

## Structural model and ligand interactions of the *Xanthomonas axonopodis* pv. *citri* oligopeptide-binding protein

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**ABSTRACT.** The oligopeptide-binding protein, OppA, ushers oligopeptide substrates to the membrane-associated oligopeptide permease (Opp), a multi-component ABC-type transporter involved in the uptake of oligopeptides by several bacterial species. In the present study, we report a structural model and an oligopeptide docking analysis of the OppA protein expressed by *Xanthomonas axonopodis* pv. *citri* (*X. citri*), the etiological agent of citrus canker. The *X. citri* OppA structural model showed a conserved three-dimensional structure, irrespective of the low amino acid identities with previously defined structures of *Bacillus subtilis* and *Salmonella typhimurium* orthologs. Oligopeptide docking analysis carried out with the proposed model indicated that the *X. citri* OppA preferentially binds tri- and tetrapeptides. The present study represents

the first structural analysis of an OppA ortholog expressed by a phytopathogen and contributes to the understanding of the physiology and nutritional strategies of *X. citri*.

**Key words:** Oligopeptide-binding protein, Opp, ABC transport, *Xanthomonas*, Structural model

## INTRODUCTION

The oligopeptide uptake system, or oligopeptide permease (Opp), represents an ATP-dependent transport system (ABC-type transporter) involved with the uptake of peptides in several bacteria and archaea species (Payne and Smith, 1994). Besides the obvious role in nutrition, the Opp systems regulate important cellular processes affecting both the physiology and virulence of several species, including gene regulation of intercellular signaling (Ruhfel et al., 1993), competence development and sporulation (Perego et al., 1991), cell wall synthesis (Park et al., 1998), and adhesion to host cells and proteins (Fenno et al., 2000; Monnet, 2003). Opp permease usually comprises five functional components: two integral pore-forming membrane proteins (OppB and OppC), two peripheral membrane-associated ATPases (OppD and OppF), and one periplasmic (or membrane-bound lipoprotein in Gram-positive bacteria) peptide-binding component (OppA), which confer high affinity but rather flexible specificity to the transported peptides (Monnet, 2003). Usually, bacterial OppAs can bind different oligopeptides with sizes ranging from three amino acid residues, such as the *Salmonella typhimurium* OppA ortholog (Hiles et al., 1987), to at least eighteen, such as one of the *Lactococcus lactis* OppA paralogs (Lanfermeijer et al., 2000). Indeed, the selective peptide-binding activity of OppAs expressed by bacterial species may reflect both cell envelope permeability restrictions and the nutritional strategy required for survival in a specific environment (Monnet, 2003).

Definition of the three-dimensional structures of the *S. enterica* sv. *typhimurium* OppA and *Bacillus subtilis* AppA, bound to oligopeptide ligands, revealed a common molecular design comprising three structural domains: domains I and III enclosing the ligand and domain II, not directly related to ligand binding and accounting for the larger sizes of OppA orthologs with regard to other ABC-transporter binding components (Tame et al., 1994, 1995; Levдикov et al., 2005). The ligand-binding site is formed by a cleft between domains I and III connected by a flexible hinge allowing the complete engulfment of oligopeptides in a “Venus flytrap-like” mechanism. Little direct interactions exist between OppA and oligopeptide side chains, explaining, at least in part, the recognition and binding to a wide variety of peptides (Tame et al., 1994, 1995; Levdikov et al., 2005). So far, the specificity of OppA orthologs has been mainly determined by *in vivo* and *in vitro* assays employing synthetic peptides and the purified protein or living cells (Sleigh et al., 1997, 1999; Detmers et al., 2000; Wang et al., 2004).

