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Characterization of a Defective Diphtheria Toxin Repressor (*dtxR*) Allele and Analysis of *dtxR* Transcription in Wild-Type and Mutant Strains of *Corynebacterium diphtheriae*

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The production of diphtheria toxin and siderophore by the *Corynebacterium diphtheriae* regulatory mutant C7(β)hm723 is resistant to the inhibitory effects of iron, and the mutant strain is defective for function of the regulatory gene *dtxR*. A 2.8-kb *Hin*dIII fragment carrying the C7(β)hm723 *dtxR* allele was cloned and characterized in *Escherichia coli*. The restriction endonuclease maps of the 2.8-kb *Hin*dIII fragment from C7(β)hm723 and the corresponding fragment from wild-type *C. diphtheriae* C7 were identical. RNA dot blot analysis with total RNA isolated from wild-type *C. diphtheriae* C7 and C7(β)hm723 indicated that the *dtxR* gene was transcribed at very low but equivalent levels in both strains and was not regulated by iron. β-Galactosidase synthesis from a *tox-lacZ* translational fusion construct in *E. coli* in high-iron medium was not repressed by the C7(β)hm723 *dtxR* allele, but was strongly repressed by the wild-type *dtxR* gene. The 28- to 29-kDa polypeptide expressed from the mutant *dtxR* allele in *E. coli* had the same electrophoretic mobility as the wild-type *dtxR* gene product in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The nucleotide sequence of the coding region and the 5′ upstream region of the C7(β)hm723 *dtxR* allele was determined and compared with the wild-type nucleotide sequence. The *dtxR* allele from C7(β)hm723 contained a single-base change located 140 nucleotides from the 5′ start of the gene, which resulted in replacement of arginine in the wild-type sequence by histidine in the mutant protein. These data demonstrate that C7(β)hm723 expresses a mutant DtxR repressor protein that is severely defective in repressor activity.

The regulation of diphtheria toxin production by *Corynebacterium diphtheriae* has been of great interest for over 50 years (25, 26). Production of diphtheria toxin by *C. diphtheriae* is regulated by an iron-dependent mechanism, with maximal toxin synthesis occurring in low-iron environments and during the late log or stationary phase of growth (25). The structural gene for diphtheria toxin, *tox*, has been found on the genome of a family of related bacteriophages, which includes the well-characterized phage β (7). Only *C. diphtheriae* strains lysogenized or infected by a bacteriophage which carries the *tox* gene are able to express diphtheria toxin.

A model describing *tox* gene regulation, proposed by Murphy et al. (23, 24), suggests that the phage-encoded *tox* gene is regulated by iron in association with a factor(s) encoded by the chromosome of the bacteria. Support for this model came from the isolation of bacterial (4, 16) and β phage (24, 35) mutants in which toxin production was resistant to the inhibitory effects of iron. Two mutations in β phage, one of which was initially mapped to the 5′ region of the *tox* structural gene (36), were recently shown to be point mutations present in the promoter-operator sequences of the *tox* gene (18). Bacterial mutants obtained by Kanei et al. (16) produced high levels of diphtheria toxin in medium with excess iron. One of these mutant strains, C7(β)hm723, had growth characteristics almost identical to that of the wild-type strain and produced toxin at equivalent levels in high- and low-iron media (16). Subsequent studies revealed that C7(β)hm723 was also moderately defective in iron transport (4). Production of the *C. diphtheriae* siderophore and diphtheria toxin is coordinately regulated by iron, and synthesis of the siderophore and toxin by the regulatory mutant C7(β)hm723 remains derepressed in high-iron medium (34). Mutants similar in phenotype to C7(β)hm723 were also isolated from the toxin-overproducing strain PW-8 (15). From these findings, C7(β)hm723 and similar *C. diphtheriae* regulatory mutants were presumed to be defective in the chromosomal gene *dtxR*, which encodes the diphtheria toxin repressor (34).

