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Involvement of oxidative DNA damage and its repair mechanisms in spontaneous chromosomal rearrangements

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Reactive oxygen species (ROS) are a major cause of spontaneous mutations in aerobic organisms. Among the ROS, hydroxyl radical attacks DNA to produce many kinds of DNA damages that have been shown to induce various kinds of mutations and chromosomal aberrations. To prevent these, organisms possess elaborate mechanisms to repair oxidative DNA damage such as base excision repair (BER), nucleotide excision repair (NER), and recombination repair. In our research group, it was recently discovered that cellular levels of ROS and oxidative DNA damage in *Escherichia coli* are significantly affected by environmental factors such as sugars and amino acids in growth media, oxygen concentration, and pH of growth media. However, frequency of base substitution mutations in wild-type *E. coli* was not significantly changed in various growth conditions differed in nutrient, oxygen concentration, and pH. This suggests that repair mechanisms against oxidative DNA damage are robust enough to counteract the elevated level of oxidative DNA damage. However, whether the environmental factors affect the recombination events, which are induced by DNA damage and cause chromosome rearrangements, is still unknown.

In order to analyze the effects of environmental factors mentioned above on spontaneous recombination events, I examined frequency of recombination events occurring in *E. coli* cells grown in different growth conditions. To do this, I first established a method for quick and accurate measurement of spontaneous recombination events in *E. coli* cells which are hemi-diploid in *rpsL* gene by using *rpsL* mutation assay and PCR analysis. I found that the frequency of spontaneous allelic recombination events was significantly increased in cells grown in minimal media (M9 + 0.2% glucose and M9 + 0.2% glycerol) and in LB under a hypoxia (low oxygen concentration (0.1%)). *E. coli* cells in these growth conditions were shown to produce more oxidative DNA damages than those grown in a standard LB medium.

Furthermore, the addition of an antioxidant, *Oxyrase*, significantly suppressed the recombination events induced under the hypoxia condition, and the recombination frequency was even lower than that under aerobic condition. From these observations, it was suggested that spontaneous recombination events were caused by spontaneous oxidative DNA damage and were affected by nutrition in growth media and oxygen concentration. Interestingly, using *lexA3* mutant strain in which the SOS response is always suppressed, I found that the spontaneous allelic recombination events depended on RecA function but not required the SOS induction.

Next, in an effort to further investigate the involvement of two major repair pathways, nucleotide excision repair (NER) and base excision repair (BER), in spontaneous recombination events, the spontaneous recombination events were examined in strains deficient in BER and/or NER repair pathways in different growth conditions. The results indicate that both NER and BER pathways are involved in suppression of the recombination events. It has been considered that the oxidative damage is mostly repaired by BER, and the contribution of NER in the repair process for oxidative DNA damage is marginal. However, an additional disruption of a NER gene, *uvrA*, in a BER deficient (*Δxth Δnfo)* strain showed a delay of cell growth and very high frequencies of recombination events and non-recombination events, both of which were much higher than that observed with cells lacking each pathway. In addition, this *ΔuvrA Δxth Δnfo* triple deletion strain was shown to be hypersensitive to H_2O_2, an oxidizing agent which induces oxidative DNA damages. Introduction of a UvrA-overproducing plasmid into the *ΔuvrA Δxth Δnfo* triple deletion strain reduced the recombination and non-recombination frequencies to those of the *Δxth Δnfo* double deletion strain and rescued the cells from killing effects of H_2O_2 treatment. These results indicate that the cellular level of oxidative DNA damage and the other types of DNA damage are huge under normal growth conditions, and an overlapped action of BER and NER pathways thoroughly removes such damages and strongly suppresses spontaneous recombination events.

Abbreviations

8-oxodG	7,8-dihydro-8-oxoguanine
AP site	Abasic site
BER	Base excision repair
CAT	Catalase
DSB(s)	DNA double strand break(s)
GGR	Global genome repair
HRR	Homologous recombination repair
LTTR	LysR-type transcriptional regulator
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TCR	Transcription couple repair

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Chapter I. Introduction

1. Reactive oxygen species and oxidative DNA damages

1.1. Oxidative DNA damages related to spontaneous mutagenesis

Reactive Oxygen Species (ROS) including superoxide anion radical ($\cdot O_2$), singlet oxygen (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) are the unfortunate products of metabolism process in aerobic organisms (Aruoma 1998; Dwyer *et. al.,* 2009). Besides that, UV irradiation, X-ray ionizing radiation or some chemical agents also contribute to generate ROS (Halliwell & Gutteridge 2007; Ercal *et. al.,* 2001; Klaunig & Kamendulis 2004; Rahman 2007; Evans *et. al.,* 2004). Among ROS, hydroxyl radical is extremely dangerous because it easily attacks DNA and induces oxidative DNA damages, hence accumulation of mutagenesis and cells death (Kashmiri & Mankar 2014; Evans *et. al.,* 2004).

Besides nucleic acids, the cellular macromolecules such as lipids and proteins are also the main substrates for ROS to produce oxidative damages. Oxidative DNA damages are the most abundant with high rate approximately 10^5 lesions per human cell per day (Bridge *et. al.,* 2014). As the most dangerous ROS, hydroxyl radical reacts with both purine and pyrimidine bases and sugar moiety of the DNA backbone (Dizdaroglu 2012) to produce many kinds of oxidative DNA damages. There are more than 20 different types of oxidative base modifications have been identified (Dizdaroglu 1993) including oxidized purine and oxidized pyrimidine. Among oxidized pyrimidine, thymine glycol and cytosine glycol are the most abundant (Najrana *et. al.,* 2000; Blaisdell *et. al.,* 1999; Slupphaug *et. al.,* 2003). Thymine glycol is lethal lesion which blocks DNA replication, while cytosine glycol is unstable lesion and quickly hydrates or deaminates to form 5-hydroxycytosine (5-OHC) or 5-hydroxyuracil (5-OHU), inducing C to T transition mutations (Blaisdell *et. al.,* 1999; Tano *et. al.,* 2001). Besides that, 7,8-dihydro-8-oxoguanine (8-oxodG) is not only the most abundant among oxidized purines but also is the most abundant oxidative DNA damages due to its low redox potential (John M. Essigmann 2006). During DNA replication, 8-oxodG is able to pair with both cytosine and adenine equally and induces G:C to T:A transversion mutations if unrepaired.

Most of oxidative DNA damages are divided into 2 groups including miscoding type and blocking type, based on characteristics in replication process (Friedberg *et. al.,* 2006). Miscoding type of oxidative DNA damages represents for the DNA lesions which induce base substitutions through the mis-incorporation of DNA polymerase and replication errors. For example, 8-oxodG pairs with both original cytosine and also adenine during DNA replication (Lu *et. al.,* 2001) and induces G:C to T:A transversion in the absence of repair proteins such as MutM and MutY (Maki 2002; Cabrera *et. al.,* 1988; Nghiem *et. al.,* 1988) or induces A:T to C:G transversion in the absence of MutT protein (Fowler *et. al.,* 1994; Maki & Sekiguchi

1992). In addition, other oxidative DNA lesions such as 5-OHC and 5-OHU can mispair with adenine during DNA replication and induce C to T transition mutations. These lesions are mostly repaired by Endonuclease III (Nth) and Endonuclease VIII (Nei) proteins in BER pathway (Blaisdell *et. al.,* 1999; Najrana *et. al.,* 2000; Tano *et. al.,* 2001; Dizdaroglu *et. al.,* 2000). However, the blocking type of oxidative DNA damages are cytotoxic lesions which are able to block DNA replication fork, hence lethality if un-repaired. Thymine glycol and abasic sites (AP sites) are well-characterized blocking lesions produced by ROS (Cooke *et. al.,* 2003; Dizdaroglu *et. al.,* 2000; Blaisdell *et. al.,* 1999), whereas thymine dimer and 6-4 photoproducts, most popular blocking lesions, are produced by UV irradiation (Boyce & Howard-Flanders 2003; McCulloch *et. al.,* 2004).

The excessive and uncontrollable amount of ROS becomes the big challenge for cells through induction of DNA damages and oxidative stress which have known as the main factors cause cancer and many diseases in human (Aruoma 1998; Maynard *et. al.,* 2009; Maluf *et. al.,* 2013). There are many human diseases related to ROS, such as Parkinson, Huntington and Alzheimer diseases (Jenner 2003; Cooke *et. al.,* 2003; Aruoma 1998), or neuron-degeneration diseases (Andersen 2004; Reynolds *et. al.,* 2007). Connections of ROS to a class of familial colorectal cancer and aging were also reported (Aruoma 1998; Waris & Ahsan 2006; Rahman 2007).

Therefore, understanding where ROS comes from and how cells avoid the ROS production as well as their consequences is an important way in preventing ourself away from ROS's damages.

1.2. Measurement of oxidative DNA damages through H_2O_2 production in *E. coli* cells

Among all the ROS, hydroxyl radical is the most dangerous ROS which highly reacts DNA, protein and lipid towards oxidative stress (Kashmiri & Mankar 2014) and most of them lead to oxidative DNA damages (Cabiscol *et. al.,* 2000). Unfortunately, it is very difficult to measure hydroxyl radical level in cells because of its unstable characteristic with an estimated half-life in cells of only 10^{-9}s (Aruoma 1998). However, it was known that hydroxyl radical is generated from hydrogen peroxide (H_2O_2) via Fenton reaction or Haber-Weiss reaction (Manda *et. al.,* 2009; Cabiscol *et. al.,* 2000; Rahman 2007; Iuchi & Weiner 1996). This hydrogen peroxide is not radical but more stable than hydroxyl radical. When *E. coli* cells are exposed to a high concentration of H_2O_2, oxidative DNA damages are produced by hydroxyl radical converted from H_2O_2 (Aruoma 1998). To protect cells against DNA damages caused by ROS, most of bacterial cells have a mechanism to control the oxidative stress, in which hydrogen peroxide responsive transactivator OxyR, a member of LTTR family, plays a key role. When the intracellular H_2O_2 level is high, this OxyR regulon is activated and turn on the

expression of defensive genes such as *katG* encoding catalase which converts H_2O_2 to water and *ahpC-ahpF* encoding alkyl hydroperoxide reductase to protect cells against the oxidative stress (Wei *et. al.,* 2012). Based on this mechanism, a DNA fragment containing promoter region of *ahpC* gene fused with GFP gene was cloned into pTN249 plasmid, and then the plasmid was introduced into *E. coli* cells. Under induction of intracellular H_2O_2, the expression of *ahpC-gfp* fusion gene was easily detected and quantified by flow cytometry (Figure 1). It has been demonstrated that the expression of *ahpC-gfp* fusion gene is dependent on H_2O_2 level (Aussel *et. al.,* 2011). Therefore, the fluorescence intensity of AhpC-GFP should reflect the intracellular H_2O_2 level in the *E. coli* cells.

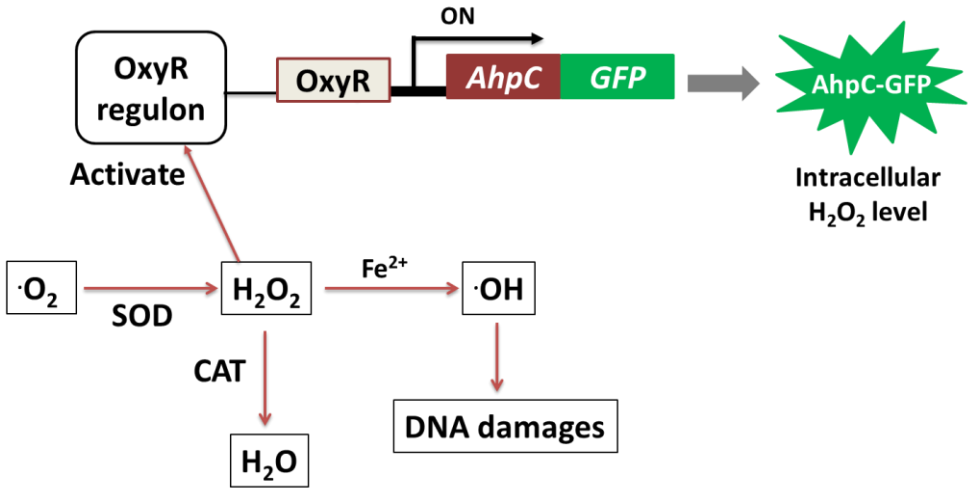


Figure 1. GFP fluorescence intensity reflects the intracellular H_2O_2 level. SOD: *Superoxide dismutase*; CAT: *Catalase*; AhpC: *alkyl hydroperoxide reductase*; GFP: Green fluorescent protein.

2. Cellular defense mechanisms against ROS in *E. coli* cells

2.1. Antioxidant defense mechanisms

The primary defense system against the damaging effects of ROS is antioxidants which convert the oxidants to less reactive species. The cellular antioxidants include enzyme group (superoxide dismutase, catalase, and alkyl hydroperoxide reductase) and non-enzyme group (thioredoxin, glutaredoxin, and glutathione) (Kashmiri & Mankar 2014). The superoxide anion and hydrogen peroxide are effectively catalyzed by the action of superoxide dismutase (SOD) and catalase. Superoxide dismutase catalyzes the conversion of two superoxide anions into a molecule of H_2O_2 and oxygen, then catalase catalyzed H_2O_2 to form water and oxygen (McCord & Fridovich 1968; Iuchi & Weiner 1996). There are two catalase enzymes in *E. coli*, including hydroperoxidase I (HPI), which is present during aerobic growth and transcriptionally controlled at different levels, and hydroperoxidase II (HPII),

which is induced during stationary phase (Von Ossowski *et. al.,* 1991). In eukaryote organisms, H_2O_2 is rapidly removed by glutathione peroxidase and catalase (Davies 2000).

Another way to avoid oxidative stress is reduction of ROS production by the addition of *Oxyrase* to growth medium (Dong *et. al.,* 2010; Fowler *et. al.,* 1994; Sakai *et. al.,* 2006). *Oxyrase* is a natural antioxidant isolated from cytoplasmic membrane of *E. coli*. In medium, *Oxyrase* removes the dissolved oxygen by capturing the oxygen and converting it to water (http://www.oxyrase.com/why-oxyrase/what-is-oxyrase). The use of *Oxyrase* to reduce oxidative DNA damages has been reported (Sakai *et. al.,* 2006; Fowler *et. al.,* 1994). In *E. coli* cells, *Oxyrase* is very powerful to produce anaerobic environment and was applied for the studies of *in vivo* functions of MutT protein (Fowler *et. al.,* 1994) as well as MutM and MutY proteins (Sakai *et. al.,* 2006) in suppression of oxidative DNA damages. In addition, it was reported that a major cause of dramatic reduction of functionality of Monkey and Mouse sperms could be oxidative damages through the production of ROS. The supplementation of *Oxyrase* into medium improved the survival and functionality of frozen sperm during the post-thaw process and cryopreservation through the reduction of ROS production (Dong *et. al.,* 2010; Mazur *et. al.,* 2000). From these observations, it is clear that the role of *Oxyrase* in suppression of oxidative DNA damages is through the reduction of ROS.

2.2. Mechanism of multiple repair pathways

In normally growing cells, the level of ROS is very low because of the effective action of antioxidants. However, when the balance between ROS and antioxidants is broken, the cellular level of ROS could be increased and induce oxidative stress. The resulting oxidative DNA damages should induce genetic changes such as mutations and chromosomal rearrangements. To protect cells against the consequences of DNA damages and to maintain genome integrity, *E. coli* cells elaborate multi pathways of DNA repair as the second defense mechanism, including nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR). These repair pathways contribute to eliminate DNA lesions and to avoid mutations. If DNA damages are not removed, damage-tolerance pathways including recombination repair and translesion synthesis pathway (TSL) help cells to overcome the consequences of DNA lesions. In this study, I focus on three main repair pathways including BER, NER, and the recombination repair pathway to investigate how these repair pathways help cells overcome the genotoxic effects of oxidative DNA damages.

2.2.1. Mechanism of Base Excision Repair (BER) pathway

Among over 20 types of oxidative DNA damages, 8-oxodG is the most abundant and efficiently removed by BER pathway. BER pathway is highly conserved from bacteria to human and becomes the most important pathway protecting cells against many kinds of oxidative DNA damages (David *et. al.,* 2007; Dizdaroglu 2005; Cooke *et. al.,* 2003; Sander & Wilson 2005). BER pathway starts when damaged or abnormal base emerges in DNA. There are three main steps in BER pathway: Firstly, damaged bases are recognized and removed by DNA glycosylase to generate abasic site (AP site) as an intermediate. The second step is incision of AP sites and removal of remaining sugar moiety by AP endonuclease. The last step is gap filling by DNA polymerase I and nick-sealing by DNA ligase (Kim & Wilson 2012; Souza *et. al.,* 2006) (Figure 2).

One of the reasons for BER pathway to be considered as the most important repair pathway is a contribution of DNA glycosylases which are able to remove a wide range of cytotoxic and mutagenic DNA bases (Krokan *et. al.,* 1997). There are two types of DNA glycosylases including mono-functional ones and bi-functional ones. The mono-functional DNA glycosylase is able to cleave the N-glycosylic bond between the target base and deoxyribose, thus releasing a free base and leaving an AP site. Typical mono-functional DNA glycosylases are MutY protein, which removes adenine opposite 8-oxodG resulting from adenine pairing with unrepaired 8-oxodG during replication (Cabrera *et. al.,* 1988; Nghiem *et. al.,* 1988; Blaisdell *et. al.,* 1999), and uracil DNA glycosylase, which is responsible for removing of uracil lesion in DNA (Krokan *et. al.,* 2002; Olinski *et. al.,* 2010; Parikh *et. al.,* 2000). On the other hand, bi-functional DNA glycosylase can further cleave the phosphodiester backbone 3' to the AP sites by AP lyase activity (Jacobs & Schär 2012), which contributes to produce a gap or nick on DNA sequence. MutM, Nei, and Nth proteins are three major proteins belong to bi-functional DNA glycosylases. The most abundant oxidative DNA damages is 8-oxodG which is specifically removed by the action of MutM protein. Other abundant oxidative DNA damages are oxidized pyrimidines such as thymine glycol, 5-hydroxycytosine and 5-hydroxyuracine are effectively removed by Nth protein (Endonuclease III) and Nei protein (Endonuclease VIII) (Saito *et. al.,* 1997; Souza *et. al.,* 2006; Melamede *et. al.,*1994).

Another important enzyme group in BER pathway is AP endonuclease because both AP site and nick, products of DNA glycosylases are further eliminated by the action of AP endonuclease. There are two major AP endonuclease enzymes, Exonuclease III (Xth) and Endonuclease IV (Nfo) in *E. coli* cells, and both enzymes are responsible for removal of AP sites by cleaving the sugar phosphate bond 5' to AP site to produce a 3'-hydroxyl group and a 5'-deoxyribophosphate. The resulting single-nucleotide gap is further filled by DNA polymerase I (Masson & Ramotar 1997; Souza *et. al.,* 2006). It should be noted that AP site

itself is one of the most frequent spontaneous lesion. It has been reported that AP sites can be generated in DNA under normal aerobic metabolism or treatment of some agents such as ionizing radiation, UV light, and H_2O_2 (Heck *et. al.,* 2003). AP site is lethal DNA lesion which blocks DNA replication (Boiteux & Guillet 2004) and causes cell death if un-repaired.

Cells lack of Nth and Nei proteins become hypersensitive to X-ray irradiation and hydrogen peroxide (Saito *et. al.,* 1997; Jiang, Hatahet, Blaisdell, *et. al.,* 1997; Jiang, Hatahet, Melamede, *et. al., 1997)*. A more sensitive to X-ray irradiation and hydrogen peroxide rather than lack of Nth and Nei has observed in AP endonuclease-deficient cell (Saito *et. al.,* 1997; Najrana *et. al.,* 2000; Saporito *et. al.,* 1989).

The base excision repair pathway can be divided into two sub-pathways including short-patch pathway and long-patch pathway (Fortini & Dogliotti 2007) based on the process of base removal by DNA glycosylases. If the repair is initiated by mono-functional DNA glycosylases, it may follow both long or short-patch while if the repair process is initiated by bi-functional DNA glycosylases, it may only follow the short-patch. In the short-patch pathway, only one nucleotide is replaced whereas about 2-10 nucleotides containing lesions in long-patch pathway are replaced during strand displacement by polymerase I and subsequently ligated (Oliveira *et. al.,* 2015).

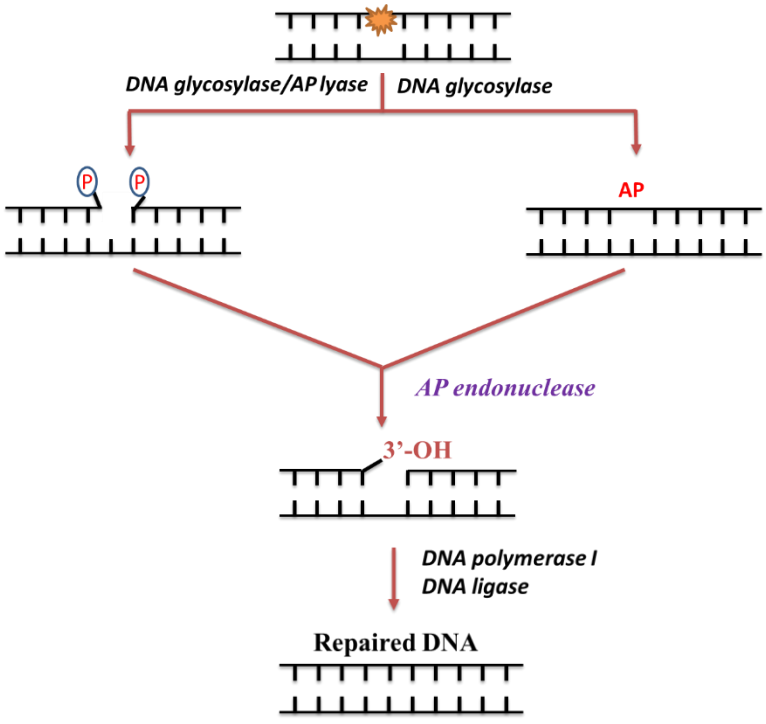


Figure 2. Mechanism of base excision repair pathway in *E. coli* cell. AP: abasic site.

2.2.2. Mechanism of Nucleotide Excision Repair (NER) pathway

Nucleotide Excision Repair pathway, which removes UV induced cyclobutane pyrimidine dimers, was first described in *E. coli* by Bill Carrier and Dick Setlow in 1964 *(Kisker et. al.,* 2013). NER pathway is responsible for removal of many types of DNA lesions in which covalently bound bulky adduct is the most common lesions (Marteijn *et. al.,* 2014). The process of NER in prokaryote and eukaryote is highly conserved and can be viewed in 5 main steps including detection of damaged site, incision, excision, re-synthesis, and DNA ligation (Kisker *et. al.,* 2013). NER process is divided into 2 sub-pathways, transcription coupled repair (TCR) pathway and global genome repair (GGR) pathway, based on the initial damage sensor proteins (Sancar 1996; Hanawalt 2002; Petruseva *et. al.,* 2014; Kisker *et. al.,* 2013; Truglio *et. al.,* 2006). In transcription coupled repair pathway, Mfd is a transcription repair coupled factor which plays a very important role in removing RNA polymerase complex stalled at the DNA lesion on the transcribed strand. Then recruiting the DNA repair proteins to the lesion, and initiating the repair process similarly to global genome repair pathway (Schalow *et. al.,* 2012; Park *et. al.,* 2002; Sidorenko *et. al.,* 2015; Smith & Savery 2005; C Kisker *et. al.,* 2013). On the other hand, GGR pathway is able to repair DNA lesions anywhere in genome DNA including un-transcribed strand (Truglio et al. 2006; C Kisker et al. 2013). Therefore, the action of TCR in repairing the lesion is much faster than the action of GGR pathway.

There are at least six proteins (UvrA, UvrB, UvrC, UvrD, DNA polymerase I and DNA ligase) are involved in GGR pathway through 5 main steps including recognition, incision, excision, re-synthesis, and ligation (Lin *et. al.,* 1997). UvrA protein exists in cells as both monomer and dimer forms which are responsible for recognition of damaged sites. UvrB protein itself cannot directly bind to DNA damage site, but $UvrA_2UvrB_2$ complex can scan along DNA sequence and stop at DNA lesion. Recently, it has been reported that UvrA itself can scan along DNA genome and stop at damaged site in a UvrB-independent manner (Stracy *et. al.,* 2016). After recognizing DNA lesion, UvrA protein is dissociated from DNA by a conformation change from open form to close form of $UvrA_2UvrB_2$ complex and leaves UvrB protein bind to DNA lesion (Kisker *et. al.,* 2013). Then, UvrC interacts with carboxy-terminal domain of UvrB protein to form a complex that leads to the subsequent incision of the damage-containing strand by UvrC function (Hsu *et. al.,* 1995; Kisker *et. al.,* 2013). After incision, UvrC dissociates from the complex and recruits UvrD DNA helicase which releases 12-13 oligonucleotides containing DNA lesion in bacteria (Truglio *et. al.,* 2006) and 24-32 nucleotide DNA fragments containing DNA lesion in higher eukaryote cells (Petruseva *et. al.,* 2014).

NER has been known as the main repair pathway for removing bulky DNA lesions such as cyclobutane pyrimidine dimers and 6-4 photoproduct, both of which are induced by

UV irradiation (de Boer & Hoeijmakers 2000; Kisker *et. al.,* 2013; Marteijn *et. al.,* 2014). However, roles of NER pathway in non-bulky DNA lesions such as oxidative DNA damages are still under controversial. In 2007, Hori and colleagues reported that UvrA and UvrB proteins in NER pathway enhanced frequency of mutations induced by oxidized deoxyribonucleotides (Hori *et. al.,* 2007). In agreement with this study, Hasegawa and colleagues found that both TCR and GGR pathways contributed to generating spontaneous mutation rather than suppressing it under normal growth condition (Hasegawa *et. al.,* 2008). Until now, there are no clear evidence to show a role of NER in repair of oxidative DNA damages in *E. coli*, except that UvrABC proteins can repair AP site *in vitro* (Lin & Sancar 1989). However, many reports showed that NER pathway contributes to repair oxidative DNA damages such as 8-oxodG and thymine glycol in human cells (Reardon *et. al.,* 1997; Melis *et. al.,* 2013). These observations suggest different mechanisms in NER pathway between prokaryote and higher organisms. Whereas bacterial cells lacking NER pathway lowered spontaneous mutagenesis (Hasegawa *et. al.,* 2008), human cells lacking NER pathway showed disposition of cancer and other symptoms such as Xeroderma Pigmentosum (XP), Cockayne syndrome (CS) and Trichothiodystrophy (TTD) (Marteijn *et. al.,* 2014; Petruseva *et. al.,* 2014; de Boer & Hoeijmakers 2000).

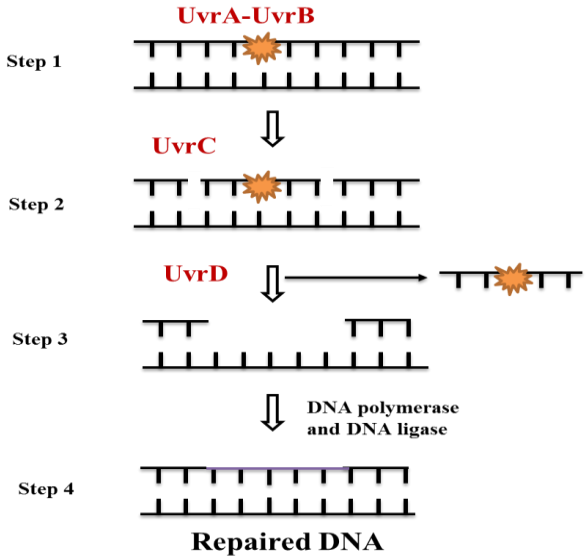


Figure 3. Mechanism of nucleotide excision repair pathway in repairing DNA lesion.
Step 1: DNA lesion is recognized by UvrA and UvrB proteins; Step 2: UvrC endonuclease comes to DNA lesion site and incises an oligonucleotide about 11 to 13 nucleotides containing the DNA lesion; Step 3: UvrD helicase comes to remove this oligonucleotide fragment; Step 4: DNA polymerase comes to synthesize a new DNA and DNA ligase seals the nick and finishes the repair process.

