Mechanism of translesional synthesis repair in *Caulobacter crescentus*

A thesis submitted towards partial fulfilment of

BS-MS Dual Degree Programme

By

Prachi Balasaheb Shinde

(201311129)

Indian Institute of Science Education and Research, Pune

Under the guidance of

Thesis Supervisor

Dr. Anjana Badrinarayanan

Reader F

NCBS-Bangalore

Thesis Advisor

Dr. Mayurika Lahiri

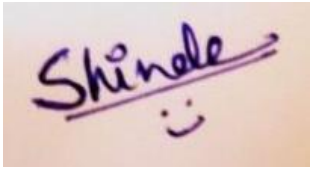
Associate Professor

Department of Biological Sciences

IISER-Pune

CERTIFICATE

This is to certify that this dissertation entitled "**Mechanism of translesional synthesis repair in *Caulobacter crescentus***" submitted towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study carried out by **Ms. Prachi Balasaheb Shinde** at **National Centre for Biological Sciences, Bangalore** under the supervision of **Dr. Anjana Badrinarayanan**, **Reader F** during the academic year **2017-2018**.



Signature of Supervisor

Dr. Anjana Badrinarayanan

Reader F

NCBS, Bangalore

Signature of Student

Ms. Prachi Shinde

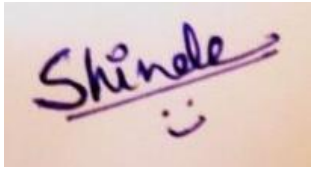
5th year BS-MS

IISER-Pune

Date: 30/03/2018

DECLARATION

I hereby declare that the matter embodied in the report entitled **"Mechanism of translesional synthesis repair in *Caulobacter crescentus*"** are results of the work carried out by me at the **National Centre of Biological Sciences, Bangalore**, under the supervision of **Dr. Anjana Badrinarayanan** and the same has not been submitted elsewhere for any other degree.



Signature of Supervisor

Dr. Anjana Badrinarayanan,

Reader F

NCBS, Bangalore

Signature of Student

Ms. Prachi Shinde,

5th year BS-MS

IISER-Pune

Date: 30/03/2018

Abstract

DNA damage in cells results from variety of exogenous and endogenous factors. Under these circumstances, DNA repair is crucial for the maintenance of genomic integrity of cells. Translesional synthesis (TLS) is a type of error-prone repair mechanism found across all domains of life. In prokaryotes, TLS has been mostly studied in the context of *Escherichia coli*, even though the components of this pathway are not conserved across all bacterial species. This study is aimed at understanding the regulation of error-prone polymerase ImuC, which is implicated in TLS in *Caulobacter crescentus* and pathogenic bacteria like *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa.* Our experiments suggest that deletion of *imuC* results in sensitivity to only certain types of DNA damaging agents like mitomycin C and ultraviolet rays indicating the specificity of the polymerase. Our preliminary experiments using bacterial two-hybrid assay shows that ImuC-mediated TLS proteins interact with replisome components like DnaN (β-clamp) and DnaE (high-fidelity replicative polymerase). We also observe that some of TLS proteins interact with the recombinase RecA as well as other putative repair protein like MmcB. Interaction of RecA with multiple repair and replisome components suggest that RecA might have a central role in recruiting repair components to the site of a lesion and thereby mediate repair pathway choice.

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Introduction

DNA damage

Bacterial cells live under diverse environmental conditions where they encounter variety of stress conditions. These stress conditions can include nutrient scarcity, pH and temperature fluctuations, DNA damage, predatory pressure etc. DNA damage is one of the prominent stresses faced by bacteria, which if not repaired can affect genome integrity, lead to genomic instability, and eventually cell death. DNA damage can be triggered by endogenous factors like reactive oxygen species or exogenous factors like UV light DNA damaging chemicals (T Lindahl, 1993; Jackson and Bartek 2010). To circumvent the deleterious effects of these DNA damaging agents cells have developed several DNA repair and damage tolerance pathways, few of which are error-prone.

DNA repair

Most pathways of DNA repair such as homologous recombination, base excision repair, non-homologous end joining, translesional synthesis, nucleotide excision repair and mismatch repair are conserved across different domains of life and have been extensively studied in an *in vitro* context **(Fig.1)** (Lusetti and Cox 2002; Hanawalt et al. 2003; Huffman et al. 2005; Yang 2006). Some of these pathways result in high fidelity repair. For example, in base-excision repair (BER), damage specific glycosylase recognises and removes damaged base resulting in an abasic site on DNA. The abasic site is cleaved by an endonuclease followed by synthesis and ligation using DNA polymerase and DNA ligase (David, Shea, and Kundu 2010; Jackson and Bartek 2010). In nucleotide excision repair (NER) lesions such as bulky base adducts and UV photo-products which distort DNA double helix are repaired. Adducts are recognised by the NER components leading to the removal of 22-30 oligonucleotides at the damage site, producing single-stranded DNA. The repair is completed through synthesis by DNA polymerase and ligation by ligase (Hoeijmakers 2001; Jackson and Bartek 2010). In mismatch repair (MMR), lesions such as insertions or deletions and mismatches that arise during DNA replication are repaired. The misincorporated base is excised, and the correct base is inserted by a DNA polymerase (Jiricny 2006).

Finally, homologous recombination, involved in double-strand break repair, is inherently error-free. Homologous recombination takes place through homology search and strand invasion followed by recombination with a homologous sequence. In the case that error-free repair is not possible, cells employ error-prone repair pathways as well. As an example, in the absence of a homologous partner, non-homologous end-joining can be opted by cells for repair of double-strand breaks. In non-homologous end joining double-strand breaks are sealed via polymerase and ligase activity (Jackson and Bartek 2010; Li and Heyer 2008). Another error-prone repair pathways is translesional synthesis, which leads to bypass of a lesion that blocks the replication fork progression (Fuchs and Fujii 2013). This repair mechanism is discussed in depth in the following section.

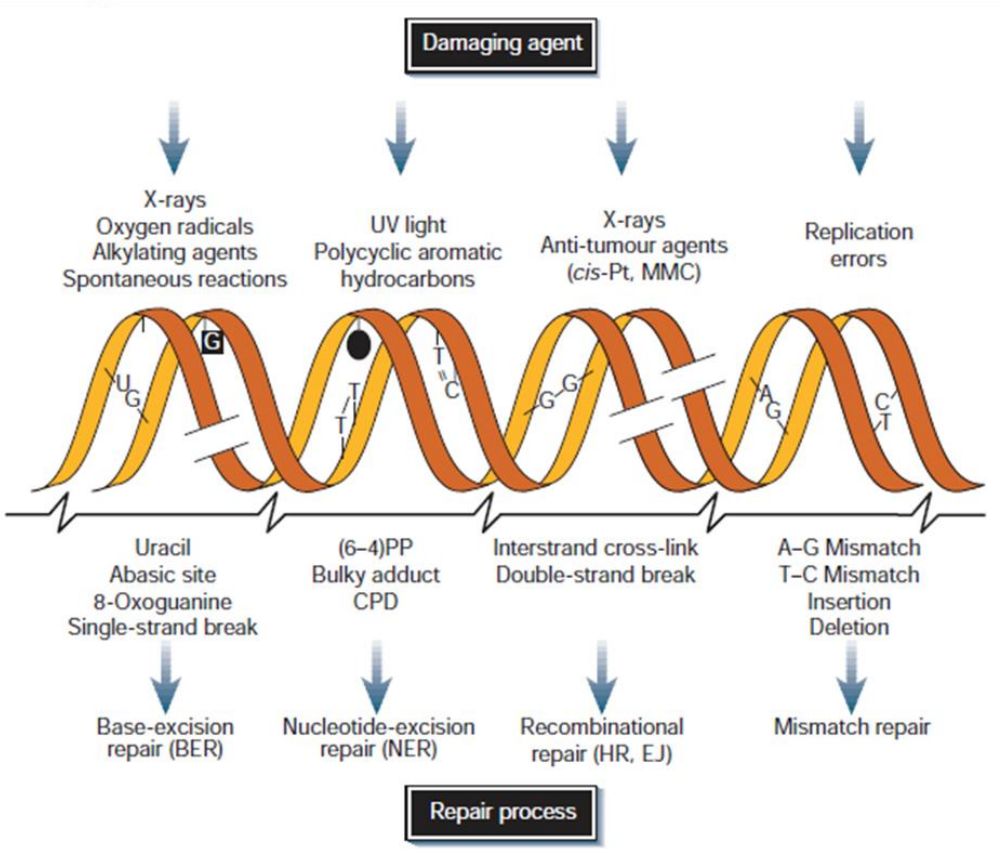

Fig.1. DNA damage and repair mechanisms [from Hoeijmakers 2001]: Different DNA damaging agents (shown on top), DNA lesions induced by these agents (shown in the center) and DNA repair mechanisms responsible for removal of these lesions (shown at the bottom).

Translesional synthesis

When the replication machinery stalls at a DNA lesion, specialized low-fidelity polymerases are recruited to the site of the lesion. These specialized low-fidelity polymerases replace the high-fidelity replicative polymerase and synthesize a short stretch of DNA across the lesion (**Fig.2**) (Vincent Page & RP Fuchs, Oncogene 2002). This process of bypass of a lesion mediated by specialized polymerases is known as translesional synthesis. Since these specialized polymerases have low fidelity, i.e. they lack proof reading activity, they can lead to incorporation of incorrect nucleotides during repair making TLS highly mutagenic (Friedberg et al. 2002). Thus TLS is a key source of mutagenesis and has far-reaching implications in processes like antibiotic resistance and pathogenesis in bacteria.

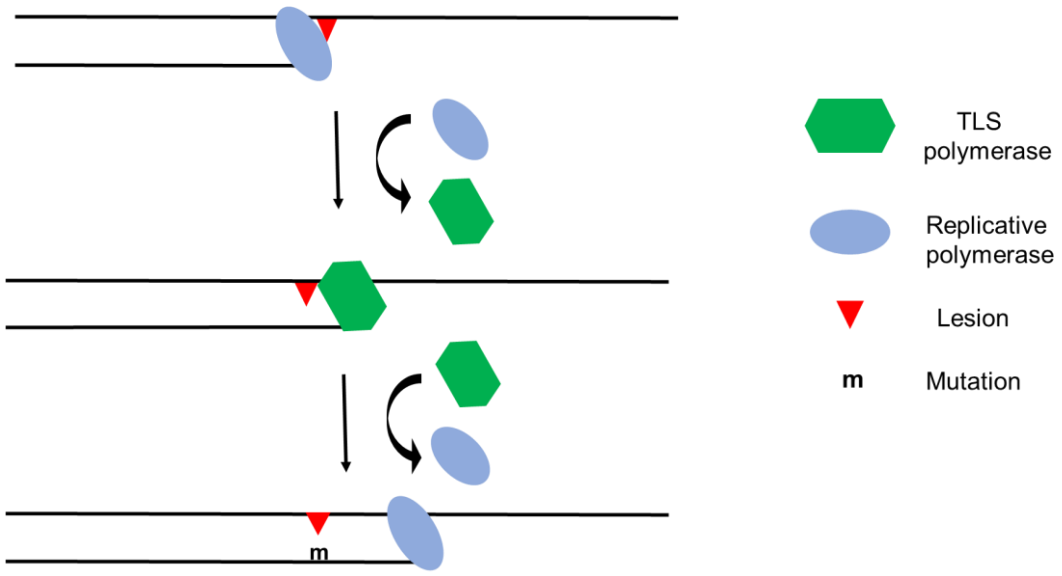

Fig.2. Switching of polymerases during translesional synthesis: This schematic represents switching of replicative polymerase (blue) with TLS polymerase (green) at the lesion site (red). TLS polymerase synthesis is mutagenic which is represented by the (**m)** (from Cordonnier and Fuchs, 1999).

Many of the error-prone polymerases studied so far are induced as a consequence of SOS response, a mechanism activated by bacterial cells facing the DNA damage. Studies suggest that SOS response is induced by formation of a RecA filament on single-stranded DNA, which in turn mediates auto-cleavage of LexA (J.W Little 1984). LexA auto-cleavage results in de-repression of the SOS regulon and expression of

genes involved in repair, including the TLS pathway. In *E.coli* approximately forty genes are upregulated upon induction of SOS response (Courcelle and Hanawalt 2001; Quillardet, Rouffaud, and Bouige 2003). These genes usually code for proteins which are involved in the repair, mutagenesis, replication and DNA metabolism (C. Janion 2008). Mechanism of translesional synthesis has been widely studied in *E. coli,* for error-prone polymerases Pol IV or DinB (Bunting, Roe, and Pearl 2003) and Pol V or UmuC (which is activated by UmuD') (Burckhardt et al. 1988).

