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Metal Ion Binding of The *Corynebacterium pseudotuberculosis* Diphtheria Toxin Repressor

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ABSTRACT

The acquisition of iron is essential to facilitate growth of bacterial pathogens. Bacteria using high-affinity iron uptake systems, siderophores, to scavenge iron from the host is regulated by the diphtheria toxin repressor (DtxR). Additionally, DtxR is a global regulator of cell metabolism and regulates the expression of diphtheria toxin in *C.* diphtheriae. The genome of *Corynebacterium pseudotuberculosis*, a related animal pathogen with *C.* diphtheriae, contain the genes for diphtheria toxin and DtxR. Our study describe the high scale heterologue expression of *Corynebacterium pseudotuberculosis* DtxR (*Cp*-DtxR) in *E.coli*, purification, and characterization of the Fe^{2+} binding using CD spectroscopy.

Key words: C. *pseudotuberculosis*; Dtxr; Heterologue Gene Expression; Purification; Iron Binding

Abbreviations: *Cp*-DtxR: *C. pseudotuberculosis* diphtheria toxin repressor*; Cd*-DtxR: *C. Diphteriae* diphtheria toxin repressor; CLA: Caseous Lymphadenitis; CD: Circular Dichroism; HTHM: Helix Turn Helix Motif; RMSD: Root Mean Square Deviation; TEV: Tobacco Etch Virus

Introduction

The acquisition of essential nutrients and ions such as iron is of primordial significance for the growth and development of bacterial pathogens [1]. Since the concentration of free iron in human serum is estimated to be 10-12 μM, which is much lower than that required for optimal bacterial growth [2], invasive pathogenic bacteria overcome this problem by using inducible, high-affinity iron uptake systems, siderophores, to scavenge iron from the host [3,4]. In gram-positive and gram-negative bacteria, the diphtheriae toxin repressor (DtxR) plays a key role in the regulation and production of siderophores and genes involved in the uptake of iron and other transition metals [5-10]. Additionally, DtxR regulates the expression of diphtheriae toxin in *C. diphtheriae* [11]. The three-dimensional structure of *C. diphtheriae* DtxR (Cd-DtxR) reveals that it exists as a dimer containing two metal binding sites per monomer [12,13]. Several transition metal ions, such as Fe(II), Ni(II), Co(II), Cd(II), Mn(II) and, to some extent, Zn(II) activate apo-*Cd*-DtxR and, the holo-enzyme binds its target DNA and blocks the transcription of the downstream genes [14-17]. Yellaboina et al. [6] predicted more than 70 DtxR-regulated operons in the *C. diphtheriae* genome and demonstrated that DtxR is a global regulator of cell metabolism.

Our study was performed on DtxR of *Corynebacterium pseudotuberculosis*, a related pathogen of *Corynebacterium diphtheriae*. Analysis of the *C. pseudotuberculosis* genome identified diphtheriae toxin and DtxR genes. However, diphtheriae toxin is not involved in the *C. pseudotuberculosis* infection. We assume that DtxR in *C. pseudotuberculosis* mainly plays a role in divalent ion uptake and in the regulation of metabolism. *C. pseudotuberculosis* causes caseous lymphadenitis (CLA) in equids, sheep, goats, and to a lesser extent in horses and cattle. This disease leads to considerable economic loss in many countries, including Brazil [18,19] and presently, no effective treatment is available to combat this disease. Based on its key role in pathogen cell metabolism, *Cp*-DtxR can be considered as a potential drug target and we present results of the expression, purification and characterization of *Cp*-DtxR and the iron binding by *Cp*-DtxR apo-protein.

Material and Methods

***In Silico* Analysis:** *Cp*-DtxR and *Cd*-DtxR sequences were retrieved from NCBI and a sequence alignment were performed using MUSCLE [20] and Box Shade web servers. The atomic coordinates from *C. diphtheriae* DtxR (PDB code: 1G3S) were used as the template for comparative modeling by the satisfaction of spatial restraints as implemented in the program Modeller 9v13 [21]. The structure of native *Cp*-DtxR (Gene ID: ADL10687.1; Uniprot: D9QAW1) was modeled.

