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Doctoral Thesis

Involvement of OxyR regulon genes in suppression of oxidative DNA damage via iron regulation in *Escherichia coli*

（酸化 DNA 損傷発生の抑制における大腸菌 OxyR レギュロン遺伝子群の鉄調節を介した役割）

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Abstract

Graduate School of Biological Sciences Abstract of Doctoral Thesis

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Title	Involvement of OxyR regulon genes in suppression of oxidative DNA damage via iron regulation in *Escherichia coli*		

Oxidative stress has been one of the leading cause of DNA damage both in prokaryotic and eukaryotic cells. In a normal physiological condition, *Escherichia coli* cells undergo a redox chain of metabolism that results in the release of various types of reactive oxygen species (ROS). Among the ROS, hydroxyl radical (•OH) is highly reactive and easily damages DNA, producing various kinds of oxidative DNA damage. •OH causes DNA lesions that affect DNA replication and transcription that ultimately causes chromosomal rearrangements, genomic instability and sometimes cell death. However, cells can undergo various repair pathways to overcome the DNA lesions caused by the ROS. Cells also possess protective mechanisms by scavenging the ROS produced or suppressing the production of the ROS. Whilst the repair pathways are extensively studied, the equally important protective mechanisms have insufficient information of its relevance to oxidative DNA damage. With that, my research purpose is to investigate how OxyR and its regulon genes are able to protect the cells from oxidative DNA damage.

Many studies have been carried out to comprehend the mechanism producing oxidative DNA damage by hydrogen peroxide (H_2O_2) which is converted to •OH by Fenton reaction. Production of H_2O_2 in *E. coli* cells occurs mainly by the autoxidation of flavoenzymes, whereas redox charges the stable H_2O_2 to the reactive •OH at the expense of oxidizing ferrous iron to ferric iron. High concentration of H_2O_2 may cause oxidative DNA damages, however, *E. coli* has its own defensive mechanisms to reduce the H_2O_2 concentration, maintaining *in vivo* H_2O_2 concentration at a very low level. This level is controlled by OxyR and some of the regulon genes involved in scavenging H_2O_2. In normally growing cells, the scavenging mechanism of the OxyR regulon is known but the mechanism of suppression of the •OH production remains elusive. Both mechanisms are related but the pathway of protection is different. Previous study from our lab has shown that the intracellular H_2O_2 is not correlated to the level of oxidative DNA damage. While H_2O_2 is scavenged, the production of •OH must be suppressed to avoid the occurrence of oxidative DNA damage. Therefore, I aimed to find out the mechanism of action of OxyR and its regulon genes in suppressing oxidative DNA damage. I also seek to find out which genes in the regulon are important to suppress oxidative DNA damage.

When cells were treated with extracellular H_2O_2, *oxyR* deletion mutant showed hypersensitivity in comparison to the wild type cells. This suggests that OxyR is very important in extreme stress condition. I determined the importance of OxyR in suppressing oxidative DNA

damage in normal growth condition. Oxidative DNA damage mostly results in relatively small changes in bases of DNA that are difficult to be directly detected. 8-oxoG, the most abundant product of oxidative DNA damage, leads to mutagenesis if left unrepaired. However, this oxidative DNA damage can be repaired efficiently and exclusively by MutM and MutY enzymes. Therefore, the mutation frequency in a *mutM* and *mutY* double deletion strain (*ΔmutM ΔmutY*) is a good measure of the cellular level of oxidative DNA damage. Together with the repair deficient *ΔmutM ΔmutY* background, deletion of the *oxyR* gene showed a significant increase in spontaneous mutation frequency, indicating the importance of OxyR to suppress oxidative DNA damage in normal growth condition. The previous study from our group showed that cells grown in minimal media showed increased oxidative DNA damage level in comparison to LB (rich) media. I found that the *ΔoxyR* strain showed more than 10 -fold increased when cells were grown in minimal media. This indicates that OxyR is very important to suppress oxidative DNA damage both in normal and low nutrient condition. To elucidate the importance of some of the genes in the OxyR regulon, the H_2O_2 sensitivities of strains with each of the genes deleted were determined. It is shown that the *katG* gene, an H_2O_2 scavenger and the *fur* gene, an iron regulator gene which controls iron import were among the most important genes in the regulon. *katG* mutants showed an increased level of oxidative DNA damage in LB but no changes in the level of minimal media. This suggests that other genes may be more important for the suppression of oxidative DNA damage in normal growth. *fur* mutant showed increased levels of oxidative DNA damage in LB, indicating that iron regulation is involved in the suppression of oxidative DNA damage in normal growth. With the deletion of *dps* and *fur* genes, the mutation frequency increased even more tremendously in cells grown in normal condition. This suggests that Dps functioning to facilitate iron storage works in a pathway different from Fur, and both are as equally important to suppress the oxidative DNA damage. Similarly, I found that deletion of *dps* and *yaaA* genes resulted in an increased mutation frequency to the level of *Δfur Δdps* strain. The *yaaA* gene previously known to be a gene of unknown function has been proposed to facilitate free intracellular iron-binding through unknown mechanisms. Further genetic analyses suggested that both *yaaA* and *dps* work in different pathways but in a synergistic manner. Surprisingly, the deletion of *fur* and *yaaA* showed no additive effect on the oxidative DNA damage level, suggesting that *yaaA* may work in the same pathway as *fur*. Through gene expression study, I showed the relationship of the *yaaA* and the *fur* gene. To confirm that oxidative DNA damage is more inclined to the high intracellular iron level, I showed that the oxidative DNA damage was salvaged by iron scavengers, through biological method via the H_2S pathway and chemical method with 2,2'-Bipyridyl.

Overall, this study showed that OxyR suppresses oxidative DNA damage in normally growing cells. OxyR regulon genes *fur*, *dps* and *yaaA* that are involved in reducing intracellular iron level contribute to the suppression of oxidative DNA damage by different pathways but relevant interactions. Findings in this study would contribute to the revelation of iron regulatory genes' functionality in the cell metabolism as a model for higher organisms.

List of abbreviations and symbols

ROS	Reactive oxygen species
O_2	Molecular oxygen
O_2^-	Superoxide anion
H_2O_2	Hydrogen peroxide
·OH	Hydroxyl radical
SOD	Superoxide dismutase
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
8-oxoG	7,8-dihydro-8-oxoguanine
BER	Base excision repair
NER	Nucleotide excision repair
MMR	Mismatch repair
DSBR	Double strand break repair
HR	Homologous recombination
Fur	Ferric uptake regulator
DUF	Domain of unknown function

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Chapter I. Introduction

1.1 Reactive Oxygen Species (ROS)

Organisms dependent on aerobic respiration use molecular oxygen as a source for energy production. However, the natural side effect of this respiration is the production of reactive oxygen species (ROS) (Imlay and Fridovich 1991). Under normal physiological condition, only 0.1–1% of the electron flux through any particular enzyme is likely to be intercepted by oxygen (Imlay 2013). Even though the percentage may seem low, because of the rapid action of superoxide and H_2O_2 towards vulnerable targets, ROS scavenging activity is activated. In the metabolic processes of respiration, redox reaction rampantly occurs, yielding to the sequential production of reactive byproducts of oxygen such as superoxide anion radical ($O^{\cdot-}_2$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radicals (·OH). These products are mainly derived from continuous univalent reductions of molecular oxygen catalyzed by several membrane–associated respiratory chain enzymes (Cabiscol, Tamarit, and Ros 2000). Leakage of electrons to molecular oxygen (O_2) from the electron transport chains consisting of flavoproteins, iron sulfur proteins, ubiquinone and cytochromes produces superoxide (O_2^-) which then undergoes dismutation by superoxide dismutase (SOD) that produces H_2O_2 (Loschen et al. 1974). A redox reaction called the Fenton reaction, involves the reduction of H_2O_2 at the expense of ferrous iron (Fe^{2+}) oxidation to ferric iron (Fe^{3+}) causes the generation of reactive ·OH (schematic diagram shown in **Figure 1**).

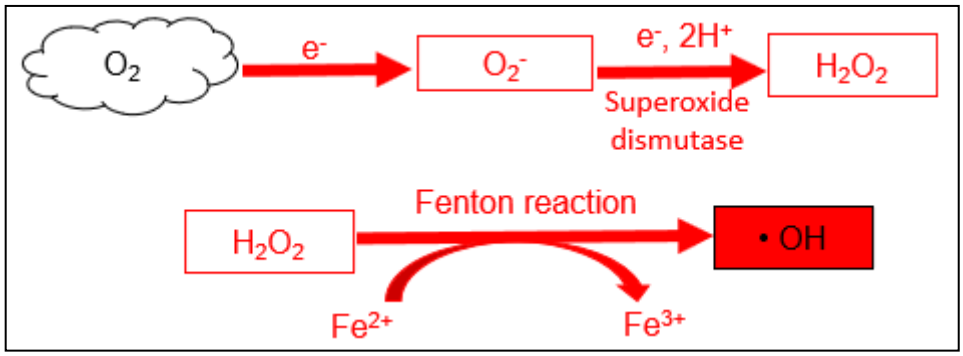

Figure 1: Formation of different reactive oxygen species during aerobic metabolic activities.

Apart from the internal production of ROS, exogenous factors such as UV irradiation, chemical agents and environmental factors contribute to the already abundant intracellular ROS load (Klaunig and Kamendulis 2004; Halliwell and Gutteridge 1999). The effects and dangers that these ROS bring could be detrimental to most biological molecules as ROS is the main cause of oxidative stress which ultimately leads to major oxidative damages. Therefore, it is crucially important that the ROS' mechanisms of production, metabolism, and distribution are properly defined to avoid dangerous circumstances throughout all living organisms.

1.2 Oxidative stress and DNA damage

ROS play a double role as both beneficial and toxic compounds to the living system. In animal cells, at moderate or low levels, ROS have beneficial effects and involve in various physiological functions such as in immune defense against pathogenic microorganisms, in cellular signaling pathways, in mitogenic response and in redox regulation (Valko et al. 2007). In plant cells, ROS play crucial roles in the signal transduction pathways that regulate plant growth, development and defense responses that provide redox control which impacts on most aspect of plant biology (Considine, Sandalio, and Foyer 2015; Bailey-Serres 2006). However, at higher concentration, ROS generate oxidative stress and can cause potential damage to biomolecules. The oxidative stress is developed when there is an excess production of ROS on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other side.

As of recent times, the best understanding of oxidative stress is found in the *Escherichia coli* model system (Imlay 2015). The biological molecules that are frequently targeted by the ROS are the DNA, RNA, proteins, and lipids (Cabiscol, Tamarit, and Ros 2000). However, the most abundant attack of ROS is on the DNA, causing oxidative DNA damage with a high rate of approximately 10^5 lesions per cell per day (Bridge, Rashid, and Martin 2014). Mutagenesis which is a consequential effect of oxidative stress is not directly caused by H_2O_2 nor O_2^- (Farr, D'ari, and Touati 1986). However, other studies suggest that H_2O_2 reacts with the cellular pool of unincorporated iron which results in association with DNA damage (Henle et al. 1999). H_2O_2 produces ·OH which can oxidize both base and ribose moieties of the DNA, giving a wide variety of lesions (Cooke et al., 2003; Hutchinson 1985).

Hydroxyl radical, being the most reactive ROS produce many kinds of damages by causing DNA lesions through reactions with both purine and pyrimidine bases, sugar moieties of the DNA backbone and tandem structures of the DNA (Dizdaroglu 2015). Many extensive studies had been carried out to characterize the types of DNA lesions (Evans, Dizdaroglu, and Cooke 2004; Dizdaroglu 2012), however, the most abundant oxidative DNA damage is through 7,8-dihydro-8-oxoguanine (8-oxoG) which may play a major role in the generation of oxygen-induced G:C to T: A transversion mutations if left unrepaired (Cheng et al., 1992). Despite this, there are many other remaining questions left unanswered regarding the intracellular etiology of oxidative DNA damage when cells are living even at the normal physiological condition. Oxygen which is abundant in the atmosphere inevitably causes the abundance of production of oxidative byproducts which causes DNA to be susceptible to damage. The understanding of how cells are able to withstand such an abundance of oxidative conditions in nature will lead to defining the factor of genomic instability across all organisms.

1.3 DNA repair mechanisms

Free radicals of the ROS are immensely reactive and can react with the different components of DNA to produce various DNA lesions. The oxidative DNA damage caused by the lesions produced from the attacks of ROS is required to be removed and repaired to protect cells against the consequences of genomic instability. Despite the broad spectrum of DNA damaging species and their involvement in many lethal processes, it is astonishing that under normal circumstances most cellular components are error free and are able to retain their functions hence maintaining the genome integrity. This is because living cells possess efficient enzymes that protect DNA from erroneous and hazardous effects by executing about 10^{16} to 10^{18} repair events per cell per day (Schärer 2003). DNA repair machineries have been proposed to function in various pathways such as base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), double strand break repair (DSBR), and homologous recombination (HR) (Friedberg et al. 2006).

As ROS' attack are reactive and rampant, the DNA bases are susceptible and among all the DNA bases, guanine has the least oxidation potential, making it frequently attacked by different reactive species. Modification of guanine can result in a variety of lethal lesions that may arise due to its oxidation, nitration, halogenation, and alkylation (Jena and Mishra 2012). Different guanine lesions formed in this way can induce mutagenesis, crosslinks between DNA strands and proteins, thereby affecting DNA replication and transcription (Abdulnur S. F. and Flurry 1976; Niles, Wishnok, and Tannenbaum 2006). Due to the extensive research that has been done on the DNA repair pathways, it is to this study's advantage that the mechanism of BER pathway is being employed.

There are numerous types of oxidative DNA damages caused by ROS of which 8-oxoG is the most abundant and efficiently removed by the BER pathway. BER pathway becomes the most important pathway which protects cells against many kinds of oxidative DNA damages (David, O'Shea, and Kundu 2007; Dizdaroglu 2005; Sander and Wilson 2005; Cooke et al. 2003) and is highly conserved among prokaryote to eukaryote. The repair mechanism of BER is initiated when cells' repair machinery detects the formation of abnormal bases during replication. DNA glycosylases which are important proteins in the BER pathway are responsible for recognizing and removing the damaged bases. One of the glycosylases, MutM can remove the 8-oxoG paired with C in DNA while adenine mispairs with 8-oxoG is eliminated by the action of MutY protein. Therefore, cells that lack MutM and MutY proteins had shown induced high frequency of G:C to T:A transversion mutations (Sakai et al. 2006; Michaels et al. 1992; Cabrera, Nghiem, and Miller 1988; Nghiem et al. 1988). It is to this knowledge of the efficient repair mechanisms of MutM and MutY that this study extends the usage of repair proteins as a tool for detecting oxidative DNA damage that arises from the formation of 8-oxoG by the attack of ROS. **Figure 2** shows the schematic illustration of how MutM and MutY repairs 8-oxoG mutation.

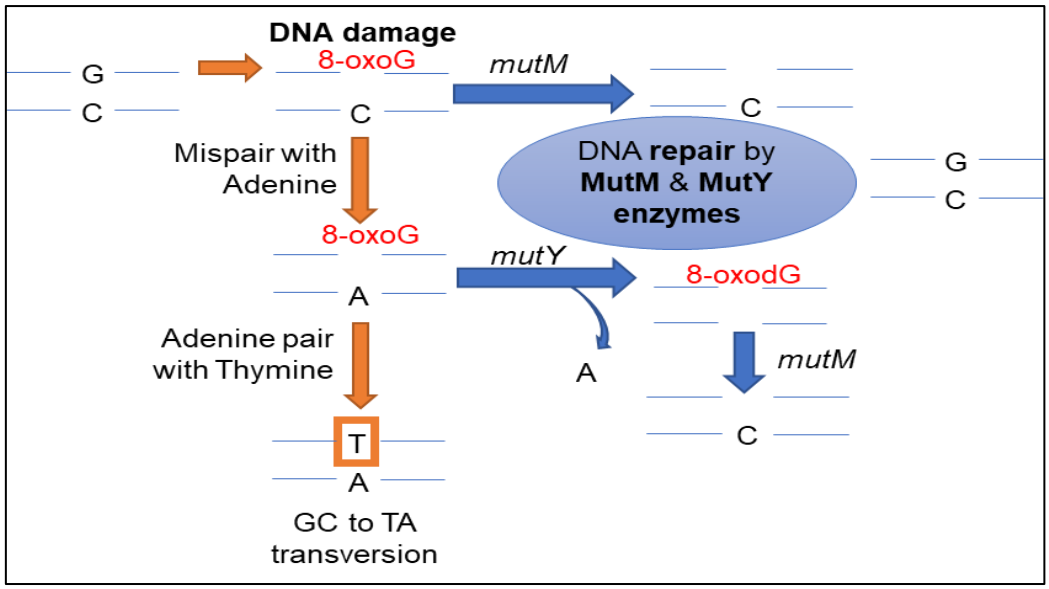

Figure 2: Schematic illustration of MutM and MutY repair mechanisms against 8-oxoG mutation.

1.4 Protective mechanisms of *Escherichia coli* by OxyR

Molecular oxygen diffuses easily into the intracellular of the cells as it is small and nonpolar and can diffuse across biological membranes as quickly as water (Ligeza et al., 1998). To this effect, contrary to the higher organisms that can withstand high oxic levels, some microorganisms suffer poor growth, elevated mutagenesis or even death when the O_2 levels exceed those of their native habitats. *E. coli*, which is a facultative anaerobe is exposed to these high O_2 levels with the effect of having to overcome the overload of the O_2 and the subsequent derivatives (Imlay 2013). The rich and saturated oxygen in the atmosphere has caused the development of defense mechanisms in organisms by various methods. In *E. coli*, the protective or defense mechanism may be by either keeping the O_2 derived radicals at an acceptable level or maintaining the concentration of the repaired oxidative damages caused by the O_2 derived radicals (Cabiscol, Tamarit, and Ros 2000). Despite the many extended studies of the oxidative stress and damage, the myriad ways of protection of the cells remain vastly unexplored. To this end, it is crucially important that the protective mechanisms of the oxidative stress and damage be fully understood.

One of the well-known oxidative stress regulators that possess protective mechanisms is through the regulation of OxyR and its regulon genes. OxyR is an oxidative transcriptional dual regulator for the activation and repression of genes particularly in elevated levels of H_2O_2. OxyR is a member of the LysR family of transcriptional regulators (Christman, Storz, and Ames 1989; Tao et al., 1989) and it is a homotetramer consisting of two domains; N-terminal domain and C-terminal domain (Kullik et al., 1995). Induction of transcription by the OxyR-dependent promoters occur when OxyR is in its oxidized state. The oxidized OxyR recognizes a motif

comprised of four ATAGnt elements spaced at 10 bp intervals and these spacing elements allow the four subunits of OxyR to bind to four adjacent major grooves on the DNA helix (Storz, Tartaglia, and Ames 1990) while on the opposite effect, reduced OxyR binds to just two major grooves (Toledano et al., 1994). In further structural studies of OxyR, it was found that sulfur residue Cys199 was oxidized to sulfenic acid in the presence of H_2O_2 and a reversible disulfide bond was formed with the residue of Cys208 (Zheng 1998). The oxidized and reduced structural conformation of the OxyR affects its regulatory domain to function as a switch in redox conditions (Choi et al., 2001). **Figure 3** shows a simple schematic illustration of the structural binding of the OxyR protein at its different forms. Oxidized OxyR binds with RNA polymerase to positively regulate dependent promoter (Tao, Fujita, and Ishihama 1993) while reduced OxyR can also function to repress certain genes functions in a negative regulation (Henderson and Owen 1999). OxyR is dependent on its own regulon members, glutathione reductase (*gorA*) and glutaredoxin (*grxA*) to return its form to the reduced state in the absence of oxidative stress (Zheng 1998). OxyR regulon system is extensive with over more than 40 genes that are controlled by it, of which some genes are this study's interest that could give insights as to which are the key players of the regulon that can protect the cells from oxidative DNA damages.

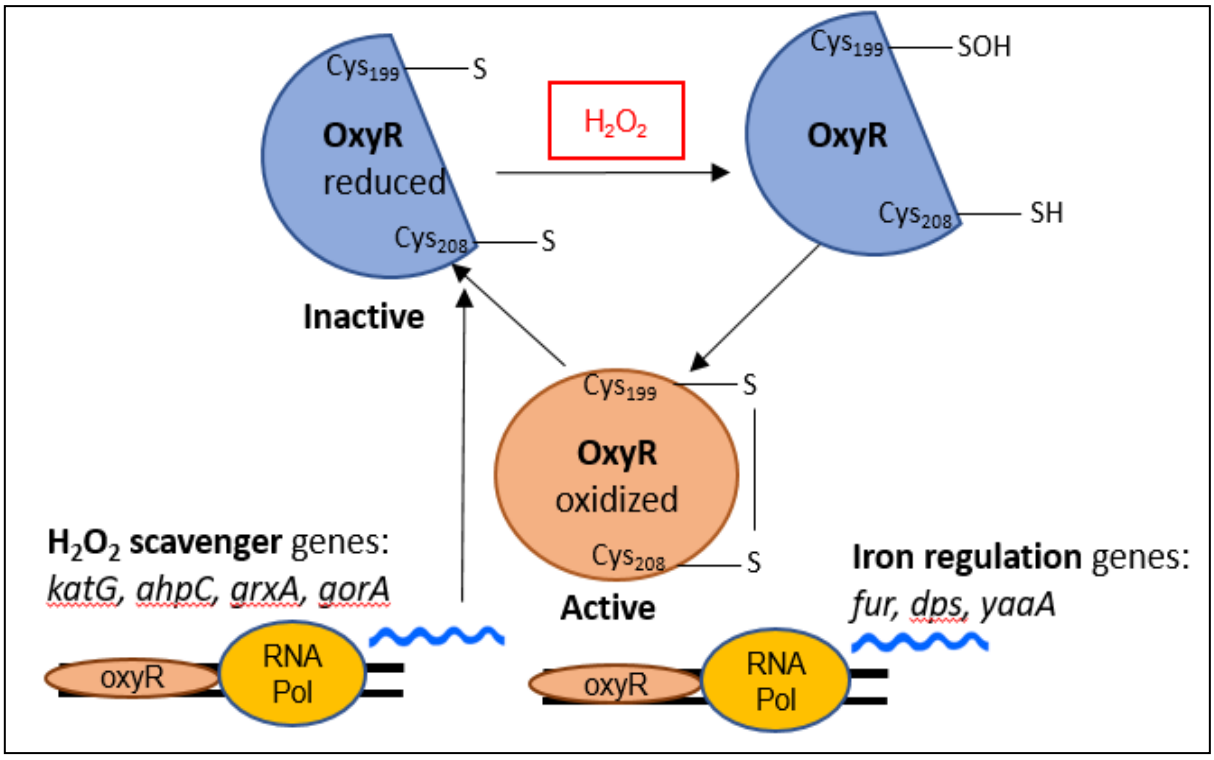

Figure 3: Schematic illustration of the structural binding of the OxyR protein at its different forms.

1.4.1 Scavenger proteins of the OxyR regulon

As mentioned earlier, OxyR is an important transcriptional factor in *E. coli* that functions both as an activator or repressor for its regulon members which mainly consists of genes that are involved in oxidative stress response and antioxidant. OxyR molecules are present in the cells at all times and are activated directly by oxidative stress signal which is in the form of elevated H_2O_2 level (Toledano et al., 1994). Upon the treatment of H_2O_2, OxyR induces its regulon to regulate the oxidative stress condition by activating the different regulon promoters (Storz, Tartaglia, and Ames 1990). OxyR controls the regulation of almost 40 genes in the presence of H_2O_2 cell toxicity. There are 28 genes that are induced by OxyR while 10 genes are being suppressed in the study of *E. coli* and *S. enterica* (Chiang and Schellhorn 2012). The OxyR system is a very effective functional defense mechanism which rapidly reacts to the induction of defense genes which have been elucidated to scavenge the high H_2O_2 levels. Among the major genes that are involved are those encoding the hydroperoxidase I (*katG*), glutathione reductase (*gorA*) and alkyl hydroperoxide reductase (*ahpCF*) (Christman et al., 1985). These genes function actively in bringing the intracellular level of H_2O_2 to the constant steady-state value of about 0.2μm so that cells can function at the optimal condition (Beatriz Gonzalez-Flecha and Demple 1997a).

Such is the effective system of OxyR and its regulon members to scavenge H_2O_2 so that cells are not further harmed by the indirect effect of high intracellular H_2O_2 level. To this end, many researchers believe that the major key players of the OxyR regulon are these H_2O_2 scavenging genes which are functioning to control the intracellular H_2O_2 level corresponding to the redox switch that determines the conformational fold of the OxyR protein. However, it should be emphasized that the OxyR regulon system is very extensive and that the protection mechanisms of oxidative DNA damage not only revolve around the controlling of H_2O_2 levels. The degradation of H_2O_2 to produce a more reactive byproduct, ·OH is not elucidated in many OxyR studies which could undermine the importance of it in relevance to oxidative DNA damage. Therefore, it is crucial to examine other factors in the OxyR regulon that may contribute to the reduction of oxidative DNA damage when cells undergo oxidative stress.

1.4.2 Iron regulation of the OxyR regulon

H_2O_2 does not directly harm DNA but causes the high production of hydroxyl radical (·OH) by the Fenton reaction (S. Park and Imlay 2003). Fenton reaction occurs in the redox state whereby transitional metal ions such as Cu^{+}, $Zn^{2+}$and Fe^{2+} are being oxidized whilst reducing the intracellular H_2O_2 to the reactive ·OH (Imlay 2015; Mahaseth and Kuzminov 2016; Abbad-Andaloussi et al., 1998). However, iron (Fe) is widely used by all types of cells in the catalysis of many essential metabolic reactions and transition (Andreini et al., 2008). Iron can be found in the soluble Fe (II) form and the insoluble Fe (III) form. The soluble Fe (II) iron donates one electron to a hydrogen peroxide molecule, causing its decomposition:

$Fe(II) + H_2O_2 \longrightarrow Fe(III) + \cdot OH + OH^-$

that produces hydroxyl radical which is capable to react with organic compound at diffusion rates (Buxton et al., 1988; Pryor 1986). To this effect, iron plays a major role in controlling the levels of intracellular H_2O_2 concentration at the expense of producing more ·OH. The relationship between the high intracellular H_2O_2 level and high iron levels which causes the oxidative DNA damage has been the interest of many researchers and remains far from conclusion. Therefore, it is crucially important that this study outlines the interaction between these two major factors and the cofounding players, such as the genes that are regulating them in relation to controlling the oxidative DNA damages.

With regards to *oxyR*, it has been well known that this gene controls most of the H_2O_2 scavenging activities which may reduce the oxidative DNA damage (as mentioned earlier). However, the iron regulators genes of the OxyR regulon has often been underestimated and less talked about in comparison with the H_2O_2 scavenger genes. As the OxyR regulon consists of almost 40 genes of which a quarter is involved in antioxidation, the others are involved in iron regulation while the rest of the genes are classified under the domain of unknown functions (DUF). Some of the genes which are involved in iron regulation are such as *fur* (DNA-binding transcriptional dual regulator), *dps* (Fe-binding and storage), and *hemH* (ferrochelatase). Other studies that have shown iron regulatory genes contribute in controlling oxidative stress (Daniele Touati 2000; McHugh et al. 2003) illustrates the importance of iron regulation by the OxyR regulon to contribute in governing oxidative DNA damage from occurring.

