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Oxidative Stress Induced HU Protein Expression in Deinococcus radiodurans

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Oxidative Stress Induced HU Protein Expression in *Deinococcus radiodurans*

by

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Introduction

Eukaryotic organisms possess chromosomal DNA that must be highly compacted, as it is thousands of times longer than the diameter of cells' nuclei (Pollard and Earnshaw, 2008). DNA is combined with structural proteins to form chromatin, which compacts the genome but also allows cellular machinery to access it during the processes of replication and transcription (Hayes and Wolffe, 1992; Pollard and Earnshaw, 2008). The first level of chromosomal DNA packaging is the nucleosome, composed of 146 base pairs of DNA coiled in a left-handed superhelix around an octamer of histone protein subunits (Pollard and Earnshaw, 2008; Ramakrishnan, 1994). Electron microscopy reveals that adjacent nucleosomes are connected by linker DNA and that the entire nucleosomal structure resembles beads on a string (Pollard and Earnshaw, 2008). This linker DNA binds a fifth histone protein H1, which is essential for folding the linear array of nucleosomes into a 30-nm chromatin fiber (Bharath *et al.*, 2002; Pollard and Earnshaw, 2008). Eukaryotic histone H1 possesses unique structural elements in its C-terminus (Bharath *et al.*, 2002). These lysine-rich repeats are responsible for the DNA condensation properties of this histone protein (Bharath *et al.*, 2002; Mukherjee *et al.*, 2008). Compaction and accessibility of the eukaryotic genome are essential for life; therefore, it was suggested that bacteria utilize a similar system (Reviewed in Saier, 2008).

Bacterial chromatin is localized within a region of the cell called the nucleoid. Electron microscopy has provided evidence for eukaryotic-type compaction of the bacterial genome into nucleosome-like structures (Reviewed in Saier, 2008). This organization of the genetic material is thought to result from the combined effects of DNA supercoiling, macromolecular crowding, polyamines, and abundant nucleoid-associated proteins, termed histone-like proteins (Drlica and Rouviere-Yaniv, 1987; Reviewed in Saier, 2008). Histone-like proteins are architectural proteins

that non-specifically bind DNA, forming bends, loops, or bridges that consequently condense chromatin (Reviewed in Saier, 2008). There are many different classes of histone-like proteins in prokaryotes, including Fis (factor for inversion stimulation), LRP (leucine-responsive regulatory protein), IHF (Integration Host Factor, a homolog of HU), H-NS (histone-like nucleoid structure protein), and HU (heat unstable protein) (Higgins, 2005; Reviewed in Saier, 2008). Research on the nucleoid of *Escherichia coli*, one of the best-characterized and most-frequently studied organisms, has revealed greater than ten nucleoid-associated proteins in substantial quantities, with HU as one of the most abundant (Reviewed in Saier, 2008). The expression level of HU suggests its vast importance in the compaction of the bacterial genome (Reviewed in Saier, 2008).

HU Proteins

HU is a small, basic protein that was first discovered in 1975 when it was isolated from *Escherichia coli* strain U93 (Higgins, 2005). Originally called factor U, the protein was later found to have similar amino acid content to eukaryotic histones, and thus, its name was changed to HU (histone-like protein from strain U93) (Higgins, 2005). HU has a highly conserved primary structure, and it can be found in virtually all eubacteria, some archaebacteria, bacteriophages, animal viruses, blue-green algae, and plant chloroplasts (Geiduschek *et al.*, 1990; Neilan *et al.*, 1993). Moreover, recent research suggests that histone-like proteins from the dinoflagellate *Crypthecodinium cohnii* are an evolutionary link between eukaryotic histone H1 and HU proteins (Wong, 2003).

HU proteins typically have a molecular weight of approximately ten kilo Daltons (kDa), range in size from ninety to ninety-nine amino acids, and exist as either homodimers or

heterodimers in solution (Chen *et al.*, 2004; Drlica and Rouviere-Yaniv, 1987; Oberto and Rouviere-Yaniv, 1996; Reviewed in Saier, 2008). While present in most bacteria as homodimers (Figure 1), HU in *E. coli* is a heterodimer of closely related alpha and beta subunits encoded by two genes, *hupA* and *hupB*, respectively (Higgins, 2005; Malik *et al.*, 1996). The dimeric structures are composed of intertwined α-helices with two β-ribbon arms (Reviewed in Saier, 2008). Proline residues found in the arms intercalate between base pairs in the DNA, forming two kinks and allowing HU to utilize its positively-charged surface to bind the minor groove in DNA (Reviewed in Saier, 2008). Research shows that the binding sites of HU homologs vary from nine to thirty-seven base pairs and that this binding induces strong bends in DNA and wraps it into nucleosome-like structures, conferring HU's contribution to DNA topology (Chen *et al.*, 2004; Malik *et al.*, 1996).

