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Importance of Nucleotides Adjacent to the Core Region of Diphtheria *tox* Promoter/Operator

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Abstract Diphtheria toxin repressor (DtxR) binds to approximately 30 to 35-bp regions containing an interrupted 9-bp inverted repeat within a 19-bp core sequence. The core sequence is fairly conserved and critical for DtxR binding. The flanking regions that are consisted of 5 to 8 more of nucleotides from the core are also required for DtxR binding. The nucleotides in both flanking regions are A-T rich. To examine whether the A-T nucleotides in both flanking regions from the core have significant roles for DtxR binding, a DNA fragment was constructed based on the diphtheria *tox* promoter/operator, and DNA fragments with substitution of A and T nucleotides in the flanking regions to G and C were also constructed. To assess the effect of these substitutions on binding of DtxR and repressibility by DtxR, β-galactosidase activity from *lacZ* fused to the region was assessed. Gel mobility shift of the region by purified DtxR was also examined. The DNA fragments containing the mutations in the flanking regions still exhibited repression and mobility shift with DtxR. The core segment with the mutation is still, therefore, recognized by DtxR. Nonetheless, the results from the assays indicated that the substitution significantly decreased repression of the operator by DtxR *in vivo* under high-iron condition and decreased binding of DtxR to the operator. These results suggest that A and T nucleotides for both flanking regions are preferred for the binding of DtxR.

Key words: *Corynebacterium diphtheriae*, DtxR, DtxR-specific promoter/operator, binding & repressibility, specific nucleotide replacement

Corynebacterium diphtheriae is the causative bacteria for the severe respiratory disease diphtheria. Diphtheria toxin is responsible for the most serious manifestations of diphtheria and is the best characterized virulence determinant of *C. diphtheriae* [1, 19]. Toxic complications of diphtheria include myocarditis and polyneuropathy [2]. This toxin, a 58-kDa protein, is encoded by the *tox* gene carried by a family of closely related corynebacteriophages, including the well-characterized phage β [1, 3, 16].

Pathogenic bacteria often use the low-iron environment of the host as a signal to induce iron-uptake systems and produce other virulence factors. Gene expression in many Gram-negative bacteria is negatively regulated by iron and Fur, the ferric uptake regulator protein. Fur is a global repressor that uses ferrous iron as its corepressor. The active holorepressor binds to regulatory DNA sequences, called the Fur-box, which are located near the promoters of the Fur-regulated genes [6, 7]. In bacteria such as *Escherichia coli*, *Salmonella typhimurium*, *Vibrio cholerae*, and *Pseudomonas aeruginosa*, more than 30 Fur-regulated genes determine a wide variety of products or phenotypes, including bacterial toxins, iron-uptake systems, the acid-tolerance response, pathways of sugar metabolism, defenses against oxygen radicals, and other virulence factors [14, 17, 28, 33].

The regulation of *tox* expression is mediated through an iron-activated *C. diphtheriae*-determined regulatory protein, diphtheria toxin repressor (DtxR) [4, 8, 22]. DtxR binds to a region upstream to the *tox* gene and represses the expression of *tox* in a manner similar to the ferric uptake regulator (Fur) in Gram-negative bacteria [4, 27, 30]. DtxR is also a global regulatory protein controlling expression of other genes such as a siderophore-dependent high-affinity iron uptake system, a heme oxygenase encoded by *hmuO* that is required for assimilation of iron from heme, and several other genes [12, 20, 23, 27]. DtxR homologs, designated iron-dependent regulators (IdeR), have also been found in other Gram-positive and acid fast bacteria, including *Mycobacterium tuberculosis* [25], *Streptomyces lividans*, and *S. pilosus* [9].

