博士論文番号 : 1581207
(Doctoral student number)

Sublethal concentration of bactericidal antibiotics induces oxidative DNA damages in *Escherichia coli* cells

（近致死濃度の殺菌性抗生物質は大腸菌細胞内で酸化 DNA 損傷を誘発する）

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Submitted on 2018/09/10

Graduate School of Biological Sciences　Doctoral Thesis Abstract

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Title	Sublethal concentration of bactericidal antibiotics induces oxidative DNA damages in *Escherichia coli* cells		

Generation of reactive oxygen species (ROS) is a natural side effect of aerobic respiration. ROS are generated through successive single-electron transfer to oxygen, which produces superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•). Among the ROS, hydroxyl radical is most dangerous, as it physically attacks DNA, causing oxidative DNA damages. One of the most abundant is 8-oxoguanine (8-oxoG), which causes G:C to T:A transversion. *Escherichia coli* possesses a set of repair enzymes, namely MutM and MutY, that effectively suppress the mutagenesis caused by 8-oxoG. Different aspects of oxidative stress as a whole has been extensively studied. Interestingly, recent studies suggested that ROS are induced by different classes of bactericidal antibiotics through complex redox alterations, regardless of their specific drug-target interactions. The increase in ROS levels seems to contribute to cell death by bactericidal antibiotics. Apart from cell-killing, low ROS stress induced by sublethal bactericidal antibiotics may on the other hand promote mutagenesis and resistance. In an important study, sublethal bactericidal antibiotics were reported to induce mutations in wild type *E. coli* strain, and this mutagenesis was suggested to be mediated by hydroxyl radical. In addition, continuous exposure to different bactericidal antibiotics at sublethal concentration subsequently induced multi-drug resistance in the wild type. However, there is no direct evidence for hydroxyl radical-induced oxidative DNA damages caused by bactericidal antibiotics at sublethal concentrations, and the mechanism is largely unknown. Thus, the purpose of my study is to clarify that sublethal bactericidal antibiotics induce oxidative DNA damages and to find a possible mechanism for this phenomenon.

In order to clarify that sublethal bactericidal antibiotics induce oxidative DNA damages, I determined the frequency of mutations caused by 8-oxoG in an *E. coli* strain (Δ*mutMY*) lacking MutM and MutY after a prolonged exposure to ½ of the minimum inhibitory concentration (MIC) of three major classes of bactericidal antibiotics. Using the *rpoB* mutation assay, I found that ½ MIC ampicillin (ß-lactam) and norfloxacin (fluoroquinolone) treatments significantly increased the level of 8-oxoG mutations, whereas gentamicin (aminoglycoside) did not. This result clearly shows that ampicillin and norfloxacin at ½ MIC induce oxidative DNA damages. The addition of hydroxyl radical scavenger, thiourea, completely suppressed the 8-oxoG mutations induced by ½ MIC ampicillin and norfloxacin. From these observations, it is evident that ampicillin and norfloxacin at ½ MIC induce oxidative DNA damages through hydroxyl radical formation. To further characterize the nature of oxidative DNA damages caused by sublethal ampicillin and norfloxacin, I determined the *rpoB* mutation frequency in

Δ*mutMY* cells treated with increasing concentrations to near MIC of ampicillin and norfloxacin. I found that the mutation frequencies for each antibiotics treatment remained at a certain level and did not increase further even at near MICs. In addition, 8-oxoG mutation levels were highest at ½ MIC for both antibiotics. This result shows that there is a threshold in the oxidative DNA damages induced by sublethal ampicillin or norfloxacin.

In order to elucidate the mechanism inducing oxidative DNA damages in cells treated with sublethal bactericidal antibiotics, I determined the important factors involved in the mechanism. Previously, it was found that the addition of glucose to LB medium significantly increased the cellular H_2O_2 level. Under this condition, mutation frequencies in Δ*mutMY* cells treated with ½ MIC ampicillin or norfloxacin were determined. Surprisingly, no significant changes in the 8-oxoG mutation levels were observed. This shows that H_2O_2 is not the limiting factor in this mechanism. Since the Fenton reaction producing hydroxyl radical requires ferrous iron (Fe^{2+}), I hypothesized that the increase in oxidative DNA damages in the antibiotics treated cells might depend on intracellular free Fe^{2+} concentration. The addition of Fe^{2+} chelator, 2,2'-bipyridyl, significantly suppressed the 8-oxoG mutation levels in Δ*mutMY* cells treated with ½ MIC ampicillin or norfloxacin, indicating that cells exposed to the antibiotics increased the intracellular concentration of free Fe^{2+}. Furthermore, the increase in mutation frequency was significantly elevated in a strain (Δ*mutMY* Δ*yaaA* Δ*dps*) lacking Dps, the major Fe^{2+} storage protein, and YaaA, an iron regulator protein, when treated with ½ MIC ampicillin or norfloxacin, indicating the importance of free Fe^{2+} in this mechanism. Most importantly, I found that the iron transport protein, TonB, is directly involved in this mechanism, in which the mutation frequency was not seen in Δ*mutMY* Δ*tonB* cells treated with ½ MIC ampicillin or norfloxacin. This observation suggests that the oxidative DNA damages induced by sublethal ampicillin and norfloxacin required TonB-dependent iron import in increasing intracellular Fe^{2+} concentration.

Overall, I showed that oxidative DNA damages are induced by sublethal concentration of ampicillin and norfloxacin, but not gentamicin. The level of oxidative DNA damages induced by the antibiotics does not increased when the antibiotics concentration was increased, suggesting that the oxidative DNA damages might not contribute to the cell-killing by bactericidal antibiotics as previously reported. I also showed that the intracellular concentration of free Fe^{2+} is a key factor in this mechanism, and TonB plays a crucial role in increasing free Fe^{2+} concentration in the sublethal ampicillin and norfloxacin treated cells.

Abbreviations

8-oxoG	8-oxoguanine
8-oxodGTP	8-oxodeoxyguanosine triphosphate
AMP	Ampicillin
NOR	Norfloxacin
GEN	Gentamicin
TET	Tetracycline
CAM	Chloramphenicol
ROS	Reactive oxygen species
OH•	Hydroxyl radical
O_2^-	Superoxide
H_2O_2	Hydrogen peroxide
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
ETC	Electron transport chain
TCA	Tricarboxylic acid
HPF	Hydroxyphenil fluorescein
PBP	Penicillin binding protein
MIC	Minimum inhibitory concentration

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Chapter 1. Introduction

1. Antibiotics

Antibiotics are types of antimicrobial drugs that are widely used in treating and preventing bacterial infections and diseases. The word '*antibiotic'* was first introduced in 1941 to describe any small molecule produced by a microorganism that antagonizes the growth of other microorganisms (Schullian, 1973). The year 1945 marked the start of 'antibiotic age' with the development of penicillin, produced by fungus *Penicillium*, and other antibiotics such as tetracycline, streptomycin, and chloramphenicol produced by soil bacteria *Streptomyces* (Conover, 1971), and until today antibiotics have been playing a big role in clinical settings. Antibiotics can be classified based on whether they are bactericidal, which induce cell death, or bacteriostatic, which only inhibit cell growth but does not cause cell death. Bactericidal or bacteriostatic antibiotics affect cellular components based on their specific mode of actions. Bactericidal antibiotics actions in causing cell death are group into three major classes, namely cell wall synthesis inhibition, nucleic acid synthesis inhibition and protein synthesis inhibition. Further explanation on major classes of bactericidal antibiotics is presented in the next sub chapter (Chapter 1.1).

Unlike bactericidal antibiotics, bacteriostatic antibiotics only inhibit cell growth mainly by inhibiting ribosome functions (targeting 30S or 50S ribosome subunits), only causing no new proteins being produced but not mistranslated proteins, thus only inhibiting cell growth (Tenson et al., 2003). Cells are not killed but cell growth and multiplication are temporarily inhibited and this effect can be reverse once the antibiotic is removed or the antibiotics effects are diminished (Weisblum & Davies, 1968). The antibiotics does not induce cell death because their mode of actions usually requires the host's defense mechanism to eliminate the infections or the microorganisms (Scheld & Sande, 1983). Examples of bacteriostatic antibiotics are such as tetracycline, chloramphenicol and macrolides (Chopra & Roberts, 2001; Tenson et al., 2003).

1.1 Bactericidal antibiotics mode of action

As mentioned earlier, bactericidal antibiotics induced cell death by interfering with normal cell functions via their specific targets. Three major classes of bactericidal antibiotics

have been extensively studied. They may inhibit cell wall synthesis, nucleic acid synthesis or protein synthesis.

The major antibiotics that inhibit cell wall synthesis are grouped in the β-lactam class, such as penicillin (including penicillin derivatives), cephalosporins and carbapenems (Wise & Park, 1965). The cell wall is an important structure to retain cell shape, preventing cell lysis due the osmotic pressure in the cytoplasm and also to anchor the membrane components and extracellular proteins (Guilhelmelli et al, 2013). Exposure to the β-lactams or other cell wall synthesis inhibitors induce changes to cell shape or size, cellular stress response induction, and ultimately, cell lysis (Tomasz, 1979). The β-lactams work by blocking the peptide bond formation reaction catalyzed by the penicillin-binding proteins (PBP) also known as the transpeptidase (Figure 1), which leads to inhibition of peptidoglycans cross-linking (Wise & Park, 1965; Tipper et al., 1965; Holtje, 1998). For example, the penicillin-derived ampicillin is used to treat many Gram-positive and Gram-negative bacteria induced infections, by acting as an irreversible inhibitor of transpeptidase, which induces cell lysis (Petri, 2011; Giguere et al., 2013).

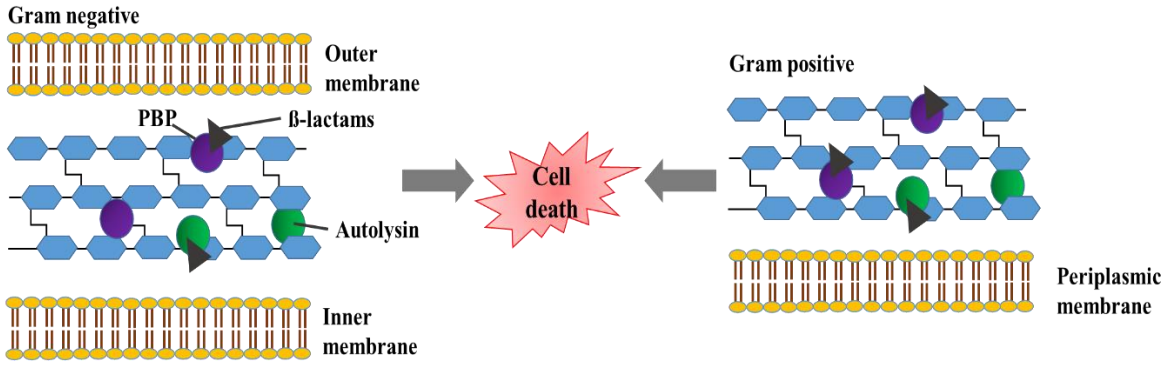

Figure 1. β-lactams mechanism of action.

β-lactams bind to PBPs to inhibit transpeptidation, causing reduction in peptidoglycans that leads to cell lysis and death.

One of the major class of bactericidal antibiotics that inhibit nucleic acid synthesis is the quinolone class. Chromosomal supercoiling via topoisomerase activity in strand breakage and rejoining reactions are important for mRNA transcription, DNA synthesis and cell division (Chen and Liu, 1994; Liwa & Jaka, 2015). DNA gyrase (DNA topoisomerase II) functions to promote negative DNA supercoiling and relaxation, and also involves in resolving knots in DNA (Ehmann & Lahiri, 2014). The quinolones and the fluoroquinolones (synthetic quinolone)

inhibit DNA gyrase functions by interacting with GyrA (DNA gyrase subunitA) to interfere with the breakage and rejoining of the DNA during supercoiling (Figure 2), producing double strand breaks which leads to cell death (Chen and Liu, 1994). Examples quinolone or fluoroquinolone antibiotics include norfloxacin and ciprofloxacin, which are widely used to treat urinary tract infections, gynecological infections, gonorrhea and bladder infections (Padaeiskaia, 2003).

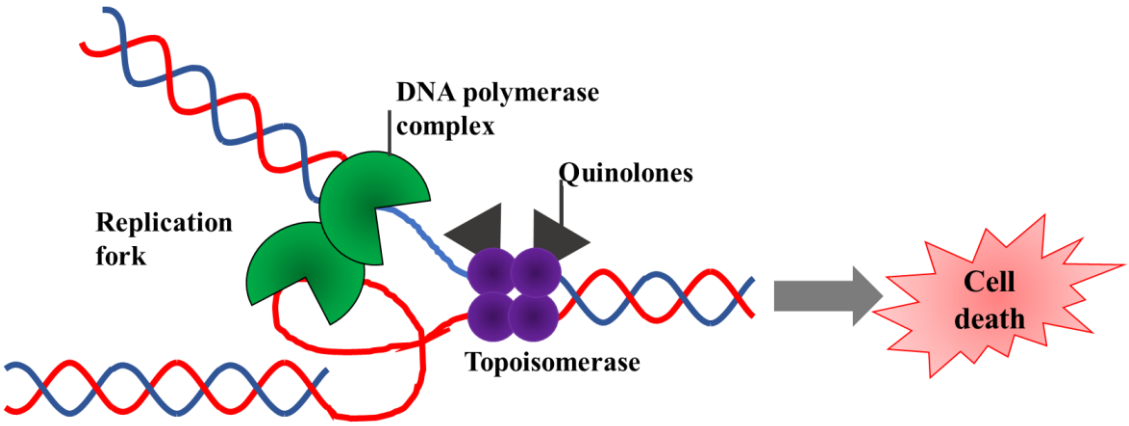

Figure 2. Quinolones mechanism of action

Quinolones binds to topoisomerase II (DNA gyrase)-DNA complex and interfere with DNA supercoiling causing double-stranded DNA breaks, leading to cell death.

Protein synthesis inhibitor commonly targets the 30S or 50s ribosome subunit, thus interfering with the normal protein synthesis processes. Microbial ribosomes, comprises of 30S and 50S subunits, directed the protein synthesis processes including the initiation phase, elongation phase and termination phases (Liwa & Jaka, 2015). The aminoglycoside class of antibiotics is the only antibiotic class of protein synthesis inhibitor that is bactericidal. Aminoglycosides act by binding to specific site on 30S ribosomes and induce cell-killing by inducing non-functional protein production and also causing misreading. Aminoglycosides can cause incorporation of wrong amino acids into elongating peptide strands, which promote protein mistranslation (Davies et al., 1965). The incorporation of mistranslated proteins into the cytoplasmic membrane may increase the cellular permeability causing an increased uptake of antibiotics molecules (Figure 3), which is lethal to the cells (Davies et al, 1986). Examples of antibiotics from the aminoglycosides include gentamicin, kanamycin and streptomycin.

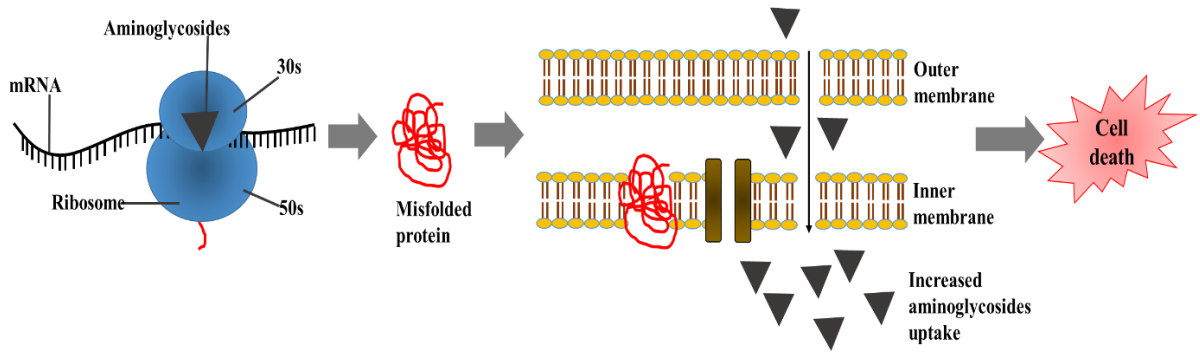

Figure 3. Aminoglycosides mechanism of action

Aminoglycosides bind to the 30S ribosome subunit, causing mistranslated proteins. These proteins can misfold and can be incorporated into the membrane, leading to an increased in drug uptake.

1.2 Bactericidal antibiotics induced cell-killing mechanism

Classically, we believe that bactericidal antibiotics induced cell death by disrupting the essential cellular functions through the primary drug-target interactions, which based on the antibiotics' specific mode of actions**.** Around 10 years ago, researchers have proposed a new idea on bactericidal antibiotics cell-killing mechanism in *E. coli*, suggested that the drug-target interaction alone is not the sole cause of bactericidal antibiotics induced cell-killing. They found that high level of reactive oxygen species (ROS) are induced by bactericidal antibiotics, regardless of their specific targets in the cell, contributing to cell killing (Kohanski et al., 2007; Dwyer et al., 2014; Belenky et al., 2015). Treatments with lethal concentrations of bactericidal antibiotics showed that the drug-target interactions resulted in common changes in metabolism and redox-related physiological alterations, regardless of their specific mode of actions, which lead to the increase in ROS level that promotes cellular damages (Kohanski et al., 2007; Dwyer et al., 2014).

Earlier findings on oxidative stress induced by lethal bactericidal antibiotics treatments involved the usage of chemical probe, fluorescent dye hydroxyphenil fluorescein (HPF), which is widely used for measuring ROS, specifically hydroxyl radicals (OH•) (Setsukinai et al., 2003). The author showed that wild type *E. coli* cells treated with high dose of ampicillin, norfloxacin and kanamycin increased the HPF fluorescence intensity, but bacteriostatic antibiotics such as chloramphenicol, tetracycline, and rifampicin does not (Kohanski et al.,

2007). Furthermore, *katG ahpCF* deletion and *katE katG ahpCF* deletion *E. coli* strains showed increased sensitivity to killing by ciprofloxacin (quinolone), indicated that ROS is involved in cell-killing by ciprofloxacin, a fluoroquinolone (Goswami et al., 2006). After these findings were reported, researchers tried to find the possible reasons for this phenomenon. It was reported that the metabolic changes causing the increase in ROS level in *E. coli* cells treated with lethal dose of bactericidal antibiotics are induced by activation of the envelope stress-response two-component sensor CpxA and redox-response two-component system ArcA (Kohanski et al., 2008). Increase in the expression of genes involved in respiration and tricarboxylic acid (TCA) cycle enzyme activated by ArcA leads to the increase in ROS formation (Liu & Wulf, 2004; Kohanski et al., 2008). Although this is used as a general consequence of lethal bactericidal antibiotics treatments, the evidence only involved treatments with aminoglycosides because this antibiotic caused incorporation of mistranslated peptides into the membrane, causing membrane disruption, which activated the CpxA-ArcA system (Kohanski et al., 2008). However, evidence with ß-lactams or quinolones regarding CpxA-ArcA induction have not been reported.

Another evidence on the involvement of TCA cycle in ROS-mediated gentamicin induced cell-killing was reported in *E. coli*, in which cells were sensitized to gentamicin when L-Serine was added, due to the increase in nicotinamide adenine dinucleotide (NADH) production (Duan et al., 2016). High level of NADH was suggested to induce ROS production, thus sensitizes the cells to gentamicin treatment (Duan et al., 2016). Other evidences on the involvement of TCA cycle in antibiotics induced ROS-mediated cell killing were also shown in other bacteria. Similar observation was reported with *Staphylococus aureus*, in which the antimicrobial peptide gramicidin A was suggested to disrupt the TCA cycle and induced hyper activation of the electron transport chain (ETC), thus inducing OH• production (Liou et al., 2015). Further evidences on lethal bactericidal antibiotics induced oxidative stress were reported. Metabolic perturbations, such as increased concentrations of central carbon metabolites, breakdown of the nucleotide pool, and reduced lipid levels were induced in *E. coli* cells treated with β-lactams, aminoglycosides, and quinolones (Belenky et al., 2015).

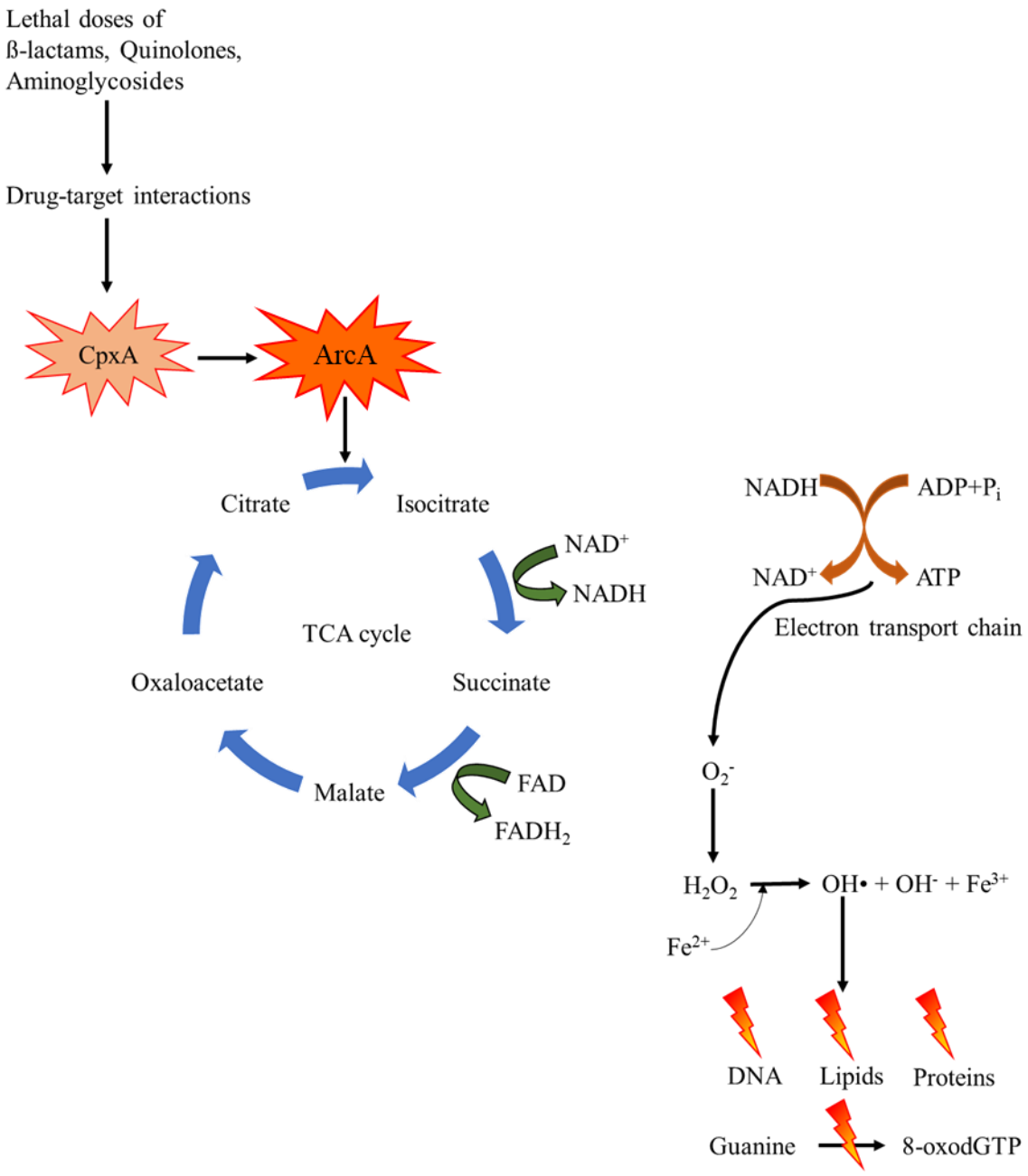

Figure 4. ROS-mediated bactericidal antibiotic cell-killing mechanism. (Modified from Van Acker & Coenye, 2017)

Bactericidal antibiotics, regardless of their specific mode of actions, activate the TCA cycle through CpxA-ArcA two-component sensor system, causing hyperactivation of the ETC that leads to the increased level of ROS which damages iron-containing proteins and dehydratases. The release of Fe^{2+} makes it available to be oxidized by H_2O_2 during Fenton reaction to produce OH• which damage cellular components.

