

The Pennsylvania State University
The Graduate School
Department of Biochemistry and Molecular Biology

**REGULATION OF IRON-SULFUR CLUSTER BIOGENESIS IN
CYANOBACTERIA**

A Thesis in
Biochemistry, Microbiology and Molecular Biology

by
Yingxian Wu

© 2008 Yingxian Wu

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

August 2008

The thesis of Yingxian Wu was reviewed and approved* by the following:

John H. Golbeck
Professor of Biochemistry and Biophysics
Professor of Chemistry
Thesis Co-Advisor

Donald A. Bryant
Ernest C. Pollard Professor of Biotechnology
Professor of Biochemistry and Molecular Biology
Thesis Co-Advisor

Alan J. Benesi
Professor of Chemistry

Richard J. Frisque
Professor of Molecular Virology
Head of the Department of Biochemistry and Molecular Biology

*Signatures are on file in the Graduate School

ABSTRACT

In this thesis, I present my studies on the two regulators for iron-sulfur cluster biogenesis in cyanobacteria, *SYNPCC7002_A0128* and SufR.

SYNPCC7002_A0128 is a homologue of IscR, the transcriptional regulator for iron-sulfur cluster biogenesis in *Escherichia coli*. To investigate the role of *SYNPCC7002_A0128*, a null-mutant strain of *SYNPCC7002_A0128* was constructed in *Synechococcus* sp. PCC 7002. Physiological features were characterized and RT-PCR analyses were conducted for the mutant. The physiological features and the changes in the transcription profiles for the *isc* genes and the *suf* genes at the deletion of *SYNPCC7002_A0128* revealed a regulatory role for *SYNPCC7002_A0128* in iron-sulfur cluster biogenesis.

SufR is found to be an iron-sulfur cluster containing, transcriptional regulator for the *suf* operon in cyanobacterium *Synechocystis* sp. PCC 6803. I continued the process of characterizing the $[4\text{Fe-4S}]^{2+,1+}$ cluster in the SufR holo protein. Chemical analysis and spectroscopic analyses showed that SufR coordinated two $[4\text{Fe-4S}]^{2+,1+}$ clusters per dimer. EPR spectroscopy revealed that reduced $[4\text{Fe-4S}]^{2+,1+}$ cluster in SufR existed in a mixture of $S = 1/2$ and $S = 3/2$ ground spin states, with the $S = 1/2$ state accounting for 44% of the total spins. These results, combined with previous studies on the cysteine variants, suggested that there is one non-cysteine ligand that binds each $[4\text{Fe-4S}]^{2+,1+}$ cluster in SufR

TABLE OF CONTENTS

LIST OF FIGURES	vi
LIST OF TABLES	vii
ACKNOWLEDGEMENTS	viii
Chapter 1 Introduction	1
1.1 Iron-sulfur clusters	2
1.2 Iron-sulfur cluster biogenesis	3
1.2.1 Three iron-sulfur cluster biogenesis systems	3
1.2.2 Regulation of iron-sulfur cluster biogenesis	6
1.2.3 Iron-sulfur cluster biogenesis in cyanobacteria	7
1.3 Organization of the thesis	9
1.4 References	11
Chapter 2 Regulatory role of <i>SYNPCC7002_A0128</i> in the cyanobacterium <i>Synechococcus</i> sp. PCC 7002	19
2.1 Abstract	20
2.2 Introduction	21
2.3 Materials and methods	24
2.3.1 Mutagenesis of <i>SYNPCC7002_A0128</i> in <i>Synechococcus</i> sp. PCC 7002	24
2.3.2 Cyanobacterial cell growth under normal and iron-limiting conditions	25
2.3.3 RNA isolation	25
2.3.4 RT-PCR analysis	26
2.3.5 Construction of an <i>SYNPCC7002_A0128</i> expression vector for overexpression in <i>E. coli</i>	27
2.3.6 Over-expression and purification of the recombinant <i>SYNPCC7002_A0128</i> encoding protein in <i>E. coli</i>	27
2.4 Results	29
2.4.1 Identification of an <i>iscR</i> homologue in cyanobacteria <i>Synechococcus</i> sp. PCC 7002	29
2.4.2 <i>SYNPCC7002_A0128</i> is not essential in <i>Synechococcus</i> sp. PCC 7002	29
2.4.3 Physiological characterization of the wild-type and the mutant strains	30
2.4.4 Gene expression under normal growth conditions in wild-type and the <i>SYNPCC7002_A0128</i> null mutant	31
2.4.5 Gene expression under iron-limiting growth conditions in the wild- type and the mutant strains	31

2.4.6 Expression and purification of the <i>SYNPCC7002_A0128</i> encoding protein in <i>Escherichia coli</i>	32
2.5 Discussion.....	33
2.5.1 The role of <i>SYNPCC7002_A0128</i> in regulation of the <i>isc</i> operon and the <i>suf</i> operon in cyanobacteria	33
2.5.2 Sequence comparison of the <i>E. coli</i> IscR and the IscR homologues in cyanobacteria.....	34
2.5.3 Future directions for the project	35
2.6 References.....	37
Chapter 3 SufR coordinates two $[4\text{Fe-4S}]^{2+,1+}$ clusters and the $[4\text{Fe-4S}]^{2+,1+}$ clusters exist in $S = 1/2$ and $S = 3/2$ ground spin states.....	49
3.1 Abstract.....	50
3.2 Introduction.....	51
3.3 Materials and methods.....	53
3.3.1 Protein purification and quantitation.....	53
3.3.2 Reconstitution of the iron-sulfur cluster in the SufR protein	53
3.3.3 Chemical analysis.....	54
3.3.4 Electron Paramagnetic Resonance (EPR) spectroscopy and spin quantitation.....	54
3.4 Results.....	56
3.4.1 The correction factor for measuring the concentration of SufR from <i>Synechocystis</i> sp. PCC 6803.....	56
3.4.2 Holo-SufR coordinates two iron-sulfur clusters per dimer	56
3.4.3 The $[4\text{Fe-4S}]^{1+}$ cluster in SufR exists in both $S = 1/2$ and $3/2$ ground spin states	57
3.4.4 The $S = 1/2$ ground spin state $[4\text{Fe-4S}]^{2+,1+}$ cluster accounts for 44% of the total spin concentration.....	57
3.4.5 The $[4\text{Fe-4S}]^{2+,1+}$ cluster in SufR may have a non-cysteine ligand.....	58
3.5 Discussion.....	59
3.5.1 The non-cysteine ligand for the $[4\text{Fe-4S}]^{2+,1+}$ cluster.....	59
3.5.2 Comparison of SufR with other iron-sulfur cluster containing transcriptional regulators.....	60
3.6 References.....	63

LIST OF FIGURES

Figure 2-1: The physical map of the <i>SYNPCC7002_A0128</i> showing the site of insertion of an antibiotic resistance cartridge (<i>aphII</i> or <i>aadA</i>).	40
Figure 2-2: Sequence alignment of IscR homologues.	41
Figure 2-3: Segregation analysis of the <i>SYNPCC7002_A0128</i> mutants in <i>Synechococcus</i> sp. PCC 7002.	42
Figure 2-4: Growth curves for the <i>SYNPCC7002_A0128</i> null mutant and the wild-type under iron-limiting conditions without CO ₂ supplementation of the bubbling air.	43
Figure 2-5: Growth curve under iron-limiting conditions for cultures bubbled with air supplemented with 1% (v/v) CO ₂	44
Figure 2-6: Transcript levels of the <i>isc</i> genes in the wild-type and the <i>SYNPCC7002_A0128</i> null mutant of <i>Synechococcus</i> sp. PCC 7002 grown under normal conditions and iron-limiting conditions.	45
Figure 2-7: Transcript levels of the <i>suf</i> genes in the wild type and the <i>SYNPCC7002_A0128</i> null mutant under normal conditions and iron-limiting conditions.	47
Figure 2-8: SDS-PAGE analysis of the purified protein encoded by <i>SYNPCC7002_A0128</i>	48
Figure 3-1: EPR spectrum from 100 mT to 410 mT of recombinant, wild-type SufR after reduction with 15 mM sodium hydrosulfite.	70
Figure 3-2: Alignment of the cysteine-containing C-terminal domains of the cyanobacterial SufR and homologous proteins from several other organisms.	71

LIST OF TABLES

Table 1–1: Assignment of iron-sulfur cluster biogenesis genes in <i>Synechocystis</i> sp. PCC 6803.	18
Table 2–1: List of the oligonucleotides used for generating the pUC19- <i>SYNPCC7002_A0128</i> construct.....	39
Table 3–1: The correction factors for measuring the concentration of SufR from <i>Synechocystis</i> sp. PCC 6803.	66
Table 3–2: Chemical analysis and spectroscopic measurement for the cluster stoichiometry for holo-SufR.	67
Table 3–3: Spin quantitation for the [4Fe-4S] ^{2+,1+} clusters in SufR.....	68
Table 3–4: Comparison of the [4Fe-4S] ^{2+,1+} cluster stoichiometry in SufR and FNR.....	69

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who gave me the possibility to complete my thesis. I am grateful to my advisors, Dr. John H. Golbeck and Dr. Donald A. Bryant, for their guidance in research and writing of the thesis.

I thank Dr. Gaozhong Shen for his ideas and suggestions throughout my experiments. I am grateful to Dr. Ramakrishnan Balasubramanian and Dr. Mark Heinnickel for their help and valuable ideas.

My colleagues from the labs of Dr. Golbeck and Dr. Bryant supported me in my research. I want to thank them for all their help and valuable hints. I want to thank Cara Lubner and Bharadwaj Jagannathan for sharing their experiment experience. I would like to thank Nithya Srinivasan, Steve Romberger, Joel Graham, Dr. Aline Gomez-Maqueo Chew, Dr. Hui Li, Yu Xu, Amaya Garcia, Dr. Fangqin Zhao for sharing funs in the lab.

Finally, I would like to give my special thanks to my parents whose support and encouragement enabled me to complete the work.

Chapter 1

Introduction

1.1 Iron-sulfur clusters

Biological iron-sulfur clusters were identified about 40 years ago and are found in all living organisms. Their role was considered only to be involved in electron transfer until nearly 20 years later, when it was discovered that the iron-sulfur cluster in aconitase plays a catalytic role [1]. Recently, iron-sulfur clusters were also found to have versatile functions including stabilization of protein structure, gene regulation, environmental sensing and radical generation [2].

Proteins containing one or more iron-sulfur clusters are called iron-sulfur proteins. The most common iron-sulfur clusters in nature are [2Fe-2S] and [4Fe-4S] clusters. Four cysteine residues usually serve as ligands to both the [2Fe-2S] cluster and the [4Fe-4S] cluster. It has also been discovered that non-cysteine residues may also serve as ligands to the [2Fe-2S] and [4Fe-4S] clusters [3]. Specialized iron-sulfur clusters such as the FeMo-cofactor in nitrogenase contain metals in addition to iron and sulfur, as an integral part of the iron-sulfur cluster [4]. Although there are certain polypeptide signature sequences that can be used to predict whether a particular protein coordinates an iron-sulfur cluster, there is no single canonical primary amino acid sequence that defines an iron-sulfur cluster binding motif [2].

1.2 Iron-sulfur cluster biogenesis

It has been shown that simple iron-sulfur clusters can be chemically reconstituted *in vitro* with reducing reagents, $\text{Fe}^{2+/3+}$ and S^{2-} [5]. However, the reconstitution cannot represent the mechanism of iron-sulfur cluster assembly *in vivo* because free $\text{Fe}^{2+/3+}$ and S^{2-} are toxic to cells. Instead, complex protein machineries are involved in the biogenesis of iron-sulfur clusters. These machineries allow the assembly and insertion of iron-sulfur clusters into target proteins *in vivo*. The major functions of the iron-sulfur cluster assembly machineries include: (1) mobilization of Fe and S atoms from their storage sources; (2) assembly of clusters; (3) transfer of cluster to the target proteins [6].

1.2.1 Three iron-sulfur cluster biogenesis systems

Three iron-sulfur cluster biogenesis systems have been discovered in bacteria. They are the *nif* (nitrogen fixation), the *isc* (iron sulfur cluster) and the *suf* (sulfur utilization factor) systems [7-9].

The first system to be discovered was the *nif* system in the nitrogen-fixing bacterium *Azotobacter vinelandii*. Two major components of the *nif* system are NifS and NifU [7]. NifS is a homodimeric pyridoxal-phosphate dependent enzyme that catalyzes L-cysteine desulfurisation. It generates enzyme-bound persulfide, which is considered to be a biologically active form of sulfur [10, 11]. The role of NifS alleviates the need to produce free sulfide for iron-sulfur cluster assembly. NifU is an iron-sulfur cluster scaffold protein [12, 13]. The N-terminal sequence contains three conserved cysteines that coordinate a “transient” [2Fe-2S] cluster. The C-terminal sequence contains two

conserved cysteines with unknown functions. The central region contains four conserved cysteines and holds a “permanent” [2Fe-2S] cluster. *In vitro* assays show that NifU can assemble a transient [2Fe-2S] cluster in the presence of NifS and that it can facilitate the transfer of this [2Fe-2S] cluster to nitrogenase in *A. vinelandii* [14].

The second iron-sulfur cluster biosynthetic system is the *isc* system[8]. Unlike the *nif* system that serves specifically for nitrogen fixation, the *isc* system is considered to be the “housekeeping” iron-sulfur cluster biogenesis system in many microorganisms. Most *isc* systems are composed of proteins encoded by the *iscRSUA-hscBA-fdx* regulon [8, 15, 16]. IscR has been shown to be a negative regulator for the *iscRSUA* operon in *E. coli*. It has sequence homology to a family of transcription factors and contains a [2Fe-2S] cluster [17]. IscS is a homologue to NifS in the *nif* system [8]. It has been shown to be a pyridoxal-phosphate-dependent cysteine desulfurase that mobilizes sulfur through desulfurization of L-cysteine, yielding alanine and IscS-bound persulfide [18, 19]. IscU is homologous to the N-terminal region of NifU in the *nif* system. It is considered to function as a scaffold protein similar to NifU. IscU and IscS can assemble and transfer [2Fe-2S] clusters to apo-ferredoxin [20, 21]. IscA was suggested to function as an alternate scaffold protein because it can assemble a transient [2Fe-2S] or [4Fe-4S] cluster and transfer the cluster to apo-ferredoxin or biotin synthase [22]. Recently, the function of IscA as a scaffold protein was challenged. It was instead suggested to play a regulatory role in redox sensing and cluster assembly in the cyanobacterium *Synechococcus* sp. PCC 7002 [23]. HscAB are molecular chaperone proteins involved in iron-sulfur cluster assembly. They exhibit intrinsic ATPase activity which can be greatly stimulated by adding the scaffold protein, IscU [24, 25]. The presence of HscAB can, in turn, stimulate

the cluster-transfer capacity of IscU to apo-ferredoxin by 20-fold if Mg-ATP is supplemented [26].