Molecular footprinting techniques demonstrated that DtxR binds symmetrically about the dyad axis of the operator [23, 27, 31] in a manner similar to the binding of several other well-characterized bacterial repressors [10,

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11, 18]. DtxR binds to approximately 30 to 35-bp regions containing a 19-bp core region. The DtxR-specific operator sequences contain a 9-bp inverted repeat within a 19-bp region, which is overlapped by proven or putative promoter sequences [12, 21, 23, 27, 32]. The sequence of the core is fairly conserved and interrupted with G or C on the center. The sequence is critical for DtxR binding, and stringency of the gene regulation is dependent on sequence similarity of the core to the consensus [12, 13, 32]. Some nucleotides of the core have been shown to be more important than others [13]. Substitution of the nucleotides with other nucleotides dramatically affected DtxR binding, and interfered with promoter and operator functions. The flanking regions that are consisted of 5 to 8 more of nucleotides in both sides from the core are required for DtxR binding in the operator. Based on the observations from all the known DtxR-specific operators, the sequences in the flanking regions have been revealed to be highly A-T rich [12, 21, 23].

The promoter/operator (P/O) of the *tox* gene in *C. diphtheriae* is the best known DtxR-specific regulatory element. The *tox* promoter contains −10 and −35 transcription initiation sequences similar to *E. coli* σ^{70} promoters [5]. DNA sequence containing a region of dyad symmetry that overlaps the −10 sequence of the *tox* promoter was proposed as a recognition site for DtxR and regulation of *tox* [29]. DtxR protected 29 nucleotides of this region, including the sequence highly homologous to consensus of the 19-bp core, from digestion by DNase I [27]. DtxR binds 4-bp more to the left and 6-bp more to the right beyond the core (Fig. 2). All the nucleotides in both flanking regions of the *tox* operator are consisted of A and T [27, 29].

The present study investigated whether these A-T rich regions flanking the 19-bp core, which apparently is bound by DtxR, were important for DtxR binding and repressibility, by substituting with other nucleotides by site-directed mutagenesis. The A and T nucleotides, protected by DtxR from DNase I digestion on the flanking regions, were substituted with G and C, respectively. The effects of these substitutions on transcriptional repressibility by DtxR and binding of DtxR were determined. The finding reported here extended the available information on the characteristics of DtxR binding to DtxR-specific promoter/operators in *C. diphtheriae*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media

E. coli K-12 DH5α [F^- φ80d*lacZ*ΔM15 Δ(*lacZ*YA-*arg*F) U169 *end*A1 *rec*A1 *hsd*R17($r_k^-m_k^+$) *deo*R *thi*-1 *sup*E44λ-*gyr*A96 *rel*A1] (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.) was used for production of DtxR protein and β-galactosidase assays. Strains were routinely cultured in Luria-Bertani broth (LB). Antibiotics and chromogenic substrates, when required, were included in the culture medium or plates at the following concentrations: ampicillin, 100 mg/l; kanamycin, 150 mg/l; and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 40 mg/l. In order to create iron-limiting growth conditions, the iron chelator ethylenediamine-di (o-hydroxyphenyl) acetic acid (EDDA) was added at 500 mg/l to LB cultures and at 50 mg/l to LB agar medium. Plasmid pQF50 promoter/probe vector was used for measuring promoter activity and repressibility of all clones [13]. Plasmid pDSK29, carrying a 5-kb fragment with the *dtxR* gene in the RSF1010-derived vector, was used for testing iron-dependent regulation of the pQF50 clones by DtxR [13]. Plasmid pTox-AT was used as the source of the pQF50 clone carrying the 60-bp fragment of the wild-type *tox* P/O (Fig. 2). Plasmid pTox-GC was used as the source of a pQF50 clone carrying the 60-bp fragment of the *tox* P/O with substitutions of A and T nucleotides in both flanking regions to G and C, respectively (Fig. 2). Plasmid pTox-GCL was used as the source of a pQF50 clone carrying the fragment with substitutions of A and T nucleotides in the left flanking region to G and C, respectively (Fig. 2). Plasmid pTox-GCR was used as the source of a pQF50 clone carrying the fragment with substitutions of A and T nucleotides in the right flanking region to G and C, respectively (Fig. 2).

