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Iron-Dependent Regulation of Diphtheria Toxin and Siderophore Expression by the Cloned *Corynebacterium diphtheriae* Repressor Gene *dtxR* in *C. diphtheriae* C7 Strains

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A regulatory gene (*dtxR*) responsible for iron-dependent repression of the toxin (*tox*) and siderophore genes in *Corynebacterium diphtheriae* was cloned and characterized. A DNA fragment carrying *dtxR* repressed expression of a *tox-lacZ* gene fusion in *Escherichia coli* DH5α in a high-iron environment but not under low-iron conditions. A protein with mobility corresponding to approximately 28 to 29 kDa was identified as the product of the *dtxR* gene by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A shuttle vector designated pCM2.6 was constructed which carries the origin of replication from *C. diphtheriae* plasmid pNG2 and confers resistance to chloramphenicol in *E. coli* and *C. diphtheriae*. DNA fragments carrying *dtxR* were cloned into pCM2.6, and the hybrid shuttle plasmids were transformed by electroporation into wild-type *C. diphtheriae* C7(β) and the regulatory mutant C7(β)hm723, which produces toxin and siderophore constitutively under high-iron conditions. Expression of the cloned *dtxR* determinant did not affect the phenotype of *C. diphtheriae* C7(β). In *C. diphtheriae* C7(β)hm723, expression of cloned *dtxR* restored full repression of siderophore production and partial repression of diphtheria toxin production during growth in a high-iron environment.

Corynebacterium diphtheriae is the causative agent of diphtheria (17). Diphtheria toxin (DT), the primary virulence factor of *C. diphtheriae*, is encoded by the *tox* gene of certain corynephages, including bacteriophage β (17). Both the *tox* gene and DT have been well characterized (17). Expression of *tox* is affected by iron, with maximal synthesis of DT occurring in low-iron environments (18). Murphy et al. (14) showed that in *Escherichia coli* extracts containing β phage DNA, production of DT is repressed by a factor from nonlysogenic *C. diphtheriae* C7. Furthermore, mutations in β phage (15, 31) or in the chromosome of *C. diphtheriae* C7 (5, 11) caused constitutive production of DT regardless of the iron concentration in the growth medium. Mutants of bacteriophage β in which the *tox* gene is resistant to iron regulation were recently shown to have point mutations in putative operator/promoter sequences of the *tox* operon (12). The C7(β)hm723 mutant of *C. diphtheriae* produces toxin constitutively in low- and high-iron media and is thought to be defective in a regulatory gene designated *dtxR* (11, 30). Boyd et al. (2) have recently cloned the *dtxR* gene of *C. diphtheriae* C7, determined its nucleotide sequence, and established that it has some homology with the *E. coli fur* gene. In an *E. coli* background, the cloned *dtxR* gene repressed expression of the cloned *tox* promoter under high-iron conditions but failed to repress *tox* expression in low-iron medium. A plausible model to explain these findings suggests that the product of the *C. diphtheriae dtxR* gene is an iron-dependent repressor that negatively regulates the corynephage *tox* operon at the level of transcription.

The product of the *fur* gene in *E. coli* regulates siderophore-dependent high-affinity iron uptake systems by a process similar to that proposed for the *dtxR* gene product of *C. diphtheriae* (1). A siderophore-dependent high-affinity iron transport system is also present in *C. diphtheriae* (19, 20), and recent studies from this laboratory (30) demonstrated that siderophore synthesis is coordinately regulated with toxin production. This evidence strongly suggests that the function of the *dtxR* gene product in *C. diphtheriae* is analogous to that of the Fur protein of *E. coli*.

