the excision and/or replication of the Fels-1 and Gifsy-1 and Gifsy-2 prophage. This was also important in later studies to show that replication of the *Salmonella* prophage was not interfering with the peptide-dependent results of the PFGE assays.

We found that the peptides inhibited the growth of bacterial cells in rich media culture. About that same time, other students in the lab showed that the peptides, in biochemical reactions, specifically inhibited several unrelated proteins that process Holliday junctions (Boldt et al., 2004, Kepple et al., 2005). They showed that the peptides bound directly to protein-free HJ, and inhibiting either by directly competing for binding with the proteins that process HJ, in the case of RecG (Kepple et al., 2005) or by altering the three dimensional structure of the substrate and preventing catalytic activity of the HJ processing enzyme, in the case of the tyrosine recombinases (Boldt et al., 2004, Cassell & Segall, 2003, Ghosh et al., 2005). We hypothesized that the peptides were inhibiting the growth of bacterial cells by inhibiting the resolution of Holliday junctions that form during every generation. Holliday junctions are formed every generation because of intrinsic DNA damage can block the progression of replication forks. This causes of this damage range from UV light to reactive oxygen species to protein-DNA adducts. Repair of stalled replication forks is thought to be as frequent as once per cell cycle (Sherratt et al., 2004, Steiner & Kuempel, 1998, Lesterlin et al., 2004).

I’ve shown that the most potent peptides have broad-spectrum antimicrobial activity and are bactericidal, but are more potent against Gram+ bacteria than Gram-
bacteria. Cells treated with peptide lose viability but can recover after extended treatment, as the peptides are either degraded, in the case of L-amino acid containing peptides, or are effluxed from cells or eventually dissociate from their targets, branched DNA. Peptide wrwycr causes bacteria to filament and mis-segregate their chromosomes, likely by interfering with the repair of DNA damage which may cause cells to attempt to divide prior to completing DNA repair, leading to the formation of anucleate cells or preventing division entirely. The peptides would also inhibit the action of the tyrosine recombinases XerC and XerD which work together to resolve dimeric chromosomes prior to cell division (Sherratt et al., 1995, Sherratt et al., 2004). We observe that the peptides cause DNA breaks, evidenced either by direct labeling of free 3’OH ends by the TUNEL method or by PFGE, and we see synergistic effects between peptide treatment and MMC or UV treatment (Gunderson & Segall, 2006). Peptide and MMS treatment have additive effects. These findings were consistent with a model of peptide action on intracellular targets.

It was necessary to further investigate both the role of DNA repair pathways in resistance to the peptides, and the reciprocal effect of the peptides on DNA repair. I thus employed a directed genetic approach, specifically testing the effect of the peptides on Salmonella mutants defective in one or more components of their repair systems. The results indicated that the DNA damage caused by the peptide is at least partially SOS-dependent, and that RecA makes targets for peptide. This is entirely consistent with less recombination in the recA mutant, and thus fewer Holliday junctions. Both RecBCD substrates and RecFOR substrates are being formed after peptide treatment, but the RecBCD substrates are either more prevalent or RecBCD
is better at repairing (or avoiding) peptide-mediated DNA damage. In the absence of RecB, it appears that RecJ (and/or RecF) systems can facilitate damage repair.

Mutant Salmonella that have lost one or more means of resolving Holliday junctions are generally more sensitive to DNA fragmentation caused by peptide treatments. RecG, according to the data, may be more involved upstream in the formation of HJ, leaving RuvABC to resolve HJ structures. The viability data also suggests that the resolution of Holliday junctions by RuvC cleavage is more necessary for the repair of peptide-mediated damage than the branch migration activities of RuvAB or RecG. Since the peptide is known to inhibit both RecG and RuvABC \textit{in vitro} (Kepple et al., 2005), how do these proteins repair peptide-dependent damage? Bacteria that are treated with the peptide, despite large decreases in viability, eventually recover. This recovery is at least partially due to efflux of the peptides from the cell, and as the peptide is effluxed, the effective intracellular concentration of peptide drops. Due to this drop in concentration, the peptides likely stabilize the Holliday junctions in a somewhat transient manner, eventually allowing their resolution to continue.

