CHAPTER 7

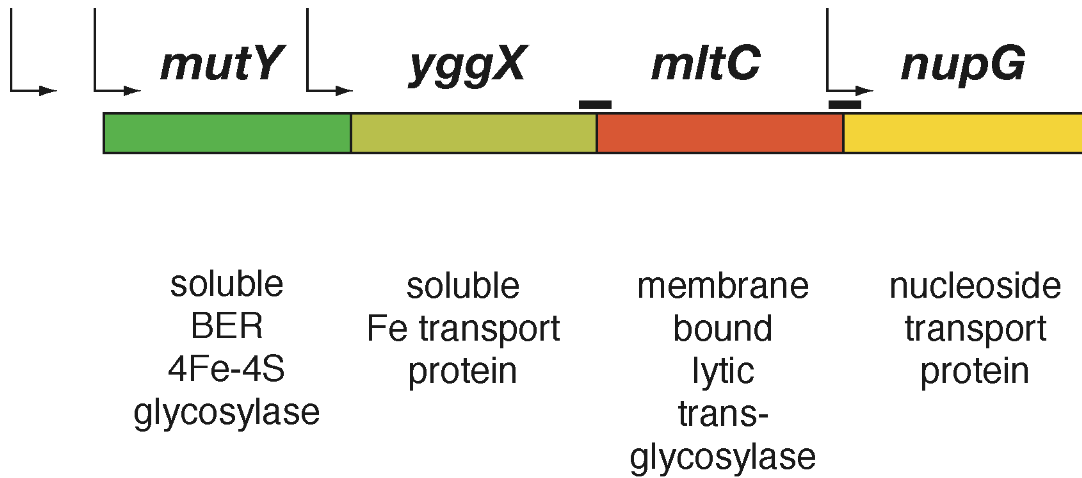
Investigating the Role of the *Rnf* Operon in DNA Repair

INTRODUCTION

MutY and Endonuclease III (EndoIII) are DNA repair glycosylases in the base excision repair (BER) pathway that are responsible for excising oxidatively damaged DNA bases from the genome (*1*). Though EndoIII and MutY do not remove the same lesions from DNA (MutY removes adenine mispaired with 8-oxo-guanine and EndoIII excises a variety of oxidized pyrimidines), these enzymes are related by their similar structures and the [4Fe4S] cluster cofactor harbored by each protein (*1-3*). The role of the iron-sulfur cluster in these enzymes is not fully understood, but it has been demonstrated that the [4Fe4S] cluster is redox active when the enzyme is bound to DNA (*4-8*). Thus, it is proposed that the iron-sulfur cluster might be used to quickly and efficiently detect damage in the genome via DNA-mediated charge transport (CT), a reaction modulated by both the structural and dynamic integrity of the DNA base-pair stack (*9-13*). Furthermore, similar redox potentials measured for MutY and EndoIII indicate that DNA CT may occur between MutY and EndoIII protein molecules; DNA CT could allow for cooperative damage detection among [4Fe4S] DNA repair glycosylases (*14*).

The discovery that MutY and EndoIII are transcribed as part of complex operons in *Escherichia coli* brings up additional questions about the role of the [4Fe4S] cluster in these proteins. The gene encoding MutY (*mutY*) is a member of an operon consisting of four genes (Figure 7.1) (*15*). The gene immediately 5` to *mutY* is *yggX*, a gene that encodes the 91aa protein YggX (*16*). YggX may function in oxidative stress protection in *Salmonella enterica* and *E. coli* (*17-19*). YggX can also sequester iron in solution and has been shown to protect DNA from damage via Fenton chemistry *in vitro*. The structure of YggX, as determined by NMR, consists of one small helical domain as well

Figure 7.1. The chromosomal arrangement of *mutY* in *E. coli*. *MutY* is the first gene in a four gene operon followed by *yggX*, which encodes a protein involved in iron trafficking and oxidative stress protection, *mltC,* the gene for a lytic membrane-bound glycosylase, and *nupG*, a gene encoding a nucleoside transport protein. Putative promoters are represented as arrows and attenuators and terminators as bars.

