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Cloning and characterization of nickel uptake regulator NUR mutants from *Streptomyces coelicolor*

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ABSTRACT

Sufficient concentrations of metal within a cell are required for proper cellular function; however, metals become toxic at very high concentrations. Therefore, it is important for an organism to have a mechanism for maintaining metal homeostasis within its cells. *Streptomyces coelicolor*, a soil-dwelling bacterium important in the production of antibiotics, utilizes the nickel uptake regulator (NUR) to maintain nickel homeostasis and oxidative response. NUR functions as a transcriptional repressor that responds to changing cytosolic concentrations of Ni^{2+}. Previous research describes two key metal-binding sites per NUR monomer. Our research seeks to analyze how changes to these binding sites affect the ability of NUR to coordinate metals and bind to DNA. NUR mutants containing single or multiple amino acid substitutions at each binding site were cloned. Biophysical characterization of these mutants and the wild-type protein will aid in understanding the role each metal site plays in the function of NUR.

INTRODUCTION

Biological Significance of Metals

Various metal ions are essential nutrients to cells, such as Fe, Zn, Mn, Cu, Co, Mo, and Ni, while other metals are toxic to a cell, such as Cd, Hg, Ag, Pb, Sn, and Cr. All metals are necessary for the cell, but become toxic at too high of a concentration, importantly Zn, Cu, and Fe. Metal toxicity often results from toxic metals displacing nutrient metals from their metabolic site as the concentration of toxic metals grows.[1] Many metals aid in the catalysis of various biological reactions necessary for cellular function, but the concentration of these metals must be tightly regulated to maintain homeostasis.[2] High concentrations of nickel, for example, become carcinogenic as a result of the formation of superoxide species and may damage DNA or oxidize lipids.[3] As a result of the dual nature of metals as both essential and toxic, it is highly important to regulate the levels of nickel, as well as other metals, within the cell.

Transcriptional Regulators

Regulation of metal concentrations within the cell begins at the level of transcription. Transcription factors are molecules, typically proteins, that control whether or not transcription occurs. These regulators often consist of a site that binds to DNA and a site that binds to a cofactor. The binding of the cofactor is related to the levels of stimuli and affects the ability of the protein to bind to DNA, thus regulating the response to these stimuli.[4] For example, as the concentration of zinc becomes too great, zinc ions will bind to a transcription factor that blocks the transcription of zinc-uptake proteins.[5] The portion of the DNA that the transcription factor binds to is called the promoter. The promoter contains a specific base pair sequence recognized by the transcription factor and the transcription initiation site that indicates where transcription of the DNA should begin.[6]

Transcription factors can either be activators or repressors. Activators promote transcription, while repressors prevent transcription. RNA polymerase is the enzyme that synthesizes mRNA from DNA to later direct the construction of proteins.[4] An activator directs RNA polymerase to begin transcription of a specific gene or quickens the rate at which RNA polymerase functions. A repressor prevents transcription, binding to activators to negate their effect or competing with activators for target DNA.[6]

Metalloregulators are a critical type of transcription factor in regulating intracellular levels of metals. Metalloregulators serve an important role in bacteria, as bacteria may experience drastic changes

in the levels of extracellular metals.[2] These proteins are necessary to direct transcription accordingly. FUR is a metalloregulator first found in *Escherichia coli* responsible for iron homeostasis and oxidative response. MntR, found in *Bacillus subtilis*, regulates the levels of manganese in the cell. CueR, also found in *B. subtilis*, maintains appropriate levels of copper. Each of these metalloregulators function at the transcriptional level to repress the production of other proteins that transport metal into the cell when there is already a sufficient concentration of metal.[5]

FUR *Family*

A family of proteins is a group of evolutionarily related proteins that share common characteristics or common functions. Proteins within a family are phylogenetically related, confirmed by sequencing. The rate of discovery of new families of proteins is not slowing, as new proteins are discovered frequently.[7] Variations within a family result from the evolution of proteins in order for an organism to best survive in its environment.[2] For example, some regulatory proteins have evolved to sense toxic heavy metals that benefit an organism living in a polluted environment.[8] There are currently seven known families of metalloregulators: ArsS, MerR, CsoR, CopY, FUR, DtxR, and NikR.[2]