1.4.2.1 Fur regulon

It is indisputable that iron availability and its level in the cells affect the redox reaction in aerobic metabolic growing cells since iron is a strong partner for the Fenton reaction that potentiates oxygen toxicity (Halliwell and Gutteridge 1984). Therefore, it is crucially important that a strict regulation of iron metabolism is employed by the cells to ensure the optimal iron levels are kept in balance so that cells cope in between iron homeostasis and that intracellular iron levels are kept at a non-toxic level to defend against oxidative stress. One of the most effective iron homeostasis machinery that is often mentioned in the OxyR regulon is the Fur protein. *fur* gene has its own promoter that is induced 10-fold by OxyR in response to H_2O_2-mediated redox stress (Zheng et al. 1999). The ferric uptake regulator (Fur) protein is itself a transcription factor that controls iron-dependent expression of more than 90 genes in *E.coli* (Klaus Hantke 2001).

Fur protein's function is like a versatile switch in controlling the iron metabolism machinery balance to maintain an optimal intracellular iron level. Fur has been identified as a regulatory protein that controls the genes that are involved in iron acquisition (Klaus Hantke 1982) which is the major pathway that depends on iron bioavailability. As illustrated in **Figure 4**, Fur acts as a positive repressor, by repressing the transcription upon interaction with its co-

repressor, Fe^{2+} and causes a de-repression in the absence of Fe^{2+}. Fe^{2+}-Fur complex normally binds between the -35 and -10 sites of the promoters of Fur-repressed genes. These Fur-binding sites were originally found to conform to a 19-bp palindromic consensus sequence known as the 'iron box' or 'Fur box' (Stojiljkovic, Bäumler, and Hantke 1994). When iron is abundant, the Fur protein blocks the expression of its downstream genes by the Fur-box inactivation on the promoter so that the genes are not transcribed and are inactive states. On the other hand, when iron is scarce, Fur is inactivated, leading to all the genes involved in iron acquisition to be de-repressed (Bagg and Neilands 1987). The vast regulations of the Fur protein are not limited to the repression of its regulon genes but it was found that Fur has positive activation on transcription of certain genes as well (Tsolis et al. 1995; Niederhoffer et al. 1990).

Due to the complexity of the Fur protein which functions to regulate its regulon members, it was found that in the absence of Fur, iron uptake and consumption were imbalanced which led to excessive free iron levels (Abdul-Tehrani et al. 1999; Keyer and Imlay 1996). These findings give insights of the importance of Fur as an all-rounder modulator in maintaining free intracellular iron levels which maybe the key point of defending cells against oxidative stress and hence a form of protection against oxidative DNA damage.

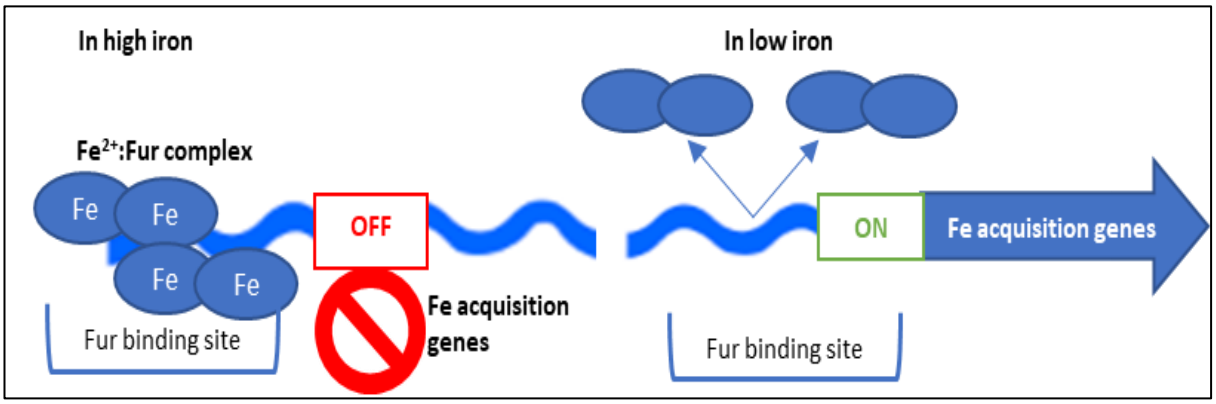

Figure 4: Schematic representation of Fur-mediated gene expression.

1.4.2.2 Dps

Another pathway that keeps iron regulation in balance is through the storage or sequestration of free iron when the intracellular iron level increases. Just like higher organisms, bacteria can deposit intracellular reserves of iron within iron storage proteins (Andrews 1998). The storage is later used to enhance growth when cells undergo iron depletion from the external sources. Iron storage proteins that are recognized in bacteria are found in three types; the archetypal ferritins (Ftn) which are also found in eukaryotes, the heme-containing bacterioferritins (Bfr) found only in eubacteria and the smaller Dps protein which is present only in prokaryotes. All these proteins have a similar molecular structure that gives them the iron-storing capability.

Dps is a ferritin-like protein that possesses ferroxidase activity which is the ability to oxidize bound ferrous ions to the ferric state (Nair and Finkel 2004). This iron storage protein has a hollow center which takes up iron in the soluble ferrous (Fe^{2+}) form but channels the iron to the central cavity where it is deposited as the oxidized ferric form (Fe^{3+}). The stored iron is tightly bound within this central cavity which is called the ferrihydrite core until cells undergo iron depletion and iron is mobilized whenever necessary for relevant biological processes.

There are not many researches that has been conducted on the expression of Dps in response to fluctuations in cellular iron levels. However, it has been shown that Dps expression is highly dependent upon the growth phase in which it is expressed. The regulation of Dps expression is dynamic, complex and varies according to growth stages. In exponential growth after exposure to H_2O_2, Dps transcription is induced by OxyR which will activate the σ^{70}-RNA polymerase. However, towards the stationary phase, Dps mRNA levels are controlled by the *rpos*-encoded σ^S which is the stationary phase-specific sigma factor (Altuvia et al. 1994). Because of its robustness in expression during different growth conditions, Dps has been targeted as a main regulatory protein that is able to protect DNA against redox stress at all times (Nair and Finkel 2004; Almiron et al. 1992).

A study has shown that Dps protein uses H_2O_2 as the oxidant instead of O_2 in aerobic conditions (Zhao et al. 2002). Through spin trap experiment, the study suggested that the primary role of Dps was to bind to the DNA and protect against the detrimental combination of ferrous iron and H_2O_2. To this effect, Dps does not only stores iron through its ferritin-like properties but protects DNA through the detoxification of H_2O_2 which ultimately weakens the lethality of Fenton reaction. Another recent study also supports that the Dps protein plays a multipurpose role in stress protection via its independent dual activities which are the DNA binding activity and the ferroxidase activity (iron storage capability) (Karas, Westerlaken, and Meyer 2015). This explains the importance of Dps in bacterial viability. Currently, there is limited experimental evidence that demonstrates the regulatory role of Dps during nutritional deprivation or oxidative stress. Therefore, the protective mechanism against oxidative DNA damage in Dps expression is further researched in this study.

1.4.2.3 YaaA

With the apparent knowledge that iron regulation in the OxyR regulon is mainly controlled by a set of noticeable genes such as Fur and Dps, it is to the interest of this study that the OxyR also induces novel genes whose function and protective mechanisms remain unknown or unexplained. Among the genes is the *yaaA* gene which was under the group of genes with domain of unknown functions (DUF) was only recently found to be involved in facilitating the binding of intracellular unincorporated iron (Liu, Bauer, and Imlay 2011). In a microarray study, this gene was shown to be induced by the transcription of OxyR (Zheng et al., 2001). Under normal growth conditions, the deficiency of YaaA does not produce a phenotype in the wild type strain MG1655. However, the YaaA deficiency showed a severe growth defect in an engineered *E. coli* strain that accumulates micromolar levels of H_2O_2 by the deletion of the scavenger genes (*Hpx*$^-$). The study showed that YaaA has the ability to reduce oxidative stress by reducing the levels of ·OH level in the *Hpx*$^-$ strain. There are not many literature reviews on this protein with regards to its structure except that the protein presents an especially challenging target for structure prediction as there are no homologs that can serve as templates (Kryshtafovych et al. 2016). In terms of functionality, limited knowledge is known from the study of Liu, Bauer and Imlay on how *yaaA* could play a role in oxidative DNA damage. In the study, it was proposed that YaaA could work in various pathways as illustrated in **Figure 5**; (1) by reducing iron import, (2) by preventing the leakage of iron from H_2O_2-damaged enzymes, (3) by decreasing free iron through sequestration, (4) by speeding iron delivery to the target proteins and (5) by increasing iron exportation. The molecular mechanisms remain unknown as there are little studies regarding this gene that has been done in recent times. With this, it is crucially important that this current study can shed some light on the potential of this particular gene and its relation to the iron regulators of the OxyR regulon.

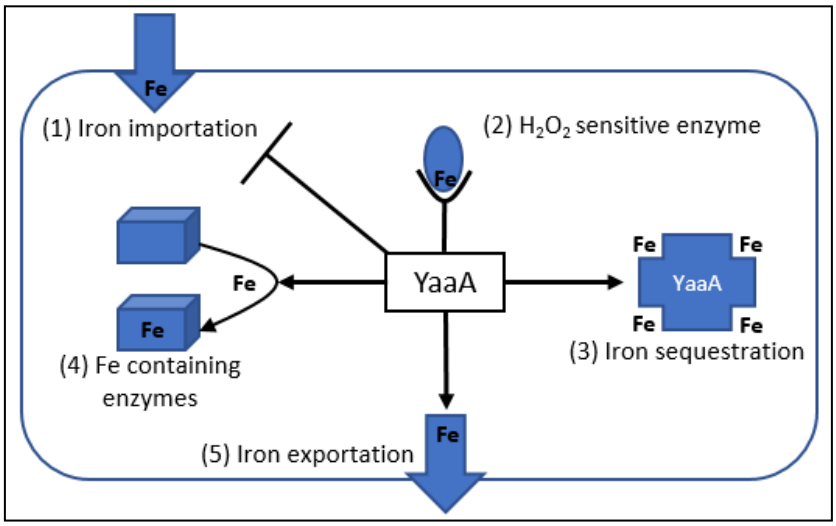

Figure 5: The potential molecular mechanisms by which YaaA can reduce levels of unincorporated iron.

1.4.2.4 TonB

As mentioned earlier, OxyR regulon directly activates the induction of iron regulatory genes such as *fur, dps* and *yaaA*. However, it is important to emphasize that the Fur protein itself controls a complex set of over 90 genes that regulated the iron metabolism machinery. One of the genes that is repressed by Fur is the *tonB* gene (McHugh et al. 2003).

TonB has been shown to be an influential factor that effects on the energy transducing system that assists in iron transportation (K Hantke and Braun 1978) through the potentiation of siderophores. Bacteria cells possess iron carriers called siderophores that has high affinity towards extracellular ferric chelators that help solubilize iron at the outer membrane to ease the transportation of iron into the intracellular of the cells (Guerinot 1994). TonB is important to provide an electrochemical charge gradient across the periplasm through its energy-transducing TonB-ExbB-ExbD protein complex that connects many siderophore receptors at the outer membrane (Larsen et al. 1994; Higgs, Myers, and Postle 1998). The TonB complex is an essential property that deems TonB to be an active player in iron transportation. However, the molecular details of the TonB action remain unresolved in its relevance to oxidative DNA damage. To this effect, I seek to investigate further the role and effect of TonB towards oxidative DNA damage.

1.5 Reduction of intracellular ferrous iron levels

Iron is one of the most important transition metal that is abundantly used by cells of all organisms. Intracellular iron metabolism is versatile because cells adopt different mechanisms to adapt to the active convertible redox states between the reduced ferrous iron (Fe^{2+}) and the oxidized (Fe^{3+}). Iron solubility is dependent on the environmental pH with ferrous iron is relative soluble but the ferric form is extremely insoluble at pH 7 (Andrews, Robinson, and Rodríguez-Quiñones 2003). Because of this, the states of intracellular iron is difficult to be differentiated and measured. Biological functions in cell mostly depend on the redox potentiation of iron to contribute to the electrochemical reaction for energy production and cells' metabolism. However, despite the usefulness of this metal, iron develops into a complex problem in aerobically growing cells. Oxygen and the reduced oxygen species interact with iron which results in a detrimental effect to living cells (Daniele Touati 2000). Therefore, cells need to achieve effective iron homeostasis by balancing the need to efficiently scavenge irons for their optimal supply while carefully guarding against iron induced toxicity. As discussed earlier, through the functionality of some genes in the OxyR regulon, cells can cope with the iron toxicity through some of the iron regulatory genes. However, with the given knowledge that because iron convertibility is rampant, the intracellular ferrous iron level which is the main cause of Fenton reaction is difficult to be measured by experimental procedures. To this end, I use two other alternative methods, the H_2S mediated Fe^{2+} reduction and the 2,2-Bipyridyl that could reduce the intracellular ferrous iron level that may reduce the oxidative DNA damage.

1.5.1 Alternative mechanism of H_2S-mediated protection against oxidative stress

Hydrogen sulfide (H_2S) was not only found to be functioning as a second messenger in many physiological processes in mammals (Kimura 2014) but also functions universally in bacterial defense against reactive oxygen species (ROS) and antibiotics-induced oxidative damage (Shatalin et al. 2011). It was shown that exogenous H_2S donor in various bacterial species could suppress H_2O_2-mediated DNA damage. Therefore, in a latest study, the mechanism of 3-mercaptopyruvate sulfurtransferase (3MST) which is responsible for the bulk of endogenous H_2S generated from L-cysteine pathway was elucidated in *E. coli* grown in LB media (Mironov et al. 2017). In that study, it was shown that 3MST- mediated endogenous production of H_2S suppresses oxidative stress in *E. coli* by sequestering free iron required to drive the genotoxic Fenton reaction. With the use of the *mstA* overexpression strain (P_{tet}-*mstA*), the H_2S was stimulated and oxidative damage was reduced. The study proposed the explanation of a model (as shown in **Figure 6**) that the increased flow of L-cysteine stimulates H_2S production by the AspC-3MST pathway which leads to the sequestration of Fe^{2+} and suppression of the Fenton reaction. The study helped to explain the mechanism of H_2S-mediated protection against oxidative stress and establish the biochemical pathway of H_2S production in response to stress in *E. coli*, which serves as an alternative protection pathway besides the earlier discussed OxyR regulon.

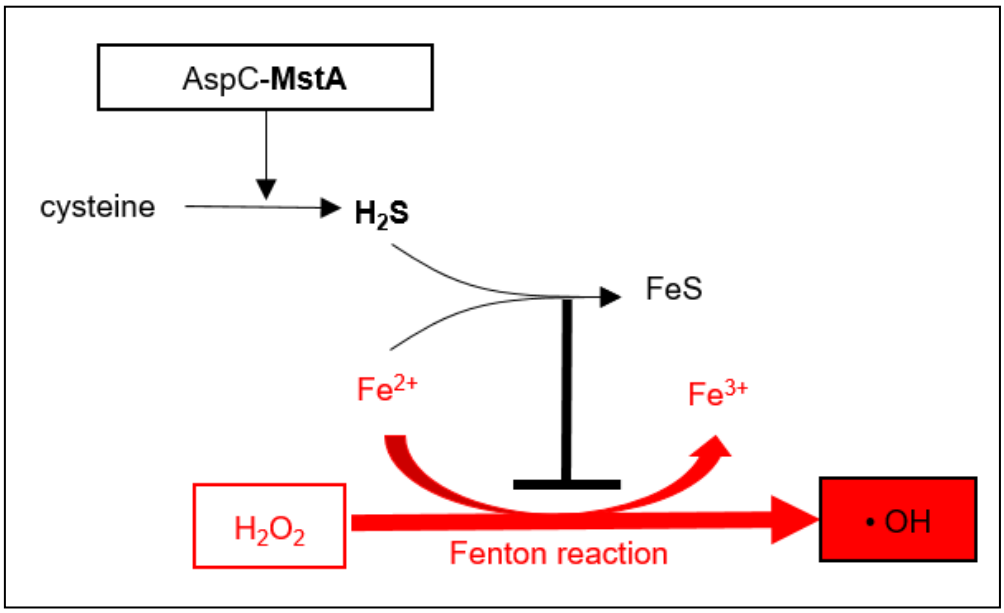

Figure 6: H_2S-mediated defense against oxidative stress in *E. coli*.

1.5.2 Iron chelation by 2,2- Bipyridyl

Genetics factors are often the main tool of study for scientists in elucidating a trait or characterization in biological relevance. However, the challenge of genetic studies is that the whole genome association is complex and that the gene interactions are overlapping with one another. To this end, this study carries out a chemical approach to study the effect of ferrous iron reduction by the addition of an iron chelator called 2,2-Bipyridyl which has been widely used in many studies that are associated to iron binding (Kaes, Katz, and Hosseini 2000).

The 2,2′-bipyridine ligand has been extensively used as a metal chelating ligand due to its robust redox stability and ease of functionalization. **Figure 7** shows the iron chelating activity of the 2,2- Bipyridyl that illustrates the ligand forming complex with the transition metal, mainly the ferrous (Fe^{2+}) iron. The apparent mechanism by which the bipyridyl chelators provide protection is by the removal of the excess iron from the cells (Crichton, Roman, and Roland 1980). Besides that, the tight binding of chelators to iron blocks the ion's ability to catalyze redox reactions. When iron is tightly bound to the chelator molecule, the reactivity of the iron is greatly dampened, hence reducing the occurrence of Fenton reaction. To this effect, the usage of 2,2-bipyridyl as an iron chelator to reduce intracellular ferrous (Fe^{2+}) iron could indicate that iron levels contribute to the cause of oxidative DNA damage.

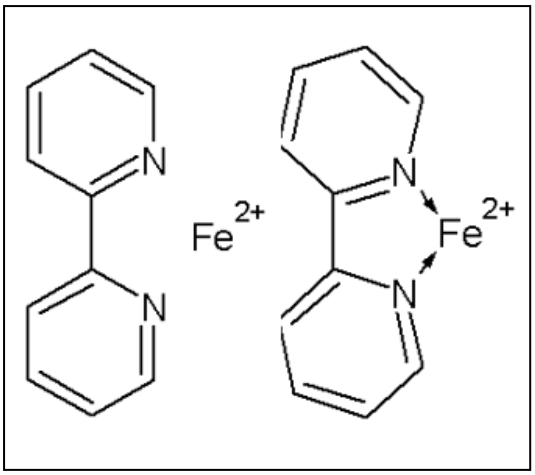

Figure 7: Iron chelating activity of the 2,2- Bipyridyl.

1.6 Rationale and objectives of the study

As mentioned earlier, ROS which is always associated with oxidative stress lead to DNA lesions that bring harmful effect towards genome instability that leads to cell death. ROS studies and effects have long been a challenge to researchers as the detrimental effect of it causes oxidative stress which leads to pathological conditions in human (Aruoma 1998; Maynard et. al., 2009; Maluf et. al., 2013). Human diseases such as cancer, Parkinson, Huntington and Alzheimer disease are all linked to oxidative stress (Rahman et al., 2012; Cooke et al., 2003). This study of ROS and oxidative DNA damage is crucially important even at the microorganism level, as most knowledge and understanding of the ROS share similarity and conservation across all aerobic organisms (Pavelescu 2015).

The knowledge about how ROS are being regulated in the cells remains to be explored. The intracellular H_2O_2 is being scavenged by known scavenger proteins but on the contrary, the reactive ·OH is left unattended to, which leads to the major cause of oxidative DNA damage that ultimately leads to mutagenesis and genomic instability. Therefore, I would like to find out how cells are able to control and regulate the balance of this ROS level, particularly the reduction of production of ·OH which is the major cause of oxidative DNA damage.

As mentioned in the literature review, ·OH production is mainly caused by the Fenton reaction which is fueled by the presence of high intracellular iron. It has been indicated that iron regulation is important to maintain the optimal metabolic state of the cells throughout all organisms (Lasocki, Gaillard, and Rineau 2014). Therefore, the control of iron regulation is crucially important to ensure that cells are not badly affected by any iron toxicity that ultimately leads to oxidative stress and DNA damage. This study illuminates how OxyR and its regulon genes play a major role in suppressing oxidative DNA damage by controlling the iron metabolism hence hampering the Fenton reaction from occurring.

In the first part of this study, I elucidate OxyR and its regulon genes in suppressing the oxidative DNA damage by imploring the strains defective of the 8-oxoG repair enzymes, *ΔmutM ΔmutY*. As shown previously in our laboratory, when *ΔmutM ΔmutY* cells are grown in minimal medium with different carbon sources, cells showed higher mutagenesis level in comparison to *ΔmutM ΔmutY* cells grown in normal nutrient condition, indicating that growth conditions affect oxidative DNA damage level. However, when the same experiment was carried out in the wild type strain, mutagenesis levels did not show any changes across all growth conditions. The study has thus given an indication that the *ΔmutM ΔmutY* strain is a good oxidative DNA damage level indicator and that low nutrient condition leads to oxidative stress that causes oxidative DNA damage. With that, I would like to show the involvement of OxyR and its regulon genes which may contribute to suppressing oxidative DNA damage when cells are grown under a normal physiological condition and low nutrient condition (minimal media) which mimics a stressed condition. I hoped to clarify that oxidative DNA damage is caused by the abundance of ·OH which can be efficiently suppressed by the OxyR and its

regulon genes.

For the second part of the study, I would like to show that the involvement of iron, particularly ferrous (Fe^{2+}) iron affects the oxidative DNA damage level. As mentioned earlier, the oxidative DNA damage is affected by the presence of the reactive ·OH that is easily produced by the presence of ferrous (Fe^{2+}) iron that drives the Fenton reaction. As OxyR and its regulon genes are one of the proposed oxidative stress regulators, I would like to show other alternative methods that could help reduce ferrous (Fe^{2+}) iron hence mediating protection to cells against oxidative DNA damage. I seek to prove that the oxidative DNA damage level is more inclined to the presence of iron rather than the high intracellular H_2O_2 which can be easily scavenged by the cells.

Continuing from the first and second part, as OxyR and its regulon genes are involved in oxidative DNA damage through the iron metabolism pathway, I would like to investigate on the gene interactions of the iron regulatory genes of the OxyR regulon. This is because the network of iron regulation transcribed by the OxyR protein is complex, especially genes that are pertaining to iron acquisition functions. In addition, I would like to concentrate on the newly found gene function, *yaaA* which is suspected to be one of the key players in reducing oxidative DNA damage. Due to the insufficient literature review of YaaA's interaction with the other iron regulatory proteins of the OxyR regulon, I seek to discover the molecular mechanisms that may give clues to the protein's functionality.

Chapter II. Materials and Methods

2.1 Materials

2.1.1 Strains and plasmids

Strains and plasmids that were used in this study are listed in **Table 1** and **Table 2** respectively.

Table 1: List of strains used in this study.

Strain	Genotype	References
MG1655 (Wild type)	F- lambda- *ilvG- rfb*-50 *rph*-1	(Guyer et al., 1981) Laboratory stock
MK7180	MG1655 *ΔmutM ΔmutY::FRT*	Laboratory stock
JW3933	BW25113 *ΔoxyR::kan*	Keio collection (Baba et al., 2006)
JW0005	BW25113 *ΔyaaA::kan*	Keio collection (Baba et al., 2006)
JW0797	BW25113 *Δdps::kan*	Keio collection (Baba et al., 2006)
JW0669	BW25113 *Δfur::kan*	Keio collection (Baba et al., 2006)
JW3914	BW25113 *ΔkatG::kan*	Keio collection (Baba et al., 2006)
JW0598	BW25113 *ΔahpC::kan*	Keio collection (Baba et al., 2006)
JW0833	BW25113 *ΔgrxA::kan*	Keio collection (Baba et al., 2006)
JW2566	BW25113 *ΔtrxC::kan*	Keio collection (Baba et al., 2006)
JW5195	BW25113 *ΔtonB::kan*	Keio collection (Baba et al., 2006)
MK9614	MK7180 *ΔoxyR::FRT*	This study: P1 (MK7180 x JW3933)
MK9624	MK7180 *ΔkatG::FRT*	This study: P1 (MK7180 x JW3914)
MK7942	MK7180 *Δfur::FRT*	Laboratory stock
MK9406	MK7180 *Δfur Δdps::FRT*	Laboratory stock
MK9404	MK7180 *Δdps::FRT*	Laboratory stock
MK9612	MK7180 *ΔyaaA::FRT*	This study: P1 (MK7180 x JW0005)
MK9616	MK7180 *Δdps ΔyaaA::FRT*	This study: P1 (MK9612 x JW0797)
MK9617	MK7180 *Δfur ΔyaaA::FRT*	This study: P1 (MK9612 x JW0669)
MK9618	MK7180 *Δfur Δdps ΔyaaA::FRT*	This study: P1 (MK9616 x JW0669)
MK9627	MK7180 *ΔtonB::FRT*	This study: P1 (MK7180 x JW5195)
AM3009	MG1655 plus p_{tet}-*mstA*-Cm	Gift from Mironov et.al, 2017
MK9628	MK9614 p_{tet}-*mstA*-Cm	This study: P1 (MK9614 x AM3009)
MK9629	MK9616 p_{tet}-*mstA*-Cm	This study: P1 (MK9616 x AM3009)

Table 2: List of plasmids used in this study.

Plasmids	Phenotype	References
pCP20	FLP, *amp*^{r}, *cm*^{r}	(Datsenko and Wanner 2000)
pTN249	*ahpC*-GFP, pSTV29	(Nakayashiki et al., 2013)
pBR322	*amp*^{r}, *tet*^{r}	(Bolivar et al., 1977)
pBR322-*oxyR*	*amp*^{r},	This study
pASK-IBA3plus	*amp*^{r}, *tet*^{r}	IBA Lifesciences
pASK-IBA3plus-*yaaA*	*amp*^{r}, *tet*^{r}	This study
pASK-IBA3plus-*dps*	*amp*^{r}, *tet*^{r}	Laboratory stock

2.1.2 Synthetic oligonucleotides

Synthetic oligonucleotides were obtained from Integrated DNA Technologies, diluted with 1x TE buffer (10mM Tris-HCl (pH8.0), 1mM EDTA) to 10mM working stock solutions and stored at -30°C.

Table 3: List of synthetic oligonucleotides used in this study.