These metabolic changes possibly induced protein carbonylation, nucleotide oxidation, and DNA double-strand breaks, which contributes to cell killing (Belenky et al., 2015). It has

been reported that the increase in superoxide (O_2^-) and hydrogen peroxide (H_2O_2), upon treatment with bactericidal antibiotics, promotes the damage of dehydratases and other iron-containing proteins, thus releasing free iron intracellularly (Kohanski et al., 2007). Hydrogen peroxide can easily oxidize the free ferrous iron (Fe^{2+}) in the Fenton reaction to produce OH• (Imlay et al., 1988). Taking all the evidences, the researchers hypothesized a new mechanism (Figure 4) in which lethal bactericidal antibiotics induce bacterial cell death by stimulating OH• production, an event dependent on metabolic and redox changes, TCA cycle, the ETC, production of O_2^- and H_2O_2 that lead to the damage of the dehydratases and iron-containing proteins, and the stimulation of Fenton reaction (Kohanski et al., 2007; Dwyer et al., 2014). The stimulation of Fenton reaction promotes the formation of OH• , causing oxidative damages to proteins, lipids and DNA, contributing to cell death in bacteria (Dwyer et al., 2014).

1.3 Sublethal concentrations of bactericidal antibiotics induced ROS

Bactericidal antibiotics, at ½ of the minimum inhibitory concentration (MIC), were reported to significantly increase the mutation rate in wild type *E. coli* cells, possibly through ROS mediation (Kohanski et al., 2010). The increase in mutation rate was correlated to the increase in HPF intensity and the mutagenesis was suppressed when cells were grown in anaerobic condition, suggesting that the mutagenesis was mediated by increase in OH• formation (Kohanski et al., 2010). Another study by Gutierrez et al. (2013) suggested the DinB-mediated incorporation of oxidized nucleotides into DNA is one a possible mechanism of mutagenesis induced by sublethal ampicillin. This finding was correlated with a hypothesis on ROS-mediated cell killing by lethal doses of bactericidal antibiotics induced the incorporation of deoxynucleoside triphosphate (8-oxodGTP) from oxidized nucleotide pool into the DNA by error-prone DNA polymerase IV (Foti et al., 2012).

The increase in ROS under sublethal bactericidal antibiotics treatments might be harmful due to the fact that ROS, specifically OH• can directly attack the DNA to produce oxidative DNA damages, especially the highly mutagenic 8-oxoguanine (8-oxoG), which is an oxidized form of guanine that can easily form mispair with adenine (Neeley & Essigmann, 2006). Even though OH• and oxidative DNA damages were suggested to be involved in sublethal bactericidal antibiotics induced mutagenesis and resistance in *E. coli* and other bacteria, the mechanisms are yet to be solved. Most importantly, there is still no direct evidence on oxidative DNA damages induced by sublethal concentrations of bactericidal antibiotics due to the fact

that the mutagenesis assays utilized wild type strains without deletions of any specific oxidative DNA damage repair genes, making it difficult to estimate the actual level of oxidative damages produced in the DNA.

Regarding OH• -mediated resistance in wild type *E. coli* cells, Kohanski et al. (2010) also observed cross-resistance to other antibiotics in cells treated with ½ MIC bactericidal antibiotics, based on the increase in the MICs (Kohanski et al., 2010). Furthermore, sublethal concentrations of vancomycin induced modest increase in ROS level, as indicated from the increase in fluorescence dye dichlorofluorescein diacetate (DCFH-DA) intensity, which possibly promote antibiotic resistance in heterogeneous vancomycin-resistant *S. aureus* strain (Li et al., 2015). While lethal dose of bactericidal antibiotics caused cell death, low concentrations of sublethal concentrations of bactericidal antibiotics may promote mutagenesis and bacterial antibiotic resistant. Bacteria are most likely to be exposed to sublethal concentrations of antibiotics in the environment and the increase in mutagenesis under this low antibiotic stress can be beneficial for them to adapt and become resistant to antibiotics, which is a dangerous consequence to the clinical world (Kummerer, 2003; Davies et al., 2006; Kohanski et al., 2010).

1.4 Antibiotics resistance

In the past years, increase in antimicrobial resistance among bacterial pathogens became threatening to global health, and jeopardize years of therapeutic accomplishments and successful results of utilizing antimicrobials to treat various diseases. Furthermore, back in 2014, antibiotic resistance has been named as one of the three most important public health threats of the 21st century by the World Health Organization (WHO) and still is a great threat today (WHO, 2014; 2018). Bacteria have an incredible ability in withstanding or adapting to various stresses and environmental threats, such as exposure to antibiotics that may endanger their viability. Bacteria utilize genetic strategies in adapting to antibiotics threat, such as mutations in genes associated with the antibiotics' mode of action and the acquisition of resistance genes from foreign DNA through horizontal gene transfer (Munita & Arias, 2016).

Through horizontal gene transfer, bacteria are able acquire external resistance gene from other bacteria or microorganisms via transformation, which is the incorporation of naked DNA; transduction, usually phage-mediated; and also conjugation (Munita & Arias, 2016). As

I mentioned earlier, another strategy for bacteria to acquire resistance is through mutations. Generally, if a group of bacterial cells from a population acquire or develop mutations in genes associated with the antibiotics mode of action, these cells can survive and adapt to the antibiotics. While the antibiotics remove the susceptible cells, only the resistant population survives and continue to multiply (Munita & Arias, 2016). Mutations can generally occur under antibiotics treatments through general mechanisms, such as direct interactions between antibiotics molecule and the specific target, recombination, as well as SOS-mediated error-prone DNA polymerase action during replication (Thi et al., 2011; Gutierrez et al., 2013). The acquired mutations may cause some modifications of the antibiotics target, the decrease in the antibiotics uptake, activation of efflux mechanism, or changes in important metabolic pathways in adapting to antibiotic stress (Campbell et al., 2001; Aldred et al., 2014).

Lethal concentrations of antibiotics may select for pre-existing resistant population through elimination of susceptible population. However, sublethal concentrations of antibiotics can accelerate acquirement of resistance by increasing the mutation rate and horizontal gene transfer (Gullberg et al., 2011; Blazquez et al., 2012). For example, *S. aureas* cells treated with sublethal β-lactams, *Enterococcus faecalis* and *Bacteroides* cells treated with sublethal tetracycline were shown to increase horizontal gene transfers (Barr et al., 1986; Stevens et al., 1993; Torres et al., 1991). A new idea on the possibility of oxidative DNA damages induced mutagenesis accelerating the acquirement of resistance in bacteria was proposed based on the observations that OH• formation increased under sublethal concentrations of bactericidal antibiotics treatments, promoting to the increase in mutation rate and cross-resistance to other antibiotics (Kohanski et al., 2010). This showed that low level of bactericidal antibiotics stress is harmful, as low stress environment permits the cells to multiply and grow normally, but at the same time have higher chances of acquiring resistance (Laureti et al., 2013).

2. Reactive oxygen species (ROS)

In this sub chapter, I will explain further on ROS, how ROS are generated in the cells and also oxidative stress protection and oxidative DNA damages repair mechanism in *E. coli*. In aerobic condition, ROS are continuously formed in *E. coli* cells through consecutive electron transfers which form O_2^- , H_2O_2 and OH• (Figure 5). It is important to know that OH• is produced through Fenton reaction, where H_2O_2 oxidizes the free Fe^{2+} to Fe^{3+}, forming OH• and hydroxide (OH^-) ion (Imlay et al., 1988). In addition, ROS are major cause of spontaneous

mutations in aerobic organisms. Among the ROS, OH• directly attacks DNA to produce oxidative DNA damages (Imlay et al., 1988).

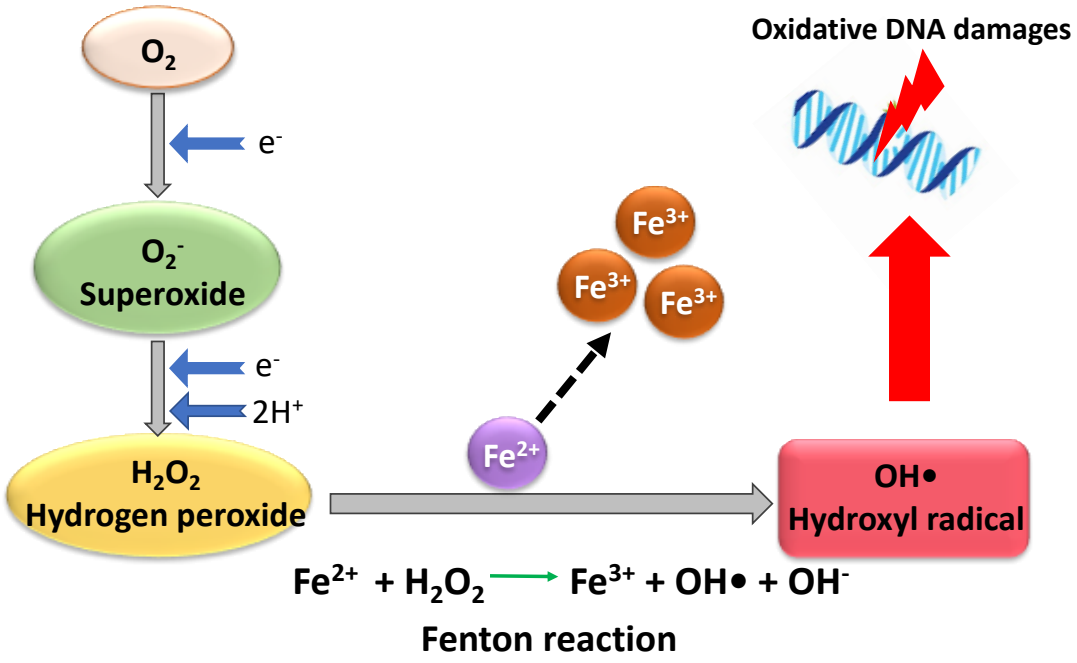

Figure 5. Formation of ROS in cells.

ROS are generated through consecutive electron transfers, forming O_2^-, H_2O_2 and OH•. In order to form OH•, H_2O_2 oxidizes the free Fe^{2+} which is called Fenton reaction.

2.1 Hydroxyl radical protection and oxidative DNA damage repair in *E. coli*

2.1.1 Scavenging enzymes

In order to protect cells from the damaging effects of OH•, *E. coli* possesses several scavenging enzymes to detoxify O_2^- and H_2O_2 since there is no known enzyme to scavenge OH• itself. The primary scavenging enzyme is alkyl hydroperoxide reductase (Ahp, encoded by *ahpCF*) controlled by OxyR regulon, which is a two-component thiol-based peroxidase that reduces H_2O_2 to water via electrons from NADH (Parsonage et al., 2005; Seaver & Imlay, 2001). Another important H_2O_2 scavenger is catalase G (encoded by *katG*), also controlled by OxyR regulon. Ahp are activated when H_2O_2 levels are low or during exponential growth phase, while catalase is highly activated when H_2O_2 levels are high or during cell starvation (Seaver & Imlay, 2001; Aslund et al., 1999). However, when cells are having high H_2O_2 stress, both *ahpCF* and *katG* were strongly induced by OxyR (Seaver & Imlay, 2001; Hillar et al., 2000).

Although O_2^- is less stable and easily converted to H_2O_2 through spontaneous dismutation, it is not enough maintain it at low concentration. Therefore, *E. coli* have three superoxide dismutases (SODs), namely cytoplasmic iron-cofactored enzyme FeSOD and the manganese-cofactored enzyme MnSOD, and a periplasmic copper-zinc-cofactored enzyme CuZnSOD (Imlay, 2008). Superoxide dismutases catalyzes the partitioning or dismutation of O_2^- into H_2O_2 or O_2 molecules (Carlioz & Tuoati, 1986).

2.1.2 Iron maintenance and regulation

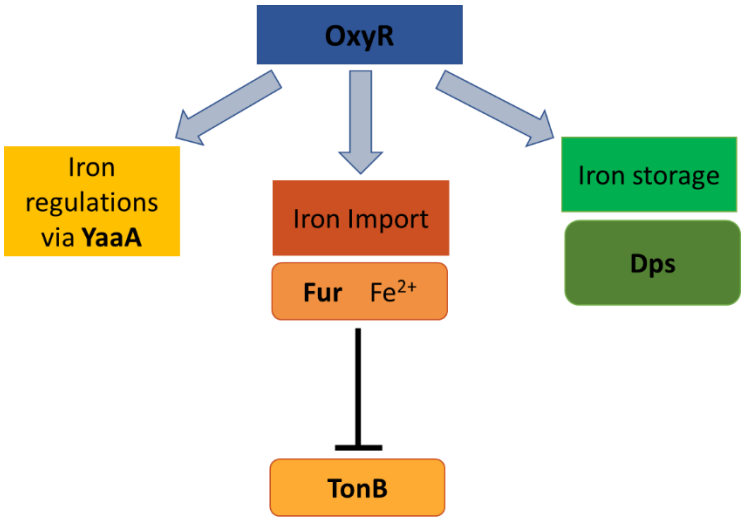

Figure 6. Main iron homeostasis regulated by OxyR in *E. coli*

As mentioned earlier, hydroxyl radical is formed when H_2O_2 oxidizes Fe^{2+} during Fenton reaction. Therefore, the production of OH• can be inhibited when intracellular free Fe^{2+} concentrations are controlled to a minimum level. In *E. coli*, the OxyR regulon controls the primary iron regulator genes, which typically regulates iron metabolism based on the intracellular iron availability (Figure 6). The important player in this regulation is ferric uptake regulator (Fur) protein (Hantke, 2001). Fur acts as a positive repressor when iron is present to the TonB-ExbB-ExbD iron import system, which is a main iron import pathway in aerobic condition (Andrews, 2003). Cells take up iron-chelate complex through outer membrane (OM) receptors which is fuelled by the energy-transducing TonB-ExbB-ExbD system (Postle, 1993). The TonB-ExbB-ExbD complex interacts and transduce energy to various OM receptors, such as FhuA, FhuE, FecA, and FepA (Andrews, 2003).

One of the most important iron storage protein is OxyR-controlled Dps, in which the primary role is to store iron and protect the DNA against Fe^{2+} and H_2O_2 action in the production of OH• (Zhao et al., 2002). During iron oxidation, Dps is shown to preferably use H_2O_2 as an electron acceptor rather than molecular oxygen, suggesting that the iron storage function is activated only during H_2O_2 stress in order to minimize oxidative damages (Zhou et al., 2002). Recently, YaaA protein of the OxyR regulon have been reported to work together with Dps and Fur, in order to suppress intracellular free Fe^{2+} concentration in *E. coli* cells (Liu et al., 2011).

2.1.3 Oxidative DNA damages repair mechanism

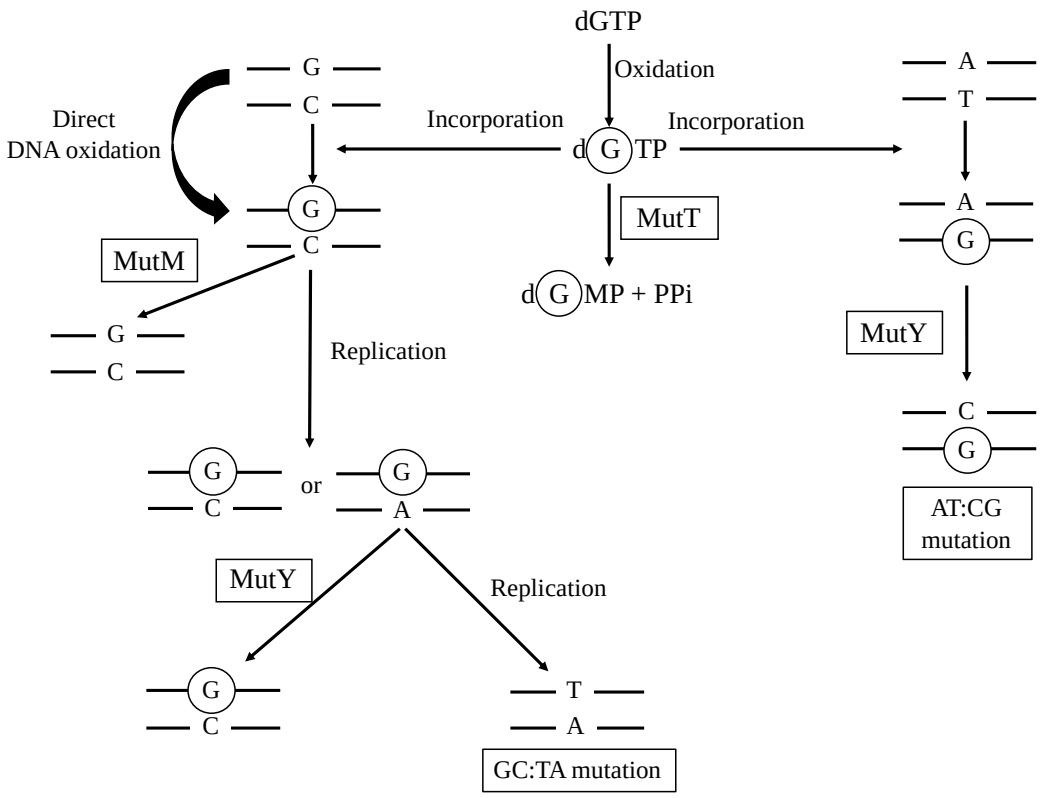

Figure 7. 8-oxoguanine-related oxidative DNA damages repair mechanisms (Modified from Fowler et al., 2003)

As mentioned earlier, OH• is highly deleterious due the fact that it can directly attacks DNA to induce oxidative DNA damages. One of the most abundant oxidative DNA damage is 8-oxoG, which is highly mutagenic base adduct, either direct oxidation in the DNA itself or

when formed in the nucleotide pool as deoxynucleoside triphosphate (8-oxodGTP) (Fowler et al., 2003). 8-oxoG can easily form mispair with adenine nucleotides that leads to G:C to T:A base substitution (Imlay et al., 1988; Wood et al., 1990). The presence of two repair enzymes in *E. coli*, MutM and MutY, almost completely suppress the mutagenesis caused by 8-oxoG (Figure 7). The MutM protein is a formamidopyrimidine-DNA glycosylase that recognizes modified purines including 8-oxoG (Chetsanga et al., 1981; Chung et al., 1991; Boiteux et al., 1992) and initiates base excision repair of 8-oxoG when paired with cytosine. If it is unrepaired, subsequent mispairing of the 8-oxoG with dATP may lead to a G-C to T-A transversion. The MutY protein is also a DNA glycosylase, but it functions by removing adenine from A-(8-oxoG) mispairing (Michaels et al., 1992). Direct incorporation of 8-oxodGTP from the nucleotide pool into the DNA commonly induces A:T to C:G base substitution (Figure 7) (Inoue et al., 1998). Sanitization of the nucleotide pool from oxidized nucleotides is carried out by MutT protein by hydrolyzing the 8-oxodGTP to produce 8-oxodGMP and pyrophosphate, thus preventing the oxidized nucleotide from being misincorporated into the DNA (Maki & Sekiguchi, 1992; Fowler et al., 2003).

3. Purpose of this study

So far, there is no direct evidence of sublethal bactericidal antibiotics induced oxidative DNA damages. Researchers used only wild type strains to examine OH• -related mutagenesis, which does not completely showed the actual level of oxidative DNA damages induced by sublethal bactericidal antibiotics. Furthermore, the use of chemical-based methods including fluorescence dyes, such as HPF, to examine the formation of OH• as a direct measure of oxidative DNA damages in cell-killing at high concentration or mutagenesis at low concentrations of bactericidal antibiotics, were reported to be inaccurate and ineffective. It is believed that HPF, a non-fluorescent dye, will generate fluorescein which is a fluorescent molecule, when reacted with OH•. Thus, the increase in HPF fluorescence in cells treated with antibiotic suggested the OH• formation (Setsukinai et al., 2003). However, recent studies have suggested that HPF does not specifically oxidized by OH• or other ROS, but redox-active metals may also react with HPF to produce fluorescence molecules, making previous findings on bactericidal antibiotics induced OH• arguable (Kalyanaraman et al., 2012; Keren et al., 2012; Liu & Imlay, 2013). A more accurate approach needs to be utilized in order to clarify whether oxidative DNA damages produced in cells treated with sublethal concentrations of bactericidal antibiotics.

In this study, I am aiming to clarify whether bactericidal antibiotics, at sublethal concentrations, induce oxidative DNA damages by using a more reliable genetic approach. In addition, I wanted to find out a possible molecular mechanism of this phenomenon. In times where the world is fighting with the spread of antibiotics resistance in bacteria, a better understanding of events leading to mutagenesis under sublethal antibiotics treatments is greatly needed, and all possible mechanisms must be elucidated. Findings obtained from this study might be an important stepping stone in uncovering a more thorough and complete mechanisms on sublethal concentrations of bactericidal antibiotics induced mutagenesis and also resistance.

Chapter 2. Materials and methods

1. Strains

All strains used this study were derived from *E. coli* strain K-12, and their genotype and origin are shown in Table 1.

Table 1: List of strains

Strain name	Genotype	Origin
MG1655	Wild type	Laboratory stock
MG1655+pTN249	MG1655 carrying plasmid pTN249 *ahpC-gfp*	Laboratory stock
MK7180	MG1655, Δ*mutM,* Δ*mutY*	Laboratory stock
MK9404	MK7180, Δ*dps*	Laboratory stock
MK9612	MK7180, Δ*yaaA*	Laboratory stock
MK9616	MK7180, Δ*yaaA,* Δ*dps*	Laboratory stock
MK9627	MK7180, Δ*tonB*	Laboratory stock
MK10102	MK7180, Δ*mazF*	Laboratory stock
MK10104	MK7180, Δ*cpxA*	Laboratory stock

2. Reagents and growth medium

All strains were grown on LB agar or LB broth. LB medium was prepared as follows; 1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract and 1% (w/v) NaCl. The pH was adjusted to pH 7.0 and then sterilized. In case of making solid agar medium, add 1.5% (w/v) Bacto agar and then sterilized. Cooled the molten LB agar to 55-50°C before pouring into petri dishes.

Bactericidal antibiotics (ampicillin, norfloxacin and gentamicin) and bacteriostatic antibiotics (chloramphenicol and tetracycline) were used for treatment. These antibiotics were purchased from Wako Pure Chemicals Industries. Bactericidal antibiotics were used at ½ MIC determined for each strain; ampicillin: 1.25 µg/ml, 1.4 µg/ml, 2.8 µg/ml; norfloxacin: 30 ng/ml, 37.5 ng/ml, 60 ng/ml; gentamicin: 0.4 µg/ml. Bacteriostatic antibiotics were also used at ½ MIC; 1.4 µg/ml chloramphenicol and 0.65 µg/ml tetracycline. For lower than ½ MIC treatment condition, 0.7 µg/ml ampicillin and 15 ng/ml norfloxacin were used. For higher than ½ MIC treatment condition, 1.9 µg/ml, 2.4 µg/ml ampicillin, and 40 ng/ml, 50 ng/ml norfloxacin were used. For lethal concentration treatment condition, 5 µg/ml ampicillin and 250 ng/ml norfloxacin were used. Rifampicin was used for determination of mutation frequency at a concentration of 100 µg/ml. Chloramphenicol were used to select *E. coli* strain carrying the plasmid pTN249 at a concentration of 25 µg/ml. Hydroxyl radical scavenger thiourea and iron

chelator 2,2'-bipyridyl were purchased from Wako Pure Chemicals Industries. Thiourea was used at 100 mM or 50 mM, while bipyridyl was used at 200 µM.

Colonies used in GFP level measurement were harvested with 1x M9 salts with sodium azide addition at final concentration of 2%. 5x M9 salts was prepared as follows; 210 mM disodium hydrogen phosphate (Na_2HPO_4), 110 mM potassium dihydrogen phosphate (KH_2PO_4), 43 mM sodium chloride (NaCl) and 94 mM ammonium chloride (NH4Cl). The solution was diluted in sterilized Milli Q water to make 1x M9 salts. In addition, sodium azide was prepared by dissolving in Milli Q water to make 20% (w/v) solution and was used at a final concentration of 2% (w/v). After colonies were harvested with 1x M9 salts, they were diluted in phosphate buffered saline (PBS) solution for GFP level measurement. The solution was prepared as follows; 2 mM KH_2PO_4, 2.7 mM sodium phosphate dehydrate ($Na_2HPO_4.2H_2O$) and 137 mM NaCl. The solution was adjusted to pH 7.2 and filtered with 0.2 µm filter (Sigma).