The FUR family, a family of metalloregulator proteins, is named after FUR, the ferric uptake regulator, originally studied in *Escherichia coli*. When each monomer on the dimeric FUR protein contains one iron atom, FUR is able to bind to DNA and repress further iron intake. FUR stops RNA polymerase from reaching DNA downstream that codes for iron-uptake enzymes. The affinity of FUR for iron is tuned to the needs of the cell; it is low enough for a small concentration of iron to accumulate but high enough to prevent too much iron buildup.[6]

Other FUR family members include ZUR, the zinc uptake regulator first found in *Bacillus subtilis* and *E. coli*; MUR, the manganese uptake regulator found in *Rhizobium leguminosarum*; and NUR, the nickel uptake regulator found in *Streptomyces coelicolor*.[6] These FUR homologs have each evolved to sense a specific metal, further enabling their respective organism to thrive in its environment.

Nickel Uptake Regulator NUR

NUR, found in *S. coelicolor*, is important in regulating nickel homeostasis and superoxide response. NUR is dependent upon nickel to be able to bind to DNA and is the only member of the FUR family that senses nickel.[9] NUR represses *nikABCDE*[10] and *nikMNOQ*[11], which are responsible for nickel uptake into the cell. NUR also directly represses *sodF1* and *sodF2*, and indirectly activates *sodN*. *SodF1* and *sodF2* code for FeSOD, an iron-dependent superoxide dismutase, while *sodN* encodes NiSOD, a nickel-dependent SOD.[10] It has been suggested that *S. coelicolor* has developed a regulatory system for nickel homeostasis that uses nickel while limiting the usage of iron in the presence of a sufficient nickel concentration. [11]

NUR is a homodimer, with each monomer containing two metal binding sites. One binding site, the Ni-site, is highly nickel specific.[11,12] The second binding site, the M-site, can bind either nickel or zinc. Mutational studies have shown that the specific amino acids involved in coordinating metal at the Ni-site are His70, His72, and His126, along with three water molecules *in vivo*, in an octahedral geometry; and that the key amino acids at the M-site are His33, His86, His88, and His90 in a square-planar geometry, along with Glu101.[11] An octahedral geometry is the favored coordination geometry of nickel, reinforcing the specificity for nickel at the Ni-site. Nickel and zinc can both achieve a square planar geometry, supporting that both metals can bind at the M-site.[13] The binding of each of these metals is highly important to the regulation of metal homeostasis within *S. coelicolor*.

This research seeks to understand the role of each M-site and Ni-site residue in metal binding and the importance of each binding site in regulating nickel homeostasis and superoxide response in *S. coelicolor*. A library of relevant single-amino acid NUR mutants and multiple-amino acid NUR mutants has been created to examine the influence of eliminating the metal-binding potential of specific residues and of entire binding sites on the functionality of NUR. Characterization of these mutants and wild type NUR as well, aids in understanding the changes in the behavior of NUR caused by the mutations.

MATERIALS & METHODS
Cloning of NUR mutants

Site-directed substitutions of NUR residues were created by a two-step PCR strategy. In the first PCR, the mutation was introduced, and in the second step, the full nur gene was obtained. Fifty microliter reactions were assembled using 1 µL template DNA, 1 µL of each primer 1 and primer 2, 10 µL Taq 5x master mix, and 37 µL of autoclaved water. Each primer used is listed in Table 1. The temperature and duration of each PCR step are described in Table 2. PCR efficiency was determined by a 1% agarose gel electrophoresis.

Fifty microliter digestions were set up using 38 µL PCR product purified by IBI PCR Clean Up Protocol, 5 µL 10x NEB4, 5 µL 10x BSA, 1 µL BamHI, and 1 µL NdeI. The digestion reaction was run at 37°C for approximately 18 hours. The desired product was selected by a 1% agarose gel electrophoresis and purified by IBI Gel Extraction Protocol. The concentration of DNA was quantified using a Thermo Scientific NanoDrop 2000c Spectrophotometer with the assumption that 1 absorbance unit corresponds to 50 ng/µL of nucleic acid.

