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: Characterization of the interaction of different sequence types of *Xylella fastidiosa* subsp*. multiplex* with olive
STSM start and end date: 02/09/2018 to 30/11/2018
Grantee name: Miguel Román Écija

PURPOSE OF THE STSM:

This short term scientific mission had three main objectives:

1. Acquisition of basic knowledge on protocols and procedures for culturing and handling of *Xylella fastidiosa,* as well as genetic manipulation. The need to acquire skills and advanced knowledge on this bacterium are motivated by the fact that in our lab we just recently started working with this pathogen, so all the knowledge acquired will be transmitted back to others.

2. Study of the ionome of xylem sap of *Olea europaea* plants growing at different conditions, from different experiments:

   - One-year old plants from two olive cultivars 'Arbequina' and 'Picual' that have been grown under controlled conditions for three months at three different incubation temperatures (18, 24 and 30°C).
   - Four-years old plants from six olive cultivars 'Arbequina', 'Arbosana', 'Changlot Real', 'Frantoio', 'Hojiblanca' and 'Picual' growing under field conditions in Córdoba, Spain that were sampled in November 2018.
   - Seven-years old plants from two cultivars 'Arbequina' and 'Picual' growing under field conditions in Córdoba, Spain sampled approximately every two months from July 2017 to August 2018.

   Xylem sap extracted from the experiment referred above was characterized for mineral nutrients by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES), with the goal of understanding the nutritional requirements of *X. fastidiosa* infecting olives.

3. Phenotypic characterization of two *Xylella fastidiosa subsp. multiplex* strains IVIA5901 and ESVL, that were isolated from infected almond trees in Spain, and differ in the presence of two plasmids (present only in ESVL isolate).
   For the characterization of these isolates we used microfluidic chambers (artificial xylem vessels). This method allow us to understand spatial and temporal processes that we are unable to observe directly inside the host in vivo.
   We compared different strains of *X. fastidiosa* subsp. *multiplex* and *fastidiosa* from Spain and United States and characterized movement, aggregation and biofilm formation, in PD2 broth and olive sap.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSMS

Two Spanish *Xf* subsp. *multiplex* wild-type isolates (IVIA5901 and ESVL), isolated from almond trees (Giampetruzzi et al. 2019), were studied and compared with two *Xf* subsp. *multiplex* strains (Alma-Em3 and BB08-1), isolated from blueberry from Georgia and Florida, respectively, and also with the reference *Xf* subsp. *fastidiosa* wild-type isolate Temecula.

To study bacterial behavior, and phenotypic characterization, the following experiments were performed:

- **Patterns of bacterial growth in liquid media,** have been studied using microfluidic chambers (Meng et al., 2005) as artificial model of xylem vessels.
- **Adhesion force experiments,** using microfluidics chambers, to study the adhesiveness of the bacteria and the role of the pili in attachment to a glass surface (De la Fuente et al., 2007).
- **Biofilm formation experiments** using the staining with crystal violet protocol (Cruz, Cobine and De La Fuente, 2012)
- **Twitching motility** assay using PW agar medium without BSA and inoculating the different *Xf* isolates. (Li et al, 2007).
- **Assessment of bacterial cell-to-cell aggregation.** Biofilm cells were obtained from growth cultures in PD2 medium, incubated with shaking at 28°C for 7 days. The cell suspension was homogenized vigorously by pipetting and the OD600 of the suspension was measured by a spectrophotometer continuously for 5 min. (Cruz, Cobine and De La Fuente, 2012) (Li et al, 2007).

Besides this set of experiments to study the movement, aggregation and biofilm formation of the isolates, a pathogenicity assay was conducted using tobacco plants of cv. SR1 and Xanthy grown in the greenhouse. Nine plants per isolate (IVIA5901, ESVL, Alma-Em3, Temecula1) were inoculated with 10^8 cfu/ml and 9 plants incolated with PBS were used as controls. After the first assement, plants will be assessed by symptoms severity every 3-4 days. At the end of the experiment, we will calculate the area under progress curve (AUDPC), derived from the incidence and severity of symptoms (De la Fuente et al., 2013) (Oliver et al., 2014).

Moreover, I have learnt several protocols, methodologies and procedures, that usually people in the Laboratory of Professor De La Fuente use, including:

- General growth, culturing and storage protocols for *Xylella fastidiosa*
- Sampling of blueberry and grape plants infected by *Xylella fastidiosa,* in commercial crops in Georgia
- Isolation of *Xylella fastidiosa* isolates from blueberry and grape field samples
- Natural transformation competence experiments (Kandel, Lopez, Almeida and De La Fuente, 2016)
- Death/live bacterial staining in microfluidics chambers
- Death/live bacterial quantification by qPCR

A total set of 236 samples of olive sap from different cultivars and crop conditions were sent from Spain, to do a mineral element composition analysis, by using a Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) with simultaneous measurements of Ca, Cu, Fe, K, Mg, Mn, Na, P, S, and Zn (De la Fuente et al., 2013).