3. Antibiotics sensitivity assay

Before determining the exact MIC, strains were tested for antibiotics sensitivity on antibiotics concentration gradient plate. Antibiotics concentration gradient plates were prepared based on the method developed by Szybalski and Bryson (1952) with modifications. Two layers of LB agar needed for each rectangular petri plate, with lower layer without antibiotics and upper layer with antibiotics (Figure 8).

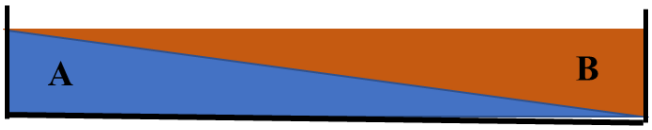

Figure 8. Antibiotics concentration gradient plate. A) LB agar B) LB agar supplemented with antibiotics

For the lower agar layer, 50 ml of molten LB agar was poured into the plate, slanted to a degree that all surface was covered and then let it hardened. Mix 50 ml LB agar with antibiotics (5 µg/ml ampicillin or 300 ng/ml norfloxacin) and poured onto the first layer. The plates were kept overnight to let downward diffusion of the antibiotics and creating a uniform concentration gradient. To prepare the *E. coli* inoculum, a single colony of each strains were

pick up and inoculated into 5 ml LB broth and incubated overnight at 37°C with shaking. The cultures were diluted at 10^{-5} and 10^{-6} dilution factor in cold LB broth. For each dilutions of each strains, spot 3 µl along the concentration gradients. The plates were incubated at 37°C for 24 hours. The range of concentrations in which the strains were sensitive to was estimated and used in determining the exact MIC.

4. Minimum inhibitory concentration (MIC) determination

In order to determine the MIC for each strain, a modified protocol developed by Andrews (2001) was used. Overnight culture for each strain were prepared and incubated at 37°C with shaking. Then, the cultures were diluted to $^1/_6 \times 10^{-6}$ dilution factor and 100 µl of the diluted cultures (about 100 cells) were plated onto LB agar supplemented with ampicillin, norfloxacin, chloramphenicol or tetracycline of different concentrations based on the concentration range obtained from gradient plate. Diluted cultures were also plated on LB agar without antibiotics as controls. The plates were incubated at 37°C for 24 hours. The MIC is defined as the lowest concentration of antibiotics to show no visible growth of the organism after 24 hours incubation (Andrews, 2001).

5. GFP reporter assay fluorescence level measurement

The GFP fluorescence levels were measured in wild type (MG1655) cells carrying pTN249 (*ahpC-gfp*) treated with lethal concentration or ½ MIC conditions for ampicillin, norfloxacin or gentamicin.

For treatment with lethal concentrations of bactericidal antibiotics, the experiments were done using liquid culture method. Overnight culture of MG1655 carrying pTN249 plasmid was prepared in LB with Cam selection and incubated at 37°C overnight. Then, the overnight culture was diluted 1:200 in 25 mL LB broth, in 300 ml flasks with Cam selection. Cultures were grown to an OD_{600} of ~0.3 before diluted 1:3 in LB broth, in 100 ml flasks. Antibiotics were added into the flasks at concentration of 5 µg/ml ampicillin or 250 ng/ml norfloxacin. No antibiotics were added in control flasks. For measurement of background fluorescence level, overnight culture of MG1655 without plasmid was diluted 1:200 in 25 mL LB broth, in 300 ml flasks. Cultures were grown to an OD_{600} of ~0.3 before diluted 1:3 in LB broth, in 100 ml flasks and without addition of antibiotics. All flasks were incubated at 37°C, with shaking at 300

RPM for 90 mins. After 90 mins incubation, flasks were taken out from the incubator, put on ice and sodium azide was added (2% final concentration) to stop ATP synthesis and preserving fluorescence intensity. Then, cultures were diluted 1:10 in PBS, in 1.5 ml microcentrifuge tubes. The diluted samples were filtered using nylon filter cloth. Keep all the tubes on ice.

For treatment with sublethal concentrations of bactericidal antibiotics, the experiments were done using plate culture method. For measurement of background fluorescence level, MG1655 without plasmid was used. Overnight culture of MG1655 carrying pTN249 plasmid was prepared in LB with Cam selection and incubated at 37°C overnight. Next, the overnight culture was diluted at $^1/_6$ x10^{-6} dilution factor in LB broth and 100 µl of culture (about 100 cells) was plated on LB agar supplemented with ampicillin, norfloxacin or gentamicin and LB agar without antibiotics as control. For measurement of background fluorescence level, overnight culture of MG1655 without plasmid was diluted at $^1/_6$ x10^{-6} dilution factor in LB broth and 100 µl of culture (about 100 cells) was plated on LB agar. All plates were incubated at 37°C until the colonies reached 1.5 mm in size (1X10^8~1X10^9 cells/ml). When colonies reached 1.5 mm in size, cells were harvested with cold 5 ml 1x M9 salts with 2% sodium azide. The harvested cultures were diluted 1:10 in PBS, in 1.5 ml microcentrifuge tubes. The diluted samples were filtered using nylon filter cloth. Keep all the tubes on ice.

Measurement of GFP fluorescence level was carried out within 30 mins of sample preparation. GFP fluorescence level was measured by using Accuri™ C6 Flow Cytometer (BD Biosciences) according to the manufacturer's protocol. All data were obtained with a 488 nm argon laser and a 515- to 545-nm emission filter (FL1). In this analysis, single-cell portion of cell population can be approximated by cell size (FSC) and internal complexity of cells (SSC) parameters (Nakayashiki & Mori, 2013). The relative fluorescence values were calculated by taking the mean fluorescence measurement for each sample. The mean fluorescence of background cells value was subtracted these values. The final value represents the average of the total fluorescent value (Nakayashiki & Mori, 2013).

During H_2O_2 stress, OxyR regulon will activate protection mechanism, including scavenger enzymes such as peroxidases and catalases to detoxify H_2O_2, as shown in Figure 9 (Zheng et al., 2001). Alkylhydroperoxide reductase (Ahp) is a primary H_2O_2 scavenger in *E. coli* controlled by the OxyR regulon (Seaver & Imlay, 2001). By using the *ahpC-gfp* reporter

assay, GFP fluorescence level was measured when cells were treated with bactericidal antibiotics, which reflects the intracellular H_2O_2 level.

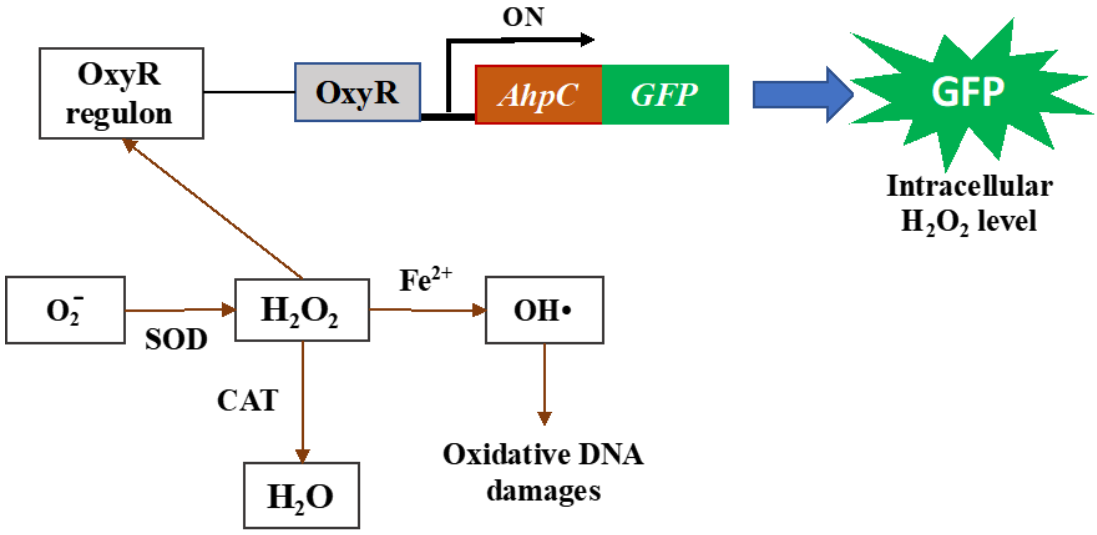

Figure 9. GFP fluorescence intensity reflects the intracellular H_2O_2 level.
SOD: superoxide dismutase; CAT: catalase; AhpC: alkylhydroperoxide reductase; GFP: green fluorescent protein

6. Measurement of spontaneous mutation by *rpoB* mutation assay

Overnight culture was prepared by picking up 1 single colony of the particular strain, inoculated in 5 ml LB broth and incubated at 37°C with shaking. The overnight culture was diluted to $^1/_6$ x 10^{-6} dilution factor and 100 µl of the diluted cultures (about 100 cells) were plated onto LB agar supplemented with sublethal concentration of ampicillin, norfloxacin, chloramphenicol or tetracycline. For experiments with thiourea, thiourea in solid form was added to liquid agar together with antibiotics before pouring into the plates for a final concentration of 50 mM or 100 mM. For iron chelation experiments, bipyridyl was added together with antibiotics to liquid agar before pouring into plates for a final concentration of 200 µM. Cells were also plated on LB agar without antibiotics as untreated populations. All streaked plates were incubated at 37°C until the colonies reached 1.5 mm in size. The growth rate was defined as the time needed for the colonies to reach 1.5 mm diameter. When colonies reached 1.5 mm in size, cells were harvested with 5 ml cold LB broth and diluted into appropriate dilutions. 100 µl of the diluted cultures were plated on LB agar (for viable cell count) and LB agar supplemented with 100 µg/ml rifampicin (for rifampicin-resistant cell count) as shown in Figure 10. LB plates were incubated at 37°C for around 16 hours, while LB

with rifampicin plates were incubated at 37°C for 48 hours. The mutation frequency values were calculated as the ratio of rifampin-resistant colonies to the total number of colony-forming unit (CFU). Statistical evaluation was done by using the Mann–Whitney *U*-test when two groups were compared (Siegel, 1956). Differences were considered significant when *p* values were <0.05 (*) or <0.01 (**).

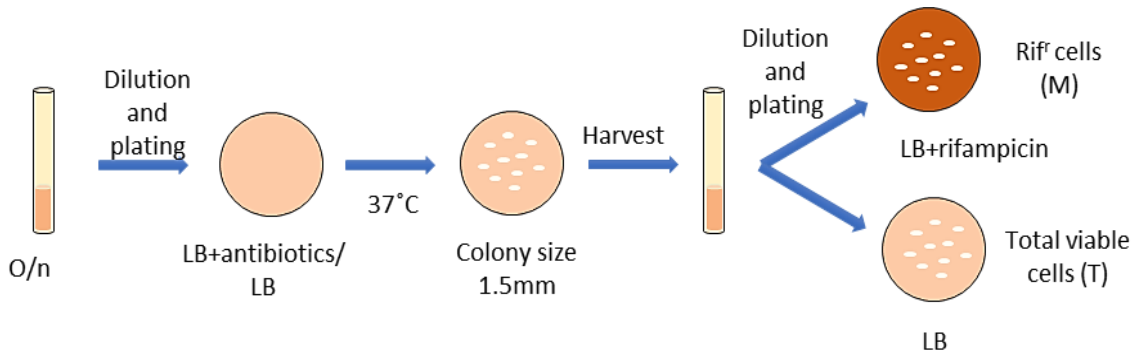

Figure 10. *rpoB* mutation assay methodology

Chapter 3. Results

Part I. Sublethal concentrations of bactericidal antibiotics induce oxidative DNA damages

1. Minimum inhibitory concentration (MIC)

First of all, antibiotics sensitivity test was done for all strains using the concentration gradient plate assay to determine the inhibitory concentration ranges (examples in Figure 11).

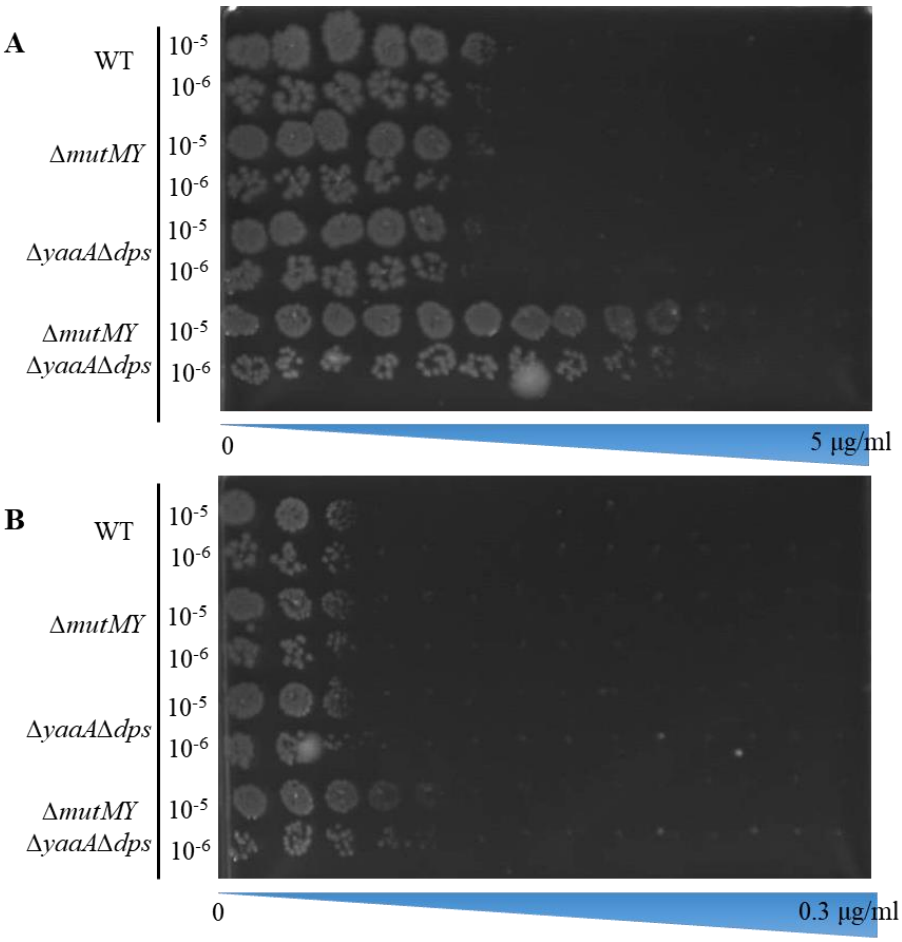

Figure 11. Antibiotics concentration gradient assay. A) Ampicillin B) Norfloxacin

Diluted cultures of each strain were inoculated along the gradient (3 μL/ spot) and incubated at 37°C for 24 hours. The concentration ranges were determined and were used to prepare LB agars supplemented with different concentrations of antibiotics. Around 100 colonies were plated and the MICs are carefully determined. In this study, the MIC is defined

as the lowest antibiotics concentration that prevents visible growth of *E. coli* cells. All the MICs of the antibiotics for each strain were determined and shown in Table 2.

Table2: MIC of antibiotics for strains used in this study

Strain	Antibiotics (μg/ml)				
	Bactericidal			Bacteriostatic	
	Ampicillin	Norfloxacin	Gentamicin	Chloramphenicol	Tetracycline
Wild type	2.8	0.06	0.8	2.8	1.3
Δ*mutMY*	2.8	0.06	0.8	2.8	1.3
Δ*mutMY* Δ*yaaA* Δ*dps*	5.6	0.12	-	-	-
Δ*mutMY* Δ*tonB*	2.4	0.075	-	-	-
Δ*mutMY* Δ*cpxA*	2.0	0.078	-	-	-
Δ*mutMY* Δ*mazF*	2.0	0.062	-	-	-

(- : MIC not determined)

Sublethal concentration is defined as antibiotic concentrations lesser than the MIC, while half MIC ($^1/_2$ MIC) is defined as antibiotic concentration at half of the MIC. On the other hand, lethal concentration is defined as concentrations that are multiple times higher than the MIC.

2. Sublethal bactericidal antibiotics induced oxidative DNA damages

My first objective was to determine whether bactericidal antibiotics, at ½ MIC, induce oxidative DNA damages. I am utilizing a genetic approach to determine the oxidative DNA damages induced by sublethal bactericidal antibiotics in *E. coli* cells in this study. I determined the mutation frequencies in Δ*mutMY* cells treated with ½ MIC bactericidal antibiotics using the *rpoB* mutation assay. 8-oxoG is the most abundant oxidative DNA damage causing G:C to T:A base substitution during replication (Dizdaroglu, 1985). However, the repair function of MutM and MutY efficiently suppress 8-oxoG-induced G:C to T:A base substitution efficiently (Michaels et al., 1992). In the absence of MutM and MutY, the 8-oxoG produced are not repaired and converted to mutations, which is reflected as the mutation frequency. Therefore, Δ*mutMY* strain provides a good measure for total produced oxidative DNA damages in *E. coli* cells. The median values of mutation frequency were used for comparison between treatments and strains, however data distributions shown in scatter plots can be found in Supplementary

figures section (page 77). As an initial finding, I determined the oxidative DNA damages induced by ½ MIC bactericidal antibiotics treatment.

I determined the mutation frequency in wild type and Δ*mutMY* strains treated with different classes of bactericidal antibiotics at ½ MIC; ampicillin (β-lactam), norfloxacin (quinolone) and gentamicin (aminoglycoside). The growth rate for both strains, with or without antibiotics treatments, were 14 hours. As shown in Figure 12, mutation frequency in untreated Δ*mutMY* was 80-fold higher than the untreated wild type. It clearly shows that MutM and MutY efficiently repaired the 8-oxoG-related mutations in the wild type, and the mutation frequency increase in Δ*mutMY* reflects the total unrepaired 8-oxoG converted to mutations produced in DNA. Δ*mutMY* cells treated with ½ MIC norfloxacin and ampicillin further increased the frequency by two to three-fold, which indicates that more oxidative DNA damages were produced. Although the mutation frequency in wild type treated with ½ MIC ampicillin and norfloxacin also showed significant increase, however the levels were small and unrelated to oxidative DNA damages. This suggested that ½ MIC ampicillin and norfloxacin may induce other types of mutation. In both strain, mutation frequencies were not changed in cells treated with ½ MIC gentamicin. This showed that gentamicin, at ½ MIC, does not induce mutagenesis and oxidative DNA damages. These findings suggested that not all bactericidal antibiotics induce oxidative DNA damages at ½ MIC, in this case only ½ MIC ampicillin and norfloxacin induce oxidative DNA damages, but not gentamicin.

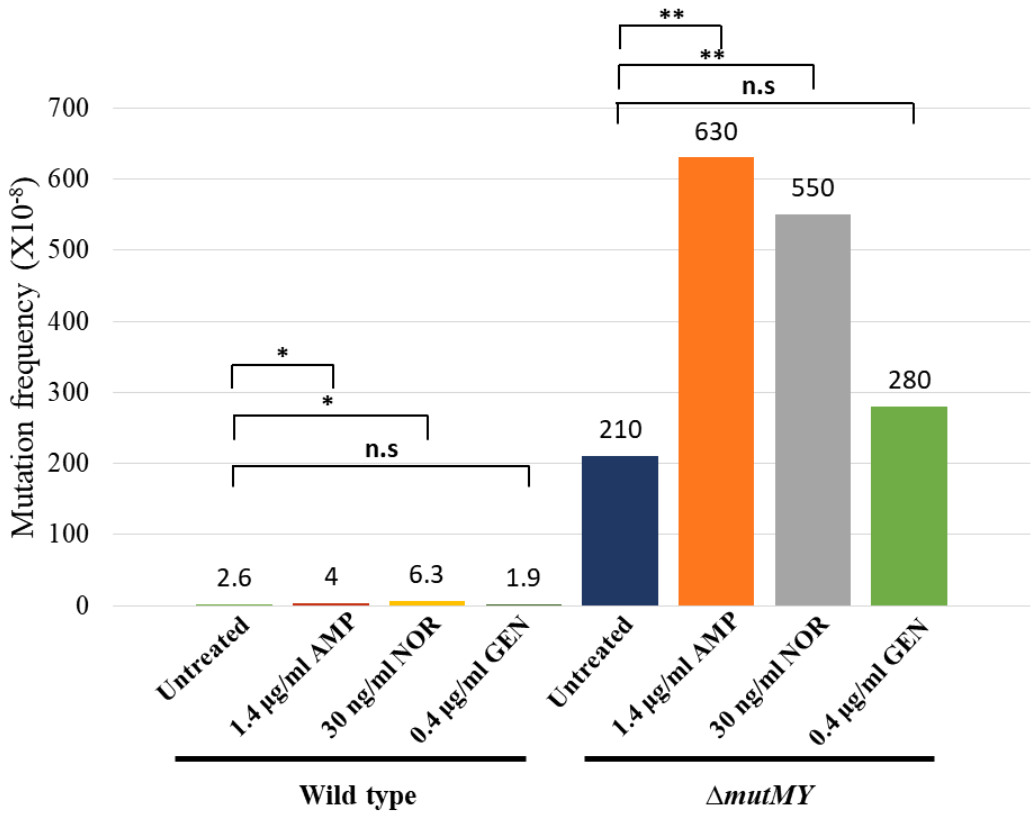

Figure 12. Mutation frequencies in wild type and Δ*mutMY* cells treated with ½ MIC bactericidal antibiotics.

Around ~100 cells were grown on LB plates supplemented with the indicated antibiotics concentration at 37°C until colony size reached 1.5 mm in diameter (growth rate was measured for each treatment). After harvesting the cells, rifampicin resistant mutant cells were selected by plating on LB supplemented with rifampicin plates at 37°C for 48 hours (see Methods). Mutation frequencies were obtained from 20 populations for each treatment and median values were used for comparison between data sets. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01, ns indicates non-significant. Growth rates are shown below.

Strain	**Wild type**				***ΔmutMY***			
Treatment	Untreated	½ MIC AMP	½ MIC NOR	½ MIC GEN	Untreated	½ MIC AMP	½ MIC NOR	½ MIC GEN
Growth rate (hrs)	14	14	14	14	14	14	14	14

(AMP: ampicillin; NOR: norfloxacin; GEN: gentamicin)

Previously, it has been reported that bacteriostatic antibiotics, such as chloramphenicol or tetracycline (ribosome inhibitors) does not stimulate ROS production (Kohanski et al., 2007). Therefore, it is possible that treatment with bacteriostatic antibiotics will not induce oxidative DNA damage, specifically 8-oxoG. To test whether the OH•-induced oxidative DNA damages is unique to bactericidal antibiotics or not, mutation frequency was measured in Δ*mutMY* cells treated with ½ MIC chloramphenicol or tetracycline. The result is shown in Figure 13.