Genetic studies in *C. diphtheriae* are very limited, due in part to the lack of an efficient system for genetic exchange. Serwold-Davis et al. (25, 26) described protoplast transformation in some strains of *C. diphtheriae* and developed an *E. coli-C. diphtheriae* shuttle vector with a chloramphenicol resistance determinant and the origin of replication from the *C. diphtheriae* plasmid pNG2 (25). This shuttle plasmid replicates stably and confers chloramphenicol resistance in both *C. diphtheriae* and *E. coli*. The efficiency of transformation of protoplasts in *C. diphtheriae* is low, however, and it appears that restriction systems in some strains of *C. diphtheriae* limit their ability to be transformed by DNA isolated from *E. coli*.

In this study, we independently cloned the *dtxR* gene from *C. diphtheriae* C7 and identified its product. The *dtxR* gene was shown to regulate the cloned *tox* gene promoter by an iron-responsive mechanism in *E. coli* DH5α. A transformation system for *C. diphtheriae*, using electroporation and a new *E. coli-C. diphtheriae* shuttle vector, was also developed, and the role of the cloned *dtxR* gene in iron-regulated expression of DT and siderophore was examined in wild-type and mutant strains of *C. diphtheriae* C7.

MATERIALS AND METHODS

Bacterial strains and plasmids. *C. diphtheriae* strains C7 and C7(β) were from our laboratory and were originally obtained from L. Barksdale (10). *C. diphtheriae* C7(β)hm723 was from T. Uchida (11), and *C. ulcerans* 712 was from N. Groman (26). *E. coli* DH5α (Bethesda Research Laboratories [BRL], Gaithersburg, Md.) was used for all cloning experiments. Stock cultures were stored at −70°C in 20% glycerol. Plasmids used are listed in Table 1 and Fig. 1.

Plasmid pTXZ184, which carries a fusion between the 5′

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference or source
pWF2B	pBR322 derivative carrying F2 fragment of *tox* gene	29
pUC18	High-copy-number cloning vector	16
pMC1403	*lacZ* promoter probe vector	3
pACYC184	Tet^r Cm^r; P15A replicon	4
pNG2	Em^r plasmid isolated from *C. diphtheriae*	N. Groman
pCM2.6	Cm^r *E. coli-C. diphtheriae* shuttle vector	This study
pTXZ184	Derivative of pACYC184 carrying *tox-lacZ* translational fusion	This study
pMS29	5-kb *Sau*3AI fragment carrying *dtxR* gene in pUC18	This study
pMS290	2.8-kb *Hin*dIII subclone of pMS29 carrying *dtxR*	This study
pMS297	1.4-kb *Pvu*II subclone of pMS290 carrying *dtxR*	This study
pMS298	1.4-kb insert from pMS297 with *dtxR* under T7 promoter control in pBluescriptKS vector	This study
pGP1-2	pACYC184 derivative carrying temperature-inducible T7 RNA polymerase	28
pCMS97	1.4-kb *Pvu*II fragment carrying *dtxR* gene in pCM2.6	This study
pCMS29	5-kb insert from pMS29 present in pCM2.6	This study
pBluescriptKS	T7 promoter expression and cloning vector	Stratagene

coding region of the *tox* operon and *lacZ*, was constructed by a multistep process. Briefly, a 316-bp *Eco*RI-*Hae*III fragment from pWF2B (29) carrying 55 bp of the 5′ coding region of the *tox* gene and approximately 250 bp of upstream sequences was ligated into the *Eco*RI-*Sma*I site of pUC19. A 333-bp *Eco*RI-*Hin*cII fragment was then excised and ligated into the *Eco*RI-*Sma*I sites of the *lacZ-lacY* promoter probe vector pMC1403 (3). This construction created an in-frame protein fusion between the first 18 amino acids of the diphtheria toxin leader peptide and β-galactosidase. A 6.5-kb *Eco*RI-*Sal*I fragment carrying the entire *tox-lacZY* fusion construct was excised from the pMC1403 derivative, an *Eco*RI linker was ligated onto the *Sal*I end, and the resulting fragment was ligated to the *Eco*RI site in pACYC184 to create pTXZ184.

