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Genetics

Molecular cloning and DNA sequence analysis of a diphtheria *tox* iron-dependent regulatory element (*dtxR*) from *Corynebacterium diphtheriae*

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ABSTRACT **Although the structural gene for diphtheria toxin, *tox*, is carried by a family of closely related corynebacteriophages, the regulation of *tox* expression is controlled, to a large extent, by its bacterial host *Corynebacterium diphtheriae*. Optimal yields of *tox* gene products are obtained only when iron becomes the growth-rate-limiting substrate. Previous studies suggest that regulation of *tox* expression is mediated through an iron-binding aporepressor. To facilitate molecular cloning of the *tox* regulatory element from genomic libraries of *C. diphtheriae*, we constructed a *tox* promoter/operator (*tox*PO)–*lacZ* transcriptional fusion in *Escherichia coli* strain DH5α. We report the molecular cloning and nucleic acid sequence of a diphtheria *tox* iron-dependent regulatory element, *dtxR*, and demonstrate that expression of β-galactosidase from the *tox*PO–*lacZ* fusion is regulated by *dtxR*-encoded protein in an iron-sensitive manner. In addition, we show that expression of the *tox*PO–*lacZ* fusion is not affected by the *E. coli* iron-regulatory protein Fur and that the *dtxR* protein does not inhibit expression of *fur*-regulated outer-membrane proteins.**

Diphtheria toxin is synthesized by *Corynebacterium diphtheriae* lysogenic for one of a family of corynebacteriophages that carries the structural gene for the toxin, *tox* (1, 2). Optimal yields of *tox* gene products have long been known to be obtained only from *C. diphtheriae* grown under conditions where iron becomes the growth-rate-limiting substrate (3, 4). In 1936, Pappenheimer and Johnson (5) showed that adding iron in low concentration to the growth medium inhibited the production of diphtheria toxin. Both biochemical and genetic evidence support the hypothesis that the corynebacteriophage *tox* gene is regulated by a corynebacterial-determined iron-binding repressor as postulated by Murphy *et al.* (6–12). This model predicted an aporepressor that in the presence of iron forms a complex; this complex then binds to the *tox* operator and blocks transcription. Under conditions of iron limitation, the iron–repressor complex dissociates, derepressing the *tox* gene.

The nucleic acid base sequence of *tox* revealed a 9-base-pair (bp) inverted repeat that overlaps the "−10" region of the promoter (13). Because many operators exhibit dyad symmetry and are positioned near their respective promoters, this region was designated the putative *tox* operator.

We here describe the genetic construction of an *Escherichia coli* host strain that carries a chromosomal diphtheria *tox* promoter/operator (*tox*PO)–*lacZ* transcriptional fusion in single copy. Because this strain constitutively expresses β-galactosidase and is phenotypically blue on 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal)-containing agar medium, we have used it to screen genomic libraries of nontoxinogenic nonlysogenic *C. diphtheriae* for determinants that repress *lacZ* expression. We report the molecular cloning and deduced amino acid sequence (25,316 molecular weight) of a diphtheria *tox* iron-dependent regulatory element, *dtxR*.* We show that this factor acts as a negative controlling element for the *tox*PO–*lacZ* transcriptional fusion in an iron-dependent fashion.

The putative *tox* operator locus was found to bear striking homology to Fur (ferric-uptake regulator)-binding sites in the *E. coli* chromosome (14–17). Fur has been well characterized (18, 19) and shown to complex with iron, functioning as a global negative controlling element. Under iron-limiting conditions, Fur releases bound iron and dissociates from its DNA-binding sites, allowing transcription of several iron-sensitive genes. Although *dtxR* from *C. diphtheriae* has 66% homology to *E. coli fur*, we have not seen *fur*-mediated repression of β-galactosidase expression from the *tox*PO–*lacZ* transcriptional fusion described here. In addition, we have been unable to detect DtxR-mediated repression of iron-regulated outer-membrane proteins in *fur* strains of *E. coli*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Coliphage. The bacterial strains, plasmids, and coliphage λ used are listed in Table 1.

Media. *E. coli* strains were grown in Luria broth (10 g of tryptone, 10 g of NaCl, and 5 g of yeast extract per liter) or lambda broth (10 g of tryptone and 10 g of NaCl per liter). As noted, medium was supplemented with ampicillin (100 μg/ml), kanamycin (35 μg/ml), X-gal (40 μg/ml), or the iron chelator 2,2′-dipyridyl (200–300 μM). *C. diphtheriae* strains were grown in low-iron C-Y medium as described (26).