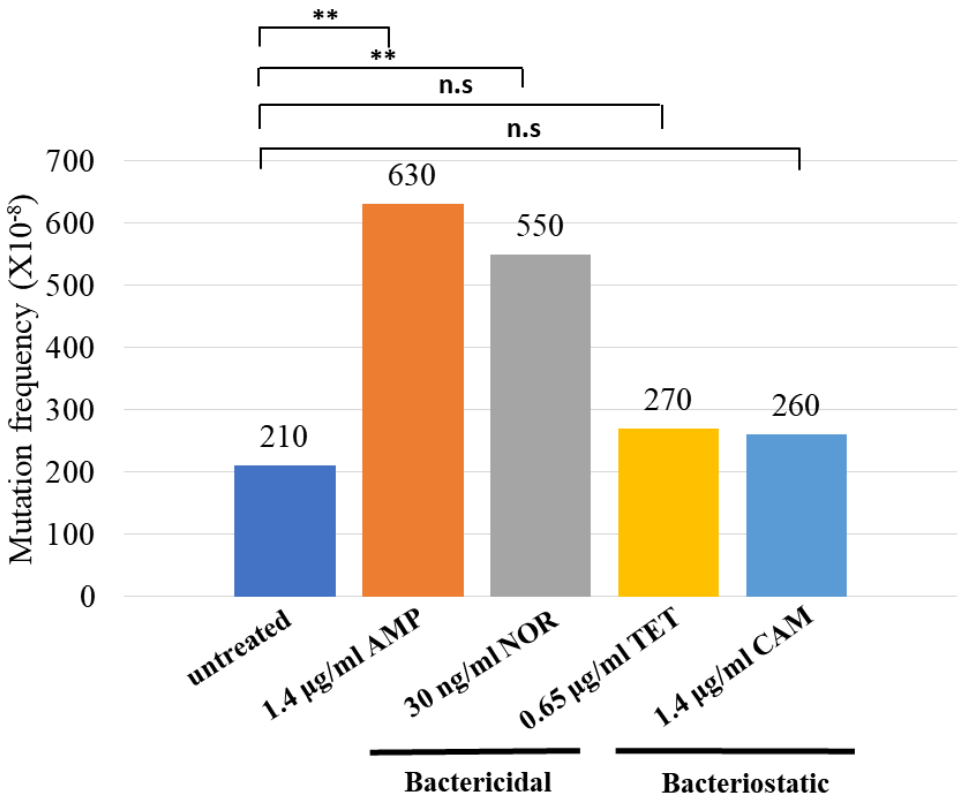

Figure 13. Mutation frequencies in Δ*mutMY* cells treated with ½ MIC bacteriostatic antibiotics.

The mutation frequencies were obtained from 20 populations for each treatment as described in Figure 12. Significance of data: (**) indicates p-value <0.01, ns indicates non-significant. Growth rates are shown below.

Treatment	Untreated	½ MIC AMP	½ MIC NOR	½ MIC TET	½ MIC CAM
Growth rate (hrs)	14	14	14	23	24

(AMP: ampicillin; NOR: norfloxacin; TET: tetracycline; CAM: chloramphenicol)

As shown in Figure 13, the mutation frequency in Δ*mutMY* cells treated with ½ MIC tetracycline and chloramphenicol does not change compared to the untreated cells, although the growth in cells treated with tetracyline or chloramphenicol was greatly delayed. This result clearly showed that sublethal bacteriostatic antibiotics does not induce oxidative DNA damages.

As I have shown in Figure 12 that ½ MIC ampicillin and norfloxacin induced oxidative DNA damages, I wanted to verify that the oxidative DNA damages were caused by OH• production. To test this, I determined the mutation frequency in Δ*mutMY* cells treated with ½ MIC ampicillin or norfloxacin in the presence of thiourea. Thiourea is a powerful OH• scavenger that diminishes the effects of OH• damage in both prokaryotes and eukaryotes

(Novogrodsky et al., 1982; Tuoati et al., 1995). Thiourea scavenges OH• by converting the oxygen radical into water (Figure 14).

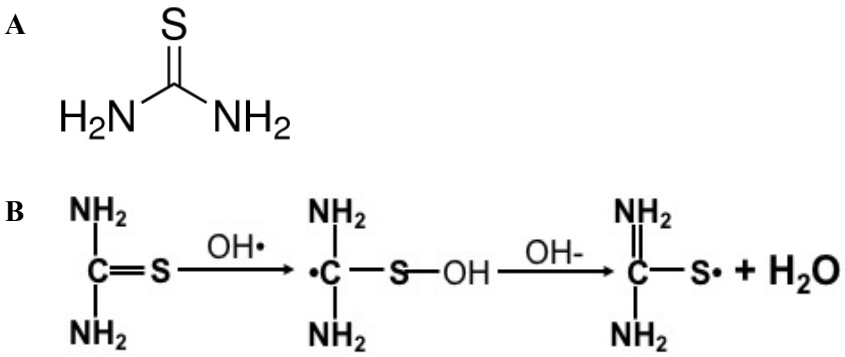

Figure 14. Thiourea structure (A) and OH• scavenging reaction (B)

Thiourea scavenges OH• through hydrogen abstraction, converting the oxygen radicals into water.

Previously, 100 mM thiourea have been shown to reduce bactericidal antibiotics-killing in the wild type (Kohanski et al., 2007). However, in this experiment ½ MIC ampicillin treated Δ*mutMY* cells could not grow and were very sensitive to 100 mM thiourea. So, thiourea concentration was reduced to 50 mM for ½ MIC ampicillin treatment. Cells treated with both thiourea and antibiotics took a slightly longer time to reach 1.5 mm size. As shown in Figure 15, thiourea suppressed the mutation frequency by two-fold in untreated cells, indicating that thiourea reduces some OH• formation, thus partially suppressing the oxidative DNA damages. The addition of thiourea significantly suppressed the mutation frequency in ½ MIC ampicillin treated Δ*mutMY* cells by three-fold and norfloxacin Δ*mutMY* cells by five-fold. Thiourea completely suppressed the antibiotics effect on producing oxidative DNA damages observed earlier in Δ*mutMY* cells without addition of thiourea. This result suggested that ½ MIC ampicillin or norfloxacin induced oxidative DNA damages through OH• formation.

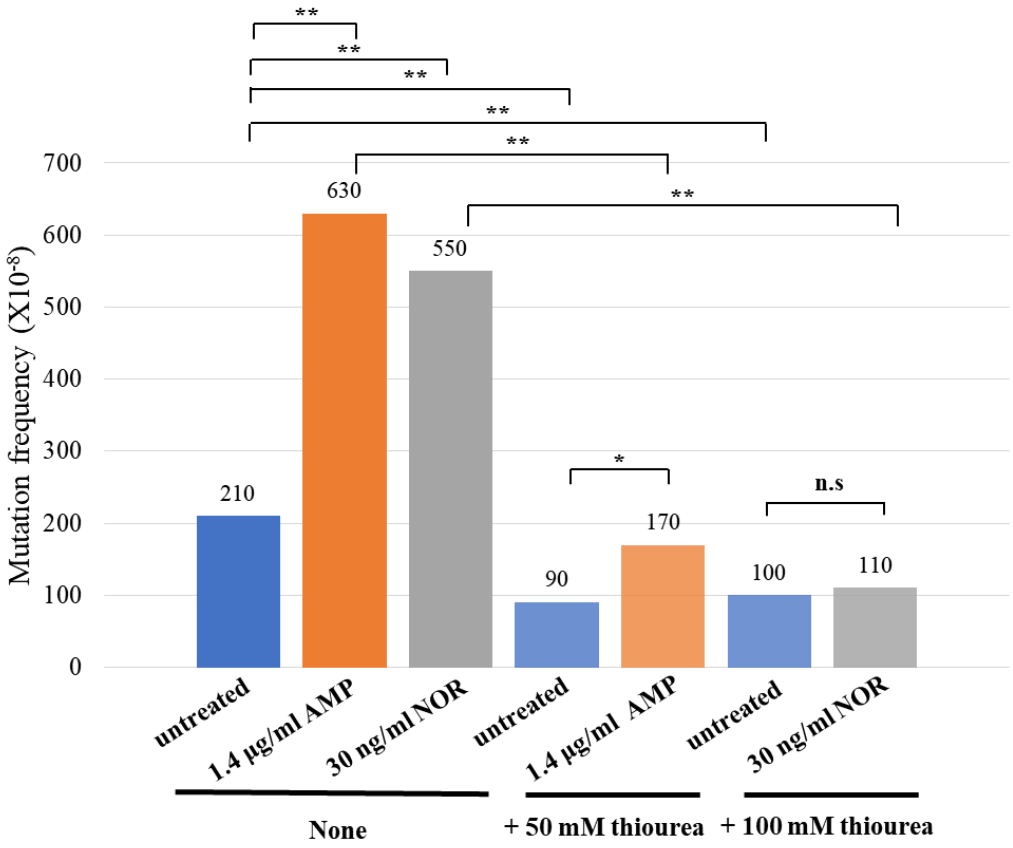

Figure 15. Role of thiourea in suppression of oxidative DNA damages in Δ*mutMY* cells treated with ½ MIC ampicillin and norfloxacin.

The mutation frequencies were obtained from 20 populations for each treatment as described in Figure 12. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01, ns indicates non-significant. Growth phenotype are shown below.

Treatment	LB			LB + thiourea		
	Untreated	½ MIC AMP	½ MIC NOR	Untreated	½ MIC AMP	½ MIC NOR
Growth rate (hrs)	14	14	14	15	16	16

(AMP: ampicillin; NOR : norfloxacin)

3. Characterization of the oxidative DNA damages induced by sublethal ampicillin or norfloxacin

So far, I have shown that ½ MIC ampicillin or norfloxacin induced oxidative DNA damages. I wanted to know whether increasing the antibiotics concentration will also increasing the level of oxidative DNA damages produced. Does the oxidative DNA damages produced depended on antibiotics concentration? To test this, I determined the mutation frequency in Δ*mutMY* cells treated with $^1/_4$ MIC or higher than ½ MIC concentrations of

ampicillin or norfloxacin. For ampicillin, the concentrations used were 0.7 µg/ml, 1.9 µg/ml and 2.4 µg/ml, while norfloxacin concentrations were used 15 ng/ml, 40 ng/ml and 50 ng/ml.

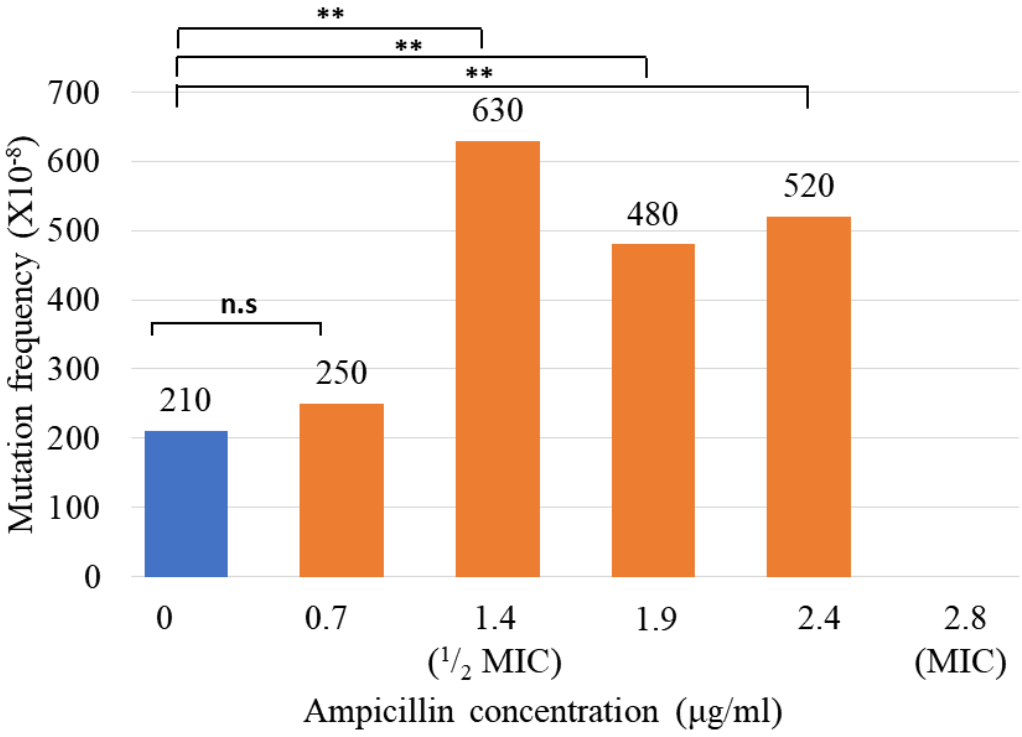

Figure 16. Characteristics of oxidative DNA damages induced by sublethal concentrations of ampicillin in Δ*mutMY* cells

The mutation frequencies were obtained from 20 populations for each treatment as described in Figure 12. Significance of data: (**) indicates p-value <0.01, ns indicates non-significant. Growth phenotype are shown below.

Treatment	**Ampicillin concentration (µg/ml)**				
	0	**0.7**	**1.4**	**1.9**	**2.4**
Growth rate (hrs)	14	14	14	16	19-20
Colony counts/ population	~100	~100	~100	~70	~60

As shown in Figure 16, no significant changes in mutation frequencies were observed in Δ*mutMY* cells treated with 0.7 µg/ml ampicillin when compared to untreated cells. This suggested that oxidative DNA damages were not induced at lower than ½ MIC ampicillin treatment. Mutation frequencies were reduced slightly in cells treated with ampicillin concentrations higher than ½ MIC (1.9 µg/ml and 2.4 µg/ml), although near MIC concentration greatly affected the growth rate and increased the killing effect. I expected that treatments with concentrations higher than ½ MIC ampicillin will induce higher oxidative DNA damages level, however this observation suggested that higher ampicillin concentrations does not induce more oxidative DNA damages, but slightly lower and a constant level. Furthermore, the oxidative

DNA damages level was the highest in cells treated with ½ MIC ampicillin. This shows that there seems to be a threshold in the oxidative DNA damages produced under sublethal ampicillin, which is a unique characteristic.

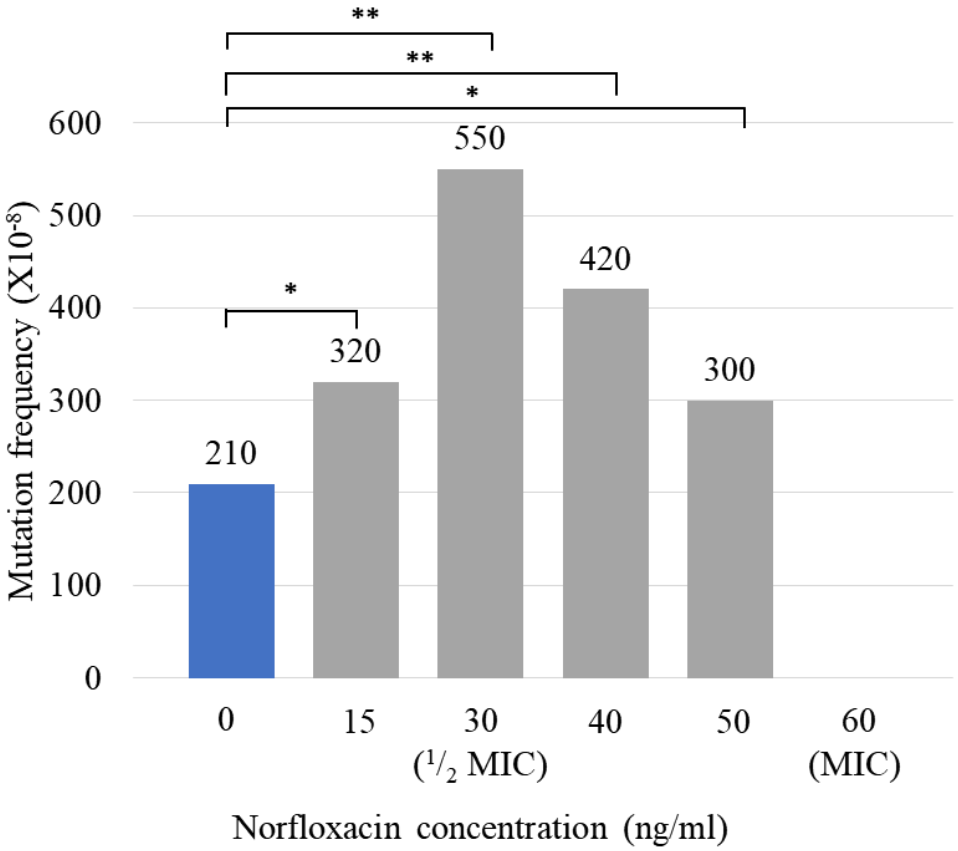

Figure 17. Characteristics of oxidative DNA damages induced by sublethal concentrations of norfloxacin in Δ*mutMY* cells

The mutation frequencies were obtained from 20 populations for each treatment as described in Figure 12. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01. Growth phenotype are shown below.

Treatment	**Norfloxacin concentration (μg/ml)**				
	0	**15**	**30**	**40**	**50**
Growth rate (hrs)	14	14	14	15	16
Colony counts/ population	~100	~100	~100	70-100	~50

There were some similarities with the mutation frequencies in Δ*mutMY* cells treated with sublethal concentrations of ampicillin and norfloxacin. The growth rates were slightly affected in cells treated with higher than ½ MIC norfloxacin concentrations, and greater killing effect was observed in cells treated with near MIC (50 ng/ml). As shown in Figure 17, no significant changes in mutation frequencies were observed in Δ*mutMY* cells treated with 15

ng/ml norfloxacin when compared to untreated cells. This suggested that oxidative DNA damages were not induced at lower than ½ MIC norfloxacin treatment. However, unlike the mutation frequency in cells treated with ampicillin at higher than ½ MIC, mutation frequencies were consistently reduced in cells treated with 40 ng/ml and 50 ng/ml norfloxacin. This observation suggested that norfloxacin at higher than ½ MIC induced lower oxidative DNA damage level. Similar to ampicillin, the oxidative DNA damages level was the highest in cells treated with ½ MIC norfloxacin. This also shows that there seems to be a threshold in the oxidative DNA damages produced under sublethal norfloxacin.

As conclusions, oxidative DNA damages were induced in cells treated with ½ MIC ampicillin and norfloxacin but not gentamicin, suggesting that not all bactericidal antibiotics at sublethal concentration induces oxidative DNA damages. Furthermore, the oxidative DNA damages induced by ½ MIC ampicillin or norfloxacin is caused by OH• production. Further characterization suggests that the level of oxidative DNA damages induced by ampicillin or norfloxacin does not increased when the antibiotics concentrations were increased, and the highest level of oxidative DNA damages induced was at ½ MIC.

Part II. Molecular mechanism of sublethal bactericidal antibiotics induce oxidative DNA damages

In the first part of my work, I confirmed that ½ MIC ampicillin or norfloxacin treatments induced oxidative DNA damages in *E. coli* cells. The next step is to find out a possible molecular mechanism of this phenomenon. In order to cause oxidative DNA damages, OH• must be produced through Fenton reaction (Davies 2000; Cooke et. al., 2003; Evans et. al., 2004). During Fenton reaction, OH• is produced via the reduction of H_2O_2 catalyzed by ferrous iron (Fe^{2+}) (Imlay et al., 1988). Therefore, in order to elucidate this mechanism, I wanted to determine the important factors involved.

1. H_2O_2 level is elevated in cells treated with ½ MIC bactericidal antibiotics treatments

During H_2O_2 stress, OxyR regulon will activate protection mechanism, including scavenger enzymes such as peroxidases and catalases to detoxify H_2O_2 (Zheng et al., 2001). Alkylhydroperoxide reductase (Ahp) is a primary H_2O_2 scavenger in *E. coli* controlled by the OxyR regulon (Seaver & Imlay, 2001). By using the *ahpC-gfp* reporter assay, relative fluorescence level was measured when cells were treated with bactericidal antibiotics, which reflects the intracellular H_2O_2 level. In the first experiment, I wanted to confirm the previous finding in which high concentration of bactericidal antibiotics induce high H_2O_2 level in cells. Here, I treated the wild type cells carrying *ahpC-gfp* reporter plasmid with similar antibiotics concentrations as the previous finding by Dwyer et al., (2014), which were 5 μg/ml ampicillin (two times MIC) and 250 ng/ml norfloxacin (four times MIC). Lethal concentrations of aminoglycosides were reported to affect bioreporter assays, such as GFP and luciferase, due to its mode of action in causing rapid inhibition of protein synthesis (Dwyer et al., 2014; Brown et al., 2012), therefore only lethal dose of ampicillin or norfloxacin were tested in the first experiment.

Figure 18 showed that high fluorescence levels were observed in cells treated with 5 μg/ml ampicillin and 250 ng/ml norfloxacin. Cells treated with ampicillin showed four-fold higher fluorescence level, while norfloxacin treatment showed seven-fold higher fluorescence level compared to untreated cells. This showed that when cells are exposed to lethal concentration of ampicillin or norfloxacin, OxyR activated the H_2O_2 stress response. This result

confirmed the previous finding on treatment of lethal concentration of bactericidal antibiotics, regardless of their specific drug-target interactions, induced high H_2O_2 level in *E. coli* cells.

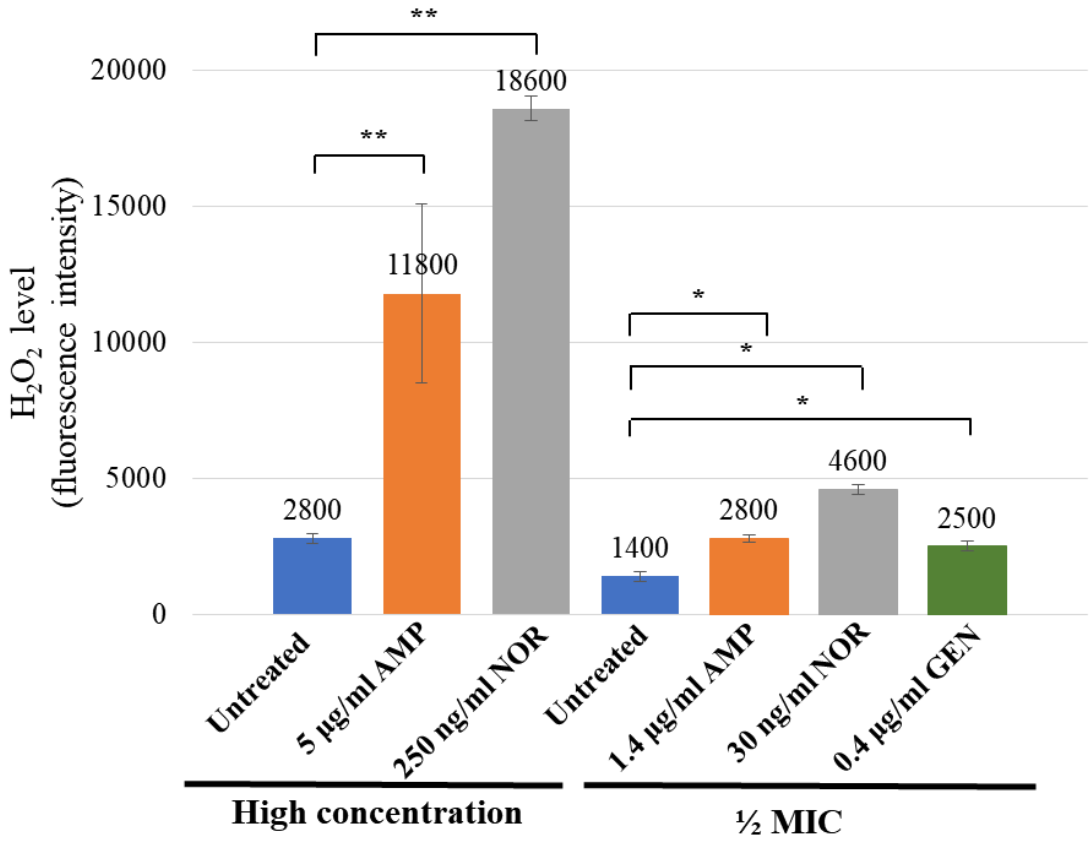

Figure 18. Intracellular H_2O_2 level in wild type cells treated with bactericidal antibiotics.

For treatment with high concentration of antibiotics, liquid culture method was used. Cells were grown in LB medium until OD_{600}: 0.3 and diluted to OD_{600}: 0.1. Antibiotics were added and grown for 90 mins. Sodium azide was added to stop the growth and cells were diluted with PBS buffer. For treatment with ½ MIC antibiotics, cells were grown on plates supplemented with the indicated antibiotics until colony size 1.5 mm in diameter. Then, cells were harvested with 1xM9 salts containing sodium azide. The cells were diluted with PBS buffer and used to measure the fluorescence level. Measurement of expression level of *ahpC*–GFP as intracellular H_2O_2 level by flow cytometer analysis. The expression of *ahpC* gene is induced when the intracellular H_2O_2 level increases, so the intensity of the fluorescence level reflects the intracellular H_2O_2 level. Data was obtained from four independent experiments. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01. (AMP: ampicillin; NOR: norfloxacin; GEN: gentamicin)

Next, I wanted to find out whether low concentration of bactericidal antibiotics induce H_2O_2 in *E. coli* cells. In this experiment, I treated the wild type cells carrying *ahpC-gfp* reporter plasmid with ½ MIC ampicillin, norfloxacin or gentamicin. Also shown in Figure 18, cells treated ½ MIC ampicillin, norfloxacin or gentamicin showed higher fluorescence level when compared to the untreated cells. This showed that the H_2O_2 scavenger gene, *ahpC*, was expressed more in cells treated with ½ MIC bactericidal antibiotics. The result suggested that intracellular H_2O_2 levels were elevated in ½ MIC ampicillin, norfloxacin or gentamicin treated cells.

