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FUNCTIONAL CHARACTERIZATION OF A NON-CYTOPLASMICALLY LOCALIZED DPS PROTEIN FROM DEINOCOCCUS RADIODURANS

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for degree of Master of Science in The Department of Biological Sciences

by
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B.S., Louisiana State University, 2004
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Abstract

The gram-positive eubacterium *Deinococcus radiodurans* is widely recognized for its extreme resistance to various DNA-damaging conditions, such as desiccation and ionizing and ultraviolet radiation. *D. radiodurans* is one of only a few bacteria which encode two Dps (DNA protection during starvation) proteins. Here I report the cloning and purification of a more soluble Dps2, Ntrunc. Also, the initial characterization of a truncated Dps2, which lacks the C-terminal metal binding loop, shows that this region is important for proper oligomerization. Furthermore, Dps2 expression was found to be moderately induced when stressed with exogenous H$_2$O$_2$, and expression levels were not altered by excess iron or iron scarcity. Dps-1 was shown to compact the *E. coli* nucleoid, similar to the *E. coli* Dps, while Dps-2 is incapable of nucleoid condensation. I also began the localization of Dps2 in *D. radiodurans*, as it is predicted to have a signal peptide. While Dps-1 may play a role in iron homeostasis and local nucleoid architecture, the function of Dps-2 may serve as a first line of defense by protecting against exogenous ROS. Together these proteins may in part contribute to the ability of *D. radiodurans* to withstand such extreme environments.
Introduction

Since its discovery in 1956 from a can of irradiated meat, *Deinococcus radiodurans* has quickly become the model organism for deciphering the cellular components that give rise to extreme radiation resistance (Anderson et al. 1956). Its ability to withstand large doses of radiation is thought to be related to desiccation resistance, as it is also isolated from arid environments (Mattimore and Battista 1996). *D. radiodurans* is a nonmotile, gram-positive bacterium containing several membrane components that share structural homology to gram-negative bacteria, including an outer membrane (Battista 1997). The genome of *D. radiodurans* was sequenced in 1999, however the sequencing did not provide any immediate clues to what mechanisms might be contributing to its radiation resistance (White et al. 1999). Another interesting characteristic of *D. radiodurans* and other radiation resistant bacteria is that they have an unusually high Mn/Fe ratio (Daly et al. 2004). Furthermore, the majority of the iron in *D. radiodurans* is located within the septal region of dividing cells, compared to other bacteria where iron is typically stored in the cytoplasm (Daly et al. 2007).

*D. radiodurans* is resistant to oxidative stress

*D. radiodurans* is able to tolerate greater than 5000 Gy of radiation without loosing viability, a level 500 fold higher than the lethal dose for humans (Battista 1997). When subjected to these levels of radiation, the genomes of all organisms become severely fragmented as a consequence of the more than 150 double strand breaks that are generated. For most organisms, this amount of damage is insurmountable and they quickly die, however *D. radiodurans* is able to reconstitute its genome completely within a span of several hours and continue normal growth (Cox and Battista 2005). Several
theories have been presented to explain *D. radiodurans*’ robustness against such damaging conditions. The most prominent of which speculates that while *Deinococcus* lacks novel repair enzymes, they have evolved to be more efficient in their repair functions (Makarova 2001), or that high intracellular Mn$^{2+}$ levels prevent protein oxidation, a primary target of irradiative stresses (Daly *et al.* 2007). Because of its impressive ability to tolerate such high levels of radiation and additional stresses, along with the ease with which it can be genetically manipulated, *D. radiodurans* has been proposed as a vehicle for bioremediation (Daly 2000).

