A Genetic Screen for the Identification of Mutants Hypersensitive to 5-Azacytidine

by

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Thesis submitted in partial fulfillment of
the requirements for the degree of Master of Science in the Department of
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ABSTRACT

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Abstract

A DNA-protein crosslink is a covalent bond between DNA and a protein. It is a type of DNA damage that is relatively understudied. This study reports on the identification of a set of transposon mutants sensitive to 5-azacytidine, a DNA–protein crosslink induction agent that induces a crosslink between DNA and a DNA methyltransferase protein. The screen showed that certain recombination, DNA repair, and tRNA modification mutants are hypersensitive to 5-azacytidine. These included the recombination recA, recC, and recG mutants. Since the recombination mutants consistently show high sensitivity to aza-C, it suggests a role for recombination in DNA-protein crosslink repair. Western blots for the levels of methyltransferase protein showed that mutants have similar levels of methyltransferase protein compared to wild type cells, arguing that the mutants’ hypersensitivity to aza-C is not because of increased methyltransferase levels. Western blots for the levels of SsrA tagging in the presence of 5-azacytidine showed that the tRNA modification transposon mutants miaA, mmmE, and mmmG are all defective in SsrA tagging, which likely explains their hypersensitivity. The SsrA tag Western blots also unexpectedly showed that the recA transposon mutant had reduced levels of SsrA tagging when treated with 5-azacytidine.
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1. Introduction

Endogenous and exogenous DNA damaging agents render the bacterial chromosome susceptible to DNA damage. One type of understudied DNA lesion is DNA-protein crosslinks (DPCs). Less archetypal than other types of lesions, DPCs are characterized by a protein covalently bound to DNA. They have many causes, and include a broad range of inducers such as chemotherapeutic agents, carcinogens, and metals (Barker et al., 2005). Interest has grown in DPCs as they have been proposed to contribute to the toxicity of some chemotherapeutic agents, such as cisplatin and mitomycin C, though these chemicals cause predominantly DNA interstrand crosslinks (3). Paradoxically, carcinogens such as arsenic and nickel compounds also cause DPC formation (Barker et al., 2005). Formaldehyde is another chemical used in previous research to generate DPCs (Quievryn & Zhitkovich, 2000, Salem et al., 2009). A type of DPC that can occur without an exogenous agent is a covalently trapped enzyme intermediate. One example is when a DNA glycosylase becomes trapped at an abasic site (Barker et al., 2005).

The variety of DPCs presents a difficulty in studying the mode of repair. They occur randomly in the chromosome, so they are not site-specific. In addition to the random placement of DPCs, different types of proteins are known to form DPCs. Actin, histones, and methyltransferase proteins all have been shown to crosslink to DNA (Coulombe et al., 1999, O'Connor & Fox, 1989). It is unclear whether any protein can be bound in a DPC, or only DNA binding proteins. Though DPCs have many causes and categories, it is still unknown how the lesions are resolved in the cell.
The drug 5-azacytidine (aza-C) was originally synthesized as an anti-metabolite to treat leukemia (Karon et al., 1973). It is a cytidine analog that induces sequence specific and protein specific DPCs. When 5-azaC is incorporated into E. coli DNA, the endogenous DNA cytosine methyltransferase activity (Dcm) is inhibited both in vivo and in vitro (Friedman, 1981, Creusot et al., 1982). The inhibition of the DNA cytosine methyltransferase is caused by the formation of a covalent bond between the DNA and protein at the carbon-6 position of 5-azaC (Santi et al., 1984). Over-expressing the endogenous E. coli methyltransferase protein on a plasmid increases sensitivity of cells to 5-azaC (Bhagwat & Roberts, 1987). However, deleting the dcm gene does not confer much resistance to 5-azaC. This may indicate that the amount of methyltransferase expressed by the chromosomal dcm gene is not high enough for a knock-out to confer resistance. The 5-azaC dependent inhibition of methyltransferase activity extends to other cytidine methyltransferase proteins such as the EcoRII, and the HpaII methyltransferase proteins (Friedman, 1981). The EcoRII and the HpaII proteins methylate specific sequences in the chromosome.

