The Characterization of Growth Curves and Evolutionary Trajectories in Hosts of *Xylella fastidiosa*

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ABSTRACT

Xylella fastidiosa is a gram-negative and non-flagellated bacterium that is detrimental to a variety of plants. This plant pathogen lives in the xylem vessel where nutrients are transported throughout the plant host and is transmitted via insect vectors that feed on xylem fluid. Since *Xylella fastidiosa* is the first plant pathogen with a fully sequenced genome, there has been incredible progress in studying the process by which it spreads. It was discovered that different phylogenetic groups of *Xylella fastidiosa* have similar transmission mechanisms. In insects, the pathogenicity mechanisms in plants are not yet understood. Thus, this study aims to observe the growth patterns and adaptations of *Xylella fastidiosa* towards characteristics that broadly mimic an insect host or a plant host by the use of the presence of polysaccharides in culture media. This will require cell culturing on various types of media and determining the extent of growth by observing and creating growth curves over a period of seven days through the presence and concentration of cells measured using a spectrophotometer.

KEYWORDS

pathogen, evolution, chitin, pectin, optical density

INTRODUCTION

Xylella fastidiosa is a gram-negative and non flagellated pathogenic bacterium that is associated with disease in a wide variety of plants and crops including almonds, grapevines, etc (Chatterjee et al. 2008). It colonizes xylem vessels and is transmitted through the mouths of insect vectors that feed on xylem sap (Chatterjee et al. 2008). This study aims to observe the growth curves and evolutionary trajectories of *Xylella fastidiosa* towards characteristics that allow for optimal fitness in environments that mimic an insect host or a plant host. To observe these phenotypes, I will culture a strain of *Xylella fastidiosa*, NAPA1, on six variations of XFM, scrape cell growth off of each media type, and measure the optical density in order to determine the concentration of cells.

A defined medium, *Xylella fastidiosa* medium (XFM) containing a single carbon source and no preformed amino acids has been currently identified as an ideal media to grow *Xylella fastidiosa* (Silva et al. 2017) for this assay. This study aims to observe the growth of *Xylella fastidiosa* on 6 versions of XFM: XFM, XFM with the addition of chitin, XFM with the addition of galacturonic acid, XFM Δ, XFM Δ chitin, and XFM Δ galacturonic acid. The purpose of XFM with the addition of chitin is to mimic insects that contain chitin exoskeletons as insect vectors of this specific bacterium including sharpshooters and spittlebugs have a chitin exterior. The purpose of XFM with the addition of galacturonic acid is to mimic plants. Units of galacturonic acid make up pectin, a starch found within the cell walls of terrestrial plants. Delta indicates that there is a lack of carbon sources, so disodium succinate and trisodium nitrate have been removed from the media. The XFM media will serve as the control, and the XFMΔ will serve as the negative control due to the lack of a carbon source. Other than XFM, there is no defined recipe for the media. Thus, the first step is to test the growth of *Xylella fastidiosa* on XFMΔ. If there is growth, the recipe must be altered to completely eradicate the carbon source.

In order to observe the growth of *Xylella fastidiosa* on each of the 6 media, I will culture the pathogen over a specific period of time and analyze DNA levels through PCR. This will enable me to create a growth curve graph for each of the six media types. Growth curves are a type of mathematical function that describes increases and decreases in the number of living cells over time (Zwietering et al. 1990). There are four distinct phases of a bacterial growth curve: lag, exponential, stationary, and death. The lag phase is the period of time in which the bacteria are

metabolically active but not dividing. The exponential phase is a time of exponential growth in the number of living cells. During the stationary phase, growth plateaus as the number of dying cells equals the number of dividing cells. Lastly, during the death phase, the number of living cells exponentially decreases (Zwietering et al. 1990). Through the data from the growth curves, I can look for adaptations on the strains of *Xylella fastidiosa* grown on the various media types, and I will be able answer my research question of whether there are any observable growth patterns of the plant pathogen *Xylella fastidiosa* in a plant host versus an insect vector environment. I will also perhaps even be able to predict future growth in various environments.

