PHENOTYPIC CHARACTERIZATION AND SEQUENCE ANALYSIS OF *pthA* HOMOLOGS FROM FIVE PATHOGENIC VARIANT GROUPS OF *Xanthomonas citri*

By

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PHENOTYPIC CHARACTERIZATION AND SEQUENCE ANALYSIS OF *pthA* HOMOLOGS FROM FIVE PATHOGENIC VARIANT GROUPS OF *Xanthomonas citri*

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Citrus canker is an economically important disease that is caused by five different groups of *Xanthomonas citri* strains: three from Asia (A, A* and A^w) and two from South America (B and C). In artificial inoculations of grapefruit, only strains of the A and B groups appear to be virulent; strains of the C and A^w group elicit an hypersensitive response (HR) and the A* strains show various levels of reduced virulence. The tested A* and A^w strains also grew to much lower concentrations in grapefruit compared to the A strain. The strains from all five citrus canker groups were virulent in Mexican lime, but the B and C strains elicited a distinctive canker lesion that was almost white in appearance. Strains from the Asiatic groups grew faster than South American B and C group strains in artificial media. Mexican lime and grapefruit can be used in artificial inoculations to readily distinguish all known strains causing citrus canker disease within 10 days without the need for other laboratory tests. Attempts to identify positive host

range determinants from *X. citri* were unsuccessful suggesting the possibility of negative factors (avirulence) being involved in determining host range of *X. citri.*

Every *X. citri* strain carries multiple DNA fragments that hybridize with *pthA*, a member of the *avrBs3/pthA* gene family from *X. citri* group A that is required for pathogenicity and growth of *X. citri* in citrus. Three new *pthA* homologs were cloned and sequenced from canker groups A^w (*pthAW*) and A* (*pthA* and pthA*-2*), and compared with *pthA, pthB* and *pthC*. Homologs *pthA, pthB, pthC pthAW* and *pthA** all have 17.5, nearly identical, direct tandem repeats of 34 amino acids and all complemented a *pthA*::Tn*5* knockout mutation in an *X. citri* group A strain B21.2. Although grapefruit is a differential host and groups A* and A^w are avirulent in grapefruit, none of the *pthA* homologs appeared responsible for the avirulence phenotype by cross complementation tests. Furthermore, none of the four *pthA* homologs from the wide host range group A strain 3213, including *pthA,* conferred an increase in host range of group A* or A^w strains to include grapefruit. *pthA*-2* carried only 15.5 repeats and did not confer either pathogenicity or avirulence to B21.2 in any citrus species tested. Phylogenetic studies separate *pthA* homologues into two groups, Asiatic and South American groups. Analysis of the predicted amino acid sequences of all sequenced *pthA* homologs from *X. citri* indicated that a specific set of amino acid residues in two variable regions of the 17^{th} direct tandem repeat may be required for pathogenicity in citrus.

CHAPTER 1
INTRODUCTION

Citrus

Citrus is one of the major fruit crops in the world. It is thought to have originated in Southeast Asia and India. Citrus was introduced into the new world in the 16th century by Spanish and Portuguese explorers (Allen, 2000). World production of citrus is estimated to be about one hundred million metric tons (FAOSTAT data, 2004, http://apps.fao.org). World citrus production has seen a substantial increase over the last 40 years (Figure 1-1). Major citrus producing countries include the United States, Brazil, China, Argentina, Spain and Mexico (Figure 1-1b). Currently, citrus produced in North and South America account for the majority of citrus production worldwide. Although citrus is mainly grown for the fresh fruit market, large citrus-based juice industries have developed in many countries such as Brazil and the United States. Generally citrus is grown between 40° North and 40° South latitudes where minimum temperatures stay above 20° – 24° F (Timmer and Duncan, 1999).

Citrus is a perennial evergreen with an expected economical production expectancy of about 50 years (Timmer and Duncan, 1999). Originally citrus was grown on its own root system, but now most citrus production plants are grafted onto various rootstocks. Rootstocks are selected for their inherent characteristics that affect production, cold hardiness, salinity tolerance, disease resistance and most importantly compatibility with scion tissue.

The majority of citrus varieties grown for commercial purposes are in the genus *Citrus* including grapefruit (*Citrus paradisi* Macfad), sweet orange (*C. sinensis* (L.) Osbeck), tangerine/mandarin (*C. reticulata* Blanco), lemon (*C. limon* Burm), lime (*C. aurantifolia* (Christm.) Swingle), pummelo (*C. grandis* Osbeck) and citron (*C. medica* L.). Other citrus relatives that are not in this genus are kumquats (*Fortunella* spp.) and trifoliate orange (*Poncirus trifoliata*). The latter is used only as a rootstock.

Florida Citrus

Total citrus production in the U.S. in 2004 is estimated at 16.2 million tons with an estimated value of $ 2.4 billion (USDA, 2004). States that produce citrus include Florida, California, Texas, Arizona, Alabama, Mississippi and Louisiana. Florida produces about 80% of the U.S. citrus, of which 20% - 25% is sold for fresh fruit consumption. In 2004, Florida produced 242 million boxes of oranges and 40.9 million boxes of grapefruit (USDA, 2004). Citrus is an important economic crop for the state of Florida, as the worth of the commercial citrus industry in Florida is estimated to be more than $8.5 billion.

Diseases play a critical role in limiting citrus production as citrus is mainly grown in the same tropical and subtropical areas that also favor the growth of microorganisms. This provides great challenges to citrus growers since they must balance cost of controlling diseases against lower projected profit margins. An example of a citrus disease that is a serious problem for citrus growers is citrus canker disease. Citrus canker has destroyed many citrus growing areas around the world. Florida authorities are putting major resources towards completely eradicating this disease. It is important to gain a better understanding of this disease because of the quarantine of citrus canker as a

pest. Citrus canker it is still spreading, despite $50 million spent between 1996 and 1999 in eradication efforts (Schubert et al., 2001).

Citrus Canker

Citrus canker is one of the major disease problems facing citrus producers in Florida and many areas of the world (Danos et al., 1981; Elgoorani, 1989; Gottwald et al., 2001). Citrus canker, also known as bacterial canker, has destroyed large areas of citrus production (Fegan et al., 2004; Schubert et al., 2001). The pathogen is thought to have originated in Southeast Asia, from where it has spread to other citrus producing areas. Asiatic citrus canker was introduced into the United States for the first time in 1912 from infected nursery material. It took approximately 20 years to eliminate this outbreak of citrus canker (Loucks and Florida. Division of Plant Industry., 1934). In 1986, citrus canker reappeared for the second time in both residential and commercial areas around Tampa, Florida. As a result of this outbreak a new citrus canker eradication program was initiated (Brown, 2001). Eight years later, Florida declared it had eradicated citrus canker at a cost of $27 million (Agrios, 1997). Citrus canker reappeared for the third time in Florida in 1995 in Dade County, and has resulted in the destruction of more than four million trees in both residential and commercial areas (FDACS data, 2005). Figure 1-2 shows the status of citrus canker disease in the state of Florida in 2004. Currently, citrus canker has been detected in 20 Florida counties. The total area under quarantine is estimated at 1,397.82 sq. miles (FDACS data, 2005, www.doacs.state.fl.us). Strong regulatory and quarantine measures were implemented in the latest effort to eradicate the disease. Healthy citrus trees anywhere within a radius of 1900 ft from infected trees are deemed exposed and are destroyed (Gottwald et al., 2002).

Citrus canker is caused by several pathogenic variants of *Xanthomonas citri*. In general, five different groups of pathogenic variants are recognized: A, B, C, A* and A^w (Gabriel et al., 1989; Stall et al., 1982; Verniere et al., 1998). In the literature two other groups of citrus canker are described: "D" and "E" strains. Although there is a single extant "D" strain that was reported in Mexico, it is thought that the fungal pathogen *Alternaria limicola* was responsible for that disease outbreak (Schubert et al., 2001). The "E" strain group was found in grapefruit only in nurseries in Florida and was described as a new "form" of citrus canker. However, strains in this group do not cause hyperplasia and do not infect fruit or mature citrus in groves. The disease is now recognized as distinct from citrus canker and was named citrus bacterial leaf spot caused by *Xanthomonas axonopodis* pv. citrumelo .

Citrus canker symptoms appear after the pathogen enters the leaves through the stomata or wounds and multiplies in the intercellular spaces of the spongy mesophyll (Gottwald et al., 1988; 1989; Graham et al., 1992; Pruvost et al., 2002). The initial symptom is the formation of water-soaked tissue followed by growth of yellow halos on the infection margins. As the disease progresses, erumpent necrotic lesions are formed on leaves, stems and fruits (Figure 1-3). At advanced disease stages, plants defoliate and fruit can drop prematurely. At the microscopic level, infected cells divide (hyperplasia) and enlarge (hypertrophy); and the pustules rupture the surface of the leaf tissue and release bacteria that become a source of inoculum for further infections (Swarup et al., 1991).

The citrus canker bacterium is transmitted by wind-blown rain, although machinery, animals and humans can also transmit it (Bock et al., 2005; Danos et al.,

1984). An important factor that contributed to the spread of citrus canker in this last infection in Florida was the Asian citrus leaf miner *Phyllocnistis citrella* (Cook, 1988). The leaf miner is probably not a vector for canker, but instead it provides wounds that allow entry of bacteria into citrus leaves (Belasque et al., 2005). Although citrus canker does not cause systemic damage, it results in reduced marketability of citrus fruit especially those produced for the fresh market.

Resistance to Citrus Canker

Citrus genotypes show differences in susceptibility to this disease. Grapefruit, sweet orange and Mexican lime are highly susceptible. Sour orange, lemon and tangelo are moderately susceptible, whereas mandarin, citron and kumquat are less susceptible (Schubert et al., 2001). It is not clear if resistance in citrus is a result of active defense responses or if it is due to physical characteristics of different citrus genotypes, e.g. number of stomata or thickness of the leaf tissue that may influence the number of bacterial particles entering citrus leaves (Goto, 1969; McLean and Lee, 1922).

Controlling Citrus Canker

The most effective control of citrus canker is application of strict regulatory and quarantine measures that will protect against the introduction of new infections (Graham et al., 2004). Most citrus producing areas put many resources into monitoring and regulating citrus canker. That is because it is so difficult to eliminate the bacteria once it has become established. Once the disease is established in an area, eradication of both infected and exposed trees and burning plant material are used to help eliminate and prevent spread of disease. Multiple applications of copper based compounds were found to help control the disease to some extent (Hwang, 1949). In some cases pruning infected branches is used to control and eliminate the source of infection. Since citrus canker

spreads by wind driven rain, wind brakes were found to be useful in controlling this disease (Gottwald and Timmer, 1995).