A secondary effect of ionizing radiation is the widespread production of damaging reactive oxygen species (ROS), in part by the radiolysis of water (Helmut 1993). As *Deinococcaceae* have long been touted for their ability to endure extremely high doses of radiation, another impressive aspect of *Deinococcus*, its ability to withstand oxidative stress, has largely gone unstudied. In comparison to *E. coli*, *D. radiodurans* is greater than eight-fold more resistant to H$_2$O$_2$ stress. While ionizing radiation is a limited source of ROS under typical growth conditions, there are a variety of more common endogenous and exogenous sources of oxidative stress that makes coping with ROS a necessity for normal cellular function and survival. The major ROS confronting organisms are H$_2$O$_2$, O$_2^-$ and OH•, the latter being the most potent of the oxidative stresses. H$_2$O$_2$ and O$_2^-$ are produced in large numbers by metabolic and other enzymatic processes and primarily damage proteins, specifically those containing Fe-S clusters. In contrast, hydroxyl radicals are typically produced by the transition metal-dependent Fenton reaction:

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}•$$
In this reaction, ferrous iron is oxidized by H$_2$O$_2$, in the process producing a hydroxide ion and hydroxyl radical. The hydroxyl radicals can damage all of the major cellular components, including DNA, RNA, lipids and proteins, and are therefore not reactively limited compared to other ROS. Furthermore, with a half-life of 10$^{-9}$ seconds, OH$\cdot$ typically reacts with the nearest biomolecule, making the defense against existing OH$\cdot$ an exceptionally difficult task (Helmut 1993).

**Dps protects against oxidative stress**

Organisms employ a variety of enzymatic and non-enzymatic antioxidants to combat oxidative stress. The most common enzyme-based defenses include catalase and superoxide dismutase, which catalyze the breakdown of H$_2$O$_2$ and dismutation of O$_2^-$, respectively. These enzymes target existing ROS and while quite effective in this capacity, the best method of protecting the cell from oxidative stress is by preventing their initial formation, especially in the case of OH$\cdot$. In this regard, the Dps (DNA protection during starvation) protein fulfills a critical antioxidant role in bacteria. Dps is a prokaryotic ferritin ortholog that forms a dodecameric assembly from monomers with a conserved 4-helix bundle domain and variable extensions protruding from either terminus of the bundle. Extensions beyond this core domain have been shown to play a role in DNA binding and proper oligomerization (Bhattacharyya and Grove 2007). The protection from ROS afforded by Dps can be mediated in a bimodal protection scheme, consisting of the physical association of Dps with genomic DNA and/or the oxidation and sequestering of Fe$^{2+}$. The former process has been studied thoroughly in *E. coli*, where it has been shown that upregulation of Dps during stationary phase results in the severe compaction of the *E. coli* nucleoid (Wolf 1999). By condensing the genome, Dps
reduces the amount of DNA surface area exposed to damaging agents, as well as serving as a physical shield from the ROS on the non-compacted portions of the DNA.

While the ability to bind and compact genomic DNA is often a major component of Dps’ protective abilities, DNA binding is a function not found in all Dps family members, as exemplified by the Dps from Agrobacterium tumefaciens (Ceci et al. 2003). In contrast to the ability to bind DNA, ferroxidase activity is typically diagnostic for these proteins and is the sole mechanism by which Dps prevents ROS proliferation. Dps oxidizes Fe$^{2+}$, frequently using H$_2$O$_2$ as the reducing agent thereby producing non-reactive Fe$^{3+}$ that then mineralizes within the core of the dodecamer. In this way the ferroxidase center not only removes Fenton reactive Fe$^{2+}$ from the cell, but also scavenges H$_2$O$_2$, another potent ROS (Helmut 1993). It should be noted, however, that this scavenging activity pales in comparison to that of catalase, the most prominent H$_2$O$_2$ scavenger.

