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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 – EuroXanth: Integrating science on Xanthomonadaceae for integrated plant disease management in Europe
STSM title: Genomic analysis of atypical clade-1 xanthomonads
STSM start and end date: 19/08/2019 to 02/09/2019
Grantee name: Daiva Burokiene

PURPOSE OF THE STSM:

(max.200 words)

The genus *Xanthomonas* represents a widely spread group of phytopathogenic bacteria that can cause serious diseases on many plant hosts. Molecular methods used for analysis of *Xanthomonas* spp. bacteria help to determine the phylogenetic position of the strains. Therefore, better understanding of the presence and structure of pathogenic bacteria populations may stimulate the development of more efficient control measures and of more advanced and specific diagnostic protocols.

Thus, the first aim of my STSM was to **analyse the genome** of an atypical *Xanthomonas* strain that was found on *Fabaceae* plant roots in Lithuania. Preliminary work, which was based on partial sequencing of two housekeeping genes, suggested that this strain belongs to clade 1 and is related to the species *X. translucens*, *X. theicola*, and *X. hyacinthi*.

The large scale comparative analyses demonstrated exceptional genome plasticity within the genus *Xanthomonas, therefore,* more and more attention is paid for pathogenesis-associated genes. Consenquently, a second aim of this study was to get trained in **reporter gene assays** that are used to identify type III-secreted effector proteins.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSM

(max.500 words)

Work carried out during my STSM was the same as it was planned:

1) The first aim of my STSM was to **analyse the genome** of an atypical *Xanthomonas* strain that was found on *Fabaceae* plant roots in Lithuania. Preliminary work based on partial sequencing of two housekeeping genes suggested that this strain belongs to clade 1 and is related to the species *X. translucens*, *X. theicola*, and *X. hyacinthi*. However, the sequencing results of genome of the strain from Lithuania was delayed. Consenquently, the complete genome sequences of *Xanthomonas theicola* Vauterin et al. (ATCC® 700184™; strain designations: LMD 8684 [ICMP 6774, Uehara TC1], CFBP 4961) isolated from canker on tea

bush (*Camellia sinensis*) in Japan at 1974 (Uehara, 1980) was used for analysis during my STSM. Data was available at the laboratory of host institution at IRD.

It was planned to perform:

- Genome annotation.
- Comparative genomics with related clade-1 species and pathovars (*X. translucens*, *X. theicola*, *X. hyacinthi*).
- Prediction of type III effector genes.
- Drafting of scientific manuscript.

Genome analysis of *X. theicola* was done for:

- Clarification of taxonomy (using *gyrB*, ANI)
- Prediction of *hrp* co-regulated genes (PIP boxes)
- Type III secretion system (T3SS) / Type III effectors
- Possible effector transposons
- Type II secretion system (T2SS) / Type IV pili
- CRISPR systems
- Non-ribosomal peptide synthases

Programs were used for analysis: Artemis, BLAST (GenBank database), Phobius, Pfam.

Data of comparative genomics of *X. theicola* with related clade-1 species and pathovars obtained during my STSM was used for the drafting of scientific manuscript.

2) The second aim of my STSM was to train on performing **reporter gene assays** for type III effectors. It was expected that genome mining of new clade-1 strains will lead to the discovery of new effector candidates. Their validation by reporter assays is a key step before further functional characterisation. The following experiments were done during my stay:

- Tri-parental conjugation.
- Plant inoculations.
- Inoculation of *X. cannabis* mutants.

Tri-parental conjugation was done as follows: strains were streaked on media (donor and helper – on LB, recipient – on PSA) (Table 1) and incubated for 2 days at 30 °C. Then, bacterial lawn (5x10 mm) was resuspended in 500 µl PSA medium and conjugation was setup (as shown in Table 1). 50 µl of the suspension was dropped onto PSA agar plate (1 % agar) and incubated over night at 30 °C. Afterwards, about 5x10 mm of bacterial lawn was washed with 400 µl PSA(±Rf)-medium. Spinned down (30 s) and bacterial pellet was resuspended in 100 µl PSA medium and drop (10–20 µl) was streaked out onto PSA-Rf-Tc plate for single colonies. Single colonies were isolated after 2–3 days at 30 °C.

