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LUCIANA COUTINHO DE OLIVEIRA

**Characterization of structure, dynamics,
function and interactions of components from
the Type IV Secretion System of *Xanthomonas
citri* by solution Nuclear Magnetic Resonance**

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RESUMO

Oliveira, L. C. - **Caracterização da estrutura, dinâmica, interações e função de componentes do Sistema de Secreção Tipo IV de *Xanthomonas citri* por Ressonância Magnética Nuclear em solução**. 2015. 135p. Tese de Doutorado – Programa de Pós-Graduação em Bioquímica. Instituto de Química, Universidade de São Paulo, São Paulo.

Bactérias usam sistemas especializados, denominados sistemas de secreção, a fim de translocar substratos para o ambiente ou para outras células, ou até mesmo para capturar moléculas do meio externo. Seis diferentes sistemas de secreção foram descritos em bactérias gram-negativas. O Sistema de Secreção do Tipo IV (T4SS) está envolvido na translocação de fatores de virulência, conjugação bacteriana, absorção e liberação de DNA, e secreção de toxinas antibacterianas. O canal do T4SS (*core complex*) corresponde a um complexo formado por 14 repetições do heterotrímero VirB7-VirB9-VirB10. A camada externa deste canal é constituída por VirB7 em complexo com os domínios C-terminal de VirB9 (VirB9^{CT}) e VirB10 (VirB10^{CT}). Os domínios N-terminal de VirB9 (VirB9^{NT}) e VirB10 (VirB10^{NT}) formam a camada interna do *core complex*. *Xanthomonas citri* pv. *citri* (Xac) é uma bactéria gram-negativa que infecta plantas cítricas causando uma doença chamada "cancro cítrico". Embora não esteja diretamente envolvido na infecção, o T4SS cromossomal secreta toxinas capazes de matar outras bactérias gram-negativas. VirB7 de Xac possui uma cauda N-terminal flexível e um domínio globular C-terminal ausente em outras proteínas VirB7. VirB7 interage com VirB9^{CT} através de sua cauda N-terminal. Nesta tese, a estrutura de RMN do complexo formado por VirB9^{CT} e um peptídeo derivado do segmento N-terminal de VirB7 foi resolvida. O complexo é estabilizado, principalmente, por interações hidrofóbicas envolvendo as cadeias laterais de determinados resíduos de aminoácidos, particularmente a Phe30, o Trp34 e a Val37 em VirB7 e a Arg250, a Tyr167 e a Tyr169 em VirB9. A substituição de alguns destes aminoácidos por alanina afeta não só a constante de dissociação do complexo *in vitro*, como também a atividade e a montagem do T4SS *in vivo*. Além disso, resíduos específicos envolvidos em oligomerização de VirB7 são essenciais para a manutenção de um T4SS funcional, embora não sejam essenciais para a montagem do sistema. Estudos estruturais, de dinâmica e de interações de um fragmento derivado da região rica em prolinas (*proline-rich region* – PRR) contida no N-terminal de VirB10 (VirB10^{NT} - resíduos 85-182) também foram realizados. Medidas de {¹H}-¹⁵N NOE heteronuclear mostraram que VirB10^{NT} é altamente flexível. Análises de deslocamentos químicos e NOEs mostrou que VirB10^{NT} forma uma hélice curta entre os resíduos 151-163. Ensaios de interação por RMN indicaram que esta hélice está envolvida em interações com VirB9^{NT}. Estes resultados são a primeira evidência convincente para a especificidade de interação entre os domínios N-terminal de VirB9 e VirB10. Estes dados apontam também para a existência de flexibilidade dentro do T4SS de Xac.

Palavras-chave: Ressonância Magnética Nuclear, Biologia Estrutural, Sistema de Secreção do tipo IV, *Xanthomonas citri*

ABSTRACT

Oliveira, L. C. - **Characterization of structure, dynamics, function and interactions of components from the Type IV Secretion System of *Xanthomonas citri* by solution Nuclear Magnetic Resonance**. 2015. 135p. PhD Thesis - Graduate Program in Biochemistry. Institute of Chemistry, University of São Paulo, São Paulo.

Bacteria use specialized systems, called secretion systems, in order to translocate substrates to the environment or to other cells, or even to uptake molecules from the exterior environment. Six different secretion systems have been described in Gram-negative bacteria. The Type IV Secretion System (T4SS) is involved in translocation of virulence factors, bacterial conjugation, uptake and release of DNA, and in the secretion of antibacterial toxins. The T4SS channel corresponds to a toroidal supramolecular complex consisting of 14 repetitions of the VirB7-VirB9-VirB10 heterotrimer. This channel, also called "core complex", is divided in two layers, an outer layer consisting of the VirB7 lipoprotein in complex with the C-terminal domains of VirB9 (VirB9^{CT}) and VirB10 (VirB10^{CT}), and an inner layer composed by the N-terminal domains of VirB9 (VirB9^{NT}) and VirB10 (VirB10^{NT}). *Xanthomonas citri* pv. *citri* (Xac) is a gram-negative bacterium that infects citrus plants causing a disease called "citrus canker". Although not directly involved in causing the disease, the chromosomally encoded T4SS is responsible for the secretion of toxins, working as a bacterial killing machine (Souza et al., 2015). The three-dimensional structure of Xac's VirB7 obtained by Nuclear Magnetic Resonance (NMR) spectroscopy (PDB 2L4W) revealed that, unlike the canonical VirB7, Xac's VirB7 consists of a flexible N-terminal domain followed by a C-terminal globular domain. The flexible N-terminal tail is involved in interaction with VirB9^{CT}. In this thesis, the NMR structure of the complex formed between VirB9^{CT} and a peptide derived from the N-terminal tail of Xac-VirB7 (VirB7^{NT}) was solved. This complex is stabilized by hydrophobic interactions involving the side chains of particular amino acid residues such as Phe30, Trp34 and Val37 in VirB7, and Arg250, Tyr167 and Tyr169 in VirB9. Mutations of such amino acids affect not only the stability of the VirB9:VirB7 complex *in vitro*, but also reduce the T4SS activity and impairs its assembly *in vivo*. Furthermore, the ability of forming VirB7:VirB7 oligomers is essential for a functional T4SS, although it is not required for assembling the complex. The structural propensity and flexibility of a fragment derived from the proline-rich region (PRR) of the N-terminal tail of VirB10 (VirB10^{NT} - residues 85 to 182) were studied. Measurements of the {¹H}-¹⁵N heteronuclear NOE showed that VirB10^{NT} is highly flexible on a sub-nanosecond time scale. Analysis of chemical shifts and NOEs showed that the ensemble and time average conformation of VirB10^{NT} consists of a short alpha helix between residues 151-163, and that this helix is involved in interactions with VirB9^{NT}. These findings provide the first compelling evidence for the interaction between the N-terminal domains of VirB9 and VirB10, and for the existence of significant flexibility within Xac's T4SS.

Keywords: Nuclear Magnetic Resonance, Structural Biology, Type IV Secretion System, *Xanthomonas citri*

Summary of Figures

Figure 1: (A) Schematic representation of the T3, T4 and T6 Secretion Systems.

The T3SS consists of approximately 25 proteins organized into 2 main sub-complexes. The machinery of translocation of the T4SS is divided in two main complexes. The Inner membrane complex (IMC) is formed by the ATPases VirD4, VirB4 and VirB11 in association with VirB3, VirB6 and VirB8. The core complex (CC) is composed by VirB7, VirB9 and VirB10. There is also a pilus composed by VirB2 and VirB5. The T6SS can be sub-divided into two main complexes. The first one is the membrane complex, which is composed by TssJ, TssL and TssM. The tail complex comprises a tail sheath (TssB and TssC), an inner tube (Hcp) and a baseplate (TssK, TssE, VgrG). Figure adapted from (Costa et al., 2015). (B) Cryo-EM structure of the T4SS encoded by the conjugative plasmid R388 (EMD 2567) (Low et al., 2014) superimposed on the crystal structure of the outer layer complex from the T4SS encoded by the conjugative plasmid pKM101 (PDB 3JQO) (Chandran et al., 2009). 27

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Chapter I - Introduction

Secretion and uptake of macromolecules play important roles in the interaction between bacteria and the environment. Cell walls of gram-negative bacteria consist of two membrane barriers, called inner and outer membranes, and a space in between, the periplasm. The complexity of the cell wall of gram-negative bacteria leads to sophisticated molecular machineries for promoting the translocation of substrates. To date, as much as six different types of gram-negative bacterial secretion systems have been described (Type I to VI Secretion Systems) (Desvaux et al., 2004; Tseng et al., 2009). Advances in DNA sequencing and annotation of new bacterial genomes, structural and functional characterization of hypothetical proteins, will eventually lead to the identification of new types of secretion systems and their roles.

Bacterial Secretion Systems of Gram-negative bacteria

The different secretion systems described to date are classified in different groups according to their biological functions as described below.

The Type I Secretion System depends on an ABC transporter

The Type I Secretion System (T1SS) is made up of three indispensable proteins: the outer membrane protein (OMP), a membrane-fusion or adaptor protein (MPF) and an ATP-binding cassette (ABC) that is embedded in the inner membrane (Delepelaire, 2004). The secretion of substrates through the T1SS occurs in a single step (Thomas et al., 2014). The T1SS is used to transport a large variety of molecules with a broad range of sizes and functions such as ions, drugs, and proteins. The ABC transporter consists of a nucleotide-binding domain (NBD), which is located in the cytoplasm, and a transmembrane domain. The ABC is responsible for the specific recognition of substrates. Usually it recognizes a C-terminal secretion signal that is present in the substrate. This C-terminal signal is

not cleaved during the secretion, it interacts sequentially with the ABC, the MPF and the OMP (Delepelaire, 2004).

Secretion systems that depend on a different transporter: T2SS and T5SS

Some protein secretion systems depend on the Sec or Tat systems for the initial transport to the periplasm. The T2SS is one of these systems. It is involved in the secretion of fully folded proteins to the extracellular milieu. The T2SS secretes a wide variety of substrates such as proteases, lipases, phosphatases or enzymes involved in the processing of carbohydrates. Once inside the eukaryotic cell, some of these substrates act as toxins and/or virulence factors, while in some bacteria the T2SS is dedicated to the secretion of a single protein, but in others such as *Vibrio cholerae* and *L. pneumophila*, the T2SS secretes over 20 different proteins (Johnson et al., 2006; Nivaskumar and Francetic; Sandkvist, 2001).

Other example of a Sec-dependent system is the T5SS. This secretion system is composed by one or two polypeptides divided in two distinct groups: the so-called autotransporters (Dautin and Bernstein, 2007), and the two-partners secretion systems (Mazar and Cotter, 2007). These are distinguished by the fact that the secreted protein and the transporter are encoded in the same polypeptide (autotransporter) or in different polypeptide chains (two-partners secretion system). In general, the T5SSs consist of three regions: a signal peptide, the passage area and a β -barrel that forms the translocation unit (Desvaux et al., 2009; Henderson et al., 2004). The type V secretion machinery is responsible for the transport of a wide variety of proteins such as proteases, adhesins and invasins (Henderson et al., 2004). One of the best studied secreted proteins by this system is the YadA from *Yersinia spp*, an adhesin that binds to components of the extracellular matrix such as collagen, fibronectin, laminin, and that plays a role in the internalization of the pathogen (Leo et al., 2012; Roggenkamp et al., 2003).

Secretion systems that inject substrates directly inside the target cell: T3SS, T4SS, and T6SS

The type III is one of the most extensively characterized secretion systems. The T3SS is essential for conferring virulence to various gram-negative bacteria such as *Salmonella* spp., *Shigella* spp., *Yersinia* spp., *Escherichia coli*, *Pseudomonas syringae*, *Ralstonia solanacearum* and *Xanthomonas* spp. (Cornelis, 2006; Lindeberg and Collmer, 2009). The T3SS was also shown to be involved in symbiotic interactions with the host cells (Galán et al., 2014). Usually, the substrates are injected inside the cytoplasm of the host cell in a single step, where they modulate a wide variety of host cellular functions by interfering with cellular processes and suppressing the host defence responses. The T3SS effectors can also be secreted to the extracellular milieu (Grant et al., 2006). Although the secretion machinery of the T3SS is highly conserved among different bacterial species, the effector proteins are specific of each pathogen (Galán, 2009). The T3SS has a syringe-like structure, which is also termed needle complex or injectisome. It is formed by approximately 25 proteins. This structure can be subdivided in two major sub-complexes. The first one is a double-membrane-spanning base, while the second is needle-like filament (PrgI) (**Figure 1**) (Costa et al., 2015). The pathogenicity of *Xanthomonas* depends on a conserved T3SS that is involved in the injection of more than 25 different effector proteins in the host plant cell (Kay and Bonas, 2009; White et al., 2009). As an example, the effectors AvrBs3/PthA are central to pathogenicity in the genus *Xanthomonas*, mimicking transcriptional activators from the plant and being able to manipulate the host transcriptome (Kay et al., 2007; Römer et al., 2007).

The Type VI secretion system (T6SS) is analogous to the secretion machineries of the types III and IV systems because the translocated protein is injected directly into the eukaryotic cell. The T6SS consists of a chaperone with ATPase activity, a channel between the inner and outer membranes, and a needle-shaped structure (Tseng et al., 2009). It is composed by 13 conserved proteins and other accessory components (Costa et al., 2015). Studies have shown that proteins secreted by this system lack a characteristic signal sequence in the amino terminal region (Cascales, 2008; Shrivastava and Mande, 2008). Proteins secreted by the

T6SS were described in pathogens such as *Burkholderia mallei*, *Salmonella typhimurium*, *Vibrio cholerae* and *Edwardsiella tarda*. In some pathogens, such as *Vibrio cholera* and *Edwardsiella tarda*, proteins secreted by the T6SS are essential to quorum sensing, stress response, biofilm formation, symbiosis, anti-bacterial phagocytosis and anti-bacterial activity (Aschtgen et al., 2008; Aubert et al., 2008; Bladergroen et al., 2003; Lauriano et al., 2004; MacIntyre et al., 2010; Pukatzki et al., 2006; Suarez et al., 2008; Weber et al., 2009). The substrate translocation machinery can be subdivided in two main complexes: a membrane complex that is formed by inner membrane (IM) embedded proteins, and a tail complex, composed by proteins that form a contractile tail (tail sheath, inner tube and a baseplate). The tail sheath forms a long tubular structure, which extends into the bacterial cytosol (**Figure 1**) (Costa et al., 2015).

The type IV Secretion system (T4SS) is a supra-molecular complex, typically formed by 12 proteins (VirB1 to VirB11 and VirD4) (**Figure 1**) (Zechner et al., 2012). The T4SS is an assembly of two major sub complexes: The inner membrane complex (IMC) and the core complex (CC). The IMC spans the inner membrane. It is composed by the ATPases VirD4, VirB4 and VirB11, and by the membrane proteins VirB3, VirB6, and VirB8. The CC is the channel through which the substrates cross the cell wall (Waksman and Orlova, 2014). The lipoprotein VirB7, VirB9 and VirB10 form the T4SS core complex, which is subdivided in two layers so-called inner and outer layers.

The inner layer of the core complex of the T4SS consists of the amino-terminal domains of VirB9 and VirB10, while the outer layer consists of the carboxy-terminal domains of VirB9 and VirB10 and the lipoprotein VirB7 (Fronzes et al., 2009a; Rivera-Calzada et al., 2013). The T4SS is the most versatile secretion system playing a variety of functions. It can be used as a conjugation machine for different bacteria such as *Escherichia coli*, *Legionella pneumophila*, *Rickettsiella grylli* and *Coxiella burnetii* (Zechner et al., 2012), for the translocation of substrates that are important to the infection promoted by *Helicobacter pylori* (Backert and Selbach, 2008), *Brucella spp* (Celli, 2006) and *Agrobacterium tumefaciens* (Jakubowski et al., 2009). The T4SS can also work in the uptake and release of DNA in *Helicobacter pylori* ComB bacterial competence system and in *Neisseria*

gonorrhoea (Cascales and Christie, 2003). We have recently described that the T4SS of *Xanthomonas citri* is involved in the secretion of toxins leading to a bacterial killing activity (Souza et al., 2015).

Figure 1: (A) Schematic representation of the T3, T4 and T6 Secretion Systems. The T3SS consists of approximately 25 proteins organized into 2 main sub-complexes. The machinery of translocation of the T4SS is divided in two main complexes. The Inner membrane complex (IMC) is formed by the ATPases VirD4, VirB4 and VirB11 in association with VirB3, VirB6 and VirB8. The core complex (CC) is composed by VirB7, VirB9 and VirB10. There is also a pilus composed by VirB2 and VirB5. The T6SS can be sub-divided into two main complexes. The first one is the membrane complex, which is composed by TssJ, TssL and TssM. The tail complex comprises a tail sheath (TssB and TssC), an inner tube (Hcp) and a baseplate (TssK, TssE, VgrG). Figure adapted from (Costa et al., 2015). **(B) Cryo-EM structure of the T4SS** encoded by the conjugative plasmid R388 (EMD 2567) (Low et al., 2014) superimposed on the crystal structure of the outer layer complex from the T4SS encoded by the conjugative plasmid pKM101 (PDB 3JQO) (Chandran et al., 2009).

Overview of structural and functional studies of the T4SS

The crystallographic structure of the outer layer of the CC from the T4SS encoded by the conjugative plasmid pKM101 from *Escherichia coli* (PDB 3JQO) (Chandran et al., 2009) shows a ring-shaped structure. This complex consists of 14 repetitions of the hetero-trimer formed by the C-terminal domains of VirB9 and VirB10 and by VirB7 (**Figure 1**). The structure of the complete CC was determined in low resolution by cryo-electron microscopy (cryo-EM) (Fronzes et al., 2009a; Rivera-Calzada et al., 2013). The cryo-EM map fits well with the X-ray data obtained for the outer-layer. Unfortunately, high-resolution structural information for the inner-layer is not yet available. A summary of the structure and function of the main components of the T4SS is given below.

VirB1 is a small periplasmic protein with 150-250 residues. It possesses a lytic transglycosylase activity, which cleaves β -1,4 glycosidic linkages between N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc). Members of this family of proteins are able to cleave the peptidoglycan layer, what can facilitate the assembly of the periplasmic channel of the T4SS (Zahrl et al., 2005). In *Agrobacterium tumefaciens*, the knockout of the *virB1* gene does not eliminate the activity of the T4SS, but attenuates the virulence. This observation indicates that, although cell wall cleavage may facilitate the assembly, it is not essential for the T4SS activity (Llosa et al., 2000).

VirB2 is a small protein and the main component of the pilus of the T4SS. VirB2 of *A. tumefaciens* and the orthologous protein encoded by the conjugative plasmid RP4 are processed through a step of cleavage followed by a cyclization, where the amino and carboxi terminals are covalently linked to the N-terminal region resulting in a cyclization of the polypeptide chain (Eisenbrandt et al., 1999, 2000).

VirB3 is small and believed to be an integral membrane protein (Shirasu and Kado, 1993). It plays an essential role in the translocation of substrates in *A. tumefaciens* (Berger and Christie, 1994). In some cases, VirB3 was identified as being expressed in fusion with VirB4, which is either located in the cytoplasm or anchored in the inner membrane (Arechaga et al., 2008; Draper et al., 2006).

VirB4 is the largest and most well conserved protein of the T4SS apparatus. It displays ATPase activity (Arechaga et al., 2008), being required for pilus biogenesis (Fullner et al., 1996), substrate transfer and virulence (Berger and Christie, 1993; Fullner et al., 1994; Watarai et al., 2002). VirB4 may also be required to energize the assembly and/or the activity of the secretion apparatus, playing regulatory roles in the substrate transfer mechanism (Alvarez-Martinez and Christie, 2009; Cascales and Christie, 2003, 2004). Negative staining electron microscopy studies of VirB4 (EMD-2137) bound to the core complex encoded by pKM101 plasmid of *E. coli* revealed that this ATPase is monomeric and located at the side of the core complex, interacting more extensively with VirB9. This location is ideal for the regulatory role that VirB4 may play in the substrate transfer mechanism (**Figure 2B and 2C**) (Walldén et al., 2012), but it is not at all clear how this would be compatible with the proposed cytosolic localization of VirB4 (**Figure 1**).

VirB5 of *Agrobacterium* is located at the end of the T-pilus (Aly and Baron, 2007). It was shown to interact with a plant cell surface protein (Aly et al., 2008), indicating that, as VirB2, VirB5 also acts as an adhesin at the interface between the T4SS and the host cell (Backert et al., 2008). The crystal structure of VirB5 from the conjugative plasmid pKM101 shows an elongated shape formed by a three helices bundle (**Figure 2D**) (PDB 1R8I) (Yeo et al., 2003).

VirB6 is an integral membrane protein embedded in the inner membrane (Cao and Saier, 2001; Jakubowski et al., 2004; Judd et al., 2005). VirB6 interacts directly with the substrate (Cascales and Christie, 2004; Jakubowski et al., 2004), what suggests that it may be part of the substrate translocation channel (Alvarez-Martinez and Christie, 2009). Recent studies of phage display, bacterial two-hybrid and fluorescence-based interaction assays revealed that the VirB6 N-terminal domain interacts with the envelope-spanning VirB10 protein (Villamil Giraldo et al., 2015). Moreover, interactions between VirB6, VirB10 and VirB8 are thought to be important for the T4SS assembly and function.