*D. radiodurans* Dps homologs

*D. radiodurans* encodes two Dps homologs, Dps1 and Dps2, which is relatively unique, as the vast majority of prokaryotes encode a single Dps. Notable exceptions to this convention include Bacillus anthracis and Mycobacterium smegmatis (Papinutto et al. 2002 and Gupta et al. 2003). Of the two Dps homologs, Dps1 has been the most thoroughly studied and it has been found to have ferroxidase activity and to bind the DNA by interacting with the major groove. DNA binding is mediated by the N-terminal extension, which contains a novel metal binding site that is also important for oligomerization. However, Dps1 does not protect DNA against OH•, which is believed to be due to an iron-exit channel in Dps1 that allows Fe$^{2+}$ to leak out of the dodecamer.
(Bhattacharyya and Grove 2007). Dps2 has not been studied as extensively, but the crystal structure for Dps2 has been solved (Figure 1 B, Cuypers et al. 2007). The structure revealed the expected four-helix bundle fold, but also that Dps2 has a unique C-terminal metal-bound loop. The aim of this project is to understand the function of the C-terminal extension of Dps2, as well as begin to uncover possible \textit{in vivo} roles for this unique Dps family member within \textit{D. radiodurans}. 
Materials and Methods

Plasmids, PCR and Primers

Ntrunc was created by performing a whole plasmid PCR, from T7/NT-TOPO vector already harboring the Dps2 gene, with primers that do not encompass the first 30 amino acid residues, forward 5’-AATGGCGTGGCCGCTCCACCAACGT-3’ and reverse primer 5’-AGCAGCCGGATCAAGCTTCAATTG-3’. The C-terminally truncated Dps2 was PCR amplified from D. radiodurans genomic DNA using forward primer 5’-CACCCAGTGCCGGCAAT-3’ and reverse primer 5’-GGGGTCTCGGTCTACGTGTTCTG-3’ and cloned into the Champion pET100 TOPO vector (Invitrogen). The Dps1 and Dps1met constructs were previously cloned (Bhattacharyya and Grove 2007).

The Dps2 promoter was PCR amplified from D. radiodurans genomic DNA using forward primer 5’-CTTCTTCCAGAGATCTCACCGTAGG-3’ and reverse primer 5’-ACAGAATGACAGATCTTTTCTCCTTATC-3’ and then digested with BglII. The PCR product was cloned into pRADZ1, a pRAD vector already containing the lacZ gene. The promoter and signal peptide of Dps2 was cloned into the pRAD1 shuttle vector using the forward primer 5’-ACAGAATGACAGATCTTTTCTCCTTATC -3’ and reverse primer 5’-ACGCCAAGCTCGCGAGGCC-3’ after digestion with AgeI and BamHI (pRAD-psp). pGFP (Clontech) and pd1EGFP-N1 (Clontech) were digested with BamHI and EcoRI and were each individually subcloned into pRAD-psp. The FLAG small epitope tag was introduced into the Dps2 reading frame using forward primer 5’-CTTCAAGCTCGACGACTACAAAGGACGACGACGACAAGAATGCGTGCCGTCCA CCAACGTCA-3’ and reverse primer 5’-ACGCGACGTCAGCAGCGCCCGCGGACT-3’ to
amplify the Dps2 gene with a FLAG tag from genomic DNA. The sequence integrity of all constructs were determined by sequencing.

**Overexpression and Purification**

pTOPO-dps2 was transformed into *E.coli* Rosetta2 and the culture grown in 1 liter of Luria-Bertani (LB) broth at 30°C containing 50 µg/ml ampicillin to an optical density of 0.2 and then induced with 1 mM isopropyl-β,D-thiogalactopyranoside (IPTG). Induction was followed by a 3 hour incubation. Cells were pelleted by centrifugation at 5000 rpm for 15 min. Cells were resuspended and incubated for 1 hour in 20 ml Lysis buffer (50 mM Tris-HCl (pH 8.0), 0.25 M NaCl, 5 mM Na2EDTA, 5% (v/v) glycerol, 5 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) and were subsequently sonicated with 15 second intervals for five minutes. Cellular debris was pelleted by centrifugation at 7000 rpm for 20 min. The supernatant for only Ntrunc was then incubated in a 70°C water bath for 30 minutes and suspension was once again pelleted by centrifugation at 7000 rpm for 30 min. Supernatants for Ntrunc and Cless were dialyzed overnight against HA buffer (50 mM Tris-HCl (pH 7.6), 50 mM KCl, 5% glycerol, 1 mM Na2EDTA, 0.2 mM PMSF, 3.5 mM β-mercaptoethanol) and centrifuged at 5000 rpm for 15 min. The supernatants were separately applied to a DEAE cellulose column equilibrated with HA buffer. Column flow-through for Ntrunc was concentrated and buffer was equilibrated to 150 mM KCl. The sample was then applied to Sephadex size exclusion column and fractions containing Ntrunc were pooled. The DEAE washes for the Cless samples were run on a CM column and the wash was concentrated and buffer was equilibrated to 150 mM KCl. Sample was then applied to Sephadex size exclusion column and fractions containing CLess were pooled. Purity was
confirmed by SDS-polyacrylamide gels stained with Coomassie brilliant blue stain. Purified protein was concentrated using a Centriprep Ultracel YM-10 centrifugal filter. Concentrations were established by comparison with 1, 0.5, 0.25, 0.125 µg/µl BSA on SDS-polyacrylamide gels stained with Coomassie brilliant blue.