During normal methylation of cytosine, the methyltransferase attacks the carbon-6 position of the pyrimidine ring and abstracts a proton from the carbon-5 position. The methyl donor, S-adenosyl methionine then donates a methyl group to the carbon-6 position, allowing the methyltransferase protein to dissociate. When aza-C replaces cytosine in the recognition sequence, the enzyme DNA-bond becomes irreversible (Santi et al., 1983, Santi et al., 1984). The drug aza-C is used in our study as a method to generate in vivo DPCs.
The use of the nucleoside analog aza-C in experiments has its advantages. Importantly, it is capable of forming a sequence specific, as well as a protein specific DPC with a cytidine methyltransferase. This is in contrast with other chemicals like formaldehyde, which creates bonds between amino side chains of proteins and DNA bases. Another advantage is that since aza-C base pairs like cytidine, it can maintain the genetic code while creating sites of the DPC in the chromosome.

Azacytidine incorporation causes secondary effects that complicate its use as a chemical DPC inducer. Protein synthesis could be inhibited since aza-C incorporates into RNA and DNA (Jones et al., 1982, Doskocil & Sorm, 1970). Research has also suggested that aza-C may interfere with the synthesis of ribosomes (Paces et al., 1968). Stability of the DNA-bound protein bond is also a concern in our experiments, but studies have shown that the covalent bond between aza-C and methyltransferase is still intact after three days incubation, or treatment with sodium dodecyl sulfate (Santi et al., 1984). However, aza-C incorporated into DNA is capable of a ring opening reaction that formylates the methyltransferase and expels the carbon-6 of the pyrimidine ring of aza-C (Walsh, 1984). This may lead to lesions in cytosine that contribute to cells’ sensitivity to aza-C.
Figure 1- Models of DPC Repair a) Nucleotide excision repair b) Proteolysis followed by nucleotide excision repair c) Endonucleolytic cleavage followed by recombination

There is no known general repair mechanism for DPCs, though there are three repair pathways suggested to resolve DPCs (Figure 1). There is evidence supporting all three models, and the variety of DPCs suggest more than one repair pathway is potentially capable of restoring genome integrity. Nucleotide excision repair is proposed to act in DPC repair because of its role removing bulky lesions from the DNA (Minko et al., 2002, Minko et al., 2005, Nakano et al., 2007). A 2009 report by Salem et al. showed that mutants of the genes \textit{uvrA}, \textit{uvrB}, \textit{uvrC}, and \textit{uvrD} are all sensitive to formaldehyde compared to wild type cells based on cell survival assays (Salem et al., 2009). However, only \textit{uvrD} showed sensitivity to 5-azaC. This is attributed to the 12-14 kDa size limitation of the proteins that NER can process; methyltransferase proteins exceed that size (Salem et al., 2009). Additionally, NER-deficient XP-A and XP-F human cells are
not particularly sensitive to exposure to formaldehyde, another DPC inducing agent (Speit et al., 2000).

Proteolysis of the DNA-bound protein, followed by nucleotide excision repair, has been suggested as another model of DPC repair (Minko et al., 2005, Reardon & Sancar, 2006). A proteolysis step could also work in conjunction with nucleotide excision repair since NER is unlikely to process lesions as large as some DPCs. Supporting this model is the observation that in human cell lines, inhibition of proteosome activity led to a persistence of DPCs in normal and XP-A cells exposed to formaldehyde (Quievryn & Zhitkovich, 2000). Additionally, DNA topoisomerase II cleavage complexes, a type of DPC, is also degraded by the 26 S proteosome pathway (Mao et al., 2001).

Previous research using aza-C tested the sensitivity of repair deficient mutants to the drug including recombination mutants and repair deficient mutants (Bhagwat & Roberts, 1987, Ide et al., 2008, Salem et al., 2009). It was shown that while recA mutants are sensitive to aza-C, in a background strain lacking overexpressing methyltransferase protein, recG and recF mutants were not sensitive to aza-C (Bhagwat & Roberts, 1987, Ide et al., 2008).

The research using DPC-inducing chemicals does not differentiate whether sensitivity to the chemicals is because a protein is involved in direct processing of the DPC, or downstream repair. Downstream damage could occur if a DPC blocks a replication fork (Kuo et al., 2007). If the fork is not restarted, a double strand break can form (Michel et al., 1997). The distinction between repair of DPCs, and proteins that may play a role in repair of double strand breaks is important, since some genes implicated in DPC repair are just as likely to play a role in downstream processing. These include the
recombination genes *recA* and *recBCD*. The secondary effects from aza-C incorporation could also contribute to sensitivity to the drug, potentially complicating the interpretation of results. Sensitivity to DPC-inducing agents should not be taken to indicate a defect in DPC repair.

