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Identification of a DtxR-Regulated Operon That Is Essential for Siderophore-Dependent Iron Uptake in *Corynebacterium diphtheriae*

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The diphtheria toxin repressor (DtxR) uses Fe^{2+} as a corepressor and inhibits transcription from iron-regulated promoters (IRPs) in *Corynebacterium diphtheriae*. A new IRP, designated IRP6, was cloned from *C. diphtheriae* by a SELEX-like procedure. DtxR bound to IRP6 in vitro only in the presence of appropriate divalent metal ions, and repression of IRP6 by DtxR in an *Escherichia coli* system was iron dependent. The open reading frames (ORFs) downstream from IRP6 and previously described promoter IRP1 were found to encode proteins homologous to components of ATP-binding cassette (ABC) transport systems involved in high-affinity iron uptake in other bacteria. IRP1 and IRP6 were repressed under high-iron conditions in wild-type *C. diphtheriae* C7(β), but they were expressed constitutively in C7(β) mutant strains HC1, HC3, HC4, and HC5, which were shown previously to be defective in corynebactin-dependent iron uptake. A clone of the wild-type *irp6* operon (pCM6ABC) complemented the constitutive corynebactin production phenotype of HC1, HC4, and HC5 but not of HC3, whereas a clone of the wild-type *irp1* operon failed to complement any of these strains. Complementation by subclones of pCM6ABC demonstrated that mutant alleles of *irp6A*, *irp6C*, and *irp6B* were responsible for the phenotypes of HC1, HC4, and HC5, respectively. The *irp6A* allele in HC1 and the *irp6B* allele in HC5 encoded single amino acid substitutions in their predicted protein products, and the *irp6C* allele in HC4 caused premature chain termination of its predicted protein product. Strain HC3 was found to have a chain-terminating mutation in *dtxR* in addition to a missense mutation in its *irp6B* allele. These findings demonstrated that the *irp6* operon in *C. diphtheriae* encodes a putative ABC transporter, that specific mutant alleles of *irp6A*, *irp6B*, and *irp6C* are associated with defects in corynebactin-dependent iron uptake, and that complementation of these mutant alleles restores repression of corynebactin production under high-iron growth conditions, most likely as a consequence of restoring siderophore-dependent iron uptake mediated by the *irp6* operon.

Corynebacterium diphtheriae is the gram-positive bacterium that causes diphtheria. Most isolates of *C. diphtheriae* from patients with respiratory diphtheria produce diphtheria toxin (DT), which catalyzes the NAD-dependent ADP ribosylation of elongation factor 2 and causes inhibition of protein synthesis and death in cells from humans or susceptible animals. DT is encoded by temperate phages, which are present in all toxin-producing strains of *C. diphtheriae*.

The diphtheria toxin repressor (DtxR), originally identified as a repressor of the gene that encodes DT, is now known to function as global regulator of metabolism in *C. diphtheriae*. Representative functions that are negatively regulated by iron are production of DT, synthesis of a siderophore (corynebactin), corynebactin-dependent iron uptake, and utilization of iron from heme. Although the physiological role of DtxR in *C. diphtheriae* is similar to that of the ferric uptake regulator protein (Fur) in several gram-negative bacteria, DtxR differs from Fur in structure and cannot substitute for Fur in function. DtxR is therefore the prototype for a novel class of metal-activated transcriptional regulators. Homologs of DtxR are present in a diverse and rapidly growing group of bacteria, as summarized by Feese et al. (5).

DtxR is synthesized as a 226-amino-acid (aa) polypeptide that forms dimers, utilizes Fe^{2+} as a corepressor, and binds to specific operator sequences in iron-regulated promoters (IRPs), thereby inhibiting transcription of downstream genes. Seven DtxR-specific, iron-regulated promoters in *C. diphtheriae*, including the *tox* promoter, IRP1, IRP2, IRP3, IRP4, IRP5, and the *hmuO* promoter, have been reported to date (10, 11, 21, 25). IRP1 to IRP5 were isolated by shotgun cloning of genomic fragments into promoterless *lacZ* reporter plasmid pQF50, followed by screening for promoters that are repressed by iron in the presence of DtxR in an *Escherichia coli* system. The open reading frame (ORF) immediately downstream from IRP1 encodes a lipoprotein that is homologous with FhuD, the ferrichrome binding protein from *Bacillus subtilis*, which is involved in ferrichrome transport (26, 28). The ORF downstream from IRP3 encodes a homolog of AraC-type transcriptional activators, which suggests that a multilevel network of iron-dependent regulation may occur in *C. diphtheriae* (11). The products of ORFs downstream from promoters IRP2, IRP4, and IRP5 do not show significant homologies to known proteins (11, 25). The *hmuO* gene, which encodes a heme

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TABLE 1. Strains and plasmids used in this study

Strain(s) or plasmid	Description	Reference or source
C. diphtheriae strains		
C7	Wild-type strain, nonlysogenic, nontoxinogenic	7
C7(β)	Wild-type reference strain, *tox*$^+$, lysogenic for phage β	7
HC1, HC3, HC4, and HC5	Siderophore uptake mutants derived from C7(β)	2
Plasmids		
pQF50	Amp^r; *E. coli* reporter vector containing a promoterless *lacZ* gene	4
pQF1	pQF50 derivative containing IRP1 promoter	25
pQF6	pQF50 derivative containing IRP6 promoter	This study
pQF1-	Reverse orientation of promoter fragment versus pQF1	This study
pQF6-	Reverse orientation of promoter fragment versus pQF6	This study
pSK6a	Library clone containing a 1.9-kb *Eco*RI insert of *irp6* region	This study
pSK6e	Library clone containing a 2.2-kb *Eco*RI insert of *irp6* region	This study
pCM2.6	Shuttle vector for *C. diphtheriae*	23
pCM6ABC	pCM2.6 derivative containing a 3.6-kb *Sal*I-*Bam*HI insert of the *irp6* operon	This study
pCM6AB	pCM2.6 derivative containing a 2.9-kb *Sal*I-*Bam*HI insert containing the *irp6A-irp6B* region of the *irp6* operon	This study
pCM6A	pCM2.6 derivative carrying a 1.9-kb *Sal*I-*Bam*HI insert containing the *irp6A* region of the *irp6* operon	This study
pNGR-6ABC	Km^r; shuttle plasmid containing the *dtxR* gene and the *irp6* operon	This study
pWR382	Amp^r; contains an 8.2-kb *Eco*RI insert of *irp1* operon	26
pDSK29	Km^r; contains the *dtxR* gene	23

oxygenase, was isolated by complementation of heme utilization in *C. diphtheriae* and *Cornynebacterium ulcerans* mutants (20, 21). The *hmuO* promoter is negatively regulated by iron and DtxR and positively regulated by heme (21).

C. diphtheriae, like many other pathogens, is capable of utilizing various forms of iron in host environments where the concentration of free iron ions is extremely low. Heme uptake (HmuTUV) (3) and utilization (HmuO) (20, 21, 22) systems are required for *C. diphtheriae* to acquire iron from heme or hemoglobin, and corynebactin is required for *C. diphtheriae* to assimilate nonheme iron during growth under low-iron conditions (17, 18). Partially purified corynebactin from *C. diphtheriae* did not react in chemical tests for catechol- and hydroxymate-type siderophores (18), and the structures of corynebactin and the components of the corynebactin-dependent iron uptake system have not been determined. *C. diphtheriae* can also utilize aerobactin from *Shigella flexneri* for siderophore-dependent iron uptake (17, 18). Another siderophore from *Corynebacterium glutamicum*, also called corynebactin, was analyzed by nuclear magnetic resonance spectroscopy and shown to be a catechol-type siderophore (1), but the structural relationships between the corynebactins from *C. diphtheriae* and *C. glutamicum* are not yet established.

Two types of mutants that are defective in corynebactin-dependent iron uptake were isolated after chemical mutagenesis of *C. diphtheriae* C7(β) (2, 18). The first mutant type, represented by strain HC6, does not produce corynebactin even under low-iron conditions and appears to be deficient in siderophore production (18). Interestingly, the PW8 strain of *C. diphtheriae*, used commercially to produce DT for conversion to diphtheria toxoid for vaccines, also lacks the ability to produce corynebactin (17). In contrast, mutants of the second type, represented by strains HC1, HC3, HC4, and HC5, overproduce siderophore even under high-iron growth conditions and exhibit much lower rates of corynebactin-dependent $^{59}Fe^{3+}$ uptake under low-iron conditions than does the parental strain, C7(β) (2). Therefore, the utilization of iron from ferric corynebactin appears to be impaired in these mutants. Attempts to complement the mutant phenotype of strains HC1, HC3, HC4, and HC5 with a plasmid clone containing the whole *irp1* operon were unsuccessful (26), suggesting that mutations in the *irp1* operon are not responsible for the defects in corynebactin-dependent iron uptake in *C. diphtheriae* strains HC1, HC3, HC4, and HC5.

