Chapter 5

Towards an *In Vivo* Assay to Examine the Effects of EndoIII Mutations on MutY Activity

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Abstract

Previously, our laboratory developed an assay to examine the effects of EndoIII mutations on the *in vivo* activity of MutY. This assay was designed to test our hypothesis that these two proteins communicate *in vivo* using DNA-mediated charge-transfer to search for sites of DNA damage. This assay showed that Y82A, an electrochemically deficient mutant of EndoIII, was less effective at helping MutY repair lesions than WT EndoIII. Later work in our laboratory discovered other mutants with varying charge-transfer properties, such as Y82S, Y75A, and W178A, and these samples were also considered good candidates for the *in vivo* assay. However, for these mutants, the assay protocol had to be modified. Methyl viologen was added to the bacteria cultures to increase oxidative stress, and bacterial strains were created in which the EndoIII mutants were expressed from the chromosome of an *E. coli* strain rather than on a plasmid. The experiments with methyl viologen showed that mutagenesis rates increase in the presence in of this oxidative-stress-inducing reagent. However, even in the presence of methyl viologen, no statistically significant differences were observed between strains expressing different variants of EndoIII. These results differ from those of previous experiments, but the discrepancy may result from differences in genetic background, growth phase at harvest, and media recipes. Importantly, the results of these experiments further elucidate the complex nature of cellular environments and their responses to mutagenesis and stress.

Introduction

Previously, our laboratory introduced a model for cooperative scanning for DNA damage using DNA-mediated charge-transfer (CT). According to this model, DNA-bound, redox-active proteins could communicate with each other by sending and receiving electrons and/or holes through the DNA helix. However, if the intervening DNA contains a lesion, then CT will not occur efficiently [1–3], signaling to the bound proteins that DNA damage is present. These proteins can then localize to the damaged site and execute repair. Our lab developed an *in vivo* assay to test this cooperative search model by examining how inactivation of *nth*, the gene that encodes Endonuclease III (EndoIII) affects the activity of MutY. EndoIII and MutY are both [4Fe-4S] cluster-containing DNA glycosylases whose *E. coli* forms have been shown to be redox-active when bound to DNA [1]. These proteins display similar midpoint potentials [1], indicating that they could transmit electrons to each other and potentially help each other search for their substrate DNA lesions.

To develop an assay to test whether MutY and EndoIII cooperate *in vivo*, the reporter strain *E. coli* CC104 was used. Developed by the laboratory of Professor Jeffrey Miller, CC104 contains an A→C mutation in the codon that encodes glutamic acid 461 of the *lacZ* gene, which encodes β-galactosidase. This mutation converts residue 461 to an alanine, rendering β-galactosidase inactive in wild-type CC104 strains [4]. However, oxidation processes within the cell can oxidize the guanine paired with the substituted cytosine, producing 7,8-dihydro-8-

oxoguanine (8-oxo-G) [5–7]. DNA replication machinery will mistakenly pair an adenine with 8-oxo-G, and then the next round of replication will place a thymine opposite this adenine. The end result of these processes is a G:C → T:A transversion at position 461 [5]. These "transverted" mutants of CC104 will then possess a functional copy of β-galactosidase and be able to grow on minimal medium supplemented with lactose as the sole carbon source.

However, the enzyme MutY removes adenine mispaired with 8-oxo-G, thus preventing CC104 from reverting to a *lac*$^+$ phenotype. Consequently, when CC104 colonies appear in low numbers on lactose-minimal media plates, the results indicate active MutY. High numbers of *lac*$^+$ revertant colonies suggest that MutY's activity has been compromised. Indeed, the number of *lac*$^+$ CC104 revertants increases 15-fold when *mutY* is genetically inactivated [8]. Our laboratory has also shown that the number of *lac*$^+$ revertants increases two fold when the *nth* gene is inactivated [8]. Given that MutY and EndoIII have not been shown to interact *in vivo*, this result suggests that EndoIII helps MutY detect and repair 8-oxo-G. This cooperation is not due to overlapping substrates, because a *mutY*$^-$*nth*$^-$ strain of CC104 produced as many *lac*$^+$ revertants, within error, as a *mutY*$^-$ strain [8].

