Structural and Physiological Analyses of the Alkanesulphonate-Binding Protein (SsuA) of the Citrus Pathogen Xanthomonas citri

Fabiano Tófoli de Araújo¹, Victor M. Bolanos-Garcia², Cristiane T. Pereira³, Mario Sanches⁴, Elisa E. Oshiro¹, Rita C. C. Ferreira¹, Dimitri Y. Chigardze², João Alexandre Gonçalves Barbosa⁵, Luís Carlos de Souza Ferreira¹, Celso E. Benedetti³, Tom L. Blundell², Andrea Balan³*

¹ Departamento de Microbiologia, Universidade de São Paulo, São Paulo, São Paulo, Brazil, ² Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom, ³ Laboratório Nacional de Bicências, Centro de Pesquisa em Energia e Materiais, Campinas, São Paulo, Brazil, ⁴ Monte Sinai Hospital, Toronto, Ontario, Canada, ⁵ Departamento de Genética, Universidade Católica de Brasília, Brasília, Distrito Federal, Brazil

Abstract

Background: The uptake of sulphur-containing compounds plays a pivotal role in the physiology of bacteria that live in aerobic soils where organosulfur compounds such as sulphonates and sulphate esters represent more than 95% of the available sulphur. Until now, no information has been available on the uptake of sulphonates by bacterial plant pathogens, particularly those of the Xanthomonas genus, which encompasses several pathogenic species. In the present study, we characterised the alkanesulphonate uptake system (Ssu) of Xanthomonas axonopodis pv. citri 306 strain (X. citri), the etiological agent of citrus canker.

Methodology/Principal Findings: A single operon-like gene cluster (ssuEDACB) that encodes both the sulphur uptake system and enzymes involved in desulphurisation was detected in the genomes of X. citri and of the closely related species. We characterised X. citri SsuA protein, a periplasmic alkanesulphonate-binding protein that, together with SsuC and SsuB, defines the alkanesulphonate uptake system. The crystal structure of SsuA bound to MOPS, MES and HEPES, which is herein described for the first time, provides evidence for the importance of a conserved dipole in sulphate group coordination, identifies specific amino acids interacting with the sulphate group and shows the presence of a rather large binding pocket that explains the rather wide range of molecules recognised by the protein. Isolation of an isogenic ssuA-knockout derivative of the X. citri 306 strain showed that disruption of alkanesulphonate uptake affects both xanthan gum production and generation of canker lesions in sweet orange leaves.

Conclusions/Significance: The present study unravels unique structural and functional features of the X. citri SsuA protein and provides the first experimental evidence that an ABC uptake system affects the virulence of this phytopathogen.

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* E-mail: andrea.balan@lnbio.cnpem.br

Introduction

All living organisms require sulphur for the biosynthesis of amino acids (cysteine and methionine) and cofactors such as glutathione, coenzyme A and coenzyme M [1]. Bacteria must either obtain these molecules directly from the environment or synthesise them using inorganic (e.g., sulphate) or organic (e.g., sulphonates) sulphur sources [2,3]. In aerobic soils, the sulphur content is almost entirely represented by sulphonates and sulphate esters of various organic compounds, with inorganic sulphur representing less than 1–5% of the available element [4]. Under such conditions, several bacterial species are known to express proteins required for the uptake of organic sulphur-containing substances such as sulphate esters, sulphonamates, sulphonates and alkanesulphonates [1,5,6].

In Escherichia coli, the uptake of sulphur-containing organic compounds requires the expression of two ATP binding cassette (ABC) transport systems: the Tau system, which is specifically involved in the uptake of taurine, and the Ssu system, which mediates the uptake of other aliphatic sulphonates [5,7]. The Ssu system is encoded by a single operon encompassing five cistrons, ssuEADCB, that encode the three components of the uptake system, which are the periplasmic alkanesulphonate-binding protein (SsuA), the membrane permease protein (SsuC) and the nucleotide-binding protein (SsuB), and two cytoplasmic proteins involved in sulphur release, NAD(P)H flavin mononucleotide oxidoreductase (SsuD) and the FMNH2-dependent sulphonate monooxygenase (SsuE) [7]. Structural analysis of the Ssu uptake system has been restricted to the recent description of the substrate-free E. coli SsuA protein [8]. The SsuA protein is similar...
to other periplasmic ABC transporters in which two globular domains form a cleft in which the ligand binds and from which it is subsequently translocated to the membrane-bound compartment. However, no information is presently available about the structure of the SsuA protein-ligand complex, the interactions of specific amino acid residues with the alkanesulphonate substrates or the molecular features that allow various alkanesulphonate molecules to fit into the protein’s ligand-binding pocket. In addition to the Ssu system of *E. coli*, functional Ssu uptake systems have also been reported in *Bacillus subtilis* and *Pseudomonas putida*, further supporting the relevant physiological role of this nutrient uptake system in soil-inhabiting bacteria [6,9]. Despite the apparently relevant physiological role of the Ssu system, no information is available regarding the uptake of organic sulphur-containing compounds by bacterial plant pathogens. Indeed, once absorbed by plants as inorganic sulphate, sulphur is rapidly converted into complex organic molecules such as proteins and sulphonates or to sulphonylumino in sulphinolipids of thylakoid membranes [7]. However, no information regarding the role of aliphatic sulphonate uptake in the growth and virulence of phytopathogens is presently available.

The *Xanthomonas* genus encompasses 27 different bacterial species and over 140 pathovars that interact with more than 400 plant species, including several economically relevant species [10,11]. *Xanthomonas citri*, the causative agent of citrus canker, is capable of infecting all citrus cultivars, although different citrus species may show distinct susceptibility to the disease, as illustrated by the high susceptibility of sweet orange (*Citrus sinensis*) and the lower susceptibility of mandarin species [12]. Canker is one of the most economically damaging diseases of citrus plants; it begins with the epiphytic colonisation of the leaf surface followed by the entrance of the pathogen into leaf tissue through stomata or wounds. Upon entrance into the plant mesophyll, the bacterium induces cell enlargement and multiplication (hyperplasia) followed by a water-soaking phenotype and the formation of blister-like lesions approximately 4 days after infection. The production of highly hygroscopic xanthan gum helps bacteria increase the water adsorption of the plant through the capillary effect from xylem, leading to disruption of the plant epidermis and formation of yellow spongy pustules that become brown and corky with time [13].

