Sulfate-Binding Protein (Sbp) from *Xanthomonas citri*: Structure and Functional Insights

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The uptake and transport of sulfate in bacteria is mediated by an ATP-binding cassette transporter (ABC transporter) encoded by *sbpCysUWA* genes, whose importance has been widely demonstrated due to their relevance in cysteine synthesis and bacterial growth. In *Xanthomonas citri*, the causative agent of canker disease, the expression of components from this ABC transporter and others related to uptake of organic sulfur sources has been shown during in vitro growth cultures. In this work, based on gene reporter and proteomics analyses, we showed the activation of the promoter that controls the *sbpCysUWA* operon in vitro and in vivo and the expression of sulfate-binding protein (Sbp), a periplasmic-binding protein, indicating that this protein plays an important function during growth and that the transport system is active during *Citrus sinensis* infection. To characterize Sbp, we solved its three-dimensional structure bound to sulfate at 1.14 Å resolution and performed biochemical and functional characterization. The results revealed that Sbp interacts with sulfate without structural changes, but the interaction induces a significant increasing of protein thermal stability. Altogether, the results presented in this study show the evidence of the functionality of the ABC transporter for sulfate in *X. citri* and its relevance during infection.

Sulfur is an ubiquitous element of the Earth that can be obtained from a variety of molecules from which sulfate is the preferred source (Aguilar-Barajas et al. 2011). In bacteria, there are different mechanisms for sulfate or organic sulfonate uptake, which involve the transport of these molecules through the inner membrane (Sekowska et al. 2000). ATP-binding cassette (ABC) transporters represent one of the biggest families of membrane proteins responsible for the transport of different molecules, including lipids, oligopeptides, sugars, amino acids, and ions. Due to the diversity of transported substrates, they are extensively reported as important for the mechanisms of virulence and pathogenesis in many species (Brown et al. 2001; Kemner et al. 1997; Li and Wang 2011; Murphy et al. 2016). One known active system for transport of sulfate is the ABC transporter SbpCysUWA, composed of a periplasmic sulfate-binding protein, Sbp, responsible for ion uptake, two permeases CysU and CysW (heterodimer) that form a pore for substrate

passage, and the ATPase CysA (homodimer) that gives energy to the process (Scheffel et al. 2005). The importance of this transporter has been directly associated to cysteine and methionine synthesis, but its absence causes generalized deficiency in growth, infection, and virulence of many bacteria (Kertesz 2001; van der Ploeg et al. 1999). Sbp, responsible for affinity and specificity of the transport, is essential for the first steps of sulfate assimilation. Due to its location in the periplasm, it has been used as antigen vaccines in bacteria (Garmory and Titball 2004; Otsuka et al. 2014) and strategic target for the development of growth inhibitors in *Mycobacterium tuberculosis* (Bhave et al. 2007; Paritala and Carroll 2013). In the *Xanthomonas* genus, the lack of sulfur-containing amino acids, sulfur compounds, or sulfate have been associated with the induction-of-pathogenicity locus in the citrus hosts and in vitro (Schulte and Bonas 1992), production of xanthan gum in *Xanthomonas campestris* (García-Ochoa et al. 2000), expansion of lesions caused by *X. phaseoli* in soybean (Laurence and Aluisio 1981), and attenuation of canker symptoms (Li and Wang 2011; Tófoli de Araújo et al. 2013). In *X. citri*, sulfur is also required for enzymes such as SufE (XAC2355), which is involved in iron-sulfur cluster biosynthesis under iron-limitation and stress conditions (Guzzo et al. 2006).

A search in the *X. citri* genome has shown more than 30 genes involved with sulfur metabolism, including most of the genes belonging to the *cys* regulon, such as cysteine pathway and sulfur-compounds uptake and assimilation genes (Pereira et al. 2015). The conservation is mainly evidenced in orthologs from *X. campestris* pv. *campestris* and the phytopathogenic bacterium *Xylella fastidiosa* (up to 95% of amino acid sequence identity), both with quite different ways of infection and pathogenesis development (Brunings and Gabriel 2003). *X. citri* Sbp also shows 60% amino acid sequence identity with the sulfur limitation-induced solute-binding protein ABC-T SMb21133 from the soil bacterium *Rhizobium leguminosarum* Rlv3841 (Mauchline et al. 2006). ABC-T SMb21133 and other transporters of sulfate and molybdate have been related to nitrogen fixation in *R. leguminosarum* (Cheng et al. 2016).

Previous polymerase chain reaction (PCR) analysis of cDNA obtained from *X. citri* showed that all the components from the *X. citri* sulfate ABC transporter are transcribed during bacterial in vitro growth (Pereira et al. 2015). Sbp has been identified by mass spectrometry analyses in different conditions (Mehta and Rosato 2001; Pegos et al. 2014; Soares et al. 2010), and the *sbp* mutant of *X. citri* showed attenuated symptoms of canker development in citrus plants, indicating the protein is important during infection and pathogenesis (Li and Wang 2011).