In agreement with data from Susan Rosenberg's lab (Magner et al., 2007), I've shown that RecQ is involved in formation of peptide targets (strand exchange intermediates) in \textit{Salmonella}, but is not the sole source of HJ. RuvC, again is frequently required to resolve recombination intermediates in the repair of damage caused by the peptides, as we see that lacking RecQ-mediated recombination has a protective effect in a \textit{ruvC} null background. Taken together, the data shows that RuvC is necessary to resolve SEIs that accumulate in a \textit{RecQ}-independent manner.
Figure 6.3: Fraction of intact DNA retained in the wells of DMSO (mock) treated cultures. The amount of intact DNA in each of the mutant *Salmonella strains* (X axis) expressed relative to the amount in the LT2 culture (wild-type, red bar).
Interestingly, the antirecombinogenic activity of UvrD affords the cell some protection from the peptides, but this effect is not cumulative with the loss of RecQ. Therefore, RecQ is involved in the formation of peptide targets, but targets will still form in its absence. Since it appears that the ultimate resolution of the SEI necessitates the activity of RuvC, it may be that the strand exchange intermediates that form cannot be resolved by RecG and branch migration (either mechanistically, or because the peptide is present), and must be cleaved by RuvC.

Peptide d-wrwycr interferes with DNA damage repair of naturally occurring lesions by binding three- and four-way DNA junctions (replication forks and Holliday junctions, respectively). Resolution of recombination normally occurs through multiple pathways, and how the DNA lesion will be repaired will depend on the DNA substrates, the affinity repair proteins have for that substrate, and the availability of the proteins to the substrate (Figures 6.1 and 6.2). Peptides alter the longevity of those substrates by preventing their resolution, disrupting the kinetics of DNA repair, and increasing the frequency with which newly initiated replication forks will collide with the site of the lesion. This process could facilitate the ‘amplification’ of double strand breaks that would continue as long as peptide is present to interfere with DNA repair intermediates, or the cell dies (Figure 6.3).
Section 6.2 Future Directions

This project has always had numerous interesting questions avenues to explore, and every experiment gave useful or interesting information. Thus, there are a number of directions that can be taken with this project, and indeed, some of them already have. There are several major questions that have yet to be resolved regarding the effect of the peptides on bacterial cells, and their application as tools to study repair and recombination. First, outside of DNA repair, what are the targets of the peptides, and by what mechanism do they enter cells? Second, can the Holliday junction-binding effects of the peptides be harnessed \textit{in vivo}, and can they be used to answer questions about recombination and repair in living bacterial cells? Finally, what is the potential therapeutic value of the peptides as an antibiotic?

Several independent transposon mutant hunts have been undertaken to find mutants resistant to peptide wrwycr. These screens have successfully identified a number of potential genes that, when mutated, confer resistance to the peptides. As expected from what we know from this study, some of the insertions that confer resistance occur in genes that encode proteins that are involved in DNA metabolism. Follow-up studies of some of the more difficult to explain insertions are underway, and these insertions include genes of unknown function, which may lead to systems responsible for peptide uptake as well as other targets of peptide activity.

Is the YqgF Holliday junction resolvase a major peptide target \textit{in vivo}? A gene I had intended to study as a potential target of the peptides was the gene \textit{yqgF}. The YqgF protein shows homology to the protein RuvC, and has been suggested to
be an essential Holliday junction resolvase (Aravind et al., 2000, Hidalgo et al., 2004). I attempted to knock out the \textit{yqgF} gene via recombineering (Sawitzke et al., 2007), while providing a copy of the gene in trans under control of an IPTG-inducible promoter. Initially, it appeared that I had created an IPTG-addicted strain that only survived in the presence of the inducer. Unfortunately, the strain lost its IPTG-dependence after being frozen into the strain collection. I abandoned creation of the strain after numerous failed attempts to re-create the IPTG-dependent strain. A postdoctoral researcher created the plasmid that expressed YqgF, and I tested the effect of overexpression of YqgF on peptide resistance or sensitivity. Overexpression of \textit{yqgF} had no effect on the strains’ susceptibility to peptide treatment. The essential YqgF protein could be a major target of the peptides, and it may be valuable to again try to create a strain that will allow the effect of the peptide to be tested in an \textit{yqgF} conditional knockout.