VirB7 is a small protein (45-60 amino acids residues) (**Figure 2A** - in green) that displays a low level of conservation and is found in only a subset of all T4SS (Alvarez-Martinez and Christie, 2009; Frank et al., 2005). In Xac, VirB7 (da Silva et

al., 2002) has 139 amino acids, from which the first 21 are removed after lipidation of Cys 22 by the LolA, LolB, LolC, LolD, and LolE system (Alegria et al., 2005). This signal peptide in the amino terminal of VirB7 is a characteristic of lipoproteins that are covalently linked to fatty acid molecules on a conserved cysteine residue (Tokuda and Matsuyama, 2004). VirB7 is located in the outer layer complex of the CC, anchored in the outer membrane via the lipid acyl chain. Unlike other VirB7, Xac's VirB7 has an additional globular domain whose topology is similar to domains present in proteins associated with the outer membrane of gram-negative bacteria. This extra C-terminal globular domain is also called N0 (Souza et al., 2011). Therefore, VirB7 of Xac can be divided into two different regions, a flexible N-terminal tail (residues 24 to 50) followed by the N0 domain (residues 51 to 139) (**Figure 2E**). A small segment in the N-terminal tail of VirB7 (residues 29 to 38) consists of the interaction site with the C-terminal domain of VirB9 (VirB9^{CT}). Some residues in the N-terminal tail (T45, E46, 47I, 48P, 49L) are involved in intermolecular interactions with residues from the N0 domain, thereby promoting oligomerization of VirB7 *in vitro*. VirB7 is responsible for the stabilization of several other proteins in the T4SS (Fernandez et al., 1996; Spudich et al., 1996), particularly VirB9 (Schröder and Lanka, 2005). The lipidation of VirB7 is not important to the assembly of the outer layer complex itself, but appears to play an important role for the localization of this system along the outer membrane (Fronzes et al., 2009a). VirB7 of *Xanthomonas citri* is essential for the correct assembly of the T4SS (Souza et al., 2011). Interestingly, VirB7 from *Brucella abortus* is not essential for the infection via the T4SS (den Hartigh et al., 2008). Structures of VirB7 from the conjugative plasmid pKM101 in complex with the C-terminal domain of VirB9 were solved by NMR (Bayliss et al., 2007) and, in the context of the outer layer complex, by X-ray crystallography (Chandran et al., 2009).

VirB8 is a highly conserved protein in all T4S systems (Christie et al., 2005). It consists of a transmembrane domain located in the inner membrane, and a large domain exposed to the periplasm (Das and Xie, 1998; Thorstenson and Zambryski, 1994). VirB8 interacts with multiple partners, including VirB1, VirB4, VirB5, VirB8, VirB9, VirB10 and VirB11 (Baron, 2006; Höppner et al., 2005; Paschos et al., 2006; Yuan et al., 2005). VirB8 interacts with the substrate during its passage through the T4SS of *Agrobacterium* (Cascales and Christie, 2004), and was proposed to be part

of the CC together with VirB7, VirB9 and VirB10 (Das and Xie, 2000; Kumar and Das, 2001). This last observation is challenged by the fact that VirB8 is not co-purified in complex with VirB7, VirB9 and VirB10 (Fronzes et al., 2009a). The three-dimensional structures of the periplasmic domain of VirB8 from *A. tumefaciens* and *B. suis* were determined by X-ray (**Figure 2F**) (Bailey et al., 2006; Terradot et al., 2005).

VirB9 is one of the main components of the CC of the T4SS. This protein possesses two domains. The N-terminal domain spans the inner layer, while the C-terminal domain spans the outer layer of the CC. VirB9 interacts with VirB10 and VirB7 in the outer layer (**Figure 2A**) (Chandran et al., 2009). The structure of the complex formed between the C-terminal domain of VirB9 and VirB7 encoded by the conjugative plasmid pKM101 was solved by solution NMR (PDB 2OFQ) (Bayliss et al., 2007). The C-terminal domain of VirB9 is a β -sandwich. This conformation was maintained in the context of the outer layer complex (PDB 3JQO). The only difference between the X-ray and the NMR structures is the presence of an extra α -helix in the amino-terminal portion of VirB9^{CT} in the context of the outer layer complex (Chandran et al., 2009).

VirB10 consists of a cytoplasmic domain, an α -helix that spans the inner membrane, a proline rich region (PRR) and a C-terminal periplasmic globular domain formed by a β -barrel (the β -barrel domain) with a modified helix-loop-helix motif projecting out from the top of the barrel, similar to an antenna (Jakubowski et al., 2009; Terradot et al., 2005). VirB10 interacts with VirD4, with VirB4/VirB8 associated with the inner membrane complex, and with VirB7/VirB9 associated with the outer layer complex (Christie et al., 2005). The crystal structure of the outer layer complex of the T4SS encoded by the pKM101 plasmid showed that VirB10 uses the antenna-shaped projection to form a pore in the outer membrane (**Figure 2A**) (Chandran et al., 2009). As VirB10 also crosses the inner membrane, this is the first report of a protein that simultaneously crosses the two membranes of Gram-negative bacteria. Thus, VirB10 may serve to relay information from the ATPases in the inner membrane to the pore located in the outer membrane. It was hypothesized that VirB10 would populate two conformational states that could

represent the putative opened and closed states of the T4SS's channel (Chandran et al., 2009).

VirB11 has ATPase activity, which is stimulated *in vitro* by the addition of lipids, what is consistent with its predicted membrane location (Krause et al., 2000; Machón et al., 2002; Rashkova et al., 1997). This protein has two domains and forms hexamers (Yeo et al., 2000). The structure of VirB11 from *H. pylori* (**Figure 2G**) was solved in the absence and presence of nucleotides, showing conformational changes that take place upon the binding of ATP (Savvides et al., 2003). VirB11 may be an important factor to provide energy in the form of ATP hydrolysis for machinery assembly, pilus production and/or secretion of substrates (Christie et al., 2005; Sagulenko et al., 2001).

VirD4 recognizes and recruits the target proteins or protein-DNA complexes to be secreted (Fronzes et al., 2009b). The orthologue of VirD4 in the *E. coli* R388 conjugative system, TrwB, has a DNA-dependent ATPase activity (Tato et al., 2005). Structures of the cytoplasmic domain of TrwB were determined in the absence and in the presence of the substrate and product analogues. TrwB forms hexameric rings, whose central channels undergo conformational changes after binding and hydrolysing nucleotides (**Figure 2H**) (Gomis-Rüth et al., 2001, 2002). The hexamer is structurally similar to the F1-ATPase, the globular domain of ATP synthase (von Ballmoos et al., 2009). Based on this similarity, it was proposed that TrwB uses the energy derived from ATP hydrolysis in order to promote the translocation of the DNA substrate through its central channel (Cabezón and de la Cruz, 2006).

Figure 2: (A) Crystal structure of outer layer complex from the *E. coli* conjugative plasmid pKM101 (PDB 3JQO). VirB7 is shown in green, VirB9^{CT} in grey and VirB10^{CT} in red. (B) C-terminal domain of VirB4 from *Thermoanaerobacter pseudethanolicus* (PDB 4AG5) (C) Negative stain electron microscopy structure of VirB4 from the pKM101 T4SS (in orange) bound to the core complex (in blue) (EMD-2137) adapted from (Walldén et al., 2012). (D) Crystal structure of VirB5 from the pKM101 plasmid (PDB 1R8I). (E) NMR structure of VirB7 from the chromosomally encoded T4SS of *Xanthomonas citri* (PDB 2L4W). (F) Crystal structure of VirB8 periplasmic domain of *Brucella suis* (PDB 2BHM). (G) Crystal structure of VirB11 from *Helicobacter pylori* (PDB 2PT7). (H) Crystal structure of the cytoplasmic domain of VirD4 encoded by the conjugative plasmid R388 (PDB 1GKI).

Scope of this thesis

Although a large amount of structural information about different parts of the T4SS has been obtained in recent years, very few structural data is available for a bacterial killing T4SS. Additionally, new experimental and structural data are needed in order to elucidate the mechanism of substrate translocation through the T4SS. As shown in the **Chapter II** of this thesis, VirB7-VirB7 self association and the intermolecular contacts of VirB7 with VirB9^{CT}, are essential for the correct functioning and/or assembly of the T4SS in Xac. The chapters III and IV of this thesis describe recent findings about Xac's VirB10. Specifically, **Chapter III** shows by NMR that a fragment derived from the proline rich region of VirB10 is highly dynamic and interacts with VirB9^{NT}. **Chapter IV** describes initial attempts to produce fragments of the C-terminal domain of VirB10 that are suitable for NMR studies.

Chapter II - VirB7-VirB7 and VirB7-VirB9 interactions are required for anti-bacterial activity of a type IV secretion system

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Abstract

The Type IV Secretion System (T4SS) from the phytopathogen *Xanthomonas citri* (Xac) is a nanomachine responsible for the secretion of toxins that kill other gram-negative bacteria. While a substantial amount of structural information has been obtained in the recent years about conjugative T4SSs, very little structural data is available for T4SSs with different functions, such as release and uptake of DNA, protein secretion and translocation. The core complex of the conjugative T4SS is a large symmetric ring, which spans the periplasm and is composed of multiple copies of the VirB7-VirB9-VirB10 heterotrimer. The outer layer of this complex, made up of VirB7 and the C-terminal domains of VirB9 and VirB10, is associated with the bacterial outer membrane. We have previously determined the NMR structure of the non-canonical Xac VirB7 protein and showed that it interacts with VirB9 and with itself. Here, high-resolution NMR spectroscopy was used to solve the solution structure of the complex between the N-terminal domain of VirB7 (VirB7^{NT}) and the C-terminal domain of VirB9 (VirB9^{CT}) from Xac's bacterial killing T4SS. VirB7^{NT} is fully disordered in the unbound state, but folds up into a short β -strand upon binding to VirB9^{CT} in a similar fashion as the TraN-TraO complex from *E. coli*'s pKM101 conjugative plasmid. Isothermal titration calorimetry (ITC) and NMR experiments highlighted specific intermolecular contacts that are critical determinants of the stability of the complex. Disruption of VirB7:VirB7 interactions by specific amino acid substitutions or by the deletion of VirB7's C-terminal globular domain, impairs the antibacterial activity of the T4SS *in vivo*. Amino acid substitutions that destabilize the VirB7:VirB9 complex also severely decrease the T4SS-dependent antibacterial activity. These findings indicate that both VirB7^{NT}-VirB9^{CT} and VirB7-VirB7 interactions are critical pre-requisites for the stability and correct functioning of the bacterial killing T4SS.

Introduction

Bacteria use specialized systems in order to secrete substrates to the environment or to deliver them to other cells. Translocation is mediated by membrane spanning supramolecular protein assemblies called secretion systems. As many as six different classes of secretion systems have been recognized in

gram-negative bacteria (Costa et al., 2015). One of them, termed the Type IV Secretion System (T4SS), has been shown to carry out a variety of functions in different bacterial species, including the translocation of virulence factors to eukaryotic hosts, plasmidial conjugation (exchange of DNA between bacteria) and uptake or release of DNA (Alvarez-Martinez and Christie, 2009; Zechner et al., 2012, Christie et al., 2014). *Xanthomonas citri* pv. *citri* (Xac) is a gram-negative bacterium that infects citrus plants causing citrus canker, a disease that has a large agricultural and economical impact worldwide (Brunings and Gabriel, 2003; Li et al., 2007; Ucci et al., 2014). Xac has two T4SSs, one encoded by the chromosomal DNA and another by the megaplasmid pXAC64. Xac's chromosomally encoded T4SS is not directly involved in infection (Souza et al., 2011), but rather responsible for the secretion of toxins that kill other gram-negative bacteria (Souza et al., 2015). The bacterial killing activity of Xac's T4SS could be an advantage in an environment where it needs to compete with a variety of other bacterial species.

A large amount of structural information has been obtained on conjugative T4SSs from a combination of X-ray diffraction, single particle cryo-electron microscopy, solution and solid-state nuclear magnetic resonance spectroscopies (NMR) (Bayliss et al., 2007; Fronzes et al., 2009a; Kaplan et al., 2015; Low et al., 2014; Rivera-Calzada et al., 2013). The prototypical T4SS is an assembly of a dozen proteins, VirB1-VirB11 and VirD4, which are organized in at least two large complexes (Waksman and Orlova, 2014). The first one is the core complex (CC), a toroidal structure formed by fourteen repetitions of the VirB7-VirB9-VirB10 heterotrimer. The CC is subdivided in two layers, an outer and an inner layer (Chandran et al., 2009; Fronzes et al., 2009a, 2009b; Low et al., 2014). The outer layer corresponds to VirB7, a lipoprotein that is anchored in the outer membrane, and the C-terminal domains of VirB9 and VirB10. The VirB10 C-terminal domain contains a membrane-spanning alpha-helix that crosses the outer membrane forming a pore with a diameter of approximately 20 Å (Fronzes et al., 2009a). The inner layer consists of the N-terminal domains of VirB9 and VirB10. The second large complex is called the inner membrane complex (IMC) (Low et al., 2014). It consists of VirB10, whose N-terminal transmembrane alpha helix spans the inner membrane, and a set of other inner membrane-associated proteins including VirB3,

VirB4, VirB6 and VirB8 and VirD4. A stalk connects the CC to the IMC (Low et al., 2014).

While a large amount of structural information is now available for the conjugative T4SSs, the only pieces of structural data obtained so far for a bacterial killing T4SS are the solution NMR and crystal structures of Xac's VirB7 (Souza et al., 2011). Differently from the canonical protein, Xac's VirB7 consists of a flexible N-terminal tail followed by a globular C-terminal domain (VirB7^{CT}), which is absent in the VirB7 from other organisms. VirB7^{CT} does not interact with VirB9 but participates in VirB7-VirB7 interactions that potentially mediate the formation of an extra-ring layer around the *Xanthomonas* T4SS core complex (Souza et al., 2011) (**Figure 3**). We showed previously that the N-terminal tail of Xac's VirB7 is intrinsically disordered in solution but forms a tight and specific complex with the C-terminal domain of VirB9 (Souza et al., 2011). Here we used high-resolution NMR spectroscopy in order to solve the three-dimensional structure of the complex formed by a peptide derived from the N-terminal tail of Xac's VirB7 (VirB7^{NT}) with the C-terminal domain of Xac's VirB9 (VirB9^{CT}). The calculated NMR conformers reveal that, while VirB9^{CT} is composed of two β -sheets forming a β -sandwich, VirB7^{NT} folds into a short β -strand that complements one of the β -sheets of VirB9^{CT} in a similar fashion as observed for the homologous TraO:TraN complex from the *E. coli* conjugative plasmid pKM101 (Bayliss et al., 2007). In order to assess the contribution of specific VirB7:VirB9 intermolecular contacts for the *in vitro* stability of the complex and for the function of the T4SS system *in vivo*, we prepared mutant complexes where those VirB7 residues involved in intermolecular contacts with VirB9 were altered. We found that a valine at position 37 and a tryptophan at position 34 of VirB7 N-terminal tail are absolutely essential to promote a tight interaction between VirB7 and VirB9 *in vitro*. Furthermore, replacement of VirB7 W³⁴ and V³⁷ side chains for alanine and glycine, respectively, reduces the T4SS antibacterial activity. Mutation of residues involved in VirB7:VirB7 oligomerization, or deletion of the VirB7 C-terminal globular domain, also abolishes the T4SS killing activity. In summary, we show that the specific VirB7:VirB7 and VirB7:VirB9 interactions that might be critical for the formation of a ring structure of the outer layer complex, are essential for a functional T4SS either because the system does not assemble or because the mechanism of substrate translocation was impaired.

Figure 3: Schematic diagram of the core complex of *Xanthomonas citri*'s T4SS (left) and *E. coli*'s T4SS (right) showing the location of the VirB7:VirB9 complex (A). Model of the outer layer complex of Xac based on a the superposition of the NMR structure of Xac's VirB7 (PDB 2L4W) (Souza et al., 2011) on the corresponding subunit of the crystal structure of the outer layer complex from *E. coli* (PDB 3JQO) (Chandran et al., 2009) (left) (B). Crystal structure of the outer layer complex from the conjugative T4SS of *E. coli* (PDB 3JQO) (right) (B).

Methodology

Protein production and purification

The recombinant C-terminal domain (residues 154 to 255) of Xac-VirB9 (GenBank code: AAM37469) was expressed in *Escherichia coli* BL21(DE3) RP cells harboring the plasmid pET28a-VirB9_{XAC2620_154-255_His}. Briefly, cells were inoculated into 2xTY supplemented with 30 µg/mL kanamycin and incubated at 37 °C under constant stirring at 250 rpm for 16 hours. After overnight cell growth, an aliquot of the pre-inoculum was diluted into 100 mL of M9 minimal medium (Tabor, 2001) containing ¹⁵N-NH₄Cl (1 g/L) and ¹³C-glucose (2 g/L) as the only nitrogen and carbon sources, respectively. After growing for 16 hours, an aliquot of 25 mL was taken and diluted into 1 L of M9 minimal medium. Expression of VirB9^{CT} was induced at 37 °C for 7 hours by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) when the optical density at 600 nm (O.D.₆₀₀) was 0.4. Cells were harvested by centrifugation, resuspended in 25 mL of 25% (w/v) sucrose and stored at -80 °C. Cell lysis was performed using a French Press and the lysis buffer consisted of Tris/HCl 20mM, pH 7.5. The lysate was clarified by centrifugation at 15,000 rpm at 4 °C for 40 minutes. The supernatant was applied into a HiLoad 26/10 SP Sepharose column (GE Healthcare) for separation by cation exchange chromatography. The protein was eluted with 330 mM to 450 mM of NaCl during a linear concentration gradient of Buffer B (20 mM Tris/HCl pH 7.5, 700 mM NaCl) over 14 column volumes (CV). The fractions containing VirB9^{CT} were pooled and applied into a Ni²⁺-HiTrap chelating column (GE Healthcare) equilibrated with buffer A (20 mM Tris/HCl pH 7.5; 200 mM NaCl, 20 mM Imidazole). VirB9^{CT} eluted with 200 mM imidazole during a linear concentration gradient from 0 to 100% of Buffer B (20 mM Tris/Cl pH 7.5, 200 mM NaCl, 500 mM Imidazole) over 20 column volumes. Fractions containing the VirB9^{CT} protein were combined and dialyzed against 4 liters of 10 mM Tris/HCl pH 7.5 for 16 hours at 4 °C under constant stirring using a dialysis membrane with cut-off of 3.5 kDa (SnakeSkin Dialysis Tubing Thermo). The sample was concentrated up to 4.5 mL using Amicon® Ultra-15 3K by centrifugation at 4,000 rpm at 4 °C. The polyhistidine-tag was removed by specific proteolysis using a thrombin-agarose resin (Sigma), followed by separation and buffer exchange to 20 mM sodium acetate pH 5.0 and 100 mM NaCl in a

HiLoad® 26/60 Superdex® 75 column (GE Healthcare). After VirB9^{CT} isolation, exchange to the NMR buffer (20 mM deuterated sodium acetate, pH 5.0, 50 mM NaCl) was performed using the Amicon ultra device. The VirB7^{NT} peptide, whose amino acid sequence corresponds to residues 24 to 46 of VirB7 (TKPAPDFGGRWKHVNHFDEAPTE; Genbank code: AAM37471), and mutant VirB7^{NT} peptides (D29A, F30A, W34A, V37G, V37A, N38A and V37F) were purchased from BIOMATIK. The concentration of the VirB7^{NT} peptide was calculated from the absorbance at 280 nm, and assuming an extinction coefficient of 5500 M⁻¹ cm⁻¹ for the wild type. NMR samples consisted of approximately 500 µM of ¹⁵N¹³C-VirB9^{CT} and unlabeled VirB7^{NT} dissolved in 20 mM of deuterated sodium acetate, pH 5.0, 50 mM NaCl, 0.5 mM TSP as a reference, 5% (w/v) sodium azide as a preservative and 10% (v/v) D₂O.