Table 1. Stains used for analysis.

Mix	Donor	Isolate	Helper	Recipient
	vector pBBR1MCS-3	Tetracyclin (Tc)	Spectinomycin (Spc)	Rifampicin (Rf)
	Insert:	10 µl	10 µl	50 µl
1	SS_{T3Ec2}::*avrBs1*$_{\Delta SS}$	Edwige-41	pRK2073	*Xcc* 8004 Δ*avrBs1*
2	SS_{T3Ec13}::*avrBs1*$_{\Delta SS}$	Edwige-44	pRK2073	*Xcc* 8004 Δ*avrBs1*
3	SS_{T3Ec14}::*avrBs1*$_{\Delta SS}$	Edwige-47	pRK2073	*Xcc* 8004 Δ*avrBs1*
4	SS_{T3Ec11}::*avrBs1*$_{\Delta SS}$	Edwige-50	pRK2073	*Xcc* 8004 Δ*avrBs1*
5	SS_{T3Ec9}::*avrBs1*$_{\Delta SS}$	Edwige-53	pRK2073	*Xcc* 8004 Δ*avrBs1*
6	SS_{T3Ec5}::*avrBs1*$_{\Delta SS}$	Edwige-63	pRK2073	*Xcc* 8004 Δ*avrBs1*
7	SS_{T3Ec8}::*avrBs1*$_{\Delta SS}$	Edwige-66	pRK2073	*Xcc* 8004 Δ*avrBs1*
8	SS_{T3Ec10}::*avrBs1*$_{\Delta SS}$	Edwige-69	pRK2073	*Xcc* 8004 Δ*avrBs1*
11	SS_{T3Ec2}::*avrBs1*$_{\Delta SS}$	Edwige-41	pRK2073	*Xcc* 8004 Δ*hrcV*
12	SS_{T3Ec13}::*avrBs1*$_{\Delta SS}$	Edwige-44	pRK2073	*Xcc* 8004 Δ*hrcV*
13	SS_{T3Ec14}::*avrBs1*$_{\Delta SS}$	Edwige-47	pRK2073	*Xcc* 8004 Δ*hrcV*
14	SS_{T3Ec11}::*avrBs1*$_{\Delta SS}$	Edwige-50	pRK2073	*Xcc* 8004 Δ*hrcV*

15	SS$_{T3Ec9}$::*avrBs1*$_{\Delta SS}$	Edwige-53	pRK2073	*Xcc* 8004 Δ*hrcV*
16	SS$_{T3Ec5}$::*avrBs1*$_{\Delta SS}$	Edwige-63	pRK2073	*Xcc* 8004 Δ*hrcV*
17	SS$_{T3Ec8}$::*avrBs1*$_{\Delta SS}$	Edwige-66	pRK2073	*Xcc* 8004 Δ*hrcV*
18	SS$_{T3Ec10}$::*avrBs1*$_{\Delta SS}$	Edwige-69	pRK2073	*Xcc* 8004 Δ*hrcV*
21	SS$_{T3Ec2}$::*avrBs1*$_{\Delta SS}$	Edwige-41	pRK2073	
22	SS$_{T3Ec2}$::*avrBs1*$_{\Delta SS}$	Edwige-41		*Xcc* 8004 Δ*avrBs1*
23	SS$_{T3Ec2}$::*avrBs1*$_{\Delta SS}$	Edwige-41		*Xcc* 8004 Δ*hrcV*
24			pRK2073	*Xcc* 8004 Δ*avrBs1*
25			pRK2073	*Xcc* 8004 Δ*hrcV*

Rf – Rimfampicin (100 µg/ml); Spc – Spectinomycin (50 µg/ml); Tc – Tetracyclin (10 µg/ml)

DESCRIPTION OF THE MAIN RESULTS OBTAINED

(max.500 words)

1) The first aim was to **analyse the genome**. Clarification of taxonomy of the *X. theicola* strain was done using the sequences of house-keeping gene *gyrB* (Fig. 1a) and the average nucleotide identity (ANI) (Fig. 1b).