2.2.3. Recombination Repair (RR)

Many types of DNA damage are always emerging in *E. coli* cells and inducing mutagenesis or cell death if unrepaired. To avoid mutagenesis, *E. coli* elaborates many repair pathways including base excision repair, nucleotide excision repair, and mismatch repair, which remove DNA lesions and protect cells against their consequences. However, during DNA replication, if DNA lesions remain unrepaired, replication fork will stall, hence cells will die if the stalled replication fork is un-recovered. Recombination repair pathway is a powerful pathway to protect cells from stalled or blocked DNA replication forks (Cox 2001). Cells lack of recombination repair machinery showed a reduced cell viability under the treatment with UV lights, X-ray or some DNA damaging agents (Kanie *et. al.,* 2007).

There are about 25 different bacterial proteins directly involved in key steps of recombination repair of stalled replication forks, excluding DNA polymerases (Cox 2002), and most of recombination events occurring in the wild type cells required RecBCD and RecF pathways (Amundsen & Smith 2003). RecBCD is responsible for repairing the double strand break DNA, and RecF pathway is responsible for repairing the single strand break DNA, but both pathways require RecA for homologous recombination. Although the RecBCD and RecF recombination machineries act separately to repair damaged DNA, they can also interchange their functions to help cells overcome the DNA lesions (Amundsen & Smith 2003). Recombination repair pathway is initiated when RecA binds to single stranded DNA to form filament from 5' to 3' direction, searches homologous sequence from intact DNA sequences, and starts strand exchange to complete recombination events.

I will explain more detail about how recombination repair pathway repairs DNA damages and induces recombination events. Until now, two recombination repair pathways involving in repairing DNA damages are well understood. In 2001, Rice and Cox reported the two recombination pathways to recover the stalled replication forks in bacteria, which are shown in Figure 4 (Rice and Cox, 2001).

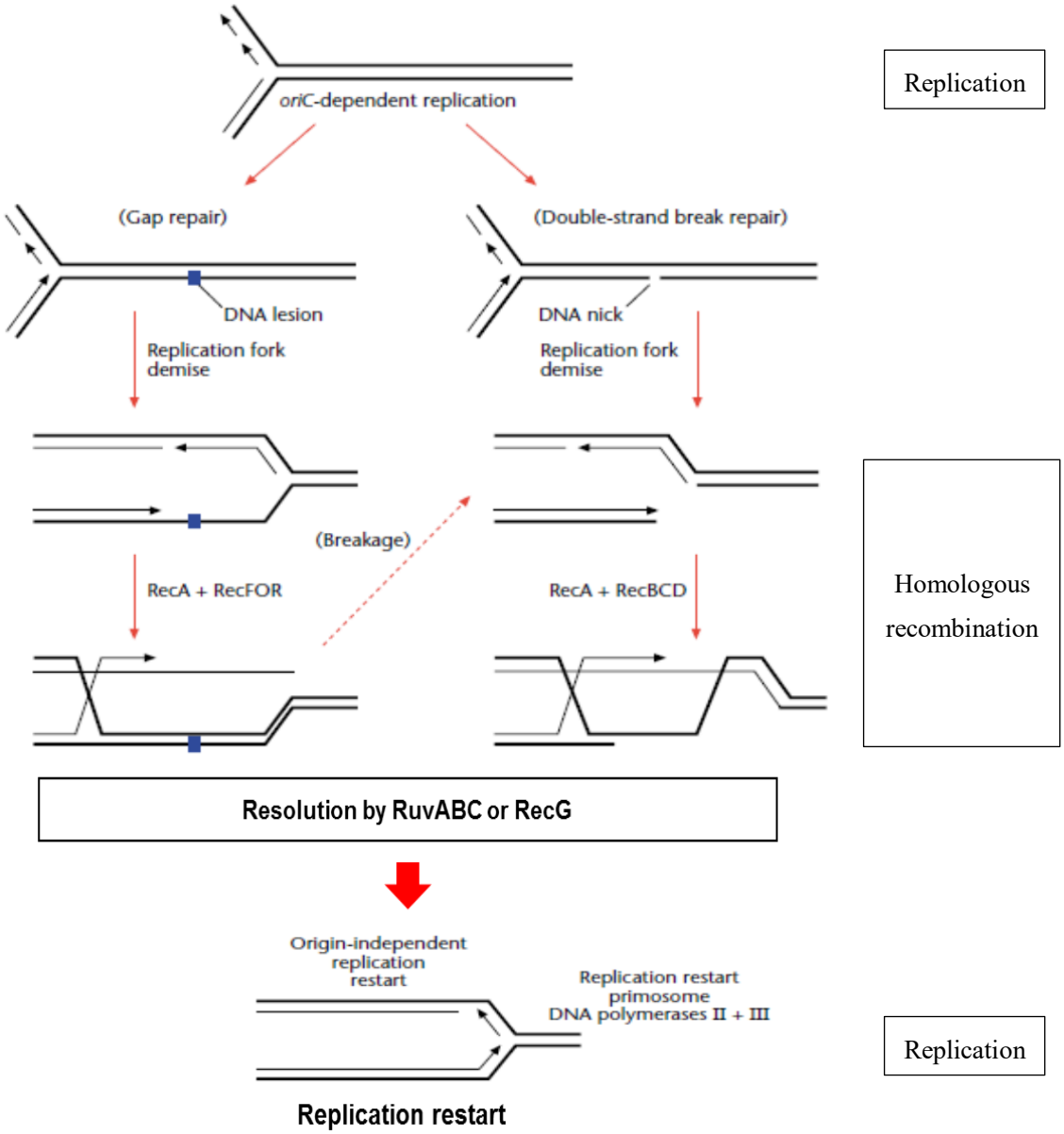


Figure 4. Pathways for reactivation of the stalled replication fork (this figure is from Rice and Cox, 2001 with some small modifications).

In the left path, the stalled replication fork occurs when ongoing replication strand meet the DNA lesion in template DNA strand. RecA and RecFOR proteins come and recruit a complementary strand. The ongoing replication uses the intact strand to continuously synthesis until it overcome DNA lesion. Then recombination intermediates are processing by the RuvABC and/or RecG activities. Then the replication proteins come and reinitiate the replication.

In the right path, if DNA template strand contains a nicked DNA, a double-strand break is produced when ongoing replication strand meet th nicked DNA. In this case, the RecBCD is major pathway together with RecA to recover the replication fork by producing recombination intermediates and restarts the replication process.

It is estimated that bacteria may suffer one of the demise of a replication fork per cell per generation under normal growing condition (Cox 2002), and perhaps 10 forks undergo recombination DNA repair in every mitotic division in mammalian cells (Lindsley & Cox 1990). Therefore, homologous recombination repair pathway becomes essential for eukaryote cell and is the most important repair function in bacterial cells for stalled replication fork (Cox *et. al.,* 2000). *E. coli* cells lacking *recA* gene, deficient in homologous recombination showed a hyper sensitivity to UV, and frequency of large deletion in the *recA* deletion strain was 10-fold higher than that in wild type cells (Kanie *et. al.,* 2007). In human, the increment in homologous recombination has been shown to be linked to tumor susceptible (Modesti & Kanaar 2001).

It is obvious that recombination repair pathway helps cells survive by maintaining genome integrity. However, the action of recombination repair also promotes genome rearrangements such as deletion, amplification, duplication, inversion and translocation (Darmon & Leach 2014), and if the genome rearrangements occur very frequently, cell viability could be affected (Rodgers & Mcvey 2016). Therefore, understanding how frequent recombination events occur helps us understand how frequent DNA replication forks are blocked in *E. coli* cells under normal growth conditions.

3. Chromosomal rearrangements

3.1. What are the chromosomal rearrangements?

Chromosomal rearrangements include various kinds of rearrangements in chromosome such as deletion, duplication, inversion or translocation (Tsai & Lieber 2010; Roth *et. al.,* 1996; Kasparek & Humphrey 2011) as shown in Figure 5. Consequences of both deletion and duplication are changes in quantity of genetic information. Deletion events occur by missing of a chromosome segment while duplication relates to increase or duplicate of some sequence (Roth *et. al.,* 1996). Phenotype of deletion events may be caused by the loss of gene function, and if an essential gene is deleted, such cells lose viability. Inversion does not lead to loss or gain of chromosome segment but is involved in the alteration of chromosome sequence due to breakage and rejoining upside down of a segment of chromosome (Roth *et. al.,* 1996). Translocation is the most dangerous chromosomal rearrangements which relate to many human diseases and cancer. In a translocation, a segment from one chromosome is transferred to a non-homologous chromosome or to a new site on the same chromosome (Clare O'Connor 2008).

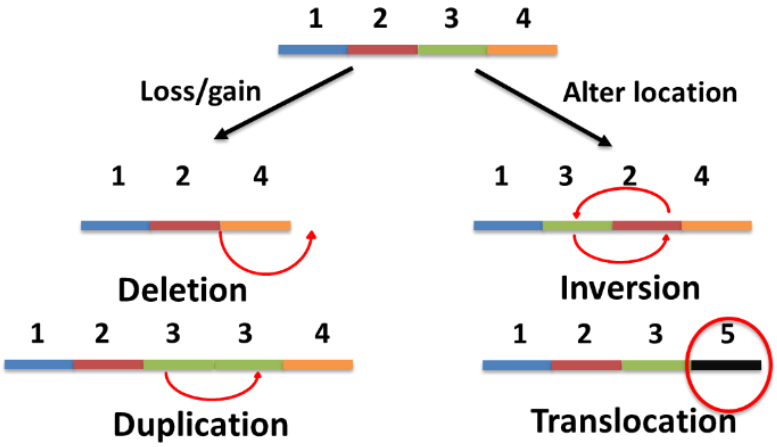


Figure 5. Types of chromosomal rearrangements.

3.2. Consequences of chromosomal rearrangements

It has been reported that consequences of chromosomal rearrangements are involved in evolution and also associated to many human diseases and cancer. The evolutionary role of chromosomal rearrangements could be that if the changes in chromosome lead to produce a new chromosome forms in the germ line, they contribute to a formation of new species or to help cells adapt with a new environmental conditions (Capilla n.d. 2015). However, chromosomal rearrangements also cause genome instability or cell death if an essential gene is lost by deletion.

In bacteria, chromosomal rearrangements play a very important role in genome diversity and evolution. For example, chromosomal rearrangements which are caused by homologous recombination have been shown to be involved in the evolution of *Helibacter pylori*, a bacterium that causes chronic inflammation (infection) in the stomach and duodenum to adapt to the host cells (Kobayashi *et. al.,* 2014). The role of chromosomal rearrangements in evolution was also reported in differentiation of yeast species (Fischer *et. al.,* 2000).

In human, it has been widely known that chromosomal rearrangements are related to many diseases and cancers, and most of them are caused by deletion and translocation events. Deletion events have been demonstrated to be involved in many diseases in human such as Cri Du Chat, which is caused by loss of sequence in chromosome 5 (Figure 6A). This disease shows very common symptoms such as cat like cry, small head, a low birth weight, slow growth, mental retardation, abnormally shaped ears (Sun *et. al.,* 2014; Niebuhr 1978). Another consequence of deletion is retinoblastoma due to loss of heterozygozity which results

in loss of Rb dominance of tumor suppressor gene (Figure 6B). The most symptom of retinoblastoma is white pupil associated to tumor in eye (Aerts *et. al.,* 2006; Classon & Harlow 2002). However, there is no evidence to show the consequence of duplication events (Roth *et. al.,* 1996).

One of the common cancer related to translocation is leukemia cancer in which a small part of chromosome 22 joins to the end of chromosome 9, forming Philadelphia chromosome. This translocation puts *c-ABL* gene encoding a protein kinase from chromosome 9 to a region controlled by BCR gene in chromosome 22. This fusion places *c-ABL* gene under the control of an ectopic promoter. Therefore, the *c-ABL* is expressed at a wrong time and a wrong place. This mis-expression of *c-ALB* gene is associated to the leukemia cancer (Nowell 2007) (Figure 6C).

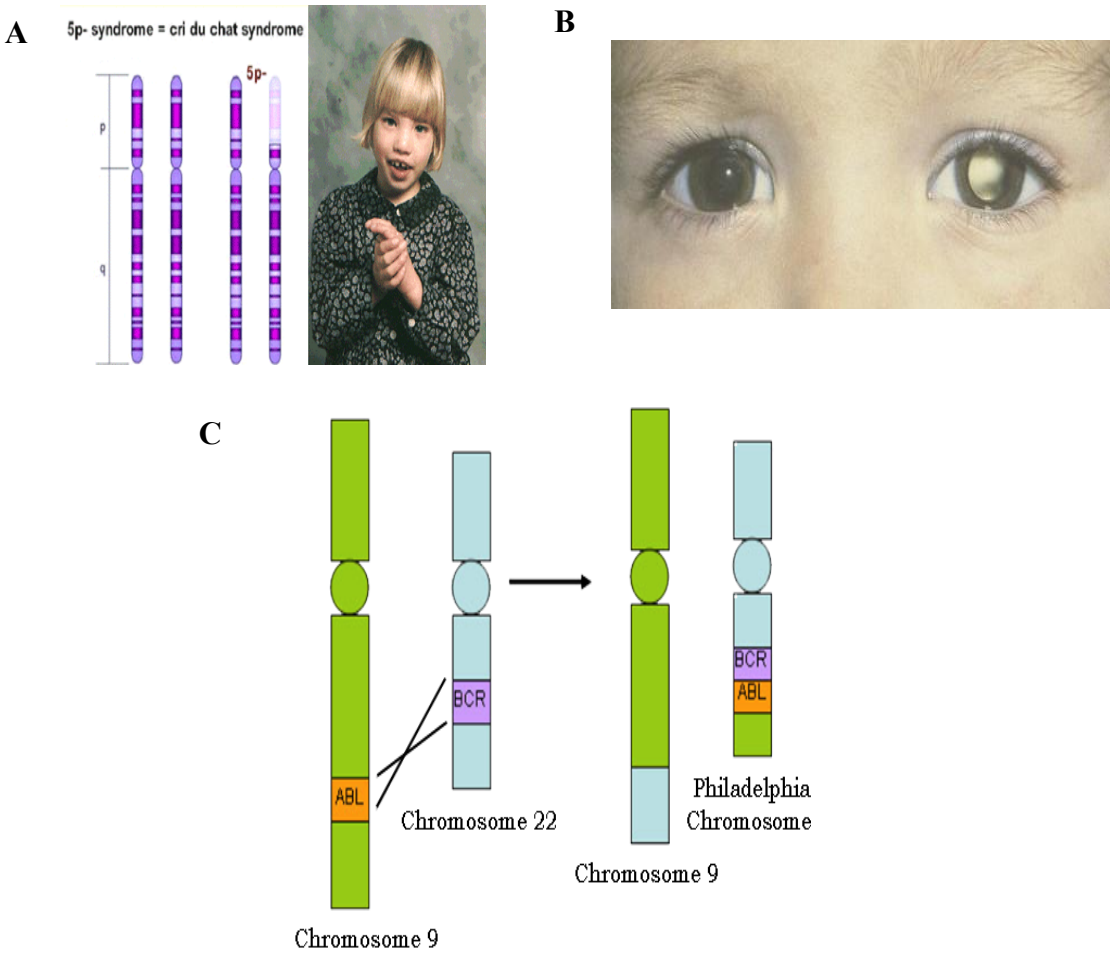


Figure 6. Human diseases related to chromosomal rearrangements.
A: Cri Du Chat disease;
B: Retinoblastoma;
C: Leukemia cancer.

3.3. Induction of chromosomal rearrangements

The process inducing chromosomal rearrangements requires one or more DNA ends which can become substrates for a range of processes including ligation, resection, annealing, invasion and/or replication (Kasparek & Humphrey 2011). Therefore, DNA double strand breaks are critical primary lesions, which induce chromosomal rearrangements through the DNA repair mechanisms (Pfeiffer *et. al.,* 2000; Kasparek & Humphrey 2011). DNA double strand breaks can be induced by exogenous sources such as ionizing radiation and UV irradiation, or by inducible expression of restriction enzymes in cells (Kobayashi *et. al.,* 2014; Helleday *et. al.,* 2007; Dudás & Chovanec 2004). Besides that, DNA double strand breaks are also able to be induced spontaneously by endogenous sources, such as DNA topoisomerase mediated DNA cleavage during cell cycles. A defective maturation of Okazaki fragment during DNA replication also contributes to accumulate DNA double strand breaks. Furthermore, the action of DNA glycosylases in elimination of multiple DNA lesions which are located closely in opposite strands may also contribute to produce DNA double strand breaks (Pfeiffer *et. al.,* 2000).

Repair of the DNA double strand break is divided into two pathways, homologous recombination repair (HRR) and non-homologous end joining (NHEJ) (Kobayashi *et. al.,* 2014; Helleday *et. al.,* 2007; Dudás & Chovanec 2004; Puchta 2005). HRR pathway strictly depends on RecA protein in *E. coli* cells (Cox 2001; Cox 2002; Cox 2003) and Rad52 and Rad51 in yeast cells (Pfeiffer *et. al.,* 2000). The rejoining of double strand break by HRR requires homologous sequences to process three sub-pathways including DNA double strand break repair, synthesis dependent strand annealing, and break induced replication. Although the repair process by HRR is basically accurate, ectopic homologous recombination between repetitive sequences in genomic DNA results in chromosomal alteration. Therefore, recombination events are considered to be the major cause of chromosomal rearrangements. On the other hand, NHEJ does not require homologous sequences and are processed by illegitimate recombination between non-homologous sequences or very short, 1-10 bp, homologous sequences simply by pasting two broken ends together (Pfeiffer *et. al.,* 2000; Hanada *et. al.,* 2000). NHEJ rescues G1 phase or non-dividing cells from degradation of chromosome initiated by double strand break, but this process is more error-prone than HRR. Thus, both HRR and NHEJ pathways contribute to induce genome instability by generating chromosomal rearrangements.

In previous studies molecular mechanisms of chromosomal rearrangements, cells treated with UV or ionizing radiations were often used to induce DNA double strand breaks. However, how frequent chromosomal rearrangements in cells normally growing without such treatments and what is the cause of double strand break in the cells are not clearly understood.

4. Thesis rationale

It is apparent that mutations and chromosomal aberrations are caused by DNA damages, which occur spontaneously during normal growth and can be induced by exposing to UV irradiation, ionizing radiation, or some chemicals. It has been reported that UV irradiation and ionizing radiation induce DNA double strand breaks that are mostly repaired by recombination repair pathway and induce chromosomal rearrangements (Kasparek & Humphrey 2011; Friedberg *et. al.,* 2006). Therefore, cells poorly grew in the absence of recombination repair system under UV irradiation or ionizing radiation (Amundsen & Smith 2003).

Among the various kinds of DNA lesions emerging in normally growing cells, oxidative DNA damages are the most abundant, which are caused by reactive oxygen species under aerobic metabolism. It has been approved that under normal growth condition every cell produces about 3,000-5,000 DNA lesions per bacteria cell per generation (Cox 2001). Among them, 8-oxodG (8-oxo-7,8-dihydroguanine) is a major oxidative DNA damage with a high production rate, about several hundreds of 8-oxodG per cell per generation (Cox 2001). 8-oxodG lesion is able to pair with cytosine and adenine as well, hence generation of G:C to T:A transversion in the absence of both MutM which eliminates 8-oxodG lesion and MutY which removes adenine from 8-oxodG:A mispair (Michaels *et. al.,* 1992; Cabrera *et. al.,* 1988; Nghiem *et. al.,* 1988). Using rifampicin-resistant mutation assay with *rpoB* as the target gene, the 8-oxodG-related mutation frequency was increased about 1,000-times in the absence of both MutM and MutY enzymes (Sakai *et. al.*, 2006).

As we have known, hydrogen peroxide (H_2O_2) is not a reactive radical, but this H_2O_2 can be quickly converted to hydroxyl radical, a highly reactive radical which attacks DNA and induces oxidative DNA damages, through Fenton reaction (Sakai *et. al.*, 2006; Cooke *et. al.,* 2003; Evans *et. al.,* 2004; Davies 2000). Recently, Nunose and Uefune in our laboratory found that the intracellular H_2O_2 levels were much higher in cells grown in minimal medium or in LB under low oxygen condition than that observed in LB under normal aerobic condition. Furthermore, they observed that when grown in minimal medium or in LB under low oxygen condition, the rifampicin-resistant mutation frequency in *ΔmutM ΔmutY* cells was further increased about 10-times as high as that observed in LB under normal aerobic condition, indicating that the growth conditions affected the amount of oxidative DNA damages produced in *E. coli* cells. However, the rifampicin-resistant mutation frequency in wild type cells was not changed in different growth conditions. This observation indicates that the action of DNA repair pathways in wild type strain very efficiently functioned to eliminate the oxidative DNA damages and to suppress spontaneous base substitution mutagenesis even when the cellular level was increased 10-times higher. As mentioned earlier, our current understanding is that oxidative DNA damages are repaired by BER pathway and

recombination repair pathway, and involvement of NER in the repair process is unclear. Therefore, no significant increase in spontaneous base substitution in cells producing higher level of oxidative DNA damages suggests that BER pathway has a very strong capacity to repair the oxidative DNA damages. However, it is not clear how efficient recombination repair pathway functions to repair double strand breaks caused by oxidative DNA damages when their level is increased in growth conditions described above. More important question is whether recombination events that induce chromosomal rearrangements are more frequent in the growth conditions that produce higher level of oxidative DNA damages. If we obtain answers to these questions, we would be able to find out the causes of spontaneous recombination events occurring in normally growing cells. Thus, I decided to investigate spontaneous recombination events in *E. coli* cells grown in different growth conditions which produce different levels of oxidative DNA damages. To this end, I hoped to clarify whether frequency of chromosomal rearrangements is affected by environmental factors such as nutrition and oxygen concentration.

About 10 years ago, our lab successfully constructed a hemi-diploid *rpsL* strain of *E. coli* with 2 *rpsL* genes and named the strain MK811. This strain can be used for genetic assay examining both forward mutations and homologous recombination events simultaneously (Kanie *et. al*., 2007). Using this *rpsL* assay, it was shown that a treatment of MK811 cells with 1 mM H_2O_2 for 30 minutes increased the frequency of total genetic changes 13-fold. Interestingly, a half of genetic changes were allelic recombination events (Sakai *et. al.,* 2006). Although the 1 mM H_2O_2 treatment is not a physiological condition, these data suggest that oxidative DNA damages can induce allelic recombination events. However, the *rpsL* genetic assay using MK811 strain cannot be used for further experiments with minimal media because the transgenic *rpsL* gene is integrated in *cysJIH* operon, resulting in a deletion of 802 bp upstream region of this operon and a cysteine auxotrophic phenotype (Kanie *et. al.,* 2007). Recently, Ohtsu and colleagues reported that the cysteine/cystine shuttle system plays an important role in oxidative stress tolerance (Ohtsu *et. al.,* 2010). Therefore, I first tried to construct a new hemi-diploid *rpsL* prototroph strain without any deletion in the genome. Then, I investigated spontaneous recombination events in the new hemi-diploid *rpsL* strain to answer these questions mentioned above. I also studied how much BER and NER contribute to the suppression of spontaneous allelic recombination events in normally growing cells.

Chapter II. Materials and Methods

1. Materials

1.1. Strains and plasmids

The information of strains and plasmids used in this study are listed in Table 1 and 2.

Table 1. List of strains used in this study

Strains	Genotype	References
MG1655	Wild type	(Guyer *et. al.,* 1981)
MK811	Wild type except *rpsL128* (*Str*^{R}) and *ΔcysJIH::rpsL*$^+$	(Sakai *et. al.,*2006)
MK9528	MG1655 *rpsL128* (*Str*^{R})	This study
MK9533	MK9528 carrying pKD46 Recombinase plasmid	This study
MK9544	MK9528 except *rpsL-rrnBT1*-kanamycin insert at upstream of *ygdH* gene and downstream of *sdaC* gene	This study
MK9547	MK9544 except *kan:: FRT*	This study
MK6006	MK811 except *ΔuvrA::kan*	Kimiko Hasegawa
JW2669	BW25113 except *ΔrecA::kan*	Keio collection (Baba *et. al.,* 2006)
JW1738	BW25113 except *ΔxthA::kan*	Keio collection (Baba *et. al.,* 2006)
MK9552	MK9547 except *ΔuvrA::FRT*	This study: P1 (MK9547 x MK6006)
MK9554	MK9547 except *ΔrecA::FRT*	This study: P1 (MK9547 x JW2669)
MK9556	MK9547 except *Δxth::FRT*	This study: P1 (MK9547 x JW1738)
JW1625	BW25113 except *Δnth::kan*	Keio collection (Baba *et. al.,* 2006)
JW0704	BW25113 except *Δnei:: kan*	Keio collection (Baba *et. al.,* 2006)
JW2146	BW25113 except *Δnfo::kan*	Keio collection (Baba *et. al.,* 2006)
MK9568	MK9547except *Δnth::FRT*	This study: P1 (MK9547 x JW1625)
MK9569	MK9547 except *Δnei::FRT*	This study: P1 (MK9547 x JW0704)
MK9572	MK9568 except *Δnei::FRT*	This study: P1 (MK9568 x JW1625)
MK9586	MK9547 except *Δnfo::FRT*	This study: P1 (MK9547 x JW2146)
MK9587	MK9556 except *Δnfo::FRT*	This study: P1 (MK9556 x JW2146)
MK9589	MK9547 carrying pTN249-*ahpC*-GFP plasmid	This study
MK9591	MK9587 except *uvrA::FRT*	This study: P1 (MK9585 x MK6006)
MK9598	MK9591 harbor pCA24N	This study

MK9599	MK9591 harbor pCA24N-UvrA	This study
SMR7467	MG1655 except *lexA3 (Ind⁻)* *malB*::Tn9*Δattλ*::P*sulA*Ω*gfp-mut2*	(Pennington & Rosenberg 2007)
MK9597	MK9547 except *lexA3 (Ind−)* *malB*::Tn9*Δattλ::PsulA*Ω*gfp-mut2*	This study: P1 (SMR7467 x MK9547)
MK9804	MK9547 except *mfd::FRT*	This study
MK9805	MK9552 except *mfd::FRT*	This study
MK9806	MK9587 except *mfd::FRT*	This study
MK7180	MG1655 except *ΔmutMΔmutY*	Haruka Uefune, 2016
MK7470	MG1655 except *ΔmutT*	This study

Table 2. List of plasmids used in this study

Plasmids	Phenotype	References
pKD46	λ Red recombinase, *araC*-P_{araB}, Ap^r	(Datsenko & Wanner 2000)
pKD13	km^r (FRT)	(Datsenko & Wanner 2000)
pCP20	FLP, amp^r, cm^r	(Datsenko & Wanner 2000)
pTN249	*ahpC*-GFP, pSTV29	(Nakayashiki *et. al.,* 2013)
pCA24N-uvrA	*UvrA* expression under T7 promoter (Aska clone)	(Kitagawa *et. al.,* 2005)

1.2. Synthetic oligonucleotides

Synthetic oligonucleotides were obtained from Integrated DNA Technologies and purified by HPLC. The oligonucleotide after purification was stored in TE buffer (10 mM Tris-HCl (pH8.0), 1 mM EDTA) and store at -30°C.

