Determination of the minimal essential nucleotide sequence for diphtheria *tox* repressor binding by *in vitro* affinity selection

(*in vitro* genetics/DtxR/gene regulation/consensus binding site)

XU TAO* AND JOHN R. MURPHY†

*Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118; and †Evans Department of Clinical Research, and Department of Medicine, Boston University Medical Center/The University Hospital, Boston, MA 02118

Communicated by A. M. Pappenheimer, Jr., May 31, 1994

ABSTRACT The expression of diphtheria toxin in lysogenic toxigenic strains of *Corynebacterium diphtheriae* is controlled by the heavy metal ion-activated regulatory protein DtxR. In the presence of divalent heavy metal ions, DtxR specifically binds to the diphtheria *tox* operator and protects a 27-bp interrupted palindromic sequence from DNase I digestion. To determine the consensus DNA sequence for DtxR binding, we have used gel electrophoresis mobility-shift assay and polymerase chain reaction (PCR) amplification for *in vitro* affinity selection of DNA binding sequences from a universe of 6.9×10^{10} variants. After 10 rounds of *in vitro* affinity selection, each round coupled with 30 cycles of PCR amplification, we isolated and characterized a family of DNA sequences that function as DtxR-responsive genetic elements both *in vitro* and *in vivo*. Moreover, these DNA sequences were found to bind activated DtxR with an affinity similar to that of the wild-type *tox* operator. The DNA sequence analysis of 21 unique *in vitro* affinity-selected binding sites has revealed the minimal essential nucleotide sequence for DtxR binding to be a 9-bp palindrome separated by a single base pair.

The expression of the diphtheria toxin structural gene, *tox*, in *Corynebacterium diphtheriae* is dependent upon the concentration of iron in the growth medium. It is well known that the production of diphtheria toxin at maximal rates only occurs during the decline phase of the bacterial growth cycle when iron becomes the limiting substrate (1). While the *tox* gene is carried by a family of closely related corynebacteriophages (2, 3), the regulation of *tox* expression is mediated through a heavy metal ion-activated *C. diphtheriae*-determined regulatory protein, DtxR (4–6). The *dtxR* gene has been cloned in *Escherichia coli*, and DtxR has a deduced molecular weight of 25,316 (4, 6). Once activated by divalent heavy metal ions, DtxR specifically binds to the diphtheria *tox* operator and protects an overlapping 33- and 27-nt region on the coding and noncoding strand from DNase I digestion (7, 8). The protected region encompasses the 27-bp interrupted palindromic sequence that composes the native diphtheria *tox* operator. In addition, DtxR has been shown to regulate the expression of siderophore genes carried on the *C. diphtheriae* chromosome (9).

In principle, the DtxR consensus binding sequence could be defined through the DNA sequence analysis of either a large number of mutant *tox* operator sequences or DtxR-responsive operators from the *C. diphtheriae* chromosome. Over the past several years, the CASTing [Cyclic Amplification and Selection of Targets (10)] technique has proven to be a powerful *in vitro* method to generate and select DNA binding sequences. Through use of variations of this method, the consensus binding sequences for myogenin (10), MyoD

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Genotype	Ref. or source
<i>E. coli</i>		
JM101	<i>supE thi Δ(lac-proAB)</i> [F' <i>proAB</i> ⁺ <i>lacI</i> ^{qZ} ΔM15]	15
DH5α	F [−] (ϕ80d <i>lacZ</i> ΔM15) Δ(<i>lacZYA-argF</i>) U169 <i>recA1</i> <i>endA1 hsdR17</i> (rk [−] , mk [−]) <i>supE44 thi1 gyrA relA1</i>	BRL
HMS174(DE3)-pDR-1		5
Plasmid		
pBluescript II KS(+)	<i>amp</i> ^r	USB
pR18-IXs	<i>amp</i> ^r	This study
pR18-X	<i>amp</i> ^r	This study
pBRtoxPO*	<i>amp</i> ^r , <i>dtxR</i> ⁺ , <i>lacZ</i> ⁺	This study
pBRtoxP-R18s	<i>amp</i> ^r , <i>dtxR</i> ⁺ , <i>lacZ</i> ⁺	This study

BRL, Bethesda Research Laboratory; USB, United States Biochemical; *amp*^r, ampicillin resistant.

*Derived from pRS551toxPO-C7 (16).

(11), c-myc (12), GCN4 (13), and SP1 (14) have been identified.