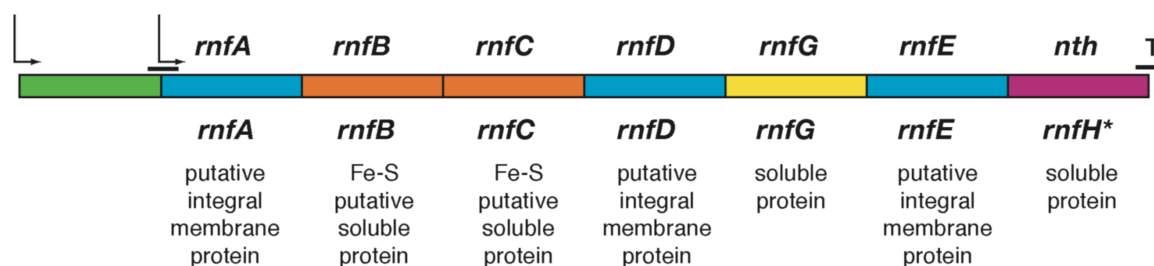


as a relatively unstructured region. The binding site for iron is not revealed in these structures, and it is postulated that YggX may bind to a partner protein inside the cell (*16*). The other two genes in the MutY operon appear to be less related in function (*15*). *mltC* encodes a membrane-bound lytic glycosylase that can hydrolyze peptidoglycans. *nupG* is the gene for a high-affinity nucleoside transporter. This genomic arrangement is conserved among many bacterial organisms, though in some bacteria only *mutY* and *yggX* are transcribed together (*15*).

The gene encoding EndoIII (*nth*) is the terminal gene in an eight-gene operon in *E. coli* (Figure 7.2) (*20*). The other seven genes have not been characterized in *E. coli*, but some of them bear sequence homology to a set of genes required for nitrogen fixation in purple photosynthetic bacteria (i. e., *Rhodobacter capsulatus*) (*21*). In *R. capsulatus*, *nth* is replaced by *rnfH*. The remaining genes are termed *rnfABCDGE* and are believed to form a membrane-bound complex probably involved in electron transport to nitrogenase or nitrogenase reductase (*22-24*). It has also been proposed that the *rnf* operon could be involved in protein-bound iron-sulfur cluster maturation in organisms that fix nitrogen.

RnfA, rnfD, and *rnfE* are predicted to encode transmembrane proteins while *rnfB, rnfC,* and *rnfG* encode largely soluble proteins. *RnfB* and *rnfC* are also predicted to bind iron-sulfur clusters. The *rnfC* gene product may contain up to two [4Fe4S] clusters while *rnfB* encodes 12 cysteine residues with potential binding sites for two [4Fe4S] clusters and one [2Fe2S] cluster. Attempts to overexpress the *R. capsulatus rnf* genes in *E. coli* have met with limited success. RnfA, RnfB, and RnfC were all able to be expressed heterologously, but they appear to associate strongly with the cell membrane, may not contain all of their iron-sulfur cofactors, and lose stability in the absence of the rest of their *rnf* counterparts.

Figure 7.2. The chromosomal arrangement of *nth* in *E. coli*. *Nth* is the terminal gene in an eight gene operon. The remaining genes are uncharacterized in *E. coli* but are homologous to a set of genes found in *R. capsulatus* and other nitrogen fixing organisms. These genes, termed *rnfABCDGE,* are required for nitrogen fixation and are believed to form a membrane-bound complex. Putative promoters are represented by arrows and terminators are indicated by bars.



Though the *E. coli rnf* genes have not been biochemically characterized, it has been demonstrated that inactivation of these genes has an effect on SoxR mediated *soxS* expression (*25*). The *soxRS* system senses oxidative stress and activates transcription of a wide variety of genes to protect against and repair oxidative damage (interestingly, one of the genes targeted is *yggX* (*19*)) (*26*). Activation of the *soxRS* regulon is mediated by SoxR, a [2Fe2S] cluster transcription factor (*27-29*). Upon oxidation of the cluster in SoxR from the 1+ to the 2+ state, transcription of *soxS* is initiated. *SoxS* transcription is transient; within minutes after administration of oxidants has ceased, SoxR is rereduced and *soxS* is no longer transcribed (*29*). The pathways for oxidation and rereduction of SoxR are not fully understood, though SoxR is activated within the cell by administration of paraquat (*29*) and it has been demonstrated *in vitro* that SoxR can be oxidized from a distance, in a DNA-mediated fashion, by guanine radicals or electrochemical methods (*30, 31*). Inactivation of the *E. coli rnf* genes slows the deactivation of *soxS* expression, indicating that the *rnf* gene products may be involved in the rereduction of SoxR (*25*).