The third system involved in the biogenesis of iron-sulfur clusters is the *suf* system [9]. The *suf* system is present in a variety of bacteria, archaea, and plastids of plants. The *suf* system consists of the *sufABCDSE* genes. In *Escherichia coli*, overexpression of the *sufABCDSE* genes can rescue the growth defect of *isc* mutants. Inactivation of the *suf* genes alone in *E. coli* does not produce any obvious phenotype, provided the cells are grown under normal conditions [5]. However, when the *isc* genes are deleted from the *E. coli* genome, five of the six *suf* genes (*sufA*, *sufB*, *sufC*, *sufS*, *sufE*) are found to be necessary for cell viability [9]. The *sufABCDSE* operon also exists in the plant pathogen *Erwinia chrysanthemi*. Inactivation of either the entire operon or each of the *suf* genes individually is not lethal, but it results in an increase in the intracellular free iron concentration and an increased sensitivity of the cell to oxidative stress [27]. Thus, the *suf* system is considered to be an alternate iron-sulfur assembly system that specifically adapts the cell to iron starvation and oxidative stress conditions in *E. coli* and *Erwinia chrysanthemi* [28, 29]. The *suf* system does not exist in all microorganisms. For example, the *suf* system is absent in *A. vinelandii*, which consequently makes the *isc* genes essential in that organism [30]. Some of the *suf* genes share functional similarity with the corresponding *isc* genes. SufA from *E. chrysanthemi* has been shown to assemble a [2Fe-2S] cluster or a [4Fe-4S] cluster and transfer it to apo-ferredoxin or a [4Fe-4S] apoprotein, such as biotin synthase [31]. Similar to IscA, the role of SufA as a scaffold protein was also questioned by its involvement in regulating cluster assembly and redox sensing [23]. SufS is homologous to IscS and also serves as

cysteine desulfurase [32]. SufE can stimulate the desulfurase activity of SufS and its stimulation involves binding and transferring of the sulfur from SufS-bound persulfide [33]. SufBCD are homologous to components of the ABC transporter complex. SufC is an ATPase that plays roles in the assembly and maintenance of the iron-sulfur cluster in *E. chrysanthemi* and plastids of *Arabidopsis thaliana* [27, 34]. No homologue of IscU, however, has been found in the *suf* operon.

1.2.2 Regulation of iron-sulfur cluster biogenesis

A feedback mechanism is involved in regulating the *isc* system. IscR is a repressor for the *isc* genes and plays a key role in feedback regulation [17, 35]. The active form of IscR contains an unstable [2Fe-2S] cluster, while the inactive form lacks the cluster. The instability of the cluster allows IscR to compete weakly with other iron-sulfur proteins for the iron-sulfur cluster. When the capacity for assembling iron-sulfur clusters is limited, IscR is in an unfavorable condition in obtaining clusters and exists predominantly in the inactive apo form. Apo-IscR derepresses the *isc* operon and leads to the full expression of the *isc* machinery. However, if iron-sulfur clusters are abundant, IscR obtains its cluster and then exists in the active form that can shut down the expression of the *isc* machinery. Thus, the regulatory role of IscR depends on the presence or absence of its [2Fe-2S] cluster. The importance of the [2Fe-2S] for IscR is also supported by the fact that the regulation of IscR is significantly impaired in the absence of the *iscS* and *hscA* genes, both of which are required for the full formation of iron-sulfur clusters [17].

A similar feedback regulatory mechanism is also found in SufR, which regulates the expression of the *suf* operon in cyanobacteria. Former members of our research group discovered that the SufR protein can negatively regulate the transcription of the *sufBCDS* operon in the cyanobacterium *Synechococcus* sp. PCC 7002 [36, 37]. Similar to the IscR in *E. coli*, an apo, inactive state and a holo, active repressor state exists for SufR [23].

1.2.3 Iron-sulfur cluster biogenesis in cyanobacteria

Cyanobacteria are photosynthetic bacteria that use water as an electron donor and produce oxygen as a by-product of photosynthesis. They have both Type I (Photosystem I, PS I) and Type II (Photosystem II, PS II) reaction centers. PS I consists of a heterodimeric core formed by the PsaA/PsaB proteins, which harbor most of the electron transfer cofactors from primary electron donor, P700, the primary electron acceptor, A₀ (a monomeric chlorophyll *a*), an intermediate electron acceptor, A₁ (a phylloquinone) and finally F_X (a [4Fe-4S] cluster) [38-40]. PsaC, a low molecular weight protein, binds to the PS I core and contains two [4Fe-4S] clusters, F_A and F_B, which function as terminal electron acceptors [41, 42]. Reduced F_A/F_B serve as strong reductants, which are capable of reducing NADP⁺ to NADPH. The reduction occurs through the involvement of the low molecular weight [2Fe-2S] ferredoxin and ferredoxin:NADP⁺ oxidoreductase [38]. The three [4Fe-4S] clusters in PS I, F_X, F_A and F_B, and the one [2Fe-2S] cluster in ferredoxin are essential for the assembly and the function of Photosystem I [23].

There are two iron-sulfur assembly systems in cyanobacteria, the *suf* system and

isc system. In addition, C-DES (cysteine desulfurylase), a group distinct from the IscS-like or SufS-like homologues, has been identified in cyanobacteria. It can also mobilize sulfur and incorporate the iron-sulfur cluster into apo-ferredoxin. However, it has a different substrate and shares a low sequence similarity with NifS [43, 44].

Unlike the *isc* systems in other microorganisms, the *isc* homologues in cyanobacteria are not organized in the form of an operon, but rather are scattered throughout the genome (Table 1-1). Two homologues to *E. coli iscS* can be identified, and these are designated as *iscS₁* and *iscS₂*. They are among the three *nifS*-like ORFs in the genome of *Synechocystis* sp. PCC 6803 [19, 45, 46]. IscS₁ and IscS₂ exhibit cysteine desulfurase activity similar to the IscS in *E. coli* [19]. A homologue to the C-terminus rather than the N-terminus of NifU, named SyNifU, is present in *Synechocystis* sp. PCC 6803 [47]. SyNifU might be the primary scaffold protein in cyanobacteria because it has been shown to transfer clusters to apo-ferredoxin [47]. No studies have been reported that delineate the functions of the HscAB homologues in cyanobacteria.

All of the *suf* components present in *E. coli* can be identified in cyanobacteria [23, 37, 45]. However, unlike the *suf* operon in *E. coli* and *E. chrysanthemii*, only the *sufBCDS* homologues form a gene cluster in cyanobacteria, while the *sufA* and *sufE* homologues are located elsewhere in the cyanobacterial genome (Table 1-1) [48]. In addition, a transcriptional repressor, named *sufR*, which does not exist in *E. coli*, is located directly upstream of the *sufBCDS* operon [36, 37]. The functions of other *suf* components are similar to those of the *suf* genes that exist in other microorganisms. Cyanobacterial SufA transfers iron-sulfur clusters, similar to the SufA in *E. coli* and *E. chrysanthemii* as mentioned above [49]. The cyanobacterial SufS is a cysteine desulfurase, similar to SufS

in *E. coli* [45]. However, unlike SufS in *E. coli*, the cyanobacterial SufS is essential [45]. No studies have been reported for the functions of the cyanobacterial SufB, SufC and SufD proteins.

As stated earlier, in most non-photosynthetic bacteria such as *E. coli*, the *isc* system is the primary machinery for iron-sulfur cluster assembly, while the *suf* system is considered to be an alternate machinery mainly responsible for iron-sulfur cluster assembly under stress conditions [28]. In cyanobacteria, however, the *suf* system is considerably more important than the *isc* system. This is supported by reverse genetic studies conducted in *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 (Table 1-1) [50]. For example, *sufS*, which encodes the cysteine desulfurase, is essential, while the *iscS₁* and *iscS₂* are both dispensable [45]. The importance of the *suf* system over the *isc* system might be generally true for other photosynthetic organisms, because the *suf* system, but not the *isc* system, is found in the chloroplasts of higher plants [23]. These results suggest that photosynthetic organisms may rely primarily on the *suf* system for assembling iron-sulfur clusters for electron transfer cofactors.

1.3 Organization of the thesis

In chapter 2, I describe my studies on a putative *iscR* gene, *SYNPCC7002_A0128*. *SYNPCC7002_A0128* was identified by a BLASTP search of the genome of the cyanobacterium *Synechococcus* sp. PCC 7002 for a homologue to the *iscR* gene of *E. coli*. A null mutant of *SYNPCC7002_A0128* was made in *Synechococcus* sp. PCC 7002 and its physiological features were characterized. RT-PCR analyses were conducted to

examine the changes in the transcription profiles of the *isc* genes and the *suf* genes as a result of the interruption of *SYNPCC7002_A0128*. The protein encoded by *SYNPCC7002_A0128* was overproduced in *E. coli* and purified by gel filtration chromatography.

In chapter 3, I continued the study of the $[4\text{Fe-4S}]^{2+,1+}$ cluster in the SufR protein from the cyanobacterium *Synechocystis* sp. PCC 6803. Chemical analysis and spectroscopic analyses showed that SufR bound one cluster per polypeptide. EPR spectroscopy revealed that the $[4\text{Fe-4S}]^{2+,1+}$ clusters in SufR existed in a mixture of two spin states, $S=1/2$ and $S=3/2$. The clusters in the $S=1/2$ ground spin state were quantified.

1.4 References

1. Ruzicka, F.J. and H. Beinert, *The soluble "high potential" type iron-sulfur protein from mitochondria is aconitase*. J Biol Chem, 1978. **253**(8): p. 2514-7.
2. Johnson, M.K., *Iron-sulfur proteins: new roles for old clusters*. Curr Opin Chem Biol, 1998. **2**(2): p. 173-81.
3. George, S.J., et al., *Electrochemical and spectroscopic characterization of the conversion of the 7Fe into the 8Fe form of ferredoxin III from Desulfovibrio africanus. Identification of a [4Fe-4S] cluster with one non-cysteine ligand*. Biochem J, 1989. **264**(1): p. 275-84.
4. Einsle, O., et al., *Nitrogenase MoFe-protein at 1.16 Å resolution: a central ligand in the FeMo-cofactor*. Science, 2002. **297**(5587): p. 1696-700.
5. Malkin, R. and J.C. Rabinowitz, *The reconstitution of clostridial ferredoxin*. Biochem Biophys Res Commun, 1966. **23**(6): p. 822-7.
6. Loiseau, L., et al., *Biogenesis of Fe-S cluster by the bacterial Suf system: SufS and SufE form a new type of cysteine desulfurase*. J Biol Chem, 2003. **278**(40): p. 38352-9.
7. Jacobson, M.R., et al., *Biochemical and genetic analysis of the nifUSVWZM cluster from Azotobacter vinelandii*. Mol Gen Genet, 1989. **219**(1-2): p. 49-57.
8. Zheng, L., et al., *Assembly of iron-sulfur clusters. Identification of an iscSUA-hscBA-fdx gene cluster from Azotobacter vinelandii*. J Biol Chem, 1998. **273**(21): p. 13264-72.

9. Takahashi, Y. and U. Tokumoto, *A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids*. J Biol Chem, 2002. **277**(32): p. 28380-3.
10. Zheng, L., et al., *Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis*. Proc Natl Acad Sci U S A, 1993. **90**(7): p. 2754-8.
11. Zheng, L., et al., *Mechanism for the desulfurization of L-cysteine catalyzed by the nifS gene product*. Biochemistry, 1994. **33**(15): p. 4714-20.
12. Fu, W., et al., *nifU gene product from Azotobacter vinelandii is a homodimer that contains two identical [2Fe-2S] clusters*. Biochemistry, 1994. **33**(45): p. 13455-63.
13. Agar, J.N., et al., *Modular organization and identification of a mononuclear iron-binding site within the NifU protein*. J Biol Inorg Chem, 2000. **5**(2): p. 167-77.
14. Yuvaniyama, P., et al., *NifS-directed assembly of a transient [2Fe-2S] cluster within the NifU protein*. Proc Natl Acad Sci U S A, 2000. **97**(2): p. 599-604.
15. Tokumoto, U. and Y. Takahashi, *Genetic analysis of the isc operon in Escherichia coli involved in the biogenesis of cellular iron-sulfur proteins*. J Biochem (Tokyo), 2001. **130**(1): p. 63-71.
16. Takahashi, Y. and M. Nakamura, *Functional assignment of the ORF2-iscS-iscU-iscA-hscB-hscA-fdx-ORF3 gene cluster involved in the assembly of Fe-S clusters in Escherichia coli*. J Biochem (Tokyo), 1999. **126**(5): p. 917-26.
17. Schwartz, C.J., et al., *IscR, an Fe-S cluster-containing transcription factor, represses expression of Escherichia coli genes encoding Fe-S cluster assembly proteins*. Proc Natl Acad Sci U S A, 2001. **98**(26): p. 14895-900.

18. Schwartz, C.J., et al., *The cysteine desulfurase, IscS, has a major role in in vivo Fe-S cluster formation in Escherichia coli*. Proc Natl Acad Sci U S A, 2000. **97**(16): p. 9009-14.
19. Behshad, E., S.E. Parkin, and J.M. Bollinger, Jr., *Mechanism of cysteine desulfurase Slr0387 from Synechocystis sp. PCC 6803: kinetic analysis of cleavage of the persulfide intermediate by chemical reductants*. Biochemistry, 2004. **43**(38): p. 12220-6.
20. Urbina, H.D., et al., *Transfer of sulfur from IscS to IscU during Fe/S cluster assembly*. J Biol Chem, 2001. **276**(48): p. 44521-6.
21. Agar, J.N., et al., *IscU as a scaffold for iron-sulfur cluster biosynthesis: sequential assembly of [2Fe-2S] and [4Fe-4S] clusters in IscU*. Biochemistry, 2000. **39**(27): p. 7856-62.
22. Krebs, C., et al., *IscA, an alternate scaffold for Fe-S cluster biosynthesis*. Biochemistry, 2001. **40**(46): p. 14069-80.
23. Balasubramanian, R., et al., *Regulatory roles for IscA and SufA in iron homeostasis and redox stress responses in the cyanobacterium Synechococcus sp. strain PCC 7002*. J Bacteriol, 2006. **188**(9): p. 3182-91.
24. Bentreop, D., et al., *Characterization of the unbound 2[Fe4S4]-ferredoxin-like photosystem I subunit PsaC from the Cyanobacterium synechococcus elongatus*. Biochemistry, 1997. **36**(44): p. 13629-37.
25. Silberg, J.J., et al., *The Fe/S assembly protein IscU behaves as a substrate for the molecular chaperone Hsc66 from Escherichia coli*. J Biol Chem, 2001. **276**(3): p. 1696-700.