Recently, the *dtxR* gene from *C. diphtheriae* C7 was cloned and characterized by Boyd et al. (2) and by Schmitt and Holmes (30). The nucleotide sequence of *dtxR* predicted a low level of homology at the amino acid level between the diphtheria toxin repressor and the *E. coli* Fur protein (2). The product of the *fur* gene regulates numerous genes in *E. coli* (including toxins [3] and siderophore synthesis and transport genes [1]) by an iron-dependent mechanism similar to that proposed for the *dtxR* gene in *C. diphtheriae*. Studies done with *E. coli* revealed that the cloned *dtxR* gene can regulate, by an iron-dependent mechanism, expression of β-galactosidase from *tox-lacZ* constructs, in which *lacZ* expression is under control of the *tox* promoter-operator sequences (2, 30). Furthermore, introducing the cloned *dtxR* gene, present on a *C. diphtheriae-E. coli* shuttle vector, into *C. diphtheriae* C7(β)hm723 results in iron-dependent repression of both siderophore and toxin (30).

Since C7(β)hm723 is the only regulatory mutant of *C. diphtheriae* which has been studied extensively, we characterized at the molecular level the regulatory defect in C7(β)hm723. In this study, the C7(β)hm723 *dtxR* allele was cloned and tested for repressor activity and transcription of the mutant and wild-type *dtxR* alleles was analyzed and compared with transcription of the gene for diphtheria toxin

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference or source
pCM2.6	Cm^r *E. coli-C. diphtheriae* shuttle vector	30
pTXZ184	Derivative of pACYC184 carrying *tox-lacZ* translational fusion	30
pCMZ100	*tox-lacZ* fusion from pTXZ184 in vector pCM2.6	This study
pMS430	2.8-kb *Hin*dIII fragment carrying C7(β)hm723 *dtxR* allele in pBluescript KS	This study
pMS431	1.5-kb *Pvu*II subclone of pMS430 carrying *dtxR* allele under T7 promoter control in pBluescript KS	This study
pMS432	1.5-kb *Pvu*II subclone of pMS430 in pBluescript KS with *dtxR* allele not under T7 promoter control	This study
pMS298	1.5-kb *Pvu*II fragment carrying *dtxR*$^+$ allele under T7 promoter control in pBluescript KS vector	30
pDSK519	Cloning and expression vector derived from RSF1010	17
pDSK29	5-kb *Sau*3AI fragment carrying *dtxR*$^+$ allele in pDSK519	This study
pGP1-2	pACYC184 derivative carrying temperature-inducible T7 RNA polymerase	32
pWF2B	pBR322 derivative carrying F2 fragment of *tox* gene	33
pBluescript KS	T7 promoter expression and cloning vector	Stratagene

(*tox*). The nucleotide sequence of the mutant gene was also determined and compared with the wild-type nucleotide sequence. This study provides definitive evidence that the *C. diphtheriae* regulatory mutant C7(β)hm723 contains a *dtxR* allele that encodes a functionally defective repressor protein.

MATERIALS AND METHODS

Bacterial strains and plasmids. *C. diphtheriae* C7 and C7(β) were from our laboratory and were originally obtained from L. Barksdale (14). *C. diphtheriae* C7(β)hm723 was obtained from T. Uchida (16). *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used for all experiments in which an *E. coli* strain was used. Stock cultures were stored at −70°C in 20% glycerol. Plasmids used are listed in Table 1.

Reagents, media, and growth conditions. *E. coli* DH5α was routinely grown on Luria broth (LB) medium (21). The following supplements were added as needed: ampicillin (50 μg/ml), chloramphenicol (34 μg/ml), kanamycin (50 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml). To ensure that iron-rich conditions were established, 20 μM $FeCl_3$ was added to LB medium in experiments in which high-iron LB medium was used. LB medium was made low iron by the addition of ethelenediamine-di-*o*-hydroxyphenylacetic acid (EDDA) at 250 μg/ml. *C. diphtheriae* strains were routinely grown in heart infusion broth (HIB) (Difco, Detroit, Mich.) containing 0.2% Tween 80. When conditions required iron-rich HIB medium, 20 μM $FeCl_3$ was added. HIB medium was made low iron by the addition of 100 μg of EDDA per ml. Concentrations of EDDA higher than 100 μg/ml caused reduced growth of *C. diphtheriae* strains. EDDA was deferrated by the method of Rogers (27) prior to use as an iron chelator.