In the present study, we investigated for the first time the presence of alkanesulphonate uptake systems in the *Xanthomonas* genus, with particular emphasis on the system encoded by *X. citri*. A single operon-like gene cluster (ssuEDACB) encoding both the uptake system and the intracellular enzymes involved in sulphur release was detected in the genome of the *X. citri* 306 strain but not in other *Xanthomonas* species with the exception of the closely related *X. fuscans*. The structure of SsuA in complex with MOPS, MES and HEPES was determined, showing the importance of a conserved dipole at the substrate cleft for sulphate group coordination and the presence of a rather spacious ligand pocket, partially filled with water molecules, that permits the binding of alkanesulphonates of quite different molecular sizes, charges and shapes. We also showed that an active alkanesulphonate uptake system is required for the growth of *X. citri* under sulphate-restricted conditions. Finally, generation of a *ssuA*-knockout mutant of the *X. citri* 306 strain showed that a defective alkanesulphonate uptake system affects both xanthan gum production and the generation of canker lesions in a susceptible citrus host. Altogether, the present work represents the first structural and functional characterisation of an alkanesulphonate uptake system of a bacterial plant pathogen and demonstrates that this nutrient uptake system plays a role in the pathogenesis of *X. citri*.

**Results**

The alkanesulphonate uptake system in *Xanthomonas*

A search of the available *Xanthomonas* genomes for genes involved in the uptake of alkanesulphonates revealed 4 species (*X. citri*, *X. fuscans*, *X. gardneri*, and *X. campestris*) in which a single operon-like gene cluster sharing similarity with *E. coli* ssu genes was found (Figure 1). The *X. citri* ssu genes showed a similar genetic organisation to that of the *ssu* operon found in *E. coli* with the exception that *ssuD* and *ssuA* were present in inverted positions in the two species. The fact that the putative functional organisations of these genes have been maintained in two citrus pathogens indicates that the *ssu* genes have a recent evolutionary history in the genus. Further structural and functional analyses of the alkanesulphonate uptake genes concentrated on *X. citri* SsuA, the alkanesulphonate-binding protein. The deduced amino acid sequence of the *X. citri* SsuA protein shared 59% identity with the *E. coli* orthologue, and the *X. citri* SsuA gene was chosen for subsequent cloning, expression and purification of a recombinant form of the protein.

Interaction of recombinant *X. citri* SsuA with various alkanesulphonate substrates

The recombinant *X. citri* SsuA protein was expressed as a soluble cytosolic protein genetically fused with a histidine tag and a thrombin cleavage site at the N-terminal end. Maximum soluble protein yields (~80 mg/L) were achieved after expression under optimum inducing conditions. The protein was purified by single-step affinity chromatography and subsequently cleaved with thrombin to remove the vector-encoded histidine (Figure 2). The purified recombinant protein remained soluble and stable at high concentrations (6–12 mg/mL) even after extended storage at −20°C. Thermal shift experiments showed increased stability of recombinant *X. citri* SsuA in the presence of MOPS, MES, CHES, or HEPES but not in the presence of taurine, sulphate, thiosulphate or hydroxylamine (Figure 3A). These results indicate that the recombinant protein binds specifically to alkanesulphonate ligands. The *Tm* of SsuA varied from 39.5°C for the unbound form to 43.5°C (CHES), 45.8°C (HEPES), 47.1°C (MES) and 51°C (MOPS) for the putative ligand-bound forms. CD and fluorescence analyses showed that the recombinant SsuA was stable at pH values of 5, 7 and 9 (Figure 3B and 3C), and that it undergoes small changes in secondary structure content upon ligand binding (Figure 3D). The quenching of fluorescence observed after the addition of alkanesulphonates corroborated the CD results and suggested the possible presence of tryptophan residues close to the ligand-binding pocket (Figure 3E). Taken together, these results show that recombinant SsuA undergoes conformational changes on binding to alkanesulphonates.

The structure of ligand-bound *X. citri* SsuA

SsuA crystallised under a variety of conditions in which ammonium sulphate and alkanesulphonates (HEPES and MES) were present (Table S1). Initial crystallisation trials of the ligand-bound form of recombinant SsuA resulted in needle-like crystals that grew after one week in the presence of ammonium sulphate, MES and PEG. Refinement of these conditions produced better crystals in the absence of PEG and the presence of sodium chloride. The best diffraction patterns (1.7 and 1.9 Å) were obtained from crystals grown in 0.1 M NaCl, 1.6 M ammonium nitrate complexes, PEG, or thioglycolic acid. The structure of the C-terminal fragment of SsuA in complex with MOPS, MES and HEPES was determined, showing the importance of a conserved dipole at the substrate cleft for sulphate group coordination and the presence of a rather spacious ligand pocket, partially filled with water molecules, that permits the binding of alkanesulphonates of quite different molecular sizes, charges and shapes. We also showed that an active alkanesulphonate uptake system is required for the growth of *X. citri* under sulphate-restricted conditions. Finally, generation of a *ssuA*-knockout mutant of the *X. citri* 306 strain showed that a defective alkanesulphonate uptake system affects both xanthan gum production and the generation of canker lesions in a susceptible citrus host. Altogether, the present work represents the first structural and functional characterisation of an alkanesulphonate uptake system of a bacterial plant pathogen and demonstrates that this nutrient uptake system plays a role in the pathogenesis of *X. citri*.
The SsuA Protein from *Xanthomonas citri*