26. Chandramouli, K. and M.K. Johnson, *HscA and HscB stimulate [2Fe-2S] cluster transfer from IscU to apoferredoxin in an ATP-dependent reaction*. *Biochemistry*, 2006. **45**(37): p. 11087-95.
27. Nachin, L., et al., *SoxR-dependent response to oxidative stress and virulence of Erwinia chrysanthemi: the key role of SufC, an orphan ABC ATPase*. *Mol Microbiol*, 2001. **39**(4): p. 960-72.
28. Tokumoto, U., et al., *Interchangeability and distinct properties of bacterial Fe-S cluster assembly systems: functional replacement of the isc and suf operons in Escherichia coli with the nifSU-like operon from Helicobacter pylori*. *J Biochem (Tokyo)*, 2004. **136**(2): p. 199-209.
29. Outten, F.W., O. Djaman, and G. Storz, *A suf operon requirement for Fe-S cluster assembly during iron starvation in Escherichia coli*. *Mol Microbiol*, 2004. **52**(3): p. 861-72.
30. Frazzon, J. and D.R. Dean, *Formation of iron-sulfur clusters in bacteria: an emerging field in bioinorganic chemistry*. *Curr Opin Chem Biol*, 2003. **7**(2): p. 166-73.
31. Ollagnier-de Choudens, S., et al., *SufA from Erwinia chrysanthemi. Characterization of a scaffold protein required for iron-sulfur cluster assembly*. *J Biol Chem*, 2003. **278**(20): p. 17993-8001.
32. Patzer, S.I. and K. Hantke, *SufS is a NifS-like protein, and SufD is necessary for stability of the [2Fe-2S] FhuF protein in Escherichia coli*. *J Bacteriol*, 1999. **181**(10): p. 3307-9.

33. Ollagnier-de-Choudens, S., et al., *Mechanistic studies of the SufS-SufE cysteine desulfurase: evidence for sulfur transfer from SufS to SufE*. FEBS Lett, 2003. **555**(2): p. 263-7.
34. Nachin, L., et al., *SufC: an unorthodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress*. Embo J, 2003. **22**(3): p. 427-37.
35. Frazzon, J. and D.R. Dean, *Feedback regulation of iron-sulfur cluster biosynthesis*. Proc Natl Acad Sci U S A, 2001. **98**(26): p. 14751-3.
36. Yu, J., et al., *Suppressor mutations in the study of photosystem I biogenesis: sll0088 is a previously unidentified gene involved in reaction center accumulation in Synechocystis sp. strain PCC 6803*. J Bacteriol, 2003. **185**(13): p. 3878-87.
37. Wang, T., et al., *The sufR gene (sll0088 in Synechocystis sp. strain PCC 6803) functions as a repressor of the sufBCDS operon in iron-sulfur cluster biogenesis in cyanobacteria*. J Bacteriol, 2004. **186**(4): p. 956-67.
38. Brettel, K., *Electron transfer and arrangement of the redox cofactors in photosystem I* Biochim. Biophys. Acta 1997. **1318**(3): p. 322-373.
39. Jordan, P., et al., *Three-dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution*. Nature, 2001. **411**(6840): p. 909-17.
40. Golbeck, J.H., *Shared thematic elements in photochemical reaction centers*. Proc Natl Acad Sci U S A, 1993. **90**(5): p. 1642-6.
41. Hayashida, N., et al., *The gene for the 9 kd polypeptide, a possible apoprotein for the iron-sulfur centers A and B of the photosystem I complex, in tobacco chloroplast DNA*. Curr Genet, 1987. **12**(4): p. 247-50.

42. Hoj, P.B., et al., *Identification of a chloroplast-encoded 9-kDa polypeptide as a [2Fe-2S] protein carrying centers A and B of photosystem I*. J Biol Chem, 1987. **262**(26): p. 12676-84.
43. Lang, T. and D. Kessler, *Evidence for cysteine persulfide as reaction product of L-Cyst(e)ine C-S-lyase (C-DES) from Synechocystis. Analyses using cystine analogues and recombinant C-DES*. J Biol Chem, 1999. **274**(1): p. 189-95.
44. Leibrecht, I. and D. Kessler, *A novel L-cysteine/cystine C-S-lyase directing [2Fe-2S] cluster formation of Synechocystis ferredoxin*. J Biol Chem, 1997. **272**(16): p. 10442-7.
45. Tirupati, B., et al., *Kinetic and structural characterization of Slr0077/SufS, the essential cysteine desulfurase from Synechocystis sp. PCC 6803*. Biochemistry, 2004. **43**(38): p. 12210-9.
46. Jaschkowitz, K. and A. Seidler, *Role of a NifS-like protein from the cyanobacterium Synechocystis PCC 6803 in the maturation of FeS proteins*. Biochemistry, 2000. **39**(12): p. 3416-23.
47. Nishio, K. and M. Nakai, *Transfer of iron-sulfur cluster from NifU to apoferredoxin*. J Biol Chem, 2000. **275**(30): p. 22615-8.
48. Wang, T., *Iron sulfur cluster assembly in photosystem I of cyanobacteria: the role of SufR (SUF) and Fdx (ISC)*. 2004, The Pennsylvania State University, University Park.
49. Wollenberg, M., et al., *A dimer of the FeS cluster biosynthesis protein IscA from cyanobacteria binds a [2Fe2S] cluster between two protomers and transfers it to [2Fe2S] and [4Fe4S] apo proteins*. Eur J Biochem, 2003. **270**(8): p. 1662-71.

50. Balasubramanian, R., *Biogenesis, Assembly, and Function of Iron-Sulfur Clusters in Photosystem I*. 2006, The Pennsylvania State University, University Park.

Table 1–1 Assignment of iron-sulfur cluster biogenesis genes in *Synechocystis* sp. PCC 6803. Genes in the *suf* system and the *isc* system are listed. Deletion mutants for each gene were constructed to evaluate whether any are essential.

Table 1–1: Assignment of iron-sulfur cluster biogenesis genes in *Synechocystis* sp. PCC 6803.

Name of the gene	ORF in 6803	Genetic study
SUF system		
SufR	SII0088	Not essential ¹
SufA	Slr1417	Not essential ¹
SufB	Slr0074	Essential ¹
SufC	Slr0075	Essential ¹
SufD	Slr0076	Essential ¹
SufS	Slr0077	Essential ¹
SufE	Slr1419	Essential ¹
ISC system		
IscR	Slr0846	Not essential ¹
IscS1	Slr0387	Not essential ¹
IscS2	SII0704	Not essential ¹
IscA	Slr1565	Not essential ¹
HscA	SII0170	(Not studied) ¹
HscB	SII0169	Not essential ¹
Fdx	Slr0148	Not essential ¹
Nfu	SII2667	Essential ¹

¹Balasubramanian, R., *Biogenesis, Assembly, and Function of Iron-Sulfur Clusters in Photosystem I*. 2006, The Pennsylvania State University, University Park

Chapter 2

Regulatory role of *SYNPCC7002_A0128* in the cyanobacterium

Synechococcus sp. PCC 7002

2.1 Abstract

IscR functions as a transcriptional regulator for iron-sulfur cluster biogenesis in *Escherichia coli*. An open reading frame, *SYNPCC7002_A0128*, in the genome of cyanobacteria *Synechococcus* sp. PCC 7002 was identified as a homologue of *E. coli* IscR by BLASTP analyses. To investigate the role of *SYNPCC7002_A0128*, a null-mutant strain of *SYNPCC7002_A0128* was constructed in *Synechococcus* sp. PCC 7002. Under normal growth conditions, the *SYNPCC7002_A0128* null mutant grew photoautotrophically with rates similar to the wild-type, which indicated that *SYNPCC7002_A0128* was not essential in cyanobacteria. However, the *SYNPCC7002_A0128* mutant grew slower or had a longer lag phase compared with the wild-type under iron-limiting conditions. RT-PCR analysis revealed transcriptional profile changes in the *SYNPCC7002_A0128* mutant for both the *isc* genes and the *suf* genes. For the *isc* genes, *iscS₁* and *iscS₂* (the cysteine desulfurase genes in the *isc* machinery) had decreased transcript levels in the mutant compared with the wild-type under normal conditions. However, under iron-limiting conditions, the transcript levels of *iscS₁* and *iscS₂* increased in the mutant. For the *suf* genes, the transcript of *sufB* increased and the transcript level of *sufR* decreased in the mutant under both normal and iron-limiting conditions. The cysteine desulfurase gene in the *suf* system, *sufS*, had an increased transcript level only under iron-limiting condition. These results indicated that *SYNPCC7002_A0128* protein in cyanobacteria was involved in the regulation of iron-sulfur cluster biogenesis genes and that its regulatory activity responded to the change in iron availability.

2.2 Introduction

Two iron-sulfur cluster assembly systems exist in *Escherichia coli*, the *isc* system and the *suf* system. The *isc* system is considered to be the housekeeping system for iron-sulfur cluster biogenesis, while the *suf* system is considered to be an alternative iron-sulfur cluster biogenesis system [1, 2]. Both the *isc* genes and the *suf* genes can be induced by H₂O₂ and iron chelation [2].

IscR was found initially to be the transcriptional repressor for the *iscRSUA* operon in *E. coli* [3]. The active form of IscR contains a [2Fe-2S]^{2+,1+} cluster and represses the expression of the *iscRSUA* operon. IscR mediates the response of the *iscRSUA* operon to both oxidative stress and iron starvation [2]. Deletion of the *iscR* gene results in constitutively high expression of the *iscRSUA* operon, and under these conditions the expression is not further induced by oxidative stress or iron starvation. In addition to its role in the regulation of the *iscRSUA* genes, IscR is also involved in the regulation of the *sufABCDSE* operon. Apo-IscR can bind to a *cis*-acting, oxidant-responsive element upstream of the *sufABCDSE* promoter [4]. Therefore, under oxidative stress, apo-IscR initiates full expression of the iron-sulfur cluster assembly machinery by both directly activating *sufABCDSE* operon transcription through binding to the *sufABCDSE* promoter and derepressing the *iscRSUA* operon [4]. In addition to the role in regulating the *iscRSUA* operon and the *sufABCDSE* operon, IscR has been discovered to regulate the iron-sulfur cluster biogenesis genes, *yarD* and *yhgI*, and the iron-sulfur cluster containing anaerobic respiratory enzymes *hyaABCDEF*, *hybOABCDEFG* and

napFDAGHBC [5]. The mixed functions of the downstream regulated genes demonstrate a role for IscR in both regulating iron-sulfur cluster biogenesis and oxidative stress.

The $[2\text{Fe-2S}]^{2+,1+}$ cluster is critical for IscR to function as a transcriptional regulator in *E. coli*. IscR with or without the cluster has the ability to bind DNA in different promoter regions so as to activate or repress different group of genes [3]. This indicates that the $[2\text{Fe-2S}]^{2+,1+}$ cluster is the primary sensor for the levels of iron-sulfur clusters and oxidative stress. An interesting feature of the $[2\text{Fe-2S}]^{2+,1+}$ cluster is that only three cysteine residues are located in IscR from *E. coli*, while four cysteines are usually involved in the coordination of a $[2\text{Fe-2S}]^{2+,1+}$ cluster [3]. This feature probably makes it more difficult for IscR in *E. coli* to obtain the iron-sulfur cluster or it makes for a less stable cluster. When conditions such as oxidative stress and iron starvation occur, IscR loses its cluster and thus becomes the apo form. Apo-IscR derepresses the transcription of the *iscRSUA* operon, leading to the complete expression of the *isc* assembly machinery [3, 6].

In cyanobacteria the only iron-sulfur cluster repressor reported at the present is SufR, a transcriptional regulator of the *sufBCDS* genes. It is located directly upstream of, and is divergently transcribed from, the *sufBCDS* operon in several sequenced cyanobacterial genomes. Interruption in this gene rescued photosynthetic competence in a *psaC* mutant that changed a cysteine residue to a serine [7]. RT-PCR analysis revealed that the deletion of the *sufR* gene resulted in an increased transcript levels of the *sufB*, *sufC*, *sufD* and *sufS* genes in *Synechocystis* sp. PCC 6803 [8]. A former member of our research group discovered that SufR harbors $[4\text{Fe-4S}]^{2+,1+}$ clusters [9]. Holo-SufR is able to bind to two sequences in the promoter region of the *suf* operon with different affinities.

In this work, I identified an IscR homologue, *SYNPCC7002_A0128*, in the genome of the cyanobacterium *Synechococcus* sp. PCC 7002 by a BLASTP search. A null mutant of *SYNPCC7002_A0128* was constructed and its physiological features were studied under both normal and iron-limiting conditions. RT-PCR analyses were conducted to compare the transcript abundances of the *isc* and *suf* genes in the wild-type and the *SYNPCC7002_A0128* mutant grown under either normal or iron-limiting conditions. This study revealed that the *SYNPCC7002_A0128* in cyanobacteria is involved in the regulation in iron-sulfur cluster biogenesis genes and that its regulation responded to the iron level in the medium.

2.3 Materials and methods

2.3.1 Mutagenesis of *SYNPCC7002_A0128* in *Synechococcus* sp. PCC 7002

An ORF with high sequence homology to the *iscR* gene in *E. coli* was identified to be *SYNPCC7002_A0128* by a BLASTP search of the genomic sequence of *Synechococcus* sp. PCC 7002. The fragment containing the *SYNPCC7002_A0128* was amplified by PCR with the primers listed in Table 2-1 with the genomic DNA of *Synechococcus* sp. PCC 7002 as template. The 1.26-kb PCR product was digested with *Pst*I and *Bam*HI and ligated to vector pUC19 digested with the same restriction endonucleases. The ligation product was transformed into *E. coli* strain DH5 α and the positive clone was screened. The pUC19 plasmid carrying *SYNPCC7002_A0128* was verified with restriction enzyme digestion and PCR amplification. *SYNPCC7002_A0128* contained in the pUC19 plasmid was interrupted at the *Eco*RV site with a kanamycin resistance gene (*aph*II) or a streptomycin resistance gene (*aad*A) (Figure 2-1). The constructs containing the *aph*II or *aad*A gene were verified by restriction digestion and PCR cloning. The pUC19-*SYNPCC7002_A0128::aph*II construct was transformed into the wild-type *Synechococcus* sp. PCC 7002 to generate a null mutant for *SYNPCC7002_A0128*. The pUC19-*SYNPCC7002_A0128::aad*A construct was transformed into the *suf*R null mutant of *Synechococcus* sp. PCC 7002 to generate the *SYNPCC7002_A0128 suf*R double mutant. Antibiotic resistant transformants were selected and grown in the A⁺ liquid medium with increasing concentration of antibiotics to allow for complete segregation of alleles. Segregation of the *SYNPCC7002_A0128*

single mutant was verified by PCR amplification of the *SYNPCC7002_A0128* locus. The *SYNPCC7002_A0128 sufR* double mutant did not segregate fully and was not studied furthering detail.