Name	Sequence (mer)
SA-1-MutM	5’- ATCCAGTTGTTCGCCAGCACGT -3’ (22mer)
SA-2-MutM	5’- CATCAGGCGCTGATGGCGAAG -3’ (21mer)
SA-3-MutY	5’- GTTGCCGGATGCAAGCATGATAAG -3’ (24mer)
SA-4-MutY	5’- CTGACCTTCTGCTTCACGTTGC -3’ (22mer)
oxyRkan Fwd	5’- AAGAGGTGCCGCTCCGTTTCTG -3’ (22mer)
oxyRkan Rvs	5’- TATTCACTGCTTTGGCGAGCGC -3’ (22mer)
katG km Fwd	5’- TCGATGTCGAAAGCTACCTGCGT -3’ (23mer)
katG km Rvs	5’- CCTGAGCTATACCATGCCGTTCT -3’ (23mer)
Fur km Fwd	5’- GGCCTTGCCGTTGTAAATGTAAGC -3' (24mer)
Fur km Rvs	5'- CTGGATTATCAGCAGTGTCTGCGT -3' (25mer)
dps Fwd	5’- TCCTGGCGAGCAGATAAATA -3’ (20mer)
dps Rvs	5’- CGATTTATAGGGCAATACCC -3’ (20mer)
yaaAkan Fwd	5'- TGACCGCCATTTTACTGCTCTC -3' (22mer)
yaaAkan Rvs	5'- CGCTAAATGACAAATGCCGGGT -3' (22mer)
gapA RT forward	5'- ACCACCGTTCACGCTACTACC -3' (21mer)
gapA RT reverse	5'- CAGTCTTTGTGAGACGGGCCA -3' (21mer)
oxyR RT Forward	5’- GGGGCGGATGAAGATACAC -3' (19mer)
oxyR RT Reverse	5’- CACCATGTTGCGCAGAGT -3' (18mer)
fur RT Forward	5’- TGAAAGTAACGCTTCCTCGTT -3' (21mer)
fur RT Reverse	5’- ATCTTCCGCACTGACGTGATT -3' (21mer)
yaaA RT Forward	5’- TGCCGGAGCTGTTAGACAA -3' (19mer)
yaaA RT Reverse	5’- CGCATCAGCGTGCTAATCT -3' (19mer)
tonB RT Forward	5’- CGCCTGACATCAAGTACAGC -3' (20mer)
tonB RT Reverse	5’- TCCTGAAGCCACACTGGTAA -3' (20mer)
dps RT Forward	5’- GCGGCGCTAACTTCATTGC -3' (19mer)
dps RT Reverse	5’- TCTGCCATGGTATCCAGATG -3' (20mer)
pBR322_infus_F	5’- AACCAACCCTTGGCAGAAC -3’ (19mer)
pBR322_infus_R	5’- TTCTTGAAGAGCAAAGGG -3’ (18mer)
GibsonN oxyR Fwd	5’- CACGAGGCCCTTTGCTCTTCAAGAATTGCTATTCTACCT ATCGCC -3’ (45mer)
GibsonN oxyR Rvs	5’- GTTCTGCCAAGGGTTGGTTTGCGCATTCACTTAAACCG CCTG -3’ (42mer)

Seq-pBR-oxyR Fwd	5'- CGCGTATCGGTGATTCATTC -3' (20mer)
Seq-pBR-oxyR Rvs	5'- GGTTATTGTCTCATGAGCGG -3' (20mer)
pASK-IBA3plus-Inverse-F1	5'- TTGGAGCCACCCGCAGTTCGAAA -3' (23mer)
pASK-IBA3plus-Inverse-R	5'- CATTTGTATATCTCCTTCTTAAAG -3' (24mer)
Gibson yaaA Fwd	5'-TTTAAGAAGGAGATATACAAATGCTGATTCTTATTTCACCTGCGAAAACG -3' (50mer)
Gibson yaaA Rvs	5'-TTTCGAACTGCGGGTGGCTCCAATTAACGCTGCTCGTAGCGTTTAAACAC -3' (50mer)
pASK Sequencing Fwd	5'- GAGTTATTTTACCACTCCCT -3' (20mer)
pASK Sequencing Rvs	5'- CGTTTACCGCTACTGCG -3' (17mer)

2.2 Chemicals and media

2.2.1 Chemicals

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

For real-time quantitative PCR: Qiagen RNeasy Protect Bacteria Mini Kit was used for total RNA extraction, SuperScript™ III Reverse Transcriptase from Invitrogen was used for cDNA synthesis and LightCycler® 480 SYBR Green I Master from Roche was used for real-time PCR amplification.

2.2.2 Media

LB medium: 1% (w/v) Bacto Tryptone, 0.5% (w/v) Bacto yeast extract, 1% (w/v) NaCl, pH 7.0. LB plate was prepared by adding LB medium with 1.5% Bacto agar. The medium was autoclaved at 121℃ for 20 minutes, cooled down at 50℃ in the water bath and poured into petri dishes.

M9 medium: Dilution was made from 5X M9 stock (210mM Na_2HPO4, 110mM KH_2PO_4, 43mM NaCl, 94mM NH_4Cl, autoclaved at 121℃ for 20 minutes and stored at room temperature. For M9 glucose agar, 1.5% Bacto agar was added with supplementation of 0.01mM $CaCl_2$ and 1mM $MgSO_4$ and 0.2% of glucose which was sterilized by 0.2μm filter ("M9 Minimal Medium (Standard)" 2010).

R-agar for P1 transduction: 1%(w/v) Bacto Tryptone, 0.1% (w/v) Bacto yeast extract,

0.8% (w/v) NaCl, 1.2% (w/v) Bacto agar were autoclaved at 121°C for 1 minute, then cool down at 50°C in the water bath before pouring onto petri dishes. For R-top agar, composition is similar except 0.8% (w/v) Bacto agar was used and 2.0mM $CaCl_2$ and 0.1% glucose was added.

Antibiotics: A stock solution of antibiotics were prepared and added when needed. The final concentration were: ampicillin 100µg/ml, kanamycin 50µg/ml, chloramphenicol 25µg/ml and rifampicin 100µg/ml. Ampicilin and kanamycin stocks were prepared by diluting with deionized water while chloramphenicol was with ethanol and rifampicin was with methanol.

2.3 Methods

2.3.1 Construction of deletion mutant strains by P1 transduction

All strains deletion was done by transducing P1 virus lysate as a donor into recipient strains of interest. Firstly, P1 virus lysate was prepared. To prepare the lysate, a donor strain with the gene of interest (from the Keio collection) was inoculated into 5ml LB broth containing kanamycin and cultured overnight. A new inoculation of 1/100 dilution in LB containing 5mM $CaCl_2$ was made the following day from the overnight culture and shaken in the water bath until OD_{600} reached 0.4-0.5 (about 10^8 cells/ml). After appropriate OD has reached, original P1 virus lysate was diluted to 10^9phage/ml and mixed with the culture. The culture was mixed with original P1 lysate with different ratios of cells to virus; 1:1, 10:1, and 100:1 to a final volume of 1ml. The culture without any phage infection served as a control. All mixtures were incubated at 37°C for 20 minutes. Then, the mixtures of bacteria cells and virus were added into 2.5ml of R-top agar, vortexed lightly and poured onto R-plates. These plates were incubated at 37°C for at least 8 hours. P1 donor lysate was collected by scraping the soft agar layer and centrifuged at 9000rpm for 20 minutes. Chloroform was added into the new P1 virus donor lysate to eliminate the remaining cells of the donor strain. The new donor virus lysate was stored at 4°C until further use.

After obtaining the donor lysate, a phage titration was made to determine the phage titer of the donor lysate. The protocol for titration of P1 virus lysate was similar as the lysate preparation except for the incubation time of infection was 10 minutes in water bath. Apart from that, the number of plaques formed on the R-top agar were counted to determine the number of phages or PFU (plaque forming unit). PFU was calculated by the following formula:

$$\text{PFU/ml} = \frac{\text{Dilution factor x number of plaques formed}}{\text{Volume of virus incubated with cells}}$$

After having known the virus titer, the donor phage was then infected into the recipient cells with the similar protocol as above. Virus were diluted to 10^9phage/ml with MC buffer (0.1M $MgSO_4$, 5mM $CaCl_2$). Donor phage and recipient cells (with OD_{600} around 0.8-0.9) were

mixed and incubated at 37°C for 30 minutes in water bath, after which 0.2ml of 0.1M sodium citrate was added to inhibit the reabsorption of the P1 virus to recipient cells and to reduce phage lysis. The mixtures were then added with 1ml LB and incubated at 37°C at a rotating shaker for another 1.5 hours. Finally, the mixtures were spread on LB containing 5mM sodium citrate and 50µg/ml kanamycin. Transductants grown on the kanamycin plates were confirmed by PCR with the appropriate primers.

Transductants of the P1 virus donor from the Keio collection carries the kanamycin fragment which was needed to be eliminated. To do so, the kanamycin cassette was popped out by using an established pCP20 plasmid transformation method (Datsenko and Wanner 2000). Electrocompetent cells were prepared from the successful transductants (Green and Sambrook 2012). Competent cells were electroporated, incubated at 30°C by rotating for 1.5 hours and spread on LB agar with 25µg/ml chloramphenicol. Plates were incubated at 30°C for overnight. The transformants were checked for their phenotype whereby the correct ones were those that were sensitive to kanamycin and resistant to chloramphenicol. After then, the pCP20 plasmid was removed by incubating at 42°C for overnight and the phenotype was checked again for kanamycin and chloramphenicol sensitive. The construct of the new strains was finally confirmed by the appropriate primers.

2.3.2 Cloning of plasmid harboring gene of interest

Plasmid was cloned with the gene of interest by using the Gibson cloning method (Gibson et al., 2009). Primers were designed for the plasmid of choice and gene of interest for PCR amplification by having a primer with 5′ end that is identical to an adjacent segment and a 3′ end that anneals to the target sequence. DNA segments were amplified separately to obtain the correct amplification size and yield. Both plasmid and gene DNA segments were then combined in equimolar concentrations in a Gibson assembly reaction. The Gibson cloning master mix consists of different enzymes and buffers with recipe according to the original method (Gibson et al., 2009). The Gibson assembly mix was incubated for 1 hour at 50°C. The DNA was transformed into competent cells of *E. coli* DH5α by electroporation. The correct plasmid product was screen by restriction digestion for proper orientation and size. Finally, the important regions of the seams between the assembled plasmid and gene were sequenced for confirmation.

2.3.3 Measurement of mutation frequency by rifampicin resistant assay

Cells were grown in overnight culture at 37°C. The overnight culture was diluted to optimal dilution factor with LB medium and 100µl of the diluted culture was spread onto LB plate to obtain about 100 colonies after overnight incubation at 37°C. When colonies formation reached to about 1.5mm in diameter, cells were harvested with 5ml cold LB medium. Optimal dilutions were made with LB and the diluted cells were spread onto LB and LB containing 100µg/ml

rifampicin plates. LB plates were incubated for 16 hours while rifampicin plates for 24 hours, after which, colonies were counted. Mutation frequency was calculated by determining the number of cells that were able to grow on the rifampicin plates (rifampicin resistance cells) over the total number of viable cells which were able to grow on the LB plates.

For the determination of mutation frequency of cells grown in minimal media, the method is similar as above with the exception that the overnight culture was diluted with a 1x M9 salt solution and spread unto M9 plates containing 0.2% glucose to obtain an optimal population for harvesting and mutation frequency determination.

Mutation frequency for all strains were determined for 20 experiments. Data obtained were subjected to statistical analysis by Mann-Whitney U test with significance level determined. Data is initially represented by a scatter distribution graph (**Figure 9**) but is then converted to bar graphs for better observation.

2.3.4 Measurement of intracellular H_2O_2 – GFP level using flow cytometer

Strains under study were transformed with the GFP fusion plasmid (pTN249-*ahpC*-GFP) according to standard electroporation methods (Green and Sambrook 2012). Cells harboring the plasmids were grown overnight in 5ml LB containing chloramphenicol 25µg/ml at 37°C for 14 hours. The overnight culture was diluted with optimal dilution (to obtain about 100 colonies per plate) with LB and spread on LB plates or similarly, diluted with 1x M9 salts solution and spread on M9 + 0.2% glucose plates. Plates were incubated at 37°C until colony formation reached 1.5mm in diameter. Cells were then harvested with 5ml 1xM9 salts containing 0.2% sodium azide and washed to 10^{-3} cells with 1xPBS (phosphate – buffered saline: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4). At least three samples were prepared from the harvested cells. Washed cells were then measured for H_2O_2 –GFP level by using a FACScan flow cytometer (Becton Dickinson). The flow cytometer measured approximated single-cell portion of the cell population and yielded the relative fluorescence values of the GFP. The relative fluorescence depicts the intracellular H_2O_2 level which was read as the GFP signals. Intracellular H_2O_2 levels were determined by the taking mean of the relative fluorescence of the three samples measured. At least two independent experiments were made for each strain of interest. The negative control used for each experiment were original strains with the gene of interest without any insertion of the GFP fusion plasmid (pTN249-*ahpC*-GFP).

2.3.5 H_2O_2 sensitivity assay by disk diffusion

Overnight cultures were made by incubation of the strains of interest in LB medium at 37°C. A 1/100 dilution was made by adding 50µl of the overnight culture into 5ml of LB medium. The inoculation was let to grow in a 160rpm shaking water bath until OD_{600} reached to about 0.35 to 0.4. Then 0.5ml of the culture was mixed with 4.5ml soft agar and poured onto LB plate. The soft agar was left to solidify upon then, a piece of disk (5mm diameter) was

placed in the middle. 10µl of 30% H_2O_2 (about 8.8M) was spotted on the disk and the plate was incubated at 37°C for overnight. The H_2O_2 sensitivity of the strain of interest was measured by calculating the mean diameter of the zone of inhibition from three different angles exhibited in the lawn of grown cells on the plate. Two independent experiments were carried out for each strain to determine the H_2O_2 sensitivity.

2.3.6 Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

E. coli cells were lysed with 1 mg/ml lysozyme in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA buffer. Total RNA was prepared based on the manufacturer's protocol of the RNeasy Mini Kit (Qiagen, Valencia, CA) and the RNase-Free DNase Set (Qiagen). The total RNA was measured using the NanoDrop™ 2000/Spectrophotometer to determine the nucleic acid concentration and the $OD_{260/280}$ ratio. Complementary DNA was then synthesized from a 10µg of total RNA using the SuperScript™ III Reverse Transcriptase Kit with random hexamers from Invitrogen. For the real-time quantitative polymerase chain reaction (qPCR), cDNA was amplified with oligonucleotide primers specific to each target gene using the LightCycler® 96 System (Roche). Reactions contained LightCycler® 480 SYBR Green I Master Mix (Roche), forward and reverse primers (0.1µM each), and a cDNA template (20ng). For the real-time PCR amplification, the following conditions were used: preincubation at 95 °C for 300seconds, 3-steps amplification with 45 cycles of PCR at 95 °C for 10 s, 55 °C for 10 seconds; melting curve dissociation for 1 cycle at 95 °C for 5 seconds, 65 °C for 60 seconds, and 97 °C for 1 second and finally cooling at 37 °C for 30 seconds. A standard curve method from genomic DNA was obtained with a 10-fold serial dilution of each of the gene of interest. The absolute quantification value of each sample was extrapolated from the standard curve. The transcript level of each genes was normalized with the *gapA* gene expression as the reference gene. Each qRT-PCR was performed at least in triplicate, and average data are reported.

Chapter III. Results

Part 1: Involvement of OxyR regulon genes in oxidative DNA damage

As mentioned in the literature review, the potential of OxyR as a suppressor in oxidative DNA damage is vast, and the mechanisms of it were investigated further. To prove that OxyR is a major stress regulator, the *oxyR* deletion mutant was first constructed by P1 transduction into the defective repair system strain *ΔmutM ΔmutY*. The strain was then subjected to H_2O_2 sensitivity test by the disk diffusion assay. As shown in **Figure 2**, the triple mutant strain *ΔoxyR ΔmutM ΔmutY* showed hypersensitivity in comparison with the *ΔmutM ΔmutY* strain. This result suggests that OxyR is crucially important in regulating extreme stress condition, particularly in the presence of elevated H_2O_2 condition. When the deletion mutant strain was complemented with a plasmid harboring the *oxyR* gene and its native promoter (*ΔoxyR ΔmutM ΔmutY* pBR322/*oxyR*), the H_2O_2 resistance returned almost to the level of the wild type strain (*ΔmutM ΔmutY*). This shows that OxyR plays an important role in the cell's defense mechanism against exogenous H_2O_2 treatment.

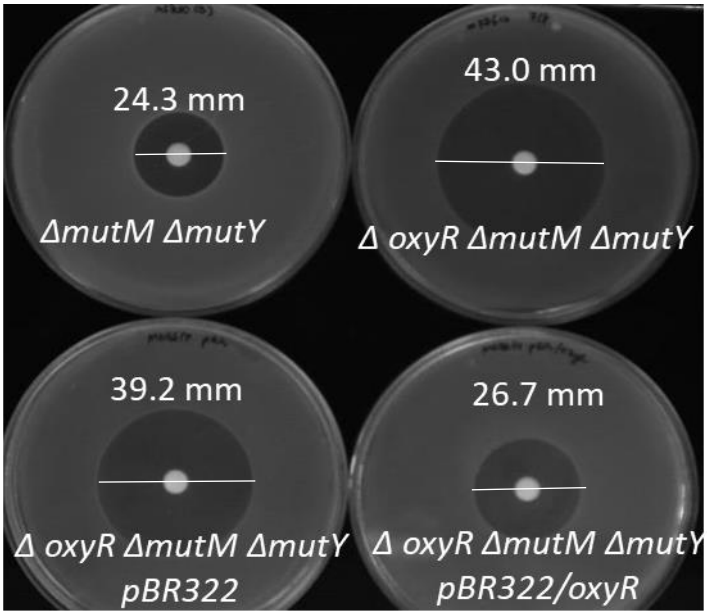

Figure 8: H_2O_2 sensitivity of *ΔoxyR ΔmutM ΔmutY* by disk diffusion assay. Data label are the average diameter of inhibition zones in mm for two independent experiments.
H_2O_2 sensitivity of *ΔoxyR ΔmutM ΔmutY* is significant against *ΔmutM ΔmutY*.
H_2O_2 sensitivity of *ΔoxyR ΔmutM ΔmutY* pBR322/*oxyR* is significant against *ΔoxyR ΔmutM ΔmutY* pBR322. Significance was calculated by independent *t*-test with $p<0.01$.

1. OxyR suppresses oxidative DNA damage

When growth phenotype was observed for the *oxyR* mutant, results showed that the mutant strain was a much slower grower both in LB and M9+glucose minimal media (**Table 4**). In addition, results showed that the oxidative DNA damage level was increased in the *ΔoxyR ΔmutM ΔmutY* strain in comparison with the *ΔmutM ΔmutY* strain by 2.7-folds and 14.2-folds in LB media and M9+glucose minimal media respectively (**Figure 9**). This data confirms that the *oxyR* gene is a major key player in reducing oxidative DNA damage. Since OxyR is a transcriptional dual regulator for its regulon members, it can be observed that the disruption of the *oxyR* gene will cause a detrimental effect as shown in the elongation period of growth and the increased in mutation frequency.

The mutation frequency of the *oxyR* mutant is increased in an exponential fold by more than 10 times in M9+glucose minimal media (**Figure 9** and **Figure 10**). In minimal media with glucose, cells use glucose as the carbon source for growth in contrast to the utilization of amino acids in LB media. The growth of cells in the minimal media utilized the catabolite repression pathway which yields a higher production of H_2O_2 by the consumption of energy in the cells and the leakage of electron in the respiration chain (B. Gonzalez-Flecha and Demple 1995). Therefore, the use of M9+glucose mimics an environment of high intracellular H_2O_2 level which also causes the increase of oxidative DNA damages. Besides that, it has been shown in previous studies of our lab members, the oxidative DNA damage increased in different folds when *ΔmutM ΔmutY* were grown in minimal media with different carbon sources. Hence, as shown in the mutation frequency of *ΔoxyR ΔmutM ΔmutY*, the oxidative DNA damage level increased tremendously in comparison to only the *ΔmutM ΔmutY* strain when the mutant strain was grown in M9+glucose minimal media.

Table 4: Growth phenotype of mutant *ΔoxyR* grown in different growth conditions.
Growth time is measured for the strain to form colonies of 1.5 mm in size in the respective media. A slow growth phenotype is indicated by a longer time while a rapid growth is indicated by a shorter time for the strain to form the 1.5 mm colony size.

Strain	**LB (hours)**	**M9+glucose (hours)**
MK7180 (*ΔmutM ΔmutY*)	12-14	43-45
MK9614 (*ΔoxyR ΔmutM ΔmutY*)	20-22	72 (3 days)

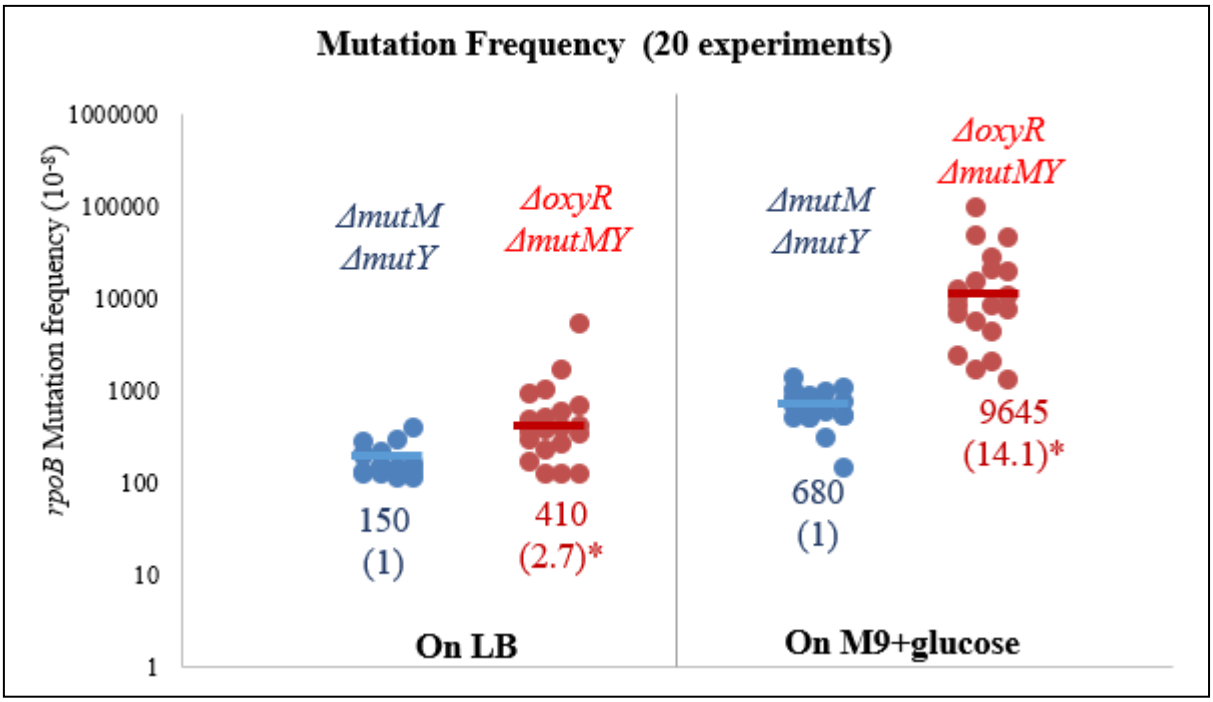

Figure 9: Distribution of mutation frequencies determined with *ΔoxyR ΔmutM ΔmutY* cells grown in LB and M9+glucose minimal media. Colored dots indicated are the individual experiments while the bars indicated represent the median levels and the data label of the colored dots indicated are the median value for 20 experiments. The values in parenthesis are the fold-change in comparison to the wild type. * indicates significance $p<0.01$ calculated by Mann-Whitney U test against the *ΔmutM ΔmutY* strain in the respective media.

For the ease of data interpretation, henceforth from this data, all mutation frequency levels are shown as median mutation frequency represented in a bar chart as shown in **Figure 10.**
All raw data with the distribution of mutation frequencies (as shown in **Figure 9**) can be found in the **Supplementary Figures** section.

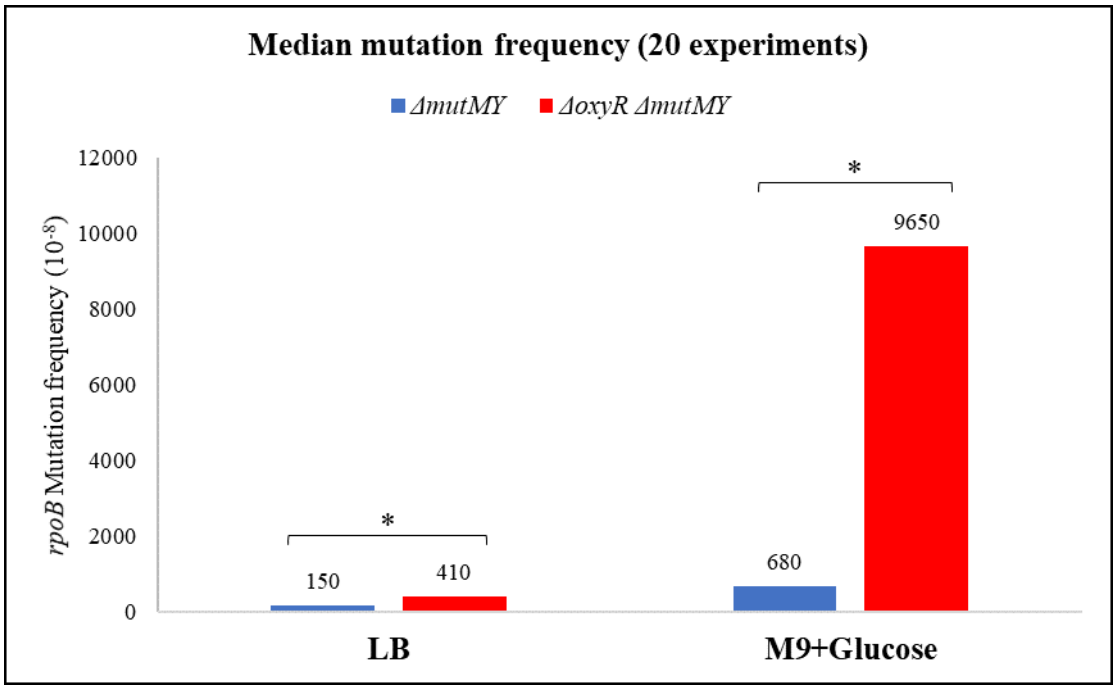

Figure 10: Representative median mutation frequency of *ΔoxyR ΔmutM ΔmutY* in LB and M9+glucose minimal media for 20 experiments. * indicates significance p<0.01 calculated by Mann-Whitney U test.

2. Important genes of the OxyR regulon

As I have shown that OxyR plays an important role as an oxidative stress regulator through the H_2O_2 sensitivity assay and can suppress oxidative DNA damage shown by the mutation frequency, I would like to know which genes in the regulon that are playing a major role in suppressing the oxidative DNA damage. To determine that, the H_2O_2 sensitivity disk diffusion assay was employed to determine which mutants in the regulon show the highest reactivity towards oxidative stress. Since OxyR is a transcriptional regulator for many genes of which some genes function in scavenging H_2O_2 and some other genes regulate iron metabolism, I chose a selection of eight major genes that are highly induced by OxyR based on a previous microarray study by (Zheng et al. 2001).

From **Figure 11**, it was revealed that *katG* deficient strain and *fur* deficient strain showed the highest H_2O_2 sensitivity. *katG* mutant showed H_2O_2 sensitivity (38.5 mm inhibition zone) that is almost equivalent to that of the *oxyR* mutant (38.7 mm inhibition zone). This indicates that KatG is indeed one of the major stress regulator when cells undergo extreme H_2O_2 concentration. This result is supported by other studies that state that KatG is a primary detoxifier that mediates redox agents such as H_2O_2 (Greenberg and Demple 1988).

On the other hand, it was found that the deletion of *fur* gene also showed H_2O_2 sensitivity of 34.9 mm inhibition zone despite the level of sensitivity is less than the *katG* deletion strain. This result suggests that Fur plays a role when cells undergo stress condition.

Even though Fur is not involved in H_2O_2 scavenging system, the stress-induced redox reaction concurrently activates the iron regulation mechanisms through the function of Fur (Stortz and Hengge-Aronis 2000).