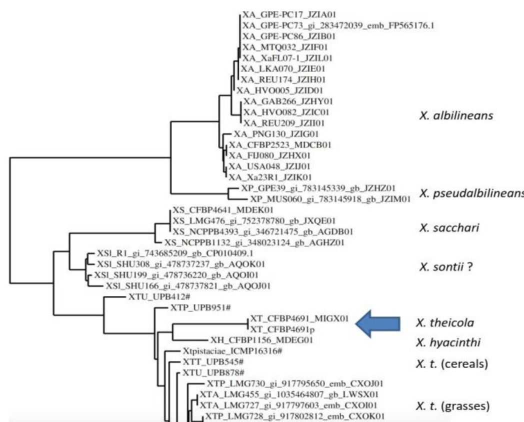

Fig. 1a

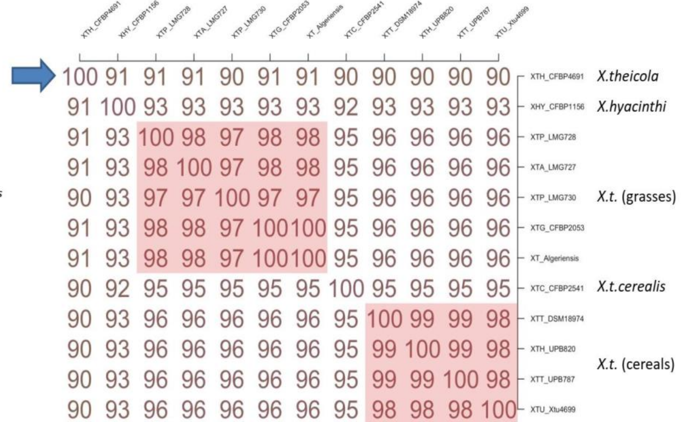

Fig. 1b

The pathogenicity of *Xanthomonas* bacteria depends on a type III protein secretion system (T3SS), where genes encoding the T3SS are referred to as hypersensitive response and pathogenicity (*hrp*) genes. It has been demonstrated that the regulation by *hrp* genes in *Xanthomonas* spp. could be mediated by the binding of Hrp proteins to a cis-regulatory element named the plant-inducible promoter (PIP) box. Prediction of *hrp* co-regulated genes was performed using motif of PIP box TTCGB-N$_{15}$-TTCGB. A survey revealed that *X. theicola* strain possessed 20 loci with PIP box and -10 region, however 4 false-positive loci were estimated using NCBI BLAST search. Finally, 10 genes (16 loci) were identified:

- Secreted serine protease
- Acyl-CoA thioesterase
- XopB
- XopG2 (2x)
- XopAD (2x)
- XopAF2
- XopE2
- RS_T3E_Hyp7

Also, prediction of *Xanthomonas* Type III effectors (T3E) was performed. Futhermore,bioinformatic analysis confirmed the presence of XopB, XopD, XopE2, XopF3, XopG2 (2x), XopL, XopM, XopX, XopZ1, XopAD (2x), XopAF2 and XopAZ (3x), some sequences homologous to effectors found in *Ralstonia*, *Pseudomonas*, *Enterobacteriaceae* (RipX, RipAG, RipAP, RipTPS, RS_T3E_Hyp7, RS_T3E_Hyp8 and HopI1) were obtained in this strain too. Moreover, two sequences of encoded transcription activator-like effectors (TALEs) were identified. A survey of genomic sequences of *X. theicola* showed two target site duplications of