\textbf{Using peptide to trap and study intermediates in the repair of stalled replication forks \textit{in vivo}.} Bacterial recombination and DNA repair have been studied extensively by both biochemical and genetic means. Many pathways of recombination proceed through transient intermediates that are difficult to quantify and study. The pathways that form these intermediates are also difficult to study due to overlap in their functions. The Holliday junction-binding effects of the peptides can be utilized to answer lingering questions about Holliday junctions, and other recombination intermediates, in living bacterial cells. There are two methods that could be useful in the study to help find the site of a collapsed fork, and where the peptides would be actively trapping intermediates. The first assay uses an ectopic \textit{ter} site to block replication forks after the overexpression of Tus (Bidnenko et al., 2002).
Tus is a protein that binds to ter sites located on either side of the terminus of replication (Bussiere & Bastia, 1999, Sharma & Hill, 1995). Tus allows replication forks to pass in one direction, but does not allow them to traverse up the opposite arm of the chromosome, to force the replication forks to meet nearer the terminus (Bussiere & Bastia, 1999, Sharma & Hill, 1995). The effect of overexpressing Tus in a cell that has an ectopically-placed ter site in the “blocking” orientation is that replication forks will frequently collapse where the ter site was inserted into the chromosome. Two-dimensional gels of restriction fragments are probed with a sequence specific to the ter site. Holliday junctions were not found by the labs using this method (Bidnenko et al., 2006, Sharma & Hill, 1995), but HJ are extremely transient (Cassell & Segall, 2003). With our peptides, it may be possible to trap and detect the Holliday junctions. Another assay that can localize a collapsed fork utilizes a strong gyrase binding site on a plasmid, along with treatment with norfloxacin, to stabilize cleavage intermediates (Pohlhaus & Kreuzer, 2005). The assay would work the same way as the Tus/ter protocol. Using these methods in various recombination-deficient backgrounds with peptide treatment may permit dissection of the steps in the repair of collapsed replication forks, and the frequency of HJ formation in the various recombination-dependent repair pathways.

Several students are working on projects looking at the efficacy of the peptides in infection models. The peptides are capable of inhibiting the growth of Salmonella within macrophages in tissue culture (Su and Segall, in preparation). The peptides have also shown promise in preventing mice from getting a Streptococcal skin infection (Naili and Segall, unpublished results). The lab has also identified small molecules that have similar in vitro and in vivo activities as the peptides (Boldt, Ranjit,
and Segall, in preparation). These small molecules may be more potent, and therefore be more ‘druggable’, than the peptides. The peptides and small molecules may or may not become clinically-relevant therapeutics, but I feel that their role as drugs may not be as important as their role as tools of molecular biology and genetics. The field of DNA repair is becoming increasingly aware of the connections between mechanisms of repair in bacteria and those of eukaryotic cells. The peptides are valuable tools in the study of DNA repair, which has far-reaching applications into topics, such as cancer research and aging.
Chapter 7: Materials and methods

**Strains and bacterial culture methods.** Bacterial strains used in this work and their sources are listed in Table 7.1. Strains were maintained on Luria-Bertani (LB) agar plates and cultures were grown in LB broth except for peptide experiments, for which we used Mueller-Hinton (MH) broth (Becton-Dickinson).

**Peptides.** All peptides were synthesized with an amidated C-terminus and purified to >95% purity at Sigma-Genosys (St. Louis, Mo.). According to convention, peptides made of L-amino acids are designated in upper case letters, while those made of d-amino acids are designated in lower case letters. Peptide stock solutions (10 mM concentration) were maintained in 50% or 100% DMSO. Final DMSO concentrations in experimental procedures were either 0.5% or 1.0%, and DMSO at the appropriate concentration was added in the absence of peptide to control for DMSO effects.

**Growth curves and minimum inhibitory concentration (MIC) determinations.** For growth curves, overnight cultures in LB were subcultured 1:100 into 96-well microtiter plates containing the desired concentration of peptide in a final volume of 150 μl (as indicated in figure legends). Alternatively, overnight cultures in LB were subcultured 1:20 into 96-well microtiter plates containing the desired concentration of peptide in a final volume of 150 microliters (as indicated in figure legends). Optical density readings (OD$_{600}$) were taken at thirty minute intervals.
Table 7.1: Bacterial strains used in this study. The Segall lab strain designation is given after the strain designation from the lab of origin.