METHODS

Culturing the pathogen

In order to test the growth of my pathogen, *Xylella fastidiosa,* on various media types, I had to begin by growing the pathogen on a standard medium known as PD3. I prepared this media a week before culturing the pathogen using my lab's standard recipe, clean glassware, an autoclave, and a fume hood in order to make sure my media was sterile and to prevent the growth of anything other than the pathogen. In order to culture the pathogen, I retrieved glycerol stocks with the pathogen inside from the -80℃ freezer. Once the Napa1 glycerol stocks had come to room temperature, I used a pipette to place 3-20 microliter streaks on each plate in the fume hood and repeated this procedure three times. After the plates had dried and wrapped up using parafilm, I incubated the plates for 3-5 days at 28℃ and waited for cell growth.

Creating my media types

While waiting for cell growth of *Xylella fastidiosa* on PD3, I made one liter of each of the six media types that I would be testing in my study. The media types include six versions of XFM (*Xylella fastidiosa* medium): XFM, XFM with the addition of chitin, XFM with the addition of galacturonic acid, XFM Δ, XFM Δ chitin, and XFM Δ galacturonic acid. To create the media, I followed my lab's standard procedure for creating XFM. For the chitin media, the only change was the addition of 10 milliliters of colloidal chitin to the solution that was vacuum

filtered. For the galacturonic acid media, the change was the addition of 0.1 grams of galacturonic acid to the solution that was vacuum filtered. Delta indicates that there is a lack of a carbon source, so there is a lack of disodium succinate and trisodium nitrate added to the media. Each of these media types was autoclaved and poured onto plates in the fume hood (50 plates per liter).

Conducting a control trial

In order to establish a control, I conducted a trial comparing growth on PD3 and XFM. XFM media tends to be hard to work with, and cells do not grow very well. Thus, it was important to run a trial on a more standard media (PD3) as a contrast to ensure that the process worked and that the optical density would be an acceptable way to measure cell growth.

To plate *Xylella fastidiosa* on XFM and PD3, I scraped the cells in the fume hood from one of the previous PD3 plates that I had grown the pathogen on. I placed the cells that I had scraped into a tube containing SCP buffer (which is a basic phosphate buffer used in many procedures), making sure to flame sterilize my loop everytime I placed it in the tube containing SCP. During this process, I continually checked the absorbance level of the tube using a spectrophotometer until the optical density reached 0.2. The optical density was utilized as a determinant of cell concentration. In order to use the spectrophotometer, I pipetted 70uL of SCP into a cuvette to use as a blank and then pipetted 70 uL of the cell solution in a cuvette to measure the absorbance. Once the absorbance reached 0.2, my cell-SCP solution was ready to be plated.

Before proceeding with plating cells, I prepared my plates in the fume hood. I utilized 7 plates of each of the two media types for a total of 14 plates. I labeled each plate in the fume hood with media type, days 1-7 to indicate cell scraping schedules, and the date. Once I labeled and organized my plates, I used a pipette to place 3-10 microliter beads of cell-SCP solution onto each of the two media types in the fume hood. I used beads instead of streaks to better standardized growth and minimize the confounding variables that streaks cause. Once the beads were dry in the fume hood, I wrapped them using parafilm and placed them in the incubator at 28℃.

In order to find growth levels, I scraped the cells from each media type in the fume hood every day for seven days. Before scraping the plates, I prepared and labeled autoclaved tubes filled with 250 microliters of SCP for each plate. Once everything was labeled, I turned on the spectrophotometer, as it needed a minimum of 30 minutes to heat up. I used a loop to scrape all the cells from each plate in the fume hood and placed the cells in their corresponding tubes. I made sure to flame sterilize my loop each time it went in a tube. Each day for 7 days, I scraped cells from 2 plates of each media type (2 plates total each day) into 250 microliters of SCP. I then made sure that the cell-SCP solution was homogenous by mixing by pipetting for a minimum of 40 times. I then measured the optical density by pipetting 70 microliters of cell-SCP solution into a UV cuvette and placed the cuvette into the spectrophotometer. I recorded each optical density value every day.

