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The Role of Mfd in Stationary-Phase Oxidative Damage Repair in *Bacillus subtilis*

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THE ROLE OF MFD IN STATIONARY-PHASE OXIDATIVE DAMAGE REPAIR IN

BACILLUS SUBTILIS

By

Katelyn Porter

Bachelor of Science-Biological Sciences
Northern Arizona University
2013

A thesis submitted in partial fulfillment
of the requirements for the

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Thesis Approval

The Graduate College
The University of Nevada, Las Vegas

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entitled

The Role of MFD in Stationary-Phase Oxidative Damage Repair in *Bacillus Subtilis*

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ABSTRACT

The Role of Mfd in Stationary-Phase Oxidative Damage Repair in *Bacillus subtilis*

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Since the 1950's it has been shown that bacterial cells accumulate mutations even in non-dividing conditions, but how this type of mutation occurs is still highly debated. In *Bacillus subtilis*, Mfd, a precursor of the nucleotide excision repair (NER) system, mediates the formation of mutations in stationary-phase or non-replicating cells. In growing cells, Mfd recruits repair when RNA polymerase is stalled during transcription; it then recruits proteins from NER to repair damage. Here, we examine the hypothesis that Mfd mediates the formation of mutations by interacting with cellular components that repair reactive oxygen species (ROS), a natural byproduct of bacteria cell respiration. Utilizing two oxidants, we tested the hypothesis that Mfd protects cell viability and mutation development after exposure to ROS in stationary-phase. Our data showed that Mfd mitigated damage caused by reactive oxygen species and that such effect is independent of the NER system. Also, Mfd, MutY and reactive oxygen species mediated the formation of mutations in stationary-phase cells.

Table of Contents

Abstract.....	iii
List of Tables.....	v
List of Figures.....	vi
List of Equations.....	viii
Chapter 1: Introduction.....	1
Chapter 2: Oxidative Damage, Mfd and Mutagenic Repair.....	6
Introduction	6
Methodology.....	9
Results and Discussion	21
Chapter 3: Conclusions and Future Directions.....	44
References.....	46
Curriculum Vitae.....	50

List of Tables

Table 1. Strains utilized in this study	11
Table 2. Stationary-phase mutagenesis induction and oxidative stress treatment conditions.....	17
Table 3. Transformation culture tube DNA additions.....	19

List of Figures

Figure 1. All plasmids used in this work.....15

Figure 2. Percent cell survival, displayed in log scale, in strains containing a functional or non-functional Mfd following exposure to ROS via the oxidizing agent *tert-butyl* hydroperoxide (TBH).24

Figure 3: Percent cell survival, displayed in log scale, in cells deficient in the Mfd, MutY or UvrA protein respectively, following exposure to ROS via the oxidizing agent *tert-butyl* hydroperoxide (TBH).....25

Figure 4: Percent cell survival, displayed in log scale, in cells containing single and double defects in Mfd and MutY deficient strains, following exposure to ROS via the oxidizing agent *tert-butyl* hydroperoxide (TBH).....26

Figure 5. Accumulation of Arg⁺ mutations over 9 days in CVarg stains deficient in Mfd, MutY or both following treatment with 1 mM TBH.....27

Figure 6. Viability of cells treated with TBH and IPTG over the 9 day SPM presented in Figure 5.....28

Figure 7. Accumulation of Arg⁺ mutations over 9 days in CVarg stains deficient in Mfd, MutY or both. Following treatment with 1 mM TBH and induction with IPTG.....29

Figure 8. Viability of cells treated with TBH and IPTG over the 9 day SPM represented in Figure 7.....30

Figure 9. The effect of Mfd and transcriptional induction on protection against 1 mM TBH of oxidant.....32

Figure 10. Diagram of UV data represented in figures 11-15.....34

Figure 11. YB955 cell survival following transcriptional induction with IPTG and or UV exposure.	35
Figure 12. KEP400 cell survival following transcriptional induction with IPTG and or UV exposure.	36
Figure 13. Cpleu cell survival following transcriptional induction with IPTG and or UV exposure.	37
Figure 14. KEP401 cell survival following transcriptional induction with IPTG and or UV exposure.....	38
Figure 15. Cpleu Mfd ⁻ cell survival following transcriptional induction with IPTG and or UV exposure.	39
Figure 16. Oxidative stress assay using diamide and transcriptional induction in strains containing an inducible <i>leuC</i> gene in a plasmid or the chromosome.	41
Figure 17. Survival of YB955 containing the empty plasmid pDG-148 after exposure to diamide.	42

List of Equations

Equation 1. Determining J/m^2 for UV damage exposure.....18

Equation 2. Formula to determine the least significant difference.....21

Chapter 1: Introduction

Evolution represents a cornerstone of science, affecting all aspects of life science.

Darwin's published works, in which he describes evolution through natural selection, quickly changed the way scientists began to think about variation in species over time (Darwin, 1859). Interestingly, while unveiling the concept of evolution was a pivotal moment in biology, the mechanisms influencing such process were yet to be described.

In the 1860's, botanist Gregor Mendel described a process for how evolution actually happens (Mendel, 1865). Mendel's experiments in which he crossed common pea plants over multiple generations showed that traits and their variants were heritable. Evolution would then work through natural selection of variants conferring a fitness advantage and passed onto the next generation. Interestingly, this concept became well accepted in higher organisms yet an understanding of how these ideas applied to bacteria, remained obscure.

It was not until the 1940's that microbiologists Salvador Luria and Max Delbruck investigated how bacteria generated mutations. Their work with multiple independent *Escherichia coli* cultures determined that bacteria acquired mutations that prevented lysis by the T1 phage. They concluded that bacteria generate mutations randomly through growth-dependent processes. This discovery described an important substrate in evolution, the formation of mutations and selection (Delbruck *et al*, 1943). These observations opened up the field of bacterial genetics and perhaps led us to neglect the possibility that mutations may also be the product of growth-independent processes.

Growth-independent mutagenesis or stationary-phase mutagenesis, (SPM) was first evidenced in the early 1950's while under study by Francis Ryan. Utilizing a strain of *E. coli* that was auxotrophic for the amino acid histidine, Ryan observed that under nutritional stress more *E.*

coli mutated and became prototrophs during stationary-phase than during growth phase (Ryan, 1954).

Stationary-phase is defined as a state in which bacterial cells experience no net growth. It is during this state that cells develop new mutations independently of genome replication. Stationary-phase is caused by many conditions such as overpopulation, toxin build up, and nutritional stress (e.g. amino acid starvation). Because bacterial cells spend much of their lives in non-replicating conditions, the generation of mutations in these conditions significantly contributes to the evolutionary process. Moreover, this type of mutagenesis has been linked to immune response evasion in pathogens as well as the accumulation of mutations conferring antibiotic resistance (Foster, 2005), which makes this a subject relevant to human health as well as evolution. Despite the importance of SPM, the underlying mechanisms driving such phenomena remain elusive.

Interestingly, stationary-phase mutagenesis stirred controversy in the scientific community in 1988. Cairns *et al.* presented the concept of directed mutation through the observation that *E. coli* Lac⁻ cells acquire mutations that conferred growth on media in which lactose was the sole carbon source but did not in other genes. Subsequent studies, showed that stochastic processes affected these types of mutation, eventually dispelled the concept of directed mutation in bacteria, and opened the door to larger questions about stationary-phase mutagenesis.

In *E. coli*, it has been proposed that during non-growing conditions, or conditions in which the stress regulon is activated, mutations occur due to the actions of error-prone polymerases repairing double stranded breaks in DNA. Repair of double-stranded breaks generate point mutations or gene amplification events that produce Lac⁺ cells. (Galhardo *et al.*, 2007).

Other models used in stationary-phase mutagenesis rely on reversion assays that confer prototrophy in amino acid biosynthesis; these constructs are based on frameshift or base substitution mutations. Bacteria carrying such mutations are then placed in an environment with a limiting growth factor and then observed for the ability to overcome their stress through the development of mutations over a prolonged time. Revertant mutants appear daily throughout 9-10 days. It is important to note that those mutants that arise within the first four days of stressed incubation are considered the product of growth-dependent processes. Colonies arising after the fourth day of incubation are considered to be stationary-phase mutants.

Stress-induced mutation in *E. coli* had focused primarily on measuring mutations that confer lactose utilization; it uses a *lacI-lacZ* gene arrangement. In this system the gene under study contains a *lac+1* frameshift mutation in the *lacI* gene. This gene arrangement is carried by an F conjugative plasmid. The *lac+1* frameshift construct results in a fusion to the *lacZ* gene and generates β -gal protein with reduced activity (2% of the wild type activity). Because of its reduced activity, Lac⁺ revertants may be generated through -1 frameshifts. Mutagenesis experiments indicated that stationary-phase mutagenesis (mutants arising after four days incubation) resulted primarily from -1 frameshifts mutations (reviewed in Galhardo *et al*, 2007).

There are two regulons that get activated during stress or stationary-phase, these are the SOS response and the cellular stress response. Both of these regulons contribute DNA repair factors acting globally and promoting mutation development. The stress response is controlled by the stress sigma factor (RpoS), which activates when environmental conditions become growth-limiting. Examples of such conditions are changes in temperature, toxin buildup, etc. In *E. coli*, RpoS up-regulates the synthesis of DNA error-prone polymerases (Foster and Layton,

2005). Error-prone polymerases participate in repair of double stranded DNA breaks and have a propensity to generate mutations.

Unlike the stress response, the SOS response is designed to respond to the internal environment of the cell. The SOS response results in arrests of DNA replication, which facilitates repair of damaged DNA, particularly double stranded breaks. In conditions of low levels of DNA damage the SOS response remains repressed as transcription-activating regions of SOS-dependent genes (about 50 genes that mediate DNA repair) are bound by a repressor, however events like double stranded breaks or paused chromosome replication lead to the formation of single-stranded DNA which interact and activate the RecA protein. The RecA nucleofilament protein has protease activity that cleaves the SOS repressor, which consequently allows transcription of SOS genes.

In the context of SPM, the activation of the stress and SOS responses lead to an increase in the error-prone DNA polymerase, pol IV. Pol IV is especially relevant in SPM because it has been shown to propagate up to 85% of point mutations in the previously referenced nutritional stress assays in which lactose is the only carbon source. (McKenzie *et al*, 2001).

Stationary-phase mutagenesis has been observed in other organisms as well, most important to my research is SPM in *Bacillus subtilis*. *B. subtilis* represents a unique model because it is a bacterium that grows and differentiates. *B. subtilis* has the ability to form subpopulations under stress. One such subpopulation displays competence, the development of which is regulated by the ComA and ComK proteins. In the early 2000's an experimental system to measure reversions to amino acid auxotrophy and SPM was developed. Experiments with this system not only concluded that ComA and ComK affected the ability of *B. subtilis* to form

revertants, but also that this method of mutation was independent of the RecA protein. This suggests that a subpopulation of differentially developed cells promotes the formation of mutations that confer escape from stress (Sung and Yasbin, 2002).

Another factor that has an effect on SPM in *B. subtilis* is Mfd (mutation frequency decline). This protein acts on RNA polymerases (RNAP) that stall during transcription elongation due to DNA damage. The function of Mfd has been extensively studied as a repair factor that prevents DNA damaged caused by ultraviolet (UV) light exposure. UV exposure causes bulky lesions in the DNA; these lesions are repaired by the nucleotide excision repair system (NER, composed of the Uvr proteins). Mfd directs NER to transcribed regions; such repair is called transcription coupled repair and is thought of as a NER sub-pathway (Hanawalt and Spivak, 2008). *B. subtilis* mutants lacking a functional Mfd protein express a decreased in SPM (Ross *et al*, 2006). However, the role of Mfd in SPM does not appear to invoke the NER pathway: SPM occurs in the absence of UV exposure and cells deficient in Uvr proteins do not show the same phenotype as those deficient in Mfd.

This thesis answers two questions about SPM in *B. subtilis*. Does Mfd interplay with repair systems other than the NER? And, does the level of transcription exacerbate the effect of Mfd in DNA repair reactions that do not involve the NER pathway? My research indicates that Mfd plays a role in the repair of oxidative damage in *B. subtilis*, and suggests that oxidative damage is a precursor to SPM.

Chapter 2: Oxidative Damage, Mfd and Mutagenic Repair

Introduction

Stationary-phase is defined as a state in which bacterial cells experience no net growth. During this phase, bacteria still perform basic cellular processes like repair of cell damage caused by reactive oxygen species generated during respiration. ROS are therefore inescapable sources of damage inflicting cytotoxic and genotoxic effects on cells. In conditions in which DNA replication and repair are limited, like those happening in cells under stress, ROS become significant intermediates in the accumulation of mutations. However the mechanisms by which mutations develop during stationary-phase remain obscure.