Ten microliter ligation reactions were assembled using 1 µL of digestion product, 1 µL of digested plasmid, 1 µL T7 DNA ligase, 5 µL of 2x T7 DNA ligase buffer, and 2 µL of autoclaved water. The pET3a, pET14, and pET15 plasmids were each extracted from E. coli containing the respective plasmid by IBI High-Speed Plasmid Mini Kit Protocol and digested as discussed. The ligation reaction was run at room temperature for approximately 24 hours. DH5α E. coli cells were transformed with the recombinant plasmid and grown on Luria-Bertani agar plates containing ampicillin. The cells were tested by a PCR screen using the T7 promoter and the T7 terminator. The DNA was sequenced off-site to confirm the success of the transformation.

Table 1. Each primer used and its sequence with mutations highlighted in grey.

Primer	Sequence
H126A-1	GACACCGACATGAAGGCCTTCGCGATCTTCGGCCGGTGCGAGAGCTGTTCCCTGAAGGG
H126A-2	CGAGAGCTGTTCCCTGAAGGGTTCAACTACCGACTCGTAAGGATCCGGG
H86A	CCTGGCCGACCGGGCCCACCACATCCAC
H86A_anti	GTGGATGTGGTGGGCCCGGTCGGCCAGG
E101Q	GCACCAACGTGATCCAGGCCGATCTGTCG
E101Q_anti	CGACAGATCGGCCTGGATCACGTTGGTGC

Table 2. The temperatures and durations of each PCR step.

PCR step	Temperature	Time
melting	95°C	30 seconds
annealing	determined by T_m of primers	30 seconds
elongation	72°C	1 minute per 1 kB

Protein purification

After expressing the mutant NUR protein, the cells are lysed and centrifuged. The supernatant is run through a nickel MCAC column using Buffer A, shown in Table 3. The protein is eluted from the column using an increasing concentration gradient of a Buffer B. Fractions containing NUR are identified by SDS-PAGE and are further purified by gel filtration chromatography. Fractions containing NUR are again identified by SDS-PAGE, and an ammonium sulfate precipitation is carried out. Protein is resuspended in a 25 mM Tris, 100 mM NaCl buffer at pH 8 and dialyzed 3x in 250 mL of the Tris buffer.

Table 3: The buffers used for the MCAC purification step.

Buffer A	Buffer B
25 mM phosphate	25 mM phosphate
100 mM NaCl	100 mM NaCl
2 mM sodium dithionite	2 mM sodium dithionite
	1 M imidazole
pH 8	pH 8

Characterization of WT NUR and NUR mutants

Spectroscopic experiments to observe whether NUR binds to DNA have been carried out on wild type NUR and various NUR mutants. The absorbance spectrum of 110 µM *sodF* promoter is taken using a Thermo Scientific NanoDrop 2000c Spectrophotometer. 100 µM NUR is then added to the DNA and the absorbance spectrum is taken again to observe the differences.

Experiments to determine the metal-exchangeability of wild type NUR have been conducted. 100 µM NUR was incubated overnight with 500 µM EDTA. 100 µM NUR was also incubated with 110 µM DNA, then 500 µM EDTA was added and left overnight. Each of the mixtures was run through a gel filtration chromatography column. The zinc content of each fraction that eluted from the column was analyzed by atomic absorption spectroscopy to determine the effects that DNA had on the ability of EDTA to strip NUR of zinc.

RESULTS & DISCUSSION
Cloning of NUR mutants

Several mutant *nur* genes have been successfully cloned. Mutant H86A, E101Q, and H126A *nur* genes were created by PCR, as these were the few mutants that had not yet been obtained. The mutant *nur* genes were successfully cloned into DH5α *E. coli* cells, shown by the PCR screen in Figure 1. Sequencing also confirmed the result. Transformation of these recombinant plasmids into NiCo21 *E. coli* cells for protein expression has been unsuccessful.
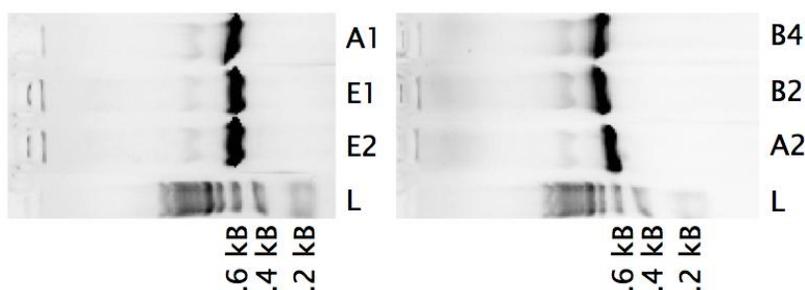

Figure 1: The PCR screen visualized on a 1% agarose gel showing successful transformation of H86A (B2, B4), E101Q (E1, E2), and H126A (A1, A2) in DH5α *E. coli* cells.