sulphate, and 0.1 M HEPES, pH 7.5 (Figures 4A and 4B). These crystals showed symmetry and systematic absence of the orthorhombic space group P2_1. Table 1 summarises the data-collection statistics. The Matthews coefficient was calculated to be 1.67, and a solvent content of 26%, corresponding to 1 molecule in the asymmetric unit, was calculated. The SsuA crystal structure revealed the characteristic folding of periplasmic-binding proteins, which consists of an α/β sandwich pattern organised into two domains (I and II) separated by a cleft at the binding site, with the ligand remaining hidden inside the pocket (Figure 4C). The N- and C-termini are present in domain I, which is connected by a hinge (Pro^{107} to Thr^{109} and Gly^{303} to Gly^{211}) to domain II. The tertiary structures of SsuA bound to MOPS and MES (PDB codes 3KSJ and 3KSX, respectively) were generated by molecular replacement using the structural coordinates of SsuA bound to HEPES (PDB code 3E4R). All crystal structures presented one molecule in the asymmetric unit. The refinement statistics, including statistics from the processing of the collected data and structure refinement, are presented in Table 1.
The SsuA ligand-binding pocket and interactions with alkanesulphonates

The crystal structure of SsuA bound to each of the three different alkanesulphonates tested showed that ligand binding is stabilised primarily through a range of polar interactions between the sulphonic acid oxygen atoms and the NH groups of main-chain peptide hydrogen bonds (Gly36, Gly86, Ser141), one side chain NH group of Gln36 and one hydroxyl group of Ser141 (Figure 5A and Table S2). Water molecules are responsible for the stability of the alkane chains. Residues in the pocket that interacts with the alkanesulphonates are highly conserved among orthologues from phytopathogens, plant-associated and soil bacteria, and enterobacteria (Figure 5B). Some residues exclusively found in X. citri SsuA confer a more apolar local environment than that observed in the other bacterial orthologues (Figure 5B, green colour).

The SsuA ligand-binding pocket has a volume of 1635±26 Å³ and an area of 2951±12 Å², but only 14% of the volume is occupied by the ligand molecule (Figure 5C). The large binding pocket and the participation of at least 12 hydrophobic residues in forming this site reveal an adaptation that could explain the binding of a rather wide range of alkanesulphonate molecules of different sizes and shapes by this protein. Corroborating the intrinsic fluorescence data, W185 faced the binding pocket, stabilising the alkane chain. Interestingly, all of the residues responsible for coordination of the sulphate (Gln36, Gly36, and Ser141) belong to two helices and one strand (a2, a5 and b3) (Figure 5G, in green); together, these residues define a positively charged cluster (Figure 5D). A similar structural organisation is found in other periplasmic ion-binding proteins such as the E. coli aliphatic sulphonate-binding protein, the Sinechocystis sp nitrate-binding protein, the E. coli phosphate-binding protein, the Thermus thermophilus glutamate/glutamine-binding protein and the X. citri molybdate-binding protein (Figure 5E).

Functional roles of X. citri SsuA

To evaluate the physiological role of SsuA during in vitro and in vivo growth of X. citri, a ssuA knockout strain (Xac::ssuA) was generated by site-specific mutagenesis (Figure 6A and B). A complementary strain (Xac::ssuAc) was generated by transformation of Xac::ssuA with the pKX33-ssuAc vector, which encoded the full length ssuAc gene under control of the native ssuA promoter, giving rise to the Xac::ssuAc strain (Figure 6C). The isogenic X. citri ssuA-deleted strain was unable to grow in minimal medium containing alkanesulphonates (HEPES, MOPS, or MES) as the sole sulphur source but grew well in the presence of sulphate (Figures 7A and 7B). This result indicated that the ssuA operon is functional and is required for alkanesulphonate uptake in X. citri. In addition, the Xac::ssuA mutant formed small bright yellow colonies after growth in LB plates for 24 h (Figure 7C). In both situations the altered phenotypes were reverted in the Xac::ssuAc strain (Figure 7C). The altered colony morphology of the Xac::ssuA strain suggests a decrease in xanthan gum production, a feature previously observed by other groups [14,15,16]. Indeed, we determined that xanthan gum production by the Xac::ssuA strain during growth in LB medium was at least three-fold lower than that of the parental strain (Figure 7D). Complementation with the pKX33-ssuAc restored in a great extent the colony morphology and production of xanthan gum by the Xac::ssuA strain (Figure 7D).

To evaluate the effects of alkanesulphonate uptake on the
pathogenicity of \textit{X. citri}, we monitored the behaviour of the wild-type, the \textit{Xac::ssuA} and \textit{Xac::ssuAc} strains after in vivo inoculation into a susceptible citrus host (\textit{C. sinensis}). As shown in Figure 8A, the \textit{Xac::ssuA} strain showed defective growth in leaf tissues 6 days after inoculation in comparison with the parental and complemented strains. In addition, the leaf lesions formed by the \textit{Xac::ssuA} strain were smaller than those formed by the parental strain (data not shown). Leaves infected with the \textit{X. citri} 306 strain

\textbf{Figure 3.} Spectroscopic analysis of \textit{X. citri} SsuA protein in the presence of alkanesulphonates and at different pH values. (A) Thermal shift assay in the presence of various alkanesulphonates (CHES, MOPS, MES, or HEPES). (B) Circular dichroism and (C) fluorescence analyses of the recombinant protein at different pH values. (D) Circular dichroism and (E) intrinsic fluorescence of the recombinant \textit{X. citri} SsuA in the presence of ligands. The stability of the protein at various pH values and in the presence of various aliphatic sulphonates was monitored by following the intrinsic fluorescence of the tryptophan residues using an Aminco BOWMAN series 2 spectrofluorometer.

