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# Deciphering the structural organization of OrfG, the VirB8-like of ICES<sub>t3</sub> Type IV secretion system (T4SS)

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## Introduction

Conjugation is one of main mechanisms allowing DNA transfer between bacteria. This process is partly responsible for the spread of antibiotic resistance, an expected outcome of bacterial adaptation. Conjugation transfer is mediated by plasmids and integrative and conjugative elements (ICEs) which encode their own T4SS, a bacterial nanomachine dedicated for their transfer. T4SS are well described in Gram- bacteria but little is known about their architecture and mode of function in Gram+. Gram- and Gram+ T4SS share certain homologous structural components including the VirB8-like proteins, a superfamily of proteins known to be essential for T4SS assembly and function<sup>1</sup>. However, their respective structural organization and their role in T4SS biogenesis and function are still poorly understood. For that purpose, we have focused on OrfG, the VirB8-like encoded by ICES<sub>t3</sub> found in *Streptococcus thermophilus*. The X-ray structure of OrfG soluble domain revealed a trimeric arrangement in the crystal, similar to that observed for its counterparts TcpC and TraM, from pCW3 and pIP501, respectively (Fig. 1). Given that OrfG, TcpC and TraM are predominantly monomeric in solution, we raised the question about the biological relevance of their trimeric assembly and wondered whether this is a common feature of VirB8-like proteins. In order to answer these questions, a part of my PhD project was devoted to the study of the structural organization of OrfG. Several approaches were therefore carried-out, demonstrating that the soluble domain of OrfG as well as the full-length protein both adopt a trimeric assembly in solution. Interestingly, this form is also functional *in vivo*.