Nucleic Acids. Oligonucleotides were synthesized by using cyanoethyl phosphoramidite chemistry on an Applied Biosystems 380A DNA synthesizer. After synthesis oligonucleotides were purified by Nensorb Prep cartridges (New England Nuclear) as directed by the manufacturer. To hybridize complementary strands, equimolar concentrations of each strand in 100 mM NaCl were heated to 90°C for 10 min and allowed to cool to room temperature.

Chromosomal DNA was extracted and purified from *C. diphtheriae* essentially as described by Pappenheimer and Murphy (27). Plasmid DNA was extracted by the alkaline lysis method and purified by ethidium bromide/cesium chloride isopynic centrifugation (28). Restriction enzymes were from Bethesda Research Laboratories and used according to the manufacturer's directions. Restriction endonuclease fragments were electrophoresed in 0.7% agarose gels in TBE (50 mM Tris/50 mM boric acid/0.5 mM EDTA, pH 8.0).

Nucleic acid sequencing was performed on both strands of plasmid DNA according to the method of Sanger *et al.* (29) as modified by Kraft *et al.* (30) using Sequenase (United States Biochemical). Oligonucleotide primers were synthesized as

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Abbreviations: *tox*PO, *tox* promoter/operator; ORF, open reading frame; X-Gal, 5-bromo-4-chloro-3-indolyl sequences β-D-galactoside; *dtxR*, diphtheria *tox* iron-dependent regulatory element.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34239).

Table 1. Bacterial strains, plasmids, and coliphage λ used in this study

Strain	Genotype	Reference or source
E. coli		
NK7047	F⁻ λ⁻ Δ*lacX74 rpsL galOP308*	Ref. 20
DH1	F⁻ λ⁻ *recA1 endA1 gyrA96 thi1 hsdR17* (rk⁻, mk⁺) *supE44 relA1*	Ref. 21
DH5α	F⁻ (φ80d *lacZ* ΔM15) Δ (*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (rk⁻, mk⁺) *supE44 thi1 gyrA relA1*	BRL
DH5α: λRStoxPO	As DH5α but lysogenic for λRS45 (*tox*PO/*lacZYA*, *kan*ᴿ)	This work
SM796	F⁻ λ⁻ *araD139* Δ(*araABC-leu*) 7696 *galE galK* Δ*lacX74 rpsL thi phoA* ΔPvu II *phoR*	Ref. 16
SBC796	As SM796 but *fur*::Tn*5*	Ref. 16
Plasmid		
pABN203	*fur*⁺	Ref. 22
pRS551	cryptic *lacZ kan*ᴿ *amp*ᴿ	Ref. 20
pRS551toxPO		This work
pHH2500		This work
pVV1500		This work
pHP900		This work
pSH1400		This work
pVN1200		This work
pRS551toxPO-VN1200		This work
pRS551toxPO-Fur		This work
Coliphage		
λRS45	*lacZ' bla'*	Ref. 20
λRS45toxPO	*tox*PO–*lacZ kan*ᴿ	This work
C. diphtheriae		
C7(−)		Ref. 23
PW8(−)		Ref. 24
1030(−)		Ref. 25

BRL, Bethesda Research Laboratories.

described. Southern blot hybridization was done as described by Ausubel *et al.* (28) on Nytran filter paper (Schleicher & Schuell). Colony blot hybridization was performed as described by Gergen *et al.* (31). The DNA probe for both techniques was the 1.5-kilobase (kb) *Pvu* II fragment from plasmid pHH2500, which was ^{32}P-labeled by using the random primers kit (8187SA, Bethesda Research Laboratories).

β-Galactosidase Assay. β-Galactosidase was measured as described by Miller (32). Briefly, 0.5 ml of an overnight culture at $A_{600\ \mu m}$ of ≈1.0 was lysed by adding 10 μl of lysis mix (toluene/0.02 M $MnSO_4$/10% SDS/2-mercaptoethanol 1:1:1:5) according to the modification of Putnam and Koch (33). Ten micrograms of lysate was transferred to 1.0 ml of Z buffer at 28°C. The reaction was initiated by adding 200 μl of *o*-nitrophenyl β-D-galactoside (ONPG) (4 mg/ml). After incubation for 30 min–3 hr, the reaction was stopped by adding 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 (32).

Preparation of Outer Membranes. Outer-membrane proteins were prepared from cells grown in Luria broth as described by Hantke (34), electrophoresed on 0.1% SDS/10% polyacrylamide gels according to the method of Laemmli (35), and stained with Coomassie blue.