The sequencing of two closely related *Xanthomonas* phytopathogens, *X. axonopodis* pv. *citri* (*X. citri*) and *X. campestris* pv. *campestris* (*X. campestris*) disclosed the presence of hundreds of specific genes, which may contribute to distinct physiological, virulence and host specificity features (Da Silva et al., 2002). A single *opp* operon formed by four genes (*oppA*, *oppB*, *oppC*, *oppD/F*) was found in the genome of *X. citri* but not in the *X. campestris* genome. The *X. citri* OppA consists of a mature polypeptide of 538 amino acid residues expressed during

active growth (Moutran et al., 2004). However, contribution of the Opp system to the *X. citri* nutrition is dubious. The elucidation of the *X. citri* OppA structure represents an important step toward a better understanding of the Opp system functionality, particularly among bacterial phytopathogens. In the present study, we report a molecular model of the *X. citri* OppA and several docking analyses carried out with different oligopeptides. The reported data show that, based on molecular modeling tools, substrate-binding features of OppA orthologs may be inferred and contribute to the design of *in vitro* or *in vivo* laboratory studies.

## MATERIAL AND METHODS

### Computational analysis

The amino acid sequences of the *X. citri oppA* gene (NP\_641208.1) are available at NCBI (<http://www.ncbi.nlm.nih.gov>). Prediction of the signal peptide was determined with the SignalP program (<http://www.cbs.dtu.dk/services/SignalP/>). Protein parameters of the *X. citri* OppA were calculated with the application of computational tools available at the ExPasy Molecular Biology Server (<http://www.expasy.org/>). The conserved OppA domains were predicted with the Conserved Domain Architecture Retrieval Tool at the NCBI. OppA sequence alignments were performed using ClustalX (Thompson et al., 1997), and the results were edited with the Genedoc program (<http://www.psc.edu/biomed/genedoc/>). The structural alignments and superimposition of structure backbones of OppA orthologs were carried out by the combinatorial extension method, as previously published (Shindyalov and Bourne, 1998) and PyMOL program (DeLano, 2002), respectively.

### Modeling the *Xanthomonas citri* OppA structure

The *X. citri* OppA structural model was determined using the X-ray structures deposited at the Protein Data Bank of *S. typhimurium* OppA (PDB code 1JEV, chain A) (Detmers et al., 2000) and *B. subtilis* AppA (PDB code 1XOC, chain A) (Levdikov et al., 2005), previously refined at 1.55 and 1.75 Å, respectively. The atomic coordinates of the *B. subtilis* AppA protein and *S. typhimurium* OppA were taken as templates to predict the three-dimensional structure of the *X. citri* OppA, using the Modeller 6v2 program to fulfill spatial restraints (Sali and Blundell, 1993). The quality of the *X. citri* OppA predicted folds, based on 10 different structural models, was evaluated with the Modeller program using the score of variable target function method (Fiser et al., 2000). The overall stereo chemical quality of the *X. citri* OppA models was assessed with the Procheck (Laskowski et al., 1993) and Ramachandran programs (Ramachandran, 1963). Model analyses, residue interactions, and comparisons with other substrate-binding proteins were performed with the Diamond Sting Suite Package (DMS 3.0), PyMOL and Coot programs (DeLano, 2002; Neshich et al., 2003; Emsley and Cowtan, 2004).