Purification of DtxR Protein

DtxR was overexpressed in *E. coli* using a T7 expression system, and the DtxR was purified from sonic extracts of

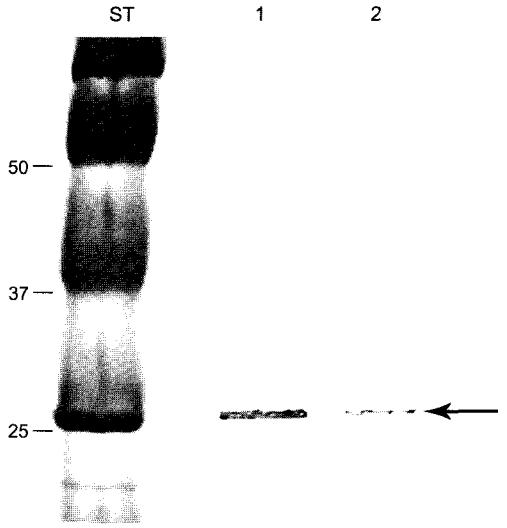

Fig. 1. SDS-PAGE of purified DtxR.
DtxR was purified by using the T7 expression system, affinity chromatography on nickel-nitrilotriacetic acid-agarose and anion-exchange chromatography on DEAE cellulose. SDS-PAGE were electrophoresed in the presence of 2-mercaptoethanol. Arrow indicates bands of the purified DtxR protein (MW 28 kDa). ST, molecular weight standards (kDa); Lane 1, 10 ng ; Lane 2, 2 ng.

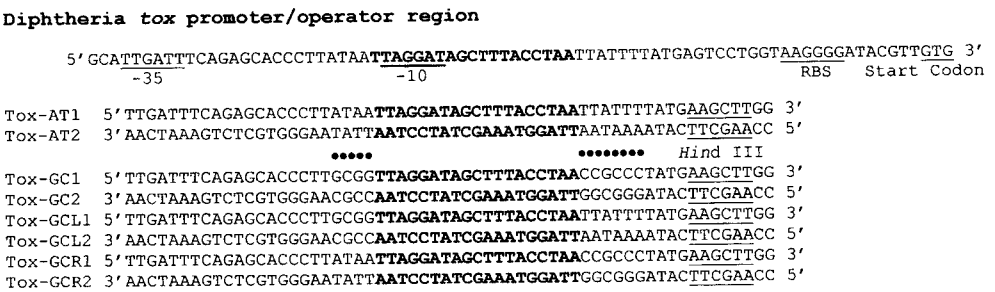

Fig. 2. Nucleotide sequence of wild-type diphtheria *tox* promoter/operator region and oligonucleotide sets designed based on the wild-type sequence.
The consensus −35 and −10 regions, the ribosome-binding site, and *tox* translational start codon are underlined [5]. DtxR binds 29-bp region including the 19-bp core region in bold case and the flanking region indicated by dots [27]. Dots indicate nucleotide pair mutated from A+T in ToxAT-12 to G+C in ToxGC-12, ToxGCL-12, and ToxGCR-12. For cloning purpose, a restriction enzyme *Hin*dIII site was added to oligonucleotide sets.

the bacteria by affinity chromatography on nickel-NTA-agarose (Qiagen Inc., Chatsworth, CA, U.S.A.) followed by anion-exchange chromatography on DEAE-cellulose [24, 27]. The purified DtxR protein appeared homogeneous by SDS-PAGE and Coomassie Blue staining (Fig. 1).

DNA Preparation and Cloning

Restriction enzymes and other DNA-modifying enzymes were used as instructed by the manufacturer (Life Technologies, Gaithersburg, MD, U.S.A.). Annealed oligonucleotides were separated by electrophoresis in low-melting-point agarose gels, excised, and purified by using Qiagen gel extraction kit (Qiagen Inc., Chatsworth, CA, U.S.A.). Promega miniprep kits (Promega, Madison, WI, U.S.A.) were used to prepare plasmid DNA for subcloning. Recombinant DNA was introduced into *E. coli* strains by electroporation.

