Final Report for CDFA Contract Number 07-0324

Project title
Evolution of *Xylella fastidiosa* avirulence

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Project history

This research project, originally submitted as a 2-year proposal, was funded for 1 year (2007-08) with a budget cut of ~20% for that year. We requested a 1-year no cost extension, thus the 2007-09 period. The recommendation made by the reviewing committee was for us to use those funds to obtain evolved lineages of *X. fastidiosa*, and request additional funding for subsequent work.

Original objectives:
1. Generation of in vitro evolved populations
2. Phenotypical characterization of populations
3. Molecular characterization of populations
4. To test avirulent populations as PD biological control agents

We used the 1-year funds to partly complete 3 of the 4 original objectives (objectives 1 to 3). We did not perform research on Objective 4. Objective 1 was completed. We have addressed several components of Objectives 2 and 3, not others, and have incorporated new methods to characterize these populations. Some of these experiments are time consuming and are still ongoing; those will be included in a research manuscript.

Introduction

Hopkins (2005) showed that avirulent *X. fastidiosa* strains may be used to control Pierce's disease. However, not all weakly virulent or avirulent isolates tested resulted in disease control, and in many cases plants eventually become symptomatic (Hopkins 2005). Understanding the biology of avirulent isolates and the mechanisms behind biological method for disease control is of importance if this approach is to be widely adopted. This project addressed these questions by comparing evolved avirulent isolates with a parent pathogenic isolate. Retrospectively comparing these isolates using high resolution tools and biological assays allowed us to determine when *X. fastidiosa* loses avirulence. Previously, Hopkins (1985) demonstrated that lab-evolved isolates moved less and caused less disease in grapevines than ancestral isolates (Hopkins 1985). Similarly, pathogenicity of *X. fastidiosa* isolate 9a5c, which causes citrus variegated chlorosis in Brazil, declined within ~50 passages (de Souza et al. 2003). Here, we produced avirulent *Xylella fastidiosa* isolates *in vitro*, in order to compare them to the original

virulent strain and better understand the changes that occurred. We have obtained less virulent lineages of *X. fastidiosa* over a period of ~18 months (80 passages) and partially characterized some of those lineages.

Results and Discussion

Objective 1: Generation of *in vitro* evolved *X. fastidiosa* lineages

We used the Temecula isolate (ATCC 700964) for this work; this isolate was obtained as lyophilized material directly from ATCC. PWG was used for our experiments, it is a complex, non-defined, rich medium, with plates maintained at 28°C for a one-week incubation period. The design for the serial passages is summarized in the figure below. A population of cells was plated on PWG. One week later, we transferred cells to a new plate and every 10 passages preserved a sample in -80°C in 30% glycerol PW medium. The procedure was repeated once a week for 80 passages (1 ½ years). Ten parallel lineages were part of the study, with 8 frozen populations per lineage. For our studies, we recovered 4 randomly selected lineages and passages 0, 10, 20, 40 and 80 for phenotypic and molecular characterization studies.

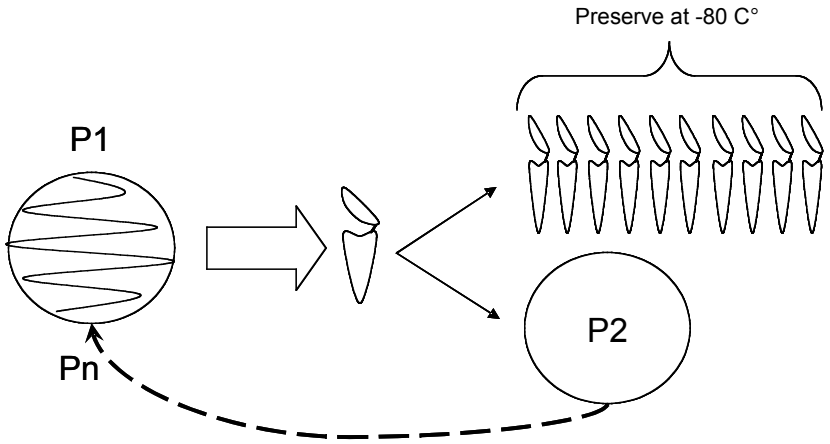


Figure 1: Method for *in vitro* passages and storage. After every tenth passage to new solid medium, we stored samples in a mixture of liquid PW broth and glycerol in the -80 freezer.

Objective 2: Phenotypic characterization of populations

Part I: *Xylella fastidiosa* infection rates *in planta*

We needle inoculated 8 replicates of each lineage/passage into almond plants. For negative controls we inoculated 8 plants with SCP buffer, 6 plants were kept in the greenhouse but not needle inoculated. We cultured from all 150 plants at 2 and 4 months after inoculation. For the culturing, 2 petioles were collected from each plant at about 15 cm above the inoculation site. We cultured according to a previously established method (Hill and Purcell 1995).