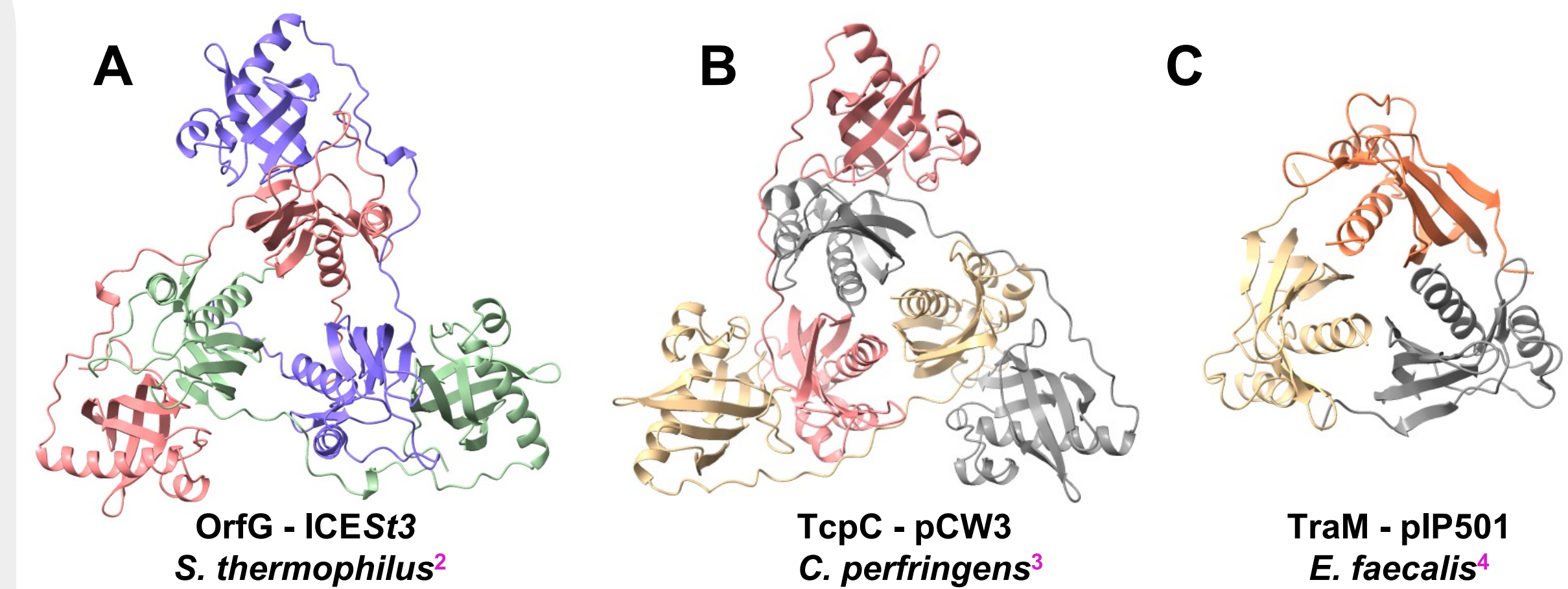


Figure 1. X-ray structures of OrfG (A), TcpC (B) and TraM (C). Each monomer is colored with a distinct color.

## 1) A cysteine cross-linking approach to analyze the binding interfaces of OrfG soluble domain trimers

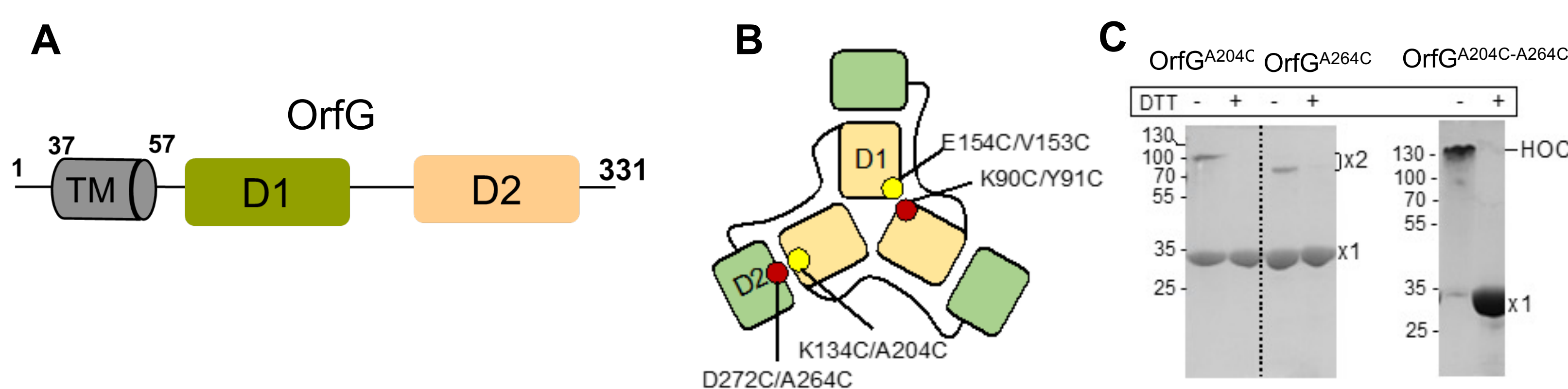


Figure 2. (A) Schematic representation of OrfG trimeric contact interfaces. (B) Localization of cysteine mutation within OrfG trimeric contact interfaces. (C) SDS page analysis of simple (OrfG<sup>A204C</sup>, OrfG<sup>A264C</sup>) and double (OrfG<sup>A204C-A264C</sup>) mutants in presence or not of DTT. HOC: High order complexes.

Despite a predominant monomeric form (band at 35kDa), a small fraction of OrfG<sup>A204C</sup> and OrfG<sup>A264C</sup> assembles into dimers in absence of DTT (bands at 70, 100kDa). On contrary, OrfG<sup>A204C-A264C</sup> mainly forms high order complexes in absence of DTT (band at 130kDa).