Re-culturing the pathogen

To plate *Xylella fastidiosa* on each of the six media types, I scraped the cells in the fume hood from one of the previous PD3 plates that I had grown the pathogen on. I placed the cells that I had scraped into a tube containing SCP buffer, making sure to flame sterilize my loop everytime I placed it in the tube containing SCP. During this process, I continually checked the absorbance level of the tube using a spectrophotometer until the optical density reached 0.2. In order to use the spectrophotometer, I pipetted 70uL of SCP into a cuvette to use as a blank and then pipetted 70 uL of the cell solution in a cuvette to measure the absorbance. Once the absorbance reached 0.2, my cell-SCP solution was ready to be plated.

Before proceeding with plating cells, I prepared my plates in the fume hood. I utilized 7 plates of each of the six media types for a total of 42 plates and performed two trials of this for a total of 84 plates. I labeled each plate in the fume hood with media type, day, and the date. Once I labeled and organized my plates, I used a pipette to place 3-10 microliter beads of cell-SCP solution onto each of the six media types in the fume hood. Once the beads were dry in the fume hood, I wrapped them using parafilm and placed them in the incubator at 28℃.

Optical density to obtain cell concentrations

In order to find growth levels, I scraped the cells from each media type in the fume hood every day for seven days. Before scraping the plates, I prepared and labeled autoclaved tubes filled with 250 microliters of SCP for each plate. Once everything was labeled, I turned on the spectrophotometer. I used a loop to scrape all the cells from each plate in the fume hood and placed the cells in their corresponding tubes. I made sure to flame sterilize my loop each time it went in a tube. Each day for 7 days, I scraped cells from 2 plates of each media type (12 plates total) into 250 microliters of SCP which I then mixed by pipetting. I measured the optical density and recorded each value every day.

Preparing the growth curves

In order to create my growth curves, I utilized the optical density data that I recorded each day. I inputted and formatted the data onto a data table in Microsoft Excel that could then be converted into a graph. When creating my graphs on Excel, I utilized time in days for my x-axis and optical density from the spectrophotometer analysis as the y-axis. I then plotted it as a line graph and made each media type a different colored line on the growth curve. I created a total of two growth curves, one from each trial.

RESULTS

Establishing a Control:

In order to establish a control, I conducted a trial comparing growth on PD3 and XFM. XFM media is hard to work with and cells don't grow as well so I ran a trial on a more standard PD3 media as a contrast to test whether the process worked and if taking optical density would be an acceptable way to measure growth. A spectrophotometer was used to take the optical density of each tube of cell-SCP solution for each media type over the course of seven days. The optical density was crucial for measuring the concentration of cells in each solution. My results in Table 1 indicated that over the course of seven days, both media types displayed an increase in the concentration of cell growth. PD3 tended to have more growth with its peak being an optical density of 1.325. Similarly, XFM had an increase in growth but peaked at a lesser value of 1.148.

Table 1. Growth of *Xylella fastidiosa* on XFM versus PD3 displayed through optical density to establish a control

	Day			
	2	**4**	**5**	**7**
PD3 Optical Density	0.0069	1.095	1.215	1.325
XFM Optical Density	0	0.6	0.717	1.148

In order to determine whether the two media types displayed a similar trajectory in growth over time, I created a growth curve (Figure 1). While the XFM had a lower concentration of cell growth over seven days, both XFM and PD3 had growth trajectories that appeared similar with a peak growth at day 7. As a result, I was able to confirm that XFM would be an acceptable medium to conduct my study with and serve as my positive control.

