DNA Sequences and Characterization of *dtxR* Alleles from *Corynebacterium diphtheriae* PW8(–), 1030(–), and C7hm723(–)

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The structural gene encoding DtxR, an iron-dependent diphtheria *tox* regulatory element, has recently been cloned and sequenced from the C7(–) strain of *Corynebacterium diphtheriae* (J. M. Boyd, M. Oza, and J. R. Murphy, Proc. Natl. Acad. Sci. USA 87:5968–5972, 1990). We report here the molecular cloning, DNA sequence analysis, and characterization of DtxR from the PW8(–), 1030(–), and C7hm723 strains of *C. diphtheriae*. While the sequence of *dtxR* from PW8(–) is identical to that of the C7(–) allele, the sequence of *dtxR* from the 1030(–) strain is only 91.4% identical; however, the deduced amino acid sequence of DtxR from 1030(–) differs by only 6 of 678 amino acids. Moreover, DtxR from all three strains is shown to regulate expression of β -galactosidase from a *tox* promoter-operator (*toxPO*)-*lacZ* transcriptional fusion. In contrast, the *dtxR* allele from the iron-insensitive *tox* constitutive mutant C7hm723 was found to have a single G→A transition, resulting in a substitution of Arg-47 to His and the loss of *tox* regulatory activity in recombinant *Escherichia coli*.

We have recently described the use of a diphtheria *tox* promoter-operator (*toxPO*)-*lacZ* transcriptional fusion in the molecular cloning of an iron-dependent *tox* regulatory element (*dtxR*) from *Corynebacterium diphtheriae* (4). DtxR is a 25,316-Da protein which was shown to be an iron-dependent negative control element in the expression of β -galactosidase from the *toxPO*-*lacZ* fusion in recombinant *Escherichia coli*. This study was recently confirmed and extended by Schmitt and Holmes (25), who demonstrated that *dtxR* controlled the iron-dependent expression of *tox* and the siderophore genes in *C. diphtheriae*.

Different strains of *C. diphtheriae* which are lysogenic for *tox*⁺ corynebacteriophage may under optimal conditions produce different yields of diphtheria toxin. The final yield of toxin produced may be influenced by the ability of a given strain to continue growth following exhaustion of iron from the medium (18) and the number of integrated *tox*⁺ corynebacteriophage genomes (22). Regardless of the final yield of toxin, the expression of the *tox* structural gene in *C. diphtheriae* is repressed by the addition of iron to the culture medium.

In contrast, the expression of diphtheria toxin from the C7hm723(*βtox*⁺) strain of *C. diphtheriae* is insensitive to iron-mediated repression (11). The mutant C7hm723 strain was isolated after nitrosoguanidine mutagenesis and was postulated to carry a defect in the diphtheria *tox* regulatory gene. In addition, Cryz et al. (6) were able to demonstrate that, relative to the parental C7 strain, C7hm723 also had a decreased rate of iron uptake. In order to gain additional insight into the regulation of diphtheria *tox* expression in both wild-type and mutant strains of *C. diphtheriae*, the *dtxR* alleles of the PW8(–), 1030(–), and C7hm723(–) strains were cloned and sequenced and the *tox* regulatory activity of

their respective DtxR was determined. In this paper, we demonstrate that the nucleic acid and deduced amino acid sequences of *dtxR* from the PW8(–) strain of *C. diphtheriae* are identical to those of *dtxR* from C7(–). In addition, we show that while the nucleic acid sequence of the *dtxR* allele from 1030(–) differs by 8.4% from that of the *dtxR* allele from C7(–), the deduced amino acid sequence of DtxR from 1030(–) differs from that of DtxR from C7(–) by only 6 of 678 amino acids. We have also determined that the DNA sequence of the *dtxR* allele from the iron-insensitive *tox* constitutive C7hm723 strain of *C. diphtheriae* differs from those of the C7(–) and PW8(–) alleles by a single base pair which results in a change of Arg-47 to His.

We also demonstrate with recombinant *E. coli* that the diphtheria *tox* regulatory activity of cloned DtxR from C7(–), PW8(–), and 1030(–) is iron dependent. In contrast, cloned DtxR from the iron-insensitive mutant C7hm723 is only partially functional, and expression of β -galactosidase from the *toxPO*-*lacZ* transcriptional fusion is constitutive in strains which carry the C7hm723 *dtxR* allele.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. *E. coli* and *C. diphtheriae* strains and plasmids used in this study are listed in Table 1.