Here, we describe the cloning and characterization of a new DtxR-dependent IRP from *C. diphtheriae*, called IRP6, by using a SELEX (systematic evolution of ligands by exponential enrichment)-like selection method that is similar to procedures used successfully with other organisms for isolation of specific target sequences in DNA (13, 33). The gene products of the corresponding *irp6* operon were found to be homologous with components of ATP-binding cassette (ABC) transporters. We show by complementation analysis that the wild-type *irp6* operon corrects the defects in repression of siderophore production by iron in *C. diphtheriae* strains HC1, HC4, and HC5 and that strain HC3 has mutations in both the *irp6* operon and the *dtxR* gene. We also completed the sequencing of the *irp1* operon and demonstrated that it encodes another putative ABC transport system in *C. diphtheriae*, whose function is currently unknown.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and β-galactosidase assays. Strains of *E. coli* and *C. diphtheriae* and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely cultured in Luria-Bertani (LB) broth (19), and *C. diphtheriae* strains were routinely grown in heart infusion broth (Difco, Detroit, Mich.) with 0.2% Tween 80 (HITW). *C. diphtheriae* strains were grown in the modified PGT medium described by Tai et al. for siderophore assay experiments (30). All bacterial strains in broth media were grown at 37°C with shaking. Antibiotics or other supplements were added at the following concentrations: ampicillin, 100 mg/liter; kanamycin, 25 mg/liter; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 40 mg/liter; chloramphenicol, 10 mg/liter for *E. coli* and 2 mg/liter for *C. diphtheriae*. To create iron-limiting conditions for *C. diphtheriae* strains in HITW, iron chelator ethylenediamine-di(*o*-hydroxylphenyl) acetic acid (EDDA) was added at a final concentration of 100 μg/ml. For β-galactosidase activity assays with *E. coli* strains, LB broth was first deferrated

TABLE 2. Linkers and primers used for cycle selection

Method	Sequence of linker or primer[a]
*Hha*I selection	
Linker	*Bam*HI *Spe*I 5′CGGATCCACTCTTGACCTCGACTAGTGCA3′ 3′GCGCC**TAGGTGAGAACTGGAGCT**GATCACGT5′ (*Hha*I)
PCR primer PAS-A	3′**TAGGTGAGAACTGGAGCT**5′
*Hpa*II selection	
Linker	(*Hpa*II) *Xba*I *Spe*I 5′CGGATCTAGACTTGACCTCGACTAGTGCA3′ 3′C**TAGATCTGAACTGGAGCT**GATCACGT5′
PCR primer PAS-B	3′**TAGATCTGAACTGGAGCT**5′

[a] Boldface letters indicate the identical sequence between the linker and its specific PCR primer. Underlined letters represent target sites for indicated restriction enzymes, and protruding ends of linkers are compatible with DNA fragments generated by restriction enzymes shown in parentheses.

by treatment with Chelex-100 (Bio-Rad, Hercules, Calif.) at 10 g/liter and then either $FeCl_3$ at 10 μM or EDDA at 200 μg/ml was added to create high-iron or low-iron growth conditions, respectively. For β-galactosidase activity assays with *C. diphtheriae* strains, untreated HITW was used for high-iron growth conditions and HITW supplemented with EDDA at 100 μg/ml was used for low-iron growth conditions. HITW with added $FeCl_3$ at 20 μM (HITW-Fe) was also used in some experiments to examine the effects of excess iron on the activity of IRPs in mutant strains of *C. diphtheriae*.

Assays for β-galactosidase activities were performed as described previously (23, 25), with some modifications for *C. diphtheriae* strains. Briefly, approximately 2×10^7 cells from overnight *C. diphtheriae* cultures were collected and resuspended in 1 ml of buffer Z (0.06 M $Na_2HPO_4 \cdot 7H_2O$, 0.04 M $NaH_2PO4 \cdot H_2O$, 0.01 M $MgSO_4 \cdot 7H_2O$, 0.05 M β-mercaptoethanol, pH 7.0). Cells were permeabilized by mixing them with 40 μl of 0.1% sodium dodecyl sulfate–80 μl of chloroform on a vortexer for 10 s. Reactions were initiated by adding 200 μl of *o*-nitrophenyl-β-D-galactopyranoside (ONPG; 4 mg/ml). When yellow was visible, the incubation times were recorded and the reactions were stopped by adding 500 μl of 1 M Na_2CO_3. For samples that did not turn yellow, reactions were terminated after 1 h. The clear aqueous phase was collected by centrifugation and used for A_{420} measurements. Miller units were used for enzymatic activity.

DNA preparation, cloning, and sequencing. Chromosomal DNA from *C. diphtheriae* strains was purified by cesium chloride gradient ultracentrifugation. The sequence of IRP6 was determined at the automated DNA sequencing facility in the Department of Biochemistry at Colorado State University, Fort Collins, Colo. The sequences of ORFs downstream from IRP6 and IRP1 and of mutant alleles of genes *irp6A*, *irp6B*, and *irp6C* in the *irp6* operon were determined at the University of Colorado Cancer Center DNA Sequencing and Analysis Core Facility, Denver, Colo. Clone Manager software was used to identify ORFs, and BLAST searches were used to search for proteins in the National Center for Biotechnology Information database that are homologous to predicted proteins encoded by ORFs.

Cycle selection procedure. Chromosomal DNA of *C. diphtheriae* strain C7 was partially digested with *Hha*I or *Hpa*II, and DNA fragments of approximately 150 to 600 bp were isolated by 1.5% agarose gel electrophoresis. Single-stranded oligonucleotides for linkers (Table 2) containing an *Hha*I- or *Hpa*II-compatible protruding end, a *Spe*I site, and an additional *Bam*HI or *Xba*I cloning site were prepared by GIBCO-BRL (Gaithersburg, Md.). After the annealing of the complementary oligonucleotides and phosphorylation, 40 pmol of the appropriate linker was ligated to 4 pmol of *Hha*I- or *Hpa*II-digested chromosomal DNA. After digestion with *Spe*I to eliminate multimeric linkers, the resulting DNA fragments were gel purified and amplified by PCR using linker-specific primer PAS-A or PAS-B (Table 2), as appropriate. A single PCR primer was used in each reaction because the complementary sequence was present on both ends of the template DNA fragments. To isolate DNA fragments capable of binding to DtxR, 2 μg of each purified PCR product was mixed with 500 μl of binding buffer (20 mM Na_2HPO_4 [pH 7.0], 50 mM NaCl, 5 mM $MgCl_2$, 100 μg of bovine serum albumin/ml, 10 μg of sonicated salmon sperm DNA/ml, 10% glycerol, and 300 μM $CoSO_4$) and purified DtxR at 200 nM. After incubation of the reaction mixture for 30 min at room temperature, 30 μl of rabbit anti-DtxR serum or a monoclonal antibody against the third domain of DtxR was added, and incubation was continued for another 30 min at 37°C. The mixture was then loaded onto a HiTrap protein G column (Pharmacia, Uppsala, Sweden) equilibrated with the binding buffer. The column was washed with 3 ml of binding buffer, and the bound DNA-DtxR-antibody complexes were eluted with 1.5 ml of 0.1 M glycine-HCl buffer (pH 2.5). Fractions of 200 μl were collected and immediately neutralized by the addition of 20 μl of 1 M Tris-HCl (pH 9.0). The DNA-containing fractions, detected by PCR amplification using primers specific for appropriate linker sequences, were combined. The amplified DNA fragments were then subjected to two more rounds of DtxR binding and PCR amplification, digested with *Bam*HI or *Xba*I as appropriate, and cloned into the *Bam*HI or *Xba*I site of vector pQF50 (4).