Stationary-phase mutagenesis is a collection of cellular mechanisms that produce genetic diversity during non-growing or stressed conditions (reviewed in Robleto *et al*, 2007). Stationary-phase mutagenesis first came to light in the early 1950's while under study by Francis Ryan. Utilizing a strain of *E. coli* that was auxotrophic for the amino acid histidine Ryan surmised that under nutritional stress more *E. coli* mutated more often than during active growth. These processes are important because they represent an underestimated area of evolution.

Key factors in stationary-phase mutagenesis in *E. coli* are the formation of double stranded breaks, repair and recombination functions as well as the activity of error-prone polymerases. The role of transcription has also been implicated in the formation of stress-induced mutations. In *E. coli*, experiments using defective genes of *argH* and *leuB* as well as reversion to prototrophy assays, Wright and coworkers showed a direct correlation between the level of transcription and mutagenesis (Wright and Minnick, 1997). It was this work that prompted the Wright lab to pose a transcription-associated mutagenesis hypothesis. In this model, transcription generates single stranded DNA and the formation of secondary, non-

canonical DNA structures; these structures prone DNA to accumulate lesions that eventually lead to the formation of mutations (Wright, 2004). However, the molecular steps of how transcription mediates the formation of mutations are less clear.

My work focuses on the role of damage caused by ROS and how *B. subtilis* repair mechanisms affect the formation of mutations in the cell during non-growing conditions. Two observations provide insight into how SPM occurs in *B. subtilis*. First SPM is potentiated by exposure to reactive oxygen species (ROS) (Pedraza-Reyes et al, 2009). Second, SPM is significantly reduced in the absence of Mfd (mutation frequency decline) (Ross *et al*, 2006). Mfd is a factor involved in transcription-coupled repair (TCR). (Reviewed in Hanawalt and Spivak 2008). While the transcription-coupled repair system has been well characterized in the context of high fidelity DNA repair caused by UV damage, it is unknown whether Mfd is involved in repair of DNA lesions caused by ROS, or if it mediates error-prone repair.

Preservation of the genome is essential for bacterial life, however DNA is constantly subjected to a wide variety of stresses that can lead to damage, such as single stranded or double stranded breaks, ultimately halting cellular processes like replication and transcription. Breaks in DNA located in highly transcribed regions of the genome may stall the transcription elongation complex. This event, if left unresolved, may lead to cytotoxicity or genotoxicity. A well-established pathway that processes stalled RNAP complexes is that of transcription coupled repair. In this pathway, the protein Mfd facilitates either the bypass of the blocking lesion or repair via the Uvr functions of the NER pathway. (Reviewed in Hanawalt and Spivak, 2008).

Repair of oxidative damage to DNA is mediated by the *mut* genes, which code for the MutT, MutM and MutY proteins respectively. Guanine residues have the lowest reduction

potential which makes them a preferred target of ROS. Oxidative damage of guanines results in the formation of an 8-oxoG (OG) lesion due to the acquisition of an electron from the guanine by the ROS. MutT prevents ROS damage to DNA by avoiding the incorporation of oxidized dGTP's during replication. MutT is a phosphatase that specifically targets 8-oxoGTP's. MutM and MutY function within in a pathway known as the Base Excision Repair (BER) system. The MutM enzyme works as a DNA glycosylase that removes 8-oxoG from the backbone of the DNA, which is subsequently replaced with an undamaged guanine by DNA PolI. If left unrepaired, 8-oxoG mispairs with adenine residues and leads to transversion mutations. The MutY glycosylase preferentially targets an 8-oxoG –adenine pair. MutY cleaves the adenine from the sugar-phosphate backbone and produces an abasic site. Both MutM and MutY create an apurinic/apyrimidinic (AP) site. AP sites are the substrate for AP endonucleases, which nick the DNA generating a 3'-OH group upstream of the AP site. This reaction primes replication by a DNA polymerase which ultimately replaces the mispaired adenine residue. The reaction is sealed by ligase.

Considering the factors affecting SPM in *B. subtilis*, we tested the novel idea that: **Mfd mediates the formation of mutations by interacting with pathways that repair oxidative damage in DNA in stationary-phase *Bacillus subtilis*.** To best address this hypothesis, I used four aims.

- 1) **Determine whether Mfd protects cell viability during exposure to ROS.**
- 2) **Determine if Mfd's function during ROS exposure is independent of the NER pathway.**
- 3) **Determine if Mfd and MutY promote the formation of mutations in stationary-phase during exposure to ROS.**

4) Determine whether changes in levels of transcription affect the repair of oxidative damage in *B. subtilis*.

These experiments improve our knowledge on the functions of Mfd, novel molecular mechanisms of mutagenesis that apply to all organisms, how cells negotiate oxidative damage, and cellular processes that are activated during stress that increase the adaptive potential of mutations.

Methodology

Media: *B. subtilis* colonies were grown on TBAB (tryptic blood agar base), liquid cultures were grown in Penassay Broth (PAB) also known as antibiotic medium 3 with the addition of 1X Ho-Le Trace Elements (Gerhardt *et al* 1994). Solid agar media containing bacteria were incubated at 37°C in the dark, liquid media containing bacterial cultures were grown at 37°C with aeration at 235 rpm. Antibiotics were added to the growth media at the following concentrations, spectinomycin 100 µg/ml, tetracycline 10 µg/ml, kanamycin 5 µg/ml, neomycin 5 µg/ml, erythromycin 1 µg/ml ampicillin 100 µg/ml. Experiments in which transcription was controlled by an inducible promoter used IPTG at a final concentration of 0.1 mM or 1 mM.

Strains: All strains are defined in Table 1. YB955 is a *B. subtilis* strain containing point mutations in genes for amino acid biosynthesis of histidine (*hisC952*), methionine (*metB5*) and leucine (*leuC427*) derived from the strain *Bacillus subtilis* 168 (Sung and Yasbin, 2002). Genetic transformation of YB955 with antibiotic resistance gene cassettes created strains with deficiencies in Mfd, MutY, UvrA, and the double knockout Mfd/MutY. Transformations were carried out as described previously (Yasbin *et al*, 1975).

Experiments to study how different levels of transcription affected the response to oxidative stress used the strain Cpleu, a YB955 derivative which contained the *leuC427* gene in the *B. subtilis* chromosome and under the control of the IPTG-inducible Hyperspank promoter $P_{\text{hyperspank}}$ (P_{hs}). The inducible *leuC427* was constructed in an integrative plasmid, transformed into YB955 and integrated into the *amyE* locus by homologous recombination (Pybus *et al*, 2010). The strain Cpleu Mfd⁻ was constructed by transforming DNA from strain YB9801 (*mfd::tc*) into Cpleu.

Strains KEP400 and KEP401 were derived from the parental strain YB955 and contain a copy of *leuC427* placed under the control of the IPTG-inducible pDG148-Stu plasmid promoter. The strain YB955 pDG148-Stu is a control strain carrying the pDG148-Stu plasmid. However this plasmid does not contain a *leuC427* gene or any other protein of interest behind the IPTG inducible promoter. The plasmid pDG148 is a shuttle vector and replicates autonomously from the *B. subtilis* chromosome (Joseph *et al*, 2001). All three strains were constructed by transformation of either the pDG148-Stu or pDG148-*leuC427* plasmid into the parental YB955 or YB955 Mfd⁻ strains respectively.

To measure mutagenesis, we used a background deficient in *argF*; a deletion of *argF* was constructed which also contained a neomycin cassette. This background also included placing a defective *argF* (Titled *argFSP*) downstream of the P_{hs} promoter (IPTG-inducible); this gene arrangement was ectopically recombined into the *amyE* chromosomal locus. Strains CVArg Mfd⁻, CVArg MutY⁻, Cv Arg⁻ Mfd/MutY⁻ containing the *argF* deletion and *argFSP* genes were constructed by transformation. (See strain table for complete strain descriptions)

Table 1. Strains utilized in this study

Strain Name	Construction	Genotype
YB955 (YB)	Sung and Yasbin, 2002	<i>metB, hisC, leuC</i>
YB955 (YB) Mfd ⁻ (YB9801)	Ross <i>et al</i> , 2006	<i>metB, hisC, leuC, mfd::tc</i>
YB955 (YB) MutY ⁻	Pedraza-Reyes <i>et al</i> , 2012	<i>metB, hisC, leuC, mutY::em</i>
YB955 (YB) Mfd ⁻ MutY ⁻	Pedraza-Reyes <i>et al</i> , 2016	<i>metB, hisC, leuC mutY::em, mfd::tc</i>
YB955 (YB) UvrA ⁻	Pedraza-Reyes <i>et al</i> , 2016	<i>metB, hisC, leuC UvrA::sp</i>
Cpleu	Pybus <i>et al</i> , 2010	<i>metB, hisC, leuC, pHS-leuC427</i>
Cpleu Mfd ⁻	Constructed for this work	<i>metB, hisC, leuC, mfd::tc, pHS-leuC427</i>
KEP400	Constructed for this work	<i>metB, hisC, leuC, pDG148-leuC427</i>
KEP401	Constructed for this work	<i>metB, hisC, leuC, mfd::tc, pDG148-leuC427</i>
CVarg	Constructed by C. Vallin	<i>metB, hisC, leuC argF:: neo, pHS-argFSP</i>
CVarg Mfd ⁻	Constructed for this work	<i>metB, hisC, leuC argF:: neo mfd::tc, pHS- argFSP</i>
CVarg MutY ⁻	Constructed for this work	<i>metB, hisC, leuC argF:: neo mutY::em, pHS- argFSP</i>
CVarg Mfd ⁻ MutY ⁻	Constructed for this work	<i>metB, hisC, leuC, argF:: neo mfd::tc, mutY::em, pHS- argFSP</i>
CVarg Phs	Constructed for this work	<i>metB, hisC, leuC argF:: neo, pHS</i>
YB955 pDG148-Stu	Constructed for this work	<i>metB, hisC, leuC pDG148-Stu</i>

Construction of plasmids with inducible gene arrangements: The plasmid pDG148-*leuC* was transformed into the YB955 and YB955 Mfd⁻ strains to create the KEP400 and KEP401 strains, respectively. The KEP400 and KEP401 strains, contain the pDG148 plasmid in which a *leuC* gene has been inserted. The promoter of the pDG148-*leuC* plasmid is controlled by IPTG (Figure 1). To construct this plasmid, pDG148-Stu was prepared from Monserate Mon1 *E. coli* cells using the Qiagen Plasmid Miniprep Kit (Qiagen, Hilden Germany). pDG148-Stu was digested with Stu1 in a 10 µl reaction containing, 1 µl 10X NEB cutsmart Buffer, 1 µl Stu1 enzyme, 1 µg plasmid DNA and 3 µl sterile Nano-pure H₂O, at 37°C for 1 hour according to manufacturers protocol (New England Biolabs, Ipswich, MA), which linearized the plasmid. The digested plasmid was subsequently de-phosphorylated in a 100 µl reaction containing: 10 µl cutsmart buffer, 5 µl phosphatase, 1 µg plasmid DNA and 35 µl sterile Nano-pure H₂O. The reaction was incubated at 37° C for 24 hours. The de-phosphorylation was stopped by heat-inactivation at 80°C for 2 minutes.

The full *leuC427* gene was PCR amplified using primers: LeuC Full Gene Stu1 Fwr 5'–TCAGGCCTATGATGCCTCGAACAATCATCA – 3' and LeuC Full Gene Stu1 Rev 5' – CCCAGGCCTCACAACTGTTTTTTCCTG – 3'. The PCR 20 µl mix contained 4 µl 5X Phusion High Fidelity (HF) Buffer, 200 µM dNTPs , 0.5 µM forward primer , 0.5 µM reverse primer, 100 ng YB955 genomic DNA, 0.6 µM DMSO and 0.2 µM Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA). PCR conditions were: denaturation at 98°C for 30 seconds, annealing at 63.5°C for 30 seconds, extension at 72°C for 1 minute, repeated for 25 cycles with a final extension of 72°C for 5 minutes. Amplification of *leuC427* was confirmed through gel electrophoresis.

The amplified *leuC* gene and digested pDG148 plasmid were ligated using 1 µl T4 ligase (Promega, Madison, WI), 100 ng pDG148 plasmid DNA, 41.34 ng *leuC427* DNA, 1 µl Ligase 10X Buffer and 2 µl sterile nano-pure H₂O. The ligation was incubated at 4°C for 48 hours. DNA ng/ml concentrations were determined via Nanovue at 260 nm (GE Healthcare Bio-Sciences, Pittsburg, PA).

The ligation reaction was transformed into Monserate Mon1 *E. coli* cells according to manufacturer's protocol (Monserate Biotechnology Group, San Diego, CA). Transformants were selected for kanamycin resistance and confirmed through PCR.

