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ClpXP-regulated Proteins Suppress Requirement for RecA in Dam Mutants of Escherichia coli K-12

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ClpXP-regulated Proteins Suppress Requirement for RecA in *dam* Mutants of *Escherichia coli* K-12

A Thesis Presented

By

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ABSTRACT

CLPXP-REGULATED PROTEINS SUPPRESS REQUIREMENT FOR RECA IN *DAM* MUTANTS OF *ESCHERICHIA COLI* K-12

September 2018

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Double strand breaks (DSB) are a common source of DNA damage in both prokaryotes and eukaryotes. If they are not repaired or are repaired incorrectly, they can lead to cell death (bacteria) or cancer (humans). In *Escherichia coli*, repair of DSB are typically accomplished via homologous recombination and mediated by RecA. This repair pathway, among others, is associated with activation of the SOS response. DNA adenine methyltransferase (*dam*) mutants have an increased number of DSB and, therefore, are notorious for being RecA-dependent for viability. Here, we show that the synthetic lethality of *Δdam*/*ΔrecA* is suppressed when *clpP* is removed, suggesting that there is a protein, normally degraded by ClpXP, which is preventing DSB from occurring.

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CHAPTER I

INTRODUCTION

Double strand breaks (DSB) are a common source of DNA damage in both prokaryotic and eukaryotic organisms. They can arise for a multitude of reasons, including ionizing radiation [36], reactive oxygen species [36], or problems in DNA replication [32]. If they are not repaired correctly, they can cause genomic rearrangements, which are hallmarks of cancerous cells in humans [12] and cell death in bacteria.

Homologous recombination is the mechanism for double-strand break repair (DSBR) in *E. coli*, and is mediated by RecA [13]. This pathway is also a dominant mechanism for DSBR in bacteria, including *E. coli*, and is one alternative in mammals, including humans [10]. Homologous recombination begins with resection of DNA at the source of the DSB on the 3'-end via RecBCD [23], an ATP-dependent helicase-nuclease complex. RecA then binds to the 3'-overhang of broken single-stranded DNA (ssDNA) [23], polymerizes to form a protein/DNA helical filament [23], and induces the SOS response [23, 33]. RecA then searches for a homologous sequence and creates a Holliday Junction to repair the break [33]. The pathway continues with strand invasion, D-loop formation, branch migration via RuvAB and RecG, and resolution of the Holliday Junction via resolvasome RuvABC [13, 32, 33] (**Figure 1**).

Methyl-directed mismatch repair (MMR) is another DNA repair pathway, which repairs improper base pairs in newly synthesized DNA (**Figure 2**). During MMR, the unmethylated strand of hemimethylated DNA is cleaved, or 'nicked', at the GATC site [11]. The system has to be able to differentiate between parent and daughter strand during this process. This differentiation is possible through the help of **D**NA **A**denine **M**ethyltransferase (Dam), which methylates adenine in 5'-GATC-3' sequences [19]. In the absence of Dam, 'nicking' occurs on

both strands of DNA, as the cell cannot differentiate between parent and daughter strand [19, 29] (**Figure 3**). This leads to an increase in DSB on the chromosome and a dependence on recombination for survival [37].

Dam is conserved across the gamma-proteobacteria and is responsible for methylation of GATC sequences. In addition to its role in DNA mismatch repair, GATC-methylation also regulates gene expression and chromosome replication [21]. Although DNA methylation occurs immediately following replication, Dam is only present at ~130 molecules per rapidly growing cell [2]. The rate-limiting level of Dam explains why there is a lag between chromosomal replication and methylation of newly synthesized DNA, or why DNA is initially hemimethylated [21]. Extensive previous work [25, 21] has shown that *dam* mutants are RecA-dependent for viability, and, therefore, that a *Δdam*/*ΔrecA* combination is synthetically lethal. One known mechanism to suppress this synthetic lethality is by removing one of the *mutH*/*L*/*S* genes [6], which constitute the 'nicking' complex in MMR (**Figure 2**).

Upon DNA damage, RecA binds to ssDNA and induces the SOS response, which is normally repressed at a transcriptional level by LexA [15] (**Figure 4A**). When DNA damage occurs, the SOS response is activated, LexA is cleaved and inactivated, and approximately 40 genes are induced for DNA repair to take place [15] (**Figure 4B-C**). Once DNA repair is complete, transcriptional repression of these genes is restored. In order for the cell to return to homeostasis, ClpXP, and other proteases, selectively degrade lingering SOS proteins [14]. This apparatus is, in part, responsible for allowing the cell to return to homeostasis after the SOS response.

Previous work [5, 24, 27] has shown that ClpXP degrades certain SOS proteins, including UvrA and RecN, which are both involved in DNA damage repair pathways.

Here, we show that by removing *clpP*, *dam* mutants are no longer dependent on RecA for survival. This would suggest that there is some protein, normally degraded by ClpXP, that when present in higher amounts, is preventing DSB from occurring.

All mutations and/or mutant strains referred to in this work are complete gene deletions, unless otherwise stated.

CHAPTER II

METHODOLOGY AND RESULTS

Strains and Media

All bacterial strains are derivatives of E. coli K-12 and are described in Table i. All P1 transductions were selected for on 2%-agar plates made with either Luria broth or 56/2 minimal media supplemented with 0.2% glucose, 0.001% thiamine, and appropriate amino acids. Selection using antibiotics used 50 μg/ml ampicillin, 25 μg/ml chloramphenicol, 50 μg/ml kanamycin, or 10 μg/ml tetracycline.

P1 Transduction

The protocol for P1 transduction has been previously described [38]. All P1 transductions were selected for on 2%-agar plates made with either minimal or rich media plus antibiotics, when appropriate. All transductants were purified on the same type of medium on which they were selected.

Turbidity (OD_{600}) Measurements

All bacterial strains were grown to mid-log phase shaking in 3mL 56/2 minimal media. 200uL of mid-log phase culture was then inoculated into shaking 10mL 56/2 minimal media. 0.75mL of culture was aliquotted into a clean, plastic cuvette for each measurement and inserted into the spectrophotometer in the proper orientation. Measurements were taken at 45-minute intervals, beginning at 0 minutes. The optical density (OD) was measured at a wavelength of 600nm. Growth curves were taken at least 3 different times. Statistical analysis was completed on growth curves using the chi-squared test.

uvrA223

To prevent degradation by ClpXP, two aspartates (DD) were added to the C-terminal end of *uvrA*, immediately prior to the stop codon. Flanking sequences with regions homologous to the end of *uvrA*, as well as immediately after, containing the two aspartates (red) were added to a portion of the pGalK plasmid (blue), encoding only the *galK* gene, via PCR. *pRSJS1645* 5' ACCGTCGCGGAGTGCGAGCATCACACACGGCACGCTTCCTTAA GCCGATGCTG**GACGAC**TAA**CCTGTTGACAATTAATCATCGGCA**-3'

prSJS1646 5'-GGAAGAAAAACGTAAATTGCTGGTGCAACTCTGAAAGGAAAAG GCCGCTCAGAAGCGGCCTTAACGA**TCAGCACTGTCCTGCTCCTTG**-3'

This fragment was then cloned onto the chromosome at the end of *uvrA* by linear transformation and standard recombineering methods [40] **(Figure 9)**. Utilization of galactose as a sole carbon source was used for selection. The construct was then verified by PCR.