TABLE 3. Primers used for PCR or inverse PCR in *irp6* cloning and sequencing

Primer	Sequence (5′–3′)
6C2	GCCGATGGAGGCTGCGAC
6C3	AGACCTCGACATGCTCGG
6D2	GGACTTCCGCCAGAAGCC
6E1	CCTGCGCCATCAGGTGGA
3Q2	CGGATCCCTCCTAGGGAT
6A	AGGATCCGGCCCCACCACAGTGG
6AB	CGGATCCCAGTGGCACTGGGCCA

Gel mobility shift and footprinting assays. DNA fragments carrying the IRP1 or IRP6 promoter/operator were end-labeled with [α-^{32}P]dCTP by using Klenow fragments (GIBCO-BRL). The labeled DNA fragments at approximately 0.5 nM were incubated with various concentrations of purified DtxR in 10 μl of binding buffer (described in "Cycle selection procedure"). For mobility shift assays, the reaction mixtures were incubated for 10 to 15 min at room temperature and then subjected to electrophoresis on 5% nondenaturing polyacrylamide gels at 4°C in 20 mM Na_2HPO_4 (pH 7.0) buffer for 1 to 1.5 h at 70 V. For footprinting assays, 1 μl of DNase I (GIBCO-BRL) at 10 ng/ml was added to each DtxR-DNA mixture at the end of the room temperature incubation. The reaction mixtures were incubated for another 1 to 3 min, at which time the DNase I treatment was terminated by phenol-chloroform extraction. The resulting DNA samples were subjected to electrophoresis on a sodium dodecyl sulfate–8% polyacrylamide gel at 70 to 90 V for 2 to 2.5 h at room temperature, and the gels were then dried and analyzed by autoradiography.

Siderophore assays. Overnight cultures of *C. diphtheriae* strains grown in modified PGT medium with or without 10 μM $FeCl_3$ were harvested by centrifugation, and the supernatants were used for siderophore assays. Chrome azurol S (CAS) assays were carried out by a slight modification of a previously described method (30). Each 500-μl sample of appropriately diluted culture supernatant was mixed with an equal volume of CAS assay buffer. The reaction mixtures were incubated for 2 h at room temperature, and their absorbance values at 630 nm were measured. A linear standard curve was constructed by testing 500-μl samples containing EDDA at concentrations of 5, 10, 20, and 40 μM. A sample of supernatant contained 1 U of siderophore/ml if its absorbance at 630 nm was equal to that of test sample containing 1 μM EDDA.

Inverse PCR. *C. diphtheriae* strain C7 chromosomal DNA was completely digested with *Hin*dIII and purified by phenol-chloroform extraction. Approximately 1.2 μg of *Hin*dIII DNA fragments was self-ligated with 5 U of T4 ligase in a final reaction volume of 250 μl, and a 0.2-μl sample of the ligation mixture was mixed with appropriate primers (Table 3) and then used for each inverse PCR using Elongase enzyme mixture (GIBCO-BRL) with appropriate Mg^{2+} concentrations as recommended by the manufacturer.

Nucleotide sequence accession numbers. The GenBank accession number for the nucleotide sequence of the *irp6* operon is AY061890. The sequence that contains an additional three ORFs downstream from the original *irp1* ORF was submitted to GenBank, and the accession number is AF176902.

RESULTS

Isolation of IRP6 from the chromosome of *C. diphtheriae* by a SELEX-like procedure. As part of an ongoing effort in our laboratory to identify and characterize the DtxR-regulated genes of *C. diphtheriae*, DNA fragments with potential DtxR binding sites were isolated from *C. diphtheriae* chromosomal DNA by a SELEX-like procedure. DNA fragments that bind

to purified holo-DtxR can be enriched exponentially, in principle, by forming DNA-DtxR complexes, purifying the complexes by use of anti-DtxR antibodies and a protein G column, using PCR to amplify the DNA fragments isolated from the complexes, and repeating the cycle several times.

DNA fragments collected after three cycles of this SELEX-like procedure in several independent experiments were approximately 200 bp. They were digested with *Bam*HI or *Xba*I, as appropriate, and cloned into pQF50 upstream from the promoterless *lacZ* gene. The resulting plasmid clones were transformed into *E. coli* strain DH5α containing pDSK29 (*dtxR*$^+$). To identify promoters that were negatively regulated by DtxR under high-iron conditions, transformants were first plated on low-iron LB agar medium containing X-Gal plus ampicillin and kanamycin to maintain positive selection for the two compatible plasmids. Colonies showing various degrees of blue, indicating that they contained cloned promoters that were active under low-iron conditions, were then picked and transferred onto high-iron LB agar plates containing X-Gal plus ampicillin and kanamycin. Colonies that were white under these high-iron conditions were considered to contain putative DtxR-regulated, iron-repressible promoters. Over 2,000 separate transformants were tested, and approximately 100 colonies that exhibited some degree of blue only under low-iron growth conditions were examined further by β-galactosidase assays after subculturing them in LB broth under high-iron and low-iron conditions. Isolates with promoters that were not consistently repressible by DtxR and iron were eliminated, as were clones that gave positive signals in Southern blots with DNA probes for the previously known IRPs. Two clones containing newly identified IRPs were identified in this manner. One of these, pQF6, containing an approximately 200-bp *Bam*HI fragment, designated IRP6, is the subject of the present study.

IRP6 promoter activity and its regulation by DtxR and iron in *E. coli*. Expression of β-galactosidase from pQF6 in *E. coli* DH5α containing or lacking *dtxR*$^+$ plasmid pDSK29 and after growth in high-iron or low-iron LB broth was examined (Table 4). Vector pQF50 and plasmid pQF1, which contained previously reported iron-regulated promoter/operator IRP1, were included as negative and positive controls, respectively. The β-galactosidase activity from pQF50 was barely detectable under any of the conditions tested and was considered to represent the baseline value for the assay system. The promoter strength of IRP6 was comparable to that of IRP1, as shown by the similar β-galactosidase activities from pQF6 and pQF1 in the absence of DtxR under low-iron or high-iron growth conditions. The activity of IRP6 was repressed to the baseline level by iron and DtxR, and the ratio of β-galactosidase activity from pQF6 under derepressing (low-iron) conditions to that under repressing (high-iron) conditions was 56. IRP6 was fully derepressed under low-iron conditions in the presence of DtxR (78.8 U of β-galactosidase activity), but IRP1 was only slightly derepressed under similar conditions (6.8 U of β-galactosidase activity). These findings raised the possibility that IRP6 has significantly lower affinity for holo-DtxR than does IRP1. To determine whether the inserts in pQF1 and pQF6 contained unidirectional or bidirectional promoters, they were excised and recloned in the opposite orientation into pQF50, generating plasmids pQF1- and pQF6-, respectively. The β-galactosidase activities from pQF1- or pQF6- in the presence or absence of pDSK29 (*dtxR*$^+$) and under high-iron or low-iron growth conditions were all comparable to the baseline level for the assay system, demonstrating that DNA fragments containing both IRP1 and IRP6 have unidirectional promoter activity.

TABLE 4. Promoter activities of IRP6 in the presence or absence of DtxR in an *E. coli* system[a]

Plasmid	Iron level	β-Galactosidase activity (Miller units)	
		− *dtxR*	+ *dtxR*
pQF50	Low	0.9 ± 0.2	1.2 ± 0.3
	High	0.7 ± 0.1	1.0 ± 0.2
pQF6	Low	87.7 ± 9.1	78.8 ± 8.6
	High	83.0 ± 0.3	1.4 ± 0.2
pQF6-	Low	1.4 ± 0.3	1.6 ± 0.6
	High	1.2 ± 0.3	0.9 ± 0.0
pQF1	Low	84.7 ± 8.7	6.8 ± 2.4
	High	69.7 ± 6.4	0.8 ± 0.2
pQF1-	Low	0.5 ± 0.1	0.8 ± 0.2
	High	0.5 ± 0.0	0.5 ± 0.2

[a] Cells were grown in LB medium treated with Chelex-100 resin, and overnight cultures were used for β-galactosidase activity assays. Low iron, addition of 200 μg of EDDA/ml; high iron, addition of 10 μM $FeCl_3$. Values represent the means ± standard deviations of assays performed on cultures grown in triplicate. −*dtxR*, without a plasmid that carries *dtxR*; +*dtxR*, with a plasmid that carries *dtxR*.

Binding of DtxR to IRP6. The DtxR-binding activity of IRP6 was investigated by gel mobility shift assays and DNase I protection assays using previously characterized DtxR-regulated promoter/operator IRP1 as a control. As shown in Fig. 1A, retardation of DNA fragments containing IRP1 and IRP6 required both DtxR at 0.1 or 0.5 μM and Co^{2+} at 300 μM. To compare the relative affinities of IRP6 and IRP1 for DtxR, additional gel mobility shift assays were performed with DtxR at concentrations ranging from 0 to 2,000 nM in the presence of 300 μM Co^{2+} (Fig. 1B). For IRP6, the lowest DtxR concentration required to show a clearly detectable mobility shift was 100 nM. In contrast, the DNA fragment containing IRP1 showed a clearly detectable mobility shift in the presence of as little as 5 nM DtxR. These findings indicate that DtxR has significantly higher affinity for IRP1 than for IRP6 in vitro, consistent with the results of the β-galactosidase activity assays for the *E. coli* system.