**β-galactosidase Assay**

Exponentially growing *D. radiodurans* was transformed with pRADZ-dps using the previously described protocol (Earl et al. 2002). Transformants were grown until the cells reached a stage of exponential growth, and were then treated with either 7.5 mM H₂O₂, 100 µM Fe(NH₄)₂(SO₄)₂ or 1 mM 2,2'-dipyridyl where indicated. Samples were taken from cultures following a 45-minute incubation period for the β-galactosidase Assay. Briefly, 500 µL culture and 500 µl Z-buffer (100 mM Phosphate buffer, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol, 1.5% (w/v) SDS and 3% (w/v) Toluene) were mixed with 1.5 mg/ml ortho-Nitrophenyl-β-galactoside (ONPG) and then incubated in a 30°C shaker until yellow color developed. Reactions were terminated with 340 mM Na₂CO₃. Absorbance values were measured at 420 and 550 nm and Miller unit activity was then calculated. The same protocol was used for stationary phase cells, except 250 µl of culture was added and Z-buffer volume was adjusted accordingly.

**In vivo Nucleoid Condensation**

Separate stocks of *E. coli* Rosetta2 were transformed with TOPO-dps2, pET5a-dps1, and a Champion pET100/D-TOPO vector containing the Dps-met gene (14,17). Transformed cultures were grown in 10 ml of LB broth containing 50 µg/ml ampicillin at room temperature until cultures reached an O.D. of 0.2. All cultures were induced with 1 mM IPTG and incubated for one hour. Two µl of each cell culture were incubated with 2
µl of 50 mg/ml 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes. Cells were viewed using a Leica DM IRE2 under 100X NA 1.4 objective. Nucleoids were visualized using a Leica A4 filter cube.

**In vivo localization**

*D. radiodurans* cells were transformed, using above method, with either pRAD-dpsGFP or pRAD-dpsEGFP and 2 µl of each cell culture were incubated with 2 µl of 50 mg/ml DAPI for 5 minutes. Cells were viewed using a Leica DM IRE2 under 100X NA 1.4 objective.
Results