<table>
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<th>Designation</th>
<th>Species and strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
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<td><em>B. subtilis</em></td>
<td>wt</td>
<td>A. Grossman</td>
</tr>
<tr>
<td>JRL585/G546</td>
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<td><em>trpC2 pheA1</em>&lt;br&gt;Δ<em>spo0KBCDE</em>::spc</td>
<td>A. Grossman</td>
</tr>
<tr>
<td>YB3000/G554</td>
<td><em>B. subtilis</em></td>
<td><em>recA260</em>::erm, cam</td>
<td>R. Yasbin via A. Grossman</td>
</tr>
<tr>
<td>SL7360/G555</td>
<td><em>B. subtilis</em></td>
<td><em>recA</em>::neo</td>
<td>R. Yasbin via A. Grossman</td>
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<td>RW138/G478</td>
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<td><em>rph-1</em></td>
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<td>MG1655 <em>ΔrecA</em>::Km</td>
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</table>
Table 7.1: Bacterial strains used in this study, continued. The Segall lab strain designation is given after the strain designation from the lab of origin.

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starting at the time of inoculation using a Molecular Devices spectrophotometer SpectraMax Plus 384 microtiter plate reader. MIC determinations were performed according to the National Committee for Clinical Laboratory Standards (NCCLS) protocol for broth microdilutions was used (Ferraro, 2000). Briefly, 100 μl aliquots of MH broth containing twice (2x) the desired final peptide concentration were added to microtiter plate wells. Cultures to be tested were grown to an OD$_{540}$ of 0.8 in MH broth, and 100 μl aliquots were added to the peptide-containing MH media. This 1:2 dilution results in the desired final concentration of peptide. Microtiter plates were incubated 16-18 hours and the lowest concentration of peptide that completely inhibited growth was identified as the MIC. These experiments were repeated a minimum of four times to determine the MIC value. Viability of the strains after treatment with peptide was determined by dilution of samples in a Tris/magnesium buffer (10 mM Tris, pH 7.4, 1 mM MgCl$_2$) in 96-well plates and plating on LB agar. Plating was performed semi-quantitatively by using a replica-plating tool to transfer inoculum from the 96-well plate to the LB plates. Plates were incubated at 37°C for 18-24 hours before they were counted.

**Microscopy.** Cultures were grown in LB broth for 90 min, then treated with peptide for 90 min. Cells were pelleted and resuspended in phosphate-buffered saline (PBS) with 4′,6-diamidino-2-phenylindole (DAPI) then viewed using epifluorescence microscopy. Cells were immobilized onto slides by use of poly-L-lysine treated cover slips (except in the case of TUNEL-treated cells, which were fixed to slides by 5 min immersion in methanol). DAPI and propidium iodide (PI) stains were obtained from Sigma Chemicals (St. Louis, MO) and Molecular Probes/Invitrogen (Carlsbad, CA),
respectively. Phase contrast and epifluorescence images were taken using a Nikon Microphot Light Microscope equipped with an Olympus digital camera controlled by Magnafire software (Optronics, Goleta, CA) in the SDSU Cell Imaging Core Facility.

**Incorporation of radiolabeled nucleosides.** Cultures of E. coli MG1655 were grown overnight and subcultured 1:20 into 96-well plates containing 150 μl LB with [3H] thymidine or [3H] uridine and with peptide (at the indicated concentration; Figure 2) or nalidixic acid (200 μg ml⁻¹) or rifampicin (200 μg ml⁻¹). Cold guanosine was added to each reaction at a concentration of 250 μg ml⁻¹ to facilitate efficient uptake and incorporation of nucleosides (Boyce and Setlow, 1962; Friesen, 1968). For each time-point, a 150 μl was precipitated with an equal volume of 10% trichloroacetic acid (TCA). Pellets were collected on pre-wetted GFC glass fiber filters (Whatman) and washed with 5% TCA. Counts per minute ([3H]) were determined in a scintillation counter after immersion of the glass fiber filters in Scinti-Safe scintillation fluid (Fisher). Each condition was tested in triplicate on two different days.