This *lac*$^+$ reversion assay was also used to test how certain EndoIII mutations affected the ability of MutY to detect its substrates. A plasmid containing D138A, an electrochemically proficient but enzymatically deficient mutant, offered the same level of assistance to MutY as WT EndoIII [8]. However, Y82A, an enzymatically active but electrochemically deficient mutant was less able

to help MutY detect lesions [8]. These data support the model that EndoIII and MutY cooperatively search for DNA damage using DNA-mediated CT.

After examining these results, it was decided that this assay should be used to examine the effect of other EndoIII mutations on the activity of MutY. However, some changes to the protocol were recommended. First, the addition of oxidative stress-inducing reagents, such as methyl viologen, was encouraged because they could increase the level of internal reactive oxygen species (ROS), cause more guanine oxidation, and possibly increase the overall revertant count. High numbers of revertants could potentially enhance subtle differences between strains of CC104 expressing different *nth* mutants.

Second, the assay needed to be adjusted such that plasmids were not required. Previously, EndoIII mutants were expressed on plasmids and then transformed into CC104*nth*$^-$ cells. However, plasmids require the use of antibiotics, which could interact with the aforementioned oxidative stress-inducing chemicals. Strains expressing plasmids may also contain slight differences in plasmid copy number from cell to cell, making data difficult to interpret and increasing the possibility of errors and outliers during the assay. Consequently, strains were constructed in which *nth* variants were expressed on the chromosomes of CC104. The *nth* variants were cloned into a plasmid that expresses genes off of a rhamnose-inducible promoter [9], and then this plasmid was allowed to integrate into the λ-phage attachment site of the strain CC104Δ*nth*. After these strains were prepared, the *lac*$^+$ reversion assay was performed to examine the effect of methyl viologen

concentration on the number of *lac*$^+$ revertants of the strain CC104Δ*nth* $P_{rha\text{-}nth}$. Then, the assay was performed using strains of CC104 expressing chromosomal copies of the *nth* variants WT, Y82A, Y82S, Y75A, and W178A.

Materials and Methods

Bacterial Strains and Plasmids. The vectors pKOV, pAH120, pINT-ts, and strain *E. coli* CC104 were obtained from the Coli Genetic Stock Center of Yale University.

Cloning. All PCR reactions were carried out using either the Failsafe PCR System with Buffers D or G (Epicentre Biotechnologies), or KOD polymerase (Merck4 Biosciences). Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen) and DNA products were isolated from agarose gels using the Wizard Kit (Promega). All primers were purchased from Integrated DNA Technologies.

Creation of CC104Δ*nth*. The methods used here were adapted from those described previously [10]. Regions 1000 bp upstream and downstream of the *nth* gene were PCR amplified from chromosomal DNA using primers CARJKB172, delnthfusrev, delnthfusfor, and CARJKB173. The resulting PCR products were used as templates in a second PCR experiment, resulting in a product of ~ 2000 bp that omits the *nth* gene. This product was ligated into the BamHI and NotI sites of the vector pKOV. Electrocompetent CC104 cells were then transformed with the pKOV plasmid containing the Δ*nth* construct and recovered at 30°C. The transformation reactions were plated onto Luria-Bertani (LB) + chloramphenicol

medium and incubated at either 30°C or 42°C. After incubation, the ratio of colony number on the 42°C plate to colony number on the 30°C indicated high integration efficiency. Cells that had been incubated at 42°C were grown in liquid culture, serially diluted, and plated onto LB + sucrose medium. Sucrose-tolerant cells were selected and examined by PCR using primers CARJKB164, CARJKB165 to verify that the *nth* gene had been cleanly deleted. After this PCR reaction, the samples that appeared to be Δ*nth* were isolated, re-streaked, and analyzed by colony PCR again. This process was repeated at least three times to ensure that the samples were not contaminated with WT CC104. Finally, sequencing with primers CARJKB174, CARJKB175, CARJKB180 was used to verify that the isolated strains were truly Δ*nth*.