In the present study, we have used CASTing to identify the consensus DtxR binding sequence. A universe of DNA sequences was synthesized *in vitro*, in which the downstream 18-nt portion of the 27-bp *tox* operator was randomized and flanked with polymerase chain reaction (PCR) primer sequences. After incubation with activated DtxR, DNA–DtxR complexes were purified by gel mobility-shift electrophoresis, PCR-amplified, and subjected to a subsequent round of *in vitro* affinity selection. After 10 rounds of affinity selection, each coupled with 30 cycles of PCR amplification, we have isolated a family of DNA targets that bind DtxR with an affinity equivalent to that of the wild-type diphtheria *tox* operator. In addition, we show that *in vitro*-selected DNA targets function as DtxR-sensitive operators in recombinant *E. coli*. Comparison of 21 unique *in vitro*-selected DNA targets with the wild-type *tox* operator has revealed that the minimal essential nucleotide sequence for DtxR binding is a 9-bp palindrome separated by 1 bp.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Medium. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria broth (LB; 10 g of tryptone, 10 g of NaCl, and 5 g of yeast extract per liter of water). As noted, LB broth or LB agar (15 g of agar per liter of broth) was supplemented with ampicillin (100 μg/ml) and 5-bromo-4-chloro-3-indolyl β-D-galactoside (40 μg/ml).

Nucleic Acids. Double-stranded plasmid DNA was prepared with the Wizard miniprep system (Promega) according to the directions of the manufacturer. The randomized *tox* operator oligonucleotide with the sequence 5'-ACACATGAATTCTAATACGACTCACTATAGGCCTATAATTAGGXXXXXXXXXXXXXXXXXXXXTTATGAGTCCTGCAGGATCCTCTCTC-3' was synthesized in an Applied Biosystems PCR-Mate synthesizer (X denotes the use of an equimolar mixture of isobutyldeoxyguanosine, benzoyldeoxyadenosine, benzoyldeoxycytidine, and thymidine phosphoramidites in the synthesis reaction mixture). The region containing the randomized bases corresponds to positions -46 to -29 upstream of the *tox* translational start signal and includes the intervening and downstream palindromic segments of the diphtheria *tox* operator (Table 2). The oligonucleotides 5'-ACACATGAATTCTAATACGACTCACTA-3' and 5'-GAGAGAGGATCCTGCAGGACTCATAAA-3' were synthesized and used as PCR primers for the 5' end and 3' end of the randomized *tox* operator oligonucleotide, respectively. The 5'-end primer used for the last round of PCR amplification was increased in size to hybridize to position -47 to generate double-stranded DNA (dsDNA) segments with a functional "-10" region of the *tox* promoter (Table 2). The randomized oligonucleotide was converted to dsDNA by extension of the 3'-end primer with Klenow fragment by standard methods (18).

DNA sequencing was performed by the dideoxynucleotide chain-termination method (19) as modified by Kraft and coworkers (20) using Sequenase (United States Biochemical). A GeneAmp DNA amplification reagent kit was used in PCR amplification according to the specifications of the

manufacturer (Perkin-Elmer/Cetus). Briefly, the reaction mixture (100 μ l) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.001% gelatin, 200 μ M each dNTP, 1 μ M each primer, a specified amount of amplification target, and 2.5 units of *Taq* DNA polymerase. The amplification cycle was 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. The cycle was repeated 30 times for each amplification.

Purification of DtxR. Expression of *dtxR* from the T7 promoter and purification of DtxR from crude extracts of recombinant *E. coli* HMS174(DE3)pDR-1 were performed as described (5).

Gel Electrophoresis Mobility-Shift Assay. The gel electrophoresis mobility-shift assay used in this study is similar to that previously described (5). For preparative electrophoresis, the gels were exposed to x-ray film without drying, and the mobility-shifted dsDNA was excised from the gel and extracted with TE buffer (10 mM Tris-HCl/1 mM EDTA, pH 8.0).

β -Galactosidase Assay. For quantitative determination of *lacZ* expression, recombinant *E. coli* transformed with various plasmids were grown in LB medium overnight in the presence of 2,2'-dipyridyl as noted. β -Galactosidase activity was measured in bacterial extracts as described (21) and modified (22). β -Galactosidase units were calculated as described by Miller (21). The results are reported as the mean of three independent assays.