In this work, we focused on the functional and structural characterization of periplasmic-binding protein Sbp, unveiling

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its mechanism of sulfate binding and important residues for the ion interaction. Furthermore, based on a gene reporter, we demonstrated that the promoter that regulates operon *sbpcysUWA* expression is highly activated during bacterial growth in vitro and during infection in vivo in *Citrus sinensis*, even after 8 days of inoculation, evidencing the relevance of Sbp for sulfur assimilation and its cognate ABC transporter.

RESULTS AND DISCUSSION

Activation of the p1017 promoter during growth of *X. citri* cells in vitro and in vivo.

The operon *sbpcysUWA*, encoding for genes XAC1017 to XAC1020, was previously described in *X. citri* and corresponds

to a sulfate ABC transporter, which was shown to be very conserved in the *Xanthomonas* genus and in other bacteria (Pereira et al. 2015). Upstream of the first gene of the operon, XAC1017 (*sbp*), we found an intergenic region in which the operon promoter, herein, named p1017 (Fig. 1A) is located. To analyze in what conditions the p1017 promoter was induced in *X. citri*, cells were transformed with the pKX33 vector containing the reporter gene *EGFP* (enhanced green fluorescent protein) under control of the p1017 promoter (pKX33.p1017EGFP). The activation of the promoter was analyzed during growth of the *X. citri* cells in Luria Bertani (LB) broth medium for 24 h and in vivo, after inoculation of transformed cells into *C. sinensis* leaves during 14 days of bacterial infection (Fig. 1B and C). The activation of the p1017 promoter in

Fig. 1. Transcriptional activation of the promoter of the *sbpcysUWA* operon in *Xanthomonas citri*. **A**, Genome organization of *X. citri* genes from the putative sulfate ABC transporter. *Sbp* encodes the periplasmic sulfate-binding protein; *cysU* and *cysW* encode the permeases of the transporter forming a heterodimer and *cysA* encodes the homodimer of ATPases. Numbers represent the genome position. The intergenic region p1017 is shown evidencing the predicted promoter -10 and -35 sequences. Data and annotation were obtained from the Kyoto Encyclopedia of Genes and Genomes database. **B**, Fluorescence micrography of an overnight culture of *X. citri* in Luria Bertani (LB) broth medium carrying the plasmid pKX33.p1017.EGFP, which sets the gene reporter under the control of p1017 promoter. Panel II, Bacteria seen under white light. Panel III, Time-course analysis of the relative fluorescence (absolute fluorescence normalized by absorbance of the culture) of *X. citri*+pKX33.p1017.EGFP and wild *X. citri* grown in liquid LB broth. **C**, Panel I, Fluorescence micrography of *X. citri* carrying the plasmid pKX33.p1017.EGFP, extracted from infected citrus leaves after 7 or 10 days or 2 weeks from infection. Panel II, Bacteria seen under white light. Panel III, Time-course analysis of the relative fluorescence (absolute fluorescence normalized by total protein content) of *X. citri*+pKX33.p1017.EGFP and wild *X. citri* extracted from infected citrus leaves.

LB broth cultures was first evidenced by fluorescence microscopy of an overnight culture of *X. citri* carrying plasmid pKX33.p1017EGFP grown in LB broth medium, which showed cells with green fluorescence due to EGFP expression in contrast with the control samples. These data were further corroborated by evaluation of the relative fluorescence in a time-course analysis of *X. citri* carrying pKX33.p1017EGFP in comparison with wild *X. citri* (Fig. 1B). The fluorescence is observed after just 2 h of cellular growth, with a significant increase after 8 h of cultivation, which corresponds to the log phase. In addition, the measurement of EGFP fluorescence during the infection of *C. sinensis* leaves also revealed p1017 promoter induction already in the early stages of infection (3 days), with an apparent increase after 10 days of infection (Fig. 1C). These results show that activation of the promoter is associated with bacterial growth in vitro and in vivo, evidencing the relevance of sulfur for maintenance inside the plant and corroborating the importance of this transporter for the bacterium. Moreover, results show that after the first induction, the promoter is kept activated, confirming sulfate levels or availability of the ion are low in the plant.

Evidences of Sbp expression in different culture media.

To evaluate the effect of culture medium in Sbp expression, *X. citri* cells were cultivated in rich medium LB broth, minimum medium M9, and M9 with a half concentration of sulfate (M9.1/2) and were submitted to mass spectroscopy analysis. Cells were grown for 72 h, when total cellular protein extracts were obtained and were applied in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), for further proteomics analyses (range of 35 to 45 kDa) (Fig. 2A and B). The effect of the sulfate concentration on *X. citri* Sbp was analyzed using as reference the results obtained in the medium