Table 3. List of oligonucleotides used in this study

No.	Name	Sequence (mer)	PCR product (kb)
1	ygdH-rpsL-rrnBT1-kana-F1	5'TGAAGTTGCCAGGCTCAGCCTACATCCCTTGCTACGAAATCTGCACGTAAGGCCTGGTGATGGCGGGATC3'(70mer)	Amplification of *rpsL-rrnBT1* fragment (0.9 kb)
2	kana(P4)-rrnBT1-R1	5'AGGTCGACGGATCCCCGGAATTAATTCTCATGTTTGACAGAAGGCCCAGTCTTTCGACTG3' (60mer)	

3	rrnBT1-kana(P4)-F2	5’CAGTCGAAAGACTGGGCCTTCTGTCAAACATGAGAATTAA3’ (40mer)	Amplification of kanamycin fragment (1.4 kb)
4	Sdac-kana-rrnBT1T2-rpsLR2	5’AGTGACCAAAAACGAGAAAACCCGGCAACAAACGTCACCGGGGGAGCGGAGAGTGTAGGCTGGAGCTGCTTC3’ (72mer)	
5	SdaC(2927829-2927852)R primer	5’CGGTTTATGAAAATAGGGAAACG3’ (23mer)	Amplification of *rpsL-rrnBT1*-kanamycin fragment (2.5 kb)
6	ygdH(2927495)-Seq (F)	5’GGCGGGTAACGTAAAAGAAGTC3’ (22 mer)	
7	Wild-rpsL-sequence-F	5’CCTTAGGACGCTTCACGC3’ (18mer)	Amplification and sequencing of authentic *rpsL* gene (0.6 kb)
8	Wild-rpsL-sequence-R	5’CGTCAGACTTACGGTTAAGC3’ (20mer)	
9	PT-F(128)adaptor:	5’TGTATATACTACCACTCCTAC3’ (21mer)	Determination of recombination events
10	uvrA-F(4274081-4274069	5’GCGATTGTACCATTACCA3’ (18mer)	Testing *uvrA* deletion (*ΔuvrA::FRT* : 0.5 kb)
11	uvrA-R(4271021-4270970)	5’GAATTTACTGCCGGAAGAA3’ (19mer)	
12	dRecA-F	5’CAGAACATATTGACTATCCGGTATTACCCGGCATGACAGGAGTAAAA3’ (47mer)	Testing *recA* deletion (*ΔrecA::FRT* : 0.3 kb)
13	dRecA-R	5’GGGCGACGGGATGTTGATTC3’ (20mer)	
14	Forward-Xth	5’TGCGCCATCGTTGACATCAT3’ (20mer)	Testing *xth* deletion (*Δxth::FRT* : 0.4 kb)
15	Reverse-Xth	5’TATAACAAAGGACGGCAGGC3’ (20mer)	
16	Nth-1711320-1711339-F	5’TTAGGTAGCTGGGCAAAAG3’ (19mer)	Testing *nth* deletion (*Δnth::FRT* : 0.6 kb)
17	Nth-1712336-1712355-R	5’CGACAGCATCGTGATCTTG3’(19mer)	
18	Nei-745755-745	5’CGGCAGAGTCAAAAGTATC3’ (19mer)	Testing *nei*

	774-F		deletion
19	Nei-74689-7470 8-R	5'TTATGACTGCCTACCTCGC3' (19mer)	(*Δnei::FRT* : 0.5 kb)
20	nfoF-primer(225 0677-2250695)	5'AAACCTCTGCTGATGGCA3'	Testing *nfo* deletion
21	nfoR-primer (2251851):	5'TCCTGCTCGGTGAGAATG3'	(*Δnfo::FRT* : 0.4 kb)
22	May-M1	5'CGCGTTAAATGCTGAATCTTTACGCATTT C3' (30mer)	Testing *dinB* deletion
23	May-M2	5'GAGAATTCGATGCATACAGTGATACCCT CA3' (30mer)	(*ΔdinB::FRT* : 0.3 kb)
24	SA-1-MutM	5'ATCCAGTTGTTCGCCAGCACGT3' (22mer)	Testing *mutM* deletion
25	SA-2-MutM	5'CATCAGGCGCTGATGGCGAAG3' (21mer)	(*ΔmutM::FRT* : 0.4 kb)
26	MFD1F	5'ACTGACACTCCTGCTGGTTTATCCC3' (25mer)	Testing *mfd* deletion
27	MFD1R	5'TGAATTATGGATGGTGACAGTGTCG3' (25mer)	(*ΔdinB::FRT* : 0.4 kb)

1.3. DNA marker

A 2.65 µg of lambda DNA was digested by 1.25 units *Eco*T14I in 50 µl of reaction buffer and incubated at 37°C for 2 hours. Then inactivated at 65°C for 20 minutes. After incubation, the solution was kept at -20°C for further usage. The DNA marker was loaded on agarose gel and stained with ethidium bromide.

1.4. Chemicals and media

** Chemicals*

For PCR reactions: ExTaq DNA polymerase, dNTPs mix, ExTaq buffer were supplied from Takara, and rTaq DNA polymerase, dNTPs mix and rTaq buffer were supplied from BioAcademia. 70% ethanol was used for DNA purification and Hi-Di Formamide was used for sequencing.

** Media*

LB medium: 1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 1% (w/v) NaCl,

pH 7.0. LB plate was LB with 1.5% bacto agar. The medium was autoclaved at 121℃ for 20 minutes, then cooled down at 55℃ for at least 1 hour in water-bath and poured into plates.

LB + *Oxyrase* medium: Contents of LB medium were put into a flask with amounts as normal, but RO water was added 10% smaller than the normal amount. After autoclaving at 121℃ for 20 minutes, then cooled down at 55℃ for at least 1 hour in water-bath. Then 10% of *Oxyrase* was slowly added (*Oxyrase* was thawed overnight at 4℃) to avoid bubble. The poured plated were kept in anaerobic box (MGC company) at 4℃ overnight.

M9 medium was prepared from 5×M9 salts stock (210 mM Na_2HPO_4, 110 mM KH_2PO_4, 43 mM NaCl, 94 mM NH_4Cl, autoclaved at 121℃ for 20 minutes and stored at room temperature) with supplement 0.01 mM $CaCl_2$, 1 mM $MgSO_4$ and 0.2% of glucose or 0.2% glycerol which was sterilized by 0.2 μm filter) (Hofnung 1993).

R-top agar for P1 transduction: 1%(w/v) bacto tryptone, 0.1%(w/v) bacto yeast extract, 0.8%(w/v) NaCl, 0.8%(w/v) bacto agar were autoclaved at 121℃ for 1 minute, then cooled down at 55℃ in water bath, and 2.0 mM $CaCl_2$ and 0.1% glucose were added.

Antibiotics: The final concentrations of ampicillin, kanamycin, chloramphenicol, streptomycin and rifampicin were used as 100 μg/ml, 50 μg/ml, 25 μg/ml, 100 μg/ml, and 100 μg/ml, respectively. The antibiotic stocks were dissolved in MilliQ water and sterilized by 0.2 μm filter tool for all of antibiotics except rifampicin and chloramphenicol. To prepare rifampicin and chloramphenicol stock solutions, rifampicin or chloramphenicol was dissolved in methanol (the chloramphenicol concentration used for construction of *lexA3* (without SOS induction) mutant strain was 10 μg/ml which is lower than normal).

2. Methods

2.1. Construction of *rpsL* hemi-diploid strain

Step 1. Isolation of MG1655 derivative having rpsL128 A-C mutation

A colony of MG1655 strain was picked up, inoculated in 5 ml LB medium, and grown with rotate at 30°C for 16 – 18 hours.

Cells in 5 ml of overnight culture were collected by centrifugation at 5000 rpm for 10 min at 4°C. The cells were resuspended in 250 μl of fresh LB medium, spread on LB plate containing 50 μg/ml streptomycin, and incubated at 30°C until colonies formed. The 8 plates in total were used for isolation of MG1655 *rpsL128*A-C strain.

The colonies grown on LB containing 50 μg/ml streptomycin medium were streaked 2 times on LB containing 50 μg/ml streptomycin medium, applied to make glycerol stocks, quickly frozen in liquid nitrogen, and stored at -84°C.

The *rpsL* gene in isolated strain (Str^R) was amplified by PCR using primer number 7 and 8 in Table 3. Then sequence of *rpsL* gene in all of the strains were determined by ABI3130 sequencing machine using primer number 7.

The isolated MG1655 strain carrying *rpsL128*A-C mutation was named MK9528 and used for further experiments.

Step 2. Integration of rpsL-rrnBT1-kanamycin fragment into MK9528 strain

Amplification of rpsL-rrnBT1 fragment by PCR:

Preparation of DNA template: A colony of MK811 strain was picked up, put into 20 μl of 1/10 TE buffer, and boiled at 99°C for 3 min. Then the cell suspension was centrifuged at 3000 rpm for 5 min at room temperature. The supernatant was used as DNA template.

Mixture of PCR reaction: 0.25 mM of dNTPs, 1x Extaq buffer, 0.5 μM of primer for each (number 1 and 2), 2.5 U of ExTaq DNA polymerase, and 5 μl of DNA template were mixed with milliQ water to be filled up to 50 μl.

PCR thermal program: 94°C for 5 minutes, (94°C for 30 seconds; 72°C for 30 seconds; 72°C for 2 minutes) x 28 cycles; 72°C for 8 minutes; kept at 4°C.

PCR products about 0.9 kb were detected by 1% agarose electrophoresis.

Amplification of kanamycin fragment by PCR using BW25113 strain carrying pKD13 plasmid

1.4 kb kanamycin fragment was amplified from pKD13 plasmid using primers number 3 and 4 in Table 3. The preparation of DNA template and PCR mixture were the same as *rpsL-rrnBT1* amplification protocol. The PCR thermal program was also the same except that the annealing temperature was 65°C.

Connection of 0.9 kb rpsL-rrnBT1 fragment with 1.4 kb kanamycin fragment to form 2.3 kb rpsL-rrnBT1-kanamycin fragment by 2 steps PCR

The *rpsL-rrnBT1* and kanamycin fragments were purified by PCR gel extraction (protocol was as gel extraction kit of QIAGEN company)

Master mix A included milliQ water, dNTPs, ExTaq buffer, DNA template (*rpsL-rrnBT1* and kanamycin products), and ExTaq *polymerase* (excluding primers). Master mix B contained all PCR components except the DNA template. Step 1 was overlapped extension reaction at 94°C for 5 minutes, (94°C for 30 seconds; 60°C for 1.5 minutes; 72°C for 2.5 minutes) x 10 cycles; Step2 was amplification of full *rpsL-rrnBT1*-kanamycin fragment at 94°C for 5 minutes, (94°C for 30 seconds; 58°C for 30 seconds; 72°C for 3 minutes) x 30 cycles; 72°C for 10 minutes; keep at 4°C. The 2.3 kb PCR product was detected by 1% agarose electrophoresis and purified by gel extraction.

Insertion rpsL-rrnBT1-kanamycin fragment into MK9528 (rpsL128A-C)

This fragment was inserted into MG9528 genome between *sda*C and *ygdH* genes using pKD46 recombinase plasmid by homologous recombination. Firstly, the *rpsL-rrnBT1*-kanamycin fragment was amplified by PCR using primer 1 and 4 containing 50 nucleotide homologous sequences of *ygdH* and *sda*C genes, respectively. Secondly, the *rpsL-rrnBT1*-kanamycin fragment with homologous sequence was transformed into MK9528 containing pKD46 recombinase plasmid by electroporation as described following.

Overnight culture of MK9528 (pKD46) was made with 5 ml LB containing ampicillin 100 μg/ml and grown with rotate at 30°C for overnight.

500 μl of overnight culture was transferred into 50ml LB + ampicillin 100μg/ml and further cultivated with shake at 250 rpm at 30°C until OD_{600} about 0.3. The pKD46 recombinase was induced by the addition of 2 ml of 10% L-arabinose. The culture was transferred to 37°C and continued to grow for 45 minutes. Cells were harvested by centrifugation at 4000 rpm for 15 minutes at 4°C. Then cells were washed 2 times with 20 ml of cold 10% glycerol and centrifuged at 4000 rpm for 10 minutes at 4°C. Finally cell pellet was dissolved in 200 μl of 10% glycerol and used as the competent cells for transformation by electroporation.

The transformants were selected on LB medium containing kanamycin 50 μg/ml. The successful transformants must be streptomycin sensitive phenotype because of the dominance of wild type *rpsL* gene.

The new strain was named MK9544, which carries *rpsL-rrnBT1*-kanamycin fragment with kanamycin resistant and streptomycin sensitive phenotypes confirmed by sequencing.

Then, kanamycin fragment was popped out using pCP20 FLP recombinase plasmid transformation.

Step 3. Pop out kanamycin fragment using pCP20 plasmid

Picking up 1 colony of MK9544 strain, and cells were grown in 5 ml LB containing 50 μg/ml kanamycin medium with rotate at 37°C for 16 – 18 hrs.

200 μl of overnight culture was transferred into 20 ml LB containing 50 μg/ml kanamycin medium (in 100 ml flask), and cells were further grown with shaking at 250 rpm at 37°C until OD_{600} 0.35 to 0.4. The flask containing cells was kept in ice-water for 20 minutes.

Cells were transferred into 50 ml conical tube and centrifuged at 4200 rpm for 10 minutes at 4°C.

Cell pellet was resuspended into 20 ml of cold 80 mM $MgCl_2$ and 20 mM $CaCl_2$

solution, and centrifuged again at 4200 rpm for 10 minutes at 4°C.

Cell pellet was resuspended into 150 µl of cold 0.1 M $CaCl_2$ solution and was ready for transformation.

100 µl of competent cells was mixed with 15 ng of pCP20 plasmid into 1.5 ml Eppendorf tube, kept on ice for 30 minutes. Then put into 42°C for 90 seconds and immediately put back on ice and kept for 2 minutes. 800 µl of LB medium was added in the Eppendorf tube containing competent cells and plasmid DNA and rotated at 30°C for 1.5 hrs. Cells were spreaded on LB plate containing 25 µg/ml chloramphenicol and grown at 30°C for 18 - 21 hrs.

Cells grown on LB containing 25 µg/ml chloramphenicol were streaked 2 times on LB medium and grown at 42°C to remove the pCP20 plasmid. The cells with Cam^S and Km^S phenotype were confirmed by PCR using primers 5 and 6. The correct strain carrying 2 copies of *rpsL* genes was named MK9547 and used for further experiments.

2.2. Construction of mutant strains by P1 transduction

Step 1. Preparation of P1 phage lysate

A single colony of donor strain with kanamycin as marker gene was picked up into 5 ml LB containing 50 µg/ml kanamycin medium, and cells were cultivated with rotate at 37°C for overnight.

50 µl of overnight culture was transferred into 5 ml LB containing 5 mM $CaCl_2$, and cells were grown at 37°C with shaking at 160 rpm in water bath until OD_{600} 0.4 - 0.5 (about 10^8 cells/ml).

The original P1 lysate was diluted with LB medium to get about 10^9 phage/ml and mixed with donor strain at different ratios of host strain to phage, 1:1, 10:1, and 100:1. The cell culture without P1 lysate was used as control. Then, the mixture was incubated at 37°C for 20 minutes. The mixture of bacteria cells and phage was mixed with 2.5 ml of R-top agar, slightly vortexed for 2 seconds, and poured on R-plates. These plates were incubated at 37°C for at least 8 hours.

The new P1 lysate was collected by scraping the soft agar layer and centrifuging at 9000 rpm for 20 minutes. Chloroform was added into new P1 phage lysate to kill the donor cells. P1 phage lysate was stored at 4°C for several months.

Step 2. Titrate of P1 virus lysate from transduction

The protocol for titration of P1 phage lysate was similar to step 1. However, the new P1 phage lysate was diluted with a series of dilution factor in LB medium, mixed with

bacterial culture as above, and then incubated at 37°C for overnight to count the PFU (number of phage per ml).

Step 3. Transduction with P1 phage lysate

This step was also done similarly to step 1 except the P1 phage lysate was mixed with recipient strain. After mixing and incubating at 30°C for 20 minutes, the mixture was mixed with 0.2 ml of 1 M sodium citrate to inhibit the reabsorption of P1 phage to recipient cells and to reduce killing of cells, then incubated for another 1.5 hours at 30°C.

Finally, the mixture was spreaded on LB plate containing 5 mM sodium citrate and 50 µg/ml kanamycin. The correct transductants were confirmed by PCR.

Step 4. Pop out of kanamycin fragment by transformation with pCP20 plasmid

The correct transductant cells were grown in 5 ml LB containing 50 µg/ml kanamycin medium with rotate at 37°C for overnight.

50 µl overnight culture was transferred into 50 ml LB containing 50 µg/ml kanamycin medium (in 100 ml flask), and cells were grown at 37°C with rotate at 220 rpm until OD_{600} 0.3 - 0.4.

35 ml of cell culture was transferred into 50 ml conical tube and kept in ice-water for 1 hour. Cells were harvested by centrifugation at 4200 rpm for 10 minutes at 4°C.

Cell pellet was resuspended in 35 ml of cold 80 mM $MgCl_2$ - 20 mM $CaCl_2$ solution, and then centrifuged at 4200 rpm for 10 minutes at 4°C.

Cell pellet was resuspended in 0.3 ml of cold 0.1M $CaCl_2$ solution and ready for transformation with pCP20 plasmid.

About 100 µl of competent cells were mixed with 10 - 20 ng of pCP20 plasmid in an Eppendorf tube, kept on ice for 30 minutes, and then heat treated at 42°C for 90 seconds. The mixture of cells and plasmid was immediately cooled down on ice for 2 minutes. 0.8 ml to 1 ml of fresh LB medium was added into the mixture, and cells were grown at 30°C with rotate for 1 hour, spread on LB plate containing 25 µg/ml chloramphenicol, and incubated at 30°C for overnight. The correct transformant showed Km^S and Cam^R phenotype.

After transformation, pCP20 plasmid in the transformant was removed by incubating at 42°C for overnight, and the resulting cells showed Km^S Cam^S phenotype.

2.3. Measurement of mutation frequency by streptomycin resistant assay

Cells were grown in 5ml LB medium at 37°C for 16 hours. The overnight cells culture was diluted about 1/3 to $1/6x10^{-6}$ with LB medium and spreaded 100µl on LB plate or

diluted with 1xM9 salts and spreaded 100µl on M9 + 0.2% glucose or M9 + 0.2% glycerol plate (about 100 colonies). Then cells were incubated at 37°C until form 1.5mm colonies in diameter. About 100 colonies were collected with 5ml cold LB medium. The total cells number was determined by growing on LB medium after 16 hours at 37°C. And the mutant cells number were determined by growing on LB containing 100µg/ml streptomycin medium after incubating at 37°C for 24 hours. Mutation frequency for each population was calculated by dividing the number of mutant cells by the total number of cells.

2.4. Measurement of mutation frequency by rifampicin resistant assay

The protocol for measuring of Rif^R mutation frequency was similar to Str^R method except 100 µg/ml rifampicin was used instead of 100 µg/ml streptomycin. For rifampicin experiment, the rifampicin containing plates were prepared within 24 hours before measuring mutation frequency, and exposure of rifampicin plate to light was avoided.

2.5. Measurement of spontaneous allelic recombination events by PCR

Step 1. Preparation of DNA template for PCR analysis

E. coli cells to be examined were grown on LB plate containing 100 µg/ml streptomycin to form colonies at 37°C for 24 hours. Then 16 Str^R colonies were picked up from a plate for PCR and sequencing analyses to identify the recombination and non-recombination events on the target *rpsL* gene. DNA template was prepared as described above.

Step 2. Optimizing the PCR annealing temperature to identify the recombination events and non-recombination events

Mixture of PCR reaction: 0.25 mM of dNTPs, 1x PCR buffer, 0.5 µM of primers (number 9 and 5 in Table 3), 2.5 U of rTaq DNA polymerase, 5µl of DNA template, were mixed with milliQ water to be filled up to 10µl. PCR thermal program: 94°C for 5 minutes, (94°C for 30 seconds; at annealing temperature for 30 seconds; 72°C for 2 minutes) x 30 cycles; 72°C for 8 minutes; kept at 4°C. PCR products about 0.9 kb and 1 kb were detected by 1.2% agarose electrophoresis.

TheVeriti™ 96-Well Thermal Cycler From Applied Biosystems was used for PCR optimization. Firstly, the series of annealing temperature from 52, 53, 54, 55, 56, 57, 58, 59, 60, 60.5, 61, 61.5, 62, 62.5, 62.6, 62.7, 62.8, 62.9, and 63°C were used for detection of recombination events using primers 9 and 5. The result indicated that with annealing temperature from 52°C to 59°C, the PCR efficiency was low and it was hard to identify recombination events and non-recombination events (data not shown). With annealing

temperature from 60°C to 62°C, DNA template with recombination or non-recombination events could be amplified well. But, with annealing temperature from 62.5°C to 63°C, I could identify recombination and non-recombination events.

Step 3. Confirming the accuracy of PCR method for detection of recombination events by sequencing.

21 colonies of Str^R derivatives of MK9547 hemi-diploid *rpsL* strain were used for the examination of accuracy of the PCR method. DNA templates from these 21 candidates and 2 control templates (wild type template and recombination event template were confirmed by sequencing) were amplified by PCR using a set of primers (9 and 5). From the PCR result, the 8 recombination events candidates and 8 non-recombination events candidates were used for sequencing analysis to confirm the accuracy of PCR method (Figure 12).

2.6. Measurement of intracellular H_2O_2 level using flow cytometer

Cells harboring a GFP fusion plasmid (pTN249-*ahpC*-GFP) were grown in 5 ml LB containing chloramphenicol 25μg/ml with rotate at 37°C for 14 hours. The cell culture was diluted with LB medium or 1xM9 salts solution, and 100 μl of cell culture was spreaded (about 100 colonies per plate) on LB plate or minimal plates, M9 + 0.2% glucose and M9 + 0.2% glycerol medium. Cells were grown at 37°C until colony size 1.5mm in diameter. Colonies were harvested with 5 ml 1xM9 salts containing 2% sodium azide. The cell culture was diluted 10^{-3}-fold with 1xPBS (phosphate-buffered saline) for measurement of expression level of *ahpC*–GFP as intracellular H_2O_2 level by flow cytometer analysis. All data were collected using a FACScan flow cytometer (Becton Dickinson) with a 488-nm argon laser and a 515- to 545-nm emission filter (FL1) at a low flow rate. The following photomultiplier (PMT) voltage settings were used: E01 (forward scatter(FSC)), 349 (side scatter(SSC)), and 736 (FL1). Flow cytometer analysis measured approximated single-cell portion of cell population defined by the FSC and SSC in order to exclude filamentous cells. The relative fluorescence values were determined by taking mean fluorescence measurement for each sample, and each value represented the average of total 6 fluorescent values. The negative control was used as cells without GFP fusion plasmid (pTN249-*ahpC*-GFP).

2.7. H_2O_2 sensitivity assay

A single colony was picked up into 5 ml LB medium, and cells were grown with rotate at 160 rpm at 37°C for overnight. 50 μl of overnight culture was transferred into 5 ml LB, and cells were grown with shaking at 160 rpm at 37°C until OD_{600} about 0.35. Then, 0.5 ml of cell culture was mixed with 4.5 ml LB soft agar and poured onto LB plate. After soft

agar and cell culture mixture became solidification, a piece of filter paper was placed in the center of plate, and 10 µl of 30% H_2O_2 (about 9 M) was spotted on the filter paper. After incubation at 37°C for overnight, the diameter of inhibition zone was measured.

2.8. Phenotypical analysis in UV irradiation

E. coli cells were grown in 5 ml LB medium with rotate at 37°C for overnight. 5 µl of serial dilutions from 10^{-1} to 10^{-6} of the cell culture were spotted on LB plate, and the plate was placed at 37°C for 15 minutes to completely dry the spot. Then cells were exposed to UV irradiation (20 J), and the plate was incubated at 37°C for overnight.

Chapter III. Results

Part 1. Involvement of oxidative DNA damages in spontaneous allelic recombination events and roles of repair pathways in suppression of recombination events in *E. coli*

1. Construction of a new hemi-diploid *rpsL* strain by PCR and transformation

In order to determine the allelic recombination events occurring in *E. coli* chromosome, I constructed an *E. coli* strain named MK9547. In this strain, a *rpsL-rrnBT1* DNA fragment is inserted at a particular site located downstream of *ygdH* gene which is located at 63 min in *E. coli* genome. There are 2 copies of *rpsL* gene in MK9547 strain, an authentic *rpsL* gene located at 74 min which makes the Str^R allele (*rpsL128*, A:T to C:G transversion at 128 location), while the transgenic *rpsL* gene was inserted at 63 min, which is a wild type sequence conferring the Str^S phenotype. The presence of *rpsL128* and wild type *rpsL* genes in the hemi-diploid *rpsL* strain results in Str^S phenotype of the strain because of the dominant phenotype of wild-type *rpsL* gene. Genetic changes which lose the function of transgenic *rpsL* gene would convert the phenotype to Str^R (Figure 7). Using this hemi-diploid *rpsL* strain, the *rpsL* mutants can easily be screened by plating cells on LB plates containing streptomycin.

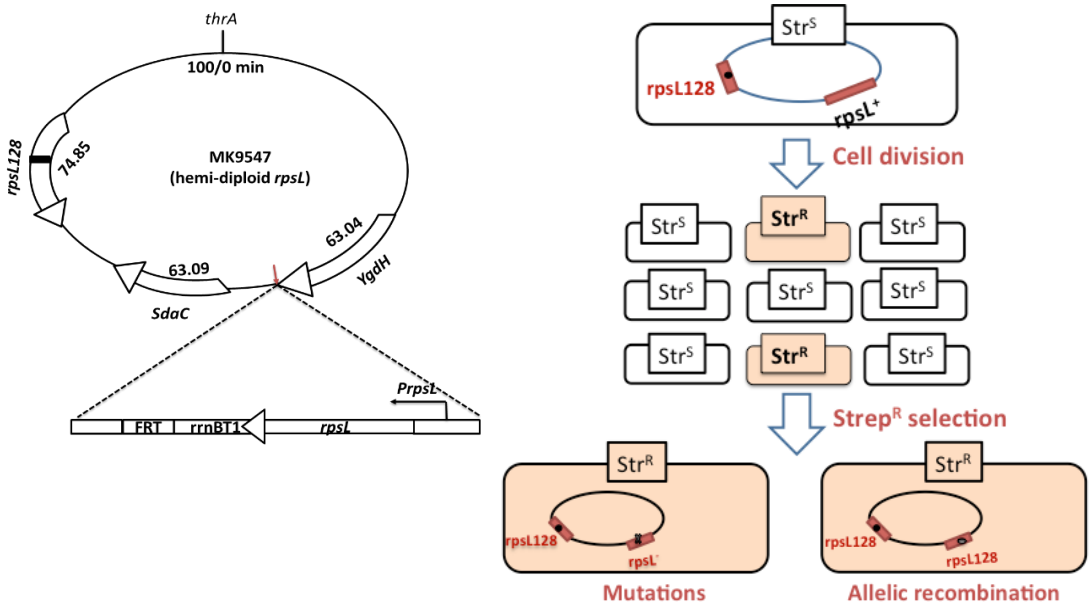


Figure 7. Schematic construction of the hemi-diploid *rpsL* strain and *rpsL* assay for the measurement of mutation frequency.