RESULTS

We have demonstrated (7) by DNase I footprint analysis that heavy metal ion-activated DtxR protects overlapping 33-nt

Table 2. DNA sequence analysis of 21 unique *in vitro* affinity-selected DtxR-responsive elements compared to the native diphtheria *tox* promoter/operator

Sequences at nucleotide positions upstream from the <i>tox</i> transcriptional start site																																		
-56	-55	-54	-53	-52	-51	-50	-49	-48	-47	-46	-45	-44	-43	-42	-41	-40	-39	-38	-37	-36	-35	-34	-33	-32	-31	-30	-29							
Native <i>toxPO</i>																																		
----- “-10” -----										----- “-10” -----																								
T	A	T	A	A	T	T	A	G	G	A	T	A	G	*	T	T	T	A	C	C	T	A	A	T	T	A	T							
										Mutant DtxR binding sites																								
										1	A	T	A	G	C	C	T	A	A	C	C	T	T	A	C	T	G	G						
										2	A	T	A	G	G	C	T	A	A	C	C	T	T	A	C	C	G	G	G					
										3	G	C	A	G	G	C	T	A	A	C	C	T	A	A	C	G	C	C						
										4	G	C	A	G	C	C	T	A	A	C	C	T	A	A	C	A	C	C						
										5	G	T	A	G	G	C	T	A	A	C	C	T	T	A	C	A	T	G						
										6	G	T	A	G	G	C	T	A	A	C	C	T	A	A	C	G	C	C						
										7	G	T	A	G	C	C	T	T	G	C	C	T	T	A	C	T	G	G						
										8	G	T	A	G	C	C	T	T	A	C	C	T	A	A	G	C	A	G						
										9	T	T	A	G	C	C	T	A	A	C	C	T	T	A	G	T	A	C						
										10	T	T	A	G	G	C	T	A	T	C	C	T	T	A	C	T	G	G						
										11	T	T	A	G	C	C	T	A	T	C	C	T	A	A	G	A	C	G						
										12	T	T	A	G	C	C	T	A	T	C	C	T	T	A	G	C	T	A						
										13	T	T	A	G	G	C	T	T	A	C	C	T	T	C	T	C	G	C						
										14	T	T	A	G	G	C	T	A	T	C	C	T	T	A	T	A	G	G						
										15	T	A	A	G	C	C	T	A	T	C	C	T	A	A	G	A	C	G						
										16	T	A	A	G	C	C	T	A	A	C	C	T	T	A	G	C	G	G						
										17	T	A	A	G	G	C	T	A	C	C	C	T	T	A	G	T	C	G						
										18	C	A	A	G	C	C	T	A	A	C	C	T	A	A	C	A	C	C						
										19	C	T	A	G	G	C	T	T	A	G	C	T	T	A	G	T	T	G						
										20	C	G	A	G	C	C	T	A	A	C	C	T	A	A	C	T	C	C						
										21	C	G	A	G	C	C	T	A	A	C	C	T	A	A	C	A	T	G						
DtxR consensus binding site																																		
. . . . T $\frac{A}{T}$ A G G T T A G $\frac{G}{C}$ C T A A C C T $\frac{A}{T}$ A																																		

The relative position of the two "-10" boxes of the *tox* promoter is indicated (17).

*Center of the palindromic sequences.

and 27-nt sequences on the coding and noncoding strand of the diphtheria *tox* operator. To determine which bases within this 27-bp region were essential for DtxR binding, we synthesized an oligonucleotide in which the 18 nt corresponding to the intervening 9 bp and downstream 9 bp of the palindromic sequence of the *tox* operator were randomized (Fig. 1A). After synthesis of the second strand with DNA polymerase in the presence of primer, nucleotide triphosphates, and [32 P]dATP, the labeled dsDNA was then mixed with DtxR in the presence of Mn^{2+} . DtxR–dsDNA complexes were then separated from unbound DNA by gel mobility-shift electrophoresis. The overall strategy that we have used for the *in vitro* affinity selection of DtxR binding sequences is shown in Fig. 1B.

