Antiparallel and Interlinked Control of Cellular Iron Levels by the Irr and RirA Regulators of *Agrobacterium tumefaciens*^{▽†}

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The plant pathogen *Agrobacterium tumefaciens* encodes predicted iron-responsive regulators, Irr and RirA, that function in several other bacteria to control the response to environmental iron levels. Deletion mutations of *irr* and *rirA*, alone and in combination, were evaluated for their impact on cellular iron response. Growth was severely diminished in the Δirr mutant under iron-limiting conditions, but reversed to wild-type levels in an $\Delta irr \Delta rirA$ mutant. The level of uncomplexed iron in the Δirr mutant was decreased, whereas the $\Delta rirA$ mutant exhibited elevated iron levels. Sensitivity of the Δirr and $\Delta rirA$ mutants to iron-activated antimicrobial compounds generally reflected their uncomplexed-iron levels. Expression of genes that encode iron uptake systems was decreased in the Δirr mutant, whereas that of iron utilization genes was increased. Irr function required a trihistidine repeat likely to mediate interactions with heme. Iron uptake genes were derepressed in the $\Delta rirA$ mutant. In the $\Delta irr \Delta rirA$ mutant, iron uptake and utilization genes were derepressed, roughly combining the phenotypes of the single mutants. Siderophore production was elevated in the *rirA* mutant, but most strongly regulated by an RirA-controlled sigma factor. Expression of *rirA* itself was regulated by Irr, RirA, and iron availability, in contrast to *irr* expression, which was relatively stable in the different mutants. These studies suggest that in *A. tumefaciens*, the Irr protein is most active under low-iron conditions, inhibiting iron utilization and activating iron acquisition, while the RirA protein is active under high-iron conditions, repressing iron uptake.

The maintenance of cellular iron homeostasis is a critical problem for nearly all forms of life. Iron is necessary for a wide range of biological processes due to its electrochemical versatility (4). However, while ~32% of the Earth is made up of iron, its bioavailability in aerobic environments at circumneutral pHs is approximately 10^{-9} M (10, 41). Additionally, atomic iron is capable of catalyzing the conversion of hydrogen peroxide to the hydroxyl radical via Fenton chemistry (28, 29). Thus, many bacteria have developed dedicated regulatory systems to tightly control the expression of iron uptake and utilization systems.

The most-well-studied bacterial iron-responsive regulator is the ferric uptake repressor (*fur*) (5, 21, 23, 49). The Fur protein is the founding member of a family of proteins containing a wide array of metal-responsive transcriptional regulators and is present in many lineages of bacteria, including *Escherichia coli* and *Bacillus subtilis* (34). Based on the *E. coli* system, Fur directly senses the cellular iron concentration by associating with a ferrous iron atom, which stimulates the protein to bind to DNA at specific sequences (Fur boxes) and repress target genes (34). The iron/Fur regulon of many organisms contains a large number of iron uptake systems, including the enzymatic machinery necessary for the synthesis and uptake of siderophores (8). Siderophores are small molecules with an extremely high affinity for iron that are produced by many or-

ganisms (42). These molecules are secreted into the environment, where they scavenge iron and are taken up by the cell in a ferrisiderophore receptor-specific, energy-dependent manner to serve as an iron source (18). Many bacteria have receptor systems to utilize siderophores produced from other sources, which can provide them with a competitive advantage in iron-limited environments (27).

In some branches of the *Alphaproteobacteria*, iron response regulation differs substantially from the canonical Fur-Fe²⁺ regulatory model. While nearly all free-living *Alphaproteobacteria* contain close homologues of *fur*, evidence suggests that in some branches, this protein is functioning predominantly to regulate the cellular response to manganese levels (3, 6, 15, 48, 53). In place of the typical Fur-based iron transcriptional response, many of the *Alphaproteobacteria* use the iron regulator Irr (53). Irr represents a distinct branch of the Fur family of transcriptional regulators that was first discovered in the soybean-nodulating organism *Bradyrhizobium japonicum* as a regulator of heme biosynthesis (22, 34). In *B. japonicum*, this protein was shown to be regulated by degradation when it bound to iron-containing heme, thereby sensing iron levels indirectly via the final step in heme biosynthesis (50, 51). In *B. japonicum*, Irr degradation was shown to require both ferric and ferrous heme, and these molecules bound to different sites in the protein, ferric heme binding to a heme regulatory motif (HRM) near the N terminus and ferrous heme binding to a triple-histidine motif near the C terminus (73). However, in contrast to the *B. japonicum* protein, Irr homologues from most *Alphaproteobacteria* do not contain the HRM, leaving the C-terminal HHH motif as the primary candidate for heme interaction (55).

In addition to Irr, all members of the *Rhizobiaceae* contain

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homologues of a different iron-responsive regulator designated RirA, for rhizobial iron regulator *A* (53). RirA was initially discovered in *Rhizobium leguminosarum* and is a member of the Rrf2 family of putative transcriptional regulators (31, 65). The expression of a large number of genes is altered in an *R. leguminosarum* *rirA* mutant (63). Although direct binding of RirA to DNA has not been reported, a DNA sequence necessary for RirA-dependent regulation was identified (63, 64, 75). The ligand that imparts iron responsiveness to RirA has not been established, although the related Rrf2-type protein IscR binds and responds to Fe-S clusters, and it has been suggested that RirA also binds Fe-S clusters (19, 31, 58).

Agrobacterium tumefaciens is also a well-studied member of the *Alphaproteobacteria*, related to both *B. japonicum* and *R. leguminosarum* (7, 53). *A. tumefaciens* is best known for its ability to cause crown gall neoplasia on plants. This occurs via cross-kingdom horizontal gene transfer (HGT) of a segment of transferred DNA called "T-DNA," to plant cells, where it is subsequently imported into the nucleus and integrated into the genome (17). Genes expressed from T-DNA in the plant cause a hormonal imbalance, uncontrolled cell proliferation, and gall formation. T-DNA also directs the synthesis of a class of molecules called "opines," which serve as a nearly exclusive food source for the infecting *A. tumefaciens*. Although T-DNA transfer has been intensively studied, other aspects of basic *A. tumefaciens* physiology have received less attention, including its acclimation to changes in iron availability. As with other terrestrial and plant-associated bacteria, in *A. tumefaciens*, the maintenance of iron at levels high enough to support growth but sufficiently low to limit toxicity is a crucial balance, and a substantial fraction of its genome appears to be dedicated to iron acquisition and utilization.

A. tumefaciens encodes homologues of *fur*, *rirA*, and *irr*, as well as a large cluster of genes that directs the synthesis of a siderophore (20, 54, 72). The *A. tumefaciens* *fur* homologue is reported to primarily regulate manganese uptake and plays only a minor role in response to iron levels (33, 53). The RirA and Irr proteins are in fact the probable iron-responsive regulatory candidates. A role for RirA in regulating several iron-related phenotypes in *A. tumefaciens*, including siderophore production, iron uptake, and oxidative stress, has been established (43). The role of Irr and its potential interplay with RirA, however, remain unstudied. In *R. leguminosarum*, transcriptional studies have shown that Irr and RirA coregulate a wide range of iron-associated genes, including *rirA* (64). It is likely that the iron response circuitry in *A. tumefaciens* is similarly intertwined, and understanding this circuitry requires examination of both regulators in parallel.