Ten to fourteen days after culturing, we checked to see which cultures were positive for *X. fastidiosa* infection. Combining the four lineages and the two culturing dates, for the initial passage, P0, 37.5% of the plants were positive. For P10, 13% were positive, for P20, 22%, for P40, 0% and for P80, 3% (Figure 2). The overall low infection rates may indicate that the initial isolate was not very pathogenic and did not multiply quickly within the almond plants. Additionally, we found some false negatives—plants that were positive in the first culturing but not the second—which can occur when *X. fastidiosa* is present in small populations and not thoroughly distributed within plants. In this case, it is possible that a larger number of plants were infected but the infections were not detected with the approach used. Regardless, our results show that the infection rates decline in later passages compared with the initial isolate and the earlier passages (Figure 2), indicating reduced *X. fastidiosa* virulence as the pathogen evolves *in vitro*. These results were similar to other previously published reports, indicating that *X. fastidiosa* consistently looses pathogenicity to plants if cultivated for long periods of time in rich medium. In addition, it provides evidence that the generation of avirulent isolates *in vitro* can be easily replicated.

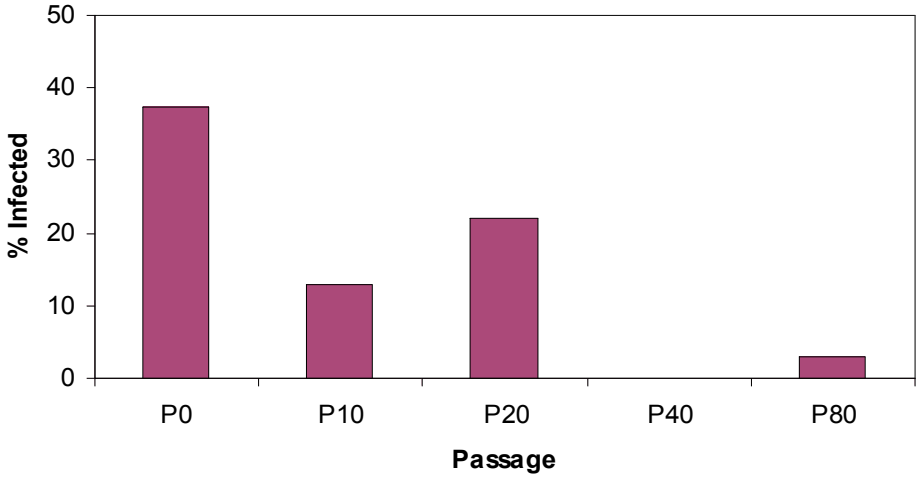


Figure 2: The percentage of positive results from cultures of inoculated plants, by passage number. Results show that sequential passages of *X. fastidiosa* in rich medium reduce pathogen virulence in plants.

Part II: Growth on medium

We noted that evolved lineages had phenotypic differences in relation to their ancestor based on its growth characteristics on PWG (Figure 3). The same phenotype was observed in all 10 lineages, suggesting parallel evolution among populations, and strong selective pressure in the conditions used. Although it is possible that evolved populations have a shorter doubling-time on PWG than ancestral populations, it is difficult to quantify that using solid media. Regardless, we have noted that colonies grow faster and bigger at passages P40 and P80. Two possible interpretations to those results are proposed. First, cells have a higher growth rate in P40 and P80 than <P20. Alternatively, gene expression is different. For example, more gum may be produced by P40 and P80 than earlier passages, although we have no data supporting either

hypotheses at this point. Lastly, the change in medium color may be a consequence of metabolic differences among passages. The medium is more basic in P40 and P80, as indicated by purple color. It is possible that *X. fastidiosa* is degrading molecules previously 'inaccessible' prior to sequential transfer. We hope to address this question in the future.

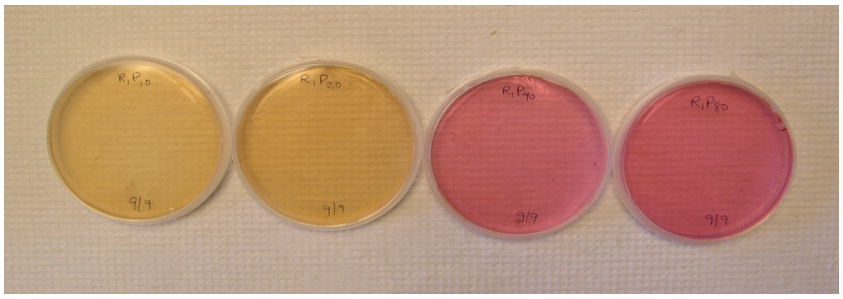
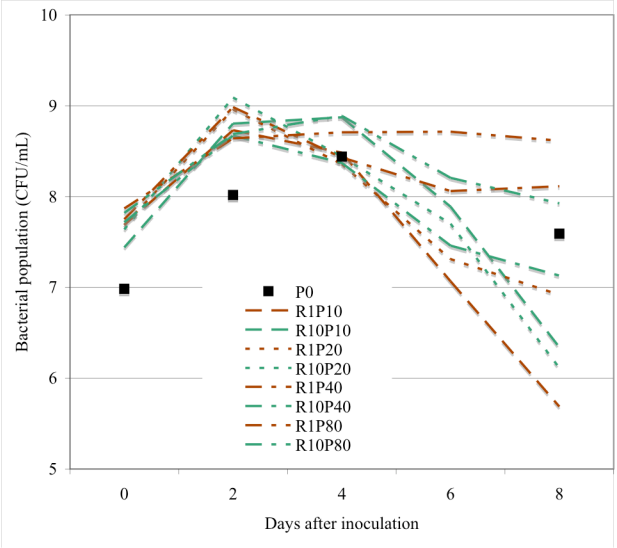