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showed typical blister-like lesions characteristic of the hyperplasia and the water-soaking phenotypes (Figure 8B, I to III). Complementation of the \textit{Xac::ssuA} with pKX33-\textit{pssuA} restored the canker lesions symptoms to the wild-type levels (Figure 8B, IV to VI). In contrast, much reduced lesions were observed in leaves inoculated with the \textit{Xac::ssuA} strain, and the lesions that formed were not blister-like (Figure 8B, VII to IX). Collectively, these results indicate that the alkanesulphonate uptake system also affects the in vivo behaviour of \textit{X. citri} in \textit{C. sinensis} plants.

**Discussion**

Although sulphonates and sulphur-containing organic compounds represent the most abundant sulphur source in most soils, there are no data regarding the role of alkanesulphonate uptake in
the behaviour of plant pathogens, particularly those belonging to the *Xanthomonas* genus. In this work, we showed that a complete set of genes required for the uptake and metabolism of alkanesulphonates is found in the genomes of *X. citri* and *X. fuscans*, two closely related citrus pathogens. We expressed and purified a recombinant form of *X. citri* SsuA that specifically binds to various alkanesulphonates in the physiology and pathogenicity of this pathogen. For that purpose, we determined the crystal structure of the *X. citri* Ssu uptake system in *X. fuscans*. Using an isogenic strain, we showed that a complete set of *ssu* genes including the ABC transporter and enzymes involved in sulphonate desulphurisation (*ssuD* and *ssuE*). This finding contrasts with the widespread occurrence of functional genes involved with the uptake and metabolism of alkanesulphonates in many bacterial genera such as *Pseudomonas*, *Shewanella*, *Streptomyces*, and *Nostoc*, these bacteria are present in soil and live in environments in which sulphate esters and carbon-bonded sulphur (sulphonates or amino acid sulphur) represent most of the available sulphur [17,1]. The paucity of *ssu* genes in *Xanthomonas* species suggests that these genes have been recently acquired in the evolutionary history of the genus, most likely by a horizontal gene transfer event. This finding also raises questions about the role of alkanesulphonate uptake in the physiology of the species in which these genes are found.

The remainder of our study focused on the investigation of the *Ssu* system in *X. citri*, the most economically relevant citrus pathogen. For that purpose, we determined the crystal structure and characterised the physiological role of the SsuA protein, the periplasmic component that confers specificity and affinity to the uptake system [1]. As a first step, we generated a recombinant form of *X. citri* SsuA that retained alkanesulphonate-binding properties similar to those expected for the native protein. As

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**Table 1.** Data processing statistics of the *X. citri* SsuA crystals and refinement data from its structures.

| Crystal | Native SsuA+/HEPES | Nal derivative | CsCl derivative | SsuA+/MOPS | SsuA+/MES |
|---------|---------------------|----------------|----------------|------------|-----------|
| **X-ray diffraction data** | | | | | |
| Wavelength (Å) | 1.433 | 1.433 | 1.433 | 1.46 | 1.46 |
| Space group | P2₁ | P2₁ | P2₁ | P2₁ | P2₁ |
| Unit cell parameters | a = 30.66 | a = 30.68 | a = 30.68 | a = 30.98 | a = 30.87 |
| | b = 85.50 | b = 85.18 | b = 85.18 | b = 86.21 | b = 86.36 |
| | c = 46.83 | c = 46.89 | c = 46.90 | c = 46.73 | c = 46.45 |
| | α = 90.00 | α = 90.00 | α = 90.00 | α = 90.00 | α = 90.00 |
| | β = 98.08 | β = 98.20 | β = 98.20 | β = 97.76 | β = 97.46 |
| | γ = 90.00 | γ = 90.00 | γ = 90.00 | γ = 90.00 | γ = 90.00 |
| Resolution range (Å) | 30.00–2.00 | 30.00–2.30 | 30.00–2.38 | 26.00–1.70 | 26.00–1.99 |
| Rsym (%) | 0.062 (0.162) | 0.062 (0.162) | 0.062 (0.162) | 0.09 (0.23) | 0.11 (0.40) |
| Completeness (%) | 99.3 (99.7) | 97.9 (95.7) | 98.0 (94.7) | 97.9 (95.7) | 95.1 (80.5) |
| Redundancy | 4.3 | 4.0 | 4.0 | 5.8 | 3.4 |
| <I/σ(I)> | 23.88 (7.48) | 13.98 (3.48) | 14.18 (3.48) | 18.30 (3.0) | 10.42 (2.1) |
| Mosaicity (deg) | 0.456 | 0.771 | 0.771 | 0.500 | 0.912 |
| Wilson Plot B-factor (Å²) | 14.6 | 22.67 | 41.04 | |
| **Refinement and model quality** | | | | | |
| Resolution range (Å) | 46.37 – 2.01 | 26.14 – 1.70 | 24.09 – 1.99 | |
| Number of reflections: work/test | 15046 | 21275 | 14798 | |
| Rvalue (%) | 0.145 | 0.162 | 0.184 | |
| Rfree (%) | 0.220 | 0.213 | 0.256 | |
| Overall mean B-factor (Å²) | 13.96 | 21.0 | 38.02 | |

Values in parenthesis correspond to data regarding the last resolution shell.

Footnote: * R-factor = \( \frac{\sum |F_o(h)| - |F_c(h)|}{\sum |F_o(h)|} \) where \( F_o(h) \) and \( F_c(h) \) are observed and calculated amplitudes for reflection h. R-free is calculated by the same equation using 5 % of the data, chosen randomly and omitted from the refinement.