To identify the DtxR-binding region of IRP6, the nucleotide sequence of IRP6 was determined (Fig. 2A) and DtxR footprinting experiments were performed with DNase I (Fig. 2B). Purified DtxR at 100 nM protected a 28-bp region in IRP6 from digestion by DNase I, but with DtxR at 500 nM the protected region extended further in both directions to include a total of 60 bp. With IRP1, DtxR at 100 nM protected a 32-bp sequence, and at 500 nM DtxR the protected region extended further in one direction only to include a total of 63 bp. These are the first reported examples of DtxR footprints that vary in size as a function of the concentration of DtxR. The putative 19-bp core sequence of the primary, high-affinity DtxR binding site in IRP6 was compared with the previously reported DtxR-specific operators from *tox*, IRP1, IRP2, IRP3, IRP4, IRP5, and *hmuO* (12) (Fig. 3). The core sequence of the IRP6 operator matched the 19-bp consensus sequence at 14 positions (74% identity), and the consensus sequence for the core DtxR binding site, based on all eight of the currently known DtxR-

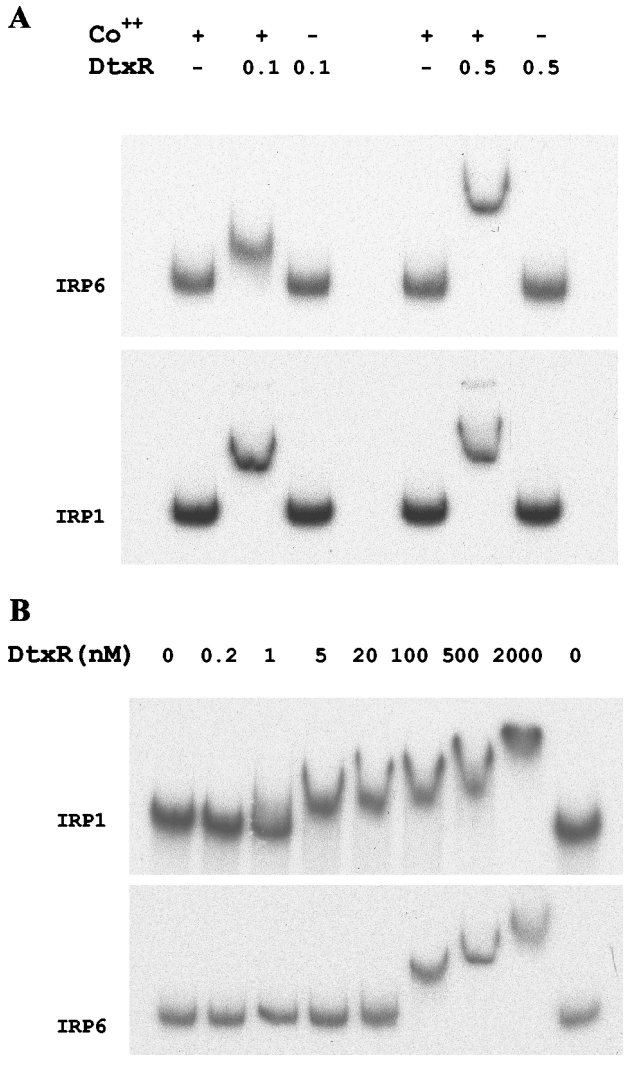

FIG. 1. (A) DtxR binding of IRP6 requires a divalent transitional metal ion, such as Co^{2+}. (B) Effect of DtxR concentration on formation of DtxR-DNA complexes in gel mobility shift assays. Detectable shifts of IRP6 occurred at 100 nM DtxR, while detectable shifts of IRP1 started at 5 nM DtxR. Co^{2+} was present in all samples at 300 μM.

regulated promoters/operators in Fig. 3, was identical with the previously deduced consensus sequence. The nucleotides in the right arm of the core region were slightly more conserved than those in the left arm, and cytidylic acid at position +6 was the only invariant nucleotide within the core regions of these eight DtxR-regulated promoters/operators. No sequences that were highly homologous with the consensus DtxR-binding sequence were found within the sequences of IRP1 and IRP6 that were protected only in the presence of high concentrations of DtxR, and the characteristics that define low-affinity DtxR binding sites have not yet been determined.

Identification of an operon downstream from IRP6. Southern hybridization experiments (data not shown) indicated that the cloned insert in pIRP6 was located on a 6.8-kb *Hin*dIII fragment from the chromosome of *C. diphtheriae* (Fig. 4A). Plasmid pSK6a (Fig. 4B), containing a 1.9-kb *Eco*RI fragment, was recovered from a λZapII genomic library of *C. diphtheriae* because it hybridized with an IRP6 probe, and the region that is homologous with IRP6 was further localized to the 680-bp *Sal*I-*Pst*I fragment within pSK6a (Fig. 4B). Sequencing this *Sal*I-*Pst*I fragment confirmed the presence of IRP6 and revealed an incomplete ORF downstream from it. To obtain more information about the genomic DNA downstream from this incomplete ORF, inverse PCR was performed using a *Hin*dIII-digested and self-ligated preparation of genomic DNA as the template (see Materials and Methods) and the primer pair 6C2 and 6D2 (Fig. 4B) to amplify the 5.6-kb segment of the circularized 6.8-kb *Hin*dIII chromosomal fragment located between the primers. The resulting 5.6-kb PCR product was also used as a probe to screen the λZapII genomic library of *C. diphtheriae* for clones containing sequences downstream from the partial ORF in pSK6a. Plasmid pSK6e, which carries a 2.2-kb *Eco*RI insert contiguous with and immediately downstream from the insert in pSK6a (Fig. 4B), was obtained by this method and sequenced. Sequences further downstream from this 2.2-kb *Eco*RI fragment in pSK6e were obtained by directly sequencing the 3.6-kb inverse PCR product, which was amplified by using primer pair 6C2 and 6E1 (Fig. 4B).

The nucleic acid sequence of a 3,650-bp segment of the *irp6* chromosomal region, determined by sequencing the two λZapII clones and the inverse PCR products described above, revealed contiguous ORFs *irp6A*, *irp6B*, and *irp6C* (Fig. 4A). BLAST searches were conducted with the amino acid sequences deduced from the three ORFs. Predicted protein Irp6A is a 395-aa polypeptide with relatively low homology to several periplasmic binding protein components of bacterial ABC transport systems, such as hypothetical protein TM0189 from *Thermotoga maritima* (27% identity; GenBank accession no. E72406). Irp6A has a typical N-terminal signal peptide and a predicted signal peptidase II recognition sequence (L-T-A-**C**-S-N), which suggests that the 27-aa N-terminal signal sequence is cleaved and that the mature form of the protein is tethered to the exterior of the plasma membrane by fatty acyl groups at the modified N-terminal cysteine residue indicated by boldface in the predicted recognition sequence shown above (14). The predicted size for the processed Irp6A protein is 372 aa. The predicted Irp6B protein is a 347-aa polypeptide with nine transmembrane segments predicted by HMMTOP analysis (http://www.enzim.hu/hmmtop). It shows homology with integral membrane components of ABC transport systems, with the highest homology being to heme uptake protein HmuU from *C. diphtheriae* (34% identity; GenBank accession no. AF109162). The *irp6C* ORF is predicted to encode a cytoplasmic protein with 252 aa residues that contains an ABC domain with well-conserved Walker A and B motifs as well as the ABC signature sequence, which is conserved among ABC transporters (8, 12, 27). Irp6C is most similar to FepC from *E. coli* (36% identity; GenBank accession no. P23878), the ATP-binding component of the ferric enterobactin transport system. ORFs *irp6A*, *irp6B*, and *irp6C* are tightly linked, with no interrupting nucleotides between the first two and only 1 bp separating the last two. The nucleotide sequence extending more than 400 bp downstream from *irp6C* did not show any significant homology to other proteins in the database. A potential stem-loop structure (with free energy level at −13.4 kcal) found 34 bp downstream from the stop codon (TAG) of *irp6C*