Preparation of cells for microscopy

Cells were grown to log phase in 56/2 minimal media. 3-6uL of culture was placed onto a 1% agarose pad with minimal media. Coverslip was placed on top of cells. Cells were allowed to incubate on pad at 37°C for 2-3 hours. Images were taken for at least 9 different fields of view (3 fields on 3 different days) and analyzed.

Analysis of microscopic images

Images were analyzed using the following software: OpenLabs 5.5.1, Oufti Version 1, and MatLabs R2016a. Strains were quantified for number of cells, cell area, number of foci, and

shape of foci using specially written MatLabs programs. Statistical analysis was completed on the number of foci with the chi-squared for homogeneity test.

CHAPTER III

RESULTS

***dam* mutants are dependent on RecA for viability, but do not show decreased recombination efficiency.** Previous work [25, 18] has shown that *dam* mutants are RecA-dependent for viability due to the increased frequency of DSB. Since a *dam*/*recA* combination is synthetically lethal, *ΔrecA* was brought in last and non-selectively in all strains via P1 transduction to avoid a negative result. A Tn10 transposon was inserted at a non-essential gene (*srlD*) nearby. This allowed for a selection for tetracycline resistance followed by a screen for kanamycin resistance. The ratio presented (**Table 2)** is the number of Δ*recA* to the total number of colonies screened. The linkage between the Tn10 insertion and *ΔrecA*(kan) is ~85%.

A recombination efficiency test was performed to show that, even though *dam* mutants are RecA-dependent for viability, they do not show decreased recombination efficiency (**Table 3**). This was demonstrated by the ability to transduce into a *dam* mutant. An allele encoding a requirement for methionine (*metB1*) with a Tn10 transposon nearby (CAG5052) was introduced non-selectively. Transductants were selected for on minimal media plus methionine in the presence of tetracycline and then screened for growth on minimal media only. Transductants that failed to grow on only minimal media carried the *metB1* allele. The null mutation in *metB1* and a transposon insertion in *btuB* used in this experiment do not affect the overall health of the cell.

Suppression of RecA-dependency in *dam* mutants is specific to the absence of the ClpXP protease complex. ClpXP is a two-module protease complex. The protease portion, ClpP, degrades proteins that contain LAA residues [8]. The ATPase chaperone component, ClpX, recognizes C-terminal residues 9-11 of an ssrA-tag (AANDENYA**LAA**) [8] and unfolds the

protein of interest. An adaptor protein, SspB, enhances the specificity of the ClpXP protease by recognizing and delivering ssrA-tagged proteins to ClpX for degradation [8]. We wanted to test whether removing *clpXP*, and having increased levels of SOS proteins, would relieve the requirement for RecA in a *dam* mutant.

The RecA-GFP (green fluorescent protein) fusion gene is one method to assess RecA activity *in vivo*. The fluorescent structures, or foci, represent RecA loaded onto damaged DNA [30]. Strains that carry RecA-GFP have all the ability of wild type RecA, though at slightly reduced levels [30]. In a *dam* mutant with RecA-GFP, around 50% of cells in a population have at least one fluorescent structure, whereas only about 6% of wild type cells do (**Table 4**). The absence of *clpP* or *sspB* in a *dam* mutant significantly decreases the number of RecA structures compared to a *dam* mutant alone ($P<0.001$), and is comparable to wild type ($P>0.999$) (**Table 4, Figure 5**). This decrease in fluorescent structures suggests that there is some protein, normally degraded by ClpXP, that when present in higher amounts, is either a) preventing DSB from occurring, or b) allowing for an alternative, RecA-independent pathway of recombination. We do not believe the latter to be the mechanism of suppression, as the literature has not shown or suggested RecA alternatives in *Escherichia coli*. For this reason, we have focused on the theory that some protein is preventing DSB from occurring.

To determine whether or not suppression is specific to the ClpXP protease complex, other protease genes were removed in a *dam* mutant and tested for RecA-dependency (**Table 5**). These include the DegP, HslUV, and Lon proteases, as well as the adaptor protein SspB. DegP is required for survival at high temperatures and has been shown to degrade mutant, oxidatively damaged, and aggregated proteins. Lon is responsible for degradation of misfolded and regulatory proteins [8], including SulA [7]. HslUV was originally identified as part of a heat

shock operon and is required for growth at high temperatures [9]. The ATPase component of HslUV, HslU, shows similar function to ClpX in aiding appropriate proteins to HslV for degradation [7]. Therefore, removing HslU is sufficient for testing the ability of suppression by the HslUV complex.

An additional recombination efficiency test (**Table 6**) was performed to show that the low co-transduction frequency present in various *dam* mutants during the RecA-dependency test was in fact due to synthetic lethality and not issues pertaining to homology in the *recA* region of the chromosome as a result of kanamycin insertions. The two genes tested, *recX* and *ygaD*, are located immediately up- and downstream of *recA*. These genes are not essential and do not affect the overall health of the cell. The same Tn10 transposon insertion was used (*srlD*) for this test. This allowed for a selection for tetracycline resistance followed by a screen for kanamycin resistance. The linkage between the Tn10 insertion and *ΔygaD*(kan) or *ΔrecX*(kan) is ~85%. There is no significant difference between wild type, *dam*, and *clpP* mutants in recombination efficiency ($P<0.001$).

***dam* mutants are still dependent on RecA for viability with recA200^{ts} mutation.** As mentioned previously, the linkage between the Tn10 insertion and *ΔrecA*(kan) is ~85%. While there was clear suppression in the *dam*/*clpP* strain, the linkage was skewed at only ~8-10%. To address this, and to further test the idea that a *dam*/*recA* combination is synthetically lethal, we utilized a temperature-sensitive *recA* mutation (*recA200*), which allows for wild-type RecA activity at 30°C, but resembles a *recA* null phenotype at 42°C [1]. The temperature-sensitive mutation resulted in a strain that grew at 30°C, but not at 42°C, as expected (**Figure 6**).

A *dam*/*clpP*/*recA200* strain and a *dam*/*sspB*/*recA200* strain was constructed and tested for viability at 42°C (**Figure 7**), as these were the two combinations of mutations in which *recA* suppression was observed. Although the *dam*/*clpP*/*recA200* and *dam*/*sspB*/*recA200* strains do not grow as well as a *dam* mutant with wild type RecA at 42°C, there is still a clear, steady upward trend in the growth curve. Therefore, these results support the previous results on transduction frequencies, even though those frequencies were skewed.

Protein responsible for suppression is not involved in GATC methylation. At least 11 proteins that are degraded by ClpXP have unknown function [5, 24]. One possibility is that suppression is linked to a protein substituting for Dam's DNA methylation activity. To test this, we began by transforming plasmid pBR322 (~4.3 kB) into wild type, Δ*dam*, and Δ*dam*/Δ*clpP* strains. In the Δ*dam* strain, GATC methylation is completely absent. Therefore, when treated with DpnI, little to no cutting should occur, as this enzyme specifically recognizes and cuts at methylated GATC sites. If appropriate GATC methylation occurs (wild type), the DpnI digest product should produce one band at ~1.5 kB, and 22 smaller bands, all ranging from 8-360bp. The *dam* and *dam*/*clpP* digests appear to be identical, which supports the theory that the protein responsible for suppression is not involved in DNA methylation (**Figure 8**).

To test which ClpXP-degraded substrates were required for suppression, we combined various mutations with a *dam*/*clpP* strain to test whether or not RecA would still be required.