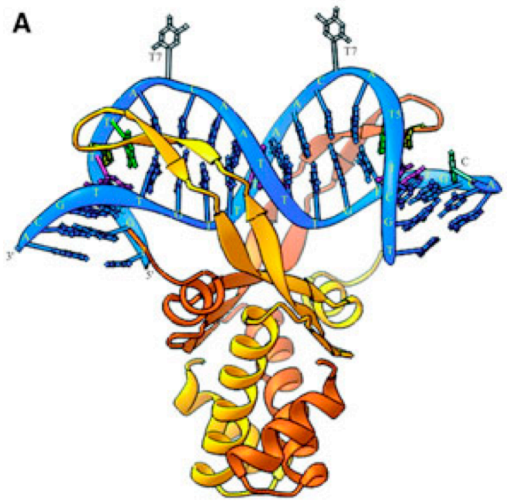

Figure 1. Ribbon diagram of the Anabaena HU-DNA complex. This HU protein exists as a homodimer (Swinger *et al.*, 2003).

E. coli HU possesses a diverse set of physiological roles, including the initiation of *oriC*-dependent DNA replication, Mu transposition, transcriptional regulation, shaping the bacterial nucleoid, and DNA recombination and double-strand break repair (Kamashev and Rouviere-Yaniv, 2000). Although HU exhibits little sequence specificity, it deforms the helical axis of DNA, and thus, it should bind preferentially to DNA that is pre-bent (Kahn and Crothers, 1992;

Reviewed in Saier, 2008). Studies reveal that HU has high affinity for structural distortions in DNA, including nicks, gaps, permanent bends, DNA repair intermediates, and three- and four-way junctions (Ghosh and Grove, 2004; Reviewed in Saier, 2008). *E. coli* HU has been shown to have one thousand-fold higher affinity for four-way junctions than for linear DNA (Bonnefoy *et al.*, 1994; Pontiggia *et al.*, 1993). The binding preferences of HU explain its presence at sites of DNA replication, general and site-specific recombination, and repair, as these processes imply the formation of intermediates that contain nicks and gaps (Castaing *et al.*, 1995). Furthermore, an implication of HU's high affinity for DNA breaks is the stabilization of damaged DNA and its protection from further degradation (Kamashev and Rouviere-Yaniv, 2000).

DNA Damage and Repair

Damaged DNA can result in cellular mutagenesis or even cytotoxicity (Reviewed in Hanawalt *et al.*, 1979). Living cells are constantly exposed to agents that have deleterious effects on their DNA (Reviewed in Hanawalt *et al.*, 1979). These agents can be either exogenous or endogenous (Reviewed in Hanawalt *et al.*, 1979). Ionizing radiation has been shown to induce cellular DNA damage through the action of hydroxyl radicals, whose mechanism is similar to that produced by damage associated with aerobic metabolism (Reviewed in Cox and Battista, 2005). Many bacteria utilize aerobic respiration to generate metabolic energy (Baatout *et al.*, 2006). A deviation from this four-electron reduction of oxygen (O_2) to water can result in the formation of reactive oxygen species (ROS) that can be toxic to cells (Baatout *et al.*, 2006; Imlay, 2003). When the accumulation of ROS (hydroxyl radicals, superoxide, or hydrogen peroxide) from aerobic metabolism exceeds the ability of cells to neutralize them, the cells become vulnerable to DNA damage, a condition termed oxidative stress (Reviewed in Ghosal *et al.*, 2005). Oxidative stress has been shown to induce a number of DNA modifications, including

