CHAPTER 1

Introduction to [Fe-S] cluster biosynthesis: the requirement of a controlled expression system in *A. vinelandii.*

Iron-sulfur [Fe-S] clusters are one of nature's simplest and most functionally versatile co-factors (Beinert et al. 1997; Fontecave 2006). Proteins that contain [Fe-S] clusters are often referred to as [Fe-S] proteins and they participate in essential biological processes such as photosynthesis, respiration and nitrogen fixation. The functions of [Fe-S] clusters can include electron transfer, catalysis, environmental sensing or structural integrity. The simplest and most common types of [Fe-S] clusters are [2Fe-2S]- and [4Fe-4S] clusters that are usually attached to their protein partners via cysteine ligands.

The biosynthesis of [Fe-S] clusters and assembly into target proteins occurs in a controlled manner involving a number of biosynthetic proteins, some of which are highly conserved (with regard to their basic features, functions and primary sequences) in both prokaryotes and eukaryotes. Proteins involved in [Fe-S] cluster biosynthesis were first identified in the Gram-negative, nitrogen-fixing bacterium, *Azotobacter vinelandii* (Zheng et al. 1993)*.* Early genetic studies identified two major gene regions involved in [Fe-S] cluster biosynthesis: the n̲itrogen-f̲ixation- (*nif*)-specific biosynthetic genes (Zheng et al. 1993) and the 'housekeeping' i̲ron s̲ulfur c̲luster (*isc)* biosynthetic genes (Zheng et al. 1998). The *nif*-specific genes encode proteins involved in the assembly of [Fe-S] clusters necessary for biological nitrogen fixation and are only expressed under diazotrophic conditions. The *isc* operon, encodes proteins required for the biosynthesis and maturation of [Fe-S] proteins necessary for general 'housekeeping' cellular activities like respiration and carbon assimilation. The Nif and Isc systems share two common core functions involving cysteine desulfurases (NifS and IscS), which act as sulfur donors, and scaffold proteins (NifU and IscU), which act as sulfur and iron acceptors. The Isc system however, has additional accessory proteins, such as molecular chaperones (HscB and HscA), a ferredoxin (Fdx) and another potential scaffold/iron binding protein (IscA) whose function in the [Fe-S] cluster biosynthetic pathway remains unclear and is the subject of intense research.

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Our current understanding of specific *in vivo* interactions that take place during [Fe-S] cluster biosynthesis is predominantly derived from *in vitro* data involving purified proteins which are often combined at concentrations that are unlikely to represent physiological quantities (reviewed in Johnson et al. 2005). Furthermore, the involvement of chaperone proteins with ATPase activity suggest that although IscS/NifS and IscU/NifU alone may be the minimum requirements for cluster formation and transfer *in vitro*, the process may prove quite different *in vivo*. Genetic studies to address some of these issues and to confirm the bona fide *in vivo* role of various components of the *isc* cluster in [Fe-S] assembly has begun in a number of laboratories. *Saccharomyces cerevisiae* and *Arabidopsis thaliana,* which contain homologs to all the *A. vinelandii* Isc components, serve as excellent genetic models for studying [Fe-S] cluster biosynthesis in eukaryotes (reviewed in Barras et al 2005 and Balk and Lobreaux 2005). Discoveries in these areas continue to have a major impact on our current understanding of the [Fe-S] cluster biosynthetic pathway. Genetic work conducted in prokaryotes has predominantly focused on the *isc* region from *Escherichia coli*. Key findings to date include the following: (i) plasmid-directed over-expression of the entire *isc* operon increases the yield of over-expressed recombinant [Fe-S] proteins that contain a correctly assembled [Fe-S] cluster (Nakamura et al. 1999); (ii) inactivation of *iscS ,iscU, iscA, hscBA* and *fdx* individually results in growth defects and a marked decrease in the maturation of a variety of [Fe-S] proteins (Takahashi and Nakamura 1999; Schwartz et al. 2000; Tokumoto and Takahashi 2001); (iii) *E. coli* contains a second [Fe-S] cluster biosynthetic region, known as Suf, that seems to serve as an alternate housekeeping [Fe-S] cluster system under conditions of oxidative stress (Takahashi and Tokumoto 2002; Outten et al. 2004); (iv) IscS activity is also necessary for the mobilization of sulfur in the biosynthetic pathways of other essential cell components such as biotin, thiamin, thiolated tRNA and Mo cofactor maturation (Lauhon and Kambampati 2000; Skovran and Downs 2000; Leimkuhler and Rajagopalan 2001; Mueller et al. 2001). Chapter 2 provides a comprehensive review, published in the Annual Reviews of Biochemistry, that summarizes our current knowledge of [Fe-S] cluster biosynthesis.

In *A.vinelandii*, unlike *E.coli*, it has not been possible to isolate strains with individual deletions in *iscS, iscU, iscA, hscB, hscA* or *fdx* (Zheng et al. 1998). This has led to the assumption that most, if not all, of these "housekeeping genes" provide the only machinery available to the cell for performing essential [Fe-S] cluster biosynthetic functions. The *A. vinelandii* genome does not contain a *suf* operon counterpart, therefore using this bacterium as our model system offers us an advantage in attempting to study the detailed roles of the *isc* proteins since we can conduct mutational analyses on the *isc* operon without the complication of a second "housekeeping" region. It also offers us the opportunity to investigate the basis of the apparent target specificity between the Isc and Nif systems, which unlike the *E. coli* Isc and Suf sytems, do not seem capable of performing redundant functions.

In order to conduct an extensive functional analysis of the essential *isc* operon of *A. vinelandii*, a non-plasmid based genetic system was developed to control the expression of the *isc* genes, providing the opportunity to investigate the physiological effects resulting from the depletion of an individual *isc* gene product under different growth conditions. This system essentially involves strains which contain two copies of the *isc* operon, the endogenous copy which is controlled by its normal promoter and a second copy controlled by an inducible promoter. The effect of deletions or mutations placed in genes within the first, endogenous *isc* copy can be monitored by repression of gene transcription from the second intact *isc* copy. Development of this controlled expression system in *A. vinelandii* was made possible by the discovery of a promoter within the sucrose metabolic region of the *A. vinelandii* genome that can be fused to any number of target proteins and whose expression can be controlled by the presence or absence of sucrose in the growth media.

The development and the application of this controlled expression system in *A. vinelandii* has been the main focus of my work. A full description of the construction of this genetic system and how it was used to identify new potential roles for certain Isc components is presented in Chapter 3 as a manuscript submitted for publication in the Journal of Bacteriology. This system was also used to further our understanding of

potential overlapping functions between the Nif and Isc systems of *A. vinelandii*. In Chapter 4, experiments which I performed in collaboration with Dr. Patricia Dos Santos, are described in two parts: Part 1 presents experimental work that was published in *Biochemical Society Transactions* showing that under normal laboratory growth conditions, the Isc and Nif systems are not functionally equivalent and therefore incapable of performing overlapping functions. Part 2 describes more recent experimental work showing conditions under which overlapping functions between the two systems can be forced. This data provides new clues as to what features of each system determines target specificity. The development of an arabinose-based protein over-expression system in *A. vinelandii* by Dr. Dos Santos has allowed substantial progress to be made in this area, and experiments in Part 2 are part of a manuscript in preparation of which I am second author. In Appendix 1, evidence is presented which highlights a potential use of this system for the purification of physiologically produced, apo-forms of [Fe-S] proteins. A full list of all the plasmids and strains constructed for the purpose of this study are described in Appendices 2 and 3., respectively.

Finally, Chapter 5 summarizes the main findings of this work and how it contributes to the field of [Fe-S] cluster biogenesis. An attempt is made to evaluate some of the key observations regarding IscA and HscBA and to propose new hypotheses for a specific role in the biosynthetic process. Future experimental work is proposed, emphasizing the value of a genetic approach in identifying possible interactions between members of the Isc machinery.

CHAPTER 2

Literature Review: Structure, Function and Formation of Biological Iron-Sulfur Clusters

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This manuscript describes the background and recent literature on the three known [Fe-S] cluster biosynthetic machineries (Nif, Isc and Suf systems) which are involved in the formation of biological [Fe-S] clusters and the maturation of [Fe-S] proteins. Emphasis is placed on the similarities and specificities of each system based on genetic, physiological, biochemical and structural data that has accumulated since the discovery of the Nif system from *Azotobacter vinelandii* in 1993. This review primarily focuses on [Fe-S] protein maturation in prokaryotes with *Escherichia coli* and *Azotobacter vinelandii* serving as the main models. However, a brief overview of the key features of eukaryotic [Fe-S] cluster biosynthesis is provided.   This chapter was written and submitted for publication with the intention for its use to satisfy the literature survey for this dissertation. As senior author my responsibility was to gather and interpret recently published work, participate in writing the document at all stages of its preparation, and to prepare the figures that are included in the review.

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2.1 - Introduction and Perspectives

Clusters of non-heme iron and inorganic sulfide ([Fe-S] clusters, Figure 1) are one of the most ubiquitous and functionally versatile prosthetic groups in nature. Since the discovery of ferredoxins in the early 1960s, the number of identified proteins that contain [Fe-S] clusters ([Fe-S] proteins) has greatly proliferated. An excess of 120 distinct types of enzymes and proteins are known to contain [Fe-S] clusters (Johnson 1994; Beinert et al. 1997; Beinert 2000). Moreover, the discovery of new types of [Fe-S] proteins and [Fe-S] clusters has resulted in an appreciation of their remarkable functional and structural diversity, which undoubtedly reflects the chemical versatility of both iron and sulfur (Beinert et al. 1997; Beinert 2000). Indeed, such considerations, coupled with laboratory demonstration of C-C bond formation on Fe/Ni/S surfaces under prebiotic conditions, have led to speculation that [Fe-S] complexes played an important role in the emergence of life on earth (Huber and Wachtershauser 1997).

2.2 - Functions of Biological [Fe-S] Clusters

A summary of the functions and types of biological [Fe-S] clusters is presented in Table 1. The ability to delocalize electron density over both Fe and S atoms (Noodleman and Case 1992; Glaser et al. 2000), makes [Fe-S] clusters ideally suited for their primary role in mediating biological electron transport. As such, [Fe-S] clusters are major components in the photosynthetic and respiratory electron transport chains, define the electron transport pathways in numerous membrane-bound and soluble redox enzymes, and constitute the redox-active centers in ferredoxins, one of the largest classes of mobile electron carriers in biology (Johnson 1994). Clusters involved in electron transfer contain [2Fe-2S], [3Fe-4S], [4Fe-4S], or [8Fe-7S] core units (Figure 1), with cysteinate generally completing tetrahedral S coordination at each Fe site. Aspartate, histidine, serine or backbone amide ligation at a unique Fe site are occasionally encountered in clusters that function in electron transport and these ligands are likely to play a role in modifying redox potential (Link 1999), gating electron transport (Calzolai et al. 1996) or coupling proton and electron transport (Hunsicker-Wang et al. 2003). While the vast

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majority of electron transfer [Fe-S] clusters are one-electron carriers, the double-cubane [8Fe-7S] cluster that is found only in nitrogenases has the potential to act as a two-electron carrier (Peters et al. 1997). Moreover, this cluster undergoes major structural changes on two-electron oxidation, involving Fe ligation by the amide N of one of the bridging cysteine residues and the O of a nearby serine, thereby providing a mechanism for coupling proton and electron transfer (Peters et al. 1997; Lanzilotta et al. 1998).

TABLE 1 Functions of Some Biological [Fe-S] Clusters

Function	Examples	Cluster Type
Electron transfer	Ferredoxins; Redox enzymes	[2Fe-2S]; [3Fe-4S]; [4Fe-4S]
Coupled electron / proton transfer	Rieske protein	[2Fe-2S]
	Nitrogenase	[8Fe-7S]
Substrate binding and activation	(de)Hydratases	[4Fe-4S]
	Radical-SAM enzymes	[4Fe-4S]
	Acetyl CoA synthase	Ni-Ni-[4Fe-4S], [Ni-4Fe-5S]
	Sulfite reductase	[4Fe-4S]-siroheme
Fe or cluster storage	Ferredoxins	[4Fe-4S]
	Polyferredoxins	[4Fe-4S]
Structural	Endonuclease III	[4Fe-4S]
	MutY	[4Fe-4S]
Regulation of gene expression	SoxR	[2Fe-2S]
	FNR	[4Fe-4S]/[2Fe-2S]
	IRP	[4Fe-4S]
	IscR	[2Fe-2S]
Regulation of enzyme activity	Glutamine PRPP amidotransferase	[4Fe-4S]
	Ferrochelatase	[2Fe-2S]
Disulfide reduction	Ferredoxin:thioredoxin reductase	[4Fe-4S]
	Heterodisulfide reductase	[4Fe-4S]
Sulfur donor	Biotin synthase	[2Fe-2S]

[Fe-S] clusters also constitute, in whole or in part, the substrate binding sites of a wide range of redox and non-redox enzymes. A site for substrate binding and activation can be established in three different ways. First, non-cysteinyl ligation at a unique Fe site of a [4Fe-4S] cluster can facilitate substrate binding and activation for dehydration/hydration reactions in a wide range of hydratases and dehydratases (Flint and Allen 1996), of which aconitase is the best characterized example (Beinert et al. 1996). A similar approach is utilized in binding *S*-adenosylmethionine (SAM), via the amino

and carboxylato groups of the methionine fragment, in order to facilitate reductive cleavage and generation of the 5'-deoxyadenosyl radical in the radical-SAM family of [Fe-S] enzymes (Cheek and Broderick 2001; Jarrett 2003). The radical-SAM superfamily comprises more than 60 different enzymes that catalyze radical reactions in DNA precusor, vitamin, cofactor, antibiotic and herbicide biosynthesis and degradation pathways. Second, incorporation of a heterometal into an [Fe-S] cluster can be required for substrate binding or activation. The [Ni-4Fe-5S] cluster in CO dehydrogenase (Dobbek et al. 2001) is the best characterized examples of this type of approach. The third mechanism involves attachment of a substrate-binding metal site to an Fe site of a [4Fe-4S] cluster via a bridging cysteinyl residue. For example, siroheme is attached to form the sulfite and nitrite reductase active sites (Crane et al. 1995), a di-nickel center is attached to form the functional form of the acetyl-CoA synthase active site (Doukov et al. 2002; Darnault et al. 2003; Svetlitchnyi et al. 2004), and a di-iron center is attached to form the Fe-hydrogenase active site (Peters et al. 1998; Nicolet et al. 2002) .

A convincing case has been made for an iron storage role for 8Fe (2x[4Fe-4S]) ferredoxins in *Clostridia* (Thauer and Schonheit 1982) and similar arguments can be made for many anaerobic bacteria and archaea. This is further supported by the presence of polyferredoxins containing up to 12 [4Fe-4S] clusters in tandemly repeated 8Fe ferredoxin-like domains in methanogenic archaea (Reeve et al. 1989; Hedderich et al. 1992). Genes encoding polyferredoxins are found in operons encoding enzymes such as hydrogenase, which contains multiple [4Fe-4S] clusters. An alternative proposal is that 8Fe ferredoxins and polyferredoxins function as scaffolds for assembly or storage of [4Fe-4S] clusters in anaerobic bacteria and archaea.
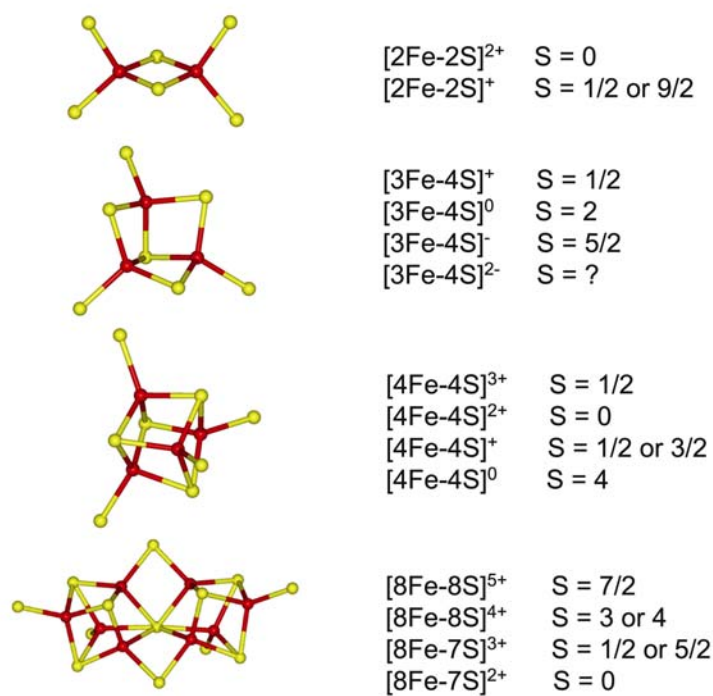
$$[2Fe-2S]^{2+} \quad S = 0$$
$$[2Fe-2S]^{+} \quad S = 1/2 \text{ or } 9/2$$

$$[3Fe-4S]^{+} \quad S = 1/2$$
$$[3Fe-4S]^{0} \quad S = 2$$
$$[3Fe-4S]^{-} \quad S = 5/2$$
$$[3Fe-4S]^{2-} \quad S = ?$$

$$[4Fe-4S]^{3+} \quad S = 1/2$$
$$[4Fe-4S]^{2+} \quad S = 0$$
$$[4Fe-4S]^{+} \quad S = 1/2 \text{ or } 3/2$$
$$[4Fe-4S]^{0} \quad S = 4$$

$$[8Fe-8S]^{5+} \quad S = 7/2$$
$$[8Fe-8S]^{4+} \quad S = 3 \text{ or } 4$$
$$[8Fe-7S]^{3+} \quad S = 1/2 \text{ or } 5/2$$
$$[8Fe-7S]^{2+} \quad S = 0$$

Figure 1   Structures, core oxidation states and spin states of crystallographically defined Fe-S clusters. Iron is shown in red and sulfur is shown in yellow. The spin state denoted by a question mark has yet to be determined and the $[Fe_3S_4]^-$ cluster has only been observed as a fragment in heterometallic $[MFe_3S_4]^+$ clusters in which M is a divalent transition metal ion.

Well-documented examples of [Fe-S] cluster-driven protein reorganization in response to medium effects (Plank et al. 1989) and cysteine substitutions (Golinelli et al. 1998) and the ability of designed and unstructured minimal synthetic peptides (maquettes) to correctly assemble [Fe-S] clusters (Mulholland et al. 1998) have elegantly demonstrated that [Fe-S] clusters control protein structure in the vicinity of the cluster. Hence, it is not surprising that [Fe-S] clusters have important structural and regulatory roles. In the case of the DNA repair enzymes, endonuclease III (Cunningham et al. 1989; Kuo et al. 1992) and MutY (Guan et al. 1998; Porello et al. 1998), the available evidence indicates that redox-inactive [4Fe-4S] clusters play purely structural roles, similar to that of Zn in Zn-finger proteins, where the cluster controls the structure of a protein loop essential for recognition and repair of damaged DNA.

There are now several well-characterized examples where [Fe-S] clusters are involved in transcriptional or translational regulation of gene expression in bacteria (Kiley and Beinert 2003). Each senses a different type of environmental stimulus and uses a distinct sensing mechanism involving cluster assembly, conversion or redox chemistry. The SoxR protein senses oxidative stress via oxidation of the $[2Fe-2S]^{2+,+}$ cluster and thereby stimulates transcriptional expression of SoxS, which is responsible for activating the transcription of numerous enzymes in the oxidative stress response (Demple et al. 2002). The FNR (Fumarate and Nitrate Reduction) protein is an oxygen sensing transcriptional regulator that functions by oxygen-induced conversion of the DNA-binding dimeric $[4Fe-4S]^{2+}$ form to a monomeric $[2Fe-2S]^{2+}$ form in order to control the expression of genes involved in the aerobic and anaerobic respiratory pathways of *Escherichia coli* (Kiley and Beinert 2003). The discovery that the apo-form of cytoplasmic aconitase is identical to the active form of IRP (Iron Responsive Protein) that binds to mRNA iron-responsive elements in eukaryotes, has revealed that translational regulation of ferritin and transferrin receptors is controlled by the assembly of a [4Fe-4S] cluster on IRP (Beinert et al. 1996). Aconitases also appear to have dual catalytic and regulatory roles in prokaryotes such as *Bacillis subtilis* (Alen and Sonenshein 1999) and *E. coli* (Tang and Guest 1999), where it has been shown that apo-forms specifically bind their cognate mRNAs.

[Fe-S] clusters have also been implicated in the regulation of enzyme activity in response to external stimuli, with the restoration of activity requiring cluster assembly or repair. The best documented example is *B. subtilis* glutamine phosphoribosylpyrophosphate amidotransferase (Smith et al. 1994), which is stabilized by the presence of a [4Fe-4S] cluster that is degraded by O_2 with concomitant proteolysis. Another intriguing example is the [2Fe-2S] cluster in mammalian ferrochelatases (Wu et al. 2001), the terminal enzyme of heme biosynthesis. The observation that the cluster is essential for activity and is readily degraded by NO, but is not present in the majority of the equivalent bacterial enzymes, has led to the suggestion that it is part of a defense mechanism that prevents the infecting organism from utilizing heme synthesized by the host organism (Sellers et al. 1996).

Two roles that have recently emerged for [Fe-S] clusters involve disulfide reduction and sulfur donation. At least two classes of disulfide reductases, ferredoxin:thioredoxin reductase in chloroplasts and heterodisulfide reductase in methanogenic archaea (Dai et al. 2000; Duin et al. 2002; Walters and Johnson 2004) have been shown to use an active-site [4Fe-4S] cluster to cleave disulfides in two sequential one-electron steps, using novel site-specific cluster chemistry, involving an intermediate with two thiolate ligands at a unique Fe site. In addition to the [4Fe-4S] cluster responsible for the reductive cleavage of SAM, biotin synthase contains a [2Fe-2S] cluster that is degraded and provides the sulfur for the conversion of dethiobiotin to biotin during a single catalytic turnover (Ugulava et al. 2001; Berkovitch et al. 2004; Jameson et al. 2004). For this process to be catalytically relevant, the [2Fe-2S] cluster must be reassembled during each catalytic cycle. This possibility indicates that [Fe-S] clusters might function as sulfur donors and that [Fe-S] cluster degradation and assembly can be part of a catalytic cycle.

2.3 - Structures and Properties of Biological [Fe-S] Clusters

Structures of homometallic [Fe-S] clusters that function exclusively in electron transfer, together with the known core oxidation states and corresponding ground state spin states, are shown in Figure 1. Of particular relevance with respect to the process of cluster biosynthesis, is the observation that $[Fe_2(\mu_2\text{-S})_2]$ rhombs can be considered as the basic building block for assembly, based on both structural and electronic considerations. Cubane-type [4Fe-4S] clusters can be assembled from two [2Fe-2S] units, whereas [3Fe-4S] and [8Fe-7S] clusters can be assembled from [4Fe-4S] units via loss of one Fe and cluster fusion, respectively. With the exception of the antiferromagnetically coupled, valence-localized $S = 1/2$ $[2Fe\text{-}2S]^+$ centers, Mössbauer studies indicate that valence-delocalized $[2Fe\text{-}2S]^+$ fragments are integral components of all higher nuclearity clusters (Beinert et al. 1997). The first examples of valence-delocalized $[2Fe\text{-}2S]^+$ centers were identified and characterized in a variant form of a 2Fe ferredoxin and shown to have ferromagnetically coupled $S = 9/2$ ground states (Crouse et al. 1995; Achim et al. 1996). Antiferromagnetic interaction between $S = 9/2$ $[2Fe\text{-}2S]^+$ fragments and $S = 2$ Fe^{2+}, $S =$

5/2 Fe^{3+}, $S = 5$ $[2Fe-2S]^{2+}$, $S = 9/2$ $[2Fe-2S]^+$, or $S = 4$ $[2Fe-2S]^0$ fragments, can be invoked to rationalize the ground state electronic properties of $[3Fe-4S]^{0,-}$ and $[4Fe-4S]^{3+,2+,+}$ clusters.

2.4 - Formation of Biological [Fe-S] Clusters

It has been known for many years that certain apo-forms of both [2Fe-2S] and [4Fe-4S] cluster-containing proteins can be activated *in vitro* by the simple addition of S^{2-} and $Fe^{2+/3+}$ (Malkin and Rabinowitz 1966). Although this observation led to a prevailing notion that [Fe-S] clusters could be spontaneously incorporated into their protein partners *in vivo*, this view was not compatible with the physiological toxicity of Fe and S at levels required for *in vitro* maturation. A more reasonable possibility was that Fe and S are delivered to apo-[Fe-S] proteins by specific carrier proteins that sequester Fe and S in non-toxic forms. This possibility was also indicated upon the emergence of protein expression systems, where it became apparent that many [Fe-S] proteins, even simple ferredoxins, often do not contain a complete complement of [Fe-S] clusters when recombinantly expressed at high levels.

2.5 - Nitrogenase Maturation – the First Paradigm for Biological [Fe-S] Cluster Assembly

Why is it that a fundamental understanding of [Fe-S] cluster biogenesis has only begun to emerge during the past decade? The answer to this question can be appreciated from the perspective that [Fe-S] cluster-containing proteins are direct players in so many key aspects of metabolism. [Fe-S] proteins also participate in the biosynthesis of essential cofactors/coenzymes. Thus, until relatively recently, it would have been extremely difficult to recognize the connection among the multiple metabolic deficiencies that are now known to be manifested when [Fe-S] cluster biogenesis is disrupted. Nevertheless, work involving various aspects of Fe metabolism (Kispal et al. 1999), the oxidative stress response (Strain et al. 1998), the role of specialized chaperones (Knight

et al. 1998; Schilke et al. 1999), and the maturation of nitrogenase (Zheng et al. 1993) has led to an understanding of the fundamental features of [Fe-S] cluster biogenesis.

Biochemical-Genetic Analysis of *nifU* and *nifS*. Nitrogenase is a complex [Fe-S] enzyme (Rees and Howard 2000) that catalyzes the nucleotide-dependent reduction of dinitrogen (nitrogen fixation) and studies on nitrogenase maturation revealed the initial insights concerning a plausible pathway for [Fe-S] cluster biogenesis. These insights were gained using the nitrogenase system for two important reasons. First, nitrogenase is a catalytically inefficient enzyme that is abundantly produced in nitrogen-fixing cells. Consequently, a specialized [Fe-S] cluster biosynthetic system is required to satisfy the high demand for [Fe-S] cluster formation under nitrogen-fixing conditions. Second, when organisms capable of nitrogen fixation are supplied a fixed form of nitrogen, such as ammonia, they do not require the synthesis of an active nitrogenase. Thus, a genetic lesion that affects nitrogenase-specific [Fe-S] cluster formation only impairs the organism's ability to grow under nitrogen-fixing conditions. In contrast, as discussed later, defects in an organism's ability to synthesize [Fe-S] clusters required for the general activation of [Fe-S] proteins are highly deleterious under all growth conditions.

The primary translation products of the structural genes encoding the nitrogenase component proteins – designated Fe protein and MoFe protein - are not active. Rather, a consortium of genes is required for the synthesis and insertion of the associated metalloclusters. Identification of nitrogen-fixation-specific (*nif*) genes directly involved in the initial assembly of [Fe-S] cluster units for nitrogenase activation were first gained by a systematic analysis of genes whose expression is coordinated with the expression of the nitrogenase structural components in the nitrogen-fixing bacterium *Azotobacter vinelandii* (Jacobson et al. 1989; Jacobson et al. 1989). The key observation relevant to the general process of [Fe-S] cluster biosynthesis was that inactivation of many *nif* genes results in defects in maturation of either the Fe protein or the MoFe protein, but deletion of either of two linked genes, *nifU* and *nifS*, uniquely results in substantial loss in activity of both the Fe protein and MoFe protein (Jacobson et al. 1989). Because the only feature shared by the Fe protein and MoFe protein is that they are both [Fe-S] proteins, the *nifU*

and *nifS* phenotypes uniquely pointed to defects in the early stages of nitrogenase-specific [Fe-S] cluster assembly. Another feature of *nifU* and *nifS* deletion strains is that, although they exhibit a substantial decrease in both Fe protein and MoFe protein activities, the activity of neither protein is completely eliminated. All of these observations resulted in the formulation of a model where NifU and NifS work together to produce [Fe-S] cluster precursors destined for nitrogenase maturation and, when NifU or NifS are inactivated, some other proteins replace their functions at a very low level.

NifS is a Cysteine Desulfurase. Purification of NifS revealed that it is a pyridoxal phosphate (PLP) containing homodimer (Zheng et al. 1993). Because PLP enzymes catalyze a diverse group of elimination and replacement reactions involving amino acids or their derivatives, the effect of the addition of individual amino acids on the UV-visible spectrum of NifS was examined, with a noticeable perturbation being uniquely associated with the addition of L-cysteine. It was also shown that NifS catalyses the elimination of S from L-cysteine to yield L-alanine and elemental sulfur or hydrogen sulfide, depending on whether or not a reducing agent was added to the reaction mixture. Further analysis of the NifS mechanism revealed the following features: (i) an active site cysteine, Cys^{325}, is extremely reactive towards alkylating reagents; (ii) the alkylated protein is inactive; (iii) substitution of Cys^{325} by alanine eliminates activity; (iv) both L-allylglycine and vinylglycine are suicide inhibitors forming covalent adducts with the active site cysteine and; (v) a persulfide could be identified at the Cys^{325} residue position upon incubation of NifS with equimolar amounts of L-cysteine substrate (Zheng et al. 1994). These results were interpreted to indicate that NifS-catalyzed desulfurization of L-cysteine involves formation of a substrate cysteine-PLP ketimine adduct with subsequent nucleophilic attack by the thiolate anion of Cys^{325} on the sulfur of the substrate cysteine (Figure 2). It is now known that NifS represents a broad class of proteins that use L-cysteine for the general mobilization of S for [Fe-S] cluster formation, as well as for other sulfur-containing prosthetic groups (discussed later). Structural and kinetic analyses of several members of this family confirmed and extended the originally proposed mechanism (Leibrecht and Kessler 1997; Mihara et al. 1997; Lang and Kessler 1999; Mihara et al. 1999; Clausen et al. 2000; Fujii et al. 2000; Kaiser et al. 2000; Mihara et al. 2000; Lima

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2002; Mihara and Esaki 2002; Mihara et al. 2002; Cupp-Vickery et al. 2003; Kaiser et al. 2003).
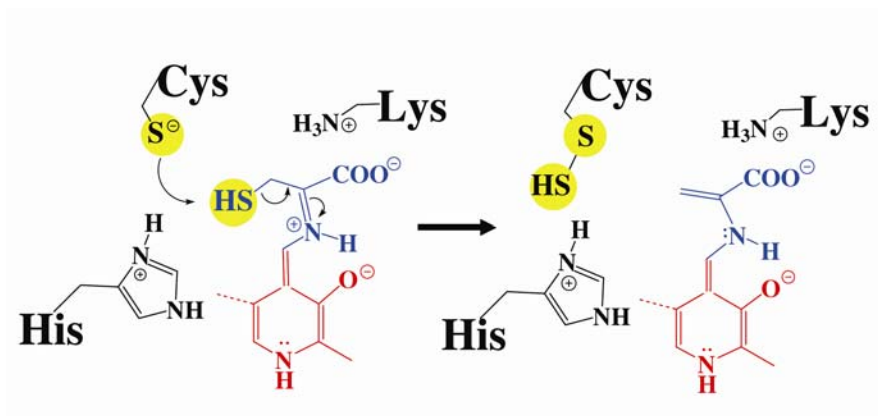

Figure 2 Salient mechanistic feature of the NifS/IscS class of cysteine desulfurases. An enzyme-bound persulfide is formed through nucleophilic attack by the thiolate anion of the active-site cysteine (located on a flexible loop) on the sulfur of the substrate cysteine (blue) PLP (red) adduct.

Based on the biochemical features of NifS, and because it is expressed only under nitrogen-fixing conditions, it was apparent that NifS has some role in the specific mobilization of S for maturation of nitrogenase. This possibility was also supported by identification of a *cysE* homolog that is co-transcribed with the *nifS* gene (Evans et al. 1991). The *cysE* gene encodes an O-acetyl serine synthase, which catalyzes the rate-limiting step in cysteine biosynthesis.

NifU is a Modular [Fe-S] Cluster Scaffolding Protein. After identification of NifS as the sulfur donor for nitrogenase maturation, two different possible functions for NifU were considered. One of these was that NifU and NifS separately provide the Fe and S required for nitrogenase maturation. The other was that NifU is a scaffold protein that provides an intermediate site for the assembly of [Fe-S] clusters, or [Fe-S] cluster precursors, destined for nitrogenase maturation. Of these two, the latter was favored because the concept of protein scaffolds functioning during complex metallocluster assembly had already been suggested on the basis that *in vivo* FeMo-cofactor

biosynthesis occurs separately from the nitrogenase MoFe protein subunits (Ugalde et al. 1984; Dean and Brigle 1985).

Purification of NifU revealed it is a homodimer that contains one $[2Fe-2S]^{2+/+}$ per monomer (Fu et al. 1994). It was initially considered that these [2Fe-2S] clusters might be destined for transfer during nitrogenase maturation. However, comparison of conserved sequences among various NifU proteins from a variety of nitrogen-fixing organisms suggested a different possibility. These comparisons indicated that NifU comprises three highly conserved primary sequences (Figure 3), connected by two stretches of relatively less conserved, acidic, linker sequences (Beynon et al. 1987; Hwang et al. 1996). Within these conserved domains, the N-terminal region contains three conserved cysteine residues (Cys^{35}, Cys^{62} and Cys^{106} [Figure 4), the central domain contains four conserved cysteine residues organized within a [2Fe-2S] cluster-ligating sequence signature (Cys^{137}, Cys^{139}, Cys^{172} and Cys^{175}), and the C-terminal domain contains two conserved cysteine residues (Cys^{272} and Cys^{275}). Thus, it appeared that either or both of the N-terminal and C-terminal domains could provide assembly sites for the formation of "transient" [Fe-S] clusters destined for nitrogenase maturation, while the central domain contains a "permanent" [2Fe-2S] cluster that could have some function in the formation or release of the transient clusters. Support for these possibilities was obtained by examination of the effects of single amino acid substitutions for each of the conserved cysteines and by expression of the individual domains, separately, and in combination (Agar et al. 2000; Dos Santos et al. 2004).

Direct evidence for the formation of transient clusters on NifU was obtained by using a combination of absorption, resonance Raman and analytical data to demonstrate the formation of a labile $[2Fe-2S]^{2+}$ cluster within the N-terminal fragment (designated NifU-1) when it is separately expressed, purified, and incubated with catalytic amounts of purified NifS, L-cysteine, and Fe^{2+} (Yuvaniyama et al. 2000). *In vitro* accumulation of [Fe-S] clusters on the proposed scaffold could also be detected on an altered form of full-length NifU that does not have the capacity to form the permanent [2Fe-2S] cluster. Furthermore, an altered form of recombinant NifU-1 that contains an aspartate residue

(Asp^{37}) substituted by alanine, is purified in a form that has partial occupancy of a labile [2Fe-2S] cluster. Finally, the potential involvement of the C-terminal domain of NifU in the assembly of transient [Fe-S] clusters is suggested by the ability of homologous proteins (designated Nfu or NifU-like proteins) from cyanobacteia, plants and higher organisms to accommodate formation of transient [2Fe-2S]$^{2+}$ or [4Fe-4S] $^{2+}$ clusters (Nishio and Nakai 2000; Leon et al. 2003; Tong et al. 2003). In aggregate, these experiments established that NifU has the capacity to accommodate formation of "transient" [Fe-S] clusters, supporting the notion that NifU serves as a scaffold for [Fe-S] cluster formation necessary for nitrogenase maturation.
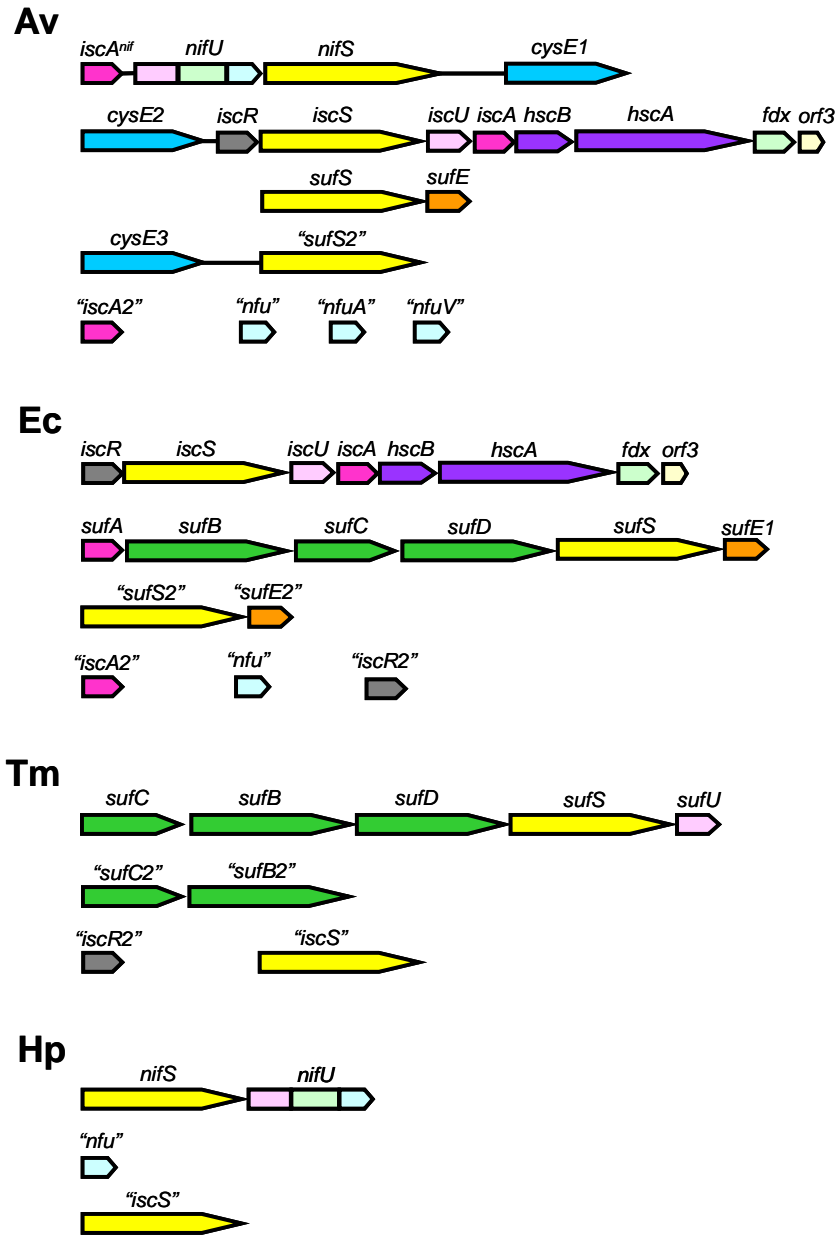
Figure 3 Organization of genes from various organisms whose products are known or suspected to be involved in [Fe-S] protein maturation. (Av, *Azotobacter vinelandii*; Ec, *Escherichia coli*; Tm, *Thermatoga maritima*; Hp, *Helicobacter pylori*) The three-domain structure of NifU is denoted by the three different colors within nifU. Genes from different organisms or different gene clusters proposed to encode products having the same function have the same color (see *nifS*, *iscS*, and *sufS*; yellow). Genes located within a single cluster that encode proteins proposed to form a macromolecular complex also have the same color (see *hscB* and *hscA*; purple; and *sufB*, *sufC*, and *sufD*: green).

```
Av-Nif  ------------------------------MWDYSEKVKEHFYNPKNAGAVEGAN---A
Av-Isc  ------------------------------MAYSDKVIDHYENPRNVGKLDAQDPDVG
Ec-Isc  ------------------------------MAYSEKVIDHYENPRNVGSFDNNDENVG
Hi-Isc  ------------------------------MAYSEKVIDHYENPRNVGSLDKKDSNVG
At-Isc  --MMLKQAAKKA----LGLTSRQSTPWSVGILRTYHENVIDHYDNPRNVGSFDKNDPNVG
Sc-Isc  MLPVITRFARPALMAIRPVNAMGVLRASSITKRLYHPKVIEHYTHPRNVGSLDKKLPNVG
Sp-Suf  -----------------------MALSK-LNHLYMAVVADHSKRPHHHGQLDG----VE
Bs-Suf  -----------------------MSFNANLDTLYRQVIMDHYKNPRNKGVLND----SI
Ca-Suf  -------------------------VMDLNSIYTEIITEHNASKHNKHKIEDA---TL
Tm-Suf  -----------------------------MMYSEAILDYANSKKFRGKLDDATVIEE


                37                               63
Av-Nif  IGDVGSLSCGDALRLTLKVDPETDVILDAGFQTFGCGSAIASSSALTEMVKGLTLDEALK
Av-Isc  TGMVGAPACGDVMRLQIKVNEQ-GIIEDAKFKTYGCGSAIASSSLATEWMKGRTLEEAET
Ec-Isc  SGMVGAPACGDVMKLQIKVNDE-GIIEDARFKTYGCGSAIASSSLVTEWVKGKSLDEAQA
Hi-Isc  TGMVGAPACGDVMQLQIKVDDN-GIIEDAKFKTYGCGSAIASSSLITEWVKGKSLEEAGA
At-Isc  TGLVGAPACGDVMKLQIKVDEKTGQIVDARFKTFGCGSAIASSSVATEWVKGKAMEDVLT
Sc-Isc  TGLVGAPACGDVMRLQIKVNDSTGVIEDVKFKTFGCGSAIASSSYMTELVQGMTLDDAAK
Sp-Suf  AVQLNNPTCGDVISLTVKFD--EDKIEDIAFAGNGCTISTASSSMMTDAVIGKSKEEALA
Bs-Suf  VVDMNNPTCGDRIRLTMKLD--GDIVEDAKFEGEGCSISMASASMMTQAIKGKDIETALS
Ca-Suf  VENGHNPSCGDDITLQLKIEN--NVIEDAAFIGVGCAISQASTSIMIDLIKGKTVEEAKK
Tm-Suf  GKNI----CGDEITLYLKV--EDGVVKDAKFEGMGCVISQASASLMLERIIGERVEEIFS


                                         106
Av-Nif  IS-------------------NQDIADYLDLPPEKMHCSVMGREALQAAVANYRGETIE
Av-Isc  IK-------------------NTQIAEELALPPVKIHCSVLAEDAIKAAVRDYKHKKGL
Ec-Isc  IK-------------------NTDIAEELELPPVKIHCSILAEDAIKAAIADYKSKREA
Hi-Isc  IK-------------------NSQIAEELELPPVKVHCSILAEDAIKAAIADYKAKQG-
At-Isc  IK-------------------NTEIAKHLSLPPVKLHCSMLAEDAIKAAVKDYKEKRVK
Sc-Isc  IK-------------------NTEIAKELSLPPVKLHCSMLAEDAIKAAIKDYKSKRNT
Sp-Suf  LADIFSEMVQ--GQENPAQKELGEAELLAGVAKFPQRIKCSTLAWNALKEAIKRSANAQHL
Bs-Suf  MSKIFSDMMQ--GKEYDDSIDLGDIEALQGVSKFPARIKCATLSWKALEKGVAKEEGGN--
Ca-Suf  LVETFIGMIKREITDEAELEQLEDALAFKNISNMPARVKCAVLAWHTFEECINEKK-----
Tm-Suf  LIEEAEKMSR---GENFDEGKLKNVTLMSDIKNYPARVKCFILAWKTLKEALKISRP----


Av-Nif  DDHEEG-------
Av-Isc  V-----------
Ec-Isc  K-----------
Hi-Isc  -------------
At-Isc  TNGAAAAGETTQA
Sc-Isc  PTMLS--------
Sp-Suf  TDQNVKEGKNV-
Bs-Suf  -------------
Ca-Suf  -------------
Tm-Suf  -------------
```

Figure 4 Comparison of primary sequences of representative members of the NifU/IscU/SufU family of proteins. Numbers refer to the IscU sequence from *Azotobacter vinelandii*. Only the N-terminal third of NifU is shown and this corresponds to the NifU-1 sequence discussed in the text. Conserved cysteines are shaded in yellow and other residues conserved in all of the shown sequences are shaded in black. The LPPVK sequences necessary and sufficient for IscU interactions with HscA are shaded in red. Av, *Azotobacter vinelandii*; Ec, *Escherichia coli*; Hi, *Haemophilus influenzae*; At, *Arabidopsis thaliana*; Sc, *Saccharomyces cerevisiae*; Sp, *Streptococcus pyogenes*; Bs, *Bacillus subtilis*; Ca, *Clostridium acetobutylicum*; Tm, *Thermotoga maritima*.

NifUS-Directed Activation of the Nitrogenase Fe Protein. An important validation of the scaffold concept for [Fe-S] protein maturation required demonstration that [Fe-S] clusters formed on a proposed scaffold are transferred to a genuine target at rates that might be expected *in vivo*. Design and interpretation of such *in vitro* "cluster transfer" experiments are difficult because many apo-[Fe-S] proteins, including the nitrogenase Fe protein, can be "spontaneously" activated by the simple addition of Fe^{2+} and S^{2-}. Indeed, incubation of apo-Fe protein, NifS, Fe^{2+}, and L-cysteine results in relatively rapid (~30 min) and efficient (>80%) Fe protein activation (Zheng and Dean 1994). Such controlled release of S^{2-} through NifS activity in combination with Fe^{2+} has gained wide application as a way to activate a variety of apo-proteins produced by recombinant techniques (Hutchings et al. 2002; Yang et al. 2002; Gubernator et al. 2003; Kamps et al. 2004; Turk et al. 2004). Nevertheless, this type of activation, in the absence of a scaffold protein, such as NifU, is unlikely to represent a physiological process.

In vitro apo-Fe protein activation involved the co-incubation of recombinantly produced NifU and NifS with L-cysteine and Fe^{2+} in order to "load" the NifU scaffold (Dos Santos et al. 2004). This sample was then mixed with separately prepared apo-Fe protein, which was then immediately assayed for activity. The results of these experiments showed that a high level of apo-Fe protein activation could be achieved and that formation of the "active" form of NifU requires L-cysteine, Fe^{2+}, and NifS. It was also shown that an approximately equimolar amount of NifU was sufficient to achieve maximum activation of apo-Fe protein and that NifS was not required to achieve cluster transfer. Cluster transfer was very rapid, occurring as fast as the assay could be formed (within several minutes). Importantly, similar *in vitro* activation could not be achieved by the co-incubation of apo-Fe protein with free Fe^{2+} and S^{2-} at concentrations equivalent to the [Fe-S] cluster-loaded form of NifU. Thus, *in vitro* activation of apo-Fe protein by using a form of NifU loaded with transient clusters appears to be rapid, efficient, and specific.