The relationship between the *rnf* gene products and EndoIII (or other [4Fe4S] cluster DNA repair enzymes) is unknown, though it is theorized that genes that are transcribed together often perform similar or related functions within the cell (*32*). To examine the possibility that the *E. coli rnf* genes might affect the activity of EndoIII within the cell, we have knocked out the *rnf* operon in an *E. coli* strain that serves as a reporter for EndoIII repair activity. Inactivation of the *rnf* operon leads to a suppression of EndoIII-associated mutations, a surprising result given that knockout of the *rnf* operon should eliminate EndoIII expression as well. Thus, further studies will be required to fully understand the relationship between the *rnf* operon and DNA repair.

MATERIALS AND METHODS

Materials

All vectors for gene inactivation were generously donated by Prof. Dianne Newman. Oligonucleotides were purchased from IDT or synthesized in-house. All enzymes were purchased from Stratgene or Roche. All strains used were derivatives of CC102 (*33*) and generated as described below. Luria-Bertani (LB) broth was used as the rich medium while NCE (*34*) medium supplemented with $MgSO_4$ (100 μM) and glucose (11 mM) or lactose (6 mM) was used as the minimal medium.

Genetic Inactivation of rnf Genes

CC102 strains were generously donated (*33*) and *RnfA* was replaced by a kanamycin resistance cassette (*kan*) in CC102 using a previously described deletion method (*35*). Primer sequences are as follows: (*rnfA* homology regions are shown in regular text and *kan* priming regions are highlighted in boldface) 5`-CTGCTCTGGATTAACGGATAATAGGCGGCTTTTTTATTTCAGGCCGAAAA**GTGTAGGCTGGAGCTGCTTC**-3`, 5`-CGCCAGGCCCAGCAGGCTCACGGCGGCAACGGCAATCCAGATAGCATTCA**CATATGAATATCCTCCTTAG**-3`. Inactivation was verified with colony PCR.

Lac⁺ Reversion Assays

Strains were streaked to LB medium and incubated overnight at 37°C. For *rnfA* knockouts, strains were streaked to LB+kanamycin (17 mg/mL). 1 mL LB cultures were started from single colonies and grown overnight in a shaking incubator at 37°C, 220 rpm. 20 mL of each starter culture was used to inoculate a 10 mL NCE+glucose culture

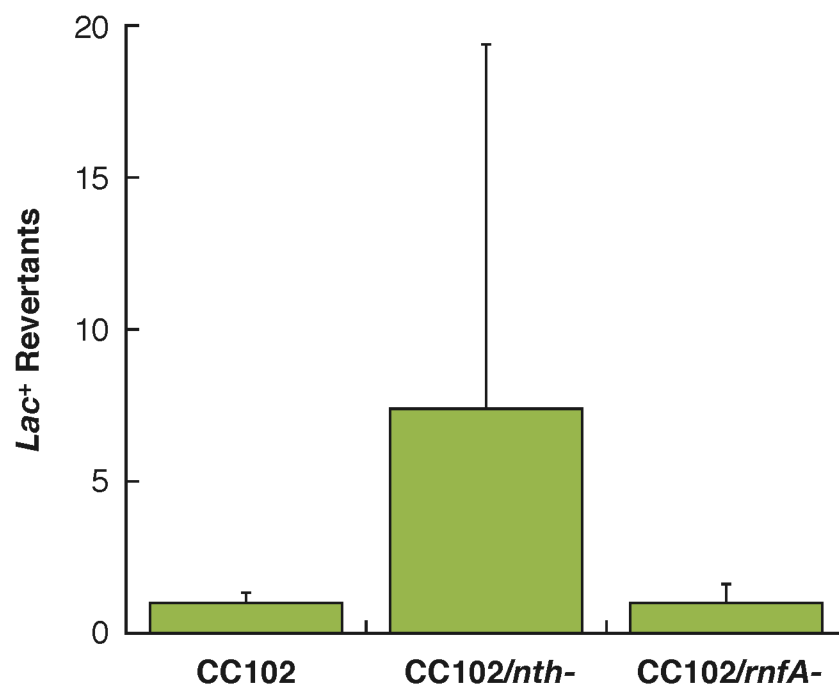
which was then grown to a density of 10^9 cells/mL at 37°C, 250 rpm. Cell density was determined by dilution plating a 10 mL aliquot of the NCE+glucose culture onto NCE+glucose solid medium followed by incubation at 37°C for 36 hours. 5 mLs of this culture was centrifuged in a clinical tabletop centrifuge at 4°C and plated on NCE+lactose solid medium and then incubated at 37°C for 36 hours. Colonies arising are reported as *lac*$^+$ revertants/mL cells plated.