The plasmid pDG148-*leuC* was prepared using the Qiagen Plasmid Miniprep Kit and then transformed into the YB955 and YB955 Mfd⁻ strains to create the KEP400 and KEP401 strains, respectively. Transformation success was first confirmed through kanamycin antibiotic selection, followed by Qiagen Plasmid Miniprep Kit. Because the Qiagen Plasmid Miniprep Kit is designed for Gram negative cells, the miniprep protocol was modified to work on *B. subtilis* cells through the addition of four steps. *B. subtilis* cells were grown in liquid culture to an OD₆₀₀ of 0.8-1.2, collected by centrifugation for 15 minutes at 3000 x g at 4°C, resuspended in buffer P1 containing lysozyme (1 µg/ml), and incubated 10 minutes at 37°C. This treatment was followed by the standard Qiagen protocol. The presence of the plasmid was confirmed through gel electrophoresis.

Construction of a defective *argFSP* inducible strain: The CVarg strain was constructed by transforming a P_{hyperspank} (pDR111) plasmid carrying the *argF* gene with a stop codon (*argFSP*) in *Bacillus subtilis*. Transformants were selected on TBAB plates with 100 µg/ml of spectinomycin. The TAA stop codon replaces the CAA codon at position 37 in the ArgF protein. The TAA stop codon was engineered into the *argF* sequence through PCR mutagenesis. This method of

mutagenesis involved a collection of primers. Primer one was designed with the previously mentioned stop codon 5'-GGTGAGCTGAAATAAAACAAAATTCAGCCTTAGTAT-3' and used in combination with the wild type *argF* reverse primer encoded with an SphI restriction site: 5'-ACCAGCATGCCCTCCTTTTTGCTGTAGTAT-3' in a PCR reaction with Vent Polymerase (New England Biolabs, Ipswich MA). This reaction product was then used as a template for a second Vent PCR reaction using a two-stop *argF* forward primer: 5'-TAAACGCCTTGCTCGCAGAAGCCGGTGAGCTGAAATAAAACAAA-3' and the same wild type *argF* reverse primer. This PCR product was then combined with an upstream region of *argF* gene for fusion. The two PCR products were mixed at a 1:1 ratio and all PCR reagents were added to the mixture excluding primers and Gotaq polymerase (Monserate Biotechnology Group, San Diego, CA). This mixture was allowed to denature and anneal for 7 cycles. After this initial step, Gotaq polymerase and primers Full Gene *argF* For 5'-GATGTCGACTAAAAGGAAGTGGCATCATGCACACAGTGACGCAA-3' and Full Gene *argF* Rev 5'-ACCAGAATGCCCTCCTTTTTGCTGTAGTATGC-3' were then added to the PCR reaction. The reaction ran for 20 cycles. The PCR products were then run on 1% Agarose gel electrophoreses and fragments corresponding to 1.2 kb size were excised from the gel and purified (Promega, Madison WI). The clean product was digested using SalI and SphI enzymes, ligated to the P_{hyperspank} plasmid and transformed into *B. subtilis*. This construct was recombined into the *amyE* region of the genome.

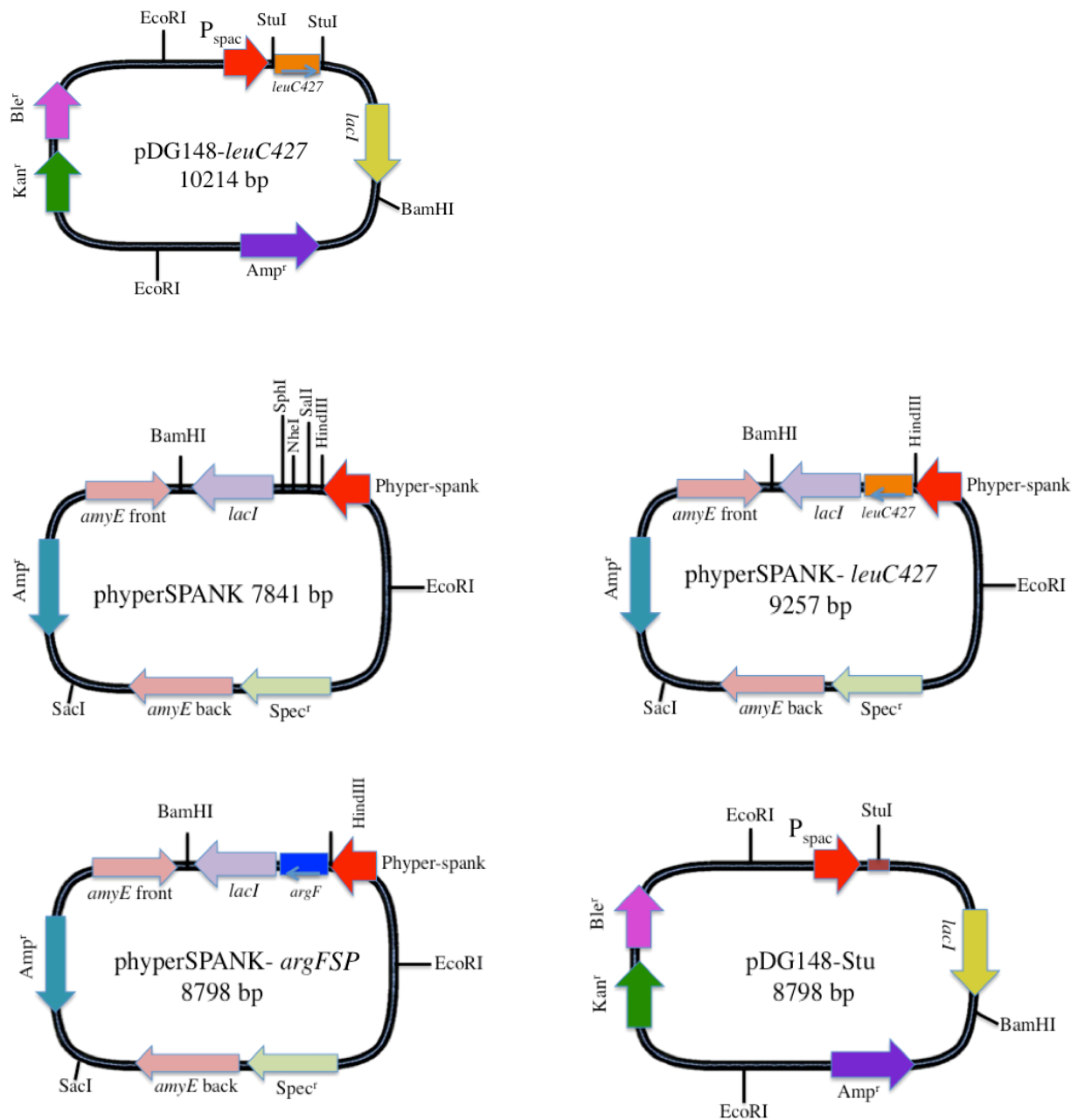


Figure 1. All plasmids used in this work. Plasmids pDG148-*leuC427* and phyperSPANK-*argFSP* were designed for this work. All other plasmids displayed in this figure were designed and published in previous works.

Preparation of oxidant: This oxidative stress assay required the preparation of a stock solution of the oxidizing agent. It is important to note that preparation of an oxidizer stock

oxidizer is affected by water quality as metal content in the water significantly reduces the strength of the oxidant. A sterile glass jar was acid washed using hydrochloric acid (1 normal), allowed to dry and then re-autoclaved for sterilization. The jar was filled with DI water and autoclaved again. To make a *tert-butyl* hydroperoxide (TBH) (Sigma Aldrich, St. Louis, MO) 1 M stock solution, two sterile microcentrifuge tubes were placed on ice. 871.8 μ l of the previously sterilized DI water was placed into one microcentrifuge tube. In the second separate microcentrifuge tube approximately 200 μ l of TBH were poured (not pipetted). From the 200 μ l aliquot of TBH, 128.2 μ l were transferred into the 871.8 μ l of DI water.

To create the diamide (Sigma Aldrich, St. Louis, MO) 1 M stock, one sterile microcentrifuge tube was placed in ice and filled with 1000 μ l of the sterile DI water. .172 g of diamide was transferred into the microcentrifuge tube containing the DI water and vortexed until diamide fully dissolved.

Cell treatment with oxidant: Cells were grown in 2 ml of PAB overnight at 37°C and 235-250 rpm. One ml of an overnight culture was transferred into 15 ml of fresh PAB containing 15 μ l of 1X Ho-Le trace elements (Gerhardt *et al* 1994) into 125 ml flasks. Growth was tracked using a spectrophotometer (OD_{600}) (ThermoFisher Scientific, Waltham, MA). When cells reach 90 minutes past the onset of stationary-phase (T90), 8 ml of culture was transferred into four 13 mm test tube (2 ml of culture in each test tube). TBH or diamide was then dispensed at different concentrations (0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM). Exposure to oxidants was at 37°C and 235-250 rpm for two hours. Following incubation, 1 ml was removed from each of the 13 mm test tubes, centrifuged at 13,000 rpm for 2 minutes, and resuspended in 1X Spizizen Minimal Salts (SMS) (Spizizen, 1958). Cells were washed and resuspended in 1X SMS to remove residual oxidizer. The resuspended culture was serially diluted in 10-fold and 0.1 ml of the final dilution

was plated onto TBAB. Plates were incubated for 24 hours at 37°C and scored for colonies to determine survival.

Stationary-Phase Mutagenesis Assay: Cells for this assay were prepared exactly as those subject to the survival assays. One ml of an overnight culture was inoculated into a culture flask with 10 ml of PAB supplemented with 10 µl trace elements. Cultures were grown to T90, with growth being tracked with a spectrophotometer (OD₆₀₀). Eight mls of the culture were transferred into four 13 mm test tube (2 ml of culture in each test tube). Each test tube was subjected to a specific condition of oxidative damage and transcriptional induction, conditions are displayed in Table 2. Cultures were incubated at 37°C and 235-250 rpm for two hours.

Table 2. Stationary-phase mutagenesis induction and oxidative stress treatment conditions

Condition Title	Condition Description
No Treatment Control (NT)	0 mM IPTG and 0 mM TBH Added
No Treatment Induced (NT-IN)	0.1 mM IPTG and 0 mM TBH Added
Treated Induced (T-IN)	0.1 mM IPTG and 1 mM TBH Added
Treated (T)	0 mM IPTG and 1 mM TBH Added

Following incubation 1 ml from each experimental condition was washed twice and resuspended in 1 ml of 1× SMS.

Aliquots of 0.1 ml were then spread plated in quintuplicate on Spizizen minimal medium (SMM) containing 1× SMS, 0.5% dextrose, 50 µg/ml isoleucine, 50 µg/ml glutamate, 1.5% agar (ThermoFisher Scientific, Waltham, MA), 50 µg/ml of histidine and methionine, and a 50 µg/ml leucine (Sigma-Aldrich, St. Louis, MO), with 0.1 mM IPTG.

The plates were incubated for 9 days at 37°C. Every 24 hours plates were observed for the appearance of Arg⁺ colonies. Titters of the *B. subtilis* cultures were measured by serially diluting the resuspended culture and plating on TBAB. Colonies were counted after 24 hours of incubation.

Viability of cells was also tracked over the 9 day period. Every odd day, a plug of agar was removed from a colony free area of each of five plates. This was done for each condition previously mentioned. The plugs were placed in 400 µl of 1X SMS, serially diluted, and plated in triplicate on SMM containing 50 µg/ml arginine, leucine, methionine, and histidine. Colonies were observed after 48 hours of incubation. The stationary-phase mutagenesis assay was originally described by Sung and Yasbin (2002).

Ultraviolet (UV) Assay: Strains were grown in 2 ml of PAB overnight at 37°C and 235-250 rpm. One ml of overnight culture is transferred into 15 ml of fresh PAB with 15 µl of 1X Ho-Le trace elements into 125 ml Neploflasks. When the culture reached T90, 1 ml was removed from the culture, centrifuged at 13,000 rpm for 2 minutes and suspended in 1X SMS. The resuspended culture was serially diluted in tenfold up to 10⁻⁷. From each dilution series three 10 µl aliquots were spotted on TBAB plates and allowed to dry for 30 minutes. Once dried, each plate was exposed to 25 J/m² of UV and incubated at 37°C for 24 hours.

In order to expose cells to the correct J/m² of UV 3 bulbs of UV-C bulbs were turned on and given 15 minutes to warm up. A Black Ray UV meter was exposed to the bulbs for 10 seconds, measuring UV intensity in µW/cm². Using this formula

$$((x \mu\text{W}/\text{cm}^2 * 0.01) * 0.06) * 1000 = \text{J}/\text{m}^2/\text{s}$$

Equation 1. Determining J/m² for UV damage exposure

the UV intensity was converted to J/m²/s. The desired UV exposure divided by the previously calculated J/m²/s determined time of exposure in seconds to reach the 50 J/m².