The possibility that transient clusters are formed within both the N-terminal and C-terminal modules of NifU, as suggested by the primary sequence and genetic

experiments, was tested by *in vitro* activation experiments where one or more of the conserved cysteine residues in both modules were substituted by alanine (Dos Santos et al. 2004). These experiments revealed that substitution by alanine for any one, or all three, of the conserved cysteine residues contained in the N-terminal module, significantly lowers the efficiency of *in vitro* NifU-directed activation of apo-Fe protein. However, substitution by alanine for any combination of cysteine residues within both the N-terminal and C-terminal modules eliminates the capacity for *in vitro* NifU-directed activation of apo-Fe protein. Why there are two potential [Fe-S] cluster assembly scaffolds within a single protein is not clear.

2.6 - Systems Involved in Generalized [Fe-S] Protein Maturation

After the initial demonstration that NifS is a cysteine desulfurase involved in the mobilization of S for nitrogenase maturation, parallel studies to those described above, were initiated in several laboratories and there are now known to be at least three different types of [Fe-S] cluster biosynthetic systems. These systems are often referred to as "Nif", "Isc" and "Suf". All three of them are unified by the involvement of cysteine desulfurases and [Fe-S] cluster scaffold proteins. In Figure 3 and in the following discussion we have designated certain genes/products as *nif*/Nif, *isc*/Isc or *suf*/Suf based on their apparent genetic and functional context, even though some of them have been given different designations in the published literature. The reason for this is to provide the reader some clarity in the following sections concerning the proposed functions of the various proteins and the general systems in which they participate (also see later discussion).

Discovery of the Isc System. The ability of *nifS* and *nifU* deletion strains to produce low levels of active nitrogenase, and to grow under nitrogen-fixing conditions at an extremely low rate, suggested that some other housekeeping function related to generalized [Fe-S] cluster biosynthesis could weakly replace NifU and NifS functions (Jacobson et al. 1989). Because these studies predated the genomics era, efforts to identify proteins involved in housekeeping [Fe-S] cluster biosynthesis required a biochemical approach where a

protein having cysteine desulfurase activity was isolated from crude extracts prepared from A. *vinelandii* cells grown under non-nitrogen-fixing conditions (Zheng et al. 1998). Information gained from microsequencing of oligopeptides obtained by tryptic digestion of this protein was used to design a strategy for isolation and DNA sequence analysis of the corresponding genomic region from *A. vinelandii*. This analysis identified 9 linked genes (Figure 3), now designated *cysE2*, *iscR*, *iscS*, *iscU*, *iscA*, *hscB*, *hscA*, *fdx*, and *orf3*, that are all likely to have some function related to [Fe-S] cluster biosynthesis. The designation "*isc*" indicates a role in *i*ron-*s*ulfur-*c*luster formation, whereas *hscB* and *hscA* (*h*eat-*s*hock-*c*ognate) were previously identified from *E. coli* and named according to their respective primary sequence similarities to *dnaJ* and *dnaK* (discussed later). Gene mapping experiments using polar insertion mutations indicated that *cysE2* is separately expressed from the other genes, all of whose expression is likely to be regulated by a single promoter preceding *iscR*. Initial gene inactivation experiments revealed that *cysE2* is not essential but that *iscS* and *hscA* are essential in *A. vinelandii*. A more thorough genetic analysis of homologous genes was performed using *E. coli* as described below.

Simple inspection of the primary sequences of genes contained in the *isc* region provided a number of important insights. The location of *cysE2* adjacent to the *isc* gene cluster indicates that, for *A. vinelandii*, there is a specialized system for the biosynthesis of L-cysteine targeted for housekeeping [Fe-S] cluster formation. However, examination of the many bacterial genomes now available indicates that this is not generally true for most other organisms. IscS and NifS bear a great deal of primary sequence similarity with a particularly high degree of conservation between their respective active site cysteine and PLP-binding regions. However, the proteins are not functionally equivalent *in vivo* because an *iscS* deletion phenotype cannot be rescued by expression of *nifS* when *A. vinelandii* grows under nitrogen-fixing conditions (Zheng et al. 1998). IscU corresponds to the N-terminal module of NifU with all three conserved NifU cysteines being conserved in IscU (Figure 4). IscA bears considerable sequence similarity to the product of a *nif*-specific gene, now designated *iscA^{Nif}*, located immediately upstream of *nifU* (Figure 3 and 5). The possibility that IscA^{Nif} might have a function in nitrogenase-specific [Fe-S] cluster formation was not appreciated until the discovery of IscA because

there is no phenotype associated with the inactivation of *iscA^Nif*. Nevertheless, the conservation of three cysteines in both IscA and IscA^Nif was interpreted to indicate this family of proteins could function in some aspect of [Fe-S] cluster biosynthesis. Another interesting aspect of the *isc* gene region is that it encodes a [2Fe-2S] ferredoxin. Although this protein does not bear similarity in primary sequence or in the organization of ligating cysteines when compared to the [2Fe-2S] cluster contained within NifU, both cluster types have similar biophysical properties, indicating they could have equivalent functions (Fu et al. 1994; Jung et al. 1999). The most unexpected feature to emerge from the physical and genetic map of the *isc* gene region was the apparent involvement of the molecular chaperones HscB and HscA in [Fe-S] cluster biosynthesis, discussed in a later section.

```
1  ---------------MAVTMTEAAARHIRRSLDGRGKGE-GIRLGVRTSGCSGLAYVLEFV
2  ---------------MITLTESAKSAVTRFISSTGKPIAGLRIRVEGGGCSGLKYSLKLE
3  -------MTVETFTPTPLLFTQGAANKVKTLISEEGNPRLKLRVFVTGGGCSGFQYGFTFD
4  ---------------MSITLSDSAAARVNTFLANRGKGF-GLRLGVRTSGCSGMAYVLEFV
5  MDMHSGTFNPQDFAWQGLTLTPAAAIHIRELVAKQPGMV-GVRLGVKQTGCAGFGYVLDSV
6  ---------MSDDVALPLEFTDAAANKVKSLIADEDNPNLKLRVYITGGGCSGFQYGFTFD

1  DEVASEDQVFESHGVKVIVDPKSLVYLDGTELDFVREGLNEGFKFNNPNVRGECGCGESFNI
2  EAGAEDDQLVDCDGITLLIDSASAPLLDGVTMDFVESMEGSGFTFVNPNATNSCGCGKSFAC
3  EDVAEDDTIVEREGVSLVVDPMSFQYLAGAEVDYQEGLEGSRFVIKNPNAATTCGCGQSFSV
4  DEPTPEDIVFEDKGVKVVVDGKSLQFLDGTQLDFVKEGLNEGFKFTNPNVKDECGCGESFHV
5  SEPDKDDLLFEHDGAKLFVPLQAMPFIDGTEVDFVREGLNQIFKFHNPKAQNECGCGESFGV
6  DQVNEGDMTIEKQGVGLVVDPMSLQYLVGGSVDYTEGLEGSRFIVTNPNAKSTCGCGSSFSI
```

Figure 5 Comparison of primary sequences of members of the IscA/SufA family of proteins from *Azotobacter vinelandii* and *Escherichia coli*. Conserved cysteines are shaded in yellow and other residues conserved in all of the shown sequences are shaded in black. (1), IscA^Nif from *A. vinelandii*; (2), IscA from *A. vinelandii*; (3), "IscA2" from *A. vinelandii*; (4), IscA from *E. coli*; (5), SufA from *E. coli*; (6), "IscA2" from *E. coli*.

Biochemical-Genetic Analysis of the *isc* Gene Cluster from *Escherichia coli*. At about the same time the *isc* gene region was discovered in *A. vinelandii* using a biochemical approach, the genome-sequencing era emerged revealing that homologous gene clusters are contained in many bacteria. Proof that genes contained within the *isc* gene region have a housekeeping function related to the maturation of [Fe-S] proteins was forthcoming from a series of elegant studies using *E. coli*, and these studies used two general approaches. In the first approach the ability to increase the yield of cluster-

containing [Fe-S] proteins produced recombinantly was examined by the co-recombinant-expression of the *isc* gene cluster (Nakamura et al. 1999). This analysis demonstrated an increased yield of cluster-containing recombinant [2Fe-2S] and [4Fe-4S] proteins upon elevated expression of the *isc* gene region. Functional inactivation of plasmid-encoded genes, separately or in combination, also indicated that *iscS*, *iscU*, *iscA*, *hscB*, *hscA* and *fdx* individually have some role in maturation of recombinantly produced [Fe-S] proteins (Takahashi and Nakamura 1999). The second approach involved the genomic inactivation of individual genes followed by comparison of the activities of enzymes that require, or do not require, [Fe-S] clusters for catalysis (Schwartz et al. 2000; Tokumoto and Takahashi 2001). These results confirmed those obtained in the first approach and showed that inactivation of *iscS*, *iscU*, *hscB*, *hscA*, or *hscA* had severe effects on [Fe-S] cluster assembly, whereas the consequences of *iscA* inactivation are relatively minor. Given the importance of [Fe-S] proteins in intermediary metabolism it was somewhat surprising that inactivation of *iscS* and *iscU* from *E. coli* resulted in mutant strains that remained viable. This is especially so because inactivation of *iscS* or *iscU* is lethal in *A. vinelandii*. This discrepancy was resolved when yet another [Fe-S] cluster biosynthetic system, called "Suf" (discussed below), was discovered in *E. coli*, whereas a complete suite of *suf* genes is not encoded in the *A. vinelandii* genome (Figure 3).

The important conclusions to emerge from biochemical-genetic studies using *E. coli* is the firm demonstration of the involvement of the Isc system in general [Fe-S] protein maturation and an indication that the Isc system has some involvement in the maturation of both [2Fe-2S] and [4Fe-4S] proteins. Nevertheless, to date, there are no genetic studies that have correlated a loss in the enzymatic activity of a protein containing only [2Fe-2S] clusters with the impairment of the Isc biosynthetic system. Perhaps the best indicator that IscU- and IscS-type proteins have a direct role in maturation of [2Fe-2S] proteins, in addition to their well documented role in maturation of [4Fe-4S] proteins, is that in *Pseudomonas putida* 86 *iscU* and *iscS* genes are located within, and co-regulated with, a cluster of genes whose products are involved in quinoline degradation (Carl et al. 2004). Some of these proteins apparently require [2Fe-2S] clusters but not

[4Fe-4S] clusters for their activities. It might be significant that homologs to *hscB*, *hscA*, and *fdx* are not contained within this gene cluster, perhaps indicating that the products of these genes endow some specificity for the maturation of specific [Fe-S] protein types.

Discovery of the Suf System. The *sufABCDSE* operon (<u>s</u>ulfur <u>u</u>tilization <u>f</u>actor) was originally identified in *E. coli* based on destabilization of the [2Fe-2S] FhuF protein in *sufD* or *sufS* mutants (Patzer and Hantke 1999). FhuF is thought to function as a ferric iron reductase protein, which permits effective utilization of ferrioxamine B or rhodotorulic acid as an iron source (Muller et al. 1998). These early genetic studies showed that *suf* expression is regulated by the iron-dependent Fur repressor and, therefore, induced under iron-deficient conditions. The *suf* operon was also found to belong to the oxidative stress OxyR-dependent regulon (Zheng et al. 2001). Sequence analysis identified SufS as a cysteine desulfurase and SufA exhibits significant identity when compared to IscA (Figure 5), including the three conserved cysteine residues, clearly indicating an involvement of the Suf system in [Fe-S] cluster biosynthesis, possibly in [Fe-S] cluster repair following iron limitation and/or oxidative stress conditions. However, *E. coli suf* mutants showed no other apparent phenotype under normal, non-stress growth conditions.

Genetic studies of the homologous *suf* operon in the plant pathogen, *Erwinia chrysanthemi*, also showed induction of *suf* genes to be iron-dependent and revealed that strains with separate non-polar disruptions in the six *suf* genes resulted in elevated levels of free intracellular iron (Nachin et al. 2001). Inactivation of *sufC*, in particular, displayed a number of other phenotypes including: (i) increased sensitivity to oxidizing agents, (ii) impaired ability to effect plant infection, (iii) decreased activity of enzymes containing oxidatively labile [Fe-S] clusters, (v) loss of an ability to assimilate iron from the ferric siderophore, chrysobactin (Nachin et al. 2001; Nachin et al. 2003). All these phenotypes were evident only under stress conditions and suggested a function for the Suf system in the activation, protection or repair of [Fe-S] proteins under conditions of oxidative stress or iron-limitation.

The importance of the Suf system in [Fe-S] cluster formation was clarified in *E. coli* by construction of strains altered in expression for different combinations of both Isc and Suf function (Takahashi and Tokumoto 2002). For example, certain *isc* mutants display severe growth defects and dramatically reduced [Fe-S] protein activities, but suppression of these phenotypes can be achieved by the elevated expression of the *suf* operon. The inactivation of both the *isc* and *suf* systems was also found to be lethal. Unlike the Nif and Isc systems of *A. vinelandii*, the overlapping roles the Suf and Isc systems in *E. coli* made it more difficult to investigate their specific functions. Nevertheless, genetic and molecular studies of the regulatory characteristics of the Isc versus the Suf system firmly established a specialized role for the Suf system under iron-starvation conditions (Outten et al. 2004). Both the *isc* and *suf* operons of *E. coli* were found to be induced during exposure to hydrogen peroxide (H_2O_2) and the iron chelator, 2,2'-dipyridyl. In the case of the *isc* operon, H_2O_2 - and 2,2'-dipyridyl- induced expression was found to be exclusively IscR-dependent. IscR is encoded within the *isc* operon and previous work established that it is a regulatory [Fe-S] protein that senses the demand for [Fe-S] cluster formation by a negative feedback loop that requires the [Fe-S] cluster-containing form to effect repression (Schwartz et al. 2001). Unlike *isc* regulation, induction of the *suf* operon in response to Fe limitation is dependent on the global regulatory protein called Fur, whereas induction in response to oxidative stress requires OxyR and integration host factor (IHF) (Cicchillo et al. 2004; Outten et al. 2004).

Comments on the Relationship of Different [Fe-S] Cluster Biosynthetic Systems. Although a comprehensive phylogenetic and genomic analysis of the different biosynthetic machineries is beyond the scope of this review, the fact that there are apparently distinct systems, some having specialized functions in certain organisms, merits some discussion. The Nif-type of [Fe-S] cluster biosynthetic system was first dicovered in *A. vinelandii* where this system is exclusively used for the maturation of nitrogenase. However, the so-called Nif-type system is not restricted to nitrogen fixing organisms because similar systems have been identified in organisms that do not fix nitrogen. For example, in *Helicobacter pylori* there is good evidence that a Nif-like system is necessary for generalized maturation of [Fe-S] proteins (Olson et al. 2000).

Also, the heterologous expression of a Nif-like system from *Entamoeba histolytica* was used to complement an *E. coli* strain deleted for the *isc* and *suf* gene clusters but only under anoxic conditions (Ali et al. 2004). These findings indicate that Nif-specific and Nif-like systems could have evolved to perform optimally under anoxic or microaerobic conditions. Nevertheless, a strict functional equivalence of various Nif-specific and Nif-like [Fe-S] cluster biosynthetic machineries cannot be inferred based only on genetic organization and primary sequence considerations because expression of *nif*-specific genes in their normal genetic context in *A. vinelandii* cannot replace the function of the Isc-type of biosynthetic machinery. Similarly, the identification of *suf* genes in a particular organism does not necessarily indicate that they represent a "back-up" system that is expressed only under specialized conditions. For example, in *T. maritima* the *sufCBDSU* unit appears to be the only intact [Fe-S] cluster biosynthetic machinery available in that organism.

A comparison of the organization of various genes whose products are known or anticipated to be involved in some aspect of [Fe-S] protein maturation in *A. vinelandii*, *E. coli*, *T. maritima*, and *H. pylori* are shown in Figure 3. The information contained in Figure 3 is intended to emphasize several important points. First, [Fe-S] cluster biosynthetic systems identified so far, share involvement of cysteine desulfurases and one or both of two proposed molecular scaffolds (see below). Second, the assignment of a protein as participating in a Nif, Isc, or Suf system should be made on the basis of genetic context. In particular, *isc* genes can be unambiguously assigned if they are contained in an operon that includes *hscB* and *hscA*, and *suf* genes can be assigned if they are in an operon that includes *sufBCD*. Third, proteins that bear primary sequence similarity to Nif, Isc, or Suf proteins, but have no known function or genetic context, can only be designated arbitrarily (see genes designated with quotation marks in Figure 3). Most organisms encode such proteins. For example, *A. vinelandii* encodes three different proteins, designated *nfu*, *nfuA*, and *nfuV* whose products bear significant sequence similarity to the carboxyl domain of NifU, including conservation of two cysteine residues. The *nfuA* and *nfuV* genes are so designated because they are contained in gene clusters related to alternative nitrogen fixation systems designated Anf and Vnf,

respectively (Joerger et al. 1989; Joerger et al. 1989). It seems reasonable to expect that proteins not obviously associated with a particular type of [Fe-S] cluster biosynthetic machinery could have specialized functions related to the maturation, protection or repair of specific [Fe-S] proteins.

2.7 - Biochemical Features of Proteins Involved in [Fe-S] Protein Maturation

Role of IscU/SufU as [Fe-S] Cluster Scaffold Proteins. IscU and SufU bear considerable primary sequence conservation, including the three conserved cysteine residues, when compared to the N-terminal third of NifU, indicating a role for these proteins as providing scaffolds for [Fe-S] cluster assembly. Nevertheless, there are several features that distinguish the SufU family of proteins from the IscU family. SufU proteins contain an 18-21 amino acid insertion located between the second and third conserved cysteines when compared to IscU or NifU (Figure 4). The SufU class also contains a strictly conserved lysine residue located immediately preceding the third conserved cysteine residue, whereas, the corresponding residue is a strictly conserved histidine in the IscU class. Another difference is that bacteria that encode a SufU do not also encode genetically linked homologs to the molecular chaperones, HscB and HscA, whereas members of the IscU class always contain HscB and HscA homologs (the role of molecular chaperones in [Fe-S] protein maturation is discussed in a later section). In line with the apparent lack of a requirement for HscB and HscA for the Suf type of [Fe-S] cluster machinery, SufU proteins also do not contain the signature sequence (LPPVK, Figure 4) that has been identified as being required for interaction of IscU proteins with HscB and HscA (Hoff et al. 2002; Hoff et al. 2003). These differences are likely to be responsible for some of the different biochemical features that have emerged during the characterization of members of the IscU and SufU class of [Fe-S] cluster assembly scaffolds.

Purified *A. vinelandii* IscU and IscS are homodimers and can form a transient $\alpha_2\beta_2$ complex as demonstrated by both size exclusion chromatography and chemical

cross-linking studies (Agar et al. 2000). IscS-mediated assembly of oxidatively and reductively labile $[2Fe-2S]^{2+}$ clusters on IscU was initially demonstrated by using a combination of absorption, resonance Raman and analytical studies (Agar et al. 2000). Subsequent studies of the time course of cluster assembly revealed a more complex situation with sequential appearance of IscU forms that contain one $[2Fe-2S]^{2+}$ cluster per dimer, two $[2Fe-2S]^{2+}$ clusters per dimer, and one $[4Fe-4S]^{2+}$ cluster per dimer, with both types of clusters having partial non-cysteinyl ligation based on resonance Raman and Mössbauer parameters (Agar et al. 2000). These data were interpreted to indicate that a $[4Fe-4S]^{2+}$ cluster can be formed on IscU by direct reductive coupling of two $[2Fe-2S]^{2+}$ clusters. Such reductive coupling has precedence in synthetic [Fe-S] cluster chemistry and could therefore represent a general pathway for formation of biological [4Fe-4S] clusters (Hagen et al. 1981).

Evidence for an *in vitro* cluster scaffolding role for IscU and the structurally and functionally related SufU protein was also gained by studies using IscU homologs from human, *Schizosaccharomyces pombe*, *E. coli*, and SufU from *T. maritima* (Foster et al. 2000; Urbina et al. 2001; Wu et al. 2002; Wu et al. 2002). The assembly of transient clusters on IscU/SufU scaffolds in these cases was accomplished using IscS-directed release of S^{2-} in the presence of Fe^{2+} or by incubation of IscU with S^{2-} and Fe^{2+}. As already mentioned, substitution of the NifU Asp[37] residue by Ala results in the stabilization of transient [2Fe-2S] clusters formed *in vitro* or *in vivo*, implicating a role for that residue in controlling the lability and, possibly, the physiological transfer of clusters assembled on NifU (Yuvaniyama et al. 2000). Substitution of the analogous Asp residue in IscU/SufU homologs from human, *S. pombe* and *T. maritima* also results in stabilization of [2Fe-2S] clusters on those proteins (Foster et al. 2000; Mansy et al. 2002; Wu et al. 2002; Wu et al. 2002). Assembly of [4Fe-4S] clusters has yet to be identified for any IscU-type species other than for the *A. vinelandii* protein. Thus, an important open question within the field involves the physiological relevance and possible target specificity, if any, of the different cluster-loaded IscU/SufU species that have been identified.

Experiments involving the *in vitro* transfer of [Fe-S] clusters from cluster-loaded forms of human, *S. pombe* or *T. martima* IscU/SufU to apo-forms of [2Fe-2S] cluster-containing ferredoxins (Fdx) have been important in providing proof-of-concept for intact cluster transfer (Mansy et al. 2002; Wu et al. 2002). Intact protein-to-protein cluster transfer was evaluated by using Mössbauer spectroscopy (Wu et al. 2002). In this case ^{57}Fe-labeled [2Fe-2S] assembled on Asp^{37} Ala human Isu (IscU homolog) was used for maturation of human apo-Fdx in a simple mixing experiment analogous to those described earlier for NifU-directed apo-Fe protein maturation. An important control was that inclusion of free ^{56}Fe in the cluster transfer reaction mixture did not dilute IscU-directed ^{57}Fe incorporation into apo-Fdx. These results indicate that *in vitro* cluster transfer does not occur via disassembly (release to solvent) and reassembly of the ^{57}Fe-[2Fe-2S] clusters contained on IscU. Cluster transfer occurs with near 100% efficiency after approximately one hour using a 10-fold excess of cluster-bound IscU. The second order rate constant was not significantly perturbed by changes in solvent viscosity, indicating the cluster transfer process is the rate-limiting step (Wu et al. 2002). These rates are at least an order of magnitude slower than for cluster transfer from NifU to apo-Fe protein (Dos Santos et al. 2004), which could reflect a requirement for accessory components for Isc-directed maturation of [Fe-S] proteins that are not required for Nif-directed maturation of the nitrogenase Fe protein. Evidence for *in vivo* participation of HscB, HscA, and Fdx in [Fe-S] cluster maturation for Isc-directed [Fe-S] protein maturation (Takahashi and Nakamura 1999; Lange et al. 2000; Lutz et al. 2001; Tokumoto and Takahashi 2001; Voisine et al. 2001; Dutkiewicz et al. 2003; Muhlenhoff et al. 2003) but not for Nif-directed Fe protein maturation supports this possibility. A 10-fold decrease in cluster transfer rates was observed using the Asp^{37}-substituted human Isu, and changes in the activation parameters and effects of pH were interpreted to indicate that the conserved aspartate plays a key role in mediating cluster transfer by maintaining a solvent-exposed and accessible cluster on IscU (Wu et al. 2002).

Investigations of the mechanism of assembly of [2Fe-2S] units on IscU/SufU have largely focused on addressing whether the initial step involves Fe or S binding. No evidence for Fe^{3+} or Fe^{2+} ion binding to cysteine residues on *A. vinelandii* IscU could be

obtained by analysis using a variety of different spectroscopic methods (Smith et al. 2001). These results are in agreement with NMR studies of *E. coli* IscU, which failed to reveal evidence for Fe^{3+} or Fe^{2+} ion binding to IscU (Adinolfi et al. 2004). In contrast, evidence for direct transfer of sulfane sulfur, S^0, from the cysteine persulfide on IscS to cysteine residues on IscU was provided by mass spectrometry (Smith et al. 2001) and ^{35}S-cysteine radiotracer studies (Urbina et al. 2001), leading to a suggestion that S transfer might precede Fe binding. In addition, mass spectrometry and other methods have provided evidence for a covalent complex between IscU and IscS monomers involving a disulfide or polysulfide linkage that is cleaved by reducing agents, indicating direct transfer of S from the cysteine persulfide on IscS to cysteine residues on IscU (Smith et al. 2001; Urbina et al. 2001; Kato et al. 2002). However, it has not been possible to complete cluster assembly on IscU/SufU by incubating the pre-sulfurated form with Fe^{2+}, suggesting that sulfuration of IscU/SufU in the absence of Fe could be artifactual (Nuth et al. 2002). In contrast to the *A. vinelandii* and *E. coli* IscU proteins, evidence for high affinity Fe^{2+} and Fe^{3+} binding to *T. maritima* SufU based on ITC and fluorescence measurements (K_D = 0.3-2.6 μM and 2.7-3.0 μM for Fe^{3+} and Fe^{2+} ion, respectively) has led to the suggestion that Fe binding precedes S transfer during [Fe-S] cluster assembly (Nuth et al. 2002). However, to our knowledge there has yet to be any direct analytical or spectroscopic evidence for Fe^{2+} or Fe^{3+} binding to any IscU/SufU protein.

There is also structural information for SufU that distinguishes it from the IscU family of proteins. Namely, NMR analysis of *T. maritima* SufU led to the conclusion that it exists in a "molten globule-like" state comprising reasonably well-defined secondary structure, but with fluxional tertiary structure and an undefined cluster binding site (Bertini et al. 2003). The structural flexibility was interpreted as being essential for the function of SufU, which is proposed to facilitate docking and cluster transfer to a wide range of acceptor apo Fe-S proteins (Mansy et al. 2004). In contrast, the NMR structure of *H. influenzae* IscU (Protein Data Bank -PDB ID: 1Q48) indicates a monomeric protein with flexibility in the N- and C-termini, but well-ordered secondary and ternary structure for a well-defined and solvent exposed cluster-binding site core that

includes the three conserved cysteine residues, as well as conserved aspartate, histidine, serine and lysine residues. A preliminary NMR analysis of *E. coli* IscU also indicates it is a well-folded monomeric protein (Adinolfi et al. 2004).

In summary, there is now compelling data to indicate that all IscU/SufU proteins function as molecular scaffolds for formation of [Fe-S] clusters destined for [Fe-S] protein maturation. Nevertheless, apparent differences between IscU and SufU proteins highlight how little is actually known concerning the molecular details of [Fe-S] cluster formation on these molecular scaffolds and their subsequent transfer during [Fe-S] protein maturation. For this reason, *in vitro* experiments involving [Fe-S] cluster formation and transfer should be interpreted cautiously. For example, when the relatively low abundance of IscU/SufU, as compared to the total amount of [Fe-S] proteins in a cell, is considered, the *in vitro* IscU/SufU-directed cluster transfer rates that have been reported cannot be considered to faithfully duplicate a physiological process. Furthermore, the nature of the [Fe-S] species that are actually assembled and transferred *in vivo* is going to be extremely difficult to establish. Specific problems relevant to this issue concern the plasticity of [Fe-S] cluster species and the facility by which free Fe and S can be used to activate apo forms of [Fe-S] proteins *in vitro*. Such problems are also confounded by the inherent lability of [Fe-S] cluster species assembled on scaffold proteins.

Another important issue that merits consideration in the evaluation of *in vitro* [Fe-S] cluster assembly and transfer experiments is that persulfides formed by the action of cysteine desulfurases can be readily converted to hydrogen sulfide by treatment with various reducing agents. Co-incubation of IscS and IscU accelerates cysteine desulfuration when compared to the same reaction in the absence of IscU (Kato et al. 2002). This and other experiments indicate direct S transfer from IscS to IscU, which is probably mechanistically relevant (Smith et al. 2001; Urbina et al. 2001). However, many *in vitro* [Fe-S] cluster assembly and transfer experiments that have been described also include a reducing agent. Thus, an uncontrolled aspect of these experiments involves the simultaneous formation of hydrogen sulfide and direct S transfer to IscU, a

situation that probably does not occur *in vivo*. This complication is highlighted by a recent kinetic analysis where it is shown that the rate of reagent-directed persulfide cleavage can vary enormously depending on the nature of the reductant used, and that the rate-limiting catalytic step from different types of cysteine desulfurases can be either persulfide formation or persulfide release (Behshad et al. 2004). In spite of these issues, the facile *in vitro* assembly of [Fe-S] clusters on IscU/SufU scaffolds and the intact *in vitro* transfer of these clusters to target proteins, even at low rates, represent an important advance in understanding the process of [Fe-S] protein maturation.

Role of IscA/SufA as [Fe-S] Cluster Scaffold Proteins. All three types of [Fe-S] cluster biosynthetic machineries discovered so far often include an IscA/SufA, and there are frequently multiple copies of genes encoding this family of proteins in many genomes. Although there is no sequence similarity between members of the IscU/SufU family and IscA/SufA family, all IscA/SufA homologs, like all IscU/SufU homologs, contain three strictly conserved cysteine residues. The conservation of three cysteines within all members of the IscA/SufA family indicated a potential role in Fe chaperone or [Fe-S] cluster scaffold activities (Zheng et al. 1998). In accord with the latter possibility, *in vitro* studies have provided abundant spectroscopic evidence for the ability of IscA/SufA proteins to assembly labile [Fe-S] clusters (Krebs et al. 2001; Ollagnier-de-Choudens et al. 2001; Morimoto et al. 2002; Wu et al. 2002; Ollagnier-De Choudens et al. 2003; Wollenberg et al. 2003; Wu and Cowan 2003; Ollagnier-De-Choudens et al. 2004). However, a consensus has yet to emerge concerning the number and type of clusters that can be assembled. For example, both $[2Fe-2S]^{2+}$ and $[4Fe-4S]^{2+}$ cluster-bound forms have been reported for *A. vinelandii* IscA[Nif] (Krebs et al. 2001), *E. chrysanthemi* and *E.coli* SufA (Ollagnier-De Choudens et al. 2003; Ollagnier-De-Choudens et al. 2004) and *E.coli* IscA (Ollagnier-De-Choudens et al. 2004) , whereas only $[2Fe-2S]^{2+}$ cluster-bound forms have been reported for the *S. pombe* Isa1 and *Synechocystis* IscA1 proteins (Morimoto et al. 2002; Wu et al. 2002; Wollenberg et al. 2003). On the basis of cluster stoichiometry and spectroscopic evidence for complete cysteinyl S ligation, subunit bridging arrangements were proposed for the $[2Fe-2S]^{2+}$ and $[4Fe-4S]^{2+}$ clusters assembled in *A. vinelandii* IscA[Nif] (Krebs et al. 2001) and the [2Fe-

2S]$^{2+}$ cluster in *Synechocystis* IscA1 (Wollenberg et al. 2003), whereas analytical data were interpreted in terms of one [2Fe-2S]$^{2+}$ per monomeric unit for *E. coli* IscA (Ollagnier-de-Choudens et al. 2001) and *S. pombe* Isa1 (Wu et al. 2002). Likewise, there is conflicting data for cysteine substitution studies. The ability to assemble unstable [2Fe-2S]$^{2+}$ clusters in forms of *S. pombe* Isa1 having conserved cysteines substituted by alanine were interpreted as indicating all three conserved cysteines are cluster ligands (Wu et al. 2002). In contrast, analogous mutagenesis studies with *Synechocystis* IscA1 failed to find evidence of cluster assembly in variants involving substitutions of the C-terminal pair of cysteines, and was interpreted to indicate these two cysteines ligate a bridging [2Fe-2S]$^{2+}$ cluster (Wollenberg et al. 2003).

The recent crystal structures of apo-forms of *E. coli* IscA (2.3 Å resolution) have not permitted unambiguous resolution of the subunit location and type of clusters assembled on IscA (Bilder et al. 2004; Cupp-Vickery et al. 2004). IscA/SufA proteins have been found to exist as a mixture of oligomeric forms in solution with dimeric and tetrameric forms predominating (Krebs et al. 2001; Ollagnier-de-Choudens et al. 2001; Wu et al. 2002; Wollenberg et al. 2003). Hence the tetrameric structure revealed in both crystal structures could be functionally relevant. Each subunit exhibits a novel, well-ordered fold in which mixed β-sheets form a compact α-β sandwich domain. The tetrameric structure produces a basket-like shape with a central cavity between the two dimers that could provide a path for delivery of Fe and S or release of clusters. The C-terminal tail that contains two of the three conserved cysteines was not ordered in either structure, indicating significant flexibility in the cluster-binding region. Nevertheless, modeling suggests that one solvent exposed [2Fe-2S]$^{2+}$ cluster could be accommodated on opposite sides of the tetramer, each coordinated between two subunits by the C-terminal CxC cysteines (Cupp-Vickery et al. 2004). The other conserved cysteine is also positioned sufficiently close to the putative cluster-binding site to participate in cluster assembly, formation or transfer.

A role for IscA/SufA proteins as scaffolds for [Fe-S] cluster assembly is further suggested by experimental demonstration of *in vitro* [Fe-S] cluster transfer from

IscA/SufA to various apo [Fe-S] proteins (Ollagnier-de-Choudens et al. 2001; Ollagnier-De Choudens et al. 2003; Wollenberg et al. 2003; Wu and Cowan 2003; Ollagnier-De-Choudens et al. 2004). In each case intact cluster transfer has been inferred based on comparison with rates and efficiency of cluster assembly using free Fe^{2+} and S^{2-} and/or the ability to assemble clusters in the presence of Fe chelating agents. In addition, cluster transfer has been shown to occur through complex formation between *S. pombe* Isa1 and Fdx (Wu and Cowan 2003), *E. coli* IscA and Fdx (Ollagnier-de-Choudens et al. 2001), and *E. coli* IscA/SufA and biotin synthase (Ollagnier-De-Choudens et al. 2004), suggesting a simple thiol exchange mechanism. Moreover, a comparative study of cluster transfer from IscA or IscU to Fdx revealed similar rates with cluster lability and solvent accessibility being major factors contributing to transfer efficiency (Wu and Cowan 2003). In aggregate these data present a strong case for a role for IscA/SufA proteins as [Fe-S] cluster scaffolds. The recent demonstration that IscA can accept clusters from IscU, but not vice-versa, has raised the intriguing possibility that IscU and IscA constitute primary and secondary cluster donors, respectively (Ollagnier-De-Choudens et al. 2004).

An alternative role for IscA as the iron donor for [Fe-S] cluster assembly within IscU has also been suggested (Ding and Clark 2004; Ding et al. 2004). This possibility was initially evaluated and considered to be unlikely based on assessment of Fe^{2+} and Fe^{3+} ion binding to *A. vinelandii* IscA^{Nif} (Krebs et al. 2001). Namely, IscA^{Nif} samples pretreated with dithiothreitol show no evidence of Fe^{3+} ion binding and only weak Fe^{2+} ion binding on the basis of absorption, VTMCD and Mössbauer studies. These experiments were conducted under anaerobic conditions in the absence of dithiothreitol. In subsequent experiments with *E. coli* IscA, an Fe^{3+}-bound species was identified that contains one Fe per dimer. In this case IscA was incubated with Fe^{2+} ion under aerobic conditions in the presence of dithiothreitol. The apparent iron association constant was assessed to be 3.0×10^{19} M^{-1} based on competition studies with citrate and the complex was shown to be an effective Fe donor for clusters assembly on IscU, even under conditions of Fe depletion resulting from the presence of citrate, a physiological Fe chelator (Ding and Clark 2004; Ding et al. 2004). However, since this Fe-bound form of

IscA can only be prepared in the presence of exogenous dithiothreitol, the physiological relevance of this species remains to be established.

Role of Chaperones in Isc-dependent [Fe-S] Protein Maturation. Heat shock chaperones and co-chaperones, for which DnaK and DnaJ, respectively, are the archetypical members, are involved in promoting a diverse array of nucleotide-dependent protein-protein interactions related to protein folding/refolding (Houry 2001). Activities of these chaperones are not necessarily restricted to functions involving temperature stress. Nevertheless, DnaK has an important function in preventing the accumulation/aggregation of misfolded proteins under heat stress conditions by promoting their folding/refolding. A general feature of this process involves an ATP-dependent DnaK interaction with short hydrophobic segments of unfolded substrate polypeptides. DnaK has an intrinsic low level of ATPase activity that is stimulated by interaction with protein substrate and the co-chaperone, DnaJ. The DnaJ protein appears to confer on the complex an ability to differentiate between misfolded proteins and peptides, thereby preventing the sequestration of DnaK into non-productive complexes.

HscB and HscA (heat shock cognate proteins) were originally identified from *E. coli* as a result of DNA sequence analysis and from genetic mapping of a mutation that partially suppresses the phenotype of an *hsn* allele, and were named on the basis of their primary sequence conservation when respectively compared to DnaJ and DnaK (Kawula and Lelivelt 1994; Seaton and Vickery 1994; Lelivelt and Kawula 1995; Vickery et al. 1997; Silberg et al. 1998). The biochemical basis of suppression of the *hsn⁻* phenotype is still not understood and the potential involvement of HscB and HscA in [Fe-S] cluster biosynthesis was not appreciated at the time of their discovery. Although a genetic association of *fdx*, *hscB*, and *hscA* was recognized early on, the involvement of HscB and HscA in [Fe-S] cluster biosynthesis only became apparent when genome sequences revealed the conserved genetic context of *iscS, iscU, iscA, hscB, hscA* and *fdx*. Evidence for a connection between molecular chaperones and [Fe-S] cluster biosynthesis was also discovered independently in yeast by the identification of mutations that suppress a superoxide dismutase deficiency (Strain et al. 1998).

Biochemical-genetic experiments have firmly established that HscB and HscA are associated with Isc-directed [Fe-S] protein maturation (Nakamura et al. 1999; Takahashi and Nakamura 1999; Tokumoto and Takahashi 2001; Tokumoto et al. 2002) and a series of thorough biochemical analyses using the *E. coli* and yeast proteins have revealed several of the important features of the system (Schilke et al. 1999; Cupp-Vickery and Vickery 2000; Hoff et al. 2000; Silberg and Vickery 2000; Voisine et al. 2000; Brehmer et al. 2001; Kim et al. 2001; Lutz et al. 2001; Schmidt et al. 2001; Silberg et al. 2001; Voisine et al. 2001; Hoff et al. 2002; Hoff et al. 2003; Cupp-Vickery et al. 2004; Tapley and Vickery 2004). HscA is able to interact with either the cluster-loaded or apo-forms of IscU (Hoff et al. 2000; Silberg et al. 2001; Hoff et al. 2002; Hoff et al. 2003; Cupp-Vickery et al. 2004; Tapley and Vickery 2004). Such interaction is assisted by HscB, which has the ability to interact with both IscU and HscA. Similar to the situation with DnaK, HscA exhibits a low level of intrinsic ATPase activity that is stimulated by interaction with HscB and greatly stimulated by a combination of HscB and IscU. A short highly conserved sequence within IscU (LPPVK), located near the third strictly conserved cysteine residue, is necessary and sufficient to stimulate HscA-dependent nucleotide hydrolysis (Hoff et al. 2003). Crystallography, mutagenesis and use of fluorescently labeled oligopeptide substrates have provided evidence for a specific HscA-IscU interaction (Cupp-Vickery et al. 2004; Tapley and Vickery 2004) and the NMR structure of the *H. influenzae* IscU (Protein Data Bank -PDB ID: 1Q48) revealed that the LPPVK motif is located within a solvent exposed loop adjacent to the apparent [Fe-S] cluster assembly site defined by the three conserved cysteines. Parallel studies performed with yeast HscB and HscA homologs are in general agreement with features of the bacterial system although there do appear to be a variety of mechanistic differences (Schilke et al. 1999; Voisine et al. 2000; Huynen et al. 2001; Kim et al. 2001; Lutz et al. 2001; Schmidt et al. 2001; Voisine et al. 2001; Dutkiewicz et al. 2003; Dutkiewicz et al. 2004).

In spite of these advances there is no direct information concerning the specific function of HscB and HscA, or their homologs, in the maturation of [Fe-S] proteins. It has been shown in yeast, on the basis of immunoprecipitation studies, that functional

depletion of the [Fe-S] cluster assembly-associated chaperones leads to an increased accumulation of Fe on IscU (Muhlenhoff et al. 2003). These results have been reasonably interpreted to indicate a requirement for the chaperones in [Fe-S] cluster transfer, but at which stage this requirement is effected is not yet clear. For example, the chaperones could be directly responsible for [Fe-S] cluster transfer during [Fe-S] protein maturation, they could be required for protein conformational changes that position clusters for transfer, or they could be involved in controlling structural rearrangements in cluster precursors leading to assembly of a species amenable to transfer. To date, there is no evidence for the direct interaction between HscB or HscA with any target [Fe-S] protein.

Biochemical Features of the SufS, SufE, SufB, SufC and SufD Proteins. SufS and SufE have been shown to constitute a class of two-component cysteine desulfurases where SufE enhances the cysteine desulfurization activity of SufS up to 50-fold (Loiseau et al. 2003; Outten et al. 2003). Mutagenesis experiments coupled with ^{35}S-radiolabeling and mass spectrometry studies have demonstrated that SufE functions as a sulfurtransferase (Ollagnier-de-Choudens et al. 2003; Outten et al. 2003). The SufE-dependent enhancement of cysteine desulfurase activity has therefore been attributed to the facile transfer of cysteine persulfides formed on SufS to a conserved cysteine residue within SufE. Addition of the SufBCD complex to SufS in the presence of SufE results in a significant additional increase in activity suggesting that SufE and SufBCD act synergistically to modulate the activity of SufS (Outten et al. 2003).

SufC has intrinsic ATPase activity and forms a tight, soluble complex with SufB and SufD (Rangachari et al. 2002; Nachin et al. 2003; Outten et al. 2003). Although the components of the SufBCD complex have similarity to the components of ATP-binding cassette (ABC) transporter proteins, the primary sequences show no indication of transmembrane segments and cell fractionation techniques show that all three proteins are localized in the cytosol (Nachin et al. 2003). The function of the SufBCD complex is still unclear and it appears unlikely that stimulation of SufSE activity is the only function of the complex, because ATP hydrolysis is not required for such stimulation (Outten et al.

2003).   Among other possibilities are nucleotide-dependent Fe release/acquisition or perhaps a chaperone function analogous or related to that of HscB and HscA. A possible role for the SufBCD complex as a scaffolding system for assembly of transient clusters and transfer to target proteins also deserves consideration because the complex contains 4 highly conserved and 6 partially conserved cysteines (Outten et al. 2003). In this context it should be noted that some archaea and bacteria contain proposed SufBCD or SufBC homologs but no IscA/SufA or IscU/SufU scaffold proteins (Takahashi and Tokumoto 2002).

2.8 - IscS has a General Role in Intracellular Sulfur Trafficking

Since the original reports that NifS and IscS have cysteine desulfurase activity this topic has received intense investigation leading to many publications concerning the mechanism and structure of members of this class of proteins (Zheng et al. 1994; Leibrecht and Kessler 1997; Mihara et al. 1997; Lang and Kessler 1999; Mihara et al. 1999; Clausen et al. 2000; Fujii et al. 2000; Mihara et al. 2000; Lima 2002; Mihara and Esaki 2002; Mihara et al. 2002; Cupp-Vickery et al. 2003; Kaiser et al. 2003). A comprehensive review of the phylogenetic, structural and functional features of the NifS/IscS/SufS family has appeared (Mihara and Esaki 2002) and a particularly thorough treatment of the catalytic mechanism has been reported (Kaiser et al. 2000). Among the various members of this family there appears to be three variations that include: (i) formation of a persulfide on an active-site cysteine located within a flexible loop (NifS/IscS class); (ii) formation of a persulfide on an active-site cysteine with immediate transfer to a second component protein (the SufSE two component class); and (iii) activation of cystine rather than cysteine with formation of cysteine persulfide as the activated species. All of these systems bear significant primary sequence and structural similarities and are unified by activation of S in the form of a persulfide. Clearly, a cellular strategy for sulfur mobilization and trafficking involves persulfide formation as way to sequester activated S in a form that is not toxic.

When the mechanism of NifS was first discovered, the possibility that enzymatic persulfide formation could represent a general mechanism for [Fe-S] cluster formation was recognized, but a potential role for the same mechanism in activation of S for other cellular processes was not appreciated. A possible role for IscS in providing the physiological S source for other metabolic processes became apparent when it was discovered that an *E. coli* or *Salmonella enterica iscS* mutant is deficient in formation of thiamine, other cofactors and thionucleotides (Lauhon and Kambampati 2000; Skovran and Downs 2000; Tokumoto and Takahashi 2001). Sorting out the involvement of IscS in such processes was complicated because different possibilities could be considered. Namely, IscS could be considered as a direct S donor, IscS could be necessary for the activation of an [Fe-S] protein that is required for some other step in the synthesis of S-containing biomolecules, or IscS could be considered an indirect S donor through an [Fe-S] cluster species. It is now known that all three situations exist. For example, inactivation of *iscS* results in the defective synthesis of all thiolated nucleotides (Mueller et al. 1998; Kambampati and Lauhon 1999; Kambampati and Lauhon 2000; Lauhon and Kambampati 2000; Mueller et al. 2001; Lauhon 2002; Nilsson et al. 2002; Wright et al. 2002; Kambampati and Lauhon 2003), yet such defects become manifested in two different ways. Thiolation of certain thionucleotides requires S transfer from IscS in pathways involving ThiI (Kambampati and Lauhon 2000; Palenchar et al. 2000; Mueller et al. 2001) or MnmA (Kambampati and Lauhon 2003) as intermediate carriers, whereas the formation of other thionucleotides requires the participation of MiaB (Esberg et al. 1999), a member of the radical-SAM family of [Fe-S] proteins (Pierrel et al. 2002), whose exact function is not yet known. Similarly thiamine biosynthesis requires IscS as a S donor via ThiI (Palenchar et al. 2000), as well as participation of an [Fe-S] protein called ThiH (Leonardi et al. 2003; Park et al. 2003; Leonardi and Roach 2004) Such divergent functions of IscS have now been clearly established by the differential impairment of different functions via amino acid substitution within the flexible loop of IscS (Lauhon et al. 2004), or by alteration of [Fe-S] cluster biosynthetic machinery other than IscS (Leipuviene et al. 2004). Biotin synthesis represents the third type of involvement of IscS in cofactor biosynthesis where it appears that a [2Fe-2S] cluster assembled within BioB directly provides the sulfur for insertion into dethiobiotin

(Ugulava et al. 2001; Jameson et al. 2004). Another interesting topic not included in this review involves a role for the IscS family of proteins in mobilization of Se (Lacourciere and Stadtman 1998; Lacourciere et al. 2000; Lacourciere 2002; Mihara et al. 2002).