RESULTS

Genetic Construction of *tox*PO–*lacZ* Transcriptional Fusion. To develop an *E. coli* host strain to screen genomic libraries of nontoxinogenic nonlysogenic *C. diphtheriae* for the putative diphtheria *tox* repressor, we constructed a transcriptional fusion between *tox*PO and *lacZ*. Each strand of the 65-bp diphtheria toxin regulatory region (−80 to −15) upstream of the *tox* translational start signal was synthesized *in vitro* (Fig. 1*A*) and modified to contain one-half *Eco*RI and one-half *Bam*HI ends to facilitate vectorial cloning into *Eco*RI and *Bam*HI sites of the promoter probe vector pRS551 (Fig. 1*B*). Plasmid pRS551 carries a cryptic *lac* operon and genes that confer resistance to both kanamycin (*kan*) and ampicillin (*bla*) (20). After ligation and transformation, recombinant *E. coli* that carried the *tox*PO–*lacZ* transcriptional fusion were selected by a blue-colony phenotype on agar medium supplemented with ampicillin and X-gal. Several clones were isolated, minilysates were then prepared, and plasmid DNA was sequenced to insure that *tox*PO was inserted in single copy and in the correct orientation. One clone was selected, and the recombinant plasmid was designated pRS551toxPO (Fig. 1*B*). As shown in Table 2, *E. coli* SM796(pRS551toxPO) was found to express moderate levels of β-galactosidase.

To introduce a single copy of the *tox*PO–*lacZ* transcriptional fusion into the *E. coli* chromosome, the *recA*⁺ NK7049 strain was transformed with plasmid pRS551toxPO and subsequently infected with coliphage λRS45. Because this coliphage carries a partial deletion of the flanking *bla* (*bla'*) and *lacZ* (*lacZ'*), the *tox*PO–*lacZ* transcriptional fusion and the kanamycin resistance gene are transferred from pRS551toxPO to λRS45 by homologous recombination. High-titer phage lysates were prepared and screened for *lac*⁺ recombinant plaques on lawns of NK7049 on X-Gal-supplemented agar medium. Because the *kan* gene from pRS551toxPO is also transferred to the recombinant phage, *lac*⁺ lysogens were subsequently isolated and colony-

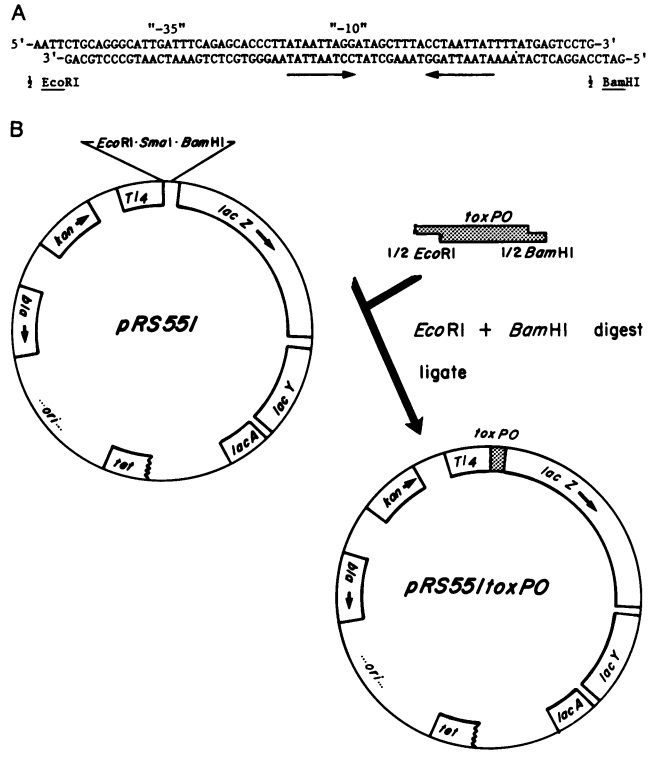

FIG. 1. (*A*) Sequence of the synthetic oligonucleotides encoding the −80 to −15 region upstream of the *tox* translational initiation signal that carries *tox*PO. The inverted arrows indicate the putative *tox* operator region. The *tox* promoter is indicated by "−10" and "−35." (*B*) Plasmids used in the construction of the diphtheria *tox*PO–*lacZ* transcriptional fusion.

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

E. coli strain	β-galactosidase, units*	
	No addition	2,2'-Dipyridyl (300 μM)
fur$^+$		
SM796	4	83
SM796(pRS551toxPO)	3,184	3,067
SM796(pRS551toxPO-Fur)	3,402	2,708
SM796(pRS551toxPO-VN1200)	73	2,682
DH1	11,159	10,617
DH1(pVN1200)	10,419	12,727
fur$^-$		
SBC796	7	36
SBC796(pRS551toxPO)	3,427	4,613
SBC796(pRS551toxPO-Fur)	3,320	3,190
SBC796(pRS551toxPO-VN1200)	68	2,771

*Average of four independent experiments.

purified on kanamycin-supplemented agar medium. One such clone was selected at random, a phage lysate was prepared, and the recombinant phage was designated λRS45toxPO (*lac*$^+$, *kan*^{R}, *amp*^{S}).