Nucleic acids. Chromosomal DNA was isolated from *C. diphtheriae* as described previously by Pappenheimer and Murphy (19). Briefly, bacterial strains were grown in 20-ml volumes in CY medium (22) containing 2% maltose at 34°C to an *A*₅₉₀ of 1.0. Penicillin and sucrose were added to 200 µg/ml and 50 mg/ml, respectively, and the cultures were incubated in a shaking water bath for 2 to 3 h. Bacteria were harvested by centrifugation, washed twice with 10 mM Tris–10 mM EDTA (pH 8.0) buffer, and then resuspended in 2 ml of the same buffer. The cells were then lysed by the addition of sodium dodecyl sulfate (SDS) to a final concentration of 1%, and the crude DNA was precipitated by the addition of 0.3 ml of 3 M sodium acetate and 2 ml of isopropanol. After incubation overnight at 4°C, the precipi-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference
Bacterial strains		
<i>E. coli</i>		
DH5 α	(ϕ 80 <i>dlacZ</i> Δ M15) Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 thi-1 gyrA96 relA1 deoR</i>	Bethesda Research Laboratories
DH5 α : λ RS551 <i>toxPO</i>	As for DH5 α but lysogenic for coliphage λ RS45(<i>toxPO-lacZYA</i> Kan ^r)	4
JM101	(F' <i>proAB lacI</i> ^q Δ M15 <i>traD36</i>) <i>supE thi1</i> Δ (<i>lac-proAB</i>)	27
SBC796	<i>araD139</i> Δ (<i>araABC-leu7696</i>) <i>galE galK</i> Δ <i>lacX74</i> <i>rpsL thi1 phoA</i> Δ PvuII <i>phoR fur::Tn5</i>	5
<i>C. diphtheriae</i>		
C7(-)		3
PW8(-)	Nonlysogenic form of PW8(ω <i>tox</i> ⁺)	T. Uchida
1030(-)(<i>belfanti</i>)		9, 12
C7hm723(-)		10
Plasmids		
pUC18	Amp ^r	27
pRS551 <i>toxPO</i>	<i>toxPO-lacZYA</i> Amp ^r Kan ^r	4
pHH2500	<i>dtxR</i> from C7	4
pPWHH2500	<i>dtxR</i> from PW8	This study
pC7hmHH2500	<i>dtxR</i> from C7hm723	This study
p1030HH1800	<i>dtxR</i> from 1030	This study
pRS551 <i>toxPO</i> -C7	<i>toxPO-lacZYA</i> Amp ^r Kan ^s <i>dtxR</i> ⁺ (C7)	4
pRS551 <i>toxPO</i> -PW8	<i>toxPO-lacZYA</i> Amp ^r Kan ^s <i>dtxR</i> ⁺ PW8	This study
pRS551 <i>toxPO</i> -hm723	<i>toxPO-lacZYA</i> Amp ^r Kan ^s <i>dtxR</i> ⁺ (C7hm723)	This study
pRS551 <i>toxPO</i> -1030	<i>toxPO-lacZYA</i> Amp ^r Kan ^s <i>dtxR</i> ⁺ (1030)	This study

tate was centrifuged and resuspended in 2 ml of TE buffer (10 mM Tris-1 mM EDTA [pH 8.0]). The DNA was then digested with proteinase K (0.5 mg) for 3 to 4 h at 37°C. Following extraction with phenol and chloroform, chromosomal DNA was precipitated with isopropanol, washed with ethanol, and dissolved in distilled water and stored frozen until use. Chromosomal DNA from *E. coli* was prepared as described previously by Ausubel et al. (2).

DNA was digested with restriction endonuclease as recommended by the manufacturer (Bethesda Research Laboratories, Rockville, Md.) and electrophoresed in 0.7% agarose gels according to standard methods (2). Following electrophoretic transfer to Nytran filter media (Schleicher and Schuell, Keene, N.H.), Southern hybridization was performed as previously described (27). Colony blot hybridization was performed as described previously by Gergen et al. (8). In both instances, the *dtxR* probe was the 2.5-kb *Hind*III fragment purified from plasmid pHH2500. The *dtxR* probe was ³²P-labelled with the random primers kit (model no. 8187SA; Bethesda Research Laboratories).