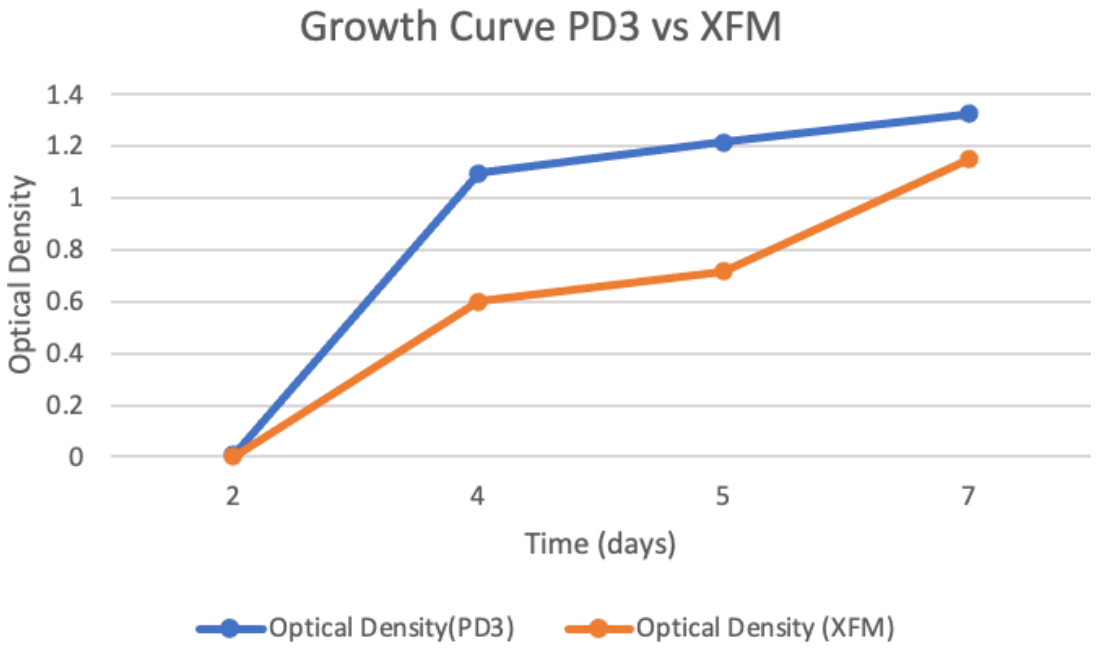

Figure 1. Growth Curve PD3 versus XFM Growth of *Xylella fastidiosa* on XFM versus PD3 displayed on a graph in order to establish a control

Optical Densities from Trials 1 and 2:

After seven days of measuring the optical density of cells for each media type, my results indicated that over the course of seven days, most of the media types in both trials except XFM chitin △ experienced some sort of growth even if it was very minimal. XFM had much more growth than any of the other five media types, with a peak at 0.915 on day 7 (Table 2a). XFM △, XFM galacturonic acid △, XFM chitin, and XFM galacturonic acid all had a similar amount of growth with peaks of 0.303, 0.225, 0.488, and 0.513 on day 7 (Table 2a). XFM chitin △ remained at values less than 0.1, which can be considered as zero growth. Trial 2 displayed an almost equivalent trend as the previous trial, however the growth was greater on day 7 for every media type (Table 2b). Again, XFM had much more growth than any of the other five media types, with a peak at 1.065 on day 7 (Table 2b). XFM △, XFM galacturonic acid △, XFM chitin, and XFM galacturonic acid all had a similar amount of growth with peaks of 0.417, 0.273, 0.672, and 0.563 on day 7 (Table 2b). XFM chitin △ reached a value of 0.14 at day 5, but quickly decreased to 0.074 soon after on day 7 (Table 2b).