Molecular Cloning of *dtxR*. Chromosomal DNA was isolated from the nontoxigenic, nonlysogenic C7 strain of *C. diphtheriae*, digested with *Hin*dIII, and after electrophoresis on agarose gels, 1- to 12-kb fragments were isolated. Chromosomal fragments were then ligated into *Hin*dIII-digested pUC18 and transformed into *E. coli* DH5α:λRS45toxPO. Transformants were selected on agar medium supplemented with ampicillin and X-gal and screened for a white-colony phenotype. From >18,000 transformants screened, 71 white and light blue colonies were isolated and colony purified for further analysis. Plasmid DNA from 20 random clones was retransformed into *E. coli* DH5α:λRS45toxPO, and 16 were found to retain their white phenotype on X-gal-containing medium. Restriction endonuclease digestion analysis of minilysate DNA with *Hin*dIII revealed that all 16 plasmids contained inserts with a common 2.5-kb segment (data not shown). One isolate was selected, and the recombinant plasmid was designated pHH2500. Fig. 2 shows a partial restriction endonuclease map of the 2.5-kb insert of pHH2500.

By using a ^{32}P-labeled 1.5-kb *Pvu* II fragment of pHH2500 as a probe for colony blot analysis, we found that all transformants retaining a white phenotype on X-gal agar medium contained DNA that hybridized to the probe under stringent conditions. Moreover, we found by Southern blot analysis that *Hin*dIII digests of chromosomal DNA from *C. diphtheriae* strains C7(−), PW8(−), and 1030(−) also contained a single band that hybridized with the 1.5-kb probe (data not shown).

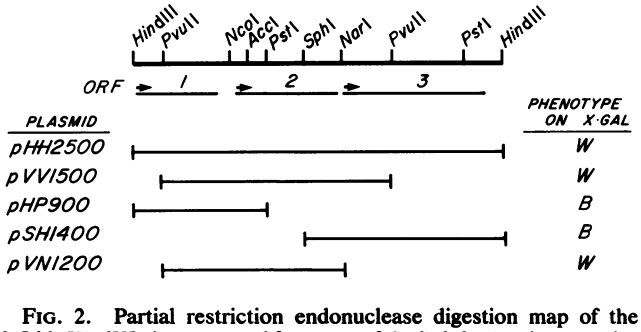

FIG. 2. Partial restriction endonuclease digestion map of the 2.5-kb *Hin*dIII chromosomal fragment of *C. diphtheriae* that encodes *dtxR*. Relative positions and direction of transcription of ORF1–3 are shown. Plasmids that carry subclones of the 2.5-kb insert and their respective phenotype in *E. coli* DH5α:λRS45toxPO transformants on X-gal medium are listed. W, white; B, blue.

DNA sequence analysis of the 2.5-kb *Hin*dIII insert revealed three nonoverlapping open reading frames (ORF1–3) (Fig. 2). To localize the putative diphtheria *tox* regulatory element, we subcloned restriction fragments into pUC18 and then transformed *E. coli* DH5α:λRS45toxPO with the derivative plasmids. As shown in Fig. 2, only those plasmids that carried ORF2 conferred a white-colony phenotype. Thus, the putative *tox* regulatory element was positioned on a 1.2-kb *Pvu* II–*Nar* I fragment of pHH2500. This segment carries the 678-bp ORF2, which is predicted to encode a 25,316-Da protein. The nucleic acid and deduced amino acid sequence of this ORF is shown in Fig. 3.

DtxR Is an Iron-Dependent Regulatory Element. Because the heterologous diphtheria *tox* promoter is relatively weak in recombinant strains of *E. coli* and the level of β-galactosidase expressed in the DH5α:λRS45toxPO lysogen is low, we recloned the 1.2-kb *Pvu* II–*Nar* I segment of pHH2500 that encodes ORF2 into plasmid pRS551toxPO. Recombinant *E. coli* carrying plasmid pRS551toxPO-VN1200 was used to study the effect of the putative *tox* regulatory element on *lacZ* expression.