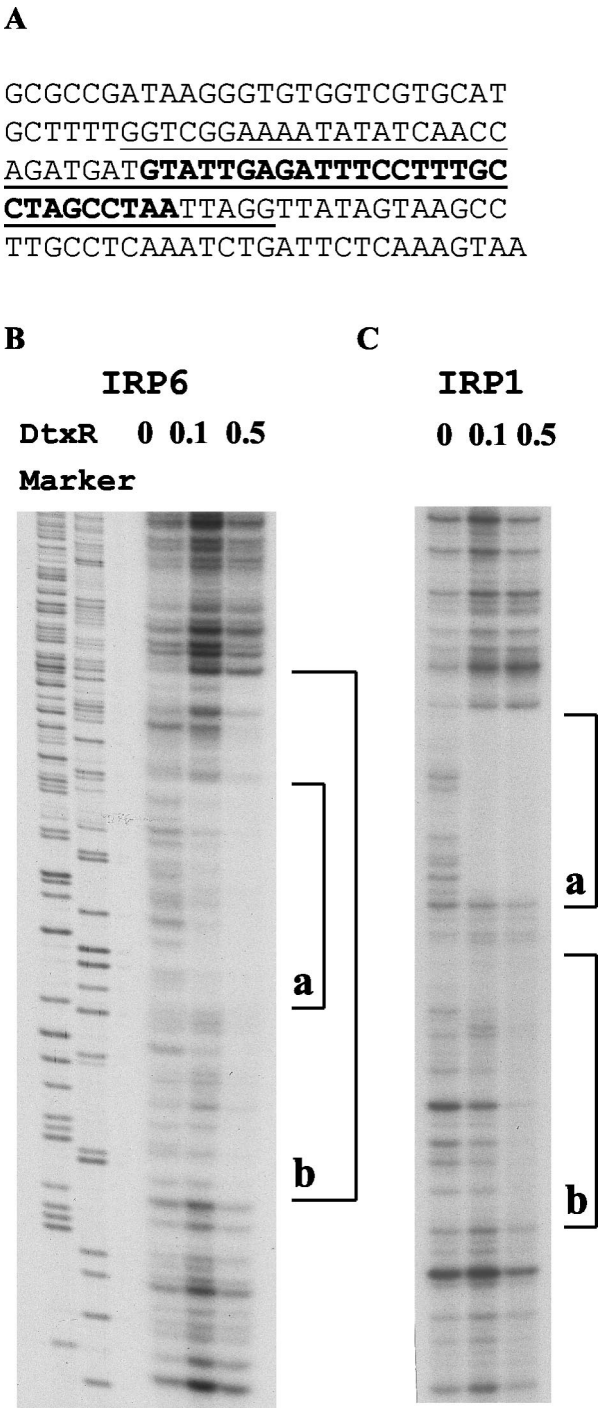

FIG. 2. (A) Nucleotide sequence of IRP6. The primary DtxR binding site defined by DNase I footprinting assays with DtxR at 0.1 μM is indicated by boldface. The longer nucleotide sequence protected from DNase I by 0.5 μM DtxR is underlined and includes the primary DtxR binding site. (B and C) DNase I footprinting assays. All the fragments were 3′ end labeled with [α-^{32}P]dCTP on one strand and incubated in the presence of Co^{2+} (300 μM) and DtxR (0, 0.1, or 0.5 μM). Brackets indicate the sequences protected by DtxR from DNase I digestion. (B) With IRP6, a longer 60-bp sequence (b) was protected by 0.5 μM DtxR and a shorter 28-bp sequence (a) that is contained within sequence b was significantly protected by 0.1 μM DtxR. Although the total radioactivity loaded in the lane with 0.1 μM DtxR was greater than that in the other lanes, the intensity of the bands within the 28-bp (a) sequence was decreased significantly both in comparison with bands in other regions of the same lane and with bands in the same 28-bp region in the lane without DtxR. (C) In IRP1, two contiguous regions (a and b) were protected by 0.5 μM DtxR but only region a was protected by 0.1 μM DtxR.

probably functions as a transcriptional terminator. Therefore, we hypothesized that genes *irp6A*, *irp6B*, and *irp6C* form a single transcriptional unit, that the *irp6* operon encodes an ABC transporter possibly involved in high-affinity iron uptake in *C. diphtheriae*, and that the Irp6A protein might function as the lipoprotein receptor for a ferric siderophore complex.

Complementation of *C. diphtheriae* iron uptake mutants by genes of the *irp6* operon. *C. diphtheriae* mutant strains HC1, HC3, HC4, and HC5 (2) were derived by chemical mutagenesis from wild-type strain C7(β) and produced large amounts of corynebacterial siderophore even under high-iron (repressing) conditions (30). ^{59}Fe transport studies showed that even though these strains were capable of producing siderophore, they were defective in siderophore-mediated ferric iron uptake. Attempts to complement the mutations in these strains with plasmid pWS382, which was thought to contain the entire *irp1* operon (26), were unsuccessful. We therefore tested whether the *irp6* operon could complement the functional defect(s) in these strains. To obtain a clone containing the entire *irp6* operon, primer pair 6C3 and 3Q2 (Table 3) was used to amplify an approximately 4.0-kb fragment (Fig. 4A and C) from chromosomal DNA of *C. diphtheriae* strain C7 using a *Pfu-Taq* enzyme mixture system (Elongase; GIBCO-BRL). The PCR product was then digested with *Bam*HI and *Sal*I, and the resulting 3.6-kb *Bam*HI-*Sal*I fragment (Fig. 4C) was cloned into the same sites in *E. coli-C. diphtheriae* shuttle vector pCM2.6, generating plasmid pCM6ABC. pCM6ABC was introduced into strains HC1, HC3, HC4, and HC5 by electroporation (24), and the transformants were tested for siderophore production under low- and high-iron conditions. Vector pCM2.6 was also introduced into these strains as a control. In wild-type strain C7(β) carrying either plasmid pCM6ABC or vector pCM2.6, siderophore production was repressed by iron to levels that were approximately 15-fold less than those under low-iron growth conditions (Table 5). All mutant strains carrying vector pCM2.6 produced much greater amounts of siderophore than wild-type strain C7(β) under high-iron conditions (≥180 U/ml) and showed slight increases in siderophore production (twofold or less) under low-iron conditions, in agreement with previous studies of these mutant strains (2, 30). When plasmid pCM6ABC was introduced into strains HC1, HC4, and HC5, production of siderophore under high-iron conditions was repressed to ≤63 U/ml, while under low-iron conditions siderophore production was derepressed to ≥236 U/ml (Table 4). These findings demonstrate that plasmid pCM6ABC was able to reverse the siderophore overproduction phenotype of strains HC1, HC4, and HC5, presumably due to the introduction of a wild-type copy of the putative siderophore uptake system encoded by the *irp6* operon. In contrast, plasmid pCM6ABC did not reverse the mutant phenotype of strain HC3.

Plasmids pCM6A and pCM6AB were constructed by cloning PCR products encoding the first one or two ORFs of the *irp6* operon with primer pair 6C3 and 6A or 6C3 and 6AB (Table

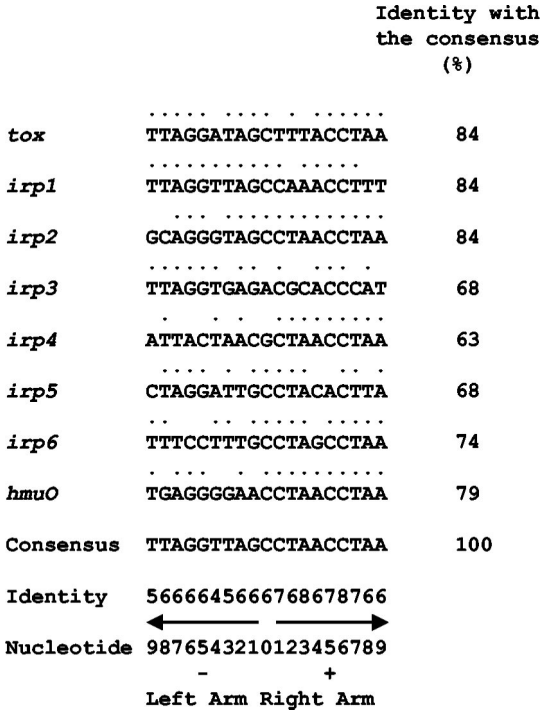

FIG. 3. Compilation of the 19-mer core sequences of DtxR binding sites and the consensus sequence. Dots above nucleotides indicate that the nucleotide matches the nucleotide of the consensus sequence. The identity score indicates the numbers of times that nucleotide was found among all the aligned DtxR binding sites. Arrows, inverted repeats within the core consensus sequence. The identity of each DtxR binding site with the consensus is indicated.