Creation of CC104Δ*nth* $P_{rha\text{-}nth}$ Strains. The methods described here are adapted from those previously published [9]. Variants of *nth* were PCR amplified from overexpression vectors using primers CARJKB164 and CARJKB165 and then ligated into the BamHI and NdeI sites of pAH120. Samples of pAH120 expressing different variants of *nth* were transformed into electrocompetent CC104Δ*nth* that were also expressing the plasmid pINT-ts. Cells were then incubated at 30ºC for one hour to allow chromosomal integration of pAH120, and then at 42ºC for 30 minutes, during which time pINT-ts should be cured. Cultures were spread onto Luria-Bertani (LB) + kanamycin media and incubated at 37ºC. This final incubation selects for cells that have integrated pAH120, since this plasmid confers kanamycin resistance. Colonies that emerged on these kanamycin selection plates

were screened using colony PCR and primers CARJKB164 and CARJKB165 to verify the presence of an *nth* variant on the chromosome. Further PCR screens were performed with primers given in reference [9] to verify that the pAH120 plasmid integrated only once.

Lac$^+$ Reversion Assay. Freezer stocks of the appropriate CC104 strains were streaked onto Luria-Bertani (LB) medium and incubated for 12 hours at 37°C. Ten different samples from each strain were inoculated into ten tubes of LB liquid medium supplemented with 1 mM rhamnose and grown for 13 hours at 37°C with shaking at 250 rpm. From each overnight culture, 200 μL of was transferred into 10 mL Minimal A Medium [11] supplemented with 0.4% glycerol and 1 mM rhamnose. Methyl viologen was added to these cultures to a final concentration of either 0 μM, 10 μM, 25 μM, or 50 μM. These cultures were grown at 37°C until OD_{600} = 0.4. The cultures were pelleted by centrifugation, rinsed in 5 mL Minimal A Medium with no carbon source, and plated onto Minimal A Medium supplemented with 0.2% lactose. The plates were incubated for 42 hours at 37°C, at which point *lac*$^+$ revertants were counted. Assays were performed at least two times for each sample, and ten replicates of each sample were used in each assay. The two highest and two lowest values were dismissed and the remaining six values were averaged. Errors are reported as standard deviations.

Results and Discussion

The effect of methyl viologen on the number of lac$^+$ revertants. One of the first assays performed with the newly created CC104Δ*nth* P*rha-nth* strain examined

whether the redox cycling reagent methyl viologen would affect the number of *lac*$^+$ revertants. The total number of revertants nearly doubled on increasing the methyl viologen concentration from 0 μM to 50 μM (Figure 5.1). This result was expected given that methyl viologen reacts with oxygen to generate superoxide [12], a reactive oxygen species (ROS). ROS can damage DNA, particularly at guanine residues since guanine is the most easily oxidized nucleobase [13–15].

Lac+ revertant counts as a consequence of nth *gene mutations*. After it was established that methyl viologen could increase the number of *lac*$^+$ revertants observed during the assay, methyl viologen was then incorporated into the protocol used to examine strains expressing different *nth* variants. The strains include CC104Δ*nth* and strains of CC104Δ*nth* P*rha-nth* expressing the *nth* variants WT, Y82A, Y82S, Y75A, and W178A. Even with the addition of methyl viologen, the results show no statistically significant differences between the number of *lac*$^+$ revertants produced by these different strains (Figure 5.2). It was surprising that these results were not consistent with results from plasmid-based assays performed previously [8]. However, the discrepancy may result from minor genetic differences in the parent CC104 strains used to produce these mutants. Other changes such as growth medium, growth phase at harvest, and nutrient source, could also affect the *in vivo* mutation rate, as discussed below. Also, expression levels of the *nth* gene under the different experimental conditions were not determined.