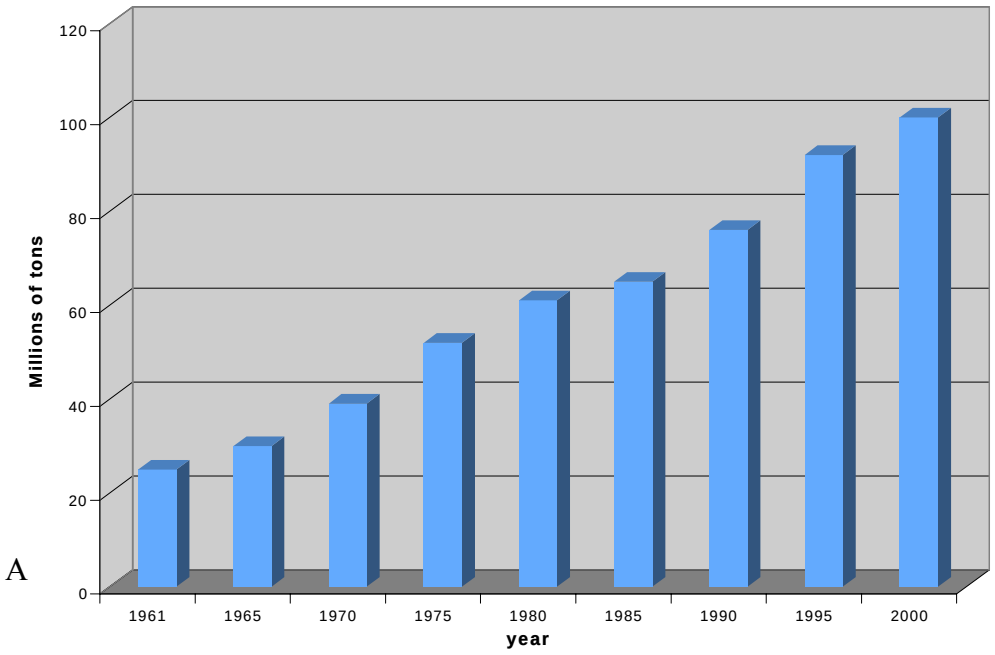
Objectives

The aim of this study was to identify host range determinants of canker causing strains. I was interested in identifying genes that are necessary for increasing the host range of canker causing strains of *X. citri*. These new strains that are limited in host range were used to screen for genes involved in host range determination. Further understanding of how host range is determined may provide important tools in developing control measures. The specific objectives of this work include the following:

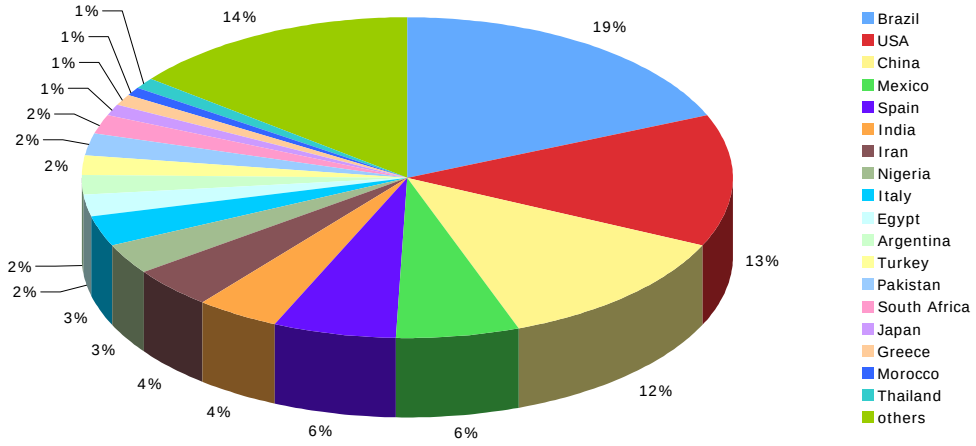
Objective 1. Characterizing canker causing *Xanthomonas citri* A* and A^w group strains.

Objective 2. Attempting to identify and characterize positive host range factor(s) in canker causing *Xanthomonas citri*.

Objective 3. Isolating pathogenicity gene (*pthA*) homologs from A* and A^w groups and characterize their role in host range determination.



A



B

Figure 1-1. World citrus production. A. world citrus production between 1961 – 2000 expressed in metric tons. B. Percent production by countries (FAOSTAT data, 2004, http://apps.fao.org).

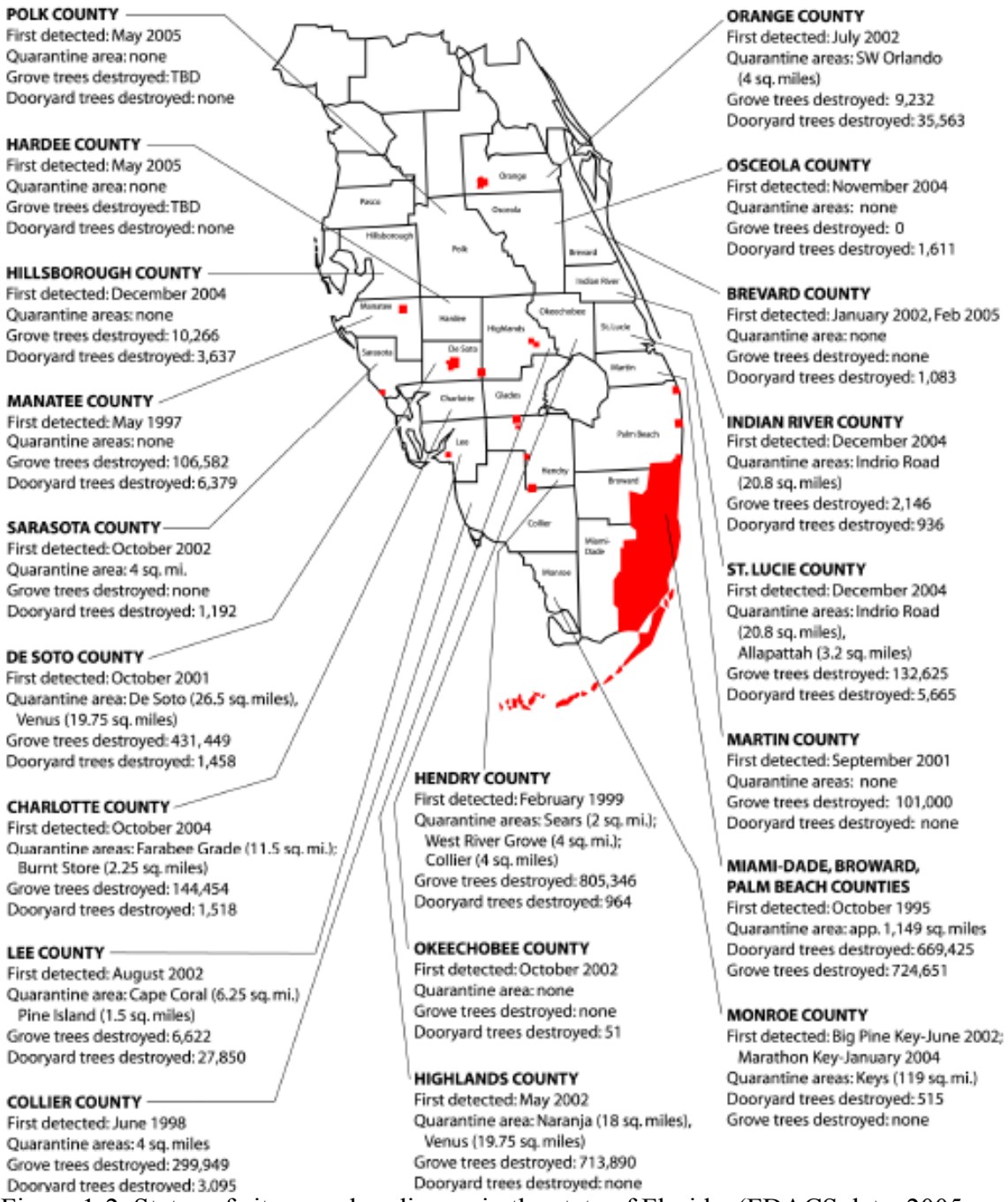


Figure 1-2. Status of citrus canker disease in the state of Florida. (FDACS data, 2005, www.doacs.state.fl.us)

Figure 1-3. Citrus canker symptoms in citrus. Citrus canker symptoms on leaves, fruit and stem. At advanced disease stages, plants defoliate and fruit can drop prematurely.

CHAPTER 2.
USE OF TWO DIFFERENT CITRUS HOSTS TO DISTINGUISH ALL FORMS OF CITRUS CANKER DISEASE

Introduction

Citrus canker disease is caused by several different pathogenic variants of *Xanthomonas citri* (ex Hasse) (Brunings and Gabriel, 2003; Gabriel et al., 1989). Although the taxonomy of these strains is controversial (Gabriel et al., 1989; Vauterin et al., 1995), five groups of pathogenic variants are recognized, based primarily on field symptoms: A, B, C, A* and A^W (Gabriel et al., 1989; Stall et al., 1982; Sun et al., 2004; Verniere et al., 1998). The Asiatic (A) group (*X. citri* pv citri A) is the most severe and widely spread throughout the world. The B and C groups (*X. citri* pv aurantifolii B and C), which are also known as cancrosis B and cancrosis C, respectively, have been found only in South America. These South American groups are phylogenetically distinct and grow more slowly on artificial media than strains from all other groups (Brunings and Gabriel, 2003; Goto, 1969; Stall et al., 1982). The B and C strains also have a reduced host range compared to the A group. In addition, the C strains elicit an hypersensitive response (HR) in grapefruit (*Citrus paradisi*) (Stall et al., 1982). Recently two new variants of the A group of citrus canker strains were identified and designated A* and A^w (Sun et al., 2004; Verniere et al., 1998). Both new groups are limited in host range to Key/Mexican lime (*C. aurantifolia*); the A^w strain causes an HR when inoculated in grapefruit at high concentrations (Sun et al., 2004). The A* and A^w strains of *X. citri* are phylogenetically most closely related to the A group (Cubero and Graham, 2002)

Various diagnostic aids have been used to confirm citrus canker disease, including PCR (Cubero and Graham, 2002; Mavrodieva et al., 2004), antibodies (Alvarez et al., 1991) and microscopy. These tests can be critical if the disease appears in regions where it has not previously been seen or has not been recently observed. Indeed, a fungal disease was misdiagnosed as citrus canker disease in Mexico (Stapleton, 1986; Stapleton and Garza-lopez, 1988), and a bacterial leaf spot disease was misdiagnosed as citrus canker in Florida in 1984 (Schubert et al., 1996). Once confirmation of citrus canker disease has been made, only host range tests can be used to reliably determine the strain group or pathovar. Historically, these studies relied on sweet orange, mandarin orange, lemon, lime and grapefruit (Stall and Civerolo, 1991). In this study, we report the use of Duncan grapefruit and Mexican lime as differential hosts to differentiate strains from all variant groups of citrus canker disease.

