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A new member of the ribbon-helix-helix transcription factor superfamily from the plant pathogen *Xanthomonas axonopodis* pv. *citri*

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ABSTRACT

XACb0070 is an uncharacterized protein coded by the two large plasmids isolated from *Xanthomonas axonopodis* pv. *citri*, the agent of citrus canker and responsible for important economical losses in citrus world production. XACb0070 presents sequence homology only with other hypothetical proteins belonging to plant pathogens, none of which have their structure determined. The NMR-derived solution structure reveals this protein is a homodimer in which each monomer presents two domains with different structural and dynamic properties: a folded N-terminal domain with βαα topology which mediates dimerization and a long disordered C-terminal tail. The folded domain shows high structural similarity to the ribbon-helix-helix transcriptional repressors, a family of DNA-binding proteins of conserved 3D fold but low sequence homology: indeed XACb0070 binds DNA. Primary sequence and fold comparison of XACb0070 with other proteins of the ribbon-helix-helix family together with examination of the genes in the vicinity of *xacb0070* suggest the protein might be the component of a toxin–antitoxin system.

1. Introduction

Xanthomonas axonopodis pv. *citri* (*Xac*) and *Xanthomonas axonopodis* pv. *aurantifolii* (*Xaa*) are responsible for citrus canker (Vauterin et al., 1995), which is considered one of the most important bacterial diseases of citrus trees (Das, 2003). Citrus canker results in a significant reduction in fruit quality and yield. Another critical feature is the socioeconomic impact of *Xac* and *Xaa* as quarantine organisms; in fact, they hinder exportation of plants and fruits to other countries and require costly eradication programs and chemical treatments (Pruvost et al., 2002). The lack of an appropriate treatment of disease control calls for a deeper comprehension of *Xac* pathogenicity.

To this aim, the *Xac* genome has been fully sequenced (da Silva et al., 2002) and its annotation revealed that 37% of the genes code for proteins of unknown function, the majority of which (80%) have homologues in other organisms. In the absence of clues regarding their function, the study of their 3D structure is considered a promising approach, since the structure of proteins with similar functions is more conserved than their primary sequence. Two identical genes, *xacb0070* located on the pXAC64 plasmid and *xaca0037* located on the pXAC33 plasmid (Fig. 1A) code for a 79 residue protein that presents significant sequence identity with a small ensemble of proteins of unknown function and found only in plant pathogens (Fig. 1B). Aiming to identify selective targets against citrus canker, we selected for structural studies the protein coded by *xacb0070*, named for simplicity XACb0070 (Galvão-Botton et al., 2003).

Here we present its NMR-derived solution structure and show that the protein belongs to the ribbon-helix-helix (RHH) superfamily of DNA-binding proteins. Consistent with this topology, we show that XACb0070 does in fact bind to DNA. Among the numerous RHH proteins characterized to date, some are the antitoxin components of toxin–antitoxin (TA) pairs (Pandey and Gerdes, 2005). Interestingly, both *xacb0070* and *xaca0037* genes are neighboured by genes (*xacb0069* and *xaca0036*) coding for a predicted nucleic acid-binding protein containing a PIN domain (homologues of the PilT N-terminal domain), which in prokaryotes are most often found in the toxic components of TA pairs (Pandey and Gerdes, 2005).

Abbreviations: *Xac*, *Xanthomonas axonopodis* pv. *citri*; *Xaa*, *Xanthomonas axonopodis* pv. *aurantifolii*); RHH, ribbon-helix-helix; TA, toxin–antitoxin pair; CSI, chemical shift index; J, spectral density function; τ_{ci}, effective correlation time; R_h, hydrodynamic radius; rmsd, root-mean-square deviation).