Site-Directed Mutagenesis

Sixty-mer oligonucleotides, designed on the basis of the *tox* P/O sequence and the *tox* P/O sequence with specific nucleotide substitutions (Fig. 2), were manufactured (Life Technologies). Double-stranded DNA fragments of ToxAT-12 were constructed by annealing oligonucleotide ToxAT-1 with ToxAT-2 in the buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$]. ToxGC-12, ToxGCL-12, and ToxGCR-12 were also constructed by annealing ToxGC-1 with ToxGC-2, ToxGCL-1 with ToxGCL-2, and ToxGCR-1 with ToxGCR-2, respectively, in the buffer. The reaction mixtures were resolved by electrophoresis in 2% agarose gels. The purified products of annealed oligonucleotides were labeled with ^{32}P and used for gel mobility shift assays. The products were also cloned into pQF50 and designated as pTox-AT, pTox-GC, pTox-GCL, and pTox-GCR, respectively. Each plasmid was transformed into *E. coli* DH5α.

Gel Mobility Shift Assays

The Klenow fragment of DNA polymerase I was used for ^{32}P-labeling of the 3'-termini of DNA fragments carrying the wild-type P/O region and the substitutions. The end-labeled DNA fragments at approximately 0.5 nM were incubated with various concentrations (0 to 50 nM) of purified DtxR in a 10-μl reaction mixture containing 20 mM Na_2HPO_4 (pH 7.0), 50 mM NaCl, 5 mM $MgCl_2$, bovine serum albumin (100 μg/ml), sonicated salmon sperm DNA (10 μg/per ml), and 10% glycerol. $CoSO_4$ was present at 300 μM as indicated in the individual experiments. The reaction mixtures were incubated for 10 to 15 min at room temperature and then subjected to electrophoresis on 5% nondenaturing polyacrylamide gels in 20 mM Na_2HPO_4 buffer (pH 7.0) at 70 V for 1 to 1.5 h at 4°C. After electrophoresis, the gels were dried and analyzed by autoradiography.

β-Galactosidase Assays

E. coli DH5α containing pTox-AT, pTox-GC, pTox-GCL, and pTox-GCR were grown overnight in LB medium with either 500 μg of EDDA per ml (low-iron conditions) or without EDDA (high-iron conditions). The reaction was initiated by adding o-nitrophenyl-β-D-galactoside (ONPG) at 4 mg/ml, absorbance was measured at 420 and 590 nm, and β-galactosidase activity was assessed by Miller units [15]. Other conditions were as described in a previous report [13]. Data presented are means and standard deviations from assays of five independent cultures grown under each set of specified conditions.

RESULTS AND DISCUSSION

Analysis on Repressibility of the Wild-Type *tox* Operator and the Mutants by Measuring β-Galactosidase Activities

The minimal length of nucleotides sequences essential for DtxR binding to the operator has been confined to a 19-bp region, most of which overlaps with promoters [12, 13, 32]. The affinity of DtxR binding is highly dependent on the sequence similarity of the operator to consensus of the 19-bp core. Footprinting assays using DNase I revealed that DtxR binds approximately 30–35 bp including the 19-

bp sequence in the near center of the binding site. Based on the observations from all the known DtxR-specific operators, the sequences in both flanking regions adjacent to the 19-bp core regions have been revealed to be highly A-T rich. In order to assess the role of the A-T rich nucleotides in both regions, a 60-bp DNA fragment was constructed based on the diphtheria *tox* promoter/operator region, and DNA fragments with substitution of A and T nucleotides in the sides to G and C, respectively, were also constructed (Fig. 2).

To examine repressibility of the wild-type operator and the mutants, the DNA fragments were cloned into pQF50, which has a promoterless *lacZ* gene, and each clone was transformed into *E. coli* DH5α with and without the compatible *dtxR*-containing plasmid pDSK29. β-Galactosidase activities of each transformant of pTox-AT, pTox-GC, pTox-GCL, and pTox-GCR were measured under high-iron (repressing) and low-iron (derepressing) conditions (Table 1). The repression ratios decreased from approximately 23-fold for the wild-type *tox* P/O to approximately 6-fold for pTox-GC, to approximately 14-fold for pTox-GCL, and to approximately 13-fold for pTox-GCR. Among the mutants, plasmid pTox-GC carrying the *tox* P/O with substitution of A and T nucleotides in both flanking regions to G and C, respectively, revealed significant reduction (approximately 4-fold) of repression ratio compared to the wild-type operator pTox-AT, while reductions of repression ratio for the other mutants were less than two-fold. These results indicated that A and T nucleotides in both regions play an important role for the *tox* operator repressibility since the mutations in both sides, rather than in one side, dramatically decreased the repression. The difference was higher (3.5 unit) for β-galactosidase production from the mutant pTox-GC than that (1.1 unit) from the wild-type *tox* P/O (pTox-AT) under high-iron condition. This suggested that the substitution decreased the repression ratio by lowering the affinity of the mutant operator to DtxR.