Material and Methods

Strains, Plasmids and Culture Media

Strains of *Escherichia coli, Xanthomonas* spp. and plasmids used in this study are listed in Table 2-1 along with their relevant characteristics and source or reference. *E. coli* strains were grown in Luria-Broth (LB) medium at 37 °C (Sambrook et al. 1989). *Xanthomonas* spp. were grown in PYGM (peptone yeast extract-glycerol-MOPS) medium at 30 °C as described by Gabriel et al. (1989). Antibiotics were used at the following final concentrations (μg/ml): rifampin (Rif), 75; spectinomycin (Sp), 35.

Recombinant DNA Techniques

Xanthomonas total DNA was prepared as described by Gabriel and De Feyter (1992) and also using Amersham Biosciences DNA Isolation Kit as described by the

manufacturer. Plasmids were isolated by alkaline lysis from *E. coli* (Sambrook et al., 1989) and *Xanthomonas* (Defeyter and Gabriel, 1991). QIAGEN's QIAprep and plasmid midi kits were also used to isolate plasmid DNA from *E. coli* and *Xanthomonas* as described by the manufacturer. Southern hybridizations were performed using nylon membranes as described (Lazo and Gabriel, 1987).

Plant Inoculations

Duncan grapefruit and Mexican lime plants were grown and maintained under natural light in the quarantine greenhouse facility at the Division of Plant Industry, Florida Department of Agriculture, in Gainesville. Temperatures in this greenhouse ranged from 25º C to 35º C, with 50 % to 100 % relative humidity. All inoculations were carried out in this facility.

Liquid cultures of the tested strains were grown in PYGM medium at 30º C for approximately 24 hr. Cultures were centrifuged @ 1000g for 3 min and cells resuspended in equal volumes of sterile tap water (saturated with $CaCO_3$) and infiltrated into the abaxial surface of young, freshly flushed partially expanded citrus leaves at two concentrations (10^5 cfu/ml for "low" and 10^8 cfu/ml for "high" levels) using the blunt end of tuberculin syringe as described (Gabriel et al., 1989). Observations were taken 5-10 days after inoculation.

***In vitro* Growth Kinetics**

Liquid cultures of *X. citri* 3213, B69, Xc270 and X0053 were prepared in PYGM medium and grown at 37º C overnight with slow shaking. The following day, 100 ml of fresh PYGM was inoculated with 50 µl of the starter culture. Optical density (OD_{600}) readings were taken at 0, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36 and 38 hr. This experiment was repeated three times.

In planta Growth Kinetics

For bacterial cell counts, whole leaves were infiltrated as described. For each strain, three leaves of each host were infiltrated. Bacterial cell counts from the inoculated leaves were taken at days 0, 1, 6, 10 and 14. A number 7 cork borer (about 1 cm^2) was used to cut one leaf disk from each inoculated leaf per time point. Each treatment, a leaf disk from each of the three inoculated leaves was placed in a mortar and pestle and macerated together (in 1 ml sterile tap water saturated with $CaCO_3$). Once homogeneity was obtained, ten-fold serial dilution were made ranging from 10^{-1} to 10^{-9}. Ten microliters droplets of each dilution were spread on PYGM plates without antibiotics and allowed to grow for 48 hr at 28 ºC. Colonies were counted from the most readily scored dilution, and the number of cfu per cm^2 of leaf tissue was calculated. The experiments were repeated three times. Populations were expressed as log cfu/cm^2 of leaf tissue.

Results

Pathogenicity Phenotypes of *X. citri* Strains

In addition to the previously described A (3213), B (B69) and C (Xc340) strains, newly described A* (Xc205, Xc270, Xc280, Xc290, Xc322 and Xc406) and A^w (X0053) strains (Sun et al., 2004; Verniere et al., 1998) were inoculated on citrus. All strains tested caused hyperplastic lesions in Mexican lime that developed 5- 9 days after inoculation (Figure 2-1). In Duncan grapefruit (*Citrus paradisi*) differential reactions were observed that distinguished each group. Strains from group A elicited typical canker symptoms in grapefruit within 6 days, but B strains elicited a whitish canker phenotype within 10 days. C and A^w strains elicited a hypersensitive response (HR) in grapefruit. The A* strains, which were originally isolated from Southwest Asia, also gave distinct phenotypes in Duncan grapefruit (Figure 2-2). Strains Xc205 and Xc322

did not elicit canker symptoms at low concentration (10^4 cfu/ml). Strain Xc406 elicited a weak canker at low concentration, but when inoculated at high concentration, elicited typical canker lesions. Strains Xc270, Xc280 and Xc290 did not elicit canker in Duncan grapefruit at either low or high inoculum concentrations. A* strains could be subdivided into three groups; A*-1, A*-2 and A*-3 (Figure 2-2). Table 2-2 summarizes symptoms of different *X. citri* groups on grapefruit and lime.

In vitro Growth

In vitro growth of *X. citri* strains in liquid medium was measured by optical density (OD_{600}) changes recorded over time (Figure 2-3). Strains 3213 (A), X0053 (A^w) and Xc270 (A*) were very similar in their growth in PYGM medium. B69 (B) strain was significantly slower in its growth compared to A, A^w and A* strains. On agar plates, the South American B and C strains grew similarly on a variety of media, and always slower than the A, A^w and A* strains.

Growth Kinetics _in planta_

Growth kinetics of *X. citri* strains 3213 (A), 270 (A*) and X0053 (A^w) were studied in Duncan grapefruit and Mexican lime leaves. In Mexican lime, growth of all three strains was similar (Figure 2-4). However the growth kinetics of these strains was different in Duncan grapefruit (Figure 2-4). Growth of the A^w strain X0053 was reduced by one order of magnitude as compared to A strain 3213. This reduction in growth was noticeable 6 days post-inoculation and continued through day 14 when the comparison ended. Growth of A* strain 270 was reduced by at least two orders of magnitude after 6 days post-inoculation as compared to strain 3213. Strain 270 did not continue to increase after 6 days growth *in planta*.

Discussion

Inoculation of different *X. citri* strains on just two citrus host species allowed the differentiation of all known *X. citri* groups based on their symptoms. All tested strains of *X. citri* caused canker in Key/Mexican lime. Lime is a very susceptible host compared to other citrus species or types. The B and C strains elicited a characteristic whitish canker in key/Mexican lime that is readily distinguished from canker symptoms caused by other strains. This is probably due to the fact that both strains are phylogeneticlly very similar and likely share common pathogenicity factors. Indeed, the pathogenicity elicitors *pthB* and *pthC* from the B and C strains were found to be closely related to each other (98% similarity) at the amino acid level and different from *pthA* homologues from A, A* and A^w strains. The latter were closely related to each other (Chapter 4).

Strains from different groups of *X. citri* exhibited quite different phenotypic responses when inoculated in Duncan grapefruit leaves. Except for the A and B groups, which elicited typical canker symptoms, strains from all other groups appeared much less virulent. X0053 from the A^w group elicited necrotic symptoms in grapefruit that took 5 to 10 days to appear. Unlike the A^W strain, the C group strain C340 elicited a relatively fast HR reaction in grapefruit that took only 3 days to appear. In both cases, the necrosis and HR, potential *avr* gene function is indicated. The A* group showed the most within-group variation among strains in grapefruit; all A* strains were characterized by reduced virulence as compared to A and B strains and lacked any evidence of eliciting necrosis or an HR. Strains Xc205 and Xc322 were only capable of causing canker in Duncan grapefruit when inoculated at high concentrations (10^8-10^9 cfu/ml). At (lower) concentrations that more closely resemble field conditions, no canker symptoms were observed with these strains. Strain Xc406 elicited a very weak canker phenotype when

inoculated at low concentration, but elicited normal canker symptoms when artificially inoculated at high concentrations. Strains Xc270, Xc280 and Xc290 were not able to elicit canker in grapefruit leaves at either concentration. All tested A* strains were unable to cause canker at low inoculum concentrations or an HR at high concentrations.

In planta growth kinetics of strains representing the fast growing A, A* and A^w groups showed interesting differences (Figure 2-4). All three strains appeared to grow similarly to each other in Mexican/Key lime. Asiatic strain 3213 inoculated in Duncan grapefruit grew to levels similar to those seen in lime. A^w strain X0053, which elicits necrotic symptoms in grapefruit, grew to a final level that was only one log lower than strain 3213. A* strain Xc270, which does not cause canker or HR in grapefruit, was unable to grow well in grapefruit, increasing only 2 logs after inoculation and grew to a final level that was more than two logs lower compared to strain 3213.

It is possible that A* strains carry an *avr* gene that specifically triggers grapefruit defenses, but without an HR. An HR is not always observed with gene-for-gene resistance (Bendahmane et al., 1999; Goulden and Baulcombe, 1993; Jurkowski et al., 2004; Lehnackers and Knogge, 1990; Ori et al., 1997; Schiffer et al., 1997; Yu et al., 2000). Indeed, a *Xanthomonas avr* gene that elicits host defense without an HR was recently reported (Castaneda, 2005). An alternative explanation is that this group is missing a factor or perhaps factors that are specifically required for growth in grapefruit, such as the extracellular polysaccharides (EPS) and lipopolysaccharides (LPS) that are needed by *X. axonopodis* pv. citrumelo for virulence on citrus (Kingsley et al., 1993). Only further experimental testing can distinguish between these explanations.

Although five different citrus host species have traditionally been used to distinguish pathovars of *X. citri*, all known groups can be readily distinguished by inoculation of only two host differentials, lime and grapefruit. Even if positive control cultures are not available, if low inoculations are used, then: 1) only the A strains elicit green cankers in both lime and grapefruit; 2) only the B strains elicit whitish cankers in lime and grapefruit; 3) only the C stains elicit whitish cankers in lime and an HR in grapefruit; 4) only the A* strains elicit green canker in lime and at best very weak cankers in grapefruit, and 5) only the A^w strains elicit green cankers in lime and an HR in grapefruit.