NMR spectroscopy

NMR experiments were performed on Bruker Avance III spectrometers operating at 800 MHz (University of São Paulo) or 900 MHz (University of Utrecht) (¹H frequency) equipped with cryogenic probes. The experiments were performed at 35 °C. NMR spectra were processed using the NMRPipe software (Delaglio et al., 1995) and analyzed using CcpNmr Analysis (Vranken et al., 2005). Assignments for backbone resonances were obtained through the analysis of the following spectra: HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, HNCO and HN(CA)CO acquired on a sample containing approximately 500 µM ¹⁵N¹³C-VirB9^{CT} in complex with excess of unlabeled VirB7^{NT}. The resonances of the nuclear spins in the side chains of VirB9^{CT} were identified through the analysis of HCCH-TOCSY, CCH-TOCSY, H(CCO)NH TOCSY, ¹⁵N-TOCSY-HSQC, and ¹³C HSQC-NOESY NMR experiments. Aromatic side chain resonances were obtained through the analysis of the previous experiments and of 2D (HB)CB(CGCD)HD and (HB)CB(CGCDCE)HE NMR experiments. The ¹H resonances of the VirB7^{NT} peptide were assigned through the analysis of 2D ¹H-¹H TOCSY and 2D-NOE experiments acquired with double filters for ¹⁵N and ¹³C. ¹⁵N longitudinal (R₁) and rotating-frame (R_{1ρ}) relaxation rates and the ¹⁵N-¹H heteronuclear steady-state nuclear Overhauser effect (NOE) were recorded at 800 MHz (¹H frequency), in the form of pseudo-3D spectra and according to standard methods (Brutscher et al., 1997; Massi et al.,

2004). The delays used for sampling ^{15}N R_1 were: 150, 300, 450, 600, 900, 1250, 1500, 1800, 2000, 2250 and 2500 ms. ^{15}N $R_{1\rho}$ was sampled during the following delays: 10, 24, 40, 54, 80, 96, 112, 124, 140, 170 and 200 ms. A spin-lock field strength of $\gamma_{\text{N}}B_1/2\pi = 1000$ Hz was applied during the $R_{1\rho}$ relaxation period, where γ_{N} is the gyromagnetic ratio of ^{15}N . The relaxation rates (R_1 and $R_{1\rho}$) were obtained by fitting the intensity decay curves to a single exponential decay function consisting of two parameters, the intensity at time zero and the rate of the decay. The relaxation rates R_2 were obtained by correcting $R_{1\rho}$ for off-resonance effects and for the contribution of R_1 using the following relation: $R_2 = R_{1\rho}/\sin^2(\theta) - R_1(\cos^2(\theta)/\sin^2(\theta))$, where $\tan(\theta) = \gamma_{\text{N}}B_1/\Delta\nu$ and $\Delta\nu$ is the resonance offset in Hz (Rule and Hitchens, 2006). The uncertainties of the fitted parameters were determined by 100 Monte Carlo simulations. $R_{1\rho}$ relaxation rates were measured at three different ^{15}N carrier frequencies: 109.2 ppm, 119 ppm and 128.8 ppm. Relaxation rates determined at more than one carrier frequency were compared, the one corresponding to the lowest χ^2 was selected. The measurements of heteronuclear NOE were carried out using saturation and inter-scan periods of 3 seconds. Residual dipolar couplings (RDCs) were measured at 800 MHz by dissolving the sample in a liquid crystalline medium consisting of 5% penta-ethyleneglycol monododecyl ether (C12E5) and n-hexanol ($r = 0.98$) (Rückert and Otting, 2000). $^1\text{D}_{\text{NH}}$ couplings were derived from a 2D ^1H - ^{15}N IPAP-HSQC experiment (Cordier et al., 1999) recorded on ^{15}N -VirB9^{CT} in complex with ^{14}N -VirB7^{NT}. The alignment tensor was calculated by singular value decomposition (Losonczi et al., 1999) using in-house scripts for MatLab. The ^{15}N relaxation data were analyzed using the extended model-free approach of Lipari and Szabo (Lipari and Szabo, 1982) using the program TENSOR2 (Dosset et al., 2000). The extraction of reliable mobility parameters requires an accurate description of the overall rotational diffusion tensor. In order to calculate the diffusion tensor, relaxation data from ^{15}N spins satisfying the following criteria were considered: i) het-NOE higher than 0.60, ii) no sign of chemical exchange, and iii) good agreement with the ^1H - ^{15}N RDCs (the conformer with the lowest Q-factor was used for this calculation). The best-fitted rotational diffusion tensor of VirB9^{CT} in complex with VirB7^{NT} is axially symmetric, characterized by a $D_{\parallel}/D_{\perp}$ ratio of 1.2 and an overall rotational correlation time of 6.6 ns. Titrations of unlabeled VirB7^{NT} and its

mutants into a solution of 275 μM ^{15}N -VirB9^{CT} were monitored by ^1H - ^{15}N -HSQC experiments. A quantitative estimation of protein:peptide binding affinities was made through the analysis of VirB9^{CT} cross-peak intensity changes as a function of the total peptide concentration assuming a 1:1 stoichiometric model (see below for details). Backbone and side chain resonance chemical shift assignments for VirB9^{CT} and VirB7^{NT} were deposited in the BMRB (access code 25512).

Structure Determination

NMR structure calculation and automated NOE assignments were performed using the Cyana 3.0 software (Güntert et al., 1997; Herrmann et al., 2002). A total of 6107 NOEs peaks were collected from 3D NOESY- ^1H - ^{15}N -HSQC, 3D NOESY- ^1H - ^{13}C -HSQC recorded separately for aliphatic and aromatic ^{13}C resonances, 2D-NOESY, 2D $^{15}\text{N}/^{13}\text{C}$ double filtered-NOE and 3D HCN-NOESY (Diercks et al., 1999) spectra and used as input to Cyana in addition to chemical shifts of assigned resonances. A total of 2188 NOEs peaks were manually assigned and given as input to CYANA as well. The chemical shifts of $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$, $^{13}\text{C}'$, $^1\text{H}\alpha$, ^{15}N , and $^1\text{H}_\text{N}$ were provided as input to Talos+ (Shen and Bax, 2013) for prediction of dihedral angles (ϕ and ψ) that were also used as dihedral angle constraints in the structure calculation protocol. Intermolecular and intramolecular hydrogen bond restraints were generated during the calculation and used as input in subsequent steps. The final distances, dihedral angles and hydrogen bond restraints generated by CYANA were used as input for recalculation in CNS (Brünger et al., 1998) followed by a final refinement in explicit solvent using the RECOORD scripts (Nederveen et al., 2005). The 20 lowest energy structures obtained after the water refinement were deposited in the PDB (2N01).

Oligomerization assays

In order to investigate the tendency of VirB7 to oligomerize *in vitro*, ^{15}N relaxation and DOSY experiments were recorded at 25 °C on a Bruker Avance III spectrometer operating at 500 MHz (^1H frequency) equipped with a TXI room temperature probe (University of São Paulo). Samples of the wild type VirB7 and a triple mutant called VirB7^{IPL} (I47A/P48A/L49A) (Table 3) were prepared at two different protein concentrations, 50 μM and 300 μM , in 20 mM Sodium Acetate pH

5, 100 mM NaCl and 10% (v/v) D₂O. Estimations of the mean ¹⁵N longitudinal (T₁) and transverse (T₂) relaxation times were performed considering the amide protons envelope. The inversion recovery delays used for sampling T₁ relaxation were 100, 200, 280, 350, 500, 750, 900, 1100, 1400, 1800 and 2000 ms. The CPMG delays used for sampling T₂ relaxation were 17, 33.9, 50.9, 67.8, 84.8, 101.8, 118.7, 135.7 and 152.6 ms. Isotropic overall rotational correlation times (τ_c) were estimated from the ratios of ¹⁵N T₁ and T₂ relaxation times by minimizing the goodness-of-fit (χ²) between experimental and calculated T₁/T₂ ratios during a grid search. Oligomerization of the wild type and the VirB7^{IP}L triple mutant was also monitored by a series of ¹⁵N-HSQC spectra recorded at 800 MHz (¹H frequency) at 40 °C as a function of the protein concentration in the range of 50 to 300 μM. Chemical shift perturbations (CSP) due to sample dilution were obtained from the weighted chemical shift changes in the ¹⁵N-HSQC spectra calculated as: $\sqrt{(\Delta\delta H^N)^2 + (0.15 \times \Delta\delta N^H)^2}$, where δH and δN are the chemical shifts of the ¹H and ¹⁵N spins, respectively. Translational diffusion coefficients (D_t) were obtained from the analysis of Diffusion-Ordered Spectroscopy experiments (DOSY) recorded using a stimulated echo pulse sequence with bipolar gradient pulses (Johnson Jr., 1999). The coefficients were obtained from the fittings of the intensity decay curves due to diffusion to an exponential decay function having two parameters (the decay rate and the intensity at time zero). The reported diffusion coefficients and uncertainties correspond to the mean and the standard deviation, respectively, of diffusion coefficients measured for different peaks along the methyl group region of the ¹D ¹H spectrum. The experiments were carried out with a 2.5 ms pulse field gradient duration, and diffusion interval of 300 ms or 280 ms, using a 500 MHz (¹H frequency) spectrometer at 25 °C.

NMR titrations

Titration to assess the binding affinity between VirB9^{CT} and VirB7 V37G were performed by adding aliquots of a 3.8 mM stock solution of the unlabeled wild type or VirB7 V37G peptide to a 275 μM solution of ¹⁵N-labeled VirB9^{CT} in the NMR tube followed by the acquisition of a ¹H-¹⁵N HSQC spectrum. An apparent binding

constant was estimated from the fitting of the peak intensity (disappearance or appearance) dependence on the total ligand concentration based on a stoichiometric model that assumes a single-site binding equilibrium:

where P and L are the free protein and free ligand, respectively; PL is the ligand-protein complex. The association binding constant is given by the ratio of the following concentrations:

$$K = \frac{[PL]}{[P][L]}. \quad (2)$$

The total concentrations of protein and ligand are given by:

$$[P_{total}] = [P] + [PL], \quad (3)$$

$$[L_{total}] = [L] + [PL]. \quad (4)$$

The binding polynomial Q for a single binding site is defined as:

$$[P]Q = [P] + [PL] = [P_{total}], \quad (5)$$

$$[P]Q = [P](1 + K[L]), \quad (6)$$

$$Q = 1 + K[L]. \quad (7)$$

The free-protein concentration can either be calculated from the definition of the equilibrium binding constant:

$$[P] = \frac{[PL]}{K[L]} = \frac{[L_{total}] - [L]}{K[L]}, \quad (8)$$

or from the definition of the binding polynomial (equation 5):

$$[P] = \frac{[P_{total}]}{Q} = \frac{[P]_{total}}{1 + K[L]}. \quad (9)$$

These two expressions (8 and 9) can be combined in order to calculate the free ligand concentration for given total protein and ligand concentrations, P_{total} and L_{total} , respectively, and a binding constant K:

$$\left(\frac{P_{total}}{1 + K[L]} \right) - \left(\frac{[L_{total}] - [L]}{K[L]} \right) = 0. \quad (10)$$

Once the free ligand concentration is known, the molar fractions of the free and bound protein (X_P , X_{PL}) are directly obtained. The molar fractions X_P and X_{PL} are related to the cross-peak intensities of the free and bound states of VirB9. This was done assuming that the cross-peak intensities ($\vec{S}$) are directly proportional to the amounts of free and bound protein given in the matrix $\tilde{P}$ (equations 11 to 13) provided that the intensities of the cross peaks at begging and at the end of the titration are known ($\vec{S}_0$).

$$\tilde{P} = \begin{pmatrix} X_P & X_{PL} \\ \vdots & \vdots \end{pmatrix}, \quad (11)$$

$$\vec{S} = \tilde{P} \vec{S}_0, \quad (12)$$

$$\vec{S}_0 = \tilde{P}^{-1} \vec{S}, \quad (13)$$

A calculated spectrum ($\vec{S}_{back}$) can be derived from the populations of free and bound protein at each ligand concentration added (equation 14):

$$S_{back} = \tilde{P} \vec{S}_0. \quad (14)$$

Repeating the calculations with different values for the binding constant K , allows the optimization of the agreement between calculated and experimental spectra. The fact that the binding regime is in slow exchange at the NMR time scale results in the intensity contribution of the free species being close to zero. In this case, this approach works for a quick estimation of the binding constant but is not robust enough to generate fine values for K .

Isothermal titration calorimetry

The calorimetric titrations were performed on a VP-ITC microcalorimeter (MicroCalTM). Measurements were obtained at constant temperature. For each titration, the cell was completed with a volume of 1.8 mL of solution containing 10 μ M to 20 μ M of VirB7^{NT} peptide (**Table 1**). The syringe of approximately 300 μ L was filled with VirB9^{CT} at a concentration of 200 to 600 μ M (**Table 1**). Protein and peptide samples were dissolved in the same buffer consisting of, 20 mM sodium acetate pH 5.0 containing 100 mM NaCl. Titrations were started with a primary injection of 0.5 μ L followed by injections of 3 to 7 μ L with 5 minutes interval. A

control experiment was made in which the buffer solution was used as the titrant. During titration, the solution in the cell was stirred at 255 rpm. The data were fitted using the program OriginLab 7.0 (OriginLab Corporation).

Table 1: Experimental details of titrations by ITC

VirB7 ^{NT} Peptide in the cell / Temperature (°C)	VirB7 ^{NT} Concentration in the cell (μM)	VirB9 ^{CT} Concentration in the syringe (μM)	VirB9 ^{CT} Injection volume (μL)	Number of injections	Maximum molar ratio during the titration (VirB9 ^{CT} /peptide)
wt / 37	20	200	3.5	86	2.24
wt / 25	20	200	5	60	2.24
D29A / 37	10	305	4	75	6.78
F30A / 37	10	300	4	75	6.78
F30A / 20	10	300	4	75	6.78
W34A / 20	10	305	4	75	6.78
V37G / 37	10	200	7	40	4.47
V37G / 20	10	200	7	40	4.47
V37A / 37	10	611	4	75	13.56
V37F / 37	10	300	4	75	6.57
N38A / 37	10	305	4	75	6.78

Interbacterial growth competition experiments

Mutations in *virB7* were carried out using the kit QuikChange® II XL (Agilent) according to the manufacturer's recommendations. The mutants were produced from the plasmid pBRA-VirB7, which contains the gene encoding VirB7 inserted between the restriction sites NcoI and SalI. The genes encoding the wild type or mutant *virB7* were under the control of a promoter inducible by arabinose. The primers used for the production of the mutants are listed in **Table 2**. The mutations were confirmed by DNA sequencing using a primer (5' ATGCCATAGCATT TTTTATCC 3') derived from the plasmid pBRA (M. Marroquin, unpublished) (**Table 3**). Xac cells labeled with GFP ("WT", "ΔB7", "ΔB7^cWT", "ΔB7^cIPL", "ΔB7^cΔCT", "ΔB7^cW34A", "ΔB7^cV37G" and "ΔB7^cIPL") (**Table 4**) and the unlabeled *E. coli* BL21(DE3)RIL ArcticExpress strains were grown in 2xTY medium until exponential phase (OD₆₀₀ = 1.0). After 12 hours of growth, cells were washed and resuspended to achieve an OD₆₀₀ of 2 for Xac and 0.2 for *E. coli*. Xac cells were then mixed with *E. coli* (Xac:*E. coli*) to a final ratio of 10:1. The mixtures were pipetted onto LB agar plates supplemented with spectinomycin (100 μg / L) and gentamicine (20 μg/mL) either in the presence or in the absence of 0.3 % of L(+)-arabinose. Competitions were carried out at 30 °C for 44 hours and evaluated by determining the CFU (colony forming unity) of each strain (Souza et al., 2015). In order to quantitative estimate the ratio between Xac and *E. coli* cells, the colonies

were excised from the plates and transferred to 1 mL of 2xTY medium. Aliquots from a serial dilution were taken and cultivated in LB 1.5 % agar plates supplemented with 150 µg/mL ampicillin or 15 µg/mL tetracycline for selection of Xac (ampicillin resistant) or *E. coli* cells (tetracycline resistant). The CFU were counted after 12 (*E. coli*) or 36 (Xac) hours of incubation at 30 °C.

Table 2: Oligonucleotides used to perform mutations in pBRA-VirB7 and/or in pET28a-VirB7. The modified codons are shown in bold.

Oligonucleotide	Sequence 5' – 3'
B7_V37G_Fw	TGGTCGTTGGAAGCAC CGG CAACCACTTTGACGAGGC
B7_V37G_Rv	GCCTCGTCAAAGTGTTG CCG TGCTTCCAACGACCA
B7_ΔCT_Fw	GCGCCACCGAAATCCCGCTCT GAA CCTCGTACACGTATCAGG
B7_ΔCT_Rv	CCTGATACGTGTACGAGGTT CAG AGCGGGATTTCCGGTGGGCGC
B7_W34A_Fw	CTCCTGACTTTGGTGGTCGT GCC AAGCACGTCAACCACTTTGACGAG
B7_W34A_Rv	CTCGTCAAAGTGTTGACGTGCTT GGC ACGACCACCAAGTCAGGAG
B7_T45A-E46A-I47A_Fw	ACTTTGACGAGGCGCC CGCCGCGCC CGCTCTACACCTCGTAC
B7_T45A-E46A-I47A_Rv	GTACGAGGTGTAGAGCGG GGCGGCGG CGGGCGCCTCGTCAAAGT
B7_I47A-P48A-L49A_Fw	GACGAGGCGCCACCGA AGCCGCGCC TACACCTCGTACACGTATC
B7_I47A-P48A-L49A_Rv	GATACGTGTACGAGGTGTAG GGCGGCGG CTTCGGTGGGCGCCTCGTC

Table 3: Plasmids used in this study

Plasmids	Description	Source/Reference
pET28a	<i>E. coli</i> expression vector, Km ^r	Novagen
pBRA	pBAD24 derivative vector for gene complementation in Xac; Sp ^r	M. Marroquin, unpublished
pBRA-VirB7	pBRA encoding the gene wt-VirB7 full length, Sp ^r	(Souza et al., 2015)
pBRA-VirB7 ^{V37G}	pBRA encoding the gene VirB7 _{XAC2622} full length, V37G mutant, Sp ^r	This study
pBRA-VirB7 ^{ΔCT}	pBRA encoding the gene VirB7 _{XAC2622} (residues 1-49), ΔCT mutant, Sp ^r	This study
pBRA-VirB7 ^{W34A}	pBRA encoding the gene VirB7 _{XAC2622} full length, W34A mutant, Sp ^r	This study
pBRA-VirB7 ^{TEI}	pBRA encoding the gene VirB7 _{XAC2622} full length, oligomerization triple mutant (T45A, E46A, I47A), Sp ^r	This study
pBRA-VirB7 ^{IPL}	pBRA encoding the gene VirB7 _{XAC2622} full length, oligomerization triple mutant (I47A, P48A, L49A), Sp ^r	This study
pET28a- VirB9 _{XAC2620_154-255_His}	pET28a encoding the gene VirB9 _{XAC2620_154-255_His} , Km ^r	(Souza et al., 2011)
pET28a-VirB7 _{XAC2622_24-139_His}	pET28a encoding the gene VirB7 _{XAC2622_24-139_His} (residues 24-139), Km ^r	(Souza et al., 2011)
pET28a-VirB7 ^{IPL}	pET28a encoding the gene VirB7 _{XAC2622} (residues 24-139), oligomerization triple mutant (I47A, P48A, L49A), Km ^r	This study

Table 4: Strains used in this study

Strains	Description	Source/Reference
<i>E. coli</i> BL21(DE3)RP	<i>E. coli</i> B F ⁻ <i>ompT hsdS</i> (r _B ⁻ m _B ⁻) <i>dcm</i> ⁺ Tet ^r <i>galλ</i> (DE3) <i>endA</i> Hte [<i>argU proL Cam</i> ^r]	Novagen
<i>E. coli</i> BL21(DE3) RIL Artic Express	<i>E. coli</i> B F ⁻ <i>ompT hsdS</i> (r _B ⁻ m _B ⁻) <i>dcm</i> ⁺ Tet ^r <i>galλ</i> (DE3) <i>endA</i> Hte [<i>cpn10 cpn60 Gm</i> ^r] [<i>argU ileY leuW Sp</i> ^r]	Agilent
<i>E. coli</i> DH5α	<i>E. coli</i> K-12 <i>fhuA2 lac</i> (del)U169 <i>phoA glnV44 Φ 80'</i> <i>lacZ</i> (del)M15 <i>gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	Life Technologies
Xac wild type pBRA GFP (WT)	<i>Xanthomonas citri</i> subsp. <i>citri</i> strain 306 (Xac 306), pBRA pBBR-5GFP; Ap ^r Sp ^r Gm ^r	(Souza et al., 2015)
Xac ΔVirB7 pBRA GFP (ΔB7)	Xac 306 ΔvirB7 _{XAC2622} pBRA pBBR-5GFP; Ap ^r Sp ^r Gm ^r	(Souza et al., 2015)
Xac ΔVirB7 pBRA-VirB7 GFP (ΔB7cWT)	Xac 306 ΔvirB7 _{XAC2622} containing pBRA-VirB7 pBBR-5GFP; Ap ^r Sp ^r Gm ^r	(Souza et al., 2015)
Xac ΔvirB7 pBRA-VirB7ΔCT GFP (ΔB7cΔCT)	Xac 306 ΔvirB7 _{XAC2622} containing the plasmid pBRA-VirB7 Δ ^{CT} pBBR-5GFP, Ap ^r Sp ^r Gm ^r	This study
Xac ΔvirB7 pBRA-VirB7 ^{W34A} GFP (ΔB7cW34A)	Xac 306 ΔvirB7 _{XAC2622} containing the plasmid pBRA-VirB7 ^{W34A} , pBBR-5GFP, Ap ^r Sp ^r Gm ^r	This study
Xac ΔvirB7 pBRA-VirB7 ^{V37G} GFP (ΔB7cV37G)	Xac 306 ΔvirB7 _{XAC2622} containing the plasmid pBRA-VirB7 ^{V37G} , pBBR-5GFP, Ap ^r Sp ^r Gm ^r	This study
Xac ΔvirB7 pBRA-VirB7 ^{IPL47_49AAA} GFP (ΔB7cIPL)	Xac 306 ΔvirB7 _{XAC2622} containing the plasmid pBRA-VirB7 ^{IPL} , pBBR-5GFP, Ap ^r Sp ^r Gm ^r	This study
Xac ΔvirB7 pBRA-VirB7 ^{IPL47_49AAA} GFP (ΔB7cTEI)	Xac 306 ΔvirB7 _{XAC2622} containing the plasmid pBRA-VirB7 ^{TEI} , pBBR-5GFP, Ap ^r Sp ^r Gm ^r	This study

Western-blotting

Xac strains were cultivated for 12 hours in 4 mL of 2xTY supplemented with 150 μg/mL ampicillin either in presence or absence of 0.3 % of L(+)-arabinose. Western blots of total cellular fractions were then carried out using the primary antibodies against VirB7 and VirB9 at 1:2,000 dilution. The primary antibodies were detected using IRDye 800CW goat anti-rabbit IgG (LI-COR Biosciences) at a 1:25,000 dilution. Nitrocellulose membranes were scanned using an Odyssey CLx infrared imaging system (LI-COR Biosciences) detection system.