2.3.2 Cyanobacterial cell growth under normal and iron-limiting conditions

For growth measurements, the segregated *SYNPCC7002_A0128* null mutant and the wild-type strain of *Synechococcus* sp. PCC 7002 were grown in liquid A⁺ medium [10]. Kanamycin at a concentration of 100 µg ml⁻¹ was added to the growth medium for the mutant. The cultures were grown at 37° C and bubbled with air with or without 1% (v/v) CO₂ supplementation. Growth rates of the mutant and the wild-type cells were monitored at 730 nm. Iron-limiting conditions were induced by a three-fold serial subculture of the wild-type and the *SYNPCC7002_A0128* mutant in iron-depleted A⁺ medium with an inoculum to produce an initial OD_{730 nm} of 0.01.

2.3.3 RNA isolation

To isolate the total RNA from the cells grown in normal conditions, the wild type and the *SYNPCC7002_A0128* null mutant of *Synechococcus* sp. PCC 7002 were grown in the A⁺ medium and harvested when the OD_{730 nm} of the culture reached 1.0 (late exponential phase). To isolate the total RNA from cells grown under iron-limiting conditions, the wild type and the *SYNPCC7002_A0128* null mutant were serially

subcultured three times in the iron-depleted A^+ medium before harvesting at $OD_{730\text{ nm}} = 1$. Total RNA was isolated with a Mini to Midi RNA preparation kit (Invitrogen). Contaminating DNA was removed by incubating the RNA sample with RNase-free DNase for 1 h at room temperature. Complete removal of DNA was verified by PCR amplification. The concentration of the RNA sample was estimated by measuring the absorption at 260 nm and using the relationship $40\ \mu\text{g RNA ml}^{-1}$ has an $A_{260\text{ nm}} = 1$. Each RNA sample was then diluted accordingly to $5\ \text{ng ml}^{-1}$ for RT-PCR analysis.

2.3.4 RT-PCR analysis

RT-PCR was performed to compare the transcript levels of genes in the *isc* system and *suf* system in both the wild-type and the *SYNPCC7002_A0128* mutant under normal and iron-limiting growth conditions. For each reaction, 5 ng of total RNA was used as template. QIAGEN OneStep RT-PCR kit was used for each analysis. The RT-PCR reactions were programmed with the same method as described previously [11]. The RT-PCR products were separated by electrophoresis on a 1% (w/v) agarose gel and the intensities of the DNA bands were compared. The *rimM* gene, which encodes the 16S rRNA processing protein, was used as a control to ensure the same amount of RNA template was used for each sample.

2.3.5 Construction of an *SYNPCC7002_A0128* expression vector for overexpression in *E. coli*

A fragment containing *SYNPCC7002_A0128* was amplified by PCR from the genome of *Synechococcus* sp. PCC 7002. The *SYNPCC7002_A0128* fragment and vector pET11a were digested with *Nde*I and *Bam*HI and separated by 0.8% (w/v) agarose gel. The DNA fragments with the expected size were then excised from the gel. After ligation at 16° C over night, the mixture was transformed into DH10β *E. coli* competent cells. Clones that carried pET11a with the *SYNPCC7002_A0128* fragment were verified by PCR amplification and restriction enzyme digestion, and subsequently confirmed by DNA sequencing.

2.3.6 Over-expression and purification of the recombinant *SYNPCC7002_A0128* encoding protein in *E. coli*

The pET11a-*SYNPCC7002_A0128* was transformed into *E. coli* strain BL21 (DE3). The over-expression of the *SYNPCC7002_A0128* encoding protein in cyanobacteria was induced by 0.5 mM IPTG at $OD_{600\text{ nm}} = 0.6$ to 0.8. After an induction period of 4 hours, the cells were harvested and lysed with a French pressure cell at 10,000 psi and the cell lysate was centrifuged at 2,000 g for 10 min. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) showed that the *SYNPCC7002_A0128* protein had formed inclusion bodies. The inclusion bodies were solublized with 8 M urea, refolded

and purified over a Sepharose S-200 column. The purity of the *SYNPCC7002_A0128* protein was verified by SDS-PAGE.

2.4 Results

2.4.1 Identification of an *iscR* homologue in cyanobacteria *Synechococcus* sp.

PCC 7002

Genomic sequencing of several cyanobacteria, including *Synechococcus* sp. PCC 7002, has provided an invaluable tool for functional genomics. IscR homologues was identified to be *SYNPCC7002_A0128* in the genomes of *Synechococcus* sp. PCC 7002 and *slr0846* in *Synechocystis* sp. PCC 6803 by using the IscR sequence in *E. coli* as a query sequence in BLASTP analyses. The IscR homologues in cyanobacteria share ~40% identity and ~60% similarity with the IscR protein in *E. coli*. As shown in Figure 2-2, *SYNPCC7002_A0128* and other cyanobacterial IscR homologues display high sequence similarity with the IscR protein of *E. coli*, especially in the putative DNA-binding region at the N-terminus. However, the cysteine residues that are the proposed ligands for iron-sulfur cluster in IscR in *E. coli* are not present in the cyanobacterial IscR homologues.

2.4.2 *SYNPCC7002_A0128* is not essential in *Synechococcus* sp. PCC 7002

The DNA fragment containing *SYNPCC7002_A0128* was cloned into vector pUC19. The *SYNPCC7002_A0128* interruption construct was generated by the insertion of a kanamycin or streptomycin resistance cartridge into the coding sequence. The construct was transformed into *Synechococcus* sp. PCC 7002 wild-type cells. After streaking and selection in a liquid growth media with increasing antibiotic concentrations,

segregated transformants were obtained. The segregation of the *SYNPCC7002_A0128* allele was verified by PCR analysis (Figure 2-3). The comparison of the transformants and the wild type showed that the mutant allele had been fully segregated. Thus, *SYNPCC7002_A0128* was not required for viability in *Synechococcus* sp. PCC 7002 under normal growth conditions.

2.4.3 Physiological characterization of the wild-type and the mutant strains

To characterize the physiological properties of the *SYNPCC7002_A0128* mutant, growth under normal condition and iron-limiting conditions was studied. Under normal growth conditions, the *SYNPCC7002_A0128* mutant grew photoautotrophically with a rate similar to that of the wild-type. When the cells were grown under iron-limiting conditions, the growth phenotype of the *SYNPCC7002_A0128* mutant depended on whether the bubbling air was supplemented with CO₂ or not. If the cells were bubbled with air without CO₂ supplementation, the *SYNPCC7002_A0128* mutant grew much slower than the wild-type, with a doubling time of 32 hrs compared to 8 hrs for the wild-type (Figure 2-4). If the cells were bubbled with air supplemented with CO₂, the *SYNPCC7002_A0128* mutant exhibited a longer lag phase compared with the wild-type. But in the exponential phase, the growth rate for the *SYNPCC7002_A0128* mutant was similar to the wild-type (Figure 2-5). Although the mechanism leading to these different phenotypes was not known, it was clear that interruption of *SYNPCC7002_A0128* caused adverse effects on cyanobacteria growth under iron-limiting conditions.

2.4.4 Gene expression under normal growth conditions in wild-type and the *SYNPCC7002_A0128* null mutant

To examine the effect of inactivating *SYNPCC7002_A0128* on the molecular level, transcript levels of the *suf* and *isc* regulons were compared. Under normal growth conditions, the mRNA levels for the cysteine desulfurase genes, *iscS₁* and *iscS₂*, in the *SYNPCC7002_A0128* mutant were lower than those in the wild-type, while transcript levels of other *isc* genes did not change significantly (Figure 2-6). In the *suf* operon, the transcript levels for *sufB* increased in the mutant, while the mRNA levels of *sufC*, *sufD* and *sufS* did not change significantly (Figure 2-7). Interestingly, the transcript level of the *sufR* gene was lower in the *SYNPCC7002_A0128* mutant. These results suggested a functional link may exist between the *SYNPCC7002_A0128* protein and SufR in cyanobacteria under normal growth conditions.

2.4.5 Gene expression under iron-limiting growth conditions in the wild-type and the mutant strains

To examine the gene expression of the *SYNPCC7002_A0128* mutant under iron-limiting conditions, total RNA was isolated from cells of the third subculture grown in the iron free A⁺ medium. Contrary to the expression profile under normal conditions, the transcript levels for *iscS₁* and *iscS₂* increased rather than decreased in the *SYNPCC7002_A0128* mutant under iron-limiting conditions compared to the wild-type

(Figure 2-6). This indicated that the *SYNPCC7002_A0128* protein might be involved in the pathway that sensed the iron level in the growth medium and regulated the levels of *iscS₁* and *iscS₂* accordingly. The third desulfurase gene, *sufS* in the *suf* regulon, also had a higher expression level in the *SYNPCC7002_A0128* mutant than in the wild-type when grown under iron-limiting conditions. On the other hand, similar to the observation of the *SYNPCC7002_A0128* mutant under normal growth conditions, the mRNA level of *sufB* was higher and the level of *sufR* was lower in the *SYNPCC7002_A0128* mutant compared to the wild-type under iron-limiting conditions (Figure 2-7). This indicated that under both normal growth conditions and iron-limiting conditions, there might be a cross talk between the *isc* and *suf* systems.

2.4.6 Expression and purification of the *SYNPCC7002_A0128* encoding protein in *Escherichia coli*

The *SYNPCC7002_A0128* protein was isolated in inclusion bodies when overproduced in *E. coli* BL21 (DE3) cells. The inclusion bodies were resolubilized in 8 M urea. The *SYNPCC7002_A0128* protein was isolated with satisfactory purity by size-exclusion chromatography (Sephacrose S-200) at pH 8.0. In the SDS-PAGE gel, the purified *SYNPCC7002_A0128* protein migrated to the position similar to the predicted molecular mass of the protein, which was ~17 kDa (Figure 2-8).

2.5 Discussion

2.5.1 The role of *SYNPCC7002_A0128* in regulation of the *isc* operon and the *suf* operon in cyanobacteria

The change in the transcript levels of the *isc* genes and *suf* genes in the absence of *SYNPCC7002_A0128* indicated that *SYNPCC7002_A0128* was somehow involved in the regulation of genes involved in iron-sulfur cluster biogenesis. The regulatory role of the *SYNPCC7002_A0128* protein could involve either direct binding to DNA to activate/repress gene expression, or it could involve changing the functionality of other regulators. Although the helix-loop-helix domain at the N-terminus of the *SYNPCC7002_A0128* protein suggests that the *SYNPCC7002_A0128* protein is more likely to bind DNA and regulate gene expression directly, no firm conclusion can be drawn until evidence of DNA binding ability is obtained.

It was interesting that only a subset of the *isc* or *suf* genes showed altered transcript levels when *SYNPCC7002_A0128* encoding gene was deleted. It is known the *iscRSUA* operon in *E. coli* and the *sufBCDS* operon in cyanobacteria are regulated by IscR and SufR, respectively [3, 8]. The different regulation patterns of the *isc* genes in cyanobacteria after deletion of *SYNPCC7002_A0128* might be due to the fact that the *isc* genes in cyanobacteria are scattered in the genome and expressed from different promoters. With respect to the *suf* operon, only *sufB* showed a significant change in the transcript level when *SYNPCC7002_A0128* was deleted. This might be due to the limited resolution of the RT-PCR analyses. Quantitative methods with higher sensitivity in

quantifying RNA might be helpful in drawing conclusion about the transcription profile for the other *suf* genes.

Another interesting feature was the role of *SYNPCC7002_A0128* protein in regulating the cysteine desulfurase genes, *iscS₁* and *iscS₂*, in the *isc* system and *sufS* in the *suf* system. Cysteine desulfurases have been shown to play essential roles in iron-sulfur cluster biogenesis by acquiring sulfur from cysteine and transferring the sulfur to scaffold proteins [12]. Changes in the levels of the *iscS₁*, *iscS₂* and *sufS* genes would result in significant change in the capacity of the iron-sulfur cluster machinery. This implied that the *SYNPCC7002_A0128* protein might play a role in adjusting the biosynthetic capacity of iron-sulfur cluster.

2.5.2 Sequence comparison of the *E. coli* IscR and the IscR homologues in cyanobacteria

Although *SYNPCC7002_A0128* and IscR homologues in several other cyanobacteria share significant overall sequence homology (~40% identity and ~60% similarity) with IscR from *E. coli*, the levels of similarity in the N-terminus and the C-terminus are quite different. The similarity is relatively high at the N-terminus in the helix-loop-helix, DNA-binding region. This implies that all IscR and IscR homologues may have similar target DNA-binding sequences. On the other hand, the homology at the C-terminus is relatively low. In the IscR homologues in cyanobacteria, 16-17 amino acids are missing at the C-terminus compared with IscR in other microorganisms. Furthermore,

the cysteine residues conserved in non-cyanobacterial microorganisms are missing in the IscR homologues in cyanobacteria. Since these conserved cysteines have been shown to be ligands to the iron-sulfur cluster contained in IscR in *E. coli* [3], the absence of conserved cysteines argues that it is unlikely for the IscR homologues to harbor an iron-sulfur cluster. Their absence raises the question of how the cyanobacterial IscR homologues, including *SYNPCC7002_A0128*, sense iron level changes without the presence of an iron-sulfur cluster. One possibility is that the IscR homologues sense iron level changes via interactions with other sensor proteins containing iron-sulfur clusters, such as IscA and SufA.

2.5.3 Future directions for the project

This represents a preliminary study to search out an IscR homologue in cyanobacteria. *SYNPCC7002_A0128* was identified to be the homologue in *Synechococcus* sp. PCC 7002 through genomic analysis. This study showed that *SYNPCC7002_A0128* was involved in the regulation of the iron-sulfur cluster related genes and that the regulatory role was responsive to iron levels. However, there still remained the question whether the regulatory role of *SYNPCC7002_A0128* involved the binding of DNA or whether it altered the functionality of other regulators. More importantly, the study presented here was not sufficiently comprehensive to reach a conclusion concerning whether *SYNPCC7002_A0128* in cyanobacteria was *iscR* or simply another paralogous regulator. These questions cannot be answered until the regulatory mechanism of the *SYNPCC7002_A0128* protein is studied in more detail.

To investigate the regulation mechanism further, DNA-binding experiments would be a direct method to study the physical interaction between the *SYNPCC7002_A0128* protein and the *cis*-acting regulatory sequences for the *isc* genes and the *suf* genes. Physical characterization and RT-PCR experiments for the *SYNPCC7002_A0128* mutant grown under oxidative stress conditions can also be carried out to determine whether the *SYNPCC7002_A0128* protein in *Synechococcus* sp. PCC 7002 plays a similar role in oxidative stress as does IscR in *E. coli*. A pull down assay might help to identify proteins that interact with the *SYNPCC7002_A0128* protein, and thus provide a clue for the mechanism of sensing. Furthermore, the double mutant with deletion of both *SYNPCC7002_A0128* and *sufR*, if it can be segregated, can be used to study the interaction between the *SYNPCC7002_A0128* protein and SufR further.