Table 2-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant Characteristics	Reference or source
Escherichia coli		
DH5α	F^-, *end*A1, *hsd*R17($r_k^-m_k^-$), *sup*E44, *thi*-1, *rec*A1	Gibco-BRL
Xanthomonas citri		
3213	Group A, wild type	Gabriel et al. 1989
3213Sp	Spontaneous Sp^r derivative of 3213, Sp^r	Gabriel et al. 1989
B21.2	*pth*A::Tn5-*gus*A, marker exchanged mutant of 3213Sp, Sp^rKn^r	Swarup et al. 1991
B69	Group B, wild type	Stall et al. 1982
B69Sp	Spontaneous Sp^r derivative of B69, Sp^r	El Yacoubi, 2005
C340	Group C, wild type	Stall et al. 1982
Xc205	Group A*, wild type	Verniere et al. 1989
Xc205Rif	Spontaneous Rif^r derivative of Xc205, Rif^r	This study
Xc270	Group A*, wild type	Verniere et al. 1989
Xc270Rif	Spontaneous Rif^r derivative of Xc270, Rif^r	This study
Xc280	Group A*, wild type	Verniere et al. 1989
Xc290	Group A*, wild type	Verniere et al. 1989
Xc322	Group A*, wild type	Verniere et al. 1989
Xc406	Group A*, wild type	Verniere et al. 1989
X0053	Group A^w, wild type	Sun et al. 2004
X0053Rif	Spontaneous Rif^r derivative of X0053, Rif^r	This study

Table 2-2. Phenotypic differences among *X. citri* strains.

	Mexican Lime		Grapefruit	
Strain	**Low** (10^4-10^5) cfu/ml	**High** (10^8-10^9) cfu/ml	**Low** (10^4-10^5) cfu/ml	**High** (10^8-10^9) cfu/ml
A	**C**[a]	**C**	**C**	**C**
B	**Wt C**[b]	**Wt C**	**Wt C**	**Wt C**
C	**C**	**C**	**HR**[c]	**HR**
A* -1	**C**	**C**	**0**[d]	**C**
A* -2	**C**	**C**	**WC**[e]	**C**
A* -3	**C**	**C**	**0**	**0**
A[w]	**C**	**C**	**0**	**HR**

a=canker, b= white canker, c=Hypersensitive response, d=no canker and e=weak canker

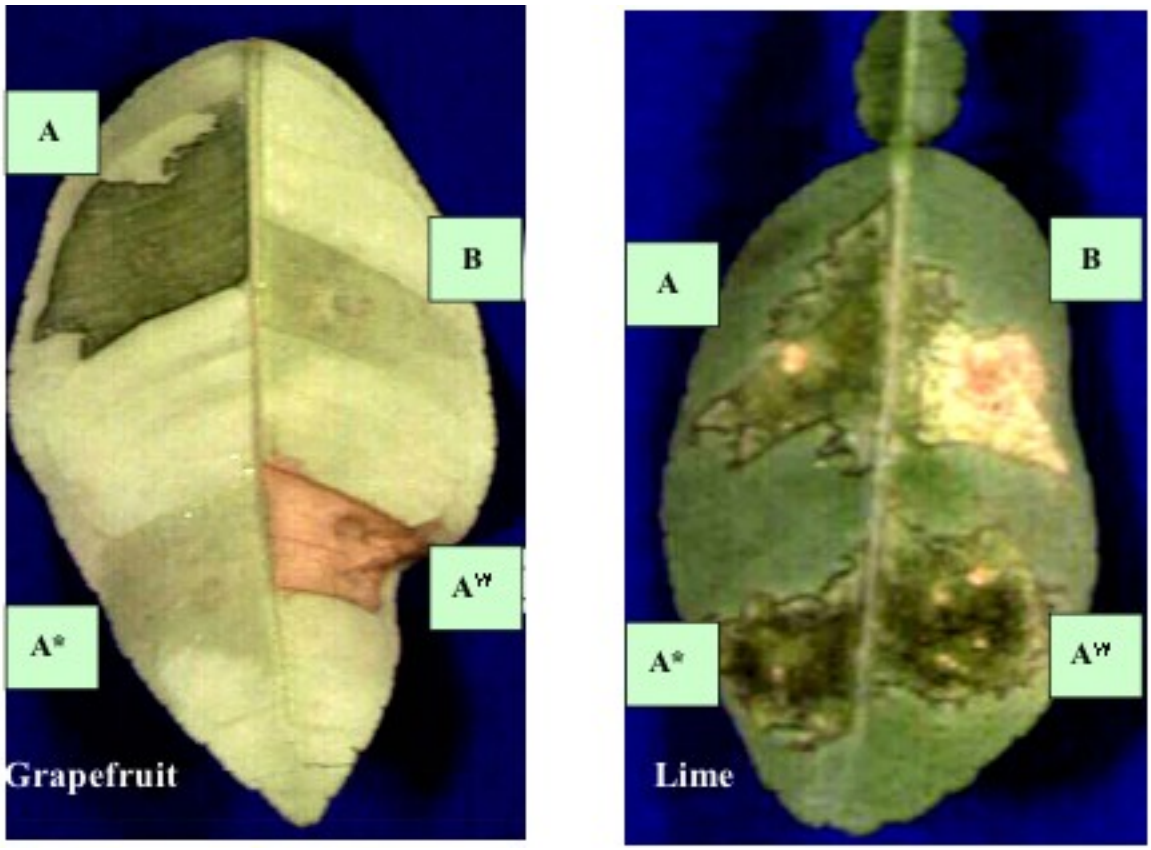


Figure 2-1. Inoculation of strains of different citrus canker groups in grapefruit and key lime. A and B strains are able to cause canker in both hosts. A* and A^w strains can only cause canker in Key lime. Note the HR in grapefruit caused by A^w

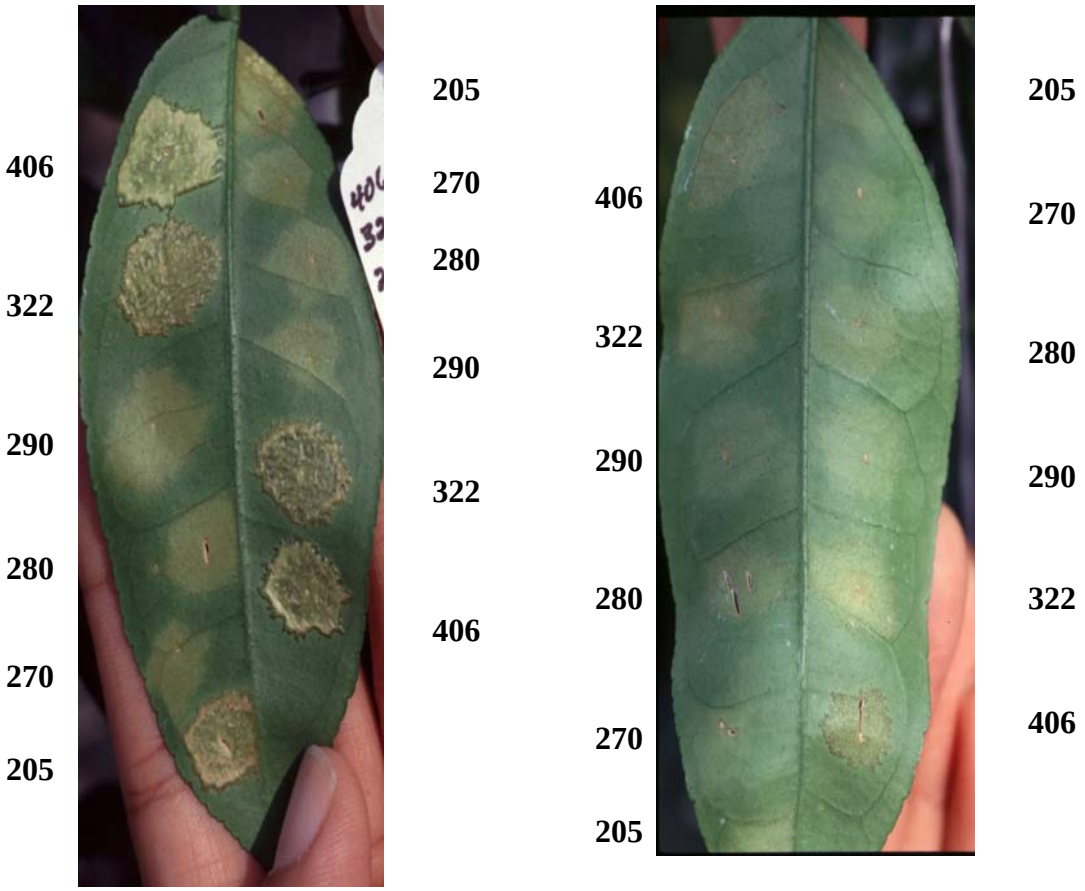


Figure 2-2. Inoculation of several different A* strains in Duncan grapefruit. High (left) and low (right) concentrations of bacteria were used for inoculation.

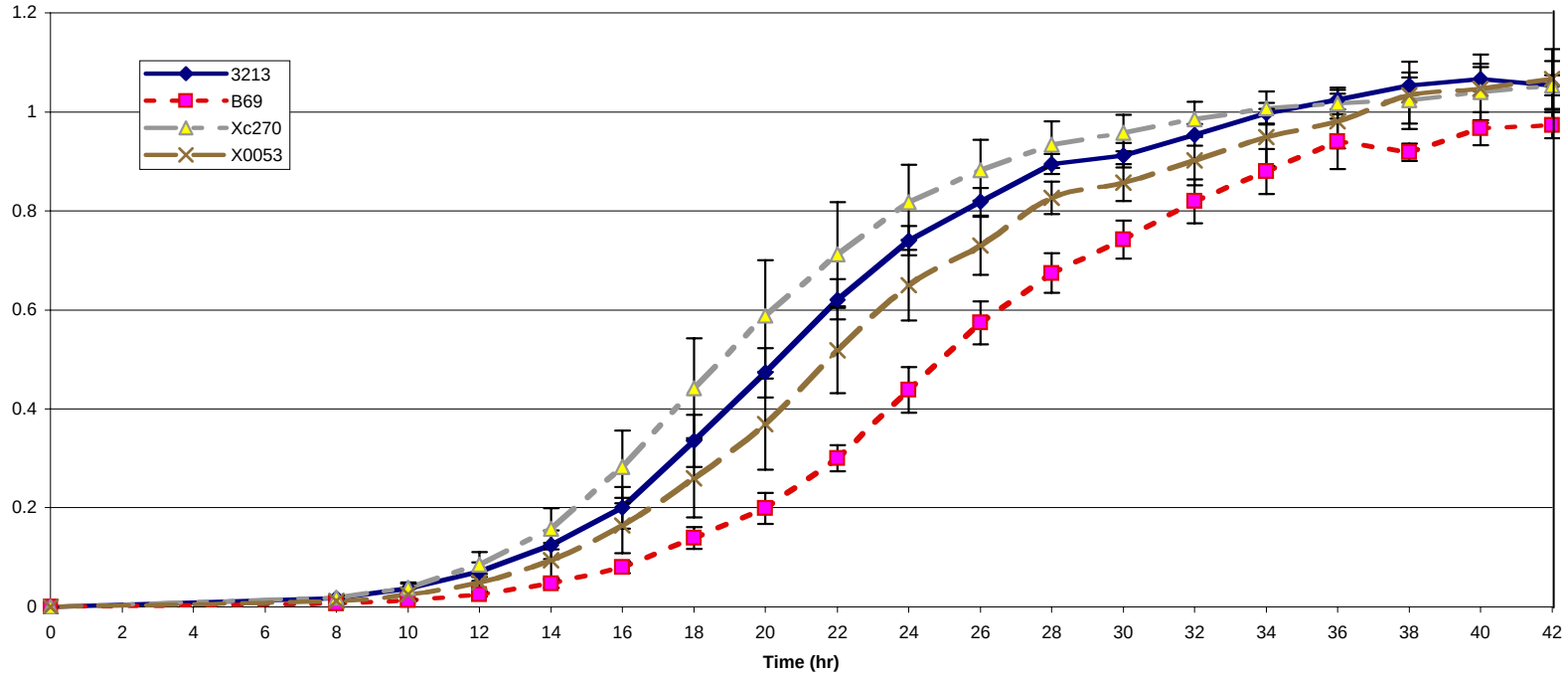


Figure 2-3. Growth of *X. citri* strains in PYGM medium. A strain 3213, A* strain Xc270, A^w strain X0053 and B strain B69 were used in this comparison.