3), respectively (Fig. 4). These two constructs were introduced into strains HC1, HC4, and HC5, and siderophore production was measured in supernatants of cultures grown under low- and high-iron conditions. For strain HC1, introduction of either plasmid pCM6AB or pCM6A improved growth in low-iron PGT medium (not shown) and complemented the defect in repression of siderophore synthesis under high-iron conditions (Table 4), indicating that strain HC1 contains a mutant allele of *irp6A*. In strain HC4, however, neither subclone complemented the mutant phenotype, suggesting that strain HC4 has a mutant allele of *irp6C*. Finally, plasmid pCM6AB, but not pCM6A, enhanced the growth of strain HC5 in low-iron PGT medium and restored repression of siderophore synthesis under high-iron conditions, indicating that strain HC5 contains a mutant allele of *irp6B*. The nucleotide sequencing of the *irp6A* allele from strain HC1 showed that it has a G-to-A substitution at position 1280, causing a Gly240Asp substitution. The sequencing of the *irp6C* allele from strain HC4 revealed that a G-to-A substitution at position 2925 resulted in a Gly45Asp substitution in the conserved Walker A motif. The sequencing of the *irp6B* allele from strain HC5 revealed base substitutions in three different codons; two of them were silent, but a G-to-A substitution at position 2325 introduced a TGA codon that resulted in chain termination at the codon for Trp193.

In mutant strain HC3, the defect in siderophore-mediated iron uptake was not complemented by the wild-type *irp6* operon alone. Since strain HC3 was produced from C7(β) by chemical mutagenesis with ethyl methane sulfonic acid ester, it was possible that HC3 harbored multiple point mutations. We observed that HC3 exhibited high sensitivity to the toxic effects of heme in HITW and that this property was shared by C7(β)hm723, a strain that harbors a mutant *dtxR* allele (24). In contrast, wild-type C7(β) and strains HC1, HC4, and HC5 were not highly sensitive to added heme in HITW. We determined the sequence of the *dtxR* allele in strain HC3, and we found that a G-to-A substitution in the codon for Trp104 caused premature chain termination of the DtxR protein. We constructed plasmid pNGR-6ABC, containing both the wild-type *dtxR* gene and the wild-type *irp6* operon, and introduced it into strain HC3. We found that strain HC3 carrying this plasmid grew well under low-iron conditions, tolerated high concentrations of heme in HITW [up to 100 μM, which is comparable to the tolerance of wild-type C7(β)], and did not overproduce siderophore and DT under high-iron conditions. Further sequence analysis showed that HC3 also has a G-to-A substitution at position 2041 in the *irp6B* gene that results in a Glu99Lys substitution in the Irp6B protein.

Activities of the IRP1 and IRP6 promoters in wild-type and mutant strains of *C. diphtheriae*. DNA fragments containing the IRP1 and IRP6 promoters were cloned into shuttle vector pCM502 (21) in front of the promoterless *lacZ* gene, and the two resulting transcriptional fusion constructs, pCM1 and pCM6, respectively, were introduced into wild-type *C. diphtheriae* C7(β) and into mutant strains HC1, HC3, HC4, and HC5. Activities of β-galactosidase in cultures grown under low- and high-iron conditions were measured. In wild-type C7(β), the β-galactosidase activities from both constructs were repressed under high-iron conditions (HITW or HITW-Fe) and derepressed in cultures grown under low-iron conditions in the presence of iron chelator EDDA (Fig. 5). As seen previously in *E. coli* (Table 4), the IRP1 promoter appeared to be more stringently repressed than the IRP6 promoter in response to iron. In all four of the mutant strains, the IRP6 and IRP1 promoter activities were derepressed when the cells were grown in HITW. Growth in HITW-Fe medium restored repression of the IRP6 and IRP1 promoters in strain HC1, but this effect was not seen with strains HC3, HC4, and HC5. These phenotypic differences between mutant strains HC1, HC3, HC4, and HC5, all of which are deficient in siderophore-mediated iron uptake, are discussed below.

Complete sequence of the *irp1* operon. Previously, an ORF named *irp1* was identified downstream of promoter IRP1 (26). More sequence downstream of *irp1* was obtained from plasmid pWR382, which contains an 8-kb *Eco*RI insert (Fig. 6). Three additional ORFs were identified. The ORF originally called *irp1* was therefore renamed *irp1A*, and the three genes that immediately follow it were named *irp1B*, *irp1C*, and *irp1D* (Fig. 6). There are only 2 bp separating the first two ORFs; the stop codon of *irp1B* overlaps with the start codon of *irp1C* (ATG). And the last two ORFs are only separated by 11 bp. Therefore, these four ORFs are tightly linked and most likely form a single transcriptional unit.

Like the *irp6* operon, the *irp1* operon also showed homology with other ABC transport systems. As shown previously (26), *irp1A* encodes a 355-aa lipoprotein that was considered to be a candidate receptor for a siderophore uptake system in *C. diphtheriae*. There is no significant homology, however, between lipoprotein receptors Irp6A and Irp1A, even in the N-terminal

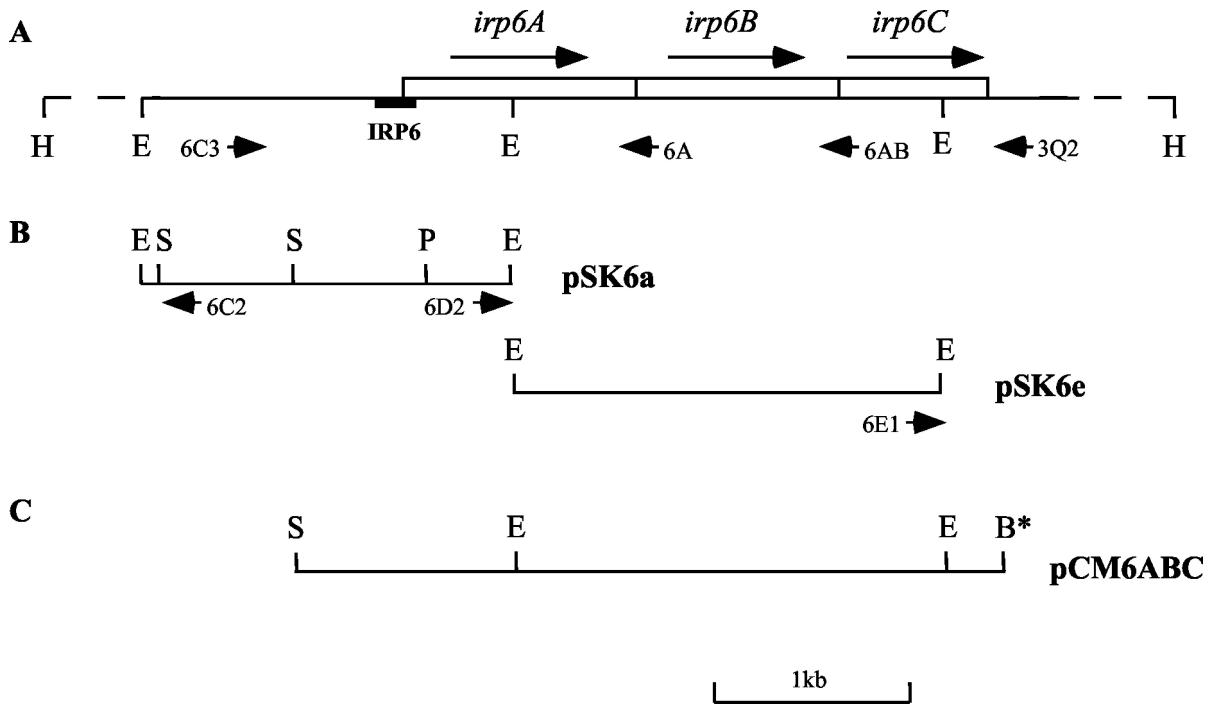

FIG. 4. Organization of *irp6* operon. (A) Restriction map of the 6.8-kb chromosomal *Hin*dIII fragment containing the *irp6* operon. Long arrows, orientations of the three ORFs; short arrows, locations and orientations of the primers used to amplify the entire *irp6* region, or regions containing only the first ORF (*irp6A*) or the first two ORFs (*irp6AB*) of the *irp6* operon; black bar, location of IRP6 promoter fragment isolated by the SELEX-like procedure. (B) Restriction map of library clone pSK6a, containing the 1.9-kb *Eco*RI fragment that hybridized to an IRP6 probe, and library clone pSK6e, containing the 2.2-kb *Eco*RI fragment that is adjacent to pSK6a on the chromosome. Short arrows, locations of the primers (6C2 and 6D2) used in inverse PCR to amplify the flanking sequence on the 6.8-kb *Hin*dIII fragment. (C) Restriction map of the 3.6-kb *Sal*I-*Bam*HI insert containing the *irp6* region in plasmid pCM6ABC. The asterisk indicates that the *Bam*HI site was generated by primer 3Q2. Restriction enzyme abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; P, *Pst*I.