## 2) Structure of OrfG<sup>A204C-A264C</sup> soluble domain

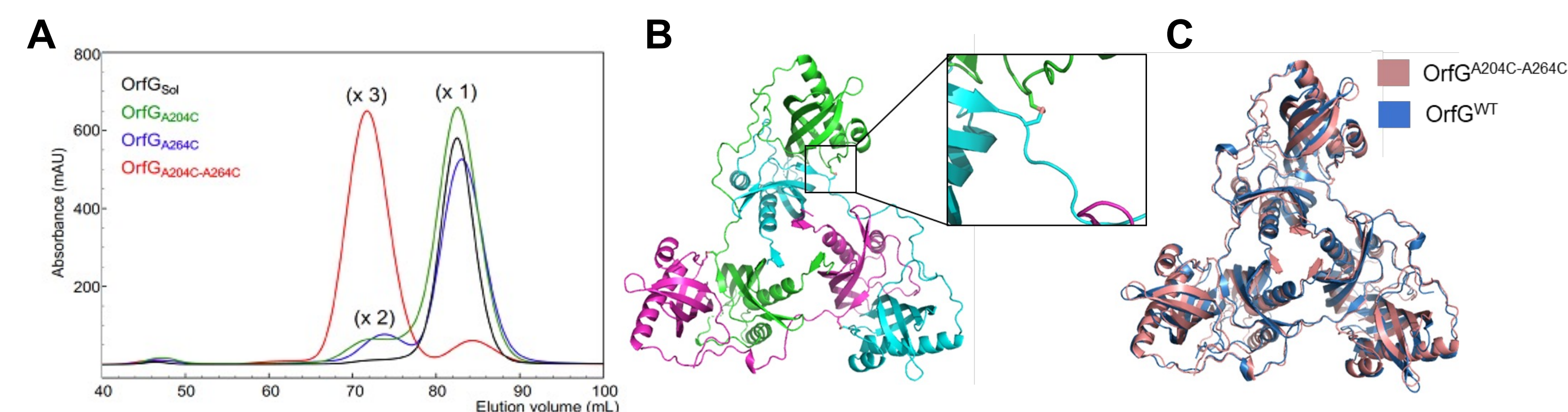


Figure 3 : (A) SEC profiles of OrfG<sup>A204C</sup> in green, OrfG<sup>A264C</sup> in blue, OrfG<sup>A204C-A264C</sup> in red and OrfG<sup>WT</sup> in black. Stoichiometries determined by SEC-MALS are indicated above each peak. (B) Crystal structure of the OrfG<sup>A204C-A264C</sup>. (C) Superposition of the structures of OrfG<sup>A204C-A264C</sup> (in brown) and OrfG<sup>WT</sup> (in blue).

Like OrfG<sup>WT</sup>, OrfG<sup>A204C</sup> and OrfG<sup>A264C</sup> are predominantly monomeric in solution. In contrast, OrfG<sup>A204C-A264C</sup> forms trimers in solution. **The structure of OrfG<sup>A204C-A264C</sup> confirms its trimeric assembly and displays the same structural organization as OrfG<sup>WT</sup> in the crystal.**