In this study, we report on a transposon mutagenesis screen designed to identify mutants hypersensitive to aza-C. We report that the screen identified knockout mutations in several DNA repair genes, recombination genes, and tRNA modification genes as sensitive to aza-C. We show that the mutants hypersensitive to aza-C were not sensitive because of an increased level of methyltransferase expression. We also show that there are three tRNA modification mutants defective in SsrA tagging, leading us to posit that a defect in tmRNA modifications may lead to a defect in SsrA tagging, causing sensitivity to aza-C.
2. Materials and Methods

2.1 Escherichia coli Strains

The screen was done using a derivative of the E. coli strain ER1793 (F' fluA2
\(\Delta(lacZ)\alpha1\) glnV44 e14 (McrA) trp-31 his-1 rpsL104 xyl-7 mtl-2 metB1 \(\Delta(mcrC-
mrr)114::IS10\), from New England Biolabs. A sulA knockout was introduced from the
Keio collection, to prevent inhibition of cell division from the SOS response of E. coli
(Baba et al., 2006). The Keio collection is a series of in frame single gene knockout
mutants in E. coli. Each gene deletion contains the kanamycin resistance marker (Baba et
al., 2006). In our background strain the kanamycin resistance cassette was removed,
following the protocol reported by Baba et al by using the FLP recombinase (Baba et al.,
2006). This new strain was named HK21 (ER1793 dinD::lacZ sulA\(^{-}\) no kan\(^{R}\)). The
Western blots were done using the HK22 strain (ER1793 sulA\(^{-}\) no kan\(^{R}\)) after the original
mutant isolates were moved to the HK22 strain using phage P1-mediated transduction.

2.2 Plasmids

Two plasmids were used in this study to express the EcoRII methyltransferase,
pR215 and pBAD-M. EcoRII. Dr. Ashok Bhagwat provided the pR215 plasmid. The
pR215 plasmid is a pACYC184-derived plasmid containing the tetracycline resistance
marker and the EcoRII methyltransferase under control of its own promoter (Bhagwat et
al., 1990). The pBAD plasmid encodes chloramphenical resistance, and the M. EcoRII
expression is under control of an arabinose inducible promoter (Guzman et al., 1995).
Expression of the EcoRII methyltransferase is repressed with glucose (0.2%) and
activated with arabinose (0.05%).
2.3 Isolation of transposon mutants

The transposon mutagenesis screen was performed using the EZ-Tn5™<KAN-2>Tnp Transposome™ Kit from Epicentre. The transposase-transposon complex was electroporated into the HK21 cells (New England Biolabs). The transformants were subsequently resuspended in Luria- Bertani (LB) growth medium and spotted onto LB agar plates with and without 5 µg/mL aza-C (Sigma Aldrich). The plates also contained kanamycin to select for the incorporation of the transposon.

2.4 Determining minimum inhibitory concentration

As a tertiary screen for aza-C sensitivity, we determined the minimum inhibitory concentration of aza-C for each mutant and compared them to wild type cells. Mutants were grown overnight in liquid media and diluted to OD<sub>560</sub> of 0.5. The cell solutions were then diluted 1:2000 in 2 ml of LB, with the addition of 12.5 µg/ml of tetracycline to select for the pR215. Then 75 ul of cell solution was then added to 75 ul of serial dilutions of aza-C in 96-well plates. The highest concentration of drug was 40 µg/ml aza-C, and each subsequent dilution was 2/3 the concentration of the previous higher concentration. The cells were grown for 18 hours and the OD<sub>590</sub> was measured.