Variations in experiments. First, the number of *lac*$^+$ revertants produced by a given sample of CC104 seems to rely upon the genotype of that strain. Even subtle, non-detectable, genetic differences can lead to differences in internal mutagenesis rates. For example, our laboratory has worked with two different "wild-type" strains for CC104 *E. coli* during the past several years. One strain came from a collaborator's laboratory (Professor Jeffrey Miller, University of California Los Angeles) and one came from a strain library (Coli Genetic Stock Center, Yale University). Although both strains have the same genotype, they produced consistently different numbers of *lac*$^+$ revertants when measured side-by-side under identical experimental conditions (data not shown). This result speaks to the fact that even small, unnoticeable, changes in the genetic background of given strains can affect their mutagenesis rates. It was also observed that the sequence of the *lacZ* gene found in the strains in our laboratory was different than that published when Cupples and Miller reported the creation of strain CC104 [4]. However, it remains to be determined whether the *lacZ* sequence discrepancy accounts for the difference between previously published assay results [8] and the data presented here.

Second, cell cultures in the CC104Δ*nth* $P_{rha\text{-}nth}$ assays were harvested in mid-exponential phase to ensure that they were actively growing while plated. By contrast, cultures in the plasmid-based assay were harvested in late exponential phase [8]. Growth phase may affect mutagenesis rates because levels of expression of oxidative stress response genes can change in stationary phase [16], as can cells' resistance to peroxide [17]. However, metabolism and expression of certain genes

slow in stationary phase [17], possibly making it more difficult for colonies to grow on lactose-minimal medium.

Third, the assay using CC104Δ*nth* $P_{rha\text{-}nth}$ strains required different growth media than the plasmid-based assay. Rhamnose had to be added to the growth medium to induce expression of the *nth* variants [18, 19]. Expression from the rhamnose-inducible promoter is inhibited by glucose [19], which was used in the plasmid-based assay. Consequently, glycerol was used as the carbon source in the experiments presented here. This change in carbon source could be significant because carbon source has been shown to affect mutagenesis rates through mechanisms thought to involve carbon catabolite repression (reviewed in references [20, 21]). Mutagenesis reporter strains produce larger numbers of mutant colonies when grown on glycerol, a non-repressive growth substrate, than when grown on glucose, a highly repressive growth substrate [22, 23]. Glucose-related compounds with even more repressive effects, such as glucose-6-phosphate and methyl-α-D-glucopyranoside, exert even more powerful anti-mutagenic effects [22]. It is unclear exactly how carbon catabolite repression influences mutagenesis rates, but authors hypothesize that the SOS DNA repair pathway (reviewed in reference [24]) may be affected. Carbon catabolite repression depresses levels of cyclic AMP (cAMP) [20, 21], an important cellular signaling molecule. In turn, low cAMP levels reduce the expression of genes involved in SOS DNA repair, which is highly mutagenic [24]. Perhaps the anti-mutagenic effects of carbon catabolite repression are part of the reason why glucose transport is induced in response to oxidative

stress in *E. coli* through the action of the *SoxRS* genes [25]. The exact relationship between metabolism and mutation remains unclear, but the change in growth medium could have impacted the outcome of the lac^+ reversion assay.

Although the goal of the experiments presented here was to determine how mutations in EndoIII affect the activity of MutY, the data do not provide such an answer. However, the process of optimizing these experiments and interpreting the results offered yet another perspective on the profound complexity of *E. coli* cells. The phenotype observed previously by Boal, et al. [8] relied upon a careful combination of cell genotype, growth phase, and growth conditions. Future work examining with *in vivo* affects of *nth* mutations will need to consider these parameters.