**Assays of SOS induction.** ELISAs to detect the amount of β-galactosidase were performed in 96-well microtiter plates. First, overnight cultures were diluted 1:20 in LB broth and grown for 90 minutes, at which time either peptide or MMC (Sigma Chemicals, St. Louis, Mo.), as a positive control, were added. After 90 minutes treatment, cultures were lysed with lysozyme on ice and cell lysates were adsorbed to medium-bind EIA plates (Costar) that had been coated with monoclonal mouse anti-β-galactosidase IgG. After washing and blocking with BSA, polyclonal rabbit anti-β-galactosidase IgG was added. After a second wash, goat anti-rabbit IgG HRP conjugate was added and the wells were washed again. The colorimetric substrate 5,
5′- tetramethylbenzidine (TMB), was used to detect the β-galactosidase-antibody complex. All binding and coating steps were performed in PBS. All antibodies and the TMB substrate were purchased from Sigma Chemical, St. Louis, Mo. Portions of the same cell lysates used in the ELISA were assayed for total protein content using the Bradford colorimetric assay reagent (BioRad). Values were calculated as the fold increase in ELISA signal at OD₄₅₀ per microgram of total protein in the cell lysate.

**Direct assay for DNA breaks.** The TUNEL assay, commonly used to measure DNA strand breaks in eukaryotic cells, was adapted by Rohwer for use with bacterial cells (Rohwer and Azam, 2000). Overnight cultures in LB broth were subcultured 1:20 into fresh media and grown 90 minutes at 37°C. Peptides and/or other DNA damaging agents were added at the concentrations indicated in the figure legends, and cultures were incubated for an additional 90 minutes (except the timecourse study, where reactions were stopped by fixation after 5, 15, 30, or 60 minutes). Immediately after incubation the cultures were pelleted, fixed with 4% paraformaldehyde and assayed using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Germany). Cells were counterstained in suspension with 0.5 μg ml⁻¹ propidium iodide (PI), a DNA-dependent fluorescent dye. After treatment, TUNEL-positive cells were quantitated using flow cytometry. For flow cytometry, between 7000 and 50000 cells were counted and the percentage of TUNEL-positive cells was calculated as a fraction of the PI-positive cells. For microscopy, cells were visualized using a Nikon Microphot Light Microscope. At least 10 representative fields of each treatment were photographed for counting and the percentage of labeled cells of the total cells in phase contrast fields was calculated.

**Flow cytometry of peptide-treated E. coli.** To study anucleate cell formation
by peptide treatment, overnight cultures in LB broth were subcultured 1:20 into fresh media and grown 90 minutes. Peptides were added at the indicated concentrations, and cultures were incubated for an additional 90 minutes at 37°C. Cell pellets were stained with 10 ml of 2 ng ml⁻¹ PicoGreen, a DNA-specific fluorophore, and then resuspended in 1 ml of 0.25 ng ml⁻¹ FM 4-64, a membrane-specific fluorophore (Molecular Probes, Eugene, OR). Flow cytometry of PicoGreen/FM 4-64 stained cells and TUNEL/PI cells were performed on a BD FACSARia desktop cell sorter (Becton-Dickinson, San Jose, Ca). The specificity of the PicoGreen dye for DNA was verified by comparing the intensity of staining of parent cells and DNA-less minicells by confocal fluorescence microscopy (Figure 7.1). A 70 μm nozzle was used during data collection for all analyses, although cells were not sorted. Data acquisition and analysis was performed using FACSDiva software (Becton-Dickinson, San Jose, Ca).

In addition to fluorescence parameters, forward scatter (FSC) and side scatter (SSC) parameters were collected in all flow cytometry experiments performed in this study. FSC can be used to give an estimation of cell size, but these measurements are affected by many parameters including absorption, wavelength, and refractive index so there are limitations to making absolute correlations (Shapiro, 2003). Side scatter (SSC), or orthogonal scatter, has been used as a measurement of granularity to distinguish various immune cell types, but has also been suggested as a possible measure of total protein content (Shapiro, 2003). Dot plots reveal peptide-dependent changes in the FSC vs. SSC population, suggesting that we are able to see abnormalities in cell morphology, such as filaments, using flow cytometry.
Figure 7.1: Confocal microscopy of PicoGreen stained minicells. 