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-230      -220      -210      -200      -190      -180
CAATAAAGCGTTTGCTTAGATATGCCTACCAATAAAGACATAAACGCCTATTAAAAGCAA

-170      -160      -150      -140      -130      -120
TCTTTAGATTAGGCGTTATAATTAAAGTCTCATCGAAAAACGCGCTGCGGGACTACAACG

-110      -100       -90       -80       -70       -60
CAACAAGAAAACTATTCCATATTTTTCACGCTACAATTCGTTGTAGATTGATAGGAATTG
                      ******                    ******

-50       -40       -30       -20       -10        1
ATCACCAGCACACAACAGTCTCCATGGCACTATAAGGAAAGAGGCTTACAATGAAGGAC
                                    ____          MetLysAsp

10        20        30        40        50        60
TTAGTCGATACCACAGAGATGTACTTGCGTACTATCTATGAGCTGGAAGAAGAGGGAGTC
LeuValAspThrThrGluMetTyrLeuArgThrIleTyrGluLeuGluGluGluGlyVal

70        80        90        100       110       120
ACCCCTCTTCGCGCTAGGATCGCTGAGCGTCTGGAACAATCTGGACCTACAGTTAGCCAA
ThrProLeuArgAlaArgIleAlaGluArgLeuGluGlnSerGlyProThrValSerGln

130       140       150       160       170       180
ACCGTTGCCCGTATGGAGCGCGATGGACTTGTCGTTGTCGCCTCAGACCGCAGTCTACAA
ThrValAlaArgMetGluArgAspGlyLeuValValValAlaSerAspArgSerLeuGln

190       200       210       220       230       240
ATGACACCGACAGGCCGCACTTTAGCGACTGCAGTTATGCGTAAACATCGCTTAGCTGAG
MetThrProThrGlyArgThrLeuAlaThrAlaValMetArgLysHisArgLeuAlaGlu

250       260       270       280       290       300
CGCCTTCTTACCGATATCATTGGTCTAGATATCAATAAAGTTCACGATGAAGCCTGCCGC
ArgLeuLeuThrAspIleIleGlyLeuAspIleAsnLysValHisAspGluAlaCysArg

310       320       330       340       350       360
TGGGAACACGTTATGAGTGACGAAGTTGAACGCAGGCTCGTGAAAGTATTGAAAGATGTC
TrpGluHisValMetSerAspGluValGluArgArgLeuValLysValLeuLysAspVal

370       380       390       400       410       420
AGTCGGTCCCCCTTCGGAAACCCAATTCCAGGTCTCGACGAACTCGGCGTAGGCAATTCT
SerArgSerProPheGlyAsnProIleProGlyLeuAspGluLeuGlyValGlyAsnSer

430       440       450       460       470       480
GACGCGGCAGCCCCCGGAACTCGCGTTATTGACGCTGCCACCAGCATGCCCCGCAAAGTA
AspAlaAlaAlaProGlyThrArgValIleAspAlaAlaThrSerMetProArgLysVal

490       500       510       520       530       540
CGCATTGTTCAGATTAACGAAATCTTTCAAGTTGAAACGGATCAGTTTACACAGCTCCTC
ArgIleValGlnIleAsnGluIlePheGlnValGluThrAspGlnPheThrGlnLeuLeu

550       560       570       580       590       600
GATGCTGACATCCGTGTTGGATCAGAAGTCGAAATTGTAGATAGAGACGGCCACATCACG
AspAlaAspIleArgValGlySerGluValGluIleValAspArgAspGlyHisIleThr

610       620       630       640       650       660
TTGAGCCACAATGGAAAAGATGTCGAACTCCTCGATGATCTGGCTCACACTATTCGTATC
LeuSerHisAsnGlyLysAspValGluLeuLeuAspAspLeuAlaHisThrIleArgIle

670       680       690       700
GAAGAACTCTAAATACTAAAGGCGGCAAATTAGATGAAACT
GluGluLeu!!!
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FIG. 3. Nucleotide and deduced amino acid sequence of the *dtxR* gene from *C. diphtheriae*. The putative −35 and −10 regions of *dtxR* promoter are designated (*), and the ribosome-binding site is underlined.

That the regulation of diphtheria *tox* expression in *C. diphtheriae* is mediated by the extracellular iron concentration is well known (4). Because 2,2'-dipyridyl can chelate extracellular iron and derepress iron-sensitive genes in *E.coli* (36, 37), we examined the effect of this agent on *E. coli* (pRS551toxPO and pRS551toxPO-VN1200).

As shown in Table 2, moderate levels of β-galactosidase are expressed by *E. coli* SM796 (pRS551toxPO), both in the presence and absence of the iron chelator dipyridyl. In marked contrast, however, the presence of *dtxR* on plasmid pRS551toxPO reduces the level of β-galactosidase expression to almost background levels in iron-rich LB medium. Moreover, the addition of dipyridyl to the growth medium of *E. coli* (pRS551toxPO-VN1200) results in a derepression of the *tox*PO–*lacZ* transcriptional fusion and results in the expression of β-galactosidase.