Table 5.1: Primers used in *lac*$^+$ reversion experiments

Name	Sequence	Purpose
CARJKB172	5'-actagtgcggccgcgaactgcgcctttctgactggatc-3'	Primer for overlap extension to create Δ*nth* construct, and for verifying absence of *nth* on CC104 chromosome. It binds to a sequence 1000 bp upstream of *nth* gene.
CARJKB173	5'-ggcgcgccggatcccataaccaatcgccagcacaatagc-3'	Primer for overlap extension to create Δ*nth* construct, and for verifying absence of *nth* on CC104 chromosome. It binds to a sequence 1000 bp downstream of *nth* gene.
delnthfusfor	5'- gcattgccaaacggtgaaacagggaatgtctgatgaagaaaaggggtaacaccgattaccccattg-3'	Primer for overlap extension to create Δ*nth* construct. It binds to a sequence immediately following the *nth* gene.
delnthfusrev	5'-caatggggtaatcggtgttacccctttttcttcatcagacattccctgtttcaccgtttggcaatgc-3'	Primer for overlap extension to create Δ*nth* construct. It binds to a sequence immediately preceding the *nth* gene.
CARJKB174	5'-acattgtttgacggtgcaga-3'	Sequencing Δ*nth* strains
CARJKB175	5'-ggtcaatatttgttgcgtgtg-3'	Sequencing Δ*nth* strains
CARJKB180	5'-gcaatggcacattgtttgac-3'	Sequencing Δ*nth* strains
CARJKB164	5'-ggcgcgcccatatgaataaagcaaaacgcctggagatc-3'	Primer for cloning *nth* alleles into pAH120, NdeI site
CARJKB165	5'-ggcgcgccggatcctcagatgtcaactttctctttgtattc-3'	Primer for cloning *nth* alleles into pAH120, BamHI site
CARJKB176	5'-cgaattcaggcgctttttag-3'	Sequencing *nth* alleles after chromosomal insertion
CARJKB177	5'- tctgctggaaccactttcagt-3'	Sequencing *nth* alleles after chromosomal insertion
CARJKB178	5'-tgaaagtggttccagcagag-3'	Sequencing *nth* alleles after chromosomal insertion
CARJKB179	5'- gggagtgggacaaaattgaa-3'	Sequencing *nth* alleles after chromosomal insertion

Figure 5.1: Lac$^+$ revertants as a function of methyl viologen concentration. Methyl viologen was added to cultures of CC104Δ*nth* P*rha-nth* growing in liquid minimal medium at the concentrations shown. Cultures were grown to OD_{600}= 0.4, pelleted, rinsed once, and then plated on minimal A medium supplemented with 0.2% lactose. *Lac*$^+$ revertants were counted after 42 hours incubation at 37°C.