Transformation Assay for *B. subtilis*: Strains were grown in 2 ml of PAB overnight at 37°C and 235-250 rpm. 1 ml of overnight culture is transferred into 10 ml of sterile GM1 with 10 µl of 1X Ho-Le trace elements into 125 ml Neploflasks. GM1 was the standard growth medium for transformation in *B. subtilis* and contained 1X SMS, 22 mM of dextrose, 0.02% casein hydrolysate, 0.1% yeast extract as well as 2 µg/ml of any amino acid(s) required for appropriate strain growth. Cultures were grown to T90, using a spectrophotometer (OD₆₀₀). 0.1 ml of culture was transferred into 0.9 ml of sterile GM2. This transfer is done three times into four individual 17 x 100 mm plastic culture tubes (Carolina Biological Supply, Burlington, NC). A fourth culture tube contained 0.9 ml GM2 without the addition of any cells. GM2 contains the same ingredients as GM1 with the addition of 0.5 mM CaCl₂ and 2.5 mM MgCl₂ (Yasbin *et al*, 1975). Culture tubes are incubated at 37°C and 235-250 rpm for 1 hour. After incubation, the transforming DNA is added at different concentrations (see Table 3), using tube 3 and tube 4 as culture only and DNA only as transformation controls respectively.

Table 3. Transformation culture tube DNA additions

Tube 1	10 µg/ml of DNA
Tube 2	1 µg/ml of DNA
Tube 3	0 µg/ml of DNA
Tube 4	10 µg/ml of DNA

Culture tubes were incubated at 37°C and 235-250 rpm for 1 hour. Transformed DNA that would not incorporate into the bacterial genome (plasmid) was incubated for an additional hour.

0.1 ml of the tube 1 culture was serially diluted in 0.9 ml 1X SMS to 10⁻⁷ and plated in duplicate on TBAB to quantify cell density. 0.1 ml of culture was removed from each tube and plated individually on TBAB plates containing the appropriate antibiotic selection. Plates were incubated at 37°C for 48 hours. All strains transformed from these works were confirmed through PCR and antibiotic selection. The primers MutY Full Fwr 5' –

ATCGTACTGTGCCCTTAGTG – 3' and MutY Full Rev 5' –

CAATGAAGCGGACTACAGCCA – 3 were used to confirmed genetic inactivation of *mutY*.

This PCR reaction was performed in a 25 µl that contained used 12.5 µl of pre-made Midas Mix which contains, buffer, dNTPs and DNA polymerase (Monserate Biotechnology Group, San Diego, CA). The reaction also included 1 µl (from 100 µM stock) forward primer, 1 µl (from 100 µM stock) reverse primer, 2 µl (45 ng/ml) DNA, and 8.5 µl sterile Nano-pure water was added. PCR conditions were: initial denaturation at 98°C for 2 minutes, denature 98°C for 30 seconds, annealing at 55.85°C for 1 minute, extension at 72°C for 3 minutes, repeated for 25 cycles with a final extension of 72°C for 5 minutes.

To confirm the *mfd* null mutant, a 25 µl PCR reaction with the primers Mfd full gene FOR 5'- CCGCATTACCACGAATATCAC – 3' Mfd full gene REV 5' - GTTGCTTTCATCTCTGGTGC – 3' was carried out. This reaction contained used 12.5 ul pre-made Midas Mix (Monserate Biotechnology Group, San Diego, CA) and followed the same protocol as listed above with the MutY procedure. PCR conditions were: Initial denaturation at 98°C for 2 minutes, denature 98°C for 30 seconds, annealing at 54°C for 1 minute, extension at 72°C for 8 minutes, repeated for 25 cycles with a final extension of 72°C for 5 minutes.

Statistical Analysis of Data: All statistical significance was determined through a combination of t-tests as well as ANOVA. ANOVA measures the variance between group means with a factor significance of $p < 0.05$. Utilizing the ANOVA analysis the least significant difference (LSD) was determined (Equation 2). If the ANOVA determined variance between means is greater than the LSD then the difference is labeled statistically significant.

$$LSD_{A,B} = t_{0.05/2DFW} \sqrt{MSW(1/n_A + 1/n_B)}$$

Equation 2. Formula to determine the least significant difference

From there, if the compared strains are determined to be significantly different statistically it is denoted by one of two ways. If only two strains were compared significant difference is denoted by a single asterisk. If the strains being compared were greater than two, significant difference is represented by a new alphabetical letter, thus graphical data labeled ‘a’ is significantly different from ‘b’ etc. A second statistical analysis was completed on the data to eliminate possible error from ANOVA. The second analysis was completed using t-tests through IBM SPSS statistics software. Statistically significant differences on the graphs derived through SPSS were found to be the same in most cases. All data shown represents the statistical analysis derived from SPSS, as it was determined to have the greatest accuracy. Statistical significance is represented as previously described using asterisks or letters respectively.

Results and Discussion

Mfd has been shown to be required for the recovery of transcription following UV damage to DNA (Schalow *et al*, 2012). Without Mfd to recruit TCR to the site of DNA bulky lesions that are left behind by UV damage, repair is substantially decreased. It has also been

observed that there is a significant decrease in the development of mutations during stationary-phase, and in cells that lack a functional Mfd and even in the absence of exogenous DNA damaging agents. (Ross *et al*, 2006). These observations prompted us to determine whether Mfd participated in DNA reactions of repair that process lesions that are not recognized by the Uvr system.

Research from (2009) Vidales *et al* demonstrated that *B. subtilis* cells under starvation conditions, (i.e. non-replicating conditions), that lack the system that processes DNA lesions caused by ROS showed increased mutagenesis. However, the mutagenic response to ROS appears complex since the single inactivation of MutY, one of the glycosylases that prevents mutations caused by ROS, promoted the formation of mutations during stationary-phase. The inactivation of error-prone DNA polymerases also lead to depressed mutagenesis in stationary-phase cells, because of this it is speculated that the repair reactions initiated by MutY are low-fidelity and mutagenic (Gómez-Marroquín *et al*. 2016).

In *E. coli*, the involvement of Mfd in repair of oxidative damage repair is multiform. A 2012 study using oxidative damaged cells deficient in Mfd, found that there was no change in the rate of transcription when compared to the wild type (Schalow *et al*, 2012). However, an in vitro study examining transcription of a luciferase reporter gene, in which they placed 8-oxoG's in the template strand of RNA polymerase, found that Mfd prevented RNAP from bypassing this lesion in non-dividing *E. coli* cells. This led to the conclusion that 8-oxoG lesions are subject to TCR in *E. coli* (Bre`geon *et al*, 2003).

Mfd protects *B. subtilis* cells against oxidative damage.

We hypothesize that Mfd mediates the formation of mutations by interacting with pathways that repair oxidative damage in DNA in stationary-phase *Bacillus subtilis*. To do this,

we subjected strains containing a functional and non-functional Mfd protein to different levels of oxidative stress (Fig. 2). We found that, the *Bacillus subtilis* strains deficient in Mfd have a decreased survival following exposure to oxidative stress.

Mfd is part of the NER (UvrA, UvrB, UvrC, UvrD) pathway, therefore we tested the idea that deficiencies in Uvr proteins affect the ability of the cell to withstand ROS stress similarly to that observed in Mfd-deficient cells. We also tested the possibility that components of the BER pathway, specifically the DNA glycosylase MutY, is involved in the response with Mfd during exposure to ROS. ROS form as a result of a missing electron in the outer most shell of an oxygen molecule, rendering the oxygen atom highly unstable. Such molecules gain stability by sharing electrons with any compound with low reduction potential. In DNA, ROS interact particularly with G residues forming the 8-oxoG lesions. If unrepaired, this lesion mispairs with adenine residues during replication and lead to transversion mutations.

We first examine whether Mfd interplays with MutY in the cytotoxicity caused from exposure to ROS by measuring cell survival in strains deficient in both factors individually (Fig. 3). Percent survival was increasingly affected by the oxidant concentration. Deficiencies in Mfd and MutY led to significantly lower percent survival than those observed in the parental strain or the UvrA⁻ mutant. Interestingly, the survival expressed by the UvrA⁻ strain indicates that the Mfd works independently of TCR in preventing cytotoxicity caused by ROS. However, the percent survival values displayed by the Mfd⁻ and MutY⁻ strains were similar, suggesting that these two factors combine to work as part of a pathway to prevent oxidative damage. To test this idea, we conducted cell survival assays with strains inactivated in Mfd, MutY, or both (Fig. 4). TBH treatment showed that single and double inactivation of Mfd and MutY led to similar values following increased oxidative stress, and all three genetic backgrounds were decreased in

survival compared to the parental strain. These experiments provided further support for the idea that Mfd and MutY work together in the interception of oxidative damage.

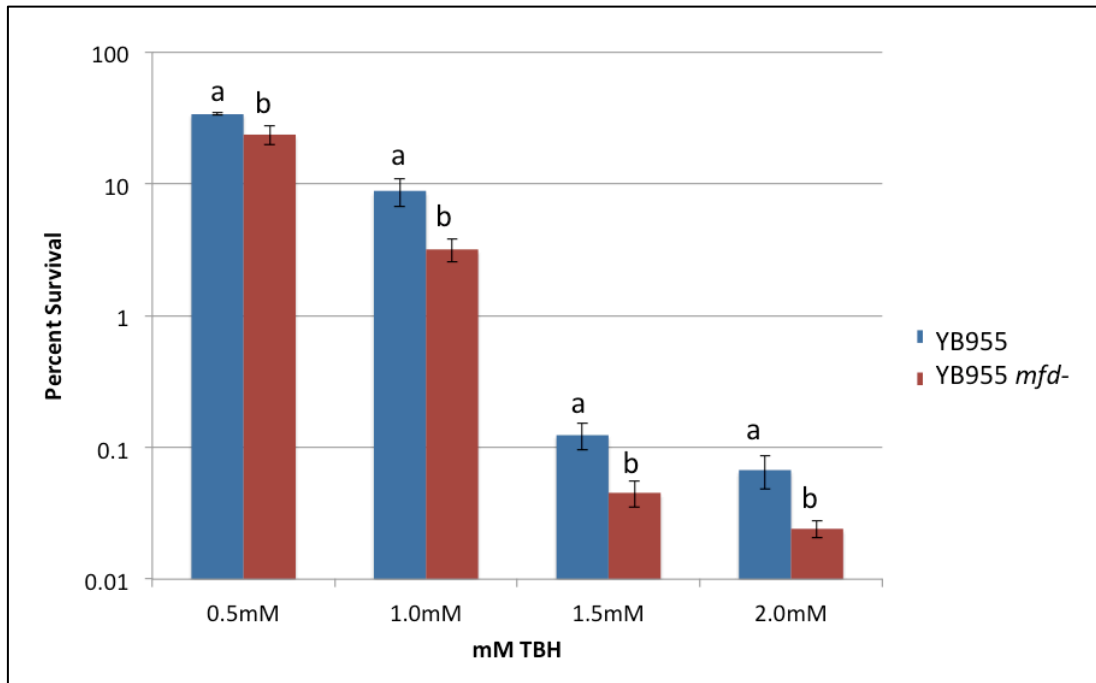


Figure 2. Percent cell survival, displayed in log scale, in strains containing a functional or non-functional Mfd following exposure to ROS via the oxidizing agent *tert-butyl* hydroperoxide (TBH). Percent survival for each strain was determined by dividing the number of colonies from each of the test concentrations by the number of colonies observed at 0 mM TBH. The error bars represent standard error, statistical significance is represented by letters within each TBH concentration group, means with different letters are significantly different. Statistical significance was measured with IBM SPSS statistical analysis software.

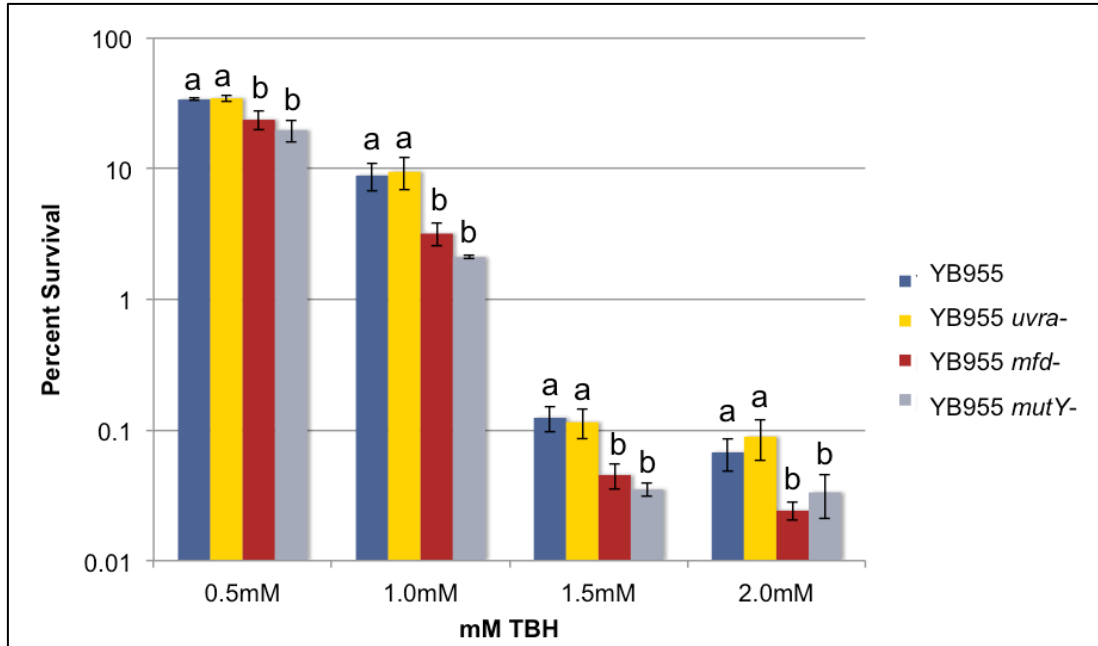


Figure 3: Percent cell survival, displayed in log scale, in cells deficient in the Mfd, MutY or UvrA protein respectively, following exposure to ROS via the oxidizing agent *tert-butyl* hydroperoxide (TBH). Percent survival for each strain was determined by dividing the number of colonies from each of the test concentrations by the number of colonies observed at 0 mM TBH. The error bars represent standard error, statistical significance is represented by letters within each TBH concentration group, means with different letters are significantly different. Statistical significance was measured with IBM SPSS statistical analysis software.