Because Tai and Holmes (14) have reported that the *E. coli* iron-dependent regulatory factor Fur partially regulates the diphtheria *tox* promoter, we subcloned the *fur* gene (1.2-kb *Acc* I–*Bgl* I fragment) from pABN203 to form pRS551toxPO-Fur in an attempt to confirm and extend their observations. As shown in Table 2, the presence of *fur* does not affect the expression of β-galactosidase from the *tox*PO–*lacZ* fusion in either the *fur*$^+$ (SM796) or *fur*$^-$ (SBC796) *E. coli* background.

Fur has been shown to regulate the expression of several outer-membrane proteins in *E. coli* (34). A shown in Fig. 4, plasmid pRS551toxPO-Fur inhibits expression of iron-regulated outer-membrane proteins in the *fur*$^-$ SBC796 background (lane H); however, the presence of cloned *dtxR* in this strain has no detectable effect on the expression of these outer-membrane proteins (lane I). Even though the putative *tox* operator shares sequence homology with *fur* operators (14), the results presented here strongly suggest that the regulatory action of Fur and DtxR are restricted to their respective target sequences.

To demonstrate that the wild-type *lac* operon is not affected by the *dtxR* gene product, pVN1200 was transformed into *E. coli* DH1. As shown in Table 2, after induction with isopropyl β-D-thiogalactoside, equivalent levels of β-galactosidase are expressed by both DH1 and DH1 (pVN1200) strains.

Because the expression of β-galactosidase from pRS551toxPO plasmid is constitutive and appears to be negatively controlled in an iron-dependent manner in *E. coli* (pRS551toxPO-VN1200), we conclude that the putative *tox* regulatory factor cloned from a genomic library of nontoxinogenic nonlysogenic *C. diphtheriae* is a diphtheria *tox* transcriptional regulatory factor and have designated it *dtxR* (*d*iphtheria *tox* *r*egulatory element). Although these results do not rigorously prove that *dtxR* is the gene encoding the diphtheria *tox* regulatory element in *C. diphtheriae*, the iron-dependent regulation of *tox*PO in *E. coli* by cloned *dtxR* is consistent with this interpretation.

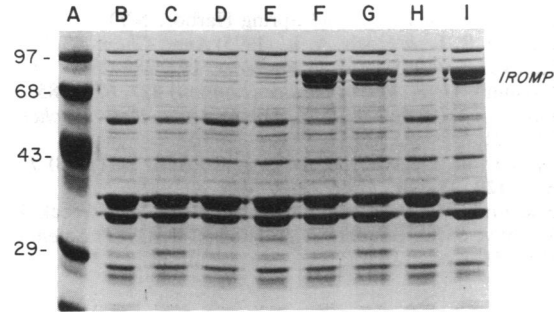

FIG. 4. SDS/polyacrylamide gel analysis of outer-membrane proteins from *fur*$^+$ (SM796) and *fur*$^-$ (SBC796) strains of *E. coli*. Lanes: A, M_r markers; B, SM796; C, SM796 (pRS551toxPO); D, SM796 (pRS551toxPO-Fur); E, SM796 (pRS551toxPO-VN1200); F, SBC796; G, SBC796 (pRS551toxPO); H, SBC796 (pRS551toxPO-Fur); I, SBC796 (pRS551toxPO-VN1200). IROMP, iron-regulated outer-membrane proteins.

Characterization of *dtxR*. We have conducted a computer search using the EMBL 21 and Swiss-Prot (PC/GENE data banks release 3.0) 13 databases for both nucleic acid and protein sequences that may share homology with *dtxR*. While *dtxR* has been found to be unique in these databases, we have found that *dtxR* shares 66% nucleic acid and 25% amino acid homology with *E. coli fur*. Inspection of the upstream noncoding region revealed a putative promoter and a ribosome-binding site for *dtxR* (Fig. 3). The sequence of the putative *dtxR* promoter in *C. diphtheriae* is similar to that described (13) for the corynebacteriophage *tox* gene. In addition, analysis of the amino acid sequence of DtxR has revealed a region that bears some homology to the helix-turn-helix motif for DNA-binding proteins (36). This region extends from Val-174 to His-201 and, according to the method of Garnier *et al.* (37), has a predicted α-helix turn α-helix secondary structure.

DISCUSSION

We have screened a genomic library of nontoxinogenic nonlysogenic *C. diphtheriae* for factors that may be directly engaged in the regulation of diphtheria *tox* expression. To facilitate screening, we have constructed an *E. coli* host strain that carries a single genomic copy of a transcriptional fusion between the diphtheria *tox* regulatory region and *lacZ*, *tox*PO–*lacZ*. We show that expression of β-galactosidase from the *tox*PO–*lacZ* fusion is constitutive (Table 2). From over 18,000 recombinant *E. coli* DH5α screened, we isolated 71 clones in which the β-galactosidase expression from *tox*PO–*lacZ* was suppressed. Of these, ≈75% retained their white-colony phenotype on X-gal-containing medium upon retransformation of their respective plasmid DNA into lysogens of *E. coli*:λRS45toxPO. It is of interest to note that all recombinant plasmids that conferred a white-colony phenotype in this strain were found to carry an insert with a common 2.5-kb *Hin*dIII segment.