2.5 Spot assays

To differentiate varying levels of drug sensitivity, spot assays were also done. Cell strains were grown overnight at 37°C and then diluted to an OD<sub>560</sub> of 0.5. Serial dilutions were done in 10-fold increments, of which 5 ul of each dilution were spotted onto LB agar plates containing 5 µg/ml aza-C and grown overnight at 37°C.
2.6 Growth curves to confirm aza-C sensitivity

To measure sensitivity to aza-C with growth curves, the ELx808™ Absorbance Microplate Reader was used. The cells were grown in liquid LB media overnight at 37°C and diluted to an OD$_{630}$ of 0.5. The cell solutions were diluted 1:2000 in 2 ml of LB, with the addition of 12.5 µg/ml of tetracycline to select for the pR215 plasmid. Then 75 µl of cell solution was added to 75 µl of serial dilutions of aza-C in 96-well plates. The highest level of drug was 4 µg/ml of aza-C. The aza-C was diluted two-fold in serial dilutions. The cells were grown overnight in the plate reader at 37°C with constant shaking. The absorbance at 630 nm was measured every 15 minutes for 18 hours.

2.7 Cell sample collection for Western blots

The Western blots were completed in the HK22 pBAD- EcoRII background. To collect cell samples for Western blots, the cell strains were grown overnight in liquid LB media at 37°C in the presence of 0.2% glucose. The cultures were then diluted to an OD$_{560}$ of 0.1 and grown until an OD of 0.5 in 10 ml cultures in the presence of 0.2% glucose to repress expression of the methyltransferase protein. Cultures with aza-C treatment contained 0.05 mg/ml of aza-C, which was added to each culture from a stock solution. Each culture was then spun down in a Sorvall centrifuge for 10 minutes to harvest the cells. The cells were washed with LB and then respun for 2 minutes. The pellet was resuspended in 10 ml LB with 0.05% arabinose to induce the methyltransferase and the cells were grown for an hour at 37 °C with shaking. Cell samples equivalent to 2 ml of 0.5 OD$_{560}$ were harvested, and incubated in an ethanol and dry ice bath for at least 15 minutes to freeze and were stored at -20 °C.
2.8 Western blots

The *EcoRII* methyltransferase Western blots were done with samples without 5-azacytidine treatment. The frozen cell pellets collected were thawed at room temperature and resuspended in 25 ul of dH2O and 25 ul of sample buffer, and boiled for 5 minutes in a water bath. Following cell lysis, 15 ul of each sample were run on a 7.5% Tris-HCl gel for approximately 2 hours in 25 mM Tris-Glycine buffer with 0.1% SDS. The portion of the gel that is over 75 kDa was cut off and stained with Coomassie blue dye to act as a loading control. The remaining portion of the gel was transferred to a nitrocellulose membrane (Protran® BA 85; Whatman®) for 60 minutes at 12 V, using a Genie Blotter transfer device (Idea Scientific Co.). The blot was blocked for 1 hour in a 20% non-fat milk powder (Biorad) solution in Tris-buffered saline (TBS). The membrane was incubated overnight at 4 °C with polyclonal *M.EcoRII* primary antibody and 0.1% Tween, and then washed three times with TBS buffer for 10 minute intervals. The membrane was incubated with secondary antibody, IRDye 800CW-conjugated goat anti-rabbit IgG (LI-COR®), for half an hour, and the washes were repeated. After air-drying, the membrane was scanned on The Odyssey® Imaging System (LI-COR® Biosciences), and quantified using the Odyssey® software.

The SsrA tag western blots were performed in the same manner as the methyltransferase blot, but the primary antibody was against the SsrA tag, provided by Dr. Tania Baker (MIT).
3. Results

3.1 Transposon mutants hypersensitive to 5-azacytidine

To identify genes and pathways potentially involved in a DPC repair pathway, a genetic screen was undertaken. The screen was performed using an *E. coli* strain containing the pR215 plasmid overexpressing the *EcoRII* methyltransferase protein to increase the likelihood of DPC formation in the presence of aza-C, and thus isolate mutants defective in DPC repair. The background strain also contains a *sulA* deletion to prevent cell filamentation in the presence of aza-C. The mutants were first generated by electroporating the EZ-Tn5™ <KAN-2> transposon™ (Epicentre) into the HK21 pR215 strain, and growing the transformants on LB agar plates with kanamycin to select for transposon incorporation.

