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Cloning, Sequence, and Footprint Analysis of Two Promoter/Operators from *Corynebacterium diphtheriae* That Are Regulated by the Diphtheria Toxin Repressor (DtxR) and Iron

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DtxR is an iron-dependent sequence-specific DNA-binding protein that binds to the *tox* operator, an inverted-repeat nucleotide sequence located upstream from the diphtheria toxin gene. In this study, two additional iron-regulated promoter/operator sequences (IRP1 and IRP2) that are controlled by DtxR were cloned from the chromosome of *Corynebacterium diphtheriae* and characterized. Operon fusions to *lacZ* were used to analyze expression from IRP1 and IRP2 in *Escherichia coli*. Transcription from both promoters was strongly repressed in high-iron medium in the presence of the cloned *dtxR* gene; however, transcription in the absence of *dtxR* was 50- to 100-fold greater, regardless of the iron concentration. Purified DtxR altered the electrophoretic mobility of DNA fragments carrying IRP1 or IRP2, and the nucleotide sequences of the two promoter/operator regions indicated that they are both homologous with the *tox* operator. DtxR protected an approximately 30-bp region on both IRP1 and IRP2 from DNase I digestion. A 19-bp consensus DtxR-binding site was derived from a comparison of the various DtxR-regulated operator/promoter sequences. Footprinting experiments using hydroxyl radicals and dimethyl sulfate demonstrated that DtxR interacted with these operators in a symmetrical manner, probably as a dimer or multimer. The deduced amino acid sequence of an open reading frame (ORF1) located downstream from IRP1 was homologous with a family of periplasmic proteins involved in iron transport in gram-negative bacteria and with the ferrichrome receptor, FhuD, from *Bacillus subtilis*. These findings suggest that ORF1 encodes a membrane-associated lipoprotein that may serve as the receptor for a ferric-siderophore complex in *C. diphtheriae*.

It has been known for more than 50 years that the production of diphtheria toxin by *Corynebacterium diphtheriae* is negatively regulated by iron (24, 25). More recently, diphtheria toxin and the corynebacterial siderophore corynebactin were shown to be coordinately regulated (3, 7, 31). This regulation is moderated by the activity of the iron-dependent diphtheria toxin repressor (DtxR), encoded by the chromosomal *dtxR* gene. The structural gene (*tox*) for diphtheria toxin is encoded on the genome of several temperate corynebacteriophages (24); however, the genes that determine siderophore production and the high-affinity iron uptake systems of *C. diphtheriae* are chromosomal (28).

Transcription of *tox* in *C. diphtheriae* is repressed in cultures grown in high-iron medium and derepressed in cultures depleted of iron (18, 22, 32). The *dtxR* gene was cloned independently by Boyd et al. (3) and Schmitt and Holmes (31). The nucleotide sequence of *dtxR* has been determined elsewhere (3), and the deduced amino acid sequence exhibited very limited homology with the ferric uptake regulatory protein (Fur) of *Escherichia coli* (3, 31). The DtxR protein was purified (34, 39), and footprinting experiments demonstrated that DtxR protected an approximately 30-bp sequence located upstream of the *tox* gene from digestion by DNase I (34, 40). This *tox* operator sequence overlapped the −10 sequence of the *tox* promoter and contained a 9-bp inverted repeat. The binding of DtxR to the *tox* operator in vitro was dependent on the presence of Fe^{2+} or any of several other divalent transition metals (33, 39).

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Hydroxyl radical footprinting revealed that DtxR bound in a symmetrical manner about the dyad axis of the *tox* operator sequence (33), in a manner similar to the binding of other well-characterized repressors, such as LexA or the phage lambda repressor, to their operators (12, 23). In contrast, studies with the *E. coli* Fur protein, which is an iron-dependent repressor like DtxR, demonstrated that it binds to the aerobactin operator (6) in a manner that does not involve symmetrical interactions about the dyad axis of the operator.

The Fur protein in *E. coli* binds to a 19-bp inverted-repeat nucleotide sequence referred to as an iron box (7, 19). Sequences with various degrees of homology to the consensus sequence for the Fur-binding site have been found upstream of numerous iron-regulated genes in *E. coli* and in other gram-negative bacteria (19, 26, 36). Although a region within the AT-rich *tox* operator has about 50% homology with the consensus Fur-binding sequence, purified DtxR does not bind to known Fur-binding sites in vitro (34), and the *tox* gene is not regulated in vivo by Fur (3, 31). Therefore, the DtxR and Fur repressors have distinctly different recognition sequences.

In *C. diphtheriae* C7(β)hm723, which carries a defective *dtxR* allele (2, 16, 32), diphtheria toxin and siderophore are produced constitutively at high levels, regardless of the iron concentration (5, 31, 37). However, when the cloned $dtxR^+$ allele is introduced into C7(β)hm723, iron-dependent regulation of the phage-encoded *tox* gene and the chromosomally encoded determinants for siderophore production and utilization is restored (31). DtxR appears, therefore, to function as a global repressor of iron-regulated genes in *C. diphtheriae*, like Fur does in *E. coli*.

In this study, we cloned two additional *C. diphtheriae* promoter/operator sequences in *E. coli*, designated IRP1 and IRP2, which are regulated by DtxR and Fe^{2+}. The nucleotide

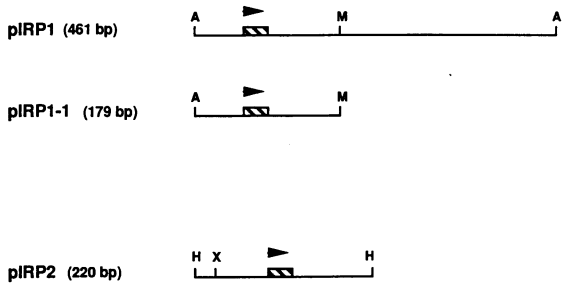

FIG. 1. Physical maps of *C. diphtheriae* chromosomal inserts that encode iron- and DtxR-regulated promoter/operator sequences. The recombinant plasmids were derived from the promoter probe vector pQF50. The size of each insert DNA is indicated in base pairs. Hatched boxes indicate DtxR-binding sites, and the directions of transcription from an iron-regulated promoter are indicated by arrows. Plasmid pIRP1-1 is a subclone of pIRP1 that contains the 179-bp *Alu*I-*Msp*I fragment. A, *Alu*I; H, *Hae*III; M, *Msp*I; X, *Xba*I.

