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Molecular Cloning, DNA Sequence Analysis, and Characterization of the *Corynebacterium diphtheriae dtxR* Homolog from *Brevibacterium lactofermentum*

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A homolog of the *Corynebacterium diphtheriae dtxR* gene was isolated from *Brevibacterium lactofermentum*. The product of the *B. lactofermentum dtxR* gene was immunoreactive with polyclonal anti-DtxR antibodies and functioned as an iron-activated repressor capable of regulating the expression of β-galactosidase from a diphtheria *tox* promoter/operator transcriptional fusion in recombinant *Escherichia coli*. The extents of induction by increasing concentrations of the chelator 2,2′-dipyridyl were identical in cells expressing DtxR from either *C. diphtheriae* or *B. lactofermentum*.

Boyd et al. (1) have described the molecular cloning of the gene for a 25,316-molecular-weight iron-dependent regulatory element, DtxR (diphtheria toxin regulatory protein), from genomic libraries of nontoxigenic, nonlysogenic *Corynebacterium diphtheriae*. In recombinant *Escherichia coli*, DtxR was shown to repress the expression of β-galactosidase from a *toxPO-lacZ* transcriptional fusion in an iron-dependent fashion. Schmitt and Holmes (7) subsequently demonstrated that *dtxR* is able to restore the iron-mediated regulation of diphtheria *tox* and siderophore gene expression in the iron-insensitive mutant C7hm723(βtox+) strain of *C. diphtheriae.*

The *dtxR* alleles from the PW8(−), 1030(−), and C7hm723(−) strains of *C. diphtheriae* have been cloned and sequenced (2, 8). While the *dtxR* allele from PW8(−) was found to be identical to that from C7(−), the *dtxR* allele from 1030(−) was found to carry six silent amino acid substitutions in the C-terminal region of the protein. On the basis of the analysis of a series of DtxR mutants (15), it is likely that the DNA-binding and metal ion activation domains of DtxR are positioned in the N-terminal 106-amino-acid region of DtxR.

Cloning of *dtxR* from *Brevibacterium lactofermentum*. In the present report, we describe the molecular cloning and characterization of a *dtxR* homolog from *B. lactofermentum*. We show that the DtxR-like protein from *B. lactofermentum* is homologous to DtxR from *C. diphtheriae*, and that the DtxR-like protein is an iron-dependent regulatory element that is able to control the expression of β-galactosidase from a diphtheria *tox* promoter/operator transcriptional fusion in recombinant *E. coli*.

During a study in which sigma factors from *B. lactofermentum* were being cloned and sequenced (6), a 786-bp open reading frame (ORF) was identified adjacent to a gene encoding a sigma factor. This ORF encodes a 228-amino-acid protein with a deduced molecular mass of 25.4 kDa. A search of the EMBL-GenBank-DDBJ databases revealed that this ORF was similar to *dtxR* from *C. diphtheriae*. In addition, sequences similar to an *E. coli* consensus promoter and a potential ribosome binding site were identified immediately upstream from the ORF (data not shown). The sequence of this putative promoter also corresponds to those of the CEP (corynebacterial *E. coli*-like) type promoters (4, 5).

Comparison of the amino acid sequences of DtxR from *C. diphtheriae* and the putative DtxR-like protein from *B. lactofermentum* revealed that the sequences are 70.8% identical (Fig. 1). Moreover, an additional 23 amino acids (10.2%) in the aligned sequences were found to be similar. Only 15 amino acids (10.5%) in the N-terminal 145-amino-acid portion of the aligned sequence were found to be dissimilar, whereas 29 (34.9%) of the C-terminal 83 amino acids were dissimilar. Since the N-terminal portion of DtxR from *C. diphtheriae* has been shown to carry the DNA- and metal ion-binding domains (14, 15), we investigated the possibility that the DtxR-related protein from *B. lactofermentum* could function as an iron-activated regulatory element.