signal sequences. The *irp1B* and *irp1C* genes are predicted to encode 343-aa and 351-aa polypeptides, respectively, and both have 10 transmembrane segments according to HMMTOP. Irp1B and Irp1C are most similar to the transmembrane components of a probable iron-siderophore uptake system in *Streptomyces coelicolor* (GenBank accession no. T36890 [41% identity] and T36890 [39% identity], respectively). There is extensive homology between membrane proteins Irp1B and Irp1C (31% identity), and the Irp1B and Irp1C proteins each have 30% homology with Irp6B. The *irp1D* gene is predicted to encode a 283-aa polypeptide which has homology with ATP-binding proteins of bacterial ABC transporters, and the Walker A and B motifs as well as the ABC signature sequence are well conserved. Irp1D is most homologous to permease protein FecE from the iron(III) dicitrate transport system in *Synechocystis* spp. (accession no. D90899, 53% identity). ATP-binding proteins Irp6C and Irp1D have 34% homology. Therefore, the *irp1* operon seems to encode another ABC transporter in *C. diphtheriae*. By comparison with the subunit organizations of other ABC transporters (8, 27), the *irp1* system most likely utilizes a heterodimer consisting of Irp1B and Irp1C as its transmembrane component, whereas the *irp6* system most likely employs a homodimer consisting of two Irp6B polypeptides as its transmembrane component.

DISCUSSION

SELEX is a powerful tool for isolation of target nucleic acid sequences when a purified, sequence-specific oligonucleotide-binding protein, such as DtxR, is available (33). To obtain biologically relevant DNA fragments, we used a SELEX-like procedure followed by in vivo screening with an *E. coli* reporter system to isolate DtxR-binding and iron-regulated promoter fragments from *C. diphtheriae*. As a result, IRP6 was obtained, and it showed DtxR-dependent iron-regulated activities in both the *E. coli* system and *C. diphtheriae* C7(β).

High-affinity uptake of iron complexes such as ferrisiderophore across the cytoplasmic membrane usually involves a binding protein-dependent ABC transport system, the acronym for a family of transporters containing ABC domains (8). In gram-positive bacteria, a binding protein-dependent ABC transport system usually consists of an extracellular lipoprotein receptor, an integral membrane protein, and an intracellular ATP binding peripheral membrane protein (31). In gram-negative bacteria, due to the existence of an outer membrane (OM), an OM porin-like receptor in conjunction with the TonB/ExbBD energy-transducing system is often used to facilitate the active transport of iron complexes through the OM (6). The ABC uptake apparatus that transports ferrisiderophore complexes across the inner membrane in gram-negative bacteria is similar to that of gram-positive bacteria, except that gram-negative bacteria employ periplasmic binding proteins as ferrisiderophore receptors and gram-positive bacteria utilize homologous membrane-attached lipoproteins as ferrisiderophore receptors. Both the *irp1* and *irp6* operons exhibit gene organization typical of ABC transport systems for

TABLE 5. Suppression of siderophore overproduction under high-iron growth conditions by complementation of mutant alleles of the *irp6* operon in *C. diphtheriae*[a]

Strain	Plasmid	Siderophore level (U/ml) in:	
		Low iron	High iron
C7(β)	pCM2.6	451 ± 30	26 ± 3
	pCM6ABC	396 ± 3	26 ± 1
HC1	pCM2.6	514 ± 12	386 ± 40
	pCM6ABC	452 ± 19	53 ± 3
	pCM6AB	372 ± 35	43 ± 0
	pCM6A	332 ± 29	60 ± 8
HC3	pCM2.6	576 ± 54	187 ± 10
	pCM6ABC	585 ± 54	236 ± 58
HC4	pCM2.6	499 ± 11	353 ± 109
	pCM6ABC	594 ± 6	63 ± 10
	pCM6AB	411 ± 61	285 ± 18
	pCM6A	333 ± 26	460 ± 141
HC5	pCM2.6	291 ± 10	194 ± 29
	pCM6ABC	236 ± 35	44 ± 5
	pCM6AB	111 ± 14	3 ± 0
	pCM6A	109 ± 21	111 ± 8

[a] Supernatants from overnight cultures grown in modified PGT medium (mPGT) (low iron) or mPGT plus 10 μM $FeCl_3$ (high iron) were used for siderophore assays. Values represent means ± standard deviations of assays performed on cultures grown in triplicate.

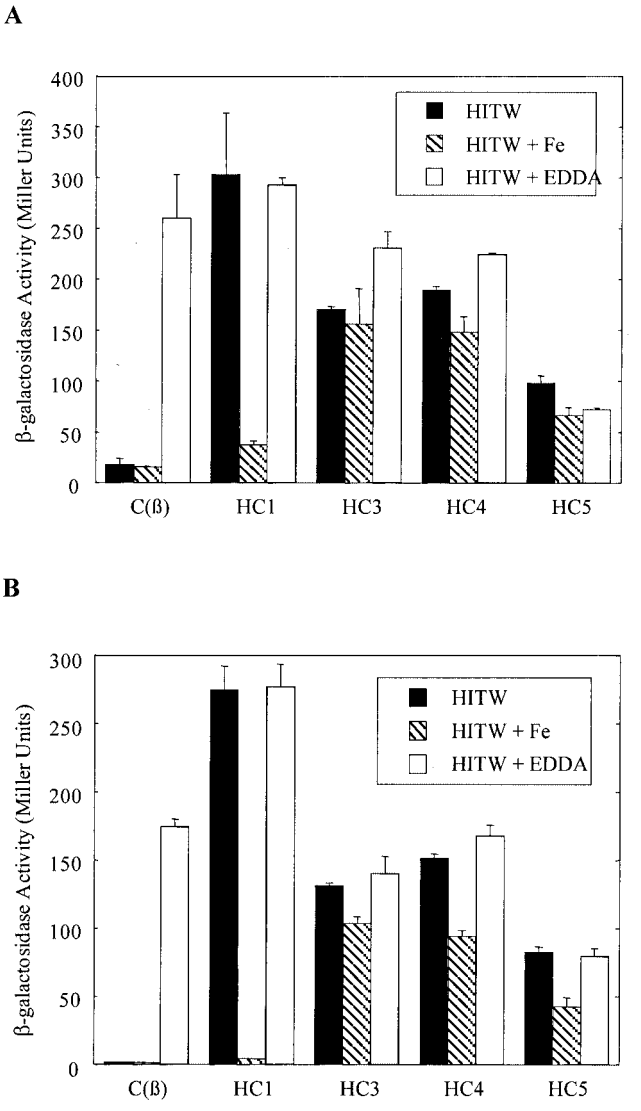

FIG. 5. β-Galactosidase activities of *C. diphtheriae* strains C7(β), HC1, HC3, HC4, and HC5 carrying promoter fusion constructs pCM6 (A) and pCM1(B) cultured in HITW, HITW-Fe, and HITW with 100 μg of EDDA/ml. Overnight cultures were used for β-galactosidase activity assays, and the activity values were the averages of samples from cultures grown in triplicate. The standard deviations are shown by the error bars.

gram-positive bacteria. Studies of several sequenced bacterial genomes indicate that genes encoding ABC transport systems are abundant and constitute one of the major gene families. For example, there are an estimated 57 ABC transporters in *E. coli* (12) and 78 in *B. subtilis* (15). Several ABC transport systems have been isolated in corynebacteria, including the *gluABCD* system for glutamate uptake in *C. glutamicum* (9), a recently characterized ABC transport system involved in heme uptake in *C. diphtheriae* (3), and an ABC export system for tetracycline in *Cornynebacterium striatum* (32). The genome sequence of *C. diphtheriae* NCTC 13129 has very recently been completed by investigators at the Sanger Centre. We performed a BLAST search of the putative proteins encoded by the *C. diphtheriae* genome using Irp6C, the ABC protein encoded by the *irp6* operon, as the probe, and we found at least 58 putative ATP-binding proteins with conserved Walker A and B motifs as well as the ABC signature sequence, which is present in ABC transport systems (8, 12, 27).

Our sequence analysis and mutant complementation experiments provided strong evidence that the *irp6* operon encodes an ABC transport system for siderophore uptake in *C. diphtheriae*. Mutations in each of the three components of this uptake system were associated with previously documented abnormalities in siderophore-mediated iron uptake (18) that indirectly affected other intracellular processes regulated by DtxR and iron. Therefore, it is apparent that siderophore-mediated iron uptake plays an important role in this organism. Siderophore overproduction under high-iron conditions was also observed in a *Rhizobium leguminosarum* mutant strain defective in the membrane component of the siderophore-dependent iron uptake system (29). The proposed role of the *irp6* transport system in *C. diphtheriae* is also consistent with previous biochemical evidence implicating ATP in ferri-siderophore uptake in *C. diphtheriae*, which was shown to be sensitive to arsenate, a phosphate homolog that inhibits ATP formation (16). Additional studies will be needed, however, to demonstrate directly that Irp6A can function as a specific binding protein for ferricorynebactin.