2. Correlation between H_2O_2 level and oxidative DNA damages induced by sublethal bactericidal antibiotics treatments.

Based on the findings in Figure 12 (Results, Part 1), I showed that treatments with ½ MIC ampicillin and norfloxacin induced oxidative DNA damages, but gentamicin does not. This indicated that not all bactericidal antibiotics, at sublethal concentrations, can induce oxidative DNA damages. In Figure 18, higher H_2O_2 level was observed in ½ MIC gentamicin treated cells. However, oxidative DNA damages was not induced in cells treated with ½ MIC gentamicin, as observed in Figure 12. This observation suggested that even though H_2O_2 level is elevated in sublethal bactericidal antibiotics treated cells, it does not mean that oxidative DNA damages were produced. For further clarification on the correlation between H_2O_2 level and oxidative DNA damages, I determined the H_2O_2 level in cells treated with increasing to near MIC concentrations of ampicillin (1.9 µg/ml and 2.4 µg/ml) using the *ahpC-gfp* reporter assay.

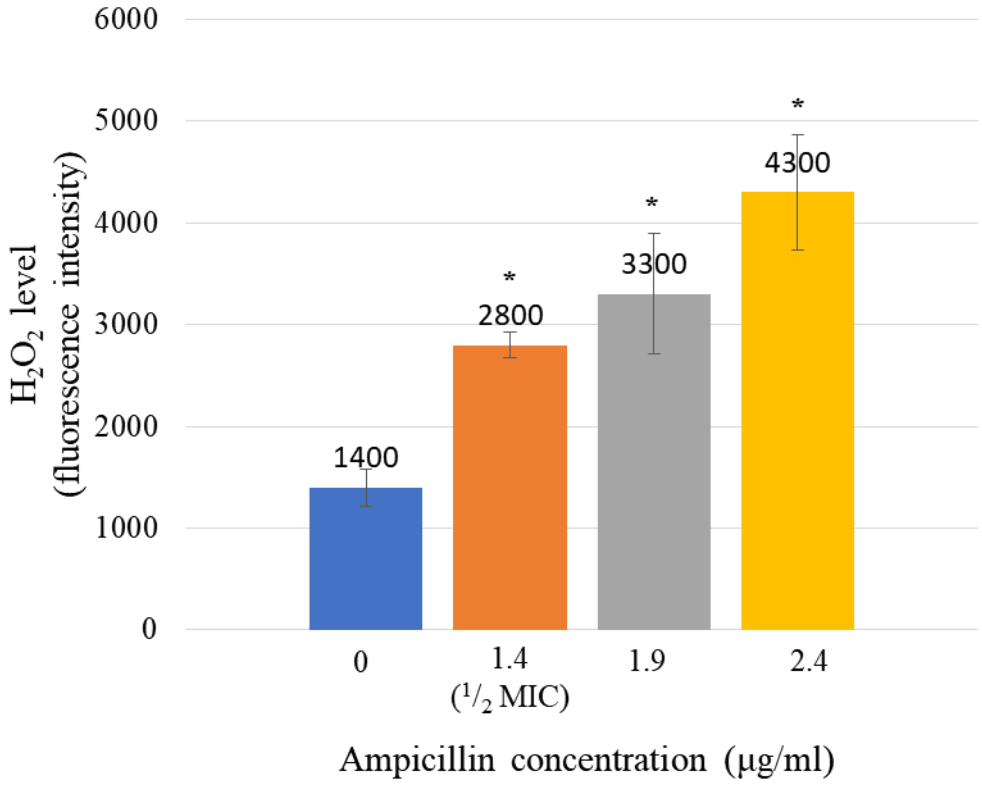

Figure 19. Intracellular H_2O_2 level in wild type cells treated with increasing sublethal concentrations of ampicillin.

Experiment was performed using plate culture method, as explained in Figure 18, except the cells were treated with 1.9 µg/ml or 2.4 µg/ml ampicillin. The data was obtained from four independent experiments. Significance of data: (*) indicates p-value <0.05.

In Figure 19, the fluorescence level continuously increased as the ampicillin concentrations were increased to near MIC. This suggested that treatments with higher ampicillin concentrations continuously elevated the intracellular H_2O_2 level, higher than cells treated with ½ MIC ampicillin. This result also supports the finding in which lethal ampicillin concentration significantly elevated the intracellular H_2O_2 level. Overall, this results suggested that the intracellular H_2O_2 level increases with the increase in ampicillin concentrations.

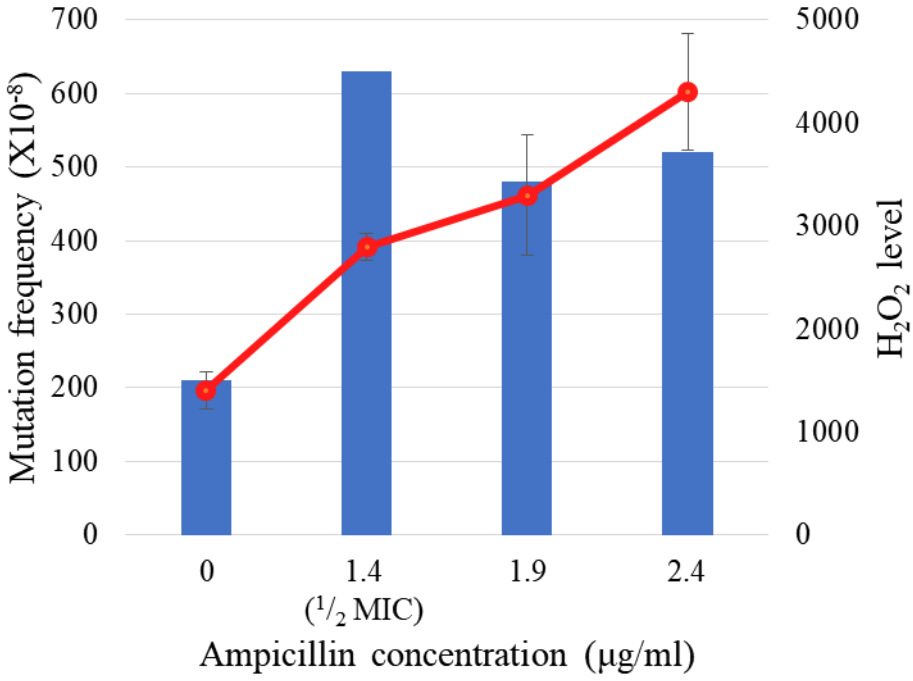

Figure 20. Correlation between intracellular H_2O_2 level and oxidative DNA damages induced by sublethal concentrations of ampicillin.

Taking together the results in Figure 16 and Figure 19, I determined the correlation between intracellular H_2O_2 level and oxidative DNA damages in cells treated with sublethal concentrations of ampicillin. In figure 20, it is clear that the intracellular H_2O_2 level continue to increase when ampicillin concentration was increased, however, the oxidative DNA damages level was the highest in cells treated with ½ MIC ampicillin (1.4 µg/ml) but reduced and remained constant at 1.9 µg/ml and 2.4 µg/ml (near MIC). This results suggested that there were no good correlation between the increase in intracellular H_2O_2 and the oxidative DNA damages level in cells treated with sublethal concentrations of ampicillin.

Previously in our laboratory, a finding showed that there was no direct correlation between high intracellular H_2O_2 level and the increase in oxidative DNA damage level (Nunose, unpublished data). High intracellular H_2O_2 level was observed in *E. coli* cells grown in LB supplemented with 0.2% glucose. However, no significant increase in the oxidative DNA damages level was observed in Δ*mutMY* cells grown on LB with glucose. Here, I utilized a similar condition to determine the effect of high intracellular H_2O_2 level on the oxidative DNA damages level in cells treated with ½ MIC ampicillin or norfloxacin. First, I determined the

intracellular H_2O_2 level in cells treated with ½ MIC ampicillin or norfloxacin grown in LB supplemented with 0.2% glucose.

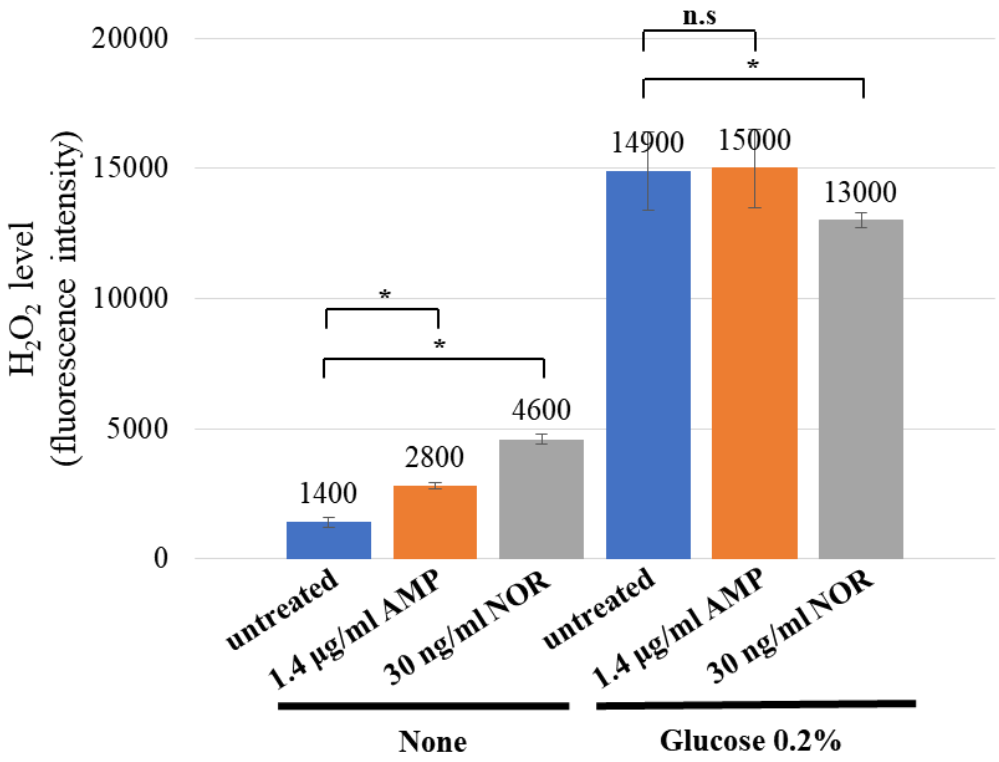

Figure 21. Measurement of intracellular H_2O_2 level in wild type cells grown in LB + glucose 0.2% and treated with ½ MIC ampicillin or norfloxacin

Experiment was performed using plate culture method, as explained in Figure 18. The data was obtained from four independent experiments. Significance of data: (*) indicates p-value <0.05 and ns indicates non-significant. (AMP: ampicillin; NOR: norfloxacin)

As shown in Figure 21, the fluorescence level in cells grown in LB with 0.2% glucose was around 10-fold higher, with or without ½ MIC ampicillin or norfloxacin treatments. The fluorescence level in ½ MIC ampicillin treated cells grown in LB with glucose was five-fold higher compared to cells grown in LB, while the intracellular H_2O_2 level in ½ MIC norfloxacin treated cells grown in LB with glucose was three-fold higher compared to cells grown in LB. This result suggested that the addition of glucose to LB medium greatly increased the intracellular H_2O_2 level, regardless of treatments with ½ MIC ampicillin or norfloxacin. Next, I determined the mutation frequency in Δ*mutMY* cells treated with ½ MIC ampicillin or norfloxacin grown in LB with 0.2% glucose, to see whether oxidative DNA damages change in high intracellular H_2O_2 level condition (Figure 22).

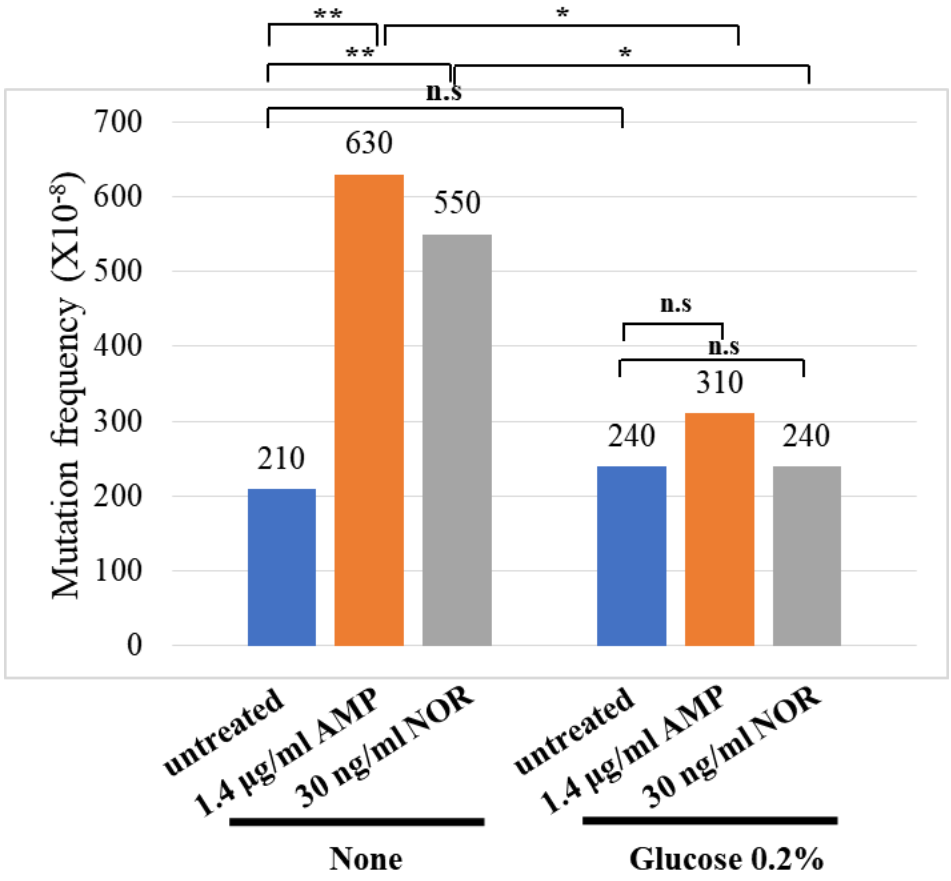

Figure 22. Mutation frequencies in Δ*mutMY* cells treated with ½ MIC ampicillin or norfloxacin grown in LB + glucose 0.2%.

The mutation frequencies were obtained from 20 populations for each treatment as described in Figure 12. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01, ns indicates non-significant. Growth phenotype are shown below.

Treatment	LB			LB + glucose 0.2%		
	Untreated	½ MIC AMP	½ MIC NOR	Untreated	½ MIC AMP	½ MIC NOR
Growth rate (hrs)	14	14	14	13	13	13

(AMP: ampicillin; NOR: norfloxacin)

As shown in Figure 22, the mutation frequency in untreated cells grown in LB or LB with 0.2% glucose does not changed, which confirmed the previous finding by Nunose in which the oxidative DNA damages level was not changed in LB with 0.2% glucose even though the intracellular H_2O_2 level was greatly elevated. Surprisingly, the mutation frequency in cells treated with ½ MIC ampicillin or norfloxacin grown in LB with glucose was completely suppressed to the untreated level. This observation suggested that the antibiotics effect on the oxidative DNA damages was suppressed by the addition of glucose.

Most importantly, this line of evidences suggested that the high intracellular H_2O_2 does not induce more oxidative DNA damages, thus intracellular H_2O_2 level is not the limiting factor in this mechanism. Although the presence of H_2O_2 is required, but it is not the only factor to be consider in order to determine whether oxidative DNA damages are being produce or not. As we all know, besides the presence of H_2O_2, the availability of free Fe^{2+} must be present in order for Fenton reaction to occur to produce OH• (Haber & Weiss, 1934). Since I found that the intracellular H_2O_2 level is not the limiting factor in the oxidative DNA damages induced in cells treated with sublethal concentrations of ampicillin or norfloxacin, I wanted to find out whether the intracellular free Fe^{2+} is the limiting factor in this mechanism.

3. Intracellular free iron as the limiting factor in the oxidative DNA damages induced by ½ MIC ampicillin or norfloxacin treatments

Hydroxyl radical formation is an important component in producing oxidative DNA damages. Two important factors, H_2O_2 and free Fe^{2+} , are needed for Fenton reaction to occur in order to produce OH•. Previously, I showed that intracellular H_2O_2 level does not affect the oxidative DNA damages in cells treated with sublethal concentrations of ampicillin or norfloxacin. So, I wanted to find out whether the intracellular free Fe^{2+} concentration is the main component and the limiting factor in this mechanism. In order to test this, I determined the mutation frequency in Δ*mutMY* cells treated with ½ MIC ampicillin or norfloxacin, in addition with an intracellular Fe^{2+} chelator 2,2'-bipyridyl (Figure 23). 2,2'-bipyridyl is a membrane-permeant, intracellular Fe^{2+} chelator that is commonly used to sequester free iron in cells (Hantke, 1981; Romeo et al., 2001; Kohanski et al., 2007). As shown in Figure 23, 2,2'-bipyridyl works by forming a three molecules complex with the Fe^{2+} ion.

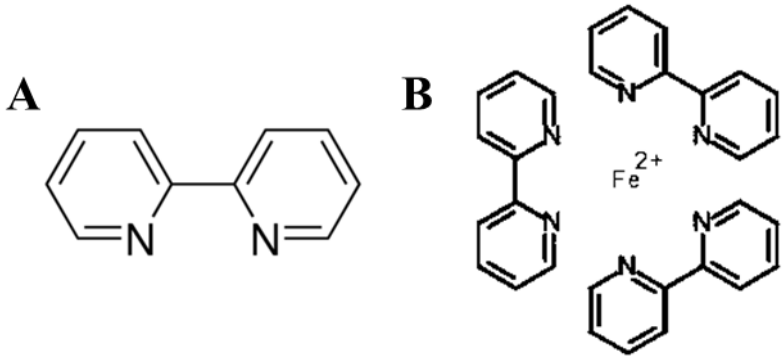

Figure 23. Structure of 2,2'-bipyridyl (A) and Fe^{2+} chelation (B)

A) 2,2'-bipyridyl is an organic compound with the formula $C_{10}H_8N_2$ B) Three bipyridyl molecules coordinate to Fe^{2+}, forming a complex.

As shown in Figure 24, the mutation frequency in untreated Δ*mutMY* cells with or without the addition of bipyridyl does not change. Cells treated with bipyridyl seems to take slightly longer time to reach 1.5 mm in size. However, bipyridyl completely suppressed the mutation frequency in cells treated with ½ MIC ampicillin and reduced the mutation frequency in cells treated with ½ MIC norfloxacin. This result suggested that exposure to ½ MIC ampicillin or norfloxacin increased the intracellular Fe^{2+} concentration. The increase in intracellular Fe^{2+} concentration is important to induce OH• formation, thus promoting oxidative DNA damages in cells treated with ½ MIC ampicillin or norfloxacin.

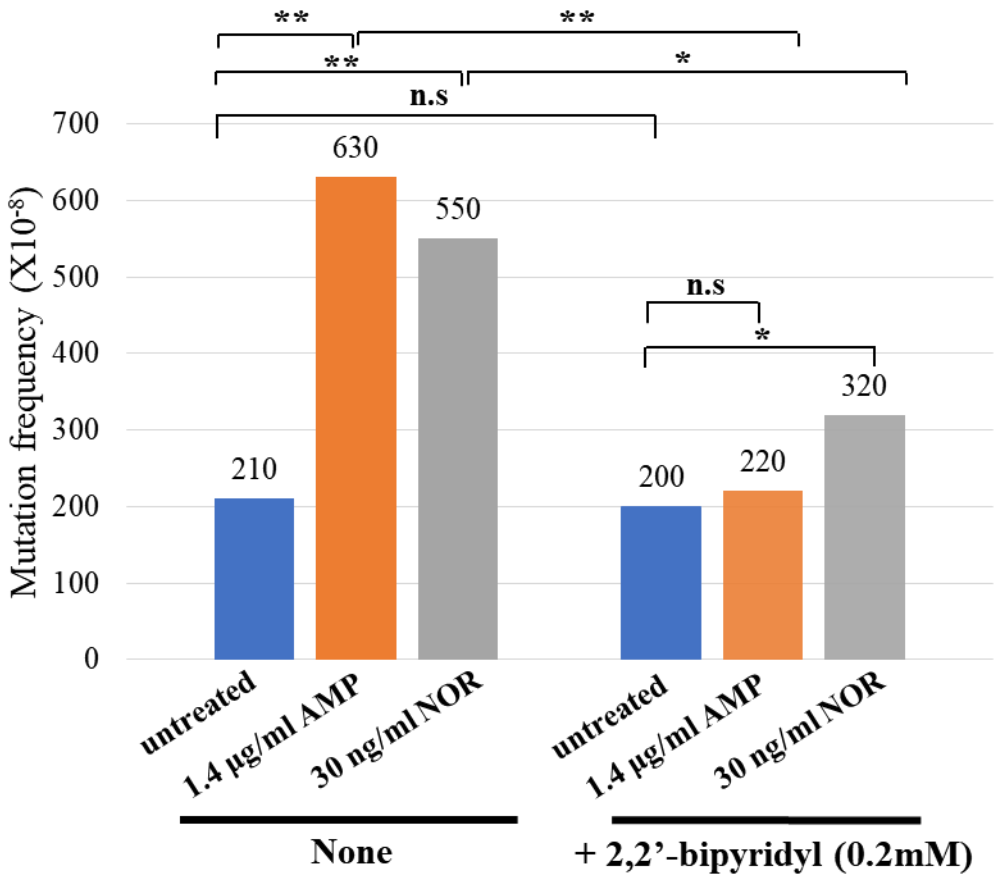

Figure 24. Mutation frequencies in Δ*mutMY* cells treated with ½ MIC ampicillin or norfloxacin in the presence of 2,2'-bipyridyl

The mutation frequencies were obtained from 20 populations for each treatment as described in Figure 12. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01, ns indicates non-significant. Growth rate is shown below.

Treatment	LB			LB + BP		
	Untreated	½ MIC AMP	½ MIC NOR	Untreated	½ MIC AMP	½ MIC NOR
Growth rate (hrs)	14	14	14	15	17	17

(AMP: ampicillin; NOR: norfloxacin; BP: 2,2'-bipyridyl)

Next, I wanted to determine the possible sources of Fe^{2+} that were involved in this mechanism. The Fe^{2+} required for OH• formation could come from intracellular sources, such as iron storage, or from extracellular sources, such as iron import. First, I wanted to determine whether the Fe^{2+} comes from the iron storage. One of the most important iron storage protein is OxyR-controlled Dps, in which the primary role is to store iron and protect the DNA against Fe^{2+} and H_2O_2 action in the production of OH• (Zhao et al., 2002). Recently, YaaA protein of the OxyR regulon have been reported to suppress intracellular free Fe^{2+} concentration in *E. coli* cells (Liu et al., 2011). In our lab, we found that the deletion of both *dps* and *yaaA* gene

increased the mutation frequency by seven-fold, suggesting that both Dps and YaaA work in complementary pathway to suppress intracellular free Fe^{2+} level, thus suppressing oxidative DNA damages (Yeo, unpublished). Therefore, I determined the mutation frequency in *E. coli* strain lacking both Dps and YaaA in the Δ*mutMY* background (Δ*mutMY* Δ*yaaA* Δ*dps*) treated with ½ MIC ampicillin or norfloxacin.