UmuC is involved in bypass of abasic sites, photoproducts such as N-2-acetylaminofluorene, thymine-thymine cyclobutane pyrimidine dimer, thymine-thymine dimer, and adducts formed from oxidized dG (Ippoliti et al. 2012). DinB is reported to bypass various lesions such as 8-oxo-dG, O6- me-dG, AP site, AAF and AF (Tang et al. 2000; Suzuki et al. 2001; Shen et al. 2002; Maor-Shoshani et al. 2003). UmuC is highly mutagenic and is inaccurate even when an undamaged template is provided (Reuven N.B et.al.1999; Tang, M; 1999), while overexpression of DinB results in minor growth defects (Kuban et al. 2005). Since both these polymerases are error-prone and lead to incorrect incorporation of nucleotides during bypass of the lesion, they have been implicated as a major cause for mutagenesis in the bacterial cells.
Studies have reported that both DinB and UmuC interact with replisome components. Both *in vivo* and *in vitro* studies have shown the interaction of UmuC with UmuD, RecA, single strand binding (SSB) protein, β-clamp and DNA pol III (Tippin, B 2004). DinB is also known to interact with Rep DNA helicase (Sladewski, Hetrick, and Foster 2011) and the β-clamp (Bunting, Roe, and Pearl 2003). Additionally, DinB has been shown to co-localize with RecA which suggest that they both might interact. (Mallik et al. 2015). Indeed, interaction of β-clamp is essential for the UmuC and DinB to carry out the TLS repair (Becherel et al. 2002; Tippin, B 2004). These observations suggest that the DinB and UmuC mediated TLS might be replication dependent and require different components of the replisome for repair.

It is also known that many bacteria have alternative polymerases for TLS, which are relatively uncharacterized compared to DinB or UmuC. Bacteria such as *Caulobacter crescentus*, *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* have another specialized polymerase known as ImuC, which is implicated in TLS repair (Ippoliti et al. 2012). Unlike DinB and UmuC, ImuC belongs to the C-family polymerase and shows close similarity to DnaE, the replicative polymerase in bacteria. Interestingly,

bacteria with ImuC seem to lack UmuC polymerase. Additionally, in many bacteria ImuC is seen to co-occur with two other proteins, ImuA and ImuB which are implicated to have accessory functions in TLS repair (Galhardo et al. 2005; Warner et al. 2010). However, in case of DinB and UmuC, no accessory proteins have been identified so far. The exact mechanism and regulation of ImuC-mediated TLS and role of accessory proteins remains unclear.

Caulobacter crescentus as a model organism to study TLS repair

In *Caulobacter crescentus imuA*, *imuB* and *imuC* genes occur as part of a single operon (**Fig.3**). ImuA is a hypothetical protein of unknown function, with homology to SulA, a cell division inhibitor in *E. coli* and RecA, the recombinase required for homologous recombination and SOS response (Alves et al. 2017). ImuB belongs to Y-family polymerases, however the catalytic site of ImuB in *M. tuberculosis* is reported to be mutated, likely leading to an inactive polymerase (McHenry 2011; Warner et al. 2010). It is known that the expression of *imuABC* gene cassette is repressed by LexA under normal growth conditions and is induced by SOS response (Da Rocha et al. 2008). Studies report that deletion of the *imuABC* cassette in *C. crescentus* results in increased sensitivity towards mitomycin C and ultra-violet rays. It was observed that individual deletions of *imuA*, *imuB* and *imuC* showed similar sensitivity to these damaging agents as deletion of the complete *imuABC* operon, suggesting that these proteins function in the same pathway (Galhardo et al. 2005). Though these genes are conserved in vast number of bacterial genomes, the exact mechanism of action in the context of TLS remains unexplored. Hence, studying ImuC-mediated TLS repair in *C. crescentus* can help in understanding the mechanism of TLS in other bacteria where ImuA, ImuB and ImuC are conserved. This study will additionally aid in identifying the differences between TLS mediated by C-family polymerases and Y-family polymerases. The specific objectives of this project are as follows:

I. Role of ImuC and accessory proteins in DNA damage repair
II. Interaction and regulation of ImuC-mediated repair pathway

Caulobacter crescentus is an ideal system to study ImuC-mediated TLS and understand role ImuA and ImuB in TLS repair as well as identify alternative functions of these proteins. *C. crescentus* is gram negative α-proteobacterium found in nutrient deficient environmental conditions. *C. crescentus* follows asymmetric cell division giving rise to a motile swarmer cell and a sessile stalked cell. This allows for easy synchronization and isolation of cells at specific cell cycle stages. Furthermore, the availability of excellent genetic tools allows for the *in vivo* study of chromosome dynamics and processes like replication and repair (Thanbichler, Iniesta, and Shapiro 2007).

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Fig.3. Genomic context of *imuABC* operon in *C. crescentus*: The genes *imuA, imuB*, *imuC* co-occur in an operon along with small non-coding RNA (CCNA_R0074).

Materials and Methods

- **Growth conditions**

Escherichia coli strains were cultured in Luria Bertani (LB) broth or LB containing 1.5% agar at 37°C. *Caulobacter crescentus* strains were routinely cultured in PYE (0.2% bactopeptone, 0.1% yeast extract, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$) broth or PYE containing 1.5% agar at 30°C. For bacterial two-hybrid assay, *E. coli* BTH101 strains were grown in M63 minimal media for 24 hours at 37°C. These strains were spotted on MacConkey agar containing 1% maltose, 1 mM IPTG, 100 µg/ml carbenicillin and 50 µg/ml kanamycin. These plates were incubated at 30°C for four days.

MacConkey plate

To prepare MacConkey plates, 40 g of MacConkey agar was dissolved in one litre distilled water and autoclaved at 121°C for 45 mins. Before pouring the plates filter sterilized maltose (1%), IPTG (isopropyl-β-D-thiogalactopyranoside 1 mM) and antibiotics carbenicillin (100 µg/ml) and kanamycin (50 µg/ml), were added to the autoclaved medium.

M63 minimal medium

To prepare 5X stock of M63 salts, 2 g of $(NH_4)_2SO_4$, 13.6 g of KH_2PO_4, 0.5 mg of $FeSO_4.7H_2O$ were dissolved in one litre distilled water, pH was adjusted to 7.0 with KOH and autoclaved. To prepare M63 minimal medium, 5X M63 stock solution was diluted to 1X and filter sterilized maltose (0.2%), glucose (0.4%), IPTG (1 mM), $MgSO_4.7H_2O$ (1 mM), 2 ml of 0.05% vitamin B1 (thiamine) and antibiotics carbenicillin (50 µg/ml) and kanamycin (25 µg/ml) were added to the 1X M63 solution.

- **Bacterial strains and plasmids**

Strains and plasmids used in this study are listed in Table 1. Primers used in this study are listed in Table 2.

Construction of deletion strains

Deletion of target genes was carried out using two-step recombination with sacB counter-selection. Flanking regions, (approximately 600 bp upstream and downstream) of the target gene was PCR amplified using primers mentioned in Table

2. These fragments were gel purified, ligated to pNPTS138 plasmid using Gibson assembly protocol and transformed into *E. coli* DH5α strain. Purified plasmids were confirmed by PCR. These plasmids were electroporated into *C. crescentus* CB15N strain and selected on kanamycin containing PYE agar. Resistant colonies were grown in media containing 3% sucrose without kanamycin to select for plasmid excision. Colonies which were sucrose resistant but kanamycin sensitive were confirmed using PCR for deletion of the target gene.

Construction of plasmids for bacterial two-hybrid assay

Plasmid constructs for bacterial two-hybrid assay were generated by cloning genes into pUT18C and pKT25 vectors at EcoRI and XbaI restriction sites using primers mentioned in Table 2. Positive clones were confirmed by restriction digestion followed by sequencing. For bacterial two-hybrid assay, pUT18C and pKT25 constructs harbouring different genes were co-transformed into *E. coli* BTH101 strain and selected on media containing antibiotics carbenicillin (50 µg/ml) and kanamycin (25 µg/ml).

- **Spotting assay**

Wild type CB15N and deletion strains were grown overnight in 10 ml PYE broth at 30˚C. Cultures were backed diluted to 0.1 OD and were incubated at 30˚C until they reached an approximate OD of 0.3. All cultures were normalized to 0.3 O.D and serially diluted from 10^{-1} to 10^{-8}. 6 µl each of all dilutions were spotted on plain PYE agar or on PYE agar containing different concentrations of the DNA damaging agents mentioned in Table 1B. The plates were incubated at 30˚C for two days and pictures were taken.

- **Bacterial two-hybrid experiment**

Protein interactions were tested using bacterial adenylate cyclase two-hybrid system (Ladant and Ullmann 1999). The genes of interest were fused to T25 and T18 fragments of adenylate cyclase in pKT25 and pUT18C vectors respectively. pUT18C construct harbouring a particular gene and pKT25 construct harbouring another gene were co-transformed into BTH101 strain and selected on LB agar containing antibiotics kanamycin and carbenicillin. For testing interactions, co-transformants were grown in M63 minimal medium with maltose, IPTG and antibiotics at 37°C until saturation. The cultures (5 µl each) were spotted on MacConkey agar containing maltose, IPTG and

appropriate antibiotics and incubated at 30˚C for four days. Pictures were taken after four days of incubation.

Table 1A. Strains and plasmids used in this study.

Strain	**Relevant features**	**Reference**
Dh5α	dlacZ Delta M15 Delta(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 relA1	
CB15N	*Caulobacter cresentus,*wild type strain	Evinger et al. 1977
BTH101	F-, cya-99, araD139, galE15, galK16, rpsL1 (Str r), hsdR2, mcrA1, mcrB1	Ladant et al. 1999
CB15N_Δ*imuABC*	Wild type strain with *imuABC* deleted	This study
CB15N_Δ*imuC*	Wild type strain with imuC deleted	This study
CB15N_Δ*recA*	Wild type strain with recA deleted; used as positive control	Badrinarayanan et al. 2017
BTH101_AB818	BTH101::pKT25+pUT18C;used as negative control	Badrinarayanan et al. 2017
BTH101_AB668	BTH101::pKT25_AddA+pUT18C_AddB;used as positive control	Badrinarayanan et al. 2017
BTH101_AB305	BTH101::pKT25_SocA+pUT18C_SocB; used as positive control	Badrinarayanan et al. 2017
Plasmids	**Relevant features**	**Reference**
pNPTS138	Integration vector (kan^r) with sacB counterselection	Dickon Alley
pKT25	Kan^r, encoding T25 fragment, multi cloning site downstream of T25	Ladant et al. 1999
pUT18C	Amp^r, encoding T18 fragment, multi cloning site upstream of T18	Ladant et al. 1999
pKT25_*imuA*	Full length CC_3213 of with T25 fragment	This study
pKT25_*imuB*	Full length CC_3212 of with T25 fragment	This study
pKT25_*imuC*	Full length CC_3211 of with T25 fragment	This study
pKT25_*dnaN*	Full length CC_0156 of with T25 fragment	This study
pKT25_*dnaE*	Full length CC_1926 of with T25 fragment	This study
pKT25_*recA*	Full length CC_1087 of with T25 fragment	Badrinarayanan et al. 2017
pKT25_*mmcB*	Full length CC_3467 of with T25 fragment	This study
pUT18C_*imuA*	Full length CC_3213 of with T18 fragment	This study
pUT18C_*imuB*	Full length CC_3212 of with T18 fragment	This study
pUT18C_*imuC*	Full length CC_3211 of with T18 fragment	This study
pUT18C_*dnaN*	Full length CC_0156 of with T18 fragment	This study
pUT18C_*dnaE*	Full length CC_1926 of with T18 fragment	This study
pUT18C_*recA*	Full length CC_1087 of with T18 fragment	Badrinarayanan et al. 2017
pUT18C_*mmcB*	Full length CC_3467 of with T18 fragment	This study

Table 1B. Concentrations of damaging agents used in this study.