sequences of the two operators revealed regions that are homologous to that of the *tox* operator. Footprinting experiments indicated that DtxR interacted with both operators in a manner similar to that of its interaction with the *tox* operator. The sequence of a partial open reading frame (ORF1) located downstream from IRP1 was also determined, and analysis of the deduced amino acid sequence suggested that ORF1 encodes a membrane-associated lipoprotein that may function as a receptor for a ferric siderophore.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* K-12 DH5α (*lac*) (Bethesda Research Laboratories, Gaithersburg, Md.) was used throughout this study for all cloning and screening assays and β-galactosidase assays and for routine isolation of plasmid DNA. Nonlysogenic, nontoxigenic *C. diphtheriae* C7(−), hereafter designated C7, is from our culture collection (14). Chromosomal DNA from C7 was used as the source for promoter/operator fragments. *E. coli* H1431 is a *fur* mutant obtained from K. Hantke.

E. coli DH5α was grown in Luria broth (LB) (21). LB medium was supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml) as required. LB cultures were made low iron by the addition of the iron chelator ethylenediamine-di(*o*-hydroxyphenyl)acetic acid (EDDA; 500 μg/ml), and LB agar medium was depleted of iron by the addition of 50 μg of EDDA per ml. EDDA was deferrated by the method described by Rogers (27).

Plasmid pQF50 (Ap^r) (10) was used for the isolation and analysis of cloned promoter sequences. DNA fragments to be screened for promoter activity were ligated into a multiple-cloning site located upstream from the promoterless *lacZ* gene in pQF50. Plasmid pDSK29 (Kn^r) carries the *dtxR* gene and has been described previously (32), and plasmid pQFtox carries the *tox* promoter/operator sequence on a 200-bp DNA fragment in pQF50. Physical maps of recombinant plasmids carrying *C. diphtheriae* iron-regulated promoter sequences are shown in Fig. 1.

DNA preparation. Chromosomal DNA used for the cloning and analysis of promoter/operator fragments was purified from *C. diphtheriae* C7 as described previously (30, 31). The boiling method described by Holmes and Quigley (13) was used to prepare small amounts of plasmid DNA. Larger quantities of purified plasmid DNA were prepared by a plasmid purification procedure developed by Qiagen, Inc., Chatsworth, Calif. The DNA fragments employed in the various DtxR-binding studies were isolated from agarose gels and purified by using the Gene Clean kit as described by Bio 101, Inc., La Jolla, Calif.

DNA fragments were end labeled with [^{32}P]dCTP (Amersham, Arlington Heights, Ill.) using the Klenow fragment of DNA polymerase I as previously described (20). DNA fragments used for sequence analysis were subcloned into the pBluescript phagemid vectors KS and SK to isolate single-stranded DNA, which was sequenced by the chain termination method described by Sanger et al. (29) using a DNA sequencing kit purchased from U.S. Biochemicals, Inc., Cleveland, Ohio.

Gel mobility shift assays and footprinting techniques. The DtxR protein used in all gel mobility shift assays and footprinting experiments was purified in our laboratory by Ni^{2+} affinity chromatography as described by Schmitt and Holmes (33). DtxR was present at approximately 500 μM in all of the gel mobility shift assays and footprinting studies. DNA fragments carrying the various promoter/operator sequences were labeled with ^{32}P at the 3′ end on either the coding or the noncoding strand and were present at approximately 0.1 to 0.5 nM in all experiments. The gel mobility shift assays and the experimental conditions for the various footprinting techniques employed in this study have been described previously (9, 33, 34, 41). Co^{2+}, Ni^{2+}, Mn^{2+}, and other divalent metals can substitute for Fe^{2+} to activate the DNA-binding activity of DtxR (34, 39). Since Fe^{2+} is rapidly oxidized to Fe^{3+} under the in vitro conditions used to analyze DtxR binding, Co^{2+} (which has greater redox stability than Fe^{2+}) was used in place of Fe^{2+} in all of the DtxR-binding studies. Therefore, $CoSO_4$ at 300 μM was used as indicated for individual experiments in the various DtxR-binding assays.

β-Galactosidase assays. Bacterial cultures supplemented with appropriate antibiotics were grown overnight in LB medium that contained 500 μg of EDDA per ml (low-iron cultures) or no added EDDA (high-iron cultures). Cultures were centrifuged briefly, supernatants were discarded, and the cell pellets were resuspended in M9 medium (21). Quantitative assays to determine β-galactosidase activity were done by using the resuspended cell pellets as described previously (21).

Nucleotide sequence accession numbers. The nucleotide sequence of the 461-bp DNA fragment in pIRP1 and the 220-bp DNA fragment in pIRP2 were assigned the GenBank accession numbers U02617 and U02634, respectively.

RESULTS

Cloning of promoters regulated by DtxR and iron. Chromosomal DNA from *C. diphtheriae* C7 was partially digested with *Sau*3AI, and DNA fragments of 1 to 4 kb were isolated and purified. The chromosomal fragments were ligated into the promoter probe vector pQF50, which had been digested with *Bam*HI. Transcription of the promoterless *lacZ* gene in pQF50 can be directed from cloned DNA fragments carrying appropriately oriented promoter sequences (10). Recombinant plasmids were transformed into DH5α/pDSK29 ($dtxR^+$) and analyzed for the expression of *lacZ* on low-iron LB agar medium containing X-Gal plus ampicillin and kanamycin to select both for pDSK29 and for the PQF50 recombinant plasmids. The formation of blue colonies indicated that the inserted fragment in pQF50 expressed promoter activity under low-iron conditions in the presence of DtxR. Blue colonies represented 5 to 15% of total Ap^r Kn^r transformants. To determine whether the promoter activities of individual plasmid clones were regulated

TABLE 1. Expression of iron-regulated promoters

Plasmid[a]	Iron[b]	β-Galactosidase activity (U)[c]	
		dtxR^{+}[d]	*dtxR*
pQF50	+	<0.5	<0.5
	−	<0.5	<0.5
pQFtox	+	<0.5	17.0
	−	15.0	23.5
pIRP1-1	+	<0.5	35.0
	−	22.0	42.0
pIRP2	+	<0.5	56.0
	−	48.0	94.0

[a] All plasmids were present in *E. coli* DH5α.
[b] +, high-iron conditions; −, low-iron conditions.
[c] Specific activity of β-galactosidase was determined according to Miller (21).
[d] *dtxR*$^{+}$, with plasmid pDSK29; *dtxR*, without pDSK29.

by iron, blue colonies were restreaked both onto high-iron and onto low-iron agar plates containing X-Gal and ampicillin and kanamycin. Colonies that were white (repressed) on high-iron medium but blue (derepressed) on low-iron medium were putative clones of iron-regulated promoters from *C. diphtheriae*. Two plasmid clones containing 1.5- and 3-kb inserts expressed this phenotype and were analyzed further.