2.6 References

1. Takahashi, Y. and U. Tokumoto, *A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids*. J Biol Chem, 2002. **277**(32): p. 28380-3.
2. Outten, F.W., O. Djaman, and G. Storz, *A suf operon requirement for Fe-S cluster assembly during iron starvation in Escherichia coli*. Mol Microbiol, 2004. **52**(3): p. 861-72.
3. Schwartz, C.J., et al., *IscR, an Fe-S cluster-containing transcription factor, represses expression of Escherichia coli genes encoding Fe-S cluster assembly proteins*. Proc Natl Acad Sci U S A, 2001. **98**(26): p. 14895-900.
4. Yeo, W.S., et al., *IscR acts as an activator in response to oxidative stress for the suf operon encoding Fe-S assembly proteins*. Mol Microbiol, 2006. **61**(1): p. 206-18.
5. Giel, J.L., et al., *IscR-dependent gene expression links iron-sulphur cluster assembly to the control of O₂-regulated genes in Escherichia coli*. Mol Microbiol, 2006. **60**(4): p. 1058-75.
6. Frazzon, J. and D.R. Dean, *Feedback regulation of iron-sulfur cluster biosynthesis*. Proc Natl Acad Sci U S A, 2001. **98**(26): p. 14751-3.
7. Yu, J., et al., *Suppressor mutations in the study of photosystem I biogenesis: sll0088 is a previously unidentified gene involved in reaction center accumulation in Synechocystis sp. strain PCC 6803*. J Bacteriol, 2003. **185**(13): p. 3878-87.

8. Wang, T., et al., *The sufR gene (sll0088 in Synechocystis sp. strain PCC 6803) functions as a repressor of the sufBCDS operon in iron-sulfur cluster biogenesis in cyanobacteria*. J Bacteriol, 2004. **186**(4): p. 956-67.
9. Balasubramanian, R., *Biogenesis, Assembly, and Function of Iron-Sulfur Clusters in Photosystem I*. 2006, The Pennsylvania State University, University Park.
10. Stevens, S.E. and R.D. Porter, *Transformation in Agmenellum quadruplicatum*. Proc Natl Acad Sci U S A, 1980. **77**(10): p. 6052-6056.
11. Balasubramanian, R., et al., *Regulatory roles for IscA and SufA in iron homeostasis and redox stress responses in the cyanobacterium Synechococcus sp. strain PCC 7002*. J Bacteriol, 2006. **188**(9): p. 3182-91.
12. Tirupati, B., et al., *Kinetic and structural characterization of Slr0077/SufS, the essential cysteine desulfurase from Synechocystis sp. PCC 6803*. Biochemistry, 2004. **43**(38): p. 12210-9.

Table 2–1 List of the oligonucleotides used for generating the pUC19-*SYNPCC7002_A0128* construct.

Table 2–1: List of the oligonucleotides used for generating the pUC19-*SYNPCC7002_A0128* construct

5' - 3'	Primer Sequence
<i>SYNPCC7002_A0128</i> F	TGC TAT CTA ACT GCA GCC CTT CTG CTT CG
<i>SYNPCC7002_A0128</i> R	GCT AAC AGC ACA GGA TCC AGA TTT TAG GT

Figure 2-1 The physical map of the *SYNPCC7002_A0128* showing the site of insertion of an antibiotic resistance cartridge (*aphII* or *aadA*). The DNA fragment containing *SYNPCC7002_A0128* was amplified by PCR with genomic DNA of wild-type *Synechococcus* sp. PCC 7002 as template. The 1.26-kb PCR product was digested with *Pst*I and *Bam*HI and ligated to vector pUC19 digested with the same restriction endonucleases. The kanamycin resistance gene, *aphII*, or the streptomycin resistance gene, *aadA*, was then inserted into the the *Eco*RV site contained in *SYNPCC7002_A0128* in the pUC19-*SYNPCC7002_A0128* plasmid.

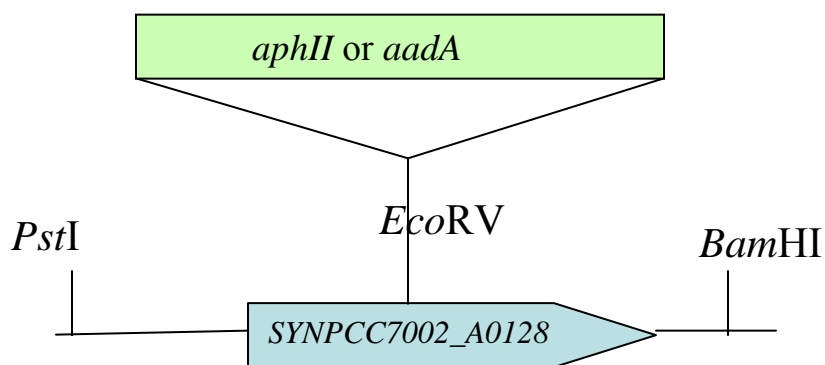


Figure 2-1: The physical map of the *SYNPCC7002_A0128* showing the site of insertion of an antibiotic resistance cartridge (*aphII* or *aadA*).

Figure 2-2 Sequence alignment of IscR homologues. 1) *SYNPCC7002_A0128* in cyanobacterium *Synechococcus* sp. PCC 7002; 2) *slr0846* in cyanobacterium *Synechocystis* sp. PCC 6803; 3) *alr2081* in cyanobacterium *Nostoc* sp. strain PCC 7120; 4) *tlr0169* in cyanobacterium *Thermosynechococcus elongatus* BP-1; 5) *glr1920* in cyanobacterium *Gloeobacter violaceus* PCC 7421; 6) *cog1959* in cyanobacterium *Crocospaera watsonii* WH 8501; 7) *caur_3222* in *Chloroflexus aurantiacus*; 8) *ct1702* in *Chlorobium tepidum*; 9) YfhP in *Salmonella enterica*; 10) *pp0841* in *Pseudomonas putida*; 11) IscR in *Xenorhabdus nematophila*; 12) IscR in *Escherichia coli*.

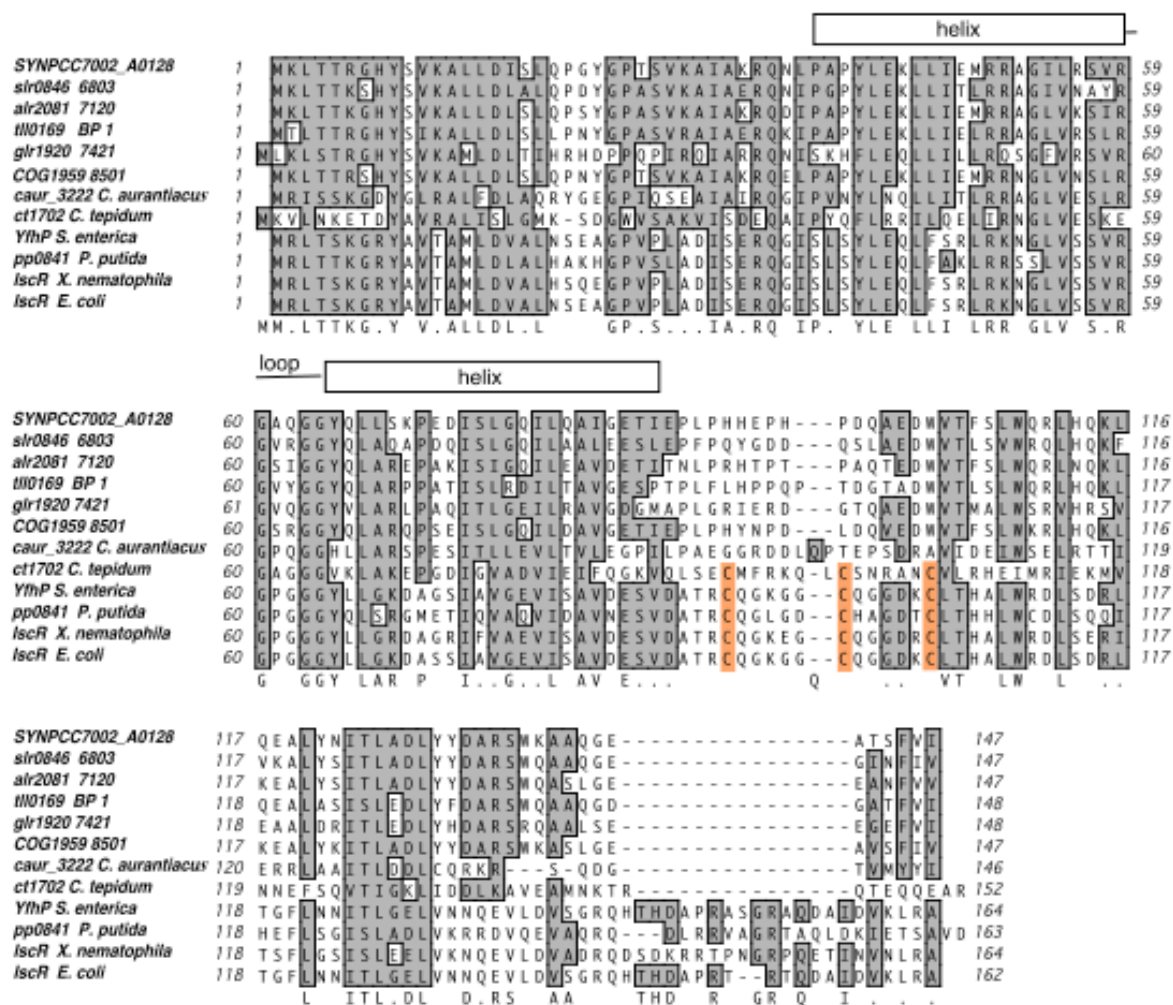


Figure 2-2: Sequence alignment of IscR homologues.

Figure 2-3 Segregation analysis of the *SYNPCC7002_A0128* mutants in *Synechococcus* sp. PCC 7002. Lane 1 and lane 4 contain DNA size markers. Lane 2 shows the product amplified by PCR from the *SYNPCC7002_A0128* locus with wild-type DNA as template. Lane 3 shows product amplified by PCR from the *SYNPCC7002_A0128* locus from a kanamycin resistant transformant. The fragment is 1.3 kb larger due to the insertion of the DNA fragment encoding the aminoglycoside phosphotransferase II gene (*aphII*), which confers resistance to kanamycin.

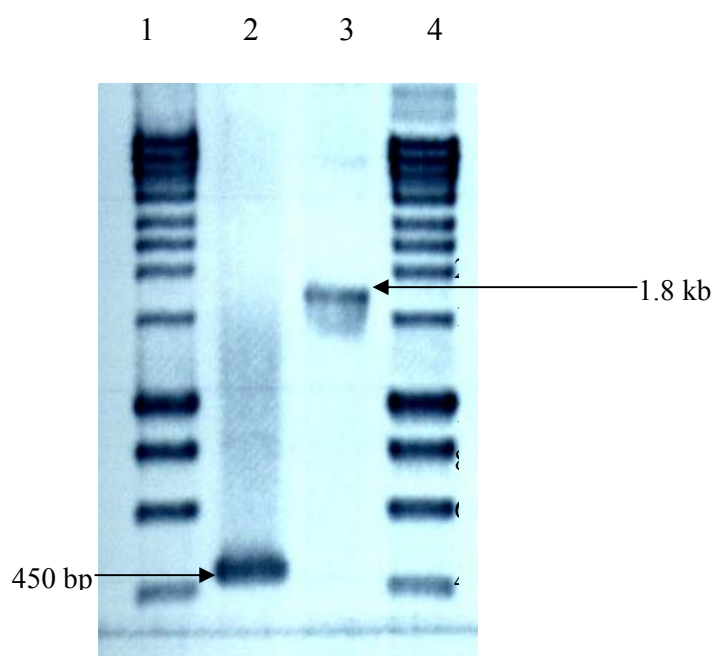


Figure 2-3: Segregation analysis of the *SYNPCC7002_A0128* mutants in *Synechococcus* sp. PCC 7002.

Figure 2-4 Growth curves for the *SYNPCC7002_A0128* null mutant and the wild-type under iron-limiting conditions without CO₂ supplementation of the bubbling air. The *SYNPCC7002_A0128* null mutant grew much slower compared to wild-type cells bubbled with air. The doubling time for the *SYNPCC7002_A0128* mutant was 32 h, while the doubling time for the wild-type was 8 h.

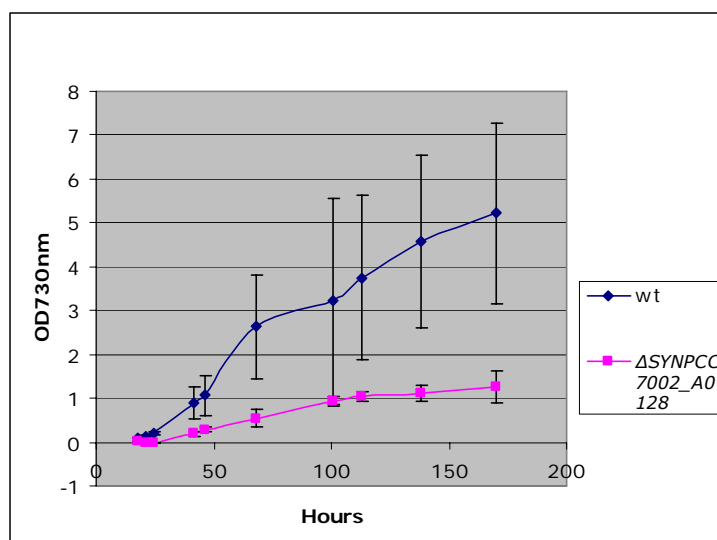


Figure 2-4: Growth curves for the *SYNPCC7002_A0128* null mutant and the wild-type under iron-limiting conditions without CO₂ supplementation of the bubbling air.

Figure 2-5 Growth curve under iron-limiting conditions for cultures bubbled with air supplemented with 1% (v/v) CO₂. The *SYNPCC7002_A0128* null mutant grew slower under iron-limiting conditions during the first 50 hours. The OD_{730 nm} for the *SYNPCC7002_A0128* null mutant was only 0.5 when the wild-type reached OD_{730 nm} = 1.3. However, the *SYNPCC7002_A0128* null mutant grew faster and with a rate comparable to that of the wild-type in the subsequent 50-hour period of growth.