Damaging Agent	**Concentrations**
Mitomycin C (MMC)	0, 0.125, 0.25, 0.5, 1 (µg/ml)
Methyl Methanesulfoante (MMS)	0, 0.25 ,0.5 ,0.75 ,1 ,1.25 ,1.5 (mM)
Norfloxacin	0, 2, 4, 6, 8, 10 (µg/ml)
Hydroxyurea (HU)	0, 2, 3, 4, 5, 6, 8, 10 (mM)
Ultra-violet (UV)	0, 10, 20, 30, 40, 50, 60, 70, 80, 90,100 (J/m^2)

Table 2. Primers used in this study.

Primer Name	**Sequences (5'-3')**
Bacterial two-hybrid primers	
*imuA*_forward_XbaI	TTATtctagaTATGGAGTTGGGAATGGCCGGAT
*imuA*_reverse_EcorI	TTATGAATTCTTATCCGAAGCGTCGTCCGGC
*imuB*_forward_XbaI	TTATtctagaTATGGGTCTCTTCCCCGGGCAG
*imuB*_reverse_EcorI	TTATGAATTCCTAACCAAACAGGCCATGGATCCACC
*imuC*_forward_XbaI	TTATtctagaTATGCGCCCGCCCGTCTATG
*imuC*_nter_reverse_EcorI	TTATGAATTCTCAATGGAAATCGCGGCTGC
*imuC*_reverse_EcoR1	TTATGGTACCTCAATGGAAATCGCGGCTGC
*dnaE*_forward_XbaI	TTATTCTAGATATGTCGGACGCGGAGGGG
*dnaE*_reverse_EcorI	TTATGAATTCCTAAACGTCTTCCAGCAGCGCCAC
*dnaN*_forward_Xba1	TTATtctagaTATGAAGCTTACGATCGAACGGGCG
*dnaN*_reverse_Ecor1	TTATgaattcTCAGACCCGCAGCGGCAT
*mmcB*_forward_XbaI	TTATtctagaTATGGACGTGATCATCGAACTGGC
*mmcB*_reverse_Ecor1	TTATGAATTCCTAAAGGCTGAGGCGCTC
Deletion primers	
del_i*muC*_up_Forward	CAAGCTTCTCTGCAGGATATCTGGACGCTGGCGCCGTTGATC
del_*imuC*_up_Reverse	ATCGCGCCCCGCTCACATGTTAGGTCCTCCCCCTCGC
del_*imuC*_down_Forward	GGAGGACCTAACATGTGAGCGGGGCGCGATCCT
del_*imuC*_down_Reverse	CGGAGACGCGTCACGGCCGAAGGCGACATGCGGGTCAGCA

Results

1) Role of ImuC and accessory proteins in DNA damage repair

Specificity of ImuC-mediated DNA repair:

Previous studies have shown that *imuABC* deletion in *C. crescentus* is sensitive to DNA damage induced by mitomycin C and ultra-violet rays (Galhardo et al. 2005). To test the specificity of ImuC-mediated DNA repair to particular types of DNA lesions, sensitivity of Δ*imuC* and *ΔimuABC* strains to various DNA damaging agents was checked. Growth of these strains under a range of concentrations of different DNA damaging agents was compared to the wild type and Δ*recA* strains using a serial-dilution spotting assay. Since SOS induction is affected in Δ*recA* strains, it is highly sensitive to DNA damaging agents, and hence serves as a positive control for the experiment. The damaging agents used in the study were mitomycin C (MMC), methyl methanesulfonate (MMS), norfloxacin, hydroxyurea (HU) and ultra-violet rays (UV) with different modes of actions, leading to a variety of DNA lesions. Methyl methanesulfonate and mitomycin C are both alkylating agents. Mitomycin C is a bi-functional alkylating agent that methylates two guanine residues located on cis or trans strands which leads to intra or inter-strand crosslinks, while methyl methanesulfonate is a mono-functional alkylating agent which methylates guanines and adenines on the DNA. Norfloxacin inhibits the activity of DNA gyrase which can subsequently result in double-stranded breaks whereas hydroxyurea depletes dNTP pool and slows down replication progression by inhibiting the enzyme ribonucleotide reductase. Ultraviolet rays cause crosslinks between thymines.

In this experiment it was observed that compared to the wild type strain, *ΔimuC* and *ΔimuABC* strains show increased sensitivity to MMC and modest sensitivity towards UV rays. However their sensitivity towards MMS, HU and norfloxacin was similar to that of the wild type strain. Interestingly, *ΔimuC* and *ΔimuABC* strains were found to be sensitive towards MMC stress but not towards MMS stress even though both of them are alkylating agents (**Fig.4**). These results invoke the possibility that ImuC is critical only in the context of DNA lesions involving crosslinks.

Fig.4.1

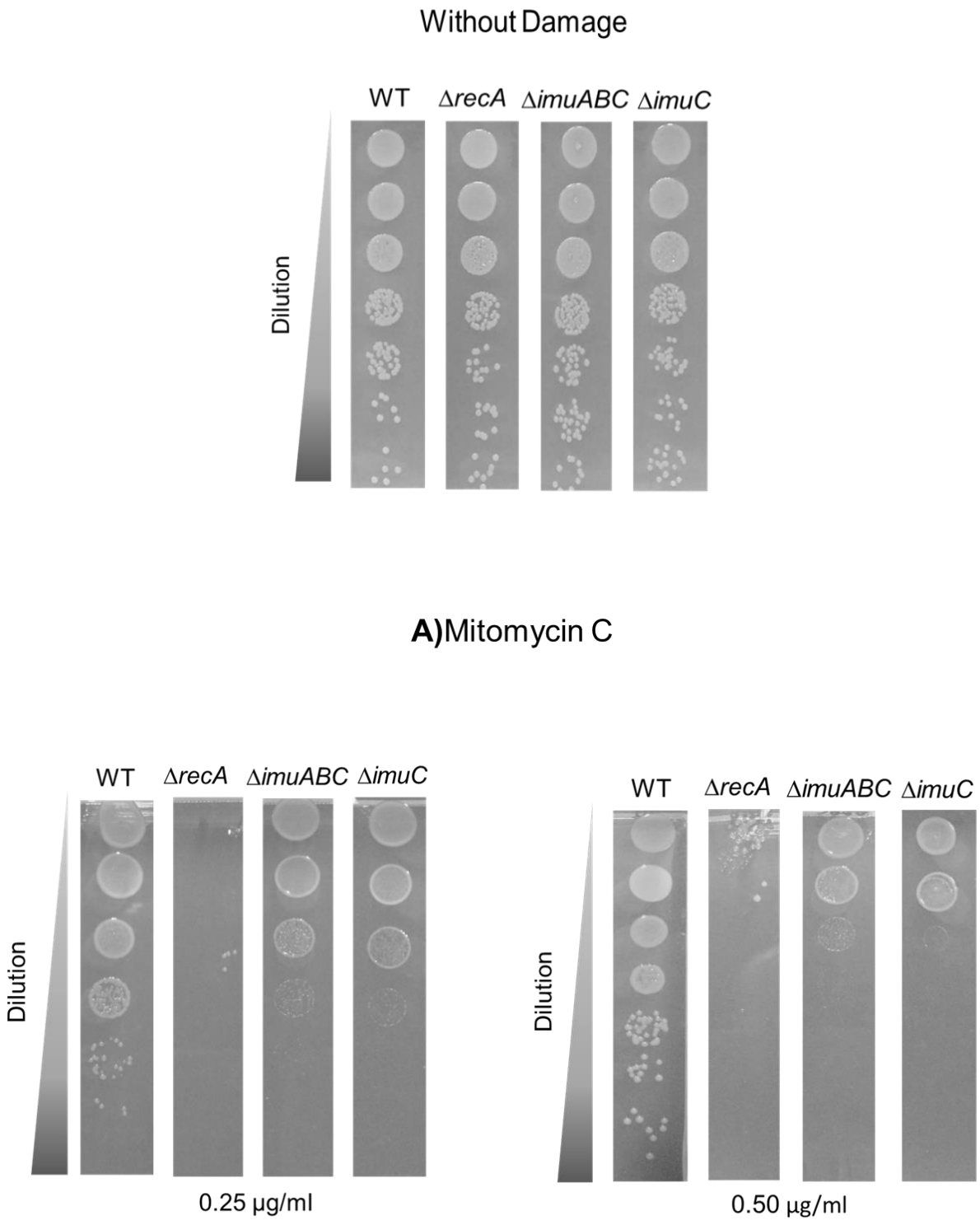
Without Damage
WT
ΔrecA
ΔimuABC
ΔimuC
Dilution
A)Mitomycin C
WT
ΔrecA
ΔimuABC
ΔimuC
Dilution
0.25 µg/ml
WT
ΔrecA
ΔimuABC
ΔimuC
Dilution
0.50 µg/ml

Fig.4.2

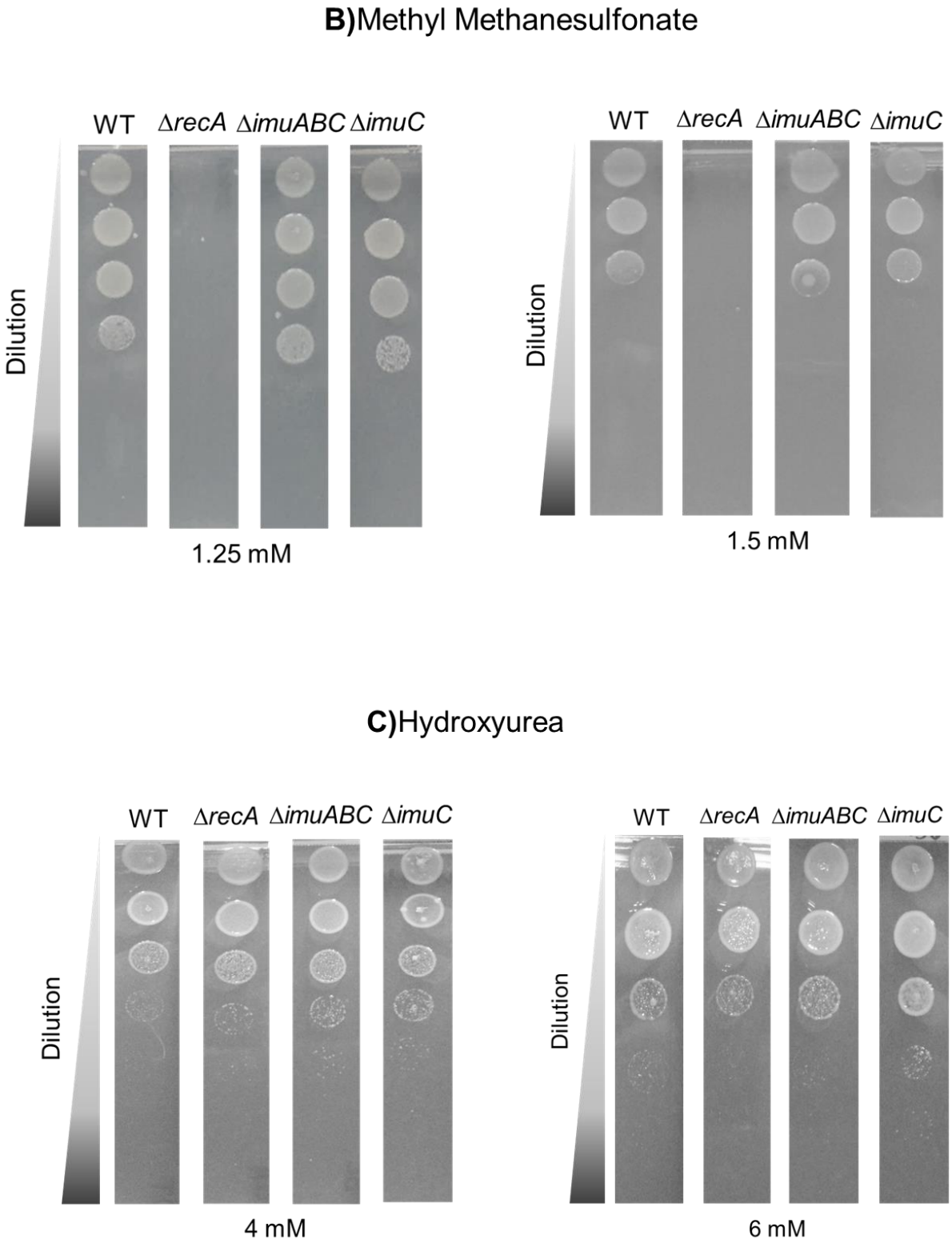
B)Methyl Methanesulfonate
WT
ΔrecA
ΔimuABC
ΔimuC
Dilution
1.25 mM
1.5 mM
C)Hydroxyurea
4 mM
6 mM