Firstly, a DNA fragment containing the kanamycin resistant gene is PCR-amplified from plasmid pKD13 in *E. coli* BW25113 (pKD13 plasmid-carrying strain), and a DNA fragment carrying *rpsL-rrnBT1* segment is also PCR-amplified from genome DNA of MK811

strain. The kanamycin fragment with 1.4 kb is then connected with 0.9 kb fragment of *rpsL-rrnBT1* to form a DNA fragment *rpsL-rrnBT1*-kanamycin with 2.3 kb by two steps PCR. The result is indicated in Figure 8.

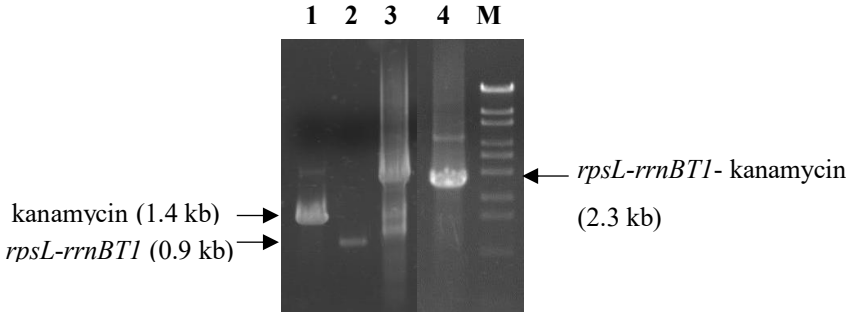


Figure 8. Preparation of *rpsL-rrnBT1*-kanamycin fragment by PCR.
Lane 1: kanamycin (1.4 kb); lane 2: *rpsL-rrnBT1* (0.9kb); lane 3: the *rpsL-rrnBT1*-kanamycin fragment (2.3 kb) after two steps PCR; lane 4: the *rpsL-rrnBT1*-kanamycin fragment (2.3 kb) after gel extraction; M: lambda DNA marker.

The *rpsL-rrnBT1*-kanamycin fragment was successfully prepared by two steps PCR as a DNA product about 2.3 kb in length together with some unspecific products (lower bands) (lane 3, Figure 8). The *rpsL-rrnBT1*-kanamycin fragment was further purified by gel extraction using QIAgen kit to remove unspecific bands which may affect transformation step (lane 4, Figure 8). Before transformation, the *rpsL-rrnBT1*-kanamycin fragment was amplified by PCR using a set of primers containing 50 nucleotides of sequences homologous to the target site. Then, the purified PCR product with homologous sequences was transformed into MK9529 (strain carrying pKD46 recombinase plasmid) by electroporation. The expression of recombinase activated the homologous recombination process between homologous sequences and resulted in integration of *rpsL-rrnBT1*-kanamycin fragment at specific site in DNA genome (between *ygdH* and *sdaC* genes). The transformants were selected on a LB plate containing 50 µg/ml kanamycin. The integrated fragment was confirmed by PCR using a set of primers which are located at *ygdH* and *sdaC* genes.

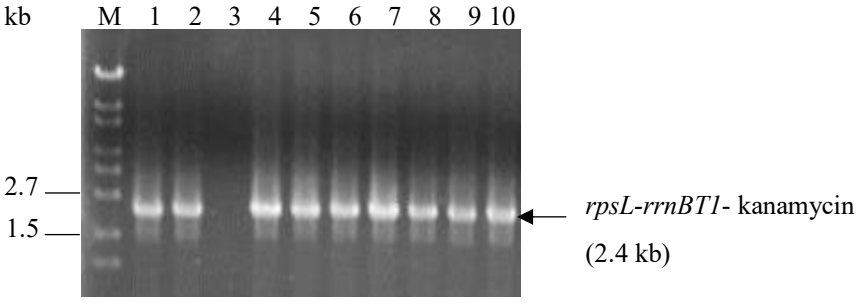


Figure 9. Testing the *rpsL-rrnBT1*-kanamycin insertion by PCR.
Lane 1-10: 10 clones after transformation, M: lambda DNA marker.

With 9 out of 10 candidates, the correct 2.4 kb band which carried the *rpsL-rrnBT1*-kanamycin fragment was successfully amplified (Figure 9). Four correct candidates were randomly selected for checking growth phenotype on LB and LB containing 100 µg/ml streptomycin plates (Figure 10). The result indicated that all of the selected candidates were streptomycin sensitive, indicating the integration of wild-type *rpsL* gene. These correct candidates were further confirmed by sequencing analysis. The sequencing data showed that the transgenic *rpsL* gene with the correct sequence was integrated at the correct position into chromosomal DNA in the cells.

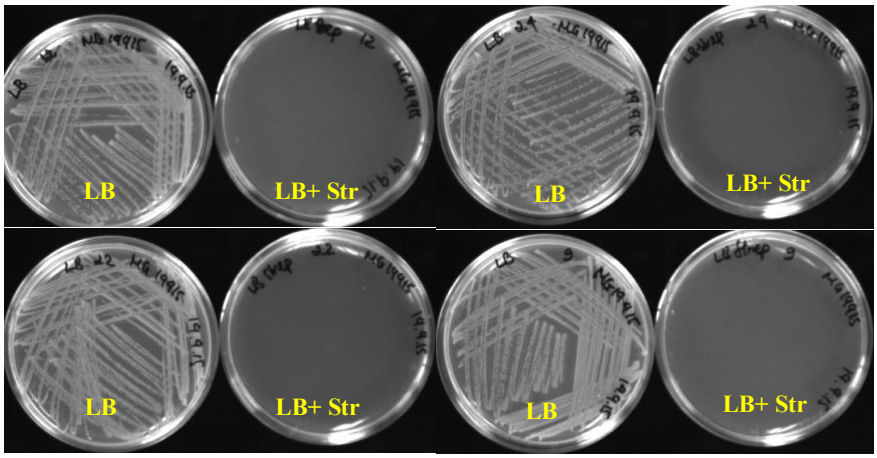


Figure 10. Growth phenotype of hemi-diploid *rpsL* strain on LB and LB + 100µg/ml streptomycin plates. Four expected candidates (colonies) were grown on LB and LB containing streptomycin plates at 37°C for about 16 hours. The corrected candidates were expected to be streptomycin sensitive.

Finally, the kanamycin resistance gene was removed by transformation of pCP20 plasmid, and the faithful removal of the DNA segment was confirmed again by sequencing. The sequencing data showed that a new hemi-diploid *rpsL* strain was successfully constructed, and I named the strain MK9547.

2. Quick and accurate method for detection of allelic recombination events

There are two copies of *rpsL* gene in the hemi-diploid *rpsL* MK9547 strain including the authentic *rpsL* gene with one base substitution A to C at site 128 called *rpsL128* and the transgenic wild-type *rpsL* gene. As a result of recombination event, the transgenic *rpsL* gene can be converted to the same sequence as authentic *rpsL128* allele (Figure 11) (Kanie *et. al.,* 2007). Therefore, to measure the frequency of recombination events, I needed to detect Str^R clones carrying A to C base substitution at 128 position in the target *rpsL* sequence.

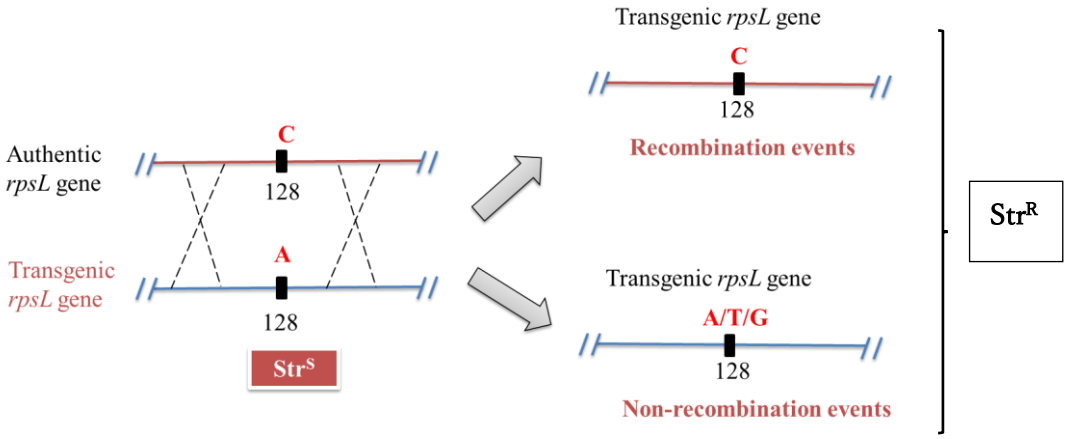


Figure 11. The recombination and non-recombination events occurring in transgenic *rpsL* gene.

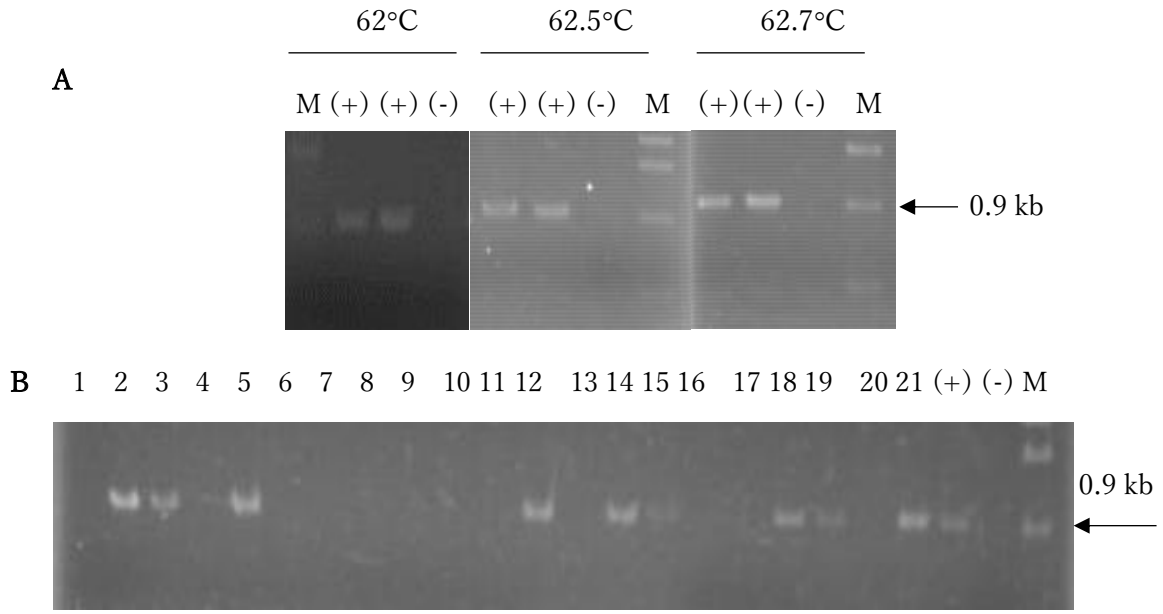


Figure 12. Optimization of PCR method for determination of the recombination events and non-recombination events.

A: PCR with different annealing temperature to identify the recombination template and non-recombination template. (+): positive control (recombination event containing DNA template); (-): negative control (Non-recombination event containing DNA template);

B: Detection of recombination events and non-recombination events by PCR using primers 9 and 5 with annealing temperature 62.5°C.

Previously, sequencing analysis was a powerful tool to detect mutations and recombination events, but this method has some disadvantages such as requirement of finances and time. In order to solve this problem, an accurate and quick method to determine the recombination events (as A to C base substitution at 128 position in the taget *rpsL* sequence) rather than the sequencing method is necessary. The key point is that the point mutation occurring at the 128 position represents recombination events while the other mutations are considered as non-recombination events. To this end, I decided to use a PCR method to identify the recombination and non-recombination events. Forward primer that contained a final nucleotide C at 3' was used for recombination events. PCR with specific annealing temperature ensures to amplify only recombination events and generates the PCR products about 0.9 kb, but this PCR was not able to amplify the non-recombination events. The result for optimizing the annealing temperature is shown in Figure 12.

The recombination events in the target *rpsL* gene which has a point mutation 128A-C can be amplified by PCR using primer 9 and 5 in Table 3. Before optimizing the PCR method, the mutations occurring in the target *rpsL* gene were determined by sequence analysis to identify the recombination events and non-recombination events templates (Figure 11). The recombination events template was used as the positive control and the transgenic wild-type *rpsL* was used as the negative control for optimization of PCR method (Figure 12).

In order to optimize the PCR reaction to detect recombination events, a set of primers including primer 9 and 5 in table 3 were used. The PCR reaction was shown in the method part. A series of annealing temperatures from 54°C to 62.7°C were used for PCR experiments. The result shows that both recombination and non-recombination events can be amplified at lower annealing temperature (data not shown). On the other hand, I found a difference in the production between recombination events and non-recombination events at 62°C, 62.5°C and 62.7°C, indicating that recombination events and non-recombination events can be clearly identified at 62.5°C and 62.7°C. Therefore, 62.5°C was used as the annealing temperature to detect the recombination events by PCR.

After optimizing the PCR method for detection of recombination events, the sequencing step was performed to confirm the accuracy of PCR method. Firstly, the 21 Str^R candidates were amplified by PCR using a set of primers 9 and 5 at 62.5°C annealing temperature to detect recombination events and non-recombination events. The data indicated that 9/21 candidates (candidates 2, 3, 5, 12, 14, 15, 18, 19 and 21) were amplified by PCR and gave the 0.9 kb band similar to the positive control, which were considered recombination events (Figure 12). Other 12/21 samples (candidates 1, 4, 6, 7, 8, 9, 10, 11, 13, 16, 17 and 20) were not able to be amplified by PCR at 62.5°C annealing temperature similar to the negative control representing non-recombination events. Finally, these recombination and non-recombination events detected by PCR were confirmed again by sequencing method.

Results showed that the detection of recombination events and non-recombination events by PCR were accurate just as the sequencing analysis, and I decided to use this method for further experiments.

3. Minimal medium induces high level of H_2O_2

In order to confirm the observation by Nunose and Uefune in our laboratory, in which intracellular H_2O_2 level was increased in cells grown in minimal medium or under hypoxia condition, I measured the intensity of AhpC-GFP, indicative of intracellular H_2O_2 level, in MK9547 strain by flow cytometry.

The result showed that the AhpC-GFP (intracellular H_2O_2) level was increased in cells grown in minimal media, M9 + 0.2% glucose and M9 + 0.2% glycerol, compared to that with LB medium, and the values of AhpC-GFP level were almost the same as previous data (Nunose, unpublished) (Figure 13). Nunose suggested that the induction of intracellular H_2O_2 level was probably due to the changes in metabolism in cells grown with different nutrition. It was demonstrated that about 87% of H_2O_2 production in cells comes from respiratory chain in *E. coli* cells, which is probably caused by leak of electron transport by NADH dehydrogenase or between ubiquinone and cytochrome b (Gonzalez-Flecha & Demple 1995). Therefore, the different nutrient components between LB and minimal medium may change the metabolism process and cause the difference in H_2O_2 production. The H_2O_2 level in cells grown in M9 + 0.2% glycerol medium was higher than that in M9 + 0.2% glucose medium, suggesting that the addition of glucose or glycerol probably changes the metabolism in *E. coli* cells. It has been reported that the metabolism in cells grown with glucose or glycerol was quite different (Abbad-Andaloussi *et. al.,* 1998). Having confirmed Nunose's finding, I decided to use the growth conditions with different media to change cellular production of H_2O_2 and oxidative DNA damages.

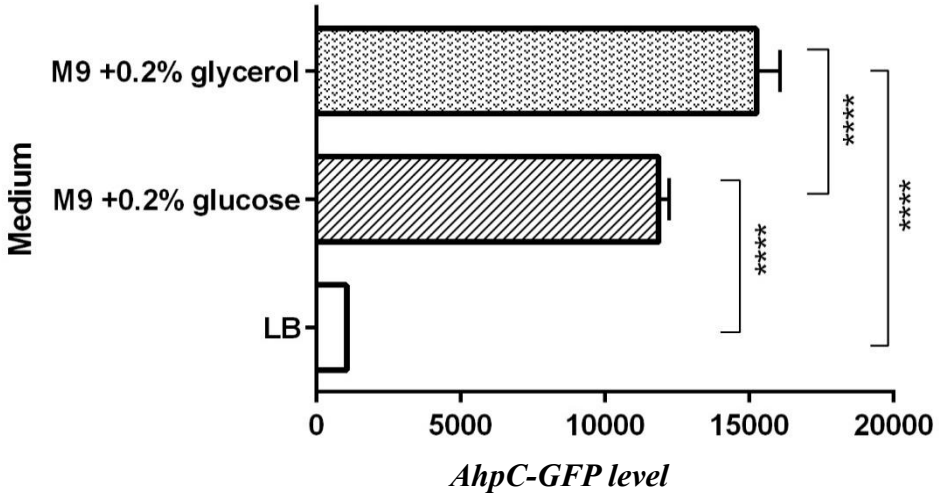


Figure 13. Measurement of intracellular H_2O_2 level in wild type (MK9547) strain grown on different medium (LB, M9 + 0.2% glucose, and M9 + 0.2% glycerol).
Cells were grown on plates containing the indicated media until colony size 1.5 mm in diameter. Then cells were harvested with 1xM9 salts containing sodium azide to stop the growth. After that, the cell culture was diluted with PBS buffer and used for measurement of expression level of *ahpC*–GFP as intracellular H_2O_2 level by flow cytometer analysis. Expression of *ahpC* gene is induced when the intracellular H_2O_2 level increases, so the intensity of ahpC-GFP level is a measure of the intracellular H_2O_2 level (see 1.2 in the Introduction part and 2.6 in Methods part for detail information). The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (****) indicates p-value <0.01.

4. Minimal medium induces spontaneous allelic recombination events

To investigate the effect of environmental factors on spontaneous allelic recombination events, cells were grown on different growth conditions including rich medium (LB) and minimal medium (M9 + 0.2% glucose or M9 + 0.2% glycerol).

As I mentioned above and in Section 4 of Chapter I, minimal medium makes cells to produce higher level of H_2O_2 than LB medium (Figure 13), and more amount of oxidative DNA damages is produced under minimal medium than LB medium (Nunose, unpublished). As shown in Figure 14, the allelic recombination events were significantly increased in the wild type (MK9547) strain grown in minimal media. Spontaneous allelic recombination events were 4-fold and 7-fold higher on minimal medium M9 + 0.2% glucose and M9 + 0.2%

glycerol, respectively, in comparison with LB medium. This data suggests that oxidative DNA damages produced in normally growing cells in minimal media would promote spontaneous allelic recombination events.

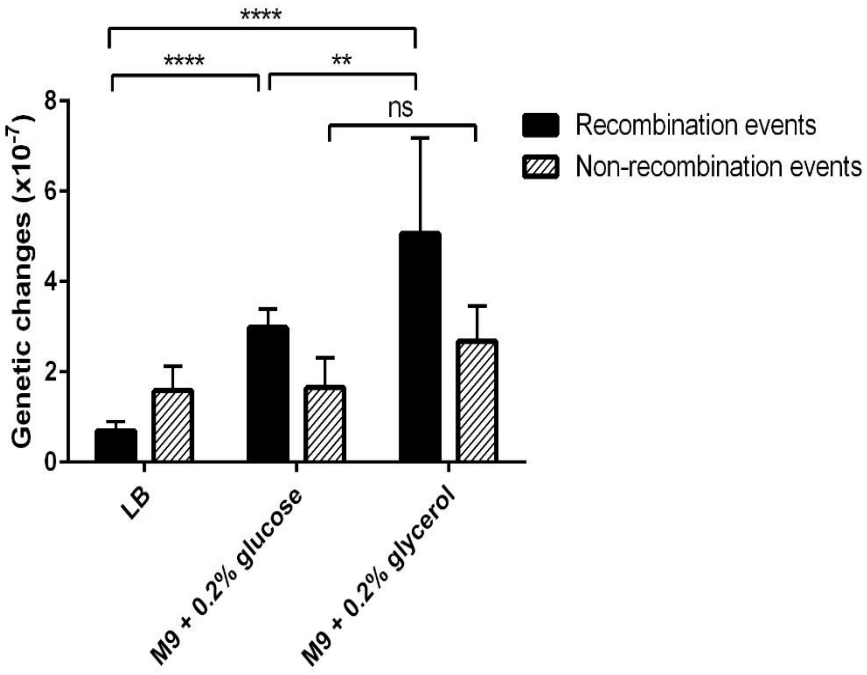


Figure 14. Nutrient factors affect spontaneous allelic recombination events in the wild type (MK9547) strain.

Cells were grown on plates containing the indicated media at 37ºC until colony size 1.5 mm in diameter. After collecting the cells, Str^r mutant cells were selected by plating on LB containing streptomycin plates at 37ºC for 24 hours to measure the mutation frequency (see 2.3 in Methods part). After that, 20 mutant colonies were used to determine the recombination and non-recombination events by PCR analysis (see 2.5 in Method part).

The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (**) indicates p-value <0.05 and (****) indicates p-value <0.01.

Medium	**LB**	**M9 Glu**	**M9 Gly**
Growth phenotype (hrs)	13	49 - 50	50
RE ($x10^{-7}$)	0.7	3.0	5.1
Non-RE ($x10^{-7}$)	1.6	1.7	2.7

(RE: Recombination events; Non-RE: Non-recombination events; M9 Glu: M9 + 0.2% glucose; M9 Gly: M9 + 0.2% glycerol).

5. Spontaneous allelic recombination events are highly induced in cells grown under hypoxic condition and suppresed by a strong antioxidant, *Oxyrase*

To verify that the allelic recombination events are caused by oxidative DNA damages, I examined effect of antioxidant on spontaneous allelic recombination events in wild type strain. As mentioned in Section 4 of Chapter I, Uefune reported that the intracellular H_2O_2 level was significantly increased when *E. coli* cells were grown under hypoxia (0.1% O_2) condition. *Oxyrase* is a natural antioxidant isolated from cytoplasmic membrane of *E. coli*, which reduces ROS production by capturing oxygen and converting it to water. *Oxyrase* was added into LB medium to see the reduction of ROS production such as H_2O_2 level in comparison to LB medium without *Oxyrase*. The AhpC-GFP level representing intracellular H_2O_2 level was measured by flow cytometry. The result indicated that hypoxia condition produced a higher level of H_2O_2 in the cells than that under normal aerobic condition. This observation was consistent with Uefune's observation, and the induction of H_2O_2 under hypoxia condition was significantly suppressed when *Oxyrase* was added (Figure 15).

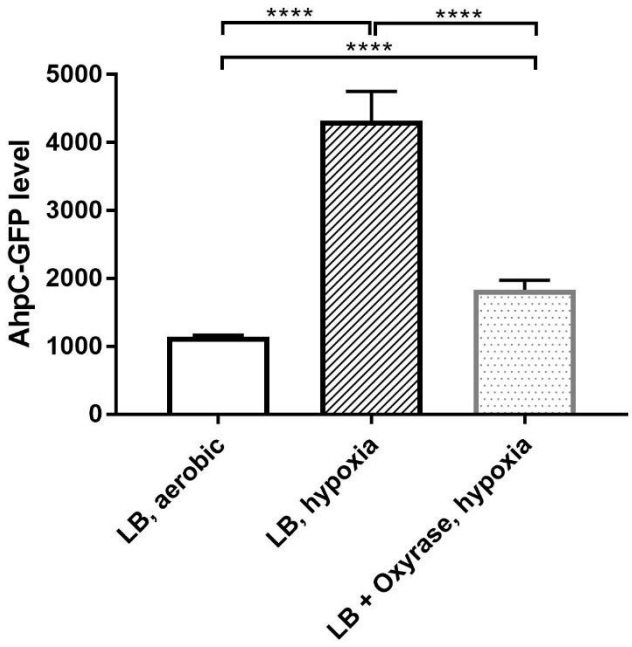


Figure 15. Intracellular H_2O_2 level in wild type (MK9547) strain grown on different oxygen conditions. Experiment was performed as shown in Figure 13. The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. Error bars represent SD. The stars indicate the significance of data: (****) indicates p-value <0.01.

As I expected, the frequency of recombination events was 3.5-fold higher in hypoxia

condition than that in aerobic condition (Figure 16). Then, I examined whether spontaneous recombination events is reduced in the addition of *Oxyrase*. Importantly, the presence of *Oxyrase* under hypoxia condition reduced the frequency of recombination events 7-fold compared to hypoxia condition and 2-fold lower than LB aerobic condition. These data clearly indicated that the recombination events induced under hypoxia condition is caused by oxidative DNA damages, and *Oxyrase* efficiently captures oxygen in medium, reducing the production of ROS, especially H_2O_2, hence reducing oxidative DNA damages in cells. However, the reduction in recombination events in the presence of *Oxyrase* (probably no oxidative DNA damages) is only 2-fold, compared to that under aerobic condition, suggesting that spontaneous DNA damages other than oxidative DNA damages also induce recombination events in cells grown under aerobic condition.

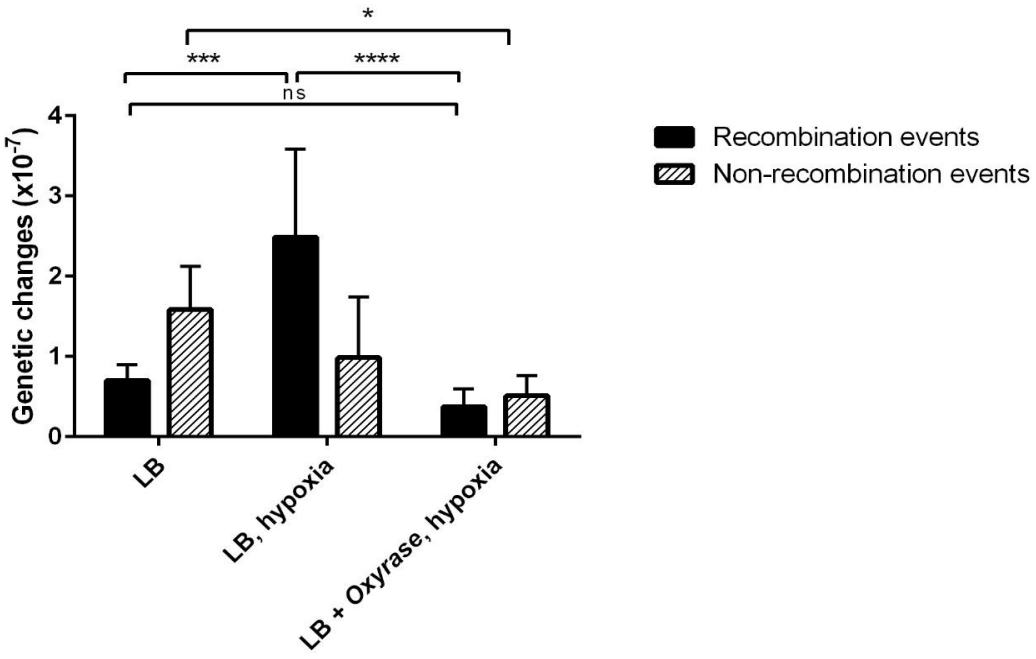


Figure 16. Role of *Oxyrase* in suppression of spontaneous allelic recombination events in wild type (MK9547) strain. The recombination and non-recombination events were determined as described in Figure 14. The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (*) indicates p-value <0.05, (***) and (****) indicate p-value <0.01.

Medium	**LB, aerobic**	**LB, hypoxia**	**LB + *Oxyrase*, hypoxia**
Growth phenotype (hrs)	13	60	24
RE ($x10^{-7}$)	0.7	2.5	0.35
Non-RE ($x10^{-7}$)	1.6	1	0.55

To obtain further evidence that oxidative DNA damages promotes recombination events in wild type strain, I investigate effects of hypoxia growth condition with and without *Oxyrase* on the production of 8-oxodG lesions in DNA and nucleotide pool in *E. coli*.

