Interim Progress Report for CDFA Agreement Number 1400139-SA

**Characterization of *Xylella fastidiosa* PhoP/Q two-component regulatory system**

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**Reporting Period:** The results reported here are from work conducted July 2014 through February 2015.

## Introduction

*Xylella fastidiosa* is a gram-negative, xylem-limited plant pathogenic bacterium and the causal agent of Pierce's disease (PD) of grapevine (Wells et al., 1981). *X. fastidiosa* forms aggregates in xylem vessels, which leads to the blockage of xylem sap movement. The formation of biofilms allows for bacteria to inhabit an area different from the surrounding environment. Furthermore, biofilm formation is an important factor in the virulence of bacterial pathogens. Biofilm formation is a result of density-dependent gene expression (Morris and Monier, 2003). Density-dependent biofilm formation is triggered by the process of quorum sensing (QS). Biofilm formation induced by QS is essential for survival and pathogenicity and may be regulated through a two-component regulatory system (TCS). TCS's are signal transduction systems through which bacteria are able to respond to environmental stimuli (Hoch, 2000). The TCS is comprised of a histidine kinase, responsible for sensing stimuli, and the response regulator, responsible for mediating gene expression (Charles et al, 1992).

The PhoP/Q TCS is a well-studied and highly conserved TCS responsible for regulation of genes involved in virulence, adaptation to environments with limiting $Mg^{2+}$ and $Ca^{2+}$, and regulation of other genes. PhoQ is a transmembrane histidine kinase protein with a long C-terminal tail residing in the cytoplasm. The periplasmic domain of PhoQ is involved in sensing of $Mg^{2+}$, $Ca^{2+}$, and antimicrobial peptides. The cytoplasmic domain contains a histidine residue that is phosphorylated when physiological signals are detected in the periplasm. The PhoP/Q TCS is a phosphotransfer signal transduction system and upon activation by environmental stimuli, PhoQ phosphorylates the corresponding response regulator PhoP. In most bacteria, environments high in $Mg^{2+}$ inhibit the PhoP/Q system through dephosphorylation of PhoP (Groisman, 2001). *Xf* contains homologs of the PhoP/Q system (Simpson et al. 2000). We have previously shown that the PhoP/Q system is essential for *Xf* survival *in planta* and plays a role in regulation of biofilm formation and cell-cell aggregation. The current aim of our research is to understand what genes are being controlled by PhoP/Q in *Xf*, especially genes involved in the early adaptation processes essential for survival in the xylem. We are also investigating factors involved in induction or repression of the PhoP/Q system such as pH, cation concentration and peptides.

## Objectives

**Objective 1:** Characterization of factors involved in induction and/or repression of the PhoP/Q system.

**Objective 2:** Identification and characterization of genes regulated by PhoP

**Objective 3:** Determine if peptides in a library provided by Prof. Carlos Gonzalez are able to bind to *Xf* PhoQ and inhibit activation of PhoP

## Objective One: Characterization of factors involved in induction and/or repression of the PhoP/Q system

We are currently working to further our understanding of factors that influence the PhoP/Q system in *Xf*. So far we have found that *XfΔphoP* and *XfΔphoQ* mutants show inhibited growth in Pim6 media containing 50 µM $Mg^{2+}$ when compared to wild-type *Xf*. We also see a reduction in growth among the mutants compared to wild-type at a lowered pH of 5.0 (instead of pH 7.0) when the media contains 500 µM $Mg^{2+}$.