DESCRIPTION OF THE MAIN RESULTS OBTAINED

- Microfluidic chambers

A different behavior have been observed among the isolates tested. Cells of *Xf* subsp. *fastidiosa* Temecula, and *Xf* subsp. *multiplex* BB08-1 and Alma-Em3, have higher motility than the Spanish *Xf* subsp. *multiplex* isolates.

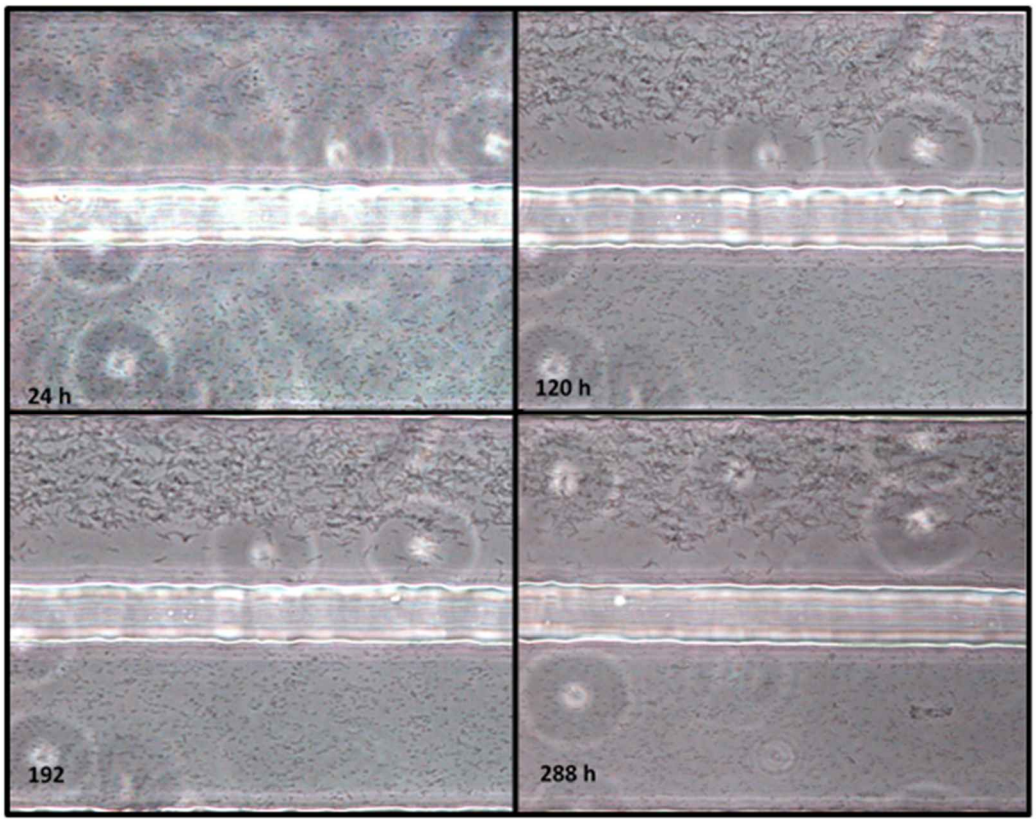

Fig 1. Time-lapse micrographs showing the formation of spherical compact autoaggregates inside microfluidic chambers (upper channel: ESVL (*subsp. multiplex*); lower channel: Temecula1 (*subsp. fastidiosa*) Images were captured at 24, 120, 192 and 288 hours.

- Adhesion force experiments

The numbers of *Xf* cells attached to the glass surface decreases as the flow rate increases (flow rate is increased 10 µl/min every minute) (fig 2). In general, cells of isolate IVIA5901 showed a lower adhesion force than isolate ESVL.

