Induced Evolution of the Temperature-Dependent Activation of Virulence Determinants in the Phytopathogenic Bacteria *Pseudomonas syringae*

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Introduction

Global climate change has become a major concern and focus across all fields of science. The reason is that the rate of global change is unexpectantly fast and is shown to be caused primarily by human activity (Tett et al. 1999). Over the past hundred years, the Earth has already warmed approximately 0.6 ℃, the rate of global warming has nearly doubled since 1975, and continues to increase (Walther et al. 2002). One of the greatest concerns relating to climate change is how organisms and ecosystems will respond to such rapid change. The effects of rising temperatures have already been observed to impact the environment at all levels of biological organization, whether it is impacting a population's habitat, organismal development, or ecosystem dynamics (Walther et al. 2002). These changes further impact species and community interactions.

Interactions especially impacted by environmental changes are ones that have experienced years of coevolution and dependence (Vie et al. 2009). A prime example of this is pathogen-host interactions. The plant-pathogen relationship is considered to be one of the clearest examples of coevolution; as pathogens evolve to infect their host, plants counter through evolving resistance (Frank 1992). In the proposed study, the system of interest is the model phytopathogenic bacteria *Pseudomonas syringae.* This species of bacteria has been sequenced and studied extensively, making it a suitable system for this study.

Like most biotrophic bacteria, *Pseudomonas syringae* aims to keep the host alive, and stealthily use plant nutrients without being detected (Cunnac et al. 2009). However, it is not easy to avoid recognition, which is why *P. syringae* and many other bacterial species have developed a complex system called the Type III Secretion System (T3SS) (Jin and He 2001). When the bacteria senses contact with the host environment, a needle like structure called the pilus (seen in Figure 1) is developed and effector proteins are secreted into the host cell cytoplasm (Jin and He 2001). Here, their role is to manipulate cellular processes to block recognition sensing and signaling (Cunnac et al. 2009). If the bacteria can avoid being recognized, the plant is considered susceptible and the infection is successful.

Temperature fluctuations impact the success and timing of host invasions, which often occur through natural openings in the plants, such as stomata and hydathodes, or through damage and wounds (Jones and Dangl 2006). Bacterial colonies tend to mobilize and induce virulence determinants when they sense that infection will be most successful, one factor impacting this is temperature (Tang et al. 2006). It has been shown that phytopathogenic bacteria induce virulence factors between 16 and 24℃ and are repressed above 28℃. The researchers also found that immune responses in the host plants are activated between 10℃ and 32℃ (Cheng et al. 2014).

From a genetic perspective, sensing contact with the host initiates expression of the hypersensitive response and pathogenicity (*hrp*) gene cluster (Tang et al. 2006). These genes encode for several T3SS proteins and effectors. They are initiated when a sigma factor targets the *hrp*-box promoters that all these genes have in common (Tang et al. 2006). This allows for specific binding of RNA polymerase to these genes, resulting in transcription into mRNA and

then translation of pilus subunits and effector proteins (Tang et al. 2006). Once translated, the Type III Secretion System is assembled and effector proteins are secreted (Tang et al. 2006).

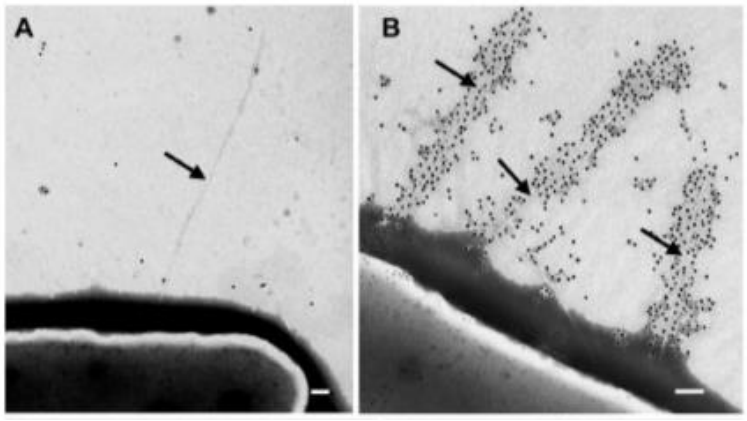


Figure 1. Showing photo of the hrp pilus from *Pseudomonas syringae* (Jin and He 2001). The long tube indicated by the arrow in 1A is the hpr pilus assembled with HrpA proteins. The dots seen in 1B are immunolabeled effector proteins secreted.
Photo from Jin and He 2001

The purpose of this study is to enhance our understanding of pathogen-host interactions as well as understand how this relationship will be affected by rising temperatures. This is critical for the use in agriculture, which is especially sensitive to environmental changes. Due to the nature of the practice, where crops experience dense cultivation and relatively low genetic diversity, pathogen outbreaks are notably detrimental to crop yield and food security (Savary et al. 2012). In fact, crop loss due to pathogens has been estimated to be between 20 and 40% of global productivity (Savary et al. 2012). As our world is inevitably changing, human practices must adjust to accommodate these changes. However, to make the necessary adjustments, the anticipated changes must first be understood. In order to predict how plant-pathogen interactions will shift, this study will use artificial evolution to characterize how phytopathogenic bacteria may evolve with rising temperatures.