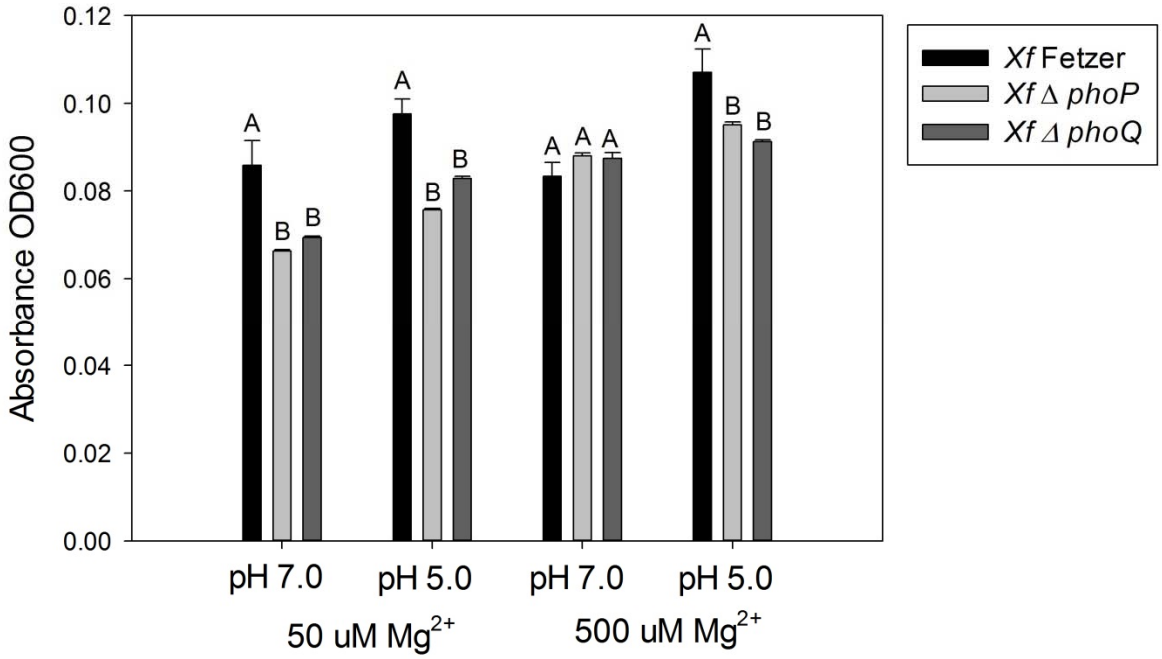


**Figure 1:** Absorbance OD600 of *Xf* fetzer, *XfΔphoP* and *XfΔphoQ* in Pim6 media containing either 50 µM or 500 µM $Mg^{2+}$ at a pH of 5.0 or 7.0. Absorbance was measured after 5 days growth at 28° C.

We are currently looking at other types of media, xylem sap and different ranges of various ions.

## Objective Two: Identification and characterization of genes regulated by PhoP

We are in the process of submitting our RNAsequencing libraries to the UC Davis Genome Center for Sequencing. Using real-time quantitative PCR, we tested a number of different conditions for growth of *Xf* in xylem sap and PD3 to identify the optimal conditions for expression of *Xf phoP*. We determined *Xf* grown on solid PD3 media for 5 days at 28° C followed by an incubation of one hour in either liquid PD3 or filter-sterilized xylem sap isolated using a pressure bomb. After the one hour incubation, RNA was isolated using the Qiagen RNAeasy mini kit with the RNAprotect bacteria reagent, to preserve the

target RNA transcripts. RNA quality was verified using the Bio-Rad Experion RNA Standard Sense Chips. After RNA was determined to be of sufficient quality, ribosomal RNA was removed from the samples using the Epicentre ScriptSeq Complete Kit for bacteria. Sequencing libraries were prepared from the rRNA depleted RNA samples according to manufactures instructions. In brief, cDNA was synthesized and purified and samples were tagged with barcodes to permit pooling of RNA samples in the sequencing lane. The libraries were purified using the Agencourt AMPure XP System (Beckman Coulter) and evaluated using the Experion DNA 1K chips (Bio-Rad).

## Objective Three: Determine if peptides in a library provided by Prof. Carlos Gonzalez are able to bind to *Xf* PhoQ and inhibit activation of PhoP

We have begun work on objective three investigating whether two potential peptides, kindly provided by Professor Carlos Gonzalez, have an inhibitory effect on *Xf*. The two peptides tested are 66-10D: FRLKFH and 77-12D: FRLKFHI (Reed *et al.*, 1997) We found the peptides have an inhibitory effect on *Xf* Fetzer when grown for 5 days in Pim6 media (Michele Igo, personal communication) modified to contain 10 uM $Mg^{2+}$. *Xf* was grown in the presence of the peptides at varying concentrations, with an inhibitory effect observed at peptide concentrations as low as 10 ug/ml (Figure 2).