2.9 - [Fe-S] Protein Maturation in Eukaryotes

Although this review has focused primarily on [Fe-S] cluster assembly in prokaryotes, it is important to note that homologs to all of the major components of the Isc machinery are also present in eukaryotes and these nucleus-encoded components are localized primarily or exclusively in the mitochondria (for reviews see (Muhlenhoff and Lill 2000; Gerber and Lill 2002; Balk and Lill 2004; Rouault and Tong 2004)). Indeed the preservation of the Isc cluster assembly machinery in mitochondria adds strong support to the proposal that mitochondria evolved from bacterial cells and has also contributed to the debate on the evolutionary relationship between mitochondria found in most eukaryotes and mitochondrion-like organelles found in certain parasitic organisms (Tachezy et al. 2001; Emelyanov 2003; LaGier et al. 2003; Tovar et al. 2003; Sutak et al. 2004; van der Giezen et al. 2004). [Fe-S] cluster biosynthesis is currently the only known biosynthetic function of mitochondria and the available evidence suggests that [Fe-S] clusters pre-assembled by the Isc machinery in the mitochondria are transported into the cytosol for the maturation of cytosolic [Fe-S] proteins, in addition to being used for the maturation of the numerous mitochondrial [Fe-S] proteins (Muhlenhoff and Lill 2000; Gerber and Lill 2002).

S. cerevisiae is the best characterized eukaryotic system and functional homologs to many of the bacterial *nif* and *isc* genes have been identified, although they are designated using a somewhat different nomenclature (Strain et al. 1998; Garland et al. 1999; Kispal et al. 1999; Schilke et al. 1999; Jensen and Culotta 2000; Kaut et al. 2000; Lange et al. 2000; Pelzer et al. 2000; Gerber and Lill 2002; Dutkiewicz et al. 2003; Gerber et al. 2004). As examples: (i) Nfu1p contains a domain corresponding to the C-terminal domain of NifU , (ii) Isu1p and Isu2p correspond to IscU, (iii) Nfs1p corresponds to NifS/IscS, (iv) Isa1p and Isa2p correspond to IscA, (v) Jac1p corresponds

to HscB, (vi) Ssq1p corresponds to HscA, and (vii) Yah1p corresponds to Fdx. The eukaryotic [Fe-S] protein maturation machinery is significantly more complex than found for prokaryotes. For example, the mitochondrial [Fe-S] cluster biosynthesis machinery includes an ABC transporter, Atm1p (ABC7 in *H. sapiens*), and a protein with sulfhydryl oxidase activity, Erv1p, which is localized in the intermembrane space (Muhlenhoff and Lill 2000; Gerber and Lill 2002). These proteins are not required for maturation of mitochondrial [Fe-S] proteins, but function in the export of a hitherto undefined [Fe-S] species into the cytosol for the maturation of cytosolic [Fe-S] proteins. Two cytoplasmic proteins, the hydrogenase-like Nar1p and a putative P-loop ATPase Cfd1p (cytosolic Fe-S cluster deficiency protein) are also essential for maturation of cytosolic [Fe-S] proteins, with Nar1p being implicated in the maturation of nuclear Fe-S proteins as well (Roy et al. 2003; Balk et al. 2004). In addition, several other mitochondrial proteins have been shown to play important roles in [Fe-S] cluster biosynthesis. These include a Fdx reductase, Arh1p (Gerber and Lill 2002), a monothiol glutaredoxin, Grx5p, that appears to be involved in the release or transfer of clusters assembled on Isu1p (Rodriguez-Manzaneque et al. 2002; Muhlenhoff et al. 2003), and a nucleotide release factor, Mge1p, which functions to stimulate Ssq1p ATPase activity (Lutz et al. 2001; Dutkiewicz et al. 2003).

The recruitment of Fe for [Fe-S] cluster biosynthesis is unclear for prokaryotic organisms but there is gathering *in vivo*, *in organello* and *in vitro* evidence indicating that frataxin could serve that function in eukaryotes (Muhlenhoff et al. 2002; Gerber et al. 2003; Yoon and Cowan 2003; Ramazzotti et al. 2004). It appears that the yeast frataxin homolog, Yfh1p, plays a specific role in the maturation of cellular [Fe-S] proteins involving an interaction with an Nfs1p/Isu1p complex necessary for assembly of clusters on Isu1p (Muhlenhoff et al. 2002; Gerber et al. 2003; Ramazzotti et al. 2004). Human frataxin has been characterized as a monomeric protein capable of binding 6-8 Fe ions, most likely at surface carboxylates (Yoon and Cowan 2003). Moreover, Fe-bound frataxin forms a tight complex with human Isu and was found to be an effective replacement for Fe^{2+} ions in the *in vitro* assembly of $[2Fe-2S]^{2+}$ clusters on human Isu

(Yoon and Cowan 2003). It remains to be seen if the bacterial frataxin ortholog, CyaY, is an effective Fe donor for cluster assembly on bacterial IscU (Bou-Abdallah et al. 2004).

Two human diseases have already been identified with defects in the mitochondrial [Fe-S] cluster assembly machinery (Gerber and Lill 2002). X-linked sideroblastic anemia and cerebellar ataxia are hereditary recessive iron storage disorders caused by a mutation in ABC7 (Shimada et al. 1998) and Friedreich's ataxia is a neurodegenerative disease associated with defects in frataxin (Campuzano et al. 1996).

The maturation of [Fe-S] clusters in photosynthetic microorganisms and higher plants is also an emerging and very active area of research (reviewed in (Shen et al. 2004)) and this process involves novel challenges when compared to other systems. For example, [Fe-S] clusters are required for maturation of the photosynthetic apparatus within plastids and the biosynthetic machinery must, therefore, be imported into the plastid and be capable of dealing with the oxidizing environment associated with photosystem I activity. It is therefore not surprising that a plethora of proteins, with significant similarities to elements of the Isc, Suf and Nif systems, as well as proteins uniquely associated with eukaryotic organisms, have already been identified in higher plants.

2.10 - Summary and Outlook

The field of study involving maturation of [Fe-S] proteins emerged when it was realized that biological [Fe-S] clusters are not formed spontaneously but require a complex biosynthetic machinery. Intense research activity in this area is evidenced by more than 200 publications that have appeared on this topic over the last decade, and many key features of the process have been discovered. Among the most significant of these are included: (i) participation of cysteine desulfurases in the mobilization of sulfur, (ii) a requirement for molecular scaffolds for assembly of [Fe-S] clusters, (iii) the participation of molecular chaperones, (iv) intact cluster transfer from scaffolds to target proteins, (v) specialized [Fe-S] cluster biosynthetic machinery for specific targets, (vi)

specialized [Fe-S] cluster biosynthetic machinery to accommodate stress conditions, (vii) feedback regulation of [Fe-S] cluster biosynthesis and regulation in response to environmental conditions, (viii) compartmentalization of [Fe-S] cluster biosynthetic machinery, (ix) intracellular trafficking of pre-formed [Fe-S] clusters and, (x) mechanisms for the protection or repair of [Fe-S] proteins (not discussed in this review). Although the main features and most of the key players have now been identified, very little mechanistic insight at the molecular level has been gained. The main challenge of the future will be to elucidate exactly how the different proteins interact together to accomplish [Fe-S] protein maturation *in vivo* and to faithfully duplicate that process *in vitro*.

CHAPTER 3

Controlled Expression and Functional Analysis and Iron-Sulfur Cluster Biosynthetic Components within *Azotobacter vinelandii*

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This manuscript describes the construction and application of a controlled gene expression system in *Azotobacter vinelandii* which was used to perform a functional analysis of the Isc [Fe-S] cluster biosynthetic machinery. The discovery of a sucrose-inducible promoter in the *A. vinelandii* genome permitted the construction of many strains which harbored two copies of the *isc* operon, one under control of the normal endogenous promoter and another under control of this sucrose-inducible promoter. A functional analysis of the *Isc* components was achieved by monitoring the physiological consequences of specific mutations within the endogenous *isc* operon under conditions which repress the expression of the second, intact *isc* operon. Using this genetic strategy, essential *isc* gene products and specific amino acids residues within Isc proteins were identified. Key findings include the effects of oxygen levels on the growth of strains depleted for IscA or HscBA, indicating a role in protection and/or repair of the Isc machinery.

I am responsible for performing most of the experiments presented except for: the construction of plasmids, strains and β-galactosidase assays pertaining to the functional analysis of IscR (Callie Raulfs and Ina Puleri); the construction of DJ1601 (Mihaela Unciuleac);and the construction of plasmids with point substitution mutations in *isc* genes (Valerie Cash). This chapter was written and submitted for publication with the intention for its use to satisfy a portion of the research completed for this dissertation. As senior author, I was involved in writing the document at all stages of its preparation and prepared all the figures that are included (except for Figure 10 and Table 3).

3.1 - Introduction

Iron sulfur clusters ([Fe-S] clusters) are simple prosthetic groups comprised of inorganic $Fe^{2+/3+}$ and S^{2-} and are found in a broad class of proteins, called [Fe-S] proteins. [Fe-S] proteins and their associated [Fe-S] clusters participate in a variety of physiological functions including metabolic transformations, electron transfer, and environmental sensing (Beinert et al. 1997; Fontecave 2006). The most common forms of biological [Fe-S] clusters, [2Fe-2S] and [4Fe-4S] clusters, can often be assembled and incorporated into the apo-forms of their cognate protein partners by the simple *in vitro* addition of $Fe^{2+/3+}$ and S^{2-} under reducing conditions (Malkin and Rabinowitz 1966). Such facile chemical assembly, together with the dynamic electronic and structural features associated with [Fe-S] clusters, has probably contributed to their emergence as one of nature's most ancient and versatile prosthetic groups.

Although certain [Fe-S] proteins can be activated using $Fe^{2+/3+}$ and S^{2-} *in vitro*, the physiological maturation of [Fe-S] proteins is considerably more complicated. It is now known that there are several different systems that can direct [Fe-S] protein maturation (reviewed in Barras et al. 2005; Johnson et al. 2005). The first system to be discovered was the so-called Nif system from *Azotobacter vinelandii*, which is required for the activation of the catalytic components of biological nitrogen fixation (Jacobson et al. 1989; Jacobson et al. 1989). The Nif system includes two proteins, NifS and NifU, which are respectively required for the pyridoxal-phosphate-dependent mobilization of S using L-cysteine (cysteine desulfurase), and for providing a scaffold for nascent [Fe-S] cluster assembly (Zheng et al. 1993; Zheng et al. 1994; Agar et al. 2000; Yuvaniyama et al. 2000; Dos Santos et al. 2004). More recently, it has been shown that the Nif system for [Fe-S] protein maturation is not necessarily restricted to nitrogen-fixing organisms because proteins bearing a high degree of similarity to NifS and NifU have been identified in non-nitrogen-fixing organisms and they appear to be required for the general maturation of [Fe-S] proteins in those organisms (Olson et al. 2000; Ali et al. 2004; Tokumoto et al. 2004). A second, more complicated system for [Fe-S] protein maturation is referred to as the Isc system, which includes eight contiguously arranged

genes encoding: IscR, IscS, IscU, IscA, HscB, HscA, Fdx and IscX (Figure 1B). For simplicity, this genomic region is generically referred to as the "*isc*" gene region. IscS and IscU bear primary sequence similarity and have functions analogous to NifS and NifU, respectively. IscA has been proposed to serve either as an alternative scaffold or as an agent of iron delivery to the IscU scaffold (Krebs et al. 2001; Ollagnier-de-Choudens et al. 2001; Ding et al. 2004; Ding et al. 2005). There is also a *nif*-encoded homolog to IscA (IscA [Nif]) but, similar to the situation with IscA, no null phenotype has yet been associated with the loss of its function (Krebs et al. 2001). HscA and HscB bear primary sequence similarity to DnaK and DnaJ, respectively and have therefore been proposed to have a chaperone function related to [Fe-S] protein maturation. This possibility is supported by an intrinsic ATPase activity of HscA, which is stimulated by HscB and by interaction of the HscBA complex with the IscU scaffold (Silberg et al. 2001; Hoff et al. 2002; Hoff et al. 2003; Cupp-Vickery et al. 2004; Tapley and Vickery 2004). Neither the function of Fdx, which contains a redox-active [2Fe-2S] cluster, nor the function of IscX is known (Jung et al. 1999; Kakuta et al. 2001; Shimomura et al. 2005). IscR is a regulatory protein that apparently controls expression of the *isc* gene cluster in a negative feedback loop that involves the assembly of a [2Fe-2S] cluster within IscR (Schwartz et al. 2001). In addition to their role in maturation of [Fe-S] proteins, Isc components have been shown to be involved in the biogenesis of certain S-containing cofactors either directly through S transfer by IscS, or indirectly, through participation of [Fe-S] proteins (Mihara and Esaki 2002).

A third system for [Fe-S] protein maturation, discovered in *Escherichia coli*, is called the Suf system (Takahashi and Tokumoto 2002). In *E. coli,* the Suf system is comprised of SufA, SufB, SufC, SufD, SufS, and SufE, and it functions under conditions of redox stress or Fe limitation, when the Isc system is apparently inadequate (Lee et al. 2004; Outten et al. 2004). SufS and SufE represent a two-component cysteine desulfurase having a function that is analogous to NifS and IscS (Loiseau et al. 2003; Outten et al. 2003), SufA bears primary sequence homology to IscA (Ollagnier-De Choudens et al. 2003), and SufB, SufC, and SufD form a complex of unknown function that has intrinsic ATPase activity (Nachin et al. 2003; Outten et al. 2003). A fourth,

rudimentary system, CsdA/CsdE, which could also have a function related to some aspect of [Fe-S] cluster formation has recently been discovered in *E. coli* (Loiseau et al. 2005).

Several features have emerged concerning the functions of [Fe-S] protein maturation systems in prokaryotic organisms. First, all of the intact systems appear to contain a cysteine desulfurase, which is required for the mobilization of sulfur, and a potential scaffold, which could be required for the pre-assembly of [Fe-S] clusters. Second, some organisms have redundant [Fe-S] protein maturation systems that operate under specific conditions. For example, in *E. coli*, the Suf system is required for generalized [Fe-S] protein maturation under conditions of oxidative stress or Fe limitation (Outten et al. 2004), whereas, in the case of the nitrogen-fixing aerobe, *A. vinelandii*, the Nif system is specifically required for maturation of [Fe-S] cluster-containing proteins required for nitrogen fixation (Johnson et al. 2004). Third, certain [Fe-S] maturation systems that have apparent specificity in one organism appear to have a more generalized function for [Fe-S] protein maturation in another organism (Ali et al. 2004; Tokumoto et al. 2004). For example, a Nif-like system and a Suf-like system appear to have generalized functions in *Helicobacter pylori* and *Thermatoga maritima*, respectively.

A number of complementary genetic and biochemical studies have established the existence of these various [Fe-S] protein maturation systems and there have also been some advances in the development of *in vitro* systems for [Fe-S] cluster assembly. Nevertheless, only limited progress has been achieved towards understanding the function of individual [Fe-S] cluster biosynthetic components at the biochemical-genetic level, or understanding the underlying basis for the specificity of [Fe-S] protein maturation in certain systems. Owing to the facile genetic manipulation of *A. vinelandii*, the fact that it does not contain an intact Suf system, and that the Nif system is expressed only under nitrogen-fixing conditions, this organism provides an opportunity for detailed analysis of the Isc components. In the present work we describe the development and application of a genetic approach for the functional analysis of the *isc*-encoded gene products.

3.2 - Materials and Methods

Bioinformatics. *Azotobacter vinelandii* genome sequence information can be obtained from the Microbial Genomics section at the Department of Energy Joint Genome Institute (JGI) website (http://genome.jgi-psf.org/mic_home). Research of protein orthologs in different genomes was performed using the BLAST program at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) and the Comprehensive Microbial Resource (CMR) at The Institute for Genomic Research TIGR website (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl). DNASTAR Lasergene software (Madison, WI) was used to analyze all DNA sequences and deduce protein sequences.

Growth of *A. vinelandii* strains and extraction of genomic DNA. All chemicals were obtained from Sigma unless otherwise stated. *A. vinelandii* strains were grown at 30°C on modified Burks minimal medium (Strandberg and Wilson 1968) containing 2% sucrose or 2% glucose as the sole carbon source. Ammonium acetate served as the nitrogen source and was added at a final concentration of 13 mM. For antibiotic resistance selection and/or screening, final concentrations were: ampicillin (0.08 μg/ml) kanamycin (0.5 μg/ml), gentamycin (0.05 μg/ml), rifampicin (5.0 μg/ml), and streptomycin (0.1 μg/ml). X-Gal (4-bromo-4-chloro-3-indoyl-β-D-galactoside) was added to a final concentration of 60 μg/ml. Chromosomal DNA was extracted from *A. vinelandii* with QuickExtract™ DNA Extraction Solution (Epicentre).

Plasmid Construction. Construction of the key parent plasmids used in this study is described below. Relevant sub-clones of each parent plasmid are listed and described in Table 1. Preparations, restriction enzyme digestion, and ligation of hybrid plasmid DNAs were performed by previously described techniques (Sambrook et al. 1987). Restriction endonucleases and Bal31 exonuclease, DNA ligase and T_4 DNA polymerase were purchased from New England Biolabs, Promega, and Invitrogen, respectively. *Escherichia coli* TB1 and XL10 GOLD® (Stratagene) were used for cloning over 30 different hybrid plasmids containing genomic DNA from *A. vinelandii*. Polymerase

Chain Reaction (PCR) was performed using a commercial kit (Failsafe^{TM} PCR PreMix Selection Kit). Cloning of PCR products into relevant vectors was achieved by using primers with engineered restriction sites or into PCR®4TOPO® vector (Invitrogen). Antibiotic gene cartridges used to create insertion mutations in specific genes were derived from: pWKR202I for gentamycin (*gn*; lab stock), pUC4-KAPA for kanamycin (*kan*; Pharmacia), and pHP45Ω for streptomycin (*str*; (Fellay et al. 1987). The 6.1 kb *lacZkan* cartridge was obtained from pLKC480 (Tiedeman and Smith 1988).

TABLE 1. Key parent plasmids and relevant derivatives of these plasmids used for the construction of *A. vinelandii* mutant strains.

Plasmids[a]	Relevant genes cloned [b,c]	Vector	Reference
pDB1264	**18 kb, BglII *A. vinelandii* chromosomal *isc* operon fragment**	**pUC119**	**This study**
pDB1286	3.5 kb HindIII *hscBAfdxiscX* fragment	pUC119	This study
pDB1291	5.2 kb BspHI *iscSUAhscBAfdxiscX* fragment with SbfI-Δ*hscBA* (a.a. HscB 78 – HscA 442)	pAra13	This study
pDB1316	5.2 kb BspHI *iscSUAhscBAfdxiscX* fragment under P$_{scrX}$ control	pUC7	This study
pDB1490	831 bp SalI fragment with SphI-Δ*iscR* (IscR a.a. 21-60)	pUC7	This study
pDB1500	831 bp SalI fragment with EcoRI-Δ*iscR* (IscR a.a. 57-119)	pUC8	This study
pDB1507	831 bp SalI fragment encoding –*iscR* codon 92, *GCA* (IscR^{C92A})	pUC8	This study
pDB1518	831 bp SalI fragment encoding – *iscR* codon 104, *GCC* (IscR^{C104A})	pUC8	This study
pDB1608	3.2 kb PciI-BglII *hscBAfdxiscX* fragment under P$_{scrX}$ control	pUC7	This study
pDB1307	**PCR-derived, 3.82 kb *scrX* gene and promoter region (P*scrX*)**	**pUC7**	**This study**
pDB1309	HincII-Kn^R from pUC-KAPA in EcoRV site of *scrX* gene	pUC7	This study
pDB1310	MCS #1[d] substituting ΔPciI-KpnI fragment of *scrX*. Introduces the following unique sites: PciI, XbaI, XhoI and KpnI.	pUC7	This study
pDB1316	5.2 kb BspHI *iscSUAhscBAfdxiscX* fragment under P*scrX* control	pUC7	This study
pDB1332	MCS #2[e] substituting ΔPciI-KpnI in *scrX*. Introduces the following unique sites: PciI, NruI, EcoRV, XhoI, BglII, XbaI, SphI, KpnI	pUC7	This study
pDB1335	6.1 kb SmaI-*lacZkan* fragment under P*scrX* control	pUC7	This study
pDB1608	3.2 kb PciI-BglII *hscBAfdxiscX* fragment under P*scrX* control	pUC7	This study
pDB945	**1 kb EcoRI *fdx* fragment**	**pUC119**	Zheng *et al*, 1998
pDB1016	HincII-*kan* from pUC-KAPA in EcoRV site of *fdx* gene	pUC119	This study
pDB1550	XhoI-Δ*fdx* (Fdx a.a. 29 – 64)	pUC119	This study
pDB933	**2.5 kb EcoRI-SstI *iscSUA* fragment**	**pUC119**	Zheng *et al*, 1998
pDB954	SalI-Δ*iscS* (IscS a.a. 14 – 182)	pUC119	This study
pDB983	NdeI-BamHI *iscSUA* fragment	pT$_7$-7	This study
pDB1058	site-directed substitution –*iscU* codon 39, *GCC* (IscU^{D39A})	pUC119	This study
pDB1209	site-directed substitution – *iscS* codon 325, *GCC* (IscS^{C325A})	pUC119	This study
pDB1215	site-directed substitution - *iscA* codon 99, *GCC* (IscA^{C99A})	pUC119	This study
pDB1216	site-directed substitution - *iscA* codon 101, *GCC* (IscA^{C101A})	pUC119	This study
pDB1227	site-directed substitution - *iscU* codon 106, *GCG* (IscU^{C106A})	pUC119	This study
pDB1228	site-directed substitution - *iscU* codon 63, *GCC* (IscU^{C63A})	pUC119	This study
pDB1236	site-directed substitution - *iscA* codon 36, *GCG* (IscA^{C36A})	pUC119	This study
pDB1250	site-directed substitution - *iscA* codon 104, *TGC* (IscA^{S104C})	pUC119	This study
pDB1350	Δ*iscU* (IscU a.a. 31-117) from Bal-31 exonuclease digestion at DraIII	pT$_7$-7	This study
pDB1351	Δ*iscUA* (IscU a.a. 34-IscA 9) from Bal-31 exonuclease digestion at SexAI in *iscA*.	pT$_7$-7	This study
pDB1391	Δ*iscA* (IscA a.a 8-125) from Bal-31 exonuclease digestion at SexAI.	pT$_7$-7	This study
pDB1404	site-directed substitution - *iscU* codon 37, *GCG* (IscU^{C37A})	pUC119	This study
pDB1538	site-directed substitution - *iscU* codon 105, *GCC* (IscU^{H105A})	pUC119	This study
pDB1546	site-directed substitution - *iscU* codon 103, *GCG* (IscU^{K103A})	pUC119	This study
pDB1611	site-directed substitution - *iscA* codon 104, *GCC* (IscA^{S104A})	pUC119	This study
pDB1023	**PCR-derived 0. 5 kb *iscR* gene with constructed NdeI-BamHI flanking sites**	**pT$_7$-7**	**This study**
pDB1212	site-directed substitution -*iscR* codon 98, *GCC* (IscR^{C98A})	pT$_7$-7	This study
Other:			
pDB528	*recA*::Tn10 (Kn^r)	pSUP102	Venkatesh *et al.,* 1990
pDB1375	2.6 kb PstI-*gn* from pWKR202I in PstI site of PCR-derived *recA* gene	pUC7	This study
pDB1400	2.6 kb blunt-ended BamHI-*gn* from pWKR202I in StuI site of PCR-derived *scrR* gene	pUC19	This study
pDB1468	6.1 kb SmaI-*lacZkan* from pLKC482 in AleI site of PCR-derived *hscA* gene	pT$_7$-7	This study
pDB1499	2.6 kb SalI-*gn* from pWKR202I in SalI site of PCR-derived *iscA2* gene	pUC119	This study

[a] Key parent plasmids are written in bold. Plasmid derivatives are written as an indented list directly below the parent.

[b] Sizes of gene deletions are described as amino acid (a.a.) deletions.

[c] Descriptions of site-directed amino acid substitutions include the corresponding DNA codon change.

[d] 45 bp Multiple Cloning Site – see Materials and Methods for sequence.

[e] 35 bp Multiple Cloning Site – see Materials and Methods for sequence.

pDB1264: A restriction digestion of chromosomal DNA from DJ1179 (Zheng et al. 1998) with BglII, served as the source of an 18 kb, genomic fragment containing the *isc* region and a *kan*-interrupted *cysE2* gene. Ligation of the BglII-chromosomal fragments with BamHI-digested pUC119, followed by selection on plates containing both ampicillin and kanamycin resulted in the isolation of an *E. coli* XL10 strain harboring pDB1264. Sub-clones of pDB1264 include pDB1286, pDB1291, pDB1316, pDB1490, pDB1500, pDB1507, pDB1518 and pDB1608 (see Table 1).

pDB1307: DNA amplification of a 3.82 kb *A.vinelandii* chromosomal region spanning the *scrX*-gene and its putative promoter,P*scrX*, was achieved using appropriate primers with flanking PstI restrictions sites: ligation of the PstI-digested PCR-product with PstI - digested pUC7 created pDB1307. To construct pDB1310 and pDB1332, a 2 kb PciI-KpnI fragment within the *scrX* open reading frame was substituted with a 45 bp or 35 bp double-stranded, DNA linker to introduce multiple cloning sites, designated MCS #1 and MCS #2, respectively, permitting the placement of desired gene/s under the control of the *scrX* transcriptional and translational regulatory elements (see Table 1). The single stranded oligonucleotide sequences that were annealed for the creation of each linker are as follows:

5'-CATGTCTAGAAAGCTTGTTAACCCGGGCTCGAGGCATATGGGTAC and 5' CCATATGCCTCGAGCCCGGGTTAACAAGCTTTCTAGA-3' for MCS #1 in pDB1310; and 5'-CATGTCGCGATATCTCGAGATCTCTAGAGCATGCGGTAC-3' and 5'CGCATGCTCTAGAGATCTCGAGATATCGCGA-3' for MCS #2 in pDB1332.

A PCR method was also used to amplify the following gene regions from the *A. vinelandii* genome using appropriate primers: the 0.5 kb *iscR* gene; the 1.7 kb *scrR* gene; the 1.5 kb *iscA2* gene; the 0.98 kb *recA* partial gene fragment; and the 1.8 kb *hscA* gene. These amplified DNA fragments were cloned into suitable vectors that served as the parent plasmids for the construction of pDB1212, pDB1400, pDB1499, pDB1375 and pDB1468, respectively.

In-frame deletions in *isc* genes were created by one of two methods: (i) restriction enzyme digestion and re-ligation, as in pDB1500 (Δ *iscR*), pDB954 (Δ *iscS*); pDB1291 (Δ *hscBA*) and pDB1550 (Δ *fdx*); (ii) Bal-31 exonuclease digestion as in pDB1350 (Δ *iscU*), pDB1391 (Δ *iscA*) and pDB1386 (Δ *iscUA*). Site-directed mutagenesis was performed using a commercial kit (GeneEditor, Promega). Altered DNA sequences were confirmed by DNA sequence analysis at the Virginia Bioinformatics Institute (VBI) sequencing facility.

Strain Construction. Over 50 different mutant *A. vinelandii* strains were constructed during completion of this work and key strains are listed in Table 2. Mutations within the *A. vinelandii* genome were achieved by transformation of competent cells with the appropriate plasmids, followed by selection and/or screening of cells which have undergone double-reciprocal recombination between genome and plasmid vector/s. Transformation of *A. vinelandii* was performed as previously described (Jacobson et al. 1989). Strains transformed with plasmids harboring genes interrupted with *lacZ* and/or antibiotic resistance cartridges were selected by plating on Burks agar plates supplemented with X-gal and/or the relevant antibiotic. Hybrid plasmids containing specific in-frame gene deletions or point mutations were transformed into *A. vinelandii* cells by congression (coincidental transfer of genetic markers) with rifampicin (Rif^r), kanamycin (Kn^r), gentamycin (Gn^r), or streptomycin (Str^r) resistance as the selection marker.

It should be noted that none of the plasmids used in the present work are capable of autonomous replication in *A. vinelandii*. Therefore, all strain constructions represent the result of recombination events that occurred between the appropriately constructed plasmid and the *A. vinelandii* genome. DJ1421 served as the parent for all strains containing a duplicated *iscSUAhscBAfdxiscX* operon under the control of the P*scrX* transcriptional and translational control elements. Construction of this strain was achieved by transformation of pDB1316 into DJ1418 (LacZ$^+$) along with a congression vector encoding rifampicin resistance. Colonies of DJ1418 formed blue colonies when grown on plates containing sucrose as the sole carbon source and supplemented with X-

Gal. Cells having undergone the desired double-crossover event were identified as Kn^s, white colonies (LacZ^-) on X-Gal-supplemented Burks (sucrose) agar plates. The correct location of the duplicated *isc* gene region was verified by PCR analysis and the integrity of this duplicated *isc* sequence was confirmed by DNA sequence analysis. This same strategy was also used to construct DJ1692 using pDB1608 (Table 1). DJ1692, which contains a duplicated *hscBAfdxiscX* under the control of the P*scrX* transcriptional and translational control elements, served as the parent strain for DJ1694 and DJ1695 (Table 2).

Most strains having in-frame deletions or point substitution mutations located in genes contained within the endogenous *isc* region were identified by their inability to effectively grow on plates containing glucose as the carbon source (Glc^- or Glc^poor phenotype). Transformants were first plated on Burks agar plates containing sucrose as the carbon source and were subsequently screened for loss/reduced growth on glucose-containing plates. Inactivation of the *recA* gene was necessary to prevent recombination between the endogenous and duplicated *isc* gene regions, and plasmids pDB528 (*recA::kan*) and pDB1375 (*recA::gn*) were used for this purpose (see Table 2). The correct location and integrity of genomic mutations was verified by PCR or DNA sequence analysis.

The recovery of recombinant strains with deletions or substitutions in the endogenous *iscA* gene, which did not result in an obvious phenotype in our preliminary analysis, required a different strategy. Construction of DJ1559 was accomplished by rescue of the Glc^- phenotype of DJ1434, which contains an in-frame deletion in both *iscU* and *iscA*, by using a plasmid (pDB1391) that contains a deletion only within *iscA*. All other strains containing point substitutions in the *iscA* gene were also constructed using the same strategy (see Table 2).

Genomic recombination with the *hscA'-lacZ* gene fusion carried by pDB1468 resulted in two different LacZ^+, Kn^r strains depending on whether the fusion was incorporated in the *isc-* or *scr*-directed copy of the *hscA* gene (see Table 2, DJ1524 and

DJ1525). Both strains containing the *hscA'-lacZ* fusion produced light blue colonies on plates containing sucrose as the sole carbon source. DJ1525 served as the parent strain for the construction of all *iscR* in-frame deletion or point substitution mutants. Transformants were plated on minimal media containing sucrose as the sole carbon source and supplemented with X-Gal and the relevant antibiotic. Strains containing the desired mutations in *iscR* were easily distinguished from the parent strain as they produced darker blue colonies on sucrose plates.

Construction of a Δ*iscR* strain with an intact *hscA* gene (DJ1601) was achieved via the replacement of the P*isc*-controlled *hscA'-lacZ* fusion in DJ1562 with an intact *hscA* gene derived from purified, chromosomal *A. vinelandii* DNA. Subsequent transformation of DJ1601 with pDB1350 (Δ*iscU*) and pDB1291 (Δ*hscBA*) could be used to rescue the slow growth phenotype associated with DJ1601 and respectively yielded strains designated DJ1603 (Δ*iscR* Δ*iscU*) and DJ1609 (Δ*iscR* Δ*hscBA*).

TABLE 2. Mutant strains constructed and/or used in this study.

Strain	Key plasmid used	*isc* gene/s mutated in endogenous, IscR-controlled *isc* operon[a] (**P*isc***)	Gene/s controlled by *scrX* promoter (**P*scrX***)	Other gene mutations[b]	Growth on Glucose[c]
DJ	n/a	none	*scrX*	none	+
DJ1411	pDB1309	none	*scrX::Kn^R*	none	+
DJ1418	pDB1335	none	*lacZKn^R*	none	+
DJ1476	pDB1400	none	*lacZKn^R*	*scrR::gn*	+
DJ1421	pDB1316	none	*iscSUAhscBAfdxiscX*	none	+
DJ1454	pDB1316	none	*iscSUAhscBAfdxiscX*	*recA::kan*	+
DJ1692	pDB1608	none	*hscBAfdxiscX*	none	+
DJ1601	none[d]	*Δ iscR*	*iscSUAhscBAfdxiscX*	none	slow[e]
DJ1696	pDB1286	*iscR* (IscR ^{C92A})	*iscSUAhscBAfdxiscX*	none	slow[e]
DJ1603	pDB1350	*Δ iscR, Δ iscU*	*iscSUAhscBAfdxiscX*	none	-
DJ1609	pDB1291	*Δ iscR, Δ hscBA*	*iscSUAhscBAfdxiscX*	none	slow
DJ1450	pDB954	*Δ iscS*	*iscSUAhscBAfdxiscX*	*recA::kan*	-
DJ1451	pDB1209	*iscS* (IscS ^{C325A})	*iscSUAhscBAfdxiscX*	*recA::kan*	-
DJ1445	pDB1350	*Δ iscU*	*iscSUAhscBAfdxiscX*	*recA::kan*	-
DJ1453	pDB1058	*iscU* (IscU ^{D39A})	*iscSUAhscBAfdxiscX*	*recA::kan*	-
DJ1452	pDB1228	*iscU* (IscU ^{C63A})	*iscSUAhscBAfdxiscX*	*recA::kan*	-
DJ1448	pDB1546	*iscU* (IscU ^{C106A})	*iscSUAhscBAfdxiscX*	*recA::kan*	-
DJ1607	pDB1538	*iscU* (IscU ^{H105A})	*iscSUAhscBAfdxiscX*	*recA::kan*	-
DJ1608	pDB1227	*iscU* (IscU ^{K103A})	*iscSUAhscBAfdxiscX*	*recA::kan*	-
DJ1434	pDB1351	*Δ iscUA*	*iscSUAhscBAfdxiscX*	none	-
DJ1559	pDB1391	*Δ iscA*	*iscSUAhscBAfdxiscX*	*recA::gn*	+
DJ1564	pDB1499	*Δ iscA*	*iscSUAhscBAfdxiscX*	*iscA2::gn*	+
DJ1570	pDB1499	none	*iscSUAhscBAfdxiscX*	*iscA2::gn*	+
DJ1656	pDB1236	*iscA* (IscA ^{C36A})	*iscSUAhscBAfdxiscX*	*recA::gn*	+
DJ1657	pDB1215	*iscA* (IscA ^{C99A})	*iscSUAhscBAfdxiscX*	*recA::gn*	+
DJ1662	pDB1216	*iscA* (IscA ^{C101A})	*iscSUAhscBAfdxiscX*	*recA::gn*	+
DJ1659	pDB1250	*iscA* (IscA ^{S104C})	*iscSUAhscBAfdxiscX*	*recA::gn*	+
DJ1699	pDB1611	*iscA* (IscA ^{S104A})	*iscSUAhscBAfdxiscX*	*none*	+
DJ1447	pDB1291	*Δ hscBA*	*iscSUAhscBAfdxiscX*	*recA::kan*	slow
DJ1694	pDB1291	*Δ hscBA*	*hscBAfdxiscX*	*recA::kan*	-
DJ1621	pDB1550	*Δ fdx*	*iscSUAhscBAfdxiscX*	*recA::kan*	-
DJ1463	pDB1016	*fdx::kan*	*iscSUAhscBAfdxiscX*	*recA::kan*	-
DJ1695	pDB1550	*Δ fdx*	*hscBAfdxiscX*	*recA::kan*	-
DJ1524	pDB1468	none	*iscSUAhscB(ΦhscA'-lacZ)fdxiscX*	none	+
DJ1525	pDB1468	*Φ(hscA'-lacZ)*	*iscSUAhscBAfdxiscX*	none	slow
DJ1532	pDB1375	*Φ(hscA'-lacZ)*	*iscSUAhscBAfdxiscX*	*recA::kan*	slow
DJ1562	pDB1490	*Δ iscR Φ(hscA'-lacZ)*	*iscSUAhscBAfdxiscX*	none	slow
DJ1580	pDB1500	*Δ iscR Φ(hscA'-lacZ)*	*iscSUAhscBAfdxiscX*	*recA::gn*	slow
DJ1582	pDB1507	*iscR* (IscR ^{C92A}) *Φ(hscA'-lacZ)*	*iscSUAhscBAfdxiscX*	*recA::gn*	slow
DJ1531	pDB1212	*iscR* (IscR ^{C98A}) *Φ(hscA'-lacZ)*	*iscSUAhscBAfdxiscX*	*recA::gn*	slow
DJ1587	pDB1518	*iscR* (IscR ^{C104A}) *Φ(hscA'-lacZ)*	*iscSUAhscBAfdxiscX*	*recA::gn*	slow

[a.] Site-directed substitutions of specific codons in *isc* genes are described as the amino acid substitution corresponding to the gene product.

[b] Gene mutations relevant to this study. Antibiotic gene cartridges are: *gn* encoding gentamycin resistance (Gn^r) and *kan* encoding kanamycin resistance (Kn^r).

[c] Refers to growth on Burks minimal media containing glucose as the sole carbon source at 30°C and 20% O_2. Unless otherwise stated, growth on media containing sucrose under the same conditions is normal.

[d] See Materials and Methods.

[e] Growth is slow on both sucrose and glucose containing media.

Depletion Experiments.   Two liters of sucrose-containing minimal medium were inoculated with the parental strain (DJ1454) or *isc* mutant strains and grown to 150 Klett (red filter) or OD_{600}=1.4 at 30°C, 300 rpm.   Cell pellets were harvested by centrifugation (4,225 x g for 5 minutes) and washed twice with Burks minimal medium containing glucose as the sole carbon source.   Re-suspended cells were diluted into fresh glucose medium at a 1:16 or 1:1000 dilution.   Depletion of the relevant *isc* gene product was allowed to progress for 11-25 hours until cell growth stopped or slowed down relative to DJ1454 (between 4-5 doubling times for DJ1450 Δ*iscS* and DJ1445 Δ*iscU* and 8 doubling times for DJ1463 *fdx::kan* and DJ1447 Δ*hscBA*).   Cells were harvested by centrifugation as above and stored at −20° C.

Aconitase and Isocitrate Dehydrogenase Assays. Crude extracts obtained from depletion experiments were prepared by sonication in degassed, argon-sparged 50 mM Tris-HCl, pH 8.0, buffer and clarified by centrifugation at 100,000 x g for 30 min. Supernatants were immediately placed in sealed air-tight vials under anoxic conditions maintained using either Schlenk lines or a Coy anaerobic chamber containing 5 % hydrogen gas balanced with nitrogen gas.   The total protein concentration of the supernatants was quantified using the Biuret method (Chromy et al. 1974). Aconitase activity was measured spectrophotometrically at 240 nm at room temperature by following the production of cis-aconitate (3.4 mM^{-1} cm^{-1} at 240 nm) (Saas et al. 2000). Assays (1 ml) were conducted in sealed, anoxic cuvettes containing 10 μl – 50 μl supernatant, 900 μl 100 mM Tris/HCl (pH 8.0) and reactions were initiated with 100 μl of 200 mM citrate.   Isocitrate dehydrogenase served as an internal control and was assayed by the production of NADPH (Cribbs and Englesberg 1964).

β-galactosidase Assays. β-galactosidase activity was determined using an assay adapted from Miller (1972). Cells were grown in sucrose or glucose containing medium to mid-log or late log phase and assays were conducted using either DMF-permeabilized whole cells (for IscR-related β-Gal assays; Table 3) or the soluble fraction of crude extracts prepared by sonication and centrifugation (all other assays; Figure 1A). β-Galactosidase activity units are expressed as $\Delta0.0001$ abs (414nm)/min/OD_{600} (for whole cells assay) or

$\Delta 0.0001$ abs (414nm)/min/mg protein (for crude extracts). Relative β-galactosidase activities represent the specific rate of absorbance change at 414 nm for the experimental samples divided by the control sample.

Growth of *A. vinelandii* under 40% or 5% Oxygen. For the growth of cells at 40% oxygen at 1 atm, inoculated Petri plates were placed in vented BBL® GasPak jars (Becton, Dickinson and Company). Evacuation of ambient air was accomplished using a Schlenk line apparatus and re-gassed by flushing with 40% O_2 balanced with 60% N_2 using a regulated gas tank (Airgas Inc.). No more than four (100 x 15mm) petri plates at a time were placed in each jar, which can hold up to 12 plates. Sealed jars were incubated at 30°C and were evacuated and reflushed with the 40% O_2/ 60% N_2 gas mixture every 2 days. Growth of cells under low-oxygen conditions was accomplished using a Coy chamber containing 5% oxygen balanced with N_2 gas from a regulated gas tank (Airgas Inc.).

Western Immuno-Blotting. Cell pellets for DJ1454 (intact *iscU*) and DJ1445 *(ΔiscU)* were obtained from the depletion experiments described above. Soluble protein extracts were prepared by French cell press, (12,000 psi) in degassed, argon-sparged 100 mM Tris-HCl, pH 8.0 and clarified by centrifugation at 100,000 x g for 30 min. Total protein concentrations were measured using the Biuret method to ensure equal loading (26 μg per lane) on 20% SDS-PAGE gels. Gels were either stained with Coomassie dye or transferred to nitrocellulose. A chemiluminescent detection system was used (LumiPhos™WB, Pierce) and the blotting protocol recommended by the supplier was followed using 4% non-fat dried milk as the blocking agent. Polyclonal primary rabbit antiserum to *A. vinelandii* IscU (Coalico biologicals, Inc.) was used at a dilution of 1:600 to probe for IscU transferred to the nitrocellulose membrane. Alkaline phosphate-conjugated anti-rabbit goat immunoglobulin G (IgG) served as the secondary antiserum (1:30,000) (Sigma-Aldrich Inc.).

3.3 – Results

Regulation of Sucrose Catabolism within *A. vinelandii*. Previous attempts to isolate mutant strains of *A. vinelandii* that have in-frame deletion or insertion mutations within the *isc* genomic region were unsuccessful, indicating that such mutations are either lethal or highly deleterious in this organism (Zheng et al. 1998). In order to assess the physiological consequences of compromising the activity of individual proteins encoded within the *isc* gene cluster, or to assess the functional importance of individual residues within those proteins, it was necessary to develop a method for conditional expression of the *isc* genes within *A. vinelandii*. The use of a multicopy plasmid system for this purpose was not considered to be an attractive approach owing to the potential complication of gene dosage effects. As an alternative strategy we exploited the natural transformation system of *A. vinelandii* to place the expression of a second genomic copy of the *isc* gene region under the control of separate regulatory elements.

To achieve this objective it was first necessary to identify a candidate system for controlled gene expression within *A. vinelandii* and then demonstrate that controlled expression could be achieved. A survey of the preliminary *A. vinelandii* genome sequence revealed a cluster of genes that we have designated as "*scr*" genes owing to the apparent involvement of their products in sucrose catabolism (Figure 1A). The *scrR* gene encodes a protein bearing sequence identity when compared to the *lacI* gene product. The *scrB* and *scrP* genes respectively encode proteins having primary sequence identity when compared to known sucrases and carbohydrate specific porins. The divergently transcribed *scrY* gene product encodes a protein having sequence identity when compared to the *lacY* gene product. The *scrX* gene, located approximately 500 base-pairs downstream from *scrY,* encodes a protein having sequence identity when compared to α-glucosidases. The organization (Figure 1A) and primary sequences of these genes suggest they represent a sucrose catabolic regulon that is negatively controlled by ScrR similar to LacI regulation of the *lac* operon from *E. coli*, with the difference being that sucrose, rather than lactose, is the metabolic effector molecule. Examination of DNA sequences preceding *scrP*, *scrY* and *scrX* for potential promoter/operator sequences also

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suggested the possibility that each of them could be coordinately regulated by ScrR but individually expressed from separate promoters.