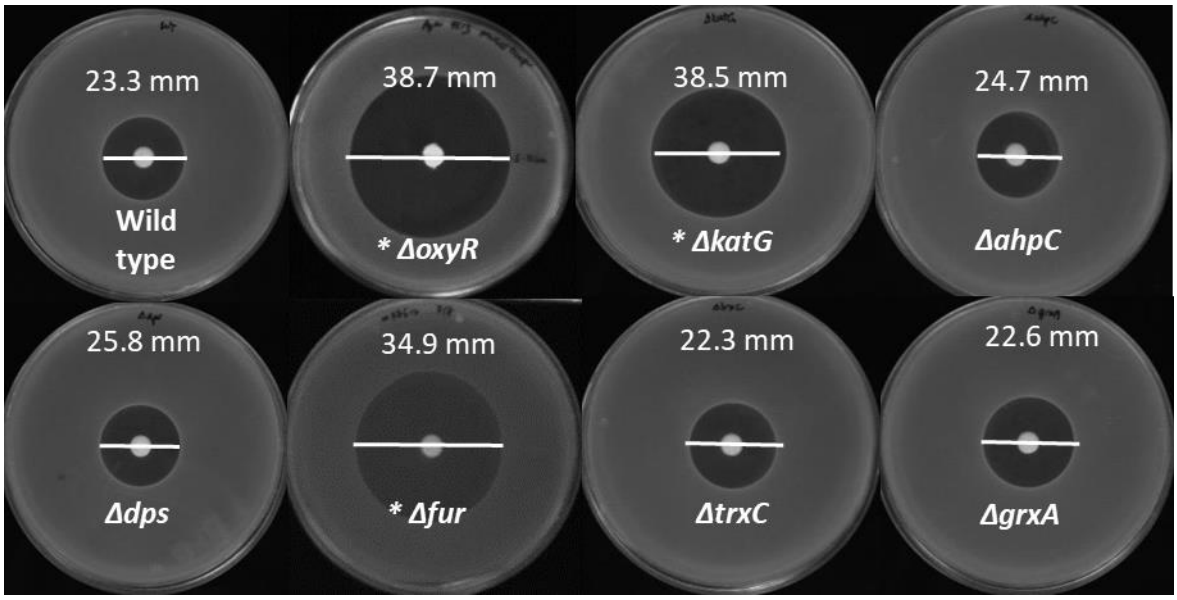

Figure 11: H_2O_2 sensitivity of genes from the OxyR regulon by disk diffusion assay.
Data label are the average diameter of the inhibition zone in mm for two independent experiment. Mutant genes tested were wild type strain (BW25113), *oxyR*::Km BW25113, *katG*::Km BW25113, *ahpC*::Km BW25113, *dps*::Km BW25113, *fur*::Km BW25113, *trxC*::Km BW25113 and *grxA*::Km BW25113. * indicates significance $p<0.01$ in comparison with the wild type, calculated with the independent *t*-test.

3. Involvement of KatG in suppressing oxidative DNA damage

With the results shown that KatG plays an important role in stress regulation, I next determined the level of oxidative DNA damage by the mutant strain *ΔkatG ΔmutM ΔmutY*. The growth phenotype of the *katG* mutant is shown in **Table 5**. Similar to the *oxyR* deletion strain, the *katG* mutant showed a slower growth phenotype than the *ΔmutM ΔmutY* strain of up to 24 hours in LB media and 46 hours in M9+glucose minimal media. In **Figure 12**, it was found that *katG* deletion strain increased mutation frequency level by a significant 2.3-fold in LB media in comparison to the *ΔmutM ΔmutY* strain. However, it was found that in M9+glucose minimal media, the *katG* deletion strain did not show much effect in the level of oxidative DNA damage in comparison to the *ΔmutM ΔmutY* strain. Even though it is expected that KatG would be able to suppress oxidative DNA damage by its H_2O_2 scavenging activity, this was only seen in LB media. In M9+glucose minimal media, where the nutrient condition is scarce, the H_2O_2 level which was supposed to be higher did not affect much on the level of the oxidative DNA damage

upon the *katG* gene deletion. This signifies that there is another important mechanism that keeps the KatG deficit in balance to control the intracellular H_2O_2 level or another mechanism that controls the excessive production of ·OH that results from the intracellular H_2O_2.

Table 5: Growth phenotype of mutant *ΔkatG* grown in different growth conditions.
Growth time is measured for the strain to form colonies of 1.5 mm in size in the respective media.

Strain	**LB (hours)**	**M9+glucose (hours)**
MK7180 (*ΔmutM ΔmutY*)	12-14	43-45
MK9624 (*ΔkatG ΔmutM ΔmutY*)	22-24	44-46

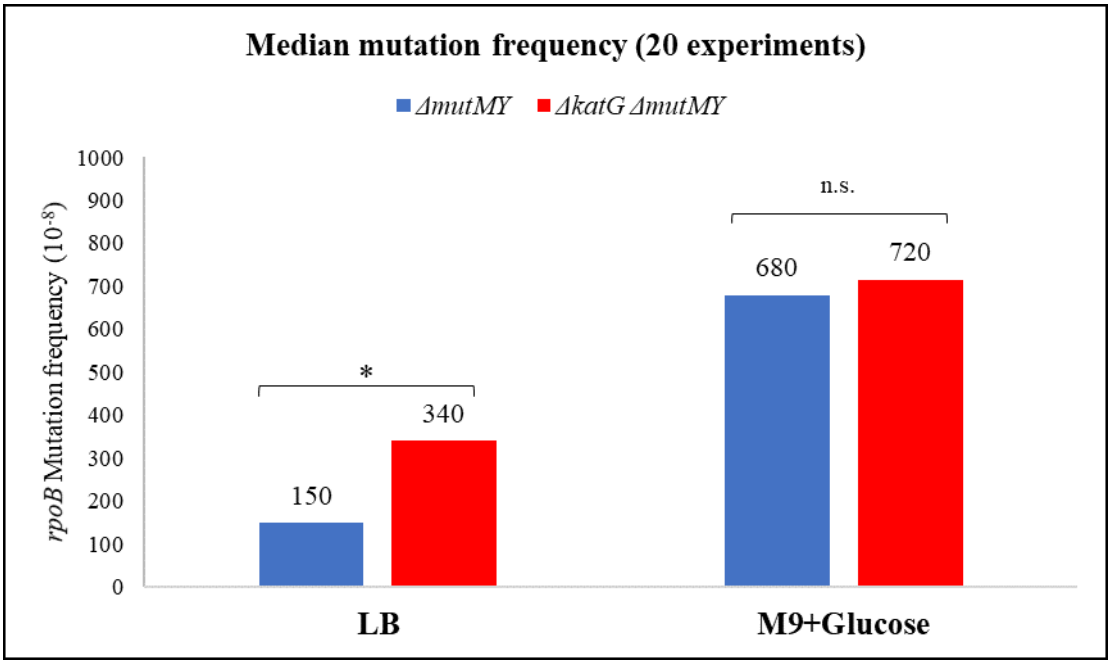

Figure 12: Representative median mutation frequency of *ΔkatG ΔmutM ΔmutY* in LB and M9+glucose minimal media for 20 experiments. * indicates significance $p<0.01$ while n.s. indicates non-significance statistically calculated by Mann-Whitney U test.

4. Involvement of Fur in suppressing oxidative DNA damage

Based on the H_2O_2 sensitivity by disk diffusion assay that has been shown, Fur may play a similar role as KatG in regulating the oxidative stress. *katG* deletion strain that showed indifference in oxidative DNA damage level in M9+glucose minimal media suggests that oxidative DNA damage level is more inclined towards Fur protein's activity.

The alternative pathway to suppress oxidative DNA damage may be through the suppression of the production of ·OH rather than the scavenging activity of the high intracellular H_2O_2 level. The suppression of ·OH involves the suppression of intracellular iron levels, hence reducing the Fenton reaction from occurring. Iron is the co-reactant in the Fenton reaction which causes the rate of DNA damage to be elevated when iron levels are high. With that, the other alternative pathway for the cell to control the oxidative DNA damage is through the regulation of the iron metabolism. Therefore, I determined the oxidative DNA damage level of the *fur* deletion strain which is the major iron regulator of the OxyR regulon.

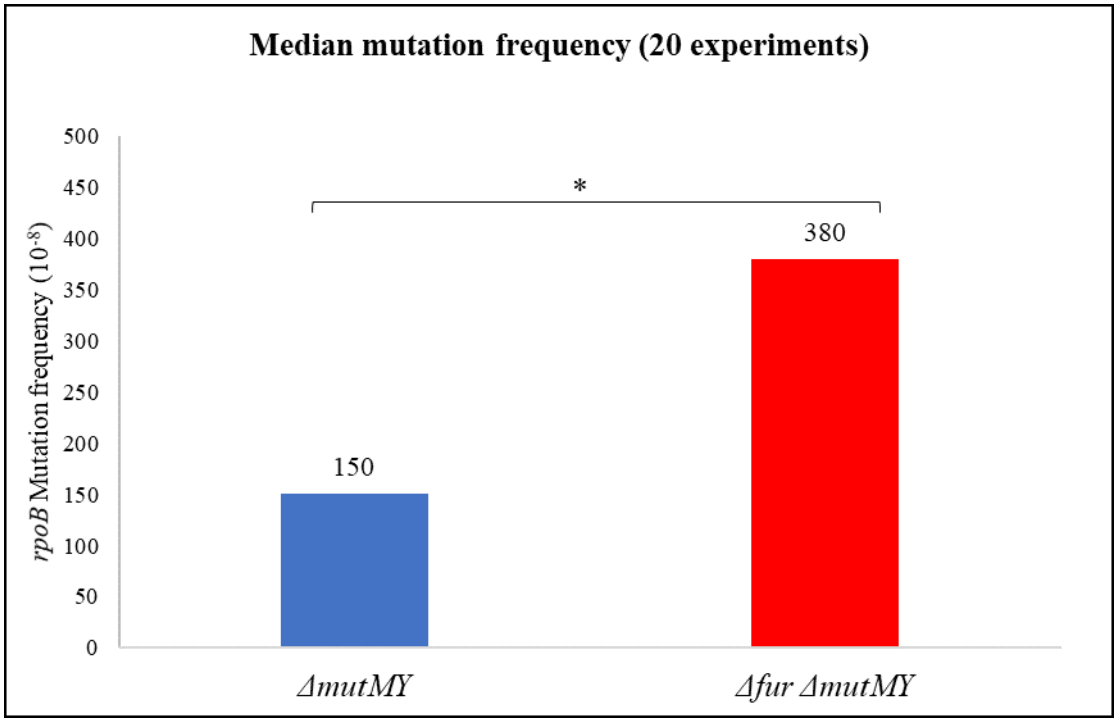

Figure 13: Representative median mutation frequency of *Δfur ΔmutM ΔmutY* in LB media for 20 experiments. * indicates significance $p<0.01$ calculated by Mann-Whitney U test.

fur mutant has a growth phenotype of 18 hours in LB media but was not able to grow on M9+glucose minimal media. This signifies that Fur is crucial as the main iron regulator and that maintenance of iron regulation is required by the Fur protein's function. **Figure 13** shows the mutation frequency of *fur* deletion strain which showed increased mutation frequency of 2.5-fold in comparison with *ΔmutM ΔmutY* strain in LB media. This suggests that Fur can suppress oxidative DNA damage in normal growth condition (LB media).

5. Involvement of Dps in suppressing oxidative DNA damage

It was difficult to prove that Fur which is the major iron regulator in the OxyR regulon is singly able to suppress oxidative DNA damage since the mutation frequency only showed a slightly significant increase of 2.5-fold where else the mutation frequency could not be determined in low nutrient condition. Therefore, I was interested to know the functionality of the other iron regulator genes that could be able to play a role in substituting for the Fur protein's function. I determined the mutation frequency of the *dps* mutant whose main function was to sequester iron that could help reduce ·OH and hence suppressing oxidative DNA damage. As mentioned in the literature review, this protein has been shown to be strongly correlated with stress response through its DNA binding and iron oxidation properties (Karas, Westerlaken, and Meyer 2015).

Table 6 shows the growth phenotype of *Δdps ΔmutM ΔmutY* in LB and M9+glucose minimal media which indicated that the *dps* deficient strain did not have a detrimental effect on the growth rate. Meanwhile, in **Figure 14**, the mutation frequency results of the *Δdps ΔmutM ΔmutY* revealed that *dps* mutant increased in mutation frequency by slight significant increase of 1.6-fold on LB media while it showed a significant increase of 1.9-fold in M9+glucose minimal media in comparison to the wild type strain (*ΔmutM ΔmutY*). This suggests that Dps could help suppress oxidative DNA damage both in normal physiological condition and in extreme low nutrient condition.

Table 6: Growth phenotype of mutant *Δdps* grown in different growth conditions.
Growth time is measured for the strain to form colonies of 1.5 mm in size in the respective media.

Strain	**LB (hours)**	**M9+glucose (hours)**
MK7180 (*ΔmutM ΔmutY*)	12-14	43-45
MK9404 (*Δdps ΔmutM ΔmutY*)	18	42-44

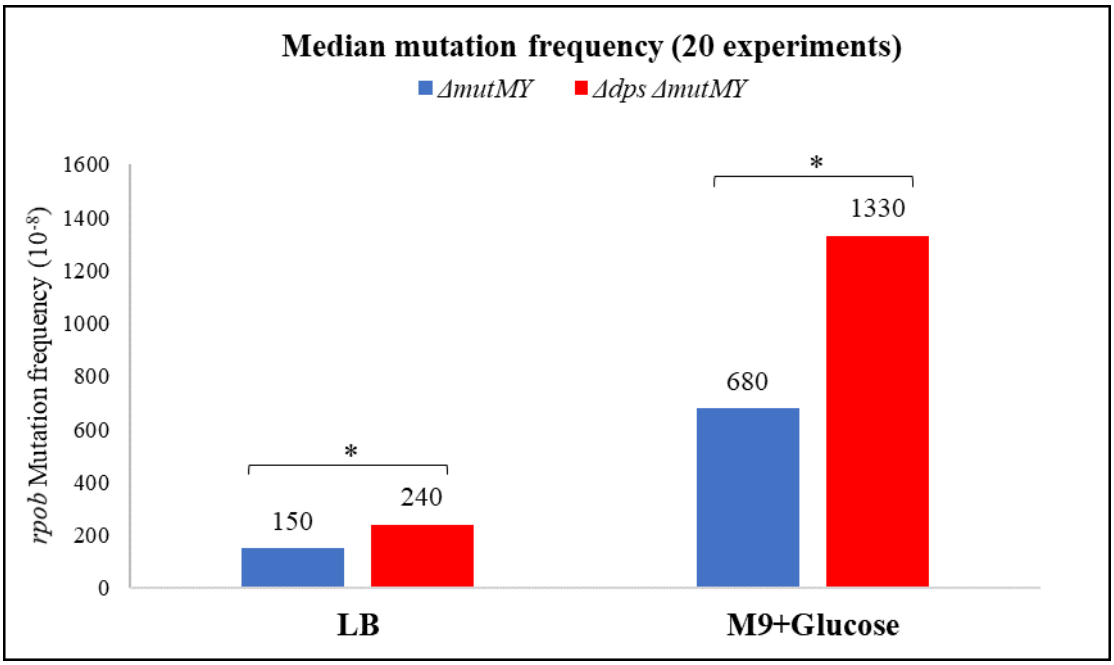

Figure 14: Representative median mutation frequency of *Δdps ΔmutM ΔmutY* in LB and M9+glucose minimal media for 20 experiments. * indicates significance $p<0.01$ calculated by Mann-Whitney U test.

6. Involvement of Fur and Dps in suppressing oxidative DNA damage

As the single deletion strains of *fur* or *dps* only showed a minimal increase in mutation frequency, it would be possible to assume that the level of oxidative DNA damage is only affected minimally in the absence of one of the genes. As the cells' oxidative response is active in defending against the ROS toxicity, it would only be more efficient if two stress defense systems would to work in a combination fashion to suppress the oxidative DNA damage. Therefore, I constructed the *Δfur Δdps ΔmutM ΔmutY* strain and tested the mutation frequency to investigate the effect of the combination of two important iron regulator genes of the OxyR regulon.

The *Δfur Δdps ΔmutM ΔmutY* showed a slow growth phenotype of 20 to 22 hours. Similar to the *fur* deletion strain, *Δfur Δdps ΔmutM ΔmutY* was not able to grow on M9+glucose minimal media.

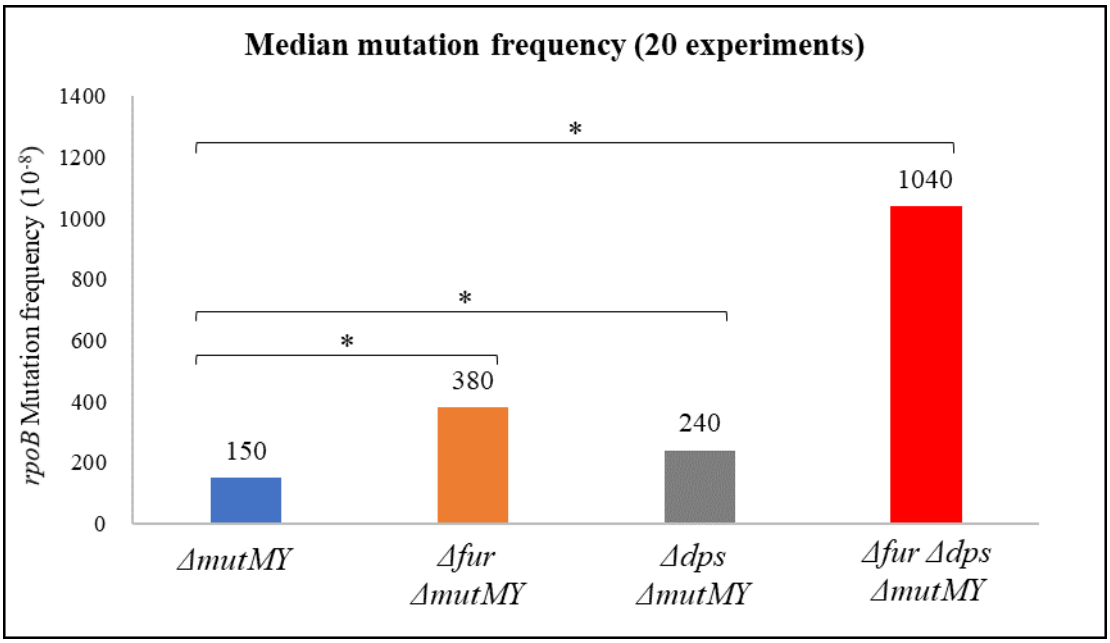

Figure 15: Representative median mutation frequency of deletion strains *Δfur ΔmutM ΔmutY, Δdps ΔmutM ΔmutY*, and combination deletion strain *Δfur Δdps ΔmutM ΔmutY* in LB media for 20 experiments. * indicates significance $p<0.01$ calculated by Mann-Whitney U test.

As shown in **Figure 15** the deletion of both *fur* and *dps (Δfur Δdps ΔmutM ΔmutY)* caused the mutation frequency to increase tremendously by 6.9-fold in comparison with the wild type strain (*ΔmutM ΔmutY*). The mutation frequency of *Δfur Δdps ΔmutM ΔmutY* also showed a significant increase by at least 2-fold in comparison with either the *fur* or *dps* deficient strain. This result suggests that both *fur* and *dps* are important to control the suppression of oxidative DNA damage independently. As both genes play their respective roles in maintaining the iron availability in the cells, the combination functionality of both genes is important. Therefore, with the deletion of these two genes, *fur* and *dps*, the iron regulation is disrupted, and this causes the increase production of ·OH and hence the increase in oxidative DNA damage.

7. Involvement of YaaA in suppressing oxidative DNA damage

As mentioned earlier, the YaaA protein's functionality is not well known except that it was proposed to facilitate in binding free unincorporated iron in the cells. With that, YaaA could reduce the production of ·OH which causes oxidative DNA damage. I investigated the level of oxidative DNA damage by constructing the *yaaA* deletion strain in the *ΔmutM ΔmutY* strain.

The *ΔyaaA ΔmutM ΔmutY* did not show much change in growth phenotype in comparison with the *ΔmutM ΔmutY* in both LB media and M9+glucose minimal media (**Table 7**). The deletion of *yaaA* showed an increase in mutation frequency by a non-significant 1.4-fold change when compared to the *ΔmutM ΔmutY* strain in LB media. However, the mutation frequency of the *ΔyaaA ΔmutM ΔmutY* strain showed 2.4 times significantly higher than the *ΔmutM ΔmutY* in M9+glucose minimal media (**Figure 16**). These data suggest that *yaaA* could only partially protect the cells in normal physiological condition (LB) but fully play an important role to suppress oxidative DNA damage in stress condition such as in low nutrient condition (minimal media).

Table 7: Growth phenotype of mutant *ΔyaaA* grown in different growth conditions.
Growth time is measured for the strain to form colonies of 1.5 mm in size in the respective media.

Strain	**LB (hours)**	**M9+glucose (hours)**
MK7180 (*ΔmutM ΔmutY*)	12-14	43-45
MK9612 (*ΔyaaA ΔmutM ΔmutY*)	12-14	46-48

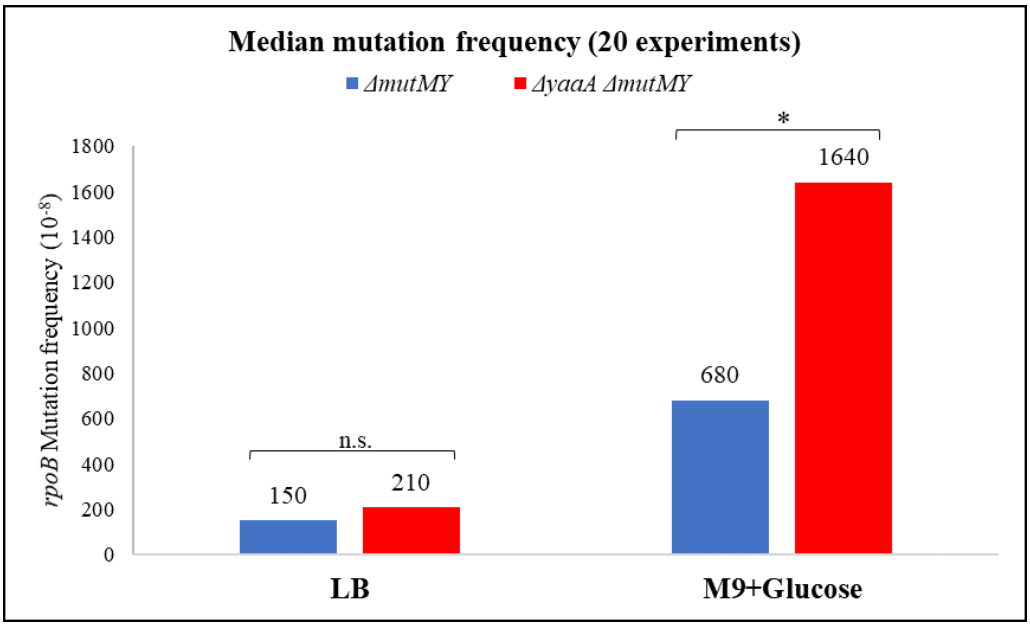

Figure 16: Representative median mutation frequency of *ΔyaaA ΔmutM ΔmutY* in LB and M9+glucose minimal media for 20 experiments. * indicates significance $p<0.01$ while n.s. indicates non-significance statistically calculated by Mann-Whitney U test.

8. Dps together with YaaA strongly suppress oxidative DNA damage

It has been shown that YaaA alone is not enough to suppress oxidative DNA damage in a normal physiological condition but fully protect cells in low nutrient condition. Since *yaaA* gene is part of the OxyR regulon which is highly effective in reducing oxidative stress, it can be speculated that YaaA's functionality can be fully potentiated in the presence of other factors. Additionally, based on the previous study (Liu, Bauer, and Imlay 2011), the mechanistic role of YaaA in reducing unincorporated iron is proposed to be with the synergistic functionality of the other iron regulatory gene. Therefore, the next approach of interest was to test the synergistic effect of *yaaA* gene with the *dps* gene by deletion of these genes in a *ΔmutM ΔmutY* strain.

The growth of the quadruple strain *Δdps ΔyaaA ΔmutM ΔmutY* is the slowest in comparison with the triple mutant strains *ΔyaaA ΔmutM ΔmutY* and *Δdps ΔmutM ΔmutY* both in LB and M9+glucose minimal media (**Table 8**). Results obtained in **Figure 17** show that deletion of both *yaaA* and *dps* genes caused a tremendous increase (7.3-fold) in mutation frequency in comparison to the *ΔmutM ΔmutY* strain in LB media. Similarly, in M9+glucose minimal media, the *Δdps ΔyaaA ΔmutM ΔmutY* strain showed a significant increase of 6.3-fold in comparison to the *ΔmutM ΔmutY* strain and more than 2-fold increase in comparison to the deletion of *yaaA* or *dps* individually. These results indicate that in the absence of *yaaA*, *dps* can compensate the functionality in iron regulation and the compensatory effect is seen to be vice versa for the *yaaA* gene towards *dps*. However, if both genes are absent even in the normal physiological condition as shown in the LB media, iron levels increase, hence driving more

Fenton reaction that leads to increase in oxidative DNA damage and mutagenesis. The functionality interaction between both genes *yaaA* and *dps* indicate a strong synergism in controlling or regulating intracellular iron levels.

Table 8: Growth phenotype of mutant *ΔyaaA* and *Δdps* grown in different growth conditions. Growth time is measured for the strain to form colonies of 1.5 mm in size in the respective media.

Strain	**LB (hours)**	**M9+glucose (hours)**
MK7180 (*ΔmutM ΔmutY*)	12-14	43-45
MK9612 (*ΔyaaA ΔmutM ΔmutY*)	12-14	46-48
MK9404 (*Δdps ΔmutM ΔmutY*)	18	42-44
MK9616 (*Δdps ΔyaaA ΔmutM ΔmutY*)	20	48-50

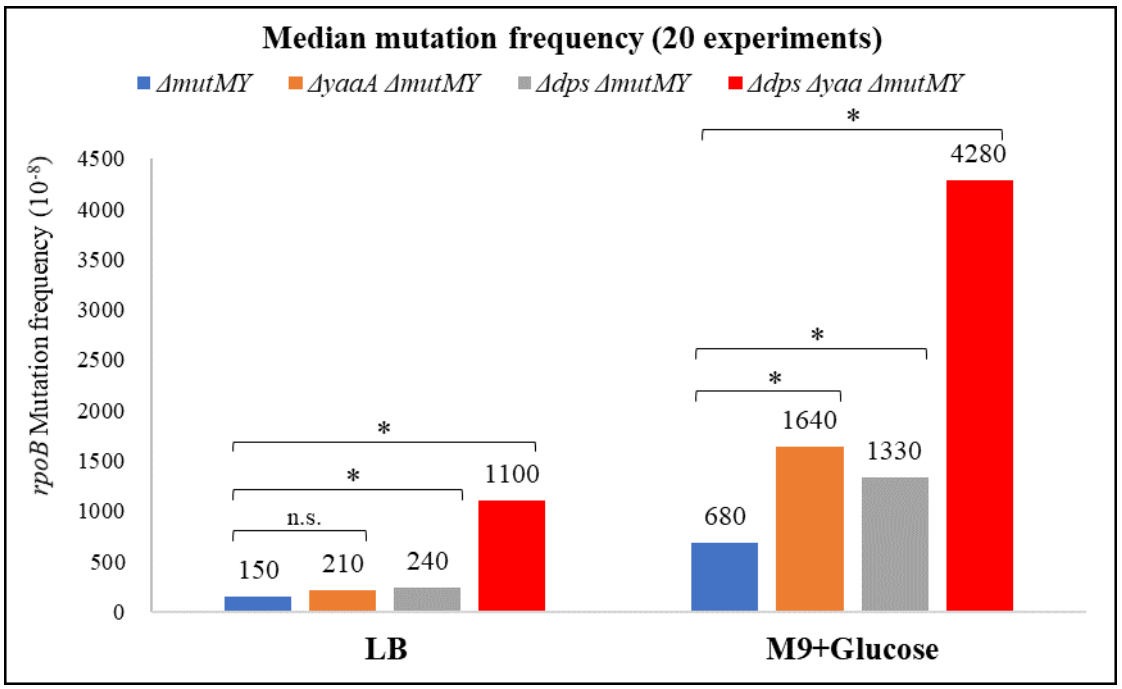

Figure 17: Representative median mutation frequency of deletion strains *ΔyaaA ΔmutM ΔmutY, Δdps ΔmutM ΔmutY*, and combination deletion strain *Δdps ΔyaaA ΔmutM ΔmutY* in LB and M9+glucose minimal media for 20 experiments. * indicates significance $p<0.01$ while n.s. indicates non-significance statistically calculated by Mann-Whitney U test.