effector transposons. Also, more *tnpT* copies were found on the putative plasmid.
Type II secretion (T2SS) and the evolutionarily related Type IV pilus (T4PS) systems are important virulence determinant in *Xanthomonas.* The analysis of the presence of *xps* and *xcs* gene clusters, which are representatives of T2SS, showed that *xcs* gene cluster is absent in *X. theicola*, but *xps* gene cluster was found in this strain. T4PS system, which is needed for cell-to-cell communication when bacteria translocate protein or DNA-protein into host cells, was found too. Bioinformatic analysis for clustered regularly interspaced short palindromic repeat (CRISPR) systems showed that *X. theicola* has both systems, I-C and I-F. It was found, that CRISPR I-C was composed by 16 spacers, where 4 of them were found to be related to phages and I-F system was found with 22 spacers. Moreover, non-ribosomal peptide synthase (NRPS) was encoded in *X. theicola* as well (Fig. 2).

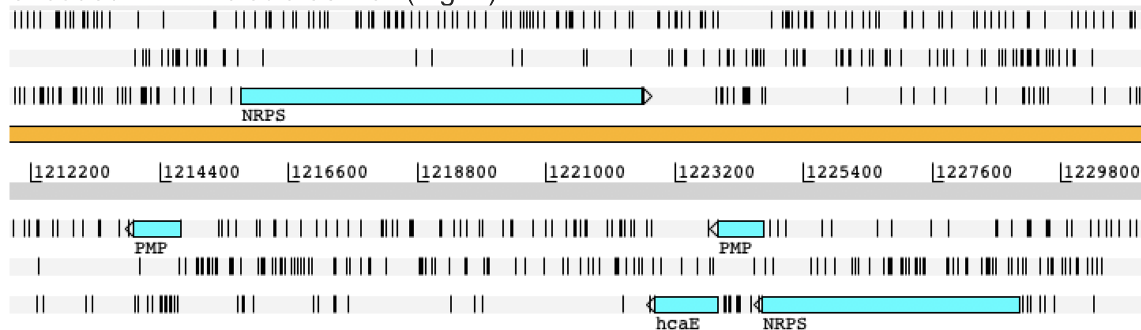

Fig. 2. Non-ribosomal peptide synthase found in *X. theicola* strain.

2) The **second aim** of my STSM was to perform the reporter gene assays.

Two single colonies were chosen after tri-parental conjugation from every mix (mentioned in the Table 1). Colonies from the rest mixes (which were not mentioned in Fig. 3) were not formed.
Mutant colonies were inoculated to the leaves of different age of pepper plants (Fig. 3a and 3b).
Preliminary results of inoculations showed hypersensitivity response (HR) of some mutants. Though, it was noticed, that HR appearance was dependant on the age of the used plants (3 or 8 weeks). Non-consistent reaction of the plants to the mutants were obtained in some cases, for example, with 1A and 1B, or 17B. Maybe experiments should be repeated with these mutants, but as plant inoculations are still in on-going process. Final result assessment will be done by my colleagues during the following 5 days, after my STSM.

Fig. 3. Leaves of paper plants inoculated with mutant colonies: a) plants of three weeks; b) plants of eight weeks.

FUTURE COLLABORATIONS (if applicable)

(max.500 words)

During my STSM, I had a possibility to visit a modern laboratory of the Institute of Research for Development (Institut de Recherche pour le Développement) in Montpellier (France) specialized in molecular research of plant-pathogenic bacteria. It was an excellent opportunity to use modern equipment and to learn new techniques. Work with the best French bacteriologists provided novel ideas, theoretical and practical knowledge, experience on different molecular and bioinformatic techniques applicable to the analysis of genomics of *Xanthomonas* spp. bacteria. The draft of a joined publication is being finalised, on the basis of my STSM results. Also, one more publication will be provided in nearest future (as soon as we get results on genome sequencing of our isolate).