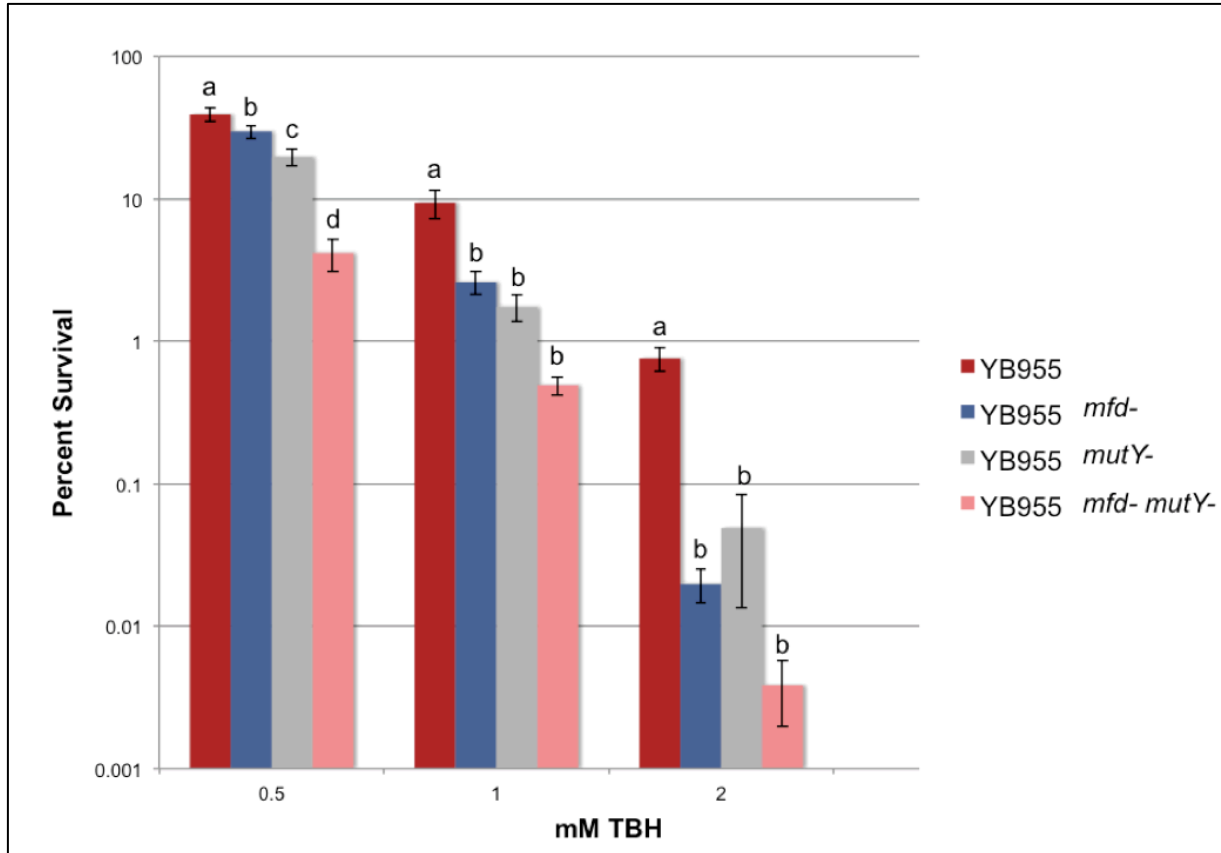


Figure 4: Percent cell survival, displayed in log scale, in cells containing single and double defects in Mfd and MutY deficient strains, following exposure to ROS via the oxidizing agent *tert-butyl* hydroperoxide (TBH). Percent survival for each strain was determined by dividing the number of colonies from each of the test concentrations by the number of colonies observed at 0 mM TBH. The error bars represent standard error, statistical significance is represented by letters within each TBH concentration group, means with different letters are significantly different. Statistical significance was measured with IBM SPSS statistical analysis software.

To determine if Mfd and MutY interplay in the formation of mutations, we utilized a stationary-phase mutagenesis assay targeting an inducible *argFSP* gene containing a stop codon in combination with single and double deficiencies in Mfd and MutY. The *argFSP* defective

gene renders cells auxotrophic for arginine, and its transcription is controlled by IPTG. The auxotrophic *argF* strains are deprived of arginine and may resume growth upon acquiring mutations at the *argFSP* locus. We scored mutation development following treatment with TBH and transcriptional induction (addition of IPTG). Control treatments were those including no transcriptional induction.

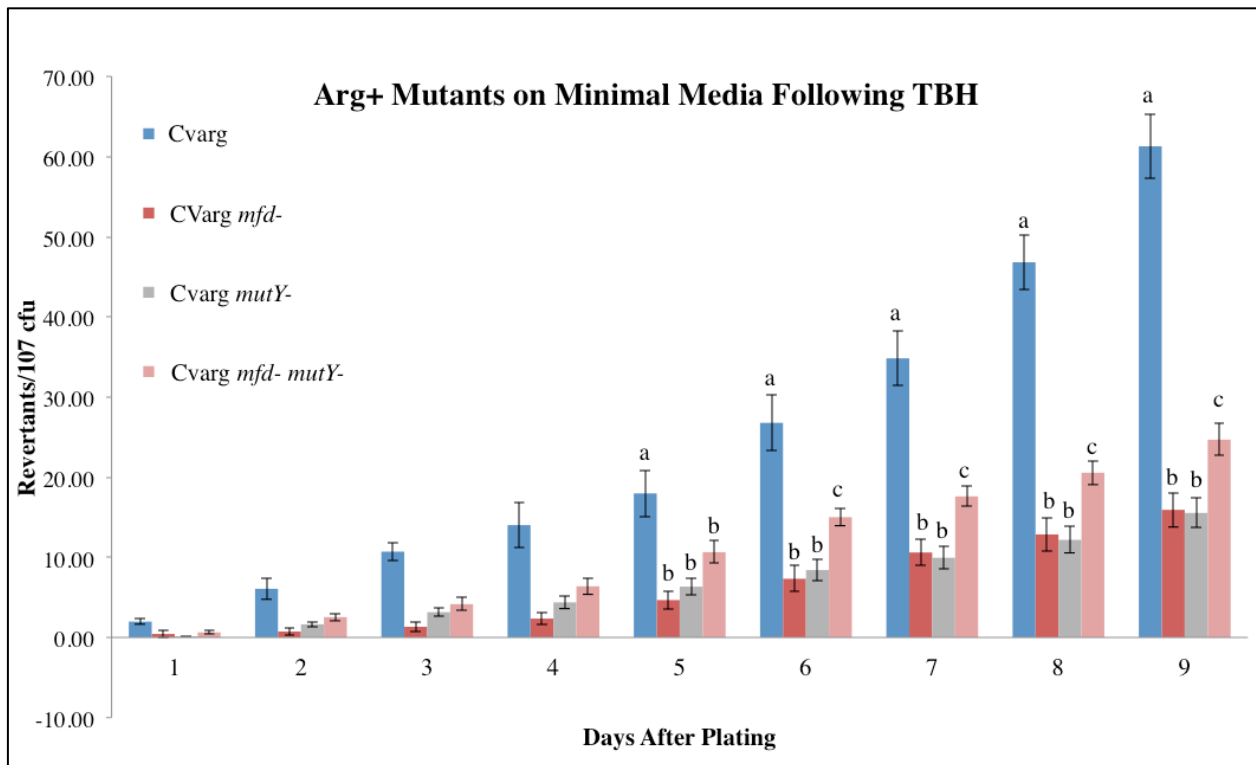


Figure 5. Accumulation of Arg⁺ mutations over 9 days in *CVarg* stains deficient in *Mfd*, *MutY* or both following treatment with 1 mM TBH. The error bars represent standard error, statistical significance is represented by letters, with each letter being statistically significant from a differing letter. Statistical significance was measured with IBM SPSS statistical analysis software.

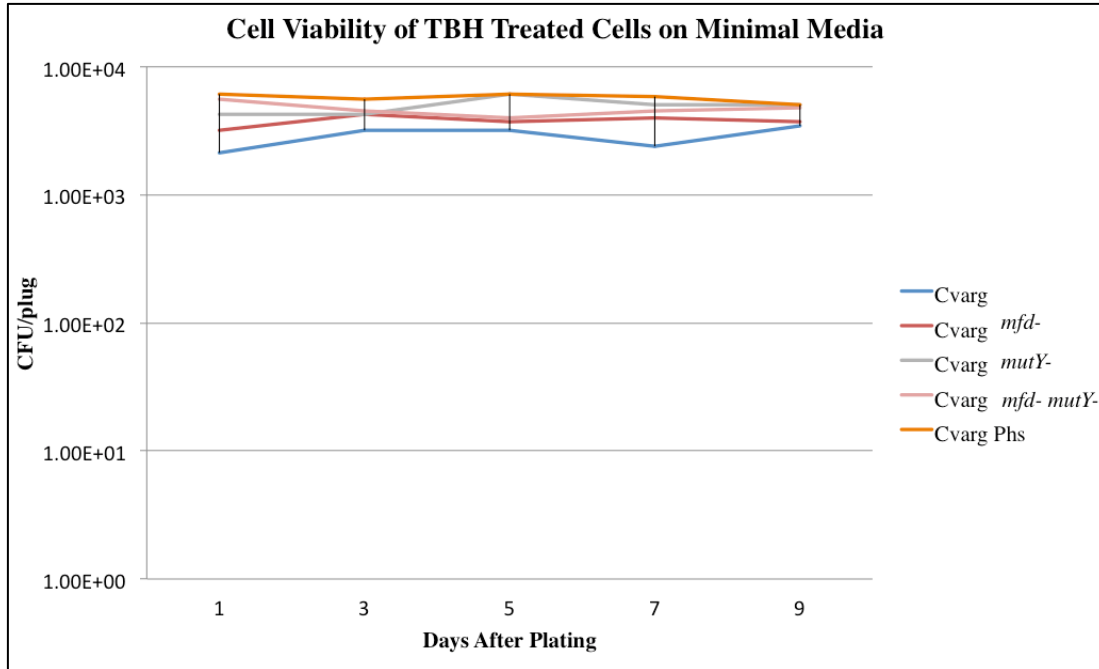


Figure 6. Viability of cells treated with TBH and IPTG over the 9 day SPM presented in Figure 5. Procedure of how viability was measured is described in materials and methods.