Cloning, DNA preparations, and DNA sequencing. Chromosomal DNA was isolated from C7(β)hm723 as described previously (29). Restriction enzymes and other DNA-modifying enzymes were purchased from Bethesda Research Laboratories. Colony blot and DNA hybridization techniques were performed by established procedures (20). Routine plasmid isolation from *E. coli* was done by the method of Holmes and Quigley (13). Plasmids were transformed into *E. coli* by the method of Hanahan (9) and into *C. diphtheriae* strains as described previously (12, 30). DNA sequence analysis was done by subcloning appropriate DNA fragments into the M13 vectors mp18 and mp19. DNA sequencing was performed by the chain termination method of Sanger et al. (28) with the Sequenase system (U.S. Biochemical Corp.), which was supplied as a kit.

Assay of β-galactosidase. *E. coli* and *C. diphtheriae* strains were grown overnight at 37°C with rotary shaking in high- and low-iron LB or HIB medium, respectively. The assay for β-galactosidase activity was done by the method of Miller (21). The procedure was slightly modified for *C. diphtheriae* strains as follows: prior to disruption of cells with 0.1% sodium dodecyl sulfate (SDS) and $CHCl_3$, cells were treated with 5 μg of lysozyme per ml for 1 h at 37°C.

RNA analysis. Total RNA was isolated from *C. diphtheriae* strains as described previously (19). RNase-free DNase I was added to each sample and allowed to incubate at 37°C for 2 h. The DNase I enzyme was removed by phenol extraction, and the RNA was precipitated with ethanol. Northern analysis of the RNA by dot blot hybridization was performed as described previously (20). To quantitate the amount of RNA hybridized to the DNA probes, a known concentration of the probe DNA fragment was placed onto the filters adjacent to the RNA samples. RNA concentrations were determined relative to the amount of hybridization detected with the control probe DNA fixed on the filter, with the assumption that hybridization of the DNA probe to homologous DNA or RNA sequences is equal.

The DNA probe for the *dtxR* gene was a 400-bp *Hae*III fragment which lies within the coding region of the gene (Fig. 1). The *tox* gene probe was a 1.4-kb *Hin*dIII fragment, excised from plasmid pWF2B (33), which carries approximately 1.2 kb of the *tox* coding region and 130 bp 5′ to the *tox* gene.

Expression of the *dtxR* gene products. The wild-type C7 and the C7(β)hm723 *dtxR* alleles were placed under transcriptional control of the phage T7 promoter present on the pBluescript KS vector. The gene products were preferentially expressed, pulse-labeled with [^{35}S]methionine, and then separated by SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (30).

RESULTS

Restriction mapping and cloning of the C7(β)hm723 *dtxR* allele. Chromosomal DNAs extracted from C7(β)hm723 and from the wild-type *C. diphtheriae* C7 were digested with *Bam*HI, *Hin*dIII, or *Pvu*II and probed with a 1.5-kb *Pvu*II DNA fragment carrying the cloned *dtxR* gene from C7 (Fig. 1). The lengths of the DNA fragments from C7 and C7(β)hm723 which hybridized to the probe were similar in each of the digests, demonstrating that no large deletion or