Figure 3: From left to right: P10, P20, P40 and P80 for lineage 1. Passages 40-80 seemed to grow faster on solid medium than earlier populations.

Using 2 of our 10 lineages, we studied the growth rate of these passages on liquid PW medium. We note that this is a different environment from PWG, and that may have affected some of the results obtained. Among other things, results were interesting as evolved populations had similar curves. First, the P0 population continued to increase in our experimental conditions up to day 4, while all other populations did not increase in size after day 2. Although it is possible that P0 grows slower, in the replicates done we initiated with a smaller population size for P0, and that may have affected our results.



Survival after day 4 on PW was similar for both lineages. Populations evolved the longest had higher survival rates on media after reaching stationary phase than early passages. Although these observations are similar to both lineages, suggesting phenotypic convergence, we are still investigating potential mechanisms behind these results. Lastly, we performed a biofilm formation test to quantify cell adhesion to surfaces. We used the protocol adapted by Meng et al. (2005), staining tissue culture tubes with crystal violet after bacterial growth *in vitro*. All evolved lineages adhered to surfaces less than the ancestral type (P0). Because *X. fastidiosa* adhesins (HxfA and HxfB) function as 'anti-virulence' surface proteins by slowing bacterial movement within plants (Guilhabert and Kirkpatrick 2005), we interpret our results as evidence of phenotypic change in our lineages, and that cell attachment to glass surfaces does not necessarily mimic cell adhesion to plant tissue. Otherwise, we would expect evolved lineages to be more pathogenic than P0. On the other hand, it is possible that adhesion is reduced but other pathogenicity pathways are also affected by the *in vitro* selective pressures.

Objective 3: Molecular characterization of populations

Short sequence repeats are tandem repetitive sections of DNA that can be used to distinguish differences between individuals or populations. We used 19 highly variable *X. fastidiosa* SSR loci identified by other researchers (Lin et al. 2005) and tested P0 and P80 for all four lineages tested biologically for pathogenicity. Those have been multiplex into 5 reactions using fluorescent-tagged primers for high-throughput population genetic analysis. This tool will be useful for studies on *X. fastidiosa* population genetic diversity and spatial/temporal distribution. One repeat deletion was found at a single locus for the P80 passage of lineage 10. The difference indicates genetic change, even in a section of DNA assumed to be under low selective pressure. We expect more significant changes in genes that are essential for growth *in vitro* on PWG. We sequenced several genes used in *X. fastidiosa*'s multi-locus sequence typing scheme (Nunney et al., UC Riverside), and found that none of the 8 genes part of that typing scheme had substitutions in any P80 population compared to P0.

We have optimized conditions for two-dimensional gel electrophoresis to compare the protein profile of P0 and other lineages *in vitro*. We still have to run those tests.

References cited

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Intellectual property issues

None we are aware of.

Pending publications

We are preparing a research manuscript that based on results obtained in this project. It will focus on the loss of *X. fastidiosa* pathogenicity by sequential in vitro transfers of this bacterium.

Contribution to Pierce's disease problem solving

The biological control of *X. fastidiosa* has gained interested after results based in Florida demonstrated that avirulent isolates reduced grapevine infection by pathogenic isolates under field conditions (Hopkins 2005). However, if this approach is to be developed into a successful Pierce's disease control strategy, it is important to understand how pathogenic isolates loose virulence, the mechanisms driving this process and the consequences to bacterial evolution. Using an approach similar to that of Hopkins, we showed that sequential transfer of *X. fastidiosa* on rich solid media resulted in avirulent isolates. In addition, phenotypic changes we observed in 10 lineages evolving in parallel showed that evolutionary changes under identical selective pressure (i.e. rich medium) were similar. Those results suggest that phenotypic (and likely genetic) changes in *X. fastidiosa* grown under the same conditions are similar, as has been shown for *Escherichia coli* and other bacteria. As such, it may be possible to consistently generate evolved lineages with the same phenotypic characteristics, which would be highly desirable for the development of a biological control strategy focused on avirulent isolates. In the future, it should be interesting to test *X. fastidiosa* lineages evolved under different conditions likely to result in loss of pathogenicity in search of a condition that generates populations capable of reducing Pierce's disease incidence. In summary, we showed that *X. fastidiosa* looses pathogenicity through serial passages on a rich medium *in vitro*, and that the 10 parallel lineages tested converged to similar phenotypes.