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Characterization of the LysR-type transcriptional regulator YcjZ-like from *Xylella fastidiosa* overexpressed in *Escherichia coli*

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ABSTRACT

The *Xylella fastidiosa* 9a5c strain is a xylem-limited phytopathogen that is the causal agent of citrus variegated chlorosis (CVC). This bacterium is able to form a biofilm and occlude the xylem vessels of susceptible plants, which leads to significant agricultural and economic losses. Biofilms are associated with bacterial pathogenicity because they are very resistant to antibiotics and other metal-based chemicals that are used in agriculture. The *X. fastidiosa* YcjZ-like (XfYcjZ-like) protein belongs to the LysR-type transcriptional regulator (LTTR) family and is involved in various cellular functions that range from quorum sensing to bacterial survival. In the present study, we report the cloning, expression and purification of XfYcjZ-like, which was overexpressed in *Escherichia coli*. The secondary folding of the recombinant and purified protein was assessed by circular dichroism, which revealed that XfYcjZ-like contains a typical α/β fold. An initial hydrodynamic characterization showed that XfYcjZ-like is a globular tetramer in solution. In addition, using a polyclonal antibody against XfYcjZ-like, we assessed the expression profile of this protein during the different developmental phases of *X. fastidiosa* in *in vitro* cultivated biofilm cells and demonstrated that XfYcjZ-like is upregulated in planktonic cells in response to a copper shock treatment. Finally, the ability of XfYcjZ-like to interact with its own predicted promoter was confirmed *in vitro*, which is a typical feature of LysR. Taken together, our findings indicated that the XfYcjZ-like protein is involved in both the organization of the architecture and the maturation of the bacterial biofilm and that it is responsive to oxidative stress.

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Introduction

Xylella fastidiosa is a gram-negative, xylem-limited bacterium whose full genome is available [1]. The *X. fastidiosa* 9a5c strain is the causal agent of citrus variegated chlorosis (CVC), which is also known as "*amarelinho*" in Brazil. This bacterium is able to form a biofilm structure inside the xylem vessels of susceptible plants, which results in hydric impairment, a decrease in nutrients and, ultimately, plant death during the later stages of disease. Therefore, this bacterium is associated with great economic losses in São Paulo, which is the highest producer of concentrated orange juice in Brazil [2]. To prevent the *X. fastidiosa* from spreading in the field, copper-based chemicals are used [3], which cause bacterial oxidative stress, among other effects [4–6]; however, living organisms have a large repertoire of responses to stress conditions, including modulation of the gene expression profile and increased production of transcriptional regulators, which allow them to protect themselves and to adapt and survive under these conditions [7].

LysR-type proteins are members of the largest family of transcription regulators (LTTRs)[1] in prokaryotes and are highly conserved in the bacteria kingdom [8,9]. The proteins in this family are involved in numerous cellular functions, including oxidative stress responses [10], cell wall shape [11], quorum sensing [12], efflux pumps, secretion, motility [13], nitrogen fixation [14], virulence [15], cell division [16], metabolism and environmental

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[1] *Abbreviations used:* LTTR, LysR-type transcriptional regulators; XfYcjZ-like, YcjZ of *X. fastidiosa*; HTH, helix-turn-helix; SEC, size-exclusion chromatography; AUC, analytical ultracentrifugation; CD, circular dichroism; EMSA, electrophoresis mobility shift assay.

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recognition [17]. Initially, LTTRs were classified as repressors [18,19]; however, as previously reported, they can also act as activators, depending on the circumstances and the signaling molecules [20]. LTTRs share a high degree of conservation in the helix-turn-helix (HTH) domain at the N-terminus, which is directly involved in DNA binding, whereas the C-terminus, which possesses the regulatory domains (RD) 1 and 2 that are co-inducer binding sites [21–23], exhibits low amino acid conservation.

In the present study, we report the initial functional and hydrodynamic characterization of a LysR-type transcriptional regulator called YcjZ-like from *X. fastidiosa* strain 9a5c (XfYcjZ-like). Recombinant XfYcjZ-like was overexpressed in an *Escherichia coli* host and purified by two-step chromatography. An initial structural analysis confirmed the secondary and tertiary structures of the recombinant and purified protein. We used a polyclonal antibody against XfYcjZ-like to confirm the expression profile of this protein during the biofilm growth of *X. fastidiosa* and demonstrated that XfYcjZ-like was able to respond to a copper shock treatment because it was upregulated in planktonic cells. In addition, the interaction of XfYcjZ-like with its own predicted promoter was analyzed *in vitro*. Our results provide new information regarding the involvement of the XfYcjZ-like transcription regulator in bacterial pathogenicity.

Materials and methods

Bacterial strain, amplification, plasmid and cloning

The coding sequence of XfYcjZ-like (975 pb; NCBI Reference Sequence: WP_010894223.1) was amplified from *X. fastidiosa* 9a5c genomic DNA by PCR using specific primers designed with a *Nde*I restriction site in the forward primer (5′-GGCCATATGGCCAG ACGCAAC-3′) and an *Xho*I site in the reverse primer (5′-CACGCTC GAGTTGGCGATGG-3′). The PCR program used for *xfycjZ*-like amplification included a denaturation step at 94 °C for 120 s, followed by an annealing step at 56 °C for 45 s and a final step at 72 °C for 90 s. This cycle was repeated 30 times. After PCR amplification, the amplicon was inserted into the pET28a vector using standard molecular biology methods [24], which added a His_6-tag to the N-terminus. The recombinant vector that was generated was subjected to DNA sequencing to confirm that the cloned sequence did not contain any base substitutions.