In this study, we examine the iron response regulatory circuitry of *A. tumefaciens* in detail. To this end, in-frame deletions of *irr* and *rirA* were generated alone (Δirr and $\Delta rirA$) and in combination ($\Delta irr \Delta rirA$), and the impact of these mutations was evaluated on a battery of iron-related phenotypes. Our data show that Irr and RirA function in concert but often in opposite directions to balance iron homeostasis via control of iron uptake and utilization. This control ensures sufficient levels of available iron for cell growth while preventing the overaccumulation of iron that would lead to toxicity and the overproduction of iron uptake systems.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids that were used in this study are listed in Table S1 in the supplemental material. The oligonucleotide primers used in this study were obtained from Integrated DNA Technologies (Coralville, IA) and are detailed in Table S2 in the supplemental material. All DNA was manipulated using standard protocols (57). All restriction enzymes and molecular biology reagents were purchased from NEB (Ipswich, MA). DNA sequencing was performed on an ABI 3730 at the Indiana Molecular Biology Institute, Bloomington, IN. DNA was purified using QIAquick spin kits (Qiagen, Germantown, MD) or E.Z.N.A. plasmid miniprep kits (Omega Bio-tek, Norcross, GA) following the manufacturers' protocols. Plasmid introduction into *A. tumefaciens* was performed via electroporation (38). Bacteria were usually grown on LB medium for *E. coli* or AT minimal medium (62) supplemented with 0.5% (wt/vol) glucose and 15 mM ammonium sulfate (ATGN) or yeast extract-mannitol (YEM) medium for *A. tumefaciens*. Iron-limited growth of *A. tumefaciens* strains was conducted in ATGN supplemented with 22 μ M $FeSO_4$ and 200 μ M ethylenediamine-di-*o*-hydroxyphenylacetic acid (EDDHA) (obtained from Complete Green Company, El Segundo, CA). The iron-limited growth of *A. tumefaciens* siderophore mutants in rich medium was conducted in YEM without added iron and with the addition of 200 μ M EDDHA (54). For standard culture conditions, the 25 mM $FeSO_4 \cdot 7H_2O$ prescribed in AT medium (62) was omitted, with no observable effect on bacterial growth, but preventing visible precipitation of iron oxides and allowing us to better control the iron levels of the medium. Sucrose (0.5%) was used to replace glucose as the sole carbon source (ATSN) during *sacB* counterselection. Cultures of *A. tumefaciens* were grown in a modified version of ATGN, ATGN_{IRP}, for the chrome azurol S (CAS) assay. ATGN_{IRP} decreases the phosphate levels from 72 mM in standard AT medium to 5 mM and buffers the solution with 5 mM imidazole (pH 7.0), to prevent high phosphate levels from interfering with the iron complexed by CAS (13, 47, 59). The following antibiotic concentrations were used for *E. coli*: 100 μ g ml⁻¹ ampicillin (Ap), 25 μ g ml⁻¹ kanamycin (Km), and 100 μ g ml⁻¹ spectinomycin (Sp); for *A. tumefaciens*, 300 μ g ml⁻¹ Km and 150 μ g ml⁻¹ Sp. For *P_{Lac}* induction, media were supplemented with 250 μ M ml⁻¹ isopropyl- β -D-thiogalactopyranoside (IPTG). Unless otherwise noted, reagents, antibiotics, and microbiological media were obtained from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (St. Louis, MO).

Generation of nonpolar markerless deletions of the iron regulatory mutants. DNA fragments of approximately 500 bp flanking the targeted gene were generated by PCR. These fragments were located upstream (amplified by primers 1 and 2) and downstream (amplified by primers 3 and 4) of the gene to be deleted and were designed to precisely remove the coding sequence without affecting adjacent genes. Primers 2 and 3 were designed with complementary sequence at the 5' ends to allow splicing by overlapping extension (SOEing), as described previously (37, 68). Briefly, the flanking amplicons were generated with Phusion high-fidelity DNA polymerase (NEB, Ipswich, MA) and agarose gel purified. The purified fragments were used as the primer and template for five cycles of PCR. A final PCR was performed using 1 μ l of the short PCR as template and primers 1 and 4. The resulting amplicon was cloned into pGEM-T Easy and confirmed by DNA sequencing. The fragment was excised using the appropriate restriction enzymes and ligated into the *sacB* counterselectable suicide vector pNPTS138, which had been cleaved with the same enzymes. The vector pNPTS138 (M. R. K. Alley, unpublished data) replicates using a *colEI* origin that is incapable of replicating in *A. tumefaciens* and contains a Km resistance marker (Km^r) and the *sacB* gene, which confers sucrose sensitivity (Suc^s). Derivatives of pNPTS138 were introduced into *A. tumefaciens* by electroporation. The only way for pNPTS138 to confer Km^r to *A. tumefaciens* is to recombine into a stable endogenous replicon. Integrants were selected for by growth on ATGN plates supplemented with Km, and plasmid integration was confirmed by PCR and sucrose sensitivity by patching onto ATSN. Excision of the integrated plasmid from the Km^r Suc^s clones was facilitated by culturing the recombinants in LB medium without Km overnight, followed by plating on ATSN. The resulting Suc^r colonies were replica plated onto ATGN supplemented with Km to confirm loss of the plasmid marker. Deletion of the appropriate region of DNA was confirmed by PCR and DNA sequencing using primers 5 and 6, which were designed to flank the deleted region.

Creation of complementation constructs. Complementation constructs were prepared by PCR and subsequent cloning of the intact wild-type coding sequences into pSRK-Km (32). The 5' primers were typically designed with stop codons in all three reading frames preceding the *E. coli* *lacZ* ribosome binding site. One exception was the 5' primer for the *Atu3692* 5' primer, which was designed to be cloned into the *NdeI* site of pSRK-Km, directly in frame with the *lacZ* start codon (see Table S2 in the supplemental material). Coding sequences

for Atu0153 (*irr*), Atu0201 (*rirA*), and Atu3692 (siderophore-associated extracytoplasmic function [ECF] σ factor) were PCR amplified from *A. tumefaciens* genomic DNA using Phusion high-fidelity DNA polymerase and the corresponding primers, designated 7 and 8. PCR products were gel purified and ligated into pGEM-T Easy. Inserts were confirmed by DNA sequencing. The coding sequence for Atu3692 was excised using NdeI and SpeI sites engineered into primers 7 and 8. For Atu0153 and Atu0201, restriction sites flanking the inserted amplicon in the pGEM-T Easy multiple cloning site (MCS) were used: SacII and XbaI for Atu0153 and SacII and NdeI for Atu0201. All of the excised fragments were ligated using T4 DNA ligase into pSRK-Km that had been cleaved with corresponding restriction enzymes. Proper fragment insertion was confirmed by PCR, and constructs were electroporated into *A. tumefaciens* competent cells.

Site-directed mutagenesis of the *Irr* HHH motif. Site-directed mutagenesis was performed on the *irr* coding sequence using a PCR approach adapted from the QuikChange protocol marketed by Stratagene Corp. Large, self-complementary primers were designed containing the desired changes located in the middle of the primers (CACCATCAC to GCCGCTGCC for AAA or CACCATCAC to GCCCAGGCC for AHA) (see Table S2 in the supplemental material). The plasmid pMEH0115 carrying the *irr* coding sequence was used as the PCR template with Phusion DNA polymerase and *irr* primers 9 and 10 for the HHH→AAA mutation or primers 11 and 12 for the HHH→AHA mutation. The reaction products were digested with DpnI to remove the parental, wild-type molecules and hemimethylated plasmid DNA, leaving only the newly synthesized, uniformly nonmethylated mutated DNA. The mutations were confirmed by sequencing, and the mutated fragment was transferred into pSRK-Km using the same restriction sites used for the generation of the *irr* complementation construct.

