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

Deborah C. Johnson, Mihaela-Carmen Unciuleac, and Dennis R. Dean*

Department of Biochemistry, Virginia Tech, Blacksburg, Virginia 24061

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A system for the controlled expression of genes in *Azotobacter vinelandii* by using genomic fusions to the sucrose catabolic regulon was developed. This system was used for the functional analysis of the *A. vinelandii isc* genes, whose products are involved in the maturation of [Fe-S] proteins. For this analysis, the *scrX* gene, contained within the sucrose catabolic regulon, was replaced by the contiguous *A. vinelandii iscS*, *iscU*, *iscA*, *hscB*, *hscA*, *fdx*, and *iscX* genes, 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 [Fe-S] protein maturation components was achieved by placing a mutation within a particular P*isc*-controlled gene with subsequent repression of the corresponding P*scrX*-controlled component by growth on glucose as the carbon source. This experimental strategy was used to show that IscS, IscU, HscBA, and Fdx are essential in *A. vinelandii* and that their depletion results in a deficiency in the maturation of aconitase, an enzyme that requires a [4Fe-4S] cluster for its catalytic activity. Depletion of IscA results in a null growth phenotype only when cells are cultured under conditions of elevated oxygen, marking the first null phenotype associated with the loss of a bacterial IscA-type protein. Furthermore, the null growth phenotype of cells depleted of HscBA could be partially reversed by culturing cells under conditions of low oxygen. Conserved amino acid residues within IscS, IscU, and IscA that are essential for their respective functions and/or whose replacement results in a partial or complete dominant-negative growth phenotype were also identified using this system.

Iron-sulfur clusters ([Fe-S] clusters) are simple prosthetic groups comprised of inorganic Fe^{2+}/Fe^{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 (5, 16). 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+}/Fe^{3+} and S^{2-} under reducing conditions (36). 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+}/Fe^{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 references 4 and 25). 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 (22, 23). The Nif system includes two proteins, NifS and NifU, which are 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, respectively (1, 13, 66, 68, 69). More recently, it has been shown that the Nif type of 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 (3, 45, 60). 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 (Fig. 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 as either an alternative scaffold or an agent of iron delivery to the IscU scaffold (11, 12, 29, 43). 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 (29). 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 the interaction of the HscBA complex with the IscU scaffold (9, 19, 21, 54, 58). Neither the function of Fdx, which contains a redox-active [2Fe-2S] cluster, nor the function of IscX is known (26, 27, 53). IscR is a regulatory protein that apparently controls the expression of the *isc* gene cluster in a negative feedback loop that involves the assembly of a [2Fe-2S] cluster within IscR (52). In addition to their role in the maturation of [Fe-S] proteins, Isc components have been shown to be involved in the biogenesis of certain S-containing cofactors ei-

* Corresponding author. Mailing address: Department of Biochemistry, Virginia Tech, Blacksburg, VA 24061. Phone: (540) 231-5895. Fax: (540) 231-7126. E-mail: deandr@vt.edu.

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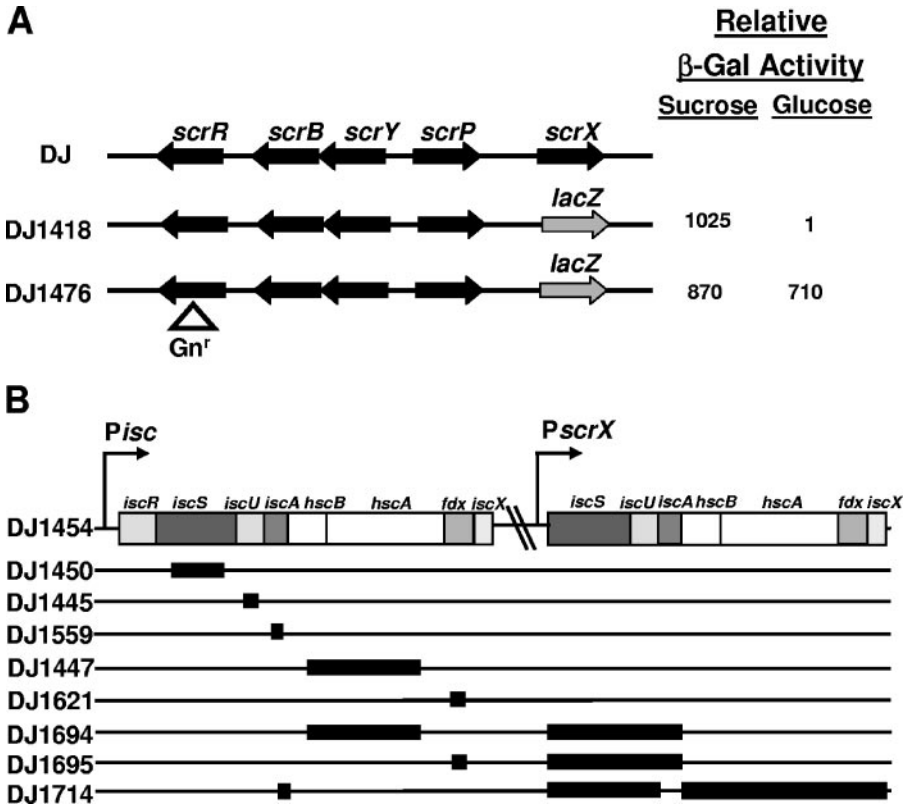

FIG. 1. Schematic representation of the relevant genetic organization of key strains used in this work. (A) Organization of the *A. vinelandii* sucrose catabolic regulon. DJ refers to the wild-type strain. DJ1418 has the *scrX* gene replaced by *lacZ*. DJ1476 was derived from DJ1418 and carries an insertion 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. β-Gal, β-galactosidase.

ther directly through S transfer by IscS or indirectly through the participation of [Fe-S] proteins (39).