Expression and Purification of DtxR: The open reading frame of *Cp*-DtxR was cloned into the vector pD441-SR by DNA 2.0 (USA). The construct contains an N-terminal TEV cleavage site and hexahistidine affinity tag. The kanamycin-resistant vector pD441-SR presents a T7 promoter inducible by IPTG and a high copy percentage provided by pUC origin of replication. DtxR-pD441-SR (DNA 2.0) vectors were transformed into *E. coli* BL21 (DE3)T1 (Sigma-Aldrich, USA) competent cells, which were grown for 16 h (overnight) at 37°C in LB medium containing sufficient amounts of kanamycin. The bacterial cultures were transferred into fresh LB medium, grown for another 3.0 h at 30°C until the OD600 reached 0.6. Subsequently, *Cp*-DtxR expression was induced by 0.4 mM IPTG, and incubated for 4 h at 30°C, being supplemented with 50 µM $FeCl_2$. Later, the culture was harvested by centrifugation at 4000 g, 5°C for 20 min, discarding the supernatant and re-suspending the *Cp*-DtxR containing cell pellet in 20 mM K_2HPO_4/KH_2PO_4 pH 7.4, 500 mM NaCl, 5% (v/v) glycerol, 2 mM imidazole.

The cell-suspension was incubated on ice for 1 h with lysozyme, subsequently being lysed by sonication in four sets of 30 s pulses of 30% amplitude, with 10 s intervals. This way obtained crude cell extract was centrifuged at 8000 g, 6°C for 90 min. The supernatant containing *Cp*-DtxR was loaded onto Ni-NTA column pre-equilibrated with 20 mM K_2HPO_4/KH_2PO_4 pH 7.4, 500 mM NaCl, 5% (v/v) glycerol, 2 mM imidazole, extensively washed with the same buffer containing 20 and 60 mM imidazole, *Cp*-DtxR eluted with 300 mM imidazole. The eluted fractions were individually pooled and injected onto a Superdex 75 10/300 GL size exclusion column (GE Healthcare), pre-equilibrated with buffer 20 mM K_2HPO_4/KH_2PO_4 pH 7.4, 150 mM NaCl. Sample purity after each purification step was assessed by 15% SDS-PAGE gels. Fractions containing *Cp*-DtxR were concentrated in a micro concentrator (MWCO: 3000Da, GE Healthcare). Protein concentrations were determined spectrophotometrically, applying the Lambert-Beer law [22].

CD Spectroscopy:

For all CD measurements, 15 repeated scans were performed, 5 scans were used to establish the baseline. The wavelength range applied for far-UV spectra was from 200 nm to 260 nm, in a time constant of 1 s and 100 nm/min continuous scanning mode, using a Jasco J-107 spectropolarimeter (Jasco, Japan). Prior to conducting the CD experiments, *Cp*-DtxR was incubated overnight with 1 mM EDTA, to remove irons from the specific binding sites. EDTA and EDTA-ion complexes were eliminated by dialysis against 20 mM K_2HPO_4/KH_2PO_4 pH 7.0. *Cp*-DtxR was diluted to a concentration of 1 µM. The effect of the iron ions was tested on the protein secondary structure. The protein was pre-incubated with 10 µM Fe^{2+} for one hour prior to the measurements. The results are presented in molar ellipticity [θ], according to: in which, θ is the ellipticity measured at a given wavelength λ (deg), c is the protein concentration (mol L^{-1}), l is the cell path length (cm) and n is the number of amino acids. CDpro software package was adopted to analyze the results [23].