containing the lower ion concentration, which was M9.1/2 (1 mM sulfate). Statistics analysis was performed applying the *t* test. Sbp was considered up- or down-regulated by sulfate when expression levels were increased more than 1.5-fold or decreased more than 0.67-fold, respectively, according to Blavillain and collaborators (2007) (Fig. 2C). The data showed that, when *X. citri* is grown in medium containing restricted amounts of sulfate, Sbp levels are increased and sulfate-dependent when compared with the rich medium. In LB broth, the presence of Sbp is reduced, probably due to the high concentration of other sources of sulfur, such as cysteine and methionine, which are described as inhibitors of the system in other bacteria (Eichhorn et al. 2000; Kertesz 2000). These data are in accordance with the lower levels of EGFP relative fluorescence observed in plants in which sulfate availability is very low (Cook et al. 1998; Sekowska et al. 2000) and most of the available sulfur comes from organic sulfonates, cysteine, and methionine and their oxidation products (Kocsis et al. 1998). In fact, organic sulfonates must constitute the major source of sulfur in the plant once *X. citri* has at least two other ABC transporters dedicated to organic sulfur uptake, the expression of which was evidenced in conditions that simulate the plant (Pereira et al. 2015). Interestingly, Sbp also has been identified by mass spectrometry analysis of enriched protein extracts from *X. citri* grown in nutrient broth NB (Soares et al. 2010), medium containing controlled concentrations of phosphate (Pegos et al. 2014), and in the leaf extracts of a susceptible host plant (*Citrus sinensis* or sweet orange) (Mehta and Rosato 2001). These data show that, even at a high or low concentration of sulfate, the protein is present, suggesting that its regulation is not so tight. In fact, the regulation of the systems

Fig. 2. The presence of sulfate-binding protein (Sbp) in *Xanthomonas citri* extracts after proteomics analysis. **A**, Growth curves of *X. citri* in Luria Bertani broth, M9 minimum medium (2 mM ammonium sulfate), and M9.1/2 minimum medium (1 mM ammonium sulfate). Samples for analyses were obtained from the 72-h culture (gray bar). **B**, Analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the three normalized extracts that were used for proteomics analysis. Black boxes show the gel region that was cut and submitted to trypsin digestion. **C**, Values obtained ($P < 0.05$) in the analysis when M9.1/2 was used as a reference.

involved with sulfur assimilation in *X. citri* is still unclear. In *Escherichia coli*, two transcriptional regulators are involved with the expression of transport systems dedicated to sulfate and organic sulfur sources CysB and Cbl (van der Ploeg et al. 1997). CysB is the transcriptional activator of the sulfate system, while Cbl is responsible for expression of *ssu* genes (sulfate starvation systems) in the absence of sulfate (Stec et al. 2006). A putative ortholog of CysB was identified in *X. citri* genome (XAC3339) but none for Cbl (Pereira et al. 2015), suggesting differences in the control of these proteins in *X. citri*. There is evidence that, in *X. citri*, the expression of components of the additional ABC transporters dedicated to organic sulfur uptake (alkanesulfonates) operates in conjunction with the expression of the SbpCysUWA components (Pereira et al. 2015). The study of the role of putative regulator CysB would be of interest for the understanding of sulfur regulation in *X. citri*.

***X. citri* recombinant Sbp is expressed as a soluble, stable, and monodisperse protein.**

Once we showed that Sbp was relevant for *X. citri* during different conditions of infection, we focused on the functional and structural characterization of this protein as a player for the sulfate uptake. After cloning and confirmation that the correct construct was obtained (Fig. 3A), the recombinant Sbp protein

was expressed and purified in the *E. coli* BL21 (DE3) strain as a soluble cytosolic protein genetically fused at the N-terminal end with a His₆ tag encoded by the pET28a expression vector. Maximum soluble protein yields, representing up to 70% of the total expressed protein, were achieved after 4 h of cultivation in LB broth medium, at 37°C, with aeration. The recombinant protein with a calculated molecular mass of 37.5 kDa was first purified by affinity chromatography after elution with different concentrations of imidazole (Fig. 3B). To eliminate small contaminants and aggregates, the purified samples were submitted to size-exclusion chromatography and were purified as a single peak (Fig. 3C). Approximately 65% of the recombinant protein was recovered at the end of the purification process and the protein remained soluble and stable at high concentrations (20 mg/ml). Dynamic light scattering (DLS) analysis revealed that Sbp was obtained as a monomer and monodisperses, allowing its use for the following experiments (Fig. 3D).

Sbp shows increased thermal stability in the presence of sulfate.

The far-UV circular dichroism (CD) spectra of *X. citri* Sbp were recorded in the absence and the presence of the oxyanions sulfate and molybdate, as a negative control (Fig. 4A). The

Fig. 3. Production of the pure recombinant *Xanthomonas citri* sulfate-binding protein (Sbp). **A**, Cloning steps of the 0.9-kb fragment corresponding to the *Sbp* gene in pGEM and pET28a vectors. Lane 1, molecular weight; lane 2, cleavage of pGEMsBp with *NdeI* and *HindIII* liberating the vector and gene fragments; lane 3, molecular weight; lanes 4 to 9, cleavage of different vectors pET28aSbp with *NdeI* and *HindIII* liberating the vector and gene fragments. **B**, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the expression and immobilized metal ion affinity chromatography (IMAC) purification of Sbp from *Escherichia coli* cells. Lanes 1 and 10, molecular weight marker; lane 2, extracts of noninduced bacterial culture; lanes 3 and 4, extracts of 2-h and 4-h, respectively, induced bacterial culture with isopropyl β-D-1-thiogalactopyranoside; lanes 5 and 6, soluble fractions I and II, respectively; lane 7, insoluble fraction; lanes 8 and 9, flowthrough from IMAC chromatography; lanes 10 to 13, eluted fractions from chromatography, using buffers with 50, 100, and 150 mM imidazole, respectively. **C**, SDS-PAGE of the fractions from the size-exclusion chromatograph of Sbp after the IMAC purification. Lane 1, molecular weight markers, lanes 2 to 9, eluted fractions from the single peak obtained in the purification. **D**, Dynamic light scattering analysis of the purified recombinant Sbp revealing the monomeric and monodisperse state of the protein.