Previous studies showed that *C. diphtheriae* can use aerobactin from *S. flexneri*, as well as its own siderophore, corynebactin, but corynebactin does not function as a siderophore for *S. flexneri* (17, 18). Corynebactin and aerobactin must therefore be different, but the structure of corynebactin from *C. diphtheriae* has not been determined. We demonstrated that strains HC1, HC4, and HC5 could use aerobactin as a siderophore to stimulate bacterial growth in the presence of iron only when

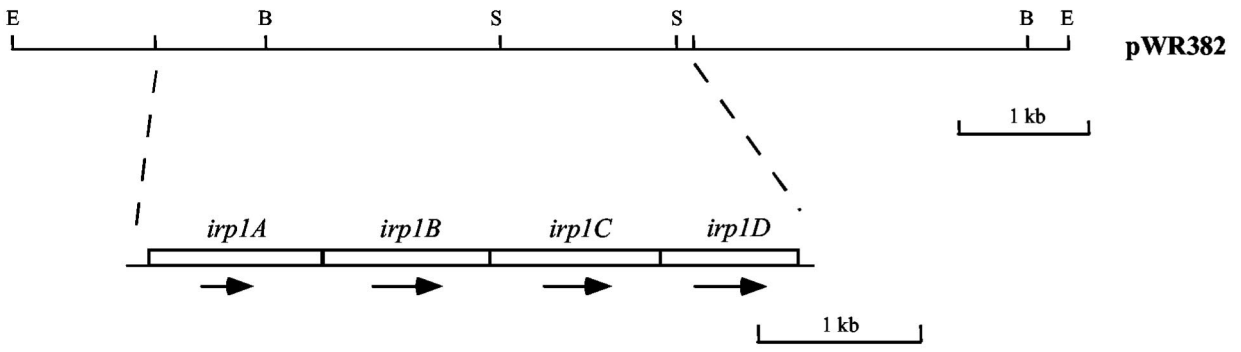

FIG. 6. Gene organization of *irp1* operon. Dashed lines indicate the location of the four ORFs (*irp1A*, *irp1B*, *irp1C*, and *irp1D*) in the 8-kb *Eco*RI insert in plasmid pWR382. Restriction enzyme abbreviations: B, *Bam*HI; E, *Eco*RI; S, *Sal*I.

the wild-type *irp6* operon was present on the complementing pCM6ABC plasmid (data not shown), indicating that the *irp6* transporter of *C. diphtheriae* is required for both corynebactin-dependent and aerobactin-dependent iron uptake. Iron assimilation by *C. diphtheriae* can also occur by siderophore-independent pathways. *C. diphtheriae* has a high-affinity system for acquiring iron from heme or hemoglobin (3). In addition, the fact that siderophore-deficient strains such as *C. diphtheriae* PW8 and C7(β) mutant HC6 can grow under high-iron conditions, but not under low-iron conditions, in heart infusion medium suggests that *C. diphtheriae* also has siderophore-independent, low-affinity iron uptake activity.

Analysis of β-galactosidase activities of our IRP1 and IRP6 promoter fusion constructs in wild-type *C. diphtheriae* strain C7(β) showed that both of these promoters were repressed during growth in HITW (high-iron conditions) and derepressed during growth in HITW broth plus 100 μg of EDDA/ml (low-iron conditions). In mutant strains HC1, HC3, HC4, and HC5, which are deficient in siderophore-dependent iron uptake, expression of the β-galactosidase reporter gene from the IRP1 and IRP6 promoter fusion constructs was derepressed during growth in HITW. For strain HC1, which expresses a Gly240Asp variant of putative ferrisiderophore receptor Irp6A, growth in HITW-Fe restored the iron- and DtxR-dependent repression of the reporter gene. In contrast, for strain HC4, which has a defect in putative ATP-binding protein Irp6C, and for strain HC5, which has a defect in putative membrane protein Irp6B, growth in HITW-Fe did not restore repression of the reporter gene. One possible explanation for these observations is that the Gly240Asp variant of Irp6A retains a low affinity for ferrisiderophores. If so, and if the concentration of siderophore produced during growth of strain HC1 in HITW exceeded the concentration of available iron, then addition of more iron to the medium might increase the concentration of ferrisiderophore complexes and permit enough siderophore-dependent iron uptake by strain HC1 to activate DtxR and cause the repression of the reporter genes in the IRP1 and IRP6 fusion constructs that we observed in Fig. 5.

When the siderophore-deficient strains HC6 and PW8 of *C. diphtheriae* were grown in HITW, however, DtxR-regulated functions were fully repressed, presumably as a consequence of iron uptake by low-affinity, siderophore-independent pathways (data not shown). The lack of repression of DtxR-regulated functions in strains HC1, HC3, HC4, and HC5, but not in strains PW8 and HC6, during growth in HITW raises the possibility that the ABC transporter encoded by the *irp6* operon may also have a role in low-affinity, siderophore-independent iron uptake in *C. diphtheriae*. Another possible explanation for the observed repression of the IRP1 and IRP6 promoters of strain HC1, but not of strains HC3, HC4, and HC5, during growth in HITW-Fe is that Irp6A is less important than Irp6B and Irp6C for low-affinity, siderophore-independent iron uptake in *C. diphtheriae*. Furthermore, the relatively high and easily measured baseline expression of the IRP6 promoter that was observed in the wild-type strain during growth in both HITW and HITW-Fe is consistent with a possible physiological role for the *irp6* operon in low-affinity, siderophore-independent iron uptake by *C. diphtheriae* under high-iron growth conditions. Additional work will be needed to investigate these hypotheses further.

Strains C(β)hm723 and HC3 both have point mutations in their *dtxR* alleles which are known to cause decreased activity or inactivation of DtxR (24, 34). We showed that strains HC3 and C(β)hm723 grew poorly in HITW supplemented with 100 μM heme, probably as a consequence of derepression of the heme uptake pathway and accumulation of toxic levels of iron or heme degradation products in the intracellular milieu. When a wild-type *dtxR* allele was introduced into strain HC3, it still overproduced siderophore under high-iron conditions (data not shown), indicating that the Glu99Lys substitution in Irp6B resulted in inactivation of siderophore-dependent iron transport.

Complementation of strain HC3 with both the wild-type *dtxR* allele and the complete wild-type *irp6* operon restored normal regulation of functions controlled by DtxR and iron. These findings demonstrated that the abnormal phenotypes of strain HC3 with respect to iron uptake and DtxR-dependent regulation were caused by mutations in *dtxR* and in the *irp6* operon and did not involve additional chemically induced mutations in other genes.

In the present study, the complete sequence of the *irp1* operon was determined and shown to encode four proteins that comprise a putative ABC transporter. Although the lipoprotein encoded by the *irp1* operon, here renamed Irp1A, is homologous to FhuD from *B. subtilis* and was previously pro-

posed as a candidate ferrisiderophore receptor, the *irp1* operon could not complement the defects in siderophore-dependent iron transport in the HC1, HC3, HC4, and HC5 variants described above (25, 26). No mutants with defects in the *irp1* operon have been described, and the function of the *irp1* operon remains unknown. It is interesting that a search of the genome of *C. diphtheriae* NCTC 13129 did not reveal the *irp1* operon from *C. diphtheriae* C7(β), indicating that this ABC transport system is not present in all isolates of *C. diphtheriae*. Since most studies of iron-dependent gene regulation in *C. diphtheriae* have been performed with strains C7(-), C7(β), and mutants derived from them, however, we have included our findings on the *irp1* operon in the present report.

In summary, we conclude that siderophore production and siderophore-dependent iron uptake are central aspects of the DtxR regulatory circuit during growth of *C. diphtheriae* in media that do not contain heme as a source of iron. These processes directly influence intracellular iron concentrations and affect the activity of DtxR, which functions as a global effector for iron-regulated cellular processes. This study demonstrates that the *irp6* operon is required for high-affinity, siderophore-dependent iron uptake in *C. diphtheriae* and that it may also have a role in low-affinity, siderophore-independent iron uptake in this pathogenic bacterium. This study also shows the effects of mutations in the *irp6A*, *irp6B*, and *irp6C* genes on the regulation of both the IRP1 and IRP6 promoters and on siderophore and toxin production (2, 30).

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