As shown in Figure 25, the growth rate was affected in the absence of YaaA and Dps, in which the untreated cells took around 17 hours, and longer for antibiotics treated cells, to reach 1.5 mm in size. The mutation frequency in untreated Δ*mutMY* Δ*yaaA* Δ*dps* cells increased by four-fold compared to the untreated Δ*mutMY* cells. The increase in the mutation frequency suggested that more oxidative DNA damages were produced in the absence of Dps and YaaA because the cells lose the functions to suppress the intracellular free Fe^{2+} level. Further increase in the mutation frequency was observed in Δ*mutMY* Δ*yaaA* Δ*dps* cells treated with ½ MIC ampicillin or norfloxacin when compared with untreated Δ*mutMY* Δ*yaaA* Δ*dps* cells. This observation suggested that the source of free Fe^{2+} required to produce OH• in ½ MIC ampicillin or norfloxacin treated cells may not come from the iron storage Dps or regulated by YaaA, since the level of oxidative DNA damages were higher in antibiotics treated cells. In addition, the actual increase in the mutation frequency was 4×10^{-6} in Δ*mutMY* cells treated with ½ MIC ampicillin, while the increasement was double the number in Δ*mutMY* Δ*yaaA* Δ*dps* cells, at 8×10^{-6}. A more striking difference was observed in ½ MIC norfloxacin treated cells. In Δ*mutMY* cells, the actual increase in mutation frequency was 3×10^{-6}, while the increasement was four-fold the number in Δ*mutMY* Δ*yaaA* Δ*dps* cells, at 1.3×10^{-5}. It showed that the increase in the oxidative DNA damages level was not just an additive effect of ampicillin or norfloxacin treatment in Δ*mutMY* Δ*yaaA* Δ*dps*, but might also be a synergistic effect to the condition in which the free iron capture and regulation mechanism was hampered.

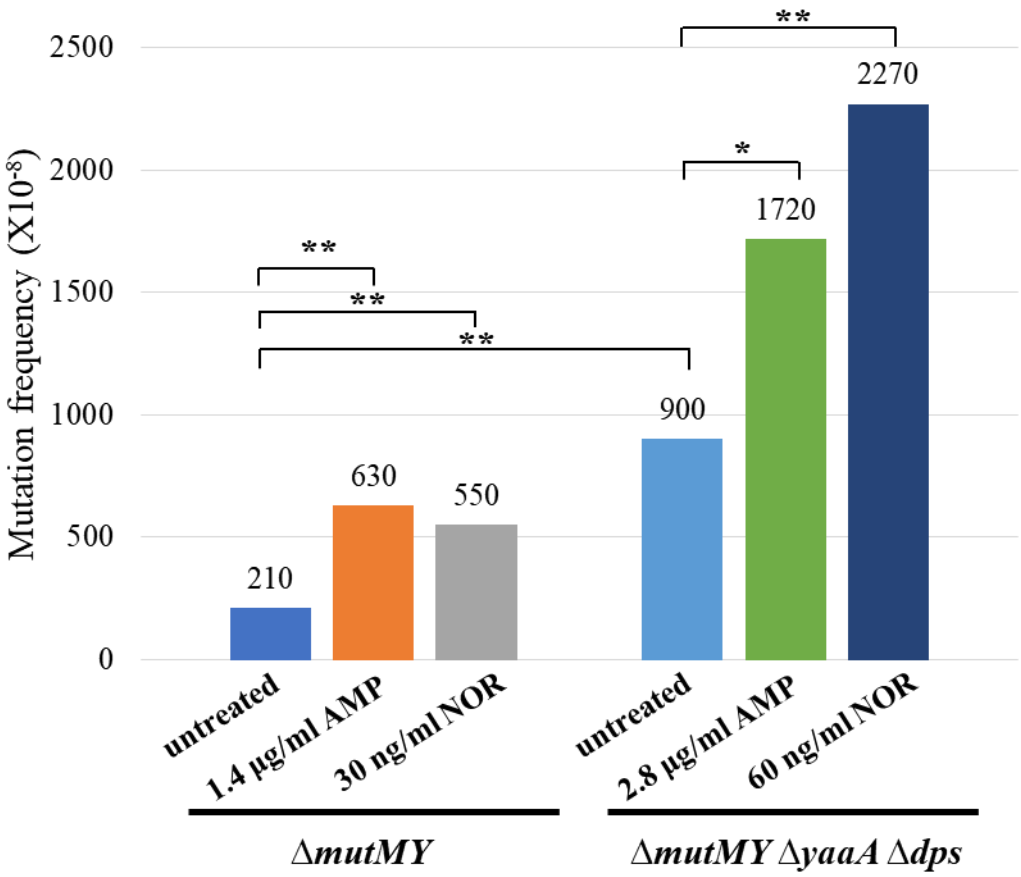

Figure 25. Mutation frequencies in Δ*mutMY* and Δ*mutMY* Δ*yaaA* Δ*dps* cells treated with ½ MIC ampicillin or norfloxacin

The mutation frequencies were obtained from 20 populations for each treatment as described in Figure 12. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01. Growth rate is shown below.

Strain	**Δ*mutMY***			**Δ*mutMY* Δ*yaaA* Δ*dps***		
Treatment	**Untreated**	**½ MIC AMP**	**½ MIC NOR**	**Untreated**	**½ MIC AMP**	**½ MIC NOR**
Growth rate (hrs)	14	14	14	17	18	19

(AMP: ampicillin; NOR: norfloxacin)

Based on my results in Figure 25, the source of free Fe^{2+} in the mechanism of oxidative DNA damages induced by sublethal concentrations of ampicillin or norfloxacin may not be from intracellular sources and is still unclear. It is possible that the increase in free Fe^{2+} during ampicillin or norfloxacin treatment comes from extracellular sources through iron import system. The main iron import system in an oxygen-rich condition is the siderophore-based iron uptake system fueled by the energy-transducing TonB system, which is repressed by Fur under the OxyR system (Postle, 1993). This system involves active transport of siderophore-Fe^{3+} complex from outer environment into the cytoplasm. The key player in this system is the

periplasm-span TonB protein which transduces energy from the cytoplasmic membrane's proton motive force to the outer membrane receptors and facilitates siderophore- Fe^{3+} transport into the periplasm (Ferguson & Deisenhofer, 2004). Here, I determined the mutation frequency in the strain lacking TonB in the Δ*mutMY* background (Δ*mutMY* Δ*tonB*) treated with ½ MIC ampicillin or norfloxacin.

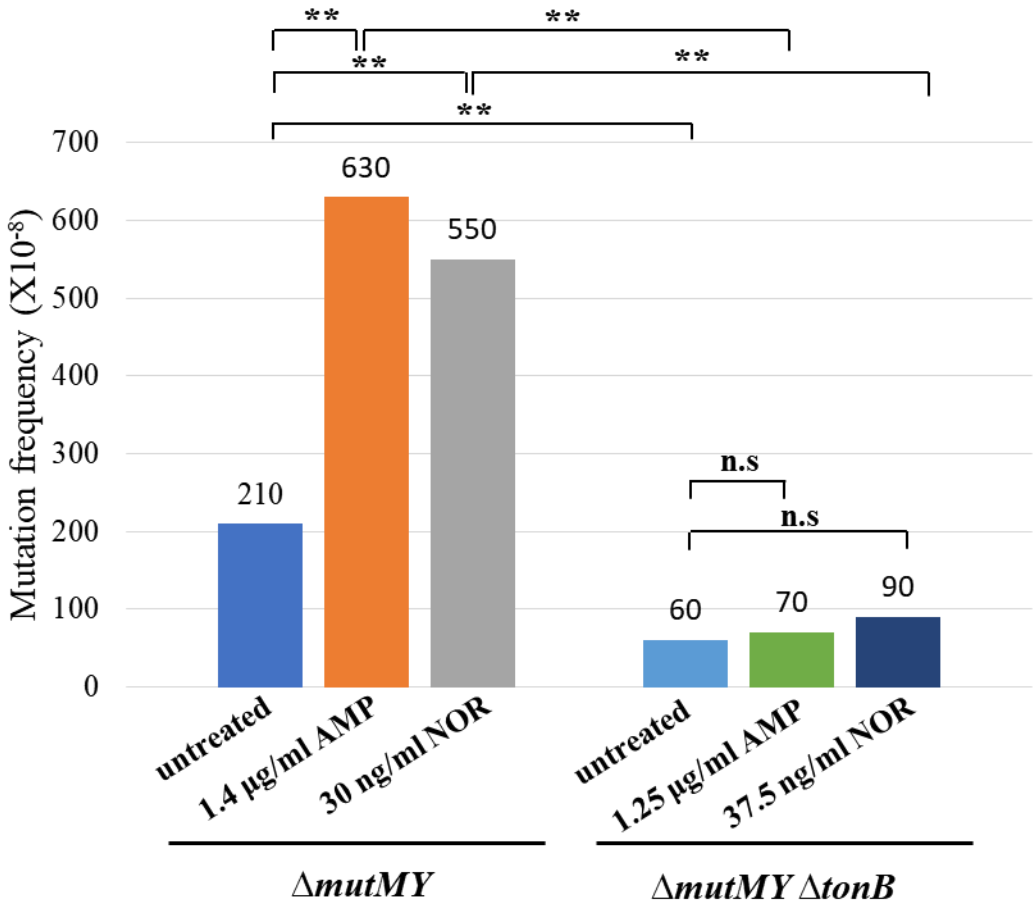

Figure 26. Mutation frequencies in Δ*mutMY* and Δ*mutMY* Δ*tonB* cells treated with ½ MIC ampicillin or norfloxacin.

The mutation frequencies were obtained from 20 populations for each treatment as described in Figure 12. Significance of data: (**) indicates p-value <0.01, ns indicates non-significant. Growth rate is shown below.

Strain	**Δ*mutMY***			**Δ*mutMY* Δ*tonB***		
Treatment	**Untreated**	**½ MIC AMP**	**½ MIC NOR**	**Untreated**	**½ MIC AMP**	**½ MIC NOR**
Growth rate (hrs)	14	14	14	20	22	23

(AMP: ampicillin; NOR: norfloxacin)

Based on Figure 26, the deletion of tonB greatly affected the growth rate, in which untreated cells took 20 hours, and antibiotics treated cells took 22 to 23 hours to reach 1.5 mm

in size. The mutation frequency in untreated Δ*mutMY* Δ*tonB* cells showed about three-fold reduction compared to the untreated Δ*mutMY* cells. This suggested that the absence of TonB prevented the iron uptake, thus reducing OH• production which helps to lower the oxidative DNA damages level. No significant changes were observed in the mutation frequency Δ*mutMY* Δ*tonB* cells treated with ½ MIC ampicillin or norfloxacin when compared to the untreated Δ*mutMY* Δ*tonB.* The oxidative DNA damages induced by ½ MIC ampicillin or norfloxacin treatments were completely suppressed in absence of TonB. This result suggested that ½ MIC ampicillin and norfloxacin effect depends on TonB function in iron uptake to increase the intracellular Fe^{2+} concentration to induce oxidative DNA damages.

As a conclusion, H_2O_2 level does not affect the oxidative DNA damages level in cells treated with sublethal concentrations of ampicillin or norfloxacin. The key component and the limiting factor in this mechanism is the intracellular free Fe^{2+} concentration and most importantly, the TonB-dependent iron uptake plays a crucial role in increasing the free Fe^{2+} concentration in cells treated with sublethal concentrations of ampicillin or norfloxacin.

Part III. Involvement of CpxA and MazF in oxidative DNA damages induced by sublethal concentrations of ampicillin

It was proposed that there is a common mechanism of bactericidal antibiotics-induced cell-killing that involves metabolic changes, the production of ROS which leads to cellular damage and cell death (Kohanski et al., 2007; Dwyer et al., 2014). The metabolic changes are thought to be caused by the activation CpxA, the envelope stress response two-component sensor, which then activated the ArcA redox-response two component system (Kohanski et al., 2008). It was also suggested that the toxin-antitoxin MazEF system was also activated upon exposure to lethal stress, such as lethal bactericidal antibiotics doses or hydroxyurea treatment (Kolodkin-Gal, 2008; Davies et al., 2009). As summarized in Figure 27, the production of toxin MazF may cleaved mRNAs, causes misfolded or truncated peptides that are inserted into the membrane, which then activates CpxA and ArcA systems (Wu et al., 2011). The activation of CpxA and ArcA may stimulates the TCA cycle and hyperactivation of the electron transport chain, which leads to elevated ROS levels (Kohanski et al., 2007; Belenky, 2015).

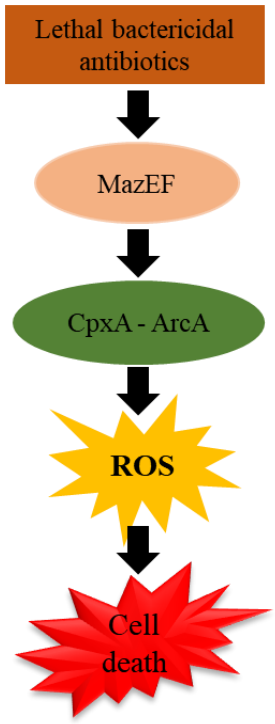

Figure 27. Hypothesized model on lethal stress, such as bactericidal antibiotics, induced cell-killing through ROS production.

Here, I wanted to examine whether similar model or some of the component can be applied to the possible mechanism of oxidative DNA damages induced by sublethal ampicillin or norfloxacin. Although the two conditions are different, one involving low bactericidal antibiotics concentration while the other involves lethal concentrations, it is possible that similar components might involve directly or indirectly. I carried the mutation frequency assay using *cpxA* deletion and *mazF* deletion in the Δ*mutMY* background, treated with ½ MIC ampicillin.

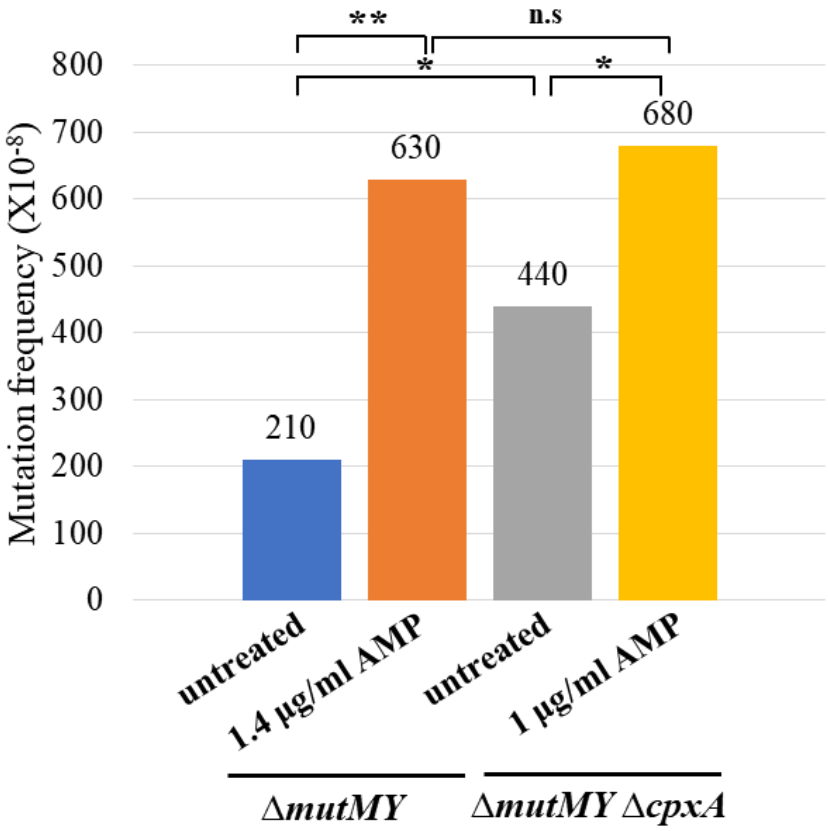

Figure 28. Mutation frequencies in Δ*mutMY* and Δ*mutMY* Δ*cpxA* cells treated with ½ MIC ampicillin

The mutation frequencies were obtained from 20 populations for each treatment as described in Figure 12. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01, ns indicates non-significant. Growth rate is shown below.

Strain	**Δ*mutMY***		**Δ*mutMY* Δ*cpxA***	
Treatment	**Untreated**	**½ MIC AMP**	**Untreated**	**½ MIC AMP**
Growth rate (hrs)	14	14	15	16

(AMP: ampicillin)

First of all, I wanted to examine the involvement of the envelope stress response two-component sensor CpxA in ½ MIC ampicillin induced oxidative DNA damages. The mutation frequency was determined in Δ*mutMY* Δ*cpxA* with or without ampicillin treatment. Deletion of *cpxA* slightly affected the growth rate by one to two hours. As shown in Figure 28, the untreated Δ*mutMY* Δ*cpxA* cells showed two-fold increase in the mutation frequency when compared to the untreated Δ*mutMY* cells. This result suggested that CpxA has a protective effect from oxidative DNA damages, in which the absence of CpxA increased the oxidative DNA damages level in the untreated condition. However, the presence of CpxA does not suppress the oxidative DNA damages in Δ*mutMY* cells treated with ½ MIC ampicillin. Furthermore, the cells treated with ampicillin in both strains showed similar oxidative DNA damages level. If CpxA is directly involved in the oxidative DNA damages induced by ½ MIC ampicillin, I expected to see a complete suppression in oxidative DNA damages in Δ*mutMY* Δ*cpxA* cells treated with ampicillin. However, Δ*mutMY* Δ*cpxA* cells treated with ½ MIC ampicillin still showed higher oxidative DNA damages. This result suggested that CpxA is not required to induce oxidative DNA damages in ½ MIC ampicillin treated cells.

Next, I wanted to examine the involvement of the toxin-antitoxin system MazEF in ½ MIC ampicillin induced oxidative DNA damages. The mutation frequency was determined in Δ*mutMY* Δ*mazF* with or without ampicillin treatment. Similar to the deletion of *cpxA*, deletion of *mazF* slightly affected the growth rate by one to two hours. As shown in Figure 29, the untreated Δ*mutMY* Δ*mazF* cells showed less than two-fold but significant increase in the mutation frequency when compared to the untreated Δ*mutMY* cells. Δ*mutMY* Δ*mazF* cells treated with ampicillin showed a slightly reduced mutation frequency when compared to Δ*mutMY* cells treated with ampicillin, but still significantly higher than the untreated Δ*mutMY* Δ*mazF* cells. This observation was quite similar to CpxA, in which the presence of MazF showed a slight protective effect against oxidative DNA damages during normal condition without antibiotics treatment as observed in the untreated cells. If MazF is required for oxidative DNA damages induced by ½ MIC ampicillin, the absence of MazF will completely suppressed the oxidative DNA damages in ampicillin treated cells. However, similar to CpxA, oxidative DNA damages were still induced by ½ MIC ampicillin treatment even in the absence of MazF. This result suggested that MazF is not required to induce oxidative DNA damages in ½ MIC ampicillin treated cells.

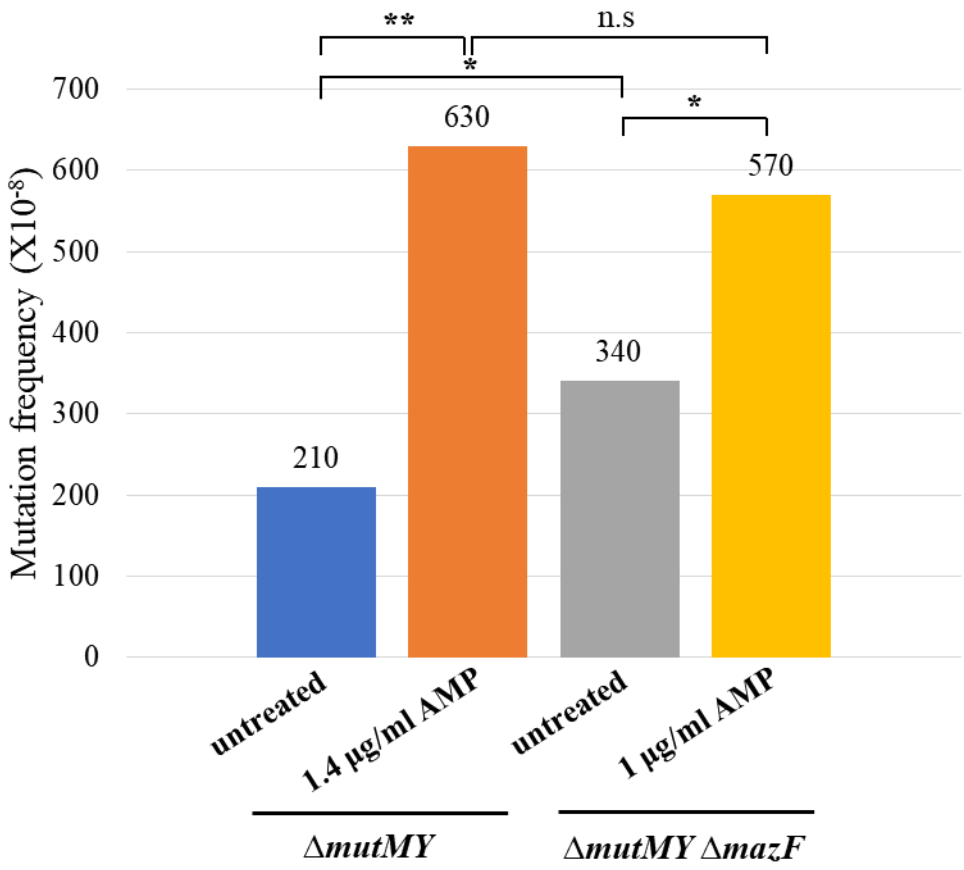

Figure 29. Mutation frequencies in Δ*mutMY* and Δ*mutMY* Δ*mazF* cells treated with ½ MIC ampicillin

The mutation frequencies were obtained from 20 populations for each treatment as described in Figure 12. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01, ns indicates non-significant. Growth rate is shown below.

Strain	**Δ*mutMY***		**Δ*mutMY* Δ*mazF***	
Treatment	**Untreated**	**½ MIC AMP**	**Untreated**	**½ MIC AMP**
Growth rate (hrs)	14	14	15	16

(AMP: ampicillin)

Taking together all findings, it seems like both CpxA and MazF are not required in ½ MIC ampicillin induced oxidative DNA damages. This suggests that some components in the previously hypothesized model on lethal concentrations of bactericidal antibiotics induced ROS may not be applicable with the sublethal concentrations of bactericidal antibiotics induced oxidative DNA damages. In addition, the complexities of MazF and CpxA actions and their roles in oxidative DNA damages protection in normal condition or sublethal bactericidal-induced stress condition are yet to be solved.

Chapter 4. Discussions

Part I. Sublethal concentrations of bactericidal antibiotics induced oxidative DNA damages

1. ½ MIC ampicillin and norfloxacin treatments induced oxidative DNA damages

A new idea on sublethal bactericidal antibiotics induced OH•-mediated mutagenesis in *E. coli* have been proposed (Kohanski et al., 2010). However, due to the lack of clear evidences, further clarification is much needed to prove and show the actual level of OH•-mediated oxidative DNA damages in cells treated with sublethal concentrations of bactericidal antibiotics. Researchers have been utilizing chemical methods, such as fluorescent dyes/ probes, to detect OH• and estimate oxidative DNA damages (Davies et al., 2009; Kohanski et al., 2010). However, several studies showed that the fluorescent dyes are non-specific, reacting with most ROS not only OH• and redox metals (Kalyanaraman et al., 2012; Keren et al., 2012; Liu & Imlay, 2013). Therefore, by utilizing a more reliable genetic approach, I clearly observed the effect of sublethal concentrations of bactericidal antibiotics on the oxidative DNA damages level in *E. coli* cells.