base and sugar lesions, DNA-protein cross-links, base-free sites, and strand breaks (Dizdar, 2005). Hydroxyl radicals are the primary agents of oxidative stress that damage DNA (Imlay, 2003). Molecules of hydrogen peroxide interact with endogenous ferrous ions to create hydroxyl radicals that are responsible for the oxidation and carbonylation of intracellular proteins, altering their structure and function, as well as strand breaks in DNA (Reviewed in Hanawalt *et al.*, 1979; Imlay, 2003). Exposure to hydrogen peroxide for an interval as short as ten minutes can severely mutate or even kill bacteria (Imlay, 2003). Mutagenic and cytotoxic effects are also observed when cells are irradiated (Reviewed in Ghosal *et al.*, 2005). Research indicates that hydroxyl radicals from ionizing radiation can cause both single-strand and double-strand DNA breaks (Reviewed in Hanawalt *et al.*, 1979). It appears that the key to surviving DNA damage induced by oxidative stress is the ability of organisms to repair their DNA (Reviewed in Ghosal *et al.*, 2005). Some organisms have a greater capacity for DNA repair than others. *Deinococcus radiodurans* seems to have evolved a metabolic design that both suppresses the formation of ROS and that allows it to reconstitute its genome even in the face of hundreds or thousands of genomic double-strand breaks, prompting some to christen it "The Consummate Survivor" (Reviewed in Cox and Battista, 2005; Reviewed in Ghosal *et al.*, 2005).

D. radiodurans

In 1956, a unique bacterium was discovered in spoiled canned ground meat that had been irradiated with two hundred fifty times the dose used to kill *Escherichia coli*, approximately four thousand Gray (Gy) (Reviewed in Cox and Battista, 2005). The organism was given the name *Micrococcus radiodurans* because of its apparent structural similarities to other species within the genus *Micrococcus* (Reviewed in Cox and Battista, 2005). However, upon further investigation, researchers discovered that this novel bacterium represented a distinct phylum

(Reviewed in Cox and Battista, 2005). The new genus given to this organism and its relatives is derived from the Greek word "deinos," meaning strange or unusual, which is a fitting description for a species capable of surviving a level of genetic damage that distinguishes it from most other organisms on Earth (Reviewed in Cox and Battista, 2005).

Bacteria within the family *Deinococcaceae* are easily cultured, vegetative, and nonpathogenic, as well as being some of the most radiation-resistant organisms ever discovered (Battista *et al.*, 1999; Minton, 1994; Minton, 1996; Reviewed in Makarova *et al.*, 2001). Despite their ubiquitous distribution, only eleven species of deinobacteria have been described, with *Deinococcus radiodurans* strain R1 having been the first characterized and most widely studied (Anderson *et al.*, 1956). *Deinococcus radiodurans* is a large (1-2 μm), non-sporulating, mesophilic, aerobic, Gram-positive coccus that is capable of forming tetrads in liquid cultures (Minton, 1994; Moseley and Evans, 1983; Wang and Schellhorn, 1995). This red-pigmented soil bacterium is best-known for its resistance to numerous agents that induce damage to DNA, including ionizing radiation, prolonged desiccation, UV light, and hydrogen peroxide (Reviewed in Makarova *et al.*, 2001; Masters *et al.*, 1991). *D. radiodurans* has been shown not only to survive acute doses of radiation, but also to flourish in its presence without displaying any effects on its growth rate or its ability to perform transcription (Reviewed in Makarova *et al.*, 2001). The existence of an efficient DNA repair system in *D. radiodurans* has been widely documented, and this bacterium appears to have an exceptional capacity for genomic restitution (Battista, 1997). Researchers suggest that *D. radiodurans* can repair genetic damage on the order of one thousand to two thousand double-strand DNA breaks, whereas *E. coli* is only able to withstand ten to fifteen double-strand breaks (Battista, 1997). However, although hypotheses abound, there still is no definitive mechanism outlined for this reconstitution of damaged DNA. In general, *D.*