Fig.4.3

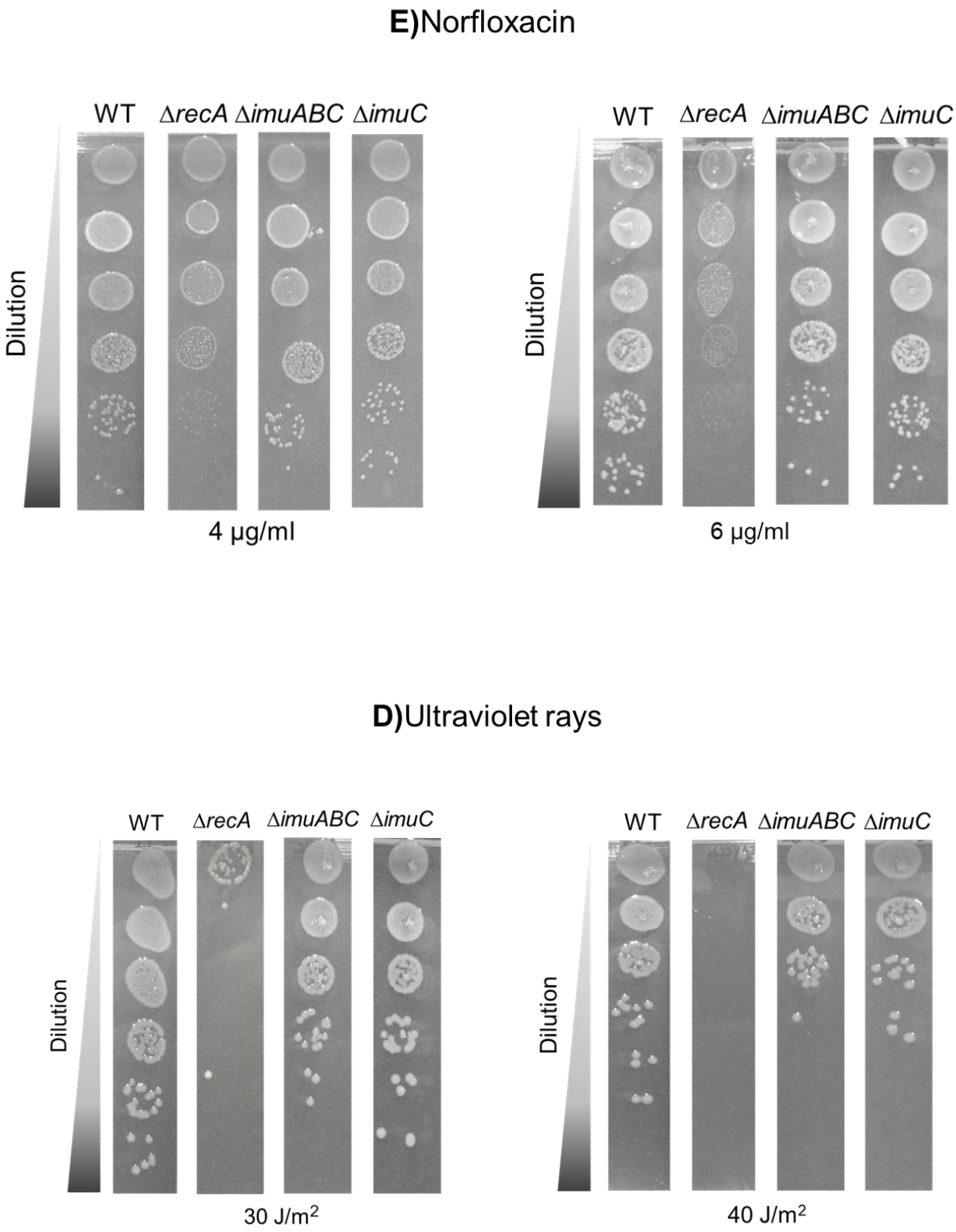

Fig.4. Sensitivity of *ΔimuABC* and *ΔimuC* strains to different DNA damaging conditions. Log fold dilutions (10^{-1} to 10^{-8}) of different strains were spotted on PYE agar 1] without damaging agents or with damaging agents such as 1] A) mitomycin C, 2] B) methyl methanesulfonate and C) hydroxyurea, 3] D) ultraviolet rays and E) norfloxacin.

2) Interaction and regulation of ImuC-mediated repair pathway

Given the specificity in repair, we wondered what interacting partners could mediate such specificity. For this, we employed a bacterial two-hybrid approach to test interactions of the TLS pathway components with each other, with the replication machinery as well as other repair components. These experiments come with the caveat that reverse combination remain to be tested in some cases, while in a small portion of positive interactions, the reverse combination did not produce results (see **Appendix A** for complete summary). In general, all interactions discussed below are going to be tested with an alternative pull-down based approach as well.

Interaction of ImuC with accessory factors ImuA and ImuB:

The *imuC* gene is seen to co-occur with two other genes, *imuA* and *imuB* which are implicated to have accessory functions in TLS repair (Galhardo et al. 2005; Warner et al. 2010). Studies in *M. tuberculosis* have shown that ImuC interacts with ImuB, and ImuB interacts with ImuA' but no interaction was observed between ImuC and ImuA. Additionally, it was shown that ImuB self-associates (Warner et al. 2010). To test

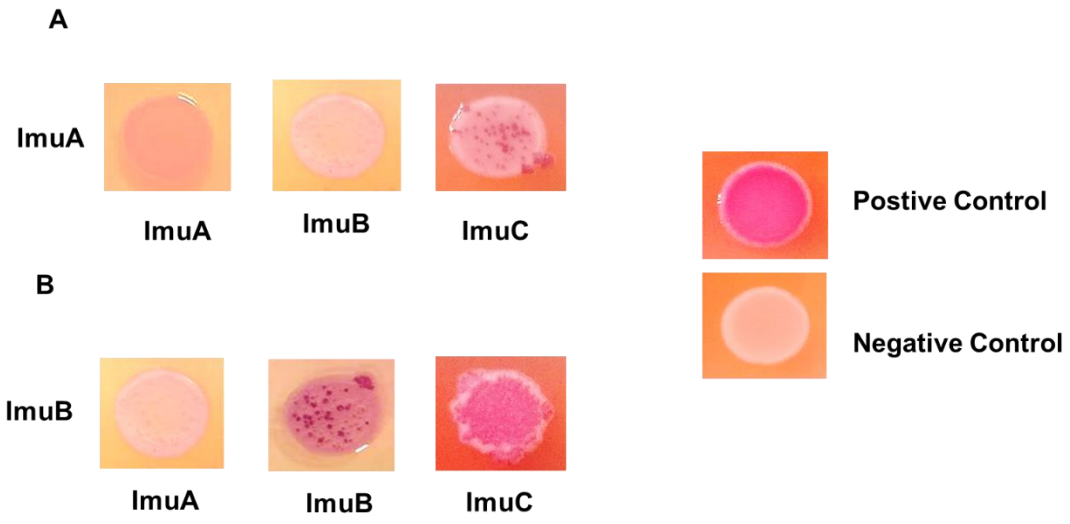

Fig.5. Interaction of ImuC with accessory factors ImuA and ImuB:
Bacterial two-hybrid assay showing interaction between different components of TLS pathway. **A.** ImuA interacts with ImuC but not with ImuB. **B.** ImuB interacts with ImuC and with itself but not with ImuA. Pink colour of the colony depicts interaction and colourless colony depicts no interaction. BTH101 co-transformed with pKT25_*addA* and pUT18C_*addB* was used as positive control and BTH101 co-transformed with empty vectors pKT25 and pUT18C was used as negative control.

similar interactions between translesional components in *C. crescentus* we performed bacterial two-hybrid assay.

Full length *imuA* and *imuB* and N-terminal region of *imuC* were cloned into both bacterial two-hybrid vectors (low-copy) and pUT18C (high-copy). We observed that ImuC interacts with ImuB as shown by the pink colouration of the spot in the BTH101 co-transformed with pKT25_*imuC* and pUT18C_*imuB* (**Fig.5B**). We did not observe interaction between ImuA and ImuB, though ImuA interacts with ImuC **(Fig.5A)** and ImuB was seen to associate with itself.

Interaction between replisome and TLS components:

Apart from physically interacting with each other, TLS components have also been shown to interact with the replication machinery. Studies in *M. tuberculosis* have reported interaction of ImuB with replisome components like DnaN and DnaE (Warner et al. 2010). Additionally, in *E.coli* it was shown that DinB, another TLS polymerase, interacts with DnaN and the Rep DNA helicase (Bunting, Roe, and Pearl 2003; Mallik et al. 2015). We tested the interaction of the TLS components in *C. crescentus* with few of the replisome components using bacterial two-hybrid assay.

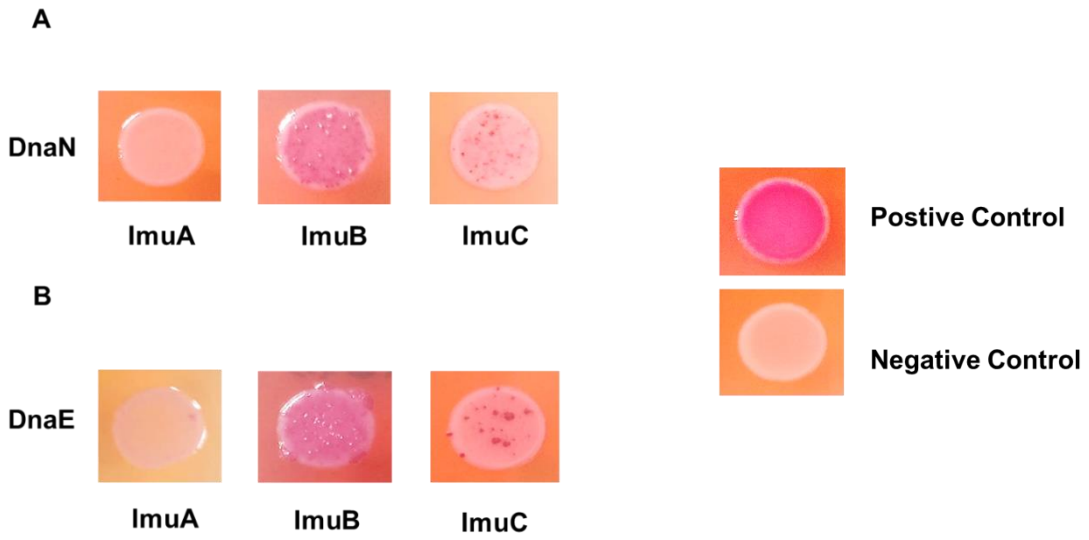

Fig. 6. Interaction of replisome and TLS components:

A. DnaN interacts with ImuB and ImuC, but not with ImuA. **B.** DnaE interacts with ImuB and ImuC, but not with ImuA. Pink colour of the colony depicts interaction and colourless colony depicts no interaction. Controls as in Fig.5.

Full length *dnaN*, *dnaE* were cloned into both bacterial two-hybrid vectors pKT25 and pUT18C. While we did not observe interaction of ImuA with DnaN or DnaE, we found that ImuB showed interaction with DnaN (in both orientations) (**Fig.6A & B**). Co-transformation of pUT18C_*imuC* with pKT25_*dnaN* also resulted in appearance of weak pink colonies, which did not reproduce in the reverse orientation, suggesting that this may not be a positive result. With respect to the replicative polymerase we found ImuB to interact strongly with DnaE (with the reverse orientation remaining to be tested).

Interaction between RecA and TLS components:

Overall, these results suggest the existence of an intricate interaction between the replisome and TLS pathway. Further, these results also suggest that TLS repair may be dependent on ongoing replication. In *E. coli,* TLS activation and activity is also dependent on RecA-mediated induction of the SOS response as well as UmuC activation (Burckhardt et al. 1988; Nohmi et al. 1988). DinB, another TLS polymerase in *E. coli* was also shown to interact with RecA (Godoy et al. 2007). In order to test the possibility of RecA being critical for TLS in *C. crescentus*, we initially checked if RecA showed physical interaction with any of the TLS pathway components.