Results and Discussion

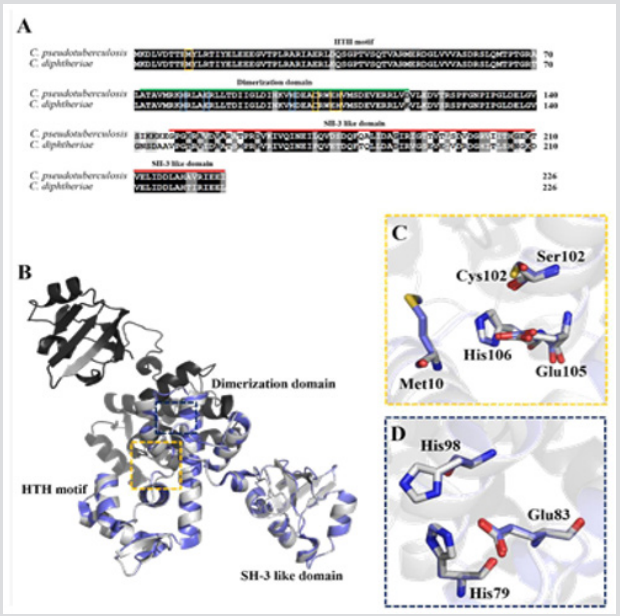

Figure 1: Sequence alignment and structural comparison of *Cp*-DtxR and *Cd*-DtxR.

a. Sequence alignment of *Cp*-DtxR and *Cd*-DtxR. DtxR domains: HTH motif (grey bar), dimerization domain (green bar) and SH-3 like domain (red bar). Yellow boxes mark the residues coordinating metal binding site 1, blue boxes mark the residues coordinating metal binding site 2.

b. Structural comparison of *Cp*-DtxR homology model (in grey) as dimer and *Cd*-DtxR crystal structure (PDB: 1G3S in blue). The domains are shown and the both ion-binding sites in the monomer are highlighted.

c. Amino acids coordinating metal binding site 1.

d. Amino acids coordinating metal binding site 2.

Sequence Alignment and Molecular Model of *Cp*-DtxR: A BLAST search for the *Cp*-DtxR sequence against the non-redundant protein sequences database [24] indicated that the protein sequence is highly conserved within the Corynebacterium genus (75% to 95%) (Figure 1A). A Blast search for the *Cp*-DtxR sequence against the data deposited with the Protein Data Bank showed a sequence identity of 78% with the *Cd*-DtxR protein structure (PDB: 1G3S), which was used to generate a *Cp*-DtxR model. The DtxR proteins are dimers and contain three highly conserved domains important for the protein function, which is characteristic of the protein family [12,13]. The HTH motif is important for DNA binding. The SH3-like domain shows more differences in the protein sequence (Figures 1A & 1B); however, the residues coordinating the metal binding sites 1 and 2 are conserved within the two species (Figures 1A-1D). The structural overlay of the *Cp*-DtxR model and the *Cd*-DtxR crystal structure resulted in an rmsd value of 0.135 (1433 atoms) and indicates the significant conservation of the protein structure within the Corynebacterium family.

Protein production and purification: The *Cp*-DtxR construct consists of 239 amino acids (including TEV cleavage site and hexa-histidine tag) with a molecular weight of 26.877 Da. The protein presented a single band on a denaturing SDS-PAGE gel (Figure 2) with an apparent molecular mass of approximately 30.000 Da, after the two-step purification (Figure 2). The size exclusion chromatography demonstrated that the protein exists in solution as a dimer, which was confirmed with a SEC calibration (supplementary Figure 1).

