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: Unravelling the molecular mechanism of copper resistance in *Xanthomonas* spp.
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PURPOSE OF THE STSM

The genus *Xanthomonas* comprises many phytopathogenic species and a total of thirteen genus members are considered as quarantine organisms. In phytopathogens, an excess of copper induces resistance mechanisms, which are of great interest since copper compounds have been widely used in agriculture for disease control. In horticulture, one of the main issues in the management of *Xanthomonas arboricola* pv. (e.g.: pv. *juglandis* and pv. *pruni*) is the presence of isolates able to grow in the presence of high copper concentration, thus making any copper treatment useless. In general, copper resistance is related to the presence of copper responsive systems, such as the *cop A/B/L* gene cluster. We isolated several xanthomonads able to detoxify very high concentrations of copper and, surprisingly, such xanthomonads do not possess any known cluster responsible for copper resistance. Therefore, it would be essential to explore the genetics of such isolates, to unveil such still unknown copper resistance determinants. The present study will be useful to study the horizontal transfer of such sequences in areas with intensive fruit cropping (stone fruits, walnuts) and the role of microbial communities in the persistence of such genetic trait. From this, we have screened various *Xanthomonas* spp. strains for *in vitro* copper resistance without possessing any *cop* cluster or genes or other known sources of copper resistance.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSM

A total of eight *Xanthomonas* spp. strains (DLS 254, 365, 236, 240, 71, 288, 103 and 450) from UNIMORE plant pathology lab were selected to study extensively for their molecular aspects of copper resistance. These strains were previously collected from symptomatic plant materials of commercial orchards and walnut plants in the year 2007-2010 in Emilio Romagna regions of Italy. These strains along with two UF (University of Florida) strains, one copper resistant (Xac44) and one copper sensitive (ME24) as controls, were used throughout this study. All these strains were extensively studied for *in vitro* copper resistance on agar plates and in liquid media. Based on the copper tolerance results, two resistant strains 254 (pv. *pruni*) and 288 (pv. *juglandis*) were sent for whole genome sequencing (WGS). WGS analysis helped to design the possible specific primers for the amplification of various gene clusters responsible for the copper resistance, for example *cop L/A/B, cus*, *pco*, and *cue*. Based on copper resistance gene cluster amplification results and bioinformatic analysis strain 254 has been selected for further analysis. Since isolate DLS 254 was found to be copper resistant and possess *cop* cluster genes, it was selected for further studies to unveil the copper homeostatis in xanthomonads. One of the UF copper sensitive strains, ME24 (*X. perforans*), was used to study the horizontal gene transfer (HGT) between strains DLS 254 and

ME24 to know whether the copper tolerance is plasmidial or chromosomal. While studying about HGT, we also studied about the inactivation of copper resistance genes to produce copper sensitive mutants using topo-knockout technique. Since it was observed that few of the strains were not xanthomonads, but they were as *Xanthomonas* like isolates, these hypothesis motivated to characterize these strains based on MLSA analysis.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

Out of eight strains it was found that five strains (DLS 254, 365, 71, 288 and 103) were tolerant to $CuSO_4$ at high concentrations like 300 and 500 ppm and remaining three strains (DLS 236, 240 and 450) were found to be sensitive. Based on copper tolerance results two resistant strains, DLS 254 and 288, were anlaysed for whole genome sequence. WGS analysis unveiled their *cop* cluster genes and its molecular identification. Based on NCBI BLAST results, it was found that strains DLS 254 and 288 as *Xanthomonas arboricola* pv. *pruni* and *Pantoea agglomerans*, respectively. It was interesting observation that these *Pantoea* strains are quite well adopted the pathogenicity and resistance against various heavy metals and over the last decade many researchers have found these *Pantoea* strains as one of the major disease causing bacterium on several plants. Our results are correlating with the findings of **Dutkiewicz et al. (2016); Yang et al. (2011)**. Based on *cop* cluster genes found from two strains and their genome annotation we designed specific primers for various copper resistance genes and used for all the remaining strains. Each strain has the different pattern of *cop* cluster genes. Since in the present study our main focus was to unveil the molecular mechanism of copper resistance in *Xanthomonas* sp., we selected strain DLS 254 for further analysis. In order to confirm if copper resistance is plasmidial or chromosomal in strain DLS 254, several *in vitro* bi-parental attempts were made. Several transconjugants were obtained using strain DLS 254 as donor and strain ME24 as recipients on NA + Cu selective medium. Also, PCR attempts for detection of *cop* genes in DLS 254 using primers specifically designed for *copA* and *copB* resistance genes, which are widespread in Cu^R xanthomonads were negative. Though plasmid profile gel image confirms the movement of plasmid from donor to recipient and subsequently positive for copper resistance on NA at 100 ppm concentration. From these results we assume that there might be a novel plasmid movement from donor to recipient and these results are correlating with Behlau et al. **(2013)**. To construct a *copA* mutant strain, the entire coding sequence was PCR-amplified from genomic DNA and cloned into the *E.coli*. The topo was inserted randomly into the plasmid using the insertion kit, according to the instructions of the manufacturer. One plasmid with the topo inserted into the *copA* coding sequence of 300 bp from the start codon was selected for further use. This plasmid was used to transform strain DLS 254 by electroporation **(Sun et al., 2003)** and recombinant clones were selected on NA medium containing kanamycin. Strain DLS 254 wild type and mutants were tested on NA plates for their copper tolerance, since *copA* gene has been inactivated which results in copper sensitivity for the mutants. Based on MLSA analysis, PCR amplification of *gyrB* and *rpoB* results which specifically target *Pantoea* spp. revealed that out of eight strains five strains (DLS 365, 236, 288, 103 and 450) belong to *Pantoea* sp.

FUTURE COLLABORATIONS (if applicable)

Conjugants plasmid profile results suggest the movement of plasmid, but specifically we were not able to tell what type of genes/plasmid has moved and we were not able to confirm the PCR amplification of *copA/copB* or other novel genes in the transconjugants; this work is still in progress. We also found few of the strains as *Pantoea* sp. and it's pathogenicity on walnut plants since these isolates were originally isolated from walnut. Dr. Jeffrey B. Jones' lab is well experienced on molecular biology and *Xanthomonas* it is necessarily important for STSM grantee and other lab members to collaborate in the future on the same or related topic. Therefore, in the framework of the Italy-USA cooperation agreement in Science and Technology, we will seek another chance to carry on research on this interesting topic, connected to the next call for bilateral projects.