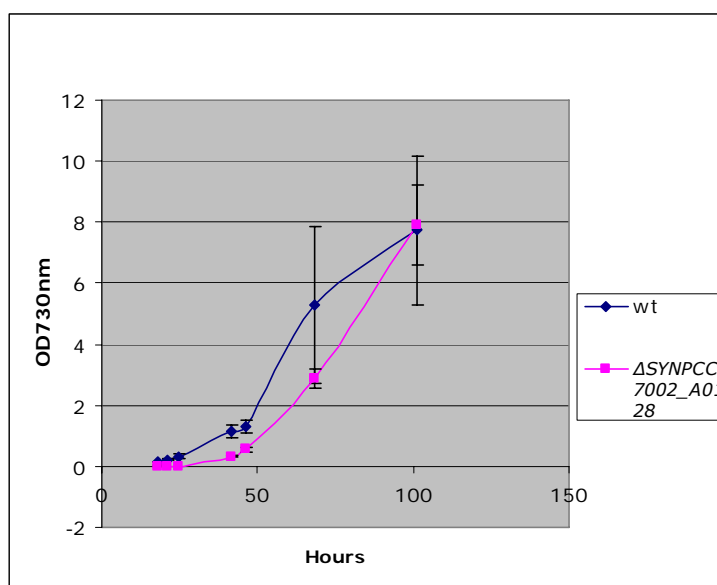


Figure 2-5: Growth curve under iron-limiting conditions for cultures bubbled with air supplemented with 1% (v/v) CO₂.

Figure 2-6 Transcript levels of the *isc* genes in the wild-type and the *SYNPCC7002_A0128* null mutant of *Synechococcus* sp. PCC 7002 grown under normal conditions and iron-limiting conditions. The *rimM* gene was used as a loading control for the total RNA amount loaded for the RT-PCR reaction. Significant changes in transcript levels for *iscS₁* and *iscS₂* were observed for *SYNPCC7002_A0128* null mutant grown under both normal conditions and iron-limiting conditions. However, under normal growth conditions, the transcript levels for *iscS₁* and *iscS₂* were lower than the wild-type, while in iron-limiting conditions, the transcript levels for *iscS₁* and *iscS₂* were higher in the mutant than in the wild-type. (Note: The RT-PCR result for *hscA* was not analyzed under iron-limiting condition.)

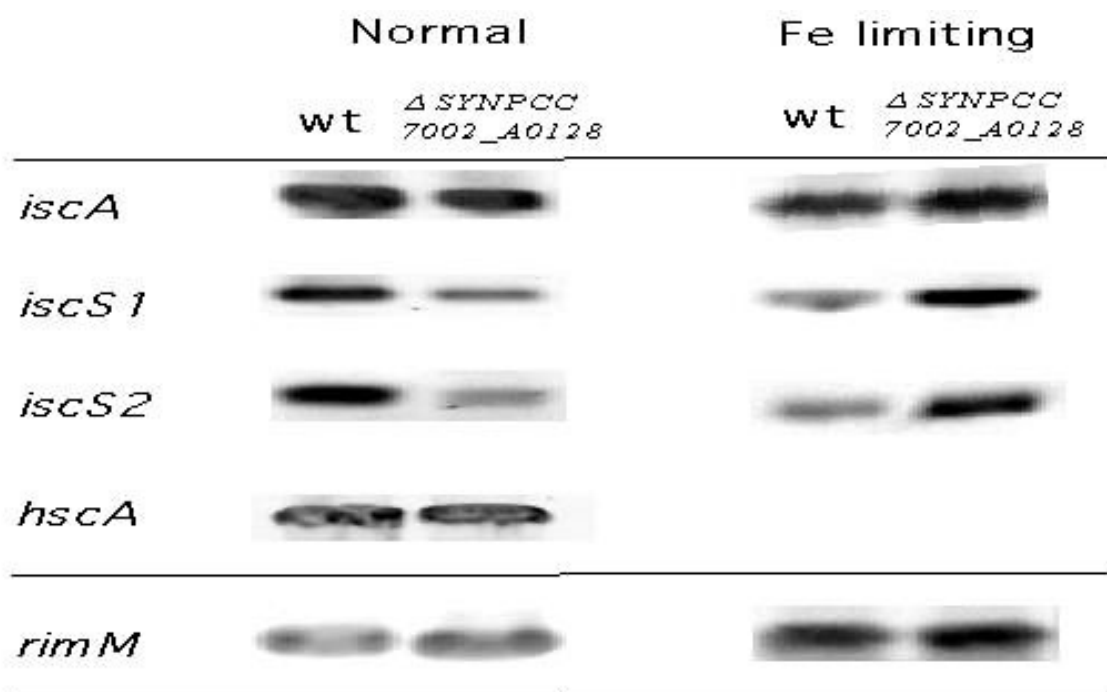


Figure 2-6: Transcript levels of the *isc* genes in the wild-type and the *SYNPCC7002_A0128* null mutant of *Synechococcus* sp. PCC 7002 grown under normal conditions and iron-limiting conditions.

Figure 2-7 Transcript levels of the *suf* genes in the wild type and the *SYNPCC7002_A0128* null mutant under normal conditions and iron-limiting conditions. The *rimM* gene was used as a control for the total RNA amount loaded for RT-PCR reaction. Significant changes in transcription levels for *sufR* and *sufB* were observed for *SYNPCC7002_A0128* null mutant grown under normal conditions, and for *sufR*, *sufB* and *sufS* under iron-limiting conditions. Under both normal and iron-limiting growth conditions, the transcript level for *sufR* decreased, and the transcript level for *sufB* increased in the *SYNPCC7002_A0128* null mutant. Under iron-limiting conditions, the transcript level for *sufS* was higher in the mutant than in the wild type.

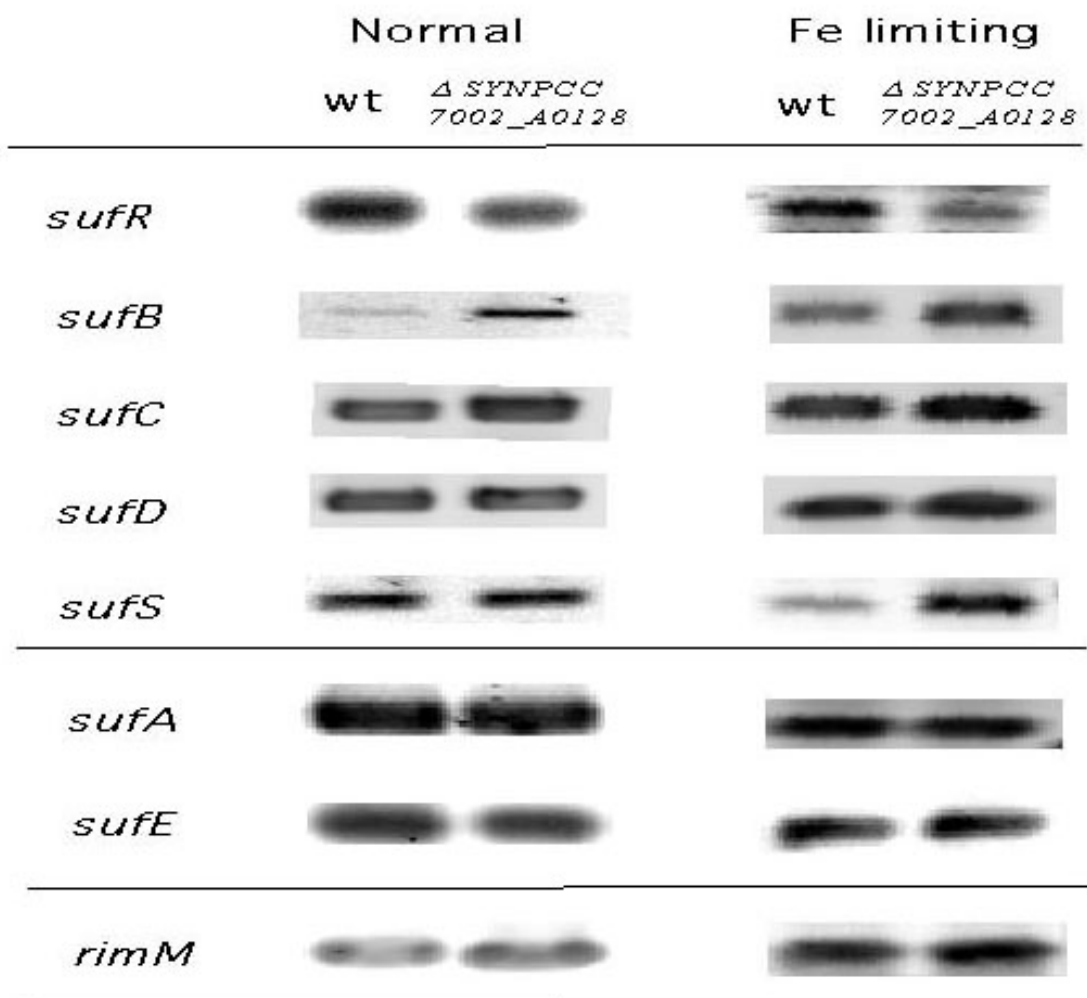


Figure 2-7: Transcript levels of the *suf* genes in the wild type and the *SYNPCC7002_A0128* null mutant under normal conditions and iron-limiting conditions.

Figure 2-8 SDS-PAGE analysis of the purified protein encoded by *SYNPCC7002_A0128*. Lane 1 was loaded with 10 μ g of the purified *SYNPCC7002_A0128* protein from gel filtration chromatography. Lane 2 was loaded with molecular mass markers, some of whose masses are indicated to the right. As indicated by the arrow, the overexpressed and purified *SYNPCC7002_A0128* had molecular mass of \sim 17 kDa.

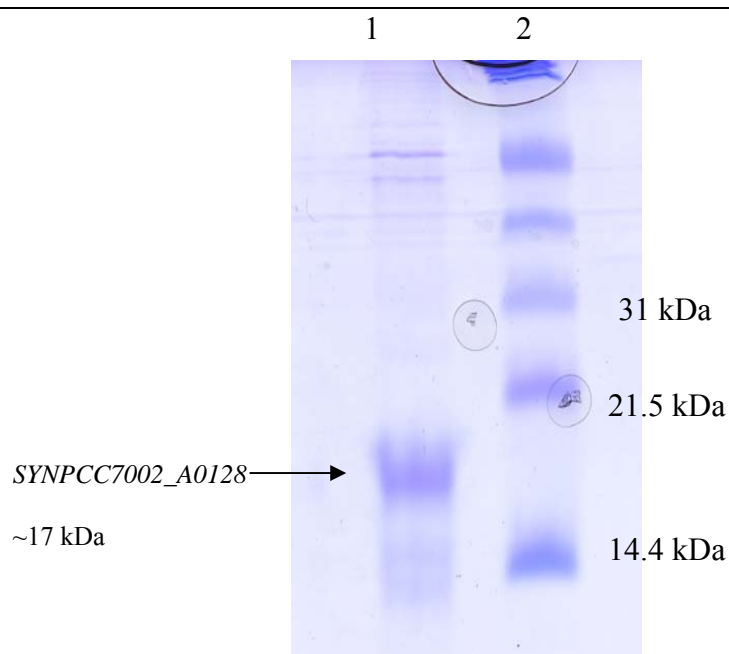


Figure 2-8: SDS-PAGE analysis of the purified protein encoded by *SYNPCC7002_A0128*.

Chapter 3

SufR coordinates two $[4\text{Fe-4S}]^{2+,1+}$ clusters and the $[4\text{Fe-4S}]^{2+,1+}$ clusters exist in $S = 1/2$ and $S = 3/2$ ground spin states

In print as: Shen G, Balasubramanian R, Wang T, Wu Y, Hoffart LM, Krebs C, Bryant DA, Golbeck JH. (2007) SufR coordinates two $[4\text{Fe-4S}]^{2+,1+}$ clusters and functions as a transcriptional repressor of the *sufBCDS* operon and an autoregulator of *sufR* in cyanobacteria. *J Biol Chem* 282 (44): 31909-31919.

3.1 Abstract

The *sufR* gene (*sl10088* in cyanobacterium *Synechocystis* sp. PCC 6803) encodes a protein proposed to be a transcriptional regulator. It is located directly upstream of the *sufBCDS* operon in several sequenced cyanobacterial genomes. The SufR protein has been shown to coordinate $[4\text{Fe-4S}]^{2+,1+}$ clusters by EPR and Mössbauer spectroscopy. The DNA binding ability of SufR is dependent on the presence and the oxidation state of the $[4\text{Fe-4S}]^{2+,1+}$ cluster. In this chapter, I continued the process of characterizing the $[4\text{Fe-4S}]^{2+,1+}$ cluster in the SufR holo protein. Chemical analysis and spectroscopic analyses showed that SufR coordinated two $[4\text{Fe-4S}]^{2+,1+}$ clusters per dimer. EPR spectroscopy revealed that reduced $[4\text{Fe-4S}]^{2+,1+}$ cluster in SufR existed in a mixture of $S = 1/2$ and $S = 3/2$ ground spin states, with the $S = 1/2$ state accounting for 44% of the total spins. The iron-sulfur cluster stoichiometry, the existence of the high-spin state, together with the previous discovery that the C191S variant has unaltered EPR spectrum around the $g = 2$ region, indicate that C191 is unlikely to provide a ligand for the cluster. Thus, there is one non-cysteine ligand that binds each $[4\text{Fe-4S}]^{2+,1+}$ cluster in SufR

3.2 Introduction

Iron-sulfur clusters play versatile roles in metabolism, including nitrogen fixation, photosynthesis, respiration and gene regulation [1, 2]. Three genetic systems, the *nif* system, the *isc* system and the *suf* system, account for iron-sulfur cluster biogenesis in bacteria, archaea and plant plastids. In photosynthetic bacteria and plant plastids, the *suf* regulon plays the primary role in iron-sulfur cluster biogenesis [3]. In the cyanobacteria *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002, the *suf* regulon consists of the *sufBCDS* operon, *sufR*, *sufA* and *sufE*.

The *sufR* gene (*sl10088* in the cyanobacterium *Synechocystis* sp. PCC 6803) encodes a protein that has been proposed to function as a transcriptional regulator. It is located directly upstream of the *sufBCDS* operon in several sequenced cyanobacterial genomes. Mutations in this gene rescue photosynthetic competence of a *psaC* mutant (C14S) [4, 5]. RT-PCR analysis reveals that deletion of the *sufR* results in increased transcription levels of the *sufB*, *sufC*, *sufD* and *sufS* genes in *Synechococcus* sp. PCC 7002 [4, 5].

A former member of our research group discovered that SufR in *Synechocystis* sp. PCC 6803 contains 216 rather than 240 amino acids [3]. SufR is predicted to have two major domains, a helix-loop-helix domain at the N-terminus and an iron-sulfur cluster binding domain containing conserved cysteines at the C-terminus. The DNA binding ability of SufR implied by the helix-loop-helix domain has been confirmed by gel mobility-shift assays [3]. DNaseI footprinting shows that both holo-SufR and reduced apo-SufR can bind the operator region between the divergently transcribed *sufR* gene and

the *sufBCDS* operon. Holo-SufR has been shown to bind to two sites, a high-affinity binding site which controls the *sufBCDS* operon and a low-affinity site which controls *sufR* expression. Apo-SufR only binds to the low affinity sequence involved in SufR auto-regulation [3].