NUR mutant and DNA interaction

Spectroscopic analyses of the *sodF* promoter alone and the *sodF* promoter after the introduction of NUR show a significant decrease in the absorbance at the absorbance peak of DNA, 260 nm, as shown in Table 4 and Figure 2. The spectral dampening appears to be caused by an interaction between NUR and DNA, as the NUR+DNA spectrum is not the addition of the NUR spectrum and the DNA spectrum. WT NUR has been shown to bind to the *sodF* promoter[11], so the decrease in absorbance from the introduction of NUR was originally thought to be caused by a binding event, but as all mutants cause a dampening, the nature of the DNA-NUR interaction cannot be confirmed from this experiment alone. Further investigation into the interactions of NUR mutants and the *sodF* promoter is necessary.

Table 4: The absorbance of the *sodF* promoter and the absorbance of the *sodF* promoter after wild type NUR or one of the various NUR mutants were introduced.

Protein	Absorbance (260 nm)	
	DNA	NUR + DNA
WT	0.595	0.202
H90A	0.444	0.316
E101A	0.528	0.129
ΔM	0.491	0.361
ΔNi	0.507	0.202
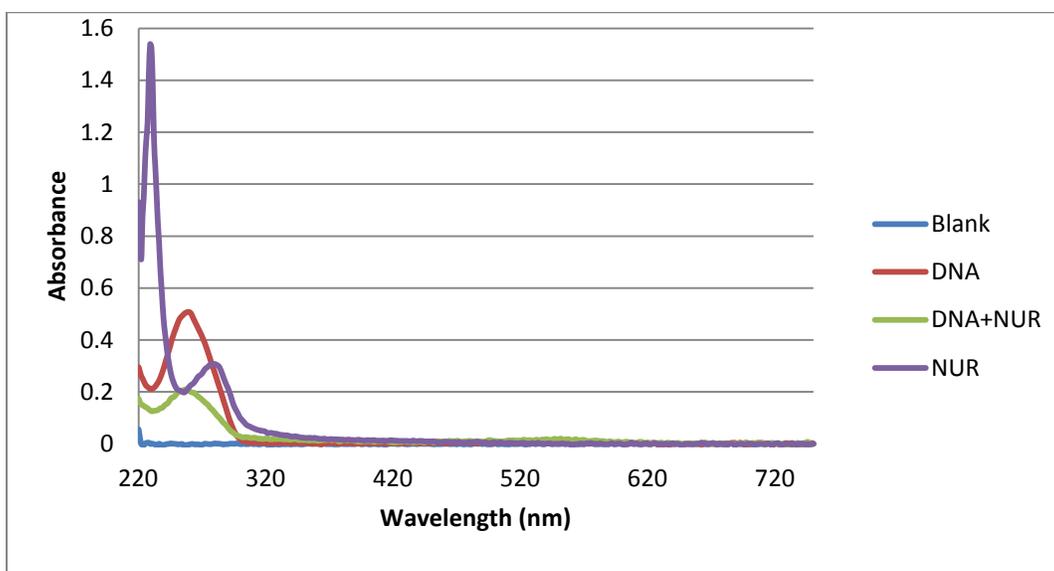
Figure 2: The absorbance spectra of the *sodF* promoter, the *sodF* promoter with NUR, and NUR alone in the UV-Vis range.

The exchangeability of metal from WT NUR

The atomic absorption spectroscopy data of WT NUR incubated with EDTA showed that very little zinc was stripped from NUR, as the largest peaks in the AAS data correspond to the gel filtration fractions that contain to NUR with a retention volume of approximately 8 mL, as shown in Figure 3. Small zinc concentrations were detected in later fractions, indicating that EDTA was able to pull some zinc from NUR, but very little relative to the amount that remained bound to the protein. Perhaps the metal is exchangeable from one binding site but not the other in the absence of DNA. Or, perhaps metal can be stripped from one monomer but not the other.