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Figure 5. Ligand-binding site and interactions of the \textit{X. citri} SsuA protein. (A) Ligand interactions of SsuA and HEPES, MES and MOPS. Domains I and II are coloured in blue and yellow, respectively; the residues involved in the ligand interaction, as well as the ligands themselves, are shown as sticks. (B) SsuA topology and conservation of the ligand-binding sites in different orthologues. The residues that form the pocket and those that interact with ligands are marked in clear and dark grey, respectively. The residues marked in green are unique to \textit{X. citri} SsuA. The numbering follows the \textit{X. citri} SsuA sequence. Xac: \textit{X. citri} (GI: 21243924); Avi: \textit{Azotobacter vinelandii} (GI: 67153214); Spr: \textit{Serratia proteomaculans} (GI: 5604713); Eco: \textit{E. coli} (GI: 90111189); Sfl: \textit{Shigella flexneri} (GI: 161486517); Ype: \textit{Yersinia pestis} (GI: 22124165); Rso: \textit{Ralstonia solanacearum} (GI: 207723295); Ppu: \textit{Pseudomonas putida} (GI: 167031279); Bph: \textit{Burkholderia phytofirmans} (GI: 187921640); and Atu: \textit{Agrobacterium tumefaciens} (GI: 159184964). (C) Structure of SsuA in ribbon diagram showing the positioning of the two helices and the beta-sheet (forest cartoon) that form the dipole. The ligand-binding pocket and HEPES inside are shown, respectively, in transparent surface and stick. (D) Electrostatic potential at the surface of the two helices (a2 and a5) and the beta-strand (b3) that contain the residues for dipole formation and sulphate group coordination. (E) Structural superposition of secondary structures of periplasmic-binding proteins (grey) with dipoles similar to that found in the SsuA structure (shown in red). The superposed
experimentally demonstrated, the recombinant protein exhibited changes in secondary structure and increased thermal stability after specific binding to MOPS, MES, CHES and HEPES but not after exposure to taurine, sulphate, thiosulphate or hydroxylamine. These results indicate that the X. citri Ssu system is apparently specific for alkanesulphonates, as previously reported for Ssu orthologues found in P. putida, E. coli and B. subtilis [18,7,6].

Previous description of the crystal structure of Ssu components has been restricted to the unbound form of E. coli SsuA [8]. The availability of large amounts of recombinant X. citri SsuA capable of binding to various alkanesulphonates permitted us to solve for the first time the crystal structure of a bacterial SsuA protein in complex with HEPES, MOPS and MES. The size of the ligand cavity and the presence of 12 hydrophobic residues within it reveal an adaptation of the SsuA protein for interaction with long-chain alkane groups. Similarly relevant is the presence of water molecules in the binding pocket, which may offer flexibility to accommodate the ligands. In agreement with the results of spectroscopic assays, the crystal structure results indicate that SsuA-ligand interactions involve conformational changes associated with movement of the protein domains. According to the three-dimensional structure of SsuA, W185 is the most probable residue involved in the observed quenching of fluorescence after ligand binding because it is in close contact with the bound alkanesulphonate and stabilises the aromatic ring of the apolar chains of the molecule. The higher thermal stability evidenced when SsuA is bound to MOPS is due to the presence of shorter hydrogen bonds with the residues belonging to the dipole. Indeed, we showed that alkanesulphonate stabilisation of SsuA is centred in residues that are conserved in different SsuA orthologues, primarily those that form a positive dipole that specifically attracts and interacts with the sulphate group of the alkanesulphonate molecule [19–21]. The mechanism of binding and the very positive electrostatic potential of the entrance of the binding pocket suggest that the ligand is attracted to the protein by the sulphate group and then stabilised inside the pocket by the water molecules and the apolar environment. The X. citri SsuA structure reported here represents the first report of a bacterial SsuA orthologue bound to different alcohols.

Figure 6. Construction of the X. citri ssuA-deleted mutant (Xac::ssuA) and complemented strain (Xac::ssuA). (A) Chromosomal deletion of the ssuA gene was obtained after electroporation of the suicide pNssuA plasmid into the X. citri 306 strain. (I) The first step in the construction of the X. citri mutant was the insertion of a 2-kb fragment encoding resistance to spectinomycin and streptomycin into the KpnI site of the ssuA gene, originating within the pNssuA (8,152 bp) vector. (II) After transformation of wild-type X. citri with pNssuA, a double recombination event generated the Xac::ssuA mutant strain (III), which was screened by selection of cells resistant to both spectinomycin and sucrose. (B) PCR amplification of ssuA genes of selected X. citri colonies using primers FssuA2Nde28a and RssuA2Hind28a. Samples: P, molecular weight markers; 1–3, colonies selected for resistance to spectinomycin and sucrose. The presence of a single 2-kb band indicates a successful gene replacement event (samples 1 and 2), while amplification of two bands of 1 kb and 2 kb in size indicates the presence of the chromosomal wild-type gene and a copy of the mutated ssuA gene (sample 3). (C) Strategy of cloning and digestion analysis of the pKX33-ssuA plasmid. The localization of ssuA gene and the promoter region in the ssu operon are evidenced in yellow and red colours, respectively. A band of 1,926 bp was generated after cleavage of the pKX33-ssuA plasmid with SalI and XbaI restriction enzymes.

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alkanesulphonates and provides a reference for future studies of alkanesulphonate ABC transporters in different bacterial species.

The results obtained in vitro showed that a bacterial strain carrying an inactive copy of the ssuA gene failed to grow in the presence of alkanesulphonates such as HEPES, MOPS and MES under inorganic sulphate starvation conditions. This observation indicates that alkanesulphonate uptake is active in X. citri and that it may be required under environmental conditions in which the availability of inorganic sulphate is restricted. Xenobiotic and naturally occurring sulphonates, including taurine, isethionate, cysteic acid, methanesulphonate, and several undefined sulphonates, make up a large part of the sulphur present in soil humus and marine sediments [2,17]. In plants, in addition to sulphonolipids found in thylakoid membranes, a variety of S-containing secondary metabolites that play important roles in defence against pathogens are synthesised [22]. Our results demonstrated that disruption of the ssuA gene resulted in an attenuated phenotype following infection of a susceptible citrus host (C. sinensis). This phenotype was attributable at least in part to the reduced production of xanthan gum, which is required for the water soaking phenotype and for the plant’s resistance to various forms of environmental stress [23,24,25]. The biosynthesis of xanthan gum involves the coordinate expression of 12 genes that are part of the gumB-gumM operon [25,26]. Twelve of these genes encode proteins with 4 or more cysteine residues. A reduction in the sulphur supply after disruption of the ssuA gene may have affected the synthesis of cysteine-enriched proteins and, thus, indirectly reduced xanthan gum production.