After the first round of *in vitro* affinity selection, the apparent concentration of DtxR–dsDNA complexes was extremely low and accounted for <0.001% of the total 32 P-labeled dsDNA added to the gel mobility-shift assay mixture (Fig. 2, lane 3). Since the 32 P-labeled wild-type *tox* operator probe used as a positive control was of similar size (Fig. 2, lane 1), the relative position of mobility-shifted dsDNA was easily located in the gel. The polyacrylamide gel from this region was excised, and the DNA was eluted from the gel and amplified by 30 cycles of PCR. Amplified dsDNA was then end-labeled with 32 P, and the dsDNA was incubated with activated DtxR and subjected to another round of affinity selection by gel mobility-shift electrophoresis. After three rounds of selection and PCR amplification, the amount of dsDNA that bound to activated DtxR was significantly increased (Fig. 3). It should be noted, however, that the average binding affinity between the *in vitro* selected dsDNA and DtxR was still low. Based upon gel mobility-shift experiments with 2-fold serial dilutions of DtxR, the concentration of activated DtxR required to cause 50% of the labeled dsDNA to shift is $\approx 20 \mu M$ (Fig. 3).

Since there is preferential enrichment of high-affinity DtxR–dsDNA complexes with each round of *in vitro* selection and amplification, we subjected this mixture of dsDNA to six additional rounds of affinity selection and PCR ampli-

FIG. 1. (A) Nucleotide sequence of the native diphtheria *tox* promoter/operator and the synthetic oligonucleotide *tox* operator with randomized bases. The 9-bp palindromic sequence of the native *tox* operator is underlined. (B) Strategy used in the *in vitro* affinity selection and characterization of DtxR binding sites.

FIG. 2. Gel mobility-shift separation of DtxR–dsDNA complexes after the first round of *in vitro* affinity selection. Lanes: 1, DtxR and labeled *toxPO* probe as a positive control; 2, randomized *tox* operator DNA alone; 3, DtxR and randomized *tox* operator DNA. The experiment of lane 3 was repeated six times to maximize the concentration of dsDNA selected after the first round of affinity selection. Arrow indicates the relative position of the mobility-shifted DtxR–dsDNA complex.

fication. The average affinity between the dsDNA and DtxR was increased by ≈ 500 -fold (Fig. 4B). The concentration of DtxR that caused 50% of the labeled dsDNA to shift was 40 nM. Moreover, this concentration of DtxR was also required to cause a 50% mobility shift of the wild-type *tox* operator probe (Fig. 4A).

The specificity of the interaction between DtxR and the *in vitro* affinity-selected dsDNA targets was demonstrated by blocking the binding of DtxR to the labeled probe by addition of either anti-DtxR antiserum or unlabeled wild-type *tox* operator probe to the reaction mixture (data not shown). These results strongly suggest that activated DtxR binds to the *in vitro* selected dsDNA at the same site as it binds to the wild-type *tox* operator.

The *in vitro* affinity-selected dsDNA targets were then digested with *Bam*HI and *Eco*RI and ligated into the *Bam*HI and *Eco*RI sites of phagemid pBluescript II KS(+) to generate pR18-IXs. DNA was then transformed into *E. coli* JM101, and single colonies were selected on LB ampicillin agar for analysis. Single-stranded DNA was prepared and DNA sequences were determined by the dideoxynucleotide chain-termination method (19, 20). The comparison of the DNA sequence of six affinity-selected dsDNA targets revealed

FIG. 3. Gel mobility-shift separation of DtxR–dsDNA complexes after three rounds of *in vitro* affinity selection. Lanes: 1, *toxPO* alone; 2, *toxPO* plus DtxR; 3, *in vitro*-selected dsDNA alone; 4–9, gel mobility-shift analysis in the presence of different concentrations of DtxR as follows: 0.6 μM (lane 4), 1.2 μM (lane 5), 2.5 μM (lane 6), 5 μM (lane 7), 10 μM (lane 8), and 20 μM (lane 9).

FIG. 4. Gel mobility-shift separation of the native diphtheria *tox* promoter/operator probe and dsDNAs after nine rounds of *in vitro* affinity selection in the presence of different concentrations of DtxR. (A) Gel mobility-shift assay of 32 P-labeled *toxPO*. (B) Gel mobility-shift analysis of 32 P-labeled dsDNAs after affinity selection. DtxR concentration in lanes: 1, 10 nM; 2, 20 nM; 3, 40 nM; 4, 80 nM; 5, 160 nM; 6, 320 nM; 7, 650 nM; 8, 1.3 μ M; 9, 2.6 μ M.

strong base preferences at several positions. Since each round of selection is coupled with 30 cycles of PCR amplification, the dsDNA was subjected to 270 cycles of PCR amplification. It is well known that PCR amplification often introduces mutations into amplified DNA. As anticipated, we found PCR induced mutations in every position of the non-randomized region of the *tox* operator probe, except for those positions corresponding to -47 to -51 and the regions covered by the 5'- and 3'-end primers (data not shown).