To confirm the synergistic effect of both genes *yaaA* and *dps*, complementation studies were done by inserting plasmids harboring either the *yaaA* or *dps* gene in the *Δdps ΔyaaA ΔmutM ΔmutY* strain. The oxidative mutagenesis level was then determined with the strains carrying the complementary plasmids harboring the genes. Results in **Figure 18** show that with the addition of the complementary gene, *yaaA* or *dps*, the mutation frequency was reduced; indicating that the presence of either one of the genes could recover the effect caused by the absence of the other gene.

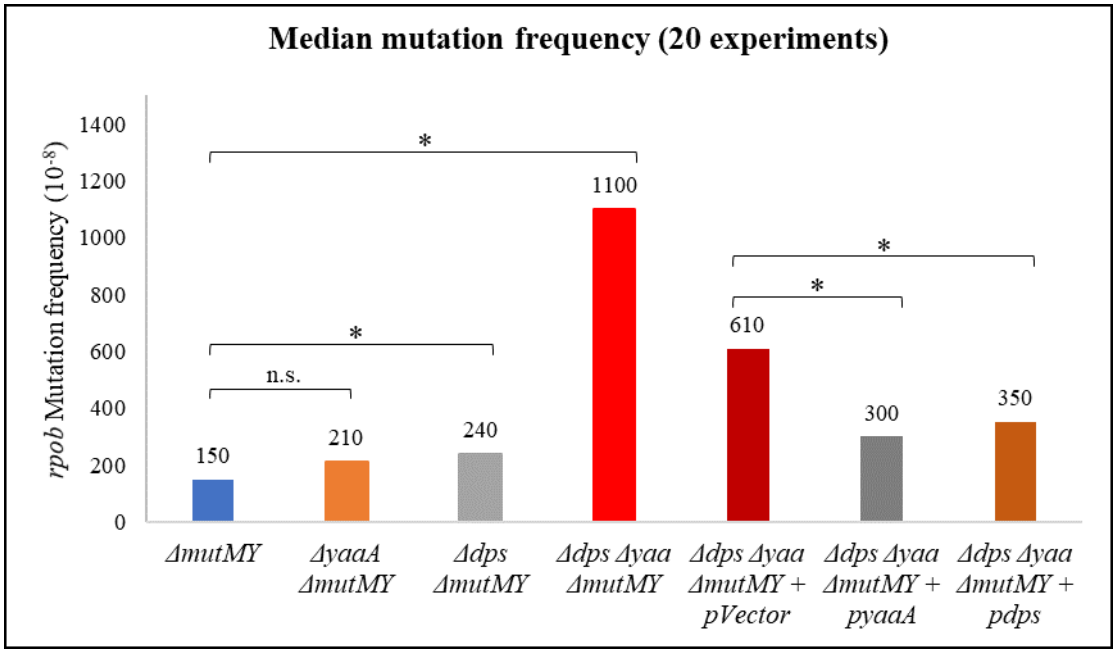

Figure 18: Complementation mutation frequency of *Δdps ΔyaaA ΔmutM ΔmutY* on LB media. * indicates significance $p<0.01$ while n.s. indicates non-significance statistically calculated by Mann-Whitney U test.

9. TonB promotes to produce oxidative DNA damage

TonB's function as one of the major components under the control of Fur repression has been explained. The expression of TonB promotes the incorporation of iron into the intracellular of the cells. For cells to maintain a low intracellular iron level, TonB activity needed to be repressed. With that, I determine the mutation frequency of mutant *tonB* gene which would indicate the decrease in oxidative DNA damage level in the absence of the gene.

The growth phenotype of *ΔtonB ΔmutM ΔmutY* showed a slow growth of 21 hours on LB media while it cannot be grown on M9+glucose minimal media. As hypothesized, the mutation frequency of *ΔtonB ΔmutM ΔmutY* showed a 3.8-fold decrease in mutation frequency in comparison with the *ΔmutM ΔmutY* strain as shown in **Figure 19**. This suggests that TonB indeed plays an important role in causing the oxidative DNA damage if its activity or expression is not repressed.

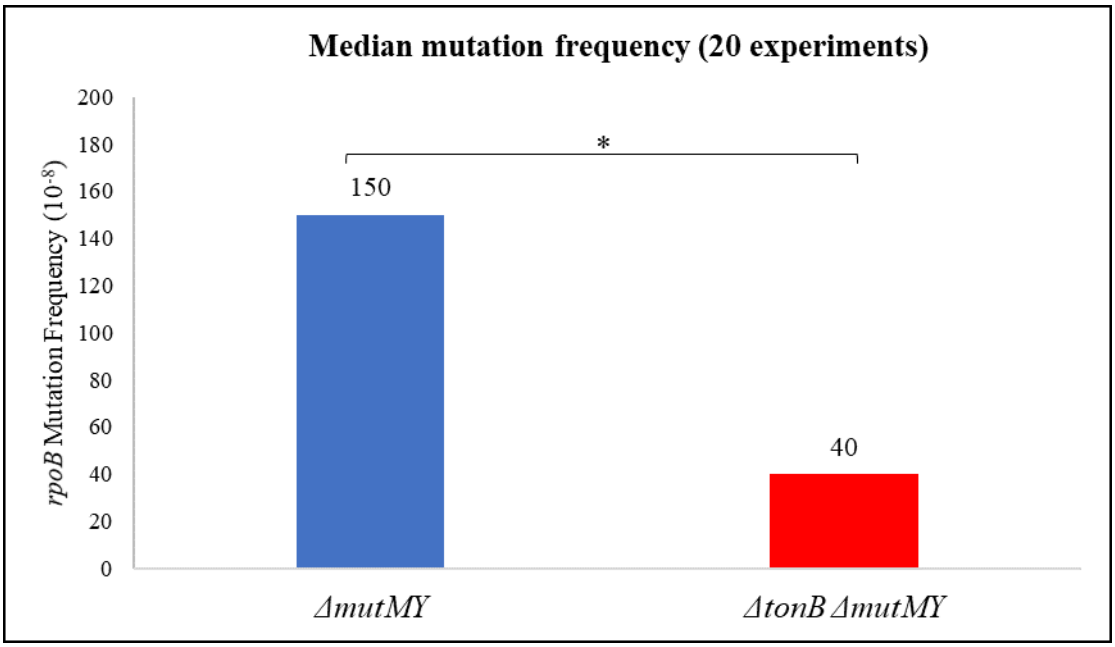

Figure 19: Representative median mutation frequency of *ΔtonB ΔmutM ΔmutY* in LB media for 20 experiments. * indicates significance $p<0.01$ calculated by Mann-Whitney U test.

Part II: Oxidative DNA damage suppression by reduced intracellular ferrous iron

Intracellular iron level is difficult to be measured with relevance to the production of ·OH and oxidative DNA damage. However, I have shown from the compiled results that iron regulatory genes of the OxyR regulon, *fur, dps,* and *yaaA* were able to suppress oxidative DNA damage while the *tonB* gene promotes oxidative DNA damage. These results through the manipulation of the iron regulatory genes' deletion indicated that iron regulation is very crucial to reduce the oxidative DNA damage by reducing the Fenton reaction, hence reducing the production of ·OH. Since intracellular ferrous (Fe^{2+}) iron plays an important role in causing oxidative DNA damage, I employed another alternative genetic method by the introduction of *mstA* overexpression which is involved in the H_2S pathway that has been explained earlier.

1. Iron binding through the alternative H_2S method

1.1 *mstA* over expression suppressed oxidative DNA damage in *oxyR* deficient mutant

P_{tet}-*mstA*-Cm was transduced into *ΔoxyR ΔmutM ΔmutY* strain to yield *mstA* over expression phenotype on the *ΔoxyR ΔmutM ΔmutY* strain which has been previously indicated with the highest level of oxidative DNA damage both in normal growth condition and in stressed condition with low nutrient condition. **Figure 20** shows that the mutation frequency of *ΔoxyR ΔmutM ΔmutY* strain that carries the chromosomal *mstA* overexpression showed a decrease in mutation frequency in comparison with the *ΔoxyR ΔmutM ΔmutY*. The suppression of oxidative DNA damage level is clearly seen when *mstA* overexpression decreased the mutation frequency almost to the wild type level both in LB and M9+glucose minimal media. This result confirms the previous study that MstA could help suppress oxidative DNA damage. Based on the previous study of MstA's functionality, the result suggests that high intracellular ferrous (Fe^{2+}) iron level was the main cause of increased ·OH production which led to the oxidative DNA damage in the *oxyR* deletion strain.

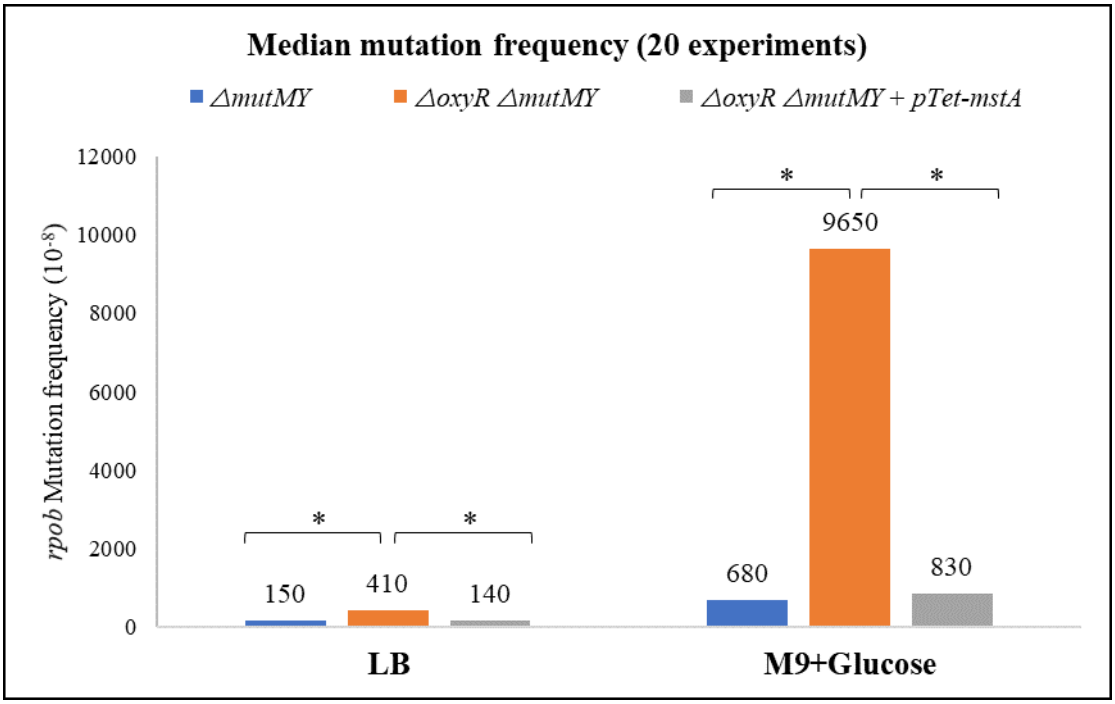

Figure 20: Representative median mutation frequency of *ΔoxyR ΔmutM ΔmutY* and *ΔoxyR ΔmutM ΔmutY* +p_{tet}-*mstA* in LB and M9+glucose minimal media for 20 experiments. * indicates significance $p<0.01$ calculated by Mann-Whitney U test.

1.2 *mstA* over expression suppressed oxidative DNA damage in iron regulators deficient strain

Earlier results indicated that the combination of *dps* and *yaaA* deletion showed a disrupted iron regulation system that resulted in increased oxidative DNA damage level. To confirm that the disrupted iron regulation's effect on the oxidative DNA damage can be compensated with the presence of an alternative system, the *mstA* overexpression was employed into the *Δdps ΔyaaA ΔmutM ΔmutY* strain.

Result shown in **Figure 21** indicates that with the overexpression of *mstA*, the *Δdps ΔyaaA ΔmutM ΔmutY* strain reduced significantly its mutation frequency to 1.6-fold in LB media but the reduction was much more clearly seen in M9+glucose minimal media at 5.2-fold. Similar to the *oxyR* deletion strain, it can be confirmed that MstA could help reduce the high intracellular ferrous (Fe^{2+}) iron level that was the main cause of increased ·OH production in the *Δdps ΔyaaA ΔmutM ΔmutY* deletion strain that led to the high oxidative DNA damage level.

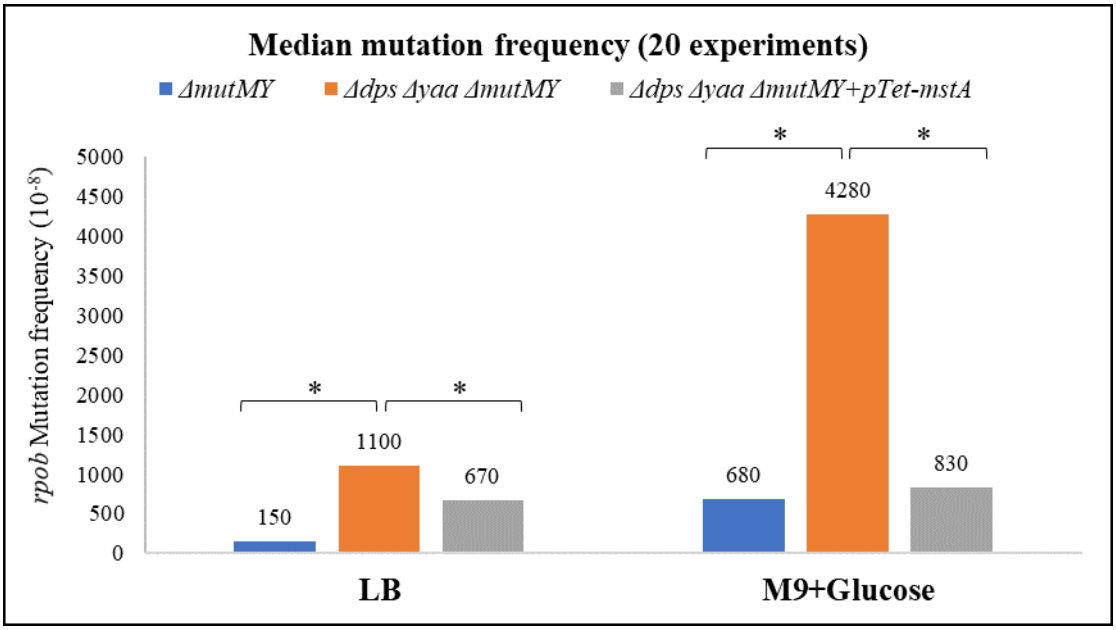

Figure 21: Representative median mutation frequency of *Δdps ΔyaaA ΔmutM ΔmutY* and *Δdps ΔyaaA ΔmutM ΔmutY* +p_{tet}-*mstA* in LB and M9+glucose minimal media for 20 experiments. * indicates significance $p<0.01$ calculated by Mann-Whitney U test.

2. Iron chelation assay by 2,2- Bipyridyl

The use of genetic method through the alternative H_2S pathway has the possibility of having metabolic overlay between the OxyR regulon genes which may not totally validate the suppression of oxidative DNA damage. As such, a chemical approach through the addition of an iron chelator, 2,2- Bipyridyl was to support the findings that iron is the main cause of oxidative DNA damage. In previous study, it was found that bipyridyl could quickly block Fenton chemistry and thereby prevent ROS from damaging DNA. Therefore, I determined the mutation frequency of *ΔoxyR ΔmutM ΔmutY* and *Δdps ΔyaaA ΔmutM ΔmutY* strains to see the effect of iron chelation that can suppress oxidative DNA damage.

2.1 Iron chelation reduced oxidative DNA damage in *oxyR* deficient strain

It was found that bipyridyl reduced oxidative DNA damage level in the *oxyR* deletion strain (**Figure 22**). When cells were grown in LB with the addition of bipyridyl, the mutation frequency of the *oxyR* deletion strain dropped to even lower level than the *ΔmutM ΔmutY* strain. This suggests that the chelation of iron could help reduce the intracellular ferrous (Fe^{2+}) level that drives the Fenton reaction that produces more ·OH that ultimately causes the oxidative DNA damage in the *oxyR* deletion strain.

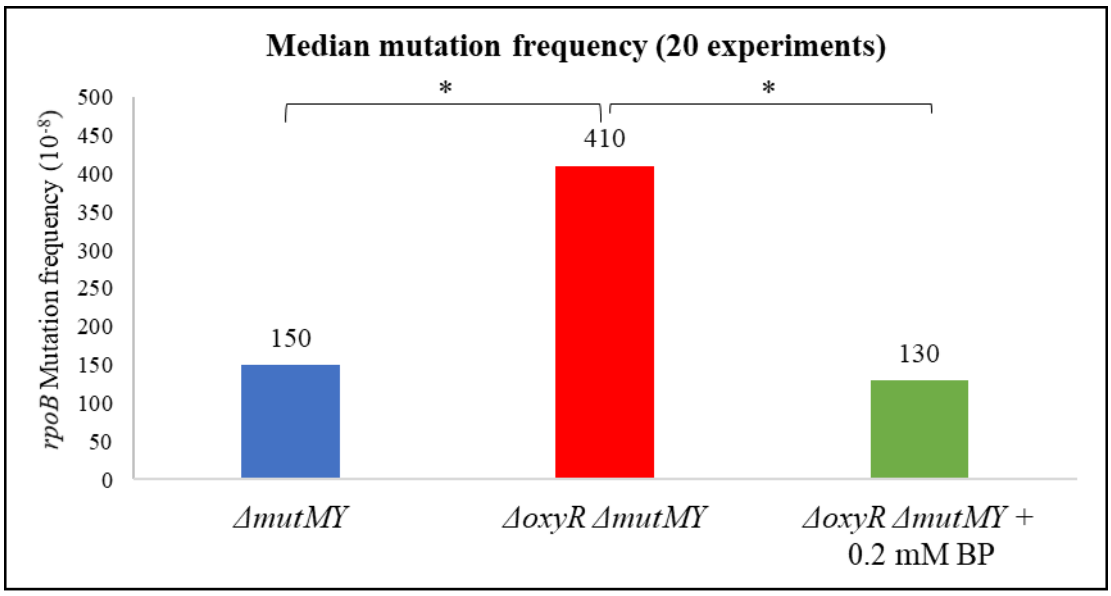

Figure 22: Representative median mutation frequency of *ΔoxyR ΔmutM ΔmutY* in LB and LB + 0.2mM 2,2- Bipyridyl for 20 experiments. * indicates significance $p<0.01$ calculated by Mann-Whitney U test.

2.2 Iron chelation reduced oxidative DNA damage in iron regulators deficient strain

In the iron regulators deficient strain, *Δdps ΔyaaA ΔmutM ΔmutY* showed a slight decrease (1.3-fold) when the cells were grown in LB media with the addition of bipyridyl in comparison to when cells were grown in just LB media (**Figure 23**). The efficiency of the iron chelation could be observed better (5.7-fold) when the *Δdps ΔyaaA ΔmutM ΔmutY* strain grew in M9+glucose minimal media with the addition of bipyridyl than compared to the strain grown in just M9+glucose minimal media. The result shown confirms that oxidative DNA damage was mainly caused by iron regulation and that the iron regulatory genes in the OxyR regulon could help suppress oxidative DNA damage by reducing the intracellular ferrous (Fe^{2+}) iron level.

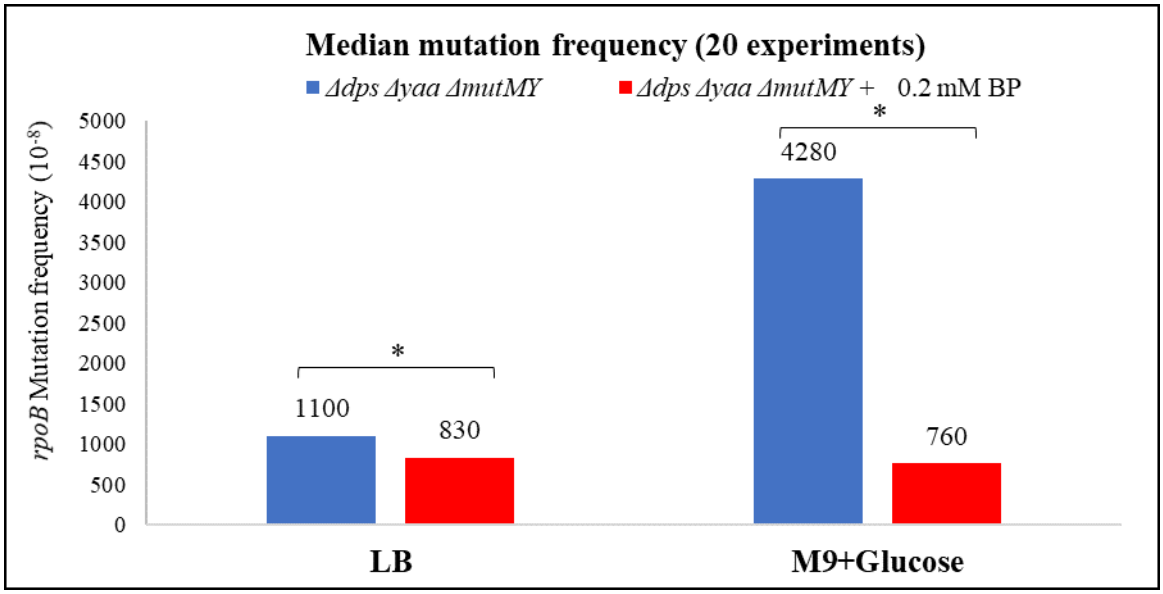

Figure 23: Representative median mutation frequency of *Δdps ΔyaaA ΔmutM ΔmutY* in LB and M9+glucose minimal media with the addition of 0.2mM 2,2- Bipyridyl for 20 experiments. * indicates significance $p<0.01$ calculated by Mann-Whitney U test.

Part III: Functional interaction of iron regulator genes in the suppression of oxidative DNA damage

1. YaaA may work in the same pathway as Fur

Having shown the combination interaction between *fur-dps* and *dps-yaaA*, I then investigated the interaction of *fur-yaaA*. **Figure 24** shows the compilation results of the mutation frequency for the iron regulatory genes with respect to their single deletion and the combination deletion in LB media. The *yaaA* gene deletion was found to show a non-significant increase in mutation frequency and the combination deletion of *fur* and *yaaA* was expected to show a much higher mutation frequency given the earlier indication that combination effect of the iron regulation was more effective in suppressing oxidative DNA damage level. On the contrary, it was surprising to find that the deletion combination of *fur* and *yaaA* (*Δfur ΔyaaA ΔmutM ΔmutY*) showed a mutation frequency that is similar to the single deletion strain *ΔyaaA ΔmutM ΔmutY*. This result suggests that YaaA may be working in the same pathway as the Fur regulon.

To support this notion, I found that the detrimental effect of the deletion of all the iron regulatory genes *Δfur Δdps ΔyaaA* was similar to the combination deletion of *fur-dps* and *dps-yaaA* deletion strain (**Figure 24**). It would be expected that with the absence of all the iron regulatory genes, the mutation frequency would have increased tremendously. Instead, with the result shown, the oxidative DNA damage level was not increased further. This finding supports the idea that YaaA may work in the same way as Fur or that YaaA protein may have the same functions as the Fur protein.

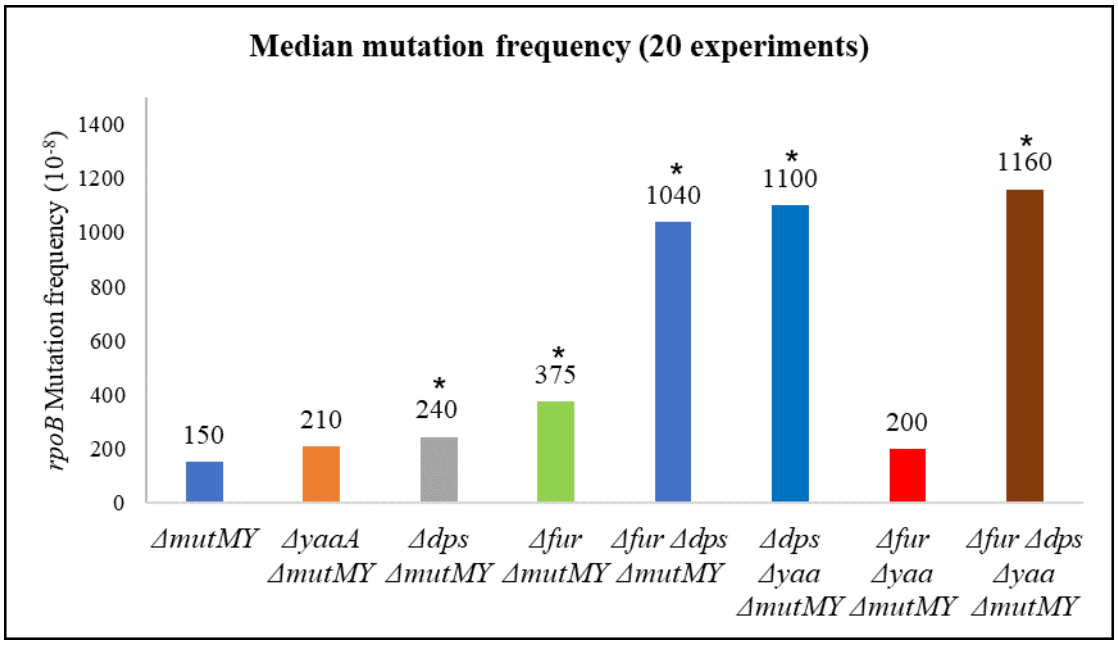

Figure 24: Representative median mutation frequency of iron regulators genes and the combination deletion genes on LB. * indicates significance $p<0.01$ against *ΔmutMY* calculated by Mann-Whitney U test.

2. YaaA functions similarly to Fur

The low mutation frequency in the *Δfur ΔyaaA ΔmutM ΔmutY* strain which was discovered earlier indicates that there is a gene interaction between the *yaaA* and *fur* genes. To further investigate the gene interaction, the relative expression of the genes were determined by quantitative PCR.

Figure 25 shows the expression level of *fur* and *yaaA* genes in four conditions, wild type (*ΔmutM ΔmutY)* cells grown in LB, wild type cells grown in LB+1mM H_2O_2 (that mimics stress condition), *Δfur ΔmutM ΔmutY* grown on LB and *ΔyaaA ΔmutM ΔmutY* grown on LB. Expression of *fur* showed a slight but not significant increase in the presence of H_2O_2. This data suggest that *fur* expression is well controlled in stress condition as Fur which is a transcription factor can express or repress its down-stream genes to control against oxidative stress condition. When *yaaA* gene was deleted, the expression level of the *fur* gene increases slightly but is not significant to the wild type strain grown on LB. This suggests that YaaA may not be regulating on the expression of the *fur* gene.