Although the precise role of alkanesulphonates during the in vivo growth of X. citri is not presently known, our results indicate that alkanesulphonates may have a previously unsuspected relevance to plant-bacterial interactions. Future studies directed at achieving a better understanding of the nutritional and physiological roles of alkanesulphonates in X. citri are warranted.

Figure 7. Lack of SsuA affects in vitro growth and xanthan gum production by X. citri. Growth curve of X. citri wild type (A) and the Xac:ssuA mutant (B) in M9 media supplemented with sulphate or different alkanesulphonate sources. Samples were taken every 2 h for measuring the growth of the samples. (C) The Xac:ssuA mutant shows altered colony morphology after growth at 30°C in LB plates. The strain recovered the normal colony morphology after complementation with the ssuA gene. (D) Production of xanthan gum by the parental and ssuA mutant strain after 24 h of growth in LB broth. Complementation with pKX33-pssuA restored the reduced xanthan gum production observed in the Xac:ssuA mutant. doi:10.1371/journal.pone.0080083.g007
period after inoculation of 7 (susceptible sweet orange cultivar Baia) during an 11-day host.

(A) Growth curves of ampicillin (100\(^{21107448}\), X. campestris protein RimM accession numbers: X. axonopodis pv. citrulli F1 (gi: 346721483), X. sacchari NCPPB 4393 (gi: 300311630), X. albilineans GPE PC73 (gi: 205011747), X. perforans 91-116 (gi: 325924357), X. fuscans subsp. aurantifoli subsp. ICPB 11122 (gi: 294625990), X. gardneri ATCC 15065 (gi: 325913092), and E. coli serotype O157:H7 (gi: 209157698). The proteins that shared high identity with the components of the Ssu transporter are the following: X. fuscans subsp. aurantifoli nitrate transport protein (XP_06732183), X. fuscans subsp. aurantifoli aliphatic sulfonates ATP-binding protein (ZP_06732181), X. fuscans subsp. aurantifoli ABC transporter permease (ZP_06703616), X. fuscans subsp. aurantifoli, X. fuscans subsp. aurantifoli oxido-reductase (ZP_06703614), X. fuscans subsp. aurantifoli nitroliotriacetate monooxygenase component A (ZP_06703613.1), X. campestris pv. m. alkanesulphonate transporter substrate-binding (ZP_06488164.1), X. campestris pv. m. ABC transporter ATP-binding subunit (ZP_06488166.1), X. campestris pv. m. ABC transporter permease (ZP_06488165.1), X. campestris pv. m. oxido-reductase (ZP_06488163.1), X. campestris pv. m. nitroliotriacetate monooxygenase component A (ZP_06488162.1), X. gardneri aliphatic sulfonates binding protein (ZP_08184321.1), X. gardneri nitrate/sulphonate/bicarbonate ATPase (ZP_08184359.1), X. gardneri nitrate/sulphonate/bicarbonate permease (ZP_08184360.1), X. gardneri oxido-reductase (ZP_08184320.1), and X. gardneri flavin-dependent oxido-reductase (ZP_08184319). Sequence alignments were performed using ClustalW2 [28] at the European Bioinformatics Institute (http://www.ebi.ac.uk/). The phylogenetic tree was accessed by the neighbour-joining method [29], and bootstrap values were obtained from 1,000 duplicates using the MEGA (Molecular Evolutionary Genetic Analysis) package, version 5 [30]. Coorindates and structure factors were deposited in the RCSB Protein Data Bank with PDB codes 3E4R (SsuA + HEPES), 3KSX (SsuA + MOPS) and 3KSh (SsuA + MES).

### Cloning of the gene encoding X. citri SsuA protein

All gene cloning steps were carried out with the E. coli DH5\(\alpha\) strain; expression of the recombinant SsuA protein was carried out with the E. coli BL21 (DE3) strain (Novagen). The DNA fragment with the X. citri ssuA gene sequence, encoding the mature protein without the first 126 base pairs (42 amino acids) corresponding to the signal peptide, was amplified by PCR (forward primer 5' gGgCATATggCCgAgCCggCgCA 3'; reverse primer 5' gGgAAgCTTGTCATTTgCgCTAC 3') with Platinum High Fidelity Taq polymerase (Invitrogen) under standard amplification conditions.

The amplicon, corresponding to 972 base pairs, was cloned into the vector pGEM T-Easy (Promega) and subcloned into the vector pET28a (Novagen) for expression in E. coli BL21 (DE3).