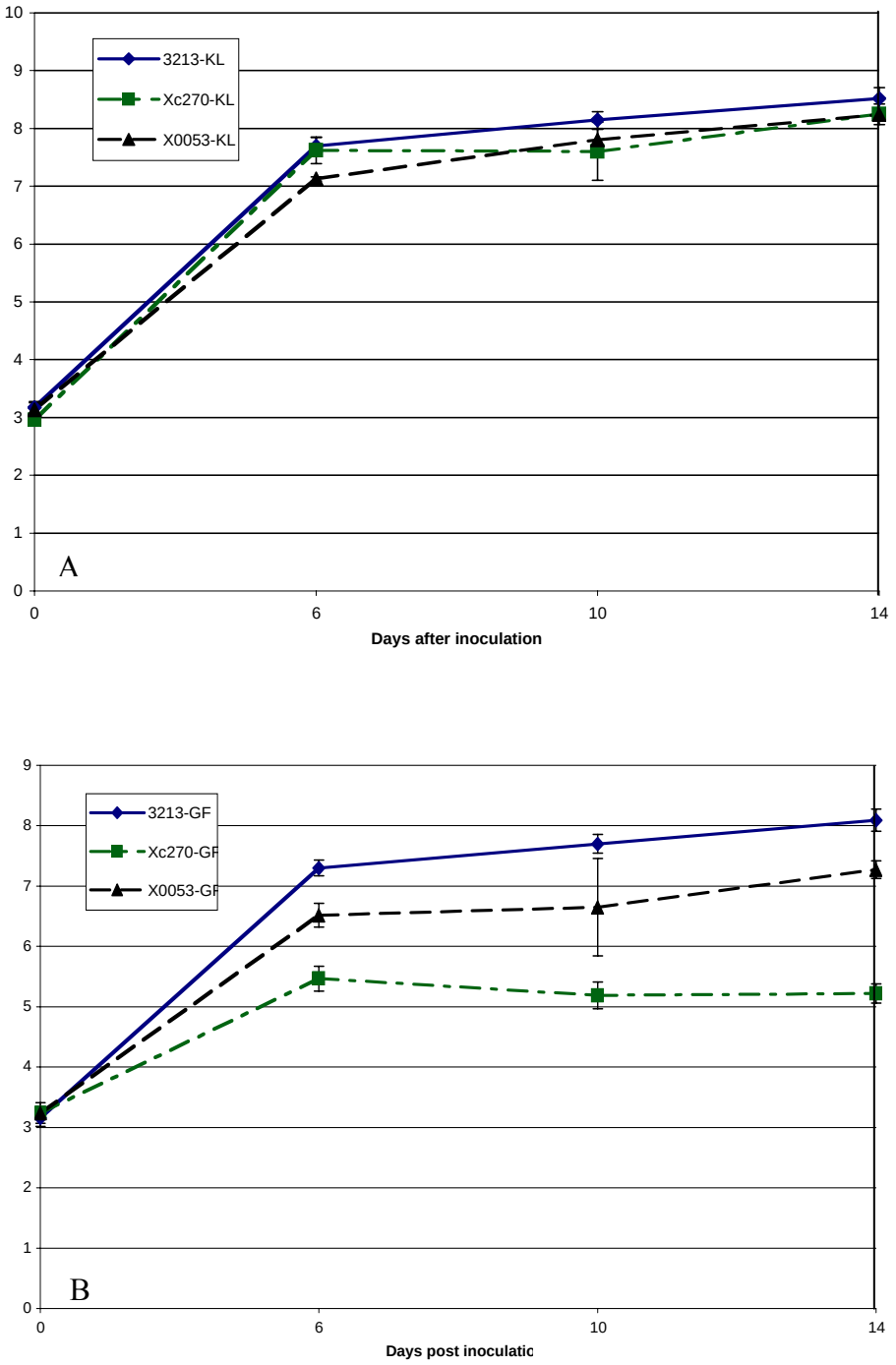


Figure 2-4. *in planta* growth of *X. citri* strains in A) Mexican/Key lime and B) Duncan grapefruit. A strain 3213, A* strain Xc270 and A^w strain X0053 were used.

CHAPTER 3
IDENTIFICATION AND CHARACTERIZATION OF HOST RANGE FACTOR(S) IN CITRUS CANKER STRAINS.

Introduction

Both positive and negative genetic factors have been found to affect the host range of phytopathogenic bacteria. For example, a given *Rhizobium* species can only nodulate a restricted number of hosts, and this specificity is determined by specific signal molecules that are exchanged between the bacteria and host plants (Fisher and Long, 1993; Kondorosi et al., 1991). Some of the host specific nodulation genes needed to actively condition the host range include NodD, NodZ, NodW, NolA and NodC (Kamst et al., 1997). Some negatively acting factors have also been found in some rhizobia and these have avirulence (*avr*) function in some hosts. For example, *nodFE* of *R. leguminosarum* bv trifolii, which is virulent in white and red clover, condition avirulence in pea (Djordjevic et al., 1987), and *nodQ* and *nodH* were found to confer avirulence to *R. l.* bv. trifolii and *R. l.* bv. viceae in their respective hosts, white clover and common vetch (Debelle et al., 1988; Faucher et al., 1989).

Agrobacterium tumefaciens and *A. rhizogenes* generally have a wide host range that includes most dicotyledonous plants. Host range in *A. tumefaciens* is thought to be generally determined by positive factors; however some negative factors of host range determination have also been found (Keen, 1990). For example, certain virulence (*vir*) genes on the Ti plasmid condition host range. Progressive deletions of the 3' end of *virE* were found to progressively reduce the number of plant species on which crown galls are

formed. *virG* from supervirulent *A. tumefaciens* strain extends the host range of certain *Agrobacterium* strains (Chen et al., 1991; Hood et al., 1986). On the other hand *virA* and *virC* appear to act as negative regulators of host range in grapevine. *virA* is involved in detection of host specific phenolics compounds. *virC* was shown to act as an *avr* gene to trigger host defenses in incompatible interactions and thus limit the number of plants *A. tumefaciens* can infect (Yanofsky et al., 1985; Yanofsky and Nester, 1986).

Strains of the genus *Xanthomonas* are always found associated with plants. Different xanthomonads attack a very wide range of plant species. However, individual species show limited host range (Gabriel, 1999b). Members of this genus are divided into species and pathovars, based on phylogeny, host range and disease symptom variation. The molecular basis of host range determination at the pathovar level is not well understood. Azad and Kado (1984) showed that elimination of the HR in tobacco to *Erwinia rubrifaciens* did not increase the host range of this pathogen to include tobacco. Similarly, Swarup et al (1992) showed that elimination of the nonhost HR did not extend the host range of *X. citri*. Factors that positively enhance the host range of *Xanthomonas* include the extracellular polysaccharide (EPS) and lipopolysaccharide (LPS); the *opsX* locus is involved in the biosynthesis of EPS and LPS, and is also needed by *X. axonopodis* pv. citrumelo for virulence in citrus (Kingsley et al., 1993).

Other positive factors that could influence the host range of pathogens are suppressors of host defenses (Ponciano et al., 2003). For example, HopPtoD2, from *Pseudomonas syringae,* was found to suppress programmed cell death in plants resulting in infection (Bretz et al., 2003; Espinosa et al., 2003; Hauck et al., 2003). Similarly, Abramovitch et al. (2003) found that the *P. syringae* effector, AvrPtoB, induced plant

disease susceptibility by preventing a programmed cell death response from occurring in tobacco plants. These results suggest that bacterial host range is determined by positive and negative acting factors.

This chapter describes attempts to identify host range determinants of *X. citri*. A virulence enhancement approach (Swarup et al., 1991) was used in an attempt to identify positive factor(s) required to increase host range of a narrow host range, A* strain Xc270, to include grapefruit.

Material and Methods

Bacterial Strains, Plasmids and Culture Media

Strains of *Escherichia coli, Xanthomonas* spp. and plasmids used in this study are listed in Table 3-1 along with their relevant characteristics, source and/or reference. *E. coli* strains were grown in Luria-Broth (LB) medium at 37 °C (Sambrook et al., 1989). *Xanthomonas* spp. were grown in PYGM (peptone yeast extract-glycerol-MOPS) medium at 30 °C as described (Gabriel et al.1989). Antibiotics were used at the following final concentrations (μg/ml): rifampin (Rif), 75; spectinomycin (Sp), 35; ampicillin (Ap), 100; gentamycin (Gm), 5.

Recombinant DNA Techniques

Xanthomonas total DNA was prepared as described by Gabriel and De Feyter (1992). Plasmids were isolated by alkaline lysis from *E. coli* (Sambrook et al. 1989) and *Xanthomonas* (De Feyter and Gabriel 1991). Restriction enzyme digestion was performed as recommended by the manufacturers. Southern hybridization was performed by using nylon membranes as previously described (Lazo et al., 1987).

Vector Preparation

To identify genes involved in determining the host range of *X. citri* pv citri, genomic DNA from the wide host range *X. citri* pv citri A strain 3213 was partially digested with *Mbo*I and size fractioned on a sucrose gradient. Cosmid vector pUFR43 was used to make a DNA library of 3213 DNA fragments. This cosmid vector (Defeyter et al., 1990) was split into two pools and cut with either *Eco*RI or *Sal*I restriction enzyme to produce the two arms and then treated with shrimp alkaline phosphatase. To create common cloning ends the arms were then cut with *Bam*HI and used for ligations to the 20 - 25kb 3213 DNA fraction (Figure 3-1 and 3-2).