The original pool of 25,861 isolates was narrowed down to a subset of hypersensitive mutants. The transposon mutants were tested for sensitivity to aza-C with a primary screen by resuspending individual colonies in 100 ul of LB liquid media, and spotting approximately 3 ul of the suspension onto LB agar plates containing 5 ug/ml aza-C. The mutants with a growth defect in the presence of aza-C were subjected to a secondary screen: the mutants were streaked onto LB agar plates containing aza-C. The mutants that still showed sensitivity were subjected to a liquid assay to compare the minimum inhibitory concentrations of each mutant and the wild type cells for aza-C in a tertiary screen. Another test for aza-C sensitivity was spot assays on LB agar plates that contained aza-C. Each cell strain was grown to an OD$_{560}$ of 0.5 and serial dilutions of
each culture were spotted onto the plates. After the spot assays there were 24 mutants remaining that still showed sensitivity to azac-C.
Figure 2- Sensitivity assays of wild type HK21 pR215 cells and two mutants: HK21 recC::Tn5 pR215 and HK21 recG::Tn5 pR215 cells. Graphs show that in the presence of aza-C, the mutants show greater growth inhibition than wild type cells.

The 24 mutants still sensitive after the spot assays were tested with overnight growth curves (Figure 2). The growth curves were done in 96-well plates using the Biotek ELx808™ Absorbance Microplate Reader. The cells were grown for 18 hours at 37°C in the presence of varying levels of aza-C. Figure 2 shows the higher levels of sensitivity of the recC::Tn5 and recG::Tn5 mutants compared to the wild type HK21 pR215 cells.

The mutants were identified using genomic isolation and a primer complementary to the transposon sequence. The mutants showing hypersensitivity to aza-C compared to
the wild type background strain are summarized in Table 1. The screen has identified genes including DNA repair genes, recombination genes, and tRNA modification genes.

**Table 1- List of genes identified from the transposon mutagenesis screen**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>recA</td>
<td>Recombination</td>
</tr>
<tr>
<td>recC</td>
<td>Recombination</td>
</tr>
<tr>
<td>recG</td>
<td>Recombination</td>
</tr>
<tr>
<td>uvrD</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>dinG</td>
<td>Helicase</td>
</tr>
<tr>
<td>ssrA</td>
<td>tmRNA</td>
</tr>
<tr>
<td>hflC</td>
<td>Protease</td>
</tr>
<tr>
<td>ftsI</td>
<td>Cell division protein</td>
</tr>
<tr>
<td>ftsK</td>
<td>Cell division protein</td>
</tr>
<tr>
<td>miaA</td>
<td>tRNA modification</td>
</tr>
<tr>
<td>mnmE</td>
<td>tRNA modification</td>
</tr>
<tr>
<td>mnmG</td>
<td>tRNA modification</td>
</tr>
<tr>
<td>tmfH</td>
<td>tRNA modification</td>
</tr>
<tr>
<td>mdtG</td>
<td>Multidrug resistance efflux transporter</td>
</tr>
<tr>
<td>pmc</td>
<td>Glutamine methyltransferase</td>
</tr>
<tr>
<td>dnaJ</td>
<td>Chaperone</td>
</tr>
<tr>
<td>sanA</td>
<td>Confers vancomycin resistance</td>
</tr>
<tr>
<td>sdaC</td>
<td>Serine transport</td>
</tr>
<tr>
<td>ynjJ</td>
<td>Unknown</td>
</tr>
<tr>
<td>yaaW</td>
<td>Unknown</td>
</tr>
<tr>
<td>ybaB</td>
<td>Unknown</td>
</tr>
<tr>
<td>pth</td>
<td>Peptidyl-tRNA hydrolase</td>
</tr>
<tr>
<td>rhsC</td>
<td>Repetitive gene</td>
</tr>
<tr>
<td>zur</td>
<td>Transcriptional regulator</td>
</tr>
</tbody>
</table>

The transposon mutants in the HK21 pR215 strain were transferred to the HK22 pBAD- *M. EcoRII* strain using phage P1-mediated transduction. The pBAD- *M. EcoRII* plasmid is induced by arabinose, and repressed by glucose (Guzman *et al.*, 1995). Spot assays were done with the mutants in the HK22 pBAD- *M. EcoRII* background strain. The mutants still showed sensitivity to aza-C, and the sensitivity was dependent on the presence of arabinose (Figure 3). Figure 3 shows the dependence of the *ynjJ::Tn5* and *miaA::Tn5* mutants on arabinose for aza-C sensitivity. However, the *recA::Tn5* and the *recC::Tn5* mutants are sensitive to aza-C even in the presence of glucose, which should repress the expression of the methyltransferase plasmid, pBAD- *M. EcoRII* (Figure 3).
The sensitivity is increased in the presence of arabinose showing that increasing methyltransferase protein increases sensitivity to aza-C. The sensitivity in the plates containing glucose and aza-C is likely because of the expression of endogenous DNA methyltransferase protein, arguing that the recA::Tn5 and recC::Tn5 mutants are more sensitive than the ynlI::Tn5 and the miaA::Tn5 mutants.