EPR. Samples of the iron regulatory mutants were prepared for electron paramagnetic resonance (EPR) as described previously (30). Briefly, 1-liter cultures were grown to an optical density at 600 nm (OD_{600}) of approximately 0.2 in ATGN. The cells were collected by centrifugation at $10,000 \times g$ for 5 min at 4°C in a Sorvall RC 5B Plus (Thermo Scientific, Ashville, NC). The cell pellets were resuspended in 8 ml LB medium plus 1 ml 0.1 M diethylenetriaminepentaacetic acid (pH 7.0) and 1 ml 0.2 M desferrioxamine and incubated at 30°C with shaking for 15 min. Cells were collected by centrifugation ($10,000 \times g$), washed twice with ice-cold 20 mM Tris-Cl (pH 7.4), and resuspended in 300 μ l of 20 mM Tris-Cl (pH 7.4)–20% glycerol. Samples were placed in EPR tubes (4-mm outside diameter, 3-mm inside diameter) (Wilmad-Labglass, Vineland, NJ) and frozen on dry ice.

The EPR analyses were performed at the Illinois EPR Research Center of the University of Illinois, Urbana-Champaign, using the following settings: microwave power of 10 mW, microwave frequency of 9.05 GHz, modulation amplitude of 12.5 G at 100 kHz, time constant of 0.032, and sample temperature of 15 K. Samples from three biological replicates for each strain prepared on different days were measured, and the data are reported as the mean and standard error.

Potato disc tumor assay. The tumor assay was adapted from the method of Anand and Heberlein (2). Potatoes were surface sterilized in 10% bleach for 30 min, followed by 30 min of UV irradiation. Potatoes were peeled, and discs were prepared using a cork borer size 6, slicing the cores into 0.5-cm-tall discs. Discs were placed on 1.5% water agar plates and inoculated with 100 μ l of culture grown to an OD_{600} of 0.6. Discs were incubated in a humidified chamber until visible tumors appeared. The discs photographed are representative of the range of tumor formation observed from three biological replicates of each strain. Fifteen potato discs were assayed for each strain.

SNG resistance assay. The streptonigrin (SNG) resistance assay was carried out as described previously (43). Briefly, cells were grown overnight in ATGN. The culture density was normalized to an OD_{600} of 0.1. Cultures were treated with 100 μ g ml⁻¹ SNG or dimethyl sulfoxide (DMSO) as a control. FeSO₄ was added to 22 μ M in all samples. Samples were incubated with shaking at 28°C for 2 h and serially diluted, and 10 μ l of each dilution was spotted on LB plates. At least three biological replicates were tested for all strains on separate days for each biological replicate, and the data are reported as the mean and standard error of these replicates. Stock solutions of SNG were prepared in DMSO at a concentration of 10 mg ml⁻¹.

H₂O₂ resistance assay. *A. tumefaciens* strains were grown in ATGN plus IPTG overnight at 28°C with shaking. Cultures were normalized to an OD_{600} of 0.2 and split into two samples: H₂O₂ was added to a final concentration of 10 mM to one sample, and the other sample was left untreated. Cultures were incubated at room temperature with shaking for 1 h and dilution plated onto ATGN. The peroxide resistance of four biological replicates was measured for each strain on separate days, and the data are reported as the mean and standard error of these replicates.

RNA extraction and RT-qPCR. Cultures were grown in ATGN to an OD_{600} of ~0.85 and split into equal samples. FeSO₄ (Amresco, Solon, OH) was added to one-half of the culture to 22 μ M, and EDDHA was added to the other half to 200 μ M. The split cultures were incubated at 28°C for 1 h. The cultures were prepared for RNA extraction using the RNeasyprotect bacterial reagent (Qiagen, Germantown, MD) following the manufacturer's protocol. RNA was extracted using Qiagen RNA minipreps (Qiagen, Germantown, MD) following the manufacturer's protocol, extending the lysis step to 2 h at room temperature on a TC-7 roller drum (New Brunswick Scientific, Edison, NJ). RNA was extracted from three biological replicates of each strain.

DNA contamination was removed from the RNA samples by DNase digestion using the TURBO DNA-free kit (Ambion, Austin, TX) following the manufacturer's protocol, but extending the 37°C incubation time to 1 h. The qScript super mix kit (Quanta Biosciences Gaithersburg, MD) was used to prepare cDNA from ~200 ng of DNA-free RNA, following the manufacturer's instructions.

Transcript quantities were measured by quantitative reverse transcription-PCR (RT-qPCR). RT primers were designed to amplify small fragments (<200 bp with a melting temperature [T_m] of 60°C) of the targeted sequences. Reactions were prepared using the PerfeCTa SYBR green FastMix LowRox kit (Quanta, Gaithersburg, MD) with 100 pM each primer and a 1:10 dilution of the cDNA reaction mixture. Reactions were run on an Mx3000P qPCR system (Stratagene, Santa Clara, CA) using the following cycling parameters: 95°C for initial denaturation for 2 min, followed by 40 cycles of 95°C for 10 s, followed by 60°C primer annealing and an extension step for 30 s. The σ^{70} subunit of RNA polymerase (Atu2167) was used as a control to normalize for the amount of cDNA in each reaction. Melt curves were performed to confirm that the fluorescent signal was the result of the desired PCR product and not primer dimers. The data are the means and standard errors of at least three biological replicates, and each replicate was assayed on a different day.

CAS assay. Chrome azurol S (CAS) assay solution was prepared as follows. CAS (605 mg) was dissolved in 500 ml water. One hundred milliliters of 1 mM FeCl₃ in 10 mM HCl was added. The resulting mixture was added slowly to 729 mg of hexadecyltrimethylammonium bromide (HDTMA) in 400 ml water for final concentrations of 1 mM CAS, 2 mM HDTMA, 100 μ M FeCl₃, and 1 mM HCl (47). Cultures of the iron regulatory mutants were grown in ATGN_{IRP} overnight at 28°C. Cells were removed by centrifugation, and 200 μ l of the culture supernatant was mixed with 200 μ l CAS solution and 10 μ l of 0.2 M 5-sulfosalicylic acid shuttle solution and incubated for 30 min at room temperature. Siderophore production was measured by a decrease in the A_{630} of the sample, and the amount of siderophore produced was calculated using the following formula: $[(A_{630}^{\text{reference}} - A_{630}^{\text{sample}})/A_{630}^{\text{reference}}] \times 100 = \text{siderophore units}$.

The $A_{630}^{\text{reference}}$ was measured from CAS assay solution mixed with ATGN_{IRP} and shuttle solution. Data are the means and standard errors of 12 biological replicates.

Pigment extraction and mass spectrometry. *A. tumefaciens* mutants overproducing pigment were densely plated on ATGN solid medium and incubated at 28°C, resulting in lawns of bacteria. These lawns were scraped off the plates and extracted with 10 ml of acetone with vigorous shaking for at least 1 h. Cell debris was removed by centrifugation ($7,500 \times g$ for 10 min). The extract was concentrated by partial evaporation, and any residual precipitate was removed by a second centrifugation in a microcentrifuge at full speed for 1 min. The absorbance spectra of the extracts were measured on a Beckman DU 640 spectrophotometer (Beckman Coulter, Brea, CA) from 300 nm to 700 nm. Purified protoporphyrin IX (PPIX) was acquired from Sigma-Aldrich (St. Louis, MO) and resuspended in a 9:1 mixture of acetone and 0.1 N NH₄OH.

The mass spectra of the acetone extracts from the wild type and the Δirr mutant were collected on a 6130-MSD quadrupole mass spectrometer (Agilent, Santa Clara, CA) in positive-ion mode. The samples were loop injected and ionized using electrospray atmospheric pressure chemical ionization (ES/APCI) mass spectrometry. The data were analyzed using Agilent's ChemStation software (Agilent Technologies, Santa Clara, CA). Mass spectrometry was performed at the Indiana University Chemistry Department Mass Spectrometry Facility.