Packaging and Transfection

The recombinant DNA was packaged using stratagene • packaging mix (Gigapack® III Gold Packaging Extract), and introduced into *E. coli* strain DH5•(mcr) via transfection as described by the manufacturer protocol. Positive white plaques were then picked and placed onto LB plates containing the antibiotic Kanamycin (20 μg/μl). Using a 48 pin replicating fork, these colonies were transferred into 96 well microtiter plates containing liquid LB (with 14% glycerol) and stored at –80º C. At the same time a replicate of each plate was made and maintained by replicating each plate once every month. DNA from eighteen randomly selected library clones was extracted and digested with *Bam*HI and electrophoresed on agarose gels in order to estimate insert size and evaluate the quality of the library. The total number of cosmid clones required to cover the entire 3213 genome (*N*) was determined using the following formula (Clarke and Carbon, 1976):

$$N = \frac{In(1 - 0.99)}{In\left\{1 - \left(\frac{insert\ size}{Total\ genome\ size}\right)\right\}}$$

Plant Inoculations

Duncan grapefruit and Mexican lime plants were grown and maintained under natural light in the quarantine greenhouse facility at the Division of Plant Industry, Florida Department of Agriculture, Gainesville, Fl. Temperatures in this greenhouse ranged from 25ºC to 35º C with 50% to 100% relative humidity. All inoculations were carried out in this facility.

Liquid cultures of all tested strains were grown in PYGM medium at 30º C for approximately 24 hr. Cultures were centrifuged and resuspended in equal volumes of sterile tap water (saturated with $CaCO_3$) and pressure infiltrated at appropriate concentrations (10^5 for low and 10^8 cfu/ml for high) into the abaxial surface of citrus leaf using the blunt end of tuberculin syringes. Observations were taken 5-10 days after inoculation. For screening of large numbers of clones, colonies were streaked onto PYGM agar plates incubated at 30º C for approximately 24 hr, resuspended in sterile tap water (saturated with $CaCO_3$) and pressure infiltrated into citrus as described.

Triparental Matings

To transfer the 3213 library to the limited host range Xc270 (A*) strain, triparental matings were performed as described by Defeyter et al (1990). Strain pRK2073 was used as a helper strain. The recipient was concentrated 50 – 100 fold. Transconjugants were screened on PYGM plates containing Rif 75µl g/ml and Gm 3µl g/ml at 28º C and 2-3 days later colonies were transferred onto new selection plates.

Results

Xanthomonas citri pv citri A 3213 Strain Genomic Library

A genomic library of *X. citri* pv citri A, strain 3213, was made and 18 randomly picked clones were evaluated for insert size and pattern (Figure 3-3). All 18 clones gave different restriction patterns indicating random insertions in the vector. The average size of the inserts was 39 kb. Based on the Clark and Carbon formula (Clarke and Carbon, 1976), 610 clones were required to cover the whole *X. citri* 3213 genome with 99% probability. Seven hundred and fifty clones were maintained in *E. coli* strain DH5α and stored in 15% glycerol at -80° C.

Screening of 3213 Library in Xc270

Five hundred and fifty clones were transferred from the 3213 library into *X. citri* pv. citri A*-3 strain Xc270 by triparental mating and transconjugants were individually screened for symptoms in Duncan grapefruit. No clones were identified that consistently increased the pathogenicity of Xc270.

Discussion

Attempts to identify positive host range determinants from *X. citri* were unsuccessful when a library from the wide host range group A strain 3213 was moved into the narrow host range group A* strain Xc270. Initially six clones (pAW377, pAW378, pAW380, pAW400, pAW413 and pAW419) seemed to elicit canker-like symptoms in Duncan grapefruit, but when those clones were re-conjugated into Xc270, the initial results were not confirmed. The *in planta* growth of Xc270 described in chapter 2 showed that the Xc270 grew poorly in Duncan grapefruit, suggesting that the only clones that would complement Xc270 and cause canker in grapefruit would be those that would increase growth. It is likely that *in planta* growth requires multiple effectors,

and that no individual cosmid would carry enough factors to reveal a strong difference. Another possibility is that Xc270 may carry *avr* genes that function in grapefruit and prevent Xc270 from growing. Avirulence is usually epistatic over virulence and therefore a screen for positive factors would fail if this were the case. Perhaps a better approach would be to construct a library of Xc270 DNA and screen in 3213 in order to identify any avirulence gene function.

Table 3-1. Strains and plasmids used in this study

Strain or plasmid	Relevant Characteristics	Reference or source
Escherichia coli		
DH5α	F^-, *end*A1, *hsd*R17($r_k^-m_k^-$), *sup*E44, *thi*-1, *rec*A1	Gibco-BRL
Xanthomonas citri		
3213	Group A, wild type	Gabriel et al. 1989
3213Sp	Spontaneous Sp^r derivative 3213, Sp^r	Gabriel et al. 1989
B21.2	*pth*A::Tn5-*gus*A, marker exchanged mutant of 3213Sp, Sp^rKn^r	Swarup et al. 1991
Xc270	Group A*, wild type	Verniere et al. 1989
Xc270Rif	Spontaneous Rif^r derivative of Xc270, Rif^r	This study
Plasmid		
pRK2013	ColE1, Km^r,Tra^+, helper plasmid	Figurski and Helinski, 1979
pRK2073	pRK2013 derivative,*npt*::Tn7, Km^sSp^r,Tra^+, helper plasmid	Leong et al. 1982
pURF043	IncW, Mob^+, *lacZ*α^+, Gm^r, Nm^r, *cos*, shuttle vector	De Feyter and Gabriel, 1991
pAW377	Fragment from *X. citri* 3213 library cloned in pUFR43	This study
pAW378	15 kb fragment from *X. citri* 3213 library cloned in pUFR43	This study
pAW380	Fragment from *X. citri* 3213 library cloned in pUFR43	This study
pAW400	Fragment from *X. citri* 3213 library cloned in pUFR43	This study
pAW413	24 kb fragment from *X. citri* 3213 library cloned in pUFR43	This study
pAW419	Fragment from *X. citri* 3213 library cloned in pUFR43	This study

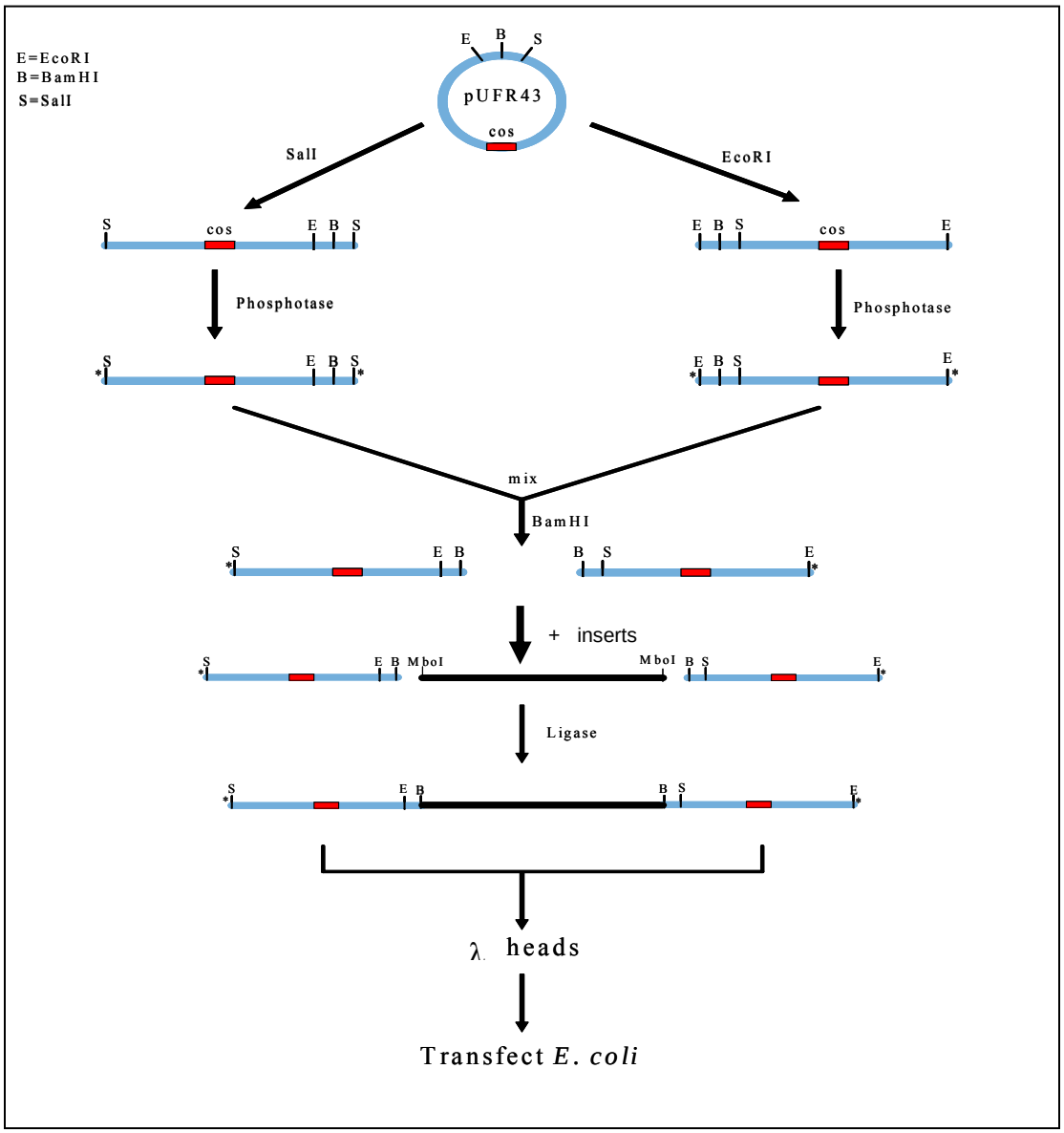


Figure 3-1. Scheme for cosmid vector preparation and DNA cloning.