Table 1. Expression of *lacZ* reporter gene in wild-type *tox* P/O and its mutants.

Plasmid	Iron	β-Galactosidase activity* (Miller unit)	
		With dtxR	Without dtxR
pQF50	High	0.4±0.2	0.5±0.2
	Low	0.4±0.1	0.4±0.2
pTox-AT	High	1.1±0.5	24.5±2.2
	Low	25.4±2.0	25.8±1.8
pTox-GC	High	3.5±0.6	21.1±1.5
	Low	20.2±1.2	20.5±1.2
pTox-GCL	High	1.7±0.5	22.7±1.4
	Low	22.9±1.8	23.0±1.2
pTox-GCR	High	1.6±0.6	21.5±1.3
	Low	20.8±1.4	21.2±1.5

*Data presented are means and standard deviations from assays of five independent cultures grown under each set of specified conditions.

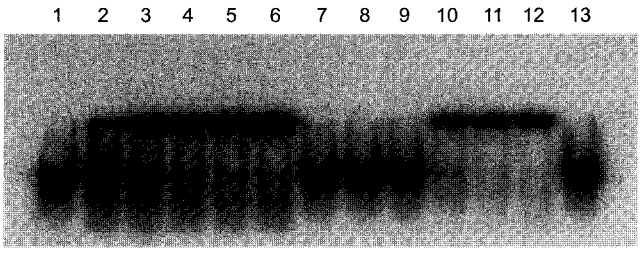

Fig. 3. Gel mobility shift assays. Length of DNA fragments was 60 bp and the fragments were end-labeled with [α-^{32}P]dCTP. DNA: lanes 1–7, ToxAT-12; 8–13, ToxGC-12. DtxR concentration: lanes 1, 7, and 13, DtxR control; 2 and 8, 10 nM; 3 and 9, 20 nM; 4 and 10, 30 nM; 5 and 11, 40 nM; 6 and 12, 50 nM.

Analysis on the Effect of the Mutation in Both Flanking Regions of the *tox* Operator on DtxR Binding by Gel Mobility Shift Assays

Plasmid pTox-GC carrying the *tox* core with substitution of A and T nucleotides in both flanking regions to G and C, respectively, exhibited significant reduction of repression compared to plasmid pTox-AT carrying the *tox* wild-type operator. β-Galactosidase production (3.5 unit) from the mutant pTox-GC under high-iron condition was significantly higher (approximately three-fold) than that (1.1 unit) from the wild-type *tox* P/O (pTox-AT), suggesting that the substitution led to lower affinity for the operator to DtxR.

To analyze whether the mutations affected the affinity of operator to DtxR, two DNA fragments, ToxAT-12 and ToxGC-12, were subjected to gel mobility shift assays with various concentration of DtxR, ranging from 0 to 50 nM in the presence of 300 μM Co^{2+} (Fig. 3). Both DNA fragments were purified and end-labeled with [α-^{32}P]dCTP. The DNA fragment containing the wild-type operator sequence initiated detectable mobility shift in the presence of 10 nM DtxR. In contrast, ToxGC-12 containing A+T to G+C substitution in both regions from the core exhibited mobility shift in the presence of 30 nM DtxR. Therefore, the substitution of G and C for A and T, respectively, on the regions caused a decrease in binding of DtxR to the DNA fragment.