Since many of the mutations occurred in the "-10" region of the diphtheria *tox* promoter, we performed a final round of *in vitro* affinity selection and PCR amplification using a 5'-end primer that also hybridized to position -47 and protected the "-10" region of the *tox* promoter. The affinity-selected dsDNA was then digested with *Bam*HI and *Eco*RI and cloned into the phagemid pBluescript to generate pR18-Xs for DNA sequence analysis as described above. Twenty-one unique sequences were found among the 34 clones that were isolated and characterized (Table 2).

The central base of the 27-bp native *tox* operator is located at position -42 from the diphtheria *tox* translational start signal. Either a C or G at this position also forms the center of the palindromic sequence that was observed in the *in vitro* affinity-selected dsDNAs. Of the 18 positions that originally contained randomized bases, 8 have a strong base preference. Positions -37, -36, -35, and -33 were almost invariably found to be C, C, T, and A, respectively. This sequence is essentially identical to a portion of the palindrome found in the native *tox* operator. In the case of the affinity-selected dsDNA, the palindromic sequence that was found extends into the middle of the intervening sequence found in the native *tox* operator. In these instances, there is a preference for T at position -40, which is complemented by an A at -44, and there is a preference for a C at position -41, which is complemented by a G at -43. Analysis of the selected dsDNAs at positions -39 and -38 shows a strong tendency for an A; and the corresponding positions at -46 and -45 show a preference for T. Other positions within the selected DtxR binding sites appear to be less important. For example, the bases A and T alternatively appear at position -34,

FIG. 5. Expression of β -galactosidase in recombinant *E. coli* that carry *lacZ* under the control of *in vitro* affinity-selected DtxR binding sites in the absence and presence of increasing concentrations of 2,2'-dipyridyl. $\circ$, Recombinant *E. coli* strains in which *lacZ* is under the control of the native *toxPO*. Solid symbols indicate *in vitro* affinity-selected DtxR binding site clones 17 ($\bullet$), 18 ($\blacklozenge$), 8 ($\blacksquare$), 7 ($\blacktriangle$), 12 ($\blacktriangledown$), and 5 ($+$).

whereas C and G are found at positions -32 and -29. Furthermore, there does not seem to be a favored base at positions -31 and -30. The results described above lead us to conclude that the minimal essential nucleotide sequence for DtxR binding has the following sequence: 5'-TWAGGT-TAGSCTAACCTWA-3', where W = A or T and S = C or G.

To demonstrate that the DtxR *in vitro* affinity-selected dsDNAs were iron-sensitive operators *in vivo*, we chose six unique dsDNA sequences and recloned them immediately upstream of the *lacZ* gene in the phagemid pBRtoxPO in which the native *tox* operator was replaced with the selected sequences. This vector also carries and constitutively expresses the *dtxR* gene. In these constructs, the "-35" element of the *tox* promoter was also inserted in the correct position such that the resulting promoter was analogous to the wild-type *tox* promoter. After ligation, phagemid DNA was transformed into *E. coli* DH5 α , and the expression of β -galactosidase was measured from strains grown in the presence of various concentrations of 2,2'-dipyridyl. In these experiments, *E. coli* DH5 α (pBRtoxPO), which carries *lacZ* under the control of the wild-type diphtheria *tox* promoter/operator, served as the positive control. The expression of β -galactosidase from *E. coli* (pBRtoxP-R18) strains, which carry the *in vitro* affinity-selected dsDNA sequences, was repressed (Fig. 5). The addition of 2,2'-dipyridyl to the culture medium to concentrations greater than 150 μ M resulted in the expression of β -galactosidase. In fact, the 2,2'-dipyridyl-induced derepression of *lacZ* in those strains that carried the *in vitro*-selected operator sequences was found to be equivalent to that observed for the strain that carried the native wild-type *toxPO/lacZ* transcriptional fusion. These results demonstrate that the *in vitro* affinity-selected DtxR binding targets function as iron-responsive operators *in vivo*.