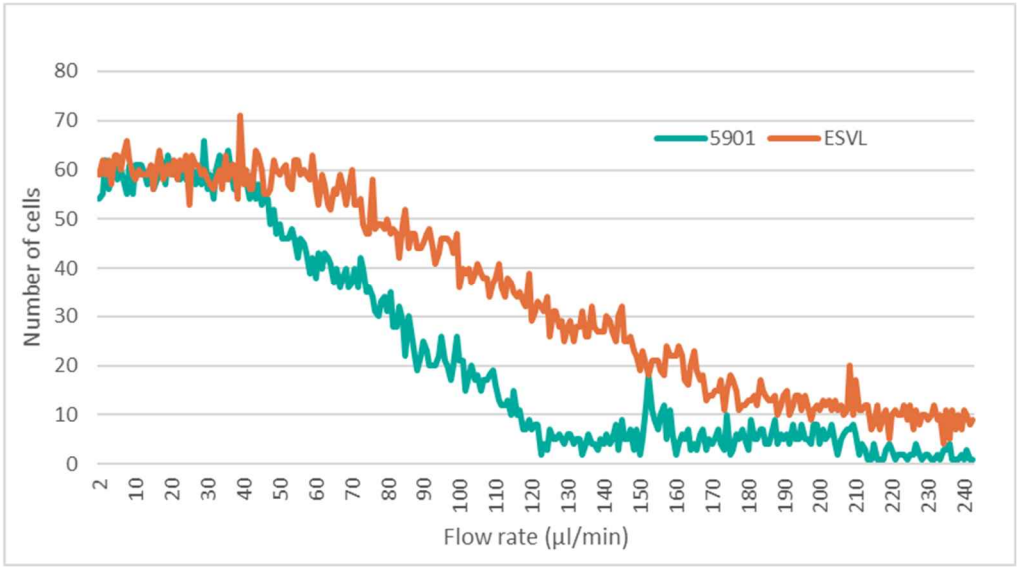

Fig. 2. Numbers of Xylella fastidiosa cells adherent to the microfluidic chamber surface as a function of the flow rate.

- Biofilm formation experiments

  All isolates were cultured without agitation in 96 well-plates and with agitation in glass tubes. Temecula1, BB08-1 and Alma-Em3 formed visible biofilms, in contrast, ESVL and IVIA5901 exhibited significantly reduced biofilms in both experiments (Figure 3 and 4).

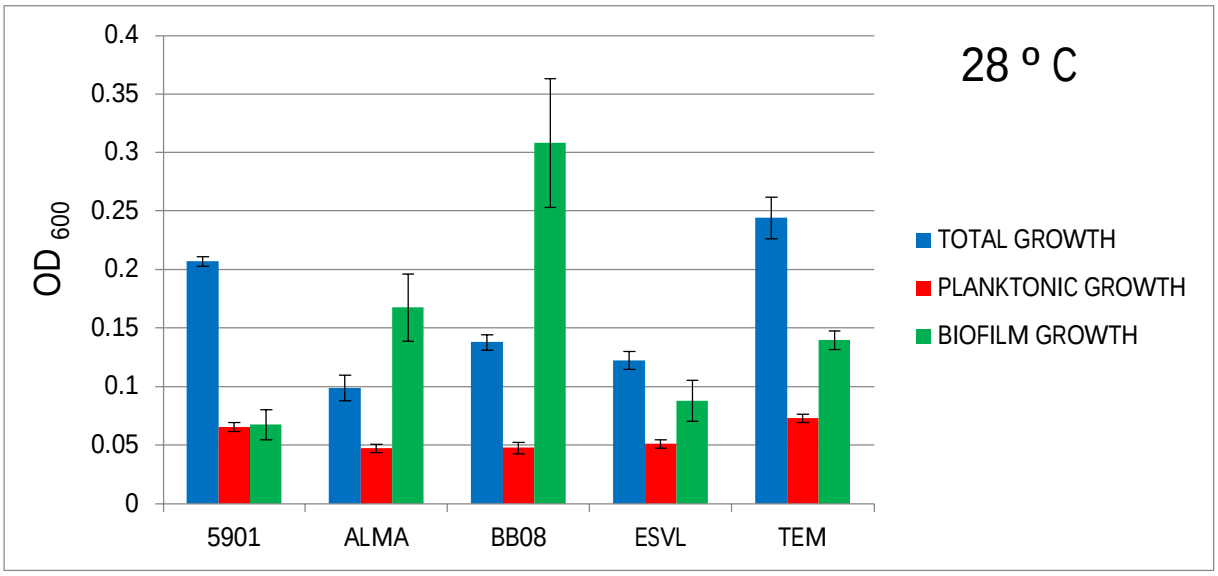

Fig. 3. Total growth, planktonic growth and biofilm formation generated by culturing the bacteria in 96-well plates and measuring optical density at 600 nm (OD600) during 8 days. Biofilm was measured, using crystal violet in the 96-well plates, at the end of the growth-curve experiment.