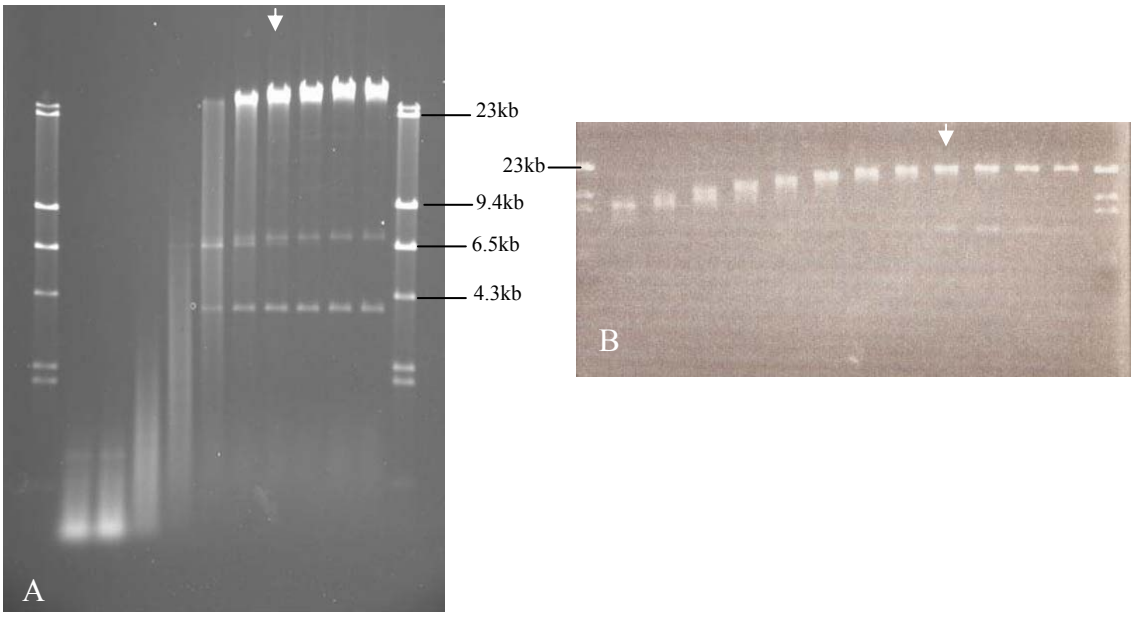


Figure 3-2. DNA fractionation. A). Partial digestion of *X. citri* 3213 genomic DNA (0.7% agarose gel). B) Size fractionation of *Mbo*I partial digest of 3213 DNA (0.7% agarose gel).

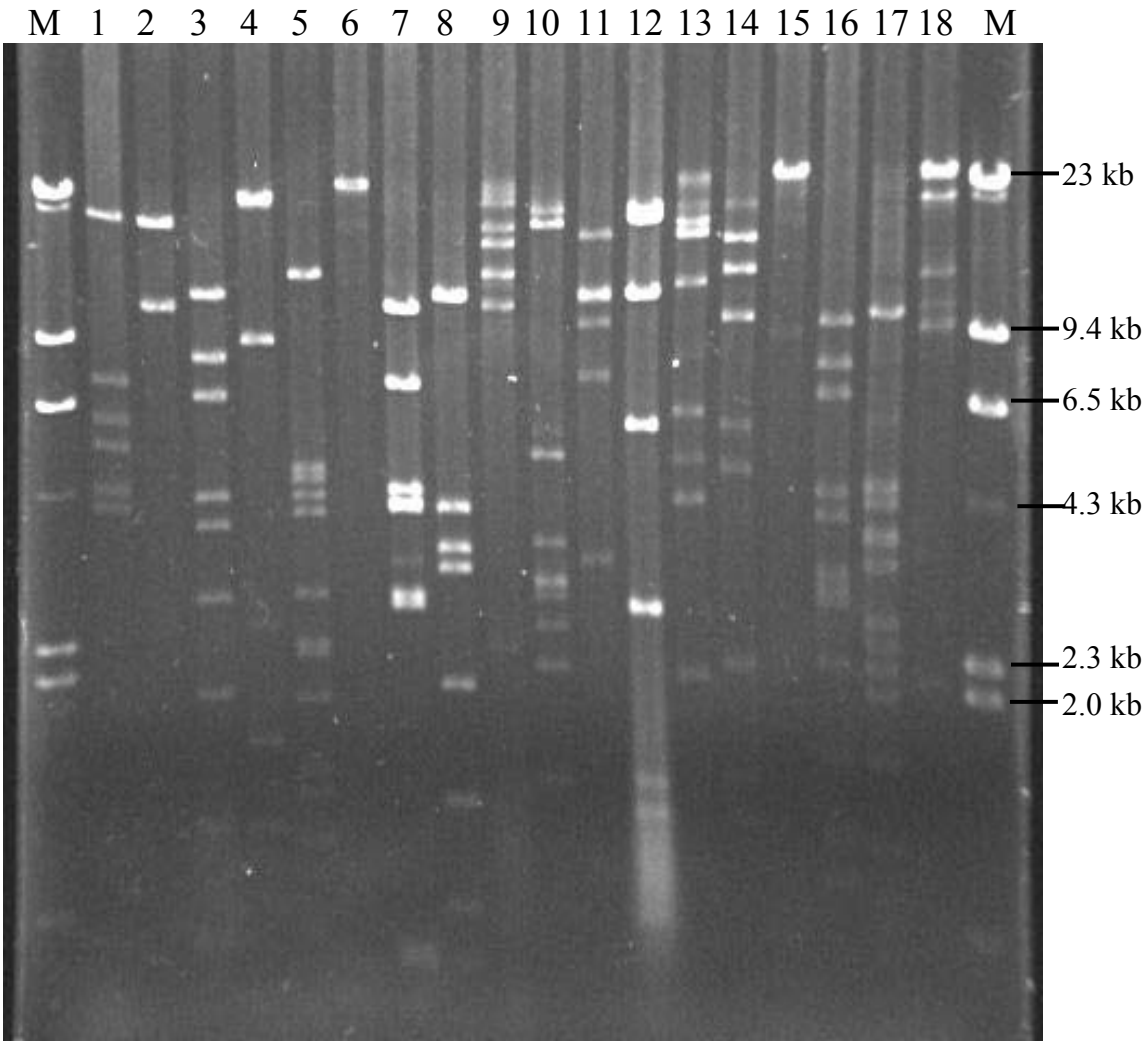


Figure 3-3. Restriction profiles of random clones from *X. citi* 3213 genomic library. DNA was digested with *Eco*RI. As a merker, λ DNA digested with *Hin*dIII (M).

CHAPTER 4.
SEQUENCE COMPARISON AND CHARACTERIZATION OF FIVE NEW *pthA* HOMOLOGS FROM FOUR DIFFERENT *Xanthomonas citri* STRAINS.

Introduction

All strains of *Xanthomonas citri* cause hyperplastic pustules in citrus that are dignostic of citrus canker disease (Gabriel, 2001). The Asiatic (A) group (*X. citri* pv citri A) has the widest host range and is widespread throughout the world. The B and C groups (*X. citri* pv aurantifolii B and C) have only been found in South America and have a reduced host range compared to the A groups (Stall and Seymour, 1983). New groups of *X. citri* pv citri (A^w from Florida and A* from Southwest Asia) were more recently identified that are primarily restricted in host range to Mexican lime (*Citrus aurantifolia*) (Stall et al., 1982b; Sun et al., 2004). Grapefruit (*C. paradisi*) serves as a differential host that is resistant to the A*, A^w and C strains; the A^w and C strains elicit a strong hypersensitive response (HR) in grapefruit, while some A* strains show reduced growth in grapefruit (Chapter 2). The molecular basis for avirulence of the A*, A^w and C strains in grapefruit and the wide host range of the A strains is unknown.

Pathogenicity gene *pthA* encodes the primary causal effector of the citrus canker disease phenotype (Duan et al., 1999; Swarup et al., 1991; Swarup et al., 1992). All strains of *X. citri* tested carry *pthA* homologs (Cubero and Graham, 2002; Mavrodieva et al., 2004). *pthA* is capable of conferring ability to cause canker-like symptoms to strains that cannot otherwise cause canker, such as *X. campestris* pv citrumelo (Swarup et al., 1991) or even *E. coli* carrying a functional *hrp* system (Kanamori and Tsuyumu, 1998).

When *pthA* is transiently expressed in citrus using either *Agrobacterium tumefaciens* or particle bombardment, small canker-like lesions are elicited (Duan et al., 1999).

pthA is the first member of the *avrBs3/pthA* gene family demonstrated to function for pathogenicity. The vast majority of cloned or described *Xanthomonas* avirulence genes belong to this family; many have demonstrated pathogenicity functions (Leach and White, 1996). Members of this gene family show very high levels of homology at the DNA sequence level (De Feyter et al., 1993; Hopkins et al., 1992; Leach and White, 1996; Yang et al., 2000). All members encode more than 11 nearly perfect, 34 amino acid, leucine rich, tandemly arranged, direct repeats. Swapping repeat regions between members of the gene family results in chimeric genes that confer the pathogenicity and/or avirulence phenotypes expected from the source genes (Herbers et al., 1992; Yang et al., 1994). Although *pthA* can confer avirulence to other xanthamonads (Swarup et al., 1992), no *pthA* homolog from *X. citri* is known to function for avirulence in citrus. Conversely, although the *pthA* homolog *aplI* from *X. citri* pv citri group A has been suggested as a suppressor of the tobaco defense response (Ponciano et al., 2003), no *pthA* homolog from *X. citri* is known to suppress citrus host defenses.

The purpose of this study was to clone, isolate, sequence and characterize *pthA* homologs that function to determine pathogenicity from all known *X. citri* groups. A secondary purpose was to determine if any of these *pthA* homologs also determined avirulence in grapefruit or could increase the pathogenicity of an A* strain in grapefruit.

Material and Methods

Bacterial Strains, Plasmids and Culture Media

Strains of *Escherichia coli, Xanthomonas* spp. and plasmids used in this study are listed in Table 4-1 along with their relevant characteristics and source or reference. *E.*

coli strains were grown in Luria-Broth (LB) medium at 37 °C (Sambrook et al., 1989). *Xanthomonas* spp. were grown in PYGM (peptone yeast extract-glycerol-MOPS) medium at 30 °C as described (Gabriel et al. 1989). Antibiotics were used at the following final concentrations (μg/ml): rifampin (Rif), 75; spectinomycin (Sp), 35; chloramphenicol (Cm), 35; ampicillin (Ap), 100; gentamycin (Gm), 5; kanamycin (Kn), 25.

Recombinant DNA Techniques

Xanthomonas total DNA was prepared as described (Gabriel and De Feyter, 1992). Plasmids were isolated by alkaline lysis from *E. coli* (Sambrook et al., 1989) and *Xanthomonas* (De Feyter and Gabriel, 1991). Southern hybridization was performed by using nylon membranes as described by Lazo and Gabriel (1987).

DNA Library Construction

Genomic DNA from the wide host range *Xanthomonas citri* pv citri group A strain 3213 was partially digested with *Mbo*I and size fractioned on a sucrose gradient. The cosmid vector pUFR43 was used to make a DNA library of 3213 DNA fragments. This cosmid vector was split into two pools and cut with either *Eco*RI or *Sal*I restriction enzyme to produce the two arms and treated with shrimp alkaline phosphatase. To create common cloning ends, the arms were then cut with *Bam*HI and used for ligations to the 20 - 25kb 3213 DNA fraction. Recombinant DNA was packaged using Stratagene • packaging mix (Gigapack® III Gold Packaging Extract), and introduced into *E. coli* strain DH5• via transfection as described by the manufacturer protocol. Positive white plaques were then picked and placed onto LB plates containing the antibiotic Kn (20 μg/μl). Colonies were transferred into 96 well micro titer plates containing liquid LB

(with 14% glycerol) and stored at –80º C. DNA from eighteen randomly selected library clones was extracted, digested with *Bam*HI and run on agarose gels in order to estimate insert size and evaluate the quality of the library.