Table 2a. Optical density of *Xylella fastidiosa* on 6 media types

	Day			
	2	**4**	**5**	**7**
XFM	0.17	0.371	0.578	0.915
XFM △	0.083	0.123	0.219	0.303
XFM galacturonic acid	0.046	0.297	0.408	0.513
XFM galacturonic acid △	0.079	0.377	0.154	0.225
XFM chitin	0.08	0.28	0.335	0.488
XFM chitin △	0.007	0.002	0.066	0.058

Table 2b. Optical density of *Xylella fastidiosa* on 6 media types for a second trial

	Day			
	2	**4**	**5**	**7**
XFM	0.213	0.476	0.608	1.065
XFM △	0.086	0.14	0.38	0.417

XFM galacturonic acid	0.065	0.308	0.412	0.563
XFM galacturonic acid △	0.102	0.386	0.18	0.273
XFM chitin	0.096	0.308	0.485	0.672
XFM chitin △	0.009	0.035	0.14	0.074

To better understand the growth trends of each media type over time, I created a growth curve for each of the two trials (Figure 2a and 2b). The graphs show that there is higher growth in XFM than XFM △. Similarly, I found that there is more growth on XFM than XFM
galacturonic acid and XFM chitin. Within the delta variants, there is also more growth on XFM △ than XFM chitin △ and XFM galacturonic acid △. Thus, I saw a pattern in both XFM and XFM △ where the addition of chitin or galacturonic acid is decreasing total growth rather than increasing it like I had hypothesized.

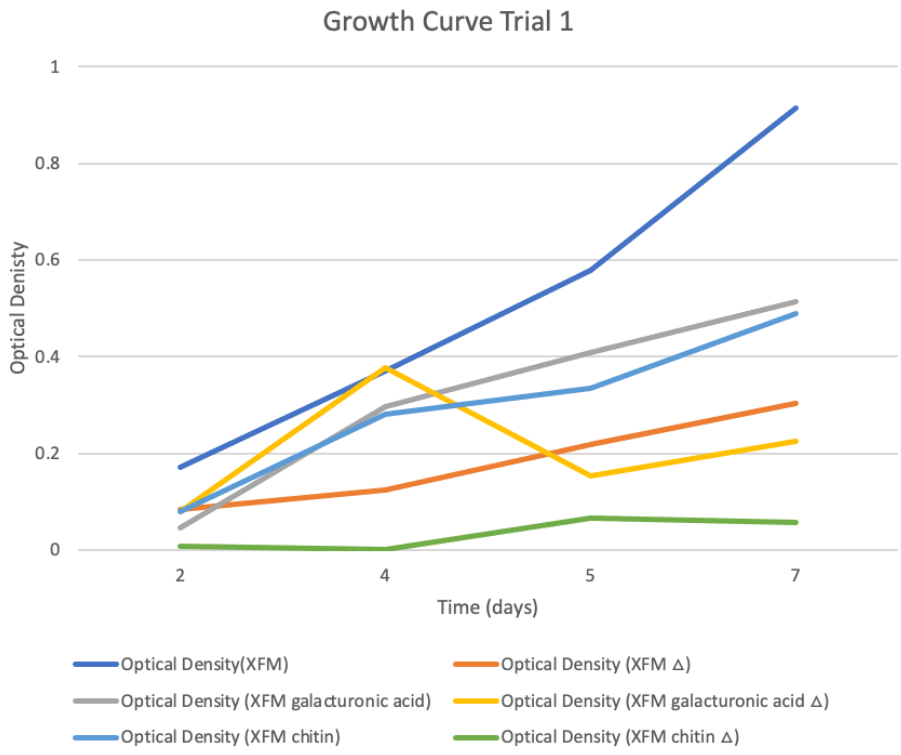

Figure 2a. Growth Curve Trial 1. Trends of *Xylella fastidiosa* growth on 6 variations of XFM