### Docking analysis

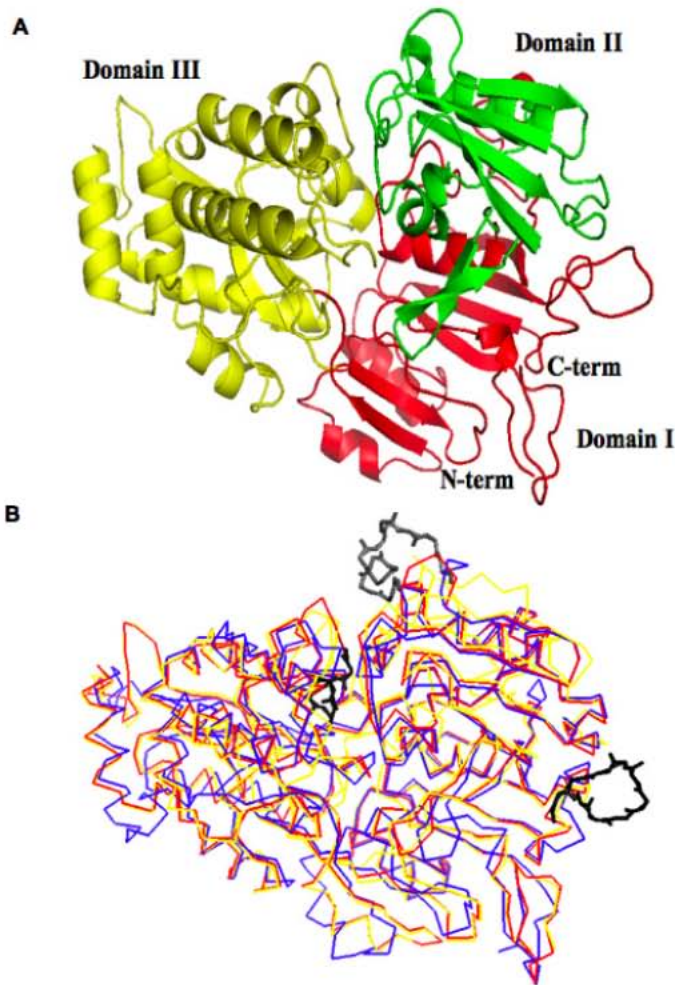
Substrate docking analyses of the *X. citri* OppA model were carried out with the GRAMM-X Protein Docking Web Server v.1.2.0 (Tovchigrechko and Vakser, 2006).

One hundred docking possibilities were generated for each ligand, and a positive event was identified whenever the ligand remained completely buried in the substrate-binding cleft delimited by the protein lateral domains as with the *S. typhimurium* OppA ortholog. Molecular interactions of protein-ligand residues were evaluated using CooT and Java Contact Table tool of the DMS 3.0. The ligand atomic coordinates of *S. typhimurium* (KKK, KDK, KPK, KHK, KGK, KCK, KWK, KNK, KIK, KMK, KAK, KEK, KFK, KHK, KLK, KQK, KRK, KSK, KTK, KVK, KYK, VKPG) and *B. subtilis* OppA (VDSKNTSSW) were retrieved from the PDB. The atomic coordinates of the *Borrelia burgdorferi* (Wang et al., 2004), *Lactococcus lactis* (Detmers et al., 2000) and different length polylysine ligands (three to seven residues) were determined with the PyMOL program (DeLano, 2002). The docking method was validated using the *S. typhimurium* OppA three-dimensional structure and the coordinates of KKK and KWK oligopeptides (Tame et al., 1995, 1996).

## RESULTS AND DISCUSSION

The *Xac* OppA structural model, based on the previously reported *S. typhimurium* and *B. subtilis* ortholog structures, revealed a similar three-dimensional fit consisting of the bilobate ellipsoidal  $\alpha\beta$  configuration characterized by mixed  $\beta$ -sheet stretches surrounded by  $\alpha$ -helices with an overall molecular dimension of 85 x 67 x 65 Å. The *X. citri* OppA model shows three major structural domains, two of them (domains I and III) delimiting the oligopeptide-binding cleft (Figure 1A). Domain I is formed by three non-contiguous sequences encompassing residues 1 to 44, 154 to 260 and 491 to 521 organized in a central 7-stranded mixed  $\beta$ -sheet stretch flanked by 4  $\alpha$ -helices. Domain III, defined by residues 261 to 490, shows a 7-stranded  $\beta$ -sheet core flanked by 9  $\alpha$ -helices. There is a disulfide bond between Cys<sup>25</sup>(P) and Cys<sup>165</sup>(P) which holds non-contiguous segments of domain I (1-44 to 154-260). OppA domain II is found only among a subset of periplasmic receptor proteins including those for dipeptides and nickel-binding proteins and confers a greater size of OppA orthologs in relation to other ABC-transporter nutrient-binding proteins (Tam and Saier Jr., 1993). *X. citri* OppA domain II encompasses amino acids 45 to 153 and forms four anti-parallel  $\beta$ -sheet strands with one side exposed at the surface of the molecule, while the other side is surrounded by seven  $\alpha$ -helices delimiting a highly hydrophobic core.