Nucleic acid sequencing was performed by the Sanger dideoxy method (24). The templates were either double-stranded plasmid DNA (12) or single-stranded M13 phage DNA (29). Oligonucleotide primers were synthesized by using cyanoethyl phosphoramidite chemistry on an Applied Biosystems 380A DNA synthesizer.

β -Galactosidase assay. β -Galactosidase was measured as previously described (14), with the modification of Putnam and Koch (21). Briefly, an overnight culture of *E. coli* was grown in either Luria broth or Luria broth containing 300 μ M 2,2'-dipyridyl to an A_{590} of approximately 1.0 and was lysed by the addition of 10 μ l of lysis mix (toluene-20 mM MnSO₄-10% SDS-2-mercaptoethanol [1:1:1:5]). A total of 10 μ l of the lysate was then transferred to 1.0 ml of Z buffer (14) at 28°C, and the reaction was initiated by the addition of 0.2 ml of *o*-nitrophenyl- β -D-galactopyranoside (ONPG) (4 mg/ml). After incubation for 30 min to 3 h, the reaction was stopped by the addition of 0.5 ml of 1 M sodium carbonate.

Absorbance was measured at 420 and 550 nm, and β -galactosidase units were calculated according to Miller (14).

Nucleotide sequence accession number. The following GenBank numbers were assigned: M80338 and M80339 [PW8(-) *dtxR*], M80337 [C7hm723(-) *dtxR*], and M80336 [1030(-) *dtxR*].

RESULTS

Southern hybridization analysis of *dtxR*. Chromosomal DNAs from nontoxicogenic, nonlysogenic *C. diphtheriae* C7(-), PW8(-), 1030(-), and C7hm723(-), as well as that from *E. coli* DH5 α , were digested with *Hind*III and electrophoresed on 0.7% agarose gels. DNA was transferred to nitrocellulose filter paper and probed under stringent conditions with a ³²P-labelled 2.5-kb *Hind*III fragment from pHH2500 which has been previously shown to carry the C7(-) *dtxR* allele (4). As can be seen in Fig. 1, *Hind*III digests of chromosomal DNA from *C. diphtheriae* C7(-), PW8(-), and C7hm723(-) show a single 2.5-kb fragment that hybridizes with the probe under stringent conditions. These *Hind*III fragments have electrophoretic mobilities identical to those of the cloned *dtxR* insert from the C7(-) strain. In contrast, Southern blot analysis of *Hind*III-digested chromosomal DNA from the 1030(-) strain of *C. diphtheriae* revealed a single fragment at 1.8 kb which only weakly hybridizes to the *dtxR* probe from C7(-) (Fig. 1). Under stringent conditions, the C7(-) *dtxR* probe failed to hybridize with *E. coli* chromosomal DNA.

Molecular cloning of *dtxR* alleles. Chromosomal fragments encoding *dtxR* alleles from the PW8(-), C7hm723(-), and 1030(-) strains of *C. diphtheriae* were cloned into plasmid pUC18 essentially as previously described (4). For both the PW8(-) and C7hm723(-) strains, following electrophoresis of *Hind*III digests of chromosomal DNA, fragments of 2 to 4 kb were extracted from agarose gels and ligated into the *Hind*III site of pUC18, whereas 1- to 3-kb fragments were used for cloning the *dtxR* allele from the 1030(-) strain.

FIG. 1. Southern blot analysis of *Hind*III-digested chromosomal DNA from *C. diphtheriae* C7(-), PW8(-), 1030(-), and C7hm723 (-); *E. coli* DH5α; and plasmid pHH2500. Following their transfer to nitrocellulose paper, DNAs were probed with the ³²P-labelled 2.5-kb insert from plasmid pHH2500 which carries the *dtxR* allele from *C. diphtheriae* C7(-). Probes (in kilobases) are indicated to the right.

Following ligation, *E. coli* DH5α:ARS551*toxPO* was transformed and screened for white-colony phenotype on agar supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and ampicillin. Following transformation with cloned inserts from either PW8(-) or 1030(-), many white colonies were apparent and a few were chosen for further investigation. As anticipated from Southern hybridization, the *Hind*III restriction endonuclease digestion pattern analyses of minilysate plasmids revealed the presence of 2.5-kb and 1.8-kb inserts from the PW8(-) and 1030(-) strains, respectively.