Objectives

The specific questions the proposed study aims to answer are: What are some of the genetic modifications leading to the induced secretion of virulence determinants at higher temperatures in the phytopathogenic bacteria *Pseudomonas syringae*? Approximately how many generations of bacteria will it take to gain the ability to infect the host at higher temperatures?

The objectives of this study are to induce the evolution of *P. syringae* in order to find possible genes involved in the temperature-sensitive induction of virulence determinants and to evaluate the number of generations it takes to evolve these adaptations.

To approach this, the researchers on this project will use the model plant-pathogen system *Pseudomonas syringae* on *Arabidopsis thaliana*, a small model plant related to mustard. The phytobacteria will experience both *in vitro* and *in vivo* conditions with temperatures above the normal range for induction of virulence determinants. The researchers will monitor the bacteria until they have evolved to be able to induce virulence factors at the higher temperature. The genes involved with this adaptation will then be identified.

The proposed project will test the following hypotheses:

1. The time it takes to evolve the ability to induce virulence factors at higher temperatures will be shorter when exposed to the host environment rather than

the laboratory environment because there is natural selection driving this adaptation.

2. The mutations determining the phenotype (able to induce virulence factors at higher temperatures) will be in the regulation and activity of sigma factors initiating *hpr* gene expression.

Research Approach

Project 1 – Induced Evolution of Bacteria in vivo

Step A: Plant and bacterial growth and preparation

We plan to grow the *A. thaliana* accession Columbia plants in growth chambers for four weeks before inoculation. The conditions are as follows: 23°C, 60% humidity, and 75 $\mu E\ m^{-2}s^{-1}$ light intensity with a 12-hour photoperiod, modeled after the growth conditions used by Cheng et al. 2014. All of the plants will be genetically identical in order to eliminate any differences in bacterial infection due to variation in plant resistance.

We will also grow some plants to use as controls for our experiments; these plants will be grown in the same conditions, but lack inoculation of bacteria. The control plants will be moved to a growth chamber the same day the experimental plants are and held at the same temperature. This will be used to compare the plants response to the presence and absence of bacteria and control for the effect temperature is having on the plants.

The bacteria will be grown in culture overnight before inoculation. This procedure will be modeled after the experiments performed by Katagiri et al. 2002 They will be incubated at 28°C on Kings medium B at pH 7 or slightly lower. The bacteria will also be genetically identical in order to compare rates of evolution. Once grown, the bacteria will then be suspended in buffer, washed, and diluted to a certain concentration. The prepared bacterial suspension will be 0.2 mol concentration of bacteria with 10mM $MgCl_2$ suspension and small amount of silwet surfactant.

Step B: Inoculation

Fifteen minutes before inoculation, the plants will be moved to a new growth chamber set to higher temperatures, so that the plants will be at the experimental temperature at the time of inoculation (Cheng et al. 2014). The temperatures tested will include 23°C, 30°C, and 34°C. The chamber set to 23°C will be the control to see the unaltered bacterial infection. The chamber with 30°C will show the bacterial infection outside of the range where virulence factors are induced, yet PTI in plants is active. Lastly, 34°C will be tested to examine the bacterial infection outside of the range where virulence factors are induced, but also outside of the range where plant resistance is activated.

In each of the three chambers, there will be 10 plants total. There will be 2 control plants; one will have no inoculation and the other will be inoculated with only the 10 mM $MgCl_2$ suspension solution. There will also be 8 plants inoculated with the bacteria, and possibly more replicates if needed.

After 15 minutes of the plants incubating in the new growth chambers, they will be inoculated with the bacterial suspension. Plants will be inoculated by dipping the leaves into the bacterial suspension for 30 seconds each (Katagiri et al. 2002). This will allow the bacteria to be placed along the surface and move into the stomata for infection. All of the plants will then be left in these chambers and the levels of infection will be monitored.

Step C: Evaluation of infection

The amount of infection on each plant will be monitored throughout the experiment. I expect that the infection will be the worst at 23°C because this is optimal for bacterial infection. I

also expect that the infection at 30℃ will be the least prominent because there will be little bacterial induced virulence factors coupled with plant resistance. The main experimental interactions will be in the chamber set to 34℃, where the bacterial evolution will be most clearly shown, given that infection is observed.

After inoculation, the bacteria will be allowed to grow on the plants and the plants will be checked on at time intervals: 1 hour, 3 hours, 6 hours, 9 hours, and every 12 hours after that. These time check points may be modified as needed. I will monitor the bacteria as they grow, reproduce, and evolve for long enough to see infection on the plant. Once infection is apparent, the next steps will be taken: examining the rate of evolution.

The amount of infection each population of bacteria cause will be calculated by percent of leaf area covered with brown bacterial spotting (Katagiri et al. 2002). This data will be recorded for later use during the comparative genomics project.