E. coli pTAC ftsZ overexpression strain was induced to create minicells, stained with PicoGreen I, and visualized by confocal microscopy. Left panel: Transmitted light image showing mother E. coli cells and minicells (indicated by black arrows). Right panel: fluorescence channel shows staining of the mother cells, but the minicells (white arrows) do not. Photos are from the center of a Z-stack taken of this field.
Analysis of chromosomal fragmentation by pulse field gel electrophoresis. Overnight cultures of the bacteria to be tested were grown in MH broth. The cultures were diluted 1:100 into 1 ml MH broth (a milliliter of culture for each treatment, typically 6), and grown at 37°C for 2 hours with shaking. Treatments were added (peptide and/or control compound), and cultures replaced to 37°C for 3 hours with shaking. To adjust the cell density, 100 μl of each treatment was diluted in 1 ml MH broth (1:10 dilution) and OD600 was taken. The remaining 900 μl was spun down at 10K rpm, 10 minutes. Pellets were resuspended in resuspension solution (10 mM Tris (pH 7.2), 20 mM NaCl, 10 mM EDTA) to achieve an OD600 of 1.3. To imbed treated bacteria into agarose plugs, 1.5% PFGE certified agarose (Bio-Rad) was prepared in sterile ultrapure water, and equal volumes of molten, cooled agarose and bacterial suspension were combined (final agarose concentration in plugs = 0.75%) and pipetted into plug molds. Plugs were allowed to harden at room temp or 4°C for 10-20 minutes. Plugs were removed from molds to 15ml conical tubes (10 plugs of one treatment per tube) for cell lysis and protein inactivation. To each tube of plugs, 4 ml of Cell Lysis Solution (10mM Tris HCl (pH 7.2), 50 mM NaCl, 100 mM EDTA, 0.2% Sodium dodecyl sulfate, 0.5% N-Lauryl Sarcosine) was added, and incubated in a 65 degrees C waterbath for 1 hour with periodic shaking. Cell Lysis Solution was decanted and 4 ml Proteinase K solution (1 mg ml⁻¹ in 100 mM EDTA, 0.2% Sodium dodecyl sulfate, and 1% N-Lauryl sarcosine) was added. Proteinase K treatment was carried out at 42 degrees C for 48-72 hours with periodic shaking. The Proteinase K buffer was discarded by decanting, and added 3 ml of 1 mM PMSF (in 20 mM tris Hcl (pH 8), 50 mM EDTA) to the plugs (to destroy Proteinase K) for 1 hour at room temperature with gentle shaking. Plugs were washed twice in 4 ml wash
solution (20 mM Tris HCl (pH 8), 50 mM EDTA) for 10 minutes at room temperature) with shaking; the wash buffer was decanted between washes. Plugs were washed twice in 4 ml storage solution (2 mM Tris HCl (pH 8), 5 mM EDTA) for 10 minutes at room temperature with shaking, then stored at 4°C in 4ml storage solution.

Digestion of agarose imbedded DNA. For each condition, plugs were removed from storage and cut in half (~5 mm square) each half was moved to a separate microfuge tube (one half was used for I-CeuI digestion, the other left undigested). One ml sterile water was added to each plug, which was then placed at 37°C for 20 min. Water was aspirated from the plugs to be digested and, to each, 200 μl 2x New England Biolabs (NEB) restriction buffer 4 (the recommended buffer for I-CeuI digestion) was added. The plugs were incubated 10 minutes at room temperature before aspirating the buffer. After washing, 200 μl 1x NEB buffer 4 with 1x BSA and 10U I-CeuI was added to each plug that was to be digested. Reactions were incubated at 37°C for 1 hour. Reaction buffer was removed from the digests and 1 ml sterile water was added and left at room temperature for 10 minutes. Digested plugs were run the same day as digestion.

Running the gel. Water was aspirated from all plugs (I-CeuI digested plugs and undigested plugs). Running buffer (500 μl of 0.5X TBE) was added to the plugs and left at room temperature until needed. The 1% PFGE certified agarose (Bio-Rad) was prepared in 0.5X TBE and set aside to cool. Each plug (5x5 mm) was placed on a tooth of the comb and the comb was placed in a casting tray, with ~1 mm space underneath plugs. Two ladders were run, a yeast chromosome (YC) ladder (NEB) and a lambda (λ) ladder (NEB), in duplicate on either side of the gel. The molten,
cooled, agarose was then poured to a level ~1 mm above the plugs on the comb (yielding a ~7 mm thick gel). The gel/platform was placed in the recess of the PFGE rig and 2.2 L of 0.5X TBE were added. All gels were electrophoresed at 14°C for 22 hours at 6.0 V cm⁻¹ with a 120° included angle and 50-90 second switch time that had a linear ramp, unless noted otherwise. Gels were stained with SYBR Green I dye (Invitrogen, Carlsbad, CA), and visualized by scanning on a Molecular Devices Storm Imager. Gels were quantified using the ImageQuant software (Molecular Devices).