RecN is required, but not sufficient, for suppression. RecN is a member of the structural maintenance of chromosomes family. This protein contains a centrally located coiled-coil domain, as well as Walker A and Walker B binding motifs in both the N- and C- terminus [35].

RecN was originally isolated in a *recBCD sbcB* mutant [17, 26] and is a key player in the RecFOR pathway of recombination [26]. Previous work [24] has provided evidence that RecN is an intrinsically good substrate for ClpXP degradation. This is expected, as RecN is 1) part of the SOS regulon, and 2) contains an LAA at its C-terminal end. We observed that the *dam/clpP* strain no longer suppresses the requirement for RecA in the absence of *recN* (**Table 7**).

Since RecN appears to be required, we tested whether removing the ability of ClpXP to recognize and degrade RecN would be sufficient for suppression. While a C-terminal tag containing two aspartates (DD) prevented degradation of RecN by ClpXP [24], we found that this alteration hindered RecN activity *in vivo* [39]. Thus, a *recN* derivative was constructed that replaced the last two alanines of the C-terminal end of RecN with serine and valine (A552S, A553V) (*recN4174)*. This mutation still prevents recognition and degradation by ClpXP, and allows for full activity of RecN in vivo [39]. Since the *dam/recN4174/recA* mutant could not be constructed, this data proposes that RecN is required, but not sufficient, for suppression, suggesting that there is/are other protein(s) aiding in suppressing the requirement for RecA in *dam* mutants (**Table 8**).

UvrA is required, but not sufficient, for suppression. The SOS regulon includes *uvrA* and *uvrB,* which are involved in the nucleotide excision repair (NER) pathway [3]. This pathway utilizes the action of the UvrABCD proteins to recognize and remove UV-induced DNA lesions. Upon DNA damage, the $UvrA_2UvrB$ complex scans DNA to locate lesions. UvrA detects a distortion in the DNA, which is then verified by UvrB [28]. UvrA then dissociates via hydrolysis, allowing for the formation of the UvrB-DNA 'pre-incision' complex. At this point, the endonuclease UvrC is recruited to the site of damage in an ATP-dependent manner [28]. UvrC binds to the pre-incision complex and cleaves only the damaged strand of DNA on either

side of the lesion [28]. Finally, helicase UvrD removes ssDNA containing the damaged site, followed by DNA synthesis and ligation [31, 34]. This mechanism of repair is rather similar to MMR. For this reason, it may be possible that the UvrABC complex/NER pathway is substituting for MMR in the *dam*/*clpP* strain, since UvrA is more readily available in the absence of *clpP*. In turn, this would allow for *ΔrecA* in the *dam*/*clpP* background. The same work [24] showed that UvrA is also a naturally good substrate for ClpXP, as it is ranked third (highly abundant) on the list of over 100 ClpXP-degraded proteins. To test if UvrA is required, we removed *uvrA* in the *dam*/*clpP* background. In doing so, we observed that the absence of *clpP* no longer suppressed the requirement for RecA in *dam* mutants, when paired with the absence of *uvrA* (**Table 7**).

Neher *et al* [24] demonstrated that a C-terminal tag containing two aspartates (DD) prevented degradation of RecN by ClpXP. Since the ClpXP recognition sequence for UvrA has yet to be identified, as it does not contain a C-terminal LAA, a similar approach was taken; two aspartates were added to the end of *uvrA* prior to the stop codon (*uvrA223*) (see materials and methods). To test the functionality of *uvrA223*, viable cell counts were taken for different exposures to UV irradiation. The addition of this C-terminal tag does not alter the functionality of the UvrA protein (**Table 8**). Since the *dam*/*uvrA223*/*recA* mutant could not be constructed, this data proposes that UvrA is required, but not sufficient, for suppression, suggesting that there is/are other protein(s) aiding in suppressing the requirement for RecA in *dam* mutants (**Table 7**).

RecN and UvrA, together, are not sufficient for suppression. Since single mutants, *recN4174* or *uvrA223*, did not suppress the requirement for RecA in *dam* mutants, we tested if both of these mutations together would suppress; perhaps the increased intracellular levels of both RecN and UvrA would be sufficient. Albeit, the combination of these mutations (*uvrA223, recN4174*) with

dam did not suppress the requirement for RecA **(Table 7)**, suggesting that there may be a third protein aiding in suppression.

DISCUSSION

Previous work [25, 21] has shown that *dam* mutants are RecA-dependent for viability, and, therefore, that a *dam*/*recA* combination is synthetically lethal. This research primarily focuses on the theory that there is a protein(s) that suppresses the requirement for RecA in *dam* mutants. It was expected that, if a specific protease were removed (ClpXP), then the protein(s) responsible for suppression would be present in higher amounts, and the *Δdam*/*ΔrecA* combination would no longer be synthetically lethal.

It is commonly known that RecA-dependency is suppressed in a *dam* mutant when one of the *mutH*/*L*/*S* genes is removed [6]. Since the mechanism of the NER pathway is quite similar to that of MMR, it may be possible that NER is substituting for MMR in the *dam*/*clpP* background because at least one of the gene products (UvrA) is more readily available. This would suppress the requirement for RecA by repairing mismatches in ssDNA without generating DSBs. Another possibility is that UvrA is simply masking the mismatch and blocking the MutHLS complex from recognizing and binding to it in the absence of *clpP*.

RecN aids in double strand break repair and is a key player in the RecFOR pathway of recombination [17]. For this reason, it is plausible, though unclear exactly how, RecN may be aiding in suppression.

Since neither of the single mutants (*uvrA223, recN4174*) alone with *dam* suppressed the requirement for RecA, this would suggest that neither RecN nor UvrA is sufficient for suppressing the requirement for RecA in *dam* mutants, although they are both required. The

combination of these mutations (*uvrA223, recN4174*) with *dam* did not suppress the requirement for RecA, suggesting that there may be a third protein aiding in suppression.

The question arose as to whether or not suppression would still be apparent if any third gene were removed in the *dam*/*clpP* background. *yfgB*, a gene of unknown function and selectively degraded by ClpXP [24], was removed in this background and suppression was still observed at the same frequency as the *dam*/*clpP* strain (data not shown). This result further supports the claim that RecN and UvrA are required for suppression.

It is also important to note that, in strains where introducing a *recA* deletion via P1 transduction was synthetically lethal, less than 10 colonies between three experimental plates grew each time, and all colonies were screened. These transductions were repeated between three and six times to obtain the appropriate number of colonies to statistically analyze the difference between them. In the *dam*/*clpP, dam*/*sspB*, and *dam*/*clpX* strains, approximately 25-50 colonies grew between three experimental plates each time, and 12-16 colonies were screened per transduction.

Future directions for this work include a) identification of all proteins required for suppression and b) identification of the mechanism of suppression. These findings may help unveil novel alternatives for DNA damage repair or provide insight for innovative cancer and gene therapies.