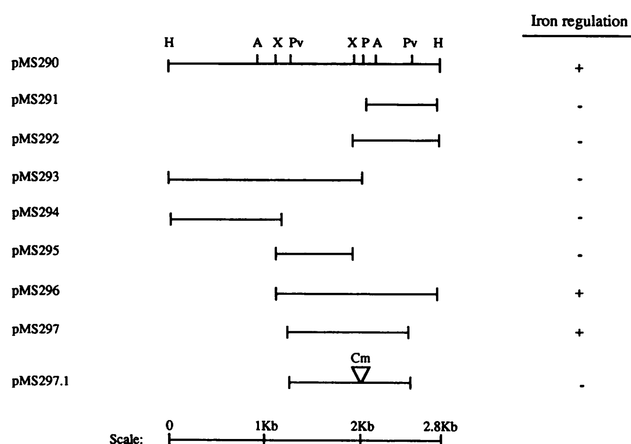

FIG. 1. Restriction endonuclease map of plasmid pMS290 and various subclones. Iron-dependent regulation for the various plasmid constructs was determined by transforming each of the plasmids into *E. coli* DH5α carrying pTXZ184 and observing the color of the colonies on iron-rich LB medium containing X-gal. White colonies indicated repression of the β-galactosidase gene fusion (+ iron regulation), while blue colonies indicated failure to repress the β-galactosidase gene fusion (− iron regulation). Cm, chloramphenicol resistance determinant. Restriction sites: A, *Acc*I; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; X, *Xba*I.

Reagents, media, and conditions for cultivation. *E. coli* DH5α was routinely cultured in Luria broth (LB) (13) or on LB agar medium. LB agar was made iron deficient by adding EDDA (ethelenediamine-di-*o*-hydroxyphenyl acetic acid) at 25 μg/ml. Heart infusion broth (HIB; Difco, Detroit, Mich.) containing 0.2% Tween 80 was used for routine growth of *C. diphtheriae* strains. Modified PGT-maltose medium, prepared as described previously, was used for growth of *C. diphtheriae* for DT and siderophore production (30). The modified PGT-maltose medium was made low iron by treating the individual components with Chelex 100 resin (Bio-Rad Laboratories, Richmond, Calif.) (2 g/liter). Siderophore and toxin production was determined from 5-ml overnight cultures grown in Erlenmeyer flasks at 37°C in Chelex 100-treated, modified PGT-maltose medium containing either 1 μM $FeCl_3$ (low iron) or 10 μM $FeCl_3$ (high iron). The following supplements were added to media for *E. coli* when necessary: ampicillin (50 μg/ml), tetracycline (12 μg/ml), chloramphenicol (34 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (40 μg/ml). Chloramphenicol was added at 2 μg/ml for *C. diphtheriae* strains harboring Cm^r plasmids.

DNA preparation and transformation. Chromosomal and plasmid DNA was isolated from *C. diphtheriae* C7 as described by Schiller et al. (22). The chromosomal DNA was further purified by CsCl-ethidium bromide density gradient centrifugation. Plasmid DNA was routinely isolated from *E. coli* by the boiling method of Holmes and Quigley (9).

Restriction enzymes and *Eco*RI linkers (BRL) and T4 DNA ligase and Klenow fragment of DNA polymerase I (Boehringer Mannheim, Indianapolis, Ind.) were used according to the manufacturers' recommendations.

E. coli was transformed with plasmid DNA by the method of Hanahan (7). *C. diphtheriae* strains were transformed with plasmid DNA by electroporation following the protocol developed for transformation of *Corynebacterium glutamicum* and *Brevibacterium lactofermentum* (8), except that all strains were grown in HIB medium supplemented with 0.2% Tween 80. A Gene Pulser apparatus (Bio-Rad) and 0.2-cm cuvettes were used for all electroporation experiments.