analysis of Sbp spectrum revealed that the protein is well-structured in solution, as it presents a relative maximum peak at 198 nm and two relative minima peaks at 208 and 222 nm, corresponding to an alpha-beta protein, as previously evidenced in the three-dimensional structure. No significant changes in the secondary structure content of the protein were observed on the addition of either sulfate or molybdate ions at a molar ratio of 1:10. On the other hand, as previously reported (Hamorsky et al. 2012), when Sbp was monitored by near-UV (250 to 320 nm) analysis, which is very sensitive to small changes in the tertiary structure, the addition of sulfate ions was able to induce changes in the protein spectrum, suggesting that structural changes, indeed, occur upon sulfate binding and, possibly, are located in the flexible regions of the protein (Fig. 4B). This effect is specific to sulfate ions, since the addition of molybdate did not induce major alterations on SBP near-UV spectrum (Fig. 4B). To further investigate the effect of sulfate binding on Sbp, we analyzed the thermal stability of the protein in the absence and the presence of ions. By evaluating the Sbp CD signal at 222 nm, as a function of temperature, in the presence or absence of oxyanions, we observed a remarkable increase of 15°C in the midpoint temperature (T_m) (55°C to 70°C) in the presence of sodium sulfate but not of molybdate (Fig. 4C), probably due to the additional hydrogen bonds that occur for sulfate binding. These data corroborate the fact that crystals of *X. citri* Sbp only were obtained in the presence of sulfate, possibly because the bound protein assumes a more stable conformation.

The three-dimensional structure of Sbp showed a highly conserved ligand-binding site with a sulfate ion inside.

Crystals of *X. citri* Sbp usually showed growth after 6 days leading to a rod shape with dimensions of 50 × 500 μm. Crystals were obtained in six different crystallization conditions containing polyethylene glycol (PEG), morpholineethanesulfonic acid (MES), or *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) in a pH range of 6.5 to 7.5, but only in the presence of sulfate. None of these crystals, however, were capable of generating diffraction patterns with resolution better than 7 Å. Crystals were grown in 0.2 M potassium sulfate, 20% PEG3350 and were diffracted at 1.14 Å resolution (Fig. 5A). After processing, crystals showed the symmetry and systematic absences of the space group C_{121} . Table 1 summarizes the data collection

statistics. The Matthews coefficient (Matthews 1968) was calculated to be 2.41 Å³/Da and the solvent content 49%, corresponding to one molecule in the asymmetric unit (Fig. 5B). The crystal structure of *X. citri* Sbp was solved by the molecular replacement method, using a 1.7 Å structural coordinate from *Salmonella typhimurium* Sbp for the initial phasing (Protein Data Base [PDB] code 1SBP). The final model consists of residues 25 to 338, which corresponds to the mature protein without the signal peptide. The first Sbp structure of a phytopathogenic bacterium revealed the same three-dimensional organization described for its ortholog. The cleft, where sulfate is bound, is delimited by N and C domains, each one encompassing two noncontiguous sequences forming a β-sheet structure surrounded by α-helices (Fig. 5C). At the N domain, residues 25 to 120 and 246 to 308 are organized in a four-stranded parallel and antiparallel β-sheet set surrounded by eight α-helices. Similarly, the C domain is also characterized by a four-stranded antiparallel β-sheet flanked by seven α-helices, defined by residues 121 to 245 and 309 to 338. The interconnection of the lobes involves two loops (121 to 123 and 24 to 245 residues) that lay at the opposite side of the cleft entrance. As in the *S. typhimurium* ortholog, the sulfate binding in *X. citri* Sbp is mediated by five conserved residues that perform seven hydrogen bonds and van der Waals' forces with the oxyanion (Fig. 5D). There are no water molecules inside the pocket after ligand interaction.

According to the three-dimensional structure of *X. citri* Sbp, at least two tryptophans, W¹⁶¹ and W²¹⁸, could be highly affected after sulfate binding, most probably W²¹⁸, which is located inside the ligand-binding pocket and directly interacting with the ion through a hydrogen bond (Fig. 6A). W²¹⁸ is located at the end of β-strand 6, making a basement for the ligand-binding site. In order to investigate this possibility, we evaluated the intrinsic fluorescence of SBP tryptophans, in the absence or in the presence of the oxyanions sulfate or molybdate (Fig. 6B). Sbp has seven tryptophan residues, but its emitted fluorescence spectrum, with excitation at 295 nm, revealed a single broad band, with λ_{max} at 327 nm (Fig. 6B), suggesting that the majority of the W residues are found in an apolar environment. The addition of sulfate ions did not alter the λ_{max} of Sbp emission; however, a clear suppression of the fluorescence in function of the increase on sulfate (but not on molybdate) concentration was observed (Fig. 6B), evidencing