I measured Rif^R mutation frequency occurring in *ΔmutM ΔmutY* mutant strain on LB, LB under hypoxia and LB + *Oxyrase* under hypoxia conditions (Figure 17). The frequency of Rif^R mutation frequency was 15-fold higher in hypoxia condition than aerobic condition. The addition of *Oxyrase* to LB medium under hypoxia condition significantly reduced the frequency about 300-fold compared to that obtained with cells grown on LB under hypoxia condition.

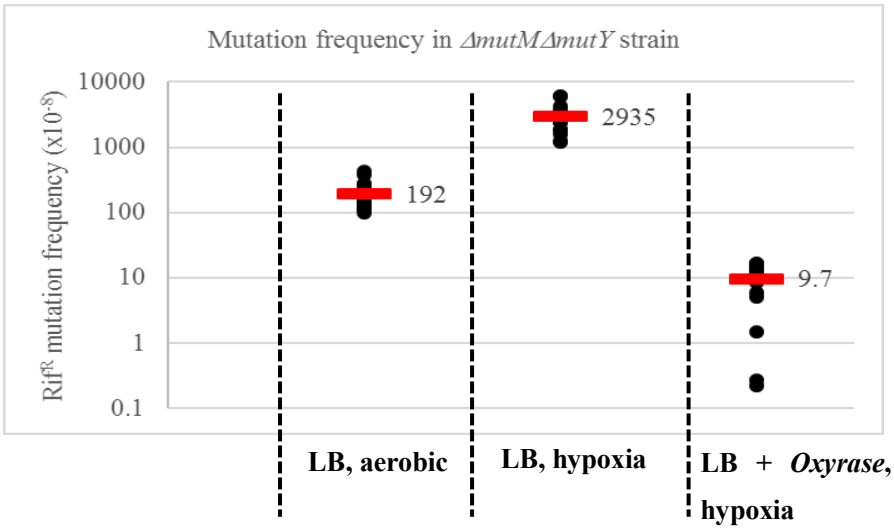


Figure 17. Suppression of 8-oxodG mutations in *ΔmutM ΔmutY* (MK7180) strain by *Oxyrase*. The data for LB and LB under hypoxia were obtained from Haruka Uefune in 2016. The mutation frequency was determined using rifampicin resistant mutation assay (see 2.4 in Methods part for protocol in detail). The number of replicated experiments and growth phenotype are shown in the below table.

Medium	**LB, aerobic**	**LB, hypoxia**	**LB + *Oxyrase*, hypoxia**
Repeat experiments	20	20	15
Growth phenotype (hrs)	14	50	24
Mutation frequency ($x10^{-8}$)	192	2935	9.7

I also measured the mutation frequency with *ΔmutT* strain under similar growth conditions. MutT is 8-oxodGTP nucleotide triphosplatase enzyme hydrolyzing 8-oxodGTP to 8-oxodGMP to avoid the incorporation of 8-oxodG to DNA, which induces A:T to G:C transversion mutations in *E. coli* cells (Maki & Sekiguchi 1992; Tassotto & Mathews 2002). The data in Figure 18 indicates that *Oxyrase* reduced the mutation frequency in *ΔmutT* strain

under hypoxia condition. This data is similar to the previous observation (Fowler *et. al.,* 1994)

From these results, I conclude that growth in minimal media (M9 + 0.2% glucose and M9 + 0.2% glycerol) and in LB under hypoxia (0.1% O_2) condition produce more oxidative DNA damages and induce spontaneous allelic recombination events.

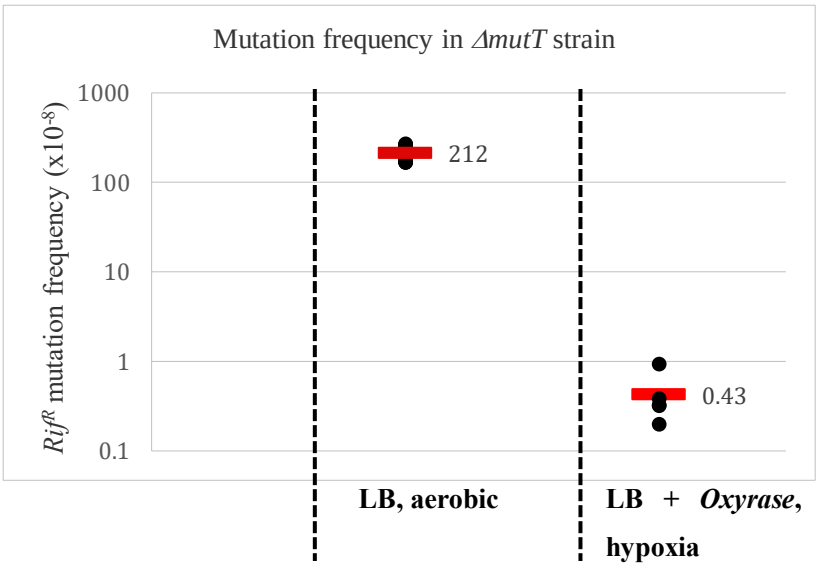


Figure 18. Suppression of 8-oxodGTP mutations in *ΔmutT* (MK7470) strain by *Oxyrase*.
The mutation frequency was determined as described in Figure 17. The number of replicated experiments and growth phenotype are shown in the below table.

Medium	**LB, aerobic**	**LB + *Oxyrase*, hypoxia**
Repeat experiments	20	5
Growth phenotype (hrs)	14	24
Mutation frequency (x10^{-8})	212	0.43

6. Base excision repair (BER) system is involved in suppression of spontaneous allelic recombination events in *E. coli*

6.1. Involvement of DNA glycosylase/AP lyase in suppression of spontaneous recombination events

BER system plays a major role in repairing small DNA lesions such as base modifications caused by oxidation, alkylation or deamination process. There are two main enzyme groups in BER pathway that is DNA glycosylases and AP endonucleases. DNA glycosylases initiate the process of BER pathway by cleaving N-glycosylic bond which links

the abnormal base to DNA backbone and leaving AP sites (apurinic/apyrimidinic sites). Then, AP sites are further processed by 2 major AP endonucleases, Exonuclease III (Xth protein) and AP endonuclease IV (Nfo protein) through cleavage of DNA phosphodiester backbone at 5' of AP sites to create a nick with a free 3'OH end for DNA polymerase repair synthesis.

Among the spontaneous DNA damages produced under normal growing condition, oxidative DNA damages are the most abundant. More than 20 types of oxidative DNA damages can be divided into mis-coding and blocking types, and most of them are repaired by action of BER pathway.

In order to investigate the involvement of two DNA glycosylase/AP lyase enzymes Nei and Nth in suppression of spontaneous recombination events, *Δnth* and *Δnei* single deletion strains as well as a *Δnth Δnei* double deletion strain were constructed by P1 transduction with MK9567 as a recipient. Frequency of spontaneous recombination events were determined in cells grown on LB or M9 + 0.2% glucose medium.

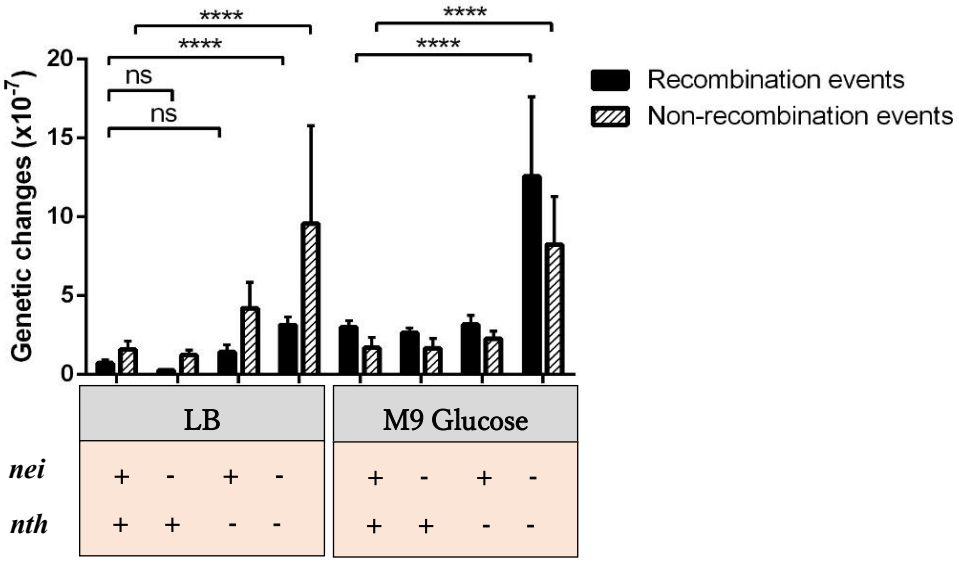


Figure 19. Involvement of DNA glycosylases/AP lyase in spontaneous recombination events *E. coli.* The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (****) indicates p-value <0.01, ns indicates non-significant. The strains used in this experiment are MK9568 (*Δnth* single deletion strain), MK9569 (*Δnei* single deletion strain), and MK9572 (*Δnei Δnth* double deletion strain). Recombination and non-recombination events were determined as described in Figure 14.

Strains	**Wild-type**		***Δnei***		***Δnth***		***Δnei Δnth***	
Medium	**LB**	**M9 Glu**	**LB**	**M9 Glu**	**LB**	**M9 Glu**	**LB**	**M9 Glu**
Growth phenotype (hrs)	13	49 - 50	13	50	13	50	13	50
RE (x10^{-7})	0.7	3.0	0.25	2.6	1.4	3.15	3.1	12.5
Non-RE (x10^{-7})	1.6	1.7	1.2	1.6	4.2	2.25	9.6	8.2

As shown in Figure 19, *Δnth* and *Δnei* single mutant strains showed no significant increment of spontaneous recombination events and non-recombination events on both LB and M9 + 0.2% glucose medium compared to the wild type. However, the frequencies of recombination events and non-recombination events in the *Δnei Δnth* double mutant strain were 4- to 5-fold higher than those with wild type strain. This data suggests that both Nth and Nei are dependent on each other to suppress both recombination and non-recombination events.

Interestingly, the characteristic of genetic changes in *Δnei Δnth* double mutant strain was different between the growth on LB and on M9 glucose medium. On LB medium, most of the genetic changes were non-recombination events. However, most of the genetic changes on M9 glucose medium was recombination events, suggesting that characteristic of oxidative DNA damages produced on LB might be different from that on M9 glucose medium. On the other hand, I observed that the frequency of non-recombination events in *Δnei Δnth* double mutant strain on LB medium was similar to that on M9 + 0.2% glucose medium. This suggests that Nth and Nei might repair a miscoding type of oxidative DNA damages, likely 5-hydroxyl cytosine, which can induce base substitution mutations in the absence of both Nei and Nth proteins.

6.2. Involvement of AP endonucleases in suppression of spontaneous recombination events

Many studies indicated that AP sites can be generated in DNA under normal aerobic metabolism or treated with some agents such as ionizing radiation, UV light or H_2O_2 (Heck *et. al.,* 2003). Besides that, the action of DNA glycosylases in eliminating base damages also contribute to produce AP sites, which can block DNA replication and induce lethality if accumulated at high level. In BER pathway, AP endonuclease enzymes play a very important role to help cells to overcome this problem by eliminating AP sites. There are two AP endonuclease enzymes in *E. coli* cells, Exonuclease III (Xth) and Endonuclease IV (Nfo). About 90% of the AP endonuclease activity in *E. coli* is performed by Exonuclease III (Xth) protein while Endonuclease IV contributes only about 10% of AP endonuclease activity (Souza *et. al.,* 2006).

As I demonstrated above, M9 + 0.2% glucose medium produced high level of H_2O_2 and induced spontaneous recombination events. The high production of H_2O_2 in cells grown on M9 + 0.2% glucose medium could induce a lot of oxidative DNA damages on DNA genome including AP sites. If this is true, cells lacking AP endonuclease (Xth and Nfo) would produce very high frequency of spontaneous recombination events.

As shown in Figure 20, *Δnfo* and *Δxth* single mutant strains did not show increment

of spontaneous allelic recombination events and non-recombination events on both LB medium and M9 + 0.2% glucose medium. However, the frequency of recombination events in *Δxth Δnfo* double mutant strain were 9- and 6-fold higher than that in wild type strain on LB and M9 + 0.2% glucose medium, respectively. This data indicates that the AP endonuclease activity of Nfo and Xth proteins is involved in suppression of recombination events. The frequency of recombination events in *Δnfo* and *Δxth* single mutant strains were as low as that in wild type strain, suggesting that either of Xth and Nfo proteins is strong enough to suppress the spontaneous recombination events.

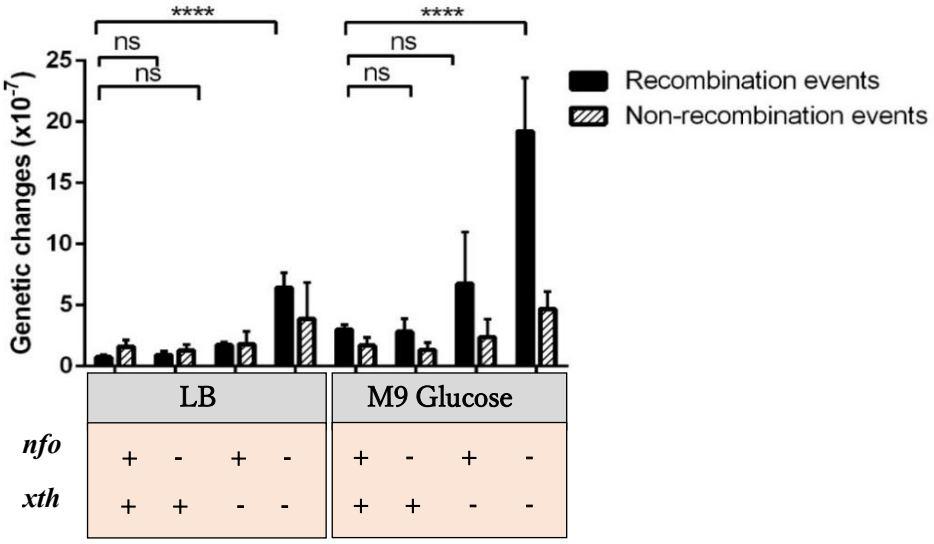


Figure 20. Involvement of AP endonuclease (Xth and Nfo) proteins in spontaneous allelic recombination events. The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (****) indicates p-value <0.01, ns indicates non-significant. The strains used in this experiment are MK9556 (*Δxth* single deletion strain), MK9586 (*Δnfo* single deletion strain), and MK9587 (*Δxth Δnfo* double deletion strain). Recombination and non-recombination events were determined as described in Figure 14.

Strains	**Wild-type**		***Δxth***		***Δnfo***		***Δxth Δnfo***	
Medium	**LB**	**M9 Glu**	**LB**	**M9 Glu**	**LB**	**M9 Glu**	**LB**	**M9 Glu**
Growth phenotype (hrs)	13	49 - 50	13	50	13	50	13	50
RE ($x10^{-7}$)	0.7	3.0	1.7	7.1	0.9	2.8	6.4	19.2
Non-RE ($x10^{-7}$)	1.6	1.7	1.8	2.1	1.3	1.3	3.8	4.6

In addition, cells lacking both Nfo and Xth proteins induced much more spontaneous recombination events compared to the wild type and *Δxth* and *Δnfo* single mutant strains, suggesting that a lot of AP sites were produced in genome DNA in normally growing cells.

7. Nucleotide excision repair (NER) system is involved in suppression of spontaneous allelic recombination events in *E. coli*

Nucleotide excision repair (NER) is one of the major DNA repair pathways involved in repair of a broad range of DNA lesions generally induced by exogenous chemicals or UV-irradiation (Sidorenko *et. al.,* 2015). NER can target single base modifications, bulky adducts, backbone modifications and inter- or intra-strand cross-links (Truglio *et. al.,* 2006). NER pathway is initiated when the DNA lesion is recognized by UvrA-UvrB complex. Recently, it has been reported that UvrA itself can scan along DNA genome and stop at DNA lesion site in UvrB dependent, suggesting how important of UvrA protein in NER pathway (Stracy *et. al.,* 2016). Therefore, this step becomes the most important step in NER pathway. I hypothesized that lack of UvrA protein may retain a large amount of DNA lesions in the DNA genome sequence and cause blocked or arrested replication forks. Then recombination repair pathways would come to rescue cells from the blockage of replication forks and induce recombination events.

In order to investigate the involvement of NER pathway in suppression of recombination events, the frequency of spontaneous recombination events occurring in *ΔuvrA* single deletion strain was determined in cells grown on LB under aerobic condition, on LB under hypoxia condition, and on M9 glucose medium. As shown in Figure 21, when cells grown on LB under normal aerobic condition, no increment in the frequency of recombination events was observed with *ΔuvrA* strain compared to the wild type strain, probably because there were not much oxidative DNA damages produced under this condition. However, the cells grown under conditions that produce higher level of oxidative DNA damages, such as on M9 + 0.2% glucose and under hypoxia condition, the frequency of recombination events in *ΔuvrA* strain was significantly increased and much higher than that in wild type strain (Figure 21). This data clearly indicates that UvrA protein in NER pathway contributes in suppression of recombination events caused by high level of oxidative DNA damages in normally growing cells.

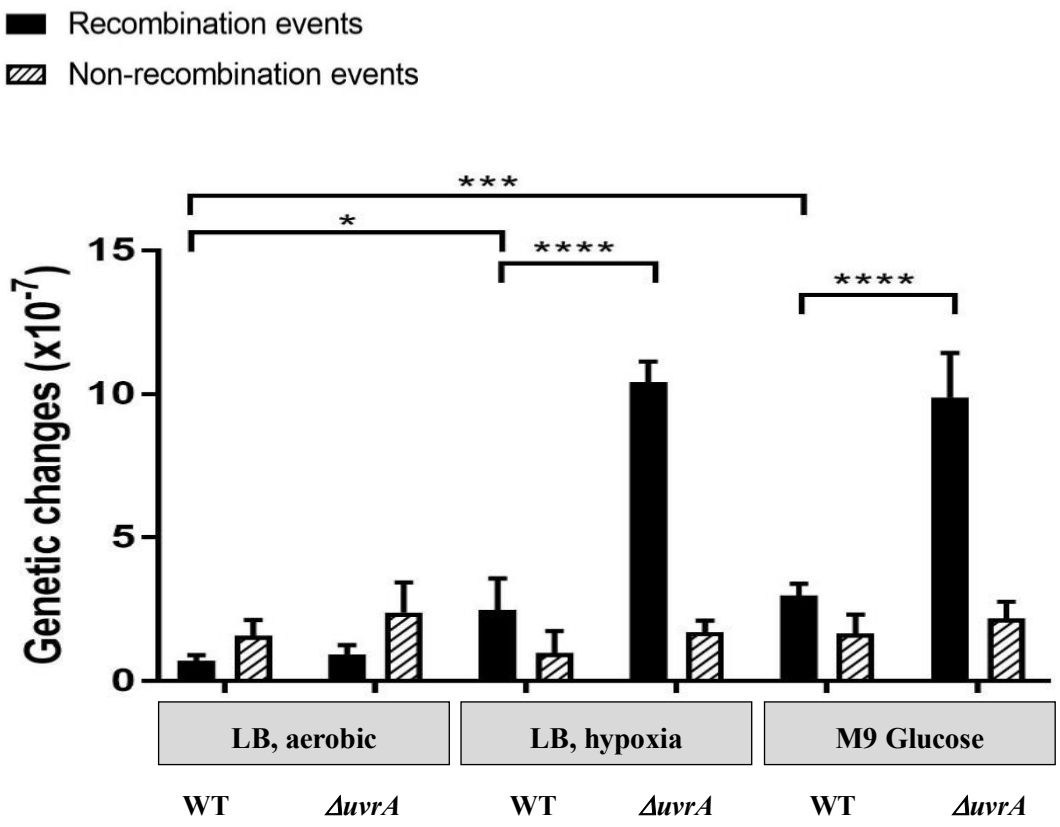


Figure 21. Involvement of NER pathway in spontaneous allelic recombination events. The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (*) indicates p-value <0.05, (***) and (****) indicate p-value <0.01. The strains used in this experiment are wild type MK9547 and *ΔuvrA* deletion strain (MK955). Recombination and non-recombination events were determined as described in Figure 14.

Strains	**Wild-type**			***ΔuvrA***		
Medium	**LB, aerobic**	**LB, hypoxia**	**M9 Glu**	**LB, aerobic**	**LB, hypoxia**	**M9 Glu**
Growth phenotype (hrs)	13	60	49 - 50	13	55	50
RE (x10^{-7})	0.7	2.5	3.0	0.9	10.4	9.9
Non-RE (x10^{-7})	1.6	1	1.7	2.4	1.7	2.2

8. Investigation of spontaneous allelic recombination events in *E. coli* cells lacking both NER and BER pathways

As described above, I showed the involvement of BER and NER pathway in suppression of spontaneous recombination events. However, the induction of recombination

events are only 3- to 9-fold higher than wild type strain in the absence of NER pathway (in *ΔuvrA* strain) or BER pathway (in *Δxth Δnfo* strain). These observations lead me to another question: Are targets of BER and NER exclusive to each other or are they overlapped?

In order to answer this question, firstly I tried to examine which enzymes in each pathway play the most important role in suppression of recombination events. Then I determined the frequency of recombination events in a strain lacking both BER and NER.

In order to clarify which enzyme group plays more important role, I compared the frequency of recombination events in strains deficient in each of the enzyme groups. The result in Figure 22 showed that AP endonuclease group seemed more important than the DNA glycosylase/AP lyase group because of the higher recombination events in AP endonuclease deficient strain on both LB under aerobic, hypoxia and M9 + 0.2% glucose medium.

Next, I tried to construct a *ΔuvrA Δxth Δnfo* triple deletion strain by P1 transduction using *ΔuvrA Δxth* double deletion strain and *Δnfo::kan* strain as the recipient and donor strains, respectively. Unfortunately, after having done transduction for 3 times, the triple deletion strain was not able to be constructed as reported by Saporito and colleagues in 1989 (Saporito *et. al.,* 1989). This might be because unrepaired spontaneous DNA damages remain at a very high level in the absence of both BER and NER pathways and kill cells during the strain construction. However, Lynn Harrison and co-workers reported that they successfully constructed the *ΔuvrA Δxth Δnfo* triple mutant strain using *ΔuvrA* deletion mutant instead of uvrA6 mutant which was used in Saporito paper and this triple mutant strain grew slower than *Δxth Δnfo* double mutant strain and *ΔuvrA* single mutant strain (Lynn Harrison *et. al.,* 2006). Therefore, to further investigate the spontaneous recombination events occurring in *ΔuvrA Δxth Δnfo* triple mutant strain, I tried to construct the *ΔuvrA Δxth Δnfo* triple mutant strain by P1 transduction again. In the fourth trial, I attempted a different approach by using *ΔuvrA::kan* strain as the donor strain and *Δxth Δnfo* double mutant strain as the recipient. After optimizing the protocol for P1 transduction, I finally obtained only one colony that showed the correct construction. However, this *ΔuvrA Δxth Δnfo* grew much slower (to obtain a 1.5 mm size of colony, it took 24 hours on LB and 72 hours on M9 + 0.2% glucose) than the *ΔuvrA* single mutant or *Δxth Δnfo* double mutant strains (14 hours on LB and about 50 hours on M9 + 0.2% glucose). I hypothesized that cells lacking both UvrA in NER and Xth and Nfo proteins in BER pathways may have kept spontaneous DNA damage at a high level enough to suppress cell growth and to induce very high frequency of recombination events.

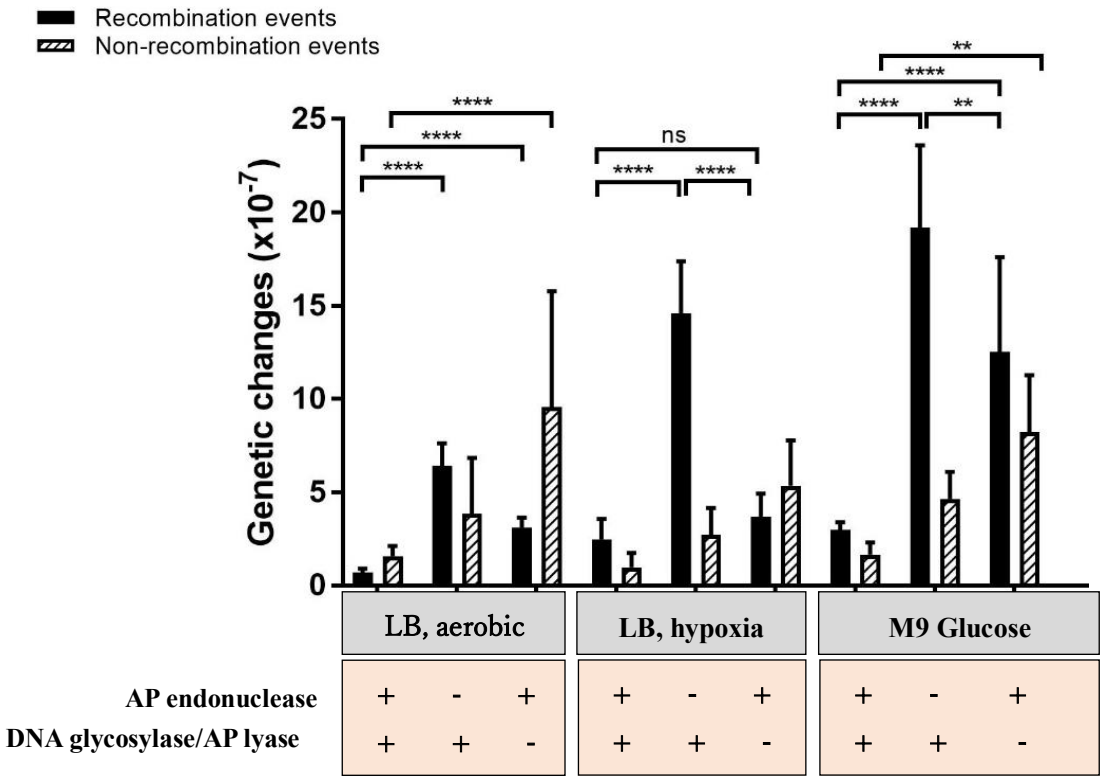


Figure 22. Recombination events in AP endonuclease (*Δxth Δnfo* double deletion strain) and DNA glycosylase/AP lyase (*Δnei Δnth* double deletion strain) deficient strains. The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (**) indicates p-value <0.05 and (****) indicates p-value <0.01, ns indicates non-significant. The strains used in this experiment are wild type MK9547, *Δnei Δnth* double deletion strain (MK9572), and *Δxth Δnfo* double deletion strain (MK9587). Recombination and non-recombination events were determined as described in Figure 14.

Strains	**Wild type**			***Δxth Δnfo***			***Δnei Δnth***		
Medium	**LB, aerobic**	**LB, hypoxia**	**M9 Glu**	**LB, aerobic**	**LB, hypoxia**	**M9 Glu**	**LB, aerobic**	**LB, hypoxia**	**M9 Glu**
Growth phenotype (hrs)	13	60	50	13-14	60	50	13	55	50
RE (x10^{-7})	0.7	2.5	3	6.4	14.6	19.2	3.1	3.7	12.5
Non-RE (x10^{-7})	1.6	1	1.7	3.8	2.7	4.6	9.6	5.3	8.2

As I expected, the frequency of recombination events was extremely very high in *ΔuvrA Δxth Δnfo* triple mutant strain, 85-fold and 160-fold higher than that in wild type strain on LB and M9 + 0.2% glucose medium, respectively (Figure 23). The delay of growth rate and the increment in recombination events suggest that a huge amount of DNA lesions remained in the genome DNA in cells lacking both NER and BER pathways even under lower

level of oxidative DNA damages in cells grown on LB. To overcome the deleterious level of spontaneous DNA lesions, cells need to recruit other repair pathways such as recombination repair or translesion synthesis pathways. In consistent with this notion, I observed sharp increment in frequencies of both recombination and non-recombination events. However, most of the genetic changes were recombination events, suggesting that recombination repair pathway may come first to rescue the cells.