Most of known DtxR-specific operators exhibiting high repressibility (such as *tox*, IRP1, and IRP5 that showed over 20-fold repression ratio by β-galactosidase activity assays) are A-T rich in the flanking regions from the core [12, 13]. *Tox* operator, especially, contains all A and T in the flanking regions. The 19-bp core sequence of DtxR-specific operators are also slightly A-T rich. Through measuring β-galactosidase activity and gel mobility shift assays, we observed that the DNA fragments containing the *tox*-A+T to G+C substitution in the flanking regions still exhibited mobility shift with DtxR (≥30 nM) and repression by measuring β-galactosidase activity assay. The mutations, therefore, regardless of the nucleotide composition in the sides, did not completely abolish the affinity of the

operator to DtxR, and the core 19-bp segment in the operator is still sufficient to be recognized by DtxR. Nonetheless, both assays showed that the presence of G and C instead of A and T in both flanking regions of the *tox* P/O was associated with significant decrease in repression of the operator by DtxR *in vivo* under high-iron condition and decrease in operator affinity. These suggested that A and T nucleotides in both flanking regions of the DtxR-specific operators from *C. diphtheriae* provide a more favorable operator site for DtxR to tightly bind.

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REFERENCES

1. Bagg, D. and J. B. Neilands. 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. *Microbiol. Rev.* **51:** 509–518.
2. Barksdale, L. 1970. Corynebacterium diphtheriae and its relatives. *Bacteriol. Rev.* **34:** 387–422.
3. Barksdale, W. L. and A. M. Pappenheimer, Jr. 1954. Phage-host relationships in nontoxigenic and toxigenic diphtheria bacilli. *J. Bacteriol.* **67:** 220–232.
4. Boyd, J., O. N. Manish, and J. R. Murphy. 1990. Molecular cloning and DNA sequence analysis of a diphtheria *tox* iron-dependent regulatory element (*dtxR*) from *Corynebacterium diphtheriae. Proc. Natl. Acad. Sci. USA* **87:** 5968–5972.
5. Boyd, J. M. and J. R. Murphy. 1988. Analysis of the diphtheria *tox* promoter by site directed mutagenesis. *J. Bacteriol.* **170:** 5949–5952.
6. Crosa, J. H. 1989. Genetics and molecular biology of siderophore-mediated iron-transport in bacteria. *Microbiol. Rev.* **53:** 517–530.
7. DeLorenzo, V., S. Wee, M. Herrero, and J. B. Neilands. 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (fur) repressor. *J. Bacteriol.* **169:** 2624–2630.
8. Ding, X., H. Zeng, N. Schiering, D. Ringe, and J. R. Murphy. 1996. Identification of the primary metal ion-activation sites of the diphtheria *tox* repressor by X-ray crystallography and site-directed mutational analysis. *Nature Structural Biology* **3:** 382–387.
9. Gunter-Seeboth, K. and T. Schupp. 1995. Cloning and sequence analysis of the *Corynebacterium diphtheriae* DtxR homologue from *Streptomyces lividans* and *Streptomyces pilosus* encoding a putative iron repressor protein. *Gene* **166:** 117–119.
10. Harrison, S. C. 1991. A structural taxonomy of DNA-binding domains. *Nature* **353:** 715–719.
11. Harrison, S. C. and A. K. Aggarwal. 1990. DNA recognition by proteins with helix-turn-helix motif. *Annu. Rev. Biochem.* **59:** 933–969.
12. Lee, J. H., T. Wang, K. Ault, J. Liu, M. P. Schmitt, and R. K. Holmes. 1997. Identification and characterization of three new promoter/operators from *Corynebacterium diphtheriae* that are regulated by the diphtheria toxin repressor (DtxR) and iron. *Infect. Immun.* **65:** 4273–4280.
13. Lee, J. H. and R. K. Holmes. 2000. Characterization of specific nucleotide substitution in DtxR-specific operators of *Corynebacterium diphtheriae* that dramatically affect DtxR binding, operator function, and promoter strength. *J. Bacteriol.* **182:** 432–438.
14. Litwin, C. M. and S. B. Calderwood. 1993. Role of iron in regulation of virulence genes. *Clin. Microbiol. Rev.* **6:** 137–149.
15. Miller, J. H. 1971. *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
16. Murphy, J. R., A. M. Pappenheimer Jr., and S. Tayart de Borms. 1974. Synthesis of diphtheria *tox* gene products on Escherichia coli extracts. *Proc. Natl. Acad. Sci. USA* **71:** 11–15.
17. Ochsner, U. A. and M. L. Vasil. 1996. Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: Cycle selection of iron-regulated genes. *Proc. Natl. Acad. Sci. USA* **93:** 4409–4414.
18. Pabo, C. O. and R. T. Sauer. 1992. Transcription factors: Structural families and principles of DNA recognition. *Annu. Rev. Biochem.* **61:** 1053–1095.
19. Pappenheimer, A. M. Jr. 1977. Diphtheria toxin. *Annu. Rev. Biochem.* **46:** 69–94.
20. Schmitt, M. P. 1997. Utilization of host iron sources by *Corynebacterium diphtheriae*: Identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin. *J. Bacteriol.* **179:** 838–845.
21. Schmitt, M. P. 1997. Transcription of the *Corynebacterium diphtheriae hmuO* gene is regulated by iron and heme. *Infect. Immun.* **65:** 4634–4641.
22. Schmitt, M. P. and R. K. Holmes. 1991. Iron-dependent regulation of diphtheria toxin and siderophore expression by the cloned *Corynebacterium diphtheriae* repressor gene *dtxR* in *C. diphtheriae* C7 strains. *Infect. Immun.* **59:** 1899–1904.
23. Schmitt, M. P. and R. K. Holmes. 1994. Cloning, sequencing, and footprint analysis of two promoter/operators from *Corynebacterium diphtheriae* that are regulated by the diphtheria toxin repressor (DtxR) and iron. *J. Bacteriol.* **176:** 1141–1149.
24. Schmitt, M. P. and R. K. Holmes. 1993. Analysis of diphtheria toxin repressor-operator interactions and characterization of a mutant repressor with decreased binding activity for divalent metals. *Mol. Microbiol.* **9:** 173–181.
25. Schmitt, M. P., M. Predich, L. Doukhan, I. Smith, and R. K. Holmes, 1995. Characterization of an iron-dependent regulatory protein (IdeR) of *Mycobacterium tuberculosis* as