### Expression and purification of recombinant X. citri SsuA

Cultures of the recombinant E. coli BL21 (DE3) strain transformed with pETSsuA were prepared aerobically in LB medium supplemented with 50 \(\mu\)g/mL kanamycin until mid-log phase (OD\(_{600}\) 0.5–0.6). IPTG was then added to a final concentration of 1.0 mM. The cultures were induced aerobically (200 rpm) for 2.5 h at 28°C. Cells were collected by centrifugation at 8,000 \(\times\) g for 15 min at 4°C and stored at –20°C for approximately 16 h before lysis. The cell pellets were suspended in buffer 1 (30 mM sodium phosphate buffer, pH 7.2, containing 100 mM NaCl, 5% glycerol and 20 mM imidazole) and incubated with lysozyme (final concentration of 100 \(\mu\)g/mL) and PMSF (1 \(\mu\)M) for 1 h in an ice bath. The cells were sonically disrupted in a Branson Digital Sonifier (Model 450), and soluble fractions were separated from the non-soluble material by centrifugation at
16,000 g for 30 min at 4°C. The recombinant SsuA protein was purified from the soluble fraction by immobilised metal affinity chromatography using a HistagHP column (GE Healthcare) according to the manufacturer’s instructions. The charged resin was washed with buffer 1 (30 bed volumes) followed by step gradient elution with buffers containing increasing concentrations of imidazole (50–500 mM). The eluted fractions were dialyzed once against 20 mM Tris-HCl at pH 7 containing 50 mM NaCl and concentrated with Ultrafree MWCO 10,000 centrifugal filters (Amicon Millipore) to a final concentration of 6 mg/mL. The eluted fractions were analysed by SDS-PAGE using 12% acrylamide gels and stained with Coomassie Blue. Protein concentration was determined spectrophotometrically using the Edelhoch method [31]. Recombinant SsuA without the His6-tag was obtained by cleavage of the purified protein with thrombin (Sigma Aldrich, USA) at room temperature. Samples of 20 mg protein were incubated for 2 h with 10 units thrombin in 50 mM Tris, pH 7.5, 10 mM NaCl and 1 mM DTT.

**Spectroscopic analyses of the recombinant SsuA protein.** The stability of the protein in solution at various pH values (20 mM Tris, pH 7.0, 50 mM NaCl; 20 mM sodium acetate, pH 5.0, 50 mM NaCl; 20 mM sodium citrate, pH 3.0, 50 mM NaCl; 20 mM glycine, pH 10.0, 50 mM NaCl) and its behaviour in the presence of aliphatic sulphonates was monitored by following the intrinsic fluorescence of the tryptophan residues using an Aminco BOWMAN series 2 spectrophotometer. The excitation and emission bandwidths were 4 and 8 nm, respectively. The fluorescence cell (1 x 1 cm) was mounted on a thermostatic holder. Tryptophan fluorescence was measured at an excitation wavelength of 295 nm, and emission spectra were recorded between 340 and 420 nm. All measurements were performed using 10 μM protein and 20 μM sulphonates in 20 mM Tris, pH 8.0. Circular dichroism measurements were carried out on a JASCO J-810 spectropolarimeter equipped with a Peltier-type temperature controller and a thermostatic cell holder interfaced with a thermostatic bath. Spectra were recorded in quartz cells with a 0.1 cm path length at a protein concentration of 10 μM in various buffers. Twenty consecutive scans were accumulated and averaged. The data were corrected for the baseline contribution of the buffer, and the observed ellipticity was converted into the averaged. The data were corrected for the baseline contribution of various buffers. Twenty consecutive scans were accumulated and recorded with a 0.1 cm path length at a protein concentration of 10 μM in various buffers. Twenty consecutive scans were accumulated and averaged. The data were corrected for the baseline contribution of the buffer, and the observed ellipticity was converted into the averaged.

**Molecular replacement and structure determination.** Diffraction data were indexed using the XDS package [34] and processed using SCALA and TRUNCATE [35]. NaI and CsCl derivative crystals were obtained by quick cryo-soaking for 15 s in a 1.433 Å resolution and recorded on a MARCCD detector (oscillation data with Δφ = 1.0°). The crystals were cooled to 110 K in a stream of nitrogen gas to minimize radiation damage. Cryoprotection was obtained by soaking crystals into drops of crystal buffer plus 10% glycerol. Data collection was performed at 100 K at the D03B-MX1 beam line Brazilian Synchrotron Light Laboratory (LNLS) using a 1.6 Å and recorded on a MARCCD25 detector (oscillation data with Δφ = 1.0°).

**Structure solution, model building and refinement.** The SsuA Protein from *Xanthomonas citri* was solved by multiple isomorphous replacement and anomalous scattering (MIRAS) using the NaI and CsCl derivative datasets. The program AutoSHARP [36] was used to calculate the phases and to determine the incorporation of iodine and caesium chloride sites using SHELX [37] and density modification procedures. The Arp/wArp [38] program was used to automatically build approximately 80% of the residues. Construction of the model was performed using COOT, and 14 cycles of refinement were realised using REFMAC [39]. The SsuA structure in the presence of MOPS and MES was obtained by molecular replacement using the structural coordinates of SsuA bound to HEPEs (PDB code 3E4R).

**Construction of the Xac::ssuA mutant.** Site-specific inactivation of the *ssuA* gene was carried out with the *X. citri* 306 strain using a suicide plasmid carrying an inactivated copy of the *ssuA* gene for the selection of a gene replacement event, according to previously published procedures [40]. The first step involved cloning of the *ssuA* gene into the pUC4 plasmid after PCR amplification with primers FssuA2Nde28a (5'-GCGCA-TATGGCGAGGC GCCGCA 3') and RssuA2Hind28a (5'-GGGAAAGCTTTCATTTGCTCACC 3') (the *NdeI* and *NdeI* restriction sites are underlined). Inactivation of the *ssuA* gene was achieved by cloning a 2 kb gene cassette derived from *Smol* cleaved Shps4Bio vector and encoding resistance to spectinomycin/streptomycin [41] into the single *KpnI* cleavage site within the *ssuA* gene. A clone carrying an inactivated copy of the *ssuA* gene (*ssuA*-sp/s) was selected and confirmed by restriction analysis and automatic DNA sequencing. The mutated copy of the *ssuA* gene was released for the next cloning step by cleavage with *NdeI* and *NdeI* restriction enzymes. The mutated *ssuA*-sp/s gene was cloned into the suicide plasmid pNPTS138 vector (originally constructed by D. Alley and kindly supplied by Dr. M. V. Marques of the Department of Microbiology, University of São Paulo). The pNPTS138 plasmid permits selection of single chromosomal integrations after plating transformed cells on plates containing kanamycin and subsequent selection of gene replacement events after a second selection round in media containing sucrose (because the plasmid carries a *sadB* gene that confers sensitivity to amine-O-sulphonic acid, 3-hydroxypropane-1-sulphonic acid and taurine.

