Crystallization and preliminary X-ray diffraction analysis of the phosphate-binding protein PhoX from *Xanthomonas citri*

Vanessa R. Pegos,^a
Francisco Javier Medrano^b and
Andrea Balan^{a,c*}

^aLaboratório Nacional de Biociências (LNBio),
Centro de Pesquisa em Energia e Materiais
(CNPEM), 13083-970 Campinas-SP, Brazil,

^bCentro de Investigaciones Biológicas, Madrid,
Spain, and ^cDepartamento de Microbiologia,
Instituto de Ciências Biomédicas II,
Universidade de São Paulo, Av. Prof. Lineu
Prestes 1374, Cidade Universitária,
05508-900 São Paulo-SP, Brazil

Correspondence e-mail: abalan@usp.br

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Xanthomonas axonopodis pv. *citri* (*X. citri*) is an important bacterium that causes citrus canker disease in plants in Brazil and around the world, leading to significant economic losses. Determination of the physiology and mechanisms of pathogenesis of this bacterium is an important step in the development of strategies for its containment. Phosphate is an essential ion in all microorganisms owing its importance during the synthesis of macromolecules and in gene and protein regulation. Interestingly, *X. citri* has been identified to present two periplasmic binding proteins that have not been further characterized: PstS, from an ATP-binding cassette for high-affinity uptake and transport of phosphate, and PhoX, which is encoded by an operon that also contains a putative porin for the transport of phosphate. Here, the expression, purification and crystallization of the phosphate-binding protein PhoX and X-ray data collection at 3.0 Å resolution are described. Biochemical, biophysical and structural data for this protein will be helpful in the elucidation of its function in phosphate uptake and the physiology of the bacterium.

1. Introduction

Periplasmic binding proteins from ATP-binding cassette transporters (ABC transporters) are essential components owing to their role in substrate uptake and delivery to the transporter. They consist of two domains connected by a hinge that form a cleft for substrate binding (Davidson *et al.*, 2008). More than 100 structures of periplasmic binding proteins have been solved in different conformations, essentially demonstrating the changes that occur after ligand binding (Berntsson *et al.*, 2010) and the essential residues for substrate binding and transport. In bacteria, the PstSABC system is the ABC transporter specific for inorganic phosphate (P_i), which contains the PstS protein as a model high-affinity phosphate-binding protein. At present, 18 structures of phosphate-binding proteins are available in the Protein Data Bank, including 12 structures of PstS from *Escherichia coli*, including the wild type and mutants (Wang *et al.*, 1994, 1997; Yao *et al.*, 1996; Hirshberg *et al.*, 1998; Ledvina *et al.*, 1998), structures of the orthologues from *Yersinia pestis* (Tanabe *et al.*, 2007), *Streptococcus pneumoniae* (PDB entry 4exl; Center for Structural Genomics of Infectious Diseases, unpublished work), *Borrelia burgdorferi* (Brautigam *et al.*, 2014) and *Clostridium perfringens* (PDB entry 4gd5; Center for Structural Genomics of Infectious Diseases, unpublished work), and the structures of PstS-1 and PstS-3 from *Mycobacterium tuberculosis* (Vyas *et al.*, 2003; Ferraris *et al.*, 2014). The structures showed similarities, including the two domains forming the cleft for the phosphate, conservation in the ligand-binding pocket and the importance of residues for high-affinity binding. The expression of *pst* genes is regulated by low phosphorus levels through the activation of the Pho regulon proteins (Van-Bogelen *et al.*, 1996), which induces an adaptative response into the cell (Lamarche *et al.*, 2008). Indirectly, the upregulation and down-regulation of many genes mediated by phosphate levels has been

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Table 1Orthologues of *X. citri* PhoX with solved structures deposited in the Protein Data Bank and sequence comparison.

Protein name	Organism	Sequence identity (%)	Query coverage (%)	E-value	PDB code and reference
Phosphate-binding protein PstS	<i>Yersinia pestis</i>	57	88	2.00×10^{-122}	2z22 (Tanabe <i>et al.</i> , 2007)
Phosphate-binding protein	<i>Escherichia coli</i>	51	88	4.00×10^{-121}	2abh (Yao <i>et al.</i> , 1996)
Phosphate-binding protein	<i>Escherichia coli</i>	56	88	4.00×10^{-121}	1ixh (Wang <i>et al.</i> , 1997)
Periplasmic binding protein	<i>Streptococcus pneumoniae</i> Canada	55	25	2.00×10^{-7}	4exl (Center for Structural Genomics of Infectious Diseases, unpublished work)
Phosphate receptor PstS-1	<i>Mycobacterium tuberculosis</i>	32	91	6.00×10^{-38}	1pc3 (Vyas <i>et al.</i> , 2003)
Phosphate-binding protein PstS-3	<i>Mycobacterium tuberculosis</i>	31	91	7.00×10^{-35}	4lvq (Ferraris <i>et al.</i> , 2014)
Phosphate-binding protein	<i>Clostridium perfringens</i>	27	59	4.00×10^{-7}	4gd5 (Center for Structural Genomics of Infectious Diseases, unpublished work)
Phosphate-binding protein PstS	<i>Borrelia burgdorferi</i>	21	75	2.10×10^{-2}	4n13 (Brautigam <i>et al.</i> , 2014)

Table 2Crystallization screening and refinement conditions for *X. citri* PhoX in the absence and presence of phosphate.