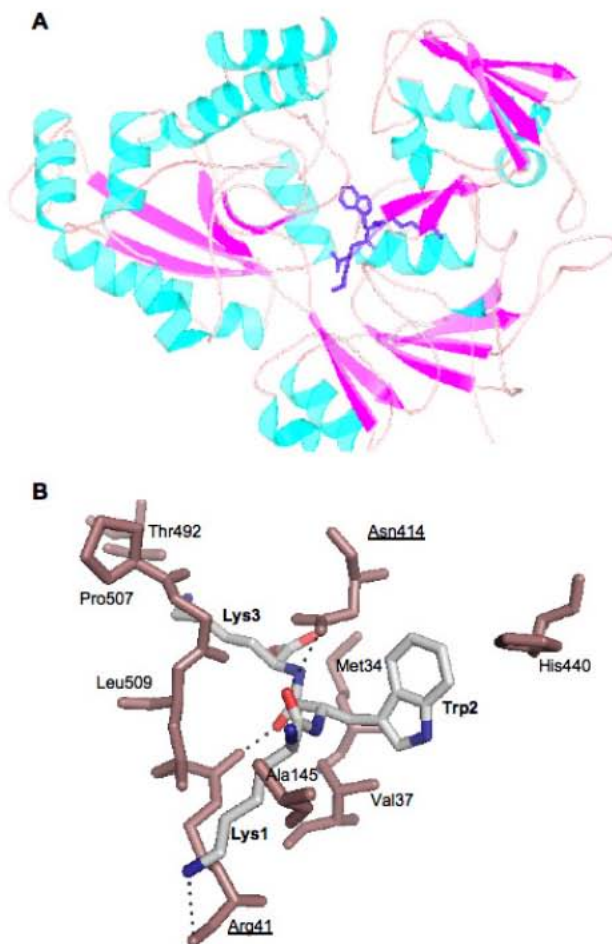
The Ramachandran plot analysis revealed that the model was validated since that 88.37% (395 residues) of the amino acid residues lay in the most favorable regions, while 11.63% (52) of them fit into allowed regions and none in disallowed regions. The *B. subtilis* and *S. typhimurium* OppA orthologs showed reduced sequence similarity with the *X. citri* OppA and identity values of 18.5 and 18%, respectively. The low sequence similarity was also observed for the recently deposited OppA structure of the Gram-negative bacterium *Thermotoga maritima* (1VR5) which shares only 12% amino acid identity with the *X. citri* OppA (r.m.s.d. 3.8 Å). Irrespective the low sequence similarity, the C $\alpha$  structural alignment revealed that the *X. citri* OppA is structurally conserved when compared with the *B. subtilis* (r.m.s.d. 1.4 Å) and *S. typhimurium* (r.m.s.d. 1.8 Å) orthologs (Figure 1B). The main differences are restricted to two extra loops in *X. citri* OppA, defined by residues Ala<sup>194</sup>-His<sup>202</sup> and Gly<sup>435</sup>-His<sup>440</sup>, which did not align in sequence and structure (indicated in black in Figure 1B). Further comparison of the three OppA models revealed twelve residues forming a loop (Asn<sup>120</sup>-Ala<sup>131</sup>, indicated in gray in Figure 1B), present only in *S. typhimurium* OppA.



**Figure 1.** The structural model of the *Xanthomonas citri* OppA. **A.** Cartoon view showing the overall *X. citri* OppA topology. The protein is formed by three domains indicated by different colors and Roman numerals: domain I encompassing amino acids 1 to 44, 154 to 260 and 491 to 521 (red), domain II delimited by amino acids 45 to 153 (green), and domain III formed by amino acid residues 261 to 490 (yellow). The N- (N-term) and C-terminal (C-term) ends of the protein are indicated. **B.** Superposition of the *X. citri* OppA (blue), *Salmonella typhimurium* OppA (red) and *Bacillus subtilis* AppA (yellow) structures. Loops present only in *X. citri* OppA or *S. typhimurium* ortholog are shown as sticks in black and gray, respectively. The three structures were overlaid using the CE program after superimposing the Ca atoms shared by the three structures.