Protein analysis. Preferential expression of *dtxR* was accomplished by cloning the *dtxR* gene downstream from the strong T7 gene *10* promoter present on the vector pBluescriptKS (Stratagene, La Jolla, Calif.). The *dtxR* gene was then transcribed by the heat-inducible T7 RNA polymerase present on plasmid pGP1-2 (27, 28). DH5α containing plasmid pGP1-2 and either pMS298 or pBluescriptKS was grown at 30°C in LB medium supplemented with 50 μg/ml each of ampicillin and kanamycin. When the culture had reached an A_{590} of 0.5, 0.2 ml of cells was centrifuged, washed with 1.0 ml of M9 medium (13), and recentrifuged. The cell pellet was resuspended in 1.0 ml of M9 medium supplemented with thiamine (20 μg/ml) and 0.01% methionine assay medium (Difco), and the culture was grown with shaking at 30°C for

60 min. The temperature was then shifted to 42°C for 15 min. Rifampin, which specifically inhibits the host RNA polymerase but does not affect the T7 polymerase, was then added to a final concentration of 200 μg/ml, and the cells were incubated at 42°C for an additional 10 min. The temperature was shifted to 30°C for 20 min, and the sample was pulsed with 10 μCi of [^{35}S]methionine for 5 min, centrifuged, and then resuspended in 120 μl of lysis buffer (60 mM Tris-HCl [pH 6.8], 1% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue). Samples were heated at 95°C, which was followed by separation of the proteins by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Siderophore assays. *C. diphtheriae* culture supernatants were assayed for siderophore by using the Chrome Azurol S (CAS) assay procedure developed by Schwynn and Neilands (23) as modified by Tai et al. (30). Samples of culture supernatants (0.5 ml) or dilutions of the supernatant were mixed with the CAS solution and incubated at room temperature for approximately 2 h. The A_{630} was measured, and dilutions of supernatants were made so that absorbance readings were between 0.2 and 0.6. Siderophore units were determined as described previously (30).

DT assay. Determination of DT in culture supernatants was done by solid-phase, competitive-binding radioimmunoassay as described previously (30), with the following modifications. ^{125}I-labeled DT was mixed with serial dilutions of culture supernatants or toxin control prior to addition to microtiter wells sensitized with anti-diphtheria toxoid antibodies. Microtiter plates were incubated for 3 h at 37°C or at room temperature overnight, and the amount of bound ^{125}I-DT was compared with a standard curve for DT.

RESULTS

Cloning the repressor gene for the *C. diphtheriae tox* operon. To identify clones carrying the *dtxR* repressor gene, we screened a chromosomal library of *C. diphtheriae* C7 DNA by using a protein fusion in which *lacZ* expression was under control of the *tox* promoter-operator sequences. The rationale for this system was that the cloned *dtxR* gene would repress expression of the *tox-lacZ* gene fusion in high-iron medium by inhibiting transcription from the *tox* promoter. The chromosomal library was constructed in the high-copy-number pUC18 vector, and the *tox-lacZ* fusion construct pTXZ184 was present on the compatible and relatively low-copy-number pACYC184 vector. The difference in copy number between the two plasmids ensured that the cloned *dtxR* gene was in excess over the *tox-lacZ* fusion construct. *E. coli* DH5α carrying pTXZ184 formed blue colonies on LB medium containing X-gal.

A chromosomal library carrying DNA fragments in the range of 2 to 5 kb obtained from a *Sau*3AI partial digest of *C. diphtheriae* C7 chromosomal DNA was constructed in the high-copy-number vector pUC18 and transformed into *E. coli* DH5α carrying pTXZ184. Approximately 8,000 to 10,000 transformants were tested for repression of *lacZ* expression on LB medium containing X-gal. Several white or pale blue colonies were identified, picked from these plates, and tested for iron-dependent regulatory activity by streaking them onto LB-X-gal medium containing either EDDA (25 μg/ml) (low iron) or no added chelator (high iron). Only one clone, designated pMS29, bred true and formed white colonies on high-iron medium and blue colonies on low-iron medium, whereas *E. coli* DH5α carrying only pTXZ184 produced blue colonies on both media. Plasmid