radiodurans appears to possess a DNA repair system similar to those in other bacteria (Reviewed in Makarova *et al.*, 2001). Functional homologs of the DNA repair genes in *D. radiodurans* have been identified in other prokaryotic species, suggesting that these genes alone do not confer the highly-resistant phenotype in *Deinococcus* (Liu *et al.*, 2003). At the same time, there are some unique features of the *D. radiodurans* repair system that may be linked with its proficiency at reconstituting damaged DNA (Reviewed in Makarova *et al.*, 2001). There have been some interesting and unusual features observed in the repertoire of DNA-associated proteins in *D. radiodurans* (Reviewed in Makarova *et al.*, 2001). *Deinococcus* encodes an ortholog of the chromosomal DNA-binding protein HU, which is believed to play a central role in DNA packaging and also as a cofactor in recombination (Reviewed in Makarova *et al.*, 2001). This protein possesses a unique N-terminal extension of lysine-rich repeats, which distinguishes it in comparison to other HU homologs and suggests its role in DNA recombination (Mukherjee *et al.*, 2008). Further, this sequence of lysine-rich repeats is similar to that found in the C-terminal domain of eukaryotic histone H1 (Figure 2) (Mukherjee *et al.*, 2008).

DrHU N-terminal extension:
`mtkkstkapa kkaapaakaa paakrgaaad sgk`

C-terminus, Eukaryotic Histone H1:
`ktpkkakkpa atrkssknpk kpktvkpkkv akspakakav kpkaakarvt kpktakpkka apkkk`

Figure 2. Lysine-rich repeats from DrHU N-terminus and Eukaryotic Histone H1 C-terminus.

As indicated above, HU exhibits a strong preference for binding breaks in DNA, which is a consequence of cellular irradiation (Reviewed in Hanawalt *et al.*, 1979; Reviewed in Saier, 2008). One suggested method of genomic restitution after ionizing radiation in *D. radiodurans* is

recombination, for which *D. radiodurans* HU (DrHU) is reported to be a cofactor (Reviewed in Makarova *et al.,* 2001; Shanado *et al.*, 1998). Moreover, hydrogen peroxide induces a spectrum of damage similar to that induced by ionizing radiation, suggesting an overlap in cells' resistance to the lethal effects of both agents (Storz and Hengge-Aronis, 2000). Presumably, the proteins necessary for ionizing radiation resistance in *D. radiodurans* will also facilitate the repair of hydrogen peroxide-induced DNA damage, including DrHU (Storz and Hengge-Aronis, 2000). Studies suggest that proteins such as DrHU are upregulated in response to oxidative stress, and cells are sensitized to DNA damage with inhibition of protein synthesis (Storz and Hengge-Aronis, 2000). Further, cells deficient in HU are shown to be extremely susceptible to radiation damage (Higgins, 2005). This study was designed to elucidate the upregulation of DrHU by quantifying the expression amount of this protein in response to oxidative stress.

Materials and Methods

Bacterial Strain and Protein Preparations

Deinococcus radiodurans strain R1 (stored at -80°C) was grown overnight in a 10-milliliter (mL) aliquot of TGY media from a 1-liter (L) solution (1000 mL double distilled water + 5 grams (g) tryptone + 3g yeast extract + 1g glucose). The cells were grown in a laboratory shaker at 30°C. Next, 25 mL of TGY from the same original 1-L solution was inoculated with 0.5 mL of the *D. radiodurans* cell culture. The cells were then grown at 30°C in the shaker until OD ~0.2. The sample was split equally into two test tubes, one serving as a control and the other as an experimental group. From an 8.8 M bottle of hydrogen peroxide (H_2O_2), H_2O_2 was added to the experimental tube so that its concentration was 5 mM. The test tubes were returned to the 30°C shaker and the cells grew for one hour. After the OD for each was recorded, each sample

was transferred to a centrifuge tube, and cell pellets were centrifuged for 10 minutes at 2200 rpm. The supernatant was discarded. Cell culture pellets can be stored at -20°C at this point, if needed.

SDS-PAGE and Western Blotting

For detection of DrHU, the cell culture pellets were each resuspended in 40 microliters (μL) protein sample dye (950 μL 10% sodium dodecyl sulfate [SDS] + 50 μL 2-mercaptoethanol) and boiled for 5 minutes at 90°C for cell lysis. Samples were electrophoresed with purified DrHU (5 μL purified DrHU + 15 μL sample dye; boiled at 90°C for 2 minutes) in a 1x Laemmli buffer on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane overnight at 60 V. The polyacrylamide gel was stained with Coomassie blue after the transfer to verify that the proteins transferred completely. The PVDF membrane was blocked with 5% nonfat dry milk dissolved in 1x tris-buffered saline (TBS) with 0.5% Tween 20 (TBST) at room temperature for one hour. The membrane was then incubated overnight at 4°C with 1:5000 dilution of mouse anti-histone H1 antibody followed by 3 10-minute washes with 1x TBST and incubation with 1:1000 dilution of goat anti-mouse immunoglobulin G (IgG) (H+L) horseradish peroxidase-conjugated secondary antibody at room temperature for one hour. The membrane was washed with 1x TBST for 10 minutes 3 times. The membrane was then incubated with a colorimetric substrate, Opti-4CN (BioRad), as per the manufacturer's protocol.