In order to test whether or not the Scr system could be used for controlled expression of other genes without compromising sucrose catabolism, the *lacZ* gene from *E. coli* was placed under the transcriptional and translational control elements of the *scrX* gene and β-galactosidase activity measured in cells that were cultured using either sucrose or glucose as the carbon source (Figure 1A). Loss of *scrX*, either by replacement with *lacZ* (DJ1418, Table 2, Figure 1A), or by interruption using an antibiotic-resistant gene cartridge (DJ1411, Table 2), did not affect the capacity of *A. vinelandii* to grow using sucrose as the sole carbon source, indicating that the *scrX* gene product is not essential for sucrose catabolism. β-galactosidase activity was repressed approximately 1,000 fold in strain DJ1418 when cultured using glucose rather than sucrose as the carbon source (Figure 1A). In contrast, the *lacZ* gene is constitutively expressed when cultured using either glucose or sucrose as the carbon source in a strain that carries both *lacZ* under control of the *scrX* promoter and an insertion mutation within *scrR* (DJ1476, Table 2, Figure 1A). This result is consistent with the hypothesis that ScrR is a trans-acting negative regulatory element functionally analogous to LacI.
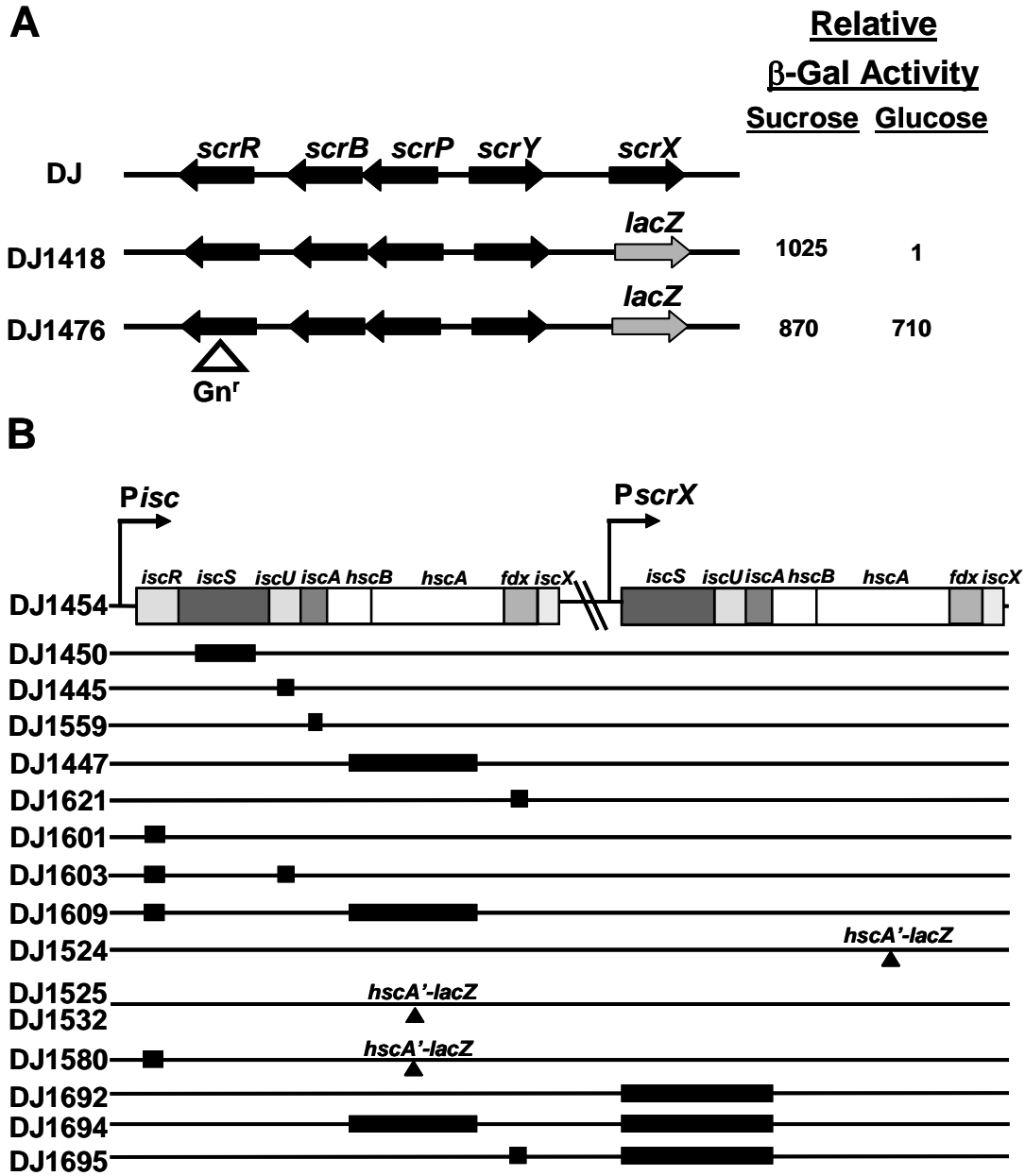
Figure 1. Schematic representation of the relevant genetic organization of key strains used in this work: (A) Organization of the *A. vinelandii* sucrose catabolic regulon. DJ refers to the wild-type strain. DJ1418 has the *scrX* gene replaced by *lacZ*. DJ1476 was derived from DJ1418 and carries an insertion mutation within the *scrR* gene. Levels of *lacZ* expression in response to carbon source or as a result of *scrR* inactivation are shown for strains DJ1418 and DJ1476. (B) Organization of *isc* genes in strains that have various *isc* genes duplicated and placed under the sucrose catabolic regulatory elements. Black boxes indicate deleted regions for a particular strain and triangles indicate the position of *lacZ* fusions.

Duplication and *scr*-Directed Expression of the *isc* Gene Region. Availability of an *A. vinelandii* strain having expression of *lacZ* under control of the *scrX* promoter (DJ1418) provided a convenient way to construct other gene fusion strains where any target gene(s) can be placed under control of the *scrX* promoter (P*scrX*). Such constructions can be achieved by using an appropriately constructed plasmid (see Table 1) and reciprocal recombination events that occur during DNA transformation. When strain DJ1418 is used for such constructions, recombinants that result in the excision of *lacZ* and replacement by a particular target gene can be identified using X-gal as an indicator. Namely, colonies that have undergone double reciprocal recombination resulting in the excision of *lacZ* and insertion of the gene of choice are white. In the present work an *A. vinelandii* strain (DJ1421) was constructed where the *iscS*, *iscU*, *iscA*, *hscB*, *hscA*, *fdx*, and *iscX* genes are duplicated and their expression placed under control of the *scrX* promoter (P*scrX*, Table 2, see Materials & Methods for details). To prevent possible recombination between the endogenous and duplicated *isc* gene regions contained within DJ1421, the *recA* gene was also inactivated to give strain DJ1454 (Table 2, Figure 1B). The location and integrity of the duplicated *isc* region within DJ1454 was confirmed by PCR and DNA sequence analysis of genomic DNA.

Conditional Depletion of *isc* Gene Products. Figure 1B shows that strain DJ1454 contains two copies of the *iscS-iscU-iscA hscB-hscA-fdx-iscX* gene regions, one whose expression is under control of P*isc* and the other whose expression is negatively controlled by P*scrX*. Our experimental rationale was that functional analysis of *isc* genes could be accomplished by placing in-frame deletions within particular P*isc*-regulated genes with subsequent examination of the effect of culturing such mutants under conditions that repress expression of the corresponding P*scrX*-regulated *isc* genes. In this system, growth of mutant strains on media containing glucose as the sole carbon source provides conditions that repress P*scrX*-controlled genes. Figure 1B shows a list of mutant strains that contain various in-frame deletions within the P*isc*-regulated region. As shown in Figures 2 and 3A, mutant strains individually deleted within P*isc*-regulated copies of *iscS*, *iscU*, or *fdx* are able to grow when using sucrose as the carbon source but

cannot grow when cultured using glucose as the carbon source. Depletion of IscU by this method was also established by western analysis (Figure 3C), although complete elimination of IscU was not observed and not expected. Namely, once an essential component is depleted below a threshold level, cells stop growing, thereby preventing further dilution of the component under study. These results establish that IscS, IscU and Fdx have essential functions in *A. vinelandii*. In contrast, no obvious growth phenotype was recognized under conditions expected to deplete IscA, and conditions expected to deplete HscB and HscA resulted in only a slower growth phenotype (Figure 2).
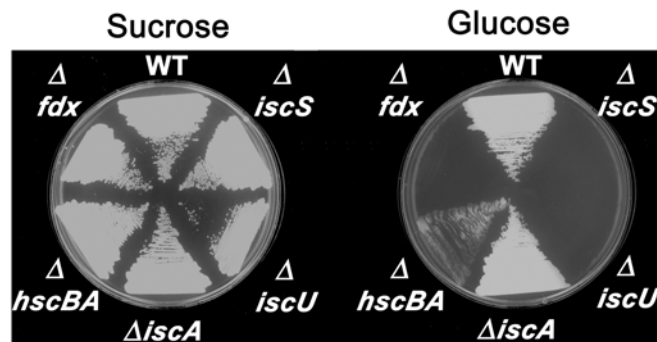

Figure 2. Effect of depletion of Isc components in *A. vinelandii*: (A) Growth phenotypes when strains are cultured using sucrose or glucose as the carbon source. Strains that carry a deletion in an IscR-regulated copy of an *isc* gene but contain an intact copy of the corresponding ScrR-regulated copy are designated as Δ*iscS* (DJ1450), Δ*iscU* (DJ1445), Δ*iscA* (DJ1559), Δ*hscBA* (DJ1447) and Δ*fdx* (DJ1621). A schematic representation of these strains is shown in Figure 1B.

In the case of *E. coli*, there is evidence for expression of *hscB-hscA-fdx-iscX* that occurs independently from expression of *iscRSUA* (Lelivelt and Kawula 1995) . We therefore tested for endogenous expression of the duplicated *hscBA* genes that could occur separately from that directed by the *scrX* promoter. This was accomplished by constructing an in-frame *hscA'-lacZ* translational fusion within the duplicated copy of *hscA* (DJ1524, Figure 1B, Table 2) and comparing the level of β-galactosidase activity when this strain was cultured using glucose or sucrose as the carbon source. In these experiments glucose-cultured cells exhibited approximately 14 fold less β-galactosidase activity when compared to the activity present in sucrose-cultured cells (data not shown).

In contrast, an approximately 1,000-fold repression is achieved under the same conditions when *lacZ* expression is placed directly under control of the *scrX* promoter (DJ1418, Figure 1A). Thus, a low level of endogenous *hscBA* expression occurs within the duplicated *isc* region that is separate from *scrX* promoter-directed expression. These results can explain the slow growth phenotype of this particular genetic construct (DJ1447; Figure 2) when cultured using glucose as the carbon source (approximately 7% expression in glucose cultures relative to sucrose cultures). In contrast, expression of the downstream *fdx* gene is sufficiently lowered in this system (DJ1463 and DJ1621, Table 2, Figures 2 and 3A) such that it accumulates below a threshold that can sustain growth. Clearly, the severity of the growth phenotype manifested upon depletion of a particular *isc* gene product could depend on the level of depletion, the nature of the specific function that is lowered, as well as the intrinsic *in vivo* stability of the protein whose expression has been limited. In order to gain an unambiguous answer to the question of whether or not HscBA are essential in *A. vinelandii*, a strain was constructed (DJ1694, Figure1B) where the P*isc*-directed *hscBA* genes are deleted and the entire intervening region between the initiation codons for *iscS* and *hscB* within the duplicated P*scrX*-regulated *isc* region is also deleted. For this strain, conditions expected to result in a more severe depletion of HscBA does result in a clear null growth phenotype (Table 2, Figure 9-left panel). As expected, a similar strain (DJ1695) for which severe depletion of Fdx is expected also results in a clear null growth phenotype (Table 2, Figure 9A). In no case could the observed growth phenotypes associated with depletion of Isc components be rescued by the addition of amino acids or the S-containing cofactors, biotin and thiamine, to the growth medium.

Effect of Depletion of Isc Components on Aconitase Activity. The effect of a sucrose-to-glucose carbon source shift on the activity of aconitase, a tricarboxylic acid cycle enzyme that requires a [4Fe-4S] cluster for enzymatic activity, was evaluated using strains deleted for various IscR-regulated Isc components. The activity of isocitrate dehydrogenase, a tricarboxylic acid enzyme that does not contain an [Fe-S] cluster for its activity, was also measured as an internal control. For these experiments, enzyme activities were measured approximately 12 to 25 hours after the appropriate strain was

shifted from sucrose to glucose as the carbon source. In the case of IscS, IscU, Fdx and HscBA depletion experiments, the time at which cells were harvested for activity measurements was dictated by the approximate time after the carbon source shift that resulted in a clear growth phenotype. Growth profiles for typical liquid culture carbon source shift experiments for depletion of IscS, IscU, HscBA and Fdx are shown in Figure 3A. The results summarized in Figure 3B reveal a very dramatic loss in aconitase activity upon depletion of IscS or IscU, a moderate effect on aconitase activity upon depletion of HscBA and Fdx, and a small, but reproducible effect on aconitase activity upon depletion of IscA. It should be emphasized that the effect on aconitase activity resulting from depletion of one particular Isc component by this method cannot be meaningfully compared to the effect of depletion of a different Isc component, because neither the absolute nor the relative depletion of individual Isc components was established in these experiments. For example, strains used to examine the effect of depletion of HscBA and Fdx shown in Figure 3B have an intact duplicated *isc* region under control of P*scrX*. Thus, there is some endogenous expression of HscBA and Fdx in these experiments and, consequently, the less severe biochemical phenotype observed for depletion of HscBA or Fdx when compared to depletion of IscS or IscU could be expected. The important result of these experiments is that, with the exception of IscA, a clear growth phenotype and a clear effect on the activity of an [Fe-S] protein having a key metabolic function can be elicited by uncoupling the expression of specific Isc components from their normal regulatory elements. Thus, this method provides an opportunity to evaluate the consequences of defects in essential Isc components. Also, because these phenotypes are only manifested by the controlled, real-time, depletion of Isc components, concern for potential secondary metabolic effects or selection of suppressor mutations that could arise as a consequence of the prolonged absence of a particular component is limited. As will be described in a following section, an unanticipated advantage of the system was that it also permitted the uncovering of previously unrecognized phenotypes associated with the depletion of IscA or HscBA.
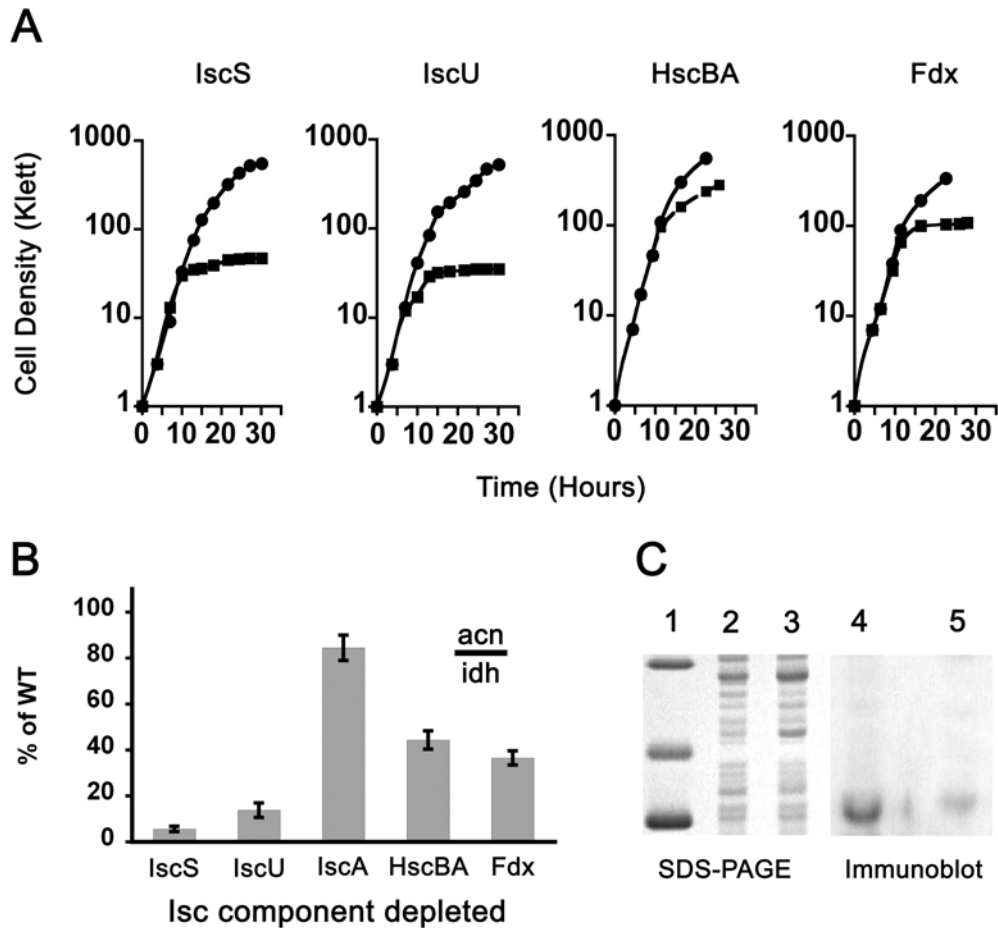
Figure 3. Depletion of Isc components in *A. vinelandii* has a detrimental effect on aconitase activity: (A) Effect on growth in liquid culture that occurs upon a carbon source shift for strains DJ1450, DJ1445 DJ1447 and DJ1463. Each strain was diluted at time 0 in liquid medium that contains either sucrose (●) or glucose (■) as the carbon source. The wild type strain grows at the same rate in liquid culture when using either sucrose or glucose as the carbon source. (B) Effect of depletion of Isc components on aconitase activity. Strains DJ1450, DJ1445, DJ1559, DJ1447, or DJ1463 were shifted from growth in liquid culture using sucrose as the carbon source to growth using glucose as the carbon source. Cells were harvested once an effect on growth was observed (see Panel A) and assayed for aconitase and isocitrate dehydrogenase activities. The ratio of aconitase to isocitrate dehydrogenase (acn/idh) activity for each sample depleted for an Isc component was individually normalized to values obtained from the wild type strain cultured under identical conditions. All data shown in the figure represent the average of two or three independent experiments. (C) Western analysis showing depletion of IscU in glucose-grown

DJ1445 cells. Left panel shows Coomassie brilliant blue staining of proteins, separated by 20% SDS-PAGE, from crude extracts of DJ1454 (WT) and DJ1445 ($\Delta iscU$) grown in glucose for 14 hours. A duplicate of this gel was used for immuno staining with antibody to IscU (right panel). *Lane 1*, M_R standards (carbonic anhydrase, soybean trypsin inhibitor, lysozyme); *lane 2*, crude extract of proteins (26μg) from DJ1454 (WT); *lane 3* crude extract of proteins (26μg) from DJ1445 ($\Delta iscU$); *Lane 4*, detection of IscU in DJ1454 (WT); *lane 5*, detection of IscU in DJ1445 ($\Delta iscU$).

The Proposed Active Site IscS Cys^{325} Residue is Essential for Physiological IscS Function. Previous biochemical studies have shown that IscS is a member of a class of cysteine desulfurases that contains an active site cysteine residue (Cys^{325}) upon which a persulfide can be formed (Zheng et al. 1993; Zheng et al. 1994; Lauhon et al. 2004). This persulfide is proposed to be the activated species used for a variety of biochemical transformations that require inorganic sulfur, including [Fe-S] cluster formation (Mihara and Esaki 2002). In the present work the *in vivo* functional role for Cys^{325} was confirmed by substituting this residue by alanine within the P*isc*-regulated copy of IscS. When cultured using sucrose as the carbon source, this mutant strain (DJ1451; Table 2) grew on Petri plates as well as an isogenic strain having both copies of IscS intact (DJ1454). However, DJ1451 could not grow when cultured using glucose as the carbon source (data not shown).

Functional Analysis of Selected Residues within IscU. *In vitro* systems have been reported where IscU, or its homologs, can be used as a scaffold for the assembly of either [2Fe-2S] or [4Fe-4S] clusters and such clusters can be transferred to the apo-forms of a variety of [Fe-S] proteins (Agar et al. 2000; Foster et al. 2000; Urbina et al. 2001; Mansy et al. 2002; Rodriguez-Manzaneque et al. 2002; Wu et al. 2002; Wu et al. 2002; Ollagnier-De-Choudens et al. 2004). A general consensus in the field is that three cysteine residues, Cys^{37}, Cys^{63} and Cys^{106}, strictly conserved among all IscU homologs (Figure 4), are likely to provide ligands for the assembly of transient clusters destined for [Fe-S] protein maturation (Garland et al. 1999; Kato et al. 2002; Smith et al. 2005). This idea is supported by the close three-dimensional juxtaposition and solvent exposure of all

three cysteines within the available IscU structures (Bertini et al. 2003; Mansy et al. 2004; Ramelot et al. 2004).
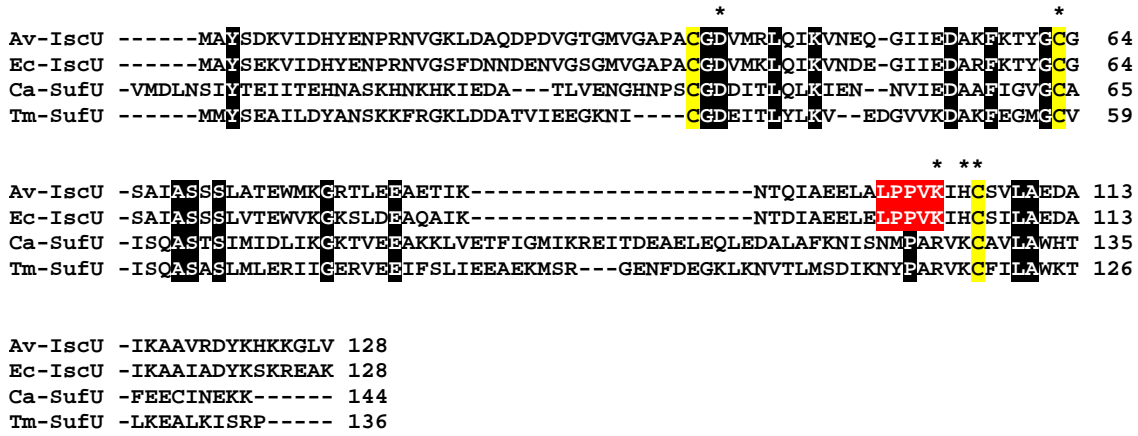
```
                                                           *                                   *
Av-IscU ------MAYSDKVIDHYENPRNVGKLDAQDPDVGTGMVGAPACGDVMRLQIKVNEQ-GIIEDAKFKTYGCG  64
Ec-IscU ------MAYSEKVIDHYENPRNVGSFDNNDENVGSGMVGAPACGDVMKLQIKVNDE-GIIEDARFKTYGCG  64
Ca-SufU -VMDLNSIYTEIITEHNASKHNKHKIEDA---TLVENGHNPSCGDDITLQLKIEN--NVIEDAAFIGVGCA  65
Tm-SufU ------MMYSEAILDYANSKKFRGKLDDATVIEEGKNI----CGDEITLYLKV--EDGVVKDAKFEGMGCV  59

                                                            *  **
Av-IscU -SAIASSSLATEWMKGRTLEEAETIK--------------------NTQIAEELALPPVKIHCSVLAEDA 113
Ec-IscU -SAIASSSLVTEWVKGKSLDEAQAIK--------------------NTDIAEELELPPVKIHCSILAEDA 113
Ca-SufU -ISQASTSIMIDLIKGKTVEEAKKLVETFIGMIKREITDEAELEQLEDALAFKNISNMPARVKCAVLAWHT 135
Tm-SufU -ISQASASLMLERIIGERVEEIFSLIEEAEKMSR---GENFDEGKLKNVTLMSDIKNYPARVKCFILAWKT 126


Av-IscU -IKAAVRDYKHKKGLV 128
Ec-IscU -IKAAIADYKSKREAK 128
Ca-SufU -FEECINEKK------ 144
Tm-SufU -LKEALKISRP----- 136
```

Figure 4. Primary sequence comparisons for IscU from *A. vinelandii* (Av) and *E. coli* (Ec), and SufU from *Clostridium acetobutylicum* (Ca) and *Thermotoga maritima* (Tm). Amino acids conserved among all alignments are highlighted in black, conserved cysteine residues are shaded in yellow, and residues conserved among IscU homologs that include the HscA interaction motif are highlighted in red. Asterisks above residues in the *A. vinelandii* IscU sequence indicate amino acids that were substituted in this study.

By using the same approach as described above for IscS, individual substitution of alanine for IscU residues Cys^{63} or Cys^{106} within the P*isc*-regulated copy of IscU, resulted in strains that could not sustain growth when cultured using glucose as the carbon source (Figure 5). In contrast, repeated attempts to isolate a strain for which the Cys^{37} residue within the P*isc*-regulated copy of IscU is substituted by alanine were not successful. The possibility that the Cys^{37} residue could be substituted by alanine without effect was excluded based on an inability to rescue the Glc⁻ phenotype of a Δ*iscU* strain in transformation experiments using plasmid DNA for which the *iscU* coding region carries a point mutation resulting in an Ala^{37} substitution (pDB1404, Table 1). Although the basis for our inability to isolate a strain that expresses both wild type and Ala^{37}-substituted forms of IscU is not yet clearly established, one explanation is that Ala^{37}-substituted IscU exerts a null dominant-negative effect on some aspect of [Fe-S] protein maturation. The likelihood for such a dominant-negative effect in the case of IscU-Ala^{37}

is supported by inspection of sucrose grown wild type and strains that produce Ala[63]- and Ala[106]-substituted versions of IscU shown in Figure 5. These comparisons reveal that the strain producing IscU-Ala[63] grows as well as the wild type control when cultured using sucrose as the carbon source, but a strain producing IscU-Ala[106] grows less effectively under these same conditions. These results suggest that a partial dominant-negative effect is exerted by the IscU Ala[106]-substituted protein and also indicate a functional inequivalence of the conserved Cys residues located within IscU. An apparent dominant-negative effect is also recognized for strains that have either the IscU Asp[39] or His[105] residues substituted by alanine. Although not apparent in Figure 5, those strains that exhibit an apparent partial dominant-negative effect when cultured using sucrose as the carbon source, are populated with both very small and relatively larger colonies, suggesting the possibility for rapid selection of strains that can either genetically or physiologically suppress the dominant-negative effect. Such capacity for the relatively rapid phenotypic suppression for these and certain other substitutions within Isc components is why growth on Petri plates, rather than growth in liquid culture, was used to evaluate growth phenotypes in the present work.
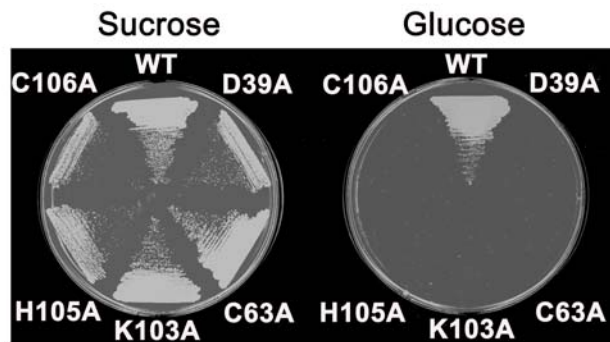

Figure 5. Growth phenotypes exhibited by strains having selected residues of the IscR-regulated copy of IscU substituted by alanine. Cells were cultured using sucrose (left panel) or glucose (right panel) as the carbon source. Each strain is designated by the single letter code for the amino acid, the residue number, and the single letter code for the substituting amino acid: (WT = DJ1454; D39A = DJ1453; C63A = DJ1452; K103A = DJ1608; H105A = DJ1607; C106A = DJ1488, see Table 2).

Although the specific function of HscBA in relation to [Fe-S] protein maturation is not yet known, IscU has been shown to dramatically stimulate the intrinsic ATPase activity of the HscBA complex (Hoff et al. 2000; Silberg et al. 2001). This stimulation has been localized to a LPPVK motif that is located two amino acids upstream from the conserved Cys^{106} residue within IscU (Hoff et al. 2002; Cupp-Vickery et al. 2004). This motif is not conserved in a class of IscU-like proteins (designated SufU in Figure 4) that are produced by certain organisms that do not encode strict HscBA homologs. In the present work substitution of alanine for the Lys^{103} residue contained within the LPPVK motif resulted in a null growth phenotype when cells were depleted for intact IscU (Figure 5), consistent with similar mutational analysis performed with *S. cerevisiae* Isu1 (Dutkiewicz et al. 2004). IscU proteins are also distinguished from the SufU class in that they have a conserved His^{105} residue that is replaced by lysine in SufU proteins. Substitution of IscU His^{105} by alanine also resulted in a null phenotype (Figure 5).

Depletion of IscA Results in an Oxygen-Sensitive Phenotype. As already discussed, the depletion strategy developed in this study did not result in a null growth phenotype under conditions expected to deplete IscA, even though depletion of Fdx, which is located downstream from IscA and HscBA, does result in a null phenotype (Figure 2 and 9). One explanation for this result is that IscA is not sufficiently depleted below a threshold required to manifest a null growth phenotype for this particular strain. Owing to the technical difficulty of the genetic construction needed to directly test this possibility, and because the loss of IscA function in *E. coli* displays no growth phenotype and only a modest effect on [Fe-S] protein maturation (Tokumoto and Takahashi 2001), we elected to explore other explanations. A second possibility is that a different cellular component might supplant the function of IscA. This possibility seemed particularly reasonable given that two proteins bearing a high degree of primary sequence similarity to IscA (designated $IscA^{Nif}$ and IscA2) are encoded within the *A. vinelandii* genome (Figure 6). A third possibility is that, under the experimental culture conditions used in these experiments, the function of IscA is dispensable. In order to distinguish among these possibilities we examined the effects of depleting IscA in a strain that is also

inactivated for IscA2, and we also searched for culture conditions that might result in a null growth phenotype under conditions of IscA depletion.
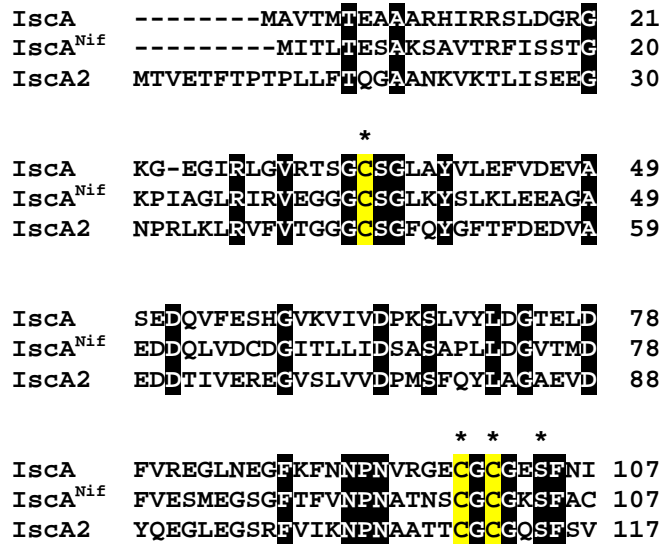
```
IscA       --------MAVTMTEAAARHIRRSLDGRG   21
IscA^Nif   ---------MITLTESAKSAVTRFISSTG   20
IscA2      MTVETFTPTPLLFTQGAANKVKTLISEEG   30

                          *
IscA       KG-EGIRLGVRTSGCSGLAYVLEFVDEVA   49
IscA^Nif   KPIAGLRIRVEGGGCSGLKYSLKLEEAGA   49
IscA2      NPRLKLRVFVTGGGCSGFQYGFTFDEDVA   59


IscA       SEDQVFESHGVKVIVDPKSLVYLDGTELD   78
IscA^Nif   EDDQLVDCDGITLLIDSASAPLLDGVTMD   78
IscA2      EDDTIVEREGVSLVVDPMSFQYLAGAEVD   88

                                * *   *
IscA       FVREGLNEGFKFNNPNVRGECGCGESFNI  107
IscA^Nif   FVESMEGSGFTFVNPNATNSCGCGKSFAC  107
IscA2      YQEGLEGSRFVIKNPNAATTCGCGQSFSV  117
```

Figure 6. Comparison of the primary amino acid sequences of three IscA homolgs encoded within *A. vinelandii*. Conserved amino acid residues are shaded in black and conserved cysteines are shaded in yellow. Asterisks above residues in the IscA sequence indicate amino acids that were substituted in this study.

As shown in Figure 7A no adverse growth effect was observed upon inactivation of IscA2 (*iscA2::gn*), under conditions expected to deplete IscA (*ΔiscA*), or under conditions where IscA2 had been inactivated and IscA was also depleted (*iscA2::gn, ΔiscA*). Because, IscA has been proposed to serve as a possible iron donor during [Fe-S] cluster assembly, we also tested for elicitation of a phenotype by culturing cells either depleted for IscA or depleted for IscA and inactivated for IscA2 under conditions of iron limitation (data not shown). No distinguishing phenotype could be associated with IscA or IscA2 by this method. In contrast, a clear null phenotype was revealed when cells depleted for IscA were cultured under conditions where the atmospheric level of oxygen was increased from ambient (~20%) to 40% (Figure 7B). When a strain that is individually inactivated for IscA2 was cultured under 40% O_2, no such oxygen sensitivity could be detected. Furthermore, the oxygen-sensitive phenotype associated with

depletion of IscA was not suppressed under nitrogen fixing conditions (data not shown), where IscA^{Nif} is expressed. Collectively, these results show that a clear null growth phenotype is associated with depletion of IscA under 40% O_2 and that neither IscA2 nor IscA^{Nif} can apparently replace the essential function of IscA manifested under conditions of elevated oxygen.
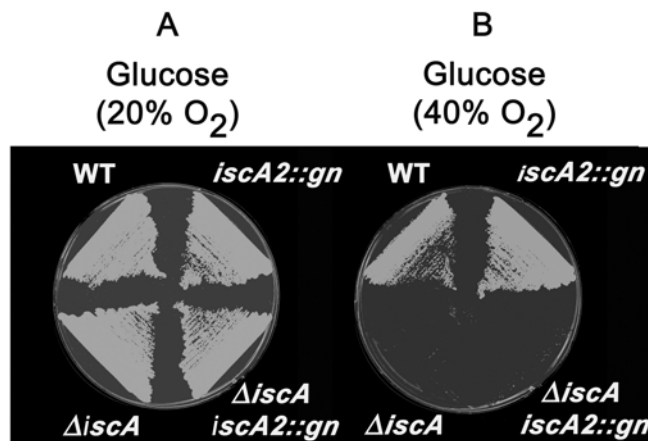

Figure 7. IscA is essential under elevated oxygen conditions and cannot be replaced by IscA2. The following strains were cultured in media using glucose as the carbon source under (A) ambient (~20%) oxygen or (B) 40% oxygen: WT = DJ1454; iscA2::Gn^r = DJ1570; ΔiscA, iscA2::Gn^r = DJ1564; and ΔiscA = DJ1559 (see Table 2). Strain DJ1564 and DJ1559 each have an intact duplicated copy of iscA whose expression is under control of the Scr regulatory elements. For both of these strains, the IscR-regulated copy of iscA is deleted. All strains show normal growth when cultured using sucrose as the carbon source under either ambient (~20%) or 40% oxygen.

The null growth phenotype associated with loss of IscA function when cultured using 40% O_2 also permitted a functional evaluation of individual residues within IscA. For these experiments strains were constructed where Cys [36], Cys[99], and Cys [101] within the P*isc*-regulated copy of IscA were individually substituted with alanine and Ser[104] was substituted with cysteine (Table 2, Figure 8). When cultured using glucose as the carbon source, which results in depletion of the P*scr*-directed expression of the wild-type copy of IscA, all of these strains exhibited the same oxygen-sensitive phenotype associated with depletion of IscA (Figure 8A). These results indicate that all four of these residues are

essential for IscA function in *A. vinelandii*. An unexpected finding, however, was the manifestation of a clear, dominant-negative phenotype for DJ1659 when cultured in the presence of 40% O_2, and under conditions where both the wild type (Ser^{104}) and substituted (Cys^{104}) forms of IscA were expressed (Figure 8B). Substitution of the IscA Cys^{99} residue by alanine also resulted in a marked dominant-negative effect although in this case the phenotype is not as severe as recognized for the Cys^{104}-substituted IscA (Figure 8B). To test whether or not the dominant-negative effect associated with the Cys^{104} substitution could be specifically related to introduction of a thiol-containing group at this position, a strain having alanine substituted for the Ser^{104} residue was also constructed (DJ1699, Table 2). In this case there was a clear loss of function evidenced by an inability of the Ala^{104}-substituted strain to grow under conditions of 40% O_2, but no dominant-negative phenotype was observed (data not shown).
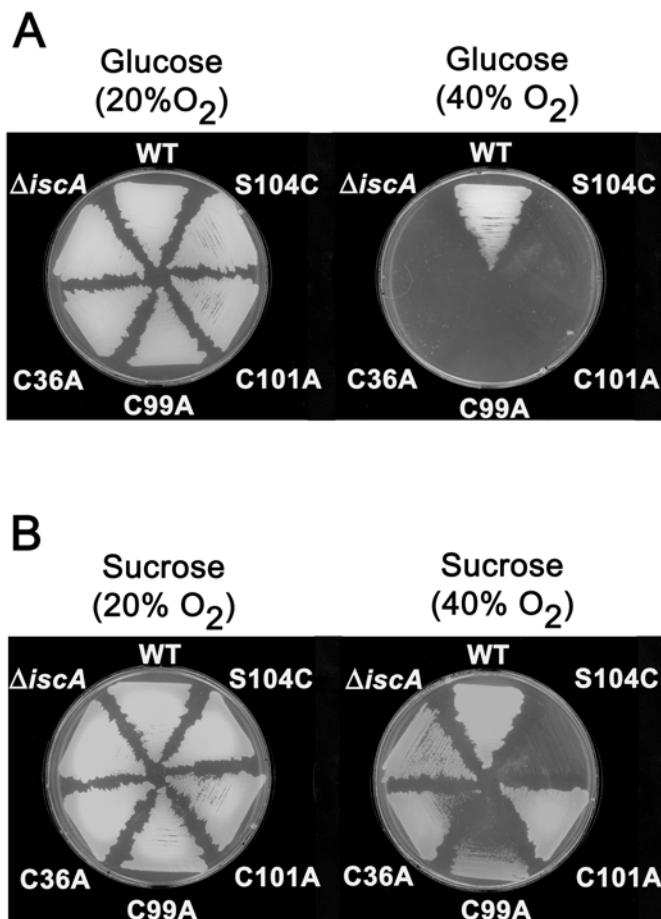
Figure 8. Growth phenotypes exhibited by strains having mutations within the IscR-regulated copy of IscA. Cells were cultured using glucose (A) or sucrose (B) as the carbon source and were incubated under ambient (~20%) oxygen or 40% oxygen as indicated in each panel. Each strain is designated using the convention described in the legend to Figure 4 and complete genotypes can be found in Table 2: (WT = DJ1454; S104C = DJ1659; C101A = DJ1662; C99A = DJ1657; C36A = DJ1656; ΔiscA = DJ1559, see Table 2).

Growth under Low Oxygen Suppresses the Phenotype Associated with HscBA Depletion. Given the oxygen-sensitive growth phenotype associated with depletion of IscA it was of interest to examine whether the null phenotype associated with the depletion of other Isc components could be rescued by culture under conditions of low oxygen availability (5% O_2). The results of this analysis (Figure 9) revealed that the null growth phenotype associated with depletion of HscBA could be partially reversed when

DJ1694 (Figure 1B) was cultured under 5% O_2, but the null phenotype associated with depletion of IscS, IscU or Fdx persisted under these same conditions.
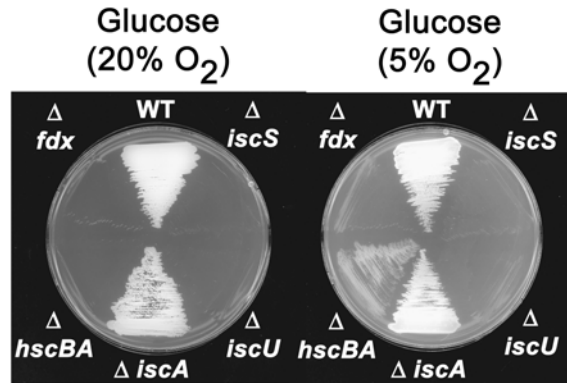

Figure 9. Effect of low oxygen on growth in cells depleted for Isc components. Strains used for depletion of IscS (DJ1450), IscU (DJ1445), or IscA (DJ1559) also encode a duplicated copy of *iscS-iscU-iscA-hscB-hscA-fdx-iscX* whose expression is controlled by the Scr regulatory elements. Strains used for depletion of HscBA (DJ1694) or Fdx (DJ1695) also have a duplicated copy of *hscB-hscA-fdx-iscX* whose expression is controlled by the Scr regulatory elements. A schematic representation of the strains used is shown in Figure 1B. All strains show normal growth when cultured using sucrose as the carbon source under either ambient (~20%) or 5% oxygen.

Functional Analysis of IscR. Previous studies using *E. coli* have indicated that IscR has the capacity to bind a [2Fe-2S] cluster and that cluster-loaded IscR is a negative regulator of *isc* expression (Schwartz et al. 2001; Frazzon et al. 2002; Kiley and Beinert 2003). In the present work, an ability to decouple expression of the *isc* genes from IscR regulation permitted a functional analysis of IscR in *A. vinelandii* and also provided an opportunity to evaluate the physiological consequences of unregulated *isc* expression. These experiments were initiated by constructing strains that have an *hscA::lacZ* translational fusion in the IscR-regulated copy of *hscA* and which also contain a second, intact copy of *isc* components whose expression is controlled by ScrR (DJ1525 and DJ1532, Figure 1B, Table 2). The IscR-regulated *hscA::lacZ* fusion in these strains is identical to the one

described previously for DJ1524 (Figure 1B, Table 2), except expression of the *hscA::lacZ* fusion in DJ1524 is controlled by ScrR. The availability of identical *hscA::lacZ* fusions in strains DJ1524 and DJ1532 also permitted direct comparison of *hscA::lacZ* expression within *A. vinelandii* when controlled by either the IscR or ScrR regulatory proteins. Results of these experiments showed that, when cultured using sucrose as the carbon source, ScrR regulated expression of the *hscA::lacZ* fusion was approximately half the level observed when compared to the IscR regulated expression of the same fusion under the same growth conditions (Table 3). This observation indicates that accumulation of Isc components in strains that contain a duplicated copy of *isc*-encoded components, whose expression is regulated by ScrR (DJ1454, Figure 1B, Table 2), probably do not result in an accumulation of Isc components substantially above that normally produced by the strain having only a single copy of intact IscR-regulated *isc* genes.

TABLE 3. β-galactosidase activity of *A. vinelandii* strains with mutations in *iscR*

Strain	Relevant Genotype[a]	Relative β-galactosidase Activity[b]
DJ1532	*Pisc-ϕ(hscA'-lacZ)*	1
DJ1524	*PscrX-ϕ(hscA'-lacZ)*	0.5 ± 0.07
DJ1562	*Δ iscR Φ(hscA'-lacZ)*	4.8 ± 0.24
DJ1580	*Δ iscR Φ(hscA'-lacZ)*	4.9 ± 0.23
DJ1582	*iscR (codon 92, GCA)*	
	Φ(hscA'-lacZ)	5.0 ± 0.14
DJ1531	*iscR (codon 98, GCC)*	
	Φ(hscA'-lacZ)	3.6 ± 0.19
DJ1587	*iscR (codon 104, TGC)*	
	Φ(hscA'-lacZ)	3.8 ± 0.14

[a]All strains contain two copies of the *iscSUAhscBAfdxiscX* gene region (see Table 2) one copy regulated by the *isc* promoter (P*isc*) and the other regulated by *scrX* promoter (P*scrX*). Except for DJ1524, the *hscA'-lacZ* fusion replaces the *hscA* gene located in the P*isc*-copy. (see Figure 1).
[b]A relative unit is defined as the $\Delta 0.0001$ abs (414nm)/min/OD$_{600}$ of the experimental sample divided by the control sample (DJ1532).

In separate experiments the consequences of deletions within *iscR* on expression of the *hscA::lacZ* fusion were examined. The effect of placing alanine substitutions for conserved cysteine residues proposed to provide [Fe-S] cluster ligands within IscR was

also examined. The results of these experiments are summarized in Table 3 and show that deletion of *iscR* results in elevated expression of *hscA::lacZ* and that individual alanine substitutions for the IscR residues Cys^{92}, Cys^{98}, or Cys^{104}, also results in elevated *hscA::lacZ* expression. These results lend strong support to previous studies in *E. coli* which indicated that a bound [Fe-S] cluster was required for the function of IscR as a repressor of the *isc* promoter (Schwartz et al. 2001).

Uncontrolled Expression of Isc Components is Deleterious. We expected that elevated expression of Isc components as a result of losing IscR function could provide an opportunity to enrich for Isc components for future biochemical analyses without gene-dosage complications that could be associated with using multi-copy plasmids. Strain DJ1601 was therefore constructed where *iscR* was deleted but all other *isc* genes remained intact. Thus, DJ1601 contained two intact copies of the [Fe-S] cluster biosynthetic components, an unregulated copy for which *iscR* has been deleted, and a second copy whose expression is regulated by ScrR (Figure1B, Table 2). It was surprising to find that DJ1601 grew much more slowly, than an otherwise isogenic wild type strain that contains a wild type *iscR* (Figure 10). The same effect was manifested when the proposed [2Fe-2S] cluster-ligating residue Cys^{92} was substituted with alanine (DJ1696, Table 2, data not shown). Repression of the Pscr-controlled *isc* operon by growth on glucose did not improve the slow growth phenotype. These results show either that unregulated elevation in the capacity for [Fe-S] formation is deleterious in *A. vinelandii* or that IscR has some other function necessary to sustain growth at wild type levels. The first of these possibilities was confirmed as the correct one by demonstrating that the slow growth phenotype exhibited by DJ1601 was suppressed (Figure 10) by combining the *iscR* deletion with a deletion in either *hscBA* (DJ1609, Figure 1B, Table 2) or *iscU* (DJ1603, Figure 1B, Table 2).
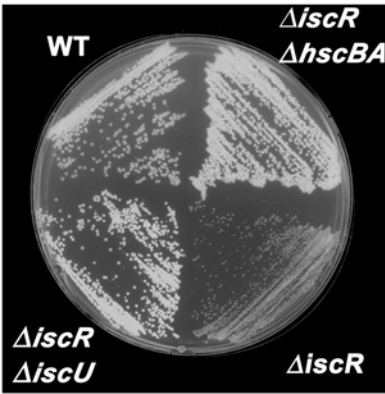
Figure 10. Deletion of *iscR* (DJ1601) results in a slow growth phenotype that can be reversed by deletion of *iscU* (DJ1603) or *hscBA* (DJ1609). A schematic representation of these strains is shown in Figure 1B.

3.4 - Discussion

Genetic tools have been developed for the controlled expression of any gene within *A. vinelandii*. This strategy is comparable to the plasmid-based, galactose-dependent controlled expression system developed in *Saccharomyces cerevisiae* (Lange et al. 2000). However, in our system, homologous recombination is used to place target genes under control of the genome-encoded sucrose catabolic regulatory elements. The specific advantage of this approach is that it obviates problems associated with increased gene dosage frequently encountered when using multicopy plasmids. Other advantages are that a simple color screen can be used to identify appropriate recombinants, as well as the availability of versatile cloning vectors (pDB1310 and pDB1332) that can be used for preparation of requisite gene fusions.