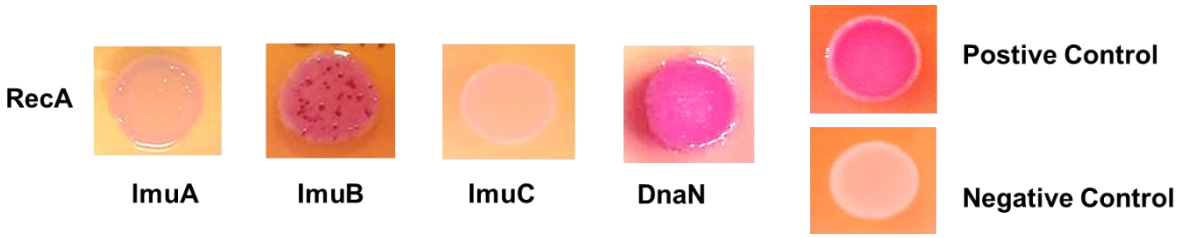

Fig. 7. Interaction between RecA and TLS components:

Bacterial two-hybrid assay showing interaction of RecA with ImuB and DnaN, Pink colour of the colony depicts interaction and colourless colony depicts no interaction. Controls as in Fig.5.

RecA did not show any physical interaction with ImuA, however showed strong interaction with ImuB. This was confirmed by co-transformation of *recA* and *imuB* in both vector backbones. Interaction between ImuC and RecA was not observed in an experiment where pUT18C_*recA* and pKT25_*imuC* were co-transformed, though interaction with full length ImuC remains to be tested (**Fig.7**). Interestingly, RecA was also seen to interact with the replisome component DnaN. These interactions are

indicative of RecA mediating or regulating crosstalk between replisome and TLS components at a DNA lesion.

Interaction of TLS and replisome with new repair component:

Previous studies have shown that putative repair gene *mmcB* is significantly expressed in *C. crescentus* on induction of SOS response (Modell, Hopkins, and Laub 2011). Epistasis analysis done in *C. crescentus* suggest MmcB might be part of the TLS pathway mediated by ImuC (Lopes-Kulishev et al. 2015). To test the physical interaction of MmcB with TLS and replisome components, bacterial two-hybrid assay was performed.

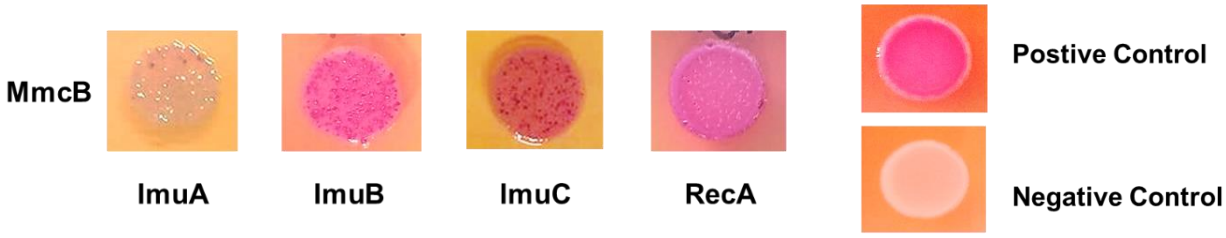

Fig. 8. Interaction of new component MmcB:

MmcB interacts with ImuB, ImuC and RecA, but not with ImuA. Pink colour of the colony depicts interaction and colourless colony depicts no interaction. Controls as in Fig.5.

We observed MmcB interaction with RecA, ImuB and ImuC when *mmcB* was cloned into pKT25. No interaction was observed between MmcB and ImuA (**Fig.8B**). However our reverse constructs appeared to be non-functional and hence the reverse interactions remain to be ascertained.

Schematic representation of the interactions observed in *C. crescentus*:

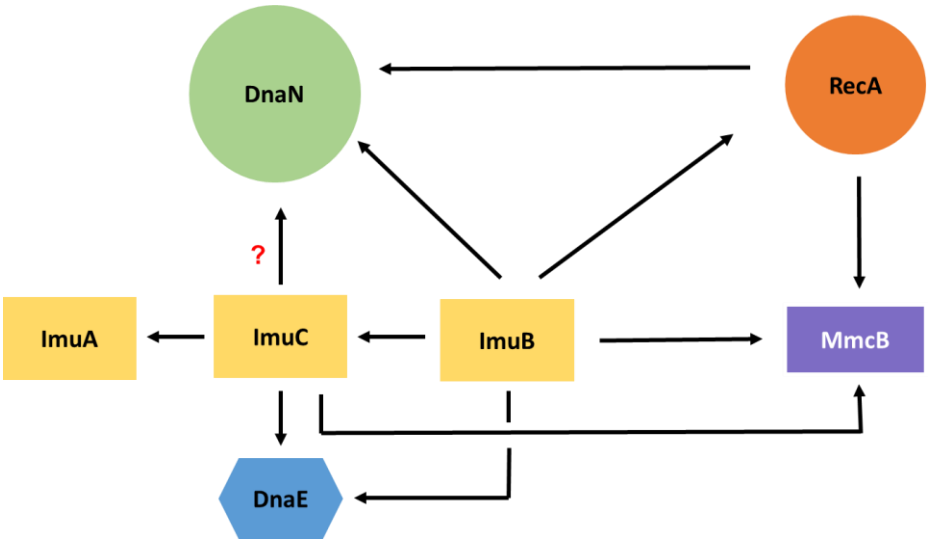

Fig.9. Interactions observed in this study: Black arrows indicate interactions observed between TLS components, replisome components, MmcB and RecA. Details of the plasmid constructs used to test these interactions are detailed in **Appendix A**.

Discussion

Though the phenomenon of TLS and error-prone polymerases have been identified in both prokaryotes and eukaryotes, there is ambiguity in the mechanism and regulation of this repair pathway. Cells encode a variety of DNA repair proteins, which carry out repair functions during damage. Some damages are repaired by the concerted action of two or more repair pathways, while a specific pathway exclusively handles some other damages. The exact mechanism of pathway choices and specificity of damage repair is an active area of research. In the first part of the study, the specificity of error-prone polymerase and TLS was addressed. Our results clearly suggest that the error-prone polymerase ImuC is associated with repair of specific types of DNA lesions, most likely those involving inter or intra-strand crosslinks. Transposon sequencing carried out in our collaborator's laboratory also support these results (Tung Le, unpublished data). Currently, the *imuC* deletion strain sensitivity to other DNA crosslinking agents are being tested. Additionally, we tested the effect of *imuABC* and *imuC* deletion on cell filamentation, replisome and divisome (using fluorescently tagged replisome and divisome associated proteins) during mitomycin C stress (**Appendix B**, **C**, **D & E**). We did not observe any significant differences between WT and deletion strains with respect to the analysis we performed. However, ongoing experiments in the lab suggest that ImuC might affect the replisome dynamics during certain types of DNA damage.

The observations in the first part of the study indicate the specificity of error prone polymerase, but it is unclear how this specificity is mediated. To gain further insight into this, we identified multiple proteins that interact with the TLS machinery. Studies have suggested that exchange of TLS polymerases with the replicative polymerase at the replication fork is stochastic, which is based on the law of mass action depending on the concentration of different polymerases and their binding affinity towards the β-clamp (Sutton and Walker 2001; Fujii and Fuchs 2004,Sutton 2010; Sale 2013). Nevertheless, few recent studies have proposed that the exchange of TLS polymerases at the replication fork is not solely dependent on laws of mass action, and there could be additional levels of regulation mediated by proteins that are present at the lesion. Our experiments show that TLS pathway components interact with few replisome as well as repair associated proteins. *In vitro* studies suggest that RecA is essential for the function of TLS polymerases (like Pol II, IV and V) and inhibit the

replicative polymerase (Indiani C, et al 2013). Physical interaction has been shown between RecA and the TLS polymerase DinB (Godoy et al. 2007), and RecA is known to be essential for activation of UmuC (Burckhardt et al. 1988). Our results show that RecA interacts with few of the replisome components as well as with repair proteins including components of ImuC-mediated TLS. Previous research has shown that RecA interacts with double-strand break repair components like AddA (Badrinarayanan, et al. 2017) and RecN (unpublished data). Our current results also suggest an interaction between recently identified repair protein, MmcB with RecA and TLS components. MmcB is predicted to be an endonuclease from PD-(D/E)XK superfamily (Lopes-Kulishev et al. 2015). While the function of this protein in repair remains unknown, recent studies have proposed a role for MmcB in TLS-mediated repair.

Together, our studies suggest a central role for RecA in DNA repair and particularly invokes the possibility of RecA mediating the recruitment of multiple repair pathways to a DNA lesion. These results raise an interesting question about the role of RecA in coordinating DNA damage repair pathway choice. Pull down of RecA will be done to validate these interactions observed in the bacterial two-hybrid assay. This experiment will also reveal the identity of other novel interacting partners of RecA which would be important in DNA repair. In order to identify the significance of RecA at the damage site, localization, and dynamics of these novel interacting partners of RecA during damage will be tested in a *recA* deletion strain with constitutive SOS induction.

To summarize, our study proposes that ImuC-mediated TLS repair is damage specific and might be critical for repair of DNA lesions involving crosslinks. Our bacterial two-hybrid assay reveals that RecA interacts with multiple repair components suggesting the role of RecA as a master regulator at a lesion and could be crucial in mediating repair pathway choice. Interaction of RecA with DnaN may also suggest that there is interplay between the processes of replication and repair. This study further shows interaction of TLS proteins with new repair component MmcB which suggests the crosstalk between different repair pathways or proteins at a lesion.

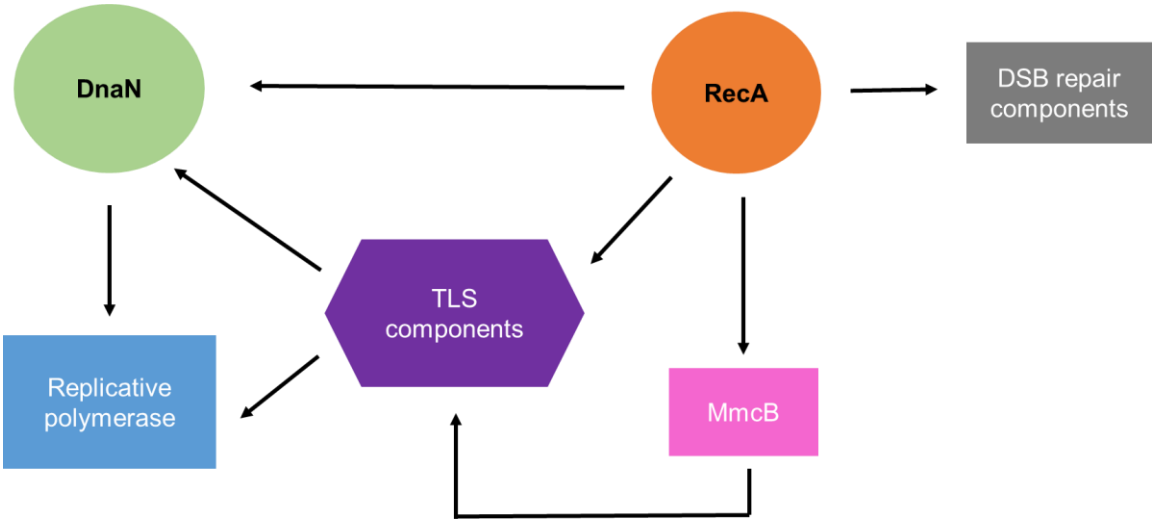

Fig.10. Model for interaction between replication and repair proteins: Schematic showing multiple interaction between RecA and other components and interaction between TLS components with the replisome components.