Subclones which maintained DtxR and iron regulation were obtained by digesting purified insert DNA with various restriction endonucleases and ligating the resulting fragments into pQF50. The subclones that maintained iron-regulated promoter activity are diagrammed in Fig. 1. Plasmid pIRP1 contains a 461-bp *Alu*I fragment derived from the 1.5-kb parental clone. Plasmid pIRP1-1 contains a 179-bp *Msp*I-*Alu*I subclone of pIRP1. Plasmid pIRP2 carries a 220-bp *Hae*III fragment derived from the 3.0-kb parental clone.

Quantitation of promoter activity. Transcription from the promoters present on plasmids pIRP1-1 and pIRP2 was measured from DH5α cultures grown under high-iron or low-iron conditions in the presence or absence of the cloned *dtxR*$^{+}$ allele on pDSK29 (Table 1). Plasmid pQFtox, which carries the *tox* gene promoter/operator sequences in pQF50, was included as a control. The *tox* promoter on pQFtox was strongly regulated by iron in DH5α, but only in the presence of the cloned *dtxR*$^{+}$ allele. The patterns of expression of the *lacZ* reporter gene from the pIRP1-1 and pIRP2 promoters were similar, but the derepressed level of transcription was slightly higher from pIRP1-1 and pIRP2 than from the *tox* promoter. These results indicate that all three promoters are similarly regulated in *E. coli* by *dtxR* and iron.

The promoter activity from pQFtox, pIRP1-1, and pIRP2 assayed in the *E. coli fur* mutant H1431, in the presence and absence of the cloned *fur*$^{+}$ allele, demonstrated that none of these promoters was regulated by *fur*, although expression of enterobactin was repressed by the cloned *fur*$^{+}$ allele as expected (data not shown).

Gel mobility shift assays. Gel mobility shift assays were used to analyze DtxR binding to DNA fragments carrying the iron-regulated promoter/operator regions from plasmids pIRP1 and pIRP2. DNA fragments of 461, 282, and 179 bp from pIRP1 and the 220-bp fragment from pIRP2 were isolated and analyzed in gel shift assays (Fig. 2). The 461-bp fragment contains the entire *Alu*I chromosomal insert from pIRP1, while the 179-bp fragment contains sequences on the left half of the insert and the 282-bp fragment carries the sequences on the right half of the insert with respect to the map shown in Fig. 1. Gel mobility shift assays demonstrated that the 179- and the 461-bp fragments from pIRP1 and the

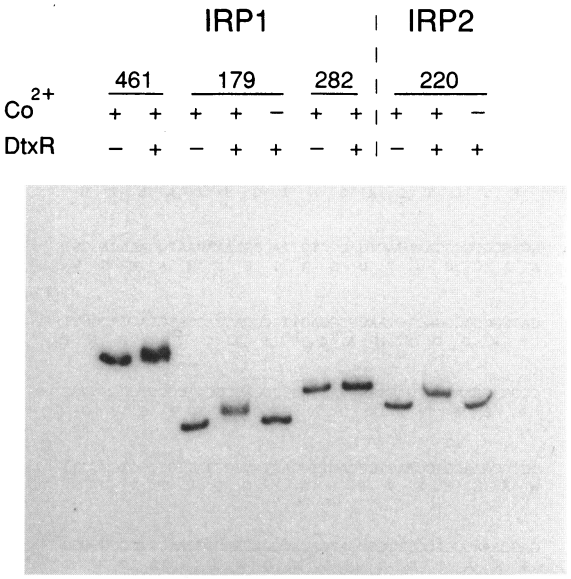

FIG. 2. Gel mobility shift assays. DNA fragments of 461 bp (*Alu*I-*Alu*I), 179 bp (*Alu*I-*Msp*I), and 282 bp (*Msp*I-*Alu*I) from plasmid pIRP1 (IRP1) and a 220-bp *Hae*III fragment obtained from plasmid pIRP2 (IRP2) were end labeled with ^{32}P and incubated in the presence (+) or absence (−) of DtxR or Co^{2+}. Experimental conditions are described in Materials and Methods.

220-bp fragment from pIRP2 had decreased electrophoretic mobility only in the presence of both DtxR and Co^{2+}, although the magnitude of the gel shift was smaller for the longer 461-bp fragment (Fig. 2). The mobility of the 282-bp fragment from pIRP1 was unaffected by the presence of DtxR. These results demonstrated that DtxR in the presence of Co^{2+} binds to the 179-bp fragment from plasmid pIRP1 and to the 220-bp fragment from pIRP2.

DNA sequence analysis. The nucleotide sequences of the *Alu*I fragment in pIRP1 (461 bp) and the *Hae*III fragment in pIRP2 (220 bp) were determined (Fig. 3A and B, respectively). Sequences present on the 461- and the 220-bp fragments that share homology with the *tox* operator and contain inverted-repeat sequences were identified, and a partial open reading frame was found downstream from the putative DtxR-binding site in the sequence from pIRP1. Putative −10 and −35 promoter sequences were identified in the sequence from pIRP2, but multiple sequences with various homologies to −10 and −35 promoter regions were present in pIRP1 in the sequence upstream from ORF1. Additional studies will be necessary to demonstrate directly which of these putative promoter sequences serves as the site for transcription initiation in pIRP1 and pIRP2 in vivo.