A third system for [Fe-S] protein maturation, discovered in *Escherichia coli*, is called the Suf system (57). 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 (32, 46). SufS and SufE represent a two-component cysteine desulfurase with a function that is analogous to those of NifS and IscS (35, 47), SufA bears primary sequence homology to IscA (42), and SufB, SufC, and SufD form a complex of unknown function that has intrinsic ATPase activity (41, 47). 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* (34).

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 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 has been achieved. Owing to the facile genetic manipulation of *A. vinelandii*, the fact that it does not contain an intact Suf system, and the fact that the Nif system is expressed only under nitrogen-fixing conditions, this organism provides an opportunity for a 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.

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 at The Institute for Genomic Research website (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl). DNASTAR Lasergene software 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 (56) 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 0.08 μg/ml ampicillin, 0.5 μg/ml kanamycin, 0.05 μg/ml gentamicin, 5.0 μg/ml rifampin, and 0.1 μg/ml streptomycin. X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) was added to a final concentration of 60 μg/ml. Chromosomal DNA was extracted from *A. vinelandii* with QuickExtract DNA extraction solution (Epicenter).

Plasmid construction. Construction of the key parent plasmids used in this study is described below. Relevant subclones of each parent plasmid are listed

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

Plasmid[a]	Relevant gene(s) cloned[b,c]	Vector	Reference or source
pDB1264	**18-kb BglII *A. vinelandii* chromosomal *isc* operon fragment**	**pUC119**	**This study**
pDB1286	3.5-kb HindIII *hscBA-fdx-iscX* fragment	pUC119	This study
pDB1291	5.2-kb BspHI *iscSUA-hscBA-fdx-iscX* fragment with SbfI-Δ*hscBA* (aa HscB 78–HscA 442)	pAra13	This study
pDB1316	5.2-kb BspHI *iscSUA-hscBA-fdx-iscX* fragment under P*scrX* control	pUC7	This study
pDB1608	3.2-kb PciI-BglII *hscBA-fdx-iscX* 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] replacing ΔPciI-KpnI fragment of *scrX*; introduces the following unique sites; PciI, XbaI, XhoI, and KpnI	pUC7	This study
pDB1316	5.2-kb BspHI *iscSUA-hscBA-fdx-iscX* fragment under P*scrX* control	pUC7	This study
pDB1332	MCS 2[e] replacing ΔPciI-KpnI in *scrX*; introduces the unique sites PciI, NruI, EcoRV, XhoI, BglII, XbaI, SphI, and KpnI	pUC7	This study
pDB1335	6.1-kb SmaI-*lacZ-kan* fragment under P*scrX* control	pUC7	This study
pDB1608	3.2-kb PciI-BglII *hscBA-fdx-iscX* fragment under P*scrX* control	pUC7	This study
pDB1634	0.4-kb PciI-BamHI *iscA* fragment under P*scrX* control	pUC7	This study
pDB945	**1-kb EcoRI *fdx* fragment**	**pUC119**	**67**
pDB1016	HincII-*kan* from pUC-KAPA in EcoRV site of *fdx* gene	pUC119	This study
pDB1550	XhoI-Δ*fdx* (Fdx aa 29–64)	pUC119	This study
pDB933	**2.5 kb EcoRI-SstI *iscSUA* fragment**	**pUC119**	**67**
pDB954	SalI-Δ*iscS* (IscS aa 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
pDB1350	Δ*iscU* (IscU aa 31–117) from Bal31 exonuclease digestion at DraIII	pT_7-7	This study
pDB1351	Δ*iscUA* (IscU aa 34–IscA 9) from Bal31 exonuclease digestion at SexAI in *iscA*	pT_7-7	This study
pDB1391	Δ*iscA* (IscA aa 8–125) from Bal31 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
Other			
pDB528	*recA*::Tn*10* (Kn^r)	pSUP102	63
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 pWKR2021 in StuI site of PCR-derived *scrR* gene	pUC19	This study
pDB1468	6.1-kb SmaI-*lacZ-kan* from pLKC482 in AleI site of PCR-derived *hscA* gene	pT_7-7	This study

[a] Key parent plasmids are written in boldface type. Plasmid derivatives are listed directly below the parent.
[b] Sizes of gene deletions are described as amino acid (aa) deletions.
[c] Descriptions of site-directed amino acid substitutions include the corresponding DNA codon change.
[d] A 45-bp multiple cloning site (see Materials and Methods for sequence).
[e] A 35-bp multiple cloning site (see Materials and Methods for sequence).