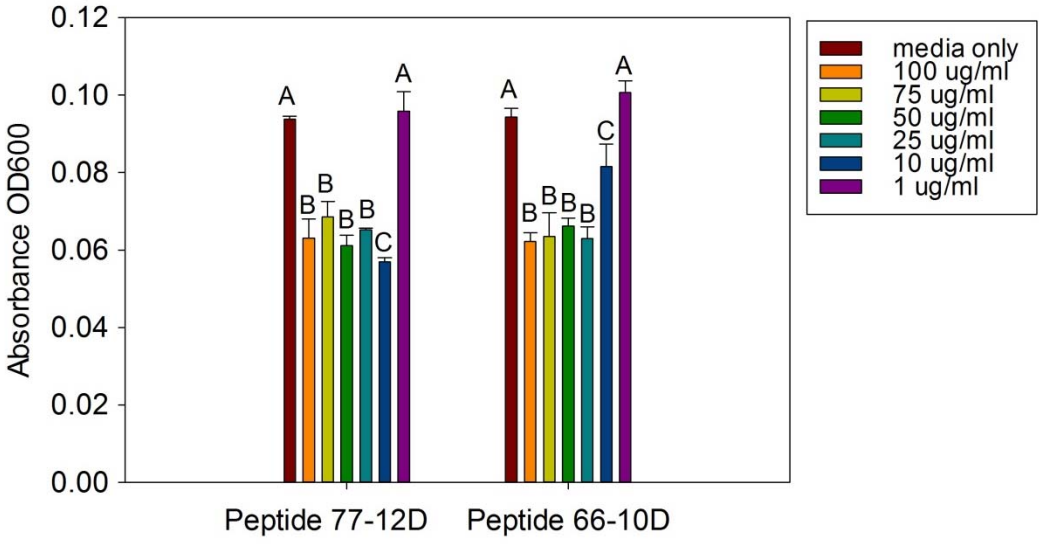


**Figure 2:** Absorbance OD600 of *Xf* Fetzer cells grown for 5 days at 28° C with shaking at 100 rpm in Pim6 media containing 10 µM $Mg^{2+}$ and varying concentrations of 77-12D and 66-10D peptides. Different letters indicate significance ($P < 0.05$) as determined by the Tukey test.

We have observed a further reduction in growth (greater inhibitory effect) of these peptides on our *XfΔphoP* and *XfΔphoQ* mutants when grown under the same conditions as above (Figure 3). It is important to note that the baseline OD600, prior to the 5 day incubation, was around 0.04.