DNase I footprinting. Footprinting experiments indicated that DtxR in the presence of Co^{2+} protected from DNase I digestion approximately 30-bp sequences on DNA fragments carrying the promoter/operator regions from either pIRP1 or pIRP2 (Fig. 4 and 5, respectively). There was no protection in the absence of Co^{2+}, and the DNA sequences within the protected regions from pIRP1 and pIRP2 include the regions that are homologous with the *tox* operator. The sequences protected from DNase I digestion by DtxR in pIRP1, pIRP2, and the *tox* operator are compared in Fig. 6, along with a DtxR-like operator sequence recently identified upstream of an iron-regulated gene in *Streptomyces pilosus* (11). Strictly conserved nucleotides within these sequences are indicated by

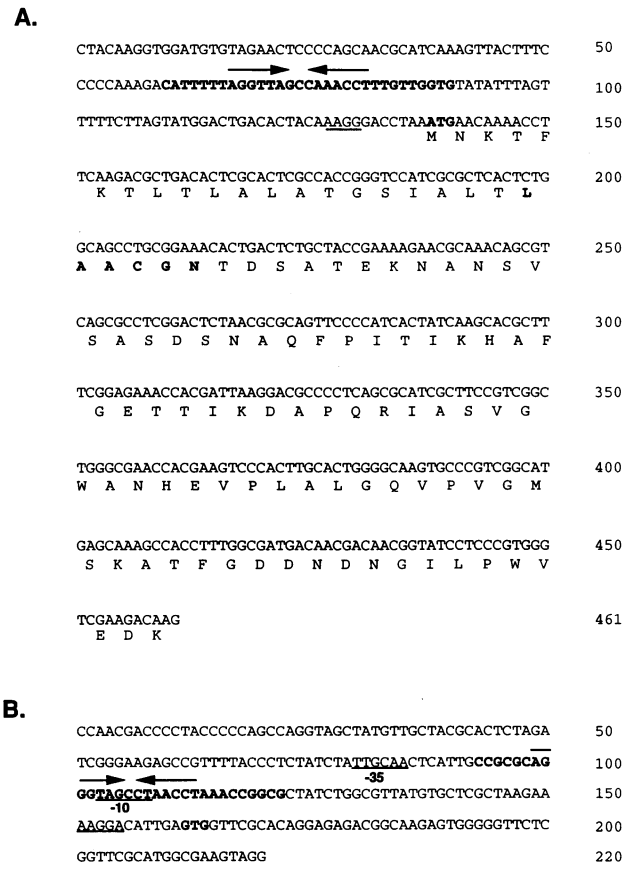

FIG. 3. Nucleotide sequences of the 461-bp *Alu*I fragment in plasmid pIRP1 (A) and the 220-bp *Hae*III fragment from plasmid pIRP2 (B). DtxR-binding sites are indicated by boldface letters with inverted arrows, which indicate inverted-repeat sequences. Putative Shine-Dalgarno sequences are underlined, and the first codons of possible open reading frames are indicated by the boldface ATG in panel A and GTG in panel B. The amino acid sequence of a partial open reading frame (ORF1) is shown in the one-letter code below the nucleotide sequence in panel A, and a possible signal peptidase II recognition sequence within this amino acid sequence is indicated by the boldface letters LAACGN. Putative −10 and −35 transcription initiation sequences are shown in panel B.

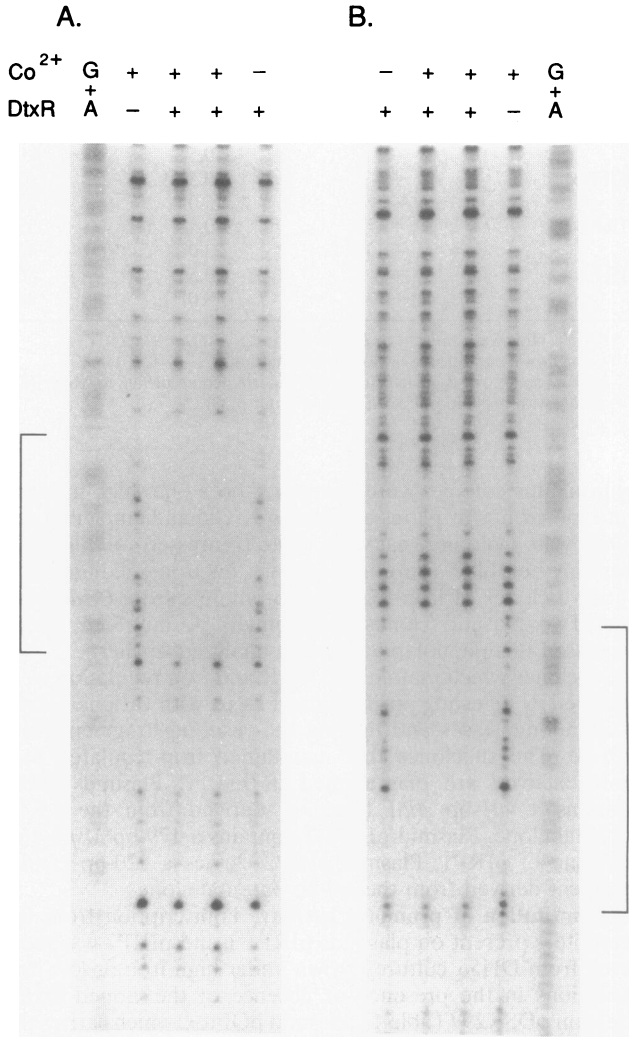

FIG. 4. DNase I protection experiments with IRP1. DNA fragments carrying the IRP1 sequences were 3′ end labeled with ^{32}P on either the coding strand (A) or the noncoding strand (B) and were incubated in a protein-binding buffer in the presence (+) or absence (−) of DtxR or Co^{2+}. Brackets indicate sequences protected by DtxR from DNase I digestion, and G + A indicates Maxam and Gilbert sequencing ladders. The experimental conditions are described in Materials and Methods.

boxes. All of the operators contain inverted-repeat sequences, which is characteristic of repressor-binding sites. The 19-bp consensus sequence within the core region of the DtxR-binding site, shown below the individual operator sequences, is a perfect palindrome (Fig. 6). All four of the operators exhibited 80 to 90% homology with the 19-bp consensus palindromic sequence for this core region. The high degree of homology among all of the operators suggests that specific base pairs within this core region are essential for recognition by DtxR. In the following sections, the iron-regulated promoter/operator sequences from pIRP1 and pIRP2 will be referred to as IRP1 and IRP2, respectively.