Future Directions

- The role of ImuA and ImuB in ImuC-mediated TLS repair
- The regulation of specificity of ImuC-mediated TLS repair
- The role of RecA and DnaN is modulating specificity of repair and subsequently pathway choices

Future experiments

- Quantitative RT-PCR to check the expression of *imuC* in *imuA* and *imuB* deletion strains
- Sensitivity of *imuC* deletion strain to other cross linking agents
- Pull down of RecA and DnaN to identify novel interacting partners
- Bacterial two-hybrid screen with RecA and DnaN to identify novel interacting partners
- Fluorescence microscopy to check ImuC localization with DnaN and RecA in damage conditions
- Dynamics of DnaN, DnaE during DNA damage in a strain with constitutive SOS induction and *recA* deletion

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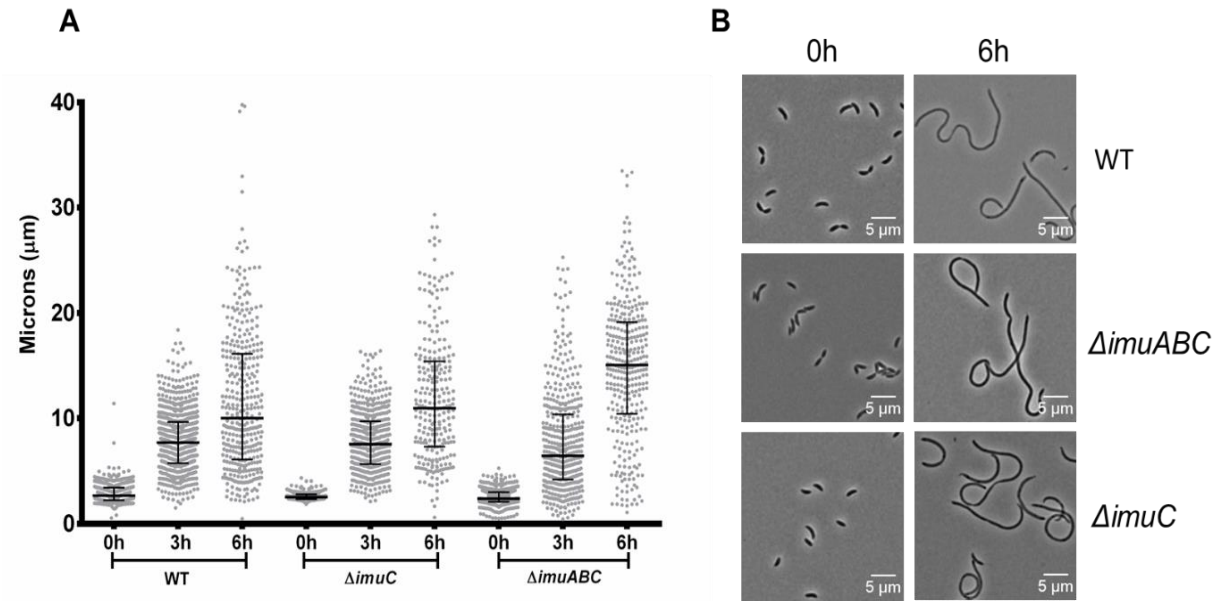
Appendix A:

Sr.No	Interactions	pKT25+pUT18C	pUT18C+pKT25
1	DNAE2+DNAN	√	×
2	DNAE2+IMUA	√	×
3	DNAE2+IMUB	√	ND
4	DNAE2+DNAE1	√	ND
5	DNAE2+DNAE2	ND	ND
6	DNAE2+RECA	ND	×
8	DNAE2+MMCB	ND	√
9	IMUB+DNAN	√	√
10	IMUB+IMUA	×	×
11	IMUB+IMUB	√	
12	IMUB+DNAE1	√	ND
13	IMUB+DNAE2	ND	√
14	IMUB+RECA	√	√
16	IMUB+MMCB	×	√
17	IMUA+DNAN	×	×
18	IMUA+IMUA	×	
19	IMUA+IMUB	×	ND
20	IMUA+DNAE1	×	ND
21	IMUA+DNAE2	ND	√
22	IMUA+RECA	×	×
23	IMUA+MMCB	×	×
24	MMCB+MMCB	x	
25	MMCB+RECA	√	×
26	MMCB+DNAN	ND	×
27	RECA+RECA	√ (known) validated by our experiment	
28	RECA+DNAN	ND	√

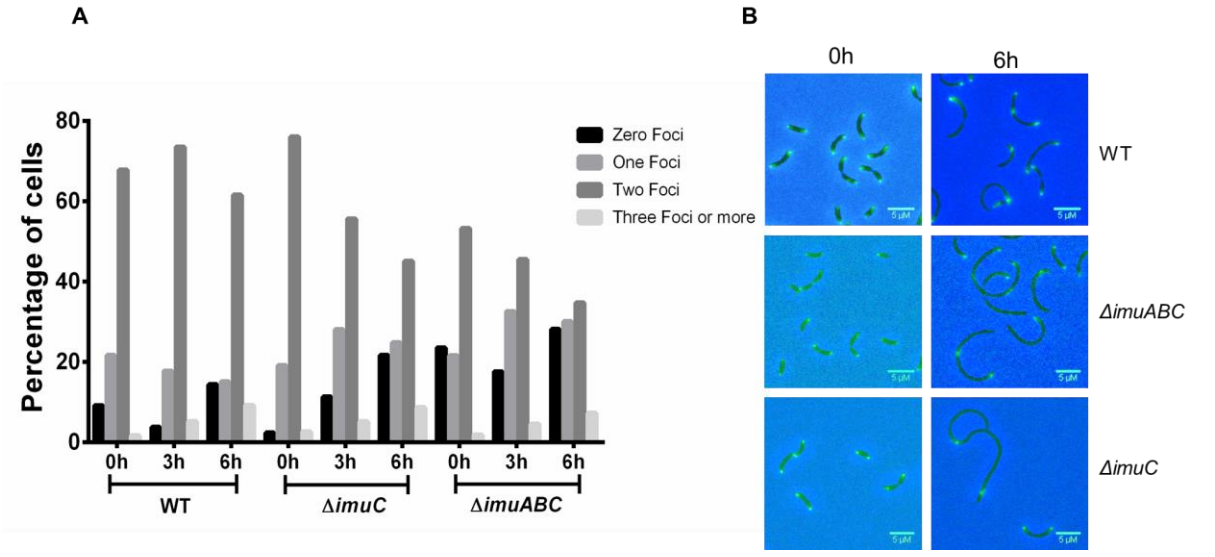
Appendix A: Table showing co-transformations done in bacterial two-hybrid assay. Co-transformations that showed interaction (√), co-transformations that did not show interaction (×), co-transformations have not been done (ND), co-transformations that did not show interaction most likely due to non-functional constructs (×) are listed here.

Appendix B:



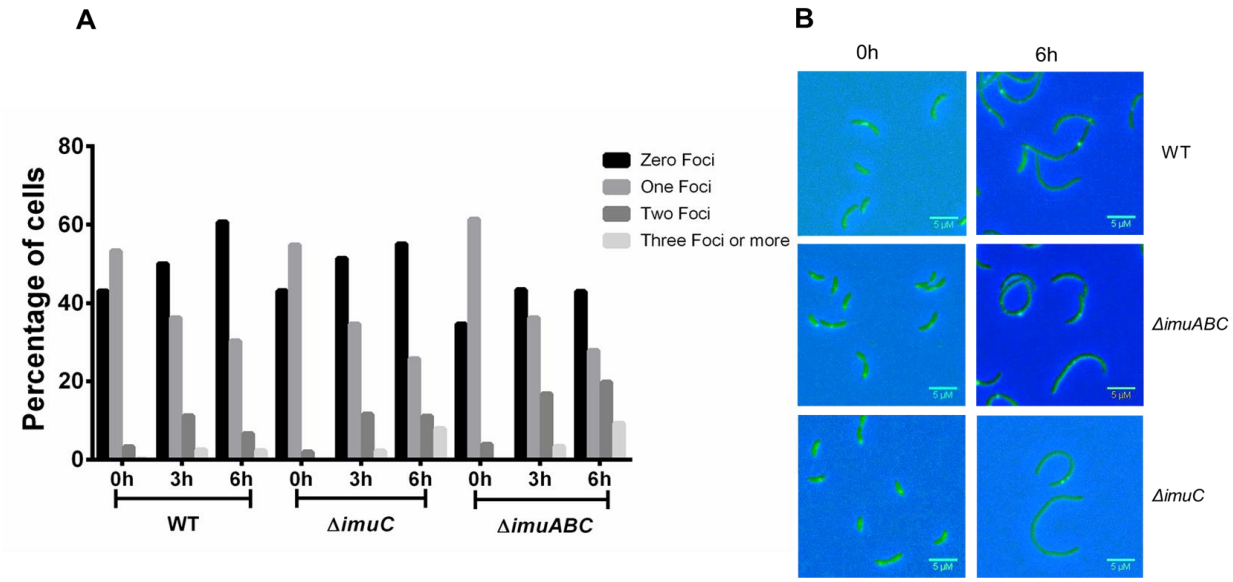
Appendix B: Cell length of WT, *ΔimuABC* and *ΔimuC* during damage. A) Quantitative analysis of cell length of different strains during damage. Y-axis represents cell length in microns and X axis represents duration of mitomycin C treatment. Each dot represents a single cell and ≥300 cells were counted in each group. B) Phase images of WT and *ΔimuABC* strains at different time points, during mitomycin C treatment (scalebar - 5 µm).

Appendix C:



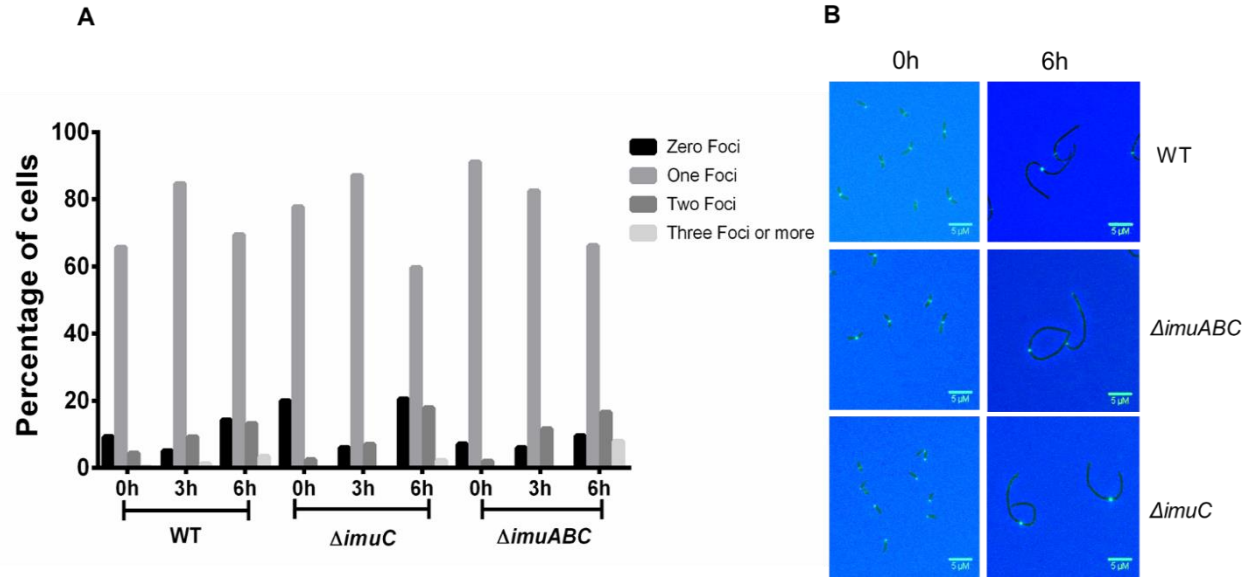
Appendix C: MipZ-YFP foci in cells to assess replication initiation in different strains during mitomycin C induced damage. A) Y-axis represents the percentage of cells with zero, one, two or three foci and X-axis represents duration of mitomycin C treatment. B) Fluorescence images of WT and *ΔimuABC, ΔimuC* strains at zero and six hour time points, during mitomycin C treatment (scalebar - 5 µm).