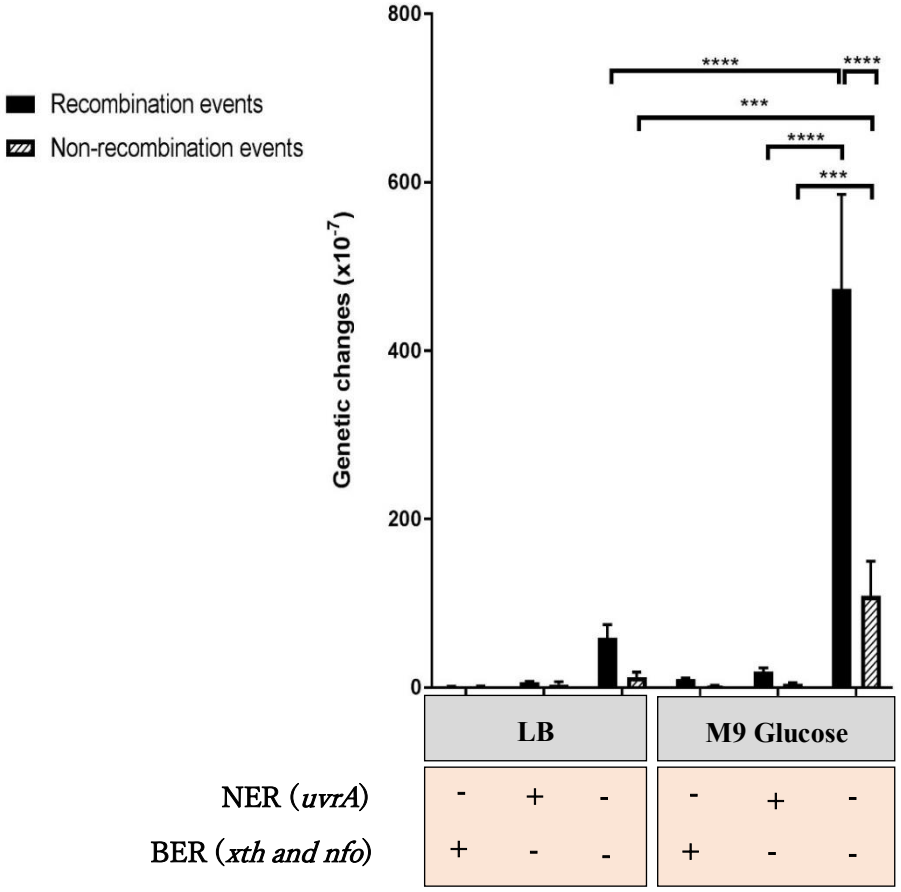


Figure 23. NER and BER play an overlapping role in suppression of spontaneous recombination events. The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (***) and (****) indicates p-value <0.01). The strains used in this experiment are *ΔuvrA* deletion strain (MK955), *Δxth Δnfo* double deletion strain (MK9587), and *ΔuvrA Δxth Δnfo* triple deletion strain (MK9591). Recombination and non-recombination events were determined as described in Figure 14.

Strains	**Wild-type**		***ΔuvrA***		***Δxth Δnfo***		***ΔuvrA Δxth Δnfo***	
Medium	LB	M9 Glu	LB	M9 Glu	LB	M9 Glu	LB	M9 Glu
Growth phenotype (hrs)	13	50	13	50	13 - 14	49 - 50	24 - 26	72
RE (x10^{-7})	0.7	3	0.9	9.9	6.4	19.2	59.3	474
Non-RE (x10^{-7})	1.6	1.7	2.4	2.2	3.8	4.6	12.3	109

In the textbook, it has been described that BER and NER pathways function independently in repair of DNA damages, and there is no overlapped action between BER and NER. BER pathway is mostly responsible for repairing the small lesions such as base modifications while NER pathway is preferentially removing the bulky lesions such as thymine dimer and DNA cross-link (Friedberg *et. al.,* 2006; Truglio *et. al.,* 2006; Lu *et. al.,* 2001). Interestingly, the big increase in the frequency of recombination events was only observed in *ΔuvrA Δxth Δnfo* triple deletion strain but not in *ΔuvrA* single deletion strain (NER deficient strain) or *Δxth Δnfo* (BER deficient strain). This indicates that NER and BER play an overlapping role in suppression of spontaneous recombination events, hence a huge amount of DNA damages are not repaired or removed when both BER and NER pathways not functioning.

In order to assert the notion that the extremely high frequency of recombination events in *ΔuvrA Δxth Δnfo* triple deletion strain is caused by unrepaired oxidative DNA damages, the frequency of recombination events was determined in cells grown on LB under hypoxia condition which produces higher level of oxidative damages. As I expected, the frequency of recombination events in *ΔuvrA Δxth Δnfo* triple deletion strain on LB under hypoxia condition was 5-fold higher than that obtained under aerobic condition (Figure 24). Importantly, the addition of *Oxyrase* significantly suppressed the occurence of recombination events. This data clearly demonstrates that the induction of recombination events in *ΔuvrA Δxth Δnfo* triple deletion strain is caused by oxidative DNA damages. Interestingly, the frequency of recombination events in LB + *Oxyrase* under hypoxia condition which mimics anaerobic condition was a level similar to that on LB under aerobic condition, suggesting that spontaneous DNA damages other than oxidative DNA damages were also produced to induce recombination events.

Taking all the data together, I conclude that the cellular level of oxidative and other types of spontaneous DNA damages are huge. However, an overlapping action of BER and NER pathways thoroughly removes such damages and strongly suppresses spontaneous recombination events.

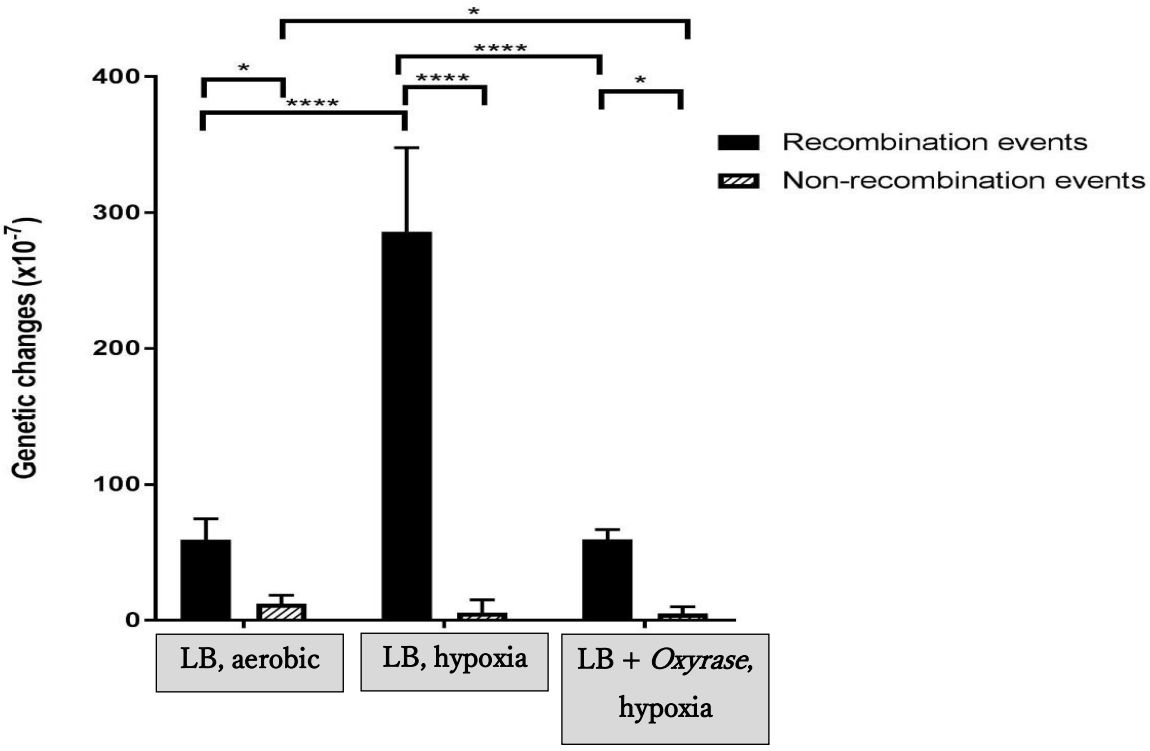


Figure 24. Spontaneous DNA damages other than oxidative DNA damages are also targets of NER and BER. The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (*) indicates p-value <0.05 and (****) indicates p-value <0.01. This experiment was done using MK9591 (*ΔuvrA Δxth Δnfo* triple deletion strain). Recombination and non-recombination events were determined as same as protocol mentioning in Figure 14.

Medium	**LB, aerobic**	**LB, hypoxia**	**LB + *Oxyrase*, hypoxia**
Growth phenotype (hrs)	24 - 26	76-80	48
RE ($x10^{-7}$)	59.3	286	59.6
Non-RE ($x10^{-7}$)	12.3	5.86	5.14

9. Overexpression of UvrA protein strongly suppresses the recombination events which are caused by oxidative DNA damages

Oxidative DNA damages such as 8-oxodG and thymine glycol are the most abundant type of spontaneous DNA damages in aerobically growing *E. coli* cells and are repaired with high efficiency by BER pathway (Maki 2002; Sakai *et. al.,* 2006). On the other hand, NER pathway plays a very important role in repairing of the bulky lesions such as thymine dimer and 6,4 photoproducts which are induced by UV irradiation (Friedberg *et. al.*, 2006). Long time ago, some reports indicated that besides the bulky lesions, NER pathway also contributes

to remove 8-oxodG or thymine glycol in vitro (Lin & Sancar, 1989; Reardon et. al., 1997). However, until now, the role of NER pathway in repairing the oxidative DNA damages are still under controversial.

In this study, I found that the addition of *uvrA* deletion in *Δxth Δnfo* double deletion strain resulted in extremely high frequencies of recombination events and non-recombination events, which were much higher than that in *ΔuvrA* single mutant and in *Δxth Δnfo* double deletion mutant strains on LB and M9 + 0.2% glucose medium (Figure 23). To further elucidate the importance of UvrA in suppression of recombination events and non-recombination events caused by oxidative DNA damages, a complementation assay was performed with the *ΔuvrA Δxth Δnfo* triple deletion strain.

A pCA24N plasmid harboring *uvrA* gene was transformed into *ΔuvrA Δxth Δnfo* triple deletion strain, and the pCA24N empty vector was used as the control. In the plasmid, *uvrA* gene is placed downstream of T5-lac promoter and is expressed by the addition of IPTG. To this end, the IPTG-induction of *uvrA* expression in cells grown on M9 + 0.2% glucose medium is not feasible because of the presence of glucose in the medium. Therefore, growth on LB medium under hypoxia condition was used for the examination of UvrA function in suppression of recombination events caused by oxidative DNA damages.

The addition of low IPTG concentration (0.1 mM) on LB medium strongly affected colony formation, making the analysis of recombination events difficult. However, we could see the reduction of recombination events in the *ΔuvrA Δxth Δnfo* triple deletion strain carrying pCA24N-UvrA plasmid compared to the control strain carrying empty vector pCA24N in the absence of IPTG (Figure 25). This might be due to a leaky expression by IPTG which have been reported (Kitagawa *et. al.,* 2005). The result of complementation experiment indicates that UvrA protein was expressed at a sufficient level in the absence of IPTG. It was clearly shown that the expression of UvrA protein completely reduced the recombination events in the *ΔuvrA Δxth Δnfo* triple deletion strain even in the absence of IPTG.

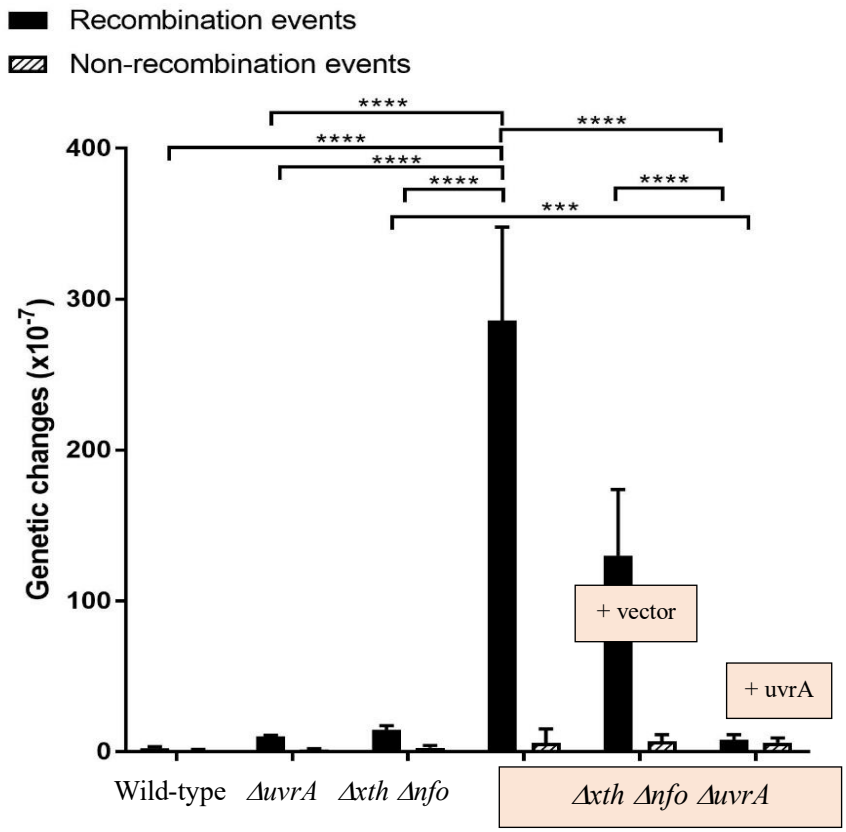


Figure 25. The overexpression of UvrA protein suppressed recombination events in *Δxth Δnfo ΔuvrA* strain grown on LB, hypoxia condition. The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (***) and (****) indicate p-value <0.01. The strains used in this experiment are wild type MK9547, *ΔuvrA* deletion strain (MK955), *Δxth Δnfo* double deletion strain (MK9587), *Δxth Δnfo ΔuvrA* triple deletion strain carrying vector PCA24N (MK9598), and *ΔuvrA Δxth Δnfo* triple deletion strain carrying PCA24N-UvrA (MK9599). Recombination and non-recombination events were determined as described in Figure 14.

Strains	**WT**	***ΔuvrA***	***Δxth Δnfo***	***Δxth Δnfo ΔuvrA***	***Δxth Δnfo ΔuvrA+ pCA24N***	***Δxth Δnfo ΔuvrA + pCA24N-UvrA***
Growth phenotype (hrs)	60	53	60	76-80	76	76
RE (x10-7)	2.5	10.4	14.6	286.03	130.07	7.97
Non-RE (x10-7)	1	1.7	2.7	5.86	6.90	5.96

To further demonstrate that the UvrA protein was expressed even in the absence of IPTG, I performed an additional experiment, in which recovery of tolerance to UV irradiation

by UvrA protein was examined. Cells lack of *uvrA* showed to be very sensitive to UV irradiation (Setlow & Carrier 1964). Therefore, the *ΔuvrA Δxth Δnfo* triple deletion strain must be very sensitive to UV irradiation as well as *ΔuvrA* single deletion strain. As I expected, the hypersensitivity to UV irradiation was clearly seen with *ΔuvrA* and *ΔuvrA Δxth Δnfo* strains compared to the wild type strain (Figure 26). The UV-hypersensitivity of *ΔuvrA Δxth Δnfo* triple deletion strain was recovered to the UV-tolerant phenotype of *Δxth Δnfo* double deletion strain as well as the wild type strain when pCA24N-UvrA plasmid but not pCA24N vector was introduced into the strain. In this experiment, cells were grown in LB media without IPTG before exposing cells spotted on LB plate to UV. This data demonstrated that a sufficient amount of UvrA protein was expressed in the absence of IPTG and hence rescued cells from killing effect of UV irradiation.

Taking the above data together, I concluded that UvrA protein plays a very important role in suppression of recombination events caused by oxidative DNA damages in normally growing cells.

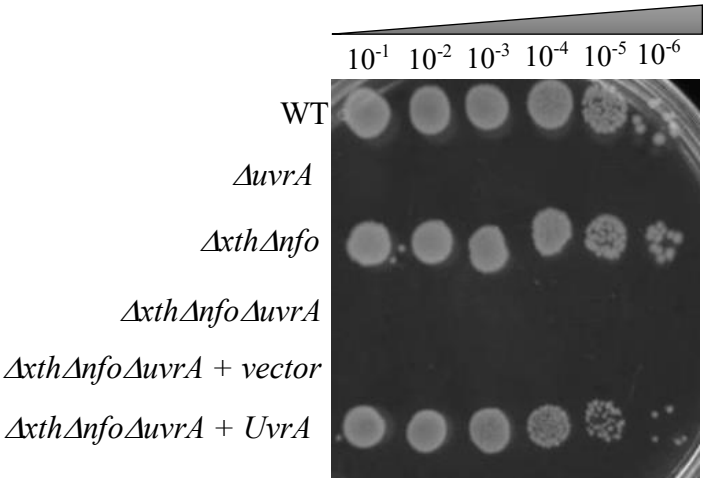


Figure 26. Phenotypical analysis of the *uvrA* deletion strain with UV irradiation (20J). The overnight culture of the cells was diluted with LB medium as dilution factors indicated. Then 5 µl of each dilution was spotted on LB plate and was irradiated under UV light (20J). The UV irradiated cells were grown at 37ºC for overnight (see 2.8 in Methods part for protocol in detail). UV sensitive or tolerant phenotype was confirmed by two independent experiments.

10. Mfd transcription couple factor is not involved in suppression of spontaneous recombination events

In this study, I obtained evidence that NER pathway is involved in suppression of spontaneous recombination events caused by spontaneous oxidative DNA damages together with BER pathway. However, the NER pathway has been considered to be repairing bulky DNA lesions but not small base modifications such as 8-oxodG which is efficiently removed by BER. Therefore, I wanted to understand how UvrA recognizes and removes the small

DNA lesions in NER pathway. It has been known that Mfd (Mutation Frequency Decline), a transcription couple factor belonging to the TCR-NER pathway, contributes to protect cells from damages during transcription. When a blocking-type DNA lesion emerges on template strand for transcription, RNA polymerase stalls at the DNA lesion site, and transcription is blocked until Mfd protein comes and dissociates RNA polymerase from the lesion site (Friedberg *et. al.,* 2006; Schalow *et. al.,* 2012). After removing the RNA polymerase from the lesion site, Mfd protein initiates the repair process by recruiting UvrA protein to the lesion site. Following repair process is the same as the global genome repair of NER pathway. If the action of UvrA in recognition of oxidative DNA damages which induce spontaneous recombination requires Mfd, BER-deficient (*Δxth Δnfo*) cells lacking Mfd protein would induce more recombination events like *Δxth Δnfo ΔuvrA* triple deletion strain.

Δxth Δnfo Δmfd triple mutant cells showed no increment in the frequencies of recombination and non-recombination events compared to those with *Δxth Δnfo* double mutant cells on both LB and M9 + 0.2% glucose medium (Figure 27a), as well as on LB under hypoxia condition (Figure 27b). In conclusion, the result suggests that Mfd protein is not involved in suppression of spontaneous recombination events caused by oxidative DNA damages. It is likely that UvrA protein by itself recognizes the oxidative DNA damages and initiates NER pathway in normally growing *E. coli* cells.

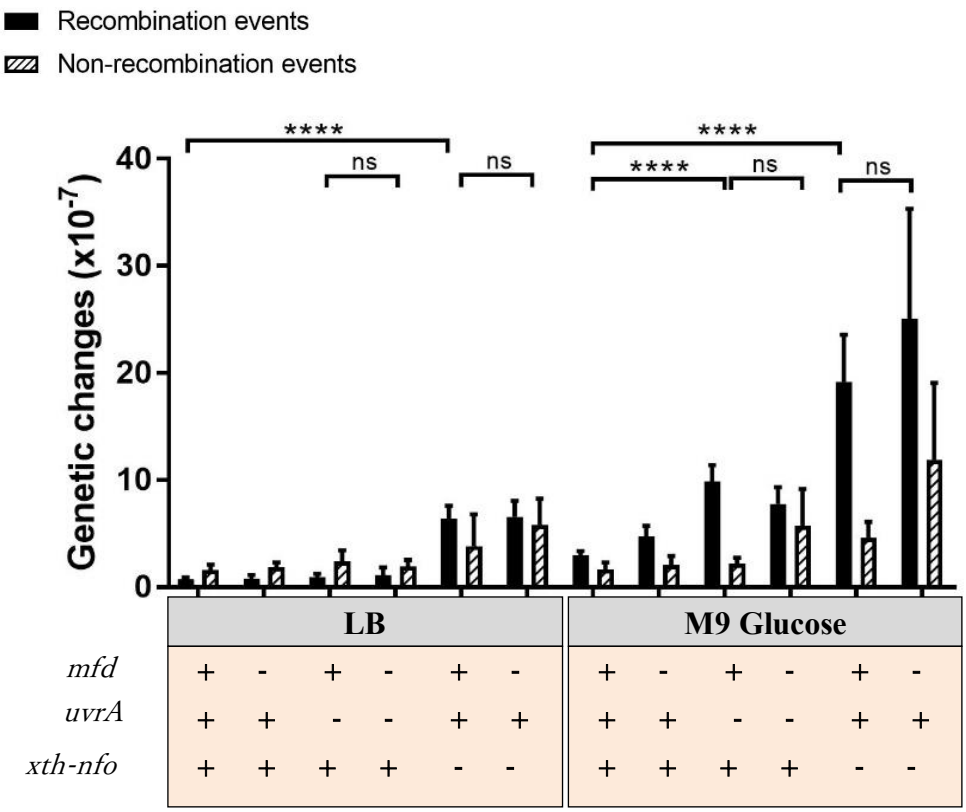


Figure 27a. Involvement of Mfd in recombination events in cells grown on LB and M9 + 0.2% glucose medium. The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (****) indicates p-value <0.01), ns indicates non-significant. The strains used in this experiment are wild type MK9547, *Δmfd* single deletion strain (MK9804), *ΔuvrA* deletion strain (MK955), *Δxth Δnfo* double deletion strain (MK9587), *ΔuvrA Δmfd* double deletion strain (MK9805), and *Δxth Δnfo Δmfd* triple deletion strain (MK9806). Recombination and non-recombination events were determined as described in Figure 14.

Strains	**Wild-type**		***Δmfd***		***ΔuvrA Δmfd***		***Δxth Δnfo Δmfd***	
Medium	LB	M9 Glu	LB	M9 Glu	LB	M9 Glu	LB	M9 Glu
Growth phenotype (hrs)	13	50	13	48	13	48	15	54
RE (x10^{-7})	0.7	3	0.8	4.8	1.1	7.8	6.6	25.1
Non-RE (x10^{-7})	1.6	1.7	1.9	2.1	1.9	5.7	5.8	11.9

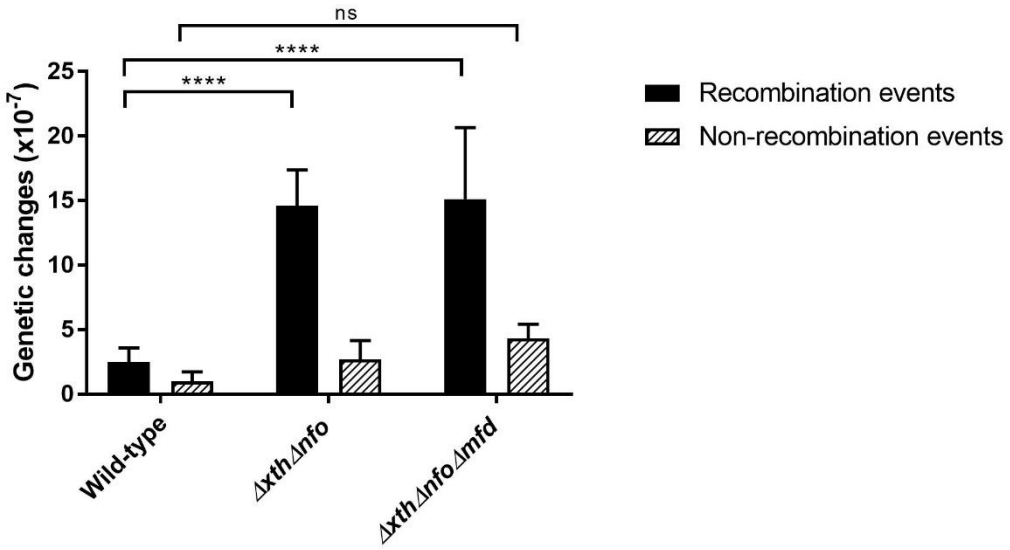


Figure 27b. Involvement of Mfd in recombination events in cells grown on LB, hypoxia condition. The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (****) indicates p-value <0.01, ns indicates non-significant. The strain used in this experiment are wild type MK9547, *Δxth Δnfo* double deletion strain (MK9587), and *Δxth Δnfo Δmfd* triple deletion strain (MK9806). Recombination and non-recombination events were determined as described in Figure 14.

Strains	**Wild-type**	***Δxth Δnfo***	***Δxth Δnfo Δmfd***
Growth phenotype (hrs)	60	60	80
RE ($x10^{-7}$)	2.5	14.6	15.1
Non-RE ($x10^{-7}$)	1	2.7	4.4

11. Spontaneous recombination events does not require SOS response

The bacterial SOS response is a global response to DNA damage promoting cell cycle arrest and inducing mutagenesis (McKenzie *et. al.,* 2001). In *E. coli*, when cells get high level of DNA damages, RecA protein is activated and turns on the SOS response by expression of over 40 genes involving multiple pathways of DNA repair such as *uvrA*, *uvrB*, and *uvrD* in nucleotide excision repair, *ssb* and *recA* in recombination repair pathway, and *dinB* and *umuCD* in translesion synthesis pathway (Janion 2008; Michel 2005). On the induction of SOS response, NER proteins and recombination proteins are initially expressed to repair DNA damages. If the DNA damages are not well repaired by NER and recombination repair pathways, TLS polymerases are induced, which can bypass the various DNA lesions (Michel 2005) and rescue cells from the deleterious effect of DNA damages.

SOS response is controlled by the action of RecA and LexA proteins. Under a low

level of DNA damages, the LexA repressor binds tightly to an operator sequence, SOS box and represses the expression of SOS genes. When the level of DNA damages is high, RecA protein is activated and then binds to LexA repressor to degrade LexA repressor, and the expression of SOS genes is switched on. RecA protein highly upregulated quickly after the induction of SOS response also plays a major role in recombination repair.