Sequence Alignment

The alignment of Dps2 with other Dps proteins reveals a large degree of homology within this family of proteins as well as some interesting factors, separating Dps2 from the common mold of most Dps proteins (Figure 1A). Firstly, the ferroxidase center composed of His70, His82, Asp97 and Glu101 (numbering referenced from Dps2 sequence) is conserved in Dps2, in line with other Dps homologs, illustrating the evolutionary importance of the ferroxidase center. Furthermore, Trp71, the sixth most conserved amino acid in Dps is present in the Dps2 sequence. This amino acid has recently been shown to be instrumental in preventing the release of radical byproducts of the ferroxidase center by capturing free electrons (Bellapadrona et al. 2010). Consistent with the sequence analysis, I have shown that Dps2 is able to oxidize and sequester iron (unpublished data). The alignment also demonstrates the level of conservation within the four-helix bundle, in contrast to the divergence of the extensions beyond this central domain. Although *D. radiodurans* Dps1 and Dps2 share a fair degree of homology, phylogenetic analysis shows that Dps2 is more closely related to other Dps proteins including both *M. smegmatis* homologs and the *E. coli* Dps. It is also interesting to note that Dps2 has both N- and C-terminal extensions, similar to that seen in Dps1 from *M. smegmatis*. In the latter protein, these extensions have been shown to play a role in oligomerization and DNA binding (Roy et al.). While there is little homology between the extensions of these proteins it is possible that the *D. radiodurans* extensions are serving a similar role in Dps2. Also, the sequence reveals that Dps2 has a signal peptide
**Figure 1.** A) Sequence alignment of Dps1 (D.rad_Dps1) and Dps2 (D.rad_Dps2) from *D. radiodurans*, and other Dps proteins from *H. pylori* (H.pyl_HNAP), *L. innocua* (L.inn_Dps), *M. smegmatis* Dps1(M.smeg_Dps1) and Dps2 (M.smeg_Dps2), *E. coli* (E.coli_Dps) and *A. tumefaciens* (A.tum_Dps). The amino acids that encompass the four-helix bundle are indicated above. The ligands for the ferroxidase centre are specified by (*) within the helices. Black boxes represent completely conserved amino acid and grey homology. The signal peptide cleavage site is indicated by an arrow. B) Dps2 dodecamer with each monomer represented by individual colors. C) Enlarged view of the iron exit pore formed by the C-terminal extension of three monomers. C-terminal extensions of each monomer are colored, starting with proline (side chain shown). Coordinated iron is represented by the red sphere.
Figure 1

A. D. rad-Dps2
M. smeg-Dps1
E. coli-Dps
A. tum-Dps
D. rad-Dps1
H. pyl-HNAP
M. smeg-Dps2
L. inn-Dps

B. D. rad-Dps2
M. smeg-Dps1
E. coli-Dps
A. tum-Dps
D. rad-Dps1
H. pyl-HNAP
M. smeg-Dps2
L. inn-Dps

C. 12
for the protein to be exported. This is the first time to our knowledge that a Dps protein has ever been found to have a signal peptide.

**Dps2 Purification**

The full length Dps2 has previously been purified and several *in vitro* experiments have been preformed, however, soluble Dps2 is difficult to attain. Therefore an attempt to clone a more soluble Dps2 was undertaken. The Dps2 crystal structure was solved using a truncated protein, lacking the first thirty amino acids, as this was found to be more stable than the full length Dps2 (Cuypers *et al.* 2007). To this end, a truncated Dps2 (Ntrunc) was subcloned and Ntrunc was overexpressed and purified by DEAE chromatography (Figure 2A and 2B). Depending on salt conditions, Dps1 and other Dps’s can exist as a dimer or dodecamer, with varying abundances. Although the crystal structure has been solved as a dodecamer, I wanted to see if it exists in a mixture of oligomeric states. However, running Ntrunc on the size exclusion column reveals that the vast majority of Ntrunc elutes with a predicted size of 240 kDa, indicating that the equilibrium is significantly in favor of the dodecamer.

**Role of C-Terminus**

In addition to the nominal comparisons between Dps2 and *M. smegmatis* Dps1 N- and C-terminal extensions, the crystal structure of Dps2 reveals that three adjacent monomers interact via their C-termini to form the iron exit channels of the dodecamer (Figure 1C). The C-terminals contain a novel metal binding site that is believed to be important in iron exit and entry. They also mediate these stabilizing contacts, and also interact with the linker regions between helix A and B and helix BC and C of the four-
helix bundle from the adjacent monomer. In light of the structural evidence suggesting a functional and architectural role for the C-terminus, a Dps2 C-terminal truncation mutant (CLess) was created that also lacks the first thirty amino acids, making the protein more