Molecular dynamics simulations

Starting models for the MD simulations were the best model of the NMR structural ensemble deposited in the PDB for TraO:TraN (2OFQ) (Bayliss et al., 2007) and of the NMR ensemble described in this thesis (PDB 2N01). The starting conformation of the unbound TraO^{CT} or VirB9^{CT} corresponded to the coordinates of the bound conformer with the peptide coordinates removed. The systems were centered in dodecahedron boxes using periodic boundary conditions. Long-range electrostatic interactions were treated using the Particle-Mesh Ewald sum method with a cut-off of 1.4 nm updating every 5 time steps. A cut-off of 1.4 nm was used for the van der Waals interactions and for the grid neighbor's list. Energy minimization was initially performed with the complex and the apo structures using steepest descent algorithm followed by conjugated gradient algorithms. SPC water molecules and counter ions (Cl⁻ and Na⁺) up to a salt concentration of approximately 0.15 M were placed randomly in the boxes to avoid superposition, followed by another energy minimization step using steepest descent and conjugated gradient algorithms. Free VirB9^{CT} and the complex VirB9^{CT}:VirB7^{NT} were equilibrated at 300 K for 200 ps with restrained atomic positions in order to allow solvent relaxation and to avoid undesirable protein-solvent contacts. The first 100 ps of MD were performed without pressure coupling (NVT ensemble) and, for the latter 100 ps the pressure-coupling bath was activated (NpT ensemble) and controlled by the Parrinello-Rahman barostat approach (Nosé and Klein, 1983) with a coupling constant of 2.0 ps. All MD simulations were coupled to an external heat bath to control temperature according to the velocity rescaling method (Bussi et al., 2007) with a coupling constant of 0.1 ps. For the startup runs, velocities were assigned to the particles randomly from a Maxwell distribution at 300 K. All covalent bond lengths were constrained by the Lincs method, which allows an integration step of 2 fs for the leap-frog algorithm that integrates the Newton's equations of motion (Hockney et al., 1974). After the equilibration was achieved, the position restraints were turned off and the system was allowed to evolve for 1 ns to assure proper temperature equilibration before the production phase. Production simulations were carried out over 50 ns with snapshots recorded at every 10 ps and data was analyzed after the equilibration step. All simulations were carried out using the

GROMACS 4.6.7 suite (Hess et al., 2008) and the GROMOS 53a6 force field (Oostenbrink et al., 2004).

Results and Discussion

Full-length Xac VirB7 tends to oligomerize in solution both in the absence and in the presence of VirB9^{CT} (Souza et al., 2011). Oligomerization involves a patch at the surface of the C-terminal globular domain (residues 63-65, 85-93, 111-119 and 131) and a region of the N-terminal tail of VirB7^{NT} (residues 42-49), adjacent to the interaction site with VirB9^{CT} (residues 29-38). These observations are consistent with the hypothesis that VirB7 takes part of a larger symmetric ring in the outer layer of the T4SS core complex. In order to avoid aggregation of VirB7 during the NMR studies, we used a peptide (VirB7^{NT}) corresponding to amino acids 24 to 46 (TKPAPDFGGRWKHVNHFDEAPTE) of the VirB7 N-terminal tail that contains the VirB9 binding site and mimics the interaction of full-length VirB7 with VirB9^{CT} (Souza et al., 2011). VirB7^{NT} binds to VirB9^{CT} in slow exchange at the NMR chemical shift time scale (Souza et al., 2011), with an apparent dissociation constant of 1 μ M as estimated by isothermal titration calorimetry (ITC) or by NMR titration (**Table 5 and Figure 4**). The interaction between VirB7^{NT} and VirB9^{CT} is enthalpy driven with a ΔH of $-26.3 \text{ kcal.mol}^{-1}$ and an entropic penalty of $17.7 \text{ kcal.mol}^{-1}$ (37 °C)(**Table 5**). Such a large entropic penalty which is probably due to the large reorganization experienced by both proteins upon binding (see below).

Table 5: Binding affinities (Kd) and thermodynamic parameters of the interaction between VirB7^{NT} and VirB9^{CT}.

Peptide	Kd (μ M)	ΔG (kcal/mol)	ΔH (kcal/mol)	$-\Delta S$ (kcal/mol)	Temperature (°C)
Wild type	0.2	-9.1	-21.7	12.6	25
Wild type	1.0	-8.5	-26.3	17.7	37
Wild type	*3.7	-7.7*	n.d.	n.d.	37
V37A	9.9	-7.1	-8.1	1.05	37
V37G	825*	-4.4*	n.d.	n.d.	37
V37F	2.5	-7.9	-13.5	5.5	37
W34A	n.d.	n.d.	n.d.	n.d.	37
N38A	6.1	-7.4	-13.5	6.1	37
D29A	4.1	-7.6	-16.5	8.9	37
F30A	6.6	-6.9	-7.21	0.3	20

* These values were estimated by NMR titration (see the methods section).

Figure 4: Raw ITC data and binding isotherms obtained from assays using VirB9^{CT} in the syringe and wild type or mutant VirB7^{NT} peptides in the cell. The temperature of each titration is indicated at the top of the graph.

VirB9^{CT} is partially folded when alone in solution but forms a tight and rigid complex upon binding to VirB7^{NT}. In agreement with previous observations (Souza et al., 2011), the ¹H-¹⁵N HSQC spectrum of VirB9^{CT} recorded at 37 °C displays fewer cross peaks than expected and a broad distribution of line widths, which are typical for a domain with significant conformational freedom. Indeed, the ¹H-¹⁵N HSQC spectrum of VirB9^{CT} recorded at 9 °C displayed considerable quality improvement, showing a greater number of cross peaks and a narrower line width distribution (**Figure 5A and B**). In contrast, the ¹H-¹⁵N HSQC spectrum of ¹⁵N labelled VirB9^{CT} in the presence of the unlabeled VirB7^{NT} peptide displays all the expected cross peaks and a narrower distribution of line widths (**Figure 5C**), indicating that complex formation stabilizes a single and well-folded VirB9^{CT} conformation. These observations are consistent with the ITC measurements. The

latter show that the free energy of binding is a small number that results from the sum of two large ΔH and $T\Delta S$ contributions (**Table 5**), which is typical from protein folding events (Lesk, 2010).

The VirB9^{CT}:VirB7^{NT} complex is monomeric in solution, as indicated by a mean isotropic overall rotational tumbling time of 6.6 nanoseconds and a translational diffusion coefficient of $11.04 \times 10^{-11} \text{ m}^2\text{s}^{-1}$ at 298 K measured by DOSY and consistent with a ~15 kDa molecule. These observations reproduce our previous findings (Souza et al., 2011) and are in marked contrast with observations that the TraO C-terminal domain (TraO^{CT}), the VirB9 homologue from the conjugative plasmid pKM101 does not undergo global conformational and dynamic changes upon binding to TraN (VirB7), it is well structured even when alone in solution (Bayliss et al., 2007). In order to gain new insights into the differential dynamics of VirB9^{CT} and TraO^{CT} in the free state, we carried out molecular dynamics simulations with the isolated domains and their complexes with VirB7^{NT} and TraN, respectively. Inspection of the MD trajectories in terms of the backbone coordinates root mean square fluctuation (RMSF) revealed that VirB9^{CT} underwent larger conformational fluctuations than TraO^{CT} during the trajectory, which is consistent with the larger dynamics observed for this domain in the free state (**Figure 6**) relative to TraO^{CT} (Bayliss et al., 2007).

Figure 5: ^1H - ^{15}N HSQC spectra of VirB9^{CT} in the unbound state or in complex with VirB7^{NT} (right) and distributions of the cross-peak line widths (left). ^1H - ^{15}N HSQC spectrum of unbound VirB9^{CT} recorded at 9 °C (A) or at 37 °C (B), or in complex with VirB7^{NT} at 37 °C (C).

Figure 6: Radius of gyration (left) and Root Mean Square Fluctuations (right) of VirB9^{CT} or TraO^{CT} in the absence or in the presence of VirB7^{NT} or TraN obtained from 50 ns molecular dynamics simulation trajectories.

Solution structure of the VirB7^{NT}-VirB9^{CT} complex

In order to obtain high-resolution structural information about the interaction of Xac-VirB7 with Xac-VirB9, we solved the NMR structure of the VirB7^{NT}:VirB9^{CT} complex. Backbone resonance assignments (¹HN, ¹⁵N, ¹³C α , ¹³C β , ¹³CO) were obtained for all residues of VirB9^{CT} (**Figure 7**) except for the amino acids belonging to the reminiscent purification tag. Spectral assignments were obtained for all amino acids of VirB7^{NT} (only ¹H resonances) except for T²⁴ and T⁴⁵. The assignments of the ¹H side chain resonances were 91 and 90 % complete for VirB9^{CT} and VirB7^{NT}, respectively (**Table 6**). The structure of the complex was calculated on the basis of 2216 NOE distance restraints obtained by automated NOE assignment using CYANA 3.0, followed by recalculation and water refinement using the RECCORD scripts for CNS (Nederveen et al., 2005). The precision of the final ensemble of the 20 lowest energy NMR models is given by a backbone RMSD of 0.37 Å when the superposition is made over VirB9^{CT} residues 163 to 252 and VirB7^{NT} residues 30 to 38, and 0.32 Å or 0.41 Å when the superposition is made over VirB9^{CT} (residues 163-252) or VirB7^{NT} (residues 30-38) alone, respectively (**Table 6**). An analysis of the stereochemistry of the NMR models using Procheck (Laskowski et al., 1993) shows that approximately 98.7 % of all residues fall within the most favored and additionally allowed regions of the Ramachandran plot (**Table 6**).

Figure 7 ^1H - ^{15}N HSQC spectrum of ^{15}N -VirB9^{CT} in complex with unlabelled VirB7^{NT} recorded at 800 MHz (^1H frequency).

Table 6: Assignment and statistics for the solution structure of the complex ^{15}N , ^{13}C -VirB9^{CT}:VirB7^{NT}

Completeness of assignment ^{15}N-^{13}CVirB9^{CT}: ^{14}N-VirB7^{NT} (%)	92.5
Backbone VirB9 ^{CT} (^{15}NH , ^{13}Ca , ^{13}CO) (%)	95.6
Backbone VirB9 ^{CT} (^1HN) (%)	99.01
Side Chain VirB9 ^{CT} (^1H) (%)	91.038
Total VirB9 ^{CT} (^1H , ^{15}N , ^{13}C) (%)	93.26, 72.152, 84.86
Backbone VirB7 ^{NT} (^1HN) (%)	94.74
Side Chain VirB7 ^{NT} (^1H) (%)	90.217
Total VirB7 ^{NT} (^1H) (%)	91.176
Total unambiguous restraints	2216
Unambiguous restraints	
Intra-residue (i=j)	535
Sequential (i-j = 1)	675
Medium range (1 < i-j < 5)	239
Long range (i-j ≥ 5)	767
Inter-molecular	87
Others restrains	
TALOS dihedral angles	140
Consistent Violations	
Distances > 0.5	0
Torsion angles > 5°	0
RMS deviation from mean (Å)	
All core residues (30-38, 163-252)	
All heavy atoms	0.827
Backbone atoms (N, Cα, C')	0.366
VirB7^{NT} core residues (30-38)	
VirB7 ^{NT} heavy atoms	1.119
VirB7 ^{NT} Backbone atoms (N, Cα, C')	0.415
VirB9^{CT} core residues (163-252)	
VirB9 ^{CT} heavy atoms	0.759
VirB9 ^{CT} Backbone atoms (N, Cα, C')	0.321
Ramachandram plot (30-38, 163-252)	
Most favored region (%)	86.31 +/- 2.51
Additionally allowed region (%)	12.36 +/- 2.59
Generously allowed region (%)	1.32 +/- 0.37
Disallowed regions (%)	0.00 +/- 0.00
WHAT CHECK structure Z-scores	
Ramachandram plot appearance	-2.42 +/- 0.26
2 nd generation packing quality	0.25 +/- 0.28
Chi1-Chi2 rotamer normality	-1.00 +/- 0.35
Backbone conformation	-4.23 +/- 0.29
G-factors	
Dihedrals	-0.26 +/- 0.05
Covalent	0.11 +/- 0.04
Overall	-0.14 +/- 0.03
Q-factor from RDC considering all NH in the backbone of VirB9^{CT}	0.57 +/- 0.01
Q-factor from RDC considering the rigid conformation parts of VirB9^{CT}	0.29 +/- 0.01
Q-factor from RDC of NHs with NOE-het higher than 0.5 in VirB7^{NT}	0.50 +/- 0.17
BMRB accession number	25512
PDB ID	2N01

In order to validate the calculated structures, ^1H - ^{15}N residual dipolar couplings (D_{NH}) of VirB9^{CT} in complex with VirB7^{NT} were measured in the presence of a nematic phase of C12E5 polyethylene glycol and n-hexanol ($r = 0.98$) (Rückert and Otting, 2000). The agreement between experimental and back-calculated RDCs is given by a mean Q-factor of 0.29 +/- 0.01 when considering $^1\text{H}^{15}\text{N}$ spin pairs located within secondary structure elements (**Figure 8**) (**Table 6**). As we lacked a ^{15}N labeled VirB7^{NT} peptide sample, D_{NH} values obtained for the full length

VirB7 in complex with VirB9^{CT} in our previous study (Souza et al., 2011) were fitted to the coordinates of the bound conformation of the VirB7^{NT} peptide (PDB 2N01). A slightly larger Q-factor of 0.50 +/- 0.17 was obtained in this case when considering only ¹H¹⁵N spin pairs displaying heteronuclear NOE values larger than 0.5 (Souza et al., 2011). In conclusion, the NMR structures of the VirB9^{CT}:VirB7^{NT} complex agree reasonably well with the ¹H-¹⁵N dipolar couplings.

Figure 8: (A) Correlation between experimental and back-calculated ¹H-¹⁵N RDCs. The reported Q-factors correspond to average values over all NMR conformers. (B) Experimental (red) and back-calculated (blue) RDCs of ¹⁵N labelled VirB9 (left) or full-length ¹⁵N-labeled VirB7 (right) in complex as a function of the amino acid number.

Description of the VirB9^{CT}-VirB7^{NT} complex NMR structure

VirB9^{CT} consists of a β -sandwich formed by two β -sheets, each containing four antiparallel β -strands (**Figure 9A**). The first β -sheet is composed of strands $\beta 1$ (residues 167-171), $\beta 8$ (residues 245-251), $\beta 7$ (residues 237-242) and $\beta 4$ (residues 206-210). The second β -sheet is formed by strands $\beta 2$ (residues 182-186), $\beta 3$ (residues 189-193), $\beta 6$ (residues 228-232) and $\beta 5$ (residues 221-225). β -strands 3 and 4 are connected by a well-defined loop, corresponding to residues 194-205 (loop 3), that contains a short 3_{10} -helix (residues 196-198). Similarly, β -strands 4 and 5 are connected by the well-defined loop 4 (residues 211-220) (**Figure 9A**).

VirB7^{NT} folds into a short β -strand, $\beta 9$ (residues 35 and 36) that complements $\beta 1$ of VirB9^{CT} forming a 5-stranded antiparallel β -sheet composed of $\beta 9$ - $\beta 1$ - $\beta 8$ - $\beta 7$ - $\beta 4$ (**Figure 9A**). The $\beta 9$ strand is stabilized by three inter-strand hydrogen bonds to $\beta 1$: Y¹⁶⁹ CO-K³⁵ NH, Y¹⁶⁹ NH-K³⁵ CO and Y¹⁶⁷ CO-V³⁷ NH. The side chain of VirB7^{NT} V³⁷ penetrates into a hydrophobic pocket of VirB9^{CT} between strands $\beta 1$ of β -sheet 1 and $\beta 2$ of β -sheet 2, making a series of important hydrophobic contacts with the side chains of Y¹⁶⁷, Y¹⁶⁹, P¹⁸⁰, V¹⁸³ and L²⁴⁹ that contribute to the stabilization of the VirB9^{CT} β -sandwich (**Figure 9B**). Additionally, the side chains of F³⁰ and W³⁴ from VirB7^{NT} penetrate into a second hydrophobic pocket located on the surface of the first β -sheet of VirB9^{CT} ($\beta 1$ - $\beta 8$ - $\beta 7$ - $\beta 4$), making contacts with the side chains of D¹⁶⁸, A¹⁷⁰, R²¹⁰, E²¹³, G²⁴⁸ and R²⁵⁰ (**Figure 9B**). Interestingly, a cation-pi interaction between W³⁴ and R²⁵⁰ was observed in all NMR conformers (**Figure 10 and Figure 11**).

Figure 9: Structure of the VirB9^{CT}:VirB7^{NT} complex. (A) Two views of the NMR ensemble consisting of the 20 lowest energy models. The conformers were superimposed on the secondary structure elements of the lowest energy structure. Disordered regions (residues 154-162 and 253-255 of VirB9^{CT}, or 24-28 and 39-46 of VirB7^{NT}) are not shown. (B) Surface representations of the best model of VirB9^{CT} in complex with VirB7^{NT}, the latter is shown in cartoon. The surface of VirB9^{CT} is colored according to the protein electrostatic potential as calculated by PyMOL (Schrödinger, LLC), where red indicates negative and blue indicates positive electrostatic potential energy. The lower representations display VirB9^{CT} in cartoons. The intermolecular contacts made by F30, W34 and V37 from VirB7^{NT} are indicated.

Figure 10: (A) Schematic representation of the intermolecular contacts between VirB9^{CT} and VirB7^{NT}. (B) Snapshots from a ¹H-¹⁵N NOESY-HSQC spectrum and a 2D-NOE spectrum showing intermolecular NOEs to R²⁵⁰ H_ε and A¹⁷⁰ H_β.

Figure 11: Quantification (A) and drawing (B) of the major intermolecular contacts involving W³⁴ of VirB7^{NT} and D¹⁶⁸ (blue), R²⁵⁰ (red), A¹⁷⁰ (yellow) and Y¹⁶⁹ (green) from VirB9^{CT} in each NMR conformer according to the criteria defined by Chimera UCSF (Pettersen et al., 2004).

A series of unexpected chemical shifts were observed and could be rationalized on the basis of the proximity between amino acids in VirB9^{CT} and the aromatic moieties from F³⁰ and W³⁴ in VirB7^{NT}. Thus, the chemical shift of A¹⁷⁰ ¹H_β is shielded to -0.34ppm (**Figure 10B and 12**), while the chemical shifts of D¹⁶⁸ ¹H_α and ¹³C_β are deshielded to 6.14 and 46.38 ppm, respectively. The chemical shift of Y¹⁶⁹ ¹H_α is deshielded to 6.55 ppm due to the proximity with W³⁴. The ¹H_α

hydrogen of E²¹³ displays the very unusual chemical shift of 2.04 ppm, possibly due to proximity with the aromatic ring currents of F³⁰ and W³⁴ of VirB7^{NT} (**Figure 12**).

Figure 12: ¹H-¹³C-HSQC spectrum showing unexpected chemical shifts due to proximity to the aromatic residues F³⁰ and W³⁴ of VirB7^{NT}.

The general topology of the VirB7^{NT}-VirB9^{CT} complex is similar to that observed for TraN:TraO^{CT} (PDB 2OFQ) (Bayliss et al., 2007). The backbone RMSD between the best NMR models from TraO^{CT}:TraN and VirB9^{CT}:VirB7^{NT} is 1.53 Å when the two conformers are superimposed on their secondary structure elements (**Figure 13A**). This is surprising considering that VirB9^{CT} and VirB7^{NT} share only 22.1% and 13.6% of identity with TraO^{CT} and TraN, respectively (**Figure 13B**). The most striking differences are restricted to the loops connecting the VirB9^{CT} β-strands, the presence of an additional hydrophobic pocket on the surface of Xac-VirB9^{CT} and specific protein-peptide contacts (**Figure 13**). Specifically, loop4 that connects β4 and β5 from VirB9^{CT} assumes a different orientation in the VirB9^{CT}:VirB7^{NT} complex, stabilized by a large number of intermolecular NOEs from F³⁰ (VirB7^{NT}) to E²¹³ and R²¹⁰ (VirB9^{CT}) (see below). Moreover the short β-strand observed in the loop4 of TraO^{CT}:TraN is absent in the Xac VirB9^{CT}:VirB7^{NT} NMR structure (**Figure 13**). In contrast with the TraO^{CT}:TraN complex, the VirB7^{NT}:VirB9^{CT} complex lacks a 3₁₀ helix in loop 1, and displays a short 3₁₀ helix in loop 3 (**Figure 13**).

Figure 13: Intermolecular contacts between VirB9^{CT} and VirB7^{NT} (top) or between TraO^{CT} and TraN (bottom) (A). The side chains that make intermolecular contacts in at least 80% of the VirB9^{CT}:VirB7^{NT} conformers are shown in sticks. These contacts are compared to similar contacts between TraO and TraN (PDB 2OFQ) (A). Amino acid sequence alignment between TraN and VirB7^{NT}, or between TraO^{CT} and VirB9^{CT}. The locations of the secondary structure elements are indicated either under or above the primary sequences (B).

In spite of the low amino acid sequence conservation detected between TraO:TraN and VirB9:VirB7, most of the intermolecular interactions observed in the NMR structure of the TraO^{CT}:TraN complex were preserved in the complex between VirB9^{CT} and VirB7^{NT} (**Figure 13**). The motif P³²V³³N³⁴K³⁵ of TraN is conserved among orthologous proteins (Souza et al., 2011), and was identified as being important for the interaction with TraO (Bayliss et al., 2007). The equivalent segment in VirB7 is H³⁶V³⁷N³⁸H³⁹. In analogy with V³³ from TraN, V³⁷ of VirB7 penetrates in the hydrophobic core of VirB9^{CT} making several intermolecular interactions with residues from the two β -sheets (**Figure 13** and **Figure 14**), while W³⁴ from VirB7^{NT} assumes a very similar orientation to W²⁷ from TraN but makes different intermolecular contacts with residues in β -strands 1 and 8, such as a cation-pi interaction with R²⁵⁰ (**Figure 13** and **Figure 12**). One of the largest differences between the binding interface of the two complexes is the presence of F³⁰ from VirB7^{NT} while a proline (P²⁵) is found in the equivalent position in the

TraN:TraO^{CT} complex (2OFQ). F³⁰ penetrates into a second pocket on the surface of VirB9^{CT} close to β 7 and β 8 (**Figure 9B**), where it contacts E²¹³ and G²⁴⁸ through hydrophobic interactions and makes a cation-pi interaction with the side chain of R²¹⁰ (**Figures 13A and 15**). While N³⁴ of TraN penetrates between the two β -sheets of the TraO^{CT} β -sandwich, the side-chain of the equivalent asparagine residue of VirB7^{NT}, N³⁸, is oriented towards the outside of the hydrophobic core not participating in intermolecular contacts (**Figure 13A**).