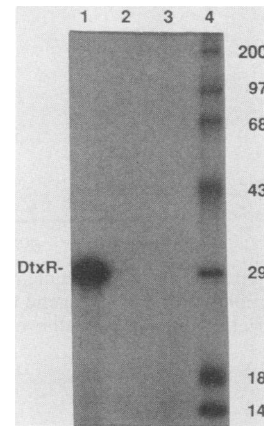

FIG. 2. Identification of the *dtxR* gene product. Shown is an autoradiograph of proteins labeled with [^{35}S]methionine and separated by SDS-PAGE as described in Materials and Methods. Plasmids carried by DH5α in each of the labeling experiments are as follows: lane 1, pGP1-2 and pMS298 (*dtxR* under T7 promoter control); lane 2, pGP1-2 and pBluescriptKS vector; lane 3, pGP1-2; lane 4, ^{14}C-labeled protein standards (in kilodaltons).

pMS29 contained a 5-kb *Sau*3AI insert in pUC18. An internal 2.8-kb *Hin*dIII fragment which retained full iron-dependent regulatory activity was subcloned into pUC18 to produce pMS290.

Mapping and subcloning of pMS290. As shown in Fig. 1, the smallest fragment of pMS290 which retained *dtxR* activity after subcloning was the 1.4-kb *Pvu*II fragment in plasmid pMS297. The chromosomal inserts in both pMS290 and pMS297 maintained their activity regardless of orientation with respect to the *lac* promoter present in pUC18. This observation suggested that the promoter for *dtxR* is present on these clones and active in *E. coli*. The *Pst*I site was chosen as a site for insertional inactivation of *dtxR* to confirm that sequences across the unique *Pst*I site in pMS297 were essential for regulatory activity. We demonstrated that inserting a Cm^r gene cassette into that site caused inactivation of *dtxR* (pMS297.1, Fig. 1). A comparison between the restriction map of our cloned fragment and the map of *dtxR* reported by Boyd et al. (2) revealed that the maps were identical for restriction enzymes used in both studies and indicated that we had independently cloned *dtxR*.

Detection of the *dtxR* gene product. The 1.4-kb chromosomal insert carrying the *dtxR* gene was excised from pMS297 and ligated into pBluescriptKS so that transcription of the *dtxR* gene could be directed by the T7 gene *10* promoter present on the pBluescriptKS vector. This new plasmid, designated pMS298, was transformed into *E. coli* DH5α which contained the heat-inducible T7 RNA polymerase on plasmid pGP1-2. This expression system allowed the *dtxR* gene to be preferentially transcribed and its product to be clearly identified by SDS-PAGE (see Materials and Methods). Figure 2 shows an autoradiograph of ^{35}S-labeled proteins after separation by SDS-PAGE. The strongly labeled band migrating at 28 to 29 kDa in lane 1 is the product of the *dtxR* gene for the following reasons: (i) the *dtxR* gene is the only full-length open reading frame present on the insert in pMS298 (2); (ii) the size of the protein corresponds approximately to the size predicted from the nucleotide sequence for the *dtxR* gene (25.3 kDa) (2); and (iii) this is the only detectable protein produced from the chromosomal insert on

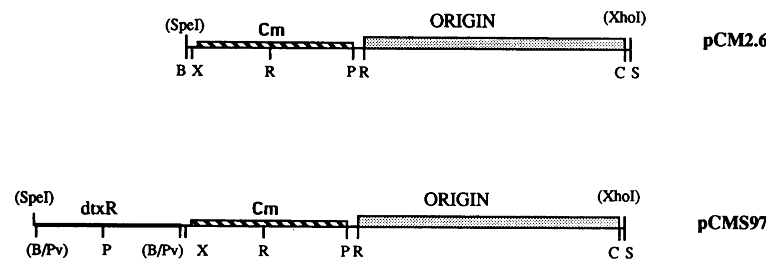

FIG. 3. Genetic map and partial endonuclease restriction map of shuttle vector pCM2.6 and plasmid pCMS97, which carries the *dtxR* gene. The pNG2 origin of replication and Cm^r gene cassette are shown. Restriction sites: B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; R, *Eco*RI; S, *Sal*I. Restriction sites in parentheses were used to construct pCM2.6 and pCMS97 (see the text), but they are not present in the final constructs.