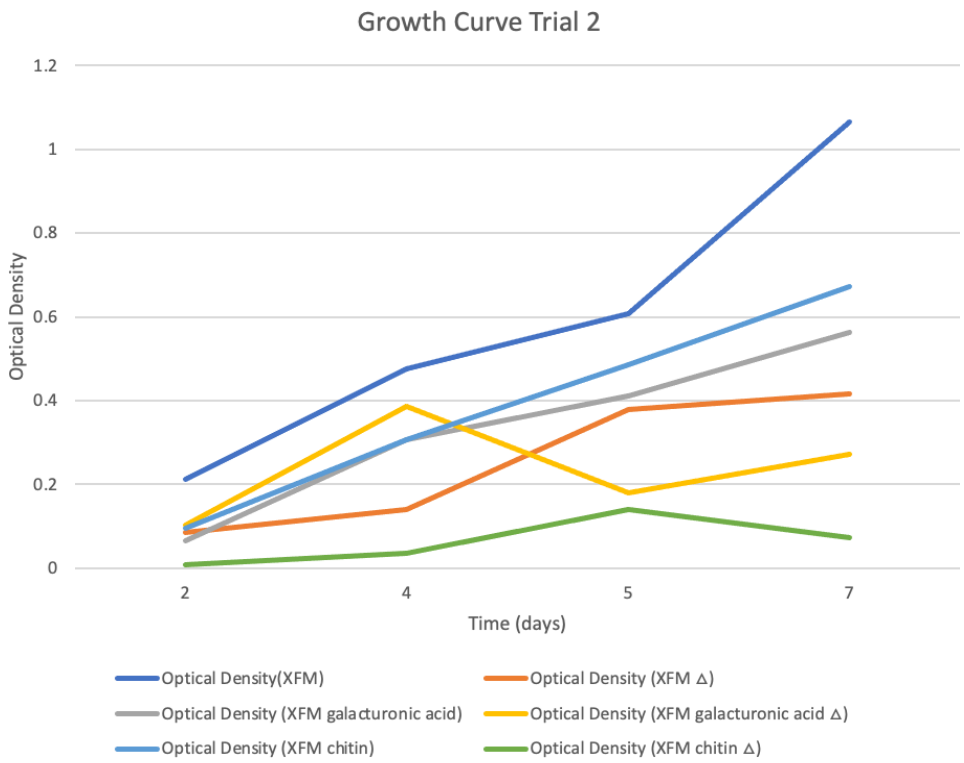

Figure 2b. Growth Curve Trial 2. Trends of *Xylella fastidiosa* growth on 6 variations of XFM during the second trial

DISCUSSION

Xylella fastidiosa colonizes both insect vectors as well as plant hosts (Lopes et al. 2009). Previous studies demonstrated an awareness that *Xylella fastidiosa* has the potential to degrade pectin contained in plant hosts and chitin within an insect vector, however, there is a lack of evidence supporting the claim that these carbohydrates are able to be utilized as carbon sources by the pathogen. A study conducted in 2010 revealed that *Xylella fastidiosa* degraded chitin when grown on an agar medium containing chitin which suggested that there was a possibility that the bacterium may use the chitin as a carbon source. Further research, however, needed to be pursued in order to determine whether the bacterium was able to grow solely on chitin lacking any other carbon source. Similarly, the pathogen is xylem-limited and interacts with plant polysaccharides such as pectin which it can then potentially also utilize as a carbon source (CABI n.d.). Our understanding of the ways in which *Xylella fastidiosa* interacts with insect vectors and plant hosts as a carbon source is significantly lagging and thus, my study aims to

reveal more about it through the creation of growth curves in various environments. When a broth culture is inoculated with bacteria, the population size of the bacteria increases showing a classical pattern. When plotted on a graph, a distinct curve is obtained referred to as the bacterial growth curve. These growth curves revealed how *Xylella fastidiosa* has evolved to incorporate the utilization of chitin and pectin as carbon sources.

Establishing a control

In order to establish a control, I conducted a trial comparing growth on PD3 and XFM. XFM media tends to be hard to work with, and cells do not grow very well. Thus, it was important to run a trial on a more standard media (PD3) as a contrast to ensure that the process worked and that the optical density would be an acceptable way to measure cell growth. Usually, this process would be conducted in liquid media, however XFM cannot be used in a liquid form due to the need for microfluidic chambers (Cobine et al. 2013). My findings indicated that both PD3 and XFM had a very similar growth curve, with PD3 expectedly having slightly more growth. These findings enabled me to continue with my study, establishing XFM as my positive control.