## 3) Interrogation of the trimeric assembly in the context of the full-length protein

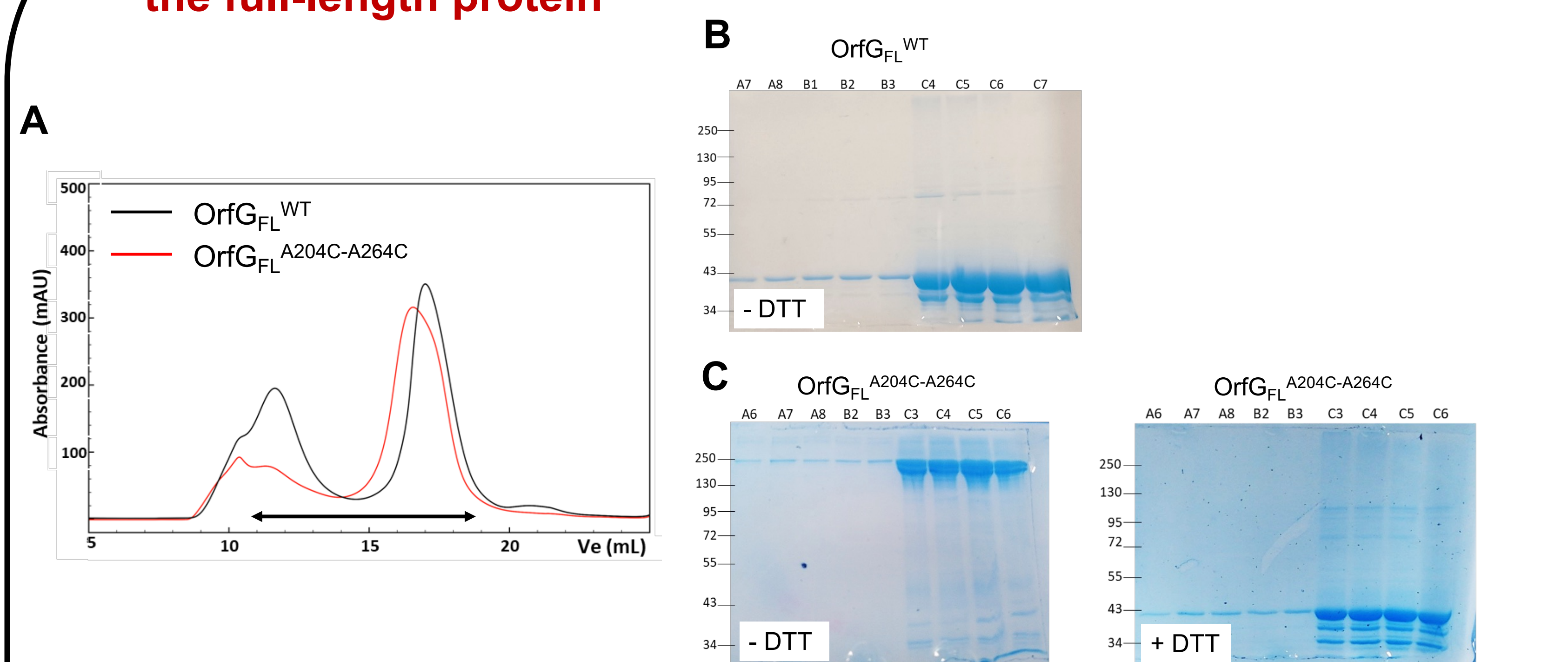


Figure 4 : (A) Superposition of SEC profiles of OrfG<sub>FL</sub><sup>WT</sup> (in black) and OrfG<sub>FL</sub><sup>A204C-A264C</sup> (in red) in presence of detergents. (B) SDS-PAGE analysis of SEC fractions without DTT for OrfG<sub>FL</sub><sup>WT</sup>, (C) in absence and presence of DTT of the OrfG<sub>FL</sub><sup>A204C-A264C</sup>. FL = Full length

In absence of DTT, a single band corresponding to the size of the OrfG<sub>FL</sub> monomer (40kDa) is detected. However, OrfG<sub>FL</sub><sup>A204C-A264C</sup> shows a high molecular weight band (250kDa) which disappear after DTT treatment. Since, this result is similar to that obtained with OrfG<sup>A204C-A264C</sup> soluble domain, **we propose that OrfG<sub>FL</sub> is able to form trimers in solution.**