β -Galactosidase assay. Fragments containing the promoter elements upstream of Atu0153 (*irr*), Atu0201 (*rirA*), and Atu1825 (*nifS*, the first gene in a putative Fe-S cluster synthesis operon) were amplified from *A. tumefaciens* genomic DNA using Phusion DNA polymerase and primers F1 and F2 (see Table S2 in the supplemental material). PCR fragments were agarose gel purified, ligated into pGEM-T Easy, and verified by sequencing. Fragments were excised with the appropriate restriction enzymes and ligated into the compatibly cleaved vector pRA301 (1). Fragments were designed to insert the promoter region and ribosome binding site with the start codon of the gene of interest in

frame with the promoterless *lacZ* gene. Fragments were confirmed by PCR, and the constructs were electroporated into *A. tumefaciens* wild-type and iron regulatory mutant strains.

Cultures were prepared for β -galactosidase assay by being cultured in ATGN containing either high (22 μ M FeSO_4) or low (22 μ M FeSO_4 plus 200 μ M EDDHA) concentrations of iron. Exponential-phase cultures were measured for OD₆₀₀ and frozen at -80°C . The β -galactosidase activity of the cultures was assayed as described previously (40). Briefly, cells were permeabilized in Z-buffer (0.06 M Na_2HPO_4 , 0.04 M NaH_2PO_4 , 0.01 M KCl, 0.001 M MgSO_4 , 0.05 M β -mercaptoethanol brought to pH 7.0) by the addition of 2 drops of 0.05% SDS and 4 drops of chloroform. β -Galactosidase reactions were initiated by addition of 100 μ l of a 4-mg ml^{-1} solution of the colorimetric substrate *o*-nitrophenyl- β -D-galactopyranoside (ONPG) and terminated with addition of 600 μ l of 1 M NaCO_2 . Intact cells and debris were removed by centrifugation, free ONP was measured by A_{420} , and specific activity was reported in Miller units. At least eight biological replicates were performed.

Regulator binding site prediction. Consensus DNA binding sites for Irr (the iron control element [ICE] box) and RirA (the iron response operator [IRO] box) have been determined for the *Alphaproteobacteria* (53). The presence of ICE and IRO boxes in the genome of *A. tumefaciens* was analyzed using the RegPredict software package with score cutoffs of 4.9 for ICE box prediction and 3.93 for IRO box prediction (45). The positional weight matrices for the ICE and IRO sequences were previously established by D. A. Rodionov (personal communication). Predicted ICE boxes upstream of Atu0201, Atu1825, Atu2613, Atu3391, and Atu4022 and predicted IRO boxes upstream of Atu0201, Atu2613, Atu3391, Atu3692, and Atu4022 were used to generate logos of the *A. tumefaciens* ICE and IRO boxes using WebLogo software version 2.8.2 at <http://weblogo.berkeley.edu/> (12).

RESULTS

***A. tumefaciens* uses multiple regulators to respond to iron availability and balance the cellular iron pool.** Several members of the *Alphaproteobacteria* have been shown to use iron-responsive regulators that are distinct from the Fur system described in *E. coli*. *A. tumefaciens* uses the Rrf2-type protein RirA (encoded on the circular chromosome of C58, Atu0201), to respond to high concentrations of iron (43). The *rirA* gene appears to be the first gene in a potential operon that is divergently transcribed from Atu0202, a gene homologous to ABC-type periplasmic binding proteins and possibly involved in iron assimilation. Additionally, *B. japonicum* has been shown to use the Fur-family iron-responsive regulator, Irr, to control cellular iron status (22). In *A. tumefaciens*, an Irr homologue is encoded on the circular chromosome by Atu0153 and is 56% identical to the Irr protein of *B. japonicum*. The *A. tumefaciens* *irr* homologue is preceded by a predicted small hypothetical open reading frame (Atu0152), which overlaps with the 5' end of *irr* by 11 bp, but is not conserved even in other agrobacterial genomes. In order to examine the iron response regulatory circuitry of *A. tumefaciens*, precise deletions of *irr* and *rirA*, (in the case of *rirA* designed to be non-polar) were generated by allelic replacement, alone and in combination ($\Delta irr \Delta rirA$), and a battery of iron-dependent phenotypes were evaluated.

The ability of the mutants to grow in defined medium was assayed, and they all exhibited equivalent growth rates in ATGN supplemented with 22 μ M FeSO_4 (Fig. 1A) or in unsupplemented ATGN with ambient iron levels (data not shown). However, when the mutants were grown under iron-limiting conditions (ATGN plus 22 μ M FeSO_4 and 200 μ M the iron-chelating compound EDDHA), the growth yield of the Δirr mutant was substantially decreased (Fig. 1B). This Δirr growth phenotype was complemented via a plasmid-borne copy of *irr* expressed from P_{Lac} (see Fig. S1A in the supple-

FIG. 1. Overall effect of iron regulators on growth and cellular iron status. Growth of the *A. tumefaciens* wild-type strain ($\blacklozenge$) and the Δirr ($\blacksquare$), $\Delta rirA$ ($\blacktriangle$), and $\Delta irr \Delta rirA$ ($\bullet$) iron regulator mutants in 22 μ M FeSO_4 (A) or 22 μ M FeSO_4 and 200 μ M EDDHA (B). Data are the means and standard deviations of three replicates and are representative of three different experiments. (C) Electron paramagnetic resonance (EPR) was used to measure the relative pool of non-protein-bound iron in the iron regulation mutants. The data are the means and standard errors of three biological replicates. Bars marked with an asterisk are significantly different from the wild type ($P < 0.05$ via unpaired Student's *t* test). Iron levels were normalized to the wild type.

mental material). Irr in *B. japonicum* is thought to carry two heme binding motifs, one of which is conserved in the *A. tumefaciens* Irr protein, comprised of a histidine triplet (positions 92 to 94). Mutations within the HHH motif (HHH $\rightarrow$ AAA and HHH $\rightarrow$ AHA) were engineered into the $P_{\text{Lac-irr}}$ plasmid, which was introduced into the Δirr mutant. In contrast to the wild-type plasmid, these alleles failed to complement the growth yield deficiency (see Fig. S1B in the supplemental material).

Deletion of *rirA* had no effect on growth irrespective of iron

levels. However, deletion of *rirA* in the Δirr mutant reversed its growth yield deficiency under iron limitation, and the $\Delta irr \Delta rirA$ mutant was indistinguishable from the wild type for growth under iron limitation (Fig. 1A and B). This indicates that the Δirr growth deficiency is somehow ameliorated in the absence of *rirA*. Constitutive expression of a plasmid-borne *rirA* gene from P_{Lac} had no effect on growth of the *rirA* mutant under either high-iron or low-iron conditions (see Fig. S1C and S2 in the supplemental material). However, introduction of the *rirA* expression plasmid into the $\Delta irr \Delta rirA$ double-mutant background resulted in a strain manifesting an iron-limited growth defect similar to that observed in the Δirr mutant (see Fig. S1D in the supplemental material).

Iron sequestration is a common mechanism used by host organisms to resist microbial infection; thus, the iron-responsive regulator mutants were tested for virulence using potato disk assays. In contrast to what was observed previously using tobacco leaves, tumor formation was found to be qualitatively indistinguishable from that of the wild type for all mutants (see Fig. S3 in the supplemental material) (43). Given the strong growth limitation of the *irr* mutant under low-iron conditions, this finding suggests that the potato disks supply sufficient iron to compensate for the deficiency in this mutant.