Method Variations

There were a total of nine protein gels (SDS-PAGE) and Western Blots performed in this study. The method for the initial trial is presented above. However, variations were made throughout the full term of this experiment in an attempt to secure better results. Elements of the

study that varied were the SDS-polyacrylamide resolving gel (12% or 17%), length of the Western Blot transfer (3.5 hours at 80 V or overnight at 60 V), dilution of the primary antibody (1:5000 or 1:500), and dilution of the secondary antibody (1:1000 or 1:500). Of the nine SDS-polyacrylamide running gels, the first four were 12%, and the last five were 17%. There were three overnight Western transfers and six 3.5-hour transfers. Only the first trial utilized a 1:5000 dilution of the primary antibody while the remaining membranes were incubated in a 1:500 dilution. Finally, the first two membranes were incubated with a 1:1000 dilution of the secondary antibody, whereas the other trials utilized 1:500.

Results and Discussion

The ultimate goal of this project was to assess the expression amount of DrHU in response to oxidative stress while the purified DrHU that was electrophoresed as well would serve as a control. The purpose of Western Blotting is two-fold. It allows an experimenter to determine the size of the protein of interest against a size marker or ladder in kDa, and it also gives information on protein expression. Ultimately, Western Blotting and incubation with the colorimetric substrate should have revealed protein bands on the PVDF membrane. Although DrHU has a molecular mass of ~14 kDa, the bands should have been at ~17 kDa because DrHU has an isoelectric point of 11, causing it to migrate more slowly (Ghosh and Grove, 2004). Additionally, the sample that had undergone oxidative stress might have exhibited a more pronounced band, indicating that the expression of DrHU was greater than in the control (i.e. in the sample that was not exposed to hydrogen peroxide). Concentrations of DrHU would have been calculated as a function of the integrated density of the bands using Alpha Imager 2200 (Alpha Innotech Corporation) (Mukherjee *et al.*, 2008). Unfortunately, no such results could be obtained for this experiment. The kDa ladder was visible on each PVDF membrane after

incubation with the colorimetric substrate. However, protein bands were never visible for either the control or for the samples, suggesting a problem in the recognition of DrHU by the primary antibody that was used. Antibody recognition is essential to obtain useful results.