In order to verify the ability of *X. citri* OppA to bind different peptides, docking analyses were performed with KWK, recognized by the *S. typhimurium* OppA (Tame et al., 1995; Sleight et al., 1997), and VDSKNTSSW, reported to bind to the *B. subtilis* ortholog (Levdikov et al., 2005). The results showed that the *X. citri* OppA has preference for the tripeptide which was docked exactly into the binding cleft between domains I and III (Figure 2A). The main residues forming the *X. citri* OppA-binding pocket were Met<sup>34</sup>, Val<sup>37</sup>, Arg<sup>41</sup>, Ala<sup>145</sup>, Asn<sup>414</sup>, His<sup>440</sup>, Thr<sup>492</sup>, Pro<sup>507</sup>, and Leu<sup>509</sup>, but KWK modeling disclosed polar interactions of Arg<sup>41</sup>/NH<sub>2</sub> and Trp<sup>2</sup>/O, Arg<sup>41</sup>/O and Lys<sup>1</sup>/NZ and Asn<sup>414</sup>/ND2 and Lys<sup>1</sup>/O (Figure 2B). Water molecules were present in the ligand-binding pocket favoring contacts with the tripeptide, similar to that seen with previously described OppA orthologs (data not shown). The modeling of tripeptide in the *X. citri* OppA showed that

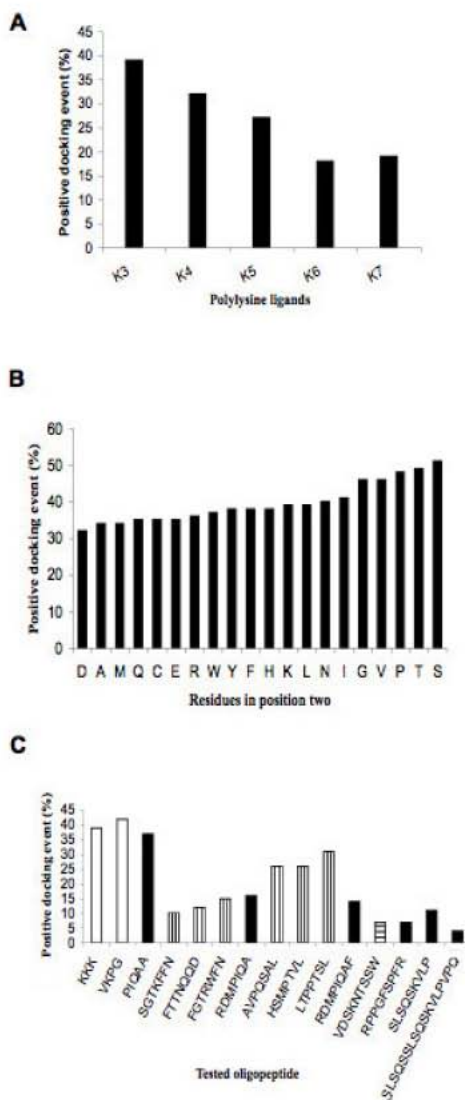
both residues that make polar contacts are conserved in the *S. typhimurium* OppA; residue Arg<sup>41</sup> is structurally aligned with the Arg<sup>41</sup> of *S. typhimurium* OppA which also interacts with the tripeptide, and the *X. citri* OppA Asn<sup>414</sup> residue, corresponding to the *S. typhimurium* Asp<sup>419</sup>, is conserved among several oligopeptide-binding proteins (Detmers et al., 2000).



**Figure 2.** Docking of KWK tripeptide into the *Xanthomonas citri* OppA ligand pocket. **A.** Schematic cartoon view of OppA and docked oligopeptide (shown in dark blue) deeply buried between domains I and III. **B.** Main residues of *X. citri* OppA that form the binding pocket. The residues that make polar interactions with KWK peptide are underlined and the interactions are shown in black dashes.