The best crystals obtained after refinement are shown in Fig. 1.

Sample	Protein concentration (mg ml ⁻¹)	Screening	Refinement	Crystals	Resolution after refinement (Å)
PhoX + sodium phosphate	10	25% PEG 3350, 200 mM sodium iodide, 100 mM bis-tris pH 5.5	20% PEG 3350, 200 mM sodium iodide	Fig. 1(a)	3.0
PhoX	8	20% PEG 3350, 200 mM sodium iodide	17% PEG 3350, 200 mM sodium iodide	Fig. 1(b)	5.0
PhoX	10	20% PEG 3350, 200 mM sodium iodide	17% PEG 3350, 250 mM sodium iodide	Fig. 1(c)	6.0
PhoX + sodium phosphate	15	20% PEG 3350, 200 mM sodium iodide	17% PEG 3350, 250 mM sodium iodide	Fig. 1(d)	2.9
PhoX + sodium phosphate	15	17% PEG 3350, 250 mM sodium iodide	15% PEG 3350, 250 mM sodium iodide	Fig. 1(e)	3.0

correlated with bacterial virulence and pathogenicity, including the *pstS* genes (Crépin *et al.*, 2011).

Curiously, in *Xanthomonas axonopodis* pv. *citri* (*X. citri*), the causative agent of citrus canker disease (Brunings & Gabriel, 2003), we identified the presence of two genes encoding phosphate-binding proteins, *pstS* (XAC1577) and *phoX* (XAC1578), which belong to the *pstSCABU* and *oprOphoX* operons, respectively (Pegos *et al.*, 2014). Sequence analysis of the proteins revealed a sequence identity of 65%. A sequence identity of greater than 50% was observed when the sequence of PhoX was compared with those of the PstS orthologues from *E. coli*, *Y. pestis* and *S. pneumoniae* (Table 1). PhoX also conserved the residues forming the putative active site found in these proteins. In previous studies, we have demonstrated that *phoX* is found in a large number of *Xanthomonas* strains and is identified in many species of proteobacteria. Recently, based on a proteomics analysis we have shown that the expression of PstS and PhoX is upregulated during depletion of phosphate, indicating that both proteins play an important function in bacterial growth (Pegos *et al.*, 2014).

In order to obtain structural information for the PhoX protein from *X. citri*, the recombinant mature protein fused to an N-terminal His₆-tag sequence was expressed in *E. coli* Tuner (DE3) cells with an apparent molecular weight of 33 kDa and was purified using immobilized metal-affinity chromatography. The purified protein was crystallized in different conditions in the presence and absence of phosphate. The crystal structure of *X. citri* PhoX will be an important achievement for the study of phosphate uptake and regulation in the

Xanthomonas genus, including the importance of the ABC transporter PstSCBA in the physiology and virulence of *X. citri*.

2. Materials and methods

2.1. Cloning, expression and purification of PhoX protein

The PhoX-encoding gene was amplified from *X. axonopodis* pv. *citri* genomic DNA (gi:21242328) using the following oligonucleotides: F_PhoX_NdeI, 5'-CATATGGCCGACGTCACCGGTG-3', and R_PhoX_XhoI, 5'-CTCGAGTTAGTGCGGCAGATTCTTGGC-3'. The amplified fragment of 951 bp consisted of the sequence encoding the mature protein without 24 amino acids from the N-terminus flanked by the restriction enzymes *NdeI* and *XhoI* at the beginning and end of the gene, respectively, and was cloned into the pET-28a vector. Recombinant *X. citri* PhoX protein fused at the N-terminal end to a His₆-thrombin cleavage site tag was obtained after expression tests in *E. coli* Tuner (DE3) strain transformed with a pET-28 derivative carrying the *phoX* gene (pET_phoX). Induction of expression occurred when the cells reached an optical density at 600 nm of 0.5 after the addition of 0.1 mM IPTG for 4 h at 30°C. Cell extracts were obtained as previously reported for the alkanesulfonate-binding protein SsuA (Araújo *et al.*, 2013) and the protein was purified by immobilized nickel-affinity chromatography with 150 mM imidazole in the purification buffer (10 mM Tris-NaCl pH 8.0, 500 mM NaCl). The purified fractions were concentrated and

Figure 1 Crystals of *X. citri* PhoX obtained in the absence and presence of phosphate (see Table 2 for details).