At this point, it was not certain whether spontaneous recombination events requires the high level of RecA protein in cells inducing SOS response. To answer this question, I used *lexA3* allele which causes strong inhibition of SOS response because LexA3 mutant protein is never degraded by activated RecA. First of all, I introduced *lexA3* mutation into the hemi-diploid *rpsL* strain, MK9547 by P1 transduction, and the resulting strain was named MK9597. It has been reported that the inhibition of SOS induction by *lexA3* mutant strain results in a UV sensitive phenotype (Friedberg *et. al.,* 2006). As shown in Figure 28, MK9597 strain showed a UV sensitive phenotype similar to the donor strain SMR7467 as a control. This implied that SOS induction was strongly inhibited in MK9597. Then, I determined the frequency of spontaneous recombination events occurring in this strain on LB under aerobic and hypoxia conditions and also on M9 + 0.2% glucose medium.

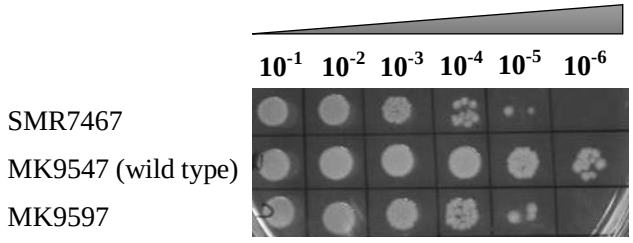


Figure 28. Checking phenotype of *lexA3* (deficient in SOS induction) strain after UV irradiation. MK9547: wild type strain, SMR7467: *lexA3* mutant strain (donor strain), and MK9597: MK9547 *lexA3* mutant strain (recipient strain). The experiment was done as described in Figure 26.

The result indicated that the frequency of spontaneous recombination events in MK9597 cells, defective in SOS induction, was not changed compared to the wild type cells on LB under aerobic and hypoxia conditions and on M9 + 0.2 glucose medium (Figure 29). Therefore, it is suggested that the spontaneous recombination events do not require SOS induction.

In addition, there was no difference in the frequency of non-recombination events in wild type strain between growth on LB and on M9 + 0.2% glucose medium. However, MK9597 (defective in SOS induction) strain showed a significant increment in the frequency of non-recombination events on M9 + 0.2% glucose medium compared to the wild type strain. This data suggests that some repair proteins are induced in wild type cells under SOS

response to repair DNA damages and suppress non-recombination events. Therefore, increased level of oxidative DNA damages produced in MK9597 cells grown on M9 + 0.2% glucose medium could not be completely repaired, hence inducing the non-recombination events.

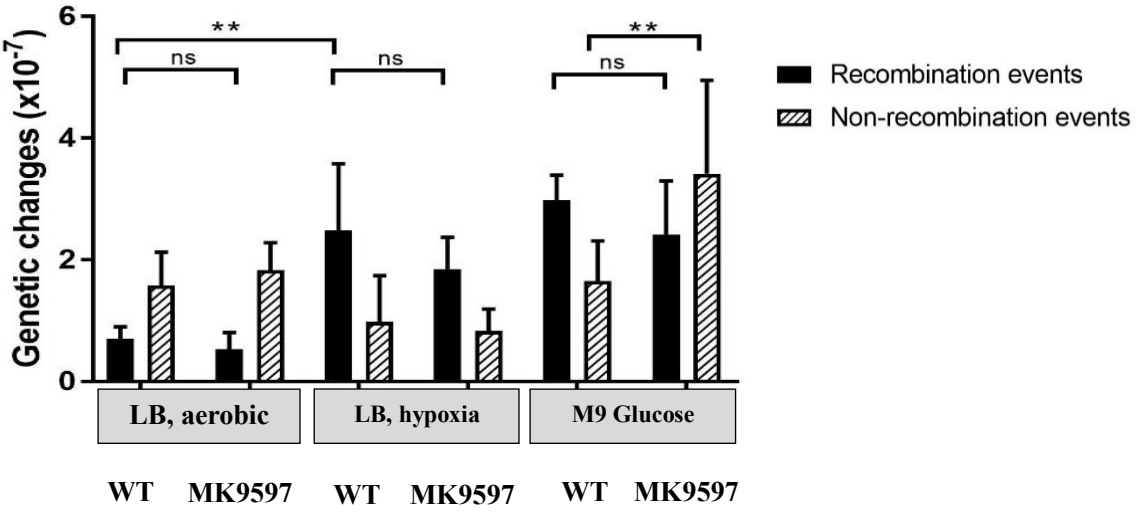


Figure 29. Investigation of spontaneous allelic recombination events in the absence of SOS response in cells grown on LB, LB hypoxia, and M9 + 0.2% glucose medium. The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (**) indicates p-value <0.05, ns indicates non-significant. The strains used in this experiment are wild type strain (MK9547) and *lexA3* mutant strain (deficient in SOS induction) (MK9597). Recombination and non-recombination events were determined as described in Figure 14.

Strains	**Wild-type**			**MK9597**		
Medium	**LB, aerobic**	**LB, hypoxia**	**M9 Glu**	**LB, aerobic**	**LB, hypoxia**	**M9 Glu**
Growth phenotype (hrs)	13	60	49 - 50	13	76	49
RE (x10^{-7})	0.7	2.5	3.0	0.5	1.85	2.4
Non-RE (x10^{-7})	1.6	1.0	1.7	1.8	0.82	3.4

12. Spontaneous recombination events are RecA-dependent

I demonstrated that spontaneous recombination events does not require SOS response or high expression level of repair proteins including RecA protein. As already mentioned, besides the activation of SOS response, RecA protein functions as recombinase to promote

homologous recombination and to protect cells against DNA damages by facilitating the recombination repair. Therefore, I wanted to confirm whether the basic level of RecA protein under normal growth condition is sufficient to induce recombination events. In order to answer this question, I determined the frequency of spontaneous recombination events in *ΔrecA* strain.

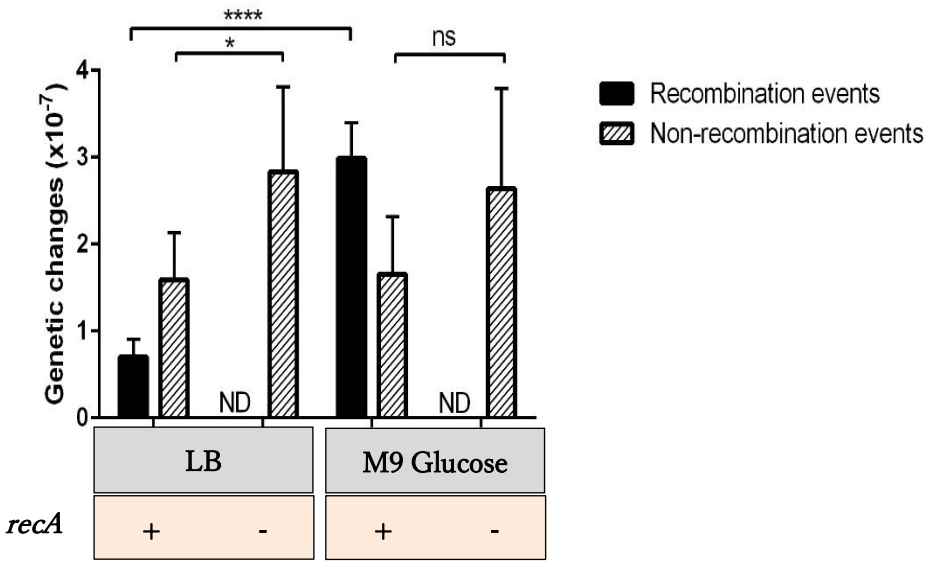


Figure 30. Recombination events depend on RecA function. The data was obtained from six independent experiments. Statistical analysis was performed using Graphpad Prism 7 software. Error bars represent SD. The stars indicate the significance of data: (*) indicates p-value <0.05 and (****) indicates p-value <0.01, ns indicates non-significant. The strains used in this experiment are wild type (MK9547) and *ΔrecA* deletion strain (MK9554). ND: not determined. Recombination and non-recombination events were determined as described in Figure 14.

Strains	**Wild-type**		***ΔrecA***	
Medium	**LB**	**M9 Glu**	**LB**	**M9 Glu**
Growth phenotype (hrs)	13	49 - 50	20	65
RE (x10^{-7})	0.7	3.0	Not determined	Not determined
Non-RE (x10^{-7})	1.6	1.7	2.8	2.6

As shown in Figure 30, no recombination events were detected with *ΔrecA* cells grown on both LB and M9 glucose medium, consistent with the previous observation by Kanie and colleagues (Kanie *et. al.,* 2007). This data indicated that most of the spontaneous recombination events occurring in wild type *E. coli* cells required function of RecA protein. It

also appeared that the basic level of RecA protein was sufficient to induce recombination events. On the other hand, *ΔrecA* cells induced more frequently non-recombination events on LB medium but not M9 + 0.2% glucose medium. These non-recombination events are probably single-base frameshifts and large deletion (Kanie *et. al.,* 2007), which are likely to be caused by error-prone DNA synthesis in TLS pathway. I concluded that the spontaneous recombination events caused by oxidative DNA damages depend on RecA protein, and the basic level of RecA protein is enough to induce recombination events in normally growing cells.

Part 2. A correlation between recombination events and H_2O_2 sensitivity

In Part 1, I have shown that oxidative DNA damages are produced in normally growing *E. coli* cells, and the intracellular level of spontaneous oxidative DNA damages vary in growth conditions different in nutrition and oxygen concentration. I have also demonstrated that spontaneous recombination events are caused by oxidative DNA damages at least in part 1, and BER and NER cooperatively suppress the spontaneous recombination events. However, I measured the capacity of BER and NER only in the suppression of recombination events, and no direct evidence for the overlapping action of BER and NER on repair of oxidative DNA damages. Therefore, I decided to perform H_2O_2 sensitivity assay to obtain direct evidence for the action of BER and NER on repair of oxidative DNA damages. I also expected that this attempt would provide evidence that spontaneous recombination events are caused by oxidative DNA damages.

1. H_2O_2 sensitivity of BER pathway mutants

1.1. DNA glycosylase/AP lyase deficient strain was slightly sensitive to H_2O_2 treatment

In the H_2O_2 sensitivity assay, cells were grown in LB liquid medium until OD_{600} about 0.3 to 0.4, then cells were plated with soft agar on LB plate, and 5μl of 9 M H_2O_2 was put on a paper disc placed at the center of the plate. After overnight incubation, the inhibition zone was measured, which reflects the sensitivity of *E. coli* strain to H_2O_2. Cells defective in repair of oxidative DNA damages would show higher sensitivity to H_2O_2 than wild type cells, resulting in a larger size of inhibition zone on the H_2O_2 assay plate. The result shown in Figure 31 indicated that the inhibition zone obtained with *Δnei Δnth* double deletion strain was similar to the wild type strain, suggesting that other repair proteins efficiently suppressed the killing effect of oxidative DNA damages.

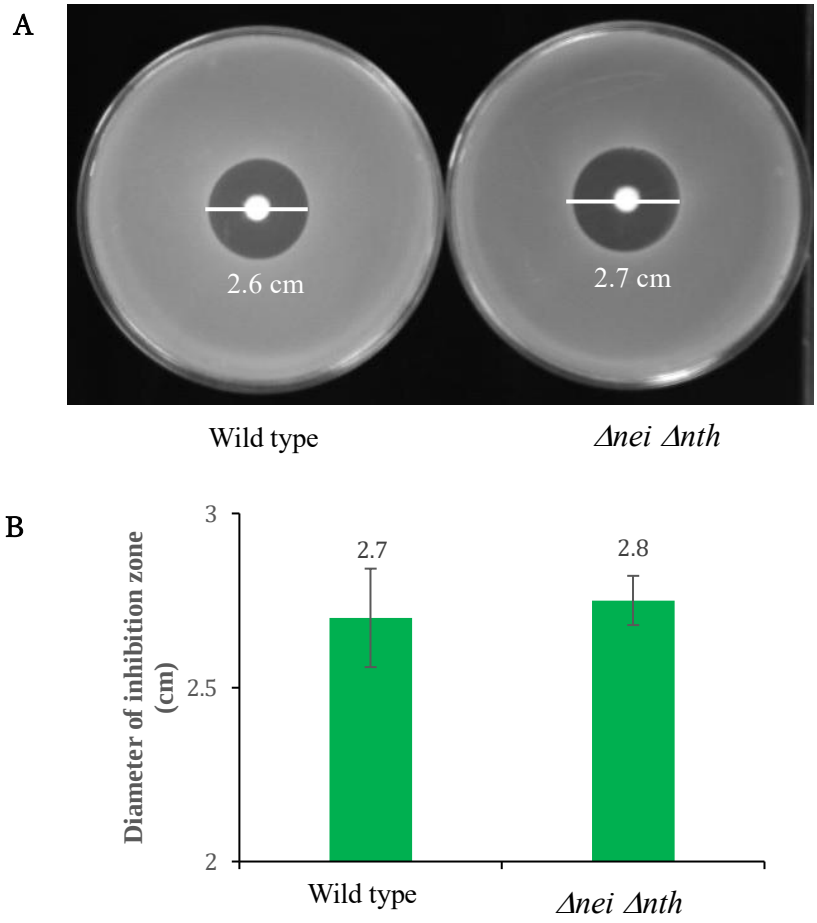


Figure 31. H_2O_2 sensitivity of wild type (MK9547) and *Δnei Δnth* double deletion (MK9572) strains.

A. Representative picture of inhibition zone for two strains.

B. Quantification of inhibition zone from two independent experiments.

1.2. Cells lacking AP endonucleases showed a higher sensitivity to H_2O_2

Cells lacking both *xth* and *nfo* genes induced more recombination events compared to cells lacking both *nei* and *nth* genes. Therefore, I expected that *Δxth Δnfo* double deletion strain would show a higher sensitivity to H_2O_2. As shown in Figure 32, *Δxth* and *Δnfo* single mutant strains showed a slightly higher sensitivity to H_2O_2 compared to wild type strain. As expected, more clear H_2O_2 hypersensitivity was observed with *Δxth Δnfo* double deletion strain. This data suggested that AP sites may be produced at a high level under H_2O_2 treatment, and both Nfo and Xth may play an important role in elimination of AP sites to rescue cells from killing effect of H_2O_2.

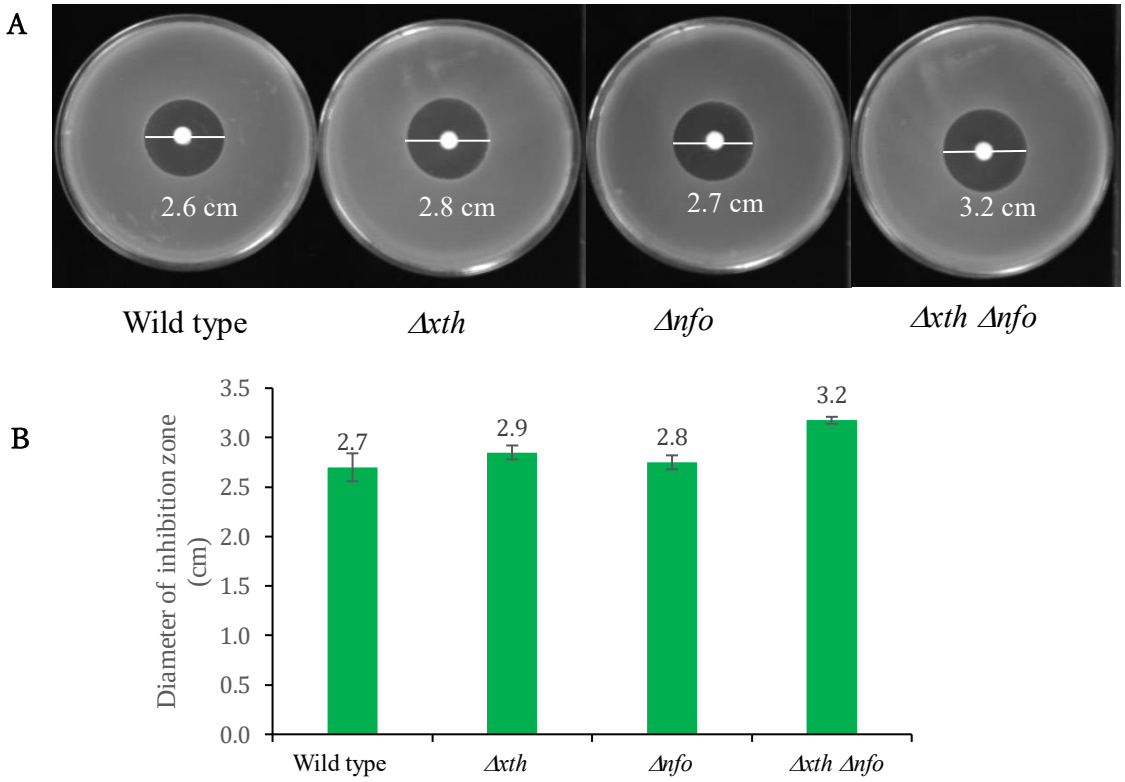


Figure 32. H_2O_2 sensitivity of wild type (MK9547) and *Δxth Δnfo* double deletion (MK9587) strains.

A. Representative picture of inhibition zone for two strains.

B. Quantification of inhibition zone from two independent experiments.

2. NER pathway and H_2O_2 sensitivity

NER pathway has been known as a major pathway in repairing bulky DNA lesions such as cyclobutane pyrimidine dimer or 6,4 photoproducts which are produced by UV irradiation (Friedberg *et. al.,* 2006). Oxidative DNA damages have been considered to be repaired mostly by BER pathway. Interestingly, in human cells, several lines of evidence indicated that NER pathway also contribute to remove oxidative DNA damages such as 8-oxodG or thymine glycol (Fortini *et. al.,* 2003; Melis *et. al.,* 2013; Menoni *et. al.,* 2012). If it is the case in *E. coli*, cells lacking UvrA protein would be more sensitive to H_2O_2.

As shown in Figure 33, cells lacking UvrA protein did not show a higher sensitivity to H_2O_2 compared to wild type strain, suggesting that the capacity of BER pathway may be sufficient to repair the oxidative DNA damages produced by H_2O_2 treatment.

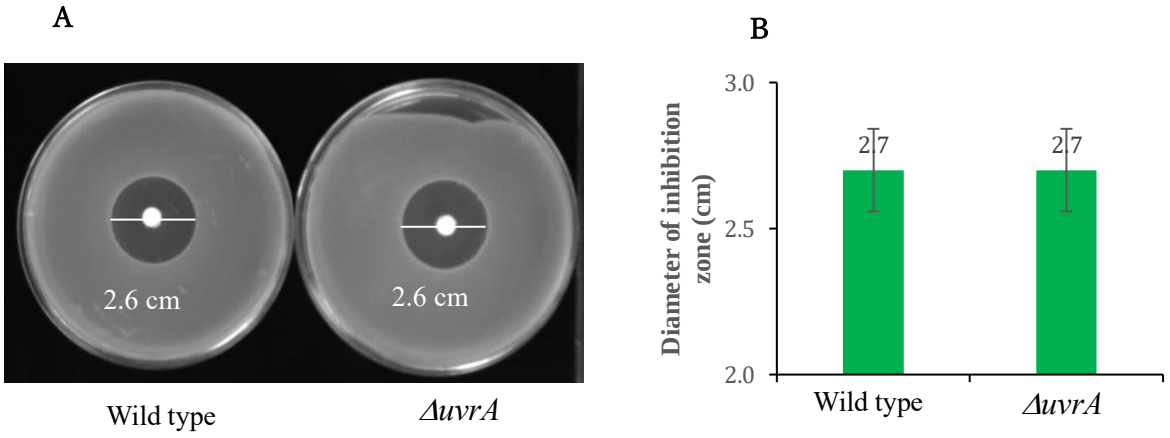


Figure 33. H_2O_2 sensitivity of wild type (MK9597) and *ΔuvrA* deletion (MK9552) strains.
A. Representative picture of inhibition zone for two strains.
B. Quantification of inhibition zone from two independent experiments.

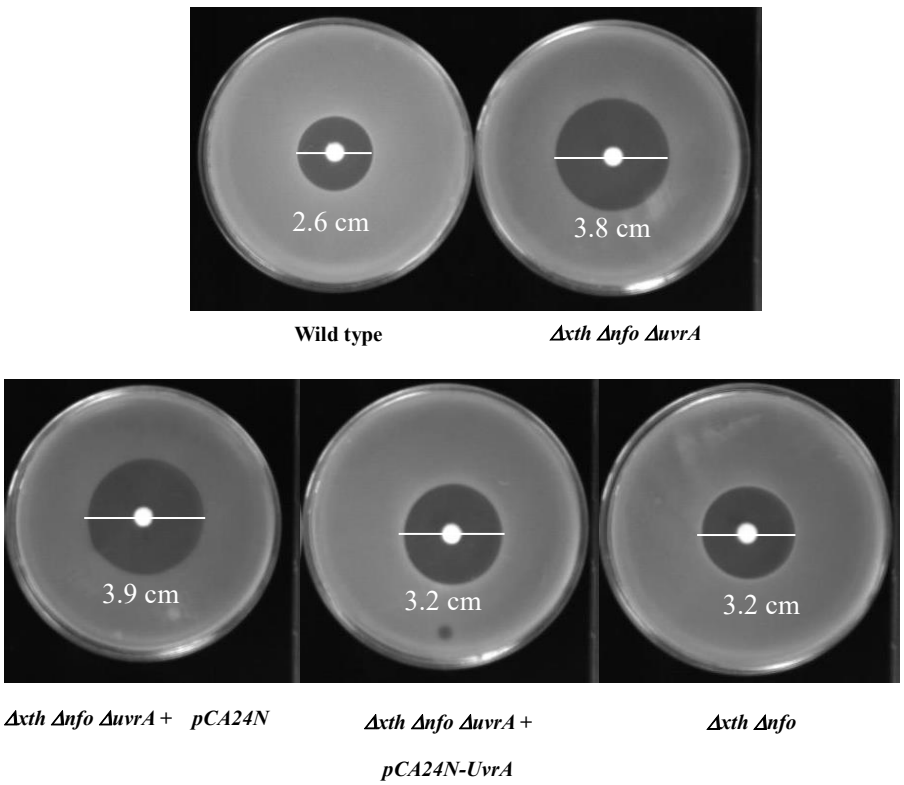
3. Cells showed hypersensitive to H_2O_2 in the absence of both BER and NER pathways

In Part 1, I have already proven that BER and NER pathways showed an overlapped action in suppression of spontaneous recombination events caused by oxidative DNA damages. I hypothesized that a huge amount of AP sites are produced in genome DNA but efficiently repaired by both BER and NER, hence reducing the frequency of recombination events. It has been reported that H_2O_2 is able to induce AP sites in DNA. In order to prove this hypothesis, cells were treated with H_2O_2 to induce a lot of AP sites in DNA. If cells lacking both BER and NER are not able to repair these induced AP sites, cells would show a very high sensitivity to H_2O_2.

As I expected, *Δxth Δnfo ΔuvrA* triple deletion strain showed a hypersensitivity to H_2O_2 treatment (Figure 34) compared to *ΔuvrA* single deletion strain in Figure 33 and *Δxth Δnfo* double deletion strain in Figure 32. This result supports my hypothesis that NER and BER pathways collaborate to eliminate oxidative DNA damages and suppress spontaneous recombination events.

Importantly, overexpression of UvrA protein rescued cells from H_2O_2 killing effect by reducing the inhibition zone to the size similar to *Δxth Δnfo* double deletion strain (Figure 34). This asserts that UvrA protein in NER pathway together with BER pathway plays a very important role in suppression of oxidative DNA damages to maintain genome integrity by suppression of spontaneous recombination events.

A



B

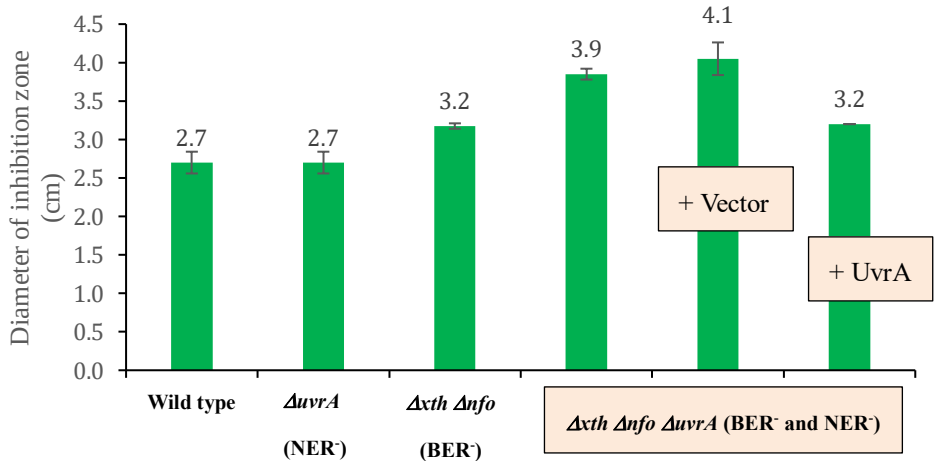


Figure 34. H_2O_2 sensitivity in BER and NER deficient strain (MK9591).

A. Representative picture of inhibition zone for two strains.

B. Quantification of inhibition zone from two independent experiments.

4. Recombination repair pathway helped cells survive under H_2O_2 treatment

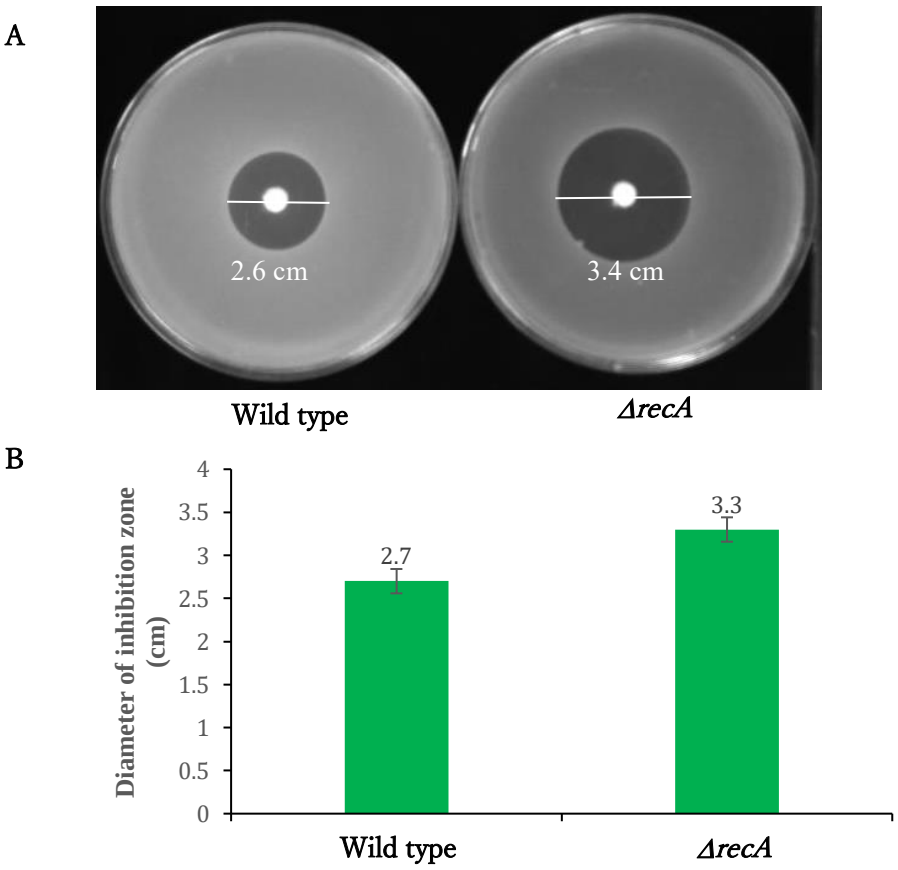


Figure 35. H_2O_2 sensitivity of wild type (MK9547) and *ΔrecA* deletion (MK9597) strains.