Step D: Estimate generation time and rates of bacterial evolution

Once infection is observed, I will calculate roughly the number of generations it took for the bacteria to evolve virulence at the higher temperature, based on the average doubling time of *P. syringae* at the set temperatures and conditions.

Project 2 – Induced Evolution of Bacteria in vitro

Step A: Design and engineering of HrpA-GPF fused protein

In order to visualize the bacterial induction of virulence factors, a major structural protein of the pilus, known as HrpA (seen in Figure 1), will be fused to a green fluorescence protein (GFP). This construct will be developed using the protocols performed by Boureau et al. 2002. When exposed to blue through ultraviolet light, the GFP protein gives off a bright green fluorescence. This will allow the researchers to clearly see when there has been induction of virulence factors in the medium because a green fluorescence will be seen. HrpA proteins are only expressed and present during infection when conditions are favorable for induction of virulence factors. HrpA has been chosen over other hrp-proteins because its presence is not size dependent (Jin and He et al. 2001). Some effector proteins secreted through the pilus may be too large to move through the pilus if fused to GFP. CRISPR-Cas9 will be used to facilitate the integration of this gene into the bacterial genome at the site where it would be fused to HrpA. Multiple control trials will be run to make sure that this GFP construct does not disrupt the activation or function of the HrpA protein.

Step B: Bacteria growth and medium

These experiments will begin with genetically identical bacteria so the rates of evolution can be compared. The bacteria will be grown on plates with the *hrp*-inducing medium. The solid minimal medium used for this experiment is M9 supplemented with 5mM mannitol, modeled after the agar used by Roine et al. 1997. The bacteria plates will be incubated at the same temperatures the *in vivo* experimental conditions: 23℃, 30℃, and 34℃. There will be 9 plates at each temperature. There will be one control plate with wild type bacteria lacking the GFP fused protein as well as 8 plates with the HrpA-GFP protein.

Step C: Presence of virulence determinants

The presence of HrpA-GFP will be monitored throughout the experiment. The bacteria will be allowed to grow on the plates and will be examined at varying time intervals by examining the plates under blue light, not to damage the bacteria. This will take place at 1 hour, 3 hours, 6 hours, 12 hours, and every 24 hours after that. These time check points may be modified as needed. I will continue monitoring the bacteria for long enough to visualize the expression of *hrp* genes through the presence of HrpA-GFP. The presence of HrpA-GFP is

evidence that the bacteria have induced virulence determinants and have evolved the ability to induce virulence factors under high temperature conditions. Once infection is apparent, observed as dark brown spotting, the researchers will go on to examine the rate of evolution.

Step D: Estimate generation time and rates of bacterial evolution

Similar to the *in vivo* experiment, the number of generations it took to evolve the ability to induce virulence factors in high temperature conditions will be estimated using the doubling time of the bacteria under certain temperatures, but otherwise optimal conditions. I expect that it will take longer for the *in vitro* experimental bacteria to develop the ability to induce virulence factors at high temperatures than it would for the *in vivo* experimental bacteria. This is because there are higher selective pressures for the bacteria surviving on the host.

Project 3 – Mapping of Possible Genes of Interest

To identify possible genes contributing to the differences in temperature dependent induction of virulence determinants, we will sequence the artificially evolved population of bacteria and compare it to the original. I will then identify the genetic modifications occurring across the genome using bioinformatic technology to align the sequences. I will cross-reference this data with the annotated sequences available online to identify modifications in genes possibly involved in creating this phenotype. I expect that the mutations leading to the adaptation of interest will be involved with the activity of the sigma factors initiating transcription of the *hrp* genes, however will examine modifications within genes known to be involved in virulence as well as undefined genes with characteristic sequences. These mutations from certain populations will be compared and examined for correlation with the strength of the infection, recorded as percent of leaf surface covered with brown spotting. If given the time, I will follow with performing mutagenesis on the following genes to test and confirm for their roles in virulence.

Intellectual Merit

The research proposed will broaden our understanding of pathogen-host relationships as a temperature-sensitive system. The coevolution between plants and pathogens has been studied extensively, however the effects of a changing environment on these dynamics needs further research. The proposed research will address this next step by determining how the interacting species will coevolve with each other as well as with rising temperatures. The experiments are designed to evaluate which species has the advantage before adaptation occurs and examine the response strategies each of these species exhibit, along with how effective these strategies will be. This work will add to what we already know regarding the plant-pathogen relationship by examining the coevolution along with a new layer of a changing environment.

Broader Impacts

The experiments described has not only scientific value, but also great economic value. The results from this research will be directly applicable to agriculture in understanding how to better protect crops in order to increase yield. The agriculture industry is always working towards increasing productivity of crops and this research will help understand how to increase crop yields in the future. Currently, potential agricultural production is reduced 20-40% worldwide due to pathogen and pests. To put this into perspective, the economic value of crops lost due to wheat rust alone values at around $5 billion USD per year (Savary 2012). This problem will likely become more detrimental in the near future due to global change. The findings of this research will help combat crop loss due to pathogens and better understand and predict how plant-microbe interactions will change in the future.

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