**Crystallisation of the *X. citri* SsuA protein.** Crystallisation conditions were screened using sparse-matrix screens in 96-well plates with protein at a concentration of 6 mg/ml in 20 mM Tris buffer, pH 7.0, containing 50 mM NaCl at 18°C. SsuA crystallisation was performed with the sitting-drop vapour-diffusion method by mixing equal volumes (3 μl) of protein solution and crystallisation solutions. Data from the first crystals obtained in HEPEs were collected at 100 K at the D03B-MX1 beam line Brazilian Synchrotron Light Laboratory (LNLS) [33] using 1.433 Å radiation and recorded on a MARCCD65 detector (oscillation data with Δφ = 1.0°). The crystals were cooled to 110 K in a stream of nitrogen gas to minimise radiation damage. Cryoprotection was obtained by soaking crystals into drops of crystal buffer plus 10% glycerol. Data collection was performed at 100 K at the D03B-MX2 beam line Brazilian Synchrotron Light Laboratory (LNLS) using a 1.6 Å and recorded on a MARCCD25 detector (oscillation data with Δφ = 1.0°).
The recombinant plasmid was named pNssuA and introduced by electroporation into the X. citri 306 strain as previously described [40]. Initial selection for the gene replacement event was carried out on plates containing kanamycin, followed by overnight growth of selected colonies in nonselective media and subsequent plating on 3% sucrose and 50 μl/ml spectinomycin to screen for cells that had undergone a second crossover event leading to excision of the plasmid carrying the wild type ssuA copy. PCR with primers FssuA2Nde28a and RssuA2Hind28a was used to confirm chromosomal deletions.

**Construction of the expression vector for complementation of the Xac::ssuA mutant.** A fragment containing a 900 base pairs upstream of the XAC2300 gene from ssu operon was amplified by PCR using primers FssuSalI-p (5’ CCAAGAAACG-GATTCTAGACCTC 3’) and RssuEcoRI-p (5’ CACGCCCCAT-CAAAAAGGACGTCA 3’). This fragment contained the promoter sequence, which was cloned into the pKX33 vector [42] for construction of the pKX33-p. The full ssuA gene (1026 bp) was amplified from X. citri genomic DNA by PCR using the primers FssuA-EcoRIe (5’ GAATTCTGAGGACGGTACGCACTGC 3’) e RssuA-Xba1c (5’ TCTAGATCATTTGCTCACCGCCTGGCG 3’) and cloned in to the pKX33 with the promoter sequence to build the pKX33-ssuA vector for expression in X. citri cells. The final plasmid was confirmed after digestion with SalI and XbaI (Figure 6C) and automatic DNA sequencing. Competent cells of Xac::ssuA strain were transformed with the final vector pKX33-ssuA by electroporation followed by selection on LB plates with 50 μg/ml kanamycin. The cells are described as Xac:ssuA.

**Xanthan gum production.** X. citri 306 strain, the isogenic Xac::ssuA mutant and the mutant complemented (Xac:ssuA) were grown in LB medium for 24 h at 30°C under aerated conditions (200 rpm) and subjected to the protocol described by Vojnov and collaborators [14]. Kanamycin (50 μg/ml) was added to the cultures of the mutant and complementary strains.

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**Supporting Information**

**Table S1** Crystallisation conditions for *X. citri* SsuA.

| Temperature | Space group | Crystal habit | Dimensions (mm) | v (Å³) | m (mg/ml) |
|-------------|-------------|---------------|-----------------|-------|-----------|
| 10°C        | P21         | Rods          | 0.5 x 0.5 x 0.5 | 1500   | 1.8       |
| 15°C        | P21         | Rods          | 0.5 x 0.5 x 0.5 | 1500   | 1.8       |
| 20°C        | P21         | Rods          | 0.5 x 0.5 x 0.5 | 1500   | 1.8       |

**Table S2** Ligand interactions formed by hydrogen bonds between *X. citri* SsuA and alkanesulphonates.

| Residue | Distance (Å) | Type | Ligand | Bond energy (kcal/mol) |
|---------|--------------|------|--------|------------------------|
| Ser    | 3.2          | H-bond | O       | 6.3                    |
| Asp    | 2.9          | H-bond | O       | 7.1                    |
| Glu    | 3.1          | H-bond | O       | 6.8                    |

**Author Contributions**

Conceived and designed the experiments: AB LCSF CB. Performed the experiments: FA CTP MS DC AB. Analyzed the data: AB LCSF TLB VBG JB. Contributed reagents/materials/analysis tools: RF EO. Wrote the paper: AB LCSF.

**Growth curves and plant infection experiments.** In vitro growth of wild-type and mutant strains was performed in 10 ml M9 minimal medium and sulphur-free M9 minimal medium supplemented with different alkanesulphonates (MES, CHES, HEPEs) as sulphur sources at 28°C. Samples were taken every 6 h for determination of the total number of viable cells. In vivo assays were performed as previously described [43]. Six-month-old plants of sweet orange (*C. sinensis*) were obtained from certified nurseries and kept in a growth room at 25–28°C with fluorescent light illumination. Leaf sectors were infiltrated with approximately 7 × 10^6 viable bacteria in a total volume of 0.3 ml. Bacterial cultures were prepared in LB without NaCl for 48 h at 28°C with shaking at 200 rpm and subsequently suspended in sterile water. The appearance of canker pustules and lesion phenotypes was monitored daily for 20 days. From the total number of bacteria inside the plant tissue, leaves were infiltrated with 7 × 10^6 viable bacteria in 6 different sectors. Every other day, three circular sections 1 cm in diameter containing the bacterial infiltrates were macerated in water and diluted aliquots were plated on LB agar plates for determination of the number of viable bacteria.
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