Uncomplexed iron levels in iron regulator mutants. We examined the cellular pool of available iron (iron not already sequestered by other cellular components) using electron paramagnetic resonance (EPR). Mutants were grown in ATGN minimal medium containing neither FeSO_4 nor EDDHA. These data show that the Δirr mutant has a greater than 50% decrease in the available iron relative to the wild type (Fig. 1C). This decrease is partially complemented by ectopic expression of *irr* (Fig. 1C). Interestingly, the $\Delta rirA$ mutant shows a 50% increase in the available iron level that is restored to near wild-type levels by ectopic expression of *rirA* (Fig. 1C). Finally, the $\Delta irr \Delta rirA$ double mutant was statistically similar to the wild type (Fig. 1C). Thus, it appears that the Δirr mutant is incapable of maintaining a bioavailable cellular iron pool that is sufficient for normal growth under iron-limiting conditions, but this iron pool is restored to sufficient levels when *rirA* is also mutated.

Iron regulatory mutants exhibit changes in sensitivity to iron-dependent growth inhibitors. Streptonigrin (SNG) is an iron-activated antimicrobial compound which kills cells through accumulation of toxic oxygen intermediates that require iron for lethality (11, 25, 70, 71). SNG has previously been used as an indirect proxy for the level of uncomplexed iron in the cell, with SNG resistance interpreted to reflect a low cellular iron content. We measured SNG resistance for the iron regulatory mutants. We found that a 2-h exposure to 100 $\mu\text{g/ml}$ SNG resulted in a wild-type survival frequency of 0.14 ± 0.03 . The Δirr single mutant was 7-fold more resistant to SNG than the wild type, and ectopic expression of *irr* resulted in increased sensitivity (Fig. 2A), consistent with lower iron levels in the mutant and increases in iron pools when *irr* is strongly expressed. The $\Delta rirA$ single mutant was previously reported to be qualitatively more sensitive to SNG than the wild type (43). However, we observed that while the $\Delta rirA$ mutant appeared to be twice as sensitive to SNG as the wild type, this difference was not statistically significant (Fig. 2A). The $\Delta irr \Delta rirA$ mutant showed a dramatic increase in resistance to SNG that was

FIG. 2. Effects of iron-dependent growth inhibitors on *A. tumefaciens*. (A) Iron response regulatory mutants change the resistance of *A. tumefaciens* to the iron-activated antimicrobial streptonigrin. The percentage of survival of the bacteria treated with SNG was determined relative to the bacteria treated with DMSO, and these data were normalized to the wild-type value. The values and error bars are the means and standard errors of at least three biological replicates. (B) Iron response regulatory mutants change the resistance of *A. tumefaciens* to H_2O_2 . The percentage of survival of the H_2O_2 -treated sample was determined relative to the untreated sample and normalized to the wild type. The values and error bars are the means and standard errors of four biological replicates. Bars marked with an asterisk are significantly different from the wild type ($P < 0.05$ via unpaired Student's *t* test).

similar to that of the Δirr single mutant (Fig. 2A), suggesting low levels of uncomplexed iron in the cell for SNG to act upon. This result disagrees with direct measurement of the cellular iron levels by EPR (Fig. 1C). This discrepancy perhaps reflects the mechanism of SNG toxicity, which requires iron but is mediated through toxic oxygen intermediates, suggesting that changes in cellular metabolism other than free iron levels could impact SNG sensitivity.

One of the major mechanisms of iron toxicity is catalyzing production of hydroxyl radicals from H_2O_2 via Fenton chemistry. Additionally, the major catalase/peroxidase of *A. tumefaciens* (Atu4642) uses heme as a catalytic cofactor in the conversion of $2\text{H}_2\text{O}_2$ to $2\text{H}_2\text{O}$ and O_2 (16). Thus, the ability of *A. tumefaciens* to resist H_2O_2 should be closely related to its iron content and metabolism, and, as with SNG, H_2O_2 has been used to determine whether or not cells are overaccumulating iron (43). Accordingly, we measured the H_2O_2 resistance of the iron regulation mutants. After 1 h of exposure to 10 mM

FIG. 3. Siderophore production by *A. tumefaciens*. (A) Gene diagram of the region encoding the proteins necessary for the synthesis, uptake, and regulation of the siderophore produced by *A. tumefaciens*. The entire cluster consists of 26 predicted genes (Atu3668 to Atu3693) occupying 52,977 bp of the linear chromosome. The 13 genes omitted from the figure are all siderophore biosynthetic genes (54). (B) Siderophore production was measured by a decrease in the A_{420} of the CAS solution relative to a reference sample consisting of CAS and ATGN_{IRP}. The decrease in A_{420} indicates iron being removed from the CAS dye. The amount of siderophore produced was normalized to the wild type. Error bars are the standard errors of 12 biological replicates with sets of three replicates performed on 4 different days. Bars marked with an asterisk are significantly different from the wild type ($P < 0.05$ via unpaired Student's *t* test).

H₂O₂, the wild-type survival frequency was 0.076 ± 0.03 . The Δirr mutant was 2.3 times more resistant to H₂O₂ than the wild type, while the $\Delta rirA$ mutant was over 18 times more sensitive, and both phenotypes could be complemented with the corresponding plasmid-borne copies of the regulators (Fig. 2B). Unlike the SNG resistance assay, the $\Delta irr \Delta rirA$ mutant was not significantly more resistant to H₂O₂ than the wild type (Fig. 2B). These results suggest that hydrogen peroxide resistance is more indicative of cellular uncomplexed iron content than SNG resistance.

The production of an *A. tumefaciens* siderophore is regulated by both Irr and RirA and requires the presence of an ECF σ factor. In order to investigate the effects of the *irr* and *rirA* deletions on the iron limitation response in *A. tumefaciens*, we examined production of a previously reported siderophore iron uptake system (54), encoded on the linear chromosome by a large cluster (~53 kb; Atu3668 to Atu3693) of nonribosomal peptide synthase/polyketide synthase (NRP/PKS) homologues (Fig. 3A). It has been shown previously that disruption of the *rirA* gene in *A. tumefaciens* resulted in elevated siderophore production (43). We observed that under ambient iron conditions, the Δirr mutant has 75% less siderophore production than the wild type and the $\Delta rirA$ mutant is elevated by 90%, consistent with the earlier study (Fig. 3B). These phenotypes were complemented with the plasmid-borne genes,

and the $\Delta irr \Delta rirA$ mutant was similar to the $\Delta rirA$ single mutant (Fig. 3B).

Examination of the region of the genome encoding the siderophore synthesis machinery revealed the presence of a putative TonB-dependent ferrisiderophore receptor (Atu3687), an iron-specific ABC transporter (Atu3688 to Atu3691), and a putative extracytoplasmic function (ECF) σ factor (Atu3692) and anti- σ factor (Atu3693) (Fig. 3A). A mutant with a precise deletion of the ECF σ factor (Atu3692; designated *SigI*) was created and evaluated for siderophore production and iron-limited growth. Deletion of the *sigI* gene decreased the siderophore to the limits of detection for the CAS assay, and the siderophore was restored when *sigI* was expressed from a plasmid (Fig. 3B). Somewhat surprisingly, this mutant did not have a growth defect in minimal media in iron-limiting conditions (see Fig. S1E in the supplemental material); however, it did show a growth defect when grown in iron-limited complex medium (YEM) (data not shown). To examine the expression and potential regulation of *sigI* by Irr and RirA, we performed RT-qPCR for this gene in low-iron and high-iron media. These data show that *sigI* is induced under iron-limiting conditions regardless of the regulatory background (Fig. 4A). However, deletion of *irr* results in a 50% decrease in *sigI* expression under iron-limiting conditions relative to the wild type (Fig. 4A). In contrast, the $\Delta rirA$ mutant expresses *sigI* at levels 10-fold higher than that of the wild type under high-iron conditions but at similar levels to the wild type under iron-limiting conditions (Fig. 4A). The $\Delta irr \Delta rirA$ mutant resulted in a derepression of *sigI* similar to that observed in the $\Delta rirA$ mutant in high-iron conditions and a wild-type level of expression under low-iron conditions.