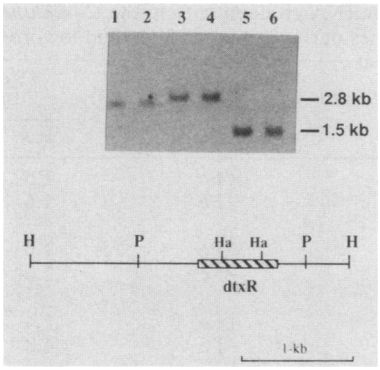

FIG. 1. Southern analysis of chromosomal DNA extracted from *C. diphtheriae* C7 (lanes 1, 3, and 5) and C7(β)hm723 (lanes 2, 4, and 6). DNA was digested with either *Bam*HI (lanes 1 and 2), *Hin*dIII (lanes 3 and 4), or *Pvu*II (lanes 5 and 6) and probed with a 1.5-kb *Pvu*II fragment which carries the C7 *dtxR*$^+$ allele. Shown below the gel is an endonuclease restriction map of the C7 *dtxR* gene and flanking sequences. Restriction sites: H, *Hin*dIII; P, *Pvu*II; Ha, *Hae*III (Only *Hae*III sites within the *dtxR* gene are shown). The 400-bp *Hae*III fragment within the *dtxR* gene was used as the probe for the RNA analysis experiments. Two *Bam*HI sites are located within the 2.8-kb *Hin*dIII fragment 26 and 497 bp from the left end. A *Bam*HI site is also located outside the 2.8-kb *Hin*dIII fragment approximately 300 bp from the right end.

rearrangement had occurred within or immediately adjacent to the *dtxR* gene in C7(β)hm723.

The 2.8-kb *Hin*dIII fragment from C7(β)hm723, identified in Fig. 1, was cloned into the pBluescript KS plasmid in *E. coli* DH5α. Chromosomal DNA from C7(β)hm723 was digested to completion with *Hin*dIII, and fragments were separated by electrophoresis through a 0.9% agarose gel. DNA fragments of 2.0 to 3.5 kb were excised, purified, and then ligated to a *Hin*dIII-cut pBluescript KS vector. The ligation mix was transformed into DH5α, and colonies containing recombinant plasmids were subjected to colony hybridization with the 1.5-kb *Pvu*II fragment carrying the *dtxR* gene to identify positive clones. One clone (pMS430) which hybridized to the *dtxR* probe was shown to carry a 2.8-kb *Hin*dIII chromosomal insert from C7(β)hm723 with a restriction endonuclease profile that was indistinguishable from the 2.8-kb *Hin*dIII fragment of wild-type C7 that encodes *dtxR* (Fig. 1) (30). The internal 1.5-kb *Pvu*II fragment that encodes the C7(β)hm723 *dtxR* allele was subcloned into pBluescript KS to construct plasmids pMS431 and pMS432, which have the *Pvu*II inserts in opposite orientations. Plasmid pMS431, which can express *dtxR* under control of the T7 promoter, was used in all subsequent analyses of the C7(β)hm723 *dtxR* allele.

Analysis of repressor activity of the C7(β)hm723 *dtxR* allele. Plasmid pCMZ100 carries a *tox-lacZ* translational fusion construct in which expression of *lacZ* is under the control of the *tox* promoter-operator sequences (Fig. 2). Additionally, plasmid pCMZ100 is derived from the *E. coli-C. diphtheriae* shuttle vector, pCM2.6 (30); it can be introduced into *E. coli* or *C. diphtheriae* so that *lacZ* expression from the *tox-lacZ* construct can be analyzed in both bacterial species.

To determine whether the *dtxR* allele from C7(β)hm723 encodes an active repressor, like that previously identified for the wild-type *dtxR*$^+$ allele (2, 30), *E. coli* DH5α carrying plasmid pCMZ100 was transformed with either pMS431 (*dtxR*) or plasmid pMS298 (*dtxR*$^+$). DH5α carrying the various plasmid constructs was initially streaked onto high- and low-iron LB agar media containing X-Gal, and the color of the colonies was observed to determine whether β-galactosidase synthesis was regulated by the concentration of iron in the medium. The presence of blue colonies indicated expression of the *tox* promoter, whereas white colonies indicated repression. DH5α carrying only pCMZ100 produced blue colonies regardless of the iron concentration, indicating that expression from the *tox* promoter in *E. coli* was not regulated by iron. When plasmid pMS298 (*dtxR*$^+$) was present in addition to pCMZ100, *lacZ* expression was iron regulated, as indicated by blue colonies (derepressed) under low-iron conditions and white colonies (repressed) under high-iron conditions. When pMS431 or pMS430 (*dtxR*) was present instead of pMS298, colonies were blue on both high- and low-iron media, indicating that the *tox* promoter was not repressed.