Subcloning of restriction fragments has identified a 1.2-kb region essential for the repression of β-galactosidase expression from the *tox*PO–*lacZ* transcriptional fusion. Nucleic acid sequence analysis has shown that the 1.2-kb insert carries a single ORF that is predicted to encode a 25,316-Da protein. The presence of this DNA fragment on plasmid pRS551toxPO reduces the level of β-galactosidase expression from the *tox*PO–*lacZ* fusion to almost background levels. The 1.2-kb *Pvu* II–*Nar* I fragment, however, has no effect on the levels of β-galactosidase expressed from the wild-type *lac* promoter in strain DH1 (pVN1200).

Because the regulation of diphtheria *tox* gene products in lysogenic *C. diphtheriae* is mediated by extracellular iron (4, 5), we examined the effect of the iron chelator, 2,2'-dipyridyl, on β-galactosidase expression by *E. coli* strains that carry the *tox*PO–*lacZ* transcriptional fusion and the putative *tox* regulatory element. We have shown that the addition of dipyridyl to the culture medium results in almost complete derepression of the *tox*PO–*lacZ* operon.

Murphy *et al.* (10) have shown that the action of the diphtheria *tox* regulatory factor was mediated at the level of transcription. In addition, the isolation of both corynebacterial (7) and corynebacteriophage (8, 9) mutants in which *tox* expression was relatively insensitive to iron suggested that the regulation of *tox* was mediated through a *C. diphtheriae* iron-binding negative controlling element (12). The molecular cloning of *dtxR* from genomic libraries of *C. diphtheriae* and the observation that this factor acts as an iron-dependent regulator of the *tox*PO–*lacZ* transcriptional fusion are consistent with the model for *tox* regulation proposed by Murphy and Bacha (12).

Recently, Fourel *et al.* (11) have shown that an iron-binding protein, DtoxR, from extracts of *C. diphtheriae* could specifically bind to the presumptive diphtheria *tox* operator locus and protect it from DNase I digestion. The putative *tox* operator locus is a 27-bp region that contains a 9-bp inverted repeat sequence separated by 9 bp (13, 38). Whether this factor is similar or identical to the corynebacterial factor we have cloned and sequenced remains to be determined. Nonetheless, DtxR protein appears to have functional properties identical to those of the iron-binding protein DtoxR.

Tai and Holmes (14) have recently described the construction of a diphtheria *tox–galK* transcriptional fusion in which the *tox* regulatory region as well as fragment A and a portion of fragment B were cloned upstream from a cryptic *galK* gene. In recombinant *E. coli* that carried this fusion, the rate of *galK* expression was increased ≈5-fold under iron-limiting conditions and appeared to be regulated by *fur*. In the present study, we have used the *fur*$^+$ SM796 strain of *E. coli* and the congenic *fur*$^-$ SBC796 strain and have not observed repression of β-galactosidase expression from the *tox*PO–*lacZ* fusion by Fur. Moreover, we did not observe an inhibitory effect of Fur on β-galactosidase expression, even when *fur* was recloned on the multicopy pRS551toxPO plasmid.

There are several possible explanations for our inability to confirm the results of Tai and Holmes (14): the genetic construct used by these investigators contains additional sequences that are 5′ to the *tox* regulatory region, and these sequences are not included in the −15 to −80 oligonucleotide encoding the *tox*PO region used in the present study. As a result, additional regulatory sites may be present on the *tox–galK* construct. Alternatively, galactokinase expressed from pTKW1 may have a longer half-life in *E. coli* under conditions of iron starvation.

Because DtxR appears to be an iron-dependent regulatory element, it was of interest to examine the effect of *dtxR* on the expression of *E. coli* iron-regulated outer-membrane proteins. We did not observe repression of Fur-regulated outer-membrane proteins in *fur*$^-$ recombinant strains of *E. coli* that carried *dtxR*. These data suggest that although Fur and DtxR appear to have similar activities in their respective hosts and that they bind to similar operator sequences, their regulatory action is likely to be restricted to their specific operators.

It is tempting to speculate that DtxR is analogous to Fur and functions as a global regulatory element in *C. diphtheriae*. Such an element would be anticipated to be directly involved in the control of the corynebacterial high-affinity iron transport system, as well as the regulation of the corynebacteriophage *tox* operon. Although the function of Fur in *E. coli* and DtxR in *C. diphtheriae* appears similar, comparison of their DNA sequence has shown only 66% nucleic acid and 25% amino acid homology, respectively.