Amino acid sequence alignment and protein structure prediction

The amino acid sequence alignment between XfYcjZ-like and the *E. coli* YcjZ protein (EcYcjZ; GenBank ID EOQ56594.1) was analyzed using the ClustalW2 server (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Structural modeling prediction of XfYcjZ-like was performed using the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) [25].

XfYcjZ-like expression and purification

Positive colonies, carrying the pET28:*xfycjZ*-like construct, were grown in a pre-inoculated 10 mL of Luria–Bertani (LB) broth containing 30 μg mL^{-1} kanamycin at 37 °C and 250 rpm for 12 h and were then transferred to 1 L of LB broth and grown at the same conditions until an OD_{600} of 0.9 was reached. The cultures were then induced with 5.6 mmol L^{-1} lactose for 12 h at 22 °C. The cells were collected by centrifugation at 8000 rpm, 8 °C.

To perform protein extraction, the cells were resuspended in 50 mmol mL^{-1} phosphate buffer, pH 7.8, 300 mmol mL^{-1} NaCl and 10 mmol mL^{-1} β-mercaptoethanol (buffer A) and sonicated using the Ultrasonic Homogenizer 4710 series instrument (Cole-Parmer instrument Co., Chicago, IL, USA) set at 70% duty cycle, with 1 min on and 5 min off on ice.

For recombinant protein purification, the cell lysate was clarified by centrifugation at 16,000×*g* for 45 min at 5 °C and loaded on a column with Ni-NTA agarose superflow (Qiagen; Hilden, Germany) and eluted with an imidazole gradient, from 0 until 500 mmol L^{-1} in buffer A. Subsequently, the purity was estimated by running the proteins on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel, which was stained using Coomassie Brilliant Blue R-250 (USB, Cleveland, OH, USA).

Analytical size-exclusion chromatography (SEC)

Analytical size-exclusion chromatography (SEC) experiments were performed on an AKTA FPLC system using a Superdex 200 10/300 GL prepacked column (GE Healthcare – Pittsburgh, PA, USA) that was previously equilibrated with buffer A. Aliquots of 500 μL of protein (approximately 25 μmol L^{-1}) were loaded onto the column at a flow rate of 0.5 mL min^{-1}, and the elution profile was monitored by the absorbance at 280 nm. To estimate the Stokes radius (R_s) of the recombinant XfYcjZ-like protein, a mix of protein standards with known R_s, including carbonic anhydrase (Mw = 29 kDa, 23.9 Å), ovalbumin (Mw = 44 kDa, 30.5 Å), conalbumin (Mw = 75 kDa, 36.4 Å), aldolase (Mw = 158 kDa, 48.1 Å) and ferritin (Mw = 440 kDa, 61 Å) were used to calibrate the column. All of the protein standards (GE Healthcare) were prepared at a concentration of 3 mg mL^{-1} in buffer A. The void volume of the column was determined using blue dextran (GE Healthcare). The elution profiles obtained for the recombinant XfYcjZ-like and standard protein were converted to the partition coefficient Kav using the following formula:

$$\mathrm{Kav} = \frac{\mathrm{Ve} - V_0}{\mathrm{Vt} - V_0}$$

where Ve is the elution volume of the protein, V_0 is the void volume of the column and Vt is the total column volume (24 mL). The XfYcjZ-like R_s was calculated by the adjusted linear fitting of the R_s of the standard proteins plotted against the $-(\log \mathrm{Kav})^{1/2}$. The experimental R_s obtained from the SEC analysis was used to estimate the frictional ratio (f/f_0) as the ratio of the experimental R_s to the radius of a sphere of the same molecular mass.

Measurement of circular dichroism

Circular dichroism (CD) spectra of the purified His_6-tagged XfYcjZ-like protein were measured using a Jasco J-810 Spectropolarimeter dichrograph (Japan Spectroscopic, Tokyo, Japan). The far-UV CD spectra were generated at 25 °C from a 13.6 μmol L^{-1} solution in 10 mmol L^{-1} sodium phosphate buffer at pH 7.8. The assays were carried out using a quartz cuvette with a 2 mm path length. Ten measurements within the 260–190 nm range at a rate of 20 nm min^{-1} were recorded. The deconvolution of the CD spectrum was performed using the DICRHOWEB server (http://dichroweb.cryst.bbk.ac.uk/).

Analytical ultracentrifugation measurements

Sedimentation velocity experiments of the XfYcjZ-like protein were carried out at concentrations ranging from 0.2 to 0.7 mg mL^{-1} in buffer A using a Beckman Optima XL-A analytical ultracentrifuge. Data acquisition during analytical ultracentrifugation (AUC) was performed at 280 nm, 20 °C, and 35,000 rpm using an AN-50Ti rotor. The AUC data analyses were performed with SedFit software (Version 12.1). The experimental *s*-value was calculated for the standard sedimentation coefficient at a concentration of 0 mg mL^{-1} of protein ($s^0_{20,w}$) to prevent interferences

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