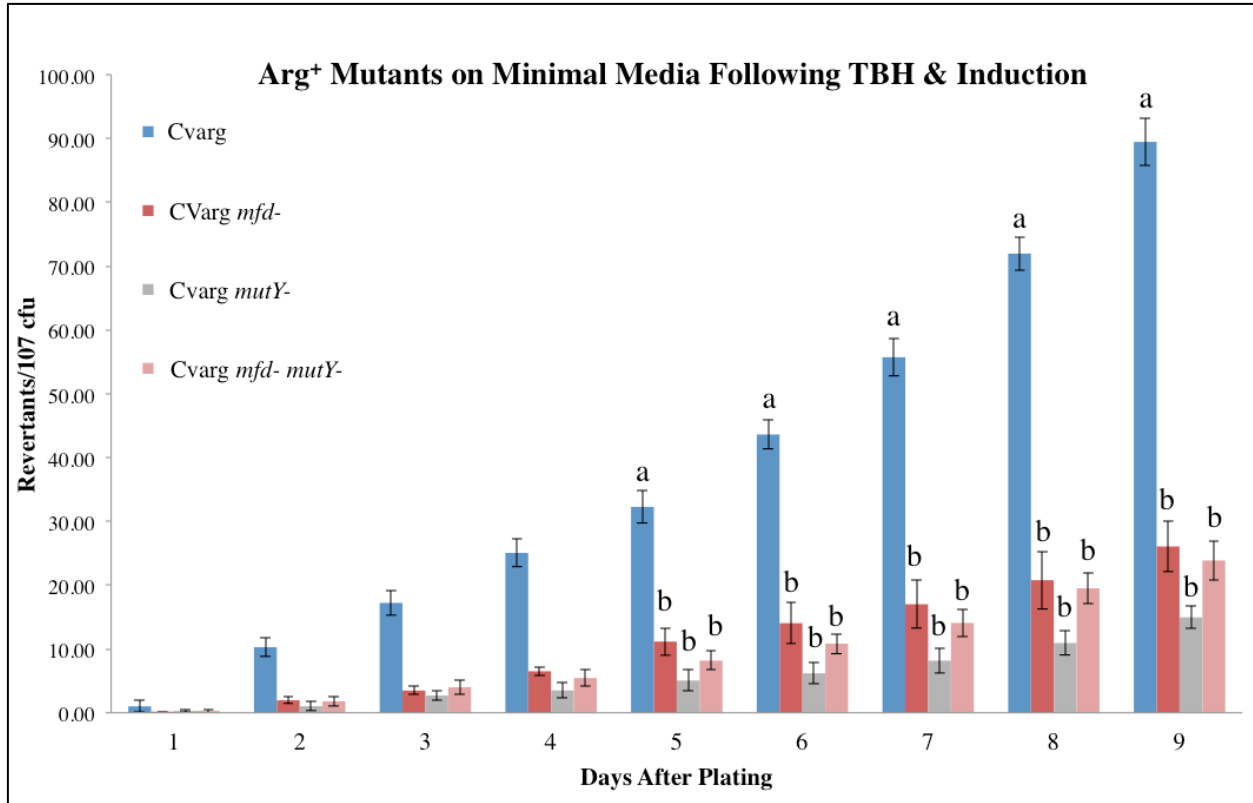


Figure 7. Accumulation of Arg⁺ mutations over 9 days in CVarg stains deficient in Mfd, MutY or both. Following treatment with 1 mM TBH and induction with IPTG. The error bars represent standard error, statistical significance is represented by letters, with each letter being statistically significant from a differing letter. Statistical significance was measured with IBM SPSS statistical analysis software.

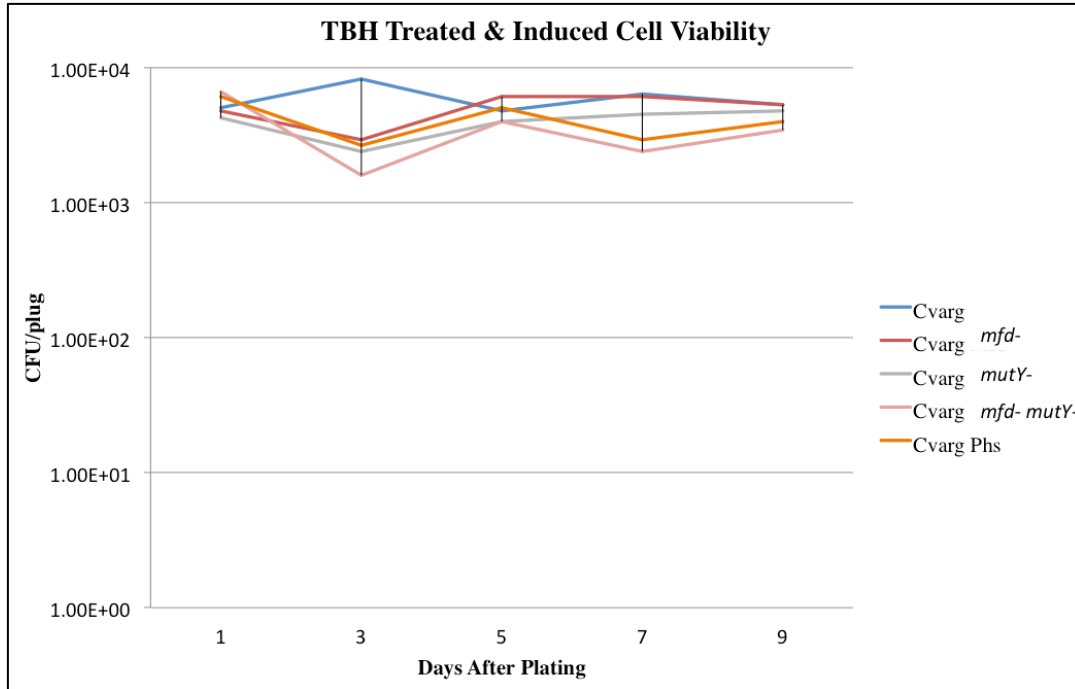


Figure 8. Viability of cells treated with TBH and IPTG over the 9 day SPM represented in Figure 7. Procedure of how viability was measured is described in materials and methods.

The results of our stationary-phase mutagenesis assay (mutants that arise on days 5-9). The CVarg strain containing a functional Mfd developed significantly more mutations than the strains containing single or double defects for Mfd and MutY. The strains with single inactivations displayed similar values in SPM throughout the 9 day period. Interestingly, the double knockout strain showed significantly lower levels of SPM than the parental strain, but higher than the strains carrying single inactivations in Mfd and MutY (Fig. 5). Cell viability throughout the assay did not vary among strains (Fig. 6).

SPM assays that included transcriptional induction of *leuC* showed a similar response to TBH treatment; however, it is important to note two differences. The level of mutagenesis in conditions of DNA damage and transcriptional induction of the gene under selection was much

higher (Fig. 7) than in the experiments with no transcriptional induction (Fig. 5). Also, the accumulation of mutations in the strain deficient in Mfd and MutY showed similar values to those shown in strains with single deficiencies in these factors (Fig. 7). Cell viability throughout the assay did not vary among strains (Fig. 8). A control strain carrying the inducible promoter but no *argFSP* yielded no mutants in these SPM assays. These results suggest that Mfd and MutY are factors that promote mutagenesis in conditions of oxidative stress. Also, in conditions of transcriptional induction these two factors worked together in the formation of mutations.

The SPM experiments with a double knockout strain in Mfd and MutY in conditions of TBH showed an interesting response. The levels of SPM were higher in the double mutant than in the strains with single inactivations in conditions of transcriptional repression. We interpret this to mean that there are other repair systems processing DNA lesions in error-prone manner. This is evident from the basal level of mutagenesis in the absence of both factors. Also, in conditions of low levels of transcription DNA may be less protected by the transcription machinery. Then, other repair systems that do not interact with the process of transcription could have more access to lesions and process them. As described above, there are other glycosylases that target oxidative damage. A new SPM pathway dependent on AP lyases and Pol X has been recently reported (Barajas-Ornelas *et al*, 2014). These possibilities could be discerned by assays in which repair reactions are reconstituted *in vitro* or by *in vivo* experiments in which cells over express competing repair factors.

Mfd-dependent protection against oxidative damage is affected by plasmid transcription.

We further investigated how Mfd protected cells against oxidative damage by altering transcriptional conditions in the cell. To do this, I used an expression vector that depended on IPTG to activate transcription. This vector contained a *leuC* gene cloned behind the Pspac

promoter (pDG148-*leuC*). The strains KEP400 and KEP401 (*Mfd*⁻) contained this plasmid and were used in these studies (Table 1, Figure 1). All strains were subjected to an oxidative survival assay with TBH and measured for survival in the presence and absence of transcriptional induction (Fig. 9).

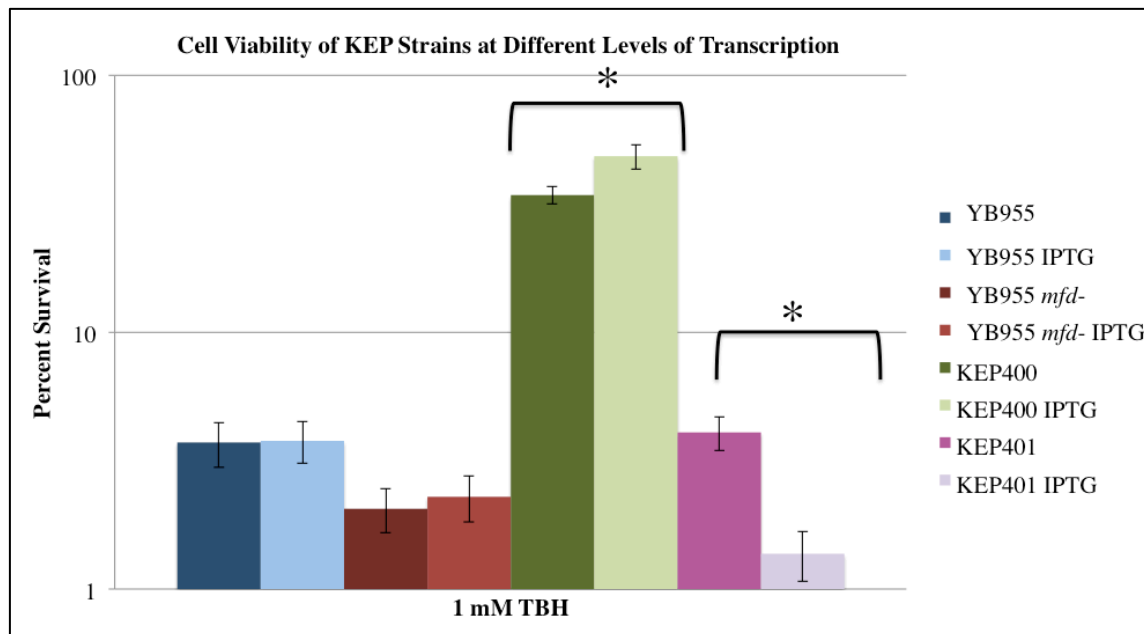


Figure 9. The effect of *Mfd* and transcriptional induction on protection against 1 mM TBH of oxidant. Statistical analysis contrasted survival in the absence and presence of IPTG within each strain. Asterisks represent statistical significance between no IPTG and IPTG supplemented conditions. Statistical significance was measured with IBM SPSS statistical analysis software.

YB955 and its *Mfd*⁻ counterpart strain were differentially affected, as previously shown, by TBH, but each of these strains was not affected by the addition of IPTG. This eliminates the possibility that cell viability is affected by the chemical inducer being used.

The response observed in the strains carrying plasmids was interesting. The strain KEP400 showed a strikingly increased percent survival compared to YB955, which suggested that transcriptional induction of pDG148-*leuC* protected the cell against oxidative damage. In the absence of the inducer this strain was significantly increased in percent survival compared to the parent strain. We attributed this protective effect to the basal level of expression previously observed in the promoter used (Pybus *et al* 2010). In the presence of the inducer, percent survival was even higher than in the non-induced condition. In the absence of Mfd and IPTG, the plasmid-dependent protection was reduced to levels observed in the parent strain (compare KEP401 and YB955) and exacerbated by induction of transcription (KEP401 IPTG). These observations strongly support the concept that increased transcription leads to increased survival following oxidative damage, but such increased survival hinges on the presence of a functional Mfd protein (Figure 9).

The plasmid experiments prompted us to determine whether the Mfd-dependent protection was specific to oxidative damage. We subjected the induced and non-induced KEP strains to a UV assay with 25 J/m² of UV-C exposure. The description of the assay is in the Materials and Methods section. We used the template seen in figure 10 to conduct our experiments in which 10-fold serial dilutions are used to measure UV sensitivity.

This assay included the strains Cpleu and Cpleu Mfd⁻, which has the *leuC427* gene controlled by the Pspac promoter integrated in the chromosome and therefore expected to display an intermediate level of transcriptional induction between the plasmid-containing strain (KEP400) and the strain that does not respond to the inducer (YB955). This assay used UV-C light, which only causes pyrimidine dimers, and not any other forms of damage traditionally associated with UV (Reviewed in Rastogi *et al* 2010).