RESULTS AND DISCUSSION

Effect of rnfA Inactivation on EndoIII Activity

The CC102 strain uses an engineered *lacZ* mutation to report the frequency of GC:AT transition mutations in a population of *E. coli* cells (*33*). EndoIII prevents these mutations through enzymatic excision of 5-hydroxy-cytosine (*1*) which will mispair with adenine if allowed to go unrepaired and to undergo replication (*36*). In the CC102 strain, a base-pair substitution has been introduced in the codon for Glu461 in *lacZ,* the gene encoding β-galactosidase, an enzyme required for lactose metabolism (*33*). Glu461 is essential for enzyme activity and the *lacZ* mutation introduced to generate the CC102 strain renders these cells *lac-* or unable to grow in lactose-containing media. A GC:AT transition mutation in the *lacZ* Glu461 codon is required for growth on lactose by CC102, thus the number of *lac*$^+$ revertant colonies reflects the GC:AT mutation rate. Inactivation of *nth* in CC102 increases the GC:AT mutation rate as shown in Figure 7.3. Note that the error bar for the CC102/*nth-* strain is large, a common phenomenon with the CC102 strain since it relys on the spontaneous oxidation of cytosine, a process that happens with somewhat low frequency inside the cell (*36*), to revert to *lac*$^+$. Inactivation of *rnfA* in CC102 does not increase the number of *lac*$^+$ revertants. Knockout of *rnfA* should

Figure 7.3. Genetic inactivation of *rnfA* in CC102. All revertants are reported as *lac*$^+$ colonies per mL (10^8 cells/mL). Inactivation of *nth* in CC102 leads to a large number of revertants, but *rnfA* inactivation suppresses the reversion rate to a level similar to the CC102 control.



inactivate the entire *rnf* operon, including *nth*. Thus, this result suggests that knockout of the *rnf* operon has a mutation suppression effect on the cell, despite the loss of the DNA repair protein EndoIII.

Discussion

It is interesting to consider the result reported here in the context of the only other proposed role for the *rnf* proteins in *E. coli*, as a reducing system for SoxR (*25*). Knockout of the *rnf* genes has been demonstrated to slow deactivation of *soxS* transcription (*25*), perhaps resulting in a more constituitive oxidative stress response state. Thus, the result observed here, that *rnf* inactivation suppresses the GC:AT mutation rate, might support the hypothesis that the *rnf* genes are involved in SoxR rereduction since a lower rate of oxidative DNA damage might be expected with a less efficient *soxS* deactivation state.

It is clear from these initial experiments that the relationship between the *rnf* genes and *nth* is complicated and will not be fully elucidated by the simple experiments performed here. Future work in this area will include experiments to determine if the mutation suppression observed with CC102 is specific to GC:AT mutations or if *rnf* inactivation reduces the general mutation rate. It must be verified, in a more rigorous manner, that inactivation of *rnfA* eliminates transcription of the downstream genes (including *nth*). Lastly, in-frame deletion of each of the *rnf* genes may reveal some of the specific roles of the individual genes.

It may also be interesting to examine the role of *yggX*, the gene immediately 5` to *mutY* in *E. coli*, in DNA repair. Reduced expression of *yggX* in *S. enterica* causes a 20–50-fold increase in the rate of GC:TA transversion mutations (the type of mutation prevented by MutY) (*18*). While YggX has been overexpressed and characterized as a

purified protein, it has not been determined if YggX binds to DNA or to MutY, or whether the iron bound by YggX is redox active.

SUMMARY

In *E. coli*, the genes encoding MutY and EndoIII, two [4Fe4S] DNA repair glycoslyases, are transcribed as operons. Each of these operons contains other putative iron-binding proteins that are currently uncharacterized in *E. coli*. Of particular interest are the *rnf* genes that precede the gene encoding EndoIII; several of the *rnf* proteins are predicted to contain multiple iron sulfur clusters and it is suggested that these proteins might play a role in reduction of SoxR, another DNA-binding [4Fe4S] cluster protein. To examine the relationship between the *rnf* proteins and EndoIII, we inactivated the entire *rnf* operon in an EndoIII activity reporter strain of *E. coli*. Interestingly, the *rnf* knockout strain displays a near wild-type level of mutations (as opposed to the elevated mutation level observed with an EndoIII genetic knockout). While this result would be consistent with the prediction that the *rnf* gene products are involved in deactivation of SoxR mediated oxidative stress protection, the relationship between the *rnf* genes and [4Fe4S] DNA repair enzymes is still not well understood and requires further examination.

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