![Image of spot assay](image)

**Figure 3-** Spot assay of HK22 pBAD- M. EcoRII mutants to test sensitivity to aza-C

### 3.2 Transposon mutants show similar levels of methyltransferase protein to wild type cells

![Image of Western blot](image)

**Figure 4-** A methyltransferase Western blot showing expression of methyltransferase protein in HK22 pBAD- M. EcoRII cells.

The *EcoRII* methyltransferase autoregulates its own transcription by binding to its promoter to halt transcription as part of the *EcoRII* restriction modification system (Friedman & Som, 1993). Cells with the *M. EcoRII* gene encoded on a plasmid show an
increased level of induction in the presence of aza-C (Friedman & Som, 1993). This is presumably because as aza-C is incorporated into the genome, the methyltransferase protein becomes associated with the DNA, and there are decreased levels of free protein to repress transcription. For a simpler system, the methyltransferase promoter of the pR215 plasmid was replaced with an arabinose inducible promoter (Guzman et al., 1995). This offers more control for the Western blot experiments since glucose can repress expression and expression is induced with arabinose.
Figure 5- Methyltransferase expression relative to wild type cells in the absence of 5-azaC. Data is average of three independent experiments.

A concern in our experiments is that the transposon mutants’ sensitivity to 5-azaC is from increased expression or stability of the methyltransferase protein. One scenario is that a mutant might be defective in protease expression, leading to an increased level of methyltransferase, thereby increasing the amount of DPCs. Western blots for the methyltransferase protein were done on the mutants without treatment with aza-C to test
that the expression levels between mutants were similar (Figure 4). The experiments were done in the HK22 pBAD-\textit{M. EcoRII} strain.

To prepare cell samples for the Western blots, the cells were grown with arabinose to induce methyltransferase expression in the absence of aza-C. The samples were then collected and Western blots were completed. The methyltransferase expression was quantified using the LI-COR\textsuperscript{®} Odyssey software. The Western blots showed that there is not a significant increase or decrease in methyltransferase expression any of the hypersensitive mutants (Figure 5). This leads us to conclude that aza-C sensitivity in pBAD-\textit{M. EcoRII} strain is not because of an aberrant increase in methyltransferase expression.
3.3 tRNA modification mutants defective in SsrA tagging

![Diagram of the tmRNA system](image)

**Figure 6- Diagram of the tmRNA system from Karzai et al. 2000**

In *E. coli*, transcription and translation are coupled. When DPCs stall transcription, translation may be stalled as well. A stalled translation complex in *E. coli* is dealt with through SsrA tagging (Karzai *et al.*, 2000). During SsrA tagging, a stalled polypeptide is tagged with a sequence by tmRNA that marks the nascent polypeptide for degradation (Figure 6). Our lab has observed that aza-C treatment of cells leads to...
induction of the tmRNA systems through evidence of increased SsrA tagging of proteins, and ssrA mutants are hypersensitive to aza-C (Kuo et al., submitted to Molecular Microbiology).

Several of the genes identified in the transposon screen are tRNA modifying genes: \textit{trmH}, \textit{mnmE}, \textit{mmnG}, and \textit{miaA} (Table 1). If these tRNA modifying proteins modify tmRNA as well, it is possible that mutations in the tRNA modifying genes lead to a defect in SsrA tagging.