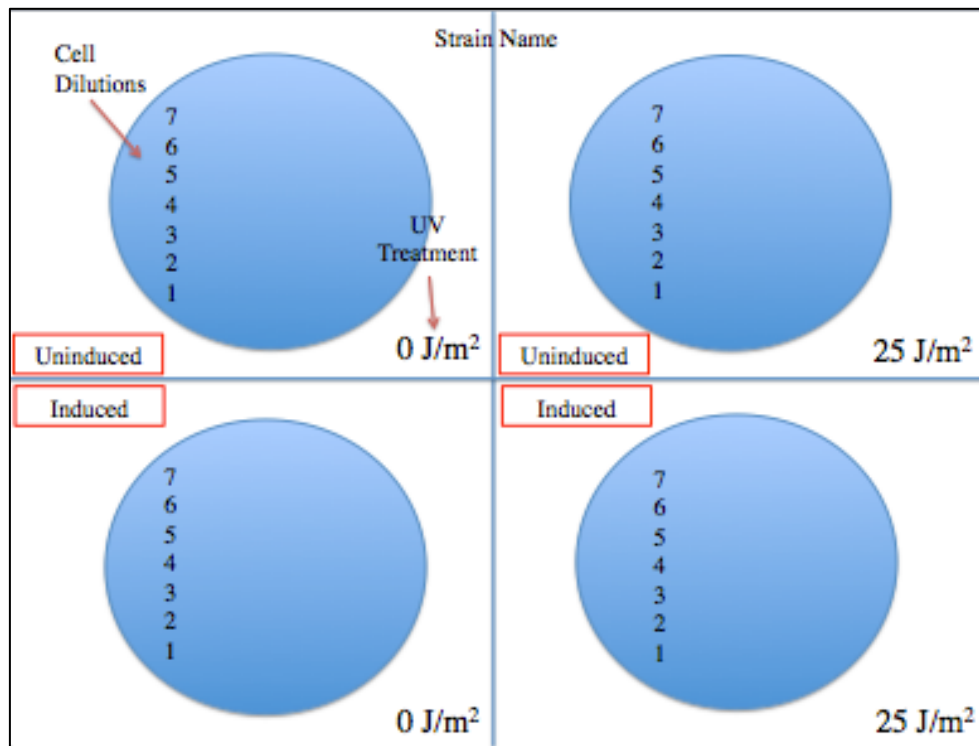


Figure 10. Diagram of UV data represented in figures 11-15. Each plates contains seven 10-fold dilutions. The number 7 denotes the most diluted spot (top).

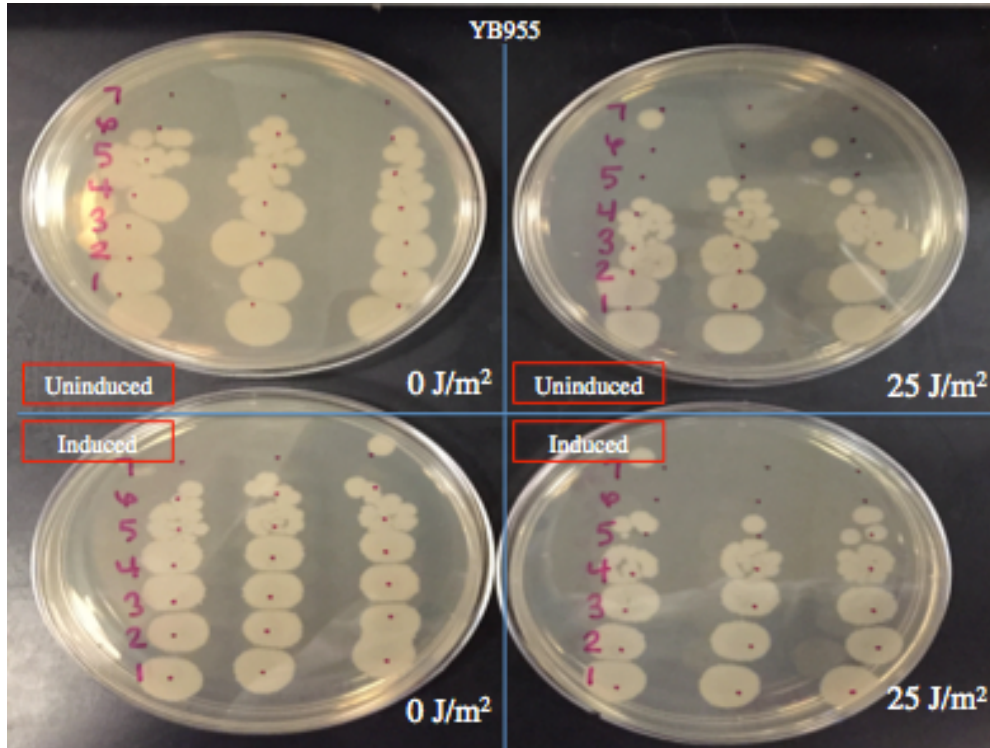


Figure 11. YB955 cell survival following transcriptional induction with IPTG and or UV exposure. Each plates contains seven 10-fold dilutions. The number 7 denotes the most diluted spot (top).

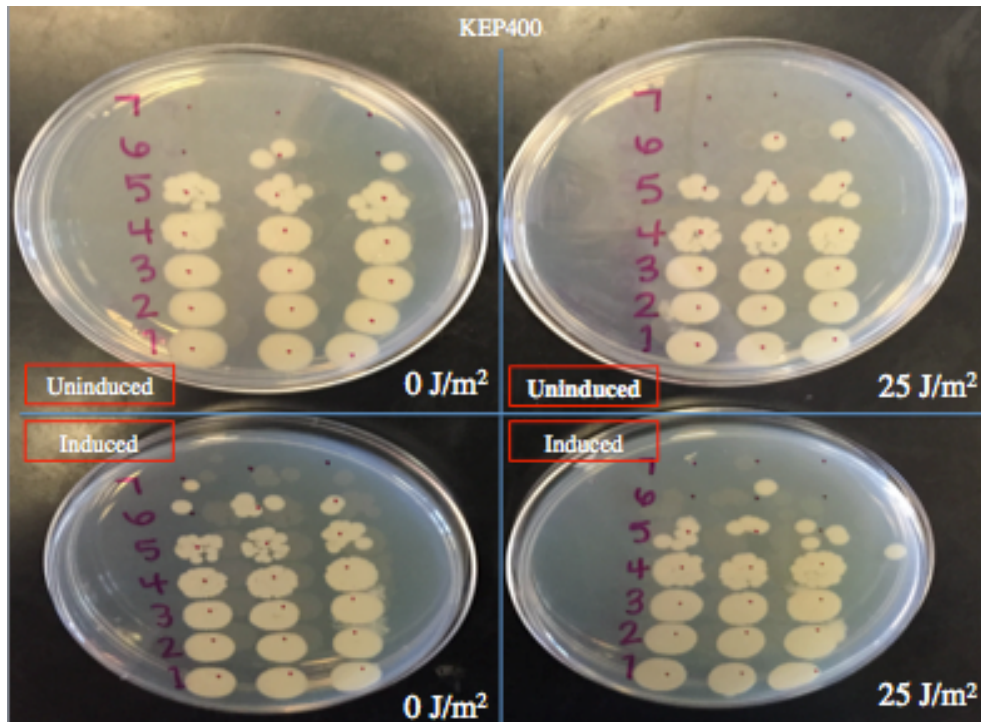


Figure 12. KEP400 cell survival following transcriptional induction with IPTG and or UV exposure. Each plates contains seven 10-fold dilutions. The number 7 denotes the most diluted spot (top).

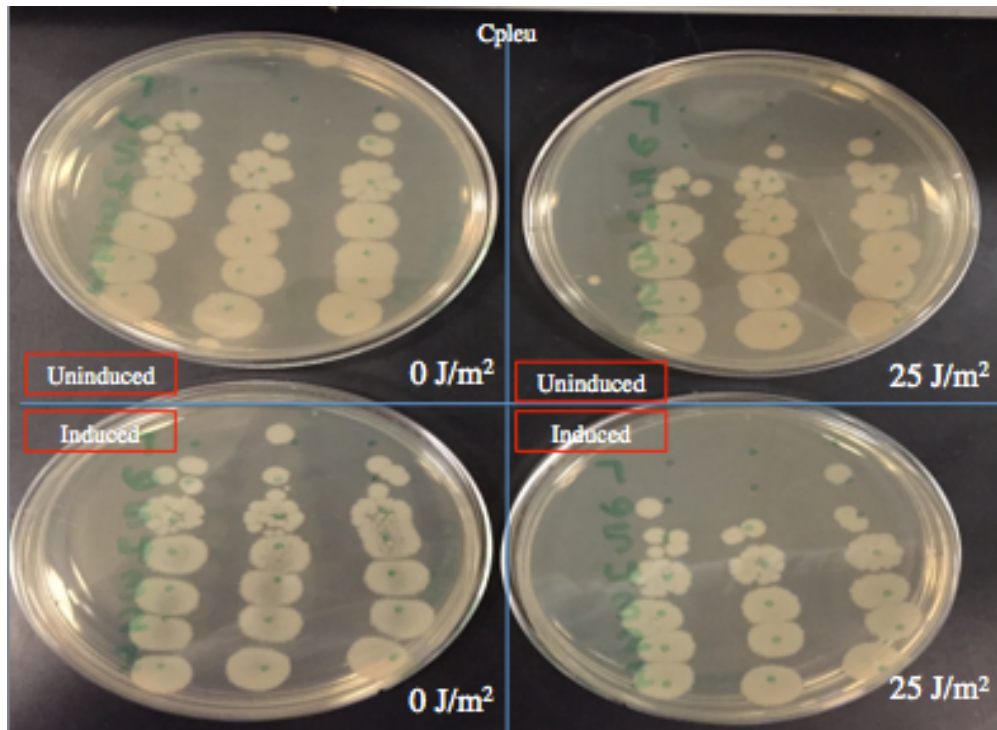


Figure 13. Cpleu cell survival following transcriptional induction with IPTG and or UV exposure. Each plates contains seven 10-fold dilutions. The number 7 denotes the most diluted spot (top).

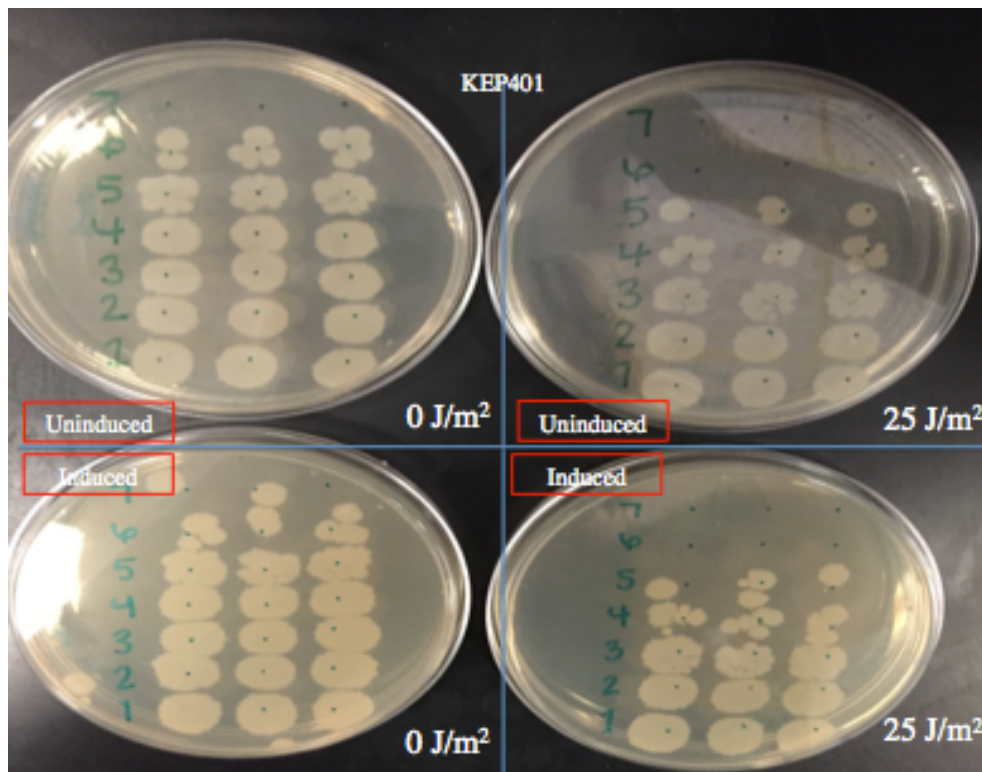


Figure 14. KEP401 cell survival following transcriptional induction with IPTG and or UV exposure. Each plates contains seven 10-fold dilutions. The number 7 denotes the most diluted spot (top).