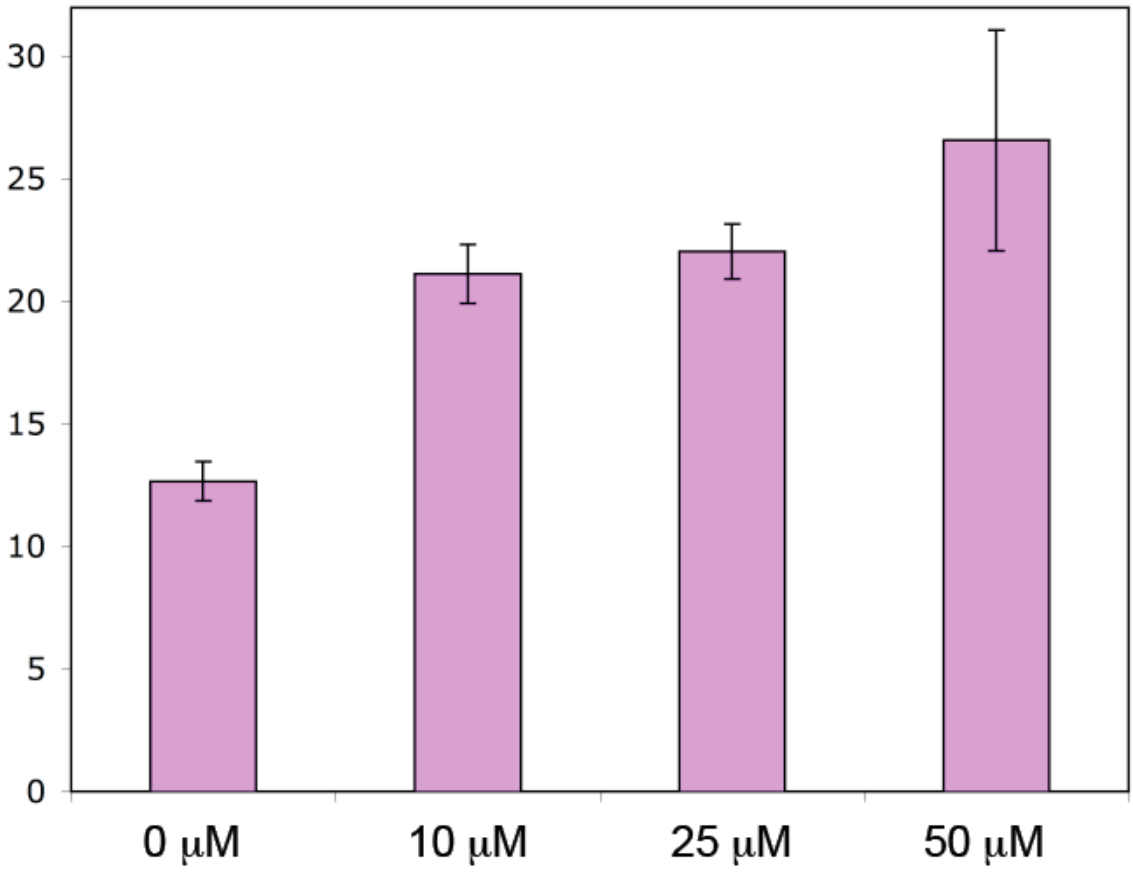

Table 5.2: Average *lac*+ revertants counted for different concentrations of methyl viologen. Results are averaged over four trials. Values are reported as the number of revertants per ~2x10^8 cells/mL.

Concentration of methyl viologen	0 μM	10 μM	25 μM	50 μM
No. of lac+ revertants	12.7 ± 0.8	21.1 ± 1.2	22.0 ± 1.1	26.6 ± 4.5

Figure 5.2: Lac$^+$ revertants produced by CC104Δ*nth* P*rha-nth* strains expressing different *nth* alleles. Strains were grown in Minimal A Medium supplemented with 0.4% glycerol, 1 mM rhamnose, and 50 μM methyl viologen. Cultures were grown to OD_{600}= 0.4, pelleted, rinsed once, and then plated on Minimal A Medium supplemented with 0.2% lactose. *Lac*$^+$ revertants were counted after 42 hours incubation at 37°C.