To quantitate β-galactosidase activity in these *E. coli* isolates, we performed assays by using LB medium under high- and low-iron conditions (Table 2). The level of β-galactosidase expressed from the *tox-lacZ* construct was also measured in *C. diphtheriae* C7 and C7(β)hm723. The results indicate that the repression of *lacZ* expression under high-iron conditions by plasmid pMS431 (*dtxR*) is very slight (approximately 4-fold repression compared with the pBluescript KS vector control), whereas the repression of *lacZ* by plasmid pMS298 (*dtxR*$^+$) is very strong (>140-fold). These results indicate that the activity of the mutant DtxR repressor in *E. coli* is much lower than that of the wild-type DtxR repressor and that the activity of the mutant repressor was not dramatically affected by the amount of iron available.

Plasmid pMS298 showed significant repression (13-fold) of β-galactosidase expressed from the *tox-lacZ* construct even under low-iron conditions. To determine whether high copy number affected the level of *lacZ* expression by pMS298

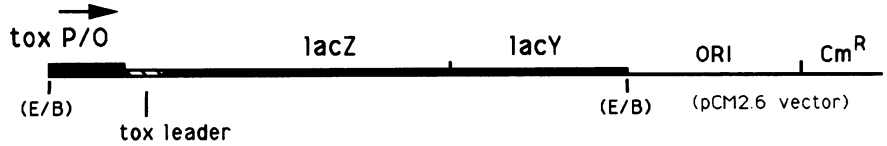

FIG. 2. Plasmid pCMZ100 carries a *tox-lacZ* gene fusion in which expression of *lacZ* is under transcriptional control of the *tox* promoter-operator (P/O) sequences. This plasmid also carries the origin of replication from the *C. diphtheriae* plasmid pNG2 (31), which enables pCMZ100 to replicate in both *E. coli* and *C. diphtheriae* strains. The pNG2 origin of replication (ORI) and the Cm^r determinant on pCMZ100 are derived from the shuttle vector pCM2.6 (30). Plasmid pCMZ100 was constructed by excising a 6.5-kb *Eco*RI DNA fragment, which carries the *tox-lacZ* gene fusion, from plasmid pTXZ184 and ligating it into the unique *Bam*HI site in the shuttle vector pCM2.6. Both the 6.5-kb fragment and the *Bam*HI-cut pCM2.6 vector were made blunt by treatment with Klenow fragment prior to ligation, and the sites designated (E/B) in pCMZ100 are no longer susceptible to *Eco*RI or *Bam*HI. The construction of the *tox-lacZ* gene fusion in pTXZ184 and of the shuttle vector pCM2.6 was described in an earlier report (30).

TABLE 2. β-Galactosidase activity in *E. coli* and *C. diphtheriae* strains grown under high- and low-iron conditions

Strain (plasmid)	β-Galactosidase activity[a] in:	
	−Fe	+Fe
E. coli		
DH5α	<0.1	<0.1
DH5α(pCMZ100)(pBluescript KS)	13.1	14.7
DH5α(pCMZ100)(pMS431)	5.2	3.4
DH5α(pCMZ100)(pMS298)	1.0	<0.1
DH5α(pCMZ100)(pDSK29)	4.6	<0.1
C. diphtheriae		
C7(pCM2.6)	<0.1	<0.1
C7(pCMZ100)	3.8	<0.1
C7(β)hm723(pCM2.6)	<0.1	<0.1
C7(β)hm723(pCMZ100)	3.5	3.7

[a] Units of β-galactosidase were determined by the method of Miller (21). Values are an average of three independent experiments, and the results of each separate experiment did not vary by greater than 25% from the average.