plasmid pMS298. No labeled proteins were detected when plasmid pGP1-2 was present alone (lane 3) or when both pGP1-2 and the KS vector were present (lane 2).

Transformation of *C. diphtheriae* with the cloned *dtxR* gene. If a single regulatory gene is required to repress toxin and siderophore production in *C. diphtheriae* (analogous to the *fur* gene in *E. coli*), then introducing the cloned repressor gene into a *fur*-like mutant of *C. diphtheriae* should restore iron regulation of siderophore and toxin. A *C. diphtheriae-E. coli* shuttle vector and a transformation system for *C. diphtheriae* were developed to introduce the cloned *dtxR* gene into *C. diphtheriae* C7(β) and the regulatory mutant C7(β)hm723. The shuttle vector pCM2.6 (3.8 kb) carries the pNG2 origin of replication (25) and a Cm^r gene cassette (24). To construct plasmid pCM2.6, a 2.6-kb *Cla*I-*Eco*RI fragment of pNG2 containing the origin of replication was first ligated into the multiple cloning site (MCS) of the *Cla*I- and *Eco*RI-digested, high-copy vector pBluescriptKS. Next, the Cm^r cassette present on a 1-kb *Pst*I-*Bam*HI fragment was ligated into the MCS of the *Pst*I- and *Bam*HI-digested pBluescriptKS plasmid carrying the pNG2 origin fragment. Finally, all pBluescriptKS DNA outside of the MCS was excised by cutting at unique *Spe*I and *Xho*I restriction sites which flanked the Cm^r gene and the pNG2 origin fragment; the *Spe*I and *Xho*I sites were converted to blunt ends; and the excised fragment was circularized to produce pCM2.6 (Fig. 3).

Plasmid pCM2.6 contains several unique restriction sites which can be used for inserting foreign DNA (Fig. 3). Two separate DNA fragments carrying *dtxR* were ligated into pCM2.6. The 5-kb insert fragment in pMS29 was excised from the MCS with *Eco*RI and *Sal*I and ligated directionally into the *Cla*I and *Sal*I sites of pCM2.6 to construct pCMS29. The *Eco*RI end of the 5-kb insert fragment and the *Cla*I end of the vector were made blunt with Klenow prior to ligating. Plasmid pCMS97 was generated by inserting the 1.4-kb *Pvu*II insert from pMS297 into the filled-in *Bam*HI site of pCM2.6 (Fig. 3).

Plasmids were transformed into *C. diphtheriae* strains C7(β) and C7(β)hm723 as described in Materials and Methods. Due to an apparent DNA restriction-modification system present in *C. diphtheriae* C7, transformation efficiencies, even with the smallest plasmid (pCM2.6), were very low (10^1 to 10^2 CFU/μg of DNA). With the larger plasmids, pCMS97 and pCMS29, transformation efficiencies were further diminished. When these plasmids were first transformed into *C. ulcerans* 712 and reisolated, the plasmids from *C. ulcerans* 712 could then be transformed into *C. diphtheriae* C7 strains at efficiencies 10- to 100-fold higher than observed with the same plasmids from *E. coli* DH5α. *C. diphtheriae* transformants were recovered after 24 to 36 h of incubation at 37°C on HIB agar plates containing chloramphenicol (2 μg/ml). Plasmids isolated from the *C. diphtheriae* strains had restriction endonuclease profiles identical to those of the original plasmids from *E. coli*, and plasmids carrying the *dtxR* gene recovered from *C. diphtheriae* retained full repressor activity when they were transformed back into *E. coli* DH5α(pTXZ184).