![Image](image.png)

Figure 7- An SsrA Western blot showing the increase of SsrA tagging in wild type cells (Lanes 1 and 2) and in the ftsK::Tn5 (Lanes 3 and 4) mutant in the presence of aza-C or a lack of SsrA tagging in the ssrA::Tn5 mutant (Lanes 5 and 6)

Cell samples were collected after treatment of aza-C to determine if aza-C treatment leads to a decrease in SsrA tagging. Western blots using an SsrA antibody (provided by Dr. Tania Baker, MIT) were done to identify mutants defective in the tmRNA pathway. The wild type strain in the presence of aza-C consistently showed SsrA tag induction. The lack of SsrA tagging in the \textit{ssrA} mutant acts as a negative control (Figure 7, 8). An interesting result was that three out of the four tRNA modifying genes are defective in SsrA tagging in the presence of aza-C (Figure 8): \textit{mnmE}, \textit{mmnG}, and
*miaA*. This supports our hypothesis that a defect in SsrA tagging causes aza-C sensitivity in tRNA modification mutants. Interestingly, the *recA* and *rhcS* mutants also show decreased levels of SsrA tagging. The *rhcS* gene is part of the *rhs* family of repetitive elements in *E. coli*, and there is no known function of the genes (Sadosky *et al.*, 1989, Zhao *et al.*, 1993). The *recA* defect in SsrA tagging is an unexpected result and could possibly be due to the poor viability of the strain.

**Figure 8- Summary of Western blots showing SsrA tagging in the presence of aza-C.** The difference in SsrA tagging in aza-C plus cells versus aza-C minus cells for each mutant. Data is an average of three independent trials. The results of the *dinG, ynjI*, and *sanA* mutants showed a high standard deviation and further experiments are needed to resolve the ambiguity.
4. Discussion

We have completed a thorough transposon mutagenesis screen to identify proteins that could potentially be involved in DPC repair. Several of the most promising genes identified from the transposon screen were previously implicated in affecting genome stability, including recombination genes. These include recA, recC, and recG (Table 1).

The RecA protein is a key recombination protein and it facilitates the alignment of homologous regions as part of the strand exchange step during recombination. The RecBCD complex acts as a helicase and exonuclease during recombination to unwind, and degrades double-stranded DNA to provide a single stranded substrate for RecA. This may implicate homologous recombination in resolving DPCs, or repairing DNA breaks that are secondary effects of DPCs.

Recombination plays a key role in the restart of stalled replication forks (Robu et al., 2001). It is possible that a DPC that stalls a replication fork requires recombination to rescue the stalled fork. A stalled replication fork can collapse and lead to a double stranded break, in which case recombination proteins would be needed to resolve the break. The endonucleolytic cleavage model also suggests that recombination would be required to restore DNA integrity in the presence of a double stranded break. It is possible that a DPC that stalls a replication fork requires endonucleolytic cleavage to remove the lesion, leaving a double stranded break that is repaired through recombination. The SbcCD protein complex has been shown to cleave proteins bound to DNA termini by creating a double strand break in vitro (Connelly et al., 2003). The sbcC
and \textit{sbcD} deletion mutants are candidates to test for aza-C sensitivity and may have a function cleaving DNA at sites of a DPC, facilitating the removal of the DPC.

Helicases are an integral part of DNA metabolism, with roles in replication, recombination, and repair. They couple NTP hydrolysis with translocation along DNA strands, unwinding DNA. There is also evidence suggesting helicase proteins play a role in removing DNA binding proteins, though these proteins were not covalently bound to DNA (Maria-Josê \textit{et al.}, 2005, Byrd & Raney, 2006).

The gene products of \textit{recG}, \textit{dinG}, and \textit{uvrD} all have helicase activity, and were all identified in the transposon screen (Table 1). Though \textit{RecG}, \textit{DinG}, and \textit{UvrD} are all helicase proteins, they have disparate functions. The \textit{RecG} protein has a role in catalyzing branch migration during recombination; its possible recombination deficiency may explain its sensitivity to aza-C. However, our results suggest it may have another function in clearing DPCs. We have shown that it is sensitive to aza-C, and that this sensitivity is not because of a greater methyltransferase expression compared to wild type cells (Figure 5).

\textit{UvrD} acts during nucleotide excision repair, unwinding DNA to release \textit{UvrC}, a repair protein bound to DNA in the same pathway. The \textit{UvrA} protein acts in the first step of nucleotide excision repair, and \textit{uvrA} knockout mutants are not sensitive to aza-C or formaldehyde (Nakano \textit{et al.}, 2007). It appears likely that nucleotide excision repair is not involved in resolving DPCs generated by aza-C. However, the DPC generated by aza-C is to a DNA methyltransferase, which is larger than the 12-14 kDa size limit of the protein that nucleotide excision repair can process. If a protease is involved in processing larger DPCs by reducing the size of the bound lesion, then nucleotide excision repair may
be involved in resolving the DPC after the action of the protease. There are *in vitro*

studies that suggest that the UvrABC protein complex can excise covalently bound


If nucleotide excision repair does not have a role repairing larger DPCs, then the

UvrD helicase could have a separate function relating to DPC repair. If the protein-DNA

bond of a DPC is cleaved and the protein is no longer covalently bound, a helicase could

physically ‘push’ the protein and clear the lesion. UvrD could also have a scanning

function; it could scan along DNA until it reaches a DPC, possibly recruiting other repair

proteins.