DtxR footprinting with hydroxyl radical and DMS. Hydroxyl radical protection experiments were used previously to identify specific contacts between DtxR and the deoxyribose-phosphate backbone at the *tox* operator (33). These studies demonstrated that DtxR binds in a symmetrical manner about the dyad axis of the *tox* operator and suggested that DtxR binds the *tox* operator as a dimer (or multimer), with each half of the functional DtxR complex contacting one end of the palindromic operator. Dimethyl sulfate (DMS) modification experiments identified three guanine residues within the *tox* operator that exhibited altered susceptibilities to methylation by DMS in the presence of DtxR. In the present study, similar hydroxyl radical protection and DMS modification experiments were performed with IRP1 (Fig. 7) and IRP2 (Fig. 8). In Fig. 7 and 8, sequences that were partially protected from hydroxyl radical cleavage by DtxR are indicated by letter designations (a through d or a′ through d′ for IRP1 and a through e or a′ through e′ for IRP2), and guanine residues that exhibited altered methylation in the presence of DtxR are indicated by arrows.

The results of the footprinting experiments with IRP1, IRP2, and the *tox* operator using DNase I, hydroxyl radicals, and DMS are compared schematically in Fig. 9A, B, and C, respectively. The hydroxyl radical protection experiments indicated that DtxR interacts in a symmetrical manner around

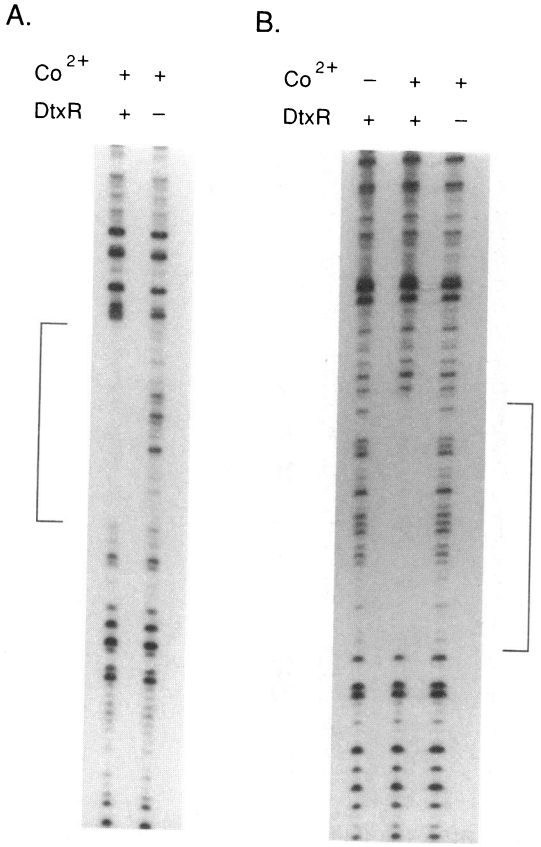

FIG. 5. DNase I protection experiments with IRP2. DNA fragments were end labeled on either the coding strand (A) or the noncoding strand (B). Reaction conditions were identical to those described in the legend to Fig. 4.

the pseudodyad axis of all three operators. However, DtxR appears to contact two regions in IRP2 (indicated by b′ and d) that are not involved in its interaction with IRP1 or the *tox* operator. In addition, the protection of sequences at one end of IRP2 (indicated by a) was much weaker (although reproducible) than protection of the corresponding region in either *tox* or IRP1. DMS modification experiments revealed that the three conserved guanine residues at positions 5, 9, and 10 in the core region of the DtxR-binding sites behaved similarly with respect to either enhanced methylation or protection from methylation in the presence of DtxR. The guanine residues at position 11 in IRP1 and IRP2 that showed slightly enhanced methylation in the presence of DtxR correspond with an adenine residue in the *tox* operator. Finally, the methylation of the two conserved guanine residues at positions 15 and 16 was slightly affected in the presence of DtxR in IRP2 but not in IRP1 or the *tox* operator, suggesting that DtxR interacts at IRP2 in a manner somewhat different from that at IRP1 or at the *tox* operator.

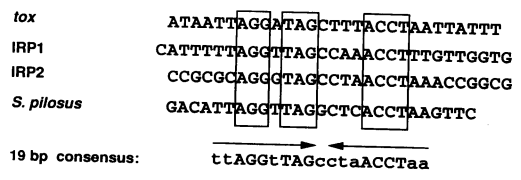

FIG. 6. DNA sequence alignment of the DtxR-binding sites from plasmids pIRP1 and pIRP2 and the *tox* operator and the putative DtxR-binding site from *S. pilosus*. Boxed sequences indicate bases that are conserved in all four operators. A 19-bp consensus sequence which was derived from a comparison of the four sequences is shown. Inverted repeats within the 19-bp consensus sequence are indicated by arrows; capital letters indicate nucleotides that are strictly conserved at that position, while lowercase letters indicate nucleotides that are less well conserved.

Amino acid homology between ORF1 and proteins involved in high-affinity iron uptake systems. An open reading frame (ORF1) was identified from the nucleotide sequence of the 461-bp *Alu*I fragment in pIRP1. ORF1 begins with an ATG codon and extends for 108 amino acids to the end of the available sequence (Fig. 3). An amino acid sequence homology search of GenBank using the Genetics Computer Group sequence programs (8) and using sequences identified from recently published studies (35) revealed that the 108 amino acids of ORF1 had significant homology with the family of binding proteins in the periplasm of gram-negative bacteria that are involved in high-affinity iron transport systems (Fig. 10) (35, 38). The recently identified ferrichrome receptor (FhuD) from *Bacillus subtilis* is also homologous with ORF1 and with this family of periplasmic proteins (Fig. 10) (35). Interestingly, the FhuD protein in *B. subtilis* was shown to be a soluble protein located on the outer surface of the bacterium. It was further postulated that FhuD is anchored to the membrane through a lipid moiety that is covalently attached to the N-terminal cysteine residue on the mature protein (35). Like the FhuD protein in *B. subtilis*, ORF1 contains an N-terminal amino acid sequence that is highly homologous with signal sequences found in lipoproteins in both gram-negative and gram-positive bacteria. A conserved signal peptidase II recognition sequence (LAACGN) is present in ORF1, and the conserved cysteine residue is presumed to serve as the lipid-modified N-terminal amino acid in a mature lipoprotein. A comparison of the 108-amino-acid residues of ORF1 with the homologous sequences from FhuD of *B. subtilis* and with each of the gram-negative periplasmic proteins indicated that they had between 15 and 25% identity and approximately 40% similarity. This level of homology is similar to that found in an earlier study in which the entire sequences of the various periplasmic proteins and FhuD were compared with one another (35). This observation suggests that ORF1 in *C. diphtheriae*, like FhuD in *B. subtilis*, may be a membrane-anchored lipoprotein that functions as receptor for a ferric-siderophore complex.