Regulation of toxin and siderophore. The iron-dependent expression of DT and siderophore was examined in wild-type *C. diphtheriae* C7(β) and in the regulatory mutant C7(β)hm723 (Table 2). Both DT and siderophore production were strongly iron regulated in C7(β), and the regulation was unaffected by the presence of the plasmids carrying the cloned *dtxR* gene or the shuttle vector alone. Thus, the phenotype of C7(β) was not altered by the presence of additional copies of the wild-type *dtxR* gene on a plasmid.

DT was synthesized at high levels constitutively in C7(β)hm723 under high-iron and low-iron growth conditions, a finding consistent with earlier studies (5, 11, 30). The presence of the pCM2.6 vector had no effect on regulation of toxin production. Plasmid pCMS97 caused 15- to 20-fold repression and pCMS29 caused about 8-fold repression of DT production by C7(β)hm723 in high-iron medium.

Under the conditions of our experiments, siderophore production by C7(β)hm723 was repressed about 5-fold under

TABLE 2. Production of toxin and siderophore by *C. diphtheriae* isolates grown in high- and low-iron media[a]

C. diphtheriae strain/plasmid	Siderophore (U/ml)			Toxin (μg/ml)		
	+Fe	−Fe	Ratio, −Fe/+Fe	+Fe	−Fe	Ratio, −Fe/+Fe
C7	2	123	62	<0.1	<0.1	NA[b]
C7(β)	2	113	57	<0.1	17.0	>170
C7(β)/pCM2.6	4	198	50	<0.1	17.3	>170
C7(β)/pCMS29	2.5	125	50	<0.1	21.3	>210
C7(β)/pCMS97	3.5	218	62	<0.1	18.9	>190
C7(β)hm723	41	229	5	15.0	16.4	1.1
C7(β)hm723/pCM2.6	52	275	5	14.8	13.2	0.9
C7(β)hm723/pCMS29	11	241	22	2.5	20.2	8.4
C7(β)hm723/pCMS97	4	202	51	0.9	15.6	17

[a] Siderophore and toxin production were assayed from the same growth flasks. Each value is the average of results from three independent experiments. Values for toxin varied by less than 10% from the average, and values for siderophore varied by less than 25% from the average.

[b] NA, not applicable. Strain C7 does not produce DT unless it is lysogenized by a tox^+ corynephage such as β.

high-iron conditions, which is much less than the 50-fold repression observed with the wild-type *C. diphtheriae* C7 or C7(β). In contrast, repression of siderophore production under high-iron conditions increased to about 25-fold in the presence of pCMS29 and about 50-fold with plasmid pCMS97.

DISCUSSION

In this study, we cloned and characterized a *C. diphtheriae* C7 gene (*dtxR*) which conferred iron-dependent regulation on the expression of a *tox-lacZ* fusion construct in *E. coli*. We also developed a *C. diphtheriae-E. coli* shuttle vector and a transformation system for *C. diphtheriae* C7 based on electroporation and used them to demonstrate the function of the cloned *dtxR* gene in wild-type and mutant strains of *C. diphtheriae*. During the preparation of this manuscript, Boyd et al. (2) reported the cloning and characterization of *dtxR* from *C. diphtheriae* C7, using a similar strategy but an independently derived reporter system involving a *tox* promoter-*lacZ* operon fusion that was present in a single copy in the chromosome of the *E. coli* strain used as the recipient for the cloned genes from *C. diphtheriae*. The gene we cloned in this study appears to be identical to the *dtxR* gene reported by Boyd et al. (2), since the two genes exhibit similar activities and possess identical restriction endonuclease cleavage maps. Boyd et al. (2) also determined the nucleotide sequence of *dtxR* and reported that it has 66% homology at the nucleotide level and 25% homology at the amino acid level with the *fur* gene of *E. coli*. We performed an independent analysis of nucleotide and deduced amino acid homologies based on the reported sequences of *dtxR* and *fur* (2, 21) using the Genetics Computer Group sequence analysis program (6), and found, under optimal sequence alignment, only 39% identity at the nucleotide level and 18% identity at the amino acid level. The limited amino acid sequence homology between the products of the *dtxR* and *fur* genes is consistent with the finding that complementation between them is weak (29) or absent (2).