**Viability after peptide treatment.** Overnight cultures in LB were subcultured 1:100 into 96-well microtiter plates containing the desired concentration of peptide in a final volume of 150 μl. Viable counts of the strains after treatment with peptide was determined by dilution of samples in a Tris/magnesium buffer (10 mM Tris, pH 7.4, 1 mM MgCl₂) in 96-well plates and plating on LB agar. Plating was performed semi-quantitatively by using a replica-plating tool to transfer inoculum from the 96-well plate to the LB plates at the time-points indicated. Plates were incubated at 37°C for 18-24 hours before they were counted. To monitor culture characteristics, optical density readings (OD_{600}) were taken at thirty minute intervals starting at the time of inoculation using a Molecular Devices spectrophotometer SpectraMax Plus 384 microtiter plate reader that took reading every half hour, after 90 seconds of shaking.
**Figure A.1:** Pulsed field gel electrophoresis of *Salmonella* LT2 recA chromosomal DNA. Cultures were treated as indicated above the gels. A) Chromosomal DNA B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. \( \lambda = \) lambda ladder.
| DMSO | 1 µg ml⁻¹ MMC | 32 µM wrrwyr | 64 µM wrrwyr | Kb | ~1800 | ~1100 | 945 | 915 | 680 | 610 | 555 | 450 | 375 | 295 | 225 | 194 | 97 | 48.5 |
| YC λ | LT2 | LT2 lexA(ind-) | LT2 lexA(ind-) | λ | YC | + | + | + | + | + | + | + | + | + | + | + | + | + |

**Figure A.2:** Pulsed field gel electrophoresis of *Salmonella* LT2 lexA3(ind-) chromosomal DNA. Cultures were treated as indicated above the gels. A) Chromosomal DNA B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. λ = lambda ladder.
Figure A.3: Pulsed field gel electrophoresis of Salmonella LT2 recD chromosomal DNA. Cultures were treated as indicated above the gels. A) Chromosomal DNA. B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. λ = lambda ladder.
Figure A.4: Pulsed field gel electrophoresis of *Salmonella* LT2 recB chromosomal DNA. Cultures were treated as indicated above the gels. A) Chromosomal DNA B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. λ = lambda ladder.
Figure A.5: Pulsed field gel electrophoresis of *Salmonella* LT2 recABC chromosomal DNA. Cultures were treated as indicated above the gels. A) Chromosomal DNA B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. λ = lambda ladder.
Figure A.6: Pulsed field gel electrophoresis of *Salmonella* LT2 recF chromosomal DNA. Cultures were treated as indicated above the gels. A) Chromosomal DNA, B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder, λ = lambda ladder.
Figure A.7: Pulsed field gel electrophoresis of *Salmonella* LT2 recJ chromosomal DNA. Cultures were treated as indicated above the gels. A) Chromosomal DNA B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. λ = lambda ladder.
**Figure A.8**: Pulsed field gel electrophoresis of *Salmonella* LT2 recBCF chromosomal DNA. Cultures were treated as indicated above the gels. A) Chromosomal DNA B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. \( \lambda \) = lambda ladder.
### Figure A.9: Pulsed field gel electrophoresis of *Salmonella* LT2 recBJ chromosomal DNA.

Cultures were treated as indicated above the gels. A) Chromosomal DNA B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. λ = lambda ladder.
Figure A.10: Pulsed field gel electrophoresis of *Salmonella* LT2 recAF chromosomal DNA. Cultures were treated as indicated above the gels. A) Chromosomal DNA B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. λ = lambda ladder.
**Figure A.11:** Pulsed field gel electrophoresis of *Salmonella* LT2 recG, ruvAB, and ruvC chromosomal DNA. Cultures were treated as indicated above the gels.