Fig. 4. Spectroscopic characterization of sulfate-binding protein (Sbp) in the absence and in the presence of oxyanions. **A**, The far UV circular dichroism spectrum of 5 μM Sbp is characteristic of an α/β folded protein. No significant differences were observed when 5 μM Sbp was treated with 10 μM of either SO_4^{2-} or MoO_4^{2-} . **B**, Comparison of near circular dichroism near UV spectra of 18 μM Sbp in the presence and absence of 36 μM SO_4^{2-} suggests that SO_4^{2-} binding on Sbp induces changes in the tertiary structure of the protein. Sbp treated with MoO_4^{2-} was tested as a negative control. **C**, Thermal denaturation of 5 μM Sbp in the absence (midpoint temperature [T_m] = 55°C) or presence of 10 μM of either SO_4^{2-} (T_m = 65°C) or MoO_4^{2-} (T_m = 55°C) was analyzed by monitoring the molar ellipticity measured at 222 nm as a function of the temperature, and the protein unfolded fraction was determined. The binding of SO_4^{2-} but not of MoO_4^{2-} promotes a significant (10°C) increase on thermal stability of the protein.

a quenching phenomenon. Based on the fluorescence intensity data of Sbp titrated with sulfate and applying equation I (provided below), we calculated the Sbp binding constant (K_b) and the number of binding sites (n). The binding parameters of SO_4^{2-} were described as $n_{\text{sulfate-SBP}} = 0.82$ and the $K_b = 0.68 \times 10^6 \text{ M}^{-1}$ (Fig. 6C), which indicates a $K_d = 1.45 \text{ }\mu\text{M}$.

As previously demonstrated (Dudev and Lim 2004), the specificity of Sbp for sulfate but not molybdate is explained by a set of features such as the interactions with solvent and residues and, also, the pocket size. Once the three-dimensional structure of *X. citri* ModA (Balan et al. 2008) (PDB code 2H5Y) was solved in the presence of molybdate, we compared the structures, focusing on the ligand-binding pocket, and observed that both proteins conserved secondary structures with significant differences at the C terminal, which is much longer in the sulfate-binding protein than ModA (Fig. 7A and B, panel I, in yellow), and that the ligand-binding pocket in Sbp is smaller than the ModA pocket (Fig. 7B, panels II and III). Moreover, the comparison of pocket residues after superimposition of the structures revealed that, despite the hydrogen bonds for interactions, residues from the pocket and those responsible for ion coordination are not conserved between both proteins (Fig. 7B, panel IV). Specifically, the presence and occupancy of the tryptophan W¹⁹⁴ and serine S¹³² in Sbp makes the pocket volume of this protein smaller than that shown in ModA, which has a tyrosine (Y¹⁷⁰) in the corresponding position (Fig. 7B, panels V and VI).

Conclusion.

The data presented in this work show, for the first time, that the putative promoter region of the operon *sbpcysUWA* is activated during all periods of growth in vitro and in vivo. In

addition, high levels of Sbp are present in *X. citri* periplasmic extracts, suggesting that the periplasmic-binding protein and its related ABC transporter are needed even during late periods of infection, indicating the relevance of the ion for bacterium growth and infection. Moreover, we solved the three-dimensional structure of Sbp at 1.14 Å resolution with a sulfate inside and showed the high affinity and specificity for the anion.

MATERIALS AND METHODS

Computational analysis.

The nucleotide sequence of the *sbp* gene was obtained at the National Center for Biotechnology Information (NCBI) (GI: 14881153). Search of Sbp ortholog sequences was carried out using the KEGG2 program of the Bioinformatics Center Institute for Chemical Research Kyoto University. Amino acid sequence alignments were performed using ClustalW (Thompson et al. 1994). Prediction of signal peptide and trans-membrane sequences were performed by SignalP and DAS programs, respectively. Protein parameters of the *X. citri* Sbp were calculated with programs available at the ExPasy Molecular Biology server.

Cloning.

The nucleotide sequence of the *X. citri sbp* gene (XAC1017), encoding the mature protein without the first 72 base pairs (bp), corresponding to a predicted 24 amino acid signal peptide, was amplified by PCR, using a forward primer (5' GTGCTGCA TATGGCGCAGACCGCC 3') and a reverse primer (5' CGCCAAGCTTTCAATCCTTCAG 3') with Platinum high fidelity *Taq* polymerase (Invitrogen), based on standard

Fig. 5. The three-dimensional structure of *Xanthomonas citri* sulfate-binding protein (Sbp). **A**, *X. citri* Sbp rod crystals diffracted at 1.14 Å; 2 μl of a sample containing 8.9 mg of *X. citri* Sbp in 1 ml of 0.05 M Tris, pH 8.0, and 0.3 μM MgSO_4 were mixed to 2 μl of 0.2 potassium sulfate and 30% PEG 3350. **B**, Part I, One molecule of Sbp as found in the asymmetric unit and, part II, detail of Sbp showing the electronic density. **C**, Three-dimensional structure of Sbp showing the N (blue) and C domains (green) and the sulfate ion in the cleft as red dot spheres. β -sheet structures are detached in darker blue and green in domains N and C, respectively. **D**, Detail of the sulfate-binding site residues in yellow stick evidencing the oxanion interactions.