Figure 14: Quantification (A) and drawing (B) of the major intermolecular contacts involving V³⁷ of VirB7^{NT} and Y¹⁶⁷ (green), Y¹⁶⁹ (blue), D¹⁶⁸ (yellow), P¹⁸⁰ (red), and V¹⁸³ (pink) from VirB9^{CT} in each NMR conformer according to the criteria defined by Chimera UCSF (Pettersen et al., 2004).

Figure 15: Quantification (A) and drawing (B) of the major intermolecular contacts involving F^{30} of VirB7^{NT} and R^{210} (red), A^{170} (blue), G^{248} (yellow) and E^{213} (green) from VirB9^{CT} in each NMR conformer according to the criteria defined by Chimera UCSF (Pettersen et al., 2004).

Backbone dynamics

In order to characterize the backbone dynamics of VirB9^{CT} in complex with VirB7^{NT}, longitudinal (R_1) and transverse (R_2) ^{15}N relaxation rates as well as the heteronuclear $\{^1\text{H}\}$ - ^{15}N NOE (het-NOE) were measured for ^{15}N -labeled VirB9^{CT} in

complex with unlabeled VirB7^{NT}. The relaxation data were analysed using the extended model free approach of Lipari-Szabo (Lipari and Szabo, 1982) as described in the methods section. The Lipari-Szabo analysis provides a generalized order parameter (S^2) and an internal correlation time (τ_i), which are measures of the amplitude and the timescale of backbone motions at a given site (Lipari and Szabo, 1982). The profile of ^{15}N order parameters along the backbone of VirB9^{CT} in complex with VirB7^{NT} is shown in **Figure 16**, and color-coded on the NMR structure of the complex (**Figure 16B**). VirB9^{CT} displays S^2 typical of a rigid protein with an average S^2 value of 0.8 (**Figure 16A**). Except for the N-terminal and the C-terminal tails, two other sites showed lower S^2 , L¹⁷⁸ in loop 1 and G²³³ in loop 6, indicating the presence of fast motions on the nanosecond time scale. Despite the presence of fast motions, loop 1 and loop 6 are well defined in the NMR ensemble (**Figure 9**). The model-free analysis indicated the presence of relaxation caused by chemical exchange (R_{ex}) due to slow motion dynamics (microsecond to millisecond) in loop 1 residues S¹⁷⁶, W¹⁷⁷ and L¹⁷⁸ as well as for D²⁴⁴ in the very short β -turn between strands $\beta 7$ and $\beta 8$. These residues all map to the same patch on the surface of the structure (**Figure 16B**). At the moment, it is not clear whether this slow dynamics have biological implications but it is interesting to note that this patch is involved in VirB9:VirB9 (TraO:TraO) interactions in the crystal structure of the pKM101 core complex outer layer (Chandran et al., 2009).

Although the stretch between D²⁹ and N³⁸ in VirB7^{NT} is well defined in the NMR structure, the tails (residues 24-28 and 39-46) display lower precision. In order to assess the correlation between VirB7 internal dynamics and the precision of the NMR ensemble along the peptide backbone, we re-analyzed ^{15}N relaxation data obtained for the full-length ^{15}N -VirB7 in complex with unlabeled VirB9^{CT} in our previous study (Souza et al., 2011) in light of our current structural data. Heteronuclear $\{^1\text{H}\}$ - ^{15}N NOE values measured on full-length VirB7 bound to VirB9^{CT} were color-coded on the structure of VirB7^{NT} in complex (**Figure 16B**). Clearly, all ^{15}N spin probes displaying NOE values greater than 0.5 fall within the better defined region of the peptide. Thus, the presence of a well-defined short β -strand flanked by more flexible regions in VirB7^{NT} is consistent with the internal dynamics of the full-length protein.

Figure 16: Backbone dynamics of VirB9^{CT}:VirB7^{NT}. (A) 1H - ^{15}N bond vector order parameters, S^2 , (top) and exchange rates (R_{ex}) (bottom) as a function of the residue number for the complex. The approximate positions of the secondary structure elements of VirB9^{CT} are shown at the top, with the β -strands indicated by black rectangles and the 3_{10} -helix by a red rectangle. (B) NMR structure of the complex color-coded with the ^{15}N S^2 values obtained from model-free analysis (VirB9^{CT}) or $\{^1H\}$ - ^{15}N NOE data (VirB7^{NT}). Residues that display R_{ex} are indicated as spheres (S^{176} , W^{177} , L^{178} , D^{244}).

Contribution of specific intermolecular contacts for the VirB7^{NT}:VirB9^{CT} interaction

In order to assess the contribution of specific intermolecular interactions for the stability of the VirB7^{NT}:VirB9^{CT} complex, the dissociation constants (K_d) between VirB9^{CT} and wild type or mutant VirB7^{NT} peptides were determined by isothermal titration calorimetry (ITC) or NMR titration experiments (**Table 5 and Figures 4 and 17**). The VirB7^{NT} residues chosen for mutation are those ones that altogether make up to 58% of all intermolecular contacts with VirB9^{CT}. We observed that substitution of W^{34} for an alanine or of V^{37} for a glycine reduced the binding affinity to

undetectable levels through ITC (**Figure 18, Table 5**). However, NMR titrations showed that the V37G mutant peptide still binds to VirB9^{CT} in slow exchange at the NMR chemical shift time scale, albeit with a much greater apparent K_d of approximately 825 μM (**Table 5, Figure 17**). When V³⁷ is replaced for an alanine instead of a glycine, the binding free energy is partially restored as indicated by a K_d of 9.9 μM in comparison with 1 μM for the wild type (**Table 5**). On the other hand, when V³⁷ is replaced for a phenylalanine the K_d increased only slightly from 1 μM to only 2.5 μM (**Table 5**), indicating that a bulkier side chain can be accommodated inside the hydrophobic pocket between VirB9^{CT} β -sheets without introducing substantial energetic penalties. It is curious that the entropy component of the binding free energy of the mutant V37A is very small, 1.05 kcal.mol⁻¹ (**Table 5**), suggesting that the binding equilibrium does not involve the folding of VirB9^{CT}. This observation is not consistent with the NMR spectrum that shows qualitatively that VirB9^{CT} is well folded when bound to VirB7^{NT} V37A.

Substitution of F³⁰ to alanine increases the K_d from 1 μM (wild type) to 6.6 μM , consistent with the fact that F³⁰ makes several intermolecular contacts with VirB9^{CT} (**Figures 10 and 15**). The substitution of other residues that also contact VirB9^{CT}, such as D²⁹ and N³⁸, increased the K_d from 1.0 μM (wild type) up to 4.1 or 6.1 μM , respectively (**Table 5**), indicating that these residues contribute to the stability of the complex. Such modest effects in terms of K_d are not easily correlated with the number of intermolecular contacts that define the orientation of each side chain in the NMR ensemble, especially in the case F³⁰ and W³⁴ (**Figure 10**). This apparent discrepancy could be rationalised in terms of the energy contribution of each interaction. For example, W³⁴ makes a strong cation- π interaction with R²⁵⁰, while F³⁰ would makes weaker and transient interactions.

Figure 17: Comparison of the ^1H - ^{15}N -HSQC spectrum of VirB9^{CT} in the unbound state and in complex with wild type and mutant VirB7^{NT} (A). Graphs of intensity increase of the cross peaks of the bound VirB9^{CT} along the titration with wild type (left) and mutant V37G (right) VirB7^{NT} (B).

Figure 18: Calorimetric titration of VirB7^{NT} wild type (dark blue), V37G (green), and W34A (red) with VirB9^{CT}.

Importance of VirB7:VirB9 and VirB7:VirB7 interactions for the T4SS's activity *in vivo*

The *virB7* gene is essential for the antibacterial activity of the Xanthomonadaceae T4SS (Souza et al., 2015). As a means of assessing the effect of disruption of VirB7:VirB9 interactions on the activity of the *Xanthomonas* T4SS, Xac cells carrying a deletion of the *virB7* gene were complemented with a plasmid harboring either the wild type or mutant *virB7* (V37G or W34A) gene, whose expression is under the control of an arabinose inducible promoter (**Figure 19A**). These Xac strains were then used in growth competition assays against *E. coli* cells in the presence of arabinose as described in the methods section.

When *virB7* is deleted from the genome, Xac cells lose their bacterial killing activity through the T4SS (**Figure 19B**). Complementation with a plasmid harboring the wild type *virB7* gene restores the antibacterial activity to levels similar to those observed for the wild type strain (**Figure 19B**). In contrast, complementing the *virB7* knockout strain with the *virB7* W34A mutant does not restore Xac's competitiveness to the same level as observed in the strain complemented with the wild-type *virB7* (**Figure 19B**). Complementation with the *virB7* V37G mutant restores Xac's competitiveness to almost the same level as the wild type complement (**Figure 19B**). These findings show a clear correlation between the antibacterial activity and the reduced *in vitro* affinity or the lack of interaction for these mutants (**Table 5**).

In order to assess whether wild type or mutant VirB7 and VirB9 proteins are present *in vivo*, a western blotting was performed using antibodies against both proteins. The expression of VirB7 constructs (~ 12.6 KDa) in Xac cells harboring the different plasmids could be revealed by the western blotting assay performed using anti-VirB7 primary antibody except for the VirB7 Δ CT due to its smaller size (~ 3.2 KDa) (**Figure 19C**) confirming that the lack of activity of the T4SS in some Xac strains was not due to the absence of expression of VirB7 proteins. A small amount of VirB9 was detected in the total cellular fraction of Xac cells complemented with the plasmid super-expressing VirB7-V37G mutant but not VirB7-W34A. In order to evaluate if the super-expression of VirB7 influences these results, the competition and the western-blotting experiments were repeated using cells cultivated in the absence of arabinose, thus allowing for basal gene expression (**Figure 20**). In this case, the mutant VirB7-V37G does not restore the activity of the T4SS in the knockout strain (**Figure 20A**), nor is VirB9 detected in these cells (**Figure 20B**). The fact that VirB9 is not detected suggests that it is being degraded possibly due to its low stability when not bound to VirB7 (**Figure 5**); consequently the T4SS channel is not being assembled. This conclusion is supported by previous co-immune precipitation experiments showing that VirB9 and VirB10 are not detected in the absence of VirB7 (Souza et al., 2011). The detection of a small amount of VirB9 in the cells grown in the presence of arabinose (**Figure 19C**) could be attributed to a larger expression level of the VirB7 V37G mutant achieved by the action of the inducer, what could compensate for its lower binding affinity relative to the wild type (**Table 5**) (**Figures 17 and 18 above**). In summary, the W34A mutation abolishes

the binding of VirB7 to VirB9 *in vitro* and *in vivo*, while binding may still be detected for the V37G mutant when it is in excess (**Table 5, Figures 19B and 20**). Disruption of the VirB7 and VirB9^{CT} complex prevents the assembly of the T4SS causing loss of bacterial killing activity.

VirB7 oligomerization is important for the T4SS's killing activity. Substitution of residues I⁴⁷P⁴⁸L⁴⁹, located at the VirB7 oligomerization interface (Souza et al, 2011) to alanines, generates a triple mutant (VirB7^{IPL}) (**Figure 19A**) that does not oligomerize *in vitro* as indicated by measurements of translational diffusion coefficients (D_t) and of overall rotational tumbling times (τ_c) by NMR at two different protein concentrations (**Table 7**), or by the lack of significant chemical shift perturbations due to dilution (**Figure 19D and 19E**). When used to complement the Xac *virB7* knockout strain, the *virB7*^{IPL} was unable to restore the T4SS-dependent killing activity (**Figure 19B**) demonstrating that VirB7:VirB7 interactions are functionally relevant. The same phenotype was observed when the competition growth assay was performed using a complementation with the mutant *virB7*^{TEI}, where the residues T⁴⁵E⁴⁶I⁴⁷ were substituted to alanines (**Figure 19A and 19B**). Expression of VirB9 at the same level as in the wild type strain was detected in the Xac *virB7* knockout cells complemented with a plasmid harbouring either the *virB7*^{IPL} or *virB7*^{TEI} mutants, indicating that the core complex is being assembled in this strain (**Figure 19C and 20B**) and the lost of T4SS's function might be due impairment of the mechanism of translocation of substrates through the core complex.

Figure 19: VirB7:VirB7 and VirB7:VirB9 intermolecular interactions are critical for a functional bacterial killing T4SS. (A) VirB7 NMR model (PDB 2L4W) showing the residues that were substituted in this study. **(B)** Quantitation of the interbacterial competition assay performed in the presence of arabinose (Top). Xac cells labeled with GFP visualized after 44 hours of cell growth in the presence or absence of *E. coli* (unlabeled) (Bottom). **(C)** Western-blotting showing the level of expression of VirB7 and VirB9 proteins in each Xac strain used in the competition experiment. Complemented strains are indicated with a red “c”. **(D)** Chemical shift perturbations of the wild type VirB7 or the VirB7^{IPL} mutant due to the increase in the protein concentration. **(E)** Residues displaying CSP greater than the mean plus 1 standard deviation are color coded red.

Figure 20: Interbacterial growth assay. Ratios of Xac/E.coli cell after 48hs of growth on plates (A). Xac cells labeled with GFP (green fluorescent protein) were visualized after 48hs growth either in the presence or the absence of *E. coli* (unlabeled) (B). Western-blotting showing the expression of VirB7 constructs in the interbacterial competition experiments. Complementation experiments are indicated with a red “c” (C).

Table 7: Mean ^{15}N relaxation rates (R_1 and R_2), isotropic overall rotational tumbling time (τ_c) and translational diffusion coefficient (D_t) measured for the wild type VirB7 and the VirB7^{IPL} at two different protein concentrations.

VirB7	R_1 (s ⁻¹)	R_2 (s ⁻¹)	τ_c (ns)	D_t (m ² s ⁻¹ × 10 ⁻¹¹)
WT 50 μ M	1.68	8.83	7.91	8.40 ± 0.40
WT 300 μ M	1.32	15.19	12.56	7.28 ± 0.12
IPL 50 μ M	1.74	9.15	7.92	9.33 ± 0.19
IPL 300 μ M	1.83	9.53	7.86	8.84 ± 0.85

As above mentioned, Xac’s VirB7 contains an additional N0 C-terminal globular domain (residues 50-139, VirB7^{CT}), which is absent in the VirB7 from other organisms. VirB7^{CT} does not interact with VirB9 but participates in VirB7-VirB7 interactions and potentially forms an extra-ring layer around the *Xanthomonas* T4SS core complex (Souza et al, 2011) (**Figure 19A**). In order to assess the functional relevance of VirB7^{CT}, we investigated whether its complete removal would alter the Xac’s T4SS bacterial killing activity. Indeed, complementation of Xac *virB7* knockout cells with a plasmid harbouring the gene encoding the mutant *virB7* Δ CT does not restore the T4SS bacterial killing activity to the same level as the wild type *virB7* complement (**Figure 19B and 20A**). Interestingly, the VirB9

protein is detected in the Xac knockout cells complemented with *virB7* Δ CT, but it is not detected when the complete *virB7* gene is removed (**Figure 19C**). These findings indicate that binding of VirB7^{NT} to VirB9 is required for the stabilization of the core complex. In contrast, interactions between Xac-VirB7 subunits and the presence of the VirB7^{CT} domain are not essential for the assembly of the system, but are still required for a functional T4SS.

Conclusions

We showed previously that Xac's VirB7 is different from the canonical VirB7 proteins due to the presence of an extra C-terminal N0 domain (VirB7^{CT}). This domain is involved in VirB7 oligomerization *in vitro*. These observations immediately suggested that Xac-VirB7^{CT} could form an extra symmetric ring around the outer layer of the core complex of the T4SS, a significant structural difference with respect to the T4SS encoded by the conjugative plasmids pKM101 and R388 from *E. coli*. Here we described the NMR structure of the complex formed between the C-terminal domain of VirB9 and the N-terminal tail of VirB7. Additionally, we showed that specific VirB7:VirB7 and VirB7:VirB9 interactions are essential for a functional T4SS. These findings are consistent with the view that VirB7 and VirB9 units assemble into a symmetric ring around VirB10 forming the core complex of Xac's T4SS. Interestingly, the VirB7 extra ring structure in the outer layer of the core complex is essential for the T4SS function. Nevertheless, whether disruption of specific VirB7:VirB7 and VirB7:VirB9 interactions leads to an inactive T4SS due to destabilization of the symmetric ring structure of the outer layer complex, or by affecting the toxin translocation mechanism in some way, remains to be investigated.

**Chapter III - Flexibility and interactions of the proline-rich region (PRR)
from VirB10**

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Abstract

The Type IV Secretion (T4S) system of the phytopathogen *Xanthomonas citri* is a multiprotein complex responsible for the transport of toxins that kill other gram-negative bacteria. The prototypical T4SS is organized in two sub-complexes: the core complex (CC) that crosses the periplasm and the outer membrane, and the inner membrane complex (IMC). A stalk, mostly composed by the N-terminal domain of VirB10, connects the CC to the IMC. Thus, VirB10 is a key T4SS subunit, since it is poised to mediate the communication between the CC and the IMC. A recent study of the full-length T4SS by cryo-electron microscopy suggested that the stalk region is dynamic. Here we used solution NMR spectroscopy to investigate the structure and the dynamics of a fragment corresponding to the N-terminal region of VirB10 located at the stalk region. Analysis of NMR chemical shifts and ^{15}N spin relaxation data indicated that the fragment is highly flexible at the sub-nanosecond time scale, what is consistent with its relatively large content of proline residues. Notwithstanding, a short and transient alpha helix was found at its C-terminal end. NMR titration experiments showed that this helical region interacts with the N-terminal domain of VirB9. These observations are consistent with recent cryo-electron microscopy data showing that VirB10 is located close to the N-terminal domain of VirB9 at the inner layer of the CC. We hypothesize that the intrinsic dynamics of VirB10 could be preserved in the stalk, where it might play a relevant functional role.

Introduction

The type IV Secretion system (T4SS) is a nanomachine specialized in the translocation of proteins and protein-DNA complexes across the bacterial cell envelope. It is present in many gram-negative bacteria where it assumes a variety of functions, such as delivery of virulence factors, conjugation, or release and uptake of DNA (Zechner et al., 2012). The best characterized T4SSs are the ones encoded by the *E. coli* plasmids pKM101 and R388, which are involved in conjugation. The T4SSs are formed by a dozen proteins, of which VirB7, VirB9 and VirB10 are part of the system's translocation chamber. Cryo-electron microscopy studies of the T4SS from the *E. coli* conjugative plasmid R388 showed that it

consists of two sub-complexes connected by a flexible stalk, the core complex (CC) and the inner membrane complex (IMC), (Low et al., 2014). The core complex consists of two layers, the inner layer and the outer layer. The latter is a ring-shaped complex anchored in the bacterial outer membrane. It is formed by fourteen repetitions of the heterotrimer made by the C-terminal domains of VirB10 and VirB9, and by VirB7. The inner layer of the CC consists of the N-terminal regions of VirB10 and VirB9 (**Figure 3**). While a X-ray structure is available for the outer layer complex, and for several of the IMC components (Waksman and Fronzes, 2010), no high-resolution structural information is available for the inner layer nor for the stalk region of the T4SS.

The chromosomally encoded T4SS from *Xanthomonas citri* pv. *citri* (Xac), a phytopathogen that infects citrus plants causing the so-called citrus canker disease (Alegria et al., 2005), was shown to be responsible for the translocation of toxins that cleave the peptide glycan layer killing other gram-negative bacteria (Souza et al., 2015). In order to increase our understanding of the architecture and the mechanism of functioning of Xac's bacterial killing T4SS, we initiated an investigation about the structural propensities of a fragment corresponding to the N-terminal region of VirB10 (VirB10^{NT}, residues 85-182), that corresponds to the proline rich region (PRR) and part of the linker to the β -barrel domain (**Figure 21**) by high-resolution NMR spectroscopy in solution. Analyses of ¹⁵N spin relaxation data and NMR chemical shifts showed that, albeit being highly flexible at the sub-nanosecond time scale, VirB10^{NT} acquires a transient alpha helical conformation at the C-terminal end (151-163). NMR titration experiments indicated that this short alpha helix is involved in interactions with the N-terminal domain of VirB9. These findings provide compelling evidence for the interaction between the N-terminal domains of VirB9 and VirB10, what is consistent with their proximity in the Cryo-EM model of the CC inner layer (Rivera-Calzada et al., 2013). Furthermore, the intrinsic flexibility of VirB10^{NT} could be preserved in the stalk. In fact Cryo-EM studies provided evidence for the existence of significant flexibility in that region. These conclusions are valid for the bacterial killing and conjugation T4SSs. We hypothesize that the dynamics between the two T4SS complexes could play a role in the substrate translocation mechanism.