In the present work controlled gene expression was used for the functional analysis of all Isc components from *A. vinelandii* with the exception of IscX. Phenotypic traits associated with functional depletion of *A. vinelandii* Isc components are consistent with results of related genetic studies using *E. coli* or *S. cerevisiae* (reviewed in (Barras et al. 2005; Johnson et al. 2005). Namely, controlled depletion of IscS, IscU, IscA, HscBA

or Fdx results in reproducible defects in the maturation of aconitase, a key metabolic protein that requires an [Fe-S] cluster for its activity. In contrast to the situation with *E. coli* (Schwartz et al. 2000; Tokumoto and Takahashi 2001), however, IscS, IscU, HscBA and Fdx were found to be essential in *A. vinelandii* and loss of their respective functions could not be reversed by nutritional supplements. In this respect, it should be pointed out that *A. vinelandii* does not encode an intact Suf system, whose expression in *E. coli* has been shown to phenotypically suppress lesions within *isc* genes (Takahashi and Tokumoto 2002; Outten et al. 2004). IscS from *E. coli* has also been shown to be a generalized agent for cellular sulfur trafficking but this function, in the case of *A. vinelandii*, was not explored in the present work.

The availability of a strain that carries two copies of intact *isc* genes, one of them having *isc* expression regulated by the *scr* control elements, also permitted the functional analysis of targeted residues within specific Isc components. Results obtained from these analyses are also consistent with and confirm the conclusions from numerous biochemical studies using modified bacterial proteins, as well as phenotypic and biochemical studies performed using *S. cerevisiae* (Barras et al. 2005; Johnson et al. 2005). In particular, conserved cysteine residues located within IscS and IscU, as well as conserved IscU residues implicated in interaction with HscA, were all found to be essential. The conserved Asp[39] residue, implicated by biochemical studies to be involved in some aspect of the release of [Fe-S] clusters from the assembly scaffold, was also found to be essential (Yuvaniyama et al. 2000). An important feature to emerge from these studies, and not anticipated from other work, is the apparent dominant negative effect exerted by certain substitutions within IscU.

Such a dominant negative effect could occur either through subunit mixing, by sequestration of some other component of the [Fe-S] protein maturation machinery, or by non-productive interaction with a target protein that requires an [Fe-S] cluster for its activity. Results reported in the present genetic analysis do not provide information about the biochemical basis for the observed dominant negative effect resulting from certain substitutions. However, the availability of such mutants isolated from a genetic

background that contains duplicated *isc* genes, now provides an opportunity for isolation of extragenic suppressor mutations as a future genetic strategy to identify the nature of interaction among certain players that participate in the process of [Fe-S] protein maturation, and possibly, their target [Fe-S] proteins.

The function of IscA is not known. On the basis of biochemical studies, it has been proposed to serve either as a scaffold protein for [Fe-S] cluster formation or as an agent of Fe delivery to the IscU scaffold (Jensen and Culotta 2000; Krebs et al. 2001; Ollagnier-de-Choudens et al. 2001; Wu et al. 2002; Ding et al. 2005). Given the available information, both of these are credible hypotheses, yet there is no genetic or physiological data to suggest that either one is correct. In the present work we found that a clear null growth phenotype is manifested in cells depleted for IscA only when challenged by elevated levels of oxygen. The same oxygen-sensitive phenotype was also observed upon substitution of alanine for any of the three conserved IscA cysteine residues. Substitution of IscA Cys^{99} by alanine also resulted in a relatively strong, but incomplete, dominant negative phenotype when cultured under elevated oxygen. An even more striking observation was a complete dominant-negative effect when IscA residue Ser^{104} was substituted by cysteine. Substitution of Ser^{104} by alanine also resulted in oxygen sensitivity but with no attendant dominant negative phenotype, indicating the dominant negative phenotype associated with cysteine substitution at this position could be related to the presence of a thiol.

The complete dominant negative effect under conditions of elevated oxygen displayed by IscA having the Cys^{104} substitution, as well as work reported by others, described below, leads us to consider a different role for IscA in [Fe-S] protein maturation than previously suggested. In a series of genome wide searches, IscA was identified as having a possible thiol/disulfide oxidoreductase function due to the presence of a CXXS motif, which is often conserved in redox enzymes, but is rarely found in other proteins (Fomenko and Gladyshev 2002). The serine residue included in the IscA CXXS motif corresponds to Ser^{104}, the same residue substituted by cysteine in the present work (Figure 4). The same CXXS motif is found in all IscA homologs, as well as in monothiol

glutaredoxins (Fomenko and Gladyshev 2002; Molina-Navarro et al. 2006). Three monothiol glutaredoxins Grx3, Grx4 and Grx5, have been identified in *S. cerevisiae* of which the first two are located in the nucleus while Grx5 is located in the mitochondria (Lopreiato et al. 2004; Molina et al. 2004). While the loss of Grx3 or Grx4 does not display dramatic growth defects, depletion of mitochondrial Grx5 results in constitutive oxidative damage and defects in [Fe-S] protein maturation (Rodriguez-Manzaneque et al. 2002; Muhlenhoff et al. 2003). Monothiol glutaredoxins are thought to participate in the deglutathionylation of mixed disulfides formed between sulphydryl groups located within the target protein and the cysteinyl residue of glutathione, by using the single cysteine residue at the active site (Bushweller et al. 1992; Tamarit et al. 2003). A yeast Grx5 homolog, known as Grx4, has recently been discovered and characterized in *E. coli* (Fernandes et al. 2005; Fladvad et al. 2005). *E. coli* Grx4 lacks *in vitro* GSH-disulfide oxidoreductase activity typical of dithiol glutaredoxins, but appears to be essential for aerobic growth in rich media and is capable of substituting for yeast Grx5 function when compartmentalized in the yeast mitochondria (Gerdes et al. 2003; Fernandes et al. 2005; Molina-Navarro et al. 2006). Yeast Grx5 homologs from humans, chickens, zebrafish and cyanobacteria have also been shown to perform yeast Grx5 functions indicating a functional conservation of Grx5 homologs throughout evolution (Wingert et al. 2005; Molina-Navarro et al. 2006). Phylogenetic profiles used to predict the participation of bacterial monothiol glutaredoxins in [Fe-S] cluster biosynthesis revealed a high evolutionary co-occurrence between Grx5 homologs and IscA and Fdx (Vilella 2004). Two-hybrid analyses have also indicated a strong interaction between Grx5 and Isa1 from *S. cerevisiae* (Vilella 2004). Finally, the phenotype associated with inactivation of Grx5 in *S. cerevisiae* can be partially rescued by elevated expression of either Isa1 or Ssq1 *(S. cerevisiae* homolog to HscA) (Rodriguez-Manzaneque et al. 2002).

In aggregate, the above information indicates a possible mechanistic connection between monothiol glutaredoxins and IscA. Given that cysteine persulfides formed on IscS must be delivered to IscU during formation of [Fe-S] clusters and that polysulfides can accumulate on IscU *in vivo* (Smith et al. 2001), there could be ample opportunity for formation of non-productive heterodisulfide (or polysulfide) species on IscU. Elevated

levels of such species (especially heterodisulfides) could also be anticipated under conditions of oxygen stress. Based on these considerations we suggest the possibility that IscA has a redox function that serves to protect or repair the IscU scaffold from oxidative damage. Such a function could also require a source of reducing equivalents. An obvious candidate to provide this function is Fdx, which is known to form a complex with IscA (Ollagnier-de-Choudens et al. 2001). It should be noted, however, that the phenotypes associated with depletion of IscA and Fdx are not the same. Nevertheless, in the case of *S. cerevisiae*, Fdx is known to have multiple physiological functions (Barros et al. 2002) and therefore, the severe phenotype associated with Fdx depletion in *A. vinelandii* might be anticipated. An alternative, but related possibility for the function of IscA is a redox role that involves the protection or repair of [Fe-S] proteins in a way similar to the one we have suggested for protection or repair of IscU. With regard to this latter possibility it is noted that the *in vitro* decomposition of the [4Fe-4S] cluster of aconitase, which occurs upon Fe release, results in the formation of bridged polysulfide species (Kennedy and Beinert 1988). The two possible functions for IscA suggested here are not necessarily mutually exclusive, nor do they exclude the possibility that IscA could also serve either as an alternative scaffold or as an Fe donor during [Fe-S] cluster assembly.

The oxygen sensitive phenotype associated with IscA depletion also led us to consider whether or not a redox sensitive phenotype could be associated with depletion of other Isc components. Because *A. vinelandii* cells depleted for IscS, IscU, HscBA or Fdx are unable to grow under ambient oxygen concentrations, we tested if lowering the oxygen concentration to 5% could rescue their respective null phenotypes. In this case, only the null phenotype associated with HscBA could be partially rescued by this method. As in the case of IscA, this result suggests a possible connection between HscBA function and oxygen sensitivity. One possibility is that HscBA could participate in protection or repair of the IscU scaffold. This possibility is consistent with recent work performed with *S. cerevisiae*, where it was concluded that functional HscBA homologs are not required for the primary *de novo* synthesis of [Fe-S] clusters on the *S. cerevisiae* IscU homolog (Muhlenhoff et al. 2003; Dutkiewicz et al. 2006). We consider

this possibility an attractive one because it has been shown that a fraction of recombinantly expressed IscU contains polymeric sulfide species that cannot be removed by cyanolysis (Smith et al. 2001). In this respect it is striking that the IscU LPPVK motif shown to be necessary and sufficient for stimulation of HscBA directed ATP hydrolysis is located adjacent to the IscU Cys^{106} residue. The fact that elevated expression of Ssq1 (HscA homolog) or Isa1 can phenotypically reverse the effect of Grx5 depletion in *S. cerevisiae* (Rodriguez-Manzaneque et al. 2002) also points to a functional connection between HscBA and IscA that is related to the capacity for [Fe-S] protein maturation to survive an oxidative challenge. An interaction between *E. coli* IscA and HscA has also been identified by two-hybrid analysis (Tokumoto et al. 2002). Whatever the specific role of HscBA and IscA, the conditional expression of null phenotypes in response to different redox conditions now provides a genetic basis to gain further insight into the complicated process of [Fe-S] protein maturation.

Finally, it was shown that elevated expression of *A. vinelandii* Isc components occurs as a result of deleting *iscR*, or as a consequence of placing substitutions for the proposed [2Fe-2S] cluster-ligating cysteine residues located within IscR. These results are consistent with a feedback regulatory model proposed for *E. coli* where cluster occupancy in IscR is used to monitor the physiological demand for [Fe-S] cluster synthesis (Schwartz et al. 2001). However, our results further demonstrate that IscR not only serves to accommodate a demand for increased [Fe-S] protein maturation but also has an important function in preventing the capacity for formation of an excess of [Fe-S] clusters beyond that required for [Fe-S] maturation. This feature is apparent from the deleterious effect associated with uncontrolled expression of Isc components, a phenotype that can be specifically suppressed by lowering the functional capacity for elevated [Fe-S] cluster formation. Although the biochemical basis for the deleterious effect is not known, one possibility is that the phenotype is a result of the uncontrolled release of iron, sulfide or iron-sulfide species from the IscU scaffold.

CHAPTER 4

Cross-Talk Studies: Analyzing Possible Overlapping Functions Between the Isc and Nif Systems

The discovery of a sucrose inducible promoter and the development of a controlled *isc* expression system in *A. vinelandii*, permitted initial studies on the possible overlapping functions between the Nif and Isc systems, hereafter referred to as "cross-talk" studies. Part 1 of this chapter describes published experiments demonstrating that under standard laboratory growth conditions, the *nif*-specific [Fe-S] cluster biosynthetic system, which is required for nitrogenase maturation, cannot functionally replace the *isc* [Fe-S] cluster system used for maturation of housekeeping [Fe-S] proteins. This finding is a clear indication that little or no functional cross-talk between Isc- and Nif-directed [Fe-S] protein maturation systems occurs when they are produced at levels necessary to satisfy their respective physiological functions.

Part 2 of this chapter describes more recent experiments designed to lend further insight into the basis for target specificity between the two [Fe-S] cluster biosynthetic systems in *A. vinelandii*. Experiments that were performed demonstrate that functional cross talk between the Nif and Isc systems can be forced if the expression levels of either system are artificially increased or the cells are exposed to low oxygen concentrations. These findings confirm that there is a high level of target specificity with regard to the Isc and Nif [Fe-S] cluster assembly systems and that each system is likely to function in concert with different accessory proteins via separate assembly pathways.

CHAPTER 4: Part 1

NifU and NifS are Required for the Maturation of Nitrogenase and Cannot Replace the Function of *isc*-Gene Products in *Azotobacter vinelandii*

Deborah Johnson, Patricia Dos Santos[1], Dennis R. Dean[1]

This part of the chapter was published with the intention for its use to satisfy a portion of the research completed for this dissertation. As primary author, I was involved in performing experimental work, writing the document and editing the figures included in this chapter. In the present work we show that the *nif*-specific [Fe-S] cluster biosynthetic system from *Azotobacter vinelandii*, which is required for nitrogenase maturation, cannot functionally replace the *isc* [Fe-S] cluster system used for the maturation of other [Fe-S] proteins, such as aconitase. These results were interpreted to indicate that in *A. vinelandii* the [Fe-S] cluster biosynthetic machineries have evolved to perform only specialized functions.

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4.1 – Introduction

Simple complexes of iron and inorganic sulphide ([Fe-S] clusters) are contained in a diverse group of proteins, called [Fe-S] proteins, which participate in a wide variety of cellular processes, including electron transfer, catalysis, and regulation of gene expression. Such functional versatility of [Fe-S] proteins is related to the structural and electronic plasticity of their cognate [Fe-S] clusters. The most familiar [Fe-S] clusters include [2Fe-2S] and [4Fe-4S] clusters, which are usually covalently attached to their protein partners through cysteine mercaptide ligands. In spite of their structural simplicity, the formation and insertion of [Fe-S] clusters into their protein partners is a complicated process.

Initial insights about the pathway for [Fe-S] cluster assembly were gained through analysis of *Azotobacter vinelandii* genes required for activation of nitrogenase, the catalytic component of biological nitrogen fixation. Nitrogenase comprises two catalytic partners, called the Fe protein and the MoFe protein, and both of these are [Fe-S] proteins (Christiansen et al. 2001). A biochemical-genetic analysis of nitrogen-fixation-specific (*nif*) genes required for nitrogenase maturation revealed that two of them, *nifU* and *nifS*, are uniquely required for the activation of both the Fe protein and the MoFe protein (Jacobson et al. 1989; Dos Santos et al. 2004). Subsequent studies suggested that NifS is a cysteine desulphurase that uses pyridoxal-phosphate chemistry to activate S in the form of an enzyme-bound persulphide (Zheng et al. 1993) and that NifU provides a molecular scaffold for assembly of "transient" [Fe-S] cluster units destined for nitrogenase maturation (Agar et al. 2000). Key observations used to validate this model include: (*i*) NifU and NifS are able to form a transient macromolecular complex (Yuvaniyama et al. 2000), (*ii*) co-incubation of NifU and NifS in the presence of L-cysteine and Fe^{++} results in the formation of labile [Fe-S] clusters on NifU (Yuvaniyama et al. 2000), (*iii*) [Fe-S] cluster-loaded NifU can be used for the effective *in vitro* activation of apo-Fe protein (Dos Santos et al. 2004), and (*iv*) placement of certain amino acid substitutions within NifU results in trapping of the transient [Fe-S] cluster on the NifU scaffold (Yuvaniyama

et al. 2000), thereby compromising the capacity for both *in vivo* and *in vitro* nitrogenase activation (Dos Santos et al. 2004).

4.2 - The *isc* and *suf* Systems also have [Fe-S] Cluster Biosynthetic Functions.

Although genetic inactivation of either NifU or NifS results in a dramatically lowered capacity for the *in vivo* maturation of nitrogenase, loss of NifU or NifS function does not completely eliminate the capacity for nitrogen fixation (Jacobson et al. 1989). This result indicated that NifU or NifS activities could be replaced at low levels by some other cellular activities. A search for other cellular components having NifU-like and NifS-like activities resulted in the identification of a group of genes proposed to be required for the maturation of other [Fe-S] proteins, for example, aconitase, that are not related to nitrogen fixation. This gene cluster (Figure 1), designated "*isc*" (*i*ron-*s*ulphur-*c*luster), encodes proteins having functions analogous to NifU (IscU) and NifS (IscS), as well as several other proteins including, an alternative scaffold (IscA), molecular chaperones (HscB and HscA), a ferredoxin (Fdx), and a negative regulator (IscR) (Zheng et al. 1998). Genes encoding homologs to IscS, IscU, IscA, HscB, HscA and Fdx are widely distributed in nature and a variety of genetic studies have clearly implicated all of them in some aspect of the maturation of [Fe-S] proteins (Muhlenhoff and Lill 2000; Tokumoto and Takahashi 2001). A third type of [Fe-S] protein maturation machinery was identified in *Escherichia coli*, which has been designated "*suf*" (Takahashi and Tokumoto 2002). In the case of *E. coli*, which also has an intact *isc* gene cluster, genetic and physiological studies have established that the *isc* system operates under "normal" growth conditions whereas the *suf* system operates under conditions of Fe limitation or oxygen stress (Outten et al. 2004). Although there appears to be specialized components that differentiate the three identified [Fe-S] cluster biosynthetic machineries they are unified by an apparent requirement for a cysteine desulphurase and [Fe-S] cluster assembly scaffold.
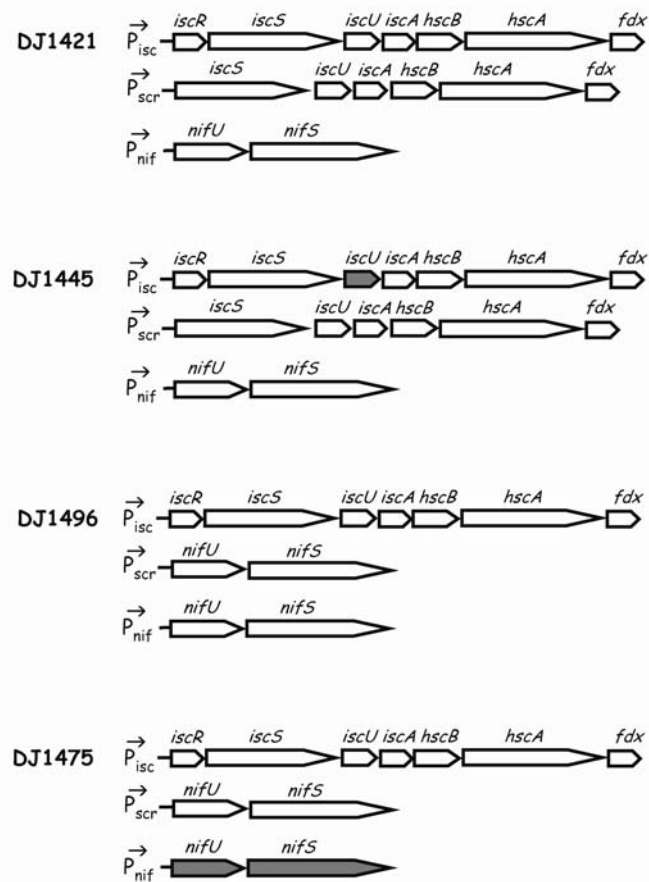
Figure 1. Organization of the *isc* gene cluster and *nifUS* genes in *A. vinelandii* strains used in this work. Individual strains are designated DJ1421, DJ1445, DJ1496 and DJ1475. The promoters designated P*isc* and P*nif* control expression of the normal *isc* and *nif* gene clusters. P*scr* is a sucrose-inducible promoter and it controls expression of the duplicated version of the genes indicated. Shaded boxes represent genes with in-frame deletions.

The picture that has emerged concerning [Fe-S] protein maturation is that some organisms have generalized "housekeeping" [Fe-S] cluster biosynthetic machinery as well as other more "specialized" [Fe-S] cluster biosynthetic machinery. However, whether or not a particular type of [Fe-S] cluster biosynthetic machinery, *isc*, *suf*, or *nif*,

operates in a "housekeeping" capacity or in a "specialized" capacity appears to depend on a particular organism. Indeed, for *Helicobacter pylori* and *Thermatoga maritima* the *nif*-like and *suf*-like systems, respectively, appear to be the only intact [Fe-S] cluster biosynthetic systems available to these organisms (Johnson et al. 2004). Interesting questions attached to the role of various [Fe-S] cluster biosynthetic machineries involves their evolutionary and functional relationships.

4.3 - Controlled Expression of *isc* and *nif* Genes.

As mentioned above the capacity for *nifU* or *nifS* deletion strains to fix nitrogen at a very low level indicated their functions could be partially supplanted by some other [Fe-S] cluster biosynthetic system (Jacobson et al. 1989). Although this hypothesis led to the discovery of the *isc*-gene cluster, the possibility that *isc* gene products can participate in the maturation of nitrogenase could not be directly tested because genetic experiments indicated that inactivation of the *isc* genes is lethal (Zheng et al. 1998). This situation also precluded the opportunity to examine whether or not a *nif*-specific [Fe-S] cluster biosynthetic component can functionally replace an *isc*-specific component. In order to overcome this problem and to develop an opportunity to examine the specific functions of different components of the *isc*-specific [Fe-S] cluster biosynthetic machinery we developed a method for the controlled expression of individual *isc*-specific and *nif*-specific components uncoupled from their normal regulatory components.

In *A. vinelandii* and many other bacteria, the *isc* genes are controlled by a negative feedback mechanism where the holo-form of an [Fe-S] cluster-containing regulatory protein (encoded by *iscR*, see Figure 1) represses transcription of the *isc* gene cluster (Frazzon and Dean 2001; Schwartz et al. 2001). The *nif* genes are controlled by a complex regulatory cascade involving regulatory proteins encoded by *nifA* and *nifL* and are only expressed under conditions that required nitrogen fixation (Little et al. 2000). In order to uncouple *isc*- or *nif*-regulated components from their normal regulatory elements, the isolated genes were placed under the control of a sucrose-inducible (*scr*) promoter *in vitro* by using recombinant techniques and subsequently reintegrated into the

chromosome in single copy by using reciprocal recombination (Figure 1). Details of the genetic constructions will be reported elsewhere. The *scr* promoter is negatively regulated by the availability of sucrose in the same way that the *lac* promoter is controlled by the availability of lactose. These constructions resulted in duplication of the genes of choice (Figure 1, strain DJ1421 and strain DJ1496), where expression of one copy is controlled by the normal regulatory elements and the *scr* promoter controls expression of the second copy.

4.4 - NifU and NifS Cannot Functionally Replace IscU and IscS.

Control experiments established that genes regulated by *scr* are expressed at a relatively high level when sucrose is present in the growth medium but are not expressed at detectable levels in the absence of sucrose. This situation permitted the placement of deletions within the normal copy of a particular gene (see Figure 1, strain DJ1445 and strain DJ1475) whose function is replaced by the second copy, providing that cells are grown in the presence of sucrose under conditions where the function of the deleted gene is required. When sucrose is removed from the growth medium, in this case replaced by glucose, products of genes controlled by the *scr* promoter are gradually depleted from the cell. In this way the physiological and biochemical consequences of the loss of function of a particular gene product can be unambiguously evaluated. The results of controlled expression experiments are shown in Figure 2. For the experiments shown in Figure 2, all cells were cultured in a medium that does not contain any nitrogen source so the cells must be capable of performing nitrogen fixation in order to grow. All strains show normal growth when cultured in the presence of sucrose (Figure 2, panels E, F, G and H). However, in the case of DJ1445 there is no growth when cells are depleted for IscU (Figure 2, panel B). In the case of strain DJ1445, depletion of IscU eliminates the capacity for growth under nitrogen-fixing conditions (Figure 2 panel B) or when a fixed nitrogen source is added to the growth medium (data not shown). These results suggest that IscU is essential under both growth conditions and that NifU cannot functionally replace IscU.
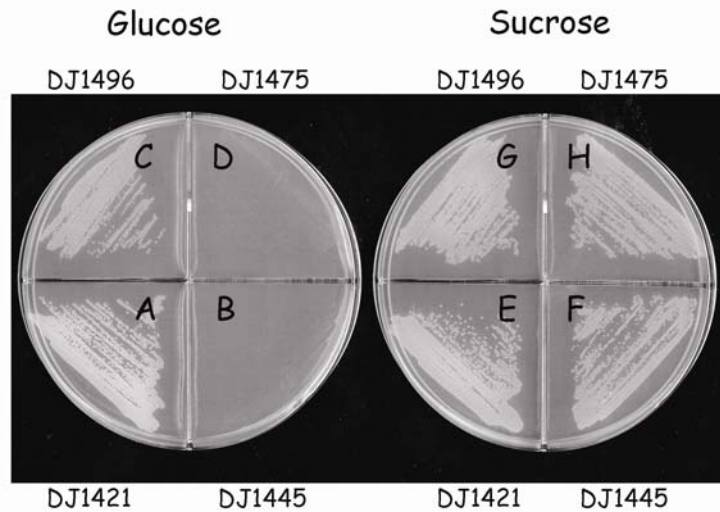
90

Figure 2. Growth of *A. vinelandii* strains cultured under different conditions. Details of the growth conditions are described in the text.

A possible explanation for the inability of NifU to functionally replace IscU is that NifU is sequestered into a macromolecular *nif*-specific complex so that it is not available for other cellular processes. To test this possibility strain DJ1496 was constructed, which contains two copies of the *nifU* and *nifS* genes, one copy whose expression is under *nif* control and the other copy under *scr* control. This strain was then used as a recipient in genetic transformation experiments where we attempted to separately delete the *iscU* gene and the *iscS* gene. In these experiments, neither the *iscU* gene nor the *iscS* gene could be deleted, even under conditions where *nifU* and *nifS* are expressed independently from the *nif*-specific components and in the absence of other *nif*-specific components. In order to confirm that *nifU* and *nifS* are actually expressed when regulated by the *scr* promoter, a derivative of DJ1496 was constructed, where the *nif*-regulated copies of *nifU* and *nifS* are deleted (Figure 1, strain DJ1475). This strain is capable of growing under nitrogen-fixing conditions when cultured in the presence sucrose (Figure 2, panel H), but not when cultured in the absence of sucrose (Figure 2, panel D), establishing that the *scr*-regulated *nifU* and *nifS* gene products have functional activity.

Although the specific function of the molecular chaperones, HscB and HscA (Figure 1) is not understood, they are required for *isc*-directed [Fe-S] cluster assembly and IscU is known to specifically interact with an HscBA complex (Hoff et al. 2000). This interaction is dependent upon an oligopeptide sequence within IscU (LPPVK) which is necessary and sufficient to stimulate intrinsic HscA-directed ATPase activity (Hoff et al. 2002). Comparison of IscU and NifU primary sequences shows that the LPPVK signature sequence within IscU is replaced by LPPEK in NifU (Hoff et al. 2002; Johnson et al. 2004). We therefore considered a second possible explanation for the inability of NifU to functionally replace IscU. Namely, that NifU does not productively interact with the molecular chaperones HscBA, and that such a specific interaction might be required for maturation of [Fe-S] proteins other than the nitrogenase components. To test this possibility, the LPPEK sequence in NifU was converted to the canonical LPPVK sequence within IscU, and experiments similar to those already described were repeated. However, this modification did not endow NifU with an ability to functionally replace IscU. In aggregate these experiments establish that, under the conditions used here, there is a high degree of specificity for [Fe-S] cluster assembly components in *A. vinelandii*. In particular, the *nif*-specific [Fe-S] cluster assembly components are required to maintain an active nitrogenase and cannot be effectively used to replace the *isc*-specific [Fe-S] cluster assembly components required for the maturation of other [Fe-S] proteins, such as aconitase.

Our results are relevant to a recent report by Takahashi and co-workers where it was shown that heterologous expression of a "*nif*-like" [Fe-S] cluster biosynthetic system from *Entamoeba histolytica* could replace the function of the *suf*- or *isc*-type of [Fe-S] cluster biosynthetic systems, but only under anaerobic conditions (Ali et al. 2004). This finding is in line with the suggestion that *nif*-like [Fe-S] cluster biosynthetic systems from non-nitrogen-fixing organisms, such as *E. histolytica* and *H. pylori,* do not have a specialized function but instead, are utilized for "housekeeping" [Fe-S] protein maturation in these organisms. It therefore appears that, in spite of considerable primary sequence identity among members of *nif*-like and genuine *nif*-specific [Fe-S] cluster biosynthetic systems, the *nif*-specific system (at least for *A. vinelandii*) has evolved an

exquisite function specialized for nitrogenase maturation. We believe it should be possible to exploit primary sequence differences between components of *nif*-like and *nif*-specific [Fe-S] cluster biosynthetic systems, differences in their corresponding three-dimensional structures (when they become available), as well as genetic strategies, to determine the basis for target specificity, a feature that is not yet understood for any [Fe-S] protein maturation process.

CHAPTER 4: Part 2

"Cross-talk" Re-visited: Conditions under which the Nif and Isc [Fe-S] Protein Maturation Systems Exhibit Full or Partial Functional Equivalence

This part of the chapter describes the results of more recent experiments I have been involved with in collaboration with my colleague, Dr Patricia Dos Santos.   The development, by Dr Dos Santos, of an arabinose-induction system to allow abundant expression of proteins in *A. vinelandii* has permitted us to re-visit our cross-talk studies with an aim of understanding the basis for the lack of cross-talk between the Nif and Isc systems in *A. vinelandii* in light of numerous findings described in Chapter 3 and published by other researchers.   Conditions have been found under which cross-talk between the two systems is possible, namely by changing the expression levels of either system or by changing oxygen conditions.   These findings are providing important clues as to what key Nif or Isc components commit each system to their respective [Fe-S] cluster assembly pathways.   My contribution to this study specifically includes the construction of strains DJ1454, DJ1450, DJ1445, DJ1620, and DJ1668. I also conducted the experiments presented in Figures 4, 7 and 8.

4.5 - Introduction

The ability of Isc components to replace Nif-specific [Fe-S] protein maturation, and *vice versa*, is referred to as functional "cross-talk". In Part 1 of this chapter, experiments were described that clearly showed that *A. vinelandii* NifU is not capable of replacing the function of IscU. Evidence to support this was based on the null growth phenotype associated with cells depleted for IscU, which cannot be rescued by expression of NifUS from its endogenous promoter (P*nif*) or the sucrose-inducible promoter (P*scrX*). Similarly, components of the Isc system were not able to fully replace the function of the Nif system since *nifU* and *nifS* deletion strains are capable of only very slow growth when cultured under conditions that require nitrogen fixation. Taken together, this data was interpreted to indicate that there is not a significant level of functional cross-talk between the two [Fe-S] cluster biosynthetic systems in *A. vinelandii* under the conditions tested, suggesting that NifS and NifU have evolved specialized functions required for nitrogen fixation. However, evidence for some functional equivalence among Nif-like and Isc components was provided by Takahashi and co-workers (Ali et al. 2004; Tokumoto et al. 2004) who showed that heterologous plasmid-directed expression of a Nif-like system from the strict anaerobe, *Entamoeba histolytica* and the microaerophile, *Helicobacter pylori* , can functionally replace the Isc and Suf machinery from *Escherichia coli*, but only under anoxic conditions. In the non-nitrogen-fixing organisms, *E. histolytica* and *H. pylori*, a two-component *nif*-like system (NifS/NifU) constitutes the only [Fe-S] cluster biosynthetic system and presumably performs housekeeping functions similar to those performed by the Isc system of *A. vinelandii* (Olson et al. 2000; Ali et al. 2004). Takahashi and co-workers concluded that hypersensitivity to oxygen, rather than target specificity, prevented the Nif-like system from efficiently performing Isc- and Suf-type [Fe-S] protein maturation functions under aerobic conditions. In light of these reports and the discovery that strains depleted for IscA or HscBA display hypersensitivity to elevated or ambient levels of oxygen, respectively, (Chapter 3) we sought to examine whether changes in oxygen concentrations or in the expression levels of the *A. vinelandii* Nif or Isc system would result in permissive conditions for cross-talk between the two systems. Our ultimate aim is to gain further insight into the basis for target specificity between the two [Fe-S] protein maturation systems in *A. vinelandii*.

4.6 - Materials and Methods

Growth of *A. vinelandii* strains - All chemicals were obtained from Sigma except for L-arabinose, which was purchased from Spectrum. *A. vinelandii* strains were grown at 30°C on solid modified Burks minimal medium (Strandberg and Wilson 1968) containing 2% sucrose or 2% glucose as the sole carbon source. A nitrogen source is omitted for growth of cells under diazotrophic (nitrogen fixation) conditions. For preparation of media containing a fixed nitrogen source, ammonium acetate was added at a final concentration of 13 mM. For antibiotic resistance selection and/or screening, final concentrations were: ampicillin (0.08 µg/ml) kanamycin (0.5 µg/ml), gentamycin (0.05 µg/ml), rifampicin (5.0 µg/ml), and streptomycin (0.1 µg/ml). X-Gal (4-bromo-4-chloro-3-indoyl-β-D-galactoside) was added to a final concentration of 60 µg/ml. Growth of cells at low oxygen was performed by placing Petri plates in a Coy chamber containing 5% oxygen at 1 atm balanced with N_2 gas from a regulated gas tank (Airgas Inc.). For the growth of cells at 40% oxygen at 1 atm, inoculated Petri plates were placed in vented BBL$^®$ GasPak jars (Becton, Dickinson and Company). Evacuation of ambient air was accomplished using a Schlenk line apparatus and re-gassed by flushing with 40% O_2 balanced with 60% N_2 using a regulated gas tank (Airgas Inc.). No more then 4 (100 x 15mm) Petri plates at a time were placed in each jar, which can hold up to 12 plates. Sealed jars were incubated at 30°C and were evacuated and reflushed with the 40% O_2/ 60% N_2 gas mixture every 2 days. Growth of liquid cultures was performed in 2000-ml or 500-ml Erlenmeyer flasks incubated at 30°C and shaken at 300 rpm. Arabinose induction was initiated when cells reached mid-log growth (OD_{600}=1.0) by the addition of L-arabinose at a final concentration of 20 mM and allowed to proceed for 4 hours. For analysis of the total protein profile of cells induced with arabinose, standard procedures for SDS-PAGE were used (Laemmlli 1970).

Plasmid and Strain Construction. Relevant plasmids and strains used in this study are listed in Table 1. The construction of new plasmids and strains (marked with an asterisk in Table 1 and not explained in Chapter 3 or Part 1 of this chapter) is described below. Restriction enzyme digestion, and ligation of hybrid plasmid DNAs were performed by

previously described techniques (Sambrook et al. 1987). Restriction enzymes, DNA ligase and T$_4$ DNA polymerase were purchased from New England Biolabs, Promega, and Invitrogen, respectively. *Escherichia coli* TB1 was used for cloning hybrid plasmids containing genomic DNA from *A. vinelandii*. Extraction of genomic *A. vinelandii* DNA and Polymerase Chain Reaction (PCR) were performed using commercial kits (QuickExtract[TM] DNA Extraction Solution and Failsafe[TM] PCR PreMix Selection Kit, Epicentre).

TABLE 1. Mutant strains used in this study.

			Genes placed in *scr* region[b]		
Strain[a]	Key plasmid used	*isc* gene/s mutated in endogenous, IscR-controlled *isc* operon (**P***isc*)	Genes under control of *scrX* promoter (**P***scrX*)	Genes under control of *E. coli ara* promoter (**P***ara*)	*nif* gene/s mutated in endogenous, *nif* operon (**P***nif*)
DJ1454	pDB1316	none	*iscSUAhscBAfdxiscX*	-	none
DJ1450	pDB954	Δ *iscS*	*iscSUAhscBAfdxiscX*	-	none
DJ1445	pDB1350	Δ *iscU*	*iscSUAhscBAfdxiscX*	-	none
DJ1620*	pDB1490	Δ *iscR*	none	-	Δ *nifU*
DJ1413	pDB1317	none	none	-	Δ *nifU*
DJ1626*	pDB1551	none	-	*nifUS*	Δ *nifUS*
DJ1639*	pDB1350	Δ *iscU*	-	*nifUS*	Δ *nifUS*
DJ1418	pDB1335	none	*lacZkan*	-	none
DJ1668*	pDB1562	none	-	*iscSUAhscBAfdxiscX*	*none*
DJ1683*	pDB1317	none	-	*iscSUAhscBAfdxiscX*	Δ *nifU*

[a] Strains constructed for the purpose of this study are marked with an asterisk (*). Strains DJ1454, DJ1450 and DJ1445 were previously described in Part 1 of this chapter and/or in Chapter 3. Construction of strain DJ1413 was published in Dos Santos *et al.*, 2004
[b] Genes placed by homologous recombination in the *scr* region can only be under P*scrX*-control or P*ara* -control , but not both.

Fusion of the *A. vinelandii nifUS* genes to the *E. coli ara* transcriptional and translational elements to create pDB1289 has been previously described (Dos Santos et al. 2004). Fusion of the *A. vinelandii iscSUAhscBAfdxiscX* gene cluster to the *E. coli ara* transcriptional and translational elements was achieved by ligation of a 5.2 kb BspHI DNA fragment containing *iscSUAhscBAfdxiscX* with an NcoI-digested pAra13 derivative to construct pDB1282 (P*ara-iscSUAhscBAfdxiscX*). The chromosomal region encoding genes involved in sucrose metabolism (*scr* region) was chosen as a suitable site for

incorporation of the P*ara-nifUS* and P*ara-iscSUAhscBAfdxiscX* fusions into the *A. vinelandii* genome. Plasmids pDB1289 and pDB1282 served as the parent plasmids for the construction of pDB1551 and pDB1562 (Figure 3) which contain the necessary flanking *scr* sequences required for double reciprocal homologous recombination into the *A. vinelandii* genome in addition to the *araC* gene required for AraC-dependent regulation of the *ara* promoter. Plasmid pDB1551 was created by ligation of the 4.1 kb SphI-XhoI DNA fragment including P*ara-nifUS* and *araC* with SphI-XhoI-digested pDB1332. Plasmid pDB1562 was created by ligation of the 6.7 kb XbaI-SpeI DNA fragment including P*ara-iscSUAhscBAfdxiscX* and *araC* with XbaI-digested pDB1332. Construction of pDB1332 has been previously described (see Chapter 3).
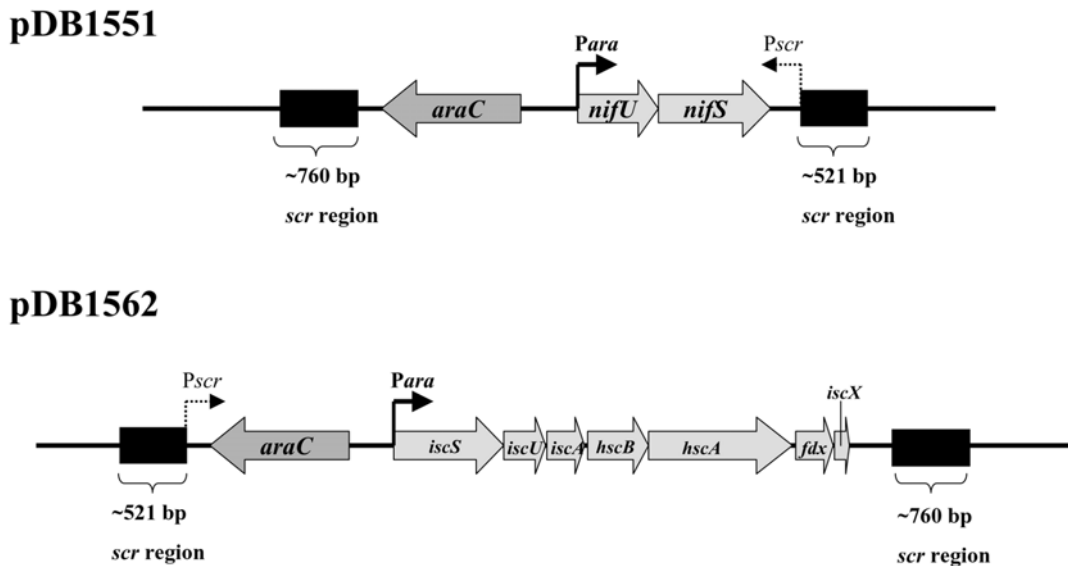
Figure 3. Key features of plasmids, pDB1551 and pDB1562, which respectively contain the *A. vinelandii nifUS* and *iscSUAhscBAfdxiscX* genes under control of the strong *ara* transcriptional and translational elements from *E. coli*. The flanking *scr* regions allow double reciprocal homologous recombination into the *A. vinelandii* genome.

Mutations within the *A. vinelandii* genome were achieved by transformation of competent cells with the appropriate plasmids, followed by selection and/or screening of cells which have undergone double-reciprocal recombination between genome and

plasmid vector/s. Details of the *A. vinelandii* transformation process have been previously described (Jacobson et al. 1989). Strains with deletions in *nifU* and/or *nifS* exhibit very poor growth under diazotrophic conditions producing visibly tiny colonies on solid media (Jacobson et al. 1989). This very slow growth phenotype (Nif $^-$) was exploited to genetically select for larger sized colonies (Nif $^+$) resulting from transformations in which expression of functional NifU / NifS has been restored or relevant mutations in the *isc* operon have been introduced. Transformation of recombinant plasmid pDB1490 (Δ*iscR*; see Table 1 in Chapter 3) into DJ1413 (Δ*nifU*;(Dos Santos et al. 2004) resulted in the isolation of DJ1620 (Table 1; Figure 8A;) which produced larger colonies on Burks nitrogen-free minimal medium when compared to the parent, DJ1413 (Table 1; Figure 8A). The cause of this growth difference can be attributed to a 120 bp deletion within *iscR* which was verified by PCR analysis of the genomic DNA. A similar strategy was used for the construction of DJ1626 (Table 1; Figure 6A) via transformation of DJ1469 (Δ*nifUS*) with pDB1551 (P*ara_nifUS*). In this case, larger sized colonies were obtained on plates containing Burks nitrogen-free minimal medium supplemented with arabinose (Table 1).

In a strategy similar to that described for the construction of DJ1445 in Chapter 3, DJ1639 (Table 1; Figure 6B), which contains an in-frame deletion in *iscU*, was identified by its inability to grow on plates lacking supplemental arabinose. Plasmid pDB1350 (Chapter 3) was recombined into the *A. vinelandii* chromosome of DJ1626 by congression with gentamycin resistance as the selected marker. Transformants were first plated on Burks minimal media supplemented with ammonium acetate and arabinose and were then screened for loss of growth on plates lacking arabinose.

Placement of a second copy of the *isc* operon in the *A. vinelandii scr* region, under P*ara* control (DJ1668), was achieved by transformation of pDB1562 into DJ1418 (*lacZ*$^+$; see Chapter 3) along with a congression vector encoding streptomycin resistance. Colonies of DJ1418 formed blue colonies when grown on plates containing sucrose as the sole carbon source. Cells which had undergone the desired double-crossover event were identified as Kn^s, white colonies (*lacZ*) on X-Gal-supplemented Burks (sucrose)

agar plates. The correct location of the duplicated *isc* gene region was verified by PCR. DJ1668 served as the parent strain for construction of DJ1683 with recombinant plasmid pDB1317 (Δ*nifU;* Dos Santos *et al*., 2004). This strain was differentiated from the parent strain by its weaker growth under diazotrophic conditions (Nif⁻) in the absence of arabinose (see Table 1; Figure 9A)

4.7 - Results and Discussion

In this study, experiments were performed to search for conditions that permit functional "cross-talk" between the Isc and Nif-specific systems of *A. vinelandii*. Strains were constructed to analyze the ability of the Nif system or the Isc system to respectively rescue the null/poor growth phenotypes associated with in-frame deletions in IscU or NifU.   Two conditions were found to improve the "cross-talk" abilities of the two [Fe-S] cluster biosynthetic systems: (i) growth under reduced oxygen conditions or (ii) growth under conditions allowing elevated expression of either the Nif or Isc systems.

Replacement of Isc [Fe-S] Protein Maturation Function by Nif-specific components. As described in Chapter 2, discovery of the respective functions of NifS (cysteine desulfurase) and NifU (proposed [Fe-S] cluster assembly scaffold) provided the first evidence that specific components are necessary for mobilization of Fe and S for biological [Fe-S] cluster formation (Zheng et al. 1993; Agar et al. 2000; Yuvaniyama et al. 2000). Because the two catalytic components of nitrogen fixation are very abundant in nitrogen-fixing cells, and both of these components contain [Fe-S] clusters, it was speculated that NifU and NifS provide a controlled way to augment the demand for [Fe-S] cluster formation under nitrogen-fixing conditions (Dos Santos et al. 2004). This idea was supported by the observation that inactivation of NifS or NifU severely compromises, but does not eliminate, the capacity for nitrogen fixation. Namely, it was suspected that the functions of NifS and NifU could be supplanted at a very low level by some other endogenous [Fe-S] cluster biosynthetic system that was primarily used for maturation of [Fe-S] proteins whose functions are not related to nitrogen fixation. This

hypothesis led to a search for such components and resulted in identification of the Isc system (Zheng et al. 1998).