**Figure 2**

A.  
B.  

**Figure 2.** Overexpression and purification of Ntrunc. A) Overexpression of Ntrunc in Rosetta2 cells. B) Purified Ntrunc with standard marker

soluble. The truncated protein was overexpressed and purified by DEAE and CM chromatography (Figure 3A and 3B). To determine if there was any change in the oligomeric state of the mutant compared to the wild type, purified CLess was run on a size exclusion column. CLess eluted in the elution volume corresponding to 18 kDa, roughly the size of the monomer (Figure 4). This is unexpected as most Dps proteins
exist as either a dimer or a dodecamer, depending on salt concentration, and the C-terminus does not make contact with any portion of the dimer interface. So truncations in this region of the protein would not be predicted to disrupt contacts between monomers.

**Figure 3**

**Figure 3.** Overexpression and purification of CLess. A) Overexpression of Cless in Rosetta2 cells. B) Purified Ntrunc with standard marker

**Dps2 Regulation**

To begin to understand the role of Dps2 in *Deinococcus*, it is important to determine what conditions regulate its expression. In other organisms, Dps has been shown to be regulated by growth phase, H$_2$O$_2$ and Fe levels (Yu *et al*.). Therefore, I cloned the Dps2 promoter into a shuttle vector harboring the *LacZ* gene (pRADZ-dps)
and set out to determine if Dps2 is regulated under similar conditions. Exponentially growing *D. radiodurans*, harboring pRADZ-dps, was subjected to H$_2$O$_2$, excess iron, or iron deficiency, and beta-galactosidase activity was subsequently measured. Under both

**Figure 4.** Oligomeric state determination of Ntrunc and CLess. Graph of standard curve from size exclusion markers. Red and Green arrows indicates elution volumes for Ntrunc and CLess, respectively.

excess Fe and conditions where Fe is scarce, the amount of activity was not statistically significant compared to the typical exponential growth. However, when exponentially growing cells were treated with H$_2$O$_2$ the Dps2 promoter activity increased by roughly 42%, indicating that Dps2 may be functioning as a first line of defense from exogenous oxidative stresses. The most pronounced change in expression levels were seen in stationary phase cells. There was a 2.5 fold decrease in overall promoter activity in stationary phase cells compared to exponentially growing cultures. The decrease in β-galactosidase activity seen during stationary phase is most likely due to differences in the
plasmid copy number. *D. radiodurans* is multigenomic and during exponential phase it has between 8 and 10 copies of its genome, however during stationary phase, there are typically 4 genome copies (Meima and Lidstrom 2000, Harsojo et al. 1981). The decrease in expression levels roughly mirrors the decrease in the number of genome copies. These findings are in direct contrast to the expression pattern of the *E. coli* Dps, where there is a thirty fold increase in expression levels associated with the transition to stationary phase (Ali Azam et al. 1999).

**Figure 5**

![Bar graph showing activity of Dps2 promoter in response to multiple stresses](image)

**Figure 5.** Activity of Dps2 promoter in response to multiple stresses. Activity of promoter was calculated in Miller units. Exponentially growing cells were treated with either 7.5 mM H$_2$O$_2$, 100µM Fe or 1mM 2’2’-dipyridyl when indicated and β-galactosidase activity measured after 45 minutes.