Additional iron uptake systems are also differentially regulated by Irr and RirA. To further examine the effects of Irr and RirA on iron uptake, the expression levels of the predicted iron transporter genes *irp6A* (Atu3391) and *fhuA* (Atu4022) were measured by RT-qPCR. Both genes exhibited similar patterns of expression in the mutants, although they varied in their strength of expression. In the wild type grown at a high iron concentration, *irp6A* and *fhuA* were expressed at low levels, and the expression of these genes was elevated under iron-limited conditions (Fig. 4B and C). The Δirr mutation decreased expression of *irp6A* by 3-fold and had no effect on *fhuA* expression under high-iron conditions (Fig. 4B and C). Under iron-limited conditions, the Δirr mutation resulted in a 5-fold drop in *irp6A* expression and a 3-fold decrease in *fhuA* expression (Fig. 4B and C). In contrast, deletion of *rirA* dramatically increased their expression in high-iron conditions relative to the wild type, but the level of induction in the *rirA* mutant with iron limitation was similar to that of the wild type (Fig. 4B and C). The $\Delta irr \Delta rirA$ mutant exhibited a mixed phenotype; the level of *irp6A* and *fhuA* expression under high-iron conditions was similar to that in the $\Delta rirA$ single mutant, but under low-iron conditions, the expression in the double mutant is similar to that seen in the Δirr single-mutant background (Fig. 4B and C). The double mutant appeared to be largely iron insensitive for expression of these iron transporter genes.

The Irr mutant accumulates a heme biosynthetic precursor. The *irr* mutant formed pink-pigmented colonies when grown on solid ATGN medium (Fig. 5A). This pigmentation was

FIG. 4. Regulation of iron uptake and utilization gene homologues in *A. tumefaciens*. The expression of *Atu3692 (sigI)*, an ECF σ factor proximal to the siderophore production and uptake cluster (A), *Atu3391 (irp6A)*, an ABC transporter substrate binding protein homologue specific for iron (B), *Atu4022 (fhuA)*, a hydroxamate-type ferrisiderophore receptor homologue (C), and *Atu2613 (hemA)*, the first dedicated enzyme in the heme biosynthetic pathway (D) was measured by RT-qPCR and is reported as the starting quantity (SQ) relative to the expression of σ^{70} . RNA was harvested from cultures of wild-type cells and the iron regulator mutants after exposure to 22 μ M FeSO_4 (Fe^{+}) or 200 μ M EDDHA (Fe^{-}) for 1 h. Error bars are the standard error of at least three biological replicates; each biological replicate was measured on a different day. Pairs of bars for the same strain marked with an asterisk are significantly different from one another, and bars marked with stars have a fold change significantly different from the wild type under high-iron conditions (open stars) and low-iron conditions (solid stars) ($P < 0.05$ via unpaired Student's *t* test).

reversed by the presence of the *irr* expression plasmid, but the *irr* plasmids with the mutated HHH motifs were unable to complement this phenotype (Fig. 5B). There was no observed colony phenotype in the $\Delta rirA$ mutant or in a wild-type strain constitutively expressing either *rirA* or *irr* (Fig. 5A) (data not shown). Production of this pigment was, however, dramatically reduced, although not eliminated, in the $\Delta irr \Delta rirA$ mutant (Fig. 5A).

In order to identify the pigment, bacterial lawns were scraped off plates and extracted with acetone, concentrated, and examined using spectrophotometry and mass spectrometry. In the mass spectrometer, the extract from the Δirr mutant showed a strong increase relative to the wild type of a molecular ion corresponding to an atomic mass of 563 Da (see Fig. S4A and B in the supplemental material). The heme precursor protoporphyrin IX (PPIX) has a molecular mass of 562 Da and has been shown to be overproduced in Δirr mutants from *B. japonicum* and *Brucella abortus*, so we examined the absorbance spectrum of extracts from wild type and the Δirr mutant as well as pure PPIX in acetone (22, 35). The wild-type extract had few discernible features other than a peak at 340 nm (Fig. 5C). The Δirr mutant extract had a similar peak at 340 nm but also had a very strong peak at 403 nm and four smaller peaks

at 502, 536, 574, and 629 nm. These major peaks correspond exactly to the absorbance of pure PPIX (Fig. 5C).

These results suggested that the Δirr mutant has elevated heme biosynthesis, saturating the cellular requirement for the PPIX cofactor, leading to its accumulation. To confirm the roles of Irr and RirA in regulating heme biosynthesis, the expression of *hemA* (*Atu2613*) was examined using RT-qPCR. HemeA is predicted to be a 5-aminolevulinic synthase, the product of which catalyzes the first dedicated step in heme biosynthesis. In the wild type, the expression of *hemA* under high-iron conditions is 10-fold higher than it is under low-iron conditions (Fig. 4D). Much of the iron-dependent regulation is lost in the Δirr mutant, with expression levels under both high-iron and iron-depleted conditions comparable to those observed for the wild type under high-iron conditions (Fig. 4D). The $\Delta rirA$ mutant expressed *hemA* similar to the wild type, while the $\Delta irr \Delta rirA$ mutant was similar to the Δirr mutant, with *hemA* expression under high-iron conditions resembling that of the wild type but with increased expression under low-iron conditions (Fig. 4D).

Expression of a putative iron-sulfur cluster synthesis operon is negatively regulated by both Irr and RirA. A cluster of genes on the circular chromosome (*Atu1819* to *Atu1825*) is

FIG. 5. Heme synthesis is misregulated in the Δirr mutant. (A) Colony phenotypes of the wild-type and the iron regulatory mutant strains and their complemented derivatives grown on ATGN; (B) colony phenotypes of the $\Delta sigI$ mutant and complement and the effects of the expression of the site-directed mutations of *irr* in the wild-type and Δirr backgrounds grown on ATGN. The brightness, contrast, and color balance of each photograph were adjusted using Adobe Photoshop CS4. (C) Absorption spectra of acetone-extracted wild-type and Δirr mutant lawns compared to the absorption spectrum of PPIX.

predicted to encode an iron-sulfur cluster synthesis system. In order to examine the effects of the iron regulators and iron concentrations on the expression of this cluster, the region upstream of *Atu1825* (encoding a homologue of the cysteine desulfurase NifS) was fused to a promoterless *lacZ* gene on a broad-host-range plasmid and evaluated for expression in the different mutants at various iron levels. In wild-type *A. tumefaciens*, changes in iron availability had little effect on *nifS* expression (Fig. 6A). The Δirr mutant expressed the *nifS-lacZ*

FIG. 6. Transcriptional regulation of a putative Fe-S biosynthesis cluster and the iron-responsive regulators. Fragments upstream of *Atu1825* (*nifS*, homologous to Fe-S cluster synthesis genes in other organisms) (A), *Atu0153* (*irr*) (B), and *Atu0201* (*rirA*) (C) were cloned upstream of a promoterless *lacZ* gene on the pRA301 plasmid and introduced into the iron regulator mutants. Transcription of *lacZ* was measured under high- and low-iron conditions using β -galactosidase assays. Data are the means and standard errors of at least eight biological replicates and are presented as Miller units. Pairs of bars for the same strain marked with an asterisk are significantly different from one another, and bars marked with stars are significantly different from the wild type under high-iron conditions (open stars) and low-iron conditions (solid stars) ($P < 0.05$ via unpaired Student's *t* test).

fusion 3-fold higher than the wild type under high-iron conditions; this overexpression increased to 10-fold under low-iron conditions (Fig. 6A). The *rirA* mutant expressed the *nifS-lacZ* fusion 4-fold higher than the wild type under high-iron conditions, but this expression returned to nearly wild-type levels

under lower-iron conditions (Fig. 6A). Plasmid-borne copies of the regulators fully complemented these mutants (data not shown). The $\Delta irr \Delta rirA$ mutant showed a combinatorial phenotype and was elevated for *nifS-lacZ* expression under high-iron conditions, similar to the $\Delta rirA$ mutant, but manifested the additional elevation observed for the *irr* mutant under low-iron conditions (Fig. 6A).