DISCUSSION

Two promoters, designated IRP1 and IRP2, were cloned from the chromosome of *C. diphtheriae* into *E. coli* and were shown to be regulated by DtxR and iron. The nucleotide sequences of DNA fragments carrying IRP1 and IRP2 showed that both were homologous with the *tox* operator. IRP1 and IRP2, as well as the *tox* promoter/operator, were not regulated in *E. coli* DH5α in the absence of the cloned *dtxR* gene, and the cloned *fur* gene could not substitute for the *dtxR* gene in regulating IRP1 or IRP2. These results support earlier studies which indicated that the iron-dependent repressors DtxR and Fur, from *C. diphtheriae* and *E. coli*, respectively, have distinctly different DNA recognition sequences (33).

DNase I footprinting experiments revealed that a 30-bp region on IRP1 and IRP2 was protected from DNase I digestion by DtxR and Co^{2+}. A 19-bp palindromic consensus DtxR operator sequence that is located within the 30-bp protected sequence was deduced by comparing the DtxR-

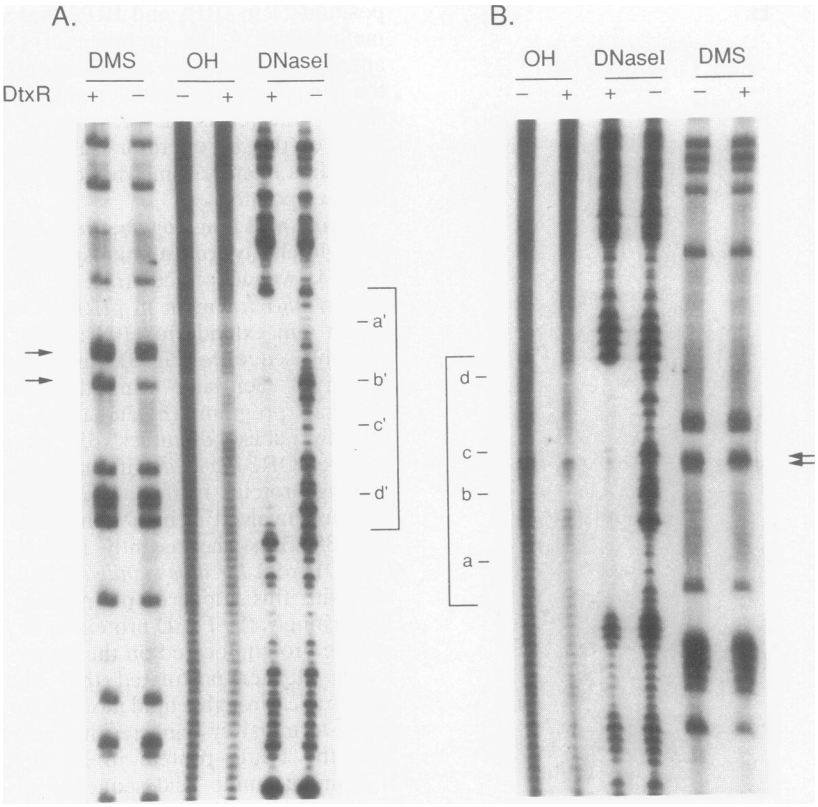

FIG. 7. Hydroxyl radical protection (OH) and DMS modification experiments with IRP1. The 461-bp (*Alu*I-*Alu*I) and the 179-bp (*Alu*I-*Msp*I) DNA fragments carrying IRP1 were 3′ end labeled on either the coding strand (A) or noncoding strand (B) and were incubated in protein-binding buffer in the presence (+) or absence (−) of DtxR. Co^{2+} was present in all assays at 300 μM. The experimental conditions are described in Materials and Methods. DNase I assays are also shown for comparison. Brackets indicate sequences protected from DNase I digestion by DtxR. Arrows indicate G residues that were altered in their cleavage in the DMS reactions in the presence of DtxR. The letters a through d and a′ through d′ indicate regions that exhibited partial protection from hydroxyl radical cleavage in the presence of DtxR.

binding sites of IRP1, IRP2, the *tox* operator, and a DtxR-like operator from *S. pilosus* (11) (Fig. 6). Although the complete 30-bp protected region for the *tox* operator has only 59 and 48% homology with the corresponding region in IRP1 and IRP2, respectively, and although IRP1 and IRP2 are only 53% homologous with each other, all four of the operators analyzed in Fig. 6 exhibit 80 to 90% homology with the 19-bp palindromic consensus sequence from the core region.

Interestingly, this 19-bp consensus sequence contains an inverted terminal repeat, which is typical for a repressor-binding site, and 10 of the 19 nucleotides within this sequence are strictly conserved in all four of the operators. These findings suggest that specific bases within these conserved sequences are essential for recognition by the activated form of DtxR.

Hydroxyl radical protection experiments demonstrated that the interaction of DtxR with IRP1 and IRP2 (the present study) and with the *tox* operator (33) is symmetrical about the dyad axis. This pattern of binding is similar to the way in which several other well-characterized prokaryotic repressors, including LexA and the phage lambda repressors, interact at their respective operator sequences (12, 23).