TABLE 3. mRNA production from the *C. diphtheriae dtxR* and *tox* genes during growth in high- and low-iron media[a]

Strain	Iron conditions	RNA hybridized to DNA probes[b]:	
		dtxR	*tox*
C7	+Fe	1.5	<0.1
C7	−Fe	1.6	<0.1
C7(β)	+Fe	1.0	2.8
C7(β)	−Fe	1.6	73.0
C7(β)hm723	+Fe	1.4	60.0
C7(β)hm723	−Fe	1.8	96.5

[a] Strains were grown as described for the β-galactosidase assay in Materials and Methods.

[b] Values represent picograms per microgram of total RNA on filter which hybridized to DNA probe. Values are an average of three independent experiments, and the results of each separate experiment did not vary by greater than 25% from the average.

under low-iron conditions, we placed the *dtxR*$^+$ gene onto a lower-copy plasmid. A 5-kb *Sau*3AI fragment which carries the C7 *dtxR* gene (30) was ligated into the RSF1010-derived plasmid pDSK519 (17), generating plasmid pDSK29. The lower-copy pDSK29 plasmid was transformed into DH5α (pCMZ100) and tested for repressor activity. The level of β-galactosidase observed with pDSK29 in low-iron medium was threefold lower than that found with the pBluescript KS control but four- to fivefold higher than that found with pMS298 in low-iron medium, suggesting that the higher copy number expected for pMS298 was probably responsible for the higher level of repression seen with pMS298.

Expression of *lacZ* from plasmid pCMZ100 in *C. diphtheriae* C7 and C7(β)hm723 appeared to mimic the results obtained with the cloned *dtxR* genes in *E. coli* DH5α (pCMZ100) (Table 2). In the wild-type C7 strain, *lacZ* expression was strongly repressed under high-iron conditions, whereas in C7(β)hm723, expression remained fully derepressed in high-iron medium.

The relatively low levels of β-galactosidase that we observed in both *E. coli* and *C. diphtheriae* are reproducible and appear to be characteristic for the protein encoded by pCMZ100. We believe that the *lacZ* expression from this recorder gene construct faithfully reflects the expression occurring at the *tox* promoter-operator sequences, however, because the iron-dependent expression of *lacZ* observed in the wild-type and mutant *C. diphtheriae* strains is consistent with previously reported regulation of toxin synthesis in these strains (16, 30, 34).

RNA analysis. To determine whether expression of the *dtxR* gene in C7(β)hm723 and in C7 is iron regulated, we measured mRNA levels for *dtxR* (Table 3). mRNA from the *tox* gene was measured simultaneously with the same preparation of RNA and served as a control for iron regulation. Whole-cell RNA was isolated from the *C. diphtheriae* strains after growth in high- and low-iron HIB media; dot blot hybridization was performed; and the amount of RNA hybridizing to specific DNA probes was quantitated as described in Materials and Methods. The RNA data indicated that *dtxR* is expressed at low but approximately equal levels in both C7 and C7(β)hm723 and that the expression was not dramatically affected by iron. Consistent with the conclusions of previous studies (22), transcription of the *tox* gene was strongly iron regulated in C7(β), but occurred constitutively in C7(β)hm723.

Expression of the C7(β)hm723 *dtxR* gene product. Because the *C. diphtheriae dtxR* gene was transcribed normally in C7(β)hm723, we tested directly for the product of the mutant *dtxR* allele. The cloned C7(β)hm723 *dtxR* gene on plasmid pMS431 is under transcriptional control of the bacteriophage T7 promoter present on pBluescript KS. When the thermally inducible T7 RNA polymerase gene present on plasmid pGP1-2 (32) is activated, the cloned C7(β)hm723 *dtxR* gene is strongly transcribed. The wild-type *dtxR* gene can be similarly expressed from plasmid pMS298 (30). The *dtxR* gene products were pulse-labeled with ^{35}S and separated by SDS-PAGE as described in Materials and Methods (Fig. 3). The C7(β)hm723 gene product (lane 2) migrated with the same electrophoretic mobility (28 to 29 kDa) as that of the wild-type gene product (lane 3). When the 1.5-kb *Pvu*II insert from plasmid pMS431 was placed in pBluescript in the opposite orientation (lane 1), this labeled protein was not