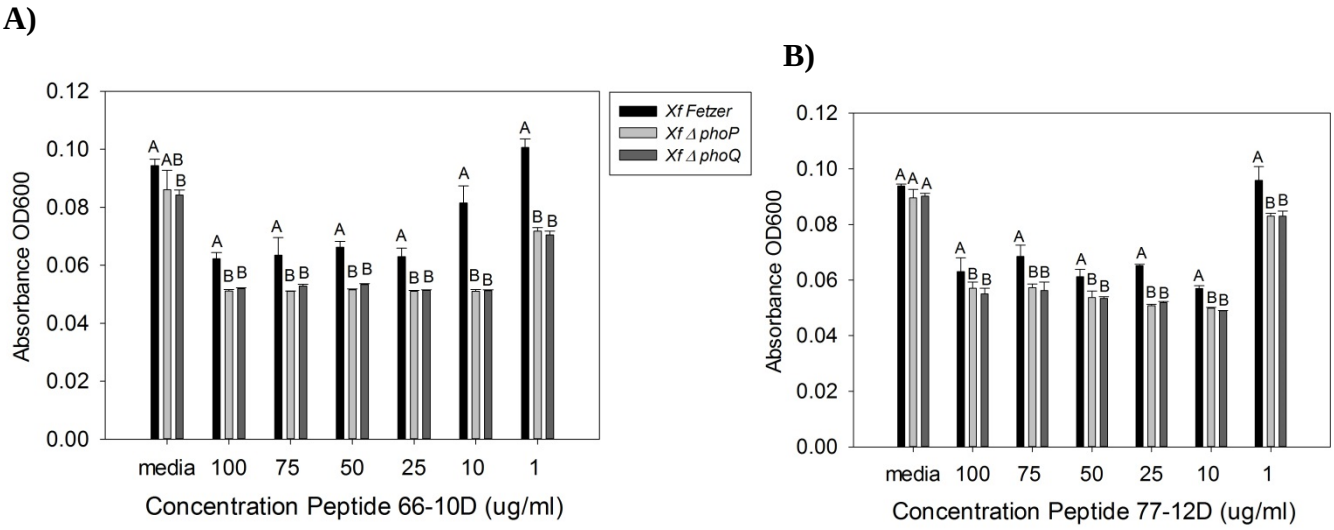


**Figure 3:** Absorbance OD600 of *Xf* Fetzer, *XfΔphoP* and *XfΔphoQ* cells grown for 5 days at 28° C with shaking at 100 rpm in Pim6 media containing 10 µM $Mg^{2+}$ and varying concentrations of A) Peptide 66-10D or B) Peptide 77-12D. Different letters indicate significance ($P < 0.05$) as determined by the Tukey test.

We also tested the effect of these peptides on *Xf* Fetzer when *Xf* was incubated in the presence of the peptide for one hour in Pim6 media containing 10 µM $Mg^{2+}$. After the incubation period, 20 µl aliquots were plated onto solid PD3 media and growth was evaluated after 7 days incubation at 28° C. We found that only peptide 77-12D was able to inhibit *Xf* growth after the one hour incubation period (Table 1).

**Table 1:** Evaluation of *Xf* Fetzer growth after 1 hour incubation with peptide 77-12D or 66-10D in Pim6 media containing 10 µM $Mg^{2+}$.

| Peptide concentration | 77-12D | 66-10D |
|---|---|---|
| **100 µg/ml** | No growth | Growth |
| **75 µg/ml** | No growth | Growth |
| **50 µg/ml** | No growth | Growth |
| **25 µg/ml** | No growth | Growth |
| **10 µg/ml** | No growth | Growth |
| **1 µg/ml** | Growth | Growth |

We are in the process of evaluating gene expression after *Xf* is incubated or grown in the presence of these peptides in order to determine whether these peptides are influencing phoP-mediated gene regulation. We are approaching this using qRT-PCR with primers for gene targets identified in objective two.

**Publications produced:**

Pierce, B. and B.C. Kirkpatrick. (2015). The PhoP/Q two-component regulatory system is essential for *Xylella fastidiosa* survival in *Vitis vinifera* grapevines. Physiol Mol Plant P. 89: 55-61.

**Research Relevance Statement:**

The research described in this report will provide relevant and significant information about the processes involved in *Xylella fastidiosa* adaptation to nutrient limited or harsh conditions such as the xylem sap. Understanding these processes will allow researchers to further identify targets for chemical and transgenic controls.

**Layperson Summary of Project Accomplishments:**

The goal of our project is to understand how the bacterial pathogen, *Xylella fastidiosa*, responds to the environment it lives in. In order to do this, we first determined how to properly mimic the response of the bacteria under laboratory conditions. This is important to insure we are evaluating the proper responses. After these conditions were determined, we prepared our samples for next generation sequencing, which will provide us with a large amount of information about all the process happening in the bacteria in that specific point in time. Using this information, we will pinpoint the process involved in adaptation and survival. We have also evaluated the ability of two small compounds (peptides) to inhibit the growth of *Xf*. We have identified one compound capable of inhibiting *Xf in vitro*.

**Status of Funds:**

**Summary and Status of Intellectual Property Associated with the Project:**

**References:**

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