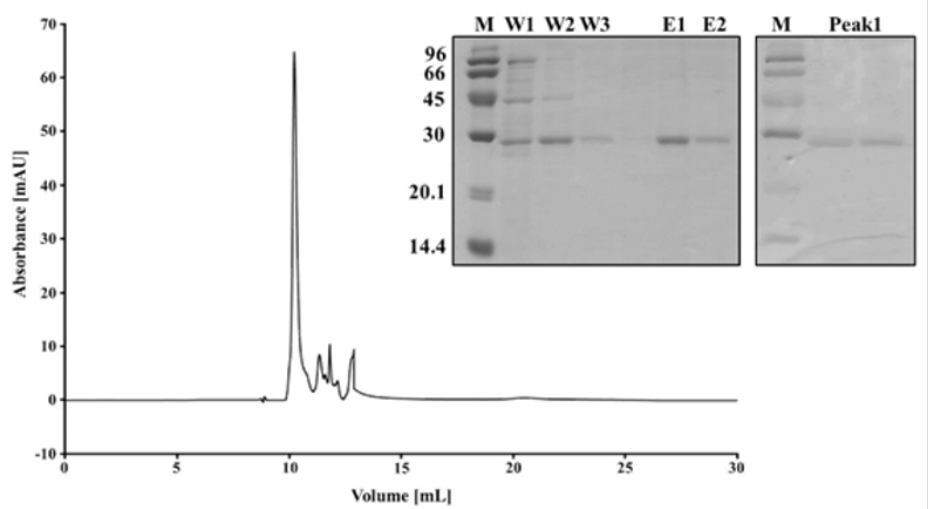

Figure 2: Purification of *Cp*-DtxR. SDS-PAGE analysis of *Cp*-DtxR after Ni-NTA purification. M: protein marker, W_{1-3}: washing steps, E_{1-2}: elution steps.SDS-PAGE of *Cp*-DtxR after size exclusion chromatography, which indicate the purity of the protein. Chromatogram of size exclusion chromatography of *Cp*-DtxR, elute at around 10.5 ml, which corresponds to a dimer of *Cp*-DtxR.

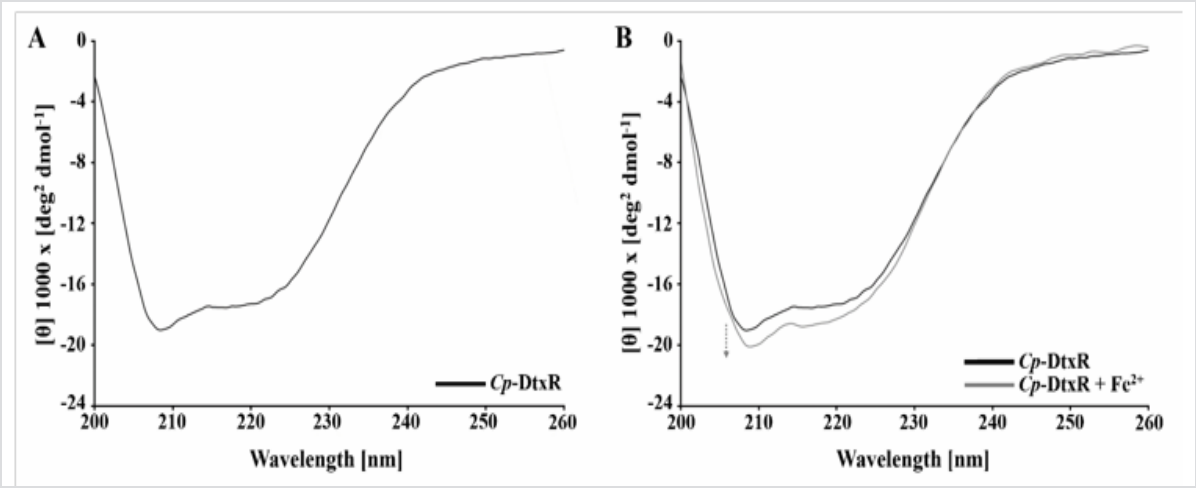

Figure 3: Conformational changes in the *Cp*-DtxR secondary structure studied by CD spectroscopy.
A: CD spectra of *Cp*- DtxR incubated with EDTA. B: Overlay of the CD-spectra from *Cp*-DxtR incubated with EDTA and after treated with Fe^{2+}.