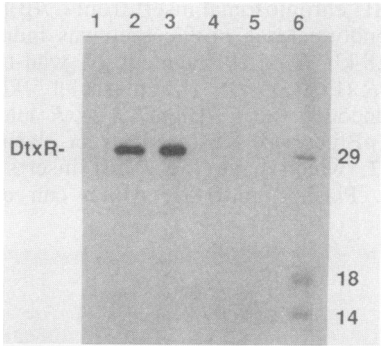

FIG. 3. Identification of [^{35}S]methionine-labeled *dtxR* gene products from *C. diphtheriae* C7 and C7(β)hm723. The *dtxR* genes were transcribed in vivo by using the T7 expression system (32), and the products were separated by SDS-PAGE as described previously (30). All labelings were done in DH5α harboring plasmid pGP1-2 (carries thermally inducible T7 RNA polymerase), unless otherwise indicated. Recombinant plasmids examined: lane 1, pMS432 (*dtxR* allele unexpressed orientation); lane 2, pMS431 (*dtxR* allele expressed orientation); lane 3, pMS298 (*dtxR*$^+$ allele expressed orientation); lane 4, pBluescript KS; lane 5, no plasmids present; lane 6, ^{14}C-labeled protein standards in kilodaltons.

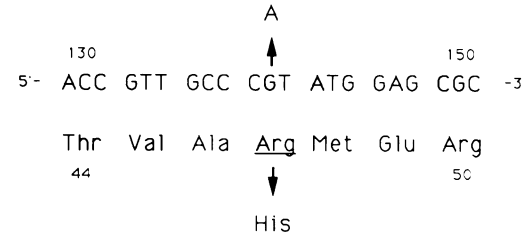

FIG. 4. Segment of the nucleotide sequence and predicted amino acid sequence of the C7 *dtxR* gene (2) where a base difference with C7(β)hm723 was found. The G residue in the wild-type sequence, which lies 140 nucleotides from the 5′ start of the gene coding region, was changed to an A residue in the C7(β)hm723 *dtxR* allele (indicated by arrow). This base substitution resulted in a change from an arginine in the wild-type amino acid sequence to a histidine in the mutant protein.

detected. Additionally, no such protein was encoded by pBluescript vector (lane 4). Expression of the *dtxR* gene product was dependent on the presence of the pGP1-2 plasmid (lane 5), confirming that genes contained within the insert are expressed preferentially under control of the T7 promoter.

DNA sequence analysis of the C7(β)hm723 *dtxR* allele. To determine whether the *dtxR* allele from C7(β)hm723 carried a mutation, we determined the entire nucleotide sequence of the coding region and approximately 175 bp of the 5′ upstream sequence. A comparison between the C7(β)hm723 *dtxR* nucleotide sequence and the published wild-type sequence (2) revealed a single-base difference 140 nucleotides from the 5′ end of the gene, which changed a G residue in the wild-type sequence to an A in the mutant sequence (Fig. 4). This base substitution resulted in a change from an arginine residue in the predicted amino acid sequence of the wild-type DtxR protein to a histidine in the mutant protein. No other differences were noted in the nucleotide sequence between the mutant and wild-type genes throughout the entire coding region or in the putative promoter sequences. We independently sequenced the wild-type *dtxR* gene over the region where the base change was found and confirmed the reported sequence of the $dtxR^+$ allele (2). Taken together, our results establish that the *dtxR* allele in C7(β) hm723 carries a missense mutation, which results in the production of a mutant diphtheria toxin repressor that lacks repressor activity but is indistinguishable in size from the wild-type repressor protein.