a functional homolog of the diphtheria toxin repressor (DtxR) from *Corynebacterium diphtheriae*. *Infect. Immun.* **63:** 4284–4289.
26. Schmitt, M. P., B. G. Talley, and R. K. Holmes. 1997. Characterization of lipoprotein IRP1 from *Corynebacterium diphtheriae*, which is regulated by the diphtheria toxin repressor (DtxR) and iron. *Infect. Immun.* **65:** 5364–5367.
27. Schmitt, M. P., E. M. Twiddy, and R. K. Holmes. 1992. Purification and characterization of the diphtheria toxin repressor. *Proc. Natl. Acad. Sci. USA* **89:** 7576–7580.
28. Stojiljkovic, I., A. J. Baumler, and K. Hantke. 1994. Fur regulon in Gram-negative bacteria. *J. Mol. Biol.* **236:** 531–545.
29. Tai, S.-P. S. and R. K. Holmes. 1988. Iron regulation of the cloned diphtheria toxin promoter in *Escherichia coli*. *Infect. Immun.* **56:** 2430–2436.
30. Tao, X., J. Boyd, and J. Murphy. 1992. Specific binding of the diphtheria *tox* regulatory element DtxR to the *tox* operator requires divalent heavy metal ions and a 9-base-pair interrupted palindromic sequence. *Proc. Natl. Acad. Sci. USA* **89:** 5897–5901.
31. Tao, X. and J. R. Murphy. 1992. Binding of the metalloregulatory protein DtxR to the diphtheria *tox* operator requires a divalent heavy metal ion and protects the palindromic sequence from DNase I digestion. *J. Biol. Chem.* **267:** 21761–21764.
32. Tao, X. and J. R. Murphy. 1994. Determination of the minimal essential nucleotide sequence for diphtheria *tox* repressor binding by *in vitro* affinity selection. *Proc. Natl. Acad. Sci. USA* **91:** 9646–9650.
33. Tsolis, R. M., A. J. Baumler, I. Stojiljkovic, and F. Heffron. 1995. Fur regulon of *Salmonella typhimurium*: Identification of new iron-regulated genes. *J. Bacteriol.* **177:** 4628–4637.