Strain List

Strain Number	*dam*	*recA*	Other	Other Relevant Genotype	Reference
CAG5052	+	+		*btuB3191::Tn10 metB1*	Singer *et al*. 1989
CAG18642	+	+		*zfj-3131::Tn10*	Singer *et al*. 1989
JC13509	+	+			Lab stock[a]
SS1576	*13*	+			Derivative of GM698
SS3922	+	+	*ΔclpX*	*del(clpX)100::kan*	Baba *et al*. 2006
SS4871	*Δ*	+		*del(dam)100::kan*	Baba *et al*. 2006
SS5129	+	+	*ΔuvrA*	*del(uvrA)100::kan*	Baba *et al*. 2006
SS5130	+	+	*ΔuvrB*	*del(uvrB)100::kan*	Baba *et al*. 2006
SS5131	+	+	*ΔuvrC*	*del(uvrC)100::kan*	Baba *et al*. 2006
SS5907	+	*Δ*		*del(recA)100::kan*	Baba *et al*. 2006
SS5983	+	+	*ΔsspB*	*del(sspB)100::kan*	Baba *et al*. 2006
SS6321	+	+			Lab stock[c]
SS7117	+	+			Lab stock[c]
SS9949	+	+	*ΔdegP*	*del(degP)100::kan*	Baba *et al*. 2006
SS9950	+	+	*Δlon*	*del(lon)100::kan*	Baba *et al*. 2006
SS9951	+	+	*ΔhslU*	*del(hslU)100::kan*	Baba *et al*. 2006
SS9988	+	+	*ΔrecN*	*del(recN)100::kan*	Baba *et al*. 2006
SS9993	+	+	*ΔclpP*	*del(clpP)100::kan*	Baba *et al*. 2006
SS10350	+	+	*ΔclpP*	*del(clpP)100::kan*	Lab stock[c]
SS10517	+	+	*ΔgalK*	*del(galK)200::frt*	Lab stock[c]
SS10970	+	+	*ΔclpP*	*del(clpP)200::frt*	Lab stock[c]
SS11399	+	+	*Δlon*		Lab stock[b]
SS11511	+	+	*ΔdegP*	*del(degP)100::kan*	Lab stock[c]
SS11512	+	+	*ΔhslU*	*del(hslU)100::kan*	Lab stock[c]
SS11637	+	*gfp-918*			Lab stock[d]
SS11644	+	*gfp-918*			Lab stock[d]
SS11748	+	*gfp-918*			SS11637→SS6321
SS11804	+	*gfp-918*	*ΔsspB*		Lab stock[d]
SS12011	*13*	*gfp-918*			SS1576→SS11748[d]
SS12023	*13*	*gfp-918*	*ΔsspB*		SS1576→SS11804[d]
SS12027	+	*gfp-918*	*ΔclpP*		SS11637→10970[d]
SS12033	*13*	*gfp-918*	*ΔclpP*		SS1576→SS12027[d]
SS12052	+	*Δ*		*zfj-3131::Tn10*	CAG18642→SS5907[c]
SS12059	+	+	*ΔsspB*	*del(sspB)100::kan*	SS5983[c]
SS12060	*13*	+			SS1576→SS6321[c]
SS12074	+	+	*ΔsspB*		SS12059[bc]
SS12075	+	+	*ΔclpP*		SS10350[bc]
SS12077	*13*	*Δ*	*ΔsspB*	*zfj-3131::Tn10*	SS12052→SS12079[c]
SS12079	*13*	+	*ΔsspB*		SS1576→SS12074[c]

SS12080	*13*	+	*ΔclpP*		SS1576→SS12075[c]
SS12083	*13*	*Δ*	*ΔclpP*	*zfj-3131::Tn10*	SS12052→SS12080[c]
SS12085	+	*Δ*	*Δlon*	*zfj-3131::Tn10*	SS12052→SS11399[c]
SS12090	*13*	+	*Δlon*		SS1576→SS11399[c]
SS12095	+	+	*ΔrecN*	*del(recN)100::kan*	SS9988→SS6321[c]
SS12097	+	*Δ*	*ΔsspB*	*zfj-3131::Tn10*	SS12052→SS12074[c]
SS12098	+	*Δ*	*ΔclpP*	*zfj-3131::Tn10*	SS12052→SS12075[c]
SS12099	+	+	*ΔdegP*		SS11511[bc]
SS12100	+	+	*ΔhslU*		SS11512[bc]
SS12290	+	+	*recN4174*		Lab stock[c]
SS12405	*13*	+	*ΔdegP*		SS1576→SS12099[c]
SS12406	*13*	+	*ΔhslU*		SS1576→SS12100[c]
SS12409	+	*Δ*	*ΔdegP*	*zfj-3131::Tn10*	SS12052→SS12099[c]
SS12410	+	*Δ*	*ΔhslU*	*zfj-3131::Tn10*	SS12052→SS12100[c]
SS12429	+	+	*uvrA223*		This work[c]
SS12430	+	+	*uvrA223*		SS12429→SS10517[c]
SS12436	*13*	*200*			SS1576→STL287
SS12445	*13*	*200*		*zfj-3131::Tn10 recA200*	CAG18642→SS12436[c]
SS12446	*Δ*	+		*del(dam)100::kan*	SS4871→SS7117[c]
SS12447	*Δ*	+		*del(dam)200::frt*	SS12446[bc]
SS12448	*Δ*	*recA200*		*del(dam)200::frt*	SS12445→SS12447[c]
SS12449	*Δ*	*recA200*	*ΔclpP*	*del(dam)200::frt*	SS9993→SS12448[c]
SS12453	*Δ*	*recA200*	*ΔsspB*	*del(dam)200::frt*	SS5983→SS12448[c]
SS12455	*Δ*	+	*ΔclpP*	*del(clpP)100::kan*	SS9993→SS12447[c]
SS12456	*Δ*	*recA200*	*recN4174*	*del(dam)200::frt*	SS12990→12448[c]
SS12457	*Δ*	+	*ΔclpP*	*del(clpP)200::frt*	SS12455[bc]
SS12459	*Δ*	+	*ΔclpX*	*del(clpX)200::frt*	SS3922→SS12447[bc]
SS12460	*Δ*	+	*uvrA223*	*del(dam)100::kan*	SS4871→12430[c]
SS12461	*Δ*	+	*ΔuvrA*	*del(uvrA)200::frt*	SS5129→SS12457[bc]
SS12462	*Δ*	+	*ΔuvrB*	*del(uvrB)200::frt*	SS5130→SS12457[bc]
SS12463	*Δ*	+	*ΔuvrC*	*del(uvrC)200::frt*	SS5131→SS12457[bc]
SS12464	*Δ*	+	*uvrA223, recN4174*		SS12290→SS12460[bc]
STL287	+	*recA200*			1

Table 1:Strain List (continued onto next page).

[a]JC13509 has the following genotype: *sulB103− lacMS286* attΦ80-*lacBK1 argE3 his-4 thi-1 xyl-5 mtl-1.*

[b]Kan resistant derivative from Keio Collection was transduced into strain as indicated in reference column. Plasmid pCP20, carrying the *flp* gene, was then introduced and Kan sensitive derivatives were screened.

[c]These strains have the following additional genotype: *hupA::mcherry FRT del(attB)::sulAp-gfp*

[d]These strains have the following additional genotype: *ygaD1:kan recAo1403 recA4155, 4136::gfp-918 (A206E) del(galK)200::frt del(attB)::sulAp-mCherry*

RecA-dependency of *dam* mutants	
	ΔrecA (Kan^R/Tet^R)
WT	28/32
Δdam	0/18

Table 2: RecA-dependency of *dam* mutants. Here, we show that *dam* mutants are RecA-dependent for viability. The data above is the number of Kan^R transductants/Tet^R transductants.

Recombination efficiency	
dam	*btuB*-met
+	14/16
Δ	13/16

Table 3: Recombination efficiency of *dam* mutants via P1 transduction. There is no significant difference between wild type and *dam* mutants in recombination efficiency ($P<0.001$).