***Xylella fastidiosa* growth on XFM variations**

My initial hypotheses were that the three XFM variations would have a similar amount of growth, if not increased growth, with the addition of a supplemental nutrient. XFM Δ would have no growth as the main carbon sources, disodium succinate and trisodium citrate, were eliminated. These hypotheses were not supported, however, as we found that the addition of chitin and galacturonic acid decreased growth instead of increasing it. This finding indicates that chitin and galacturonic acid are not being used as a nutrient source as other organic acids were also available in the medium (Killiny and Almeida 2009). This finding suggests that there could potentially be something about chitin or galacturonic acid that is toxic to cells and is inhibiting growth in some way. Similarly, this could also mean that perhaps the pathogen isn't feeding on these substances, but it may be feeding on something that is inhibiting growth which could have further implications in the study of this pathogen (Killiny and Almeida 2009).

In a medium with chitin or galacturonic acid as the sole carbon source, XFM chitin Δ and XFM galacturonic acid Δ, *Xylella fastidiosa* grew to decreased optical densities compared to XFM chitin and XFM galacturonic acid, which has two other organic acids as carbon sources. Together with other evidence presented, my findings indicate that chitin and galacturonic acid exploitation as a carbon source while colonizing insect vectors and plant hosts seems unlikely in *Xylella fastidiosa* (Killiny et al. 2009).

In a medium lacking a carbon source (due to disodium succinate and trisodium citrate being eliminated), XFM Δ, we found an increased amount of growth compared to XFM chitin Δ and XFM galacturonic acid Δ. This suggests that there was some chemical in the medium that was still contributing conditions to sustain bacterial life. The media used included glutamine and asparagine, which are both of paramount importance for cellular metabolism and can sometimes serve as an alternative nitrogen and carbon source for the cell (Kirsch et al. 2022). While the experiments I conducted did not test for this hypothesis, the potential for other unintended amino acids to serve as a carbon source is a vital consideration when recreating these media variations during another trial of the study.

Growth on the negative control, XFM Δ

One factor that my study did not account for was growth on XFM Δ which was initially intended to act as my negative control. As stated previously, this can be attributed to amino acids incorporated into the media that, under certain conditions, can act as a carbon source (Killiny and Almeida 2009). This means, however, that we cannot answer whether *Xylella fastidiosa* can grow only on chitin versus galacturonic acid since XFM Δ was supposed to act as my negative control. During a future trial of this study, a negative control lacking any sort of additives to sustain life should be used. This could be, for example, a plate containing only agar in which *Xylella fastidiosa* would lack the proper nutrients to grow.

Limitations with an early procedure

During the first trial ever conducted, I measured cell growth by taking the cells I scraped and proceeding with a DNA extraction and subsequently qPCR to obtain a Ct value. The Ct

(cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (Bell et al. 2020). Ct levels are inversely proportional to the amount of target nucleic acid in the sample, which means the lower the Ct level, the greater the amount of target nuclei in the sample (Bell et al. 2020). My results displayed an increase in Ct value over time which was illogical considering that at least my positive control, XFM, should have shown a decreased Ct level over time to indicate growth. I suspect that with the accumulation of various procedures, samples were contaminated which inclined me towards the decision of adhering to the simple yet effective method of measuring the optical density.

Broader implications and future research

Although my hypotheses were not supported by the data, I learned about *Xylella fastidiosa* and the patterns with which it grows as well as refined my methods. Through a culmination of various trials including failed attempts at qPCR, using liquid media, and attempting to measure biofilm, I learned there is a lot more to the mechanisms by which *Xylella fastidisoa* functions than we initially thought. I am excited to reveal more about the pathogen and to understand the evolution of genes related to chitin and pectin. Just in California itself, there are countless strains of *Xylella fastidiosa* (Hernandez-Martinez et al n.d.). As a result, my future research will be looking at genes involved in the chitin degradation pathway as well as the pectin degradation pathway in order to determine whether there is evidence of evolution within the genes in different strains.

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