Various footprinting studies suggest that the LexA repressor, which controls numerous genes involved in the SOS regulon in *E. coli* (42), recognizes a 4-bp inverted-repeat sequence that is similarly situated in each half of the palindromic operator, separated by an 8-bp intervening region (15). The 4-bp recognition sequence for LexA is highly conserved among LexA-regulated operators, and it is part of a larger inverted-repeat region that is less well conserved among the various operators. Additionally, hydroxyl radical footprinting and ethylation interference experiments indicate that the 4-bp recognition sequence is located in a window between two LexA contact positions on the deoxyribose-phosphate backbone within the operator (15). DtxR produces a hydroxyl radical footprint similar to LexA, and the corresponding windows in the *tox* operator, IRP1, and IRP2 consist of the 4- to 5-bp sequences between the regions indicated by a′ and b in Fig. 9A through C at which DtxR contacts the deoxyribose-phosphate backbone of the operator. The 3-bp inverted-repeat sequence 5′-AGG(N_9)CCT-3′, which is conserved in all four operators, lies completely within this window in IRP1 and the *tox* operator but partially overlaps the protected region designated b in IRP2 (Fig. 9). Most well-studied prokaryotic repressors, including LexA, the *lac* repressor, and the lambda repressors, all have either a 3- or a 4-bp primary recognition sequence which is palindromic and separated by a 5- to 8-bp intervening sequence (12, 23).

Two mutations within the *tox* operator that interfere with regulation by iron and DtxR, designated *tox-201* and *tox-202*, have been characterized by sequence analysis (17). The *tox-201* and *tox-202* mutations were G-to-A transitions of the second and third positions, respectively, within the conserved 5′-

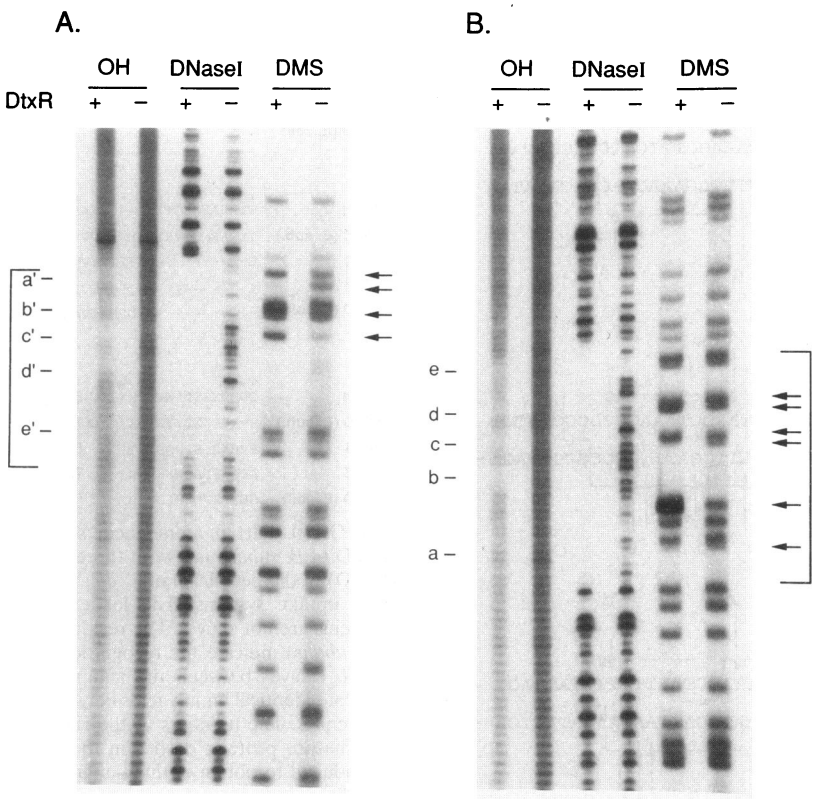

FIG. 8. Hydroxyl radical protection (OH) and DMS modification experiments with IRP2. The 220-bp DNA fragment carrying IRP2 was end labeled on either the coding (A) or the noncoding (B) strand. Figure labels and reaction conditions are identical to those described in the legend to Fig. 7.

AGG-3′ sequence that lies within the window region described above. In vivo, both mutants exhibited a significant decrease in the iron-dependent repression of diphtheria toxin synthesis (43), which suggests that DtxR binds inefficiently to these mutant operators. DNase I footprinting experiments with the *tox-201* operator sequence confirmed that DtxR bound very poorly to this operator in vitro (34). In summary, these observations suggest that the 5′-AGG(N_9)CCT-3′ conserved palindromic sequence within the DtxR-regulated operators is essential for the sequence-specific recognition of DNA by DtxR.

DtxR appears to interact with IRP2 in a slightly different manner from the way in which it interacts with IRP1 or the *tox* operator. Hydroxyl radical footprinting indicated that DtxR contacts the deoxyribose-phosphate backbone of IRP2 at two regions, designated b′ and d in Fig. 9, that are not contacted in either IRP1 or the *tox* operator. Furthermore, DMS modification experiments revealed that the two guanine residues at positions 15 and 16 were affected in their susceptibilities to methylation in the presence of DtxR; however, the corresponding guanine residues in both IRP1 and in the *tox* operator were unaffected by DtxR binding (Fig. 9). The reason for the differences in the footprinting pattern between IRP2 versus IRP1 and the *tox* operator is not clear. Although the level of homology among all of these operators is very high within the 19-bp core region, the flanking sequences that are outside the core but within the region protected by DtxR from DNase I digestion vary greatly. In IRP2, the flanking sequences have a very high GC content, whereas in the *tox* operator, the corresponding region has a very high AT content and in IRP1 this region is slightly more than half AT. It is possible that these large differences in flanking sequences may contribute to the differences in the binding of DtxR to IRP2 versus IRP1 and the *tox* operator. Additional studies are required to determine the molecular basis for this finding.

An open reading frame of 108 amino acids (ORF1) was identified downstream of the DtxR-binding site in pIRP1 (Fig. 6), but no significant open reading frame on the 220-bp fragment was identified in pIRP2. The partial sequence of ORF1 has homology with a family of binding proteins in the periplasm of gram-negative bacteria that are involved in the transport of ferric siderophores (35, 38) and with the recently identified ferrichrome receptor, FhuD, from *B. subtilis*, which is a membrane-associated lipoprotein (35) (Fig. 10). The FhuD signal sequence is typical for lipoproteins in both gram-negative and gram-positive bacteria. It contains a LAACGN sequence that is covalently modified at the cysteine residue and then cleaved by signal peptidase II to generate a mature lipoprotein that contains an N-terminal lipid-modified cysteine that functions as a membrane anchor. ORF1 also has a 23-amino-acid signal sequence that contains the LAACGN recognition sequence for signal peptidase II.