Percentage of Cells with Fluorescent RecA Structures				
Genotype	Avg Cell Area	% Cells with 0 Foci	% Cells with 1 Foci	% Cells with 2+ Foci
Wild type	440	94.2	5.2	0.6
*Δdam**	886	50.9	34.9	14.2
*ΔclpP***	544	64.3	24.4	11.3
*ΔsspB***	458	93.7	5.2	1.1
*Δdam/ΔclpP***	487	64.5	27.0	8.5
*Δdam/ΔsspB***	787	86.1	5.7	8.2

Table 4: Percentage of Cells with Fluorescent RecA Structures. Cells were grown to log phase in minimal media and placed on 1% agarose pad for microscopy. Cells were allowed to grow to log phase once placed on pad. * = Significant difference in the number of foci compared to wild type ($P<0.001$). ** = Not a significant difference in the number of foci compared to wild type ($P>0.025$).

The effects of mutations in protease genes on RecA-dependency		
	dam+	*Δdam*
WT	28/32	0/18
ΔclpP	22/32	4/54
ΔclpX	24/32	4/43
ΔsspB	24/32	6/56
ΔdegP	14/16	0/19
ΔhslU	11/14	0/17
Δlon	16/20	0/18

Table 5: The effects of mutations in protease genes on RecA-dependency. Here, we show that RecA is no longer required for survival in *dam* mutants in the absence of *clpP* or *sspB*. The data above is the number of Kan^R transductants/Tet^R transductants.

Recombination efficiency				
clpP	*dam*	*recX* (Kan^R/Tet^R)	*ygaD* (Kan^R/Tet^R)	*recA* (Kan^R/Tet^R)
+	+	15/16	16/16	28/32
Δ	+	13/16	15/16	22/32
+	*Δ*	14/16	14/16	0/15
Δ	*Δ*	13/16	14/16	4/54

Table 6: Recombination efficiency of *dam* mutants via P1 transduction. There is no significant difference in recombination efficiency ($P<0.001$). The data above is the number of Kan^R transductants/Tet^R transductants.

Contribution of ClpXP-degraded Proteins to Suppression			
	clpP	*dam*	*recA* (Kan^R/Tet^R)
	+	*Δ*	0/18
	Δ	*Δ*	4/54
ΔclpP	*Δ*	*Δ*	0/17
recN174	+	*Δ*	0/19
ΔuvrA	*Δ*	*Δ*	0/24
ΔuvrB	*Δ*	*Δ*	0/26
ΔuvrC	*Δ*	*Δ*	0/22
uvrA223	+	*Δ*	0/23
recN4174, uvrA223	+	*Δ*	0/19

Table 7: Contribution of RecN to suppression. The data above suggests that RecN and UvrA are required, but not sufficient, for suppressing the requirement for RecA in *dam* mutants.

Assessment of UvrA Function with C-terminal Tag			
	WT	*ΔuvrA*	*uvrA223*
0J	138	124	127
10J	129	3	118
20J	122	-	109
30J	117	-	104
40J	110	-	97

Table 8: Assessment of UvrA function with C-terminal tag. The addition of two aspartates on the C-terminal end of *uvrA* does not significantly alter the functionality of the protein up to 40J of UV exposure ($P<0.001$). The data above is the total number of colonies from 100uL of 10^{-6} diluted culture.

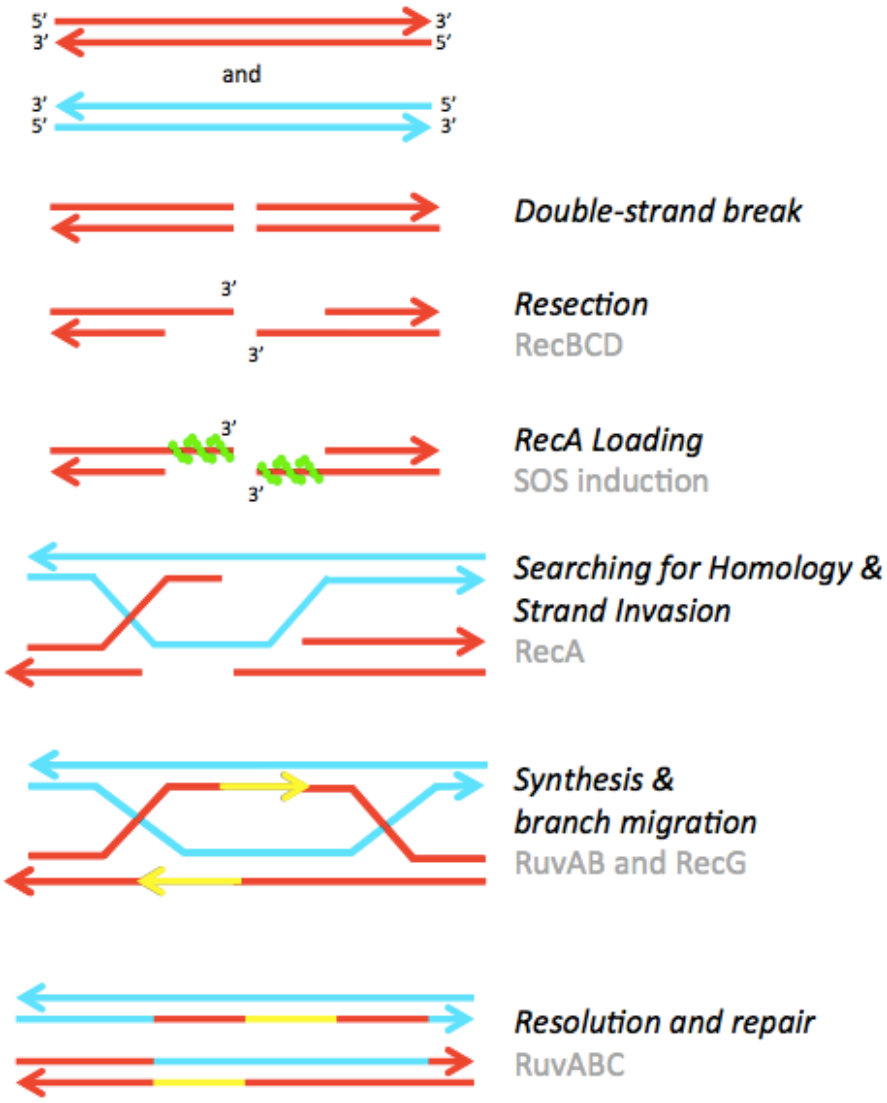

Figure 1: Homologous Recombination Pathway (HR). RecBCD resects ssDNA on the 5'-end of a DSB. RecA then loads onto ssDNA and activates the SOS response. After RecA filaments onto damaged DNA, searches for homology, and exchanges strands, RuvAB and RecG carry out synthesis and branch migration to generate a Holliday Junction. Finally, RuvABC resolves the Holliday Junction, and DSBR is complete.