A) Chromosomal DNA. B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. \( \lambda \) = lambda ladder.
**Figure A.12:** Pulsed field gel electrophoresis of *Salmonella* LT2 recG ruvAB chromosomal DNA. Cultures were treated as indicated above the gels. A) Chromosomal DNA. B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. λ = lambda ladder.
Figure A.13: Pulsed field gel electrophoresis of *Salmonella* LT2 recG ruvC chromosomal DNA. Cultures were treated as indicated above the gels. A) Chromosomal DNA. B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. λ = lambda ladder.
Figure A.14: Pulsed field gel electrophoresis of *Salmonella* LT2 recQ, uvrD, and recQ uvrD chromosomal DNA. Cultures were treated as indicated above the gels.

A) Chromosomal DNA. B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. $\lambda$ = lambda ladder.
Figure A.15: Pulsed field gel electrophoresis of *Salmonella* LT2 recQ ruvC chromosomal DNA. Cultures were treated as indicated above the gels. A) Chromosomal DNA. B) I-CeuI digested chromosomal DNA. The size of standard bands in kilobases are labeled to the left of the gel. YC = yeast chromosome ladder. λ = lambda ladder.
Figure A.16: Viability analysis of Salmonella recA, lexA(ind-), recD, recBC, and recABC strains. Photographs of LB plates used for dilution series have dilutions labeled across the top, and treatment conditions labeled down the side. From top to bottom, panels are wild-type LT2, recA, lexA(ind-), recD, recBC and recABC. Timecourse of treatment is represented left to right. Time '−2' is the time of subculture, and time '0' is the point at which the cultures were treated. Time '3' and '22' are three and 22 hours after treatment. Photographs represent data from triplicate independent cultures from at least 2 different days (n of at least 6, LT2 n=54).
Figure A.17: Viability analysis of *Salmonella* recB, recF, recJ, recBC recF, recB recJ, and recA recF strains. Photographs of LB plates used for dilution series have dilutions labeled across the top, and treatment conditions labeled down the side. From top to bottom, panels are wild-type LT2, recB, recF, recJ, recBC recF, recB recJ, and recA recF. Timecourse of treatment is represented left to right. Time ‘-2’ is the time of subculture, and time ‘0’ is the point at which the cultures were treated. Time ‘3’ and ‘22’ are three and 22 hours after treatment. Photographs represent data from triplicate independent cultures from at least 2 different days (n of at least 6, LT2 n=54)
Figure A.18: Viability analysis of *Salmonella* recG, ruvAB, ruvC, recG ruvAB, and recG ruvC strains. Photographs of LB plates used for dilution series have dilutions labeled across the top, and treatment conditions labeled down the side. From top to bottom, panels are wild-type LT2, recG, ruvAB, ruvC, recG ruvAB, and recG ruvC. Timecourse of treatment is represented left to right. Time ‘-2’ is the time of subculture, and time ‘0’ is the point at which the cultures were treated. Time ‘3’ and ‘22’ are three and 22 hours after treatment. Photographs represent data from triplicate independent cultures from at least 2 different days (n of at least 6, LT2 n=54).
**Figure A.19:** Viability analysis of *Salmonella* recQ, uvrD, ruvC, recQ uvrD, and recQ ruvC strains. Photographs of LB plates used for dilution series have dilutions labeled across the top, and treatment conditions labeled down the side. From top to bottom, panels are wild-type LT2, recQ, uvrD, ruvC, recQ uvrD, and recQ ruvC. Timecourse of treatment is represented left to right. Time ‘-2’ is the time of subculture, and time ‘0’ is the point at which the cultures were treated. Time ‘3’ and ‘22’ are three and 22 hours after treatment. Photographs represent data from independent triplicate cultures from at least 2 different days (n of at least 6, LT2 n=54)
Figure A.20: Viability analysis of Salmonella recG, ruvC and recA strains. Photographs of LB plates used for dilution series have dilutions labeled across the top, and treatment conditions labeled down the side. From top to bottom, panels are wild-type LT2, recG, ruvC and recA. Timecourse of treatment is represented left to right. Time ‘-2’ is the time of subculture, and time ‘0’ is the point at which the cultures were treated. Time ‘3’ and ‘22’ are three and 22 hours after treatment. Units for all MMC and peptide concentrations are µg/ml and µM, respectively. Photographs represent data from triplicate independent cultures from at least 2 different days (n of at least 6).
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