EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

SHORT TERM SCIENTIFIC MISSION (STSM) SCIENTIFIC REPORT

This report is submitted for approval by the STSM applicant to the STSM coordinator.

Action number: CA16107
STSM title: Role of conjugative plasmids in biofilm formation by the phytopathogen *Xylella fastidosa*
STSM start and end date: 03/09/2019 to 29/11/2019
Grantee name: Carolina Palencia Gándara

PURPOSE OF THE STSM

X. fastidiosa (Xf) is a phytopathogen with a wide host range, causing chronic vascular infections in more than 550 plant species. Xf main mechanism of pathogenicity is systemic vessel colonization of infected plants by multiplication and movement followed by formation of biofilms. Bacteria within a biofilm develop adaptive advantages, such as increased resistance to antibiotics and persister cell formation. Xf codifies several secretion systems (type I, II, IV and V). Type I, II and V secretion systems of Xf have been characterized, but there is no information about the type IV secretion system (T4SS). T4SS is composed by a series of membrane proteins and ATPases that allow conjugative and mobilizable plasmids transfer among bacterial cells. Conjugative plasmids encode the whole machinery needed for their self-transmission. Our preliminary analysis revealed that most Xf sequenced strains contain conjugative plasmids. Furthermore, it has been demonstrated that biofilm formation is linked to bacterial conjugation, since the conjugative pilus is involved in both primary adhesion and biofilm assembly in *E. coli*. Our objective is to determine if conjugative plasmids present in Xf have a role in adhesion and virulence.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSM

Our objective during the STMS was to abolish the conjugative function of the plasmids from Xf in order to evaluate whether they have a role in Xf pathogenicity or not. Previously to the STMS, we tried to synthesize a CRISPR-Cas9 based system that would target Xf's conjugative plasmids specifically, but some issues were encountered and we are currently working on this approach. Our other approach, which was carried out during the STMS, was to inhibit Xf conjugative plasmids by creating strains defective in conjugative pilus synthesis, without which conjugation would not occur.

We chose to work with the strain *Xylella fastidiosa* subsp. *fastidiosa* M23 (Rogers and Stenger, 2012), that contains the conjugative plasmid pXFAS01, which is identical to the plasmid contained in a strain of Xf isolated in Spain, *Xylella fastidiosa* subsp. *fastidiosa* IVIA5235 (Landa et al., 2018).

For conjugative pili to form and thus, conjugation to occur, the membrane protein with ATPase activity VirB4 is essential (Rabel et al., 2003). For this reason, we chose to knock out the ATPase TrbE from pXFAS01, which is a homolog of VirB4. We knocked out the gene *trbE* by following the protocol of homologous recombination mutation developed in the host laboratory and described in Kandel, Chen, and De La Fuente (2018). Briefly, 800-1000 bp of the upstream and downstream regions of the gene of interest are amplified separately, then a kanamycin resistance cassette is inserted between them, substituting the

selected CDS. The selected strain is then transformed naturally with this construct and plated on PD3 with kanamycin for mutant selection.

After obtaining the mutant, some experiments for comparison between the wild-type and mutant strains were performed, following the protocols described in (Cruz, Cobine, and De La Fuente, 2012):

- Cell to cell aggregation.
- Twiching motility.
- Biofilm formation in 96-well plates.
- Biofilm formation and adhesion in microfluidic devices.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

Xf conjugative plasmids encode all the machinery necessary for their self-transfer between cells. Also, conjugative pili have and adhesive role in biofilm formation by other gram negative microrganisms (Beloin, Roux, and Ghigo, 2008).

We aimed to abolish conjugative pili synthesis by knocking out the gene *tbrE*, which encodes for an essential ATPase. We carried out the protocol for homologous recombination mutagenesis and checked mutant colonies with specific primers that would confirm presence/absence of the targeted gene (Figure 1.)

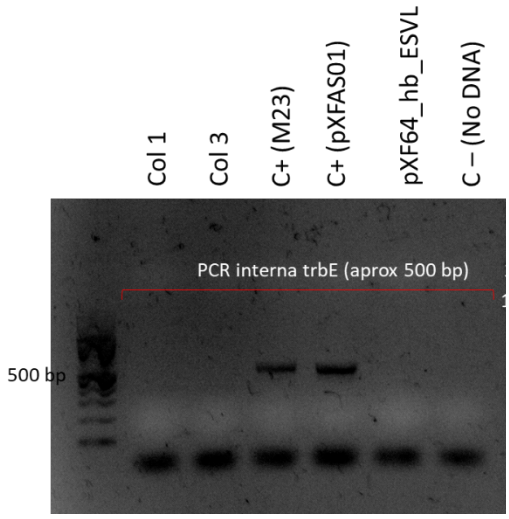

Figure 1. PCR confirming *trbE* absence in the mutant colonies. If trbE was present, a band of approx. 500 bp would be amplified (seen in Controls).