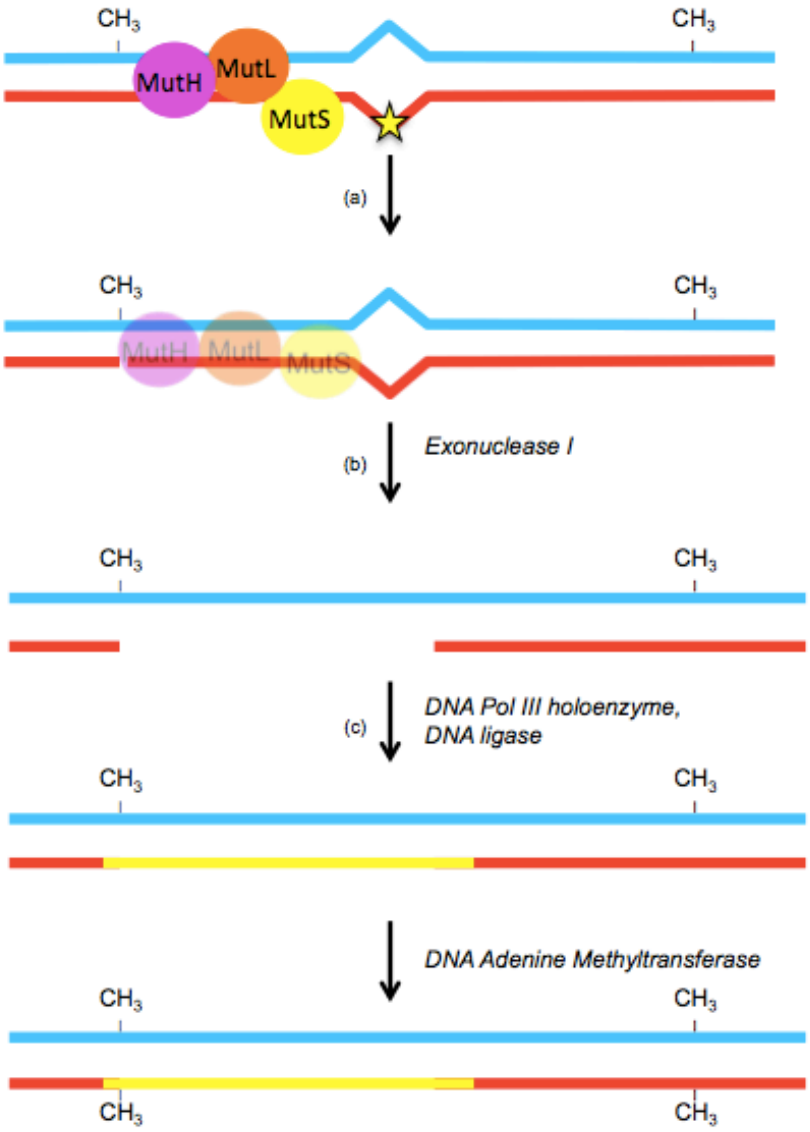

Figure 2: Methyl-directed Mismatch Repair Pathway (MMR). Base mismatches in newly synthesized, unmethylated DNA are recognized by the MutSLH complex. The complex cleaves the newly synthesized strand of DNA to remove the mismatch, and the gap is then synthesized and filled. Dam then methylated the newly synthesized strand of DNA.

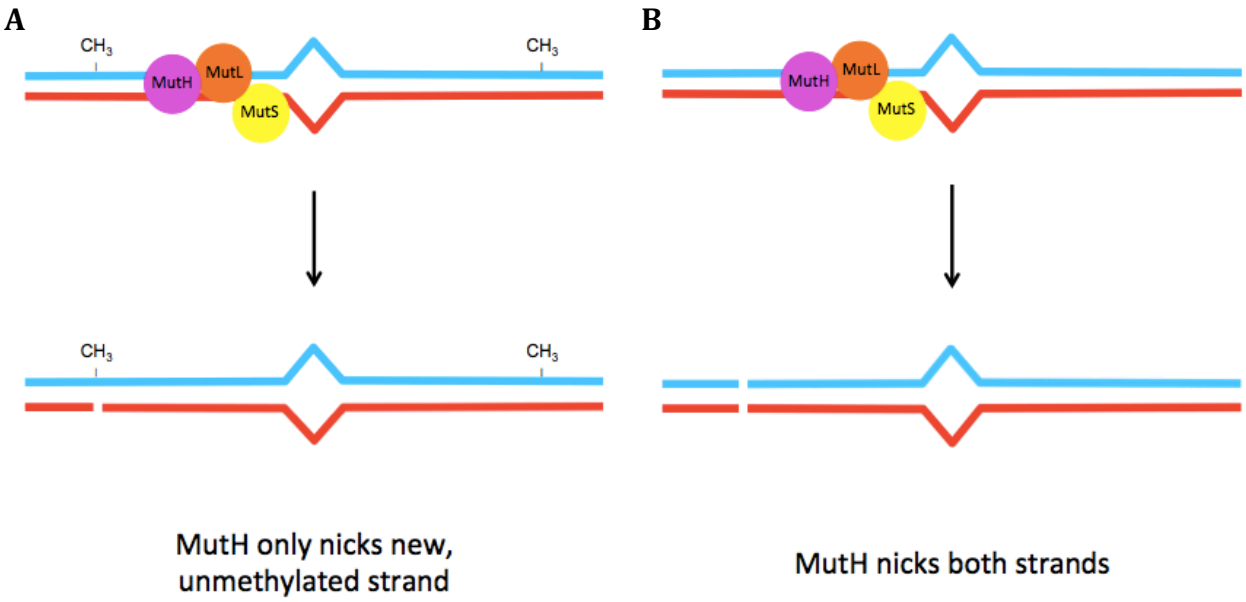

Figure 3: MMR in *dam* mutants. A. In wild type cells, the MutSLH complex only nicks the unmethylated, newly synthesized strand. B. In *dam* mutants, the MutSLH complex cannot differentiate between parents and daughter strand and nicks both strands, causing a DSB.