Docking of VDSKNTSSW into the *X. citri* OppA showed that 17 amino acid residues interact with the ligand, but the oligopeptide did not fit properly into the OppA model and part of the peptide N-terminal region lay outside the ligand-binding cleft (data not shown). Indeed, the low affinity of *X. citri* OppA for nonapeptides was confirmed in subsequent docking analyses. Additional ligand interaction analyses of *X. citri* OppA were carried out with the GRAMM-X Protein Docking Web Server using a set of oligopeptides previously shown to interact with different OppA orthologs. Initial results based on the KKK tripeptide showed that correct docking events into the *X. citri* OppA-binding cleft occurred much more frequently (38% positive events in 100 docking trials) than dockings carried out with peptides containing five or more residues

(less than 20% positive events). To evaluate the role of oligopeptide size on the binding to OppA, different polylysine ligands, ranging in size from 3 up to 7 residues, were docked into the *X. citri* OppA model. The results clearly show that *X. citri* OppA preferentially binds short peptides, as demonstrated by the number of positive docking events (Figure 3A). Increasing the ligand size from 3 to 6 lysine residues showed a 50% reduction in positive docking events, further supporting the protein's higher affinity for shorter tripeptides. We also investigated the role of specific amino acid residues on the binding of *X. citri* OppA to KKK derivatives in which the central lysine residue was replaced by different amino acids (Figure 3B).



**Figure 3.** Docking analysis of *Xanthomonas citri* OppA with different oligopeptides. **A.** Polylysine peptides ( $K_n$ , where  $n$  is the number of lysines). **B.** Relevance of the second residue in the docking of OppA with tripeptides (KXX). **C.** *X. citri* OppA docking trials with oligopeptides recognized by different bacterial OppA orthologs. Black bars - oligopeptides recognized by the *Lactococcus lactis* OppA orthologs; white bars - oligopeptides recognized by the *Salmonella typhimurium* OppA ortholog; hatched bars with horizontal stripes - oligopeptides recognized by the *Bacillus subtilis* OppA ortholog, and hatched bars with vertical stripes - oligopeptides recognized by the *Borrelia burgdorferi* OppA orthologs.

Replacement of lysine residue by glycine, valine, proline, threonine, or serine increased by approximately 10% the number of positive docking events with OppA in comparison with the KWK ligand suggesting that both the ligand size and amino acid composition contribute to protein binding. Finally, using the coordinates of peptides known to bind *S. typhimurium* (Hiles et al., 1987; Fenno et al., 2000; Monnet, 2003), *B. subtilis* (Monnet, 2003), *B. burgdorferi* (Lanfermeijer et al., 2000) and *L. lactis* (Tame et al., 1995) OppA orthologs, it was possible to demonstrate that the tetrapeptide VKPG, originally recognized by the *S. typhimurium* OppA ortholog (Figure 3C, white bar), generates the highest number of positive docking events (43%) with the *X. citri* OppA, followed by KKK (39%) and PIQAA (37%) (Figure 3C). Consistent with the observations based on the KKK tripeptide, the replacement of specific amino acids with positively charged residues containing small side chains increased significantly the affinity of *X. citri* OppA for specific oligopeptides. Previous comparative amino acid sequence analyses of different bacterial OppA orthologs demonstrated that *X. citri* OppA is more closely related to the ortholog expressed by *Streptomyces roseofulvus*, a Gram-positive bacterium species, than other orthologs expressed by Gram-negative species (Moutran et al., 2004). Nonetheless, the present structural analyses employing oligopeptide docking computational tools showed that the *X. citri* OppA protein shares functional features with orthologs expressed by Gram-negative species, such as *S. typhimurium*. Shorter oligopeptides (3 to 5 residues) containing preferentially positively charged amino acid residues with small side chains fit better into the peptide-binding cleft of the protein. Collectively, these results indicate that the application of molecular modeling approaches, as reported in the present study, may contribute to the study of protein-ligand interactions of OppA orthologs without experimentally determined structures, supporting further *in vitro* and *in vivo* studies aimed at a better understanding of the *X. citri* nutritional strategies.

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