EPR and Mössbauer spectroscopy show the presence of a stable $[4\text{Fe-4S}]^{2+,1+}$ cluster in the chemically reconstituted recombinant SufR protein [3]. Gel filtration chromatography and non-denaturing polyacrylamide gel electrophoresis showed that both holo-SufR and apo-SufR are dimeric, indicating that the presence of the $[4\text{Fe-4S}]^{2+,1+}$ cluster does not affect its oligomeric state [3]. The four conserved cysteines, in the motif $\text{CX}_{12}\text{CX}_{13}\text{CX}_{14}\text{C}$ at the C-terminus of SufR, are potential candidates for ligands of the $[4\text{Fe-4S}]^{2+,1+}$ cluster. Although all four cysteines are conserved in cyanobacteria, only C164, C177 and C206 are conserved in non-photosynthetic bacteria. The absence of C191 in non-photosynthetic bacteria implies that C191 may play a different role than C164, C177 and C206. A divergent function of C191 is further supported by an EPR spectroscopic study on four SufR variants (C164S, C177S, C191S and C206S). The C164S, C177S and C206S SufR variants exhibit an altered EPR spectrum of the $[4\text{Fe-4S}]^{2+,1+}$ cluster around the $g = 2$ region compared with the wild type, but the spectrum of the $[4\text{Fe-4S}]^{2+,1+}$ cluster in the C191S variant is the same as the wild type [3].

In this work, I continued to characterize the $[4\text{Fe-4S}]^{2+,1+}$ cluster in holo-SufR. My work showed that one $[4\text{Fe-4S}]^{2+,1+}$ cluster per polypeptide was present in the dimeric SufR protein. Both $S = 1/2$ and $S = 3/2$ ground states were present for the reduced $[4\text{Fe-4S}]^{1+}$ cluster in SufR. However, only the $S = 1/2$ ground spin state was quantified.

3.3 Materials and methods

3.3.1 Protein purification and quantitation

The wild-type SufR protein was expressed in *E. coli* strain BL21(DE3) and purified using a procedure described previously [5]. The purity of the protein was verified by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. The protein concentration was measured using the Bradford assay [6]. A correction factor for the Bradford assay was estimated by calculating the ratio of the concentration determined by quantitative amino acid analysis of SufR (amino acid facility, University of Iowa) with the concentration measured by the Bradford assay using albumin standard (BSA). A correction factor of 1.36:1 was applied for SufR.

3.3.2 Reconstitution of the iron-sulfur cluster in the SufR protein

The purified recombinant SufR protein was chemically reconstituted with iron-sulfur clusters using a procedure described earlier [5]. The reconstituted SufR protein was concentrated by ultrafiltration over a 10-kDa cut-off membrane (Millipore). Excess iron and sulfide were removed by repeated dilution of the protein with Tris-HCl buffer (50 mM, pH 8.3) and by concentrating the diluted sample by ultrafiltration. UV-Vis spectroscopy and EPR spectroscopy were carried out to verify the presence of iron-sulfur clusters in the reconstituted, concentrated SufR.

3.3.3 Chemical analysis

The iron and sulfide content of the reconstituted SufR protein was measured using a procedure described previously [3, 7]. Ferrous ammonium sulfate and sodium sulfide were used as standards for the iron analysis and sulfide analysis, respectively. Different dilutions of standard and sample were measured. The iron and sulfide concentrations of the sample were determined by comparing the slope of the dilution curve of the sample with the slope of the standard. For both iron analysis and sulfide analysis, multiple analyses were conducted to ensure accuracy.

3.3.4 Electron Paramagnetic Resonance (EPR) spectroscopy and spin quantitation

Reconstituted SufR was reduced with sodium hydrosulfite prior to analysis by EPR spectroscopy. The EPR spectrum of SufR was recorded using a Bruker ECS 106-X-band spectrometer. The spectrum for the $S = 1/2$ ground spin state $[4\text{Fe-4S}]^{1+}$ cluster was taken at a temperature of 16 K and a microwave power of 50 mW [3]. The spectrum for the $S = 3/2$ ground spin state cluster $[4\text{Fe-4S}]^{1+}$ cluster was taken at 5 K and a microwave power of 126 mW.

Spin quantitation of the $S = 1/2$ ground spin $[4\text{Fe-4S}]^{1+}$ clusters in SufR was carried out by using the well-characterized $[4\text{Fe-4S}]^{2+,1+}$ protein, PsaC, as standard [8]. Both the PsaC standard and the SufR sample were treated with 15 mM dithionite to completely reduce the iron-sulfur clusters for EPR spectroscopy. The EPR spectra of PsaC and SufR were recorded at a temperature of 16 K and microwave powers of 1 mW, 10 mW, 25 mW and 50 mW. The number of spins in the $S = 1/2$ ground spin state iron-

sulfur cluster was estimated by measuring the area under the integrated EPR signal. The percentage of the iron-sulfur clusters in the $S = 1/2$ spin state was calculated by comparing the number of spins in SufR and PsaC and measuring the iron-sulfur cluster concentrations by the optical absorbance at 400 nm.

3.4 Results

3.4.1 The correction factor for measuring the concentration of SufR from *Synechocystis* sp. PCC 6803

The correction factor for SufR was calculated by dividing the concentration based on amino acid analysis by the concentration based on the Bradford assay using BSA as the standard. Three measured correction factors are listed in Table 3-1. The average correction factor was 1.36 ± 0.27 .

3.4.2 Holo-SufR coordinates two iron-sulfur clusters per dimer

Iron and sulfide analyses along with protein concentration measurements on two independent preparations of the holo-SufR protein resulted in similar values of iron and sulfide per polypeptide. The average iron to protein ratio for SufR of the two preparations was 3.7, while the sulfide to protein ratio was 3.5 (Table 3-2). This indicated that holo-SufR coordinated one $[4\text{Fe-4S}]^{2+,1+}$ cluster per polypeptide, or two $[4\text{Fe-4S}]^{2+,1+}$ clusters per dimer.

The cluster to protein ratio was confirmed by estimating the concentration of oxidized $[4\text{Fe-4S}]^{2+}$ cluster. The concentrations of the oxidized clusters were determined by the optical absorbance at 400 nm. The extinction coefficient used for an oxidized $[4\text{Fe-4S}]^{2+}$ clusters was $15,000 \text{ cm}^{-1}\text{M}^{-1}$ [9]. The protein concentration of SufR was measured using the Bradford assay with BSA as the standard. The average cluster to protein ratio calculated by the method was 0.93 (Table 3-2). Thus, both chemical analyses and the

oxidized $[4\text{Fe-4S}]^{2+}$ cluster concentration of holo-SufR indicated that SufR contained one $[4\text{Fe-4S}]^{2+,1+}$ cluster per polypeptide.

3.4.3 The $[4\text{Fe-4S}]^{1+}$ cluster in SufR exists in both $S = 1/2$ and $3/2$ ground spin states

At a temperature of 5 K and a microwave power of 126mW, a broad and intense EPR signal appeared between $g = 5$ and 6 in addition to the known set of resonances around $g = 2$ (Figure 3-1). These EPR signals were similar to the $[4\text{Fe-4S}]$ cluster in the Fe protein of nitrogenase from *Azotobacter vinelandii* [10], which existed in a mixture of $S = 1/2$ and $S = 3/2$ ground spin states. There were no other resonances between the signal at $g = 5$ and 6 and the signal around $g = 2$. Thus, the $[4\text{Fe-4S}]^{1+}$ cluster in SufR exists exclusively as a mixture of ground spin states $S = 1/2$ and $S = 3/2$.

3.4.4 The $S = 1/2$ ground spin state $[4\text{Fe-4S}]^{2+,1+}$ cluster accounts for 44% of the total spin concentration

Spin quantitation for the $S = 1/2$ ground spin state in SufR was estimated by comparing the area under integrated EPR signal around $g = 2$ with that of the PsaC standard. Conditions were: temperature of 16 K and microwave powers of 1 mW, 10 mW, 25 mW and 50 mW. It has long been known that PsaC binds two $S = 1/2$ ground spin state $[4\text{Fe-4S}]^{2+,1+}$ clusters [8]. Thus, by comparing the total number of spins in the

SufR sample with that of the PsaC sample at identical $[4\text{Fe-4S}]^{2+,1+}$ cluster concentrations (determined by optical absorbance 400 nm), the percentage of the clusters in $S = 1/2$ ground spin state in SufR could be estimated. As shown in Table 3-3, those clusters in the $S = 1/2$ ground spin states accounted for 44% of the $[4\text{Fe-4S}]^{2+,1+}$ clusters in SufR.

3.4.5 The $[4\text{Fe-4S}]^{2+,1+}$ cluster in SufR may have a non-cysteine ligand

The presence of one $[4\text{Fe-4S}]^{2+,1+}$ per polypeptide implies that four ligands are provided by each polypeptide. The four cysteine residues at the C-terminus of SufR in the form of $\text{CX}_{12}\text{CX}_{13}\text{CX}_{14}\text{C}$ are potential candidates. However, contrary to the assumption that all four cysteines serve as ligands, the EPR spectrum of the single cysteine variants of SufR (C164S, C177S, C191S and C206S) revealed altered EPR signal in the $g = 2$ region only for the C164S, C177S and C206S variants, and not for the C191S variant. This implies that the $[4\text{Fe-4S}]^{2+,1+}$ cluster can be assembled without C191 [3]. Thus, C191S is unlikely to be a ligand for the $[4\text{Fe-4S}]^{2+,1+}$ cluster. Either a non-cysteine residue or a small molecule, such as H_2O , must serve as the fourth ligand. The non-cysteine hypothesis is further substantiated by the existence of $S = 3/2$ ground spin state for $[4\text{Fe-4S}]^{2+,1+}$ in SufR, which is commonly observed in iron-sulfur proteins that have a non-cysteine ligand. Thus, I conclude that SufR is ligated by three of the four cysteines (C164, C177 and C206) at the C-terminus and one non-cysteine residue or a small molecule.

3.5 Discussion

3.5.1 The non-cysteine ligand for the $[4\text{Fe-4S}]^{2+,1+}$ cluster

Characterization of cysteine to serine variants of SufR suggests that C191 is not a ligand for the $[4\text{Fe-4S}]^{2+,1+}$ cluster. This conclusion is also supported by the fact that C191 is conserved only among SufR homologues in cyanobacteria. With the single exception of the SufR homologue from *Vibrio cholerae*, there is no cysteine in this position or in any other nearby position in predicted SufR-like proteins from non-photosynthetic bacteria (Figure 3-2). Thus, it is likely that there is one non-cysteine ligand to the $[4\text{Fe-4S}]^{2+,1+}$ cluster.

The existence of a non-cysteine ligand is consistent with the fact that the $[4\text{Fe-4S}]^{2+,1+}$ cluster in SufR exists in a mixture of $S = 1/2$ and $S = 2/3$ ground spin states. Those proteins that contain $[4\text{Fe-4S}]^{2+,1+}$ clusters with a non-cysteine ligand usually display high ground spin states. *Pyrococcus furiosus* ferredoxin is a known example of a ferredoxin containing a single $[4\text{Fe-4S}]^{2+,1+}$ cluster that has three cysteines and one aspartic acid as ligands [11]. In the reduced form, the cluster exists with a spin mixture of $S = 1/2$ (20%) and $S = 3/2$ (80%) ground spin states. The 8Fe-8S ferredoxin III from *Desulfovibrio africanus* is a monomeric protein that contains two $[4\text{Fe-4S}]^{2+,1+}$ clusters, one of which is coordinated by one aspartate and three cysteines [12]. This cluster has a mixture of $S = 1/2$ and $S = 3/2$ ground spin states when reduced. The iron-sulfur cluster is labile and can readily and reversibly lose one Fe under oxidative conditions to yield a $[3\text{Fe-4S}]^{1+,0}$ cluster. If the aspartate is changed to a cysteine, the protein contains two

$[4\text{Fe-4S}]^{2+,1+}$ clusters, with only a $S = 1/2$ ground spin state represented in the reduced form [13]. In PsaC, a spin-state crossover from $S = 1/2$ to higher ground spin states occurs when the second cysteine in the CXXCXXCXXXCP motif of PsaC is changed to either serine or aspartic acid [14]. All of these examples connect the presence of the non-cysteine ligand to the occurrence of a high ground-spin state.

The identity of the fourth ligand to the $[4\text{Fe-4S}]^{2+,1+}$ cluster in cyanobacterial and proteobacterial SufR remains unknown, although one potential candidate is the near-conserved glutamate residue (one exception is SufR in *Corynebacterium glutamicum*) that follows two residues after the 2nd conserved cysteine residue (Figure 3-2).

3.5.2 Comparison of SufR with other iron-sulfur cluster containing transcriptional regulators

The transcription factors, FNR, SoxR and IscR also employ iron-sulfur clusters as sensors [15-17]. A comparison of SufR with these transcriptional regulators reveals some features common to iron-sulfur cluster containing regulators.

SufR shares functional and structural features with the oxygen regulator FNR in *Bacillus subtilis*. Both have a helix-loop-helix domain at the N-terminus and a sensory domain with conserved cysteine residues at the C-terminus, and both sense redox changes via the $[4\text{Fe-4S}]^{2+,1+}$ cluster [15]. Both form a homodimer, and the dimeric form does not depend on the presence of the cluster [15]. A comparison of the UV and visible spectra of

the FNR and SufR confirms the conclusion of one iron-sulfur cluster per polypeptide in SufR. The absorption spectra of the holo-proteins show a ratio of $A_{420}/A_{280} = 0.42$ for FNR and 0.33 for SufR (Table 3-4) [15]. Considering that the molar extinction coefficient at 280 nm for SufR is 1.33 times as that of FNR (calculated on a per protein basis using ProtParam), the protein-to-cluster ratios for FNR and SufR are nearly identical (1:1.0). One $[4\text{Fe-4S}]^{2+,1+}$ cluster per protein monomer is known to be present in FNR [15]. Hence, this analysis is consistent with our assessment that one $[4\text{Fe-4S}]^{2+,1+}$ cluster per protein monomer is present in SufR. The similarities between SufR and FNR can be extended to the ligands for the $[4\text{Fe-4S}]^{2+,1+}$ cluster. Both proteins are proposed to contain three cysteine ligands and one non-cysteine ligand to the $[4\text{Fe-4S}]^{2+,1+}$ cluster, although the nature of the non-cysteine ligand is unclear in both SufR and FNR [15]. It is also interesting that at least two of these features shared by SufR and FNR in *B. subtilis* are not shared by FNR in *E. coli*, which has an N-terminal, rather than a C-terminal, extension rich in cysteine residues, and a dimeric oligomeric state that is dependent on the presence of the iron-sulfur cluster [15, 18].