In gram-negative bacteria, ferric siderophores are transported through the cell envelope by a process that involves several proteins, including an outer membrane receptor and a soluble binding protein in the periplasm that are specific for the ferric siderophore and two or more cytoplasmic membrane-associated proteins (1, 4). Gram-positive bacteria do not have outer membranes and, therefore, do not encode proteins equivalent to the outer membrane receptor proteins of gram-

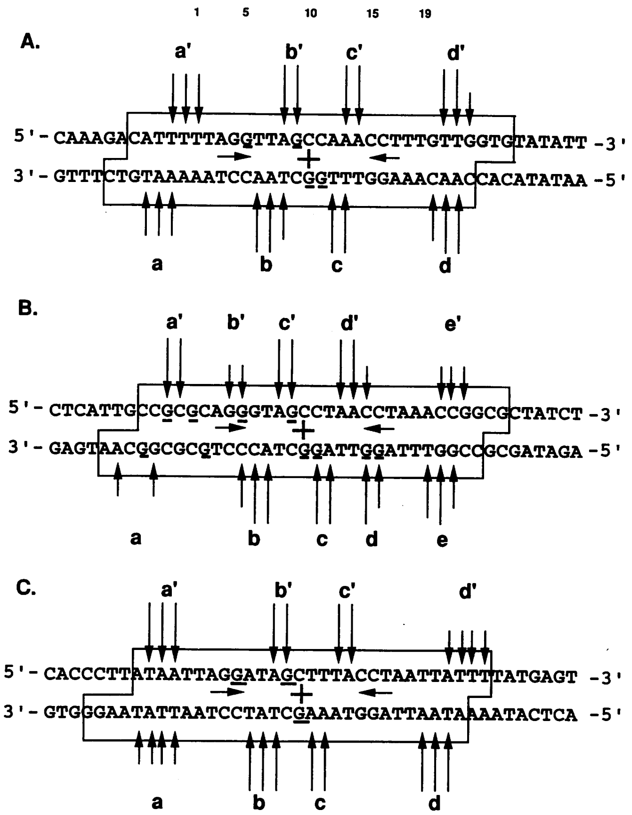

FIG. 9. Summary of footprinting experiments with DNase I, hydroxyl radicals, and DMS with IRP1 (A), IRP2 (B), and the *tox* operator (C). The top sequence is the coding strand, and the location of the 19-bp sequence is indicated by numbers at the top of the figure. Arrows indicate sequences protected from hydroxyl radical cleavage by DtxR. The lengths of the arrows indicate whether the protection was strong (long arrows) or weak (short arrows). The letter designations adjacent to the arrows in panels A and B correspond to the letters shown in Fig. 7 and 8, respectively. The results with the *tox* operator are from our previous publication (33). The boxed regions indicate sequences protected from DNase I digestion by DtxR. Inverted arrows indicate palindromic sequence (AGG[N_9]CCT) with strictly conserved ends that may be essential for recognition by DtxR. The crosses at the center of this palindromic sequence denote the pseudodyad axis of the operators. The G residues that had either enhanced or decreased cleavage in the DMS reactions in the presence of DtxR are underlined.

```
                   *   *   *    *     *   ****** *        **** *      *
ORF1             MNKTFKTLTLA-LATGSILTLAACGN-TDSATEKNANSVSASDSNAQFPI   26
FhuD (B. sub.)   MTHIYKKLGAAFFALLLIAALAACGNNSESKG-------SASDSKGAETF   20
FecB                                   AM--------------VQDEHGTFTL   12
FepB                                   AVQAADWPR-------QITDSRGTHTL   20
FhuD (E. coli)                         A-------------------------    1

                 * *   *   *   **** *  *   ** ****  ***   *
ORF1             TIKHAFGETTIKDAPQRIASVGWANHEVPLALGQVPVGMSKAT-----FG   70
FhuD (B. sub.)   TYKAENGNVKIPKHPKRVVVMADGYYGYFKTLGINVVGAPEN--------   62
FecB             E-----------KTPQRIVVLELSFADALAAVDVSPIGIADDNDAKRILP   50
FepB             E-----------SQPQRIVSTSVTLTGSLLAIDAPVIASGATTPNNRV-A   57
FhuD (E. coli)   -----------AIDPNRIVALEWLPVEVLLALGIVPYGVADTINY-RLWV   38

                 **          *
ORF1             DDNDNGILPWVEDK    85
FhuD (B. sub.)   ---VFKNPYYK-GK    72
FecB             EVRAHLKPWQSVGT    65
FepB             DDQGFLRQWSKVAK    72
FhuD (E. coli)   SEPPLPDSVIDVGL    53
```

FIG. 10. Partial amino acid sequence alignment of ORF1 with FhuD of *B. subtilis* and with three binding proteins (FecB, FepB, and FhuD) in the periplasm of *E. coli* that are associated with high-affinity iron uptake systems. The leader sequences for the three *E. coli* proteins are not shown. The residues in boldface for ORF1 and FhuD (*B. subtilis*) indicate the recognition sequence for signal peptidase II, which cleaves between the alanine and cysteine residues. Asterisks indicate identical amino acids between ORF1 and any of the other four sequences. The numbers to the right indicate amino acid residues in the mature protein; dashes in the sequence indicate gaps that were introduced to obtain optimal alignment.

negative bacteria. Instead, it has been proposed (35) that the receptors for ferric siderophores in gram-positive bacteria are lipoproteins that are functionally homologous to the periplasmic proteins involved in iron uptake in gram-negative bacteria. The lipoprotein receptors in gram-positive bacteria are thought to be tethered to the outer surface of the cell through attachment of their lipophilic membrane anchors to the plasma membrane (35). Although the amino acid sequence homologies in Fig. 10 suggest that ORF1 in *C. diphtheriae* encodes an iron- and DtxR-regulated lipoprotein that may serve as a membrane receptor for a ferric-siderophore complex, additional studies are required to prove this hypothesis.

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