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1. Uchida, T., Gill, D. M. & Pappenheimer, A. M., Jr. (1971) *Nature (London) New Biol.* **233**, 8–11.
2. Buck, G. A., Gross, R. E., Wong, T. P., Lorea, T. & Groman, N. B. (1985) *Infect. Immunol.* **49**, 679–684.
3. Pappenheimer, A. M., Jr. (1955) in *Mechanisms of Microbial Pathogenicity*, eds. Howie, J. W. & O'Hea, A. J. (Cambridge Univ. Press, New York).
4. Pappenheimer, A. M., Jr. (1977) *Annu. Rev. Biochem.* **46**, 69–94.
5. Pappenheimer, A. M., Jr., & Johnson, S. J. (1936) *Br. J. Exp. Pathol.* **17**, 335–341.
6. Murphy, J. R., Pappenheimer, A. M., Jr., & Tayart de Borms, S. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 11–15.
7. Kanei, C., Uchida, T. & Yoneda, M. (1977) *Infect. Immunol.* **18**, 203–209.
8. Murphy, J. R., Skiver, J. & McBride, G. (1976) *J. Virol.* **18**, 235–244.
9. Welkos, S. & Holmes, R. K. (1981) *J. Virol.* **37**, 936–945.
10. Murphy, J. R., Michel, J. L. & Teng, M. (1978) *J. Bacteriol.* **135**, 511–516.
11. Fourel, G., Phalipon, A. & Kaczorek, M. (1989) *Infect. Immun.* **57**, 3221–3225.
12. Murphy, J. R. & Bacha, P. (1979) in *Microbiology 1979* (Amer. Soc. Microbiol., Washington), pp. 181–186.
13. Boyd, J. & Murphy, J. R. (1988) *J. Bacteriol.* **170**, 5949–5952.
14. Tai, S.-P. S. & Holmes, R. K. (1988) *Infect. Immunol.* **56**, 2430–2436.
15. Calderwood, S. B. & Mekalanos, J. J. (1987) *J. Bacteriol.* **169**, 4759–4764.
16. Calderwood, S. B. & Mekalanos, J. J. (1988) *J. Bacteriol.* **170**, 1015–1017.
17. de Lorenzo, V., Wee, S., Herro, M. & Neilands, J. B. (1987) *J. Bacteriol.* **169**, 2624–2630.
18. Hantke, K. (1984) *Mol. Gen. Genet.* **197**, 337–341.
19. Schaffer, S., Hantke, K. & Braun, V. (1985) *Mol. Gen. Genet.* **200**, 111–113.
20. Simons, R. W., Houman, F. & Kleckner, N. (1987) *Gene* **53**, 85–69.
21. Hanahan, D. (1983) *J. Mol. Biol.* **166**, 557–580.
22. Bagg, A. & Neilands, J. B. (1985) *J. Bacteriol.* **161**, 450–453.
23. Barksdale, W. L. & Pappenheimer, A. M., Jr. (1954) *J. Bacteriol.* **67**, 220–232.
24. Park, W. H. & Williams, A. W. (1896) *J. Exp. Med.* **1**, 164–185.
25. Bezjak, V. (1954) *Antonie von Leevwenhook* **20**, 269–272.
26. Pappuoli, R., Michel, J. L. & Murphy, J. R. (1983) *J. Bacteriol.* **153**, 1202–1210.
27. Pappenheimer, A. M., Jr., & Murphy, J. R. (1983) *Lancet* **ii**, 923–926.
28. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1989) *Current Protocols in Molecular Biology* (Wiley, New York).
29. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
30. Kraft, R., Tardiff, J., Krauter, K. S. & Leinwand, L. A. (1988) *BioTechniques* **6**, 544–547.
31. Gergen, J. P., Stern, R. H. & Wensink, P. C. (1979) *Nucleic Acids Res.* **7**, 2115–2136.
32. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
33. Putnam, S. L. & Koch, A. L. (1975) *Anal. Chem.* **63**, 350–360.
34. Hantke, K. (1981) *Mol. Gen. Genet.* **182**, 288–292.
35. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
36. Pabo, C. O. & Sauer, R. T. (1984) *Annu. Rev. Biochem.* **53**, 293–321.
37. Garnier, J., Osguthorpe, D. J. & Robson, B. (1978) *J. Mol. Biol.* **120**, 97–120.
38. Kaczorek, M., Delpeyroux, F., Chenciner, N., Streeck, R. E., Murphy, J. R., Boquet, P. & Tiollais, P. (1983) *Science* **221**, 855–858.