Table 3
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Diffraction source	W01B-MX2, LNLS
Wavelength (Å)	1.459
Temperature (K)	110
Detector	MAR Mosaic 225 CCD
Crystal-to-detector distance (mm)	120
Rotation range per image (°)	0.5
Total rotation range (°)	164.5
Exposure time per image (s)	30
Space group	$P2_1$
a, b, c (Å)	81.56, 115.60, 133.30
α, β, γ (°)	90.00, 90.33, 90.00
Mosaicity (°)	0.45
Resolution range (Å)	87.36–3.00 (3.11–3.00)
Total No. of reflections	131283 (12689)
No. of unique reflections	46810 (4633)
Completeness (%)	94.3 (95.5)
Multiplicity	2.8 (2.7)
$\langle I/\sigma(I) \rangle$	8.4 (2.5)
R_{meas}	0.145 (0.554)
Overall B factor from Wilson plot (Å ²)	39.95

dialyzed against 10 mM Tris–HCl pH 8.0 and kept at -20°C at a final concentration of 10 mg ml^{-1} .

2.2. Protein crystallization

Samples of recombinant *X. citri* PhoX were submitted to crystallization trials at the automatized crystallization facility at the Brazilian National Laboratory of Biosciences (LNBio; CNPEM, Campinas, SP, Brazil) using the hanging-drop vapour-diffusion method, mixing equal volumes ($1:1\text{ }\mu\text{l}$) of protein and reservoir solution and incubating at 290 K. Initial screening with protein at 10 mg ml^{-1} was performed using commercial crystallization screening kits from Hampton Research and Jena Biosciences. Conditions that showed crystalline structures or needles were refined, varying the pH, precipitant concentration and protein concentration to yield suitable crystals. A list of all of the conditions that allowed crystal growth and refinement is shown in Table 2 and crystals are shown in Fig. 1.

2.3. Data collection and processing

PhoX crystals were mounted in nylon loops and flash-cooled in mother liquor using liquid nitrogen; the high PEG concentration was sufficient to provide cryoprotection. The PhoX data set was collected

Figure 2
Expression and purification of *X. citri* PhoX. PhoX was expressed from *E. coli* Tuner (DE3) cells after IPTG induction and was purified by immobilized metal-affinity chromatography using nickel columns. Lane MW, molecular-weight markers; lane E, cell extract; lane P, cell pellet; lane FT, flowthrough of the immobilized nickel-affinity chromatography; lane 1, eluted samples from washing with 5 mM imidazole; lanes 2 and 3, eluted samples using 50 and 150 mM imidazole, respectively.

on the W01B-MX2 protein crystallography beamline at Laboratório Nacional de Luz Síncrotron (LNLS), Brazil using a wavelength of $1.459\text{ }\text{\AA}$ and a MAR Mosaic 225 CCD detector. Diffraction data were indexed and integrated using *XDS* (Kabsch, 2010) and merged and scaled using *AIMLESS* (Evans, 2011) from the *CCP4* package (Winn *et al.*, 2011). Molecular replacement was performed with *PHENIX* (Adams *et al.*, 2010) using PstS from *E. coli* K12 (PDB entry 2abh; Yao *et al.*, 1996), which shares 51% amino-acid identity with PhoX, as a model. The phosphate, water molecules, H atoms and atoms with zero occupancy were removed from the 2abh structure to provide a better search model. Data-collection statistics are shown in Table 3.

3. Results and discussion

PhoX was produced from *E. coli* cells in a soluble form and purified in a single step of purification using immobilized metal-affinity

Figure 3
Crystal and diffraction pattern of *X. citri* PhoX. (a) Crystal of PhoX obtained in 20% PEG 3350, 200 mM sodium iodide and sodium phosphate with protein at 10 mg ml^{-1} . (b) Diffraction pattern of the crystal to $3.0\text{ }\text{\AA}$ resolution (circle).

chromatography (Fig. 2). The purified protein was submitted to crystallization trials in the absence and presence of phosphate, resulting in needles and very small crystals that were less than 15 μm in the longest dimension. Most of the crystals were grown in PEG 3350 and sodium iodide. Refinement of these conditions improved the size and shape of the crystals, especially reducing the percentage of PEG 3350 and increasing the concentration of sodium iodide (Table 2). The presence of phosphate also increased the hardness of the crystals, suggesting a possible rearrangement of the structure upon ligand binding. The best diffraction pattern was obtained from a crystal grown in 200 mM sodium iodide, 20% PEG 3350 (Fig. 3). This crystal diffracted to a maximum resolution of 3.0 Å and the data were processed using XDS (Kabsch, 2010). The crystals showed the symmetry and systematic absences of the primitive monoclinic space group $P2_1$. All data-collection statistics are shown in Table 3.

The range of possibilities for the Matthews coefficient (Matthews, 1968) of between 1.83 and 3.30 Å³ Da⁻¹ indicated the presence of between nine and five protein molecules in the asymmetric unit, respectively. After molecular replacement and analysis of the data, the best number of molecules in the asymmetric unit with no packing clashes was eight, for which the Matthews coefficient was calculated to be 2.01 Å³ Da⁻¹ and the solvent content to be 40.3%. The molecular-replacement procedure was performed using the *E. coli* phosphate-binding protein structure modified by CHAINSAW (Stein, 2008) as a search model (Yao *et al.*, 1996; PDB entry 2abh) in Phaser (McCoy *et al.*, 2007). The initial maps produced are encouragingly good, clearly showing electron density for the phosphate. The model is being rebuilt and refined.

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