A. Representative picture of inhibition zone for two strains.

B. Quantification of inhibition zone from two independent experiments.

Recombination repair pathway has been known as the main pathway to rescue the stalled or broken replication fork and to induce recombination events. In order to investigate the role of recombination repair pathways in tolerance to oxidative stress, *ΔrecA* deletion strain was treated with 9 M H_2O_2 to induce oxidative DNA damages. In Figure 32, the *recA* knock out strain showed a higher sensitivity to H_2O_2 compared to wild type strain. I concluded that the recombination repair pathway plays a very important role in helping cells survive under oxidative stress.

Chapter IV. Conclusions

Part 1. Involvement of oxidative DNA damages in spontaneous allelic recombination events and roles of repair pathways in suppression of recombination events in E. coli

1. Aerobic growth in minimal medium and hypoxic growth in LB medium lead to production of oxidative DNA damages and induction of spontaneous recombination events in *E. coli* cells.
2. An overlapped action of BER and NER pathways thoroughly removes spontaneous oxidative DNA damages and strongly suppresses spontaneous recombination events in *E. coli*.
3. Spontaneous recombination events in *E. coli* depend on RecA protein but not SOS induction.

Part 2. A correlation between recombination events and H_2O_2 sensitivity

1. Recombination repair pathway helps cells survive under H_2O_2 treatment.
2. Cells lacking Nei and Nth proteins, major DNA glycosylases/AP lyase enzymes, are only slightly sensitive to H_2O_2.
3. Cells lacking Xth and Nfo proteins, major AP endonucleases, are more sensitive to H_2O_2 than those lacking Nei and Nth proteins.
4. Cells lacking both BER (Xth and Nfo proteins) and NER (UvrA protein) pathways are hypersensitive to H_2O_2 compared to those lacking either of NER and BER pathways.

Chapter V. Discussions

Part 1. Involvement of oxidative DNA damages in spontaneous allelic recombination events and roles of repair pathways in suppression of recombination events in *E. coli*

1. Chromosomal rearrangements are affected by environmental factors

Reactive oxygen species (ROS) including superoxide ($\cdot O_2$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2) are generated under normal cellular metabolism in aerobic condition (Cooke *et. al.,* 2003) and induce more than 20 types of oxidative DNA damages in cells (Kashmiri & Mankar 2014). Among the reactive oxygen species, hydroxyl radical is highly reactive and extremely dangerous because it can directly attack DNA to induce oxidative DNA damages. But, it seems impossible to detect the intracellular hydroxyl radical because of its unstable nature in cells. On the other hand, although hydrogen peroxide is less reactive, it also damages DNA by quickly converting to hydroxyl radical by Fenton reaction. Because of its stable nature in cells, we can easily determine the intracellular level of hydrogen peroxide by a flow cytometry method. Using this method, studies in our laboratory revealed that the cellular H_2O_2 level is variable in different growth conditions such as different nutrition in media and oxygen concentration for cultivation (Andoh, 2013; Uefune, 2015; Nunose *et. al*., 2016).

It was recently found in our laboratory that cells grown on minimal media, M9 glucose and M9 glycerol, produced a very high level of H_2O_2 compared to those on a rich medium, LB. This observation suggested that oxidative DNA damages might be significantly induced in cells grown in minimal media. Using rifampicin resistant mutation assay and a *ΔmutMΔmutY* strain, the frequency of mutations caused by 8-oxodG which represents for oxidative DNA damages was examined with cells grown in different growth conditions. As expected, the cellular level oxidative DNA damages measured by the mutation frequency was much higher in minimal media than in rich medium. The oxidative DNA damage level in M9 glucose medium was about 10-fold higher than in LB, and that of M9 glycerol was about 10-fold higher than in M9 glucose medium (Nunose *et. al.*, 2016). However, the intracellular H_2O_2 level in M9 glycerol was only slightly higher than M9 glucose, suggesting that the intracellular H_2O_2 level does not simply reflect the level of oxidative DNA damages. As I mentioned above, H_2O_2 can damage DNA by quickly converting to hydroxyl radical through Fenton reaction which requires free iron ion (Halliwell & Gutteridge 2007; Nunoshiba *et. al.,* 1999). Therefore, it is likely that in cells grown in M9 glucose and M9 glycerol, concentration of free iron might be increased to a level depending on the carbon source, resulting in an elevated level of hydroxyl radical which induces oxidative DNA damages. More importantly, a key to explain why cells grown in minimal medium induce more oxidative DNA damages than in rich medium is the presence of amino acid in LB medium. LB medium is very rich in amino acids while M9 minimal medium does not contain amino acids. Nunose in our lab

found that when casamino acids were added to M9 glucose medium, the cellular level of oxidative DNA damages were reduced to that in cells grown on LB medium. This observation suggests that amino acid in LB medium may suppress free iron concentration, hence suppressing the production of oxidative DNA damages.

It was also found in our laboratory that cells grown under a hypoxia condition, 0.1% oxygen concentration, produced a high level of intracellular H_2O_2 and induced more oxidative DNA damages compared to aerobically growing cells (Uefune, 2015). This phenomenon can be explained by an increased auto-oxidation in cells under low oxygen concentration. As widely known, electron is produced and transferred during the respiratory process, and oxygen molecule captures electron to produce reactive oxygen species including superoxide, hydrogen peroxide and hydroxyl radical (Figure 36). To avoid the production of superoxide from oxygen molecule, terminal oxidase captures the electron to form water (Gonzalez-Flecha & Demple 1995). *E. coli* cell has three *terminal oxidases* listed in a table below.

Name	Genes	Induced condition
Cytochrome bd-I terminal oxidase	cydAB	Under low oxygen concentration
Cytochrome bd-II terminal oxidase	cbdAB	Starvation for carbon and phosphate
Cytochrome bo terminal oxidase	cyoABCD	Under aerobic condition

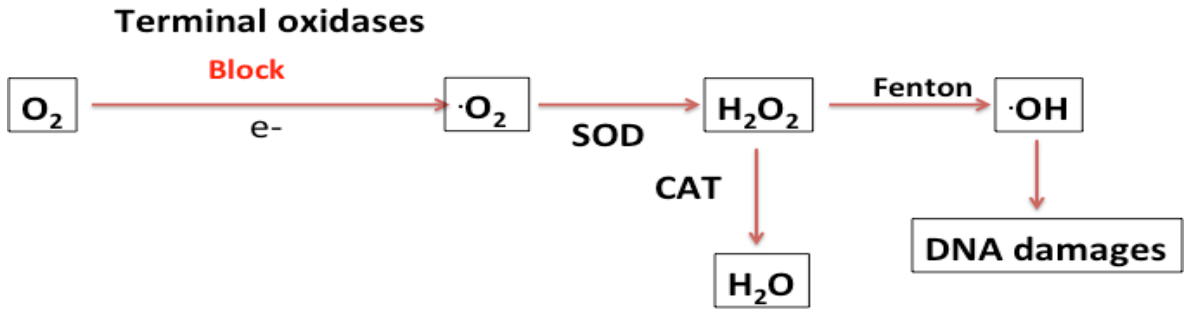


Figure 36. Reactive oxygen species production is blocked by action of *terminal oxidase*.

In order to find out why low oxygen concentration results in production of more oxidative DNA damages in *E. coli* cells, Uefune in our lab constructed strains lacking each of three *terminal oxidases* or all *terminal oxidases*. Cells lacking all *terminal oxidase* produced an extremely high level of intracellular H_2O_2 under aerobic condition, but the level of intracellular H_2O_2 was only slightly increased under low oxygen concentration. This observation suggested that most of electrons transferred within the respiration chain are properly captured by the action of *terminal oxidase* under normal aerobic condition, avoiding

the unfavorable auto-oxidation that produces H_2O_2. However, the activity of *terminal oxidase* in capturing the electrons seems to be lowered under the hypoxia condition, only a small amount of electrons are captured by *terminal oxidase*, and many remaining electrons promote auto-oxidation, producing a high level H_2O_2.

As described above, it is now evident that environmental factors including nutrition and oxygen concentration affect the intracellular level of H_2O_2 and the level of oxidative DNA damages in chromosomal DNA in *E. coli*. However, frequency of spontaneous mutations is unchanged in wild type cells grown in different growth conditions. This is due to a cellular capacity of DNA repair efficiently and sufficiently repairing the oxidative DNA damages. On the other hand, frequency of recombination events in wild type *E. coli* cells varies in different growth conditions. More recombination events are induced in cells grown in minimal medium or under hypoxia condition. In the present study, I demonstrated at the first time that some environmental factors affect chromosomal rearrangement in normally growing cells. The increased level of oxidative DNA damages under these conditions are subjects of BER and NER pathways, but a part of oxidative DNA damages that remains unrepaired blocks DNA replication and induces recombination repair. Therefore, chromosomal rearrangement caused by spontaneous recombination events is affected by the cellular capacity of DNA repair as well as the cellular level of oxidative DNA damages that is affected by environmental factors.

2. Base excision repair pathway significantly suppresses recombination events

It has been believed that most of oxidative DNA damages are repaired by BER pathway because most of oxidative DNA damages are base modifications such as 8-oxodG, which is the most abundant oxidative DNA lesions and repaired by MutM-dependent BER. Other oxidative DNA damages, thymine glycol, 5-hydroxycytosine and 5-hydroxyuracil are efficiently repaired by BER initiated by Endonuclease III (Nth) and Endonuclease VIII (Nei) (Saito *et. al.,* 1997; Souza *et. al.,* 2006; Melamede *et. al.,*1994). I found that oxidative DNA damages promoted the production of spontaneous recombination events, suggesting that BER pathway probably plays a very important role in suppression of recombination events. To investigate the involvement of BER pathway in suppression of recombination events, two main enzyme groups, DNA glycosylase and AP endonuclease were studied. The result indicates that both DNA glycosylase and AP endonuclease are involved in suppression of recombination events.

It has been reported that 90% of AP endonuclease activity is performed by Xth, and Nfo contributes only about 10% the activity (Souza *et. al.,* 2006; Saporito *et. al.,* 1989; Daley *et. al.,* 2010). However, in this study, the frequency of recombination events was not changed

in the absence of either Xth or Nfo (Figure 20), indicating that contributions of Xth and Nfo in suppression of spontaneous recombination events are equal. Both Xth and Nfo proteins are very important in processing the AP sites and suppression of spontaneous recombination events.

Similarly to the case of AP endonuclease, among two major DNA glycosylase/AP lyase for oxidative pyrimidine damages, Nth (Endonuclease III) and Nei (Endonuclease VIII), Nth was reported to mainly function to repair the spontaneous mutagenic lesions (Saito *et. al.,* 1997). However, the frequency of recombination events was not much changed in the absence of either Nth or Nei protein, but cells lacking both Nth and Nei proteins increased the frequency of recombination events (Figure 19). This observation suggests that both Nth and Nei proteins play equally an important role in suppression of recombination events.

The frequency of spontaneous recombination events in the AP endonuclease deficient strain was higher than that in the DNA glycosylase/AP lyase deficient strain, suggesting that AP endonuclease seems more important than DNA glycosylase/AP lyase in suppression of recombination events (Figure 22). Since AP endonuclease is responsible to eliminate the AP site, a cytotoxic lesion which can block replication fork during replication (Souza *et. al.,* 2006; Korolev 2005), it is likely that many AP sites are produced in normally growing cells and efficiently induce recombination events in cells lacking AP endonuclease.

3. Nucleotide excision repair suppresses spontaneous recombination events

In contrast to the role of BER pathway in repair of the oxidative DNA damages, involvement of NER pathway in repairing the oxidative DNA damages is under controversial. In NER reaction reconstituted *in vitro* with UvrA, UvrB, and UvrC proteins, AP sites and thymine glycol were removed from DNA containing the oxidative DNA damages (Lin & Sancar 1989). However, *uvrA* and *uvrB* deletion mutant strains showed decreased frequencies of spontaneous mutation, suggesting that the NER pathway contributes to induce mutations rather than to avoid them (Hori *et. al.,* 2007; Hasegawa *et. al.,* 2008). In this study, I obtained clear evidence that UvrA protein in NER functions to suppress spontaneous recombination events only under the growth conditions that induce high level of oxidative DNA damages. Although I did not show the data for UvrB protein, UvrB is also involved in suppression of spontaneous recombination events. Interestingly, the action of UvrB is lower than UvrA. It is probable that UvrA is able to recognize the DNA lesion and to activate UvrC in a way UvrB-independent (Stracy *et. al.,* 2016).

I also showed that NER pathway functions together with BER pathway in repairing oxidative DNA damages and suppressing the spontaneous recombination events. This is one of the most important finding in my study because it has not been considered for long time

that NER pathway is important for DNA repair of the oxidative DNA damages in normally growing cells. An extremely high frequency of spontaneous recombination events in cells lacking both BER (Xth and Nfo) and NER (UvrA) pathways was sharply decreased to the level observed with wild type cells when UvrA protein was overexpressed. This clearly demonstrates that, depending on the concentration of UvrA in cells, NER can repair oxidative DNA damages much better than BER pathway. In addition, frequency of recombination events comparable to that in wild type strain was seen in *uvrA* deficient strain aerobically grown in LB but not in *xth* and *nfo* deficient strain, suggesting that the cellular level of UvrA is much lower than Xth and Nfo.

4. Overlapped action of BER and NER pathway in suppression of spontaneous recombination events

In 2002, Marie Guillet and Serge Boiteux reported that endogenous AP sites caused cell death in the absence of BER (AP endonuclease) and NER in yeast cell, suggesting that many recombination events are induced and caused cell death (Guillet & Boiteux 2002). Because the absence of these two main repair pathways caused lethality in yeast cells, the spontaneous recombination events could not be determined. Fortunately, the BER and NER deficient strain was able to be constructed in *E. coli*, and I could determine the frequency of spontaneous recombination events in the absence of these two pathways. The result indicated that a huge number of recombination events are occurring in cells lacking both BER and NER pathways but not in the absence of each pathway.

Taken all the data together, I hypothesized that an overlapped target for BER and NER is AP site. AP sites are directly produced by reactive oxygen species or indirectly by the action of DNA glycosylases which remove oxidative DNA damages such as thymine glycol (Saito *et. al.,* 1997; Gifford & Wallace 2000) and 8-oxodG (Nghiem *et. al.,* 1988; Fowler *et. al.,* 2003; Kanie et. *al.,* 2007) as well as non-oxidative DNA damages such as uracil, a product of cytosine deamination (Parikh *et. al.,* 2000; Krokan *et. al.,* 2002; Olinski *et. al.,* 2010), and alkylating lesions such as 3-meA or 7-meA (Sedgwick *et. al*., 2007; Mielecki *et. al.,* 2015). Therefore, many AP sites seem to be produced in cells grown under normal growth condition. To prove my hypothesis, I performed H_2O_2 sensitivity analysis. It has been suggested that more AP sites are induced by H_2O_2 treatment because lack of AP endonuclease leads to hypersensitivity to H_2O_2 (Souza *et. al.,* 2006). Therefore, in the absence of both BER and NER pathways, cells could not repair well and die. As I expected, *ΔuvrA* strain was not sensitive to H_2O_2, suggesting that the activity of BER pathway is sufficient to repair the AP sites and other oxidative DNA damages induced by H_2O_2 treatments. However, *Δxth Δnfo* double deletion strain showed a hypersensitivity to H_2O_2, suggesting that the activity of NER pathway alone was not sufficient to rescue the cells from the killing effect of H_2O_2. More

importantly, *ΔuvrA Δxth Δnfo* triple deletion strain appeared to be more hypersensitive to H_2O_2 than *Δxth Δnfo* double deletion strain, clearly demonstrating that both BER and NER pathways function in repairing the AP sites and other oxidative DNA damages under H_2O_2 treatment.

To further prove the notion that AP site is the main DNA lesion in cells grown under normal growth condition, it should be constructed and examined *Δnei Δnth ΔuvrA* triple deletion strain which also lacks both BER and NER pathways. The frequency of spontaneous recombination events in this *Δnei Δnth ΔuvrA* triple deletion strain could be much lower than *Δxth Δnfo ΔuvrA* triple deletion strain because most of AP sites are efficiently removed by Xth and Nfo in the *Δnei Δnth ΔuvrA* cells.

A new important question must be how UvrA protein recognizes the oxidative DNA damages. To obtain a clue for this question, I examined the recombination events in *Δmfd* deletion strain. Mfd (Mutation frequency decline) protein is a transcription couple factor, which contributes to counteract to the effect of DNA damage on transcription process by dissociation of RNA polymerase that stalls at DNA lesion and recruiting UvrA to the lesion site to initiate NER pathway. However, the results showed that Mfd is not involved in the suppression of recombination events. Recently, Graciela Spivak reported another transcription couple repair pathway called UvrD mediated TCR pathway, which does not require Mfd protein (Spivak 2016).

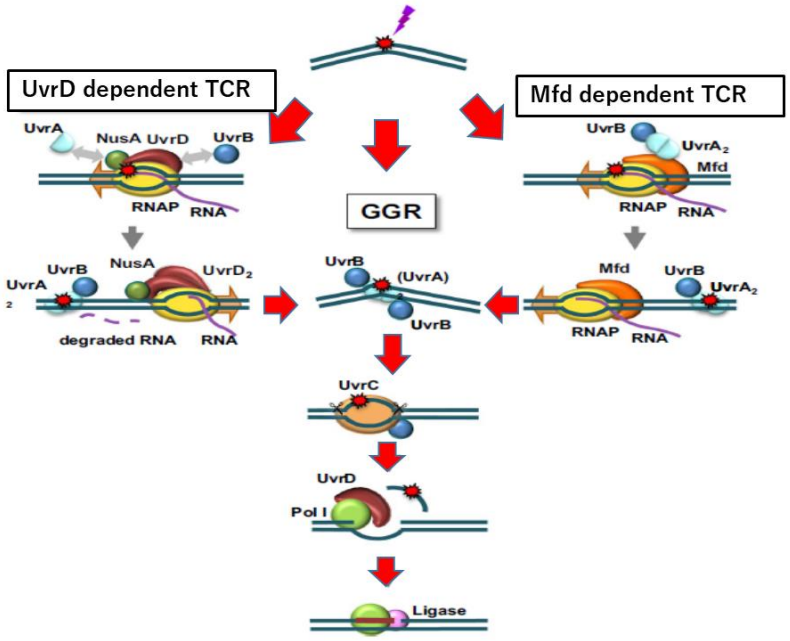


Figure 37. NER pathway in *E. coli* cell (this figure is from Graciela Spivak, 2016 with some small modifications). Middle path is Global Genome Repair (GGR) which detects lesion throughout genome and left and right paths are Transcription Couple Repair (TCR) which is mediated by Mfd (right path) and UvrD (left path).

This process requires the interaction of UvrD helicase and NusA transcription factor. The unique point of this process is to allow the recovery of transcription after DNA lesion is removed. Similar to Mfd protein, UvrD/NusA complex recruits UvrA and UvrB proteins to the lesion site and facilitates the repair process (Figure 37). To further investigate how UvrA protein works in the recognition of oxidative DNA lesions such as AP sites, it is obvious to carry out the study on NusA protein and/or UvrD helicase.

5. A hypothesized model for repair of AP sites by BER and NER pathways

Considering the overlapped action of BER and NER pathways in repair of oxidative DNA damages, I propose a hypothesized model to explain the overlapped action of BER and NER pathways (Figure 38). Under normal growth condition, many AP sites are spontaneously produced in cells. Most of AP sites are produced directly by reactive oxygen species, but some of them are also produced by the action of many kinds of DNA glycosylase such as MutM for 8-oxodG, MutY for A from 8-oxodG:A mispair (Cabrera *et. al.,* 1988; Nghiem *et. al.,* 1988), and Nei and Nth for 5-hydroxylcytosine (Saito *et. al.,* 1997; Jiang, Hatahet, Blaisdell, *et. al.,* 1997; Gifford & Wallace 2000). Other than those, AlkA eliminates alkylating lesion such as 3-meA (Sedgwick *et. al.,* 2007; Mielecki *et. al.,* 2015), and uracil DNA glycosylase removes uracil in DNA (Parikh *et. al.,* 2000; Olinski *et. al.,* 2010; Krokan *et. al.,* 2002). In BER pathway, AP sites are processed by AP endonucleases, Xth and Nfo proteins.

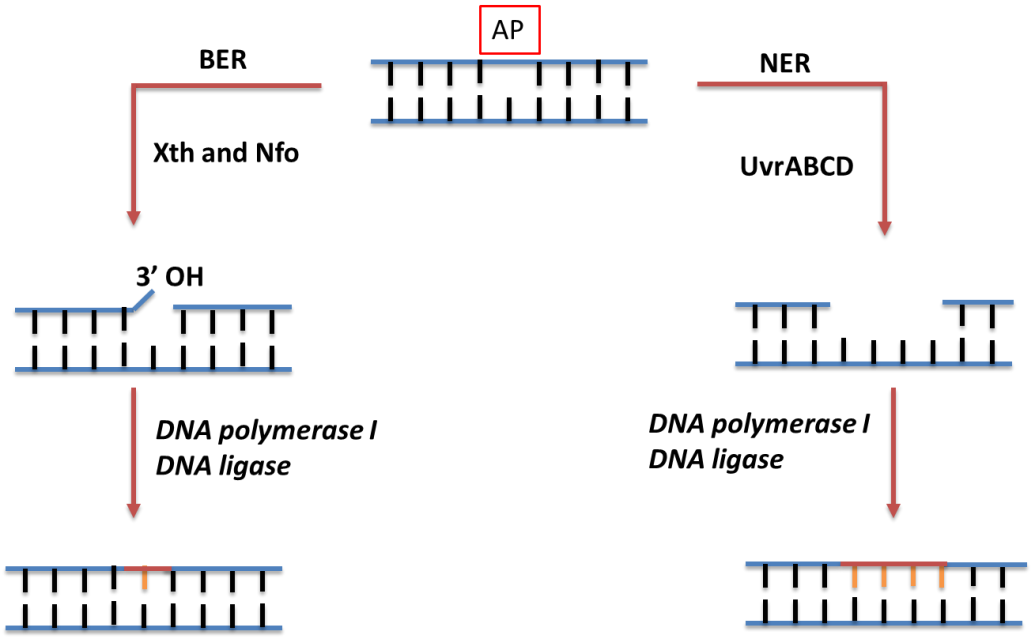


Figure 38. BER and NER collaborate to repair AP site.

In NER pathway, UvrA protein recognizes AP sites by itself or through the action of UvrD-NusA interaction, and then AP site is removed by the action of UvrBCD together to remove about 11-13 oligonucleotides fragment containing AP site. At the next step for both pathways, DNA polymerase I comes to the gap and synthesizes DNA. Finally DNA ligase comes to seal the nick, and intact DNA is obtained.

Part 2. H_2O_2 sensitivity

1. BER pathway and H_2O_2 sensitivity

It was reported that *xth* deficient strain showed hypersensitivity to H_2O_2 but not in case of *nfo* deletion strain (Souza *et. al.,* 2006). In agreement with this, I found that *Δnfo* single deletion strain showed the same sensitivity to H_2O_2 as wild type strain. However, I found that *Δxth* strain was slightly more sensitive to H_2O_2 than wild type strain but not as hypersensitive as Sauza group's observation (Souza et al. 2006). A significant hypersensitivity to H_2O_2 was only observed with cells lacking both Xth and Nfo (Figure 32). This sensitivity data is consistent to the recombination events data, in which the frequency of recombination events was very high in cells lacking both Xth and Nfo but not in cells lacking each enzyme.

In case of two DNA glycosylase/AP lyase enzymes, Nei and Nth, it has been reported that cells lacking either Nei or Nth showed the same sensitivity to H_2O_2 as wild type cells, but cells lacking both Nei and Nth were extremely sensitive to H_2O_2 (Saito *et. al.,* 1997). In contrast, cells lacking both Nei and Nth showed an only slightly more sensitivity to H_2O_2 compared to the wild type strain in my experiment (Figure 31), which is consistent to the recombination events data. Therefore, it is likely that the base damages that are substrate of Nei and Nth proteins might be repaired by other pathways like NER.

On the other hand, it was clearly shown that cells lacking AP endonuclease were more sensitive to H_2O_2 than cells lacking DNA glycosylase/AP lyase, suggesting that most of oxidative DNA damages produced under H_2O_2 treatment might be AP sites.

2. NER pathway and H_2O_2 sensitivity

Several lines of evidence indicated that NER pathway in human protects cells against oxidative DNA damages both *in vivo* and *in vitro (Menoni et. al., 2012; Reardon et. al.,* 1997). However, the involvement of NER pathway in repairing the oxidative DNA damages in *E. coli* cells has been under controversial, suggesting that role of NER pathway might be different between *E. coli* and higher organism. NER pathway is responsible for repair of bulky DNA lesions such as thymine dimer and intra-strand-cross links (Petruseva *et. al.,* 2014). Therefore, it was not surprising that NER deficient strain showed the same sensitivity to H_2O_2 as wild type strain (Figure 33).

However, in the absence of both BER and NER pathways, cells showed an extremely high sensitivity to H_2O_2. This indicates clearly that both BER and NER function to repair oxidative DNA damages and protect cells against the killing effect of H_2O_2. Therefore, I concluded that NER pathway in *E. coli* cells protects cells against oxidative DNA damages as like in human cells (Menoni *et. al.,* 2012).

3. Recombination repair pathway and H_2O_2 sensitivity

Recombination repair pathway has been known to be responsible for rescuing cells from stalled or blocked replication forks by facilitating homologous recombination process (Cox 2001; Cox 2002). Whereas spontaneous base substitution mutation was not changed in wild type strain under different growth conditions that produce different levels of oxidative DNA damages (Nunose *et. al*., 2016), recombination events in wild type strain was much higher in M9 glucose medium than in LB medium. Thus, it seems that recombination repair pathway participates in repair of oxidative DNA lesions which are not repaired by BER or NER and induces recombination events. I expected that very high level of oxidative DNA damages in cells treated with H_2O_2 would lead cells more sensitive to H_2O_2 in the absence of recombination repair pathway. Despite a previous report that RecA had a little function in repairing the hydrogen peroxide induced DNA damages compared to the other repair pathway (Hagensee & Moses 1989), in my experiment, *ΔrecA* cells clearly showed hypersensitivity to H_2O_2, suggesting that the action of recombination repair pathway rescues cells from killing effects of H_2O_2.

Chapter VI. Future plans

As I mentioned, besides oxidative DNA damages, other DNA damages also contribute to induce spontaneous recombination events in normally growing cells. Alkylation and deamination of nucleotide bases have been known as popular DNA lesions which are induced under normal growth condition, hence accumulate mutations. The alkylation lesions are produced by alkylating agents which are widespread in the environment and also as products of metabolism process (Sedgwick *et. al.,* 2007; Mielecki *et. al.,* 2015). The most abundant alkylating lesions are N3-methyladenine (3meA) and N7-methylguanine (7meA), which are effectively repaired by AlkA DNA glycosylase and Tag DNA glycosylase. 3meA is cytotoxic, blocks DNA replication, and may be involved in production of recombination events while elimination of 7-meA leads to produce AP sites which are cytotoxic lesions. Therefore, I would like to investigate the involvement of these two DNA glycosylase enzymes, AlkA and Tag proteins, in the suppression of recombination events.

In addition, deamination of cytosine has been known to produce uracil in DNA, resulting in U:G mispair, hence toxic to cells if not repaired (Parikh *et. al.,* 2000; Olinski *et. al.,*2010; Krokan *et. al.,* 2002). Uracil DNA glycosylase (UDG) is one of the most well studied DNA glycosylase, which removes uracil from DNA. Further investigation should be carried out on deamination of cytosine occurring in normally growing cells, especially their association with induction of recombination events.

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