If there is effective functional cross-talk between the Isc and Nif [Fe-S] cluster assembly components in *A. vinelandii*, it was expected that a strain inactivated for Isc components would be able to grow if cultured under nitrogen-fixing conditions, which results in expression of the Nif-specific [Fe-S] cluster assembly components. However, our previous unsuccessful attempts to rescue the null growth phenotype of an *iscU* deletion strain, when cultured under nitrogen-fixing conditions, was interpreted to indicate that there is not a significant level of functional cross-talk under these conditions (Part 1 of this chapter). In this study, we examined whether or not expression of the Nif-specific [Fe-S] cluster biosynthetic components could rescue the null growth phenotype exhibited upon depletion of either IscU or IscS when cultured under low levels of oxygen (~5% at 1atm). Results of these experiments are shown in Figure 4 and reveal that a strain depleted for IscU cannot grow under nitrogen-fixing conditions when cultured in the presence of ambient (~20%) oxygen but can grow when cultured under ~5% oxygen. This effect is specific to nitrogen fixation because a null growth phenotype occurs upon depletion of IscU when cells are cultured under either 20% or 5% oxygen, if expression of the nitrogen fixation components is repressed (data not shown). In contrast, a strain depleted for IscS cannot grow under any of the conditions described here. An inability of NifS to functionally replace IscS could be related to a specific role for IscS in mobilizing S for formation of certain sulfur-containing cofactors and thiolation of certain tRNAs, which is not dependent upon [Fe-S] cluster formation (Mihara and Esaki 2002). This data is consistent with data from Takahashi's group whose work also indicated that the *E. histolytica and H. pylori* Nif-like components were not sufficient to replace the function of the *isc* machinery in the absence of IscS (Ali et al. 2004; Tokumoto et al. 2004).
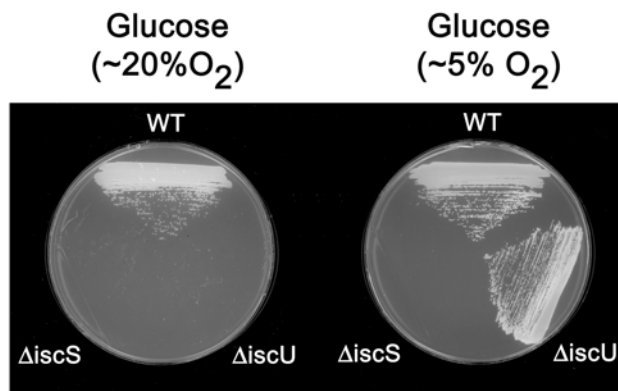
Figure 4. Expression of the Nif system under low oxygen concentrations rescues the null growth phenotype of a strain with an in-frame deletion in *iscU*. Strains DJ1454 (WT), DJ1450 (Δ*iscS*) and DJ1445 (Δ*iscU*) above were streaked on Petri plates containing Burk's (glucose) minimal medium in the absence of a fixed nitrogen source. The construction and genotype of these strains have been described in Chapter 3 (see Materials and Methods and Table 2). Duplicate plates were incubated at 30°C at ambient (~20%) or low (~5%) oxygen. Under the above conditions, *nif* genes such as *nifS* and *nifU* genes are expressed from the *nif*-promoter while repression of the intact, P*scrX* –controlled *iscSUAhscBAfdxiscX* genes results in the depletion of the relevant Isc component (in this case, IscS or IscU).

One explanation for the rescue of the null growth phenotype of IscU-depleted cells only under nitrogen-fixing and low-oxygen conditions described above could be that the activities of the Nif-specific [Fe-S] cluster assembly components are hypersensitive to oxygen. This explanation is probably not correct because NifU and NifS are fully capable of supplying [Fe-S] clusters for nitrogenase maturation when cultured under ambient oxygen concentrations (~20%) or even when cultured at elevated (40%) oxygen concentrations. An alternative explanation is that functional cross-talk between the Nif and Isc [Fe-S] cluster biosynthetic systems is relatively ineffective. Namely, the Nif-specific [Fe-S] cluster biosynthetic system is so ineffective at replacing Isc function that maturation of [Fe-S] proteins does not occur at a sufficient level to maintain cellular metabolism when growing under ambient oxygen concentrations. In contrast, under low oxygen conditions, where the demand for formation and maturation of oxygen labile [Fe-S] proteins, such as aconitase, is expected to be lower, Nif-directed replacement of Isc-dependent [Fe-S] protein maturation is apparently above the threshold required to sustain

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growth. This model implies inherent target specificity with respect to Nif- versus Isc-directed [Fe-S] protein maturation.

If the hypothesis that the Nif-system is simply ineffective with respect to replacing Isc function is correct, we expected that such inefficiency might be compensated by high-level expression of the Nif-specific components. In order to test this possibility, expression of the *nifUS* genes was decoupled from the *nif* regulatory elements and placed under the strong *E. coli ara* control elements (pDB1551; Figure 3), which were incorporated in the *A. vinelandii* genome within the *scr* region via homologous recombination (strain DJ1626; Table 1; Figure 5A). Figure 5B shows that addition of arabinose (which cannot be metabolized by *A. vinelandii*) to the growth medium results in a very high level of accumulation of NifU and NifS. The accumulation of NifU or NifS cannot be detected by SDS-PAGE when wild type cells are cultured under normal nitrogen-fixing conditions (data not shown). The high-level, arabinose-induced expression of NifU and NifS by strain DJ1626 is also evidenced by the observation that, several hours after arabinose induction, cell pellets acquire a black color, probably as a consequence of the accumulation of insoluble iron-sulfides (Figure 5C). The elevated production of [Fe-S] clusters as a result of *ara*-regulated *nifUS* hyperexpression did not affect the capacity of strain DJ1626 for normal growth (Figure 6A). This result is in contrast to the deleterious effect that accompanies a much more modest elevation in expression of the Isc components recognized for strain DJ1601 (Figure 10 in Chapter 3). Although it remains unclear why elevated expression of intact Isc components is deleterious, the lack of a similar effect upon hyperexpression of *nifUS* suggests the effect cannot be explained by uncontrolled release of iron-sulfides.
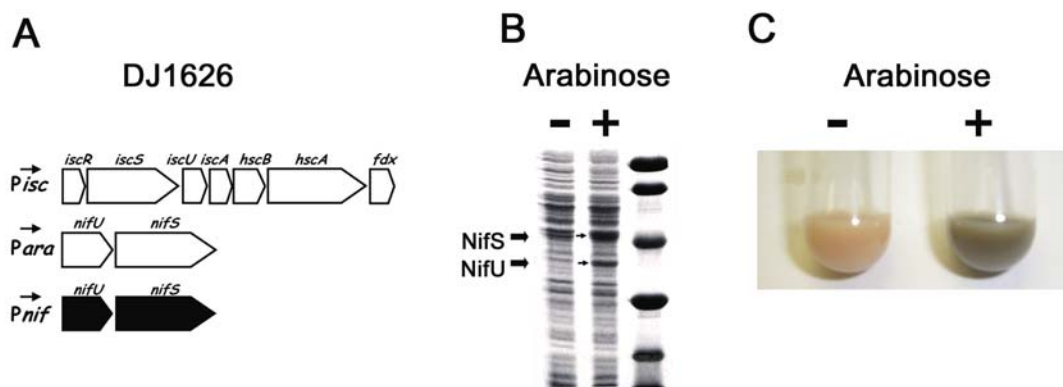
Figure 5. Arabinose-dependent and abundant expression of NifU and NifS in *A. vinelandii* strain DJ1626. (A) Organization of the *isc* and *nifUS* genes in DJ1626. Deleted genes are shaded in black. The promoters P*isc* and P*nif* control expression of the normal *isc* and *nif* gene clusters. P*ara* is the *ara*-inducible promoter located in the *scr* region of the *A. vinelandii* genome. (B) SDS polyacrylamide gel electrophoretic analysis of DJ1626 crude extracts form cells induced with L-arabinose (+) compared to cells grown in the absence of L-arabinose (-). Proteins were separated by 15% SDS-PAGE and stained with Coomasie brilliant blue. Migration positions of NifS (~45 kDa) and NifU (~33 kDa) are indicated by arrows. The right lane shows M_r standards (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme). (C) Cell pellets of DJ1626 induced with L-arabinose (+) and grown in the absence of L-arabinose (-).

Our ability to delete the essential *iscU* gene in DJ1626 and obtain a stable arabinose-dependent strain (DJ1639) confirmed that elevated expression of NifU/NifS could fully compensate for loss of IscU. This data is presented in Figures 6A and 6B in which the growth of DJ1626 and DJ1639 in the presence and absence of arabinose are compared. Figure 6B shows that a strain deleted for *iscU* can grow under ambient oxygen concentrations provided there is a high level of *nifUS* expression driven by the *ara* control elements. Because no other *nif*-regulated components are expressed under the conditions used for these experiments (expression of nitrogen fixation components is repressed) it is clear that NifU and NifS are the only *nif*-specific components required to replace Isc function when expressed at high levels. Attempts to delete the *iscS* gene in DJ1626 were not successful. This result is consistent with our previous conclusion that

NifS cannot replace the S trafficking functions of IscS that are separate from its role in mobilizing S for [Fe-S] cluster formation. The apparent accumulation of iron-sulfides upon hyperexpression of *nifUS* in DJ1626, may offer a possible explanation for how the Isc machinery can be functionally circumvented. Namely, direct transfer of clusters assembled on the NifU scaffold to various target proteins normally serviced by the Isc components might not occur in this case. Rather, it could be that free iron-sulfides released from the NifU scaffold – or intact [Fe-S] clusters released from NifU and subsequently coordinated by small thiol-containing entities (thioredoxins, for example) - are used for the quasi spontaneous maturation of [Fe-S] proteins.

Figure 6. Arabinose-induced, elevated expression of NifU and NifS provides permissive growth conditions for a strain with an in-frame deletion in *iscU*. (A) *Top*, Organization of the *isc* and *nif* genes in DJ1626. Deleted genes are shaded in black. Promoter designations have been described in the legend for figure 5. ***Bottom***, growth of DJ1626 in flasks containing 500 ml Burks minimal media supplemented with 13 mM ammonium acetate with (▲) or without arabinose (●). (B) *Top*, Organization of the *isc* and *nif* genes in DJ1639. ***Bottom***, growth of DJ1639 with (▲) or without arabinose (●).

The observation that hyper-expressed NifU cannot replace IscU function at elevated (~40%) oxygen concentrations, as shown in Figure 7B lends further support to the notion that NifU lacks the target specificity required to effectively replace IscU functions. This lack of cross talk under oxidative stress conditions is unlikely to be due to NifUS hypersensitivity to oxygen since *ara*-expressed NifUS is capable of nitrogen fixation functions under these same conditions (DJ1626, Figure 7B). Reduced levels of NifUS due to sequestration of the *ara*-expressed NifUS components into a Nif protein complex is also excluded since growth of DJ1639 under non-diazotrophic conditions at ~40% oxygen also does not permit *ΔiscU* rescue (data not shown). A number of explanations can be put forward to interpret why NifU cannot replace IscU at 40% oxygen: (i) at high oxygen levels, the demand for [Fe-S] clusters may increase above a threshold level which can no longer be accommodated by NifU; (ii) accessory proteins such as IscA and HscBA, which may be required for the protection and/or repair of housekeeping [Fe-S] protein maturation during conditions of oxidative stress (Chapter 3), could be highly specific for IscU and unable to interact with the much larger NifU protein and; (iii) if free iron-sulfide release from hyper-expressed NifU in DJ1639 allows quasi spontaneous maturation of [Fe-S] proteins, then the complete functional replacement of the IscU scaffold in DJ1626 is partly the result of the by-products released from an 'over-active' NifU scaffold. At these high oxygen levels, iron released from NifU clusters may be predominantly in an oxidized form that is unsuitable for spontaneous [Fe-S] cluster assembly. None of these possible explanations are mutually exclusive and further experimentation is required to identify which explanation, if any, is most likely to be the correct one. Taken together, however, this study continues to indicate that the NifU scaffold in *A. vinelandii* has evolved to perform a specialized nitrogen-fixation function with such superb target specificity that it is simply ineffective at performing more generalized [Fe-S] maturation functions.

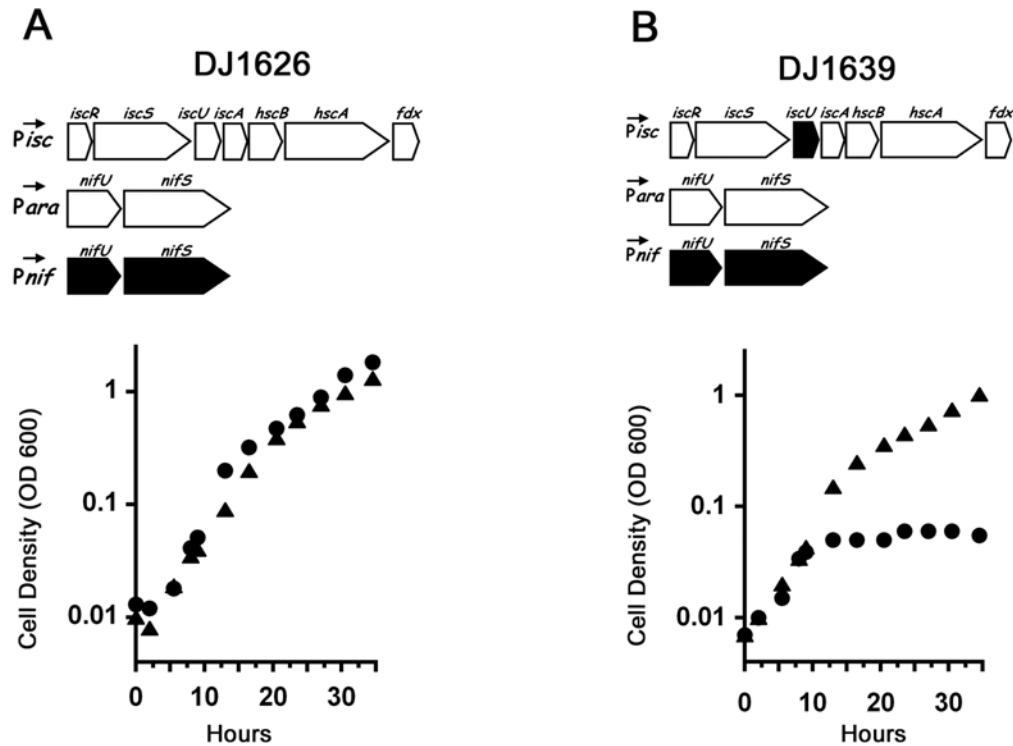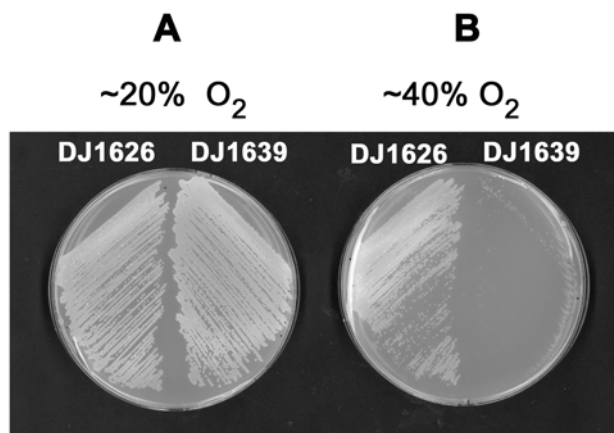**Figure 7**. Elevated expression of NifU and NifS does not provide permissive growth conditions for a strain with an in-frame deletion in *iscU* under oxidative stress conditions. Strains DJ1626 and DJ1639 were cultured in nitrogen–free Burk's minimal media supplemented with 20 mM L-arabinose under (A) ambient (~20%) oxygen or (B) 40% oxygen.

Replacement of Nif-specific [Fe-S] protein maturation function by the Isc components. As previously mentioned, a search for, and the subsequent discovery of the Isc system was prompted by the hypothesis that Isc components might supplant the function of NifUS, although at a very low level (Zheng et al. 1998). This hypothesis was based on the observation that *nifU* or *nifS* deletion strains remained capable of very slow growth when cultured under conditions that require nitrogen fixation. It is not possible to directly test this hypothesis by examining the effect of combined inactivation of both the Isc and Nif components because the Isc components have an essential function. However, the elevated level of expression of Isc components when IscR is inactivated (chapter 3) permitted an evaluation of the potential functional replacement of Nif-specific [Fe-S] protein maturation by Isc components in a different way. Namely, we reasoned that if the capacity for very slow growth observed for a *nifU* deletion mutant under conditions that require nitrogen fixation is supplied by the Isc components, then elevated expression of the Isc components as a consequence of IscR inactivation should increase the growth rate of a *nifU* deletion mutant under these conditions. Data shown in Figure 8 confirmed this possibility and show that a strain deleted for *nifU* and *iscR* (DJ1620)

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grows better under nitrogen-fixing conditions than a strain deleted for only *nifU* (DJ1413). This effect is further enhanced when cells deleted for *nifU* or both *nifU* and *iscR* are cultured under nitrogen-fixing conditions and low (5%) oxygen. These results can be explained in the same way as previously suggested for enhanced replacement of Isc-directed [Fe-S] protein maturation by Nif-specific components. Namely, when cultured under lower levels of oxygen there could be a lower demand for machinery required for maturation of the nitrogenase catalytic components.
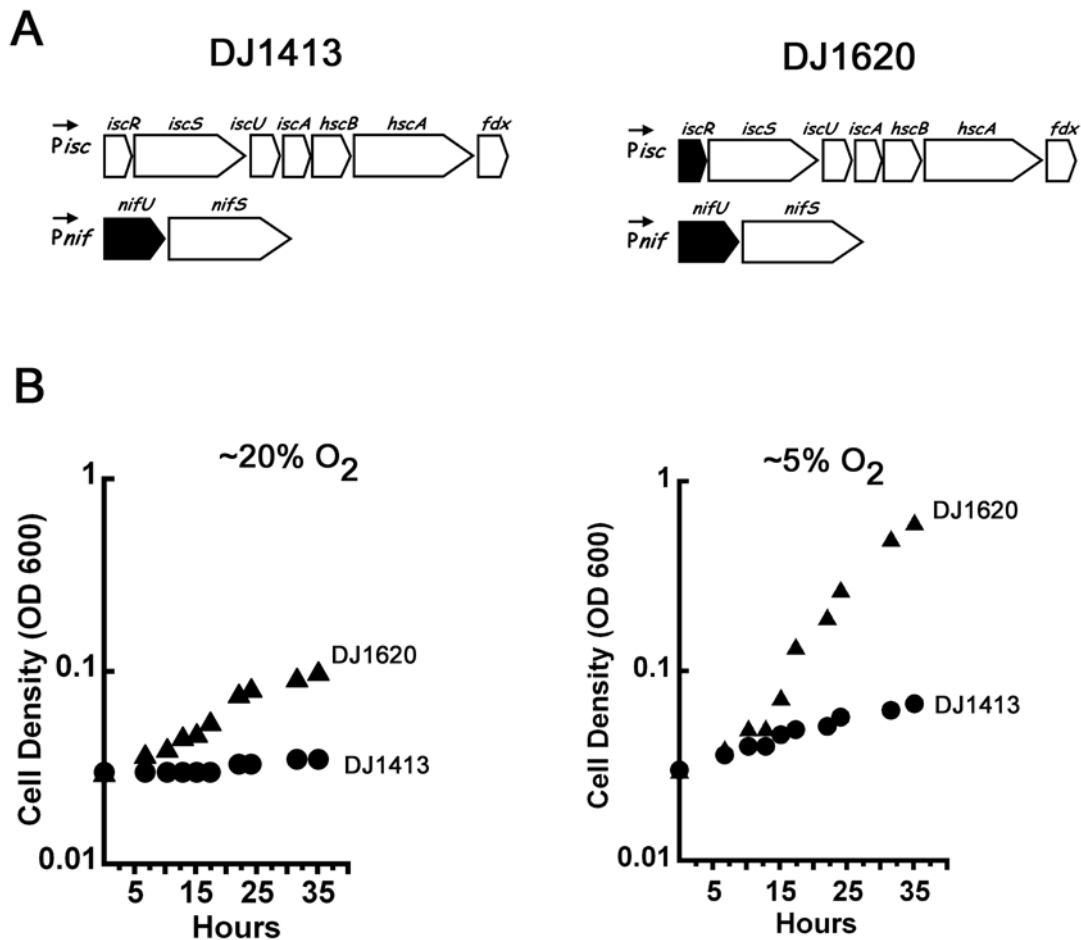
Figure 8. Elevated expression of the Isc system, resulting from inactivation of *iscR*, improves the growth rate of a strain deleted for *nifU*. (A) Organization of the *isc* and *nifUS* genes in DJ1413 compared to DJ1620. (B) Strains DJ1413 (●) and DJ1620 (▲) were grown in nitrogen-free Burks minimal media. Duplicate flasks were incubated in a shaker at 30°C at 300 rpm under ambient oxygen conditions (~20%) or under low (~5%) oxygen conditions.

Placement of the *iscSUAhscBAfdxiscX* genes under the strong *E. coli ara* control elements (pDB1562; Figure 3), and incorporation in the *A. vinelandii* genome within the *scr* region followed by deletion of the *nifU* gene (strain DJ1683, Table 1) also resulted in improved growth in the presence of arabinose (Figure 9). The limited ability of the Isc system to replace the function the Nif system implies that nitrogen fixation involves machinery that interacts poorly with the Isc system. More specifically, it could also suggest that the central and C-terminal domains of NifU (Dos Santos et al. 2004) which are not possessed by IscU are the key components required for target specificity to the *nif*-specific [Fe-S] cluster maturation pathway. The Isc system does not contain proteins homologous to these two domains in NifU. However, since elevated expression of the Isc system in general (either by deleting *iscR* or induction of P*ara- iscSUAhscBAfdx* with arabinose) results in an unexplained slow growth phenotype, more specific conclusions as to why the Isc system is not functionally equivalent to the NifUS system will require further analysis.
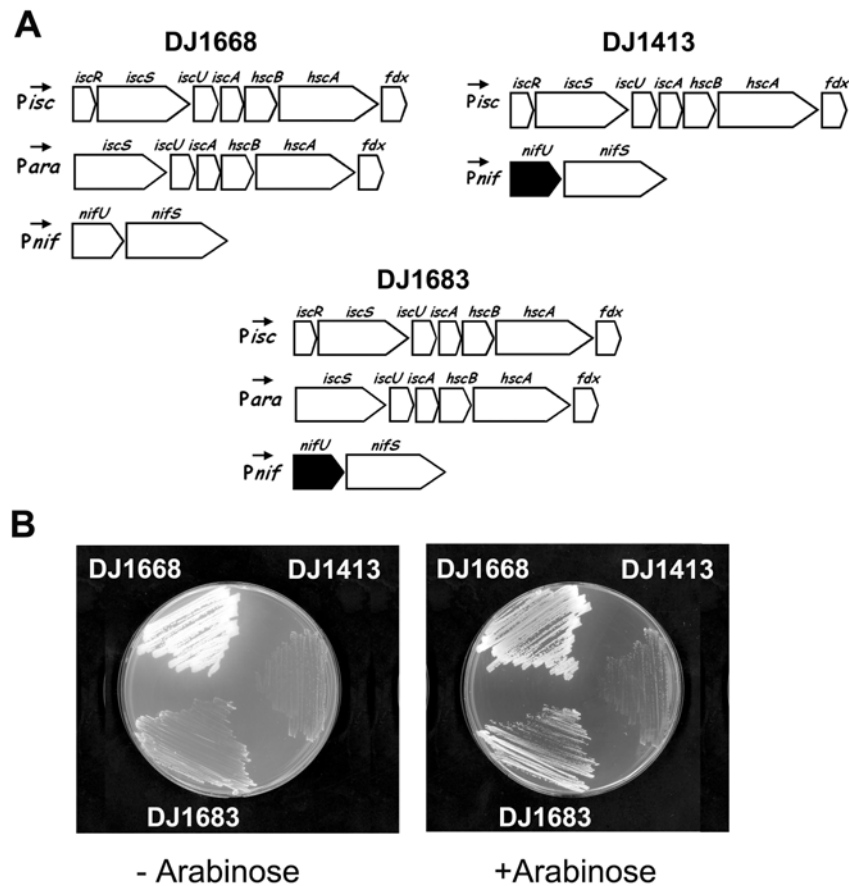
Figure 9. Arabinose-induced, elevated expression of the *isc* operon provides permissive growth conditions for a strain with an in-frame deletion in *nifU*. (A) Organization of *isc* and *nifUS* genes in strains DJ1668 and DJ1683. See figure 6 for DJ1413 gene organization. Deleted genes are shaded in black. Promoter designations have been described in the legend for figure 3. (B) Growth phenotypes of DJ1668, DJ1683 and DJ1413 cultured on Burks minimal media containing ammonium acetate with (left panel) and without (right panel) the addition 20 mM L-arabinose.

In summary, cross-talk studies to date confirm that the Nif –specific and Isc systems have evolved specialized functions and the basis for this target specificity can now be investigated given the powerful genetic system we have established in *A. vinelandii.*

CHAPTER 5

Summary and Future Directions

Proteins bound to iron-sulfur ([Fe-S]) clusters have been found in nearly all living organisms including archaea, bacteria and eukaryotes. The diversity of functions these clusters endow on respective protein partners is testament to the unique redox properties that result from protein bound Fe ions linked to each other through sulfide bridges (Beinert et al. 1997). Since the coordination environment within a protein can dramatically change the properties of a resident [Fe-S] cluster, it is no surprise that new chemical capabilities and biological roles for these co-factors continue to be discovered (Fontecave 2006).

It is well established that the simplest [Fe-S] clusters can spontaneously assemble and attach to proteins in reductive aqueous solutions containing ferrous and sulfide salts (Malkin and Rabinowitz 1966). [Fe-S] clusters were probably among the first biocatalysts at the origin of life, however their sensitivity to oxygen and the toxicity of their components, seems to have forced many aerobic organisms to evolve complex ways to assemble, protect and repair essential, but delicate [Fe-S] clusters (Huber and Wachtershauser 1997; Imlay 2006). The different [Fe-S] cluster biosynthetic machineries (Nif, Isc and Suf) identified to date all share two common features: a cysteine desulfurase (NifS, IscS, SufS), which allows the use of cysteine as a stable source of sulfur atoms and a potential scaffold protein (NifU, IscU, SufA) upon which clusters are thought to assemble prior to transfer to recipient proteins (reviewed in Johnson et al. 2005 and Barras et al. 2005). There is evidence that all three systems are capable of synthesizing [Fe-S] clusters for the assembly of "housekeeping" Fe-S proteins since organisms have been identified which rely exclusively on only one of the three systems (Olson et al. 2000; Ali et al. 2004; Johnson et al. 2005). However, in organisms that contain more then one system, such as *E. coli* (Isc and Suf) and *A. vinelandii* (Isc and Nif), specialized functions seem to have evolved for each system (Johnson et al. 2004; Outten et al. 2004). Our work aims to contribute answers to one of the major questions

that is yet to be elucidated in this field: What are the specific molecular and chemical features of each system that allow adaptation to specific functions or specific growth conditions ? A detailed answer to this 'big picture' question will ultimately result in the identification of common [Fe-S] cluster biosynthetic pathways in cells and involve a combination of different experimental approaches including genetics, physiology, gene regulation, enzymology, biophysics and structural biology.

This study describes the construction and application of a controlled expression system in *A. vinelandii* which has made it possible to perform an *in vivo* functional analysis of the Isc system. By exploiting the essential requirement of the Isc machinery it is now possible to genetically identify functional relationships and possible interactions between Isc components. Development of this system was possible based on the discovery of a sucrose-inducible promoter located within a cluster of four genes encoded within the *A. vinelandii* genome, whose products are proposed to be involved in the acquisition and catabolism of sucrose. These genes and their proposed products include *scrB* (sucrase), *scrP* (porin), *scrY* permease, and *scrX* (α-glucosidase) and appear to be organized into a regulon with independent transcription units that include *scrPB*, *scrY* and *scrX*. In this work, the product of a gene located upstream from *scrB*, designated *scrR*, was shown to act as a negative regulatory protein controlling expression of *scrX* in a sucrose-dependant manner. Because ScrX is not required for sucrose catabolism it can be replaced, via homologous recombination, by other genes of interest, which results in their expression being effectively repressed or induced by using glucose or sucrose as the carbon source. Versatile cloning vectors (pDB1310 and pDB1332) were constructed for the preparation of desired gene fusions. The construction of an *A. vinelandii* strain (DJ1418) containing the *E. coli lacZ* gene under *scrX*-promoter control, provides a simple color screen that can be used to identify appropriate recombinants. One of the advantages of this novel genetic tool in *A. vinelandii* is that the expression of target genes is controlled from a genome-encoded promoter and obviates problems associated with increased gene dosage effects frequently encountered when using multicopy plasmids.

The cloning of a 5.7 kb *A. vinelandii* genomic fragment containing the whole *isc* operon permitted the construction of strains in which the *scrX* gene was specifically replaced by *iscS, iscU, iscA, hscB, hscA, fdx,* and *iscX,* resulting in duplicate genomic copies of these genes, one whose expression is directed by the normal *isc* regulatory elements (P*isc*) and the other whose expression is directed by the *scrX* promoter (P*scrX*). Functional analysis of individual [Fe-S] protein maturation components was achieved by placing a mutation within a particular P*isc*-controlled gene and allowing the cell to remain viable by complementing this loss with induced expression of the intact *isc* gene under P*scrX* control. By removing the inducer from the growth medium, which in this case is sucrose, cells were depleted of the relevant *isc* gene product, and the physiological effects of this depletion were investigated. This experimental strategy was used to confirm that IscS, IscU, HscBA and Fdx are essential for growth in *A. vinelandii* and their depletion results in a deficiency in the maturation of aconitase, an enzyme that requires a [4Fe-4S] cluster for its catalytic activity. The importance of these proteins in *A. vinelandii* was consistent with similar genetic analyses conducted in *E. coli* and yeast (reviewed in Barras et al. 2005).

The major novel findings of this work include the following:

(i)   the discovery of a null phenotype associated with the loss of IscA that cannot be rescued by two other *A. vinelandii* IscA homologs, IscA[nif] and IscA2. This represents the first null phenotype associated with a prokaryotic IscA homolog;

(ii)   the dispensability of the chaperones, HscBA under conditions of low oxygen, providing the first *in vivo* evidence for a possible role of HscBA in [Fe-S] cluster biosynthesis;

(iii)   the identification of conserved amino acid residues in IscU and IscA which, when substituted, exhibit partial or full dominant-negative growth phenotypes, indicating the requirement of these residues in IscU or IscA function and/or interaction with other Isc components.

(iv)   the observation of a slow growth phenotype resulting from the inactivation of IscR and associated with the increased production of

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IscU, HscB and HscA suggesting that IscR may have evolved regulatory functions to prevent, rather then activate increased levels of [Fe-S] clusters in the cell ;

(v)   the discovery that NifU and IscU display partial functional equivalence only if either protein is abundantly expressed or expressed under low oxygen conditions permitting a more fine-tuned analysis of the basis for target specificity between the Isc and Nif systems;

(vi)   the indication of the presence of an endogenous promoter within the *isc* operon that may direct differential gene expression of certain components of the *isc* machinery such as *hscB* and *hscA*.

Together, these findings have opened up a large number of research possibilities in this field. It is not the aim of this section to exhaustively describe all the possible future directions, rather an attempt is made to evaluate the observations that are most interesting to the author and offer specific suggestions for future work.

The over 150 plasmids and strains constructed for the purpose of this study provide a solid resource for future investigations in *A. vinelandii* [Fe-S] cluster biosynthesis. In addition to providing experimental tools for specific genetic and physiological analyses of the Isc or Nif systems, many of these strains can be used as a source from which proteins of interest can be purified and analyzed when expressed in the absence of an essential Isc component or when expressed under conditions of elevated levels of Isc components. It is speculated that the Isc machinery may function as a multi-protein complex (Frazzon et al. 2002). This genetic system has permitted the construction of strains containing *isc* genes fused to polyhistidine tags, facilitating the purification of individual Isc gene products along with potential Isc partners to which they are bound.

The effect of oxygen levels on the requirement or dispensability of Isc components such as IscA and HscBA constitutes one of the most exciting discoveries of this work. Although *in vitro* data has suggested a role of IscA as an alternate scaffold

protein (Krebs et al. 2001; Ollagnier-De Choudens et al. 2003) or as an Fe donor to IscU (Ding et al. 2005; Ding et al. 2005), the lack of supporting *in vivo* evidence has made it difficult to ascertain its role in the biosynthesis of [Fe-S] clusters. The role of HscBA also remains elusive although *in vitro* data showing IscU-dependent ATPase activity (Hoff et al. 2000) and the severe growth defects indicated by yeast and *E. coli* strains lacking these chaperones (Tokumoto and Takahashi 2001; Voisine et al. 2001; Dutkiewicz et al. 2003), has led to the assumption that *in vivo*, IscU relies on the function of HscBA. In this work, data is presented that suggests a role of IscA and HscBA in the protection and / or repair of nascent or assembled [Fe-S] clusters during cluster biosynthesis. This hypothesis seems plausible given the sensitivity of [Fe-S] clusters to oxidative damage and the fact that phylogenetic studies show a correlation between the presence of IscA homologs and a capacity for rigorous aerobic growth (Vilella 2004).   It is possible that these proteins may prevent or repair the formation of unwanted inter-disulfide bonds that may inactivate a significant portion of IscU regularly exposed to oxygen. Alternatively, IscA and/or the HscBA may prevent or repair dead-end forms of IscU that result from the IscS-dependent accumulation of polymeric sulfide species (Agar et al. 2000). Biochemical experiments with recombinantly-produced, purified components can be designed to investigate these possibilities. Another complementary strategy, however, could involve the characterization (via mass spectrometry, for example) of *A. vinelandii* IscU purified from a genetic background depleted for IscA and/ or HscBA.

    The above hypotheses suggest an essential role for IscA during aerobic growth, yet the phenotypes of strains depleted for IscA and HscBA are not the same. Namely, HscBA, but not IscA is essential at ~20% oxygen. Numerous attempts to isolate a strain with a deletion in *iscA* in the absence of a second, intact *isc* operon have failed and this strongly suggests that IscA may be performing essential functions under normal oxygen (20%) levels. The reason that strain DJ1559 (*ΔiscA*) may be capable of growth on glucose media at normal oxygen levels, may be due to a low level of endogenous *iscA* expression (as was observed for *hscBA* in DJ1447) that may occur separately from P*scrX* -directed *iscA* expression within the duplicated *isc* region. The construction of a strain

with a deletion in the P*isc*-controlled operon and the placement of a second copy of *iscA* under P*scrX*-control in the absence of any putative endogenous promoter is underway and will be required to unambiguously resolve this issue.

The existence of a CXXS motif in all IscA homologs, which is also a typical motif of monothiol glutaredoxins, has raised interesting questions about a possible thiol redox function for IscA (Fomenko and Gladyshev 2002). The location of the CXXS motif is well within the putative active site that was structurally determined for *E. coli* SufA, which shows high sequence identifty with IscA (Wada et al. 2005). However, if this cysteine is one of the ligands thought to bind iron or an [Fe-S] cluster, how can it also be involved in performing thiol-redox functions similar to monothiol glutaredoxins ? To attempt to answer this question, it is tempting to speculate that IscA may actually have an inbuilt redox sensing system. Under low oxygen conditions, iron or an [Fe-S] cluster may be stably bound to the protein and inactivate any thiol-redox function. Under conditions of elevated oxygen, however, loss of the bound iron or cluster may activate the thiol-redox function necessary to protect /repair the Isc machinery from rising oxygen levels. The recent novel finding of a novel human glutaredoxin, Grx2, which uses a [Fe-S] cluster as a redox sensor does set precedence for this mode of function (Lillig et al. 2005). The identification of the trans-dominant negative growth effect resulting from substitution of the serine residue within this motif to a cysteine residue, lends further support to this possibility. This strain, like many of the *iscU* mutant strains which displays partial dominant-negative effects, can now be used to search for intragenic or intergenic supressors of this mutation which can shed light on the mechanistic function of IscA or help identify interacting Isc partners.

A possible involvement of a monothiol glutaredoxin in this protection/repair process should not be overlooked. In yeast, a monothiol glutaredoxin known as Grx5 has been found to play an essential role in [Fe-S] cluster biosynthesis and mutant strains lacking *grx5* display sensitivity to oxidative damage, a phenotype which is partially rescued by over-expression of the yeast IscA or HscA homolog (Rodriguez-Manzaneque et al. 2002). Two sequences showing high percentage identity to yeast Grx5 have been

identified in the fully sequenced *A. vinelandii* genome, and future work in elucidating the role of IscA and HscA in protecting and/or repairing [Fe-S] clusters should not exclude a genetic assessment of *A. vinelandii*'s requirement, if any, for each one of these Grx5 homologs.

Of particular interest is the inability of IscA^{Nif} or IscA2 to replace the function of IscA in *A. vinelandii*. If it is assumed that this CXXS motif endows IscA with thiol-based redox properties, then small variations within this motif may dramatically alter this function. Close observation and comparison of the CXXS motif in the three *A. vinelandii iscA* homologs (See Figure 3.6, Chapter 3) indicates a conserved CGXS motif where the X corresponds to a glutamate residue in IscA, a serine residue in IscA2 and a lysine residue in IscA^{Nif}. Recent findings indicate that substitution of the glutamate in IscA to a lysine residue results in a trans-dominant-negative phenotype. This data indicates that the glutamate is essential for IscA function and offers at least one possible explanation why IscA^{Nif} is incapable of replacing IscA function under normal physiological growth conditions. It is possible that sequence variations between IscA homologs determines different functions. The lack of cross-talk between the IscA homolgs in *A. vinelandii* makes this a suitable system to identify growth conditions or specific mutations which can permit overlapping functions.

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APPENDIX I

Evidence of the Accumulation of Apo-Forms of the [2Fe-2S] Enzyme, Benzoate Dioxygenase, in Cells Depleted for IscU.

The development of a method for the conditional expression of the *isc* genes in *A. vinelandii* (described in chapter 2), has allowed us to analyze the physiological consequences of compromising the activity of essential *isc* gene products and to therefore, further our insight into the *in vivo* role of individual Isc biosynthetic components. An added feature of this controlled expression system is that cells depleted for an Isc component are potential reservoirs of apo-forms of [Fe-S] proteins, assuming such apo-forms remain stable in the cell. Purification of such apo-proteins would provide a source of naturally produced and physiologically relevant target proteins for future *in vitro* [Fe-S] cluster reconstitution experiments using purified *A. vinelandii* Isc components or the Isc components from *A. vinelandii* crude extracts. The importance of obtaining naturally produced, apo-forms of [Fe-S] proteins is highlighted by the fact that most published *in vitro* reconstitution studies aimed at elucidating the process of *de novo* assembly of [Fe-S] clusters on target proteins rely on the use of apo-forms of the target protein that have been prepared by the chemical removal (using strong chelating agents) of the specific [Fe-S] cluster prior to reconstitution by purified Isc or Nif components (reviewed in Barras et al. 2005; Johnson et al. 2005). Although such studies are important in establishing the general requirement of the Isc or Nif systems in cluster assembly, it has not been possible to establish with certainty if the observed reconstituted cluster is the product of *de novo* [Fe-S] cluster formation or [Fe-S] cluster repair. *De novo* cluster formation or repair may require different subsets of the Isc machinery because an apo-protein that has lost its resident cluster due to artificial extraction or oxidation for example, may assume a different conformation compared to a newly synthesized apo-protein that has yet to receive a cluster.

In searching for a suitable target protein that could be isolated in its 'newly synthesized' apo-form from cells depleted for a specific Isc component, three criteria had

to be satisfied: (i) the protein had to require a [2Fe-2S] cluster for an enzymatic function that could be measured; (ii) the protein had to be naturally abundant in the cells since no reliable protein over-expression system was currently available in *A. vinelandii*; and (iii) the apo-form of the target protein should remain stable in depleted cells and during purification for use in *in vitro* reconstitution studies. The choice of a [2Fe-2S] protein was aimed at trying to address one of the key questions in our field: does the formation and assembly of both [2Fe-2S] and [4Fe-4S] clusters require all of the Isc components or does each cluster type require a different subset of this machinery *in vivo*. Previous *in vitro* data from our lab has clearly demonstrated the requirement of the cysteine desulfurase/scaffold pair (e.g. NifS/NifU and IscS/IscU) in the *in vitro* reconstitution of the [4Fe-4S] clusters required by respective apo- target enzymes (Dos Santos et al. 2004; Unciuleac 2006). In all of these cases, functional reconstitution was confirmed by reactivation of the cluster-dependent enzymatic activity. It is not certain however, if *in vivo*, other accessory Isc or Nif components are also required during this assembly process. Reconstitution studies directed towards [2Fe-2S] proteins, however, have been restricted to ferredoxins (Ollagnier-de-Choudens et al. 2001; Mansy et al. 2002; Wu et al. 2002; Ollagnier-De Choudens et al. 2003), for which there were no activity assays available to confirm if the reconstituted cluster resulted in a functional holo-ferredoxin. One of our aims was therefore to select a [2Fe-2S] enzyme that would allow us to start establishing suitable reconstitution protocols with a view of eventually conducting comparative analysis of the similarities and differences in the assembly requirements between [2Fe-2S] and [4Fe-4S] clusters.

Benzoate-1,2-dioxygenase, a [2Fe-2S] enzyme involved in aerobic benzoate catabolism seemed to be a suitable candidate for these studies since *A. vinelandii* is capable of growth on media containing benzoate as the sole carbon source. Benzoate-1,2-dioxygenase catalyzes the first step in the degradation of benzoate by utilizing NADH to reductively activate and cleave O_2 for incorporation into the benzoate aromatic ring to form a nonaromatic cis-diol 3,5-cyclohexadiene-1,2, diol-1-carboxylic acid (benzoate diol, Figure 1;(Gibson and Parales 2000). Benzoate diol is subsequently rearomatized by benzoate diol dehydrogenase (XylL), with the loss of carbon dioxide, to

form catechol, which is further metabolized to tricarboxylic acid cycle intermediates by enzymes of the β-ketoadipate pathway. The benzoate dioxygenase system consists of an oxygenase component (XylXY), which is responsible for the oxygenation of benzoate, and a reductase component (XylZ), which oxidizes NADH and transfers the resulting electrons to the oxygenase component (Wolfe et al. 2002). The reductase is a flavoprotein containing FAD as a cofactor as well as a [2Fe-2S] cluster (Yamaguchi and Fujisawa 1978). The oxygenase component has a $\alpha_3\beta_3$ subunit structure which includes a [2Fe-2S] Rieske cluster and a mononuclear iron site (Wolfe et al. 2002). This enzyme system has been well characterized in several gram negative bacteria including *Pseudomonas putida* (Jeffrey et al. 1992; Wolfe et al. 2002) and *Pseudomonas arvilla* (Yamaguchi and Fujisawa 1978; Yamaguchi and Fujisawa 1980)
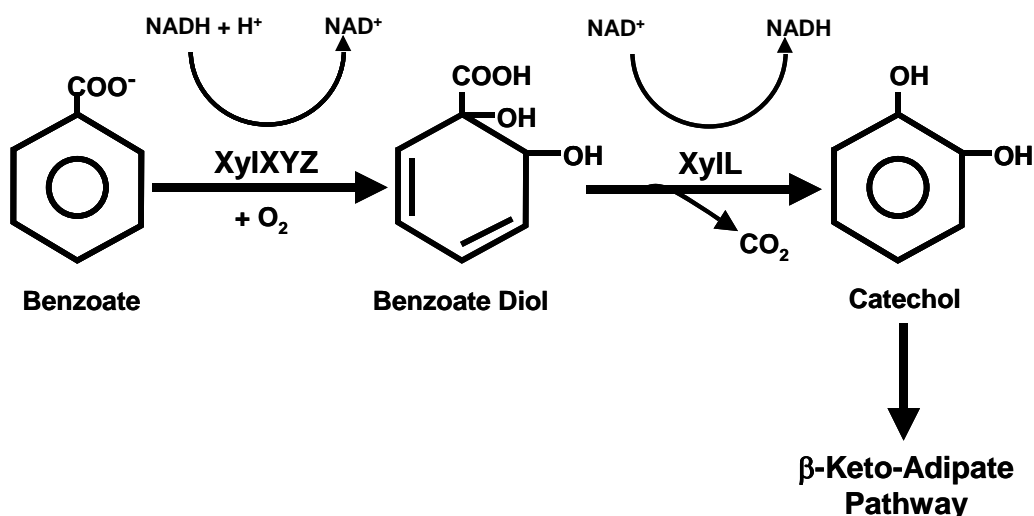
Figure 1: The complete degradation of benzoate by aerobic bacteria is initiated by the conversion of benzoate to benzoate diol by a multicomponent benzoate 1,2-dioxygenase (XylXYZ).

The *A. vinelandii* genome contains gene regions that show high sequence homology with the benzoate dioxygenase genes of *P. putida*. We have annotated these gene regions which are now referred to as *xylX, xylY,* and *xylZ* (Figure 2). The products of these genes have sizes similar to the benzoate dioxygenase subunits of *P. putida:* ~52

kDa and ~19 kDa for the α (XylX) and β (XylY) subunits of the dioxygenase component and ~ 36 kDa for the reductase component (XylZ).   Preliminary genetic studies indicated that these genes were essential for benzoate catabolism in *A. vinelandii* because strains containing null mutations in *xylX* or *xylZ* were incapable of growth on media containing benzoate as the sole carbon source (Ina Puleri, unpublished results).
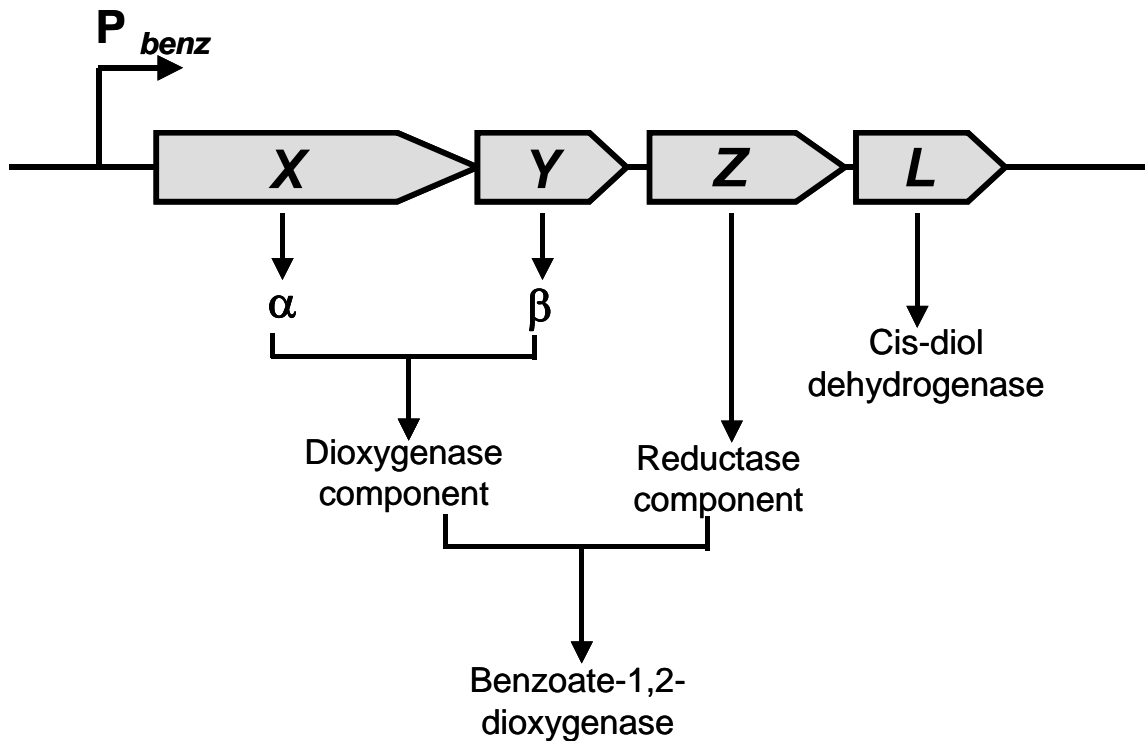
Figure 2: Transcriptional organization of the *xyl* genes in *Azotobacter vinelandii* whose products catalyze the initial reactions of the benzoate catabolic pathway.