and described in Table 1. Preparations, restriction enzyme digestion, and ligation of hybrid plasmid DNAs were performed according to previously described techniques (50). Restriction endonucleases and Bal31 exonuclease, DNA ligase, and T4 DNA polymerase were purchased from New England Biolabs, Promega, and Invitrogen, respectively. *Escherichia coli* TB1 and XL10 GOLD (Stratagene) were used for the cloning of over 30 different hybrid plasmids containing genomic DNA from *A. vinelandii*. PCR was performed using a commercial kit (Failsafe PCR PreMix selection kit). Cloning of PCR products into relevant vectors was achieved by using primers with engineered restriction sites or the PCR4TOPO vector (Invitrogen). Antibiotic gene cartridges used to create insertion mutations in specific genes were derived from pWKR202I for gentamicin (*gn*; laboratory stock), pUC4-KAPA for kanamycin (*kan*; Pharmacia), and pHP45Ω for streptomycin (*str*) (15). The 6.1-kb *lacZ-kan* cartridge was obtained from pLKC480 (59).

pDB1264. Restriction digestion of chromosomal DNA from DJ1179 (67) 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. Subclones of pDB1264 include pDB1286, pDB1291, pDB1316, and pDB1608 (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* coding sequence was replaced with a 45-bp or 35-bp double-stranded DNA linker to introduce multiple cloning sites (MCS) designated MCS 1 and MCS 2, respectively, permitting the placement of the desired gene(s) under the control of the *scrX* transcriptional and translational regulatory elements (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 1.7-kb *scrR* gene, the 0.98-kb

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 P*scrX*	Other gene mutation(s)[b]	Growth on glucose[c]
DJ	NA	None	*scrX*	None	+
DJ1411	pDB1309	None	*scrX*::*kan*	None	+
DJ1418	pDB1335	None	*lacZ-kan*	None	+
DJ1421	pDB1316	None	*iscSUA-hscBA-fdx-iscX*	None	+
DJ1434	pDB1351	Δ*iscUA*	*iscSUA-hscBA-fdx-iscX*	None	−
DJ1445	pDB1350	Δ*iscU*	*iscSUA-hscBA-fdx-iscX*	*recA*::*kan*	−
DJ1447	pDB1291	Δ*hscBA*	*iscSUA-hscBA-fdx-iscX*	*recA*::*kan*	Slow
DJ1448	pDB1227	*iscU* ($IscU^{C106A}$)	*iscSUA-hscBA-fdx-iscX*	*recA*::*kan*	−
DJ1450	pDB954	Δ*iscS*	*iscSUA-hscBA-fdx-iscX*	*recA*::*kan*	−
DJ1451	pDB1209	*iscS* ($IscS^{C325A}$)	*iscSUA-hscBA-fdx-iscX*	*recA*::*kan*	−
DJ1452	pDB1228	*iscU* ($IscU^{C63A}$)	*iscSUA-hscBA-fdx-iscX*	*recA*::*kan*	−
DJ1453	pDB1058	*iscU* ($IscU^{D39A}$)	*iscSUA-hscBA-fdx-iscX*	*recA*::*kan*	−
DJ1454	pDB1316	None	*iscSUA-hscBA-fdx-iscX*	*recA*::*kan*	+
DJ1463	pDB1016	*fdx*::*kan*	*iscSUA-hscBA-fdx-iscX*	*recA*::*kan*	−
DJ1476	pDB1400	None	*lacZ-kan*	*scrR*::*gn*	+
DJ1559	pDB1391	Δ*iscA*	*iscSUA-hscBA-fdx-iscX*	*recA*::*gn*	+
DJ1607	pDB1538	*iscU* ($IscU^{H105A}$)	*iscSUA-hscBA-fdx-iscX*	*recA*::*kan*	−
DJ1608	pDB1546	*iscU* ($IscU^{K103A}$)	*iscSUA-hscBA-fdx-iscX*	*recA*::*kan*	−
DJ1621	pDB1550	Δ*fdx*	*iscSUA-hscBA-fdx-iscX*	*recA*::*kan*	−
DJ1656	pDB1236	*iscA* ($IscA^{C36A}$)	*iscSUA-hscBA-fdx-iscX*	*recA*::*gn*	+
DJ1657	pDB1215	*iscA* ($IscA^{C99A}$)	*iscSUA-hscBA-fdx-iscX*	*recA*::*gn*	+
DJ1661	pDB1351	Δ*iscUA*	*iscSUA-hscBA-fdx-iscX*	None	−
DJ1662	pDB1216	*iscA* ($IscA^{C101A}$)	*iscSUA-hscBA-fdx-iscX*	*recA*::*gn*	+
DJ1670	pDB1391	Δ*iscA*	*iscSUA-hscBA-fdx-iscX*	None	+
DJ1692	pDB1608	None	*hscBA-fdx-iscX*	None	+
DJ1694	pDB1291	Δ*hscBA*	*hscBA-fdx-iscX*	*recA*::*kan*	−
DJ1695	pDB1550	Δ*fdx*	*hscBA-fdx-iscX*	*recA*::*kan*	−
DJ1702	pDB1468	Δ*iscA*	*iscSUA-hscB*(ϕ*hscA′-lacZ*)*fdx-iscX*	None	+
DJ1714	pDB1634	Δ*iscA*	*iscA*	None	+

[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. Antiobiotic gene cartridges are *gn*, encoding gentamycin resistance (Gn^r), and *kan*, encoding kanamycin resistance (Kn^r).
[c] Refers to growth on Burks minimal medium containing glucose as the sole carbon source at 30°C and 20% O_2. Growth on medium containing sucrose under the same conditions was normal.

recA partial gene fragment, the 0.4-kb *iscA* gene, 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 pDB1400, pDB1375, pDB1634, and pDB1468, respectively.