By using the rifampicin resistant mutation assay and *ΔmutMY* strain, the frequency of mutations caused by 8-oxoG which represents for oxidative DNA damages was examined in cells treated with sublethal concentrations of bactericidal antibiotics. I showed that out of the three bactericidal antibiotics used in this study, ampicillin (β-lactam) and norfloxacin (fluoroquinolone) treatments at ½ MIC significantly increased oxidative DNA damages level in *ΔmutMY* cells. The addition of potent OH• scavenger thiourea significantly suppressed the oxidative DNA damages induced in *ΔmutMY* cells treated with ½ MIC ampicillin and norfloxacin. These results confirmed that ½ MIC ampicillin and norfloxacin induced oxidative DNA damages through OH• formation. However, no significant increase in oxidative DNA damages level in *ΔmutMY* cells treated with ½ MIC gentamicin (aminoglycoside), suggesting that not all bactericidal antibiotics from different classes induce oxidative DNA damages. This result is in contrast with the proposed hypothesis that all three main bactericidal antibiotic classes, at ½ MIC, induced OH•-mediated mutagenesis (Kohanski et al., 2010). Furthermore, wild type cells treated with ½ MIC gentamicin does not show significant increase in rifampicin resistant mutant, suggesting that gentamicin is less likely to induce mutations at ½ MIC in *E.*

coli. Small increase in rifampicin resistant frequency were observed in wild type cells treated with ½ MIC ampicillin and ½ MIC norfloxacin, but 100-fold lower than the increase in *ΔmutMY*. In the wild type strain, MutM and MutY are present to suppress 8-oxoG-related mutations (Fowler et al., 2003), suggesting that the increase in the mutation frequency was not caused by oxidative DNA damages but might arise from other types of mutations. I also showed treatments with bacteriostatic antibiotics, tetracycline and chloramphenicol, does not induce oxidative DNA damages at ½ MIC.

2. Characteristics of oxidative DNA damages induced by sublethal concentrations of ampicillin and norfloxacin

Kohanski et al. (2010) also reported that treatment with near MIC concentration of norfloxacin increased the mutation rate in wild type *E. coli*, which was correlated with higher increase in HPF fluorescent dye intensity, suggesting that higher concentration of norfloxacin induced higher OH• formation, thus promoting more mutations. Different from their observation, I found that increasing the concentrations of ampicillin or norfloxacin to near MIC does not further increase the oxidative DNA damages level in *ΔmutMY* cells. Furthermore, oxidative DNA damages level was the highest at ½ MIC ampicillin or norfloxacin treatments. Unique characteristics were observed, in which the oxidative DNA damages in cells treated with higher sublethal concentrations of ampicillin (1.9 and 2.4 μg/ml) was constant but lower than that of ½ MIC while the oxidative DNA damages induced by higher sublethal concentrations of norfloxacin (40 and 50 ng/ml) continuously decreased. It indicated that oxidative DNA damages induced by sublethal concentrations of ampicillin and norfloxacin is threshold-based. This finding might also suggest that high level of oxidative DNA damages might not be a main factor contributing to bactericidal antibiotics-induced ROS lethality as previously proposed (Kohanski et al., 2007, Dwyer et al., 2014). The highest level of oxidative DNA damages was observed at ½ MIC treatments, suggesting that presence of ampicillin or norfloxacin at ½ MIC in nature is dangerous as bacteria can acquire mutations through the induced oxidative DNA damages that might be beneficial for adaptation and resistance (Andersson & Hughes, 2014).

Part II. Molecular mechanism for induction of oxidative DNA damages by sublethal bactericidal antibiotics induce

1. Intracellular H_2O_2 level does not affect the oxidative DNA damages level in cells treated with sublethal concentrations of ampicillin and norfloxacin

Hydroxyl radical (OH•) is produced when H_2O_2 oxidizes free Fe^{2+} in the Fenton reaction (Imlay et al., 1988). Unfortunately, it seems impossible and very difficult to detect the intracellular OH• because of its unstable characteristic in cells (Aruoma, 1998). However, H_2O_2 is less reactive and stable, I can easily determine the intracellular level of H_2O_2 by a flow cytometry method using the *ahpC*-gfp reporter assay. The expression of *ahpC-gfp* fusion gene has been shown to be 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. Treatments with ½ MIC ampicillin, norfloxacin or gentamicin significantly increased the H_2O_2 level, suggesting the cells exposed to these antibiotics at ½ MIC produced more H_2O_2. Although the H_2O_2 level increased in cells treated with ½ MIC gentamicin, the oxidative DNA damages level was not changed compared to the untreated cells. Furthermore, treatments with higher than ½ MIC and near MIC concentrations of ampicillin did not induced more oxidative DNA damages compared to ½ MIC treatment, even though the H_2O_2 levels were higher than that of ½ MIC. This shows that the level of intracellular H_2O_2 does not affect the oxidative DNA damages in cells treated with sublethal concentrations of bactericidal antibiotics.

It was found in our laboratory that cells grown on LB supplemented with glucose produced a very high level of H_2O_2 compared to those grown on only LB medium however oxidative DNA damages level was not changed. This observation also suggests that H_2O_2 level does not affect oxidative DNA damages level. To check whether high H_2O_2 level increases the oxidative DNA damages level in antibiotic treated cells, I examined the H_2O_2 level and oxidative DNA damages level in cells grown on LB glucose treated with ½ MIC ampicillin or norfloxacin. Using the *ahpC-gfp* reporter assay, I found that cells grown in LB glucose with or without antibiotics produced very high level of H_2O_2. However, I could not see any significant changes in the oxidative DNA damages in cells grown in LB glucose with or without antibiotics. This results further suggest that H_2O_2 level, although very high, does not affect the oxidative DNA damages level. Surprisingly, addition of glucose completely suppressed the oxidative DNA damages in ½ MIC ampicillin or norfloxacin treated cells. One possible explanation is that in response to the greatly elevated intracellular H_2O_2 level in cells grown in LB glucose,

the OxyR transcription factor may activates the expression of antioxidants including ahpCF and iron regulation genes to protect cells from oxidative stress and collectively suppressed the oxidative DNA damages in cells treated with antibiotics (Zheng et al., 1999; Aslund et al., 1999). It has been reported that glucose, lactose or sucrose addition to LB medium significantly increased *ahpCF* and *dps* inductions while *ΔoxyR* suppressed the induction of both genes, suggesting that glucose and other carbohydrates induced the OxyR regulons (Rothe et al., 2012).

2. Intracellular free Fe^{2+} concentration is a limiting factor in oxidative DNA damages induced in cells treated with ½ MIC ampicillin and norfloxacin

The presence of H_2O_2 is required to form OH• but is not a limiting factor in the oxidative DNA damages induced by ½ MIC ampicillin and norfloxacin. In the Fenton reaction, free Fe^{2+} is also required. It seems likely that intracellular Fe^{2+} concentration is important to limit OH• formation and oxidative DNA damages in cells treated with ½ MIC ampicillin and norfloxacin. Addition of 2,2'-bipyridyl, a free Fe^{2+} chelator, suppressed the oxidative DNA damages in cells treated with ½ MIC ampicillin or norfloxacin, while the untreated cells grown with or without 2,2'-bipyridyl exhibited similar oxidative DNA damages level. This observation suggested that free Fe^{2+} concentration was increased in cells treated with ½ MIC ampicillin and norfloxacin, thus promoting oxidative DNA damages production. However, in order to clarify that free Fe^{2+} concentration is indeed increase, measurement of intracellular free Fe^{2+} needs to be carried out. A method to specifically measure intracellular free Fe^{2+} was described by Keyer & Imlay (1996) by using electron paramagnetic resonance (EPR) analysis. Unfortunately, it is not possible to carry out the analysis at this current moment due to the lack of facility and tools. The free Fe^{2+} required for OH• formation could originate from intracellular sources, such as iron storage proteins, or extracellular sources, such as iron import. In *E. coli*, the OxyR regulon controls the primary iron regulator genes, which typically regulates iron homeostasis based on the intracellular iron availability.

Recently in our laboratory, it was found that the deletion of both *dps* and *yaaA* gene significantly increased oxidative DNA damages, suggesting that both Dps (iron storage protein) and YaaA (intracellular iron regulation) work in different pathways to suppress intracellular free Fe^{2+} level. When exposed to ½ MIC ampicillin or norfloxacin, significant increase in oxidative DNA damages levels was still observed suggesting that the source of Fe^{2+}

may not come from intracellular sources. Furthermore, the results also suggests that the increase in the oxidative DNA damages levels was not just an additive effect of ampicillin or norfloxacin treatment in Δ*mutMY* Δ*yaaA* Δ*dps*, but might also be a synergistic effect to the condition in which the free Fe^{2+} capture and regulation mechanism were hampered. It is possible that if the free Fe^{2+} level in cells treated with ½ MIC ampicillin and norfloxacin increases through extracellular sources, such as iron import, the intracellular iron overload due to iron uptake cannot be controlled in the absence of Dps and YaaA, which reflected the higher relative oxidative DNA damages observed in antibiotics treated *ΔmutMY ΔyaaA Δdps* cells when compared to antibiotics treated *ΔmutMY* cells.

Using *ΔmutMY ΔtonB*, I showed that the oxidative DNA damages level in untreated cells was significantly suppressed compared to *ΔmutMY*, and the oxidative DNA damages in ½ MIC ampicillin or norfloxacin treated cells were also significantly suppressed to the untreated level. These results suggested that TonB function is required for ½ MIC ampicillin and norfloxacin induced oxidative DNA damages. TonB protein functions as an energy transducer to the OM receptors to transport Fe^{3+} into the cytoplasm (Ferguson & Deisenhofer, 2004). Thus, the absence of TonB prevented iron uptake, reducing OH• production and suppresses the oxidative DNA damages, as observed. The result suggests that the source of free Fe^{2+} required for OH• formation under ½ MIC ampicillin and norfloxacin treatments might originate from TonB iron uptake system. This result is different from Kohanski et al. (2007)'s work, in which intracellular iron-containing proteins are the source of Fe^{2+} required for stimulation of Fenton reaction in their proposed cell-killing mechanism, while deletion of *tonB* does not show protective effect against lethal ampicillin and norfloxacin induced cell-killing. It also suggests that different stress level, lethal or sublethal stress, perturbs iron homeostasis differently. Overall, the results suggest the importance of intracellular free Fe^{2+} concentration as a limiting factor in ½ MIC ampicillin and norfloxacin induced oxidative DNA damages.

3. Hypothesis on ½ MIC ampicillin and norfloxacin induced oxidative DNA damages in *E. coli* cells

Overall, I showed that ampicillin and norfloxacin, at ½ MIC, induced oxidative DNA damages in *E. coli*, through OH• formation via Fenton reaction. I found that although intracellular H_2O_2 level was increased, it does not affect the oxidative DNA damages level in treated cells. Then, further investigations indicated that intracellular free Fe^{2+} concentrations were increased in cells treated with ½ MIC ampicillin and norfloxacin, thus suggesting that the intracellular free Fe^{2+} concentration is the limiting factor for oxidative DNA damages induced by ampicillin or norfloxacin at ½ MIC. The source of free Fe^{2+} is thought to come from TonB-mediated iron import. Based on this hypothesis, I proposed a model for ½ MIC ampicillin and norfloxacin induced oxidative DNA damages in *E. coli* cells (Figure 30).

However, question on how the exposure to ½ MIC ampicillin or norfloxacin increased TonB-mediated iron import activity is yet to be solved and need to be addressed in future. There are several possibilities to how ½ MIC ampicillin or norfloxacin may perturb the regulations of TonB-mediated iron import. The expression of *tonB* gene is always repressed by Fur when intracellular iron is available (McHugh et al. 2003). When bind to Fe^{2+} forming Fur-Fe complex, Fur controls the expression of iron regulation genes, in which mostly involved in iron uptake and metabolism (Stojiljkovic et al. 1994; McHugh et al. 2003). The antibiotics drug-target interaction may inactivate Fur, thus de-represses *tonB* expression, or may directly affect TonB activity without going through Fur. One way to test this hypothesis is to determine the effect of ampicillin or norfloxacin on oxidative DNA damages in strain lacking *fur* (*ΔmutMY Δfur*). Another possibility is that direct interactions with antibiotic molecules inactivates Fur, leads to de-repression of *tonB* expression, or direct interaction between TonB and antibiotic molecules activates TonB function. In order to determine the effect of antibiotics on *tonB* expression, it is important to carry out transcriptional analysis of *tonB* in the presence of ampicillin and norfloxacin.

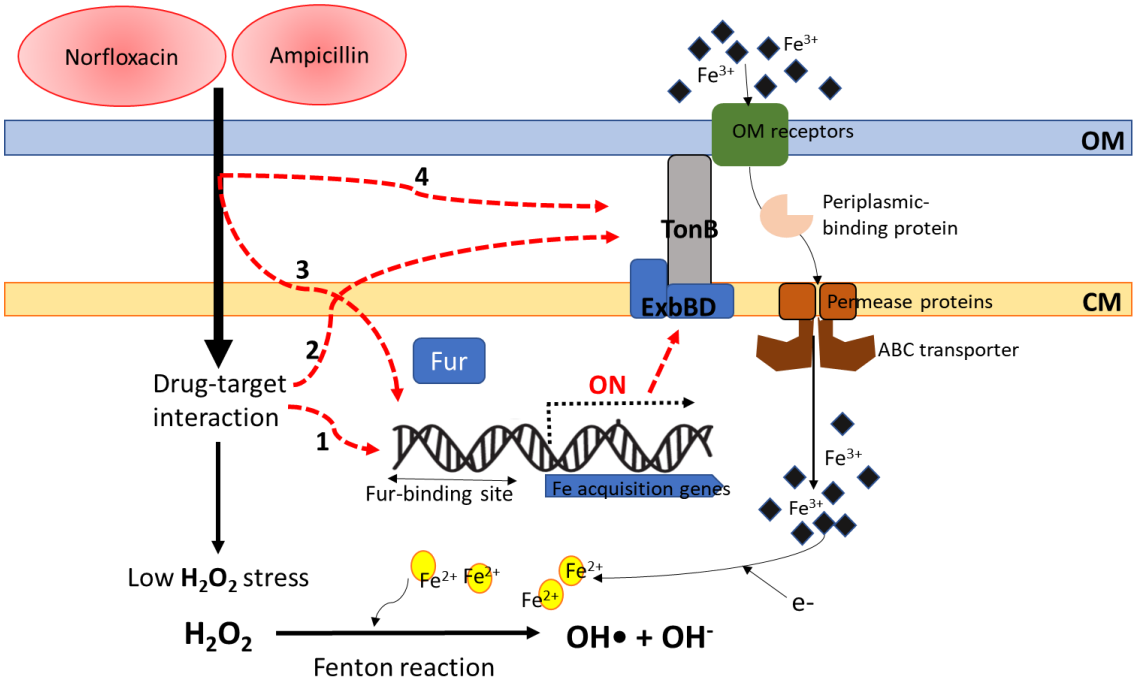

Figure 30. Proposed model for oxidative DNA damages induced by ½ MIC ampicillin and norfloxacin.

Cells exposed to ½ MIC ampicillin or norfloxacin increase H_2O_2 level, elevate the intracellular free Fe^{2+} concentration probably through TonB-mediated iron uptake and stimulating Fenton reaction to produce OH•, thus inducing oxidative DNA damages. Further clarification is needed to solve whether TonB function is activated via inactivation of Fur by antibiotics drug-target interaction **(1)**, direct activation through antibiotics drug-target interaction **(2)**, inactivation of Fur through interactions with antibiotic molecule **(3)** or direct interactions of TonB with antibiotic molecules **(4)**.

Part III. Involvement of MazF and CpxA in oxidative DNA damages induced by sublethal concentrations of ampicillin

Previously, a common mechanism of stress-induced ROS and bacterial cell killing was hypothesized. Stress, such as lethal bactericidal antibiotics or hydroxyurea, causes degradation of antitoxin MazE to occur leading to increase in toxin MazF, which induces mRNA cleavages, causes misfolded or truncated peptides that are inserted into the membrane, which then activates envelope stress response, CpxA and ArcA two-component sensor systems (Zhao & Drlica, 2014; Davies et al., 2009; Wu et al., 2011). The activation of ArcA through CpxA may stimulate the TCA cycle and hyperactivation of the electron transport chain, which leads to elevated ROS levels (Kohanski et al., 2007; Belenky, 2015). Using *ΔmutMY ΔmazF* and *ΔmutMY ΔcpxA*, I found that the oxidative DNA damages was still induced in cells treated with

½ MIC ampicillin. If the MazF and CpxA functions are required for ½ MIC ampicillin induced oxidative DNA damages, I expected a complete suppression of the oxidative DNA damages level but that was not the case. I also found that the oxidative DNA damages increased in the untreated cells of both *ΔmutMY ΔmazF* and *ΔmutMY ΔcpxA* strains, suggesting that MazF and CpxA may protect the cells from oxidative DNA damages in untreated or less stress condition. MazF have been reported to promote both cell-killing and helping stressed cells to enter persistency or dormancy in order to tolerate stress (Pedersen et al., 2002). Cpx also exhibited dual-functionality involving live-or-die stress response, in which giving protection at low stress conditions but inducing cell-killing at high stress level conditions (Dorsey-Oresto et al., 2013). It is suggested that roles of MazF and Cpx change at different stress levels, where they are protective in no-stress or less stress conditions, but destructive at high stress levels (Dorsey-Oresto et al., 2013; Engelberg-Kulka et al., 2006; Pedersen et al., 2002).

Based on my findings, it might suggests that in no stress condition, MazF and CpxA protect the cells from oxidative DNA damages. In relation to the previous bactericidal antibiotics induced ROS model, MazF and CpxA does not seem to be involved in promoting oxidative DNA damages in cells treated with ½ MIC ampicillin.

Chapter 5. Conclusions

Part I. Sublethal concentrations of bactericidal antibiotics induce oxidative DNA damages

1. Ampicillin (ß-lactam) and norfloxacin (fluoroquinolone), at ½ MIC, induced oxidative DNA damages through OH• formation.
2. ½ MIC gentamicin (aminoglycoside) does not induce oxidative DNA damages.
3. ½ MIC bacteriostatic antibiotics, tetracycline and chloramphenicol, does not induce oxidative DNA damages.
4. Oxidative DNA damages level does not increase in cells treated with higher than ½ MIC and near MIC concentrations of ampicillin and norfloxacin

Part II. Molecular mechanism of sublethal bactericidal antibiotics induce oxidative DNA damages

1. No good correlation between intracellular H_2O_2 level and the oxidative DNA damages level in cells treated with sublethal concentrations of ampicillin and norfloxacin
2. Intracellular free Fe^{2+} concentration is the limiting factor in oxidative DNA damages induced by ½ MIC ampicillin and norfloxacin
3. TonB-mediated iron uptake function is required in oxidative DNA damages induced by ½ MIC ampicillin and norfloxacin

Part III. Involvement of MazF and CpxA in oxidative DNA damages induced by sublethal concentrations of ampicillin

1. MazF and CpxA exerted slight protective effect in the untreated cells.
2. MazF and CpxA does not seem to be involved in the oxidative DNA damages induced by ½ MIC ampicillin.

Chapter 6. Future Plans

I have proposed a hypothesis on the mechanism of oxidative DNA damages induced by ½ MIC ampicillin and norfloxacin. There are still some questions need to be addressed. First of all, how does the exposure to ½ MIC ampicillin or norfloxacin increased TonB-mediated iron import activity? As I mentioned earlier, there are several possibilities to how ½ MIC ampicillin or norfloxacin may perturb the regulations of TonB-mediated iron import. As *tonB* gene expression is always repressed by Fur when intracellular iron is available (McHugh et al. 2003), it is possible that antibiotics might indirectly affect TonB function by targeting Fur to de-repress TonB. Another possibility is direct activation of TonB by the antibiotics without perturbing Fur. Ampicillin or norfloxacin may affect Fur or TonB through their specific drug-target interaction effects or the interaction between antibiotics molecules and Fur or TonB. Although the current findings suggest that the main source of iron in the oxidative DNA damages caused by ½ MIC ampicillin and norfloxacin may come from iron import, it is also important to examine the possibility of other iron sources involved in this mechanism, such as the cytochrome BD oxidase and iron sulfur clusters (Fe:S) (Davies et al., 2009; Kohanski et al., 2007).

Acknowledgements

It is a pleasure to express my sincere and deepest gratitude to my supervisor Professor Dr. Hisaji Maki for his patience, motivation, enthusiasm, great knowledge, and also for giving me the opportunity to do my PhD study in his laboratory. His involvement has triggered and nourished my intellectual maturity that I will benefit from, for a long time to come. Above all, he provided me huge encouragement and support in various ways. His guidance helped me in all the time of research and writing of this thesis.

I would also like to say thank you to my dissertation committees, Professor. Dr. Hiroshi Takagi and Associate Professor Dr. Yukio Kimata who have been giving me a lot of helpful advices and comments during my PhD study. Not forgetting Dr. Satoko Maki, my gratitute for her encouragement, suggestions and helpful advices. I would like to thank NAIST, Graduate School of Biological Sciences and International Students Affair Section for the support throughout my studying and living in Japan. I would also like to express my appreciation to Associate Professor Dr. Masahiro Akiyama and Dr. Asako Furukohri for their advices, suggestions and guidance throughout the years. A big thank you to all Microbial Molecular Genetics Laboratory members for all the help. This study was made possible by the financial support from The Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT).

Last but not least, I wish to express my sincere appreciation to my mother (Zakiah Othman) and father (Iskandar Vijaya Abdullah) who encourage and cheer for me all the way to the end. This thesis is dedicated to them who had given me the opportunity of an education from the best institutions. Their prayers and moral support will always be in my heart.

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Supplementary figures

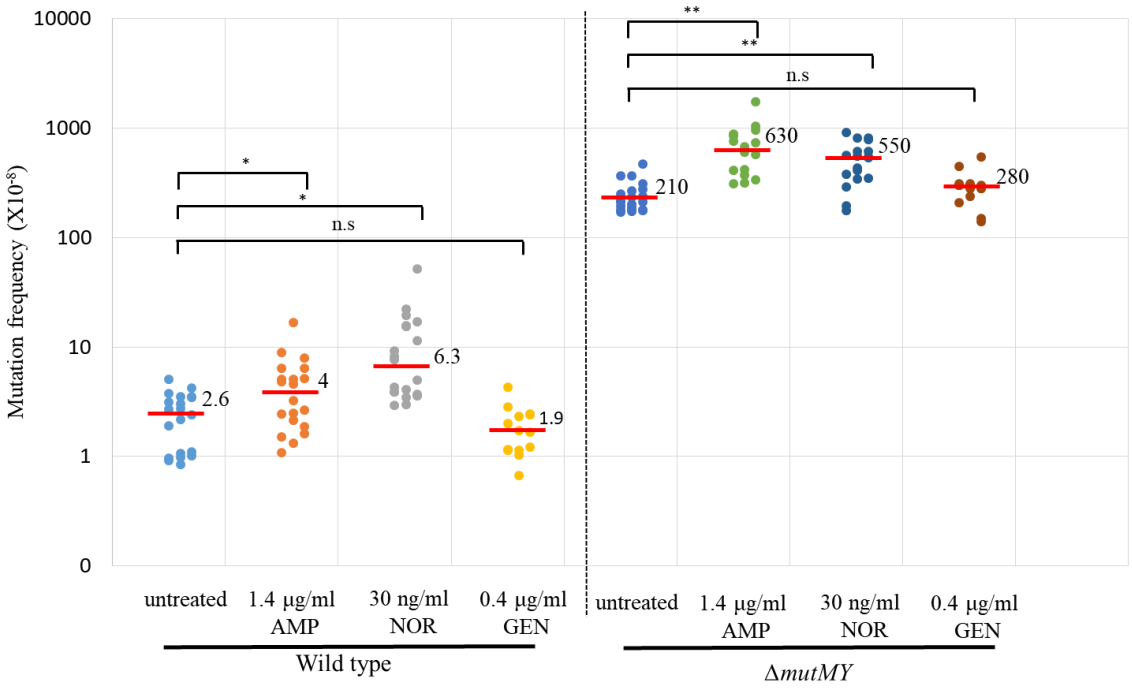

Figure S1. Mutation frequencies in wild type and Δ*mutMY* cells treated with ½ MIC bactericidal antibiotics. Mutation frequencies were obtained from 20 populations for each treatment and values were plotted against log-scale y-axis. The median values were used for comparison between data sets. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01, ns indicates non-significant.