Appendix D:



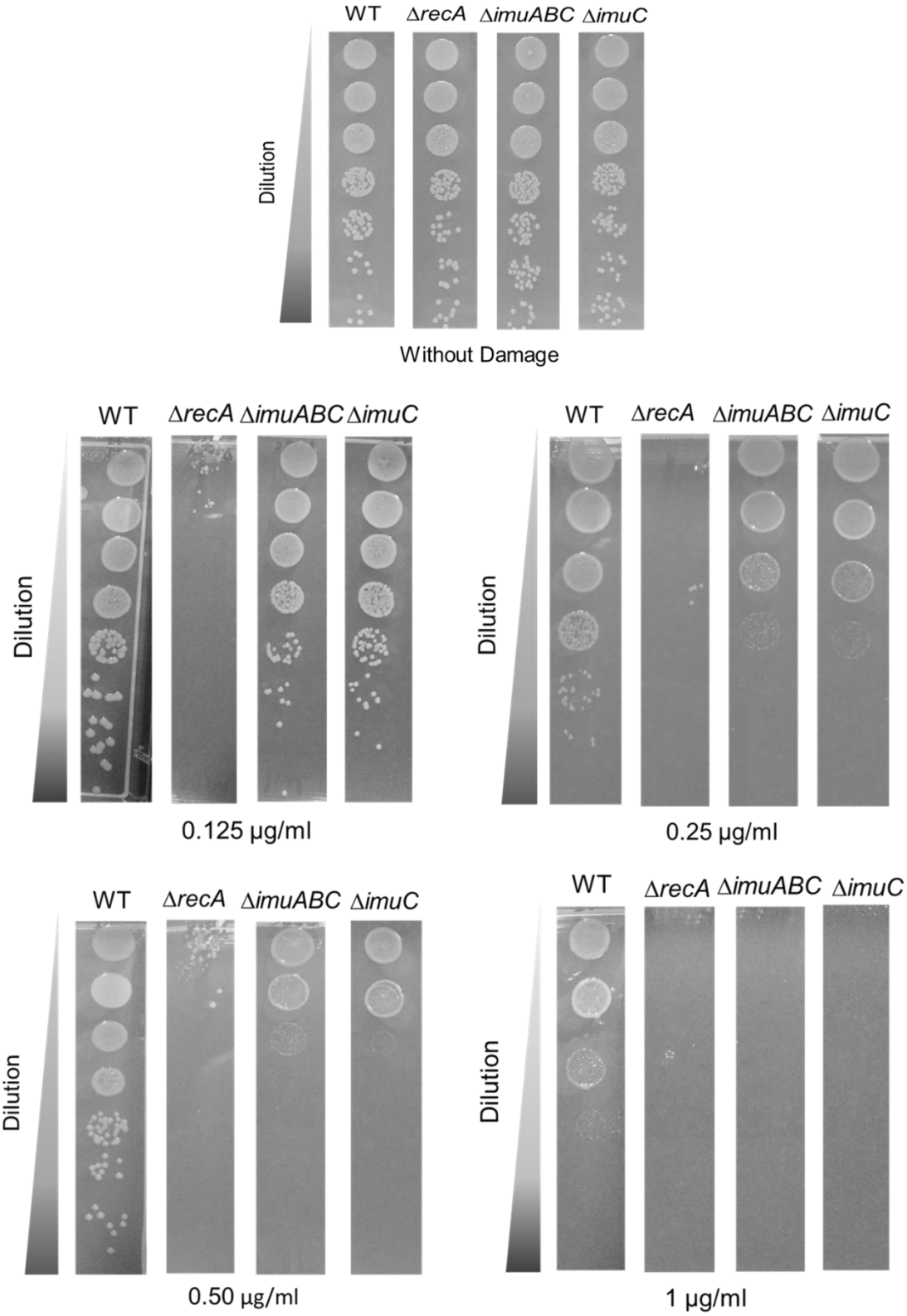
Appendix D: DnaN-mcherry foci in cells to assess replication in different strains during mitomycin C induced damage. A) Y-axis represents the percentage of cells with zero, one, two or three foci and X-axis represents duration of mitomycin C treatment. B) Fluorescence images of WT and *ΔimuABC, ΔimuC* strains at zero and six hour time points, during mitomycin C treatment (scalebar - 5 µm).

Appendix E:



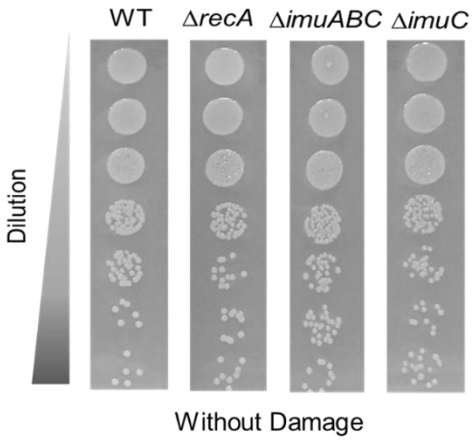
Appendix E: FtsZ-YFP foci in cells to assess division in different strains during mitomycin C induced damage. A) Y-axis represents the percentage of cells with zero, one, two or three foci and X-axis represents duration of mitomycin C treatment. B) Fluorescence images of WT and *ΔimuABC, ΔimuC* strains at zero and six hour time points, during mitomycin C treatment (scalebar - 5 µm).

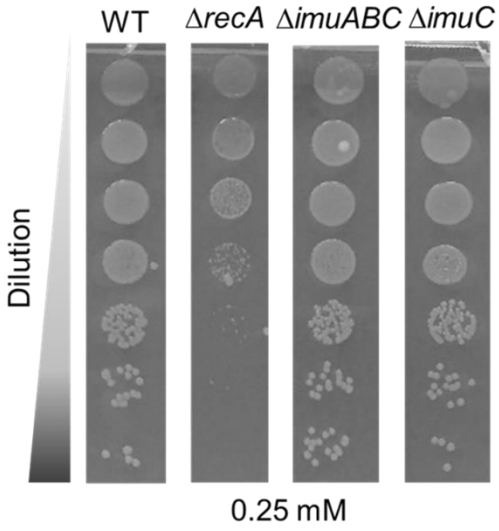
Appendix F: Mitomycin C stress

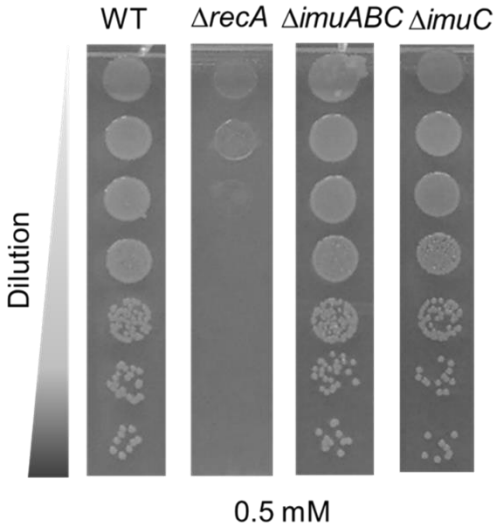


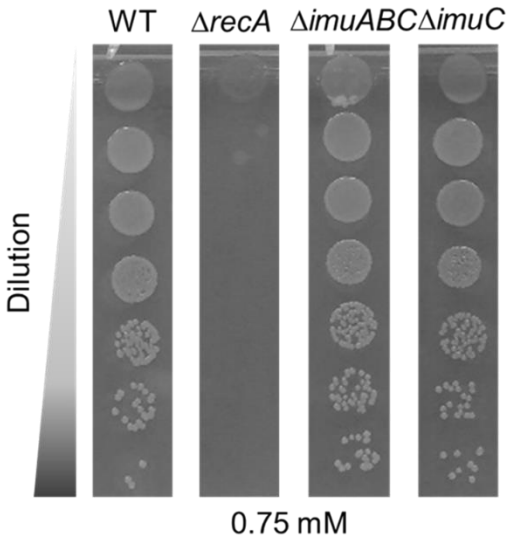
Appendix F: Sensitivity of *ΔimuABC* and *ΔimuC* strains to mitomycin C stress: Log fold dilutions (10^{-1} to 10^{-8}) of different strains were spotted on PYE agar with varying concentrations.

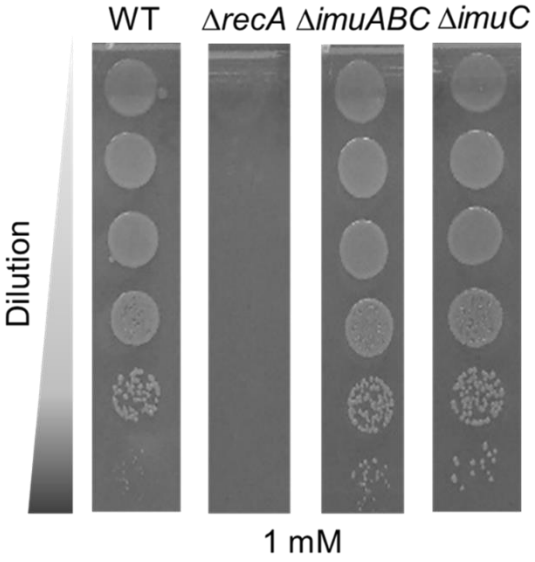
Appendix G: Methyl Methanesulfonate stress