Chromosomal DNA from the C7hm723 strain of *C. diphtheriae* was digested with *Hind*III as described above, and fragments of 2 to 4 kb were isolated and ligated into the *Hind*III site of pUC18. Plasmids were then transformed into DH5α:ARS551*toxPO*, and colonies were screened on X-Gal and ampicillin-containing agar. Since the mutant C7hm723 has an iron-insensitive *tox* phenotype and since it was likely that DtxR from C7hm723(-) was defective, transformants with a white phenotype were not expected. In order to isolate clones carrying the C7hm723 *dtxR* allele, a ³²P-labelled 2.5-kb *Hind*III probe from pHH2500 was used in colony blot hybridizations. Interestingly, transformants which had a blue phenotype on X-Gal agar were found to hybridize with the 2.5-kb *Hind*III probe. These results suggested that DtxR from C7hm723(-) was unable to regulate the expression of β-galactosidase from the *toxPO-lacZ* transcriptional fusion in recombinant *E. coli*.

DNA sequence analyses. The DNA sequence of the *dtxR* allele from the PW8(-) strain of *C. diphtheriae* was found to be identical to that of *dtxR* from C7(-) (data not shown). In contrast, the DNA sequence of the *dtxR* allele from the C7hm723(-) strain was found to differ by a single G→A transition at nucleotide 139, resulting in a change from Arg-47 to His (Fig. 2).

As described above, after Southern hybridization analysis under stringent conditions the *dtxR* allele from the 1030(-) strain of *C. diphtheriae* showed little similarity to that from the C7(-) strain (Fig. 1). It is of interest that by DNA

amino acid	M K D L V D T T E M Y L R T I Y E L
C7(-)	ATGAAGGACTTAGTCGATACACAGAGATGTACTTGCCTACTATCTATGAGCTG
1030(-)	ATGAAGGACTTAGTCGATACACAGAGATGTACTTGCCTACTATCTATGAGCTG
amino acid	E E E G V T P L R A R I A E R L E Q
C7(-)	GAAGAAGAGGGAGTCACCCCTCTTCGCGCTAGGATCGCTGAGCGCTCTGGAACAA
1030(-)	GAAGAAGAGGGAGTCACCTCCCTTCGCGCTAGGATCGCTGAGCGCTCTGGAACAA
amino acid	S G P T V S Q T V A R M E R D G L V
C7(-)	TCTGGACCTACAGTTAGCCAAACCGTTGCCGTATGGAGCGCGATGAGCTTGTG
1030(-)	TCTGGACCTACAGTTAGCCAAACCGTTGCCGTATGGAGCGCGATGAGCTTGTG
amino acid	V V A S D R S L Q M T P T G R T L A
C7(-)	GTTGTCGCTCAGACCGCAGCTCTACAAATGACACCGACAGCGCGCTTATAGCG
1030(-)	GTTGTCGCTCAGACCGCTAGCTCTACAAATGACACCGACAGCGCTTATAGCG
amino acid	T A V M R K H R L A E R L L T D I I
C7(-)	ACTGCAGTTATGCGTAACATCGCTTAGCTGAGCGCTTCTTACCGATATCAT
1030(-)	ACTGCAGTTATGCGTAACATCGCTTAGCTGAGCGCTTCTTACCGATATCAT
amino acid	G L D I N K V H D E A C R W E H V M
C7(-)	GGTCTAGATATCAATAAAGTTCCAGCTGAAAGCTGCGCTGGGAACACGTTATG
1030(-)	GGCCTAGATATCAATAAAGTTCCAGCTGAAAGCTGCGCTGGGAACATGTATG
amino acid	S D E V E R R L V K V L K D V S R S
C7(-)	AGTGACGAAGTTGAACGCGAGCTCGTGAAAGTATTGAAAGATGTGAGCTGGTCC
1030(-)	AGTGACGAAGTTGAGCGCGAGCTCGTGAAAGTGTGAAAGATGTGAGCTGGTCC
amino acid	P F G N P I P G L D E L G V G N S D
C7(-)	CCCTTCGGAACCCCAATTCAGGCTCTGACGAACCTGCGCTAGGCAATTCAGC
1030(-)	CCCTTCGGAACCCCAATTCAGGCTCTGACGAACCTGCGCTAGGCAATTCAGC
amino acid	A A A P G T R V I D A A T S M P R K
C7(-)	GCGGACGCCCCGGAACCTCGCTATTTGACGCTGCCACAGCATGCCCCGCAAA
1030(-)	GCGGACGCCCCGGAACCTCGCTATTTGACGCTGCCACAGCATGCCCCGCAAA
amino acid	V ² R I V Q I N E I F Q V E T D Q F T
C7(-)	GTACGCTATGTTTCAGATTAAACGAAATCTTCAAGTTGAAACGGATCAGTTTACA
1030(-)	GTACGCTATGTTTCAGATTAAACGAAATCTTCAAGTTGAAACGGATCAGTTTACA
amino acid	Q L L D A D I R V G S E V E I V D R
C7(-)	CAGCTCCTCGATGCTGACATCCGTGTTGATCAGAAAGTGAAGTTCGATGATAGA
1030(-)	CAGCTCCTCGATGCTGATATCCGTGTTGATCAGAAAGTGAAGTTCGATGATAGA
amino acid	D G H I T L S H N G K D V E L L D D
C7(-)	GACGGCCACATCAGCTTGAAGCCCAATGGAAAGATGTGCAACTCTCGATGAT
1030(-)	GACGGCCATATCAGCTTGAAGCCCAATGGAAAGATGTGCAACTCTCGATGAT
amino acid	L A H T I R I E E L
C7(-)	CTGGCTCACACTATTCGTATCGAAGAACTC
1030(-)	CTGACTCACACTATTCGTATCGAAGAACTC