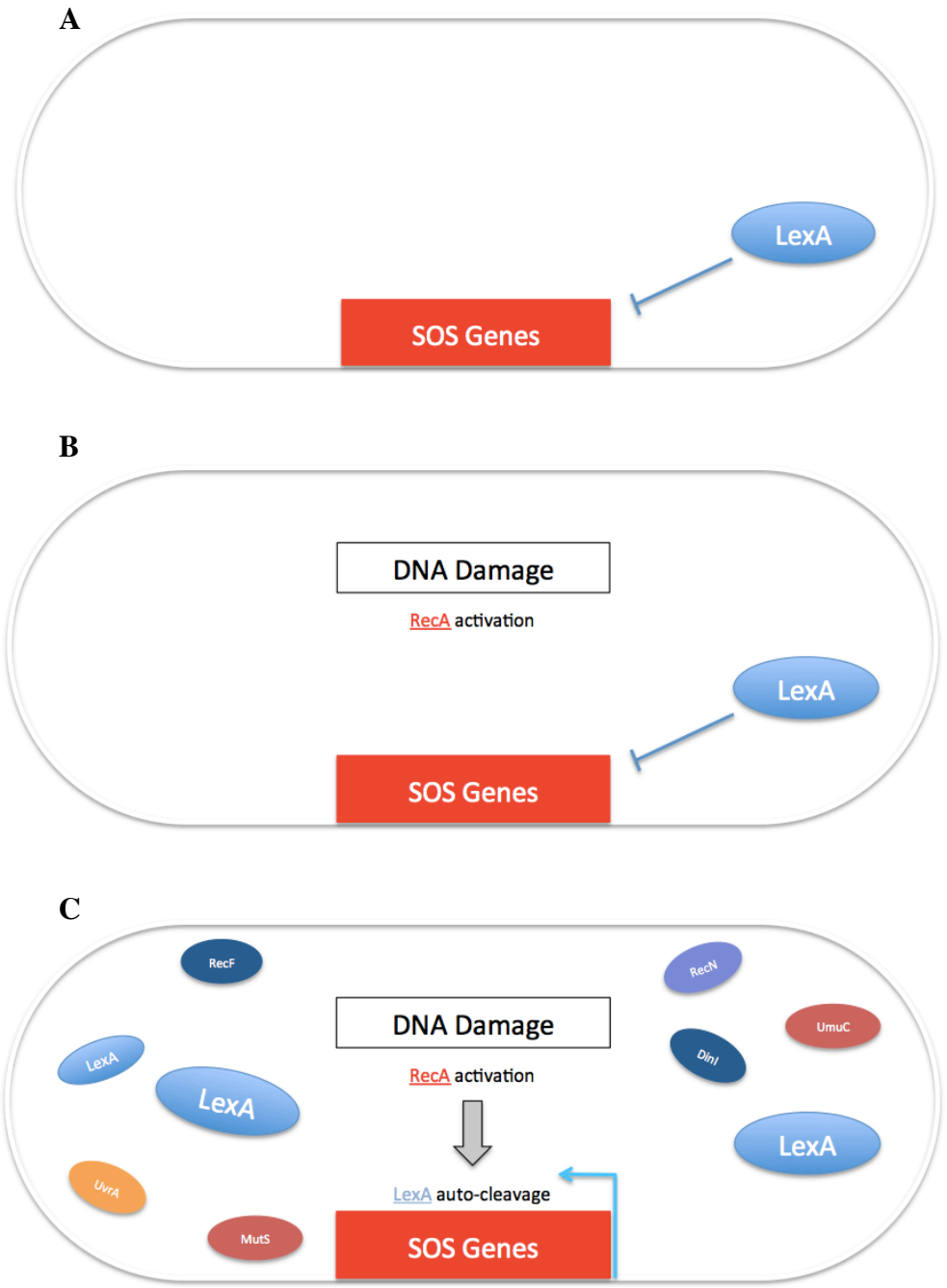

Figure 4: SOS response in *E. coli*. A. Genes are normally repressed at transcriptional level by LexA protein. B. Upon DNA damage, RecA binds to ssDNA and activates the SOS response. C. LexA autocleaves and induces transcription of approximately 40 genes.

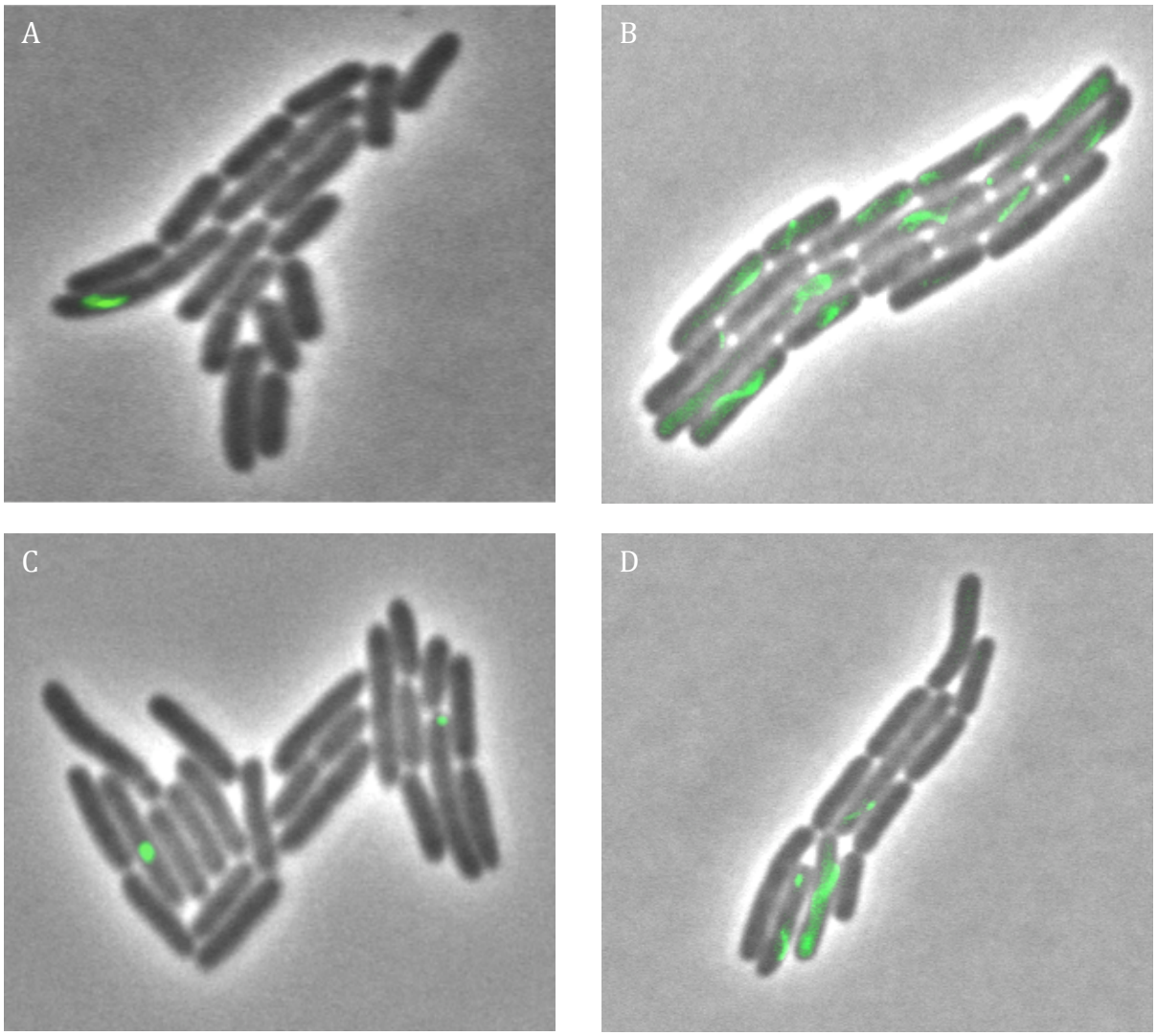

Figure 5: RecA-GFP in *dam* mutants. A. Wild type with RecA-GFP. B. *dam* with RecA-GFP. C. *dam*/*clpP* with RecA-GFP. D. *dam*/*sspB* with RecA-GFP.

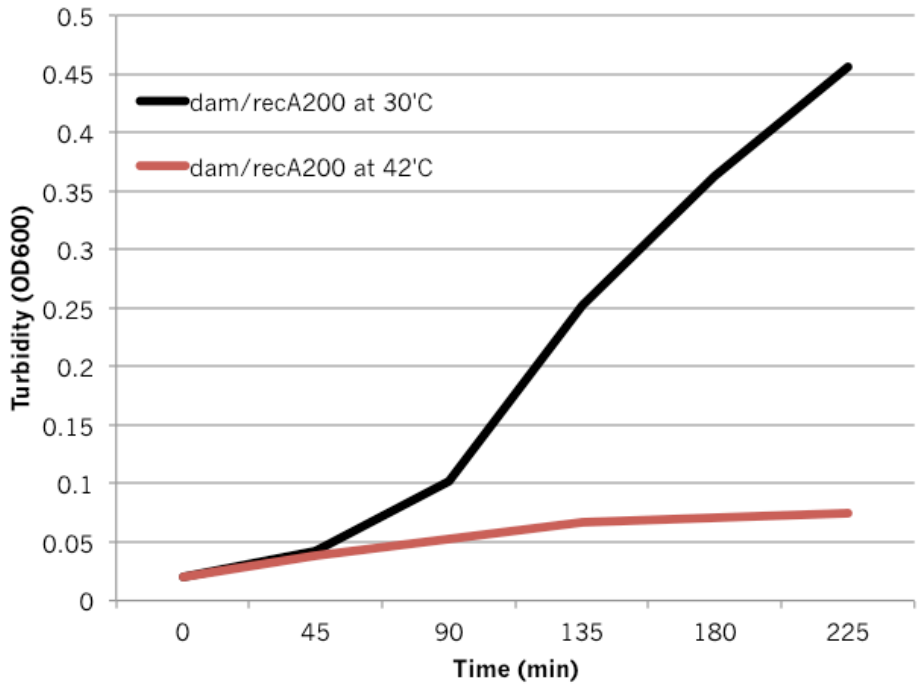

Figure 6: Growth of *dam/recA200* at 30°C and 42°C. The strain was grown shaking in 56/2 minimal media at 30°C and 42°C. See materials and methods for turbidity measurement protocol.