For the expression of the *yaaA* gene, it was found that the induction of H_2O_2 increased the expression significantly in comparison to the wild type strain that was grown in LB media. This suggests that *yaaA* gene expression is H_2O_2 stress-inducible and that *yaaA* gene may play a role in controlling oxidative DNA damage in stress condition. Besides that, it was also found that in the deletion of *fur*, the expression level of *yaaA* increased in comparison to the wild type strain grown in LB media. This suggests that in the absence of Fur, *yaaA* gene is expressed because the induction of *yaaA* gene's expression may compensate for the absence of Fur protein's functionality.

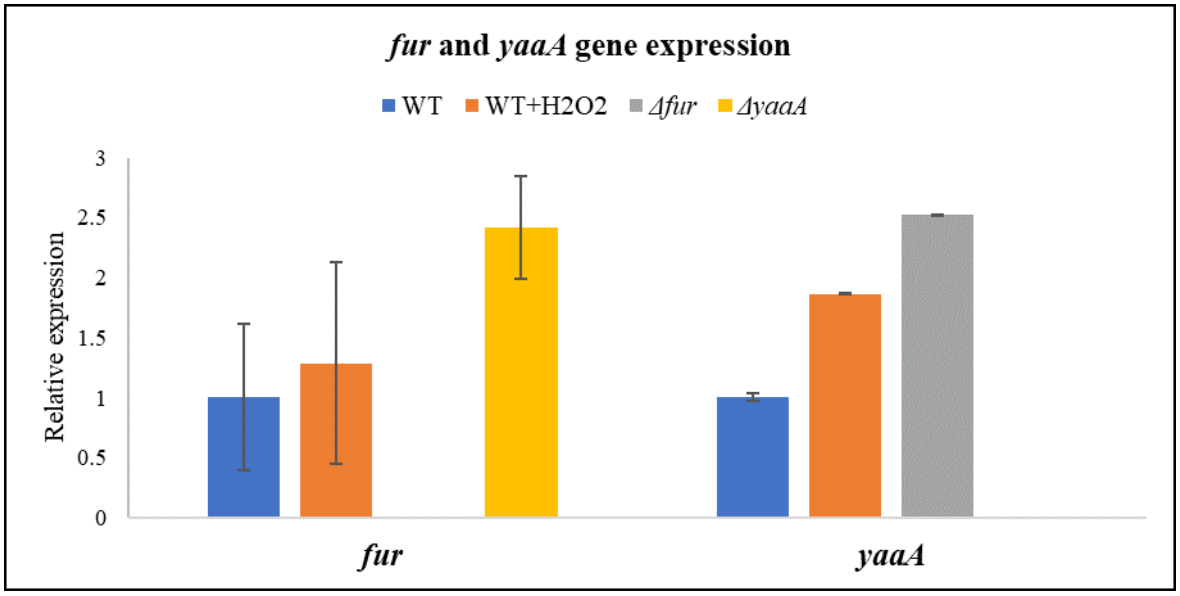

Figure 25: Expression levels of *fur* and *yaaA* genes. Data representation is the mean of three replicates measurement with the ± standard deviation indicated by error bars.

3. TonB is regulated by Fur but not by YaaA

It has been shown in the above results that the deletion of *tonB* gene was able to reduce the mutation frequency. This result suggests that the induction of *tonB* could bring detrimental effect towards oxidative DNA damage. As mentioned, the *tonB* gene expression is repressed by Fur, however, the expression level of *tonB* in relation to YaaA is not known. The functionality of YaaA towards Fur-controlled genes such as the *tonB* gene has not been investigated. Since it was found that the expression of the *yaaA* gene may be related to the Fur protein, I determined the *tonB* expression to investigate the correlation of YaaA on the Fur-regulated expression of the *tonB* gene.

In **Figure 26**, it is shown that when wild type cells were grown in LB+H_2O_2, *tonB* was induced. This suggests that during stress condition, induced TonB increases incorporation of iron into the cells that may cause the increase in oxidative DNA damage. It is clearly shown that the deletion of *fur* also causes the increased expression of *tonB* which confirms that *tonB* expression is heavily dependent on the repression of Fur in regulating the incorporation of iron into the cells. Finally, it is also shown that in the absence of YaaA, *tonB* gene is not induced. This result suggests that YaaA does not control the *tonB* expression. With the current result, it can be suggested that YaaA does not function to repress the *tonB* gene in the same manner as how Fur protein does. There may be other possibilities of the YaaA protein's function which will be discussed in the later section.

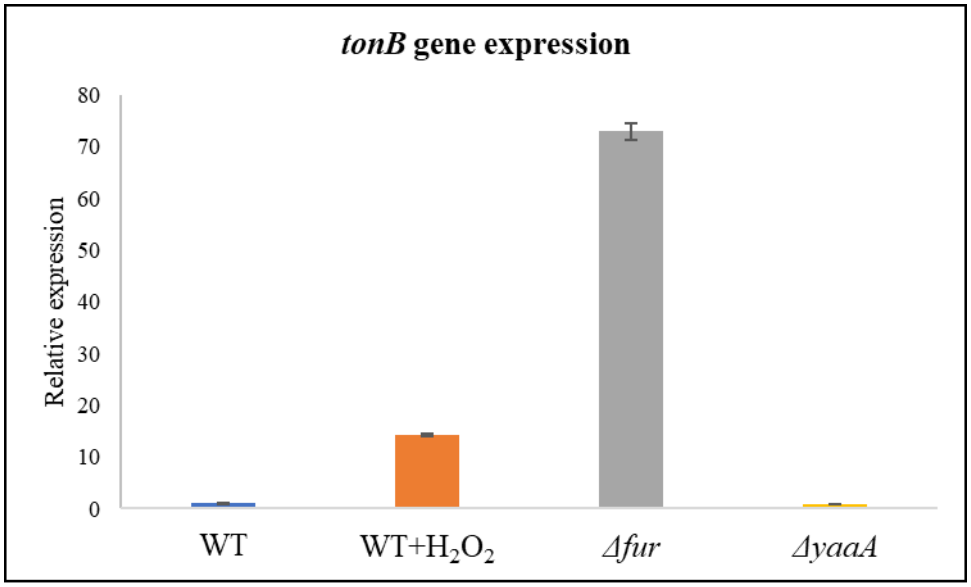

Figure 26: Expression level of *tonB* gene. Data representation is the mean of three replicates measurement with the ± standard deviation indicated by error bars.

4. Dps is induced when intracellular iron is increased

I have shown the interaction of *fur, yaaA* and *tonB* expressions that suggest iron importation through the Fur regulon profoundly regulated the *tonB* repression and that YaaA protein does not affect on the *tonB* gene expression. These findings have not been correlated with the iron storage pathway that involves the expression of the *dps* gene. I then determined the expression of *dps* gene to correlate the functionality of *fur, dps* and *yaaA* whose mutation frequency of the combination deletion strains have been determined earlier.

When wild type cells were grown in H_2O_2, the *dps* expression was increased **(Figure 27)**. This phenomenon is typical of the Dps protein that is highly induced by OxyR to protect cells against oxidative stress condition. Besides that, it was found that in the absence of Fur (*Δfur*) *dps* gene was expressed to the level similar to when cells undergo H_2O_2 stress. This indicates that the disruption of Fur iron regulation causes the *dps* gene to be induced. Likewise, in the *yaaA* gene deletion, it was found that *dps* expression was highly induced. This may suggest that the disruption of YaaA may cause a backup mechanism that is through the highly expressed *dps* gene to overcome the detrimental effect that has been caused. This data can be correlated to the mutation frequency results that showed the combination deletion of *fur-dps* and *dps-yaaA* revealed a profound increased in oxidative DNA damage level. Dps may work as a backup mechanism when the Fur regulon iron importation regulation or the mechanistic effect of the YaaA protein is disrupted.

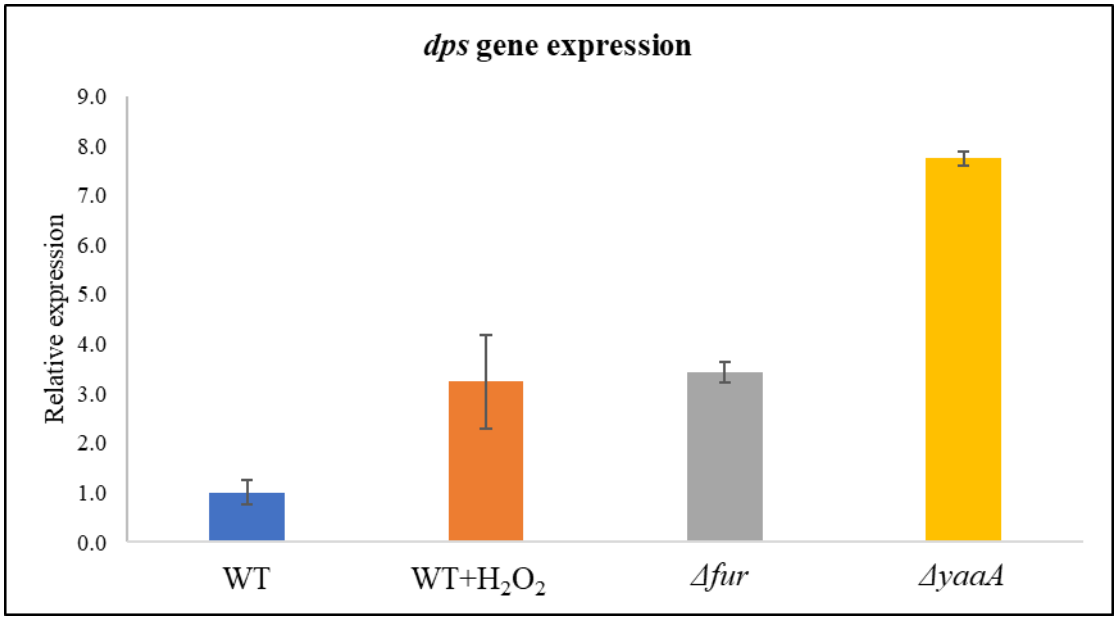

Figure 27: Expression level of *dps* gene. Data representation is the mean of three replicates measurement with the ± standard deviation indicated by error bars.

[#]Comprehensive data representation of the expression for all the genes *fur, yaaA, tonB* and *dps* is shown in **Supplementary Figure 1.**

Chapter IV. Discussions

The studies of oxidative stress, oxidative DNA damage, and the various defense mechanisms of cells have always been the focus across all organisms. Many studies had been carried on ROS and its detrimental effect in causing biological lesions that ultimately causes pathological conditions and diseases especially to human health. (Rahman et al., 2012). The imbalance between the overproduction of ROS and insufficient antioxidant defenses that protect the cells causes pathogenesis of a variety of diseases, such as cancer, cardiovascular diseases and neurodegenerative disorders (Phaniendra, Jestadi, and Periyasamy 2015). Consequence to this, it is essential that an outline of how cells can protect against the detrimental effect of the ROS should be laid out. Because of the conservation of ROS mechanisms across all aerobic organisms (Pavelescu 2015), it is to this study's advantage that a prokaryote model is used to elucidate the mechanisms in a more comprehensive manner. To this end, I investigated on the protection mechanisms of ROS and found that OxyR through its interplay of iron regulatory genes could suppress ·OH production which is the major cause of oxidative DNA in *E. coli* cells.

Part 1: Suppression of oxidative DNA damage by OxyR and its iron regulatory genes

1. **OxyR suppresses oxidative DNA damage.**

It is a known knowledge that OxyR is a good stress regulator in *E. coli* and throughout all bacteria cells (Dubbs and Mongkolsuk 2016). It has been shown that OxyR provides a strong protection against the action of H_2O_2 (**Figure 8**). Nevertheless, the experiment does not explain that OxyR can protect cells against oxidative DNA damage. For the cells to rid of mutagenesis contributed by the presence of oxidized bases of the DNA, the effective MutM and MutY enzymes are required to repair the lesions caused. By disruption to the two genes *ΔmutM ΔmutY* and *oxyR*, I managed to show that the oxidative DNA damage level significantly increased in a normal nutrient condition (LB media). This result is opposing to the previous finding (Yamamura et al. 2000) that states that *oxyR* and the *oxyR* regulated genes do not play a significant role in defense against spontaneous mutagenesis. However, it should be noted that, the study did prove that G:C to T:A transversion mutagenesis was abundant but the mutations detected were that of the wild type strain which is different from my study that showed the mutation frequency in the MutM and MutY deficient strain. In whole, this suggests that without an efficient repair mechanism (such as that of MutM and MutY), that removes the bulk of 8-oxoG, the protective mechanisms of OxyR against oxidative DNA damage cannot be clearly seen. Henceforth, it is clear that ·OH production that causes an abundance of 8-oxoG is the major cause of oxidative DNA damage that can be efficiently suppressed by OxyR.

Previous findings by Nunose, 2016 (unpublished data) showed that *ΔmutM ΔmutY* cells exhibited different levels of oxidative DNA damage when cells were grown in different

nutrient growth conditions (**Supplementary Figure 2**). LB media provides a rich nutrient condition with the presence of essential amino acids for growth where else in M9 minimal media+glucose, cells require a different growth metabolism by inducing catabolite repression pathway by using the available carbon source (Brückner and Titgemeyer 2002). Catabolite repression pathway causes the inhibition of cAMP and the inhibition of transcription of OxyR that subsequently increases H_2O_2 levels (Beatriz Gonzalez-Flecha and Demple 1997b) which ultimately effects on the production of ·OH, seen by increased mutation frequency. Also, previous study by Nunose showed that the addition of casamino acids into the M9 minimal media+glucose showed that the oxidative DNA damage level decreased back to when cells were grown on LB media. This suggests that the amino acid deprivation leads to nutritional stress response that causes oxidative DNA damage. This proposal is supported by a review that noted *E. coli* cells elicits a stringent response to conserve energy under nutritional stress (Chatterji and Kumar Ojha 2001).

2. Reduction in ·OH production is important in suppressing oxidative DNA damage.

It is undeniable that many researches have shown that the confounding factor of oxidative stress and DNA damage is the production of H_2O_2 that resulted from the aerobic respiration activity (Asad et al. 2004). Owing to this reason, *E. coli* cells or many other bacterial cells have efficient H_2O_2 scavenging activity which could help bring down the intracellular H_2O_2 level to the optimum state. The H_2O_2 may seem to be abundantly produced as a byproduct of respiration but it is important to mention that H_2O_2 is poorly reactive unless in the presence of transition metals (Halliwell, Clement, and Long 2000). I have shown that the deletion of KatG only increased mutation frequency by 2.3-fold which is not as high as the deletion of OxyR (**Figure 12**). On the other hand, in M9+glucose minimal media, the mutation frequency did not increase and is quite similar to the wild type strain. One would expect that KatG's deletion which causes high intracellular H_2O_2 level would cause high oxidative DNA damage especially in stress condition (minimal media). Instead, the result shown suggests that H_2O_2 level itself is not the main determinant of increased oxidative DNA damage. The probable explanation would be that the intracellular iron level is kept at a safe and constant level so that ·OH that causes oxidative DNA damage is less produced. Similarly, in the past experiment of my lab mate, it was found that intracellular H_2O_2 level does not affect oxidative DNA damage (**Supplementary Figure 3**). When glucose was added into LB medium, the intracellular H_2O_2 level was tremendously increased but on the contrary, oxidative DNA damage level which was tested by mutation frequency did not change much. The results proved that cells were able to control a stable intracellular H_2O_2 level and that the oxidative DNA damage is most likely caused by the effect of iron regulation.

3. Iron regulatory genes of the OxyR regulon suppress oxidative DNA damage individually and by combined functions.

Iron regulatory genes of the OxyR regulon have each their respective role in maintaining the cellular iron level at the optimum concentration. With the induction of exogenous H_2O_2 or in a presence of high oxidative stress, *fur* gene which is under the regulation of OxyR is induced to regulate iron levels. Other study (Varghese et al. 2007) supports that OxyR must induce Fur to avoid the excessively high levels of intracellular iron. Another study also stated that in high intracellular H_2O_2 level, Fe^{2+}: Fur complex is reduced because ferrous (Fe^{2+}) iron in the complex is speedily oxidized by Fenton reaction (S. Park, You, and Imlay 2005). Consequence to that, Fur repressor activity which depends upon maintaining the bound ferrous (Fe^{2+}) iron, encounter the problem of ferrous iron deficit, hence causing the de-repression of the Fur regulon. Ultimately, the de-repression of Fe^{2+}: Fur complex causes the iron uptake systems to be activated. To this end, I have shown that with the deletion of *fur* gene, oxidative DNA damage is increased, indicating that Fur is a key player in suppressing the oxidative DNA damage in the OxyR regulon in normal physiological condition (**Figure 13**). In the presence of defined media like the M9+glucose minimal media, iron deficiency led to the growth inhibition of the *fur* mutant. This suggests that, iron itself plays and overall expect of growth factor for the cells and without the proper maintenance, cells are deemed inviable. Besides that, other "non-iron" functions such as respiration, the TCA cycle, glycolysis, purine metabolism and redox stress resistance genes are controlled by Fur's function which makes Fur as a global regulator or modulon that supports why *fur* mutant did not grow in the M9+glucose minimal media (D. Touati 1988; Stojiljkovic, Bäumler, and Hantke 1994; S. J. Park and Gunsalus 1995; Vassinova and Kozyrev 2000). Fur protein is not only transcribed by OxyR but is also regulated by its *fur* gene product and the cAMP-CAP system (Lorenzo et al. 1988) which means that the deficient gene product of *fur* causes the inhibition of growth in the catabolite repression pathway of Fur's other modulon functions.

Dps was shown to suppress oxidative DNA damage (**Figure 14**) that supports the functionality of Dps as an iron storage protein by reducing high intracellular ferrous iron or that Dps could bind to the DNA by giving a shielding effect against the oxidative stress. The latter reasoning is supported by a finding that Dps exhibits protection of DNA during oxidative stress (Martinez and Kolter 1997) through experiments that showed Dps protects the DNA backbone from breakage and prevents base damages by using *dps* overexpression plasmid on mutator phenotype of null alleles of *mutM* and *mutY*. The study has similarity to my study; however, I determined the oxidative DNA damage level through the abundance of 8-oxoG by the usage of mutant defective of the *dps* gene in the absence of the repair genes *mutM* and *mutY*. The *dps* deficient mutant exhibited increased levels of spontaneous mutations by the production of more ·OH that causes oxidative DNA damage. With this, my data is supported by another study that states the Dps protein has multipurpose functionality whereby it not only supports the DNA by

binding to it but it is able to neutralize and sequesters iron toxicity by its ferroxidase centers (Karas, Westerlaken, and Meyer 2015).

As Fur is the major iron regulator of the OxyR regulon and Dps has been shown to play a major part in iron storage and sequestering, I found out that the combination of these two genes led to a detrimental effect in their oxidative DNA damage level. This harmful effect is worse than the *oxyR* deficient strain, which indicated that these two iron regulators play a crucial role in keeping the maintenance of the intracellular iron level and ·OH level at a harmless state. The reason is because even though OxyR may be in control of the regulon genes, the Fur and Dps may still be constitutively expressed (Geisel 2011) and because both Fur and Dps are not only controlled by the OxyR as the main transcription factor, other transcription factors effects on the expression of the Fur (Lorenzo et al. 1988) and Dps (Altuvia et al. 1994) as well. This suggests that there are gene interactions within the OxyR regulon itself and that the deletion of the genes would result in disastrous effect than the deletion of the activator or repressor protein.

The molecular details of TonB protein activity remain unknown with relevance to oxidative DNA damage. However, I have found that with the deletion of the *tonB* gene, oxidative DNA damage is reduced. This indicates that TonB brings detrimental effect to the cells if it is overtly expressed by causing more energy transduction across the periplasm to the membrane receptors of the siderophores whereby the iron ligand of the siderophores would bring in more iron molecules into the cells, hence causing the increase in Fenton reaction and oxidative DNA damage. The deletion of TonB alone is strong enough to reduce the oxidative DNA damage level which indicates that TonB protein is the key player under the regulation of the Fur regulon which could regulate the iron transportation system. This is supported by another study that the hypermutability of a strain deficient of *fur, sodA* and *sodB* which induced excess iron and excess superoxide production was able to be salvaged with the deletion of the *tonB* gene (Nunoshiba et al. 1999). In addition to that, the *yaaA Hpx*$^{-}$ deficient strain also showed recovery to the cell viability when *tonB* gene was deleted (Liu, Bauer, and Imlay 2011). These evidences support that iron regulation through the transportation and importation system through the TonB pathway stimulates the ·OH production that leads to oxidative DNA damage.

4. YaaA reduces oxidative DNA damage.

Little is known about the protein YaaA in relations to its molecular mechanisms except that it is regulated by OxyR (Zheng et al. 2001) and that it helps reduce the amount of intracellular unincorporated iron proven by whole-cell electron paramagnetic resonance (EPR) analysis (Liu, Bauer, and Imlay 2011). I investigated this gene's contribution to find out its role in oxidative DNA damage and found that YaaA can suppress oxidative DNA damage especially in low nutrient condition or stress condition (**Figure 16**). The deletion of the *yaaA* gene itself did not exhibit any specific growth phenotype which indicates that the absence of YaaA in the wild type or repair deficient strain (*ΔmutM ΔmutY*) did not have any effect on the physiological

growth of the cells. Also, I found that in the deletion of the *yaaA* gene, the strain showed no difference in growth in the presence of 1mM H_2O_2 but the addition of plasmid overexpressing the gene makes the cells more sensitive towards H_2O_2 (**Supplementary Figure 4**). Nevertheless, YaaA was clearly shown to be affecting on the *Hpx*$^-$ strain (Seaver and Imlay 2004) which lacks all the H_2O_2 scavengers and deemed the cell to be highly exposed to H_2O_2 stress. Hence, in comparison to my study, YaaA protein can be suggested to suppress oxidative DNA damage in oxidative stress condition caused by high intracellular H_2O_2 level which is contributed by the low nutrient condition in M9+glucose minimal media. However, the intracellular H_2O_2 level does not directly affect the oxidative DNA damage level, but rather, intracellular iron level was increased due to the absence of YaaA that led to the main factor of ·OH production and hence the increased DNA damage level. When *yaaA* deletion in the *ΔmutM ΔmutY* strain was grown in M9+glucose minimal media, the oxidative DNA damage level increased by 2.4-folds higher than the wild type *ΔmutM ΔmutY* strain. However, when the *ΔyaaA ΔmutM ΔmutY* strain was tested for the intracellular H_2O_2 level, there was no increase in comparison with the *ΔmutM ΔmutY* strain (**Supplementary Figure 6**). Hence, it can be brought to light that the oxidative DNA damage that was measured is more inclined to the production of ·OH that is caused by the increased Fenton reaction rather than the high intracellular H_2O_2 which can be scavenged and less reactive than the ·OH counterpart.

I found that the YaaA protein and Dps protein may work to suppress oxidative DNA damage in an independent manner after showing the increase in mutation frequency level in the absence of those genes (**Figure 17**). With the deletion of both genes, I would expect the oxidative DNA damage level to have an additive effect, however, the deletion of both genes elucidated a synergistic effect with the mutation frequency shown to be tremendously increased (7.3-fold). Even though the molecular mechanism of YaaA remains unknown, I showed that with the absence of Dps protein, YaaA can help to compensate its function to maintain the cells in oxidative stress condition through the complementation assay. This functionality works in a vice versa manner where the absence of YaaA will then be compensated by the functionality of Dps to control oxidative DNA damage. To the best of my knowledge, this finding is novel and that the previous results only mentioned that the double deletion of *yaaA* and *dps* in the *Hpx*$^-$ strain made cells' growth defect to be more exacerbated. This finding may be important to give insights to further studies especially in the structural binding and the interactive functionality of these two proteins.

Part 2: Oxidative DNA damage is suppressed by iron suppression.

After having shown that OxyR and its regulon genes are able to suppress oxidative DNA damage level through the reduction of ·OH production by the iron regulatory genes, I confirm with other approaches; another genetic method that is different from the OxyR regulon and the chemical method that applied the common chelator 2,2-Bipyridyl. In my study, I have

shown the protective effect of the *mstA* overexpression through the construction of p_{tet}-*mstA* in the deletion of *oxyR* (**Figure 20**) and the deletion of *dps yaaA* strain (**Figure 21**). However, the deletion of the *mstA* could not be seen to give a disastrous effect of oxidative DNA damage level to both above strains (data not shown). This may be because the overexpression strain has a higher rate of L-cysteine utilization via the sequential action of aspartate transferase (AspC-MstA complex). Based on previous study (Awano et al. 2005), of all the cysteine desulfhydrases, it can be suggested that the major enzyme which is responsible for L-cysteine conversion to H_2S would be the 3MST. The high L-cysteine degradation in the p_{tet}-*mstA* cells helps the suppression of Fenton reaction which is said to be promoted by the L-cysteine effectiveness in reducing Fe^{3+} to Fe^{2+} (S. Park and Imlay 2003). Therefore, to that effect, Fe^{2+} can be suppressed, causing the lesser production of ·OH and reduced level of oxidative DNA damage.

The usage of cell permeating iron chelator such as bipyridyl has been widely used (Imlay, Chin, and Linn 1988) to prove that ferrous iron is the causing factor of Fenton reaction and the consequence of it is the increased in oxidative DNA damage. In my study, it was shown that the iron chelation activity is clearly seen especially when cells are grown in the M9+glucose minimal media (**Figure 23**). This may suggest that genes that are important in iron regulation such as *yaaA* and *dps* which are deleted and grown in defined M9+glucose minimal media would cause the increased production of intracellular ferrous iron. The cells produced high endogenous ferrous iron by Fenton reaction that can be salvaged by chelating with the bipyridyl. However, in LB media, the addition of bipyridyl only showed a slight difference in oxidative DNA damage level. This maybe because LB media is a complex media that may reduce the iron chelation activity due to the saturation on the chelation sites of the bipyridyl. This is supported by another study that showed in an iron-rich condition, the iron-dependent repression was fully saturated (Lim et al. 2008).

Part 3: Suggested molecular mechanism of YaaA's interaction to the other iron regulatory genes is through the activity of iron transport mechanisms.

The surprising discovery that was found was the combination deletion of *fur* and *yaaA* which showed an unexpected similarity of oxidative DNA damage level with the *yaaA* deletion strain. One possibility would be that the functionality of YaaA protein would be under the control of the Fur regulon. From the gene expression study (**Figure 25**), it was shown that the *yaaA* gene may seem to be under the control of Fur. However, the induction of *yaaA* gene by Fur was not high and additionally, the transcription of YaaA by the Fur protein remains ambiguous as no research has been done on the activity of the Fur-box on the *yaaA* promoter site. Besides that, the expression of *yaaA* under the deletion of *fur* may indicate that YaaA protein's function may substitute for the Fur protein's functionality to regulate iron importation and transportation. This would be a more likely possibility because the mutation frequency level of the deletion of both genes *fur-yaaA* was not exacerbated as the functionality of these two

genes are similar. Meanwhile, the data also suggest that Fur expression is independent of YaaA, indicating that both genes may work in the same way in reducing oxidative DNA damage.

In relevance to the similarity of YaaA and Fur proteins' function, I attempted to discover if YaaA may have any effect towards the Fur-regulated gene, of which *tonB* gene was shown to be the most prominent one (Noinaj et al. 2010). As expected, *tonB* gene is strongly repressed by Fur, however on the contrary, YaaA does not show regulation on the transcription of the *tonB* gene. The data suggest that YaaA protein's function to regulate *tonB* gene expression may be different from the Fur protein's function in repressing the *tonB* gene. One possibility is that YaaA may affect the activity of TonB protein interaction with the other iron transporter activity (Liu, Bauer, and Imlay 2011), such as the ferric enterobactin uptake (Ent) system. Protein-protein interaction experiments may uncover these possibilities of the YaaA protein. It can also be hypothesized that YaaA may affect other genes regulated by the Fur regulon. However, more experiments needed to be done as the Fur-regulon's network of iron homeostasis involves many genes in a complex interaction (Seo et al. 2014).