In-frame deletions in *isc* genes were created by one of two methods: (i) restriction enzyme digestion and religation, as in pDB954 (Δ*iscS*), pDB1291 (Δ*hscBA*), and pDB1550 (Δ*fdx*), and (ii) Bal31 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 sequencing facility.

Strain construction. Key strains constructed during the completion of this work are listed in Table 2. The placement of mutations within the *A. vinelandii* genome was achieved by transformation of competent cells with the appropriate plasmids, followed by selection and/or screening of cells that have undergone double-reciprocal recombination between the genome and plasmid vector(s). Transformation of *A. vinelandii* was performed as previously described (22). Strains transformed with plasmids harboring genes interrupted with *lacZ* and/or antibiotic resistance cartridges were selected by plating onto Burks agar plates supplemented with X-Gal and/or the relevant antibiotic. Specific in-frame gene deletions or point mutations were transferred to the *A. vinelandii* genome using congression (coincidental transfer of genetic markers) using rifampin (Rif^r), kanamycin (Kn^r), gentamicin (Gn^r), or streptomycin (Str^r) resistance as the selection marker as previously described (22).

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 double-reciprocal recombination events that occurred between the appropriately constructed plasmid and the *A. vinelandii* genome. DJ1421 served as the parent for the initial construction of all strains containing a duplicated *iscSUA-hscBA-fdx-iscX* operon under the control of the P*scrX* transcriptional and translational control elements. Construction of DJ1421 was achieved by transformation of DJ1418 ($LacZ^+$) using pDB1316 DNA. Colonies of DJ1418 formed blue colonies when grown on plates containing sucrose as the carbon source and supplemented with X-Gal, whereas cells having undergone the desired double-crossover events to yield DJ1421 were identified as 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 *hscBA-fdx-iscX* operon under the control of the P*scrX* transcriptional and translational control elements, served as the parent strain for DJ1694 and DJ1695 (Table 2). A blue/white screening process was also used to construct DJ1714, which contains only a duplicated *iscA* gene copy under the control of the P*scrX* transcriptional and translational control elements. DJ1714 is the result of a double reciprocal recombination event between plasmid pDB1634 and the *hscA′-lacZ* gene fusion located within the P*scrX* region of the parent strain, DJ1702, and produces white colonies ($LacZ^-$) on X-Gal-supplemented Burks (sucrose) agar plates. PCR and DNA sequencing analysis confirmed that the duplicated *iscA* gene was placed under the control of P*scrX*.

Most strains with 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^{slow} phenotype). Transformants were first plated onto Burks agar plates containing sucrose as the carbon source and were subsequently screened for loss of growth or 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 (Table 2). The correct location and integrity of genomic mutations were verified by PCR or DNA sequence analysis (63).

The isolation of 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 and DJ1670 was accomplished by rescuing the Glc^- phenotype of DJ1434 and DJ1661, respectively, using plasmid pDB1391, which restored an intact *iscU* gene but maintained a

deletion only within *iscA*. Other strains containing point substitutions in the *iscA* gene were also constructed by using the same strategy (Table 2).

Depletion experiments. Two liters of sucrose-containing minimal medium was inoculated with the parental strain (DJ1454) or *isc* mutant strains and grown to 150 Klett units (red filter) or an optical density at 600 nm of 1.4 at 30°C and 300 rpm. Cell pellets were harvested by centrifugation (4,225 × *g* for 5 min) and washed twice with Burks minimal medium containing glucose as the sole carbon source. Resuspended cells were diluted into fresh glucose medium at a 1:16 or 1:1,000 dilution. Depletion of the relevant *isc* gene product was allowed to progress for 11 to 25 h until cell growth stopped or slowed down relative to that of DJ1454 (between four and five doubling times for DJ1450 Δ*iscS* and DJ1445 Δ*iscU* and eight doubling times for DJ1463 *fdx*::*kan* and DJ1447 Δ*hscBA*). Cells were harvested by centrifugation as described 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 × *g* for 30 min. Supernatants were immediately placed into sealed airtight 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 (7). Aconitase activity was measured spectrophotometrically at 240 nm at room temperature by monitoring the production of *cis*-aconitate (3.4 mM^{-1} cm^{-1} at 240 nm) (49). Assays (1-ml volume) were conducted using sealed, anoxic cuvettes containing 10 μl to 50 μl supernatant and 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 measuring the production of NADPH (8).

β-Galactosidase assays. β-Galactosidase activity was determined using an assay adapted from a method described previously by Miller (40). Cells were grown in sucrose- or glucose-containing medium to mid-log or late log phase, and assays were conducted using the soluble fraction of crude extracts prepared by sonication and centrifugation. Relative β-galactosidase activities represent the specific rate of the 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 regassed by flushing with 40% O_2 balanced with 60% N_2 using a regulated gas tank (Airgas Inc.). No more than four (100- by 15-mm) 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 immunoblotting. Cell pellets for DJ1454 (intact *iscU*) and DJ1445 (Δ*iscU*) were obtained from the depletion experiments described above. Soluble protein extracts were prepared by French cell press (12,000 lb/in^2) in degassed, argon-sparged 100 mM Tris-HCl (pH 8.0) and clarified by centrifugation at 100,000 × *g* for 30 min. Total protein concentrations were measured using the biuret method to ensure equal loading (26 μg per lane) on 20% sodium dodecyl sulfate-polyacrylamide gels. Gels were either stained with Coomassie dye or transferred onto nitrocellulose. A chemiluminescent detection system was used (LumiPhosWB; Pierce), and the blotting protocol recommended by the supplier was followed, using 4% nonfat 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 served as the secondary antiserum (1:30,000) (Sigma-Aldrich Inc.).