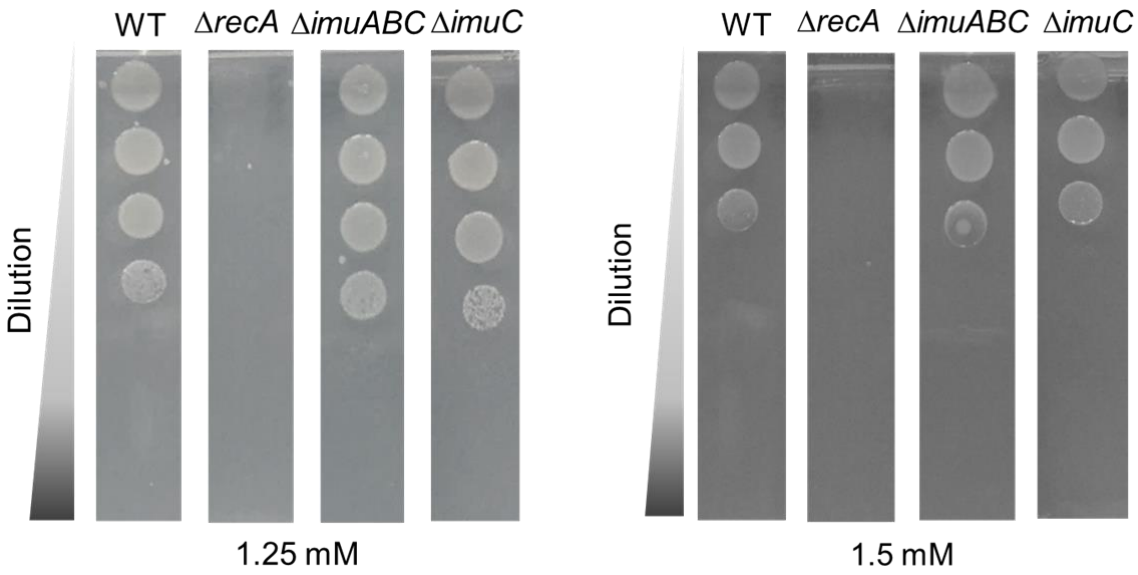






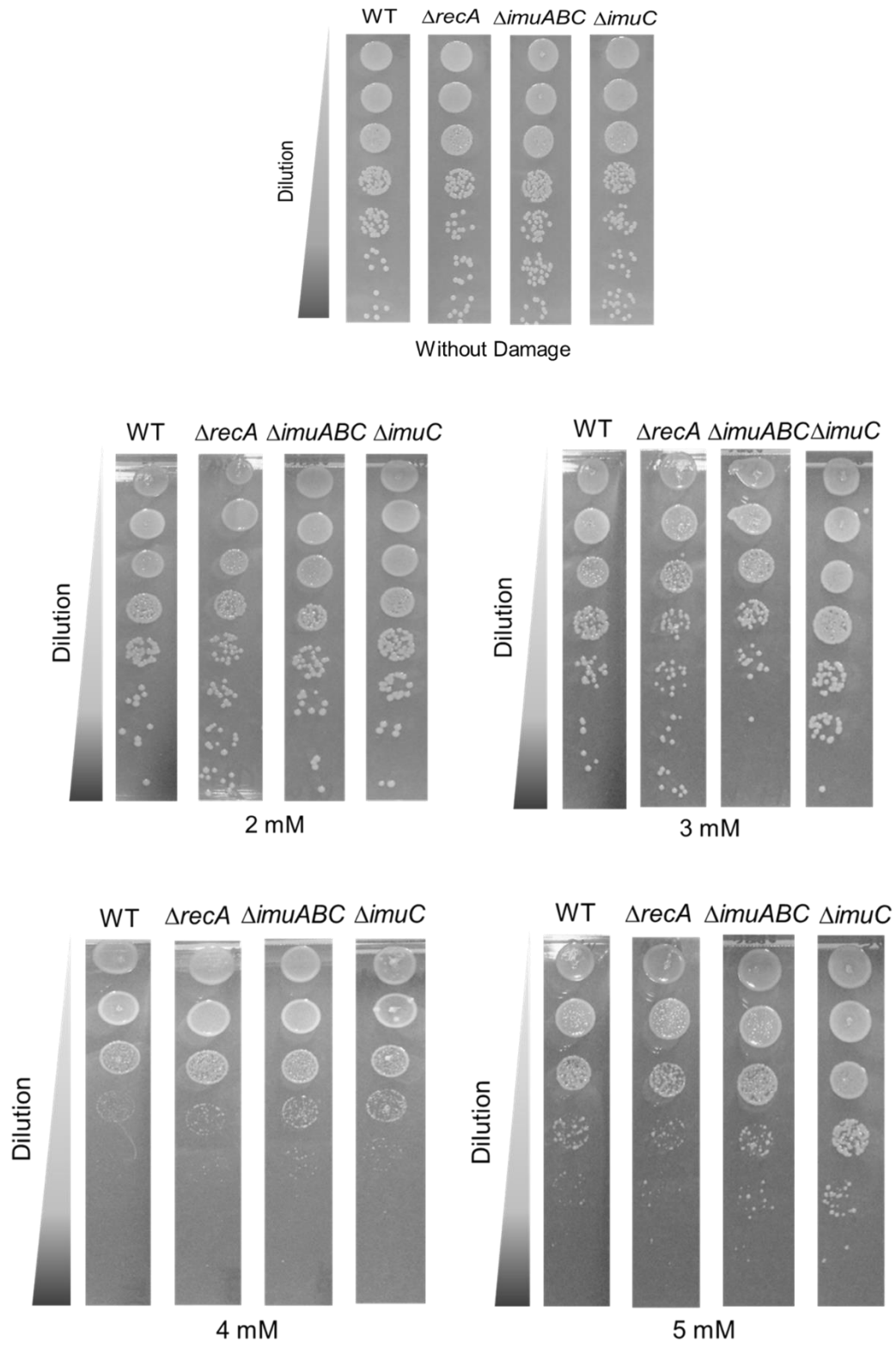


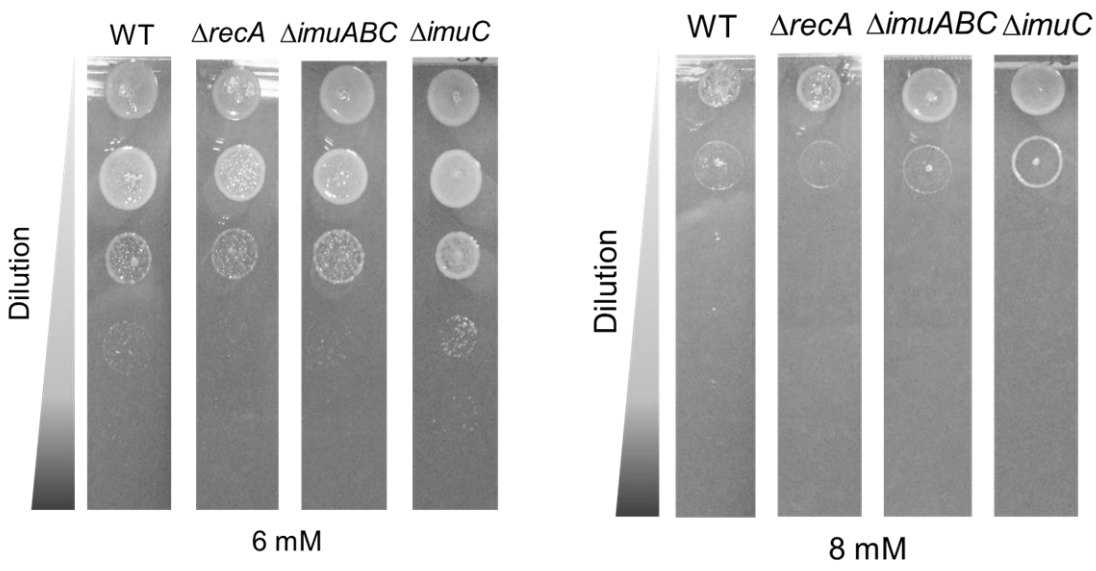




Appendix G: Sensitivity of *ΔimuABC* and *ΔimuC* strains to methyl methanesulfonate stress: Log fold dilutions (10^{-1} to 10^{-8}) of different strains were spotted on PYE agar with varying concentrations.

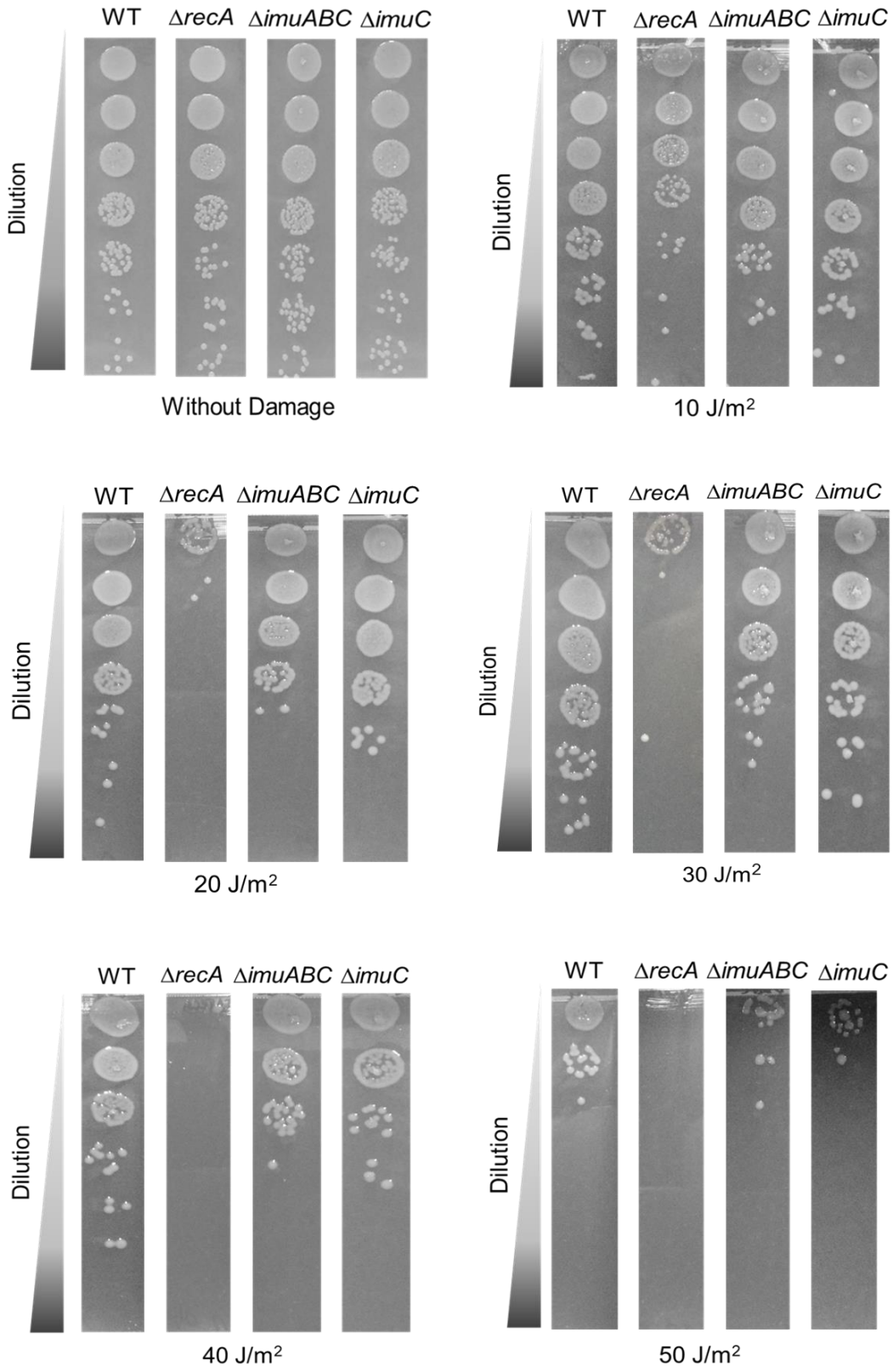
Appendix H: Hydroxyurea stress





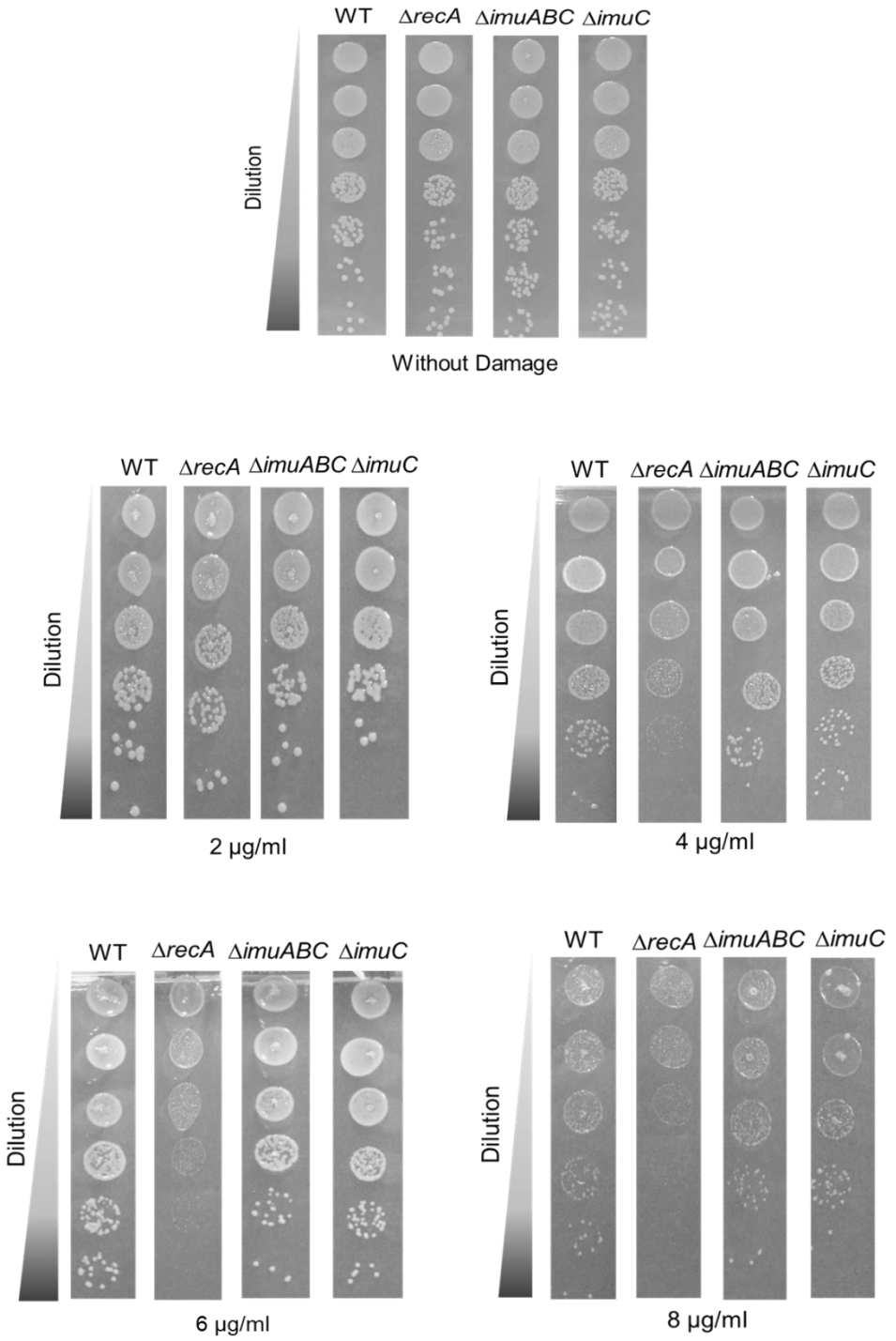
Appendix H: Sensitivity of *ΔimuABC* and *ΔimuC* strains to hydroxyurea stress: Log fold dilutions (10^{-1} to 10^{-8}) of different strains were spotted on PYE agar with varying concentrations.

Appendix I: Ultraviolet rays stress



Appendix I: Sensitivity of *ΔimuABC* and *ΔimuC* strains to ultraviolet rays stress: Log fold dilutions (10^{-1} to 10^{-8}) of different strains were spotted on PYE agar with varying concentrations.

Appendix J: Norfloxacin stress



Appendix J: Sensitivity of *ΔimuABC* and *ΔimuC* strains to norfloxacin stress: Log fold dilutions (10^{-1} to 10^{-8}) of different strains were spotted on PYE agar with varying concentrations.