Plasmid pULJSX4, which carries the *B. lactofermentum dtxR* gene, was introduced by transformation into *E. coli* DH5α: λRS45*toxPO-lacZ* (1). In this strain, expression of β-galactosidase is under the control of the native diphtheria *tox* promoter/operator and has been shown previously to be regulated by DtxR from *C. diphtheriae* in an iron-dependent fashion (1). Transformants were selected on Luria-Bertani medium supplemented with ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and clones which carried pULJSX4 were found to retain a white phenotype on this medium.

Detection of the DtxR-like protein in *B. lactofermentum* and recombinant *E. coli*. Immunoblot analysis following sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation of crude extracts of *E. coli* DH5α(pULJSX4) and *B. lactofermentum* showed nearly identical protein bands that were immunoreactive with polyclonal anti-DtxR antisera (Fig. 2). The electrophoretic mobility (M_r, 27,000) of the DtxR-like protein from *B. lactofermentum* was found to be slightly greater than that of DtxR purified from *C. diphtheriae* (11). The three *B. lactofermentum* DtxR-like proteins detected in crude extracts of recombinant *E. coli* were found to have M_rs of 27,000, 26,500, and 18,000. The appearance of smaller immunoreactive species suggests that the *B. lactofermentum* 27,000-M_r DtxR-like protein may be partially degraded in *E. coli*.

Iron-dependent repression of a diphtheria *tox* promoter/

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Cd-DtxR    MKDLVDTTEMYLRTIYELEEEGVTPLRARIAERLEQSGPTVSQTVARMER    50
           .|||||||||||||||||||||. ||||||||||||||||||||||||||
Bl-DtxR    VKDLVDTTEMYLRTIYELEEEGIVPLRARIAERLEQSGPTVSQTVARMER    50

Cd-DtxR    DGLVVVASDRSLQMTPTGRTLATAVMRKHRLAERLLTDIIGLDINKVHDE   100
           |||| |. |||| ||| ||.|| ||||  ||||||||||||||| |||||
Bl-DtxR    DGLVHVSPDRSLEMTPEGRSLAIAVMRNDRLAERLLTDIIGLDIHKVHDE   100

Cd-DtxR    ACRWEHVMSDEVERRLVKVLKDVSRSPFGNPIPGLDELGVGNSDAAAPGT   150
           ||||||||||||||||| || || ||||||||||| |.|. ..     | 
Bl-DtxR    ACRWEHVMSDEVERRLVEVLDDVHRSPFGNPIPGLGEIGLDQADEPDSGV   150

Cd-DtxR    RVIDAATSMPRKVRIVQINEIFQVETDQFTQLLDADIRVGSEVEIVDRDG   200
           | ||       | ||||.||| ||. .||  | || . .|.||.|.   |
Bl-DtxR    RAIDLPLGENLKARIVQLNEILQVDLEQFQALTDAGVEIGTEVDIINEQG   200

Cd-DtxR    HITLSHNGKDVELLDDLAHTIRIE--EL     226
            . ..|||  |||.|||||..|.|  |
Bl-DtxR    RVVITHNGSSVELIDDLAHAVRVEKVEG     228
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FIG. 1. Comparison of the deduced amino acid sequences of DtxR from *C. diphtheriae* (Cd) and the DtxR-like protein from *B. lactofermentum* (Bl). The amino acid sequences are presented in the one-letter code. Bars indicate identity, and dots indicate the following conservative changes: A-S-T, D-E, N-Q, R-K, I-L-M-V, and F-Y-W.