Methodology

Expression and Purification

The gene encoding VirB10^{NT} (residues 85 to 182) (GSHMQSGEDSAPPKPRTE TVVAPALPQSMTAPVEEAPVPLAQQPSLPPLPPMPTDNSEEVSSAPERQRGPTLLERRILAESAANGGGVPGQLGAQPAPTQED) was chemically synthesized and cloned into the expression vector pET28(a) by GenScript USA Inc. with codon optimization for host expression in *Escherichia coli*. Uniformly ¹⁵N/¹³C doubly labelled VirB10^{NT} was expressed as a His-tag fusion protein in *E. coli* BL21(DE3) RP cells in M9 minimal medium containing 1.0 g/L of ¹⁵NH₄Cl and 2.0 g/L of ¹³C-glucose as the only nitrogen and carbon sources, respectively. Expression of His-VirB10^{NT} was carried out at 37 °C for 7 hours upon the addition of 0.5 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) when the optical density at 600 nm (O.D.₆₀₀) was at 0.4. Cells were harvested by centrifugation and resuspended in the lysis buffer (20 mM Tris/Cl pH 8, 200 mM NaCl). Cell lysis was performed using a French Press. The lysate was centrifuged at 15,000 rpm at 4 °C for 40 minutes, after that the supernatant was loaded onto a Ni²⁺-HiTrap chelating column (GE Healthcare) equilibrated with buffer A (20 mM Tris/HCl pH 8; 200 mM NaCl, 10 mM Imidazole). The bound His-VirB10^{NT} was eluted with approximately 75 mM of Imidazole during a linear concentration gradient from 0 to 100 % of buffer B (20 mM Tris/Cl pH 8, 200 mM NaCl, 500 mM Imidazole) over 20 column volumes (CV). Fractions containing the His-VirB10^{NT} were combined, transferred to a dialysis membrane bag with cut-off of 3.5 kDa (SnakeSkin Dialysis Tubing Thermo) and dialyzed against 4 liters of dialysis buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl) overnight at 4 °C. The His-tag was removed by incubation with thrombin-agarose resin (Sigma) for 5 hours at ambient temperature. The cleavage reaction product was loaded onto a Hiload 26/600 Superdex 75 pg column for separation and buffer exchange to 20 mM sodium acetate pH 5.5 and 150 mM NaCl. The fractions containing doubly labeled ¹⁵N/¹³C VirB10^{NT} were collected and concentrated using the Amicon® Ultra-15 device with a

membrane NMWL of 3 kDa. The protein quality was checked by SDS-PAGE and the concentration was estimated by DC Protein Assay Reagents Package (Biorad). NMR samples consisted of 300 μM of uniformly doubled labeled $^{15}\text{N}/^{13}\text{C}$ -VirB10^{NT} dissolved in 20 mM sodium acetate, pH 5.5, containing 150 mM of NaCl, 5% of sodium azide as a preservative and 10% of D₂O.

The recombinant N-terminal domain of VirB9 (VirB9^{NT} - residues 27-150) was expressed in *Escherichia coli* BL21(DE3) RP cells harboring the plasmid pET28a- VirB9_{XAC2620_27-150_His}, where the VirB9 gene fragment was cloned between the NdeI and BamHI restriction sites. Cells were grown in 1 liter of 2xTY medium supplemented with 30 $\mu\text{g}/\text{mL}$ kanamycin and 100 $\mu\text{g}/\text{mL}$ chloramphenicol and incubated at 37 °C under constant stirring at 250 rpm for 16 hours. Expression of VirB9^{NT} was induced at 30 °C for 6 hours by the addition of 0.4 mM of IPTG when the O.D.₆₀₀ was 0.6. Cells were collected by centrifugation and the lysis was performed using a French Press. The lysis buffer consisted of Tris/HCl 20mM, pH 8, 200 mM NaCl. The lysate was clarified by centrifugation at 15,000 rpm at 4 °C for 40 minutes. The supernatant was applied onto a Ni²⁺-HiTrap chelating column (GE Healthcare) equilibrated with buffer (20 mM Tris/HCl pH 8; 200 mM NaCl, 20 mM Imidazole). The protein was eluted using a linear concentration gradient of imidazole over 20 CV up to a final imidazole concentration of 500 mM. The fractions containing VirB9^{NT} were pooled and applied on a Superdex S75 26/600 gel filtration column (GE Healthcare) equilibrated with 20 mM Tris-HCl pH8, 150 mM NaCl. Fractions containing VirB9^{NT} were combined and concentrated up to 270 μM using an Amicon® Ultra-15 (*cutoff* 3 kDa) device by centrifugation at 4,000 rpm at 4 °C. During the concentration, the buffer was exchanged to 20 mM sodium acetate pH 5.5, 150 mM NaCl.

NMR spectroscopy

All NMR spectra were acquired at 280 K on a Bruker Avance III spectrometer operating at 800 MHz (1H frequency) and equipped with a cryogenic TCI probe. Backbone resonances assignments were obtained from the analysis of a set of standard triple-resonance NMR experiments (Cavanagh et al., 1995): HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, HNCO and HN(CA)CO. NMR spectra were processed with NMRPipe (Delaglio et al., 1995) and analyzed using CcpNMR

Analysis 2.4.1 (Vranken et al., 2005). The backbone resonances were used as input for the prediction of secondary structure propensity using SSP (Marsh et al., 2006) and of backbone dihedral angles (Φ and Ψ) using TalosN (Shen and Bax, 2013). The heteronuclear $\{^1\text{H}\} - ^{15}\text{N}$ -NOE experiment was performed in interleaved fashion using saturation time and inter-scan relaxation delay of 3 seconds. The heteronuclear NOE values were obtained from the cross peak intensity ratios of spectra recorded starting from the pure ^{15}N polarization with or without pre-saturation of the ^1H spins. The mixture of VirB10^{NT} with VirB9^{NT} (residues 27-150) was performed by adding 500 μL of unlabelled VirB9^{NT} at 270 μM to 500 μL of an NMR sample consisting of 150 μM of ^{13}C - ^{15}N -VirB10^{NT} in order to achieve a molar ratio of 1:1.8 (VirB10^{NT}:VirB9^{NT}), followed by concentration until a final volume of 500 μL .

Mass spectrometry

The mass spectrometry analysis was performed using the MALDI TOF-TOF method and the equipment Ultraflextreme – Bruker. The matrix was DHB (2,5-Dihydroxy benzoic acid) 10mg/ml. The sample was diluted in trifluoroacetic acid (TFA) 0.1% and mixed with the matrix on 5:1 (matrix:sample) proportion.

Results and discussion

In order to get insights on the topology of Xac's VirB10, we employed amino acid sequence analysis using SPOCTOPUS that predicts the location of transmembrane helices (Viklund et al., 2008), and IUpred that predicts disorder (Dosztányi et al., 2005). Topology predictions using SPOCTOPUS (Viklund et al., 2008) showed that Xac's VirB10 has two transmembrane alpha helices (TM) (**Figure 21**). The first TM comprises residues 62 to 82 and the second TM residues 297 to 317 (**Figure 21**). The second TM coincides with TraF's transmembrane helix that crosses the outer membrane of the bacterium envelope (PDB 3JQO) (Chandran et al., 2009). The N-terminal TM helix, in contrast, is expected to cross the inner membrane (Jakubowski et al., 2009). The presence of this helix was

recently detected by solid-state NMR spectroscopy within the context of the complete T4SS isolated from bacterial membrane extracts (Kaplan et al., 2015). IUPred predicts the existence of a domain within the C-terminal periplasmic portion of VirB10, which must correspond to the C-terminal β -barrel domain (**Figure 21**) (Jakubowski et al., 2009). Crystal structures for VirB10's C-terminal β -barrel domain were obtained for the orthologues ComB10 from *Helicobacter pilori*'s T4SS (PDB 2BHV) (Terradot et al., 2005), and TraF in the outer layer of the CC of the pKM101 conjugation plasmid (PDB 3JQO) (Chandran et al., 2009). IUPred also predicts significant flexibility at the proline-rich region (PRR), comprising residues 82 to 185, although a somewhat reduced disorder tendency is predicted for residues 150 to 163 (**Figure 21**). These observations are consistent with the view that the PRR of VirB10 forms extended structures (Jakubowski et al., 2009).

Figure 21: Prediction of disorder by IUPred (Dosztányi et al., 2005) as a function of VirB10 amino acid sequence. The cytoplasmic N-terminal domain (residues 1-61) is shown in black. Periplasmic amino acids are shown in red (residues 83-296 and 318-389). Putative transmembrane helices (residues 62-82 – inner membrane; and residues 297-317 – outer membrane) are shown in green based on predictions by SPOCTOPUS (Viklund et al., 2008).

With the goal of obtaining detailed structure and dynamic information about VirB10's PRR region, we designed a fragment corresponding to residues 85 to 182 of Xac-VirB10, hereafter called VirB10^{NT}, and characterized its conformational properties and the dynamics by solution NMR spectroscopy. The ¹H-¹⁵N HSQC spectrum of VirB10^{NT} displays a narrow dispersion of amide resonances along the ¹H dimension (ω_2), which fall close to 8.5 ppm as usually observed for intrinsically disordered polypeptides (**Figure 22**) (Tomba and Fersht, 2009).

Figure 22: ¹H-¹⁵N-HSQC spectrum of VirB10^{NT}. The spectrum was recorded at 800 MHz ¹H frequency, at 7 °C.

Although the observed number of ^1H - ^{15}N cross peaks in the HSQC spectrum is in agreement with what is expected based on the amino acid sequence of VirB10^{NT}, a closer analysis reveals the presence of extra peaks with lower intensity (**Figure 23**). The extra peaks might indicate the presence of different fragments in the sample (**Figure 23**). This conclusion is in agreement with the mass spectrometry analysis, where a major signal at 11099.5 m/z corresponds to the full-length VirB10^{NT} sample (expected mass of 11089.6 m/z) and the other signals at 4331.1 and at 6757.7 m/z might correspond to two peptides produced by proteolytic cleavage of VirB10^{NT} (**Figure 23**). In fact, data obtained in our group suggests that VirB10 is susceptible to proteolysis at the PRR region (data not shown). Additionally, structural studies of the core complex of the pKM101 T4SS of *E. coli* showed that the N-terminal region of VirB10 is digested when treated with proteases such as trypsin, chymotrypsin or elastase (Chandran et al., 2009; Fronzes et al., 2009; Rivera-Calzada et al., 2013). The significant susceptibility to proteolytic digestion shown by this region of VirB10 is consistent with the presence of backbone flexibility (vide infra). From hereafter we will analyse only the main set of cross peaks observed in the ^1H - ^{15}N HSQC spectrum of VirB10^{NT}.

Figure 23: ^1H - ^{15}N -HSQC spectrum of Xac's VirB10^{NT} plotted at a low contour level (A). Mass spectrometry analysis of the doubled labelled NMR sample of VirB10^{NT} (B). SDS-PAGE of the NMR sample of VirB10^{NT} (C). The molecular weight standards were applied in the well on the left and the NMR sample was applied in the well on the right.

In order to assess VirB10's propensity for acquiring secondary structure, we attempted to extract structural information from NMR chemical shifts. All backbone amide (^1H and ^{15}N) resonances were assigned, except for the first Gly (**Figure 22**), which is part of the reminiscent tag. A total of 97.1 and 95.7 % of $^{13}\text{C}_\alpha$ and $^{13}\text{C}_\beta$ resonances were assigned. The secondary structure of a protein can be assessed from the dependence of the NMR chemical shifts, in particular $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$ and ^{13}CO , with the backbone conformation using the SSP and TALOS-N (Marsh et al., 2006; Shen and Bax, 2013). Both approaches use secondary chemical shifts, which are obtained from the deviations of the experimental chemical shifts from the random coil values ($\Delta\delta = \delta_{\text{observed}} - \delta_{\text{coil}}$) for $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$ and ^{13}CO (Silvia Spera, 2002). The TALOS-N program uses artificial neural networks trained on a large set of high-resolution X-ray structures in order to predict the backbone dihedral angles (Φ and Ψ). This prediction is based on secondary chemical shifts and on amino acid

sequence homology between the target and selected proteins within a database (Shen and Bax, 2013). The SSP is a very simple method that is based on secondary ^{13}C chemical shifts and adjusts the chemical shifts references. The SSP has been reported to be an effective method for the analysis of secondary structure in folded as well as for the investigation of the secondary structure propensity (SSP) in disordered proteins (Marsh et al., 2006).

The resultant secondary structure prediction obtained using both programs was consistent. Positive SSP values indicate helix propensity, while negative values indicate β -strand formation. Thus, an alpha helix comprising residues 151-163 of VirB10^{NT} was found by the SSP analysis (**Figure 24**), what is in agreement with the dihedral angles predicted for residues 153 to 160 by TALOSN (**Figure 24C and 24D**).

Figure 24: Secondary Structure Prediction by (A) SSP (Marsh et al., 2006) or (B) TalosN (Shen and Bax, 2013). (C) Ramachandran Plot for VirB10^{NT}. (D) ψ and ϕ dihedral angles classified as “strong” by TalosN (Shen and Bax, 2013). Only predictions classified as “Strong” are shown.

To investigate the backbone dynamics of VirB10^{NT} at fast time scales, we recorded a $\{^1\text{H}\}$ - ^{15}N heteronuclear NOE (het-NOE) experiment (**Figure 25**). The het-NOE is highly sensitive to motions of the backbone ^1H - ^{15}N bond vectors at fast time scales (sub-nanosecond). Thus, a high het-NOE (close to 0.7) is compatible with rigid regions, while lower het-NOE values are indicative of a more dynamic structure. NOE values close to 0.2 were detected along the stretches between amino acids 85-150 and 165-182, much lower than what would be expected for a rigid amide bond vector on an isotropic tumbling molecule at 800 MHz, ~ 0.77 (Kay et al., 1989). In contrast, the segment formed by amino acids 151-163 is characterized by het-NOEs close to 0.5, indicating a lower degree of flexibility and consistent with the acquisition of a more rigid structure as indicated by the chemical shift analysis (**Figure 24**). In conclusion, chemical shifts and ^{15}N relaxation data indicate that VirB10^{NT} is intrinsically disordered in solution, but support the

formation a short and transient α -helix within residues 151-163. An α -helix at this location is consistent with a previous study where Rivera-Calzada and co-workers (Rivera-Calzada et al., 2013) modelled a short helix in a similar region of TraF (alpha-helix C) using *in silico* secondary structure predictions and a cryo-EM map of the T4SS encoded by the pKM101 conjugation plasmid (**Figure 26**).

Figure 25: Profile of the $\{^1\text{H}\}$ - ^{15}N -NOE values as a function of the amino acid sequence. The α -helical region is highlighted in yellow.

Figure 26: Sequence alignment between TraF and VirB10 inner layer regions. The underlined sequence represents the transmembrane α -helices. The α -helices proposed for TraF and observed in VirB10^{NT} are highlighted in orange (Rivera-Calzada et al., 2013).

Since the three-dimensional structures of the N-terminal regions of VirB9 and VirB10 are unknown, Rivera-Calzada and co-workers used several structure-predictors such as PSIPRED (Jones, 1999), Phyre2 (Kelley and Sternberg, 2009),

HHpred (Söding, 2005) and I-TASSER (Kelley and Sternberg, 2009; Roy et al., 2010), in order to predict and then fit the coordinates of the secondary structure elements of *E. coli*'s VirB9 and VirB10 (residues 24-159 and 1-160 respectively) on the cryo-EM map of the T4SS (Rivera-Calzada et al., 2013). Docking of structural models predicted for TraO/VirB9 and TraF/VirB10 on the cryo-EM map of the full length pKM101 core complex positioned the alpha-helix C of TraF/VirB10 close to the N-terminal domain of TraO/VirB9 (Rivera-Calzada et al., 2013). In order to test whether VirB10^{NT} would interact with the N-terminal domain of VirB9 we recorded the ¹H-¹⁵N-HSQC spectrum of VirB10^{NT} in the absence and in the presence of a construct corresponding to the N-terminal domain of VirB9 (VirB9^{NT}) unlabelled. We found a decrease in peak intensity for a specific set of cross peaks after the addition of VirB9^{NT} (**Figure 27A and 27B**). A quantitative analysis of intensity ratios before and after the addition of unlabelled VirB9^{NT}, is shown in **Figure 27C**. The specific decrease of cross peak intensity is a clear evidence of binding, and indicates that residues 151-163 of VirB10^{NT}, located in the α -helical region, are involved in the interaction with VirB9^{NT} (**Figure 27C**). A decrease in peak intensity upon binding could be explained by line broadening due to chemical exchange between free and bound forms at the intermediate exchange regime, or considering that formation of a tight complex results in a longer overall tumbling time leading to line broadening. Such line broadening effect, however, would not be observed for the residues that are not at the interface with VirB9^{NT} due to their intrinsic flexibility. The latter option is less probable, since in the case of a tight complex new cross peaks due to the bound species should have been observed.

Figure 27: ^1H - ^{15}N -HSQC spectra of ^{15}N -VirB10^{NT} in absence (pink) or in the presence of unlabelled VirB9^{NT} (green) at a molar ratio of 1:1.82 (VirB10^{NT}:VirB9^{NT}) (A) and (B). Those peaks that showed decreased intensity after adding VirB9^{NT} are indicated. (B) Intensity ratios of cross-peaks from ^{15}N -VirB10^{NT} in the absence and presence of unlabelled VirB9^{NT} (C).

Conclusions

VirB10 connects the CC to the IMC and it must play a pivotal role in the mechanisms of substrate translocation and allosteric communication between the CC and the IMC (Jakubowski et al., 2009). While the C-terminal domain of VirB10, around which assemble VirB7 and VirB9, is well folded and forms the outer layer complex (Chandran et al., 2009), VirB10's PRR displays significant tendency for being disordered (**Figure 21**) (Jakubowski et al., 2009) and had not yet been characterized by high-resolution biophysical techniques. Here we showed that a fragment corresponding to the PRR region of the bacterial killing T4SS (VirB10^{NT}) is highly flexible in solution but forms a short and transient alpha-helix close to the linker with the C-terminal β -barrel domain. The α -helix corresponds to α -helix C of TraF (Rivera-Calzada et al., 2013). We further showed that helix C interacts with VirB9^{NT}. These findings are consistent with a model of the inner layer of the pKM101 core complex, which positioned the alpha-helix C of TraF/VirB10^{NT} close to TraO/VirB9^{NT}. VirB10's PRR is located at the stalk region, where it might be in complex with other VirB proteins such as VirB3, VirB5, VirB6 and VirB8 (Low et al., 2014). It is likely that the intrinsic dynamics of the PRR will be preserved within the stalk. This proposal is supported by cryo-EM data that revealed significant flexibility within the stalk, and by observations that the N-terminal segment of VirB10 is highly susceptible to proteolytic digestion even when in context of the complete core complex (VirB7-VirB10).

Chapter IV – Cloning, expression and purification of VirB10^{CT} for structural studies^{*1}

¹ *The studies reported in this chapter had the participation of the exchange student Angy Lizeth Dávalos from the Universidad del Valle, Colombia, and were part of her undergraduate thesis.

Introduction

The Type IV Secretion System (T4SS) is a nanomachine used to translocate DNA and protein substrates across the cell wall of gram-negative bacteria and to deliver them to prokaryotic or eukaryotic cells (Cascales and Christie, 2003). The T4SS machinery is subdivided in three major sub-complexes. The inner membrane complex (IMC) consists of the ATPases (VirB4, VirD4 and VirB11) in association with other components located at the cytoplasmic side of the inner membrane (IM). The second is the core complex (CC), which is composed by VirB7, VirB9 and VirB10. The CC is assembled in two layers, the inner and the outer layer. The last sub-complex is the pilus, which is formed by the pilin VirB2 and by VirB5 (Costa et al., 2015).

VirB10 seems to be a key player in the translocation process, since it is the only subunit that spans the inner and the outer membranes (Chandran et al., 2009). VirB10 may play a role in coupling the energy produced by the ATPases in the IMC with the formation of a stable and productive CC assembly. VirB10 is organized in three different regions: the cytoplasmic domain, followed by a transmembrane helix (TM) that spans the inner membrane, a proline-rich region (PRR) and a β -barrel domain (Jakubowski et al., 2009), which contains a second TM that spans the outer membrane (Chandran et al., 2009). Garza and Christie (Garza and Christie, 2013) observed that the first transmembrane helix of VirB10 of *A. tumefaciens* has a putative dimerization motif. Deletion of this region blocks the production of the T-pilus, indicating that the transmembrane helix of VirB10 may be involved in the assembly of the system or in the translocation activity through its dimerization motif (Garza and Christie, 2013).

Xanthomonas citri's VirB proteins have low sequence identity with its orthologues in *Escherichia coli* and in *Agrobacterium tumefaciens*, the best characterized T4SSs (da Silva et al., 2002). In order to understand the function and architecture of the T4SS from *Xanthomonas citri* (Xac), we have investigated different components of this system, particularly VirB7, VirB9 and VirB10 that form the outer layer complex. We showed, in the Chapter II, that stable interactions between VirB9 and VirB7 are essential for a functional T4SS. In Chapter III, we investigated the structure, the dynamics and interactions of the proline-rich region

(PRR) of Xac's VirB10. In this chapter we designed different constructs of Xac's VirB10 in order to obtain a soluble fragment suitable for NMR studies.

The amino acid sequence alignment of VirB10 and the orthologous proteins whose structures are available (ComB10 and TraF), indicated that they share similar topologies. In order to investigate the validity of this hypothesis, and to obtain structural information about the inner layer complex, we initiated a study of the PRR and β -barrel domains of Xac's VirB10. Since VirB10 makes a large number of intermolecular contacts in the outer layer complex, two different constructs corresponding to the β -barrel domain were designed in order to obtain a fragment that does not aggregate in solution. However, these constructs showed not to be soluble in the preliminary tests. In order to study the structure of the PRR we prepared a construct related to this domain. Unfortunately, expression of the PRR construct was not observed. Alternatively, we made a chimeric construct consisting of the PRR and the β -barrel domains of VirB10 separated by a thrombin cleavage site.

Methodology

Cloning

The plasmids and the oligonucleotides used in this study are listed in **Table 8** and **Table 9** respectively. The DNA fragments encoding specific residues of VirB10 (XAC2619) were amplified by PCR from *Xanthomonas citri* genomic DNA and cloned in a plasmid using restriction enzymes (**Table 8**). All clones were confirmed by DNA sequencing. The clones used in this study are shown in the **Table 9**.