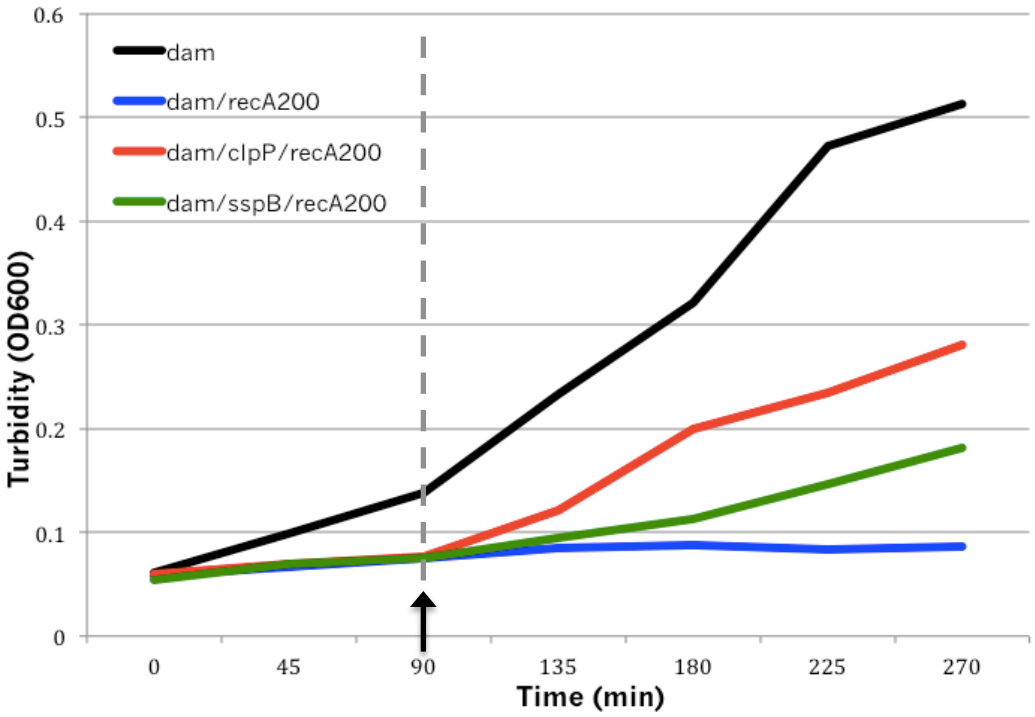

Figure 7: Growth of *dam* mutants carrying *recA200* mutation at 30°C and 42°C. The strain was grown shaking in 56/2 minimal media at 30°C and 42°C. The upward arrow at 90 minutes indicates the temperature shift from 30°C to 42°C. See materials and methods for turbidity measurement protocol.

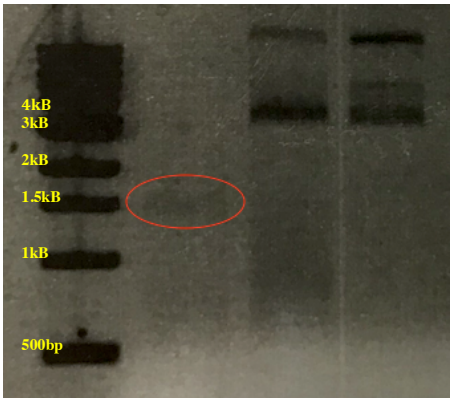

Figure 8: DpnI digest of pBR322 in wild type, *dam*, and *dam*/*clpP* strains. 10kB ladder, pBR322 isolated from wild type strain and digested with DpnI, pBR322 isolated from Δ*dam* strain and digested with DpnI, and pBR322 isolated from Δ*dam*/Δ*clpP* strain and digested with DpnI ran on 1% agarose gel.

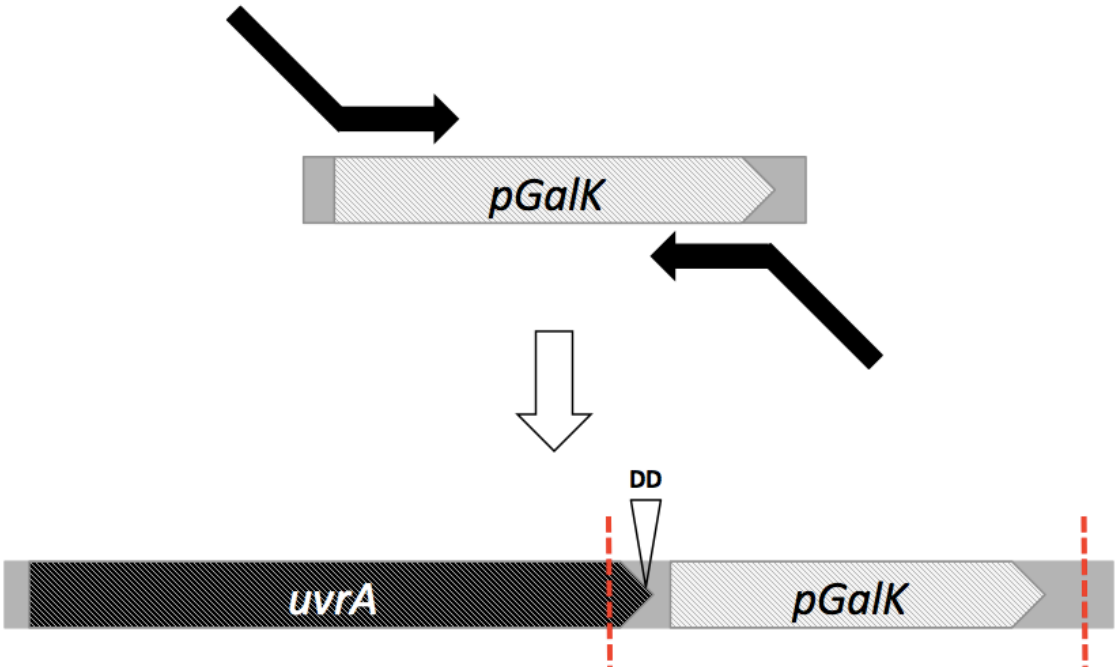

Figure 9: Recombineering – *uvrA223* construct. Two aspartates (DD) were added to the C-terminal end of *uvrA*, immediately prior to the stop codon, via PCR. This fragment (also encoding for *galK)* was then cloned onto the chromosome at the end of *uvrA* by linear transformation and standard recombineering methods [40].

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