Feedback and transcriptional regulatory interactions of the iron response circuitry of *A. tumefaciens*. Much of the data presented above imply an epistatic interaction between Irr and RirA. To examine the transcriptional regulatory interactions between Irr and RirA, the *irr* and *rirA* upstream regions through their start codons were fused in-frame to a promoterless *lacZ* gene on a broad-host-range plasmid and analyzed in the different mutants. The expression of the $P_{irr}::lacZ$ fusion showed only very small changes, depending on the mutant background and iron availability. Overall, there was a slight but insignificant increase in the $P_{irr}::lacZ$ activity under iron-limited conditions, which is especially notable in the Δirr mutant. None of the other mutants varied dramatically for *irr* expression from the wild type under either high- or low-iron conditions (Fig. 6B). The regulation of *rirA* appears to be more pronounced. In the wild type, $P_{rirA}::lacZ$ was expressed at a relatively constant amount at all of the iron concentrations tested (Fig. 6C). Deletion of *irr* resulted in a large increase in $P_{rirA}::lacZ$ expression irrespective of iron concentration, and this increase could be complemented by ectopic expression of *irr* (data not shown). In the $\Delta rirA$ mutant, expression of *rirA* was increased under high-iron conditions but returned to nearly wild-type levels under iron-limited conditions. Ectopic expression of *rirA* in the $\Delta rirA$ mutant background restored expression from $P_{rirA}::lacZ$ to wild-type levels (data not shown). The $\Delta irr \Delta rirA$ mutant strongly expressed $P_{rirA}::lacZ$, similarly to the Δirr single mutant.

DISCUSSION

In this study, we examined the iron response regulatory network of *A. tumefaciens* using defined mutations in the *irr* and *rirA* regulatory genes, evaluating iron levels, iron-dependent phenotypes, and expression of iron acquisition and iron utilization genes. We have shown that Irr and RirA work in parallel, but in opposite directions, to maintain proper iron homeostasis. Irr represses expression of *rirA*, thereby connecting the two regulators in a regulatory hierarchy that allows Irr to act indirectly on a large number of genes via its control of *rirA*. This results in several phenotypes of the *irr* mutant that are due in part to its regulation of *rirA*. A model for the deduced iron response regulatory circuitry of *A. tumefaciens* is shown in Fig. 7.

Irr and RirA control iron homeostasis. In order to acclimate *A. tumefaciens* to growth in low-iron environments, Irr inhibits the transcription of genes involved in iron-consumptive processes such as heme and Fe-S cluster biosynthesis. Reducing the rate of iron utilization spares iron stores and ensures that the scarce iron available to the cell is consumed only by essential processes. In addition to conserving iron by reducing utilization, Irr plays a positive role in the activation of iron uptake systems (Fig. 7). The ability of the Irr from *A. tumefaciens* to positively and negatively regulate target genes is similar to that

FIG. 7. Model for the deduced iron response regulatory circuitry in *A. tumefaciens*. Under iron-replete conditions, the concentrations of heme and Fe-S clusters rise, resulting in ligand binding by both Irr (to heme) and RirA (to Fe-S clusters). This leads to a decrease in Irr regulatory activity and an increase in RirA regulatory activity, acclimating the cell to high-iron conditions by derepression of iron utilization systems and repression of iron uptake systems. Under low-iron conditions, the concentrations of heme and Fe-S clusters decrease, leading to an increase in Irr regulatory activity and a decrease in RirA regulatory activity. These changes acclimate the cell to low-iron conditions by inhibiting iron utilization systems and activating/derepressing iron uptake systems. Solid lines indicate strong transcriptional activation (arrowheads) or repression (bars). Dashed lines indicate weaker control; the dashed arrow indicates Irr activation of *irpA6* only. Solid serpentine lines indicate gene expression or siderophore production. Dashed serpentine lines indicate posttranscriptional modifications that regulate the Irr and RirA proteins.

observed in *B. japonicum* (44, 56). As was observed in *R. leguminosarum*, Irr also inhibits the transcription of *rirA*, the other iron-responsive regulator in *A. tumefaciens* (64). RirA inhibits iron uptake systems, as shown here and in previous work by Ngok-Ngam et al. (43). Thus, Irr further adapts the cell to low-iron conditions by reducing the transcription of a regulator that functions to repress the expression of iron uptake systems (43). This reciprocal regulation via Irr and RirA allows *A. tumefaciens* to accumulate sufficient cellular iron pools and grow efficiently under iron-limited conditions. The *irr*-null mutant cannot mount the proper low-iron regulatory response, resulting in a decreased cellular iron pool similar to the decrease in total iron content observed in *B. japonicum*, as well as a dramatic growth yield defect (74). This is presumably through a combination of unregulated consumption of iron and the limited expression of iron uptake systems. Elevated *rirA* expression in the Δirr mutant may acutely exacerbate the compromised growth under low-iron conditions, because deletion of *rirA* in the *irr* mutant returns a wild-type growth yield under iron-limited conditions and in an elevated cellular iron pool. Under high-environmental-iron conditions, Irr appears to have little effect on the transcription of iron uptake and utilization genes, although it still appears to exert a notable repressive effect on expression of the Fe-S cluster synthesis operon (*nifS*). Our data clearly indicate that Irr is not fully

deactivated even at the highest iron concentrations used in this study.

The patterns of gene expression observed here correlate well with predicted binding sites for Irr and RirA in the *A. tumefaciens* C58 genome (31, 53). Predicted binding sites for Irr (ICE boxes) are found in the upstream nontranslated regions of *rirA*, the iron transporter homologues *irp6A* and *fhuA*, and presumptive iron utilization genes *hemA* and *nifS*. Our expression data suggest that Irr represses *rirA*, *hemA*, and *nifS*. Notably, there are no predicted ICE boxes upstream of *irr* or *sigI*, consistent with our expression data. Predicted RirA binding sites (IRO boxes) were identified upstream of *rirA* itself, *irp6A*, *fhuA*, *sigI*, and *nifS*. This is again consistent with our expression data, including the lack of RirA regulation on *hemA*. It is interesting that both Irr and RirA repress *nifS* expression, under iron-limited and iron-replete conditions, respectively, and the gene has both an ICE box and an IRO box separated by 13 bp, both of which overlap a putative σ^{70} promoter sequence. ICE and IRO box consensus sequences generated from the regions upstream of genes that have been shown to be regulated by Irr and RirA, respectively, in this work are shown in Fig. S5A and B in the supplemental material.

In the $\Delta rirA$ mutant, *nifS* expression is derepressed under high-iron conditions, but under iron-limiting conditions, *nifS* expression is similar to that of the wild type. Expression of *nifS* is elevated in the Δirr mutant under high-iron conditions, and even more so under low-iron conditions (Fig. 6A). This observed increase in *nifS* expression at lower iron levels suggests additional repression at high iron levels. This additional repression under high-iron conditions could be readily explained by RirA, which is inactive at lower iron levels. However, if this were correct, levels of *nifS* expression in the $\Delta irr \Delta rirA$ mutant should be similar under both high- and low-iron conditions, yet it retains a significant induction under low-iron conditions. This observation suggests an additional level of iron-responsive regulation of *nifS* that is independent of both Irr and RirA.