amplification conditions. The forward primer included one *NdeI* restriction site, while the reverse primer encoded one *HindIII* restriction site (underlined on primer sequences). The resulting amplified fragment, corresponding to 945 bp, was

Table 1. Data processing statistics of the *Xanthomonas citri* sulfate-binding protein (SBP) crystal and refinement data from its structure^a

Parameters	Values
Data collection and processing	
Synchrotron and beamline	Diamond Light Source I02
Date of data collection	30.04.2016
Data collection temperature (K)	100
Wavelength (Å)	0.979490
Resolution range (Å)	30.16 – 1.14 (1.181 – 1.14)
Space group	C121
Unit cell (a, b, c) (Å)	183.06, 54.54, 34.2
(α, β, γ) (°)	90.0, 98.9, 90.0
Total number of reflections	371,345 (16,855)
Number of unique reflections	112,643 (6,232)
Multiplicity	3.7 (2.7)
Completeness (%)	93.3 (70.3)
Mean I/sigma(I)	15.2 (1.9)
Wilson B factor (Å ²)	11.4
R _{merge}	0.039 (0.650)
R _{meas}	0.046 (0.800)
R _{pim}	0.025 (0.459)
CC _{1/2}	0.998 (0.527)
Refinement	
R-work	0.1776 (0.2933)
R-free	0.1905 (0.2981)
Number of nonhydrogen atoms	2725
Macromolecules	2499
Ligands	10
Water	216
Protein residues	314
RMSD ^b (bonds) (Å)	0.006
RMSD (angles) (°)	1.06
Ramachandran favored (%)	98
Ramachandran allowed (%)	2
Ramachandran outliers (%)	0
Clashscore	1.61
Average Bfactor (Å ²)	18.10
Macromolecules	17.60
Ligands	15.60
Solvent	23.50

^a Values in parenthesis correspond to data regarding the last resolution.

^b RMSD = root mean square displacement.

inserted into pGEM T-Easy vector (Promega) and was cleaved with *NdeI* and *HindIII* enzymes (Fermentas) for subsequent cloning into the expression vector pET28a (Novagen), previously digested with the same enzymes. After transformation of chemically competent *E. coli* DH5α cells, 10 recombinant colonies were subjected to restriction analysis and nucleotide sequencing, using the methods of Sanger et al. (1977). The recombinant plasmid pET28a.sbp was further purified in larger quantities and was transformed into the *E. coli* BL21 (DE3) strain (Novagen).

Production of the recombinant *X. citri* Sbp.

Cultures of the recombinant pET28a.sbp-transformed *E. coli* BL21 (DE3) strain were prepared aerobically in Erlenmeyer flasks, with LB broth medium supplemented with 50 µg of kanamycin per milliliter until midlog phase (optical density at 600 nm of 0.5 to 0.6), when isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma) was added to a final concentration of 0.1 mM. The synthesis of the recombinant protein was induced aerobically (200 rpm) for 2 h at 37°C. Cells were collected by centrifugation at 8,000 × g for 15 min at 4°C and were stored at –20°C for approximately 16 h before lysis. The cell pellets were suspended in 20 ml of solubilization buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1% Triton X-100 [final concentration of 30 mg/ml]) for 30 min in an ice bath in the presence of lysozyme (final concentration of 0.1 mg/ml). Cells were sonically disrupted in a Branson digital sonifier (model 450), and soluble fractions were separated from the nonsoluble material by centrifugation at 16,000 × g for 40 min at 4°C. The Sbp protein was purified, in batch, from 20 ml of soluble fraction by immobilized metal affinity chromatography, using nickel-charged resin from GE (HiTrap HP, according to manufacturer's instructions). The charged resin was washed with 10-column volumes of buffer 1 (10 mM Tris-HCl, pH 8.0, 500 mM NaCl), followed by step gradient elution with buffers containing increasing concentrations of imidazole (20 to 500 mM). To eliminate aggregates and contaminants from the previous purification, a size-exclusion chromatography was performed in buffer 2 (10 mM Tris-HCl, pH 8.0, 50 mM NaCl) with a Superdex 16/60 200 column (GE Healthcare). The fractions containing eluted protein were concentrated with Ultrafree MWCO 10,000 centrifugal filters (Amicon Millipore), to a final concentration of 20 mg/ml. Samples of 15 µl of the eluted protein fractions containing approximately 2 mg per milliliter

Fig. 6. Fluorescence and analysis of sulfate binding on *Xanthomonas citri* sulfate-binding protein (Sbp). **A**, Detail of the Sbp ligand-binding site evidencing the two tryptophans close to the ligand. **B**, Fluorescence emission spectra of Sbp in the presence of different concentrations of SO_4^{2-} . Sbp (1 µM) was titrated with SO_4^{2-} (0, 60, 80, 90, 110 nM) or MoO_4^{2-} (100 and 500 µM) and was excited at 295 nm at 20°C. **C**, Determination of K_b and n for sulfate binding to Sbp by fluorimetric data.

were analyzed by SDS-PAGE, using 12% acrylamide gels stained with Coomassie blue. Protein concentration was spectrophotometrically determined, using the method by Edelhoch (1967). To evaluate the polydispersity of the samples, a 1 mg/ml concentration of size-exclusion chromatograph-purified Sbp was centrifuged at $10.000 \times g$ for 30 min and was then analyzed by DynaPro99 DLS equipment (Wyatt Technology Corp.) at 18°C. It was collected for 300 accumulations with 10 s each. The samples that presented less than 20% polydispersity were selected for crystallization trials.