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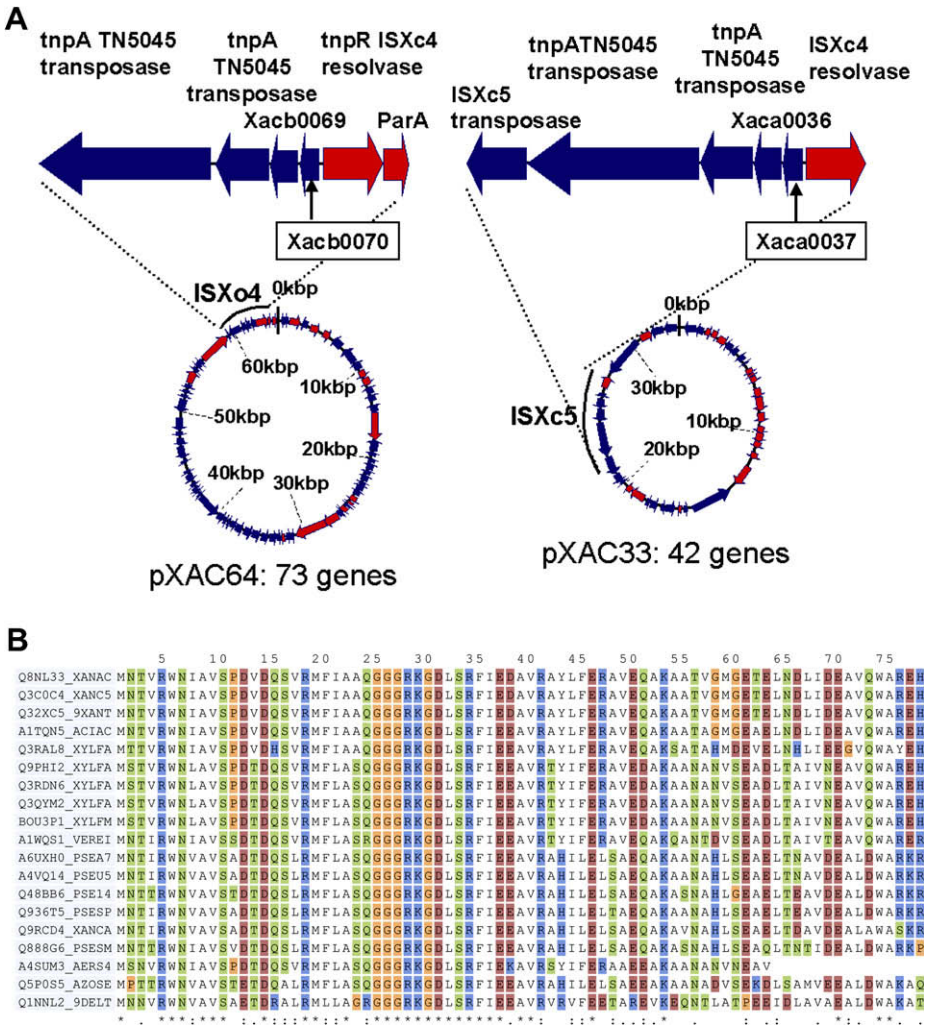

Fig. 1. (A) Graphical representation of the plasmids of *Xanthomonas axonopodis* pv. *citri* (strain 306). The two plasmids pXAC64 (right) and pXAC33 (left) are of 64.92 and 33.70 kb, respectively. The regions in the genome where *xacb0070* and *xaca0037* are located are expanded in the upper region of the figure. (B) Multiple alignment of XACb0070 with amino acid sequences of closest sequence homologues. Amino acids are shaded by property: cyan, positively charged; red, negatively charged; white, hydrophobic and aromatic; green, neutral hydrophilic; orange, glycine or proline. The protein sequences are denoted using the UniProtKB/TrEMBL accession number. The species abbreviations are: XANAC, *Xanthomonas axonopodis* pv. *citri*; XANC5, *Xanthomonas campestris* pv. *vesicatoria*; 9XANT, *Xanthomonas axonopodis* pv. *glycines*; ACIAC, *Acidovorax avenae* subsp. *citrulli*; XYLFA and XYLFM, *Xylella fastidiosa*; VEREI, *Verminephrobacter eiseniae*; PSEA7, *Pseudomonas aeruginosa*; PSEU5, *Pseudomonas stutzeri*; PSE14, *Pseudomonas syringae* pv. *phaseolicola*; PSEP, *Pseudomonas* sp.; XANCA, *Xanthomonas campestris*; PSESM, *Pseudomonas syringae* pv. *tomato*; AERS4, *Aeromonas salmonicida*; AZOSE, *Azoarcus* sp.; 9DELT, *delta proteobacterium* MLMS-1. (For interpretation of colour mentioned in this figure, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Sample preparation and NMR conditions

Uniformly ^{15}N-labeled and uniformly ^{15}N–^{13}C-labeled recombinant XACb0070 were expressed and purified as previously described (Galvão-Botton et al., 2003). The NMR experiments were performed at 35 °C in 40 mM sodium phosphate buffer (pH 6.7), 50 mM NaCl, and 0.05 % (m/v) NaN_3 and 300 μM protein concentration; at higher protein concentration aggregation was observed. Another set of experiments was carried out at pH 9.0, 300 mM NaCl and 0.05 % (m/v) NaN_3 and 500 μM protein concentration; in these conditions the protein presented greater stability over time.

2.2. Intrinsic fluorescence spectroscopy

Thermal denaturation experiments were performed using a JASCO FP-6200 fluorimeter, equipped with a temperature controlled single cell holder. The protein concentration was 2 μM in 10 mM sodium phosphate buffer (pH 6.7), 100 mM NaCl. Tryptophan was excited at 295 nm and the fluorescence spectra were recorded over the 310–400 nm range. Temperature scan was performed in the range 25–95 °C, with increments of 5 °C. Spectra were measured allowing 5 min for temperature equilibration at each given temperature. The protein melting curve was obtained by plotting the wavelength of maximum fluorescence emission as a function of temperature.

2.3. Separation techniques

Samples of 1.5 μg of XACb0070 were subjected to 15% polyacrylamide gel electrophoresis under denaturing (SDS–PAGE) and native (PAGE) conditions and stained with Coomassie blue.

Gel filtration chromatography was performed using a Sephacryl S200 HiPrep 16/60 column (GE Healthcare Life Sciences) equilibrated with 10 mM potassium phosphate buffer (pH 6.8), 300 mM NaCl, and a flow rate of 0.5 ml/min.