The hypothesis that YaaA and Fur may work in a similar manner was supported by the Dps expression study that suggested Dps works as a compensatory protein in the absence of either the Fur or the YaaA expression (**Figure 27**). It was discovered that the deletion of *yaaA* increased the expression of the *dps* gene prominently. This novel finding can advocate to the mutation frequency of the *dps-yaaA* deletion combination that showed both the genes work in a synergistic manner. What is interesting is that it was shown in a study using a *Salmonella* model that in the deletion of Dps, survival against oxidative H_2O_2 stress condition under iron replete condition (high iron level) was similar to the wild type strain (Yoo et al. 2007). The study suggested that apart from the Fur's function as a subsidiary iron regulator, there is an induction of an unknown protective mechanism against the oxidative stress. This projects a possibility that YaaA can be the suggestive unknown protective mechanism since Fur and YaaA are highly induced in the absence of the Dps protein. Besides that, it was evidenced in the same study that the *S. typhimurium* showed a 95% homology with other enteric Gram-negative bacteria such as *E. coli, Shigella* and *Yersinia.*

From all the data that have been gathered, it can be summarized that, like Dps and unlike Fur, YaaA seems not to play a prominent role in routine growth such as in the LB media. However, YaaA expression level and functionality in suppressing oxidative DNA damage level can be seen to be highly influenced by the presence of H_2O_2 stress as seen in the mutation frequency data and gene expression study data. Because of the lack of literature review on the molecular mechanisms and functionality of YaaA protein, with the current data, I can assume that YaaA plays an important role as an accessory protein of iron importation and mediator to iron trafficking machinery under the regulation of the OxyR regulon. I hypothesize the working mechanisms of the iron regulation to reduce oxidative DNA damage through the following proposed model as shown in **Figure 28**: (1) upon the induction of H_2O_2 stress, activated OxyR

induces the expression of the iron regulatory genes of its regulon. (2) Fur being the major iron regulator in iron importation represses the regulation of *tonB* expression so that TonB protein would not be able to energize the other iron importation mechanisms that bring in Fe^{3+} into the cell. Fur also represses other iron acquisition genes. (3) Meanwhile, YaaA may work to suppress the activity of TonB by inhibiting translocation activity of ferrisiderophore (iron transporter proteins) that bring in iron from the outer atmosphere of the cell. (4) YaaA may also be regulating on the other iron acquisition genes under the Fur regulon. (5) YaaA protein protects cells against oxidative DNA damage when cells are induced under oxidative H_2O_2 stress. (6) Finally, the Dps protein as an iron storage protein, functions to help control the iron regulation under the OxyR expression to reduce oxidative DNA damage when cells undergo stress condition.

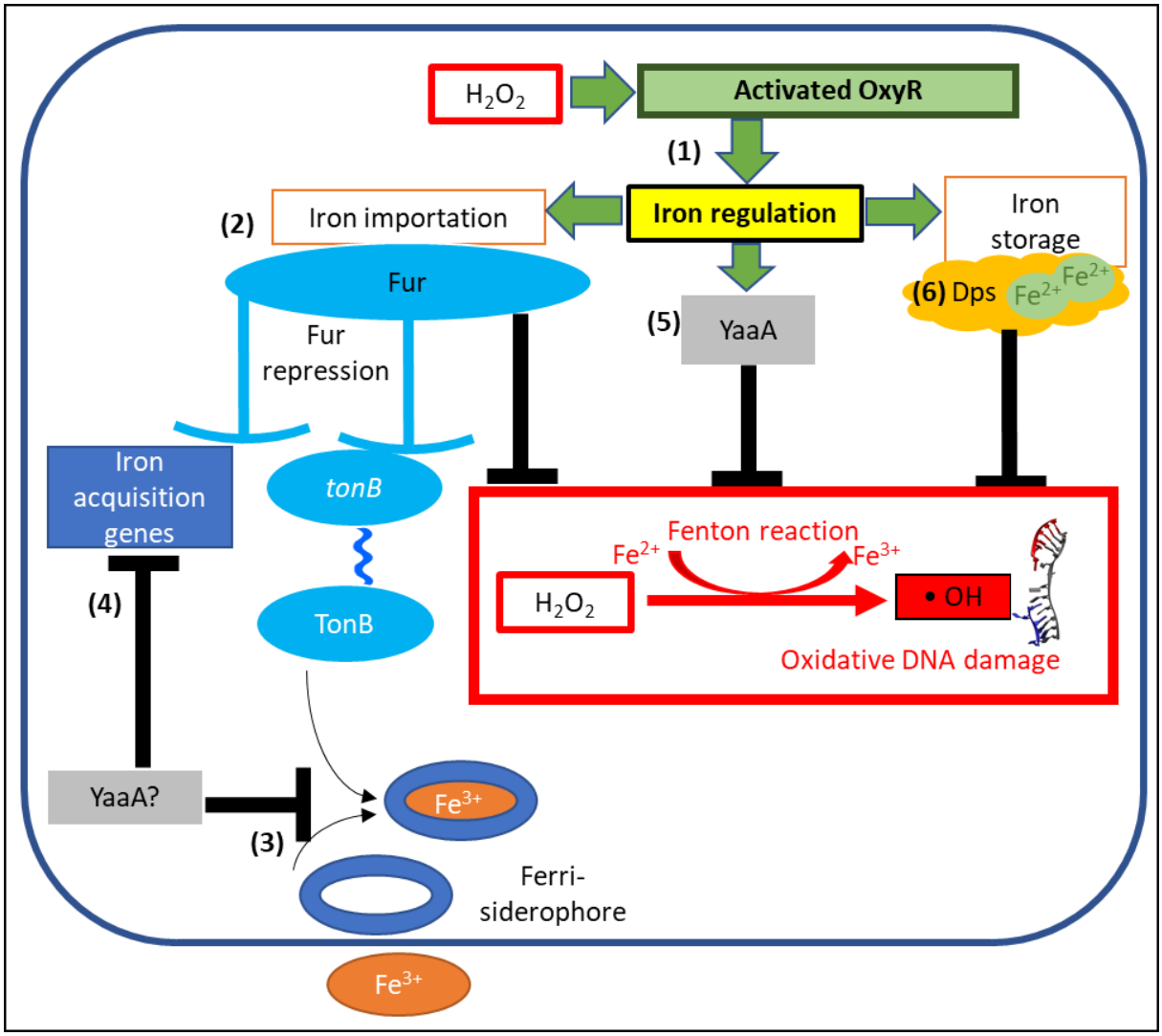

Figure 28: A hypothesized model of mechanisms of the iron regulation to reduce oxidative DNA damage.

Conclusion

As a conclusion from the investigation of this study, I have shown that, OxyR regulon, specifically its iron regulatory genes are able to protect against oxidative DNA damage. Genes that are involved in the iron acquisition and transportation such as the *yaaA* gene have potentials in helping cells survive in oxidative stress, but its molecular mechanisms remain to be explored. As the OxyR protein shares homology across most enteric bacteria, this study is suggestive that the iron regulation mechanism is the main pathway of how bacteria cells survive and retain their resistance towards the oxidative stress. The scope of this study does not limit itself to prokaryotes level only albeit some genes such as *fur* and *yaaA* are only found in bacterial cells, however, the concept of how cells are adaptable in protecting against the ROS attack by the iron homeostasis machinery and how the repair mechanisms are involved, provide crucial information on how aerobic organisms maintain genomic stability.

Future plans

There are much more aspects that remain to be explored in the research of iron regulation systems of the *E. coli* cells. The factors of iron importation, trafficking and disposition within the cell mostly remain elusive. With more genes with unknown functions such as that of *yaaA* are discovered, the biological activity and functionality in relevance to the protection of oxidative DNA damage are widely vague. Nevertheless, from this study, I have found an important clue that YaaA protein may function mostly through the iron importation or trafficking mechanisms by maybe inhibiting the function of other iron acquisition genes or inhibiting the activity of TonB protein which will then reduce the mobility of translocating ferric irons into the cells. My future plans would be to find out how YaaA can interact towards these kinds of activities by: finding the gene interaction of *yaaA* to the other iron receptors and traffickers such as *fecA* (ferric citrate outer membrane transporter), *fhuF* (hydroxamate siderophore iron reductase) and *fepA* (ferric enterobactin outer membrane transporter). Much more studies can be done to progress the understanding of iron metabolisms.

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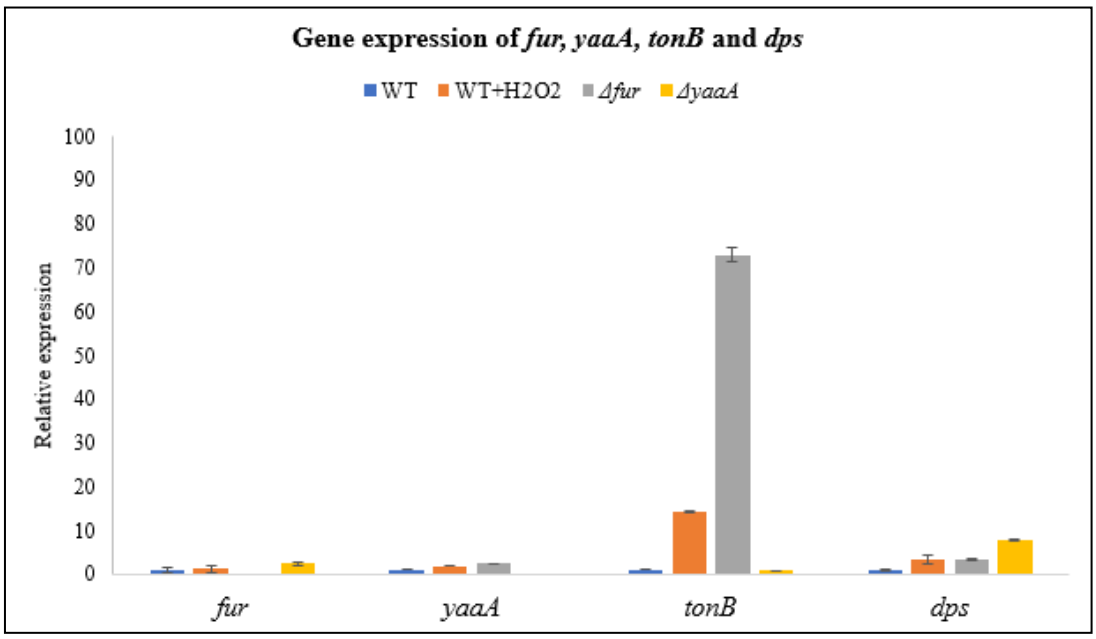
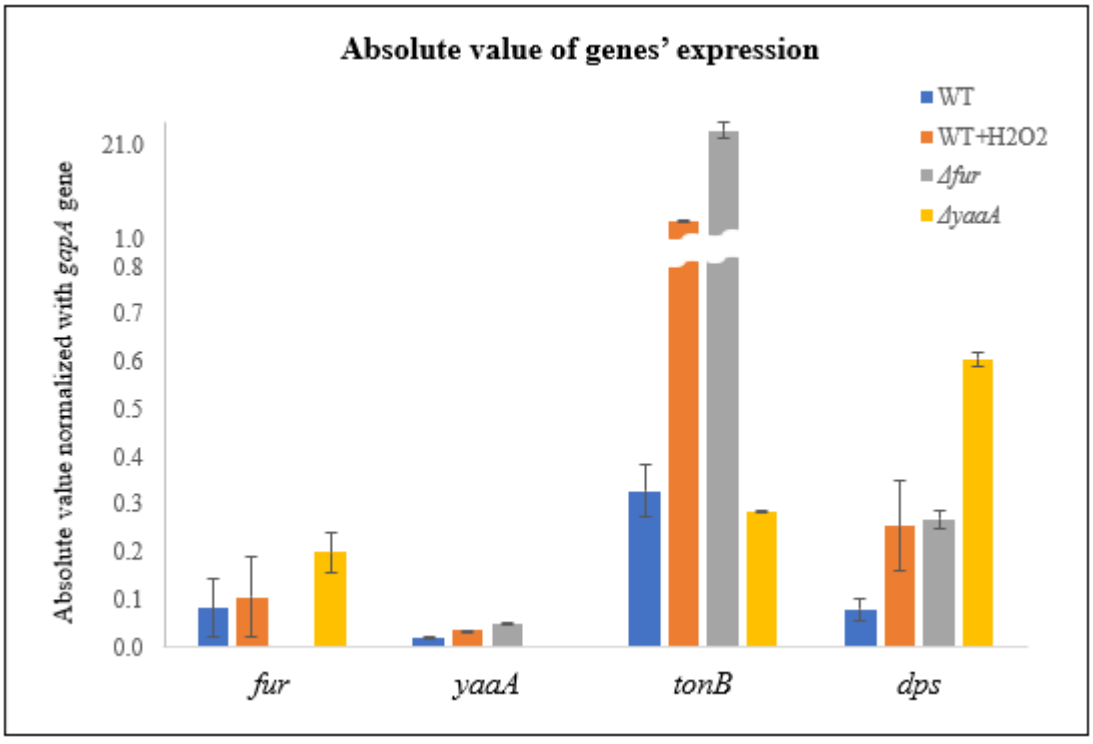
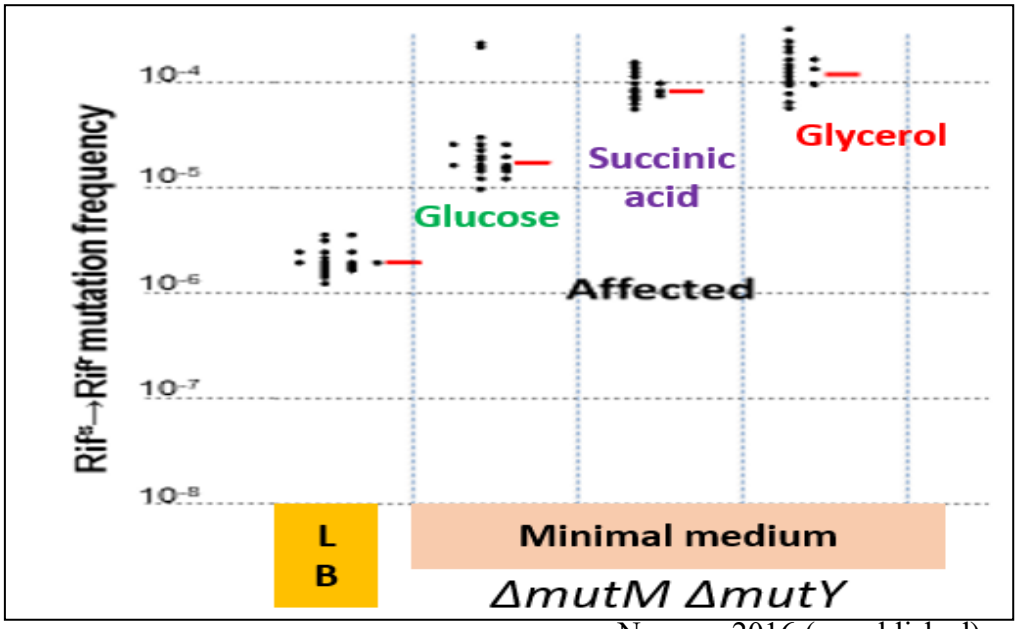
Supplementary figures



Supplementary Figure 1(a): Expression levels of *fur, yaaA, tonB* and *dps.* Data representation is the mean of three replicates measurement with the ± standard deviation indicated by error bars.



Supplementary Figure 1(b): Absolute value (transcript concentration) before conversion to relative expression. Data representation is the mean of three replicates measurement with the ± standard deviation indicated by error bars.



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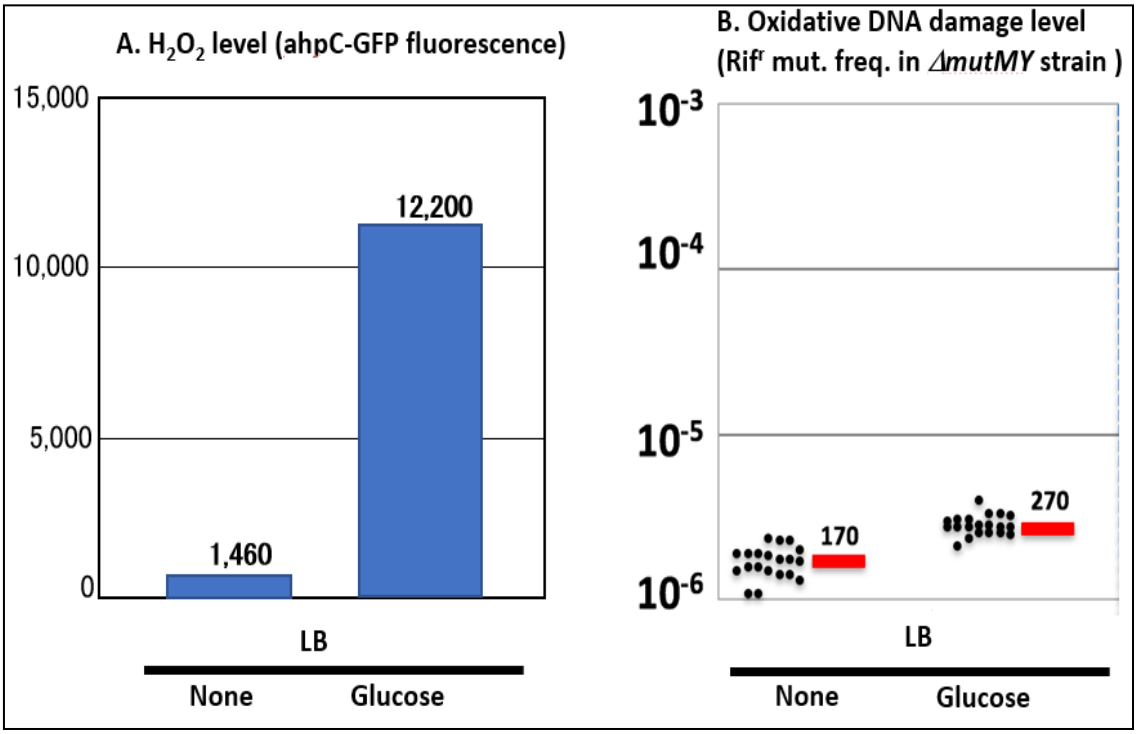
Supplementary Figure 2: Distribution mutation frequency of *ΔmutM ΔmutY* in LB and M9 minimal media with different carbon sources for 20 experiments in each condition.

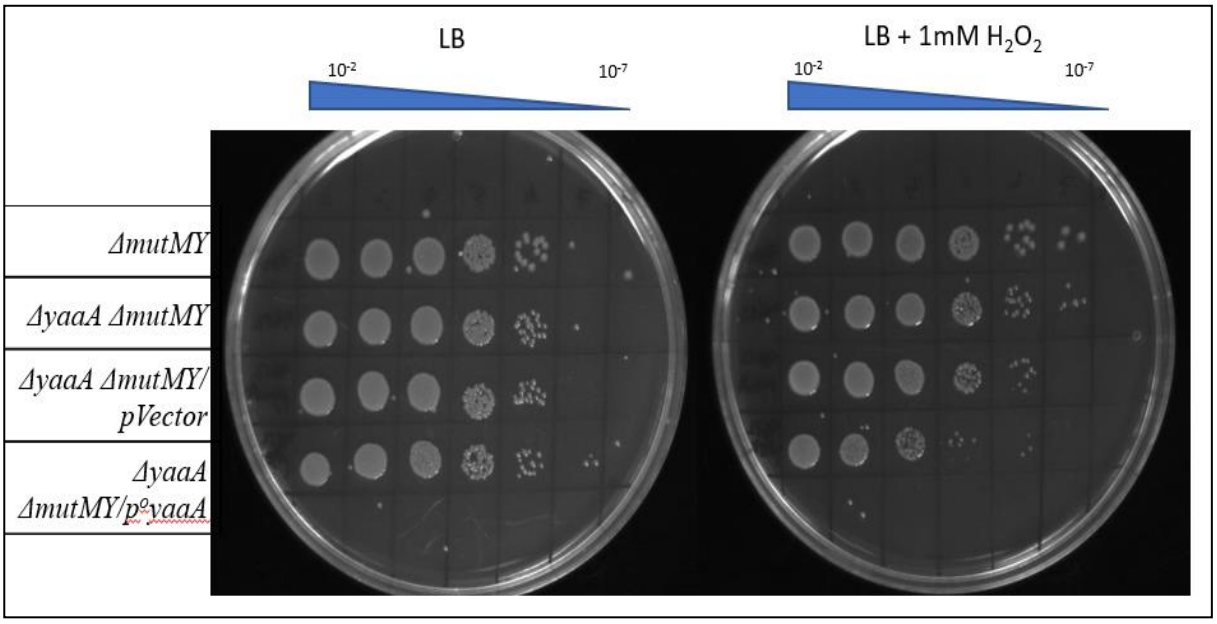


Nunose, 2016 (unpublished).

Supplementary Figure 3:

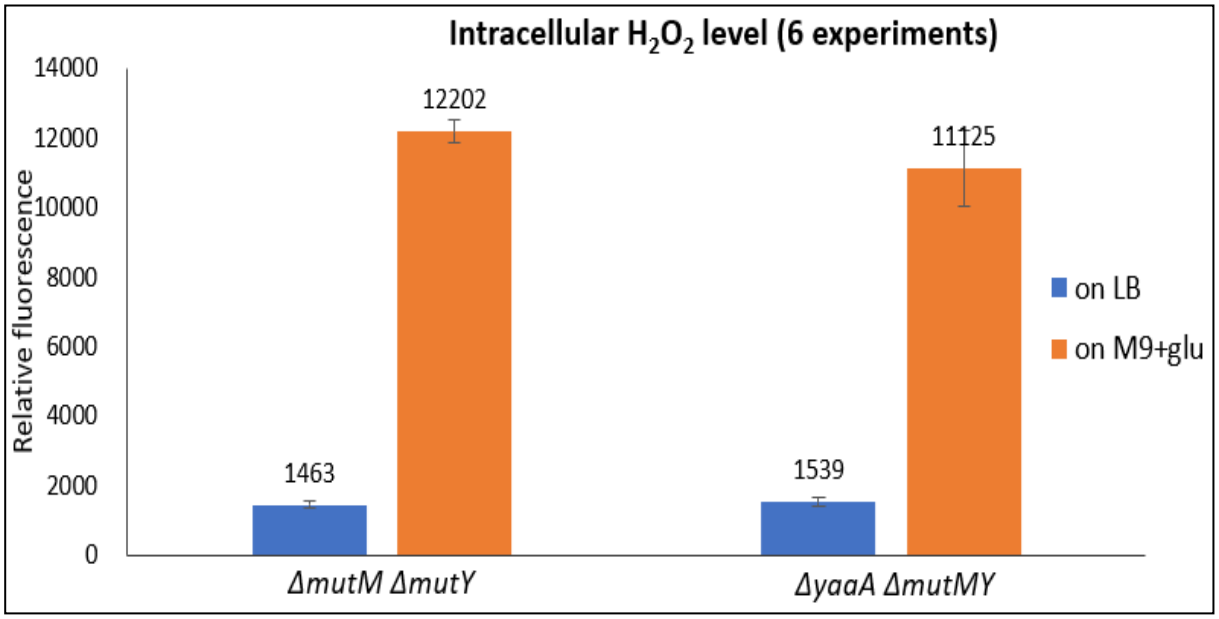
A. Intracellular H_2O_2 level of *ΔmutM ΔmutY* in LB media and LB+glucose media.

B. Distribution mutation frequency of *ΔmutM ΔmutY* in LB and LB+glucose media.



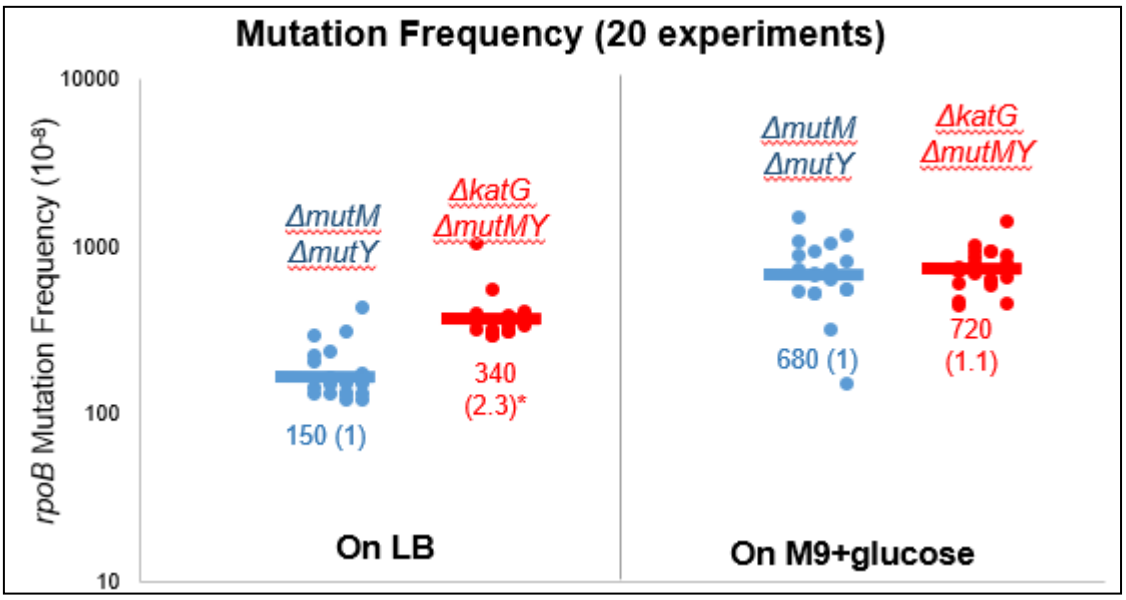
Supplementary Figure 4:

Disk diffusion assay of H_2O_2 sensitivity of *yaaA* deletion strain and its complementary overexpressing plasmid in LB (left plate) and LB + 1mM H_2O_2 (right plate).



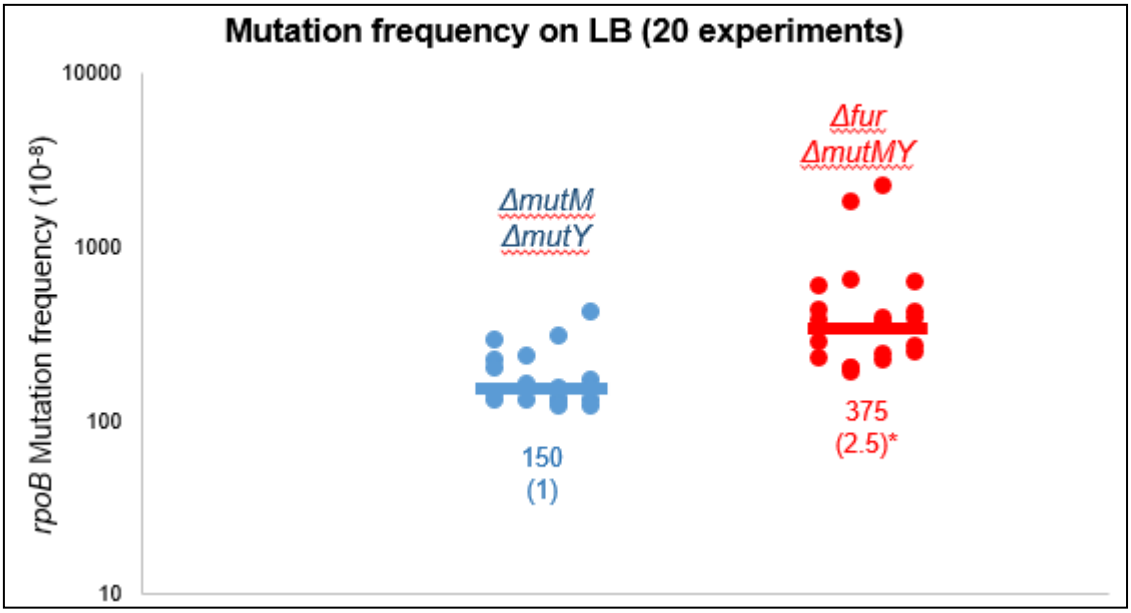
Supplementary Figure 5:

Intracellular H_2O_2 level of *ΔmutM ΔmutY* and *ΔyaaA ΔmutM ΔmutY* on LB and M9+glucose minimal media. Data representation is mean for 6 experiments with ± standard deviation indicated by error bars.