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 (67). 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 the 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 necessary to first identify a candidate system for controlled gene expression within *A. vinelandii* and then demonstrate that controlled expression could be accomplished. A survey of the preliminary *A. vinelandii* genome sequence revealed a cluster of genes that we have designated "*scr*" genes owing to the apparent involvement of their products in sucrose catabolism (Fig. 1A). The *scrR* gene encodes a protein bearing sequence identity to the *lacI* gene product. The *scrB* and *scrY* genes encode proteins that have primary sequence identity compared to known sucrases and the *E. coli* LacY protein, respectively. The divergently transcribed *scrP* gene product encodes a protein with sequence identity to carbohydrate-specific porins. The *scrX* gene, located approximately 500 bp downstream from *scrY*, encodes a protein with sequence identity to α-glucosidases. The organization (Fig. 1A) and primary sequences of these genes suggest that they represent a sucrose catabolic regulon that is negatively controlled by ScrR in a way similar to that of 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 suggested the possibility that each of them could be coordinately regulated by ScrR but individually expressed from separate promoters.

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 of the *scrX* gene, and β-galactosidase activity was measured in cells that were cultured using either sucrose or glucose as the carbon source (Fig. 1A). Loss of *scrX*, either by replacement with *lacZ* (DJ1418) (Table 2 and Fig. 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 (Fig. 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 and Fig. 1A). This result is consistent with the hypothesis that ScrR is a *trans*-acting negative regulatory element functionally analogous to LacI.

Duplication and *scr*-directed expression of the *isc* gene region. The availability of an *A. vinelandii* strain with expression of *lacZ* under the 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 the control of the *scrX* promoter (P*scrX*). Such constructions can be achieved by using an appropriately constructed plasmid (Table 1) and re-

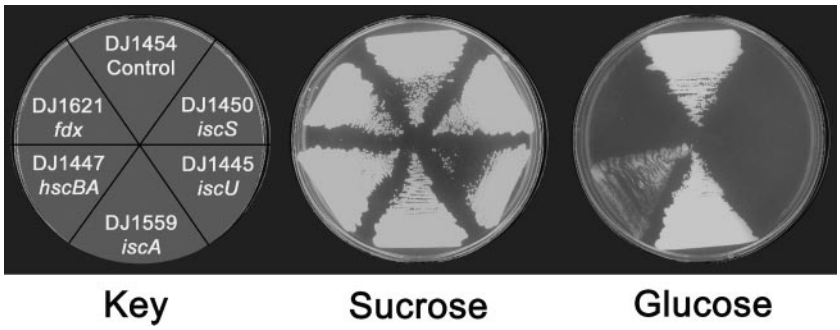

FIG. 2. Effect of depletion of Isc components in *A. vinelandii*. Shown are growth phenotypes, using sucrose or glucose as the carbon source, of strains that carry a deletion in an IscR-regulated copy of an *isc* gene but that contain an intact copy of the corresponding ScrR-regulated copy. A schematic representation of these strains is shown in Fig. 1B.

ciprocal recombination events that occur during DNA transformation. When strain DJ1418 is used for such constructions, recombinants that result in the excision of *lacZ* and the 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 the insertion of the gene of choice are white. In the present work, an *A. vinelandii* strain (DJ1421) where the contiguous *iscS*, *iscU*, *iscA*, *hscB*, *hscA*, *fdx*, and *iscX* genes were duplicated and their expression was placed under the control of the *scrX* promoter (P*scrX*) was constructed (Table 2) (see Materials and 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 create strain DJ1454 (Table 2 and Fig. 1B). The location and integrity of the duplicated *isc* region within DJ1454 were 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 region, one whose expression is under the control of P*isc* and the other whose expression is 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 a subsequent examination of the effect of culturing such mutants under conditions that repress the expression of the corresponding P*scrX*-regulated *isc* genes. In this system, growth of mutant strains on medium 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 Fig. 2, mutant strains individually deleted within P*isc*-regulated copies of *iscS*, *iscU*, or *fdx* are able to grow when cultured using sucrose as the carbon source but cannot grow when cultured using glucose as the carbon source. 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 HscBA resulted in only a slower-growth phenotype (Fig. 2).

In the case of *E. coli*, there is evidence for expression of *hscBA-fdx-iscX* that occurs independently from the expression of *iscRSUA* (33). In the present work, *lacZ* fusions were used to show that a very low level of expression of *hscBA-fdx-iscX* also occurs independently from *iscRSUA* (data not shown). Thus, the low level of growth recognized under conditions expected to deplete HscBA could be the consequence of a low level of endogenous expression that occurs independently from the *scrX* promoter. It should be noted that because the depletion of *fdx* by this method results in a clear null growth phenotype, there must also be a significant level of depletion of HscBA in these experiments. We therefore considered the possibility that HscBA are essential but not sufficiently depleted by this method to elicit a clear null growth phenotype. This possibility was tested by the construction of a strain where the transcription of *hscB* and downstream genes is placed directly under the control of the *scrX* promoter and where the *iscR*-regulated copy of *hscBA* is deleted (DJ1694) (Fig. 1B). Another strain was constructed where the expression of *iscA* was similarly placed directly under the control of the *scrX* promoter and the IscR-regulated copy of *iscA* was deleted (DJ1714) (Fig. 1B). Growth of these strains when cultured using either sucrose or glucose as the carbon source (Fig. 3) shows that HscBA are essential in *A. vinelandii* but that IscA is not.