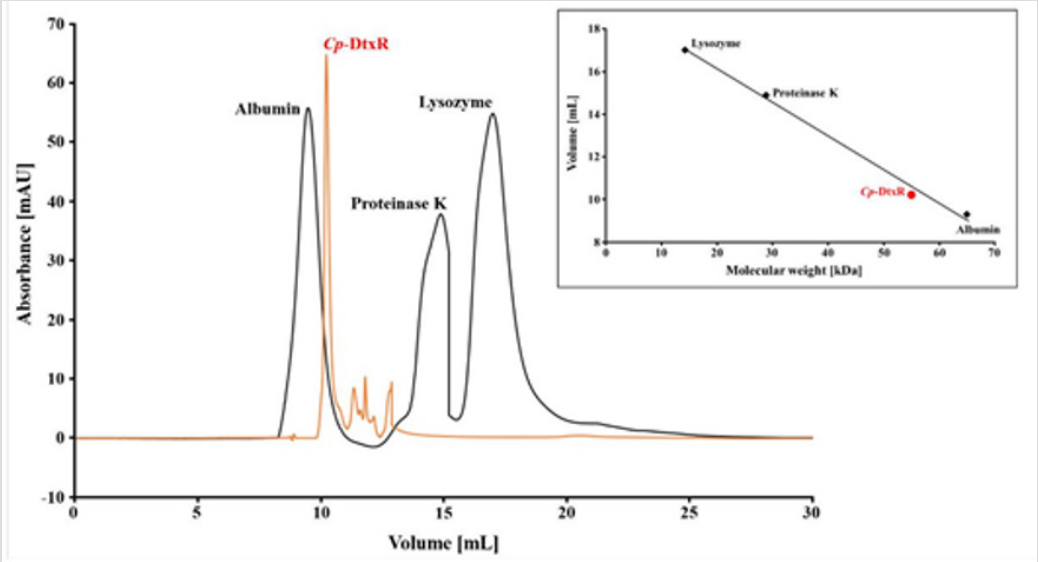

Figure S1: Calibration of the SEC column used for the *Cp*-DtxR purification. The chromatogram show the elution peaks of Albumin (65 kDa), Proteinase K (29.7 kDa) and Lysozyme (14.3 kDa). *Cp*-DtxR elute at around 10.5 ml, the protein monomer has a MW of around 27 kDa, but the protein elute as dimer, near a MW of 60 kDa as demonstrated in the linear elution plot.

CD spectroscopy: Protein conformational changes induced by iron ions were monitored using far-UV CD spectroscopy. Thereby the effect of Fe^{2+} binding to apo-*Cp*-DtxR were investigated (Figure 3). The results of the secondary structure prediction of *Cp*-DtxR by CDpro [23] based on the results of the far-UV CD measurements indicated 34% α-helices, 61% random coiled regions and 5% β-sheet in the protein secondary structure. The results indicated a high α-helical and random coiled content in the secondary of the protein, these results are in agreement with the existing 3D structures of DtxR proteins for example from *C. diphtheriae*(PDB: 1G3S), 43% α-helices and 14% β-sheet. Secondary structural change due to Fe^{2+} binding was observed and analyzed using the CDpro software [23]. The results demonstrated that following Fe^{2+} binding, an increase in the α-helical content (34% to 38%) of the protein secondary structure is observed. Local structural rearrangements following metal ion binding to apo-DtxR have been correlated with helix-to coil transitions [25], which are in close agreement with our CD results.

Conclusion

The diphtheriae toxin repressor from *C. diphtheriae* is an intensively investigated cellular protein. The protein controls diphtheriae toxin production and the production of siderophores and is a global regulator of the cell metabolism. *C. pseudotuberculosis* is a related pathogen of *C. diphtheriae* and contains the diphtheriae toxin and DtxR genes, but diphtheriae toxin is not considered to be directly involved in the infection mechanism of *C. pseudotuberculosis* rather, DtxR seems to play regulatory function in the transition metal uptake and the cell metabolism. The function and importance of *C. pseudotuberculosis* DtxR remains poorly understood and research in this area can contribute to narrow the search for molecular targets against caseous lymphadenitis.Our results describe for the first time a protocol for the heterologous gene expression of *Cp*-DtxR in *E.coli* cells and a subsequent two-step purification process. The production of protein in a high amount and quality are necessary for further investigations of *Cp*-DtxR.

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