Crystallization and structure determination.

The purified recombinant Sbp, diluted in 6.7 mM Tris-HCl, pH 8.0, at the final concentration of 8.9 mg/ml, was submitted to crystallization trials at 18°C, using the sitting-drop vapor-diffusion technique, using the Oryx6 (Douglas Instruments, Ltd.) automatic protein crystallization system at the Crystallographic X-Ray Facility of the Department of Biochemistry, University of Cambridge, United Kingdom. The solution in which Sbp formed a rod crystal was 0.2 M potassium sulfate, 20% (wt/vol) PEG3350, which provided protection against ice formation. Crystallographic data from this single frozen crystal were collected at the protein crystallography beamline I02 from Diamond Light Source, using a Pilatus 6M detector. Datasets of

Sbp were indexed and integrated using XDS (Kabsch 2010) and molecular replacement was carried out with Phaser MR34 (McCoy et al. 2007), taking the *S. typhimurium* Sbp (PDB code 1SBP) (He and Quioco 1993), which shares 65% amino acid sequence identity, as starting model. The model was refined using PHENIX 1.11.1 (Adams et al. 2010) and was checked by PROCHECK (Laskowski et al. 1993). The structural coordinates for *X. citri* Sbp were deposited in PDB with the accession code 5UM2. The program COOT (Emsley and Cowtan 2010) was used for model building. All pictures were generated with the PYMOL software (Delano 2002).

CD assays.

Measurements were carried out with a JASCO J-810 spectropolarimeter equipped with a Peltier-type temperature controller and a thermostated cell holder, interfaced with a thermostatic bath. For far-UV analysis, spectra were recorded in 0.1-cm path length quartz cells at a protein concentration of 5 μ M in 10 mM Tris-HCl buffer at pH 8.0, in the absence or presence of 10 μ M of sodium sulfate or sodium molybdate. For near-UV analysis, spectra were recorded in 1-cm path length quartz cells at a protein concentration of 18 μ M in the absence or presence of the ligands at 36 μ M. Twenty consecutive scans were compiled and the average spectra was stored. The data

Fig. 7. Comparison between *Xanthomonas citri* sulfate-binding protein (Sbp) and ModA proteins. **A**, The amino acid sequence structure-based alignment of ModA and Sbp. Amino acids forming secondary structures as β -sheets and α -helices are colored in gray and yellow, respectively. Residues that interact with the oxanions are shown in red and a black box shows the location of W¹⁹⁴ and Y¹²⁵. **B**, Structural comparison between Sbp and ModA. Panel I, Structural alignment of Sbp (blue) and ModA (gray) shown as cartoon. Extra secondary structures in Sbp are shown in orange. Panels II and III, The internal surfaces and pockets (red circles) of Sbp and ModA, respectively. Panel IV, Superimposition of the ligand-binding pocket and residues (stick) in the two structures. Panels V and VI, Detail of the residues in Sbp and ModA pockets, respectively. The residues W¹⁹⁴ and Y¹²⁵ and pockets are shown in red (full for Sbp and dashed line for ModA).

were corrected for the baseline contribution of the buffer and the observed ellipticities were converted into the mean residue ellipticities (θ) based on a mean residue molecular mass of 37.5 Da. The T_m represents the temperature at the midpoint of the unfolding transition. The secondary structure was estimated from fitted far-UV CD spectra, using the CDNN software package (Böhm et al. 1992).

Intrinsic tryptophan fluorescence.

Sulfate binding was followed by fluorescence measurements using an Aminco BOWMAN series 2 spectrofluorometer. Excitation and emission bandwidths were 4 and 8 nm, respectively. The fluorescence cell (1 × 1 cm) was mounted on a thermostatic holder. Tryptophan fluorescence was measured with an excitation wavelength of 295 nm and the emission spectra were recorded between 300 and 450 nm. All measurements were performed in 10 mM Tris-HCl, pH 8.0, at 20°C, with the protein at a concentration of 1 μ M, calculated using the extinction coefficient for a molecular weight of 37.5 kDa. Sodium sulfate was titrated to the protein solution to give a final ligand concentration in the range of 60 to 110 nM and the respective emission spectra were recorded. As a control, putative effects on Sbp fluorescence caused by the sulfate analog sodium molybdate up to 500 μ M were also evaluated. The measured fluorescence data were corrected for dilution and inner filter effects (Mertens and Kägi 1979). The obtained fluorescence data were normalized and, in order to estimate the number of sulfate binding sites to Sbp, n and the binding constant (K_b) data were further evaluated, using equation 1:

$$\log \left[\frac{(F_0 - F)}{F} \right] = \log K_b + n \log [\text{ligand}],$$

where F_0 and F are the values of Sbp fluorescence intensity on $\lambda_{\max}$ (wavelength of maximum intensity of apo-Sbp fluorescence) in the absence and presence of the ligand, respectively, K_b is the binding constant, and [ligand] is the ligand concentration (Bourassa et al. 2010; Pinto et al. 2010).