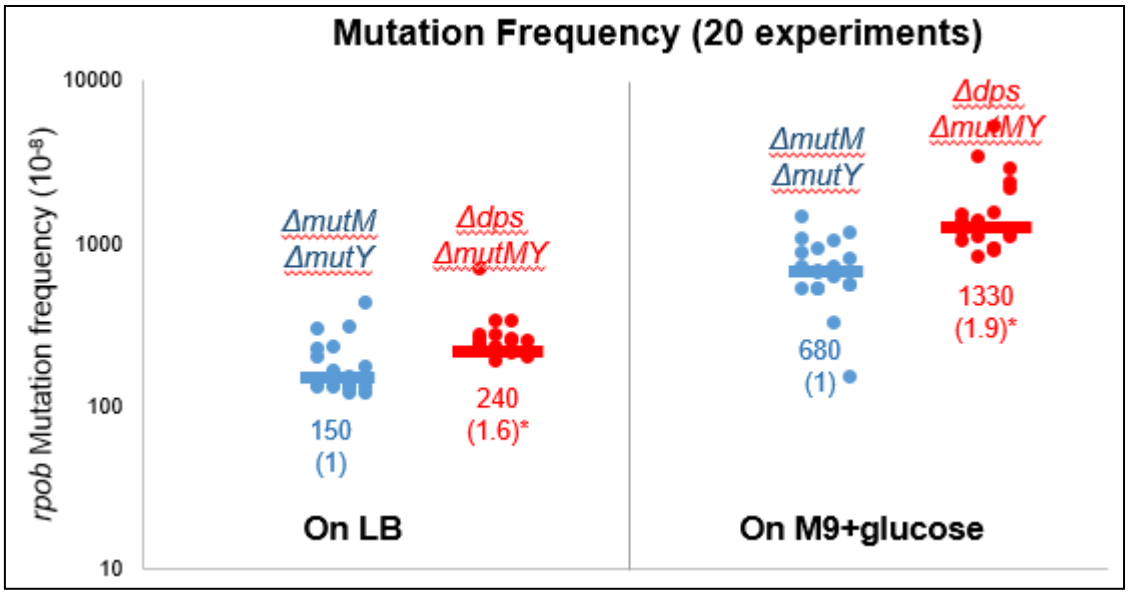
Supplementary Figure 6:

Distribution of mutation frequencies determined with *ΔkatG ΔmutM ΔmutY* cells grown in LB and M9+glucose minimal media. Colored dots indicated are the individual experiments while the bars indicated represent the median levels and the data label of the colored dots indicated are the median value for 20 experiments. The values in parenthesis are the fold-change in comparison to the wild type. * indicates significance p<0.01 calculated by Mann-Whitney U test against the *ΔmutM ΔmutY* strain in the respective media.



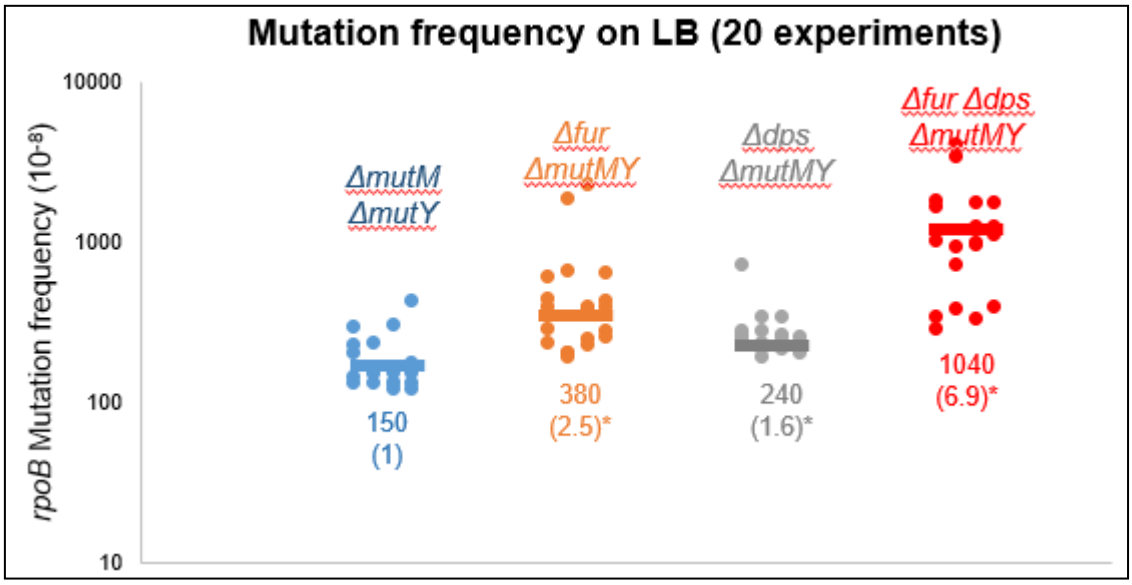
Supplementary Figure 7:

Distribution of mutation frequencies determined with *Δfur ΔmutM ΔmutY* cells grown in LB media. The values in parenthesis are the fold-change in comparison to the wild type. * indicates significance p<0.01 calculated by Mann-Whitney U test against the *ΔmutM ΔmutY* strain.



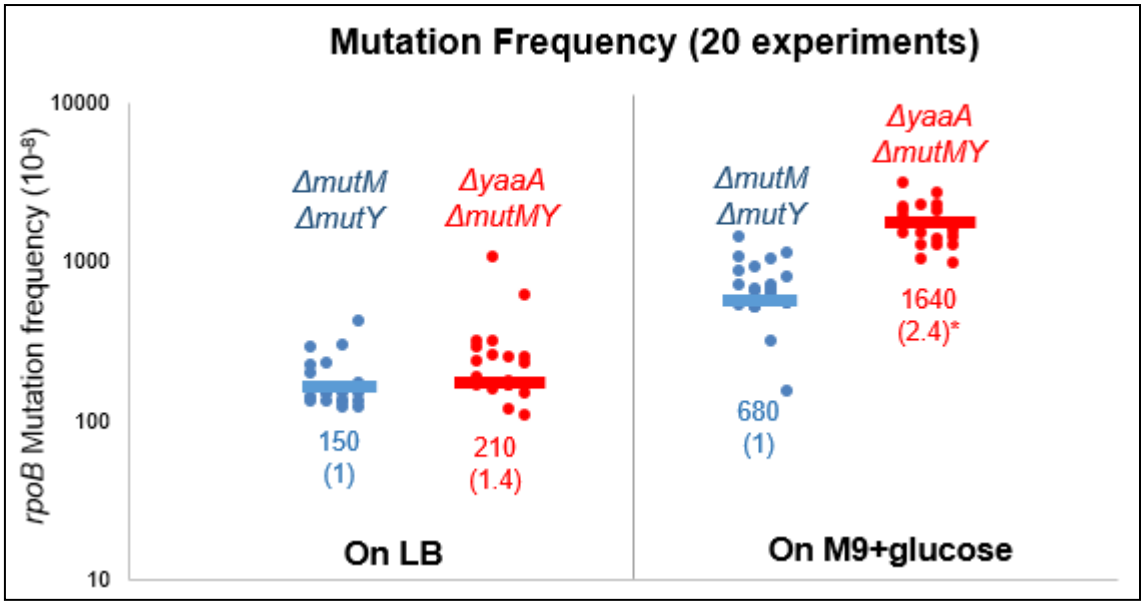
Supplementary Figure 8:

Distribution of mutation frequencies determined with *Δdps ΔmutM ΔmutY* cells grown in LB and M9+glucose minimal media. The values in parenthesis are the fold-change in comparison to the wild type. * indicates significance p<0.01 calculated by Mann-Whitney U test against the *ΔmutM ΔmutY* strain in the respective media.



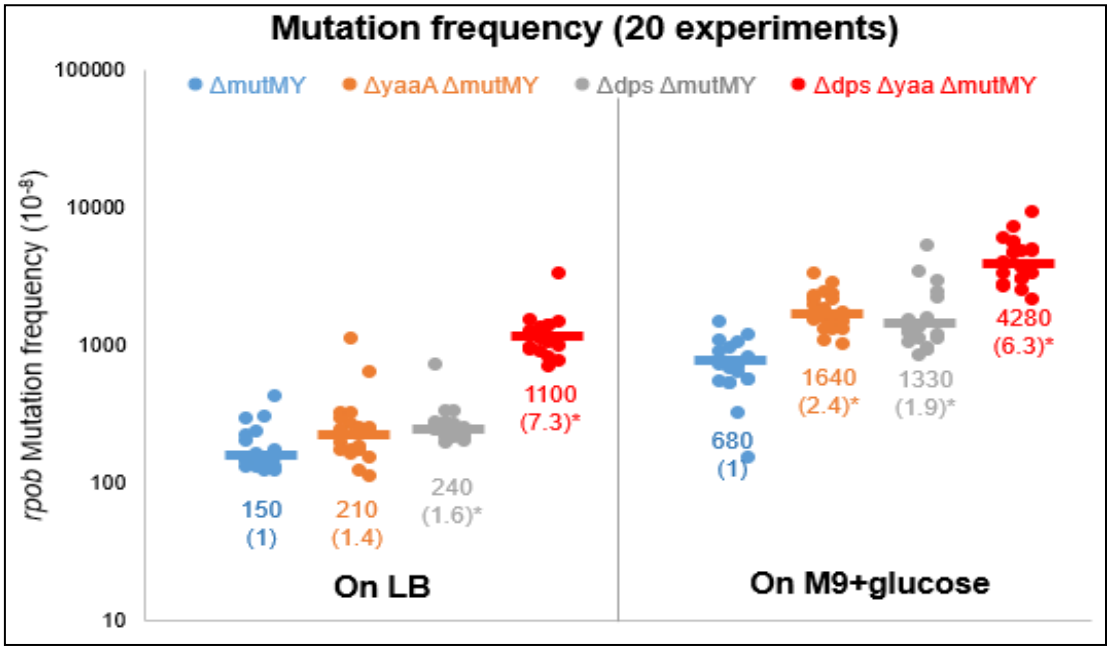
Supplementary Figure 9:

Distribution of mutation frequencies determined with cells grown in LB media. The values in parenthesis are the fold-change in comparison to the wild type. * indicates significance p<0.01 calculated by Mann-Whitney U test against the *ΔmutM ΔmutY* strain.



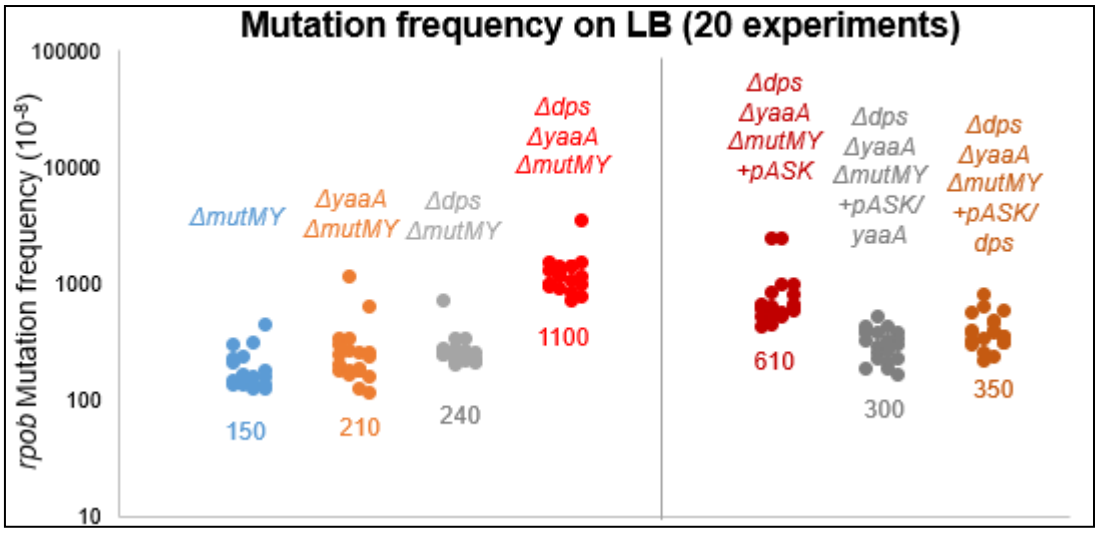
Supplementary Figure 10:

Distribution of mutation frequencies determined with cells grown in LB and M9+glucose minimal media. The values in parenthesis are the fold-change in comparison to the wild type. * indicates significance p<0.01 calculated by Mann-Whitney U test against the *ΔmutM ΔmutY* strain in the respective media.



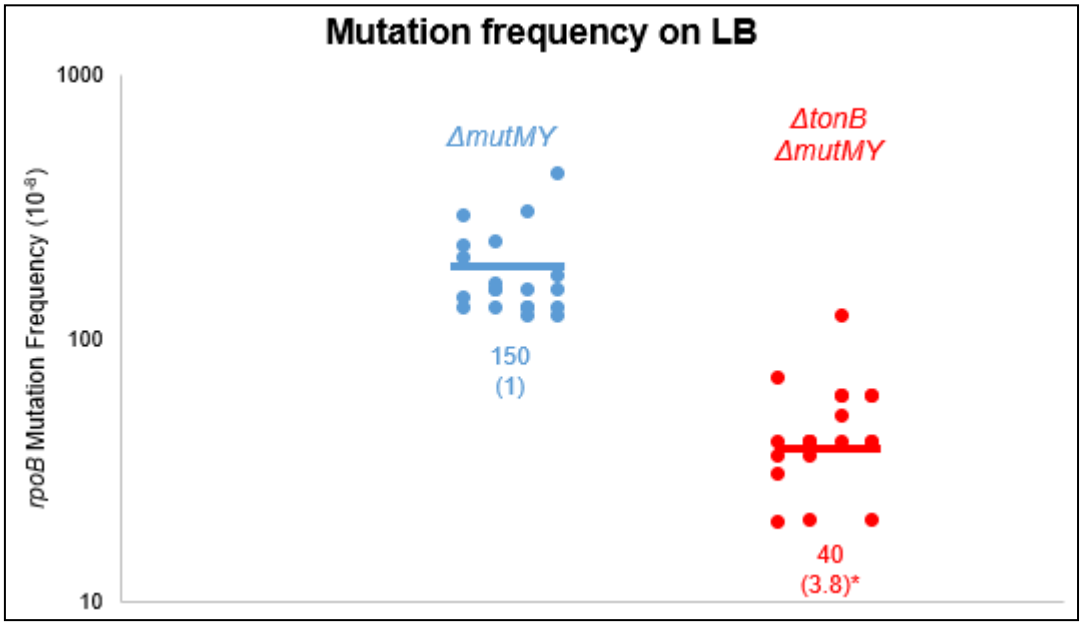
Supplementary Figure 11:

Distribution of mutation frequencies determined with cells grown in LB and M9+glucose minimal media. The values in parenthesis are the fold-change in comparison to the wild type. * indicates significance p<0.01 calculated by Mann-Whitney U test against the *ΔmutM ΔmutY* strain in the respective media.



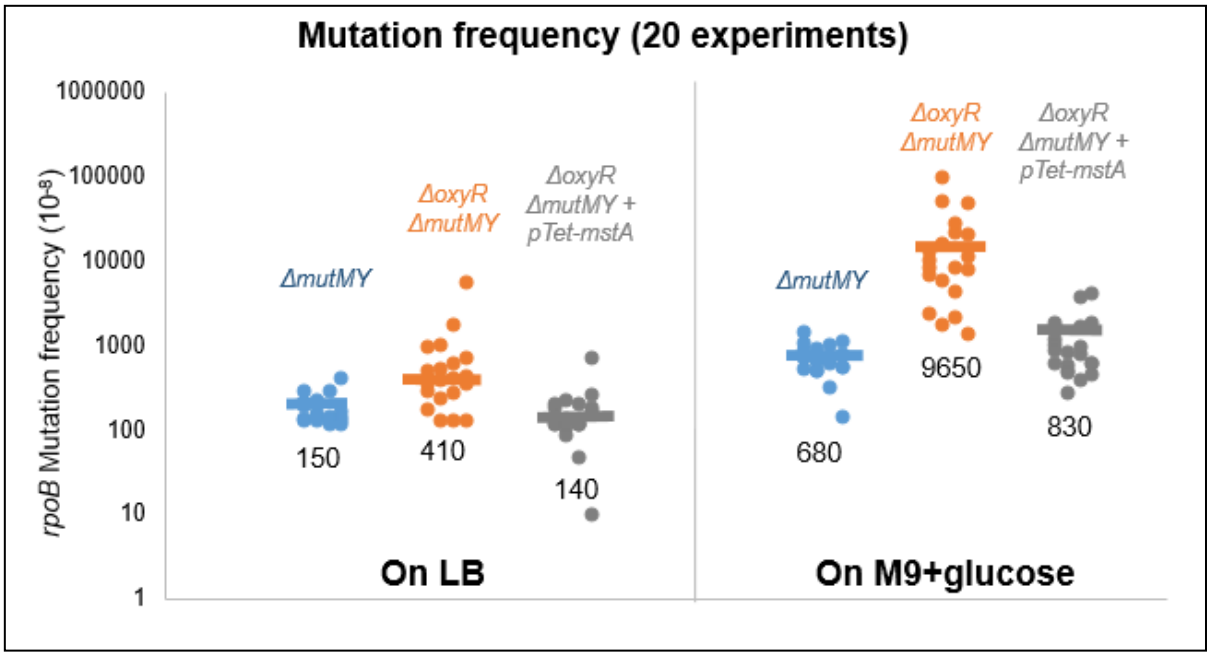
Supplementary Figure 12:

Distribution of mutation frequencies determined with cells grown in LB media.

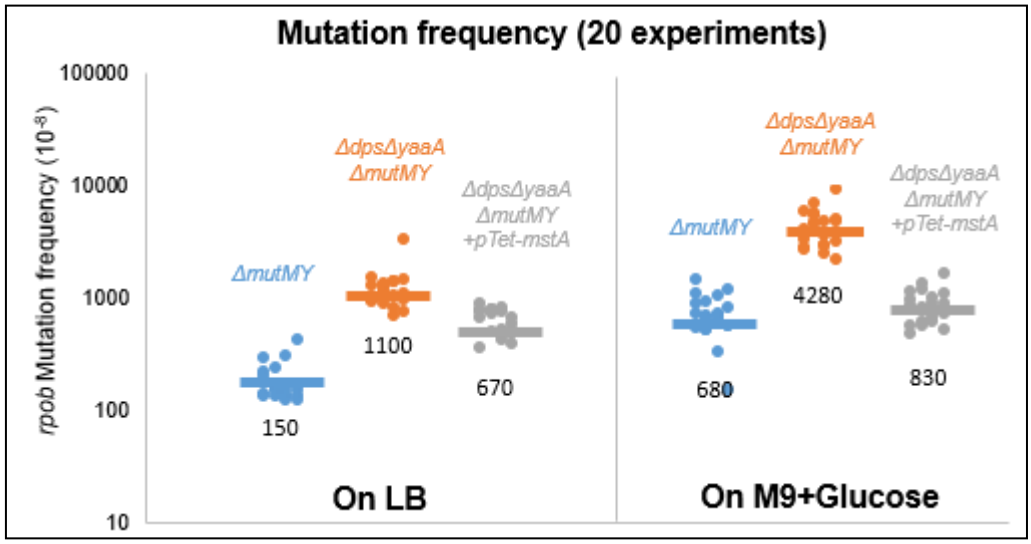


Supplementary Figure 13:

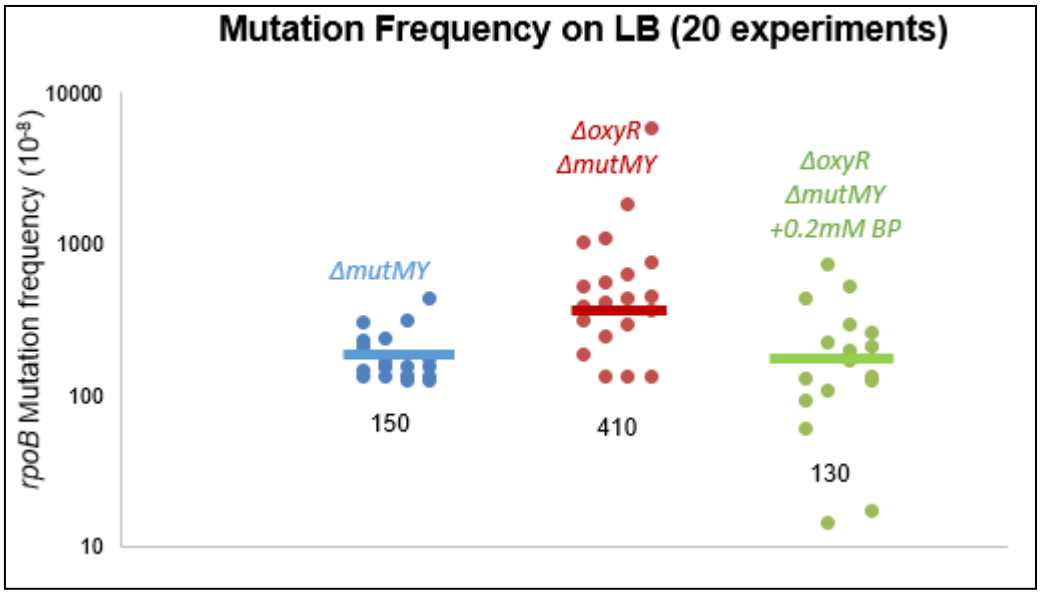
Distribution of mutation frequencies determined with cells grown in LB media. The values in parenthesis are the fold-change in comparison to the wild type. * indicates significance $p<0.01$ calculated by Mann-Whitney U test against the *ΔmutM ΔmutY* strain.



Supplementary Figure 14:
Distribution of mutation frequencies determined with cells grown in LB and M9+glucose minimal media.

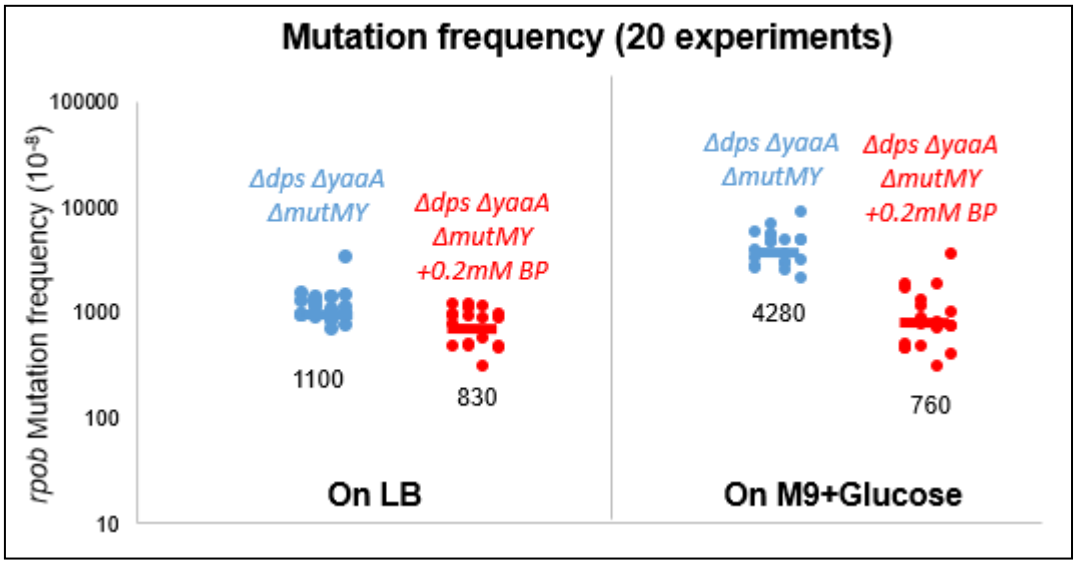


Supplementary Figure 15:
Distribution of mutation frequencies determined with cells grown in LB media and M9+glucose minimal media.



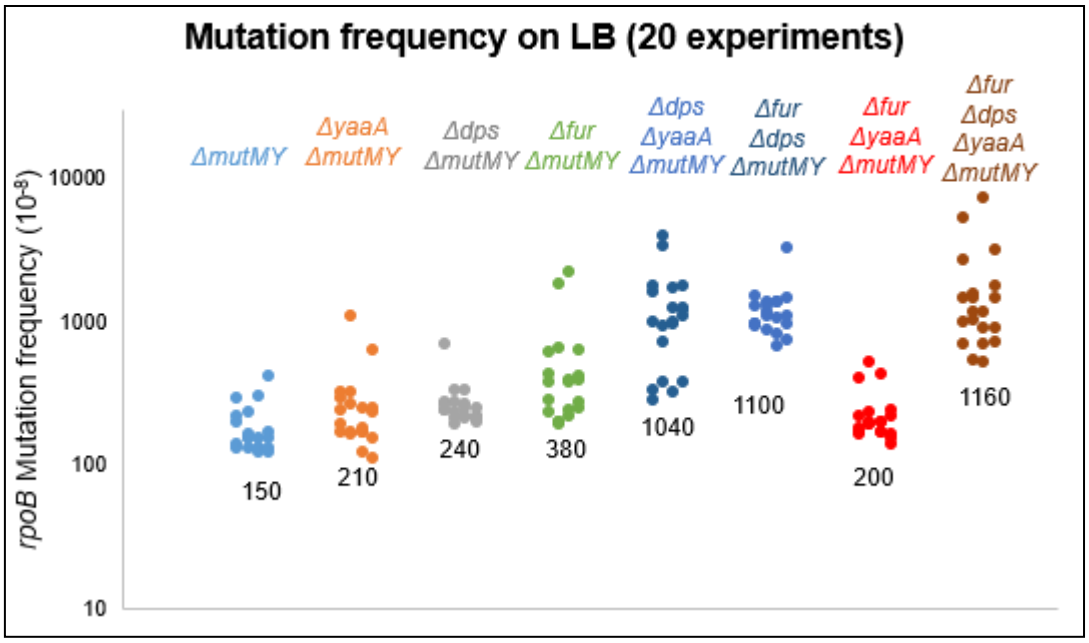
Supplementary Figure 16:

Distribution of mutation frequencies of *ΔoxyR ΔmutMY* determined with cells grown in LB media and LB + 0.2mM 2,2- Bipyridyl.



Supplementary Figure 17:

Distribution of mutation frequencies of *Δdps ΔyaaA ΔmutMY* determined with cells grown in LB and M9+glucose minimal media with the addition of 0.2mM 2,2- Bipyridyl.



Supplementary Figure 18:

Distribution of mutation frequencies of iron regulator genes and the combination deletion genes on LB media.