DISCUSSION

The nitrosoguanidine-induced mutation in C7(β)hm723 results in a phenotype in which high levels of diphtheria toxin are produced during growth in medium containing excess iron (8, 16). Although the mutant strain has growth characteristics in both high- and low-iron media that are very similar to those of the parental strain, the iron uptake system of C7(β)hm723 is two- to threefold less active than that of wild-type C7 (4). The reason for the altered iron transport activity of C7(β)hm723 has not been determined, although it was suggested that either the mutation that affects the regulation of diphtheria toxin also causes the abnormal iron uptake phenotype or there is more than one mutation in the strain. The *fur* gene from *E. coli* has been shown to directly regulate many genes in *E. coli* by an iron-dependent mechanism similar to that proposed for the *C. diphtheriae dtxR* gene (1). Mutations in the *E. coli fur* gene do not appear to interfere with the ability of this organism to transport iron or ferric-siderophore complexes (10).

The predominant phenotype associated with *E. coli fur* mutants is the constitutive production of all iron-regulated gene products (1, 10). However, studies by Hantke (11) have also shown that *E. coli fur* mutants are impaired in their ability to utilize certain carbon sources. Additionally, the *fur* gene in *E. coli* was shown to be weakly autoregulated and also regulated by catabolite repression (5). These observations suggested a link between the metabolic status of the cell and the Fur protein (and also possibly the expression of iron-regulated genes). Although the defect in the *C. diphtheriae* C7(β)hm723 strain also results in the constitutive expression of two iron-regulated products in this species, diphtheria toxin and siderophore, the possible association between metabolic status or utilization of carbon sources and the *dtxR* gene in *C. diphtheriae* has not yet been examined. The transcriptional studies in this report indicate that expression of the *dtxR* gene is not regulated by the iron concentration in the medium, which is in contrast to findings with other iron-dependent regulatory genes (including *fur* in *E. coli* [5] and *regA* in *Pseudomonas aeruginosa* [6]). The RNA studies also indicate that the *dtxR* gene in both C7 and C7(β)hm723 is transcribed very weakly, suggesting that the DtxR protein may be required at only low levels within the cell.

Recent reports from our laboratory showed that synthesis of the *C. diphtheriae* siderophore, corynebactin, is coordinately regulated with diphtheria toxin production by an iron-dependent mechanism (34). Unlike toxin synthesis, siderophore production was not fully derepressed in high-iron medium in C7(β)hm723 (30). This observation suggested that siderophore regulation might involve additional factors or that the *dtxR* mutation in C7(β)hm723 might be leaky, such that DtxR had no detectable activity at the *tox* operator, but maintained partial repressor activity at the operator(s) for the siderophore gene(s), or both. This study provided additional evidence that the diphtheria toxin repressor from C7(β)hm723 has very slight repressor activity (Table 2), since the mutant *dtxR* allele on the high-copy pBluescript KS plasmid pMS431 was able to repress expression of *lacZ* from the *tox-lacZ* construct three- to fourfold relative to the vector alone.

The DNA sequence of the *dtxR* allele from C7(β)hm723 revealed a single-base difference from the wild-type sequence, which results in a change from an arginine in the deduced wild-type amino acid sequence to a histidine in the mutant protein. By analogy to the Fur protein in *E. coli*, three primary activities could be associated with an iron-dependent repressor protein such as DtxR: (i) ability to bind DNA and inhibit transcription from specific promoters, (ii) ability to bind iron, and (iii) ability to form dimers or multimers (1). The DtxR protein produced by C7(β)hm723 may be altered in one or any combination of the above properties. Although the mutant *dtxR* gene produces a polypeptide with an electrophoretic mobility identical to that of the wild-type DtxR polypeptide in SDS-PAGE, this comparison provides no information concerning the secondary, tertiary, or quaternary structure of the mutant and wild-type repressor proteins or their biochemical functions. Studies with the *E. coli* Fur protein have not yet identified specific amino acids associated with active sites for any of these functions. The specific effects of the mutation in the *dtxR* allele in C7(β)hm723 on the mutant DtxR protein remain to be determined.

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