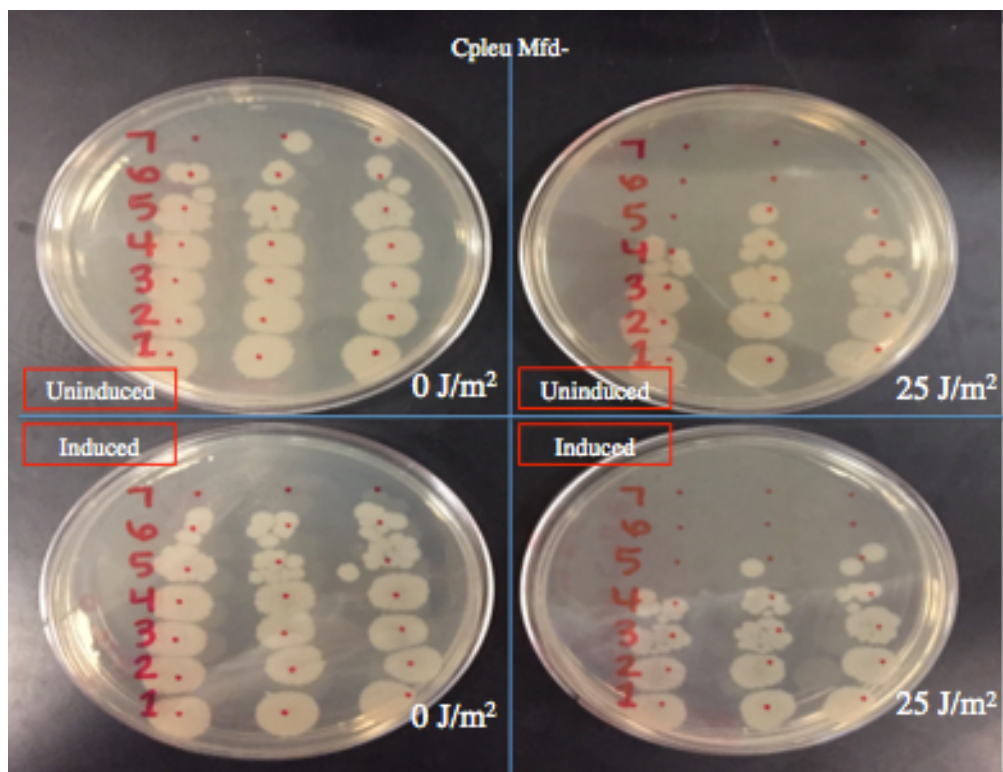


Figure 15. Cpleu Mfd⁻ cell survival following transcriptional induction with IPTG and or UV exposure. Each plates contains seven 10-fold dilutions. The number 7 denotes the most diluted spot (top).

The UV assay displayed differences in cell survival between the strains with and without a functioning Mfd. However, there were no differences attributed to transcriptional induction of the KEP strains. We also added a genetic background in which the *leuC*-inducible gene was integrated into the chromosome (Cpleu and Cpleu Mfd⁻ strains). These strains showed the same response as the KEP, YB955 and YB955 Mfd⁻ strains. Altogether, these results suggest that transcriptional induction and Mfd combine to specifically protect against oxidative damage.

To examine whether Mfd and transcriptional activation protected against oxidative damage that targets DNA, we conducted experiments that exposed cells to diamide, an oxidant that targets thiol groups and thus limits oxidative damage to proteins (Kosower *et al* 1969).

Surprisingly, the results observed in diamide exposure experiments followed the same pattern as the one observed in TBH experiments (Fig. 16).

This is an exciting result because it points to the idea that Mfd protects the cell against oxidative stress in at least two different ways. The experiments in Arg⁺ mutagenesis indicated that Mfd and MutY combine to mediate repair of oxidative damage. The experiments using diamide as an oxidant lend support to the idea that Mfd protects the cell by facilitating expression of a protective proteome that quenches ROS. At overexpressed levels, LeuC would quench ROS and prevent damage to other proteins. This is consistent with the drastic increase in survival observed when pDG148-*leuC* plasmid is present in the cell compared to the survival seen in the parent strain. The results presented here is that Mfd functions beyond facilitating DNA repair and that it is involved in gene expression that consequently creates an increase of proteins that quench ROS during stationary-phase. It is also possible that Mfd directly or indirectly regulates the cellular response to ROS. Perhaps the protective effect exerted by Mfd against protein damage caused by ROS is by directly or indirectly regulating the genes controlled by PerR. PerR, the homolog of OxyR, primarily exists in Gram positive organisms. In *B. subtilis* PerR acts as a regulator of the inducible peroxide stress response. It functions as a ferric uptake regulator (Fur) that, when induced, acts as a repressor of genes such as those responsible for catalase and zinc uptake.

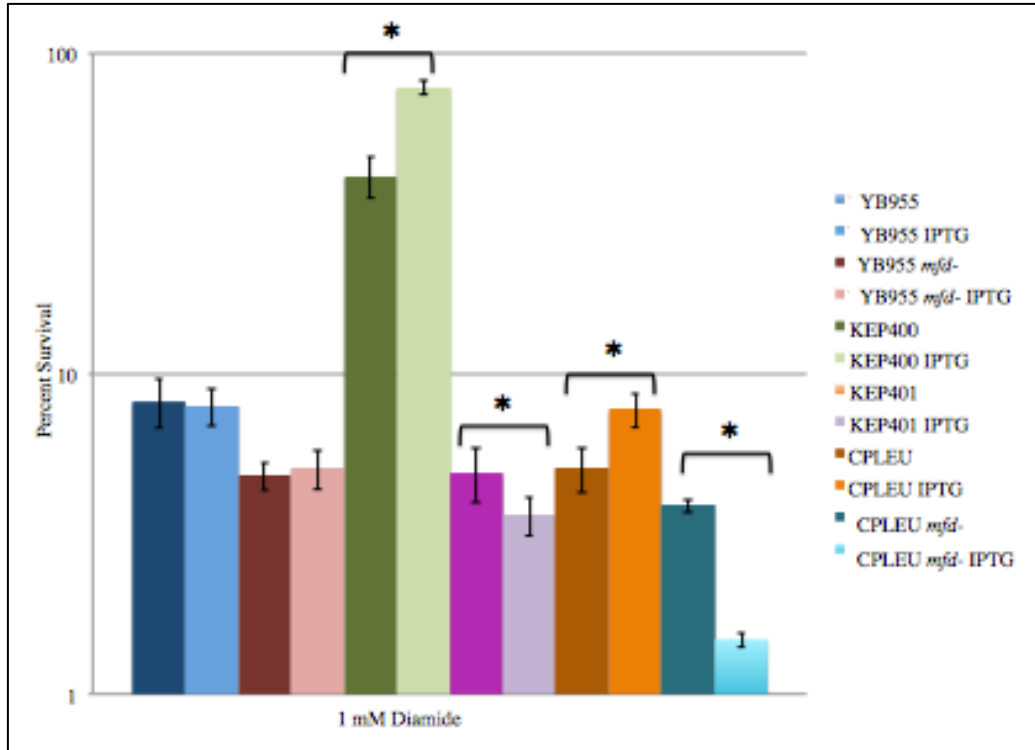


Figure 16. Oxidative stress assay using diamide and transcriptional induction in strains containing an inducible *leuC* gene in a plasmid or the chromosome. Asterisks represent statistical significance between two difference levels of transcription in one strain.

Statistical significance completed with IBM SPSS statistical analysis software.

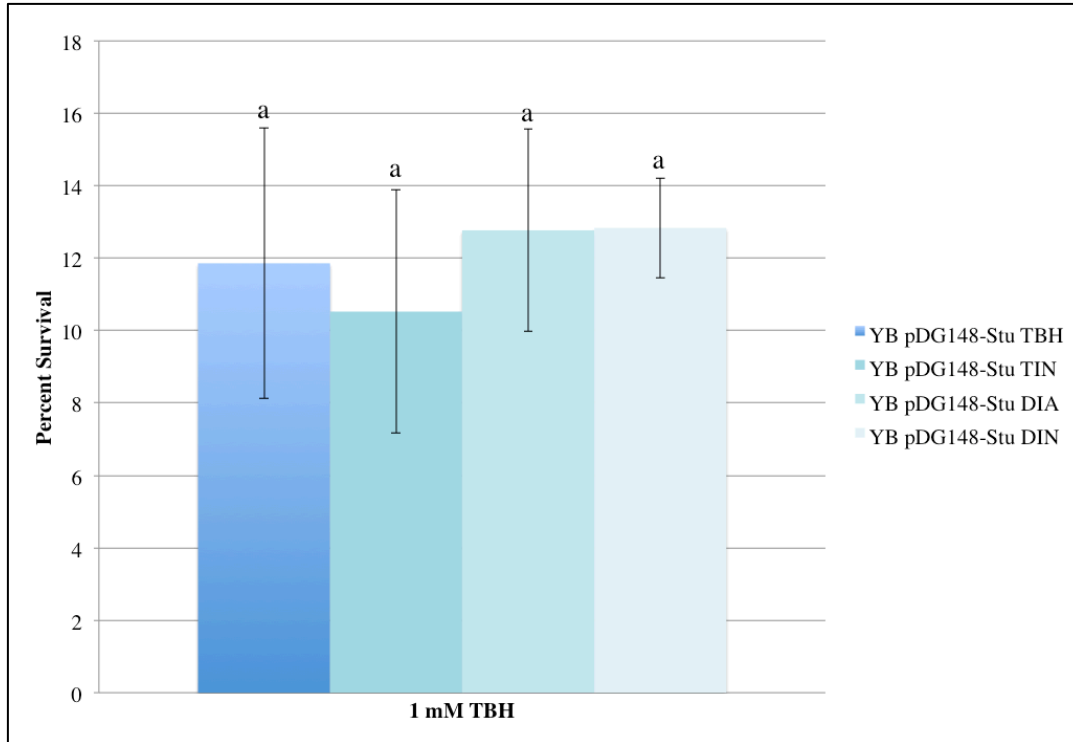


Figure 17. Survival of YB955 containing the empty plasmid pDG-148 after exposure to diamide. Cells were treated in four different ways: 1 mM tert-butyl hydroperoxide (TBH), 1 mM tert-butyl hydroperoxide and IPTG induction (TIN), 1 mM diamide (DIA), 1 mM diamide and induction with IPTG (DIN). The error bars represent standard error, statistical significance is represented by letters, different letters denote significant differences. Statistical significance measured with IBM SPSS statistical analysis software.

As a control we measure survival in YB955 containing the pDG148-Stu1 plasmid (Fig. 17) without the cloned *leuC* gene under induction with IPTG. Without the *leuC* gene and in the presence of IPTG there was no protective effect following exposure to both TBH and diamide. This further suggests that Mfd is functioning in a secondary protective role by facilitating expression of a protective proteome that absorbs ROS preventing more extensive cell damage.

In these studies we have displayed a clear connection between Mfd and oxidative damage repair in *B. subtilis* and that Mfd protects against oxidative damage into two ways.

First, our data provides evidence that Mfd's role in the processing of ROS is not within the NER pathway. Instead, Mfd interplays with the BER pathway, most specifically the DNA glycosylase MutY and error-prone synthesis during DNA repair. It is interesting to note that AP sites, an intermediate formed during repair of 8-oxoG, were shown to be mutagenic and cause the RNAP to stall in *E. coli* (Zhou and Doetsch, 1993). The occurrence for this interplay to produce mutations with high adaptive potential would be substantial since genes under selection are highly transcribed and error-prone DNA polymerases are increased during stationary-phase.

The second aspect of Mfd's effect against the cytotoxic effects caused by ROS involves protein expression. The increase of protection seen in the KEP400 strain following induction indicates that increased levels of protein in the cell protects against ROS, perhaps by facilitating quenching of such species. This was evident from the experiments that used diamide as an oxidant that does not target DNA.

Chapter 3: Conclusions and Future Directions

This dissertation demonstrated that Mfd mediates the formation of mutations by interacting with cellular components that repair reactive oxygen species. This study was originally aimed to determine if Mfd promoted SPM following damage by ROS in *B. subtilis* through the utilization of inducible constructs that measure mutagenesis. However, I also found a new function of Mfd that goes beyond TCR. This new function may be one of a global transcriptional modulator that facilitates the production of ROS quenchers that protect cell viability in *B. subtilis*.

Further research originated by this study will illuminate how the interplay between Mfd and MutY occurs. This could be done through *in vitro* assays that involve repair of oxidative lesions and the actions of Mfd and MutY. The interplay of Mfd and MutY may not require direct protein interactions as is the case between Mfd and NER. An indirect interaction may take place via a stalled of RNAP that has encountered an 8-oxoG residue. This lesion may be occupied by MutY repair reactions, which would present an obstacle for the RNAP. Then, Mfd could dislodge the stalled RNAP thereby making the lesion available for further processing. Evidence for this type of repair has been presented recently (Gómez-Marroquín *et al.*2016).

The results from the plasmid experiments suggest that Mfd facilitates the expression of ROS protein quenchers. Further experiments using expression vectors with genes coding for peptides with no cysteine residues will be very informative on this potential new function of Mfd. It is also possible that Mfd may indirectly affect prevention of ROS formation by controlling expression of PerR. The latter possibility could be addressed by examining expression of PerR as a function of Mfd.

Oxidative damage has also been linked to activation of programmed cell death. In *E. coli*, there are toxin-antitoxin modules that are activated by oxidative stress (Hazan *et al* 2004). This lends to an interesting line of experiments that can probe whether Mfd affects activation of such modules therefore influencing survival upon exposure to ROS.

In conclusion, my work has expanded the functions of Mfd beyond its classic role of DNA repair. Since Mfd is very well conserved in bacteria and active during stationary-phase conditions, future studies of the effects of Mfd on the biology of bacteria may uncover further novel aspects of bacterial evolution.

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