The data presented below provides the first evidence that an apo-form of the dioxygenase component of the benzoate-1,2-dioxygenase system accumulates in cells depleted for IscU. Crude extracts of *A. vinelandii* cells grown in the presence of sodium benzoate and passed through a Q-Sepharose anion exchange column produce an elution profile at 405 nm that indicates a dramatic overall increase in the expression of [Fe-S] proteins and other 405 nm-absorbing chromophores during benzoate metabolism. This increased absorbance can be partly attributed to the abundant expression of benzoate metabolic enzymes, including benzoate-1,2-dioxygenase, as shown by the SDS-PAGE gel in Figure 3 in which two distinct protein bands, with molecular weight sizes

corresponding to the expected sizes of XylX and XylY, are the main constituents of an elution fraction corresponding to one of the major peaks of the profile.
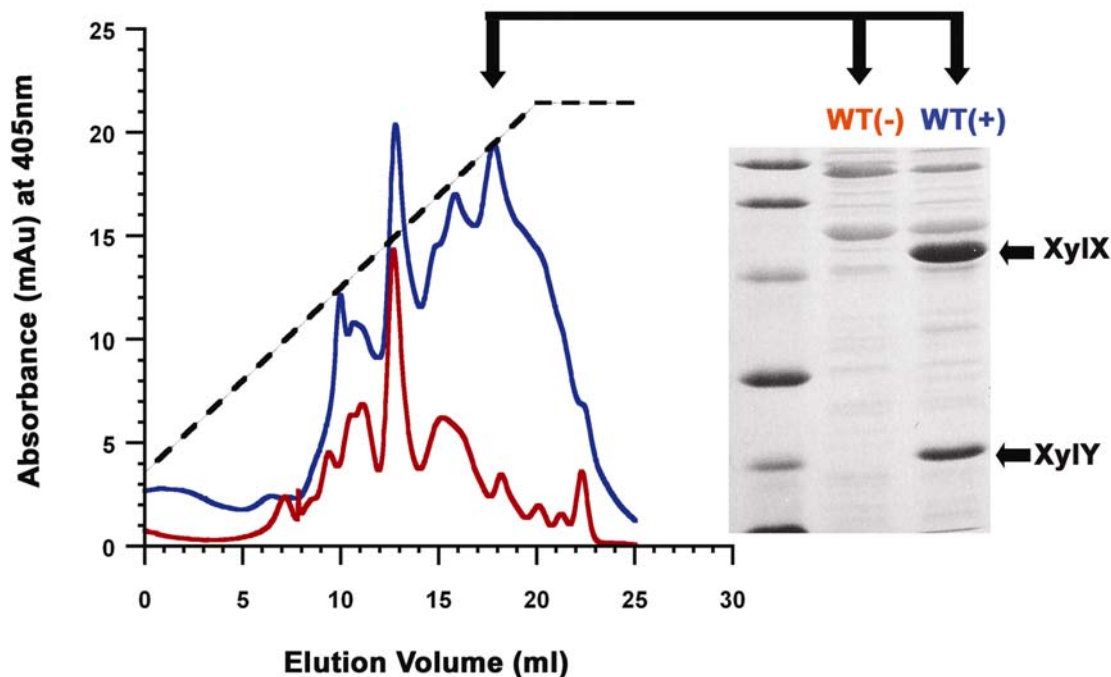
Figure 3: Q-sepharose elution profiles of the crude extracts of WT cells grown in the presence (+benz; blue) and absence (-benz; red) of 25 mM sodium benzoate. Crude extracts were prepared by 1 passage through a French Cell Press (12,000 psi) and centrifugation at 100,000xg for 30 min. A total of 5 mg of protein for each crude extract was separately applied to a 1ml Hi-Trap Q-Pharmacia® column equilibrated with 50 mM Tris-HCl, pH 7.45. Following a wash with 8 volumes of the same buffer, proteins were eluted in a 20 ml gradient of 0-1 M NaCl. Elution profiles were monitored at 405nm. The bold arrows indicate the 405 nm peak and fraction where benzoate 1,2,-dioxygenase proteins, XylX (51.6 KDa) and XylY (19.5 KDa), co-elute from the column and migration in 15% SDS-PAGE. The left lane shows M_r standards (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme).

Crude extracts of cells grown in benzoate but depleted for IscU, however, do not show this increased absorbance at 405nm suggesting that IscU is required for the maturation of the abundant [Fe-S] proteins required during benzoate metabolism (Figures

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4). SDS-PAGE analysis of the elution fraction containing XylX and XylY clearly shows that despite the loss of the absorbance peak at 405 nm of this fraction, XylX and XylY do not appear to be degraded and are present at concentrations comparable to the wildtype cells. This suggests that the apo-form of benzoate-1,2-dioxygenase is stable and amenable to purification. Verification that the protein bands observed in Figures 3 and 4 actually correspond to XylX (~52 kDa) and XylY (19 kDa) was supported by comparison of the SDS-PAGE migration pattern of affinity purified, his-tagged XylXY from wildtype *A. vinelandii* cells (data not shown; Ina Puleri, unpublished results). The UV-Visible spectra of these purified samples were characteristic of a protein containing a [2Fe-2S] Rieske cluster (data not shown). Presumably, it is this cluster that fails to get assembled in XylX when expressed under conditions in which IscU is depleted resulting in the accumulation of apo-XylX. This evidence is also a clear indication that IscU is not only involved in maturing [4Fe-4S] enzymes such as aconitase but also in the maturation of [2Fe-2S] enzymes like benzoate-1,2-dioxygenase.
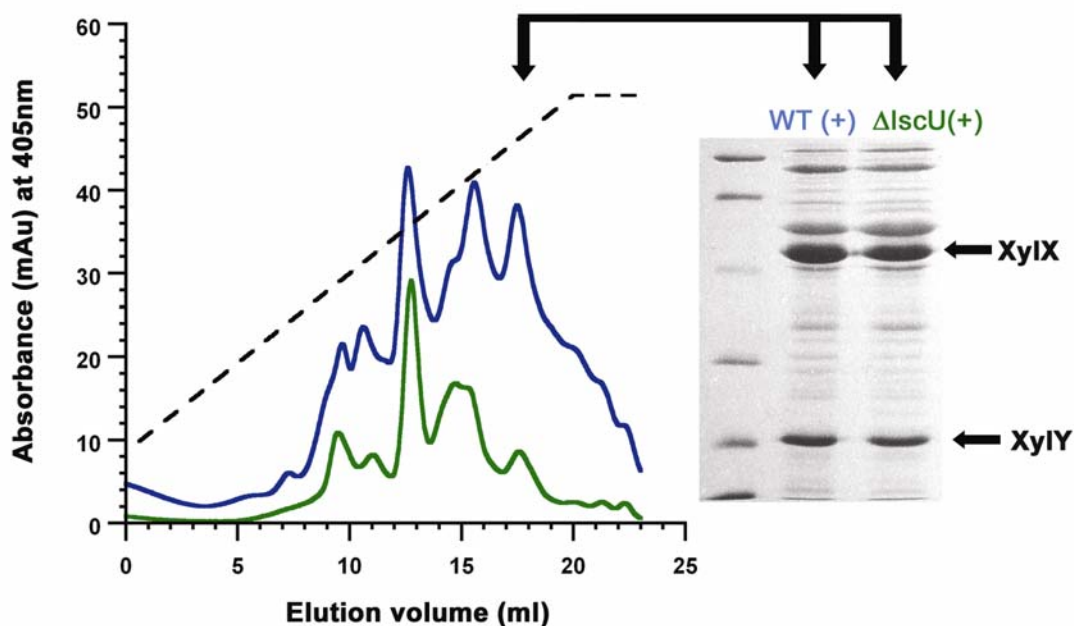
Figure 4: Q-sepharose elution profiles of the crude extracts of WT cells (blue profile) compared to cells depleted for IscU (Δ*iscU;* green profile) in glucose media supplemented with 25mM sodium benzoate (+ benz). A total of 8 mg of protein for each crude extract was applied to the column. The bold arrows indicate the 405 nm peak and fraction corresponding to the elution of benzoate 1,2,-dioxygenase proteins (XylX, XylY) and migration in 15% SDS PAGE. The left lane shows M_r standards (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme)

Purification of this presumed apo-form of XylXY was stalled by two unresolved technical problems: (i) the lack of a suitable enzymatic assay to test the functionality of benzoate-1,2-dioxygenase; (ii) difficulties in concentrating purified forms of holoXylX and XylY for further use in an assay and / or reconstitution studies. Benzoate-1,2-dioxygenase activity is usually measured by monitoring the oxidation of NADH or the consumption of O_2 in the presence of excess levels of the reductase, XylZ (Wolfe et al. 2002). Despite numerous attempts to adapt the various published assays for this system, we were not able to measure activity using purified XylX (with or without a His tag), XylY and XylZ. No activity could be measured directly from the crude extracts of cells grown in benzoate due to high levels of background NADH oxidation. The low

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concentrations of purified components available for these assays may be responsible for the lack of activity. Assuming these hurdles can be overcome, future work will involve testing the effects of depleting different Isc components on benzoate dioxygenase activities and subsequently establishing a suitable [2Fe-2S] cluster *in vitro* enzyme activation system using naturally produced apo-XylXY from *A. vinelandii*.

APPENDIX II

Plasmids constructed during this project[*]

pDB1264   ***isc* operon (P*isc-iscR,iscS,iscU,iscA,hscB,hscA,fdx,iscX*)**. 18 kb BglII
fragment from DJ1179, containing the whole _isc_ operon and upstream region,
ligated into pUC119 BamHI. The ligation reaction consisted of BglII-
digested DJ1179 chromosomal DNA mixed with BamHI-digested pUC119.
The kan^R cartridge located in KpnI site of *cysE2* permitted selection on LB
plates containing ampicillin and kanamycin. Plasmid purified in ultra-
competent *E.coli* strain XL10 (Stratagene). *E.coli* cells harboring this
plasmid grow more slowly. Amp^R and Kn^R

pDB1265   ***isc* operon (P*isc-iscR,iscS,iscU,iscA,hscB,hscA,fdx,iscX*)**. Same as
pDB1264, but DNA purified in *E.coli* strain TB1. Amp^R and Kn^R.

pDB1266   ***isc* operon and *cysE2*::*kan***. 8.5 kb ApaLI fragment from pDB1264, blunt-
ended with T4 polymerase and ligated into pUC119 SmaI-site. Contains _isc_
operon and *cysE2::kan*. *E.coli* cells harboring this plasmid grow more
slowly. Plasmid purified in *E.coli* strain XL10. Amp^R and Kn^R.

pDB1267   ***isc* operon and *cysE2*::*kan*** Same as pDB1266, but DNA purified in *E.coli*
strain TB1. *E.coli* cells harboring this plasmid grow more slowly. Amp^R and
Kan^R.

pDB1271   ***hemK***. 1.7 kb PCR-derived *A. vinelandii* genomic fragment containing *hemK*
gene homolog ligated into pUC7 EcoRI-site. Contains about 300bp upstream
and downstream of *hemK* gene. Primers used were designed with EcoRI
sites. Sequences are as follows: HemK5'(forward primer) –

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[*] Plasmids constructed by undergraduate research assistant, Milagros Perez.

CATGGAATTCTCCGAGACGCGCAGGCTGCTG ($T_M = 68°C$) and

HemK3'(reverse primer)

GATCGAATTCCGAGAGTTGGCCTTCGAGGCG-3'($T_M = 67°C$) .

TRANS *A. vinelandii* DNA used with Failsafe™PCR Pre-mix kit (Epicentre)

using 2-step cycle with annealing/extension temp. = 65°C and buffers G or H.

Amp^R.

pDB1275   **Δ*hemK*** . In-frame deletion in *hemK* gene. 432 bp KpnI-NruI deletion in pDB1271 ligated with oligonucleotide cartridge containing BamHI site. Sequence of cartridge: 5' CGGA TCCCG . Amp^R.

pDB1276   ***hemK::kan*.**1.3 kb HincII pUC4-KAPA cartridge ligated into blunt-ended KpnI-NruI deletion in hemK. Same deletion as in pDB1275. Kan^R cartridge in opposite direction to *hemK*. Amp^R, Kan^R.

pDB1277   ***hemK::kan***   Same as pDB1276, but Kan^R cartridge in same orientation as *hemK*. Amp^R, Kan^R.

pDB1278   **P*isc-isc* operon** 1.5 kb NruI deletion in pDB1267, removing most of *cysE2* and its kanamycin cartridge. *isc* promoter and operon are intact. Amp^R.

pDB1279   **P*isc-isc* operon** 36 bp cartridge ligated into NruI site of pDB1278 creating the following restriction sites: SalI-BglII-XbaI-NdeI-BamHI-SstI . Not sure of orientation of cartridge. Amp^R.

pDB1281   **P*isc-isc* operon** 6.1 kb XbaI fragment from pDB1279 inserted into XbaI site in pDB1201. Places *isc* operon in a broad host range plasmid. Kn^R.

pDB1282   ***isc* operon (*iscS* – *orf3*).** 5.2 kb BspHI fragment from pDB1267 ligated into NcoI site of pDB1280 (a derivative of pAra13). Puts under arabinose control. Amp^R.

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pDB1285   **P*isc*-*isc* operon**. 6.1kb XbaI fragment from pDB1279 inserted into XbaI site in pUC119. Amp^R.

pDB1307   **P*scrX*-*scrX* gene** 3.28 kb PCR-derived *A. vinelandii* genomic fragment containing scrX gene and upstream promoter ligated into PstI site of pDB1308 (pUC7 with ΔPciI). Primers used were designed with PstI sites. Sequences are as follows: Gluc-5'(forward primer) – TAG CCA TGC ACA TTG C (T_M = 43°C) and Gluc-3' (reverse primer) TGC TGG CGA TCA TCT G (T_M = 46°C) . TRANS *A. vinelandii* DNA used with Failsafe™PCR Pre-mix kit (Epicentre) using 3-step cycle with annealing temp. = 45°C and buffers D or E. Amp^R.

pDB1308   PciI deletion in pUC7. Site filled in with T_4 DNA polymerase.Amp^R.

pDB1309   **P*scrX*-*scrX*::*kan*.** HincII fragment from pUC4-KAPA inserted into EcoRV site of pDB1307 to interrupt scrX gene Amp^R, Kn^R.

pDB1310   **Cloning vector for P*scrX* gene fusions**. 45 bp cartridge with PciI 5'overhang and KpnI 3' overhang, ligated into ΔPciI-KpnI sites of pDB1307. Linker introduces the folowing unique restriction sites: PciI, XbaI, XhoI, KpnI. Cartridge sequence created by annealing oligonucleotides deb-1 CATG TCT AGA AAG CTT GTT AAC CCG GGC TCG AGG CAT ATG GGT AC and deb-2: AGA TCT TTC GAA CAA TTG GGC CCG AGC TCC GTA TAC C. Amp^R.

pDB1316   **P*scrX*-*iscS,iscU,iscA,hscB,hscA,fdx,iscX*.** 5.2 kb BspHI fragment from pDB1279 (containing *isc* operon) ligated into PciI site of pDB1310. Orientation confirmed so that *isc* operon is under control of *scrX* promoter from *A.vinelandi*. Amp ^{R}

pDB1321 **P*isc*-*isc* operon** 7.5 kb NruI fragment from pDB1264 containing isc operon ligated into SmaI site of pDB279 (pUC19 with ΔEcoRI). Plasmid purified from JM109. Amp^R.

pDB1322 **P*isc*-*isc* operon** Same as pDB1321, except NruI fragment cloned in oposite direction.

pDB1328 ***isc::kan.*** 1.2kb EcoRI fragment from pUC4-KAPA ligated into 5.1 kb ΔEcoRI pDB1321. KAPA fragment replaces the isc operon. Orientation of KAPA fragment opposite to orientation of <u>isc</u> promoter. Amp^R, Kan^R.

pDB1329 ***isc::kan.*** Same as pDB1328, but KAPA fragment in opposite orientation.

pDB1332 **Cloning vector for P*scrX* gene fusions:** 35 bp linker (oligos: deb-3, deb-4) with PciI 5' overhang and KpnI 3' overhang, ligated into ΔPciI-KpnI sites of pDB1307. Linker introduces the following unique restriction sites: PciI, NruI, EcoRV, XhoI, BglII, XbaI, SphI, KpnI. Cartridge sequence created by annealing oligonucleotides deb-3, C ATG TCG CGA TAT CTC GAG ATC TCT AGA GCA TGC GGT AC and deb-4, AGC GCT ATA GAG CTC TAG AGA TCT CGT ACG C. Amp^R.

pDB1335 **P*scrX*-*lacZY::kan.*** 6.1 kb SmaI fragment from pLKC480 ligated into EcoRV site of pDB1332. Places <u>lac**Z**</u>, <u>lac**Y**</u>::Kan^R under control of putative scrX promoter Amp^R, Kan^R.

pDB1357 ***nasA*-*nasB* gene fragments** 1.8 bp BclI-BamHI fragment, from a 2.3 kb PCR fragment of *A.v.*, ligated into BamHI site of pUC19. Includes last 331 bp of *nasA* (large fragment), all of *nas A* (small fragment), first 935 bp of *nasB* gene. Primers: NasB-5' (forward primer) – CGG GCA GCA ACA GGT CGG CG ($T_M = 62°C$) and NasB-3' (reverse primer) CAT GTG GTC GCC GCA CAA GG ($T_M = 58°C$) . TRANS *A. vinelandii* DNA used with

Failsafe™PCR Pre-mix kit (Epicentre) using 3-step cycle with annealing temp. = 62°C and buffers D,E or F. Amp^R.

pDB1362   ***nasB::kan***. 1.3kb SmaI fragment from pUC4-KIXX ligated into blunt-ended NcoI site of pDB1357. Should interrupt reading frame of *nasB* gene. Amp^R, Kan^R.

pDB1363   ***nasB::gn*** Blunt-ended BamHI fragment from pWKR202I ligated into blunt-ended NcoI site of pDB1357. Should interrupt reading frame of *nasB* gene Plasmid purified from JM109. Amp^R, Gn^R.

pDB1364   ***his-nasB* gene fragment.** Insertion of polyHis tag cartridge into NcoI site of *nasB* gene fragment in pDB1357. Places seven histidines after initial methionine. Amp^R. Cartridge: CATG CAT CAC CAC CAT CAC CAC CA.

pDB1370   ***isc::gn.***   Blunt-ended 2.6 kb PstI Gn^R cartridge from pWKR202I ligated into blunt-ended ΔSfiI-EcoRV in pDB1321. The Gn^R cartridge replaces majority of the *isc* operon from *iscS* (aa49) to *fdx* (aa36). Amp^R, Gn^R.

pDB1371*   **Congression vector: Δ*orf* (*nif*)4-11::gn.** Blunt-ended 2.6kb PstI Gn^R from pWKR202I ligated into blunt-ended ΔSmaI of pDB137. Constructed for use as a congression vector for transformations with *A. vinelandii*. Complementary congression vectors include pDB137 (Kn^R) and pDB1371 (Gn^R) . Amp^R Gn^R.

pDB1383*   **Congression vector: Δorf (*nif*)4-11::str.** 2 kb SmaI fragment from pHP45Ω ligated into ΔSmaI in pDB137. Constructed for use as a congression vector for transformations with *A. vinelandii*. Complementary congression vectors include pDB137 (Kn^R) and pDB1371 (Gn^R) . Amp^R, Str^R

pDB1400   _scrR::gn._   2.7 kb BamHI fragment from pWKR202I, blunt-ended with T4 DNA polymerase, ligated into StuI site of pDB1385. Interrupts scr**R** gene with gentamycin cartridge. Amp^R, Gn^R.

pDB1403*   _nasB_ **gene (whole)**. 2.7 kb _nasB_ gene with PCR-engineered NdeI and BamHI sites, ligated into NdeI-BamHI digested pT$_{7-7}$ vector Places nasB under control of T7 promoter.   Primers used: nasB-5'(full) CCGTCATATGGCCCACCAGACCACC (T$_M$ = 55°C) and nasB-3' (full)GATCGGATCCCGGATTCAGCTATTCGC (T$_M$ = 54°C) . TRANS _A. vinelandii_ DNA used with Failsafe™PCR Pre-mix kit (Epicentre) using 3-step cycle with annealing temp. = 50°C and buffers D or E. NasB over-expresses well in _E. coli_ but does not remain soluble following cell breakage via sonication or osmotic shock. Amp^R.

pDB1419*   _his-nasB_ **gene**. Poly-histidine tag inserted into NdeI site of pDB1403. Puts his tag at N-terminus of _nasB_ gene. 7 histidines placed just after intiation methionine: TATG CAT CAC CAC CAT CAC CAC CA. Amp^R.

pDB1468   **φhscA'-lacZY::Kn^R.** 6.1 kb SmaI lac**ZY**::Kan^R fragment from pLKC482 ligated into AleI site of pDB1303 (hscA gene under pT7 control). _lacZ_ cartridge is in frame with _hscA_. Amp^R, Kan^R

pDB1479   _iscA2_ **gene**. 1.5 kb PCR fragment containing _iscA2_ from _A. vinelandii_ genome ligated into TOPO vector. Contains entire _iscA2_ gene. Primers: IscA2-5' (forward primer) – TAGGATCCGTGGATCTG CAGGCG (T$_M$ = 61°C) and IscA2-3' (reverse primer) TAGGATCCCCCGCAGCAATTGCTCCC (T$_M$ = 61°C) . TRANS _A. vinelandii_ DNA used with Failsafe™PCR Pre-mix kit (Epicentre) using 3-step cycle with annealing temp. = 58°C and buffers E or F. Amp^R.

pDB1494   *iscA2* <u>gene</u>. 1.5kb EcoRI fragment from pDB1479 ligated into EcoRI sites of pDB1006. Puts *iscA2* gene into pUC119 without SalI site. Amp^R.

pDB1499   <u>***iscA2::gn.***</u> 2.6kb SalI Gn^R cartridge inserted into SalI site of pDB1494, interrupting *iscA2* gene. Amp^R, Gn^R.

pDB1532   <u>**P*benz*- *iscU37CA*.**</u> 383 bp NdeI-BamHI fragment from pDB1214 ligated into NdeI-BamHI sites of pDB1497. Puts *iscU37CA* under benzoate promoter control. Amp^R.

pDB1550   <u>**Δ*fdx*.**</u> 96 bp XhoI deletion in pDB945. Creates an in-frame deletion in the *fdx* gene of the *isc* operon. This deletion is flanked by 270 bp downstream (*fdx* and *iscX*) and 670 bp upstream (*fdx* and *hscA*) suitable for recombination in *A. vinelandii* genome. Amp^R.

pDB1564   1.9 kb BspHI-SphI fragment from pAra13 (containing the *araC* gene, *ara*-promoter with multiple cloning site), ligated into PciI-SphI site of pDB1332. This plasmid has the elements required for Ara-dependent gene expression flanked by *scr* gene regions (~500 bp on P*scr* promoter side and 759 bp of *scr* region downstream to *scrX* gene), useful for recombination into *scr* operon of the *A. vinelandii* chromosome. Amp^R.

pDB1565   Deletion of NdeI site in pDB1564 through T4 polymerase fill-in. Amp^R.

pDB1567   1.9 kb BspHI-SphI fragment from pAra13 ligated into NcoI-SphI sites of pDB1565. This plasmid is very similar to pDB1564 except the elements required for Ara-dependent gene expression are flanked by smaller sized *scr* regions (~200 bp on P<u>*scr*</u> promoter side and 759 bp of *scr* region downstream to *scrX* gene). Frequency of double reciprocal recombination into *A. vinelandii* chromosome may be low. See plasmid map for pDB1568. Amp^R.

pDB1568   **Cloning vector for P*ara*-gene fusions.** 42 bp NcoI linker (NcoI-NdeI-EcoRV-XhoI-BamHI-NcoI) inserted into NcoI site of pDB1567. Only NdeI site in this linker is unique. This plasmid allows fusion of NdeI-BamHI gene fragments from pT7-7-derived vectors in our lab into the NdeI-BglII sites of the P*ara* multiple cloning region. Transformation into *A. vinelandii* will result in integration of this fusion into the scr region via homologous recombination permitting Ara-dependent expression of desired proteins. Recombination space is : ~200 bp on P*scr* promoter side and 759 bp of *scr* region downstream to *scrX* gene. Frequency of double reciprocal recombination into *A. vinelandii* chromosome may be low. Oligos used for linker: Nco-pHis'5 CAT GGC CCA TAT GGA TAT CAT GCA TCT CGA GGG ATC CT and Nco-pHis-3' CG GGT ATA CCT ATA GTA CGT AGA GCT CCC TAG GAG TAC. Plasmid has been confirmed by sequencing using P*scr*-5' primer: GTC TGG CAC GGT TGC CCT ATG (T_M=58°C). Plasmid map available. Linker is located in the NcoI site of the Para multiple cloning site in following orientation: GCC ATG GCC CAT ATG GAT ATC ATG CAT CTC GAG GGA TCC T , where ATG = start codon from MCS of P*ara*13; ATG = start codon corresponding to fused NdeI fragments. Amp^R.

pDB1572   1.9 kb blunt-ended HindIII-SphI fragment from pDB1568 ligated into ΔEcoRV sites of pDB1307 (5.9 kb). This plasmid contains all the essential components of pDB1568 but is flanking *scr* regions are larger: ~1.5 kb on P*scr* promoter side and ~1.6 kb of scr region downstream to *scrX* gene. This allows more space (on either side of a pAra-controlled gene fusion) for recombination into the *A.v*inelandii chromosome. As in pDB1568, direction of the pAra promoter is opposite to the P*scrX* promoter. **Note:** cannot be used for insertion of pT7-7 − derived NdeI-BamHI fragments since there is an NdeI site in pDB1307. Amp^R.

pDB1573   This plasmid is the same as pDB1572 except the HindIII-SphI fragment is in the opposite orientation, so that the P*ara* and P*scr* promoters are in the same direction. **Note:** cannot be used for cloning of pT7-7 – derived NdeI-BamHI fragments. Amp^R.

pDB1584   4.6kb SalI fragment from pDB1572 ligated into SalI site of pDB1474 (ΔNdeI in pUC7). This plasmid is similar to pDB1572 except it now has a unique NdeI site which is part of the linker. **Note:** cannot be used for cloning pT7-7 – derived NdeI-BamHI fragments due to the presence of an extra, unwanted BglII site. Amp^R.

pDB1588   **Cloning vector for Para-gene fusions (derivative of pDB1568)** 1 kb SacI-XmaI deletion in pDB1584, followed by blunt-end ligation with T4 DNA polymerase. Removal of this 1 kb fragment from pDB1584 deletes an unwanted BglII site. This plasmid is like pDB1568, and has unique NdeI and BglII sites suitable for cloning pT7-7 – derived NdeI-BamHI fragments for P*ara*-controlled expression and recombination into *A.v*. The difference is the much larger recombination space: ~1.5 kb on P*scr* promoter side and ~727 bp of *scr* region downstream to *scrX* gene. Frequency of double reciprocal recombination into *A. vinelandii* chromosome should be higher then for pDB1568. Plasmid map available. Amp^R.

pDB1593   ***Para-iscU37CA*** 0.3kb NdeI-BamHI fragment from pDB1214 ligated into NdeI-BglII sites of pDB1588. Places *iscU37CA* under pAra control. Collaborative effort with Callie Raulfs. Amp^R.

pDB1608   ***PscrX- hscB,hscA,fdx,iscX.*** 3.2kb PciI-BglII hscB-hscA-fdx-iscX fragment from pDB1299 ligated into PciI-BglII sites of pDB1332. Places *hscB-hscA-fdx-iscX* under control of P*scrX*. Amp^R.

APPENDIX III

Strains constructed during this project

DJ1405   **Δ*hemK***

DJ1370 x pDB1275 x pDB303. Has 432bp deletion in gene show which shows high sequence homology to *hemK* from *E. coli*. Increase in expression of fusion protein, CysE2'-LacZ, observed on BN plates supplemented with X-gal, compared to DJ1370 with wt *hemK*. Rif^R.

Reason for construction: *functional analysis of HemK and its possible involvement in IscR-regulated [Fe-S] cluster biosynthesis.*

DJ1406   ***hemK::kan***

DJ1370 x pDB1276. Has *hemK* gene disrupted with KAPA cartridge. Increase n expression of fusion protein, CysE2'-LacZ, observed on BN plates supplemented with X-gal, compared to DJ1370 with wt hemK. Rif^R, Kn^R.

Reason for construction: *functional analysis of hemK and its possible involvement in IscR-regulated [Fe-S] cluster biosynthesis.*

DJ1411   ***scrX::kan***.

Trans x pDB1309. Has a Kn^R cartridge insertion in *scrX* gene. Kn^R.

Reason for construction: *functional analysis of scrX to determine if it is essential for A. vinelandii viability.*

DJ1418   **P*scrX* - *lacZY::kan***

Trans x pDB1335. Places *lacZY::kan* cartridge under control of putative *scrX* promoter (P*scrX*). Strain produces blue colonies when grown on Burk's media containing sucrose or raffinose. Strain produces white

colonies when grown on Burk's media containing glucose, maltose, melibiose and fructose.

Kn^R.

Reason for construction: *to determine if sucrose induces gene expression from this promoter. Color screen allows use as a parent strain for easy isolation of recombinants containing genes of interest under PscrX control.*

DJ1421   ***PscrX - iscSUAhscBAfdxiscX.***

DJ1418 x pDB1316 x pDB303. Places second copy of isc operon (*iscS-iscX*) under control of *scrX* promoter. Strain grows normally on sucrose and glucose.

Rif^R

Reason for construction: *to control the expression of a second set of Isc gene products independent of those under IscR-control. This strain permits placement of potentially lethal mutations, in the endogenous, IscR-controlled isc operon. Cells should remain viable if plated on media containing sucrose as the main carbon source.*

DJ1422   ***Pisc -iscR iscS::kan iscUAhscBAfdxiscX* and *PscrX - iscSUAhscBAfdxiscX.***

DJ1421 x pDB952. Places Kn^R cartridgein in endogenous, IscR-controlled *iscS* gene. Strain grows fine on sucrose, but cannot grow on glucose. Colonies resulting from recombination between the two *iscS* genes appear on glucose plates after 4-5 days.

Kn^R, Rif^R.

Reason for construction: *Functional analysis of Isc gene products.*

DJ1423   ***Pisc-iscRΔiscS iscUAhscBAfdxiscX* and *PscrX - iscSUAhscBAfdxiscX.***

DJ1421 x pDB954 x pALMZ1'K15. A 547 bp in-frame deletion placed in endogenous, IscR-controlled *iscS* gene. Removes amino acids: 14-182.

Strain cannot grow on glucose. Colonies resulting from recombination between the two *iscS* genes appear on glucose plates after 4-5 days Kn^R, Rif^R.

Reason for construction: *Functional analysis of IscS gene product.*

DJ1424   **P*isc - iscR iscS325CA iscUAhscBAfdxiscX* and P*scrX - iscSUAhscBAfdxiscX.***

DJ1421 x pDB1209 x pALMZ1'K15. The variant *iscS325CA* gene replaces wt *iscS* gene. Strain cannot grow on glucose. Colonies resulting from recombination between the two *iscS* genes appear on glucose plates after 2-3 days.

Kn^R, Rif^R.

Reason for construction: *Functional analysis IscS gene product.*

DJ1425   **P*isc - iscRSUAΔhscBAfdxiscX* and P*scrX - iscSUAhscBAfdxiscX.***

DJ1421 x pDB1291 x pALMZ1'K15. A 1652 bp in-frame deletion placed in endogenous, IscR-controlled *hscBA* genes. Removes amino acids: HscB 78 – HscA 442) Strain grows slowly on glucose. Colonies resulting from recombination between the two *hscBA* genes appear on glucose plates after 4-5 days

Kn^R, Rif^R

Reason for construction: *Functional analysis of HscBA gene product.*

DJ1426   **P*isc - iscRSUAhscBA fdx::kan iscX* and P*scrX - iscSUAhscBAfdxiscX.***

DJ1421 x pD1016 Kn^R-insertion mutation placed in endogenous, IscR-controlled *fdx* gene. Strain grows extremely slowly on glucose. Colonies resulting from recombination between the two *fdx* genes appear on glucose plates after 4-5 days

Kn^R, Rif^R

Reason for construction: *Functional analysis of fdx gene product.*

DJ1427   ***Pisc- iscRSiscU::kaniscAhscBAfdxiscX* and P*scrX -***
           iscSUAhscBAfdxiscX.

           DJ1421 x pD1018. A Kn^R-insertion mutation placed in endogenous, IscR-
           controlled *iscU* gene. Strain does not grow on glucose. Colonies resulting
           from recombination between the two iscU genes appear on glucose plates
           after 4-5 days.

           Kn^R, Rif^R

           Reason for construction: (i) *Functional analysis of isc gene products.(ii)*
           Serve as a potential parent strain for placement of substitution mutations
           in PiscR - iscU gene.

DJ1428   ***Pisc - iscRSiscU106CAiscAhscBAfdxiscX* and P*scrX -***
           iscSUAhscBAfdxiscX.

            DJ1421 x pDB1227 x pALMZ1'K15. The variant gene *iscU106CA*
           replaces wt *iscU* gene in endogenous, IscR-controlled operon. Strain
           cannot grow on glucose. Colonies resulting from recombination between
           the two *iscU* genes appear on glucose plates after 2-3 days.

           Kan^R, Rif^R

           Reason for construction: *Functional analysis of conserved cysteine*
           residue106 in IscU gene product.

DJ1429   ***Pisc – iscRSiscU63CAiscAhscBAfdxiscX* and P*scrX -***
           iscSUAhscBAfdxiscX.

           DJ1421 x pDB1228 x pALMZ1'K15. The variant gene *iscU63CA*
           replaces wt *iscU* gene in endogenous, IscR-controlled operon. Strain
           cannot grow on glucose. Colonies resulting from recombination between
           the two *iscU* genes appear on glucose plates after 2-3 days.

           Kan^R, Rif^R RecA$^+$

           Reason for construction: *Functional analysis of conserved cysteine*
           residue 63 in IscU gene product.

DJ1434   **P*isc* - *iscRSΔiscUAhscBAfdxiscX* and P*scrX* - *iscSUAhscBAfdxiscX.***
DJ1421 x pDB1351 x pALMZ1'K15. A 788 bp in-frame deletion placed
in endogenous, IscR-controlled *iscU* and *iscA* genes. Removes amino
acids: IscU 34 – IscA 9). Strain cannot grow on glucose. Deletion
confirmed by PCR with primers *iscR-5*'(CATG CAT ATG CGA CTG
ACA ACC AAA GGC) and *iscA-3*'(CTAC GGA TCC ATG CGA TGA
TCT C) using Buffer D and annealing temp.=52°C. Expected product size
= ~2.2 kb (instead of ~2.5 kb for DJ1454). Colonies resulting from
recombination between the two *iscU* and *iscA* genes appear on glucose
plates after 4-5 days
Kn^R, Rif^R

Reason for construction: (i) *Functional analysis of IscU and IscA gene
products.(ii) to serve as a parent strain for construction of a recombinant
with just ΔiscA.*

DJ1435   **P*isc* - *iscRSΔiscUiscAhscBAfdxiscX* and P*scrX* - *iscSUAhscBAfdxiscX.***
DJ1421 x pDB1350 x pALMZ1'K15. A 700 bp in-frame deletion placed
in endogenous, IscR-controlled *iscU* gene. Removes amino acids: 31 –
117). Strain cannot grow on glucose. Colonies resulting from
recombination between the two *iscU* genes appear on glucose plates after
4-5 days.
Kn^R, Rif^R

Reason for construction: *Functional analysis of IscU gene product.*

DJ1439   ***nasB::kan* and P*scrX* - *iscSUAhscBAfdxiscX***
DJ1421 x pDB1362 x pDB62   A Kn^R gene cartridge placed between *nasB*
gene (nitrate reductase) and *nasA* gene (nitrite reductase). Concomitant
deletion of *nifNX3,4* inactivated strain's nitrogen-fixation ability so that
the presumed loss of nitrate reductase activity following the mutation in
nasB could be analyzed by plating on media containing potassium nitrate
(10mM) as the sole nitrogen source (BNO$_3$). However, strain is

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apparently able to grow on BNO_3 and displays sensitivity to chlorate (Chl^+) so n*asB/nasA* have not been appropriately inactivated.

Rif^R, Kn^R, Nif^-, Chl^+, NO_3^+

Reasons for construction: *(1) Functional analysis of nasB/nasA gene products. (2) To create a parent strain for placement of an N-terminal His tag on nasB to facilitate purification of nitrate reductase from A. vinelandii (3) To create a parent strain for placement of specific isc mutations in Pisc-isc operon, with a view of purifying nitrate reductase from cells depleted for a specific Isc component.*

DJ1440   ***nasB::kan***

Trans x pDB1362 x pDB62. A Kn^R gene cartridge placed between *nasB* gene and *nasA* gene with concomitant deletion of *nifNX3,4* genes which are required for nitrogen fixation. See explanation above for DJ1439. Strain is apparently able to grow on BNO_3 and displays sensitivity to chlorate (Chl^-) so *nasB/nasA* have not been appropriately inactivated.

Kn^R, Nif^-, Chl^-, NO_3^+

Reasons for construction: *(1) Functional analysis of nasB/nasA gene products. (2) To create a parent strain for placement of an N-terminal His tag on nasB to facilitate purification of nitrate reductase from A. vinelandii*

DJ1441   ***nasB::gn*** and ***PscrX - iscSUAhscBAfdxiscX***

DJ1421 x pDB1363 x pDB31. A Gn^R gene cartridge placed between *nasB* and *nasA* with concomitant deletion of *nifD*gene which is required for nitrogen fixation. See explanation for DJ1439 above. This strain is unable to grow on BNO_3 and displays resistance to chlorate (Chl^+) so *nasB/nasA* have been appropriately inactivated.

Rif^R, Kn^R, Nif^-, Chl^+, NO_3^-

Reasons for construction: *same as described above for DJ1439.*

DJ1443   **_his (x7)-nasB_ gene**.

DJ1440 x pDB1364 x pDB58. Poly (x7)-histidine linker cloned into the NcoI site of *nasB* gene creating a gene product with an N-terminal His tag. Oligos used to create linker: NasB-his, CATGTGGTGGTGATGGTGGTGATG and CATGCATCACCACCATCACCACCA. Since the parent strain DJ1440 retained nitrate reductase activity it was not possible to select for transformants using BNO_3 plates and available congression vectors were very limited (congression vectors containing gentamicin and streptomycin were unavailable at this time). Selection was therefore performed via congression with pDB58 which contains intact *nifNX3,4* and therefore restored a Nif$^+$ phenotype allowing growth on B plates. Nif$^+$ recombinants were screened for Kn^S and Amp^S. This strain displayed sensitivity to chlorate, indicating the his-tag did not affect nitrate reductase activity. Nif$^+$, Chl$^-$, (NO_3^+ - on the basis of Chl$^-$ phenotype).

Reason for construction: (1) to facilitate purification of NasB directly from its natural host organism for enzyme characterization and spectroscopic analysis of its putative [2Fe-2S] and [4Fe-4S] cluster types.

DJ1444   **_his (x7)-nasB gene_** and **P_scrX - iscSUAhscBAfdxiscX_**

DJ1439 x pDB1364 x pDB58 - description is the same as for DJ1443, except the parent strain contains duplicate copies of the *isc* [Fe-S] cluster biosynthetic genes.

Rif^R, Nif$^+$, Chl$^-$, (NO_3^+ - on the basis of Chl$^-$ phenotype)

Reason for construction: (1) to facilitate purification of NasB directly from its natural host organism for enzyme characterization and spectroscopic analysis of its putative [2Fe-2S] and [4Fe-4S] cluster types. (2) To create a parent strain for placement of specific isc mutations in Pisc-isc operon, with a view of purifying hi-tagged nitrate reductase from cells depleted for a specific Isc component.

DJ1445   ***Pisc - iscRS⊿iscUiscAhscBAfdxiscX* and *PscrX - iscSUAhscBAfdxiscX***
          and ***recA::kan***

          DJ1421 x pDB1350 x pDB528. A 700 bp in-frame deletion placed in
          endogenous, IscR-controlled *iscU* gene. Removes amino acids: 31 –117.
          Absolutely no growth on glucose under diazotrophic or non-diazotrophic
          conditions at 21% or 40% oxygen. Does grow at 5% oxygen under
          dizaotrophic conditions only. Unknown if recombination of pDB528
          (*recA::kan*) is single or double since *A. vinelandii* displays natural
          resistance to tetracycline up to 4μg/ml.   Colonies on BN (sucrose) plates
          are of uniform size

          Rif^R, Kn^R,$RecA^-$

          Reason for construction: *Functional analysis of IscU gene product*
          without complication of phenotypes resulting form allele recombination
          between two iscU alleles.

DJ1446   ***nasB::gn***

          TRANS x pDB1363 x pDB31. A Gn^R gene cartridge placed between
          nasB and *nasA* with concomitant deletion of *nifD* gene which is required
          for nitrogen fixation. No growth on BNO_3 plates. This strain displays the
          same phenotype as DJ1441, except it does NOT contain two copies of the
          isc [Fe-S] cluster biosynthetic genes.

          Gn^R, Nif^-, Chl^R, NO_3^-.

          Reasons for construction: *same as described above for DJ1440.*

DJ1447   ***Pisc - iscRSUA⊿hscBAfdxiscX* and *PscrX – iscSUAhscBAfdxiscX* and**
          recA::kan

          DJ1421 x pDB1291 x pDB528. A 1652 bp in-frame deletion placed in
          endogenous, IscR-controlled *hscBA* genes. Removes amino acids: HscB
          78 – HscA 442. Strain grows slowly on glucose. Glucose growth
          improves a lot on nitrogen-free glucose media or in a Coy chamber with

~5% oxygen. No growth at 40% oxygen. Colonies on BN (sucrose) plates are of uniform size. Tetracycline sensitivity cannot be confirmed. Kn^R, Rif^R, $RecA^-$

Reason for construction: *Functional analysis of HscBA gene product without complication of phenotypes resulting form allele recombination between two hscBA alleles.*

DJ1448 **P*isc – iscRSiscU^{106CA}iscAhscBAfdxiscX* and P*scrX – iscSUAhscBAfdxiscX* and *recA::kan***

DJ1421 x pDB1227 x pDB528. The variant gene *iscU106CA* replaces wt *iscU* gene in endogenous, IscR-controlled operon. Absolutely no growth on glucose under diazotrophic or non-diazotrophic conditions at 21% or 40% oxygen. Not done at 5% oxygen. Tetracycline sensitivity cannot be confirmed. Colony sizes on BN (sucrose) plates are mixed and start growing slowly on BN plates - suggesting a negative transdominance effect. Growth apparently "catches up" after a few days and becomes indistinguishable from WT (i.e. DJ1421 or DJ1454). Slow growth is NOT observed when grown in liquid BN culture.

Rif^R, Kn^R, $RecA^-$

Reason for construction: *Functional analysis of cysteine 106 in iscU gene product without complication of phenotypes resulting form allele recombination between two iscU alleles.*

DJ1449 ***his (x7)-nasB gene* and P*scrX - iscSUAhscBAfdxiscX***

DJ1441 x pDB1364. Poly (x7)-histidine linker cloned into the NcoI site of nasB gene creating a gene product with an N-terminal His tag as described for DJ1443 and DJ1444. However, parent strain DJ1441 did not have nitrate reductase activity so it was possible to select transformants by plating on BNO_3 plates and screen for Gn^S and Amp^S. This particular strain was selected for its "faster growth" phenotype on BNO_3 plates, compared to other transformants. It was the preferred strain for nasB

purification attempts since it was expected that with an inactivated nitrogen fixation system, the cell may produce relatively elevated expression of nitrate reductase (compared to DJ1443, for example) when grown on media containing mitrate as the sole nitrogen source.

Rif^R, Nif$^-$, Chl$^-$, NO$_3^+$

Reasons for construction: *exactly as described for DJ1444.*

DJ1450   **P*isc* - *iscRΔiscS iscUAhscBAfdxiscX*** and **P*scrX – iscSUAhscBAfdxiscX*** and ***recA::kan***

DJ1421 x pDB954 x pDB528. A 547 bp in-frame deletion placed in endogenous, IscR-controlled *iscS* gene. Removes amino acids: 14 –182. Absolutely no growth on glucose under diazotrophic or non-diazotrophic conditions at 5%, 21% or 40% oxygen. Colonies on BN (sucrose) plates are of uniform size. Tetracycline sensitivity cannot be confirmed.

Rif^R, Kn^R,RecA$^-$

Reason for construction: *Functional analysis of iscS gene product without complication of phenotypes resulting from allele recombination between two iscS alleles.*

DJ1451   **P*isc* - *iscRiscS^{325CA}iscUAhscBAfdxiscX*** and **P*scrX – iscSUAhscBAfdxiscX*** and ***recA::kan***

DJ1421 x pDB1209 x pDB528. The variant gene *iscS325CA* replaces wt *iscS* gene in endogenous, IscR-controlled operon. Absolutely no growth on glucose under diazotrophic or non-diazotrophic conditions at 21% or 40% oxygen. Not tested at 5% oxygen. Colony sizes on BN (sucrose) plates are mixed and grow quite slowly on BN plates relative to DJ1421 or DJ1454 - suggesting a negative transdominance effect. Growth in liquid BN culture not done.Tetracycline sensitivity cannot be confirmed.

Rif^R, Kn^R,RecA$^-$

Reason for construction: Functional analysis of the active site cysteine 325 in iscS gene product without complication of phenotypes resulting form allele recombination between two iscU alleles.