DISCUSSION

In the present study, we have used the CASTing method (see Introduction) to identify the minimal essential nucleotide sequence required for DtxR binding. Beginning with a pool of dsDNA in which 18 bp corresponding to the intervening and downstream inverted repeat sequences of the native *tox* operator were randomized, we have selected *in vitro* a family of dsDNAs from a universe of 6.9×10^{10} potential binding sites in which the apparent DtxR binding affinity is equivalent to that of the wild-type diphtheria *tox* operator.

DNA sequence analysis of the selected dsDNAs reveals that the minimal essential nucleotide sequence for DtxR binding is a 9-bp palindrome separated by either a single C or G. The minimal essential nucleotide sequence for DtxR binding is as follows:

The analysis of 21 unique *in vitro* affinity-selected targets suggests that the sequence 5'-T.AGG..AG.CT..CCT.A-3' represents the essential DtxR binding site. All of the dsDNAs that were analyzed fit into this profile except for clone 13, which carries a C at position -33, and clone 19, which carries a G at position -37.

Welkos and Holmes (23) isolated corynebacteriophage β mutants, β^{201} and β^{202} , in which *tox* expression is less sensitive to iron-mediated repression. It was later found that each of these mutants carries a single G $\rightarrow$ A mutation respectively at position -47 (β^{201}) and -48 (β^{202}) (24). These results are consistent with our observations on the DNA sequence requirements for the formation of a DtxR binding site. In a recent report, Schmitt and Holmes (25) have cloned and characterized two iron-responsive promoters from *C. diphtheriae* and propose a 19-bp DtxR consensus binding site that is identical to the minimal essential nucleotide sequence required for DtxR binding described in this report.

It is of interest to note that a T occurs at position -41 of wild-type *tox* operator, whereas in the affinity-selected sequences a C is strongly preferred at this position. These results suggest that the native *tox* operator may not be an optimal target for DtxR binding. It is likely that naturally found DtxR-sensitive binding sites may not reflect sequences that have the highest binding affinity for this regulatory element. Since the affinity between DtxR and given targets has to meet the requirements imposed by sensing an environment signal, it is likely that a variety of DtxR binding sites, each with a different binding affinity, will be found.

We have also demonstrated in this study that the *in vitro* affinity-selected dsDNA sequences are able to function as iron-responsive operators *in vivo*. It should be noted that the expression of β -galactosidase from six unique, independently isolated, transcriptional fusions that we have analyzed have only a 2- to 3-fold difference from the wild-type *tox* promoter/operator-*lacZ* fusion. There are at least two factors that may lead to differential levels of *lacZ* expression. The first is the relative strength of the promoter. It has been demonstrated previously that there are two "-10" elements that overlap with an upstream palindromic sequence in the native diphtheria *tox* operator (17). Mutations in the downstream "-10" element result in a 50% decrease in *tox* gene expression. In the case of the affinity-selected targets, the randomized region covers positions -45 and -46, which belong to the downstream "-10" element. The insertion of bases that are different from those found in the wild type in these positions might influence promoter strength. Alternatively, the avidity of interaction between DtxR and the affinity-selected targets could also affect the level of β -galactosidase expression in a given isolate. For example, since the DNA sequences of the promoter region in clones 7 and 8 are identical, the higher level of *lacZ* expression in clone 7 is apparently due to a lower affinity of DtxR binding. In this instance, it is possible that the G at position -38 directly affects the affinity of DtxR binding.

It is of particular interest to note that Günter *et al.* (26) have recently reported that a repressor-binding site in the promoter region of *desA* gene is responsible for iron-mediated gene regulation in Gram-positive *Streptomyces pilosus* and

Streptomyces lividans. This dyad symmetrical element is homologous to the native diphtheria *tox* operator. The *desA* gene encodes lysine decarboxylase, which catalyzes the first reaction of siderophore desferrioxamine B synthesis and is induced by iron deficiency (27). Although the negative controlling element for *desA* has not been identified in *Streptomyces* spp., the remarkable similarity of the regulatory sequence to the consensus DtxR binding site suggests that a DtxR-like regulatory protein may exist in *Streptomyces* spp. Accordingly, it is possible that DtxR is a member of a family of regulatory proteins that have homologous amino acid sequences and DNA binding targets and that control the expression of iron-responsive genes in their respective Gram-positive host.

We thank Dr. Johanna vanderSpek for her critical reading of the manuscript. This work was supported by Public Health Service Grant AI-21628 from the National Institute of Allergy and Infectious Diseases.

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