Depletion of IscA results in an oxygen-sensitive phenotype. Because IscA has been proposed to serve as a possible iron donor during [Fe-S] cluster assembly, we tested for the elicitation of a phenotype by culturing cells depleted of IscA under conditions of iron limitation. However, no distinguishing phenotype was recognized under these conditions. In contrast, a clear null growth phenotype was revealed when cells depleted of IscA were cultured under conditions where the atmospheric level of oxygen was increased from ambient (~20%) to 40% (Fig. 3). The oxygen-sensitive phenotype associated with the depletion of IscA using strain DJ1714, where the expression of *iscA* is directly under the control of the *scrX* promoter, was also observed under the same conditions using DJ1559, where a low level of *scrX* promoter-independent expression of *iscA* might occur.

Growth under low-oxygen conditions partially suppresses the growth phenotype associated with HscBA depletion. Given the oxygen-sensitive growth phenotype associated with the depletion of IscA, it was of interest to examine whether or not the null phenotype associated with the depletion of other Isc components could be spared by culture under conditions of low oxygen availability. The result of this analysis revealed that the null growth phenotype associated with the depletion of HscBA

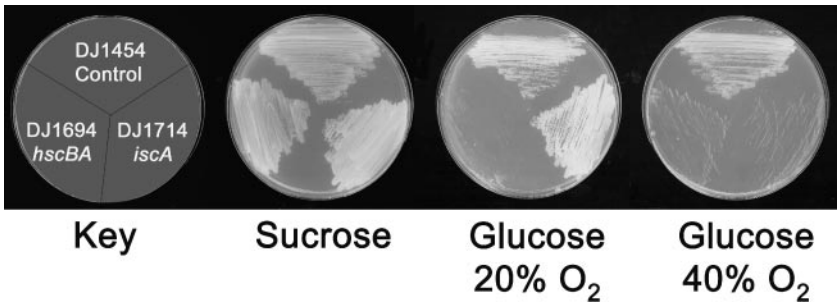

FIG. 3. HscBA are essential Isc components, while IscA is essential only under conditions of elevated oxygen. Strains were cultured in medium using glucose as the carbon source under ambient (~20%) oxygen or 40% oxygen. Strain DJ1714 has a deletion in the IscR-regulated copy of *iscA* and an intact duplicated copy of *iscA* whose expression is directly controlled by Scr regulatory elements. Strain DJ1694 has a deletion in the IscR-controlled *hscBA* copy and a duplicated copy of *hscB-hscA-fdx-iscX* whose expression is controlled by P*scrX*. A schematic representation of the strains used is shown in Fig. 1B. All strains showed normal growth when cultured using sucrose as the carbon source under ambient (~20%) oxygen.

could be partially reversed under conditions of low oxygen availability (Fig. 4), but the null growth phenotype associated with depletion of IscS, IscU, or Fdx persisted under these same conditions.

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 of various IscR-regulated Isc components and whose growth phenotypes are shown in Fig. 2. 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 h after the appropriate strain was shifted from using 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 the depletion of IscS, IscU, HscBA, and Fdx are shown in Fig. 5A. Results summarized in Fig. 5B reveal a very dramatic loss of aconitase activity upon the depletion of IscS or IscU, a moderate effect on aconitase activity upon the depletion of HscBA and Fdx, and a small but reproducible effect on aconitase activity upon the depletion of IscA. It should be emphasized that the effect on aconitase activity resulting from the depletion of one particular Isc component by this method cannot be meaningfully compared to the effect of the 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 that were used to examine the effect of the depletion of HscBA and Fdx shown in Fig. 5A and B have an intact duplicated *isc* region under the control of P*scrX*. Thus, as described above, there is some endogenous expression of HscBA and Fdx in these experiments, and consequently, the less severe biochemical phenotype observed for the depletion of HscBA or Fdx than that observed for the depletion of IscS or IscU could be expected. Depletion of IscU using the method described in this work was also established by Western analysis (Fig. 5C). In this case, a 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.

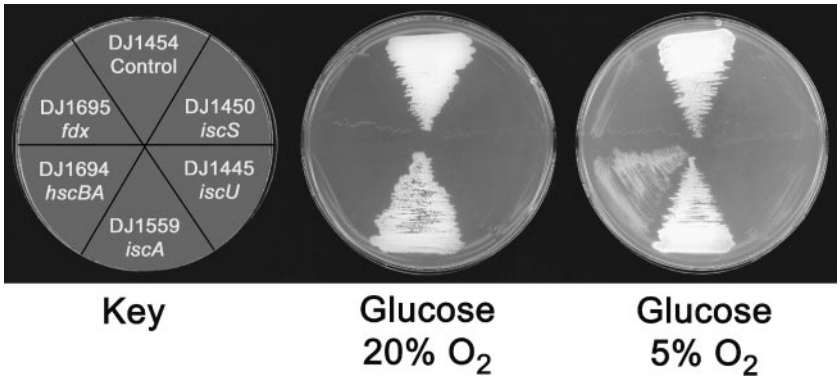

FIG. 4. Effect of low oxygen on growth in cells depleted of Isc components. Strains used for the depletion of IscS (DJ1450), IscU (DJ1445), or IscA (DJ1559) also encode a duplicated copy of *iscS-iscU-iscA-hscB-hscA-fdx-iscX* whose expression is controlled by the Scr regulatory elements. Strains used for the 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 (Table 2). A schematic representation of these strains is shown in Fig. 1B. All strains show normal growth when cultured using sucrose as the carbon source under either ambient (~20%) or 5% oxygen.