**Nucleoid Compaction**

As mentioned earlier, during stationary phase in *E. coli*, Dps becomes the most prominent nucleoid associated protein in the cell and this drastic increase in Dps levels
results in the severe compaction of the *E. coli* genome (Ali Azam et al. 1999). These finding laid the framework for speculating that one of the roles of Dps is to compact the bacterial nucleoid tightly to protect it from various stresses. However, in *D. radiodurans* it has recently been shown that knocking out both Dps1 and Dps2 has no observable effect on nucleoid morphology. This group also demonstrated that the essential HU protein played a major role in maintaining a compact genome (Nguyen et al. 2009). Our lab has found the HU promoter to be much more active then either of the Dps promoters (unpublished data). This led us to speculate that perhaps the lack of observable alteration in genomic morphology is due to the overwhelming quantity of HU with respect to Dps1 and Dps2 and that the Dps homologs may be affecting nucleoid morphology in a more subtle manner. To test whether Dps1 and Dps2 are acting in this capacity, both homologs along with Dps1met, a mutant Dps1 that is unable to bind DNA, were individually expressed in *E. coli* and the nucleoids were visualized by fluorescence microscopy. In the control cells expressing Dps1met, 30% of cells showed some level of nucleoid condensation. These levels of compaction were very similar to those seen in cells expressing Dps2, roughly 34%, which is interesting because Dps2 is able to bind DNA *in vitro*. When Dps1 was expressed in *E. coli* almost 90% of the cells had a condensed nucleoid, similar to the compacted nucleoid phenotype seen in stationary phase *E. coli* (Figure 6). These findings indicate that Dps1 may in fact be contributing to local nucleoid architecture.
Dps2 Localization

The presence of a signal peptide in the Dps2 sequence predicts a non-cytoplasmic localization, which would explain why Dps2 is unable to condense the genome (Figure 7A). The signal peptide would theoretically lead to Dps2 being exported to the

**Figure 6**

**Figure 6.** *In vivo* nucleoid condensation. *E. coli* Rosetta2 expressing Dps-met (A), Dps-1 (B), and Dps-2 (C) were overexpressed. Nucleoid condensation is visualized by DAPI staining. Overlay images are a combination of both DIC and DAPI images.
Figure 7. Signal sequence prediction and EGFP codon analysis. A) Signal sequence prediction using Dps2 sequence using SignalP 3.0 Server. Probable cleavage sites are indicated with red lines. B) First fifty-seven EGFP codons with the problematic valine codon indicated in red. C) Codon usage table for valine (from Liu, Q. 2006)
*Deinococcus* periplasm. In order to determine the location of Dps2, a fusion construct was generated with the promoter and signal peptide of Dps2 fused to Green Fluorescent Protein (GFP), such that GFP transcription and cellular location should mirror that of the native Dps2. Attempts to localize the fusion construct however were unsuccessful as no discernable fluorescence was detected from the cells harboring the fusion construct.

Lack of expression was not due to deficiencies in promoter activity as β-galactosidase assays demonstrated the Dps2 promoter to be active (as discussed above). In *Streptomyces coelicolor*, a bacterium with a GC-rich genome similar to *Deinococcus*, GFP reporter constructs failed to give detectable signal as well. An enhanced GFP (EGFP) variant, containing over 190 silent mutations for optimal use in mammalian systems, was shown to have strong fluorescence in *Streptomyces* (Sun, J. *et al.* 1999). On this line of reasoning an EGFP Dps2 promoter/signal peptide construct was made and localization was once again attempted. Although EGFP has been engineered to be better suited in mammalian systems and by extension contains modified codons that are optimal for GC-rich organisms, it failed to fluoresce in *Deinococcus*. Analysis of EGFP codon map shows that in addition to containing a large number of methionines, it contains a valine whose codon, GUA, is rarely used in *Deinococcus* (Figure 7B). Since GFP fluorescence is evidently not an easily viable option for *in vivo* localization in *Deinococcus radiodurans*, the use of the small epitope FLAG tag was implemented. The FLAG tag was cloned into the Dps2 gene such that it is in-frame between the proposed signal peptide cleavage site and the starting amino acid in the Ntrunc protein. In this way processing of the signal peptide during export would yield an exposed FLAG tag on the N-terminus. At present, detection of the fusion is being attempted in *D. radiodurans*. 
Discussion

Importance of C-terminus in oligomerization

The metal-coordinating N-terminus of Dps1 is needed for assembly into dodecamer and mutants that disrupt this site results in the formation of dimers. As the C-terminus of Dps2 has a metal-coordination loop, the expectation was to see dimers if indeed it has a role in dodecamer formation. Removal of the C-terminus resulted in a shift from dodecamers to monomers, as seen by size exclusion chromatography, which was an unexpected observation since the dimer interface is isolated from interactions with the metal bound C-terminus. It is important to note that CLess was cloned into a plasmid encoding a His-tag at the amino terminal end of the protein. There is precedent for analyzing this data with a skeptic eye, since a Dps protein from *Mycobacterium smegmatis* was purified with a His-tag as well and it caused the formation of Dps trimers, which was later shown to be an artifact of the His-tag (Gupta and Chatterji 2003 and Ceci et al. 2005).