In vivo and in vitro transcriptional activation of the p1017 promoter.

To analyze the activation of the *sbpcysUWA* operon, a fragment of 776 bp upstream of the start codon from the *sbp* gene (XAC1017), containing the p1017 promoter sequence as predicted by BProm Software, was amplified by PCR, using the oligonucleotides 5'-GGGCCCATGTACCAGCGCGTTG TATTTGCG-3' and 5'-CCATGGAACGGAACGGGAA TATGTCACGACG-3' as forward and reverse primers, respectively. The amplified fragment was cloned into pGEM/EGFP (Barbosa and Benedetti 2007), using the restriction enzymes *Apa*I and *Nco*I to obtain the pGEM.p1017EGFP vector, which had the p1017-EGFP cassette subcloned into the pKX33 vector (Baldini et al. 1999). The final vector for expression in *X. citri* was pKX33.p1017EGFP and contained the *EGFP* gene (Chalfie et al. 1994) under control of the p1017 promoter. To measure the activity of the promoter, *X. citri* cells were transformed with the pKX33.p1017EGFP vector and were inoculated in LB broth for growth for 24 h. The EGFP fluorescence was measured in 96-well plates, using 100 μ l of cultures in the EnSpire Multimode Plate Reader (PerkinElmer) and were plotted as a relative quantification of the fluorescence. The bacteria also were observed in a fluorescence microscopy. Similarly, to analyze the p1017 promoter activation in vivo, *Citrus sinensis* leaves were infected with *X. citri* wild type carrying the pKX33.p1017EGFP vector. The fluorescence of *X. citri* carrying the plasmid pKX33.p1017EGFP and extracted from infected citrus leaves, according to Vanini and collaborators

(2008), was measured after 7 or 10 days or 2 weeks from the infection.

Samples for proteomics analysis.

Production of native Sbp in *X. citri* was monitored in three different growth conditions. After cell growth during overnight incubation at 30°C and 200 rpm in LB broth, cultures were washed three times with sterile water to remove media contamination and were normalized for inoculation at a rate of 10⁵ CFU/ml in LB broth (rich medium), M9 minimum medium (with 2 mM magnesium sulfate), and M9.1/2 minimum medium (with 1 mM magnesium sulfate). Cultures were maintained at 30°C, 200 rpm, for 72 hs. After growth, cells were harvested by centrifugation 6700 × *g* for 20 min and were resuspended, according to Pegos and collaborators (2014). The supernatants were quantified using the method by Bradford (1976). Samples containing 100 μ g of protein were applied in 12% SDS-PAGE running at 120 V for 2 hs and were stained with Coomassie blue. The regions corresponding to the range between 25 and 45 kDa were excised from gel and were submitted to trypsin digestion, as previously described (Paes-Leme et al. 2012). The runs were performed at the Mass Spectrometry Laboratory facility at the Brazilian National Laboratory of Biosciences (LNBio), Centro de Pesquisas em Energia e Materiais, Campinas, Brazil.

Tandem mass spectrometry (MS/MS) and data analysis.

The peptide mixtures were separated using a column C18 Rp-nano UPLC (nanoAcquity; Waters Corp) coupled to a Q-TOF Ultima API mass spectrometer (Waters Corp.) with a nano electrospray source at a flow rate 0.6 nl/min. The digested proteins were submitted to a gradient of 2 to 90% of acetonitrile in 0.1% formic acid over 45 min. The equipment was set to 3.5 kV, the cone voltage 30 V, and the temperature 100°C. The mode was 'top three', in which one MS spectrum is acquired, followed by MS/MS of the three most intense peaks. Technical samples and spectra were acquired using MassLynx software and the raw data files were converted by Mascot Distiller v.2.3.2.0 (Matrix Science Ltd.) for searching against the *Xanthomonas axonopodis* pv. *citri* 306 database (NCBI taxonomy identification number 190486), using mascot with carbamidomethylation as fixed modification, oxidation of methionine as a variable modification, one trypsin missed cleavage, and a tolerance of 0.1 Da for the precursor and fragment ions. For protein quantity measurement, the mascot output files were analyzed in Scaffold Q+ (version 3.6 Proteomic Software Inc.), also used to calculate the normalized spectral counts and to validate peptide and protein identifications considering the parameters to obtain a false discovery rate of less than 1% than protein and peptides, as previously described (Nesvizhskii et al. 2003).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Bioinformatics Center Institute for Chemical Research Kyoto University
 GenomeNet database: www.genome.jp
 DAS Transmembrane Prediction server:
<http://www.sbc.su.se/~miklos/DAS>
 Expasy Molecular Biology server: <http://www.expasy.org>
 SignalP server: <http://www.cbs.dtu.dk/services/SignalP>
 Softberry's BProm software: <http://www.softberry.com>