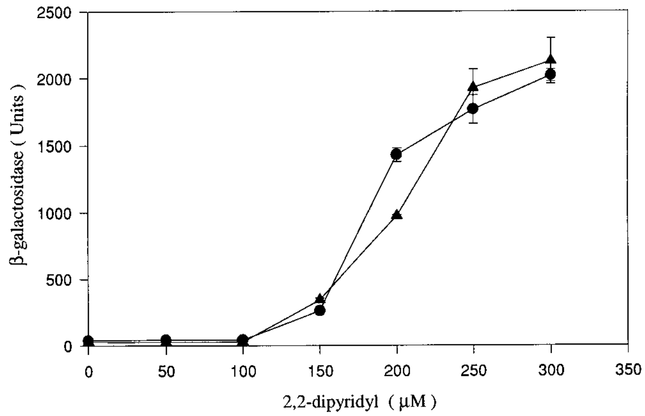

FIG. 3. Expression of β-galactosidase from a diphtheria *toxPO-lacZ* transcriptional fusion in *E. coli* DH5α(pRS551*toxPO*-3XH1) (●) and in *E. coli* DH5α (pRS551*toxPO*-VN1200) (▲) in the absence of 2,2′-dipyridyl and in the presence of increasing concentrations of 2,2′-dipyridyl.

operator-*lacZ* transcriptional fusion by *B. lactofermentum* DtxR. Since the level of β-galactosidase expression in *E. coli* DH5α:λRS45*toxPO-lacZ* is low, we recloned a 1.3-kb *Xho*I-*Hin*dIII fragment of pULJSX4 which encodes the *B. lactofermentum dtxR* gene into multicopy plasmid pRS551*toxPO* (1). The resulting plasmid, pRS551*toxPO*-3XH1, was then introduced into *E. coli* DH5α in order to study the iron-dependent regulation of *lacZ* expression. As a positive control, we used plasmid pRS551*toxPO*-VN1200 (1). As shown by the results in Fig. 3, the DtxR-like protein from *B. lactofermentum* was found to regulate the expression of β-galactosidase in an iron-dependent fashion. In the absence of the chelator 2,2′-dipyridyl, the *lacZ* reporter gene was completely repressed. The addition of dipyridyl to concentrations of above 150 μM resulted in the derepression of *lacZ*. Moreover, in the presence of increasing concentrations of 2,2′-dipyridyl, the derepression of *lacZ* in the presence of the DtxR-like protein from *B. lactofermentum* was found to be essentially identical to that observed for DtxR from *C. diphtheriae* (Fig. 3).

Günter et al. (3) have reported that a repressor binding site in the promoter region of the *desA* gene is responsible for the iron-mediated regulation of gene expression in *Streptomyces pilosus* and *Streptomyces lividans*. This dyad symmetry element was found to be homologous to the native diphtheria *tox* operator, and essentially identical to the minimal essential nucleotide sequence required for DtxR binding (9, 12). The *desA* gene encodes lysine decarboxylase, and is induced by iron deficiency (10). Although a negative controlling element for *desA* has not been identified in *Streptomyces* spp., the remarkable similarity of the regulatory sequence to the consensus DtxR binding site suggests that a DtxR-like regulatory protein(s) may exist in *Streptomyces* spp. Using the polyclonal anti-DtxR antibody, we have observed by immunoblot analysis the presence of a reactive protein with an M_r of 28,000 to 32,000 in crude extracts of *S. lividans* and *Mycobacterium tuberculosis* (data not shown). On the basis of these preliminary observations, we anticipate that a family of DtxR-like regulatory elements may be found in at least some species in the *Corynebacterium*, *Streptomyces*, and *Mycobacterium* genera of gram-positive organisms. Accordingly, these regulatory proteins are likely to share similar DNA-binding domains, transition metal ion activation domains, and DNA binding targets.

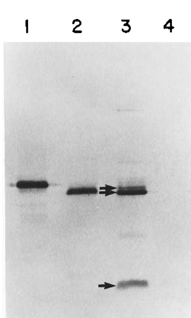

FIG. 2. Immunoblot analysis of crude protein extracts of *B. lactofermentum* and *E. coli* strains. Lanes: 1, *C. diphtheriae* purified DtxR; 2, *B. lactofermentum* BL13869; 3, *E. coli* DH5α(pULJSX4); 4, *E. coli* DH5α(pBluescript II KS+). The arrows indicate different immunoreactive forms present in crude extracts of *E. coli* DH5α(pULJSX4).

Nucleotide sequence accession number. The DNA sequence of the *B. lactofermentum dtxR* gene has been deposited in the EMBL-GenBank-DDBJ nucleotide sequence data libraries under accession number L35906.

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