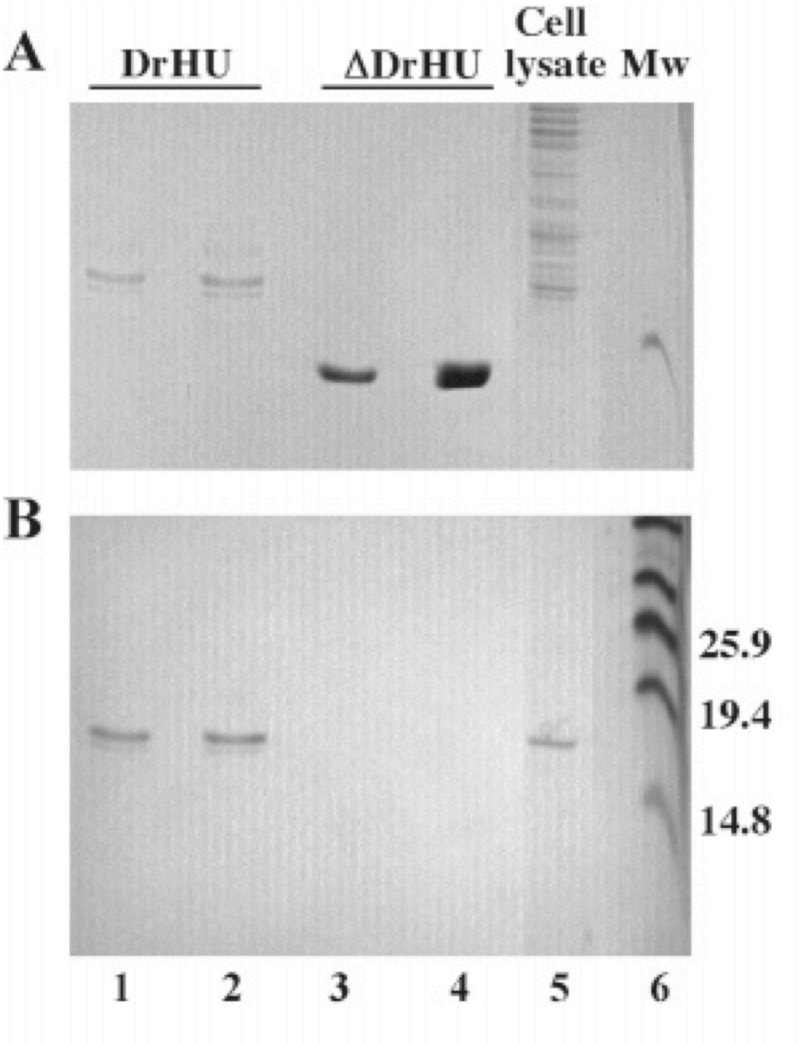

Figure 3. (A) Stained SDS-PAGE gel. Lanes 1 and 2 contain purified DrHU that has migrated to ~23 kDa. Lanes 3 and 4 contain DrHU from which the N-terminal lysine-rich repeats have been removed. The modified protein samples migrate further than purified DrHU. The *D. radiodurans* cell lysate in lane 5 exhibits a band at ~23 kDa that corresponds to DrHU. The molecular weight marker is in lane 6.

(B). Western Blot of the gel in (A), using an antibody against eukaryotic histone H1. The antibody recognizes DrHU in lanes 1 and 2 and in the cell lysate, but it does not recognize the modified DrHU in lanes 3 and 4 due to its lack of lysine-rich repeats (Ghosh and Grove, 2006).

In order to detect a protein transferred to a PVDF membrane through Western Blotting, a primary antibody is added at an appropriate dilution and is incubated with the membrane (Baldwin *et al.*, 2004). If this primary antibody is directed against the blotted protein, the antibody will bind to the protein. Detection of the primary antibody occurs upon incubation of the membrane with an anti-immunoglobin antibody, or secondary antibody, that has been coupled to a reporter group such as the enzyme horseradish peroxidase (HRP) (Baldwin *et al.*,

2004). The colorimetric substrate precipitates when it reacts with the reporter group, or conjugate. The result is a visible band at the location of the primary antibody bound to the protein of interest. Ultimately, the entire protocol relies on the recognition of the protein by the primary antibody.

The primary antibody used in this experiment is a mouse monoclonal to histone H1. It recognizes the unique structural motifs found in the C-terminus of human histone H1, namely a series of lysine-rich repeats. As previously indicated, HU shares structural similarities to histone H1 (Bharath *et al.*, 2002; Wong *et al.*, 2003). Specifically, the N-terminal extension of DrHU also contains repetitive sequences rich in lysine (Ghosh and Grove 2006). Other studies used a similar primary antibody with success, and the antibody was shown to exhibit cross reactivity between the lysine-rich repeats in histone H1 and those in DrHU (Figure 3) (Mukherjee *et al.*, 2008). However, that antibody no longer exists, and thus this experiment used one with similar specifications. Unfortunately, no cross reactivity was observed which affected the results of this study.

Although this experiment yielded unexpected results, useful information can be gleaned from this project and used in additional studies. The dilutions of the primary antibody used here were within the limits of the suggested protocol. However, perhaps a more highly concentrated dilution of the same antibody, such as 1:100, could produce better results. Conversely, different antibodies that recognize lysine-rich repeats of histone H1 could be administered in additional tests if available, and the outcomes could be compared to the results of this experiment. Perhaps this would offer a method of elimination to identify an antibody that actually recognizes DrHU. Additionally, studies instead could produce an integration host factor (IHF) for *D. radiodurans* and analyze its expression in response to oxidative stress. IHF is not found in *D. radiodurans*,

yet this nucleoid-associated protein exhibits greater than 45% homology with HU in other prokaryotes (Higgins, 2005). Ultimately, this project may offer valuable information to enhance the success of future experiments.

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