DJ1452 **P*isc* – *iscRSiscU*^{63CA}*iscAhscBAfdxiscX* and P*scrX* – *iscSUAhscBAfdxiscX* and *recA::kan***

DJ1421 x pDB1228 x pDB528. The variant gene *iscU63CA* replaces wt *iscU* gene in endogenous, IscR-controlled operon. Absolutely no growth on glucose under diazotrophic or non-diazotrophic conditions at 21% or 40% oxygen. Not done at 5% oxygen. Tetracycline sensitivity cannot be confirmed. Colony sizes on BN (sucrose) plates are mixed but do not show an initial slow growth phenotype on BN plates as observed for DJ1448.

Rif^R, Kn^R,RecA$^-$

Reason for construction: Functional analysis of cysteine 63 in iscU gene product without complication of phenotypes resulting form allele recombination between two iscU alleles.

DJ1453 **P*isc* – *iscRSiscU*^{39DA}*iscAhscBAfdxiscX* and P*scrX* – *iscSUAhscBAfdxiscX* and *recA::kan***

DJ1421 x pDB1058 x pDB528. The variant *iscU39DA* replaces wt *iscU* gene in endogenous, IscR-controlled operon. Absolutely no growth on glucose under diazotrophic or non-diazotrophic conditions at 21% or 40% oxygen. Not done at 5% oxygen. Tetracycline sensitivity cannot be confirmed. Colony sizes on BN (sucrose) plates are mixed and start growing very slowly on BN plates - suggesting a negative transdominance effect. Growth apparently "catches up" after a few days and becomes indistinguishable from WT (i.e. DJ1421 or DJ1454). Slow growth is NOT observed when grown in liquid BN culture.

Rif^R, Kn^R,RecA$^-$

Reason for construction: Functional analysis of aspartate 39 in iscU gene product without complication of phenotypes resulting form allele recombination between two iscU alleles.

DJ1454   **P*scrX* - *iscSUAhscBAfdxiscX*** and ***recA::kan***

DJ1421 x pDB528. Strain is just like DJ1421 except has *recA::kan*. Tet sensitivity cannot be confirmed.

Kn^R, Rif^R.

Reason for construction: to serve as the 'WT' for functional studies conducted with strains containing mutations in the P*isc*-controlled operon

DJ1455   **P*isc*–iscR*Δ(iscSUAhscBAfdx::gn)iscX*** and **P*scrX* – *iscSUAhscBAfdxiscX*** and ***recA::kan***

DJ1421 x pDB1370 x pDB528. A 4.4 kb in-frame deletion placed in endogenous, IscR-controlled *isc* operon. Removes *isc* region in between codons encoding amino acid 49 in IscS and amino acid 36 if Fdx. IscR gene is intact. Absolutely no growth on glucose under diazotrophic or non-diazotrophic conditions at 21%. Not tested at 40% or 5% oxygen. Selected on plates containing both kanamycin and gentamycin. Grows reasonably slowly on sucrose – when compared to DJ1454 on glucose - not a particularly healthy strain. Indicates that expression level from P*scrX* promoter not as good as P*isc* promoter.

Rif^R, Gn^R, Kn^R, RecA$^-$

Reason for construction: To qualitatively compare levels of isc expression from the PiscR promoter and PscrX promoter.

DJ1456*   ***Δnif4-11::gn***

Trans x pDB1371. A Gn^{R}cartridge inserted into a non-essential section within the *nif* gene region of *A. vinelandii*.

Reason for construction: To test effectiveness of congression vectors, pDB1383 (Str^R) and pDB137 (Kn^R), which are complementary to pDB1371 (Gn^R).

DJ1460   ***his (x7)-nasB gene*** and ***Pisc - iscRSUAΔhscBAfdxiscX*** and ***PscrX - iscSUAhscBAfdxiscX***

DJ1449 x pDB1291 x pDB1375. Strain has same genotype and phenotype as DJ1447 (slow growth on glucose) except it also contains a his-tagged *nasB* gene and a deletion an in-frame *nifD* gene, conferring a Nif⁻ phenotype.

Gn^R, Rif^R. Nif⁻ RecA⁻, Chl⁻, NO$_3^+$

Reasons for construction: *(1) to characterize the cluster content of NasB when purified from cells depleted for HscB and HscA.*

DJ1462   ***his (x7)-nasB gene*** and ***Pisc -iscRSΔiscUiscAhscBAfdxiscX*** and ***PscrX - iscSUAhscBAfdxiscX***

DJ1449 x pDB1350 x pDB1375. Strain has same genotype and phenotype as DJ1445 (no growth on glucose) except it also contains a his-tagged *nasB* gene and a deletion an in-frame nifD gene, conferring a Nif⁻ phenotype.

Gn^R, Rif^R. Nif⁻ RecA⁻, Chl⁻, NO$_3^+$

Reasons for construction: *to characterize the cluster content of NasB when purified from cells depleted forIscU*

DJ1463   ***Pisc- iscRSUAhscBA fdx::kan iscX*** and ***PscrX – iscSUAhscBAfdxiscX*** and ***recA::gn***

DJ1421 x pDB1016 x pDB1375. A Kn^R cartridge placed in endogenous, IscR-controlled *fdx* gene. Strain grows extremely slowly on glucose as observed for DJ1426. Glucose growth improves a little on nitrogen-free glucose media or in a Coy chamber with ~5% oxygen. No growth at 40% oxygen. Colonies on BN (sucrose) plates are of uniform size.

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Gn^R, Kn^R, Rif^R, $RecA^-$

Reason for construction: *Functional analysis of fdx gene product without complication of phenotypes resulting form allele recombination between two fdx alleles.*

DJ1464   **P*scrX – iscSUAhscBAfdxiscX*** and ***recA::gn***

DJ1421 x pDB1375. Strain is just like DJ1421 except has *recA::gn.* Seems to grow slower then DJ1421- not sure why - did not use as WT strain when conducting depletion experiments with *isc* mutant strains. Tet sensitivity cannot be confirmed.

Gn^R, Rif^R, $RecA^-$

Reason for construction: *to serve as the 'WT' for functional studies conducted with strains containing mutations in the Pisc-controlled operon.*

DJ1476   ***scrR::gn*** and **P*scrX – lacZY::kan***

DJ1418 x pDB1400. A Gn^R cartridge interrupting the *scrR* (the putative P*scrX* repressor protein). Produces blue colonies when grown on both glucose and sucrose plates containing X-gal. β-galactosidase assays confirm that this deletion results in approximately equal levels of LacZ expression from P*scrX* when grown in sucrose and glucose. liquid media. Gn^R, Kn^R.

Reason for construction: *to test hypothesis that ScrR is a LacI-type repressor and can repress the scrX promoter.*

DJ1488   **P*isc - iscRSΔiscUAhscBAfdxiscX*** and **P*scrX - iscSUAhscBAfdxiscX*** and ***recA::kan***

DJ1421 x pDB1386 x pDB528. An in-frame deletion in *iscU* and *iscA* in the IscR controlled copy of the *isc* operon. Absolutely no growth on glucose plates at 21% or 40% oxygen. Tet sensitivity cannot be confirmed.

Kn^R, Rif^R.

Reason for construction: *Functional analysis of iscU and iscA gene products without complication of phenotypes resulting form allele recombination between two iscU/iscA alleles.*

DJ1489 ***scrB::gn* and *PscrX – lacZY::kan***

DJ1418 x pDB1416. A Gn^R cartridge interrupting *scrB* gene. No change in phenotype observed – continues to grow well on sucrose. As for DJ1418, LacZ expressed only on sucrose plates.

Kn^R, Gn^R

Reason for construction: *to investigate if ScrB encodes an essential sucrase in A. vinelandii.*

DJ1501 ***ΔsufSE::kan***

Trans x pDSE13. Plasmid constructed by Janet Donohue (Tim Larson's lab). Kn^R resistance cartridge interrupting *sufSE* genes. Stability check confirms that cartridge is stably integrated into *A. vinelandii* chromosome. Grows well at 40% oxygen.

Kn^R

Reason for construction: *to check if SufSE gene products are essential for A. vinelandii viability.*

DJ1502 ***ΔcysE3::kan***

Trans x pDER76. Plasmid constructed by Janet Donohue (Tim Larson's lab). Kn^R resistance cartridge interrupting *cysE3* gene. Stability check confirms that cartridge is stably integrated into *A. vinelandii* chromosome. Grows well at 40% oxygen.

Kn^R

Reason for construction: *to check if CysE3 gene product is essential for A. vinelandii viability.*

DJ1503   ***Δrhd::kan***

Trans x pDE66.   Plasmid constructed by Janet Donohue (Tim Larson's lab). Kn^R resistance cartridge interrupting *rhd* gene located immediately upstream of *cysE3*. Stability check confirms that cartridge is stably integrated into *A. vinelandii* chromosome. Grows well at 40% oxygen. Kn^R

Reason for construction: *to check if Rhd gene product is essential for A. vinelandii viability.*

DJ1504   ***φ(orf1'-lacZ)cysE2*** and **P*scrX - iscSUAhscBAfdxiscX***

DJ1421 x pDB1179 x pDB1383. An *orf1'lacZ* fusion under control of the *cysE1* promoter speculated to be controlled by IscR. Strain is light blue on sucrose, X-gal plates.

Rif^R, Str^R.

Reason for construction: *to create a parent strain for investigating the effects of depleting isc components on maturation of IscR – and [Fe-S] protein.*

DJ1505   ***φ(orf1'-lacZ)cysE2*** and **P*isc - iscRSΔiscUiscAhscBAfdxiscX*** and

PscrX – iscSUAhscBAfdxiscX and ***recA::gn***

DJ1504 x pDB1350 x pDB1375. In-frame deletion in *iscU* gene in P*isc* controlled copy of *isc* operon. Strain does not grow on glucose as for DJ1445. On sucrose plates, level of β-gal activity seems the same as diploid parent, DJ1504.

 Gn^R, Rif^R, Str^R, recA$^-$.

Reason for construction: *to test the effects of depleting IscU on the maturation of the [2Fe-2S] cluster on IscR by measuring the change in β-galactosidase activity following a carbon-source shift from sucrose to glucose.*

DJ1506   **ɸ(orf1'-lacZ)cysE2** and **P*isc* - *iscRΔiscSiscUAhscBAfdxiscX*** and

                P*scrX* – *iscSUAhscBAfdxiscX* and **recA::gn**

                DJ1504 x pDB954 x pDB1375. In-frame deletion in *iscS* gene in P*isc*

                controlled copy of *isc* operon. Strain does not grow on glucose as for

                DJ1450. On sucrose plates, level of β-gal activity seems the same as

                diploid parent, DJ1504.

                Gn^R, Rif^R, Str^R, recA$^-$.

                Reason for construction: *to test the effects of depleting IscS on the*

                maturation of the [2Fe-2S] cluster on IscR by measuring the change in β-

                galactosidase activity following a carbon-source shift from sucrose to

                glucose.

DJ1507   **ɸ(orf1'-lacZ)cysE2** and **P*isc* - *iscRSUAΔhscBAfdxiscX*** and

                P*scrX* – *iscSUAhscBAfdxiscX* and **recA::gn**

                DJ1504 x pDB1291 x pDB1375. In-frame deletion in *hscBA* genes in P*isc*

                controlled copy of *isc* operon. Strain does not grow on glucose as for

                DJ1450. On sucrose plates, level of β-gal activity seems the same as

                diploid parent, DJ1504.

                Gn^R, Rif^R, Str^R, recA$^-$.

                Reason for construction: *to test the effects of depleting hscBA on the*

                maturation of the [2Fe-2S] cluster on IscR by measuring the change in β-

                galactosidase activity following a carbon-source shift from sucrose to

                glucose.

DJ1524   **P*isc* – *iscRSUAhscBAfdxiscX*** and **P*scrX* – *iscSUAhscBɸ(hscA'-lacZ)fdxiscX***

                DJ1421 x pDB1468. A <u>lac**ZY**</u>Kan cartridge fused to the *hscA* gene of the

                P*scr* controlled *isc* copy. Strain grows on sucrose and glucose. Never

                goes blue on B(G)NX plates; takes a very long time to turn blue on BNX

                plates. β-gal activity shows only a 14-fold decrease in activity when

                grown in liquid media containing glucose instead of sucrose. This is in

contrast to DJ1418, in which there is a 1000-fold decrease when grown in glucose.

Rif^R, Kn^R.

Reason for construction: _to investigate the presence of a weak internal promoter (upstream of hscBA) within the isc operon._

DJ1525   **_Pisc – iscRSUAhscBϕ(hscA'-lacZ)fdxiscX_** and **_PscrX – iscSUAhscBAfdxiscX_**

DJ1421 x pDB1468. A _lacZYkan_ cartridge fused to the _hscA_ gene of the P_isc_ controlled _isc_ copy. Strain is light blue on sucrose plates. Turns dark blue on glucose and grows slowly, as observed for DJ1447. Allele recombination _between hscA_ genes obvious after 4-5 days on glucose.

Rif^R, Kn^R.

Reason for construction: _to construct a parent strain to allow analysis of IscR repressor function at isc promoter_

DJ1531   **_Pisc – iscR^{98CA}SUAhscBϕ(hscA'-lacZ)fdxiscX_** and **_PscrX– iscSUAhscBAfdxiscX_** and **_recA::gn_**

DJ1525 x pDB1212 x pDB1375 (<u>rec**A**</u>::Gn^R). The variant _iscR98CA_ gene placed under control of P_isc_ controlled _isc_ operon. Turns darker blue on sucrose plates compared to parent, DJ1525. Turns dark blue on glucose and growth is slow, like DJ1425. β-gal activity measured from crude extracts of sucrose-grown cells show ~14-fold increase compared to DJ1525.

Gn^R, Kan^R, Rif^R.

Reason for construction: _Functional analysis of cysteine 98 in IscR gene product._

DJ1532 **Pisc – iscRSUAhscB**φ**(hscA'-lacZ)fdxiscX** and **PscrX –**

iscSUAhscBAfdxiscX

recA::gn

DJ1525 x pDB1375. Strain shows same phenotype as DJ1525 except it

has an inactivated *recA* gene.

Gn^R, Kan^R, Rif^R.

Reason for construction: *to use as a "WT" strain for comparison with*

other strains containing mutations in the iscR gene. No complication from

allele recombination between the hscBA alleles.

DJ1551 **Pisc – iscRSUΔiscAhscBAfdxiscX** and **PscrX – iscSUAhscBAfdxiscX**

DJ1434 x pDB1391. A 285 bp in-frame deletion placed in endogenous,

IscR-controlled *iscA* gene. Removes amino acids: IscA 8 – IscA 25.

Strain can grow on glucose at 21% oxygen but not at 40% oxygen. Strains

isolated by rescuring the glucose$^-$ phenotype of DJ1434. Deletion

confirmed by PCR with primers *iscR-5'* and *iscA-3'*as described for

DJ1434. Expected product size = 2.2 kb.

Kn^R, Rif^R

Reason for construction: *Functional analysis of IscA gene products.*

DJ1558 **Pisc – iscRSUΔiscAhscBAfdxiscX** and **PscrX – iscSUAhscBAfdxiscX**

and **recA::gn** and **ΔnifDK**.

DJ1551 x pDB1375 x pDB31. Introduces Gn^R cartridge into *recA* gene

and inactivates nif**DK**. Grows well on sucrose, glucose and

glucose/nitrate plates. Kn^R, Rif^R, Gn^R Nif-

Reason for construction: *To see if IscA is specifically involved in [2Fe-*

2S] cluster formation which means it would be required for nitrate

reductase function since NasB protein is thought to contain both [2Fe-2S]

and [4Fe-4S] clusters.

DJ1559 **P*isc* – *iscRSU*Δ*iscAhscBAfdxiscX*** and **P*scrX* – *iscSUAhscBAfdxiscX***
and ***recA::gn***

DJ1551 x pDB1375. Strain was obtained from the above transformation
(DJ1558), except it is nif+ and recA ⁻. Used for depletion experiments.
PCR used to confirm integrity of deletion in *iscA* as described for DJ1551.
Kn^R, Rif^R, Gn^R.

Reason for construction: *Functional analysis of IscA gene product
without complication of phenotypes resulting from allele recombination
between two iscA alleles.*

DJ1564 **P*isc* – *iscRSU*Δ*iscAhscBAfdxiscX*** and **P*scrX* – *iscSUAhscBAfdxiscX***
and ***iscA2::gn***

DJ1551 x pDB1499. A Gn^R cartridge interrupting the *iscA2* gene. Strain
grows well on glucose and sucrose at 21% oxygen. No growth at 40%
oxygen.
Kn^R, Rif^R, Gn^R

Reason for construction: *to investigate if IscA2 can replace IscA function.*

DJ1565 **P*isc* – *iscRSU*Δ*iscAhscBAfdxiscX*** and **P*scrX* – *iscSUAhscBAfdxiscX***
and ***iscA2::gn*** and ***ΔnifDK***.

DJ1551 x pDB1499 x pDB31. Same as DJ1564 above, except is Nif⁻ due
to a deletion in *nifDK* genes. Strain grows on nitrate plates.
Kan^R, Rif^R, Gn^R

Reason for construction: *to investigate if IscA2 can replace IscA function.*

DJ1566 **P*isc*–*iscR*Δ*(iscSUAhscBAfdx::gn)iscX*** and **P*scrX* –
*iscSUAhscBAfdxiscX*** and ***recA::kan***

DJ1421 x pDB1370. Strain has a Gn^R cartridge replacing a 4.4 kb
deletion in P*isc*-controlled operon. Strain cannot grow on glucose. No
allele recombination observed after 4 days. Strain is the *recA*⁺ version of
the previous DJ1455

Gn^R, Rif^R.

Reason for construction: *functional analysis of isc operon and for use as a parent strain for future constructions.*

DJ1568   ***Pisc – iscRSUAhscBAfdxiscX*** and ***PscrX – iscSiscU::kaniscAhscBAfdxiscX***

DJ1421 x pDB1018.   Strain selected on glucose plates supplemented with kanamycin, so the Kn^R cartridge is in the P*scr* copy.

Kan^R, Rif^R

Reason for construction: *to use as a parent strain for placement of mutations withing the Pscr-controlled iscU copy.*

DJ1570   ***Pisc – iscRSUAhscBAfdxiscX*** and ***PscrX – iscSUAhscBAfdxiscX*** and ***iscA2::gn***

DJ1421 x pDB1499.   Places a Gn^R cartridge in the *iscA2* gene in a strain diploid for the *isc* genes.   No particular phenotype observed relative to DJ1421.

Gn^R, Rif^R.

Reason for construction: *functional analysis of IscA2 in an "isc diploid" background.*

DJ1585   ***Pisc – iscRSUAhscBAfdxiscX*** and ***PscrX – iscSiscU*** ^{37CA}***iscAhscBAfdxiscX***

DJ1568 x pDB1404 x pDB1383.   Strain has an *iscU37CA* substitution in the P*scr* copy of the *iscU* gene.   Grows well on glucose and sucrose (like DJ1421).   Strain obtained via congression and screen for Kn^S.   Confirmed by DNA sequencing (PCR primers: Pscr-5': GTC TGG CAC GGT TGC CCT ATG and IscA-3':CTAC GGA TCC ATG CGA TGA TCT C using Buffer D and annealing temp.=55°C.   Sequencing primer: IscS-5' GCC ATC CAG GTA GCC (Tm=39°C)

Rif^R, Str^R.

Reason for construction: *functional analysis of cysteine 37 in IscU. It has not been possible to obtain a strain with this substitutionin the Pisc-controlled copy of the isc operon. It is speculated that this may be due to a negative transdominant effect of this mutation on WT iscU. However, this strain does not show sucrose sensitivity.*

DJ1599   **P*isc*-*iscRSUAhscB*ϕ(*hscA'-lacZ*)*fdxiscX*** and
P*scrX*-*iscSiscU*^{37CA}*iscAhscBAfdxiscX*

DJ1585 x pDB1468. An ϕ(*hscA'-lac*Z)::kan fusion inserted into P*isc* copy of *hscA* and with *iscU37CA* mutation in *Pscr* copy (verified by sequencing as done for DJ1585). This strain seems to be a little darker blue on sucrose plates compared to DJ1525 (which does not have the *iscU37CA* mutation in the P*scr* copy).

Kn^R, Rif^R, Str^R

Reason for construction: *functional analysis of cysteine 37 in IscU. Construction to check if a speculated negative transdominant effect exerted by the Pscr-expressed IscU37CA variant is overcome by elevated expression of the Pisc_controlled WT IscU.*

DJ1601   **P*isc* -Δ*iscRiscSUAhscBAfdxiscX*** and **P*scrX* - *iscSUAhscBAfdxiscX*.**
DJ1562 x chromosomal DNA from DJ1588 x pDB1371. Strain constructed by Mihaela Unciuleac and verified by PCR by Deborah Johnson, using assorted primers. Chromosomal DNA from DJ1588 (which contains ϕ (*iscU'-GFP*) in the P*isc* copy of *iscU* gene) was transformed, via gentamicin congression, into DJ1562 which contains a 120bp in-frame deletion in *iscR* and ϕ(*hscA'-lacZkan*) under P*isc*-control. White colonies that are Kn^S were identified. All other *isc* genes in both copies are intact. It does not carry the *iscU'-GFP* fusion. This strain grows slowly on BN media at 21%, 40% and 5% oxygen.

Rif^R, Gn^R

Reason for construction: original reason was to obtain a strain with Pisc-controlled ϕ (iscU'-GFP) and an intact hscA gene. However, double homologous recombination between incoming DJ1588 chromosomal DNA and parent strain 1562 chromosomal DNA only occurred in the iscU-hscB-hscA region resulting in the 'removal' of ϕ (iscU'-GFP) and ϕ(hscA'-lacZkan) but NOT the 120 bp deletion in iscR gene.

DJ1602   **P*isc* -*iscRSUAhscBAfdxiscX* and P*scrX* - *iscSUAhscBAfdxiscX.***
DJ1601 x pDB1481. DJ1601 was transformed from slow growth on BN to normal growth by transformation with pDB1481, which contains the wild type *iscR* gene.
Rif^R, Gn^R
Reason for construction: to investigate if the cause of the slow growth phenotype observed for DJ1601 can be directly linked to the deletion in *iscR*.

DJ1603   **P*isc* -Δ*iscRiscS*Δ*iscUAhscBAfdxiscX* and P*scrX* - *iscSUAhscBAfdxiscX.***
DJ1601 x pDB1350. DJ1601 was transformed from slow growth on BN to normal growth by transformation with pDB1350. Strains carries an in-frame deletion in *iscU* and an in-frame deletion in *iscR*. Integrity of deletions confirmed by PCR using assorted primers. No growth on glucose.
Rif^R, Gn^R
Reason for construction: to investigate if the cause of the slow growth phenotype observed for DJ1601 can be directly linked to increased expression of the Isc biosynthetic machinery.

DJ1604   **P*isc* -*iscRSUA*Δ*hscBAfdxiscX* and P*scrX* - *iscSUAhscBAfdxiscX.***
DJ1421 x pDB1291 x pDB1383. Strain just like DJ1425 except does not harbour Kn^R – has Str^R instead. Strain grows slowly on glucose. Colonies

resulting from recombination between the two *hscBA* genes appear on glucose plates after 4-5 days

Kn^R, Rif^R

Reason for construction: *Functional analysis of HscBA gene products.*

DJ1605   **P*isc* -*iscRSUAhscBAfdxiscX*** and **P*scrX* - *iscSUAhscBAfdxiscX.***

DJ1421 x pDB1383 (Str^R). Strain isolated from transformation carried out for DJ1604 above.

Rif^R, Str^R.

Reason for construction: Serves as a "parent" control for experiments done with DJ1604 for *functional analysis of HscBA gene products.*

DJ1607   **P*isc* – *iscRSiscU*^{105HA}*iscAhscBAfdxiscX*** and **P*scrX* – *iscSUAhscBAfdxiscX*** and **recA::kan**

DJ1421 x pDB1538 x pDB528. The variant gene *iscU105HA* replaces wt *iscU* gene in endogenous, IscR-controlled operon. Absolutely no growth on glucose under diazotrophic or non-diazotrophic conditions at 21% or 40% oxygen. Not done at 5% oxygen. Tetracycline sensitivity cannot be confirmed. Colony sizes on BN (sucrose) plates are mixed but do not show an initial slow growth phenotype on BN plates as observed for DJ1448 i.e. no apparent transdominance.

Rif^R, Kn^R,RecA$^-$

Reason for construction: *Functional analysis of histidine 103 in iscU gene product without complication of phenotypes resulting form allele recombination between two iscU alleles.*

DJ1608   **P*isc* – *iscRSiscU*^{103KA}*iscAhscBAfdxiscX*** and **P*scrX* – *iscSUAhscBAfdxiscX*** and **recA::kan**

DJ1421 x pDB1546 x pDB528. . The variant gene *iscU103KA* replaces wt *iscU* gene in endogenous, IscR-controlled operon. Absolutely no growth on glucose under diazotrophic or non-diazotrophic conditions at 21% or

40% oxygen. Not done at 5% oxygen. Tetracycline sensitivity cannot be confirmed. Colony sizes on BN (sucrose) plates are mixed but do not show an initial slow growth phenotype on BN plates as observed for DJ1448 i.e. no apparent transdominance.

Rif^R, Kn^R,RecA$^-$

Reason for construction: *Functional analysis of lysine 103 in iscU gene product without complication of phenotypes resulting form allele recombination between two iscU alleles.*

DJ1609   **P*isc -ΔiscRiscSUAΔhscBAfdxiscX* and P*scrX - iscSUAhscBAfdxiscX.***
DJ1601 x pDB1291 x pDB528. DJ1601 was transformed from slow growth on BN to normal growth by transformation with pDB1291. Strains carries an in-frame deletion in *hscBA* and an in-frame deletion in *iscR*. Integrity of deletions confirmed by PCR using assorted primers. Slow growth on glucose.
Rif^R, Gn^R, RecA$^-$

Reason for construction: *to investigate if the cause of the slow growth phenotype observed for DJ1601 can be directly linked to increased expression of the Isc biosynthetic machinery.*

DJ1619   **P*isc -ΔiscRiscSUAhscBAfdxiscX* and Δ*nifU***
DJ1413 x pDB1500. Strain has 186 bp in-frame deletion in *iscR* (codons 57-119) and an in-frame deletion in *nifU* (codons 23-270), obtained by diazotrophic rescue of the very, very slow growth phenotype of DJ1413. Grows slowly under diazotrophic conditions. Grows very slowly on BN plates but well in liquid BN. Deletion in *iscR* confirmed by PCR.
Kn^R, Rif^R, Nif ^{slow}

Reason for construction: *to investigate if elevated expression of Isc machinery (as a result of a deletion in iscR), will allow some level of "cross-talk" (i.e. functional replacement) between the Isc and Nif systems – specifically of the nifU gene product.*

DJ1620   ***Pisc -ΔiscRiscSUAhscBAfdxiscX*** and ***ΔnifU***

DJ1413 x pDB1490. Strain has 120 bp in-frame deletion in *iscR* (codons 21-60) and an in-frame deletion in *nifU* (codons 23-270), obtained by diazotrophic rescue of the very, very slow growth phenotype of DJ1413. Grows slowly under diazotrophic conditions. Grows very slowly on BN plates but well in liquid BN. Deletion in *iscR* confirmed by PCR. Kn^R, Rif^R, Nif^{slow}

Reason for construction: *to investigate if elevated expression of Isc machinery (as a result of a deletion in iscR), will allow some level of "cross-talk" (i.e. functional replacement) between the Isc and Nif systems – specifically of the nifU gene product.*

DJ1621   ***Pisc- iscRSUAhscBA Δfdx iscX*** and ***PscrX – iscSUAhscBAfdxiscX*** and ***recA::kn***

DJ1421 x pDB1550 x pDB528. An in-frame deletion in *fdx* (codons 29-64) placed in endogenous, IscR-controlled *fdx* gene. No growth on glucose at 21% or 40% oxygen. Some growth on glucose possible on nitrogen-free glucose media or in a Coy chamber with ~5% oxygen – very limited growth though. Colonies on BN (sucrose) plates are of uniform size.

Gn^R, Kn^R, Rif^R, RecA$^-$

Reason for construction: *Functional analysis of fdx gene product without polar effects on downstream iscX gene and without complication of phenotypes resulting form allele recombination between two fdx alleles.*

DJ1622   ***Pisc- iscRSUAhscBA Δfdx iscX*** and ***PscrX – iscSUAhscBAfdxiscX***

DJ1421 x pDB1550 x pDB1383. A RecA$^+$and Kn^S version of DJ1621. Phenotype same as DJ1621.

Str^R, Rif^R, RecA$^+$

Reason for construction: *Functional analysis of fdx gene product*

DJ1628   **P*isc* -Δ*iscRiscSUAhscBAfdxiscX*** and **P*scrX* - *lacZY::kan***

DJ1601 x pDB1335. This strain has one copy of the *isc* operon under
P*isc*-control with Δ*iscR*. Second *isc* copy under P*scr*-control was replaced
by *lacZ::kan*. Grows poorly on BN plates (slightly slower than DJ1601).
Kn^R, Rif^R, Gen^R, RecA$^+$

Reason for construction: *to obtain an "isc haploid" with deletion in
iscR. Serves as a parent strain for placement of genes of interest under
Pscr-control in a background with elevated Isc expression.*

DJ1644   **P*isc* -Δ*iscRiscSUAhscBAfdxiscX*** and **Δ*nifU***

DJ1620 x pDB1556. Strain contains a Gn^R cartridge interrupting the *cydR*
gene. Strain shows same slow growth phenotype of DJ1601. Growth on
B plates improved (as for DJ1601).
Kn^R, Gn^R, Rif^R.

Reason for construction: *to investigate if an increased cell respiration
rate due to inactivation of cydR gene product, will create a more
anaerobic intracellular environment which might improve growth of
DJ1601 as is observed under diazotrophic growth conditions.*

DJ1645   **P*isc* - *iscRS*Δ*iscUiscAhscBAfdxiscX*** and **P*scrX* - *iscSUAhscBAfdxiscX***
          and **P*benz*-(*His*)-*xylX***

DJ1637 x pDB1350 x pDB528. Placement of an in-frame deletion in *iscU*
gene under P*isc*-control. No growth on glucose, as for DJ1445. This
strain also contains his-tagged *xylX*.

Kan^R, Rif^R, benz+

Reason for construction. *to characterize the cluster content of XylX
(component of benzoate dioxygenase enzyme) when purified from cells
depleted forIscU.*

DJ1647 ϕ(orf1'-lacZ)cysE2 and **P*isc* - *iscRSUΔiscAhscBAfdxiscX*** and

P*scrX* - *iscSUAhscBAfdxiscX*

DJ1551 x pDB1179 x pDB1383. Inactivation of cysE2 gene product in
genetic background containing an in-frame deletion in *iscA* gene in P*isc*
controlled copy of *isc* operon. Strain grows well on glucose as for
DJ1551. On sucrose plates, level of β-gal activity seems the same as
DJ1504. No growth at 40% oxygen.
Str^R, Kn^R, Rif^R.

Reason for construction: *(i) to test the effects of depleting IscA on the
maturation of the [2Fe-2S] cluster on IscR by measuring the change in β-
galactosidase activity following a carbon-source shift from sucrose to
glucose (ii) to see if it is possible to rescue the 40% oxygen, null growth
phenotype associated with ΔiscA by inactivating cysE2. Accumulation of
cysteine in cells has been associated with oxidative stress.*

DJ1650   **P*isc* – *iscRSUiscA^{36CA}hscBAfdxiscX*** and **P*scrX* – *iscSUAhscBAfdxiscX***
DJ1434 x pDB1236.. The variant *iscA36CA* replaces wt *iscA* gene in
endogenous, IscR-controlled operon. Strain obtained by rescuing glucose$^-$
phenotype of DJ1434 and screening for 40% O_2 sensitivity. Absolutely no
growth on glucose at 40% oxygen. Grows like WT at 40% oxygen on
sucrose plates. Growth not checked at 5% oxygen.
Rif^R, Kn^R

Reason for construction: *Functional analysis of cysteine 36 in iscA gene
product.*

DJ1651   **P*isc* – *iscRSUiscA^{99CA}hscBAfdxiscX*** and **P*scrX* – *iscSUAhscBAfdxiscX***
DJ1434 x pDB1215. The variant *iscA99CA* replaces wt *iscA* gene in
endogenous, IscR-controlled operon. Strain obtained by rescuing glucose$^-$
phenotype of DJ1434 and screening for 40% O_2 sensitivity. Absolutely no
growth on glucose at 40% oxygen. Shows some level of transdominance

at 40% oxygen on sucrose plates because colonies take longer (compared to WT) to grow – growth eventually catches up. Growth phenotype not checked at 5% oxygen.

Rif^R, Kn^R

Reason for construction: *Functional analysis of cysteine 99 in iscA gene product.*

DJ1652   **P*isc* – *iscRSUiscA^{101CA}hscBAfdxiscX*** and **P*scrX* – *iscSUAhscBAfdxiscX***

DJ1434 x pDB1216. The variant *iscA101CA* replaces wt *iscA* gene in endogenous, IscR-controlled operon. Strain obtained by rescuing glucose$^-$ phenotype of DJ1434 and screening for 40% O$_2$ sensitivity. Mutation confirmed by PCR using promers / reactions conditions described for DJ1551. Absolutely no growth on glucose at 40% oxygen. Grows like WT at 40% oxygen on sucrose plates. Growth phenotype not checked at 5% oxygen.

Rif^R, Kn^R

Reason for construction: *Functional analysis of cysteine 101 in iscA gene product.*

DJ1653   **P*isc* – *iscRSUiscA^{104SC}hscBAfdxiscX*** and **P*scrX* – *iscSUAhscBAfdxiscX***

DJ1434 x pDB1250. The variant *iscA104SC* replaces wt *iscA* gene in endogenous, IscR-controlled operon. Strain obtained by rescuing glucose$^-$ phenotype of DJ1434 and screening for 40% O$_2$ sensitivity. Mutation confirmed by PCR using promers / reactions conditions described for DJ1551. Absolutely no growth on glucose OR sucrose at 40% oxygen showing strong negative transdominance. Growth phenotype not checked at 5% oxygen.

Rif^R, Kn^R

Reason for construction: *Functional analysis of serine 104 in iscA gene product and effect of introducing a 4th potential thiolate [Fe-S] cluster ligand.*

DJ1655   **φ(orf1'-lacZ)cysE2** and **Pisc - iscRSUΔiscAhscBAfdxiscX** and

PscrX – iscSUAhscBAfdxiscX and **recA::gn**

DJ1647 x pDB1375. Same phenotype as described for DJ1647 except it is

RecA⁻

Str^R, Kn^R, Rif^R, Gn^R, RecA⁻

Reason for construction: *as for DJ1647. This strain eliminates issue*

associated with allele recombination between two iscA copies.

DJ1656   **Pisc – iscRSUiscA^{99CA}hscBAfdxiscX** and **PscrX – iscSUAhscBAfdxiscX**

and **recA::gn**

DJ1650 x pDB1375. This strain is a *recA⁻* version of DJ1650. No growth

on glucose at 40% oxygen. Poor growth on sucrose at 40% oxygen..

Kn^R, Rif^R, Gn^R.

Reason for construction: *Functional analysis of cysteine 99 in iscA gene*

product without complications assoicaited with iscA allele recombination.

DJ1657   **Pisc – iscRSUiscA^{36CA}hscBAfdxiscX** and **PscrX – iscSUAhscBAfdxiscX**

and **recA::gn**

DJ1651 x pDB1375. This strain is a *recA⁻* version of DJ1651. No growth

on glucose at 40% oxygen. Good growth on sucrose at 40% oxygen..

Kn^R, Rif^R, Gn^R.

Reason for construction: *Functional analysis of cysteine 36 in iscA gene*

product without complications assoicaited with iscA allele recombination.

DJ1659   **Pisc – iscRSUiscA^{104SC}hscBAfdxiscX** and **PscrX – iscSUAhscBAfdxiscX**

and **recA::gn**

DJ1653 x pDB1375. This strain is a recA- version of DJ1653. No growth

on 40% oxygen on either glucose or sucrose, therefore displays

transdominant phenotype. Kn^R, Rif^R, Gn^R.

Reason for construction: *Functional analysis of cysteine 36 in iscA gene product without complications assoicaited with iscA allele recombination.*

DJ1661   **P*isc* - *iscRS∆iscUAhscBAfdxiscX* and P*scrX* - *iscSUAhscBAfdxiscX.***
DJ1421 x pDB1391 x pDB1383. Strain has *∆iscUA* in P*isc* controlled copy of *isc* operon. It shows same phenotype as DJ1434 except it was constructed to have Str^R, instead of Kan^R. No growth on glucose. Rif^R, Str^R.

DJ1662   **P*isc* – *iscRSUiscA^{101CA}hscBAfdxiscX* and P*scrX* – *iscSUAhscBAfdxiscX* and *recA::gn***
DJ1652 x pDB1375. This strain is a *recA$^-$* version of DJ1652. No growth on glucose at 40% oxygen. Good growth on sucrose at 40% oxygen. Kn^R, Rif^R, Gn^R.
Reason for construction: *Functional analysis of cysteine 101 in iscA gene product without complications assoicaited with iscA allele recombination.*

DJ1666   **P*ara* – *iscSUA(∆hscBA)fdxiscX***
DJ1418 x pDB1563 x pDB1383. This strain has *iscS, iscU, iscA and fdx* under control of Para in the α-glc region (P*scr*) of *A.v*. Construction of this strain required plating of 80 BNXStr^R plates – only 7 Amp^S, Kan^S strains obtained. PCR confirms location and intergrity of this construct. Primers used:Pscr-5', GTC TGG CAC GGT TGC CCT ATG and AraC-3', TCCCGGCAATAGCGGATC using Buffer D and annealing temp.=55°C. IscS, IscU, IscA and Fdx barely visible in SDS-PAGE of crude extracts prepared following 3 hour arabinose induction at OD$_{600}$ = 1.0. Grows quite well on BN-Ara at 30°C, but weakly at room temperature. Grows quite well at 40% oxygen. Str^R

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Reason for construction: to use arabinose-induction as a method of controlling expression of Isc proteins for genetic analsysi or for purification of Isc components.

DJ1668   ***Para – iscSUAhscBAfdxiscX***

DJ1418 x pDB1562 x pDB1383. This strain has whole *isc* operon under pAra control in the α-*glc* region (P*scr*) of *A.vinelndii*. Construction of this strain required plating of 80 BNXStr^R plates – only 9 Amp^S, Kn^S strains obtained. PCR (as described for DJ1666) confirms that the *isc* operon is under P*ara* control. Could not really see evidence of abundant expression of Isc proteins on SDS-PAGE gel of crude extracts. Strain grows visibly slower on BN at 30°C (not as slow as DJ1601), but barely grows at room temperature. Very poor growth at 40% oxygen.
Str^R.

Reason for construction: to use arabinose-induction as a method of controlling expression of Isc proteins for genetic analsysi or for purification of Isc components.

DJ1670   ***Pisc - iscRSUΔiscAhscBAfdxiscX*** and ***Pscr – iscSUAhscBAfdxiscX***

DJ1661 x pDB1391. Strain is similar to DJ1551 except has Str^R, instead of Kan^R A 285 bp in-frame deletion placed in endogenous, IscR-controlled *iscA* gene. Removes amino acids: IscA 8 – IscA 25. Strain can grow on glucose at 21% oxygen but not at 40% oxygen. Strains isolated by rescuring the glucose$^-$ phenotype of DJ1661. Deletion confirmed by PCR with primers *iscR-5'* and *iscA-3'*as described for DJ1434. Expected product size = 2.2 kb.
Str^R Rif^R

Reason for construction: Functional analysis of IscA gene product and to serve as a parent strain for the construction of an isc haploid strain (with ΔiscA) by removal of the Pscr-controlled isc copy using pDB1335 (Pscr_lacZY::kan).

DJ1684   ***Pisc – iscRSUAhscBAfdxiscX*** and ***Para –iscU^{37CA}***

DJ1418 x pDB1593 x pDB1383. Constructed in collaboration with Callie
Raulfs. This strain places *iscU37CA* under control of the arabinose
promoter located in the α-glc region (P<u>scr</u>) of *A.vinelndii*. There is no
discernable growth phenotype on BNara plates. Confirmed by PCR and
sequence analysis.

Str^R

Reason for construction: *to test the hypothesis that substitution of*
cysteine 37 to alanine in IscU results in a negative transdominat
phenotype that is detrimental for the cell.

DJ1692   ***PscrX –hscBAfdxiscX***

DJ1418 x pDB1608 x pDB1383. Strain contains *hscB-hscA-fdx-iscX*
under P*scr* control. Transformation required 3 liters BNXStr plates.
Obtained ~14 white colonies in total. Only 3 were Amp^S and Kan^S. Not
confirmed by PCR.

Str^R

Reason for construction: *to serve as the parent strain for investigating if*
HscB and HscA are essential for A. vinelandii viability. Placement of
hscBAfdxiscX genes directly under Pscr-control removes the putative
internal promoter within the isc operon that may account for the slow
growth phenotype if DJ1447.

DJ1694   ***Pisc - iscRSUAΔhscBAfdxiscX*** and ***PscrX –hscBAfdxiscX*** and ***recA::kan***

DJ1692 x pDB1291 x pDB528. Strain contains P*scr*-*hscBA-fdx-iscX* and
an in-frame deletion in the *hscBA* genes under P*isc*-control as in DJ1447.
Absolutely no growth on glucose at 21% or 40% oxygen. Slow growth on
glucose at 5% O$_2$.

Kn^R, Str^R, RecA$^-$.

Reason for construction: to investigate if HscB and HscA are essential for A. vinelandii viability in the absence of a putative internal promoter within the isc operon.

DJ1695   **P*isc* - *iscRSUAhscBAΔfdxiscX*** and **P*scrX* –*hscBAfdxiscX*** and **recA::kan**

DJ1692 x pDB1550 x pDB528. Strain contains *Pscr-hscBA-fdx-iscX* and an in-frame deletion in the *fdx* gene under P*isc*-control as in DJ1621. No growth on glucose. "Slower than WT" growth on sucrose. Correct recombinants were visibly smaller colonies.   Very, very slow growth on glucose at 5% O_2.
Kn^R, Str^R, RecA$^-$.

Reason for construction: functional analsysi of fdx in the absence of a putative internal promoter within the isc operon. Also to investigate if loss of fdx may affect other cellular functions.

DJ1699   **P*isc* – *iscRSUiscA^{104SC}hscBAfdxiscX*** and **P*scrX* – *iscSUAhscBAfdxiscX*** and **recA::gn**

DJ1661 x pDB1611. The variant *iscA104SA* replaces wt *iscA* gene in endogenous, IscR-controlled operon. Strain obtained by rescuing glucose$^-$ phenotype of DJ1661. Mutation confirmed by PCR using promers / reactions conditions described for DJ1551. Absolutely no growth on glucose at 40% oxygen. Unlike DJ1653 (*iscA104SC*), this strain grows well on sucrose at 40% oxygen. Growth phenotype not checked at 5% oxygen.
Rif^R, Str^R

Reason for construction: Functional analysis of serine 104 in iscA gene product andto verify if the negative transdominance observed for DJ1653 (iscA104SC), is a result of the addition of cysteine or loss of serine.

DJ1700   **_Pisc – iscRSUiscA^{103EK}hscBAfdxiscX_** and **_PscrX–iscSUAhscBAfdxiscX_**

         and

recA::gn

DJ1661 x pDB1612. The variant *iscA103EK* replaces wt *iscA* gene in endogenous, IscR-controlled operon. Strain obtained by rescuing the glucose⁻ phenotype of DJ1661. Strain grows well on glucose at 20% O_2, but does <u>not</u> grow on glucose at 40% O_2 or on sucrose at 40% O_2. It therefore exhibits a transdominant phenotype as observed for DJ1653 (*iscA*104SC).

Rif^R, Str^R, RecA⁻

Reason for construction: *Functional analysis of glutamate 103 in iscA gene product. This substitution to lysine converts the CG**E**S motif in IscA into the CG**K**S motif found in IscA^{Nif}.*

DJ1701   **_Pisc - iscRSUΔiscAφ(hscA'-lacZ)fdxiscX_** and **_Pscr–iscSUAhscBAfdxiscX_**

DJ1670 x pDB1468. Inactivation of both *iscA* and *hscA* in Pisc-controlled isc operon. Strain is light blue on sucrose after 4-5 days. No growth on glucose under normal ambient conditions.

Rif^R, Str^R, Kn^R

Reason for construction: *to investigate the effects of inactivating both IscA and HscA on A. vinelandii viability at 21% oxygen. My inability to obtain a strain 'haploid'f for the isc genes and with a deletion in iscA has led me to believe that a small amount of IscA is actually essential under normal growth conditions. This construction lends some support to that hypothesis since a null growth phenotype is displayed that is different from DJ1551 and DJ1447.*

DJ1702   ***P*isc - iscRSUΔiscAhscBAfdxiscX* and **P*scr–iscSUA*ϕ(hscA'-lacZ)**
          fdxiscX

DJ1670 x pDB1468. Inactivation of *iscA* in the P*isc*-controlled operon
and *hscA* in the P*scr* controlled operon. Stain is light blue on sucrose after
4-5 days. Normal growth on glucose under normal ambient conditions.
Rif^R, Str^R, Kn^R, RecA+.

Reason for construction: *to serve as a parent strain for replacing the*
Pscr-controlled isc operon with just the iscA gene under Pscr control.

VITA

Deborah Cumarsawmy Johnson was born on November 10th, 1970 in Kuala Lumpur, Malaysia, daughter of Param and Rosy Cumaraswamy. She moved to Milan, Italy in 1983 where she completed her secondary education at The International School of Milan. Deborah received a B.S. degree in Microbiology from the University of Warwick (UK) in 1992 and a Postgraduate Diploma in Education (PGDE) from the University of Southampton (UK) in 1996. She worked as a science teacher at Priory Secondary School in Portsmouth (UK) before moving to Blacksburg, Virginia (USA) in 1997 where she taught Biology at Blacksburg High School. She returned to graduate school in 2000 and completed her M.S. and Ph.D at Virginia Tech in the Departments of Biology and Biochemistry, respectively. She is married to Professor Martin E. Johnson and has an 18-month old son named Liam.