IscR in *E. coli* has been found to form a homodimer and binds a $[2\text{Fe-2S}]^{2+,1+}$ cluster [16]. Similar to SufR, the DNA-binding ability of IscR in *E. coli* depends on the presence of the iron-sulfur cluster [16]. Furthermore, there are only three cysteines in the protein sequence, which implies that a non-cysteine ligand may also be present in IscR [16]. Thus, the feature of a non-cysteine ligand is shared among all three transcriptional regulators, IscR, SufR, and FNR. This feature might make the clusters more labile and facilitate their function as sensors to respond to oxidative stress and cluster abundance.

SoxR is a homodimer containing a pair of $[2\text{Fe-2S}]^{2+,1+}$ clusters. Similar to other transcriptional regulators, its activity is controlled by its $[2\text{Fe-2S}]^{2+,1+}$ cluster [17, 19-21]. However, unlike the other regulators discussed above, SoxR is activated by the redox potential of the $[2\text{Fe-2S}]^{2+,1+}$ cluster rather than the presence of the cluster [19]. Apo-SoxR and reduced holo-SoxR can still specifically bind DNA without activating transcription. It should be noted that, unlike the other regulators, the $[2\text{Fe-2S}]^{2+,1+}$ clusters in SoxR are ligated by four cysteines [22]. The different pattern of residues ligating the iron-sulfur cluster in SoxR may be related to its different regulatory role.

Thus, the existence of a protein homodimer and the presence of two $[2\text{Fe-2S}]^{2+,1+}$ clusters or two $[4\text{Fe-4S}]^{2+,1+}$ clusters per dimer may be common features in iron-sulfur cluster-containing transcription factors, including SufR.

3.6 References

1. Johnson, M.K., *Iron-sulfur proteins: new roles for old clusters*. *Curr Opin Chem Biol*, 1998. **2**(2): p. 173-81.
2. Beinert, H. and P.J. Kiley, *Fe-S proteins in sensing and regulatory functions*. *Curr Opin Chem Biol*, 1999. **3**(2): p. 152-7.
3. Balasubramanian, R., *Biogenesis, Assembly, and Function of Iron-Sulfur Clusters in Photosystem I*. 2006, The Pennsylvania State University, University Park.
4. Yu, J., et al., *Suppressor mutations in the study of photosystem I biogenesis: sll0088 is a previously unidentified gene involved in reaction center accumulation in Synechocystis sp. strain PCC 6803*. *J Bacteriol*, 2003. **185**(13): p. 3878-87.
5. Wang, T., et al., *The sufR gene (sll0088 in Synechocystis sp. strain PCC 6803) functions as a repressor of the sufBCDS operon in iron-sulfur cluster biogenesis in cyanobacteria*. *J Bacteriol*, 2004. **186**(4): p. 956-67.
6. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*. *Anal Biochem*, 1976. **72**: p. 248-54.
7. Golbeck, J.H. and B. Kok, *Further studies of the membrane-bound iron-sulfur proteins and P700 in a photosystem I subchloroplast particle*. *Arch Biochem Biophys*, 1978. **188**(2): p. 233-42.
8. Hoj, P.B., et al., *Identification of a chloroplast-encoded 9-kDa polypeptide as a [4Fe-4S] protein carrying centers A and B of photosystem I*. *J Biol Chem*, 1987. **262**(26): p. 12676-84.

9. Antonkine, M.L., et al., *Chemical rescue of a site-modified ligand to a [4Fe-4S] cluster in PsaC, a bacterial-like dicluster ferredoxin bound to Photosystem I.* Biochim Biophys Acta, 2007. **1767**(6): p. 712-24.
10. Lindahl, P.A., et al., *Mossbauer, EPR, and magnetization studies of the Azotobacter vinelandii Fe protein. Evidence for a [4Fe-4S]1+ cluster with spin S = 3/2.* J Biol Chem, 1985. **260**(20): p. 11160-73.
11. Conover, R.C., et al., *Spectroscopic characterization of the novel iron-sulfur cluster in Pyrococcus furiosus ferredoxin.* J Biol Chem, 1990. **265**(15): p. 8533-41.
12. George, S.J., et al., *Electrochemical and spectroscopic characterization of the conversion of the 7Fe into the 8Fe form of ferredoxin III from Desulfovibrio africanus. Identification of a [4Fe-4S] cluster with one non-cysteine ligand.* Biochem J, 1989. **264**(1): p. 275-84.
13. Busch, J.L., et al., *[3Fe-4S] <--> [4Fe-4S] cluster interconversion in Desulfovibrio africanus ferredoxin III: properties of an Asp14 --> Cys mutant.* Biochem J, 1997. **323** (Pt 1): p. 95-102.
14. Yu, L., et al., *Modified ligands to FA and FB in photosystem I. II. Characterization of a mixed ligand [4Fe-4S] cluster in the C51D mutant of PsaC upon rebinding to P700-Fx cores.* J Biol Chem, 1995. **270**(47): p. 28118-25.
15. Reents, H., et al., *Bacillus subtilis Fnr senses oxygen via a [4Fe-4S] cluster coordinated by three cysteine residues without change in the oligomeric state.* Mol Microbiol, 2006. **60**(6): p. 1432-45.

16. Schwartz, C.J., et al., *IscR, an Fe-S cluster-containing transcription factor, represses expression of Escherichia coli genes encoding Fe-S cluster assembly proteins*. Proc Natl Acad Sci U S A, 2001. **98**(26): p. 14895-900.
17. Hidalgo, E., et al., *Binuclear [2Fe-2S] clusters in the Escherichia coli SoxR protein and role of the metal centers in transcription*. J Biol Chem, 1995. **270**(36): p. 20908-14.
18. Lazazzera, B.A., et al., *DNA binding and dimerization of the Fe-S-containing FNR protein from Escherichia coli are regulated by oxygen*. J Biol Chem, 1996. **271**(5): p. 2762-8.
19. Pomposiello, P.J. and B. Dimple, *Redox-operated genetic switches: the SoxR and OxyR transcription factors*. Trends Biotechnol, 2001. **19**(3): p. 109-14.
20. Hidalgo, E. and B. Dimple, *An iron-sulfur center essential for transcriptional activation by the redox-sensing SoxR protein*. Embo J, 1994. **13**(1): p. 138-46.
21. Ding, H., E. Hidalgo, and B. Dimple, *The redox state of the [2Fe-2S] clusters in SoxR protein regulates its activity as a transcription factor*. J Biol Chem, 1996. **271**(52): p. 33173-5.
22. Bradley, T.M., et al., *Cysteine-to-alanine replacements in the Escherichia coli SoxR protein and the role of the [2Fe-2S] centers in transcriptional activation*. Nucleic Acids Res, 1997. **25**(8): p. 1469-75.

Table 3–1 The correction factors for measuring the concentration of SufR from

Synechocystis sp. PCC 6803.Table 3–1: The correction factors for measuring the concentration of SufR from *Synechocystis* sp. PCC 6803.

TRIAL	CONCENTRATION MEASURED BY AMINO ACID ANALYSIS	CONCENTRATION MEASURED BY BRADFORD	CORRECTION FACTOR
1	1.64 ± 0.03	0.980 ± 0.013	1.67
2	1.22 ± 0.03	0.980 ± 0.013	1.24
3	0.313 ± 0.006	0.270 ± 0.030	1.16
Average			1.36 ± 0.27

Table 3–2 Chemical analysis and spectroscopic measurement for the cluster stoichiometry for holo-SufR. Two different preparations of holo-SufR sample were prepared. Each preparation was measured for three times for the chemical analysis and protein quantitation. An average number of 3.7 for Fe/SufR and an average number of 3.5 for sulfide/SufR were obtained. Spectroscopic measurement of Fe/S cluster contents in SufR reveals result consistent with chemical analysis, with 0.93 clusters per protein.

Table 3–2: Chemical analysis and spectroscopic measurement for the cluster stoichiometry for holo-SufR.

	Prep1	Prep2	Average
Chemical assay of Fe and S contents in SufR			
Iron (mM)	0.509 ±0.015	0.635±0.009	
Sulfur (mM)	0.456±0.084	0.603±0.077	
Holo-SufR (mM)	0.128±0.001	0.185±0.002	
Fe/SufR	4.0±0.1	3.4±0.1	3.7
S/SufR	3.6±0.7	3.3±0.4	3.5
Spectroscopic measurement of Fe/S cluster contents in SufR			
Fe/S (mM)	0.045	0.030	
Protein (mM)	0.043	0.037	
Fe/S per SufR	1.05	0.81	0.93

Table 3–3 Spin quantitation for the $[4\text{Fe-4S}]^{2+,1+}$ clusters in SufR. The number of spins in the $S = 1/2$ ground spin state iron-sulfur clusters in the SufR sample and the PsaC standard are listed in the table. The EPR spectra are taken at a temperature of 16 K and microwave powers of 1 mW, 10 mW, 25 mW and 50 mW. The SufR sample has an absorbance of 0.93 at 400nm and the PsaC standard of 0.67. After adjusting the cluster concentration difference, the SufR sample has 44% of the $[4\text{Fe-4S}]^{2+,1+}$ clusters in the $S=1/2$ ground spin state.

Table 3–3: Spin quantitation for the $[4\text{Fe-4S}]^{2+,1+}$ clusters in SufR.

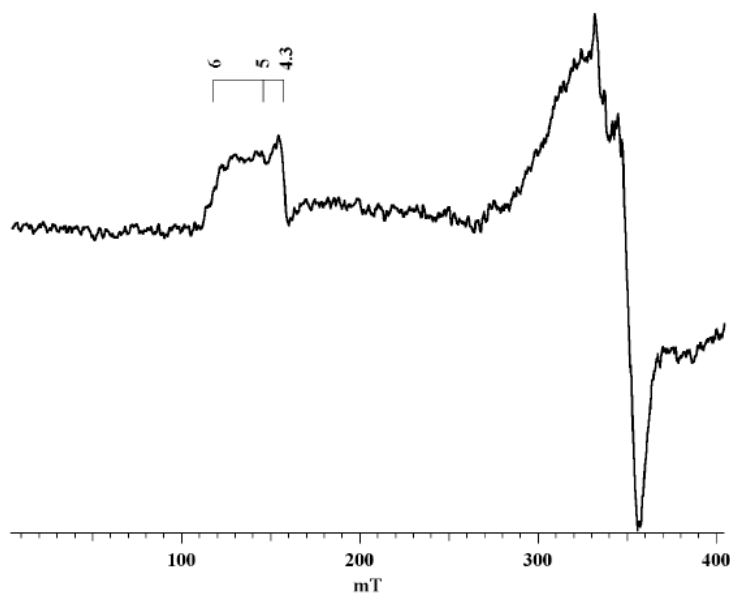
MICROWAVE POWER	1 MW	10 MW	25 MW	50 MW	AVERAGE
Total spins of SufR sample with $A_{400} = 0.93$	10031	31312	49938	68643	
Total spins of PsaC sample with $A_{400} = 0.67$	15528	51711	82904	116950	
Percentage of $S = 1/2$ ground spin state clusters in SufR	47%	44%	43%	42%	44%

Table 3–4 Comparison of the $[4\text{Fe-4S}]^{2+,1+}$ cluster stoichiometry in SufR and FNR. The absorption spectra of the holo-proteins show a ratio of $A_{420}/A_{280} = 0.42$ for FNR and 0.33 for SufR [15]. The molar extinction coefficient at 280 nm is 21680 for SufR and 16305 for FNR (calculated on a per protein basis using ProtParam). The protein-to-cluster ratios for FNR and SufR are nearly identical (1:1.0).

Table 3–4: Comparison of the $[4\text{Fe-4S}]^{2+,1+}$ cluster stoichiometry in SufR and FNR.

	SufR	FNR	SufR: FNR
Absorption Ratio (A_{420}/A_{280})	0.33	0.42	0.786
Extinction coefficient at 280 nm (Calculated by ProtParam)	21,680	16,305	1.330
Protein-to-cluster ratio			1.0 :1

Figure 3-1 EPR spectrum from 100 mT to 410 mT of recombinant, wild-type SufR after reduction with 15 mM sodium hydrosulfite. The spectrometer conditions were: temperature, 5 K; power, 126 mW; microwave frequency, 9.47 GHz; receiver gain, 2×10^4 ; modulation amplitude, 10 G at 100 kHz. The g -value range from 5 to 6 is indicated; the sharp $g = 4.3$ resonance is likely derived from a small population of non-reduced, octahedrally-coordinated Fe^{3+} ions in the rhombic limit of $E/D = 1/3$. The apparent g -values are depicted above the brackets.



Place

Figure Here

Figure 3-1: EPR spectrum from 100 mT to 410 mT of recombinant, wild-type SufR after reduction with 15 mM sodium hydrosulfite.

Figure 3-2 Alignment of the cysteine-containing C-terminal domains of the cyanobacterial SufR and homologous proteins from several other organisms. The conserved sequences of the C-terminal part were from: 1) cyanobacterial consensus sequence that was drawn from sequence analysis of SufR from *Synechococcus* sp. PCC 7002 and published sequences of the SufR-like proteins in other cyanobacteria in the databases; 2) *Vibrio cholerae*; 3) *Chloroflexus aurantiacus*; 4) the DeoR protein of *Bacillus cereus* 14579; 5) *Shewanella oneidensis* MR-1; 6) *Mycobacterium tuberculosis*; 7) *Corynebacterium glutamicum* ATCC 13032; 8) *Yersinia pestis*; and 9) *Streptomyces coelicolor*.

```

-----C-----C-----C-----C-----
1      XLXEHHCAXX-IVAESFPXVCGHE-LEMFXXVLP-DCXVEXRTHXXWGXHCGYLIQX
2 149  ILIENHCPIC-KAATRCPSLQSE-LNVFRRLGEECHIERREHIVAGERRCTYFIAA 205
3 157  VLRLFACPYY-DVAADYPEVCSME-RRMIEYVLG--EKLVL EDTIRAGAHNCRFVLRP 210
4 154  VIEKQNCPLK-EIAERFPQVCEDE-KEMKRLFTN-AEVKTLANMCEGDCSCSYQIKE 208
5 148  WLFENHCPIC-SAAATKCQNF CRSE-LQLFQQLFADIARVSREEHIVEGARRCAYRIAP 204
6 194  EICQHHCPVS-HVAEEFPELCETE-QQAIAEVLG--THVQRLAMIVNGDCACTTHVPL 247
7 173  SLALHSCPFFVNDKRPSAFVCAIH-AGFIQESLGENNRIQLELKPLNAPGTCKVHVFS 229
8 160  LLIENHCPIW-AAAASCQRF CRTE-LAVFQQALG--VPVERVEHILSGARRCTYRIHP 213
9 172  QLCQHHCPVA-HVAEQFPQLCEAE-TEFFAELLG--THVQRLATIAHGDTVCTTFIPK 224

```

Figure 3-2: Alignment of the cysteine-containing C-terminal domains of the cyanobacterial SufR and homologous proteins from several other organisms.