The size of the *dtxR* gene product predicted from the nucleotide sequence of the *dtxR* gene is 25.3 kDa (2). We have used a T7 expression system to identify the product of the *dtxR* gene. The DtxR protein migrates in SDS-PAGE with a mobility corresponding to 28 to 29 kDa, which is within the limits of accuracy of SDS-PAGE for predicting the molecular mass of proteins.

Previous studies by Kanei et al. (11) and by Tai et al. (30) suggested that C7(β)hm723 lacked the putative iron-dependent repressor for DT and siderophore. Consistent with this hypothesis, C7(β)hm723 grows nearly as well as wild-type *C. diphtheriae* C7 and produces toxin constitutively under high-iron and low-iron growth conditions. Tai et al. (30) also reported constitutive production of siderophore by C7(β)hm723, in contrast to the slight repressibility of siderophore production by C7(β)hm723 observed here, but the high-iron and low-iron growth conditions were not identical in these two studies. The nitrosoguanidine-induced mutation(s) in C7(β)hm723 has not been characterized, and C7(β)hm723 is also slightly defective in its rate of iron uptake compared with wild-type C7(β) (5).

Our present studies demonstrated that the introduction of the cloned *dtxR* gene into *C. diphtheriae* C7(β)hm723 restored the repressibility of toxin and siderophore production by iron, although toxin repression was less than in wild-type C7(β). These results strongly suggest that strain C7(β)hm723 is defective in *dtxR* activity. The failure of the cloned *dtxR* gene on plasmid pCMS29 or pCMS97 to restore iron-dependent regulation to the wild-type level for both toxin and siderophore production could have many possible explanations. These include (i) low expression of the cloned *dtxR* gene; (ii) elements present on the shuttle vector or on flanking sequences that interfere with repressor expression or activity; (iii) additional uncharacterized mutations in C7(β)hm723; (iv) involvement of multiple regulatory systems in control of synthesis of toxin or siderophore, or both; and (v) interactions between the wild-type and mutant *dtxR* gene products that interfere with repressor activity. The last possibility would be consistent with the hypothesis that the functional state of the *dtxR* gene product is a dimer or multimer, analogous to the proposed active form of the Fur protein in *E. coli* (1). Additional studies will be required to distinguish among these and other possibilities.

Our present studies also suggested that C7(β)hm723 may not be completely defective in *dtxR* activity, because slight repression of siderophore production was noted during growth under high-iron conditions. If this slight iron-dependent repression of siderophore production is due to residual activity of *dtxR* and not some other regulatory mechanism, it suggests that toxin production is not as tightly regulated by the *dtxR* product and iron as is siderophore production. The observation that plasmid pCMS97 or pCMS29 in C7(β)hm723 caused more stringent repression of siderophore production than of toxin production is consistent with that hypothesis. Tai et al. (30) also reported that the concentration of iron needed to repress siderophore production by *C. diphtheriae* C7(β) is less than that needed to repress toxin production.

Transformation of *C. diphtheriae* and *C. ulcerans* by electroporation is faster, more efficient, and technically easier than the protoplast transformation system used previously by other investigators (25, 26), and vectors for use in *C. diphtheriae* are under development in several laboratories. Although restriction-modification barriers among corynebacteria are still a problem, the progress that has been made recently in genetic analysis of *C. diphtheriae* clearly demonstrates that toxinogenesis and virulence in this well-studied pathogen can be analyzed by the techniques of molecular biology.

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