Dps2 Cellular Localization

The discovery that the iron in *D. radiodurans* is not located in the cytoplasm, but rather in the region between dividing cells brings about some interesting questions about iron homeostasis within *Deinococcus*. Firstly, it is important to note that in typical bacteria, iron is spread out in a relatively diffuse manner throughout the cytoplasm, whereas the iron in *D. radiodurans* is more concentrated, giving it the potential to cause a great deal of local oxidative damage. So, while it is undoubtedly beneficial to have Fenton reactive Fe$^{2+}$ away from DNA and critical cytoplasmic proteins, the Fe$^{2+}$ must be sequestered in some capacity, so as to prevent damage to membrane proteins and other
periplasmic components. *D. radiodurans* does not encode genes corresponding to the two major iron storage proteins in bacteria, ferritin or bacterioferritin. However, it does encode the two Dps proteins. Furthermore, the presence of the signal peptide on Dps2 makes it the prime candidate for serving this iron-sequestering role in the cell. Locating Dps2 will be important to begin to piece together the puzzle of understanding its role in the cell. If its predicted location holds true, it will also be interesting to see if Dps2 is exclusively co-localized with the iron in the septum or if it will be dispersed in a more punctate fashion around the perimeter of the cell.

**Prospects of EGFP fluorescence in Deinococcus**

The application of the commonly used GFP and EGFP reporter gene was unsuccessful in our present efforts to localize Dps2, which was most likely due to the codon preferences of *D. radiodurans*. It could prove to be very beneficial having an EGFP reporter system that worked in *Deinococcus*, because unlike small epitope tags which require the cells to be fixed and then incubated with antibodies, the use of other reporter systems do not lead some of the structural rearrangements that can be caused by fixing the cells. Furthermore, they can be used for time course experiments that can be extremely informative when observing a dynamic process. Since Val23 seems to be the most problematic residue in the sequence, replacing the rare codon with the optimal *Deinococcus* codon, GUG might help solve the problem of detection.

**Possible functions of Dps homologs**

Comparisons of both Dps homologs reveal a large degree of functional differences between the two, arguing for each homolog to play a distinct role in
*Deinococcus.* The ability of Dps1 to compact the *E. coli* genome along with its inability to protect DNA from ROS, suggests that it may be influencing local genome structure and possibly be involved in iron homeostasis. Conversely, Dps2 being localized in the periplasm could serve as a first line of defense against exogenous ROS and it could also sequester the non-cytoplasmic iron. The upregulation of Dps2 by H$_2$O$_2$ is consistent with this hypothesis. Together these two proteins may afford *D. radiodurans* a multilayered protection framework that in sum contributes to its ability to withstand such high levels of oxidative stress.

With the increase in toxic waste sites in the United States, cost effective methods for removing contaminated soil and standing water samples are being sought out. *D. radiodurans* has come to the forefront of the argument for bioremediation vehicles. By beginning to understand the proteins and pathways that give rise to *Deinococcus*’s extreme resistance to the plethora of damaging agents it encounters in these environments, I can perhaps modulate these existing factors, making *Deinococcus* even better suited to survive in such extreme environments.
References


Vita

Brian Reon is a native of Lake Charles, Louisiana, born in 1985 to Alus and Catherine Reon. He graduated from Alfred M. Barbe High School on May 2004 and enrolled in Louisiana State University in August of 2004. Brian is a graduate of Louisiana State University where he studied biological chemistry. Following the completion of the master’s degree, he will enroll at the University of Virginia Medical School in the fall of 2010.