## 4) Demonstration/evidence of *in vivo* activity of the orfG trimer

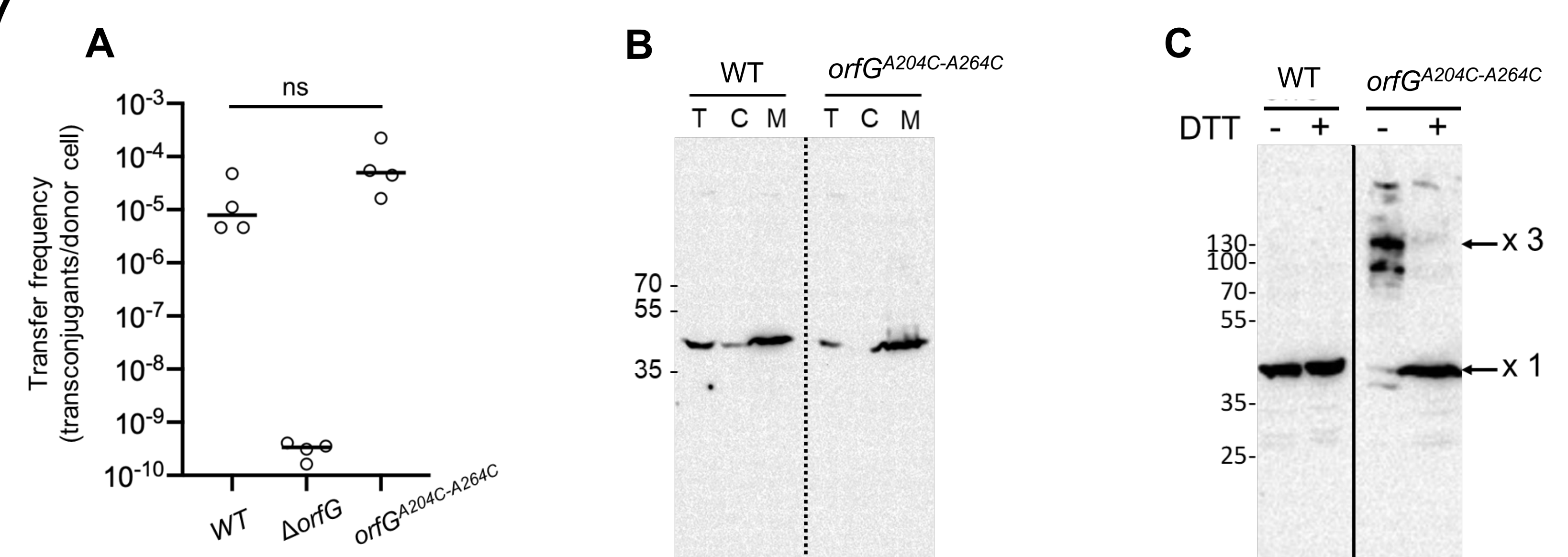


Figure 5 : (A) Conjugative transfer frequencies of ICES<sub>t3</sub> using *S. thermophilus* WT,  $\Delta$ orfG or orfG<sup>A204C-A264C</sup> as donor strains. (B) Western blot analysis of total (T), cytoplasmic (C) and membrane (M) fractions from strains expressing OrfG<sub>FL</sub><sup>WT</sup> (WT) and OrfG<sub>FL</sub><sup>A204C-A264C</sup> (orfG<sup>A204C-A264C</sup>) in reducing conditions. (C) Western Blot (WB) analysis of membrane fractions with and without DTT.

Mating experiments revealed that **OrfG is essential for ICES<sub>t3</sub> transfer** and that **the cysteine mutant is functional *in vivo***. By using WB, we showed that **OrfG<sub>FL</sub><sup>WT</sup> and OrfG<sub>FL</sub><sup>A204C-A264C</sup> are mainly located in *S. thermophilus* membranes**. More interestingly, the profile of the orfG<sup>A204C-A264C</sup> strain without DTT shows a band at 130kDa which disappears in the presence of DTT. Given that the size of the OrfG<sub>FL</sub> monomer is 40kDa, this band at 130kDa (40x3) would be the OrfG<sub>FL</sub> trimer. **This indicates that OrfG trimer is functional *in vivo*.**

**Conclusions/perspectives:** Although predominantly monomeric in solution, the data presented above show that OrfG forms functional trimers *in vivo* with the same structural organization observed in its crystal structure. Since TraM and TcpC show also the same packing in their crystal structures, this suggests that **VirB8-like in Gram+ operate as trimers in their corresponding T4SS**. This family of proteins in Gram- bacteria has been a target of choice for the design of conjugation inhibitors. Our study of OrfG structural organization and cellular localization which indicates that the soluble domain of OrfG is located in the cell wall and accessible on the surface of *S. thermophilus* (data not shown), **makes this protein a very interesting target for the design of conjugation inhibitors targeting the trimer contact interfaces.**

## References

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