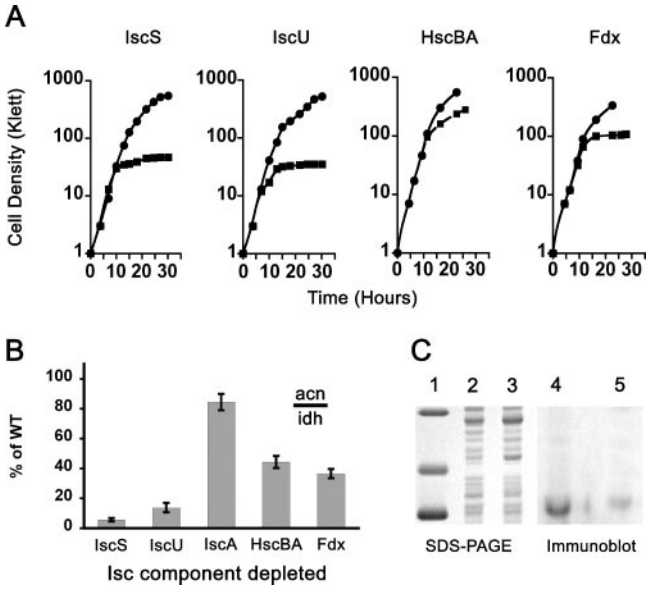

FIG. 5. Depletion of Isc components in *A. vinelandii* has a detrimental effect on aconitase activity. (A) Effect on growth in liquid culture that occurs upon a carbon source shift for strains DJ1450, DJ1445, DJ1447, and DJ1463. Each strain was diluted at time zero in liquid medium that contains either sucrose (●) or glucose (■) as the carbon source. The wild-type strain grows at the same rate in liquid culture when either sucrose or glucose is used as the carbon source. (B) Effect of depletion of Isc components on aconitase activity. Strain DJ1450, DJ1445, DJ1559, DJ1447, or DJ1463 was 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 of an Isc component was individually normalized to values obtained from the wild-type strain (DJ1454 [WT]) 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. The left panel shows Coomassie brilliant blue staining of proteins, separated by 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), from crude extracts of DJ1454 (wild type) and DJ1445 (Δ*iscU*) grown in glucose for 14 h. A duplicate of this gel was used for immunostaining with antibody to IscU (right panel). Lane 1, M_r standards (carbonic anhydrase, soybean trypsin inhibitor, and lysozyme); lane 2, crude extract of proteins (26 μg) from DJ1454 (wild type); lane 3, crude extract of proteins (26 μg) from DJ1445 (Δ*iscU*); lane 4, detection of IscU in DJ1454 (wild type); lane 5, detection of IscU in DJ1445 (Δ*iscU*).

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 with 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 manifested only 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.

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 contain an active-site cysteine residue (Cys^{325}) upon which a persulfide can be formed (31, 68, 69). 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 (39, 68, 69). In the present work, the in vivo functional role of Cys^{325} was confirmed by replacing 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) grows on petri plates as well as an isogenic strain that has both copies of IscS intact (DJ1454). However, DJ1451 cannot grow when cultured using glucose as the carbon source (data not shown).

Functional analysis of selected residues within IscU. In vitro systems where IscU, or its homologs, can be used as a scaffold for the assembly of either [2Fe-2S] or [4Fe-4S] clusters and where such clusters can be transferred to the apo forms of a variety of [Fe-S] proteins have been reported previously (2, 17, 37, 44, 62, 64, 65). A general consensus in the field is that three cysteine residues, Cys^{37}, Cys^{63}, and Cys^{106}, strictly conserved among all IscU homologs (see reference 25 for a phylogenetic comparison of the IscU family of proteins), are likely to provide ligands for the assembly of transient clusters destined for [Fe-S] protein maturation (18, 28, 55). This possibility is supported by the close three-dimensional juxtaposition and solvent exposure of all three cysteines within the available IscU structures (6, 38, 48). By using the same approach as described above for IscS, the individual replacement of alanine for IscU residues Cys^{63} or Cys^{106} within the P*isc*-regulated copy of IscU resulted in strains that cannot sustain growth when cultured using glucose as the carbon source (Fig. 6). In contrast, repeated attempts to isolate a strain for which the Cys^{37} residue within the P*isc*-regulated copy of IscU is replaced by alanine were not successful. The possibility that the Cys^{37} residue could be replaced 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 the sucrose-grown wild-type strains and strains that produce the Ala^{63}- and Ala^{106}-substituted versions of IscU shown in Fig. 6. 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 partial dominant-negative effect is also recognized for strains that have either the IscU Asp^{39} or His^{105} residues replaced by alanine. Although not apparent in Fig. 6, 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 rela-