(AMP: ampicillin; NOR: norfloxacin; GEN: gentamicin)

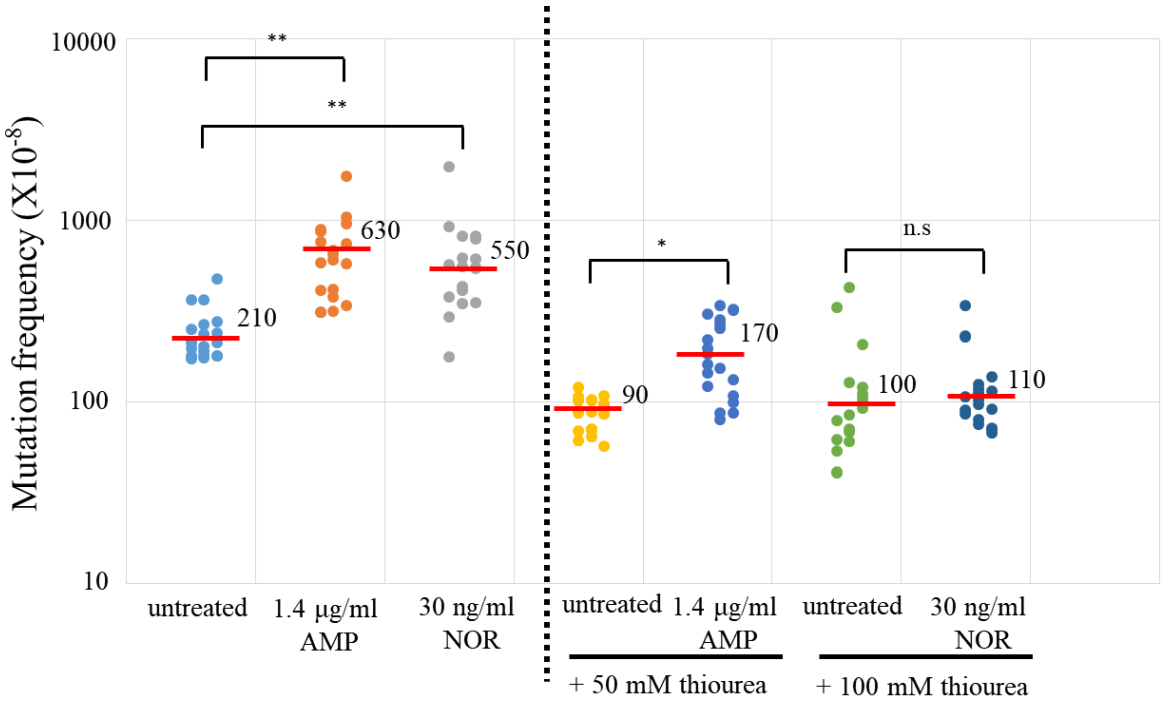

Figure S2. Thiourea suppressed the oxidative DNA damages in Δ*mutMY* cells treated with ½ MIC ampicillin and norfloxacin. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01, ns indicates non-significant. (AMP: ampicillin; NOR: norfloxacin)

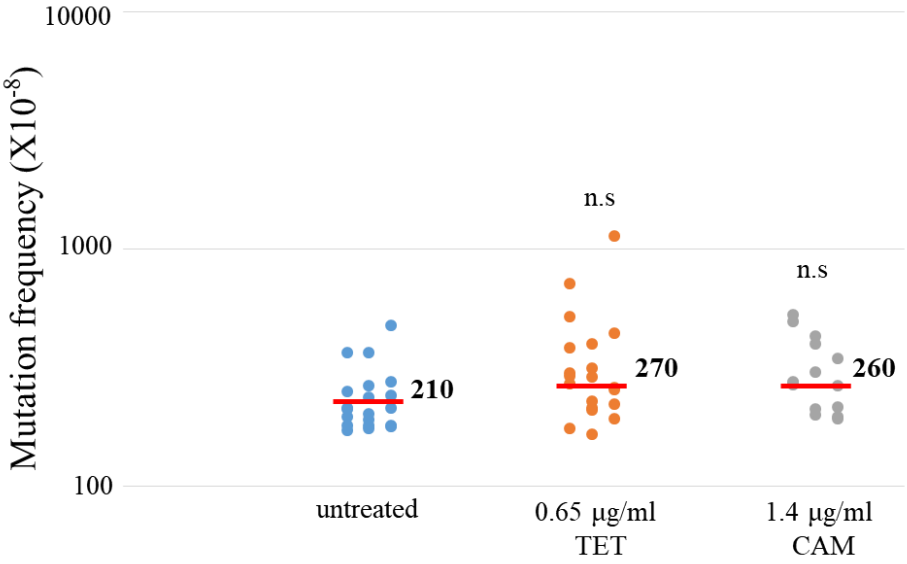

Figure S3. Mutation frequencies in Δ*mutMY* cells treated with ½ MIC bacteriostatic antibiotics. Significance of data: n.s indicates non-significant. (TET: tetracycline; CAM: chloramphenicol)

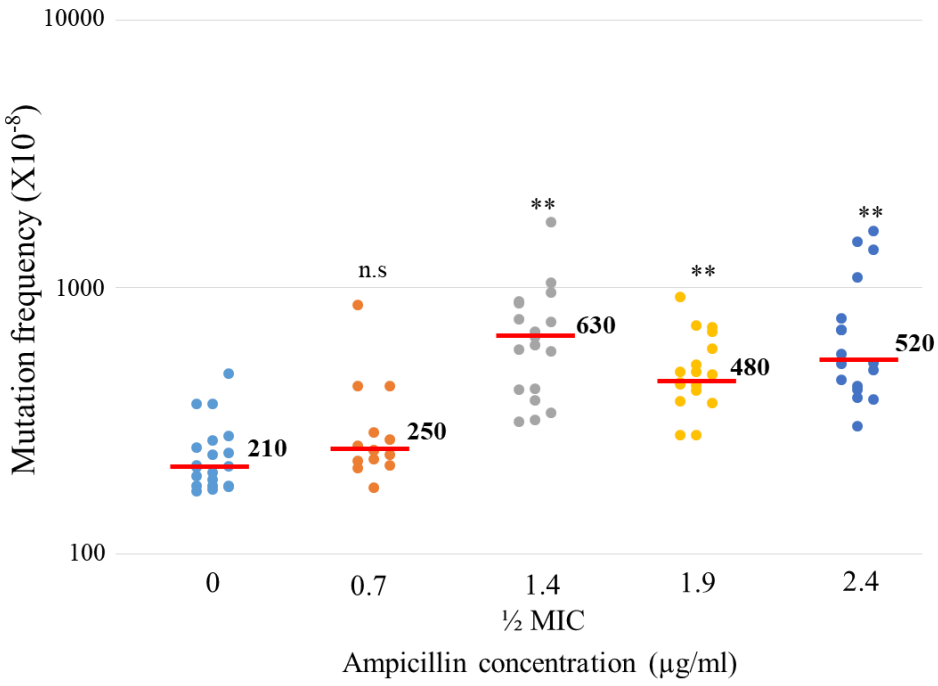

Figure S4. Characteristics of oxidative DNA damages induced by sublethal concentrations of ampicillin in Δ*mutMY* cells. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01, ns indicates non-significant.

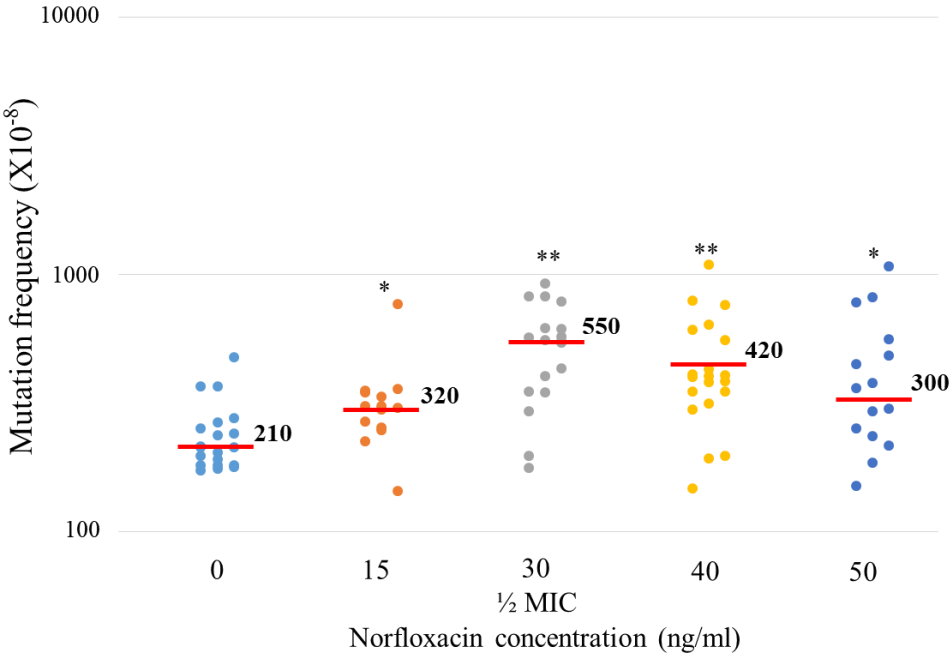

Figure S5. Characteristics of oxidative DNA damages induced by sublethal concentrations of norfloxacin in Δ*mutMY* cells. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01

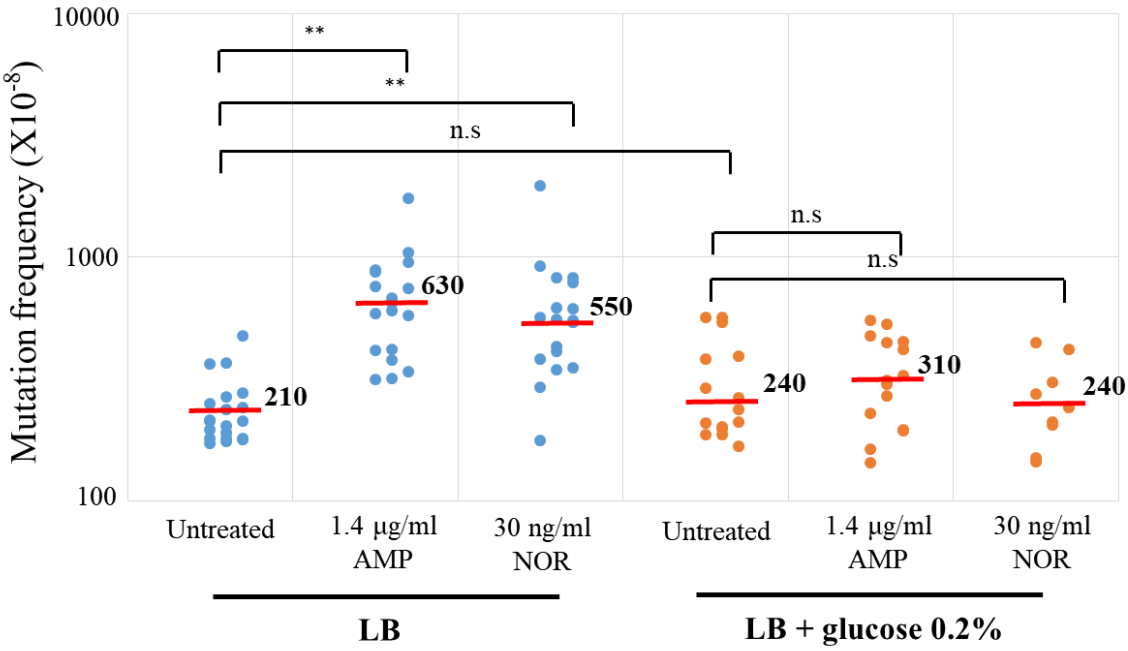

Figure S6. Mutation frequencies in Δ*mutMY* cells treated with ½ MIC ampicillin or norfloxacin grown in LB + glucose 0.2%. Significance of data: (*) indicates p-value <0.05 and n.s indicates non-significant. (AMP: ampicillin; NOR: norfloxacin)

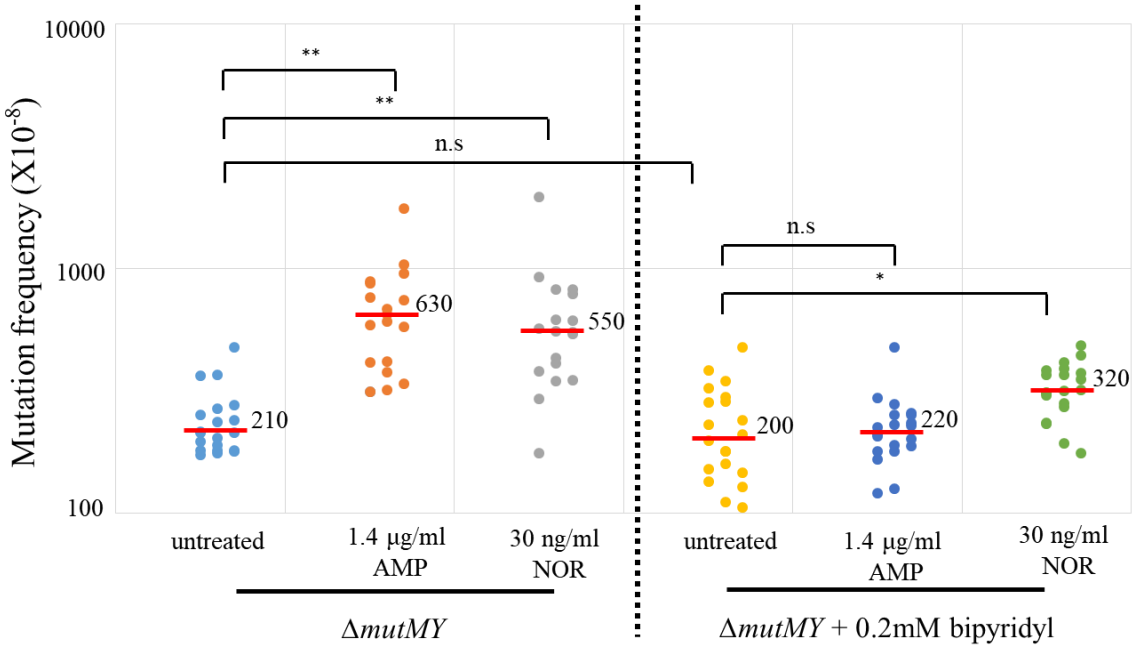

Figure S7. Mutation frequencies in Δ*mutMY* cells treated with ½ MIC ampicillin or norfloxacin in the presence of 2,2'-bipyridyl. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01, ns indicates non-significant. (AMP: ampicillin; NOR: norfloxacin)

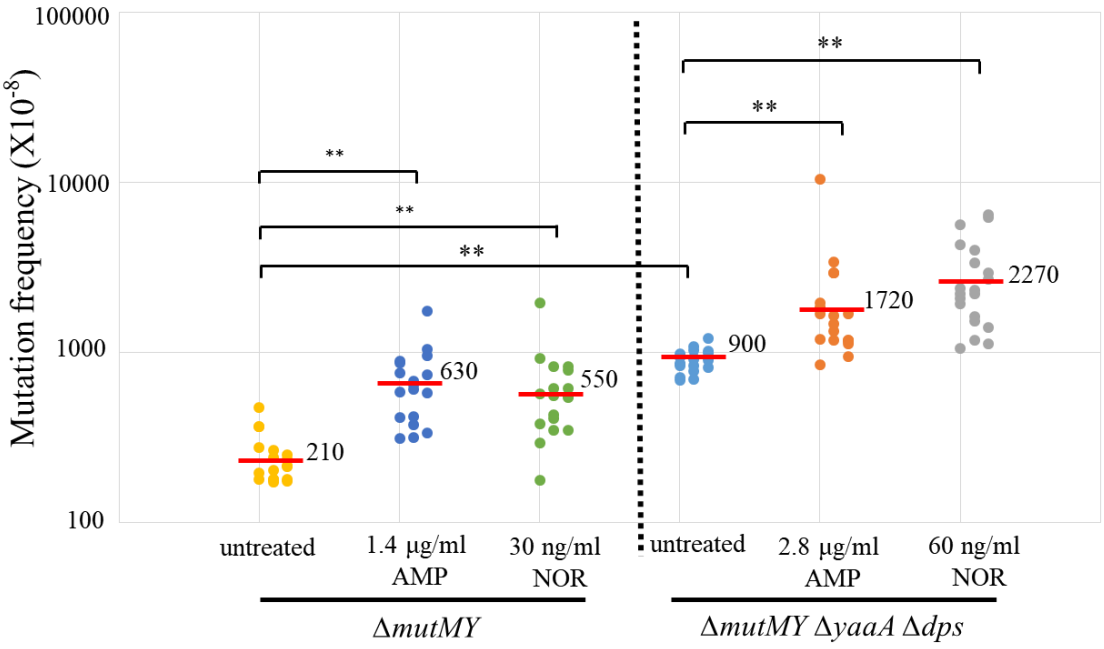

Figure S8. Mutation frequencies in Δ*mutMY* and Δ*mutMY* Δ*yaaA* Δ*dps* cells treated with ½ MIC ampicillin or norfloxacin. Significance of data: (**) indicates p-value <0.01 (AMP: ampicillin; NOR: norfloxacin)

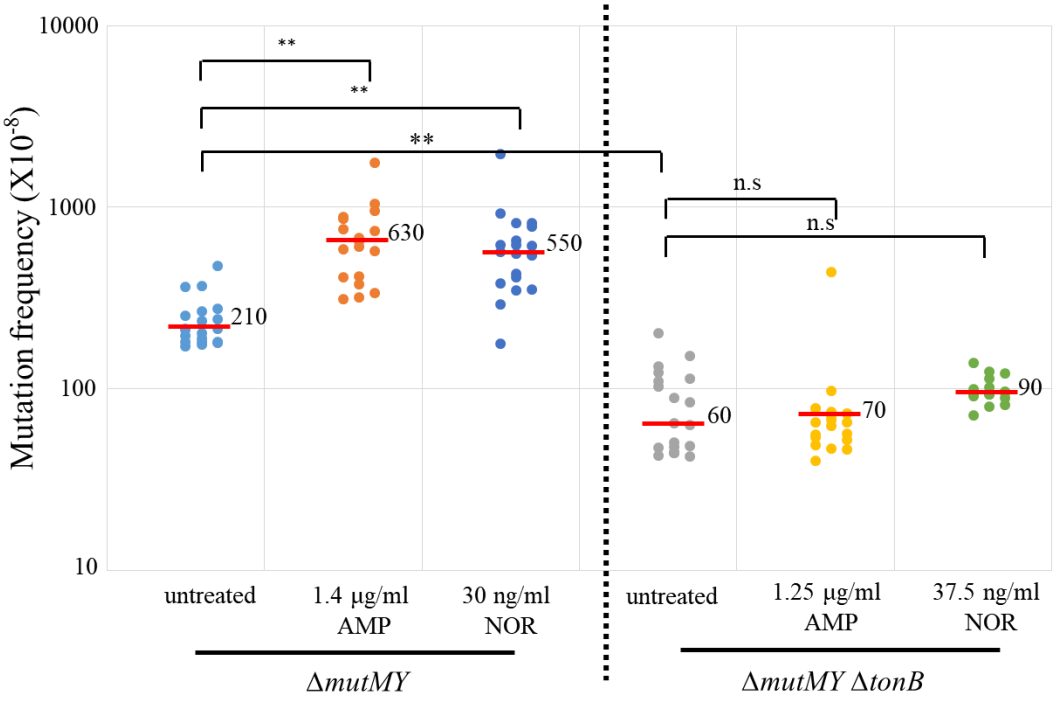

Figure S9. Mutation frequencies in Δ*mutMY* and Δ*mutMY* Δ*tonB* cells treated with ½ MIC ampicillin or norfloxacin. Significance of data: (**) indicates p-value <0.01, ns indicates non-significant. (AMP: ampicillin; NOR: norfloxacin)

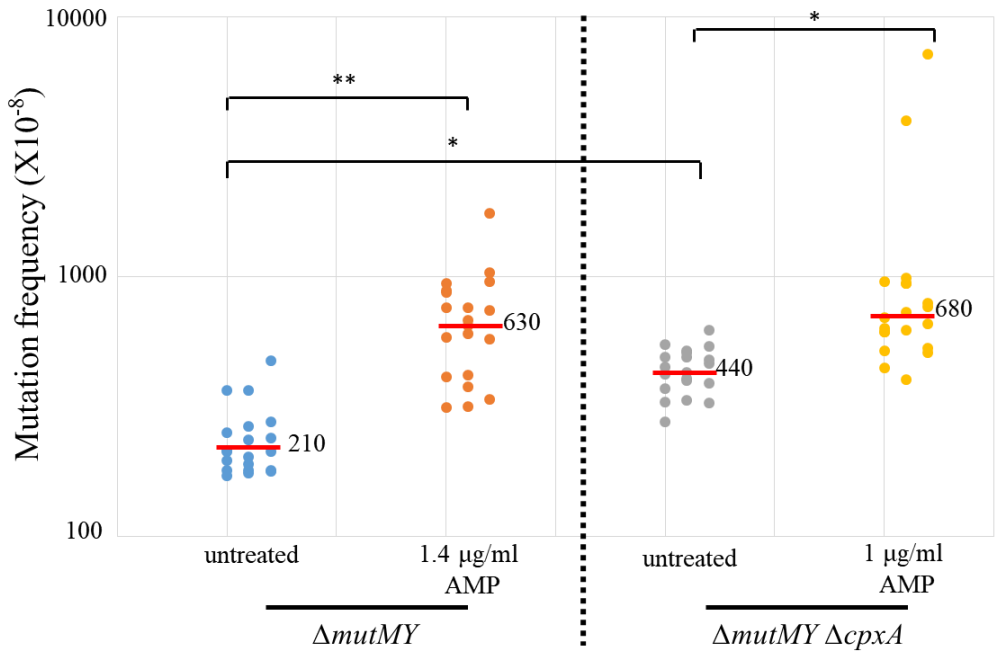

Figure S10. Mutation frequencies in Δ*mutMY* and Δ*mutMY* Δ*cpxA* cells treated with ½ MIC ampicillin. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01 (AMP: ampicillin)

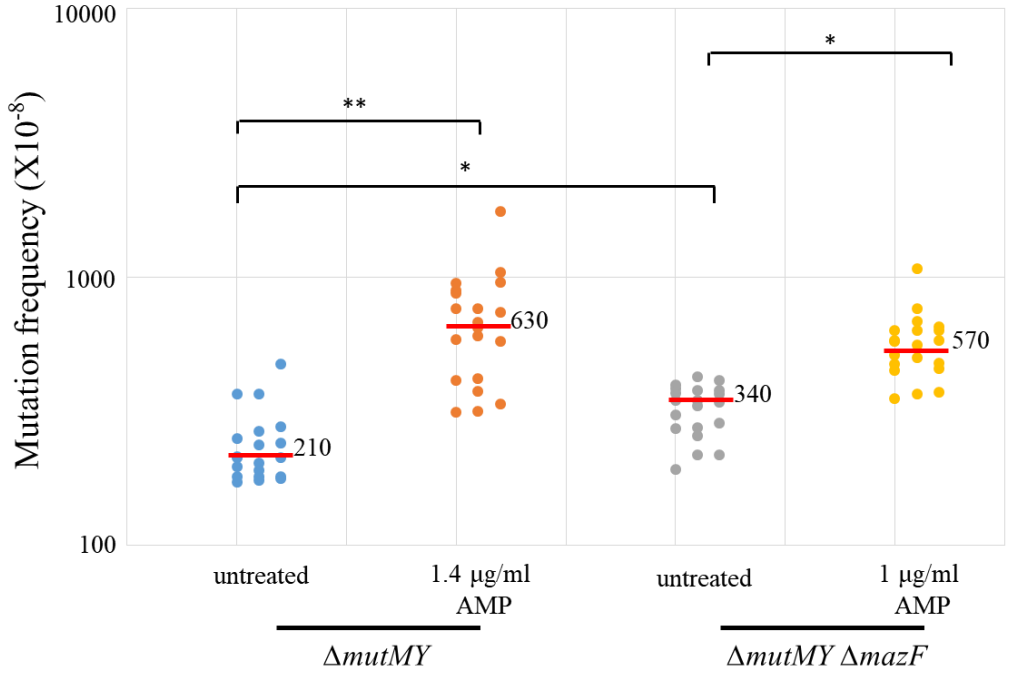

Figure S11. Mutation frequencies in Δ*mutMY* and Δ*mutMY* Δ*mazF* cells treated with ½ MIC ampicillin. Significance of data: (*) indicates p-value <0.05 and (**) indicates p-value <0.01 (AMP: ampicillin)