After confirming the mutants obtained by PCR, we performed a conjugation experiment from mutant Xf M23 to *E. coli* following the protocol described by Burbank and Van Horn (2017). No *E. coli* transconjugants were obtained, so we confirmed the conjugation inhibition by knocking out *trbE.*

Some experiments that evaluate phenotypic characteristics of Xf were performed in order to compare the strain carrying the wild-type plasmid with the strain carrying the mutant Δ*trbE* plasmid. Experiments were carried out in presence or absence of Ca^{2+}. Ca^{2+} promotes biofilm formation and cell aggregation by Xf (Cruz, Cobine, and De La Fuente, 2012; Cruz et al., 2014), so if conjugation absence would have an effect on these functions, in Ca^{2+} it would be more evident.

Cell-cell aggregation. These experiments asseses the capacity of Xf cells to aggregate, this means, to bound between each other though different bacterial appendixes. As shown in Figure 2, no difference in aggregation when comparing the wild-type and the mutant strains was observed.

Twitching motility. This experiments gives insight on the motility capacities of the strains assessed, we show in Figure 3 that neither the wild-type nor the mutant strain are capable of moving on agar plates.

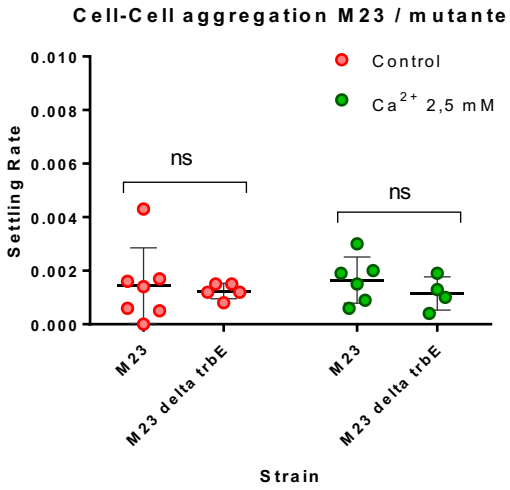

Figure 2. Cell-cell aggregation experiments. In red are shown results for unsupplemented PD3 medium, while in green are shown results for PD3 supplemented with Ca^{2+} (2 mM). The "settling rate" measurement comes from the equation

$$Settling\ rate = \frac{ln((OD600_{t=5min}) - (OD600_{t=30min}))}{25\ min}.$$ ns = not significant

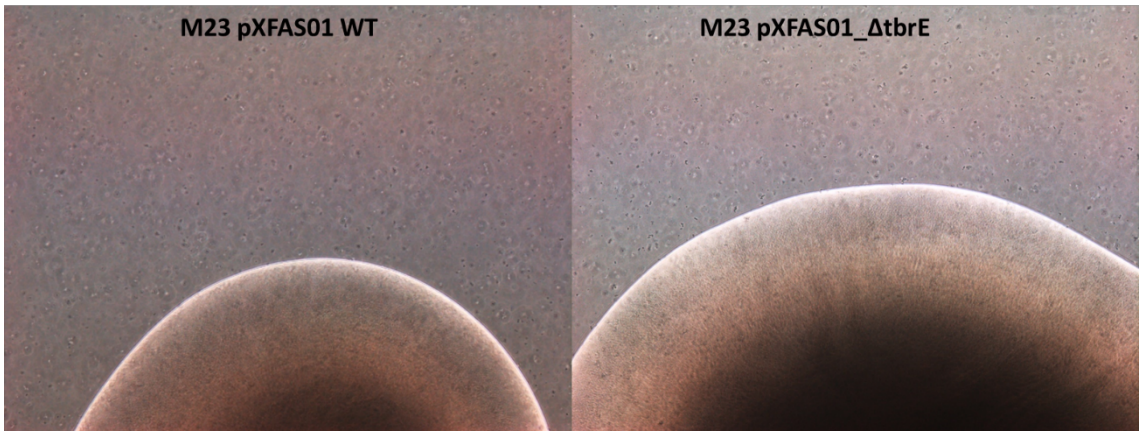

Figure 3. Twitching motility experiments. On the left, wild-type strains. On the right, strain M23 containing the Plasmid deficient on pili synthesis and conjugation.

Biofilm formation in plates. Biofilm formation experiments give us information about adherence capacity of a bacterial strain to a surface and between cells. Biofilms experiments were carried out at room temperature (approx. 22 °C) and 140 rpm for 13 days. Wells were rinsed with ds H_2O and dyed with cristal violet 0.1 % to measure cell attachment. In Figure 4, we can see that there is no difference between the wild-type and the mutant strain in their biofilm formation capacity, neither in absence or presence of Ca^{2+} (2 mM).