Irr and RirA influence the sensitivity of *A. tumefaciens* to oxidative damage. Iron plays a critical role in the free radical chemistry of many bacteria. One major mechanism of iron toxicity is the catalysis of H_2O_2 conversion into the hydroxyl radical via Fenton chemistry (26, 28, 52). In addition, many oxygen radical detoxification proteins use iron or iron-containing cofactors such as heme. Thus, it is not surprising that perturbations of bacterial iron homeostasis often result in changes in H_2O_2 resistance profiles. In particular, increased sensitivity to H_2O_2 correlates with increased available cellular iron (66). The iron-activated antimicrobial streptonigrin has also been used to approximate the iron state of several bacterial species, with an increased SNG sensitivity implying an increased available cellular iron (33, 43, 70). In *A. tumefaciens*, it has previously been shown that a disruption of *rirA* results in an increased sensitivity to both H_2O_2 and SNG, and it has been suggested that increased sensitivity is due to increased iron concentrations in the cell (43). We measured the sensitivity of our mutants to SNG and H_2O_2 and found that the sensitivity profiles of the single mutants corresponded to the amount of uncomplexed iron as measured by EPR; decreased iron in the *irr* mutant correlated with increased SNG/ H_2O_2 resistance, and increased iron in the *rirA* mutant correlated with decreased resistance. Increased H_2O_2 resistance in an *irr* mutant has also

been observed in *Brucella abortus* (36). The $\Delta irr \Delta rirA$ double mutant does not universally follow the rule of increased uncomplexed cellular iron resulting in increased sensitivity, as it has a slightly increased EPR-detectable iron concentration but low SNG sensitivity. This suggests that in *A. tumefaciens*, SNG sensitivity cannot be used to approximate the cellular uncomplexed-iron concentration. It is worth noting that the cytotoxic activity of SNG functions via oxygen radical generation (11, 14). It is plausible that Irr represses a toxic oxygen defense mechanism, and in the absence of this repression, *A. tumefaciens* can exhibit increased radical resistance even in the presence of elevated levels of iron.

A putative heme binding motif in Irr is required for activity. The existing model for Irr iron responsiveness established in *B. japonicum* proposes that Irr indirectly responds to cellular iron levels by associating with the ferrichelatase responsible for ligating an Fe atom to a PPIX molecule, the final step in heme biosynthesis (46, 51). The newly synthesized heme is passed to Irr, marking it for oxidation and proteolytic degradation (50, 51). The Irr protein from *B. japonicum* appears to bind two heme molecules—a ferric heme at the N-terminal heme regulatory motif (HRM) and a ferrous heme at a C-terminal HHH motif (73). Mutation of the HHH motif results in a stable Irr protein, while mutation of the HRM reduces the rate of degradation (73). The general applicability of this model to other Irr proteins has recently been called into question (60). The N-terminal heme regulatory motif of the *B. japonicum* Irr protein is absent in many *irr* homologues (61). In addition, it has been shown in *R. leguminosarum* that while Irr appears to be a heme binding protein, it differs from the *B. japonicum* homologue in that it is stable in the presence of heme (60). Finally, it has been shown that the HHH motif in the Irr protein from *R. leguminosarum* is necessary for binding to DNA, while the role of the HHH motif from *B. japonicum* remains untested (60). In *A. tumefaciens*, site-directed mutants of Irr that modify its C-terminal HHH motif to either an AAA motif or an AHA motif eliminate this protein's ability to complement either the growth defect or the PPIX overproduction phenotype of the Δirr mutant. Although we have not tested whether this protein binds to heme or DNA, these data are consistent with the findings from *R. leguminosarum* that show that the HHH motif is necessary for regulatory activity.

RirA regulates uptake and utilization under high-iron conditions. In contrast to Irr, RirA does not appear to have a strong effect on the expression of iron uptake genes under iron-limited conditions. However, when iron is replete, RirA has a strong repressive effect on the iron uptake systems examined in this study. The influence of RirA on iron utilization is less clear. Heme biosynthesis and the expression levels of *hemA* under high- and low-iron conditions are relatively unchanged by the $\Delta rirA$ mutation. In contrast, the expression of the Fe-S cluster synthesis operon (*nifS*) appears to be inhibited by RirA under high-iron conditions. RirA is thought to respond to iron indirectly via association with Fe-S clusters and is a member of the Rrf2 family of transcription regulators, which also includes the *E. coli* regulator IscR (64). Members of this family often contain three conserved cysteine residues, which in the case of RirA from *A. tumefaciens* are located at residues 91, 99, and 105 (55). In *E. coli*, IscR inhibits the transcription of the *isc* Fe-S cluster synthesis operon through

an unknown mechanism that requires formation of an IscR-[2Fe-2S] cluster complex (58). Homologues of *iscR* are present in most of the *Alphaproteobacteria*; however, none of the sequenced *Alphaproteobacteria* contain both *iscR* and *rirA*, and those with *rirA* are a subset of the *Rhizobiales* (53). It is plausible that RirA initially regulated Fe-S cluster synthesis specifically, but has evolved to control diverse iron-related functions, using Fe-S clusters as a proxy for elevated iron. Future phylogenetic work examining the sequence and distribution of RirA binding sites and the evolutionary history of the Rrf2 family of regulators will contribute to the understanding of the evolution of iron-responsive transcriptional regulation. In addition, biochemical studies focused on confirming the ability of RirA to bind Fe-S clusters and examining the nature and oxidation state of the ligand will be useful in dissecting the mechanism of RirA function.

Siderophore regulation. The production of siderophores is a metabolically costly process that is tightly regulated to ensure that production only occurs when siderophores are needed (24, 39, 67). One mechanism that bacteria use to regulate siderophore production is by controlling expression of the siderophore production genes via an extracytoplasmic function (ECF) σ factor (39, 67). The siderophore produced by *A. tumefaciens* (a polyketide/nonribosomal peptide molecule encoded by a large gene cluster of almost 53 kb) is controlled by such a σ factor, which we have designated σ^I (54). The expression of *sigI* is regulated by RirA and indirectly by Irr through its control of *rirA* expression, thus tightly linking siderophore production to iron availability (Fig. 3B). Under iron-limited conditions, Irr represses *rirA* expression, thereby preventing its repression of *sigI*, leading to elevated *sigI* expression and siderophore synthesis. The regulation of σ^I function (perhaps mediated by an anti-sigma factor) beyond RirA-dependent expression control, is not yet understood. Under iron-replete conditions, *rirA* expression is not repressed by Irr, and RirA (presumptively through Fe-S association) represses *sigI* expression, thereby limiting siderophore synthesis when iron is plentiful. There are also recognizable IRO elements upstream of several of the siderophore biosynthetic genes, suggesting the possibility of direct control of these genes by RirA, as well as its effect on *sigI* expression.

Conclusions. Iron uptake and iron utilization activities in *A. tumefaciens* are precisely balanced under conditions of both paucity and plenty. These regulatory effects combine to control the pool of internal iron available to the cell. Irr ensures that there is sufficient uncomplexed iron for bacterial growth by stimulating iron uptake systems and inhibiting iron-utilizing processes. RirA prevents the overaccumulation of iron that would lead to iron toxicity by repressing iron uptake systems. The two regulatory pathways are linked by the Irr repression of *rirA* expression, with Irr at the top of the regulatory hierarchy. The combination of the Irr and RirA inputs on the cellular iron pool maintains iron homeostasis in the cell, ensuring that levels are maintained within a physiologically relevant range.

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