Table 8: Oligonucleotides and plasmids used in order to clone specific regions of VirB10 in expression vectors. *Fw* = Forward; *Rv* = Reverse

Oligonucleotide	Sequence 5' – 3'	Restriction enzyme	Amplicon	Plasmid/antibiotic	Final peptide
XacVirB10 ¹³⁷⁻³⁸⁹ – Fw	CCGACCCATATGAGCGAAGAGGTCAGCTCC	NdeI	783 pb	pET28a, Kan	274 aa 29,05 Kda pI= 6,59
XacVirB10 – Rv	GCAGGATCCT CA CTTCGGCAGGACAGC	BamHI			
XacVirB10 ¹⁹⁵⁻³⁸⁹ Fw	ATCAGCCATATGGACGGTCTGCTAGTGCGC	NdeI	529 pb	pET28a, Kan	216 aa 23,17 Kda pI=7,19
XacVirB10 ⁸¹⁻¹⁸² – Fw	CTCGCCCATATGTGGCTAGCGACGCAAAGC	NdeI	330 pb	pET28a, Kan	123 aa, 12,8 Kda pI= 5.53
XacVirB10 ⁸¹⁻¹⁸² – Rv	GACGGATCCT TA ATCTTCCTGAGTAGGCGCAGGC	BamHI			
Operon VirB7 – Fw	GAGAACATATGGTGAATCCGATGTATGTGTCCAAGCTG	NdeI	3528 pb	pET30, Kan	XacVirB7 XacVirB8 XacVirB9 XacVirB10
Operon VirB10 – Rv	GCAGCGGCCGCCCACTTCGGCAGGACAGCCG	NotI			
VirB10Nt ₈₅₋₁₈₂ _NdeI_Fw (E) para pET	TCCCATATGCAAAGCGGGGAGGATTCCGCG	NdeI	324 pb	pET28a, Kan ou pGEX-4T1, Amp	His-VirB10 ^{NT} - tromb- VirB10 ^{CT} ΔTM 269 aa 28,32 Kda pI= 5,97
VirB10Nt ⁸⁵⁻¹⁸² -Fw (E) para pGEX	TGGCTAGAATTCCAAAGCGGGGAGGATTCCGCG	EcoRI			
VirB10Nt ⁸⁵⁻¹⁸² _tromb-Rv (F)	GCTGCCGCGCGGCACCAGATCTTCCTGAGTAGGCGCAG	Overlap			
VirB10Ct1 ¹⁹⁵⁻²⁹⁵ _tromb-Fw (A)	CTGGTGCCGCGCGGCAGCGACGGTCTGCTAGTGCGC	Overlap	333 pb		
VirB10Ct1 ¹⁹⁵⁻²⁹⁵ -Rv (B)	CAGTGTGGCAGGATGTGCGTTGTAGTTACCCGGATGACC GGA	Overlap			
VirB10Ct2 ³⁶²⁻³⁸⁹ -Fw (C)	TACAACGCACATCCTGCCACACTGACGATC	Overlap	107 pb		
VirB10Ct2 ³⁶²⁻³⁸⁹ _Sall-Rv (D)	TTCGTCGACTCACTTCGGCAGGACAGC	Sall			

Table 9: Plasmids used in this study

Clone	Expression, resistance	Source/ Reference
pET28a- XacVirB10 ¹³⁷⁻³⁸⁹	His-VirB10 ¹³⁷⁻³⁸⁹ , Kanamycin®	This study
pET28a- XacVirB10 ¹⁹⁵⁻³⁸⁹	His-VirB10 ¹⁹⁵⁻³⁸⁹ , Kanamycin ®	This study
pET28a- XacVirB10 ⁸¹⁻¹⁸²	His-VirB10 ⁸¹⁻¹⁸² , Kanamycin ®	This study
pET28a- XacVirB9 ¹⁵⁴⁻²⁵⁵	His-VirB9 ¹⁵⁴⁻²⁵⁵ , Kanamycin®	(Souza, 2010)
pET11a- XacVirB9 ¹⁵⁹⁻²⁵⁵	VirB9 ¹⁵⁹⁻²⁵⁵ , Kanamycin®	(Souza, 2010)
pET28a- B10 ^{NT} -B10 ^{CT}	His-VirB10 ^{NT} _trombina-VirB10 ^{CT} ΔTM, Kanamycin®	This study

Cloning the Chimera His-VirB10^{NT}-thrombin-VirB10^{CT}ΔTM

A chimeric DNA fragment was made using traditional cloning strategies and the PCR overlapping method (Heckman and Pease, 2007). The template DNA used in the PCR reaction that amplified a fragment of the N-terminal region of VirB10, containing the proline-rich region (PRR) (nucleotides that code for amino acid residues 85-182 – Reaction 1, **Figure 28**), was the plasmid pET28a- XacVirB10⁸¹⁻

¹⁸² (**Table 9**). This PCR reaction resulted in a product of 324 base pairs. The clone pET28a- XacVirB10¹⁹⁵⁻³⁸⁹ was used as a template in order to amplify the regions of β -barrel domain of VirB10 (segments 195-295 and 362-389 – Reactions 2 and 3, respectively, **Figure 28**). This strategy was used in order to obtain a construct with the transmembrane helix deleted. These two PCR products were mixed and used as template in a fourth PCR reaction, where the overlapping method was applied using primers A and D for amplification of region that codes for the segment 195 to 389 without the transmembrane region (residues 296-361) (**Figure 28**). The forward and reverse primers used in the Reactions 2 and 3, respectively, display complementary regions in their extreme ends. Reaction 4 generated a product corresponding to the C-terminal region lacking the transmembrane helix (B10^{CT} Δ TM – **Figure 28B**) by mixing the products from Reactions 2 and 3. Then, the products from Reactions 1 and 4 were used as template in the Reaction 5, where the oligonucleotides “VirB10NT⁸⁵⁻¹⁸²-Fw” and “VirB10CT2³⁶²⁻³⁸⁹-Rv” (**Table 8**) represented as “E” and “D” respectively (**Figure 28**), were used to amplify the PCR product. The complementary regions in the extreme ends of the DNA products from reactions 1 and 4 consist of the nucleotides coding for the thrombin recognition site. At the end of reaction 5, a product of PCR consisting of DNA fragments that code for the N-terminal region of VirB10 (residues 85-182), followed by a thrombin cleavage site and the C-terminal region of VirB10 lacking the transmembrane helix (296 to 361), was obtained (**Figure 28C**).

Figure 28: Schematic representation of the cloning strategy used in order to clone the chimeric construction of VirB10.

Expression

The recombinant proteins were expressed in *Escherichia coli* BL21(DE3) or alternatively in *ArcticExpress* (Agilent) harbouring the plasmids showed in the **Table 9**. Typically, an inoculum of 5 mL of *E. coli* cells containing the plasmid of interest (**Table 9**) was diluted in 500 mL of 2xTY medium supplemented with kanamycin® at 50 µg/mL. The expression was induced when the culture optical density at 600 nm (O.D.₆₀₀) was 0.6 by the addition of 0.5 mM of IPTG. Different conditions of induction were tested in order to evaluate the better situation for each protein in terms of expression level and solubility. Cells were harvested by centrifugation at 5000 rpm and 4 °C for 30 minutes. The pellet was resuspended in the lysis buffer (typically 50 mM Tris pH 8,0; 200 mM NaCl; 1mM 2-mercaptoethanol; 20 mM Imidazole, 5% glycerol and protease inhibitors cocktail). Cell lysis was performed by French Press. After centrifugation of the lysate at 15000 rpm for 40 minutes at 4 °C, the supernatant (soluble fraction) was separated from the pellet (insoluble fraction).

Fractionation of the lysate and solubility test of His-VirB10¹⁹⁵⁻³⁸⁹

After cell lysis, the soluble fraction (S.F.) was transferred to a new tube. A volume of 20 mL of washing solution (1M urea; 0,5M NaCl, 5 mM 2-mercaptoethanol, 50 mM Tris pH 8,0) was added into the pellet followed by centrifugation for 15 minutes at 12000 rpm at 4 °C. The supernatant was transferred to a new tube (washing), while the pellet was solubilized with 15 mL of solubilisation buffer (8M Urea; 0,5 M NaCl; 5 mM 2-mercaptoethanol; 50 mM Tris pH 8,0). This last fraction was called insoluble fraction (I.F.). The collected samples were submitted to electrophoresis in a 16% SDS-PAGE Tris-Tricine gel (Schagger, 2006) in order to evaluate the solubility of the recombinant protein.

Purification of His-VirB10¹⁹⁵⁻³⁸⁹ by affinity chromatography under denaturant conditions

The insoluble fraction from the lysate containing VirB10¹⁹⁵⁻³⁸⁹ was submitted to a centrifugation step at 15000 rpm. The supernatant was applied in a 5mL HiTrap column chaged with Ni²⁺ (GE Healthcare) and equilibrated with 50 mM sodium phosphate pH 8, 500 mM NaCl, 8M urea. A linear gradient of 30 column volumes (CV) from 0 to 100 % of 50 mM sodium phosphate pH 4, 500 mM NaCl, 8M Urea was used in order to elute the adsorbed proteins. The chromatography was carried out in a flow of 1 mL/minute. The fractions were collected and an aliquot was applied in SDS-PAGE in order to evaluate the quality of the purified samples of VirB10¹⁹⁵⁻³⁸⁹.

Co-expression of His-VirB10¹⁹⁵⁻³⁸⁹ and VirB9¹⁵⁹⁻²⁵⁵

E. coli cells harbouring the plasmids pET28a-His-Xac-VirB10¹⁹⁵⁻³⁸⁹ (Kanamycin® resistance) and pET11a-Xac-VirB9¹⁵⁹⁻²⁵⁵ (ampicillin® resistance) were used in order to co-express the two recombinant proteins: His-VirB10¹⁹⁵⁻³⁸⁹ and VirB9¹⁵⁹⁻²⁵⁵. Protein expression was induced by the addition of 0.5 mM of IPTG when the cells reached the optical density at 600 nm of 0.6. The protein expression was carried out for 5 hours at 37 °C.

Results and discussion

VirB10 is the only protein that crosses both the inner and the outer membranes of the cell wall. Amino acid sequence analysis using Spoctopus (Viklund et al., 2008), shows that VirB10 has two transmembrane helices (TM), the inner TM (residues 62 to 82) and the outer TM (residues 297 to 317). The region between the TMs is predicted to be located at the periplasm. Residues 81 to 182 form the proline-rich region (PRR) and display a high tendency for disorder as predicted by IUpred (Dosztányi et al., 2005). In contrast, the region between residues 195 to 389, which would correspond to the β -barrel domain, has a lower disorder tendency as indicated in **Figure 29 A**. Based on these observations, VirB10 can be divided in two periplasmic domains: VirB10^{NT} (residues 85-182), which was discussed in the Chapter III of this thesis, and VirB10^{CT} (residues 195-398).

Figure 29: Prediction of disorder by IUPred (Dosztányi et al., 2005) as a function of VirB10 (A) and VirB9 (B) amino acid sequences. The cytoplasmic N-terminal domain (residues 1-61 in VirB10) is shown in black. Periplasmic amino acids are shown in red (residues 83-296 and 318-389 in VirB10) and in blue for VirB9 (residues 26 to 255). Putative transmembrane helices (residues 62-82 – inner membrane; and residues 297-317 – outer membrane in VirB10) are shown in green and the signal peptide of VirB9 in orange (residues 9-25). The predictions of transmembrane helices and signal peptides were performed with SPOCTOPUS (Viklund et al., 2008).

Xac's VirB10 has low sequence identity with known orthologues whose three-dimensional structures are known. The amino acid sequence alignment of VirB10 and COMB10 from *Helicobacter pylori*, whose structure is known (PDB

2BHV), and TraF, whose crystal structure was solved in the context of the outer layer of the CC (PDB 3JQO), reveals 30% and 31% of identity, respectively. The first and second TMs are located in the same region as in COMB10 and TraF, they are supposed to cross the inner and outer membranes, respectively (**Figure 30**).

A

VirB10	--KALVFLAAIAALLILAIFWLA	TQSGEDSAPPK-PRTEVVAP-----	103
COMB10	LKGAIVFVGGFATITISLIY	HQKPKAPLNNQPSLLNDEVKYPLQDYTFQTNPQPTNTE	64
	*:***:***: : : : : * . : *		
VirB10	-----ALPQSMAPVEE---	APVPLAQQPSLPPLPPMPTDNSEEVSSAPERQ---RG	149
COMB10	SSKDATIKALQEQLKAALKALNSKEMNYSKEET-FKSPMDLKTN---	TTPPKDFSLKQ	120
	** : : * : : : : : : : * * . . : * : : :		
VirB10	PTLLERRILAESAAANGGVPGLGAQPAPTQEDGPVTLAKPISNPDGL-----		197
COMB10	LDLLAARITPF-----	KQSPK---NYEENLIFVDNPNPIDGFTNLKEKDIA	164
	** ** : * : * * : * : *		
VirB10	----L----VRGTYIRCILETRIISDFGGYTSCIVTEPVYSINGHNLLLPKGSKMLGQY		248
COMB10	TNENKLLRTITADKMIPAFILITPISQIAGKVIAQVESDIFAHMGKAVLIPKGSKVIGYY		224
	* . * . * * * * : * : * : * : * : * : *		
VirB10	SAGEP-TSHRLQVVDVVTPTGLDVTLMGP-GIDTLGSSGHPGNYNAHWG	NKIASALFI	306
COMB10	SNNKMGYRLDIVWSRIITPHGINIMLTNAKGADIKGYNGLVGELIERNF	ORYGVPLLI	284
	* : : * : * : * : * * : * : * * * : : : * : *		
VirB10	SLLSDAFKYAA---A	EYGPET-TTIGVGSGIVTQQPFESNTARSMQQLAEQAVEKSGRR	361
COMB10	STLTNGLLIGIT	SALNSRNGEGATNFFGDYLLMQ--LMRQSGMGINQVNVNQLRDKSKI	342
	* * : : . . : * : * : * : * : * : : : : * : * : * : *		
VirB10	PATLTINQGTVLNVYVAKDVDFS	AVLPK-----	389
COMB10	APIVVIREGSR--VFISPTDIFFP	IPRENEVIAEFLK	378
	: . * : * : * : * : * : *		

B

VirB10	--KALVFLAAIAALLILAIFWLA	TQSGEDSAPPKPRTE-----	VVAPALPQSMAPVEE	115
TraF	GMAFVILMALLALVFIGITVM	-----	GKIRAPAKAEADKGGKAQQANTLPNYSFNSDPD	93
	*:***:***: : : : : * : * * . . . : * : * : :			
VirB10	APVPLAQQPSLPPLPPM-----	PTD---NSEEVSSAPERQ	RGP--TLERRILAESAA	163
TraF	VNKPATASATDARAVQAAQADADAGSSNTGARTSNKRKEPSPEELAMQRRLLGELAQ			153
	. * : * * : : * : * : * : * : * : * : * : * : * : *			
VirB10	-NGGGVPGQLGAQPAPT-QEDGPVT-----	LAKPISNPDGLLVRGTYIRCIL		208
TraF	TNQAATSNSPGVQPDNETSEGSSALAKNLT	PARLKASRAGVMANPSLTVPGKMI	PCGT	233
	* . . . * . * . * . : * : : * : * : * : * : * : *			
VirB10	ETRIISDFGGYTSCIVTEPVYSINGHNLLLPKGSKMLGQYSAGEPT-SHRLQVVDVVT			267
TraF	CTELDTTVPGQVSCRVSQDVYADGLVRLIDKGSWVDGQITGGIKDQARVFLWERIRN			273
	* . : . * . * * : : * * : * : * : * : * : * : *			
VirB10	P-TGLDVTLMGPGIDTLGSSGHPGNYNAHWG	NKIASALFISLLSDAFKYAAA	EYGPETTT	326
TraF	DQDGTIVNIDSAGTNSLGAGIPGQVDAHME	ERLRGATMISLFSDTLTALVN	QTQSNNI-	332
	* . : . * : * : * * : * : * : * : * : * : * : *			
VirB10	IGVGSGIVTQQPFESNTARSMQQLAEQAVEKSGRRPATLTINQGTVLNVYVAKDVDFS	AV		386
TraF	-----QY-NSTENSGQLASEALRSYMSIPTLYDQGD	AVSIFVARDLDFS	GV	380
	: . * . * * : * : * : * * : * : * : * : * : *			
VirB10	LPK--		389	
TraF	YTLADN		386	

Figure 30: Amino acid sequence alignments of VirB10 and COMB10 (A) and VirB10 and TraF (B). The amino acid sequences corresponding to the putative cytoplasmic regions were not considered. The transmembrane helices, according to SPOCTOPUS (Viklund et al., 2008), are shown in green.

In contrast to VirB10, VirB9 has no transmembrane alpha-helix. Sequence analysis using SPOCTOPUS (Viklund et al., 2008) indicates that this protein has a signal peptide comprising amino acids 9 to 25 (**Figure 29**). The segment likely to be located at the periplasm starts from the residue 26. Disorder prediction indicates that, similarly to VirB10, VirB9 has an N-terminal and a C-terminal domain (VirB9^{NT} and VirB9^{CT}, **Figure 29**) but these two domains are folded and possibly separated by a flexible segment. The presence of a flexible linker connecting the N-terminal

and C-terminal domains of VirB9 and VirB10 would be consistent with the absence of electron density between the inner and outer layers in the T4SS's core complex (Fronzes et al., 2009).

Cloning and definition of the studied domains

In order to investigate the structure of VirB10 we cloned two fragments from the C-terminal domain (**Figure 31**). The first fragment consists of residues from 195 to 389, and the second consists of residues 137 to 389. The choice of these two constructs was done based on the amino acid sequence alignment between VirB10 and TraF (PDB 3JQO). The smaller construct, VirB10¹⁹⁵⁻³⁸⁹ (**Figure 31C**), does not have the “extended arm” that is involved in a large number of intermolecular contacts between VirB10 subunits in the outer layer complex of the conjugative T4SS (Chandran et al., 2009). The absence of this extended arm could increase the chances of obtaining a soluble fragment, which is essential for solution NMR studies. The periplasmic N-terminal region of VirB10 (VirB10^{NT}) has a high tendency for disorder (**Figure 29A**), which is consistent with the high content of prolines in the PRR (Jakubowski et al., 2009). A fragment of VirB10 (VirB10⁸¹⁻¹⁸² - residues 81-182) was cloned in order to test its expression as a soluble domain (**Table 9**). The clones were confirmed by DNA sequencing (data not shown).

Figure 31: Cloned fragments from Xac-VirB10. (A) Heterotrimer from outer layer complex of pKM101's T4SS (PDB 3JQO). VirB10^{CT}, VirB9^{CT} and VirB7 are shown in red, grey and green, respectively. (B) Schematic representation of Xac's VirB10 regions. The residue numbers are shown below the scheme. The putative periplasmic region is shown in red and the transmembrane helices (TM) in green. (C) Schematic representation of the cloned fragments.

Expression and solubility assays for His-VirB10¹⁹⁵⁻³⁸⁹

The tests for expression and solubility of His-VirB10¹⁹⁵⁻³⁸⁹ were carried out using different *E. coli* strains and induction conditions: *E. coli* BL21(DE3) at 37 °C (5 hours of induction) or at 18 °C (overnight expression) and alternatively in *E. coli* ArcticExpress (Agilent) at 12 °C (overnight expression). Unfortunately, the fragment

consisting of the residues 195-389 of VirB10 was not observed on the soluble fractions of all tested conditions (**Figure 32**).

Figure 32: Expression and solubility assay of His-VirB10¹⁹⁵⁻³⁸⁹. SDS-PAGE 16% showing that His-VirB10¹⁹⁵⁻³⁸⁹ was expressed by induction with 0,5 mM of IPTG in *E. coli* BL21(DE3) at 18 °C and at 37°C (left and middle gel respectively) and in *E. coli* Arctic at 12 °C (right gel). His-VirB10¹⁹⁵⁻³⁸⁹ was found in the insoluble fraction (I.F.) under all induction conditions. . MW) Molecular weight; N/I) Not induced; I) Induced; S.F.) Soluble Fraction; W) Washed fraction with 1 M Urea; I.F.) Insoluble fraction.

Partial purification of VirB10¹⁹⁵⁻³⁸⁹ under denaturants conditions

Due to the low solubility of VirB10¹⁹⁵⁻³⁸⁹ in all tested conditions, the insoluble fraction from the overnight expression at 18 °C in *E. coli* BL21(DE3) (**Figure 32**) was suspended in a buffer containing 8M of urea and applied in a Ni²⁺ Hitrap Chelating HP / 5mL (GE) in order to perform a purification at denaturant conditions. The domain was eluted with a linear pH gradient. The final purified domain shows a high degree of purity, as indicated by SDS-PAGE (**Figura 33**).

Figure 33: Affinity chromatography purification of His-VirB10¹⁹⁵⁻³⁸⁹. The chromatogram is shown at the top and the 15% SDS-PAGE at the bottom. The linear pH gradient along 30 column volumes is evidenced in green. The absorbance at 280 nm and the collected fractions are shown in blue and red respectively. Arrows in the chromatogram evidence the fractions corresponding to pure protein. M) Molecular weight; N/I) Not induced; I) Induced.

Co-expression of His-VirB10¹⁹⁵⁻³⁸⁹ and VirB9¹⁵⁹⁻²⁵⁵.

E. coli expresses VirB10¹⁹⁵⁻³⁸⁹ in a high level as shown above. Although this domain could be obtained from re-folding assays, its expression in a soluble form would be desirable for solution NMR. Since VirB10 interacts extensively with VirB9 in the outer layer complex of T4SS (Chandran et al., 2009), we hypothesized that VirB10¹⁹⁵⁻³⁸⁹ could become soluble in the presence of VirB9¹⁵⁹⁻²⁵⁵. Based on this hypothesis, a co-expression assay was designed. The C-terminal domains of VirB10 and VirB9 were expressed from pET28a and pET11a respectively (**Table 9**). This approach allowed the expression of a His-tagged VirB10¹⁹⁵⁻³⁸⁹ and VirB9¹⁵⁹⁻²⁵⁵ untagged. After expression, a solubility assay was performed to verify the solubility of each domain. While most of VirB9¹⁵⁹⁻²⁵⁵ was found in the soluble fraction (S.F.) of

the bacterial lysate, His-VirB10¹⁹⁵⁻³⁸⁹ continued to be found in the insoluble fraction (I.F.) (**Figure 34**) indicating that the interaction between these two domains is not strong enough to bring VirB10^{CT} to the soluble fraction.