Biofilm formation in microfluidic chambers. Biofilms formed in microfluidic chambers give information about the process during its development. Experiments were performed in microfluidic chambers following the protocol described by De La Fuente, Burr, and Hoch (2008). In Figure 5, after 3 and 5 dpi (days post inoculation), it is observed that bacterial aggregates start to form both by the wild-type and the mutant strains, which suggest that biofilms are starting to build at this time point, but longer experiments are needed to determine if there is difference between them.

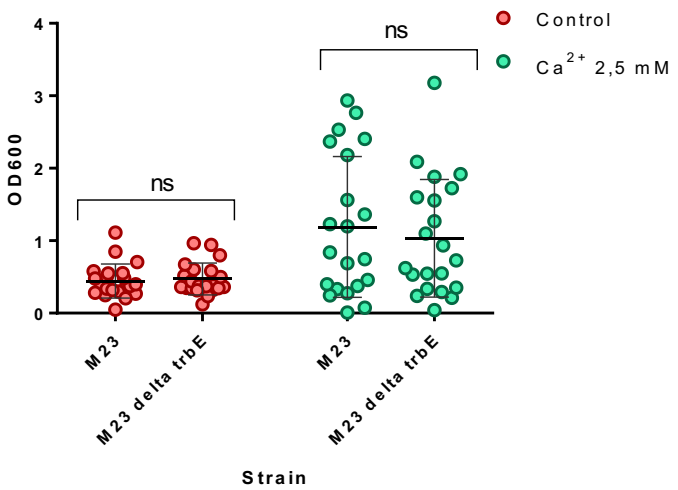

Figure 4. Biofilm formation by Xf M23. In red, biofilm formation by wild-type and mutant strain in unsupplemented PD3 medium. In green, biofilm formation in PD3 medium supplemented with Ca^{2+} (2 mM). ns = not significant.

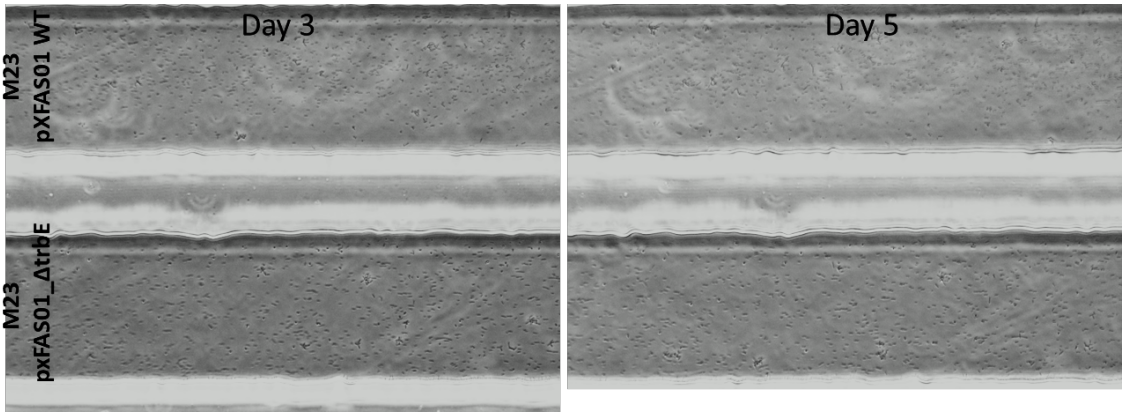

Figure 5. Biofilms in microfluidic chambers. Upper channel, wild-type strain. Lower channel, mutant strain. Pictures were taken at 3 and 5 dpi.

FUTURE COLLABORATIONS (if applicable)

After the STMS, a collaboration has been setup between Dr. de la Cruz and Dr. De La Fuente's laboratories. The combination between the knowledge in plasmid biology and Xf biology will make a profitable partnership out of this collaboration.

Moreover, experiments in microfluidic devices, which are not available to be made in University of Cantabria's laboratory yet, will be performed by Dr. De La Fuente's lab members. Also, if differences in microfluidic devices are observed between wild-type and mutant Xf M23, in planta experiments will also be performed in Auburn University's greenhouse facilities.

On the other hand, in Dr. de la Cruz laboratory we are working on constructing conjugation-deficient mutants in order to study different Xf strains isolated in Spain, which together with the results obtained by Carolina during her stay in Auburn University will lead to the production of high impact publications, as no knowledge is available about the role of conjugative plasmids in Xf biology and pathogenicity up to now.