FIG. 2. Comparative nucleotide and deduced amino acid sequences of the *dtxR* alleles from the C7(-), C7hm723(-), and 1030(-) strains of *C. diphtheriae*. Amino acid substitutions encoded by the *dtxR* alleles from C7hm723(-) and 1030(-) are designated by a superscript one and superscript two, respectively.

sequence analysis the *dtxR* allele from 1030(-) shows 91.4% identity to that from the C7(-) strain. The first 30 of 54 changes in the nucleic acid sequence are conservative and do not result in changes of the deduced amino acid sequence in the N-terminal 73% of DtxR. All of the nucleic acid changes which result in the six amino acid substitutions are positioned in the C-terminal region of this regulatory protein (Fig. 2).

DtxR activity in vivo. As shown in Table 2, cloned DtxR proteins from the C7(-), PW8(-), and 1030(-) strains of *C. diphtheriae* were able to repress the expression of β-galac-

TABLE 2. Expression of β -galactosidase by *E. coli* strains

<i>E. coli</i> strain	β -Galactosidase units ^a	
	Strain alone	Strain and 2,2'-dipyridyl ^b
SBC796	12	36
SBC796(pRS551)	101	83
SBC796(pRS551toxPO)	7,522	8,503
SBC796(pRS551toxPO-C7)	93	2,474
SBC796(pRS551toxPO-PW8)	74	1,509
SBC796(pRS551toxPO-hm723)	2,687	6,531
SBC796(pRS551toxPO-1030)	161	1,616

^a Units are averages of six independent experiments.^b 300 μ M was added.

tosidase from the *toxPO-lacZ* transcriptional fusion in recombinant *E. coli*. Moreover, the regulation of the *toxPO-lacZ* fusion by DtxR from the four corynebacterial strains was found to be iron dependent, since addition of the iron chelator 2,2'-dipyridyl to the growth medium was found to result in the derepression of *lacZ*.

As anticipated from the colony phenotype of *E. coli* SB796(pRS551toxPO-C7hm723), cloned DtxR from the iron-insensitive C7hm723 mutant strain of *C. diphtheriae* was found to only weakly regulate the expression of β -galactosidase (Table 2). It is interesting that the addition of 2,2'-dipyridyl to the growth medium of this strain resulted in an increase in the level of β -galactosidase expressed in recombinant strains of *E. coli* which carry the *dtxR* allele from C7hm723(-). These results suggest that cloned DtxR from the C7hm723 strain has a low but detectable level of *toxPO* regulatory activity.

DISCUSSION

The studies reported here have shown that the *dtxR* alleles from the C7(-) and PW8(-) strains of *C. diphtheriae* have an identical sequence and that their respective DtxR proteins are iron-dependent *tox* regulatory elements. Interestingly, DtxR from the 1030(-) strain is functionally identical to the C7(-) and PW8(-) regulatory proteins, but the nucleic acid sequence of the *dtxR* allele from this strain shows an 8.6% divergence from that of the *dtxR* in either C7(-) or PW8(-).