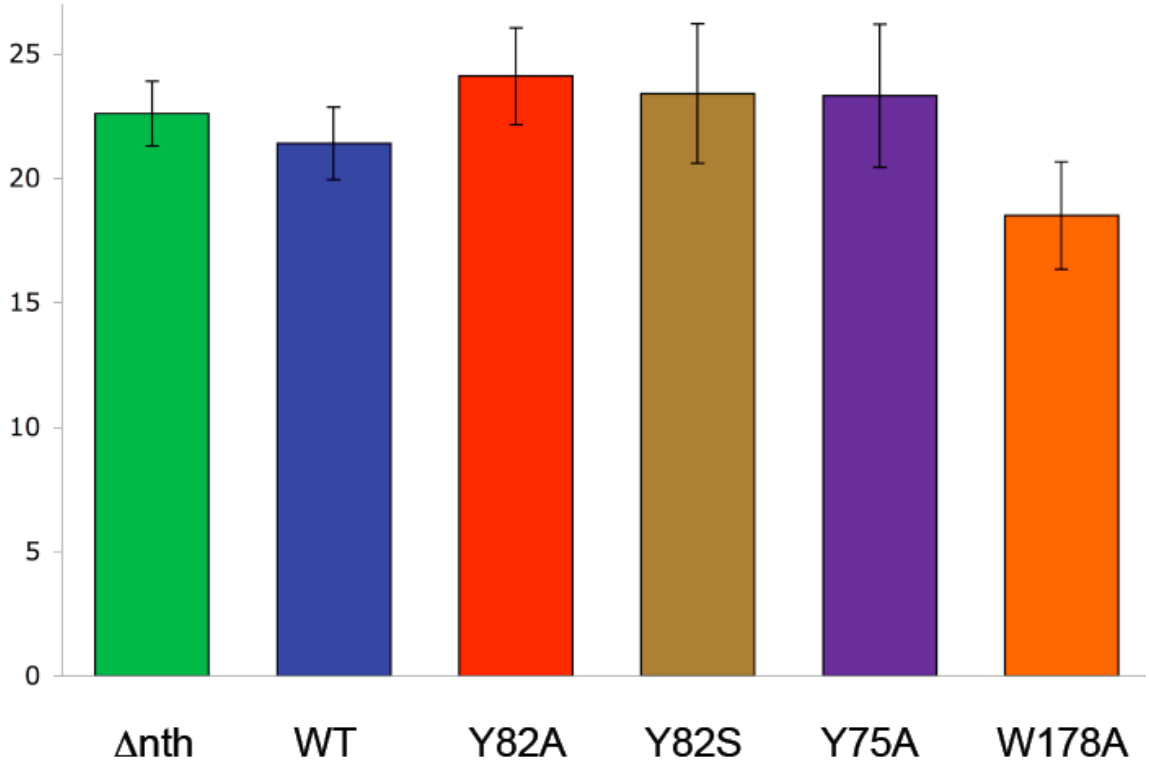

Table 5.3: Average *lac*$^+$ revertants counted for strains expressing different variants of *nth*. Values are reported as the number of revertants per ~ 2x10^8 cells/mL.

Strain	CC104Δ*nth*	CC104Δ*nth* P*rha-nth*	CC104Δ*nth* P*rha-Y82A*	CC104Δ*nth* P*rha-Y82S*	CC104Δ*nth* P*rha-Y75A*	CC104Δ*nth* P*rha-W178A*
No. of lac$^+$ revertants	22.6 ± 1.3	21.4 ± 1.5	24.1 ± 1.9	23.4 ± 2.8	23.3 ± 2.9	18.5 ± 2.2

References:

1. Boal, A.K., et al., *DNA-Bound Redox Activity of DNA Repair Glycosylases Containing [4Fe- 4S] Clusters.* Biochemistry, 2005. **44**: 8397–8407.
2. Boal, A.K., and J.K. Barton, *Electrochemical Detection of Lesions in DNA.* Bioconjugate Chem., 2005. **16**: 312–321.
3. Boon, E.M., et al., *Mutation detection by electrocatalysis at DNA-modified electrodes.* Nature Biotechnology, 2000. **18**: 1096–1100.
4. Cupples, C.G., and J.H. Miller, *A set of lacZ Mutations in Escherichia coli that Allow Rapid Detection of Each of the Six Base Substitutions.* Proc. Natl. Acad. Sci. USA, 1989. **86**: 5435–5439.
5. Neeley, W.L., and J.M. Essigmann, *Mechanisms of formation, genotoxicity, and mutation of guanine oxidation products.* Chemical Research in Toxicology, 2006. **19**(4): 491–505.
6. Burrows, C.J., and J.G. Muller, *Oxidative nucleobase modifications leading to strand scission.* Chemical Reviews, 1998. **98**(3): 1109–1151.
7. David, S.S., V.L. O'Shea, and S. Kundu, *Base-excision repair of oxidative DNA damage.* Nature, 2007. **447**: 941–950.
8. Boal, A.K., et al., *Redox signaling between DNA Repair Proteins for efficient lesion detection.* Proc. Natl. Acad. Sci. USA, 2009. **106**(36): 15237–15242.
9. Haldimann, A., and B.L. Wanner, *Conditional-Replication, Integration, Excision, and Retrieval Plasmid-Host Systems for Gene Structure-Function Studies of Bacteria.* Journal of Bacteriology, 2001. **183**(21): 6384–6393.
10. Link, A.J., D. Phillips, and G.M. Church, *Methods for Generating Precise Deletions and Insertions in the Genome of Wild-Type Escherichia coli: Application to Open Reading Frame Characterization.* Journal of Bacteriology, 1997. **179**(20): 6228–6237.
11. Miller, J.H., *Experiments in Molecular Genetics*. 1972, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
12. Bus, J.S., and J.E. Gibson, *Paraquat—Model for Oxidant-Initiated Toxicity.* Environmental Health Perspectives, 1984. **55**(APR):37–46.
13. Steenken, S., and S.V. Jovanovic, *How easily oxidizable is DNA? One-electron reduction potentials of adenosine and guanosine radicals in aqueous solution.* Journal of the American Chemical Society, 1997. **119**(3): 617–618.
14. Saito, I., et al., *Photoinduced DNA Cleavage Via Electron-Transfer—Demonstration That Guanine Residues Located 5′ to Guanine Are the Most Electron-Donating Sites.* Journal of the American Chemical Society, 1995. **117**(23): 6406–6407.
15. Genereux, J.C., A.K. Boal, and J.K. Barton, *DNA-Mediated Charge Transport in Redox Sensing and Signaling.* Journal of the American Chemical Society, 2010. **132**(3): 891–905.

16. Demple, B., *Regulation of Bacterial Oxidative Stress Genes.* Annual Review of Genetics, 1991. **25**: 315–337.
17. White, D., *The physiology and biochemistry of prokaryotes*. 2000, New York: Oxford University Press.
18. Cardona, S.T., and M.A. Valvano, *An expression vector containing a rhamnose-inducible promoter provides tightly regulated gene expression in Burkholderia cenocepacia.* Plasmid, 2005. **54**(3): 219–228.
19. Haldimann, A., L.L. Daniels, and B.L. Wanner, *Use of new methods for construction of tightly regulated arabinose and rhamnose promoter fusions in studies of the Escherichia coli phosphate regulon.* Journal of Bacteriology, 1998. **180**(5):1277–1286.
20. Gorke, B., and J. Stulke, *Carbon catabolite repression in bacteria: many ways to make the most out of nutrients.* Nat. Rev. Microbiol., 2008. **6**(8): 613–624.
21. Deutscher, J., *The mechanisms of carbon catabolite repression in bacteria.* Curr. Opin. Microbiol., 2008. **11**(2): 87–93.
22. Ambrose, M., and D.G. Macphee, *Catabolite repressors are potent antimutagens in Escherichia coli plate incorporation assays: experiments with glucose, glucose-6-phosphate and methyl-alpha-D-glucopyranoside.* Mutation Research—Fundamental and Molecular Mechanisms of Mutagenesis, 1998. **398**(1–2): 175–182.
23. Ambrose, M., and D.G. MacPhee, *Glucose and related catabolite repressors are powerful inhibitors of pKM101-enhanced UV mutagenesis in Escherichia coli.* Mutat. Res., 1998. **422**(1): p. 107-112.
24. Rattray, A.J., and J.N. Strathern, *Error-prone DNA polymerases: when making a mistake is the only way to get ahead.* Annu Rev Genet, 2003. **37**: p. 31-66.
25. Rungrassamee, W., X. Liu, and P.J. Pomposiello, *Activation of glucose transport under oxidative stress in Escherichia coli.* Archives of Microbiology, 2008. **190**(1): p. 41-49.