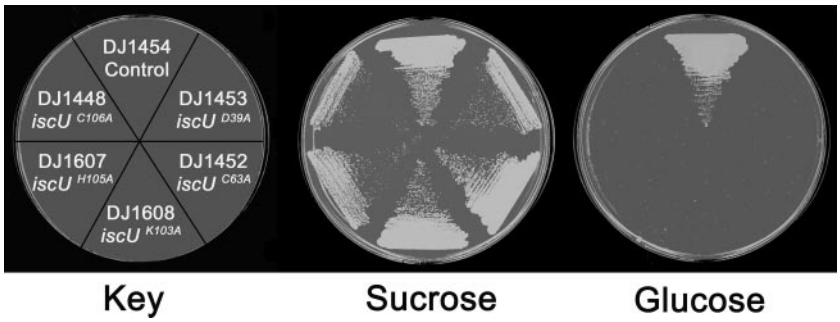

FIG. 6. Growth phenotypes exhibited by strains with selected residues of the IscR-regulated copy of IscU replaced by alanine. Cells were cultured using sucrose or glucose as the carbon source.

tively larger colonies, suggesting the possibility of rapid selection of strains that can either genetically or physiologically suppress the dominant-negative effect. Such a capacity for the relatively rapid phenotypic suppression of 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.

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 (20, 54). This stimulation has been localized to an LPPVK motif that is located two amino acids upstream from the conserved Cys^{106} residue within IscU (9, 21). This motif is not conserved in a class of IscU-like proteins, designated SufU (25), that are produced by certain organisms that do not encode strict HscBA homologs. In the present work, replacement of the Lys^{103} residue contained within the LPPVK motif with an alanine resulted in a null growth phenotype when cells were depleted of intact IscU (Fig. 6), consistent with similar mutational analyses performed with *Saccharomyces cerevisiae* Isu1 (14). 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. Replacement of IscU His^{105} by alanine also results in a null phenotype (Fig. 6).

Conserved cysteine residues within IscA are essential for its function. The null growth phenotype associated with the depletion of IscA when cultured using 40% O_2 also permitted a functional evaluation of individual residues within IscA. For these experiments, strains where strictly conserved Cys^{36}, Cys^{99}, and Cys^{101} residues within the P*isc*-regulated copy of IscA were individually replaced by alanine were constructed. When cultured using glucose as the carbon source, all of these strains exhibited the same oxygen-sensitive phenotype associated with the depletion of IscA (data not shown; see reference 25 for primary sequence comparisons of the IscA family of proteins).

DISCUSSION

Genetic tools for the controlled expression of any gene within *A. vinelandii* have been developed. This strategy is comparable to the plasmid-based, galactose-dependent controlled expression system developed in *Saccharomyces cerevisiae* (30). However, in our system, homologous recombination was used to place target genes under the control of the genome-encoded sucrose catabolic regulatory elements. The specific advantage of this approach is that it obviates the problems associated with the increased gene dosage frequently encountered when multicopy plasmids are used. 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 the preparation of requisite gene fusions.

In the present work, controlled gene expression was used for the functional analysis of IscS, IscU, IscA, HscBA, and Fdx from *A. vinelandii*. Phenotypic traits associated with the functional depletion of *A. vinelandii* Isc components are consistent with results of related genetic studies using *E. coli* or *S. cerevisiae* (reviewed in references 4 and 25). Namely, the 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* (51, 61), however, IscS, IscU, HscBA, and Fdx were found to be essential in *A. vinelandii*, and a 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 (46, 57). 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, with 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* (4, 25). In particular, conserved cysteine residues located within IscS and IscU, as well as conserved IscU residues implicated in interactions 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 (66), was also found to be essential. 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 through subunit mixing, by sequestration of some other component of the [Fe-S] protein maturation machinery, or by nonproductive interactions 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 the isolation of extragenic suppressor mutations as a future genetic strategy to identify the nature of interactions among certain players that participate in the process of [Fe-S] protein maturation and, possibly, their target [Fe-S] proteins.

The function of IscA has not been firmly established. However, on the basis of biochemical studies, it has been proposed to serve as either a scaffold for [Fe-S] cluster formation or an agent of Fe delivery to the IscU scaffold (10, 24, 29, 43, 64). In the present work, a clear null growth phenotype was manifested in cells depleted of IscA only when challenged by elevated levels of oxygen. It therefore appears that if IscA does serve as a scaffold for [Fe-S] cluster assembly, this function could be specific to the maturation of [Fe-S] proteins under conditions of elevated oxygen, or, perhaps, IscA could function in the repair of [Fe-S] proteins whose corresponding clusters have been oxidatively damaged. If IscA functions in the specific acquisition of Fe for cluster assembly on the IscU scaffold, the oxygen sensitivity of IscA depletion could indicate that the pool of available Fe in *A. vinelandii* is depleted under conditions of an oxygen challenge. However, our inability to elicit a phenotype associated with the depletion of IscA under conditions of Fe limitation does not support this possibility. Another possibility is that IscA has a specific function related to the protection or repair of the IscU scaffold under conditions of an oxygen challenge. A final significant finding was that the null growth phenotype associated with the depletion of HscBA is partially spared by lowering the ambient oxygen concentration. Although the specific function of HscBA is not yet known, it has been established they have an intrinsic ATPase activity that is greatly stimulated upon interaction with IscU. These observations lead us to speculate that HscBA could have a chaperone function that is specifically related to the protection or repair of the IscU scaffold under oxidative conditions.

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