The 1030(-) strain has recently been reclassified from *C. belfanti* to a subspecies of *C. diphtheriae*, *C. diphtheriae* (*belfanti*) (10, 13). Strains of *C. diphtheriae* (*belfanti*) differ from *C. diphtheriae* by serology as well as by the lack of nitrogen reductase activity (10). It is of interest that while the amino acid sequence of DtxR from the 1030(-) strain differs from that of DtxR from both the C7(-) and PW8(-) strains by 6 of 678 amino acids (0.8%), there is considerable difference (8.4%) between the nucleic acid sequences of their respective *dtxR* alleles. This difference suggests considerable evolutionary divergence-convergence between *C. diphtheriae* (*belfanti*) 1030(-) and the C7(-) and PW8(-) strains of *C. diphtheriae*. It should be noted that more than 50 years had passed between the isolation of the PW8 strain in New York and that of the C7(-) strain from a patient in California (3, 20). Nonetheless, the nucleic acid sequences of their respective diphtheria *tox* alleles carried by corynebacteriophage ω and β have been shown to be identical (9, 23). In the present report, we show that the nucleic acid sequence of the corynebacterial gene encoding the *tox* regulatory factor, DtxR, carried by C7(-) and PW8(-) is also identical.

The iron-insensitive C7hm723 mutant strain of *C. diphthe-*

riae was derived from the wild-type C7(β -*tox*) strain following nitrosoguanidine mutagenesis and selection for the ability to produce diphtheria toxin under conditions of excess iron (11). While the nature of the mutation was not known, the mutant phenotype segregated with the corynebacterial host rather than with corynebacteriophage β . As a result, Kanei et al. (11) suggested that the C7hm723 strain might carry a defect in the gene encoding the diphtheria *tox* repressor. Indeed, both DNA sequence analysis of the *dtxR* allele and functional studies of DtxR in recombinant *E. coli* have shown that this is the case. Cloned DtxR from C7hm723 carries a single point mutation, Arg-47 to His, and is unable to fully repress the synthesis of β -galactosidase from the *toxPO-lacZ* transcriptional fusion. The G $\rightarrow$ A transition mutation detected in the C7hm723 *dtxR* allele is consistent with the mechanism of nitrosoguanidine mutagenesis (26).

There are many potential explanations for the failure of DtxR from C7hm723 to regulate the *tox* promoter. Since histidine residues have been shown to be involved in the iron-binding pocket of many iron-binding proteins (1), it is possible that the substitution of Arg-47 with His may interfere with iron binding by DtxR and thereby cause alterations in secondary structure which result in a loss in the ability to recognize or bind to the *tox* operator. Alternatively, since Arg-47 is positioned near one of the potential helix-turn-helix regions predicted by the REGULAT program (release 6.0; Intelligenetics), the introduction of the His residue at position 47 may disrupt the helix, making DtxR from C7hm723 unable to bind to the *tox* operator. Another possible explanation is that this mutation interferes with the potential dimerization of the repressor, causing destabilization of the putative repressor-iron-DNA complex.

We have shown that the single mutation carried by the C7hm723 *dtxR* allele is sufficient to almost completely abolish the *tox* regulatory activity of DtxR from C7hm723(-), whereas all six amino acid substitutions in the C-terminal region of DtxR from 1030(-) are silent mutations. On the basis of the properties of DtxR from C7hm723(-) and 1030(-), it is tempting to speculate that the N-terminal region of this regulatory protein may be involved in iron binding and may associate with the *tox* operator.

The hypothesis that the corynebacteriophage *tox* gene is regulated by an iron-binding repressor (15) is supported by both biochemical and genetic evidence (4, 7, 11, 16, 17, 25, 28). The molecular cloning of the *dtxR* alleles and the partial characterization of their respective DtxR from both wild-type strains of *C. diphtheriae* and mutant iron-insensitive strains further support this hypothesis. Nonetheless, it should be noted that, as yet, there is no direct evidence which demonstrates that DtxR is capable of directly binding iron and that the putative DtxR(Fe) complex will selectively bind to the diphtheria *tox* operator.

ADDENDUM IN PROOF

After this article was submitted for publication, M. P. Schmitt and R. K. Holmes (Infect. Immun., 59:3903-3908, 1991) reported a single G $\rightarrow$ A transition mutation in *dtxR* from the C7hm723 strain of *C. diphtheriae*. This mutation results in a substitution of Arg-47 with His and a loss of *tox* regulatory activity. The results reported in this article confirm and extend these observations.

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