Figure 34: Co-expression and solubility assays of His-VirB10¹⁹⁵⁻³⁸⁹ and VirB9¹⁵⁹⁻²⁵⁵. SDS-PAGE 16% showing that His-VirB10¹⁹⁵⁻³⁸⁹ was co-expressed with VirB9¹⁵⁹⁻²⁵⁵ by induction with 0,5 mM of IPTG in *E. coli* BL21(DE3) at 37°C. His-VirB10¹⁹⁵⁻³⁸⁹ was found in the insoluble fraction (I.F.). MW) Molecular weight; N/I) Not induced; I) Induced; S.F.) Soluble Fraction; W) Washed fraction with 1 M Urea; I.F.) Insoluble fraction.

VirB10^{NT} cloning

The purpose of this part of the study was to clone a VirB10^{NT} fragment in order to perform interaction studies with VirB9^{NT}. A fragment from the amino-terminal portion of VirB10 (amino acid residues 81 to 182) was cloned in pET28a (**Table 9 and Figure 31 C**). Various expression conditions for His-VirB10⁸¹⁻¹⁸² were tested at two temperatures (at 37 °C and 18 °C) and the expression strains (*E. coli* BL21 (DE3), *E. coli* BL21 (DE3) RP, *E. coli* BL21 (DE3) pLysS, *E. coli* BL21 (DE3) RIL and ArcticExpress). However, expression of the protein was not observed in none of the tested conditions (data not shown). Alternatively, VirB10⁸¹⁻¹⁸² was cloned in the pGEX-4T1 expression plasmid in fusion with GST (Glutathione-S-

Transferase) in its N-terminal end and the expression of this new construct, GST-VirB10⁸¹⁻¹⁸², was tested. Likewise, there was no expression of GST-VirB10⁸¹⁻¹⁸² under any conditions tested (data not shown). As an alternative to achieve the expression of the N-terminal fragment and, at the same time, to obtain soluble VirB10^{CT} fragment, a chimeric construct containing VirB10^{NT} (amino acids 85 to 182) and VirB10^{CT} (amino acids 195 to 389) separated by a thrombin site and the transmembrane helix (amino acids 296 to 361) deleted, was designed.

Expression of the chimera VirB10^{NT}-VirB10^{CT}ΔTM.

Since the expression of VirB10^{NT} could not be observed and VirB10^{CT} was only detected in the insoluble fraction of the bacterial lysate, another strategy was chosen. The chimera pET28a-VirB10^{NT}-VirB10^{CT}ΔTM, which could provide both the N-terminal (VirB10^{NT}) and C-terminal (VirB10^{CT}) fragments separated by a thrombin cleavage site, was cloned. To increase the chances that VirB10^{CT} could be found in the soluble fraction, part of the gene responsible for coding the second transmembrane helix of VirB10 (amino acids 296-361) was deleted (see materials and methods, **Figure 28**).

Expression of the chimera His-VirB10^{NT}-VirB10^{CT}ΔTM was performed in *E. coli* BL21 (DE3) at 37 °C for 5 hours or at 18 °C overnight by induction with 0.5 mM of IPTG. A reasonable level of expression was observed in these two conditions, but the chimera was only found in the soluble fraction when the expression was carried out at lower temperature (**Figure 35A**).

After expression, the soluble fraction was applied in a Ni²⁺ HiTrap column (GE). The purification under denaturant conditions was carried out. Aliquots from the fractions collected during the purification were applied in a 16% SDS-PAGE in order to verify the level of purity (**Figure 35B**). Although a large amount of the chimera His-VirB10^{NT}-VirB10^{CT}ΔTM was not adsorbed in the column and could be found in the flowthrough (fractions 1, 2 and 3 – **Figure 35B**), a great amount of the protein was successfully purified (elution in the fractions 11 to 23 – **Figure 35B**). After the affinity purification, His-VirB10^{NT}-VirB10^{CT}ΔTM was submitted to a thrombin cleavage assay. The cleavage was not so efficient (data not shown) and

other cleavage conditions must be tested in the future. These results show that it is possible to obtain a good quality expression and purification of VirB10 fragments in order to carry out structural studies of this protein by solution NMR in the future.

Figure 35: Solubility and affinity purification assays of His-VirB10^{NT}-VirB10^{CT}ΔTM. (A) SDS-PAGE 16% showing that when expressed at 37 °C for 5 hours, His-VirB10^{NT}-VirB10^{CT}ΔTM was mainly found in the insoluble fraction (I.F.) (left), while when the expression was carried out at 18 °C overnight (right) part of His-VirB10^{NT}-VirB10^{CT}ΔTM was found in the soluble fraction (S.F.). MW) Molecular weight; N/I) Not induced; I) Induced; Lis) Lysate; S.F.) Soluble Fraction; W) Washed fraction with 1 M Urea; I.F.) Insoluble fraction. (B) Fractions from affinity chromatography.

Conclusions

Fragments corresponding to the C-terminal domain of VirB10 displayed satisfactory expression levels but were insoluble. In contrast, no expression was detected for the N-terminal domain of VirB10, which contains a proline-rich region (PRR), in various *E. coli* strains and under various conditions. The fragments cloned in this chapter were amplified from the Xac's genomic DNA. However, we observed in the previous chapter (Chapter III) that a gene coding for the N-terminal region of VirB10 but with the DNA sequence optimized for the codon usage of *E. coli*, showed good expression levels. The undetectable expression of the N-terminal fragment of VirB10, whose DNA was amplified from Xac's genomic DNA sequence, could be explained considering that translation rates proportionated by the rare codon usage may not be fast enough to compensate the inherently low stability of the proline-rich region of VirB10 (see chapter III), resulting in protein degradation. In order to improve the solubility of the C-terminal domain and the expression levels of the N-terminal domain, we designed a chimera that consists of the N-terminal fragment (VirB10NT⁸⁵⁻¹⁸²) in fusion with the C-terminal fragment (VirB10CT¹⁹⁵⁻³⁸⁹), and separated by a thrombin cleavage site. In addition, the C-terminal TM helix was deleted. The chimera displayed excellent expression levels and good solubility. However, thrombin cleavage was very inefficient.

Chapter V - General Conclusions

Here, the NMR structure of the complex formed between the C-terminal domain of Xac's VirB9 (VirB9^{CT} - amino acid residues 154 to 255) and the N-terminal tail of Xac's VirB7 (VirB7^{NT} - amino acid residues 24 to 46) was determined. The NMR structure reveals that VirB9^{CT} consists of two β -sheets forming a β -sandwich. VirB7^{NT} forms a β -strand that complements the first β -strand of VirB9^{CT}. This complex is stabilized by hydrophobic contacts. Mutation of residues involved in intermolecular contacts such as the Trp34 and the Val37 in VirB7, affected the stability of the complex *in vitro*. A tight interaction between VirB7 and VirB9 was shown to be essential for a functional T4SS. Disruption of this complex impaired the assembly of the T4SS, explaining the inactivation of the system *in vivo*.

Previous studies showed that unlike the canonical VirB7, Xac's VirB7 consists of a flexible N-terminal domain followed by a globular C-terminal domain called N0 domain. While the N-terminal tail of Xac-VirB7 is involved in binding to VirB9, the N0 domain is involved in interactions with other VirB7 molecules via specific residues at the N-terminal tail. Here we investigated the importance of the self-oligomerization of VirB7 for the function and assembly of the T4SS *in vivo*. Measurements of chemical shift perturbations (CSP), of the overall rotational tumbling time (τ_c) and of the translational diffusion coefficient (D_t) of a triple VirB7 mutant, showed that the substitutions I47A, P48A and L49A generate a mutant VirB7 that does not oligomerize *in vitro*. Furthermore, although they do not affect the assembly of the system, disruption of these specific VirB7:VirB7 interactions leads to an inactive T4SS *in vivo*. Altogether, these findings suggest that a tight interaction between VirB7 and VirB9 is essential for a stable and functional T4SS, while the self-association of VirB7 subunits is relevant for the translocation of substrates across the core complex.

VirB10 is a key component of the T4SS since it connects the core complex to the inner membrane complex (IMC) in the cytoplasmic side of the bacterial cell wall. This protein has a well folded β -barrel domain (Jakubowski et al., 2009) that

interacts with VirB9 and VirB7 forming a symmetric ring-shaped assembly in the outer layer of the core complex (Chandran et al., 2009). On the other hand, the N-terminal segment of VirB10 (VirB10^{NT}) contains a proline-rich region (PRR) that spans the inner layer of the core complex (Jakubowski et al., 2009). Here, we showed that a fragment corresponding to the PRR of Xac's VirB10 (VirB10^{NT} – residues 85-182) is indeed highly flexible. Analysis of chemical shifts showed that the ensemble and time average structure of VirB10^{NT} consists of a short alpha helix between residues 151-163. This α -helix is involved in binding to the N-terminal domain of VirB9 (VirB9^{NT}). These findings provide, for the first time, compelling evidence for the interaction between the N-terminal domains of VirB9 and VirB10. Our data point to the existence of significant flexibility within Xac's T4SS as suggested by cryo-electron microscopy data obtained on the *E. coli* conjugative systems (Fronzes et al., 2009; Rivera-Calzada et al., 2013). The presence of flexibility between the two layers of the T4SS could be relevant for the substrate translocation mechanism.

Fragments corresponding to the C-terminal domain of VirB10 displayed satisfactory expression levels but were insoluble. No expression was detected for a fragment derived from the N-terminal domain of VirB10 in various *E. coli* strains and under various expression conditions. However, we observed that a DNA fragment optimized for the codon usage in *E. coli* displayed good expression levels. A chimera consisting of the PRR (VirB10^{NT}- residues 85-182) in fusion with the C-terminal β -barrel domain (VirB10^{CT}- residues 195-389) and containing the transmembrane helix deleted was cloned. This chimera displayed excellent expression levels and good solubility. However, thrombin cleavage was very inefficient. Other approaches should be tried in order to obtain a sample of the β -barrel domain of Xac's VirB10 for solution NMR studies.

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Curriculum Vitae

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EDUCATION

University of São Paulo, São Paulo, Brazil.
PhD in Biochemistry, expected December 2015
Federal University of Viçosa, Minas Gerais, Brazil.
Bachelor's Degree in Biochemistry, June 2010

RESEARCH EXPERIENCE

07/2010 – Present

Graduate Research Assistant, University of São Paulo, USP, Brazil.
Thesis title: Structural studies of the periplasmic channel of the Type IV Secretion System
Advisor: Prof. Roberto Kopke Salinas

11/2013 – 01/2014

Visiting Student, Bijvoet Center for Biomolecular Research, Utrecht University, The Netherlands
Topic: Structure determination of proteins with NMR Spectroscopy
Advisor: Prof. Rolf Boelens

03/2009 – 06/2010

Undergraduate Research Assistant, Universidade Federal de Viçosa, UFV, Brazil
Thesis: Heterologous expression of domains of NRP-B soy protein and production of polyclonal antibodies to specific recombinant domains.
Advisor: Prof. Elizabeth Pacheco Batista Fontes.

02/2006 – 02/2009

Undergraduate Research Assistant, Universidade Federal de Viçosa, UFV, Brazil
Topic: Histology studies of testicular parenchyma of bats and adult Wistar rats
Advisor: Prof. Sérgio Luis Pinto da Matta

HONORS AND AWARDS

2010 - PRESENT PhD fellowship from CNPq (Conselho Nacional de Desenvolvimento Científico e tecnológico), São Paulo, Brazil
2014 - "1st SBBq-Biochemical Society AWARD" for best oral presentation, XLIII Annual Meeting of Brazilian Society for Biochemistry and Molecular Biology (SBBq). Title: Structural Studies of Periplasmic Proteins from the Type IV Secretion System of *Xanthomonas citri* (Xac)
2013 - Award for best Extra-European NMR Students – Awarded to visit one of the Bio-NMR Research Infrastructures

2013 - Isotec student sponsorship to attend the 18th International Society Of Magnetic Resonance conference

2010 and 2011 - Approved with the highest score among the candidates in masters and PhD levels in exam for entrance in Biochemistry program from the University of São Paulo

2009 - 2010 Undergraduate Fellowship from FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais), Viçosa, MG, Brazil

2007 – 2008 Undergraduate Fellowship from CNPq (Conselho Nacional de Desenvolvimento Científico e tecnológico), Viçosa, MG, Brazil

PUBLICATIONS

(In preparation) “VirB7-VirB7 and VirB7-VirB9 interactions are required for anti-bacterial activity of a type IV secretion system” **Oliveira, L.C.**; Souza, D.P.; Oka, G.U.; Lima, F.S.; Oliveira, R.J.; Favaro, D.C.; Wienk, H.; van Ingen, H.; Boelens, R.; Farah, C.S.; Salinas, R.K.

(In preparation) “NMR studies of the periplasmic proteins of the Type IV Secretion System.” **Oliveira, L.C.**; Dávalos, A.L.; Souza, D.P., Farah, C.S.; Salinas, R.K.

“Organization and seasonal quantification of the intertubular compartment in the bat (Pallas, 1776) testis. Morais, D. B.; **Oliveira, L.C.**; Carmo Cupertino, M.; Freitas, K.M.; Freitas, M.B.D.; Paula, T.A.R.; Matta, S.L.P. Microscopy Research and Technique (Print), v. 76, p. 94-101, 2012.”

“The binding protein BiP attenuates stress-induced cell death in soybean via modulation of the N-rich protein-mediated signaling pathway. Reis, P.A.B.; Rosado, G.L.; Silva, L.A.C.; **Oliveira, L.C.**; Oliveira, L.B.; Costa, M.D.L.; Alvim, F.C.; Fontes, E.P.B. Plant Physiology (Bethesda), v. 157, p. 1853-1865, 2011.”

“The effects of *Tynnanthus fasciculatus* (Bignoniaceae) infusion on testicular parenchyma of adult Wistar rats. Melo, F.C.S.A.; Matta, S.L.P.; Paula, T.A.R.; Gomes, M.L.M.; **Oliveira, L.C.** Biological Research (Print), v. 43, p. 445-450, 2010.”

ORAL PRESENTATIONS

2015 – Selected for an oral presentation in the Young Scientist Program (YSP) in the 23rd IUBMB Congress and 44th Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology (SBBq), 2015

2014 – Selected for oral presentation at XLIII Annual Meeting of Brazilian Society for Biochemistry and Molecular Biology (SBBq). Title: Structural Studies of Periplasmic Proteins from the Type IV Secretion System of *Xanthomonas citri* (Xac)

TEACHING AND MENTORING

2012 - 2014 Graduate Teaching Assistant, Department of Biochemistry, University of São Paulo (USP)

Semester courses taught: Nutrition Biochemistry, Biochemistry and Molecular Biology, Biochemistry: Structure of Biomolecules and Metabolism.

2012 - 2014 Training of undergraduate students in laboratory techniques, USP Department of Biochemistry

2012 Training and supervision of graduate students in the VII Winter School in Biochemistry and Molecular Biology, USP Department of Biochemistry

2011 Training and supervision of undergraduate students in the VI Summer School in Biochemistry and Molecular Biology, USP Department of Biochemistry

2006 - 2010 Undergraduate Teaching Assistant, Department of Biology and Department of Biochemistry, Universidade Federal de Viçosa

Semester courses taught: Basic Biochemistry, Biochemistry of Macromolecules, Metabolic Biochemistry, Cellular Biochemistry, Histology and Embryology, Basic Embryology, Animal Embryology, Cell Biology.

WORKSHOPS

2014 Multidimensional NMR in structural biology. (European Molecular Biology Organization. 50h).

2012 Introduction Into Advanced NMR (Dr. Christian Griesinger, Max Plank Institute; organized by the Federal University of the Rio de Janeiro, Brazil).

2012 5th School in Biological Physics. (Centro Brasileiro de Pesquisas Físicas, Brazil)

2009 VI Summer School in Cellular and Molecular Biology. (University of São Paulo, USP, Brazil).

TECHNICAL SKILLS

Solution NMR spectroscopy

Protein NMR techniques for elucidation of structure and dynamics

NMR structure calculation

Isothermal titration calorimetry

Protein expression and purification

Molecular cloning

Real-time PCR

Antibody production

RNA isolation

Yeast two-hybrid

SELECTED POSTER PRESENTATIONS

"Characterization Of The Structure, Dynamics, Function And Interactions Of Components From The Type Iv Secretion System Of *Xanthomonas Citri* By Solution Nuclear Magnetic Resonance. **Oliveira, L. C.**; Souza, D.P.; Oka, G. U.; Wienk, H.; Lima, F. S.; Van Ingen, H.; Boelens, R.; Oliveira, R. J.; Favaro, D. C.; Farah, C. S.; Salinas, R. K. . In: 15th Nuclear Magnetic Resonance Users Meeting, 2015, Angra dos Reis"

"Structural Studies of Periplasmic Proteins from the Type IV Secretion System of *Xanthomonas citri* (Xac). **Oliveira, L. C.**; Souza, D.P.; Wienk, H.; Van Ingen, H.; Favaro, D. C.; Boelens, R.; Farah, C. S.; Salinas, R. K. . In: XLIII Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology (SBBq), 2014, Foz do Iguaçu."

"A NMR Study of the Calcium-Binding Domains of *Drosophila melanogaster* s Na⁺/Ca²⁺ Exchanger Abiko, L. A.; Vitale, P. M.; Favaro, D. C.; **Oliveira, L. C.**; Bruschweiler-Li, L.; Bruschweiler, R.; Salinas, R. K.. In: XLIII Annual Meeting Of The Brazilian Society For Biochemistry And Molecular Biology (Sbbq), 2014, Foz do Iguaçu."

"Solution NMR studies of protein components of the Type IV secretion system (T4SS) of a phytopathogen. **Oliveira, L. C.**; Souza, D.P.; Farah, C. S.; Salinas, R. K. In: Chemistry in Relation to Biology and Medical Research, 2013, Eindhoven."

"Solution structure of the VirB9^{Ct}-VirB7^{Nt} complex from the *Xanthomonas* Type IV Secretion System. **Oliveira, L. C.**; Souza, D.P.; Favaro, D. C.; Davalos, A. L.; Farah, C. S.; Salinas, R. K. . In: 18th International Society Of Magnetic Resonance Meeting, 2013, Rio de Janeiro. p. 81-81."

“NMR study of the calcium-binding domains of the Na⁺ /Ca²⁺ exchanger of *Drosophila melanogaster* by solution NMR. Abiko, L. A.; Hauk, P.; Favaro, D. C.; **Oliveira, L. C.**; Bruschweiler-Li, L.; Bruschweiler, R.; Salinas, R. K. In: 18th International Society Of Magnetic Resonance Meeting, 2013, Rio de Janeiro. p. 82-82.”

“Characterization of ncRNAs and T-UCRs in Human Cell Lines. **Oliveira, L. C.**; Pereira, C.O.; Fachel, A.A.; Verjovski-Almeida, S. . In: XL Annual Meeting of The Brazilian Biochemistry and Molecular Biology Society, 2011, Foz do Iguaçu.”

“Production, Partial Purification and Characterization of Amylase of Rice (*Oryza sativa*). Oka, G. U.; **Oliveira, L. C.**; Bruckner, F. P.; Lopes, K. V. G.; Maia, T. F.; Queiroz, J. H.; Fietto, L. G. . In: XXXIX Annual Meeting of The Brazilian Biochemistry and Molecular Biology Society, 2010, Foz do Iguaçu.”

“Expression and Purification of the N-Rich and DCD Domains from Soybean N-rich Protein-B in Bacterial System. **Oliveira, L. C.**; Reis, P. A. B.; Rosado, G.L.; Fontes, E. P. B.; Fietto, L. G. . In: XXXIX Annual Meeting of The Brazilian Biochemistry and Molecular Biology Society, 2010, Foz do Iguaçu.”

“Identification of Proteins that interacts with Asparagine Rich Proteins (NRP-A and NRP-B) of Soybean (*Glycine max*). **Oliveira, L. C.**; Reis, P. A. B.; Rosado, G.L.; Alves, M. S.; Fontes, E. P. B.; Fietto, L. G. . 2009. In: XIX DE INICIAÇÃO CIENTÍFICA SIC, Viçosa, 2009.”

“Morphometric variation among seasonal elements testicular of intertubular compartment of the bat *Molossus molossus* (Chiroptera: Molossidae), **Oliveira, L. C.**; Danielle Barbosa Moraes; Marli do Carmo Cupertino; Sérgio Luis P. da Matta; Mariella Bontempo Ducca Freitas . In: XVIII Simpósio de Iniciação Científica SIC, Viçosa, 2008.”

“Preliminary testicular morphometry of adult Wistar rat treated with water infusion of cipó-cravo (*Tynnanthus elegans*). **Oliveira, L. C.**; Fabiana Cristina Silveira Alves de Melo; Sérgio Luis P. da Matta . In: XVI Simpósio de Iniciação Científica, 2007, Viçosa.”

“Evaluation of the use of semen electronic defroster in Artificial Insemination With Fixed Time in cows nellore. **Oliveira, L. C.**; Vinicio Araujo Nascimento; Ciro Alexandre Alves Torres; Marcia Dias; Clóvis Gomes de Carvalho Júnior; Alexandro Rubim Dias; Daniel Macedo Rates . In: XV Seminário de Iniciação Científica da UFOP, 2007, Ouro Preto.”