The function of DinG is unknown. It may be that DinG is involved in the removal

of DPCs, and thus have a role in DNA repair. It is possible that these helicases, though

with disparate functions, are all involved in scanning along DNA and recognizing DPCs.

In our screen we identified the *hflC* and *dnaJ* genes. The *hflC* gene encodes the

HflC gene product that modulates the HflIB (FtsH) protease (Karzai *et al.*, 2000). The

*hflC* gene is part of the tmRNA system as the ATP-dependent FtsH protease is one of

several that degrade SsrA-tagged proteins (Karzai *et al.*, 2000). The DnaJ protein is a

chaperone that works in conjunction with the DnaK chaperone and may promote

degradation of certain molecules by the FtsH protease (Yura & Nakahigashi, 1999). The

dnaJ gene has also been proposed to have interactions with the *ssrA* gene (Munavar *et

al.*, 2005). In the tmRNA system in *E. coli*, SsrA requires the SmpB (small protein B)

(Karzai *et al.*, 2000). A previous study from our lab has tested the sensitivity of *smpB*

dnaJ, and *smpB hflC* double mutants to aza-C (Kuo *et al.*, submitted to Molecular

Microbiology). The study showed that both double mutants are more sensitive to aza-C
than the dnaJ, or hflC single mutants. The sensitivity of each single mutant was dependent on the presence of the overexpressing methyltransferase. This suggests that the dnaJ and hflC mutants have a role other than their function in the tmRNA pathway, and that this role is related to the presence of DPCs. It is a very intriguing possibility that DnaJ or HflC is involved in the proteolysis of a covalently bound protein.

The rhsC transposon mutant was identified in our screen. Currently the function of the rhsC gene product is not well understood. It is part of a family of repetitive genes within the E. coli chromosome (Zhao et al., 1993). Together, they make up almost 1% of the E. coli chromosome (Zhao et al., 1993). There are five genes in the family: rhsA, rhsB, rhsC, rhsD, and rhsE (Sadosky et al., 1989). The rhsA, rhsB, rhsC, and rhsD share a 3.7 kb homologous core region (Zhao et al., 1993). The rhsC transposon mutant presents an interesting result because it showed the highest level of methyltransferase protein compared to wild type cells, as well as a defect in SsrA tagging in the presence of aza-C (Figure 5, 8). These two results are possibly related, but are unexpected since an excess of methyltransferase protein should increase the number of DPCs, and thus increase SsrA tagging. However, the methyltransferase Western blots only show the total level of methyltransferase and does not differentiate the protein covalently bound to the DNA and the free levels of protein. A quantitative DPC assay would be necessary to determine the levels of protein bound to DNA.

Through a systematic transposon mutagenesis screen, we have identified gene candidates for the repair of DPCs. Experiments show that transposon mutants’ hypersensitivity to aza-C, compared to wild type cells, is not because high levels of methyltransferase protein. We have also shown that the tRNA modification transposon
mutants miaA, mnmE, and mnmG are defective in SsrA tagging, which likely explains their sensitivity to aza-C (Figure 8).

Azacytidine is a cytidine analog, so it is capable of incorporating into DNA and RNA. The drug 2’-deoxy-5-azacytidine only incorporates into DNA, but was unsuitable for screening because of its high cost. Currently, we are testing the 24 mutants remaining for sensitivity to 2’-deoxy-5-azacytidine in overnight growth curves. Preliminary results suggest the majority of the mutants are sensitive to the drug, strengthening our screen results (data not shown).

Future experiments involve quantifying the number of DPCs formed in the presence of 5-azaC, and identifying which gene candidates are responsible for the decrease of DPCs in wild type cells. In our experiments thus far, there is a lack of a physical DPC assay. Further research must focus on developing a method to quantify the number of in vivo DPCs generated by induction agents.
5. References