As shown in Figure 4, when WT NUR is incubated with the *sodF* promoter and EDTA, there remains a large amount of zinc bound to the protein. But, a substantially larger amount of zinc was detected in later fractions, indicating that EDTA is able to remove more metal from WT NUR in the presence of DNA. This supports the hypothesis that NUR being bound to DNA increases the exchangeability of metal from the binding sites of NUR.

Where the metal is removed, however, is yet to be determined. As purified by the methods described above, NUR is not 100% metal-loaded. It is likely that one binding-site, probably the M-site, has a higher metal occupancy than the Ni-site, as metal coordination at the Ni-site is weaker than at the M-site. The small amount of metal bound at the Ni-site may be exchangeable before NUR is bound to DNA. The metal removed by EDTA with DNA may be from the M-site of one monomer of NUR, while the M-site of the other monomer remains metal-loaded and non-exchangeable. The AAS peaks corresponding to NUR-bound metal and EDTA-bound metal are relatively equal, supporting this hypothesis of metal being pulled from one M-site of the dimer but retained by the other. Additional metal-binding experiments may be conducted to further analyze the exchangeability of metal from NUR.
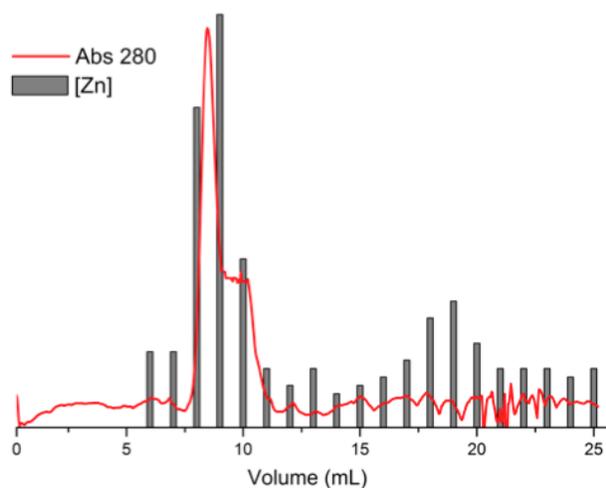

Figure 3: The gel filtration chromatogram of WT NUR incubated with EDTA with the relative zinc content of each fraction measured by the atomic absorption spectro-photometer also shown.
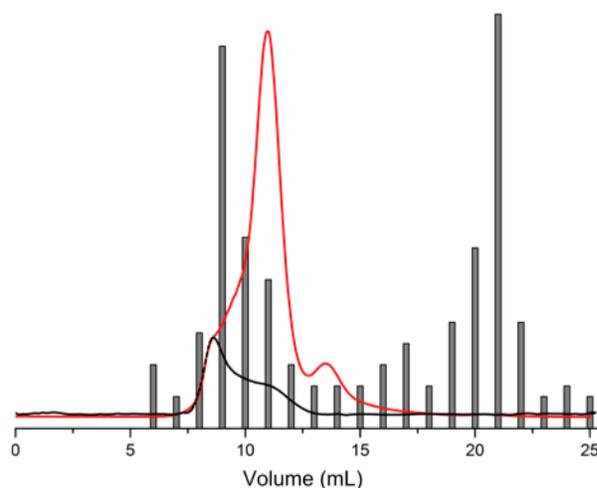
Figure 4: The gel filtration chromatogram of WT NUR and DNA incubated with EDTA with the relative zinc content of each fraction also shown. A chromatogram of NUR alone (black) was overlaid to show the volume at which NUR elutes from the column.

CONCLUSION

The mutant NUR library is near completion. Mutant *nur* genes H86A, E101Q, and H126A have been successfully cloned, but transformation into NiCo21 *E. coli* cells for protein expression has been unsuccessful. Spectroscopic experiments of several mutants incubated with the *sodF* promoter indicate that all examined mutants interact with DNA. How they interact will be determined with further experiments. The metal-exchangeability experiment showed that EDTA pulled some metal from NUR when incubated without DNA, but when NUR was incubated with DNA, EDTA removed much more metal from the protein, suggesting that metal is more exchangeable from NUR once bound to DNA. Further exploration will uncover more about the behavior of NUR.

ACKNOWLEDGEMENTS

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