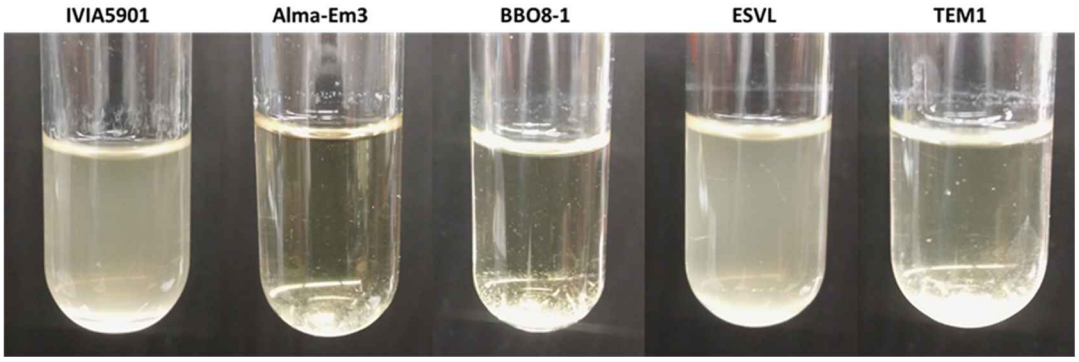

Fig 4. Biofilm formation in glass tubes

- Twitching motility

  The morphology of the edge of the colonies was examined using a microscope. Colonies with a peripheral fringe were designated as twitch positive. Temecula is the studied isolate with the biggest fringe. Regarding the isolates from subsp. *multiplex,* the Spanish isolates (IVIA5901 and ESVL) have a smooth colony margin indicating the lack of twithing motility.

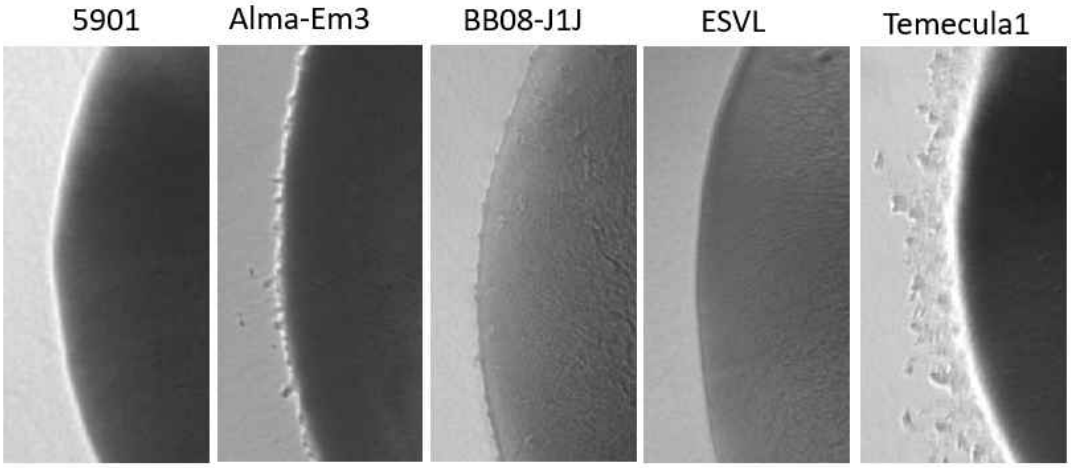

Fig. 5. Colony morphologies of studied isolates on a modified PW agar surface for 5 days

- Assessment of bacterial cell-to-cell aggregation
  Alma and BB08-1 has similar settling rates (between 9 and 11 mAbs/min), while Spanish isolates had a zero settling rates, but after a hour and two hours the OD600 was continuously measured for 5 min again, the settling rate of this isolates goes up to 3-4 mAbs/min

Análisis of olive sap samples by ICP-OES

Initial analysis of the mineral composition of xylem sap extracted from *Olea europaea* var. *europaea* has indicated the existence of differences among olive crop varieties, age of the plant and also during the growing season. Table 1 shows the results of minimum and maximum concentrations of the mineral nutrients in the analyzed samples.

Table 1. Minimum and maximum concentrations of the mineral nutrients in the analyzed samples

Mineral Nutrients	Min-max Concentration (u.M)
Ca	125,3-1233,3
Cu	0,24-15,34
Fe	0,14-9,27
K	680,5-8752,3
Mg	34,7-1090,4
Mn	0,27-5,5
Na	20,5-6948,8
P	132,9-2092,7
S	36,5-1120,2
Zn	0,29-10,98

References

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FUTURE COLLABORATIONS (if applicable)

We are currently finishing the pathogenrticy essay in tobacco plants.

With the data obtained in the professor De La Fuente lab, we are going to publish a paper.

Both working groups will follow a close relationship and collaboration between them.