Plant Inoculations

Duncan grapefruit and Mexican lime plants were grown, maintained and inoculated under natural light in the quarantine greenhouse facility at the Division of Plant Industry, Florida Department of Agriculture in Gainesville, Fl. Temperatures in this greenhouse ranged from 25ºC to 35º C with 50% to 100% relative humidity.

Liquid cultures of tested *Xanthomonas* strains were grown in PYGM at 30 ºC for approximately 24 hr. Cultures were centrifuged @ 1000g for 3 min at room teperature, and resuspended in equal volumes of sterile tap water (saturated with $CaCO_3$) and pressure infiltrated at appropriate concentrations (10^5 for low and 10^8 cfu/ml for high) into the abaxial citrus leaf surface using the blunt end of a tuberculin syringe. Observations were taken 5- 10 days after inoculation.

Southern Hybridization Analysis

Genomic DNA from all canker causing strains were isolated as described, digested with either *Eco*RI or *Bam*HI restriction enzyme and the digested DNA was analysed by electrophoresis on 0.6% agarose gels. DNA was then transferred onto GeneScreen Plus® (DuPont, Wilmington, Delaware) nylon membranes as described by the manufacturer. Membranes were hybridized with a ^{32}P-labeled *Bam*HI internal fragment of *pthA*.

Colony Hybridization

Plasmid DNA from A^W strain X0053 was digested with *Eco*RI *and Kpn*I and ligated into shuttle vector pUFR047. Recombinant DNA was transformed into DH5• competent cells, and transformed clones were selected on Ap100 and X-Gal/IPTG in LB

agar. White colonies were transferred onto a registry plate and pZit45 was included at specific positions as a control. Plasmid DNA from A* strain Xc270 was digested with *Eco*RI and *Hind*III and ligated into shuttle vector pUFR71. Recombinant DNA was transformed into DH5α and selected on Cm35 LB and X-Gal/IPTG plates. White colonies were transferred from registry plates onto Colony/PlaqueScreen™ hybridization transfer nylon membranes and placed colony side up on plain LB plates and incubated for 2- 4 hr at 37 ºC. DNA was fixed on membranes as described by the manufacturer and hybridized with a ^{32}P-labeled *Bam*HI fragment of *pthA*. Group B strain B69 plasmid DNA was digested with *Eco*RI, and a 23 kb and a 4.3 kb fragment that hybridized with *pthA* were cloned in pUFR53 resulting in pQY93.3 and pQY22.1, respectively. A 14kb *Hin*dIII fragment within the pQY93.3 *Eco*RI fragment was subcloned in pUFR53, resulting in pQY96. Group C strain C340 plasmid DNA was digested with *Sal*I, and a 20 kb and a 6 kb fragment that hybridized with *pthA* were cloned into pUFR53, resulting in pQYC2.1 and pQYC1.1, respectively. The 6 kb insert from pQYC1.1 fragment was cloned into the high copy vector pUC119 resulting in pQY103.5.

Triparental Matings

Clones that hybridized to *pthA* were conjugated into *Xanthomonas* strain B21.2 (*pthA*::Tn*5*) by triparental mating as described by Defeyter et al (De Feyter et al., 1990). Strain pRK2073 was used as a helper strain. The recipient strain was concentrated 50 – 100 fold for higher conjugation rate. 10 µl of each recipient, donor and helper were mixed together on PYGM plate and allowed to grow for 6 hr to overnight at 28º C. Transconjugants were screened on PYGM plates containing Sp 35 µl g/ml and Gm 5µl g/ml at 28º C. Two to three days later colonies were transferred onto new selection

plates. Successful transconjugants were infiltrated into Duncan grapefruit and Mexican/Key lime. Southern blot analysis was used to further analyze clones.

Marker Integration Mutagenesis

The mutants BIM2 (*pthB*::pUFR004) of B69Sp and CIM1 (*pthC*::pUFR004) of C340 were created by the integration of pYY40.10 (2.0 kb internal *Stu*I-*Hinc*II fragment of *pth*A in pUFR004), and Cm resistant colonies were selected.

Sequence Analysis of *pth* Genes

pthA homologs from A*, A^w, B and C strains were sequenced using primers based on the sequence of *pthA* (Swarup et al., 1992) and designed to cover the entire gene. Seven primers were used for sequencing reactions; DG8: gaggtggtcgttggtcaacgc, DG35: agttatctcgccctgatc, DP35: caggtcactgaagctgcccgc, DP36: gcgggcagcttcagtgacctg, DP37: ccgaaggttcgttcgaca, DP38:ctgtcgaacgaaccttcg, DP45: gcatggcgcaatgcactgac, and YP03: tagctccatcaaccatgc. Sequencing was done at the UF ICBR DNA Sequencing Core, Gainesville, FL. When necessary, fragments were cloned into high copy vectors such as pUC119 or pUC19 to obtain larger amounts of DNA.

Sequence information of these genes was used to construct the full DNA sequence using Vector NTi software (Invitrogen, Carlsbad, California). Nucleotide and predicted amino acid sequence alignments were carried out with the program CLUSTAL W. Percent amino acid identity was calculated using the needle program in EMBOSS package which uses the Needleman-Wunsch algorithm to do global alignment of sequences. The DNA sequences of *pthA1, pthA2, pthA3* and *pthA4* (da Silva et al., 2002) were taken from GenBank Accessions # NC_003921, NC_003921, NC_003922 and NC_003922, respectively. The DNA sequences of *apl1,apl2* and *apl3* (Kanamori and

Tsuyumu, 1998) were taken from GenBank Accessions # AB021363, AB021364 and AB021365, respectively. Dendograms showing phylogenetic relationships of these genes were generated with TREECON (version 1.3b) (Van de Peer and De Wachter, 1994) using neighbor-joining algorithm with Poisson correction. RSc1815, an *avrBs3/pthA* gene from *Ralstonia solanacearum* was used as an outgroup for phylogenetic tree construction. The percentage of trees from 100 bootstrap resamples supporting the topology is indicated when the percentage is above 70.

Results

Southern Blot Analysis

Southern blot analyses revealed that all tested *X. citri* strains have at least two *Bam*HI DNA fragments that strongly hybridized to an internal *Bam*HI fragment from *pthA* (Figure 4-1a; some data not shown). With the exception of group A strains, which had four *Bam*HI fragments that hybridized with *pthA*, all other strains from all other groups, including the A*, A^w, B and C groups, had only two such *Bam*H1 fragments. All strains tested appeared to share a 3.4 kb *Bam*HI hybridizing fragment of a size similar or identical to that of *pthA*. Otherwise, each of the different phenotypic groups exhibited distinct and characteristic banding patterns. Based on the hybridization intensity of both *Bam*HI and *Eco*RI digested DNA fragments and other results (not shown), the C and A^w strains appeared to carry their two hybridizing DNA fragments on a single plasmid (Figure 4-1).

Cloning, Characterization and Sequencing of *pthA* Homologs from *X. citri* A*, A^w, B and C Strains

Using an internal fragment of *pthA* as a probe, colony hybridization of *E. coli* carrying cloned group A^w strain X0053 plasmid DNA revealed eight colonies with

hybridizing inserts (Figure 4-2). The plasmids from these colonies were designated as pAW5.1 – 5.8, and all carried hybridizing inserts of identical size (Table 4-1). Four of these inserts were separately introduced into strain B21.2 (*pthA*::Tn*5*) and screened for pathogenicity. All four clones complemented the knockout phenotype of B21.2 and restored ability to cause canker in both Duncan grapefruit and Mexican lime (Figure 4-3; Table 4-2). The *pthA* homolog encoded on pAW5.2 was sequenced and designated *pthAW*.

Similarly, colony hybridization of cloned group A* strain Xc270 plasmid DNA revealed three hybridizing clones, designated as pAW12.1 - 12.3. The inserts carried on pAW12.1 and 12.2 were identical in size; pAW12.3 was smaller. When transferred to B21.2, pAW12.1 complemented B21.2 and resulted in canker symptoms in both grapefruit and lime (Figure 4-4; Table 4-2). The *pthA* homolog encoded on pAW12.1 was sequenced and designated *pthA**. pAW12.3 did not complement B21.2 in either host (Table 4-2). The *pthA* homolog from pAW12.3 was sequenced and designated *pthA*-2*. *pthA*-2* carried only 15.5 internal repeats. To verify that the lack of evident activity of *pthA*-2* was not due to a cloning artifact, the promoter region and Shine-Dalgarno (SD) sequence were verified to be present on pAW12.3. In addition, no premature stop codons or frame shifts were found in *pthA*-2*.

Colony hybridization of cloned group B strain B69 plasmid DNA revealed several hybridizing clones of two different sizes. Representative clones of both sizes were selected for complementation tests. pQY93.3 (23 kb insert) and pQY22.1 (4.3 kb insert) were mobilized by conjugation into B21.2; only pQY93.3 was found to complement B21.2, resulting in canker symptoms in both grapefruit and lime (Table 4-2). The *pthA*

homolog was subcloned from pQY93.3 on pQY96, verified as functional in B21.2 designated as *pthB* and sequenced.

Finally, colony hybridization of cloned group C strain C340 plasmid DNA revealed several hybridizing clones of two different sizes, and again representative clones of both sizes were selected for complementation tests. pQYC2.1 (20 kb insert) and pQYC1.1 (6 kb) were mobilized by conjugation into B21.2; only pQYC1.1 was found to complement B21.2, resulting in canker symptoms in both grapefruit and lime (Table 4-2). The *pthA* homolog encoded on pQYC1.1 was designated as *pthC* and sequenced.

Even when inoculated at high concentrations, none of the *pthA* homologs (*pthAW, pthA*, pthA*-2, pthB* or *pthC*) in B21.2 elicited an HR in grapefruit.

Inactivation and Complementation of Genes *pthB* and *pthC* in *X. citri* pv aurantifolii

In order to determine the role of *pthB* in the pathogenicity of *X. citri* pv aurantifolii group B strain B69Sp in citrus, marker integration mutagenesis was carried out. Southern blot analysis showed that *pthB* had been interrupted in BIM2 (*pthB*::pUFR004). BIM2 was unable to cause canker (data not shown). BIM2 was fully complemented by pAB2.1, pZit45, pAB18.1 (all carrying *pthA*), pQY96 (carrying *pthB*) and pQYC1.1 (carrying *pthC*) to elicit wild type response in grapefruit and lime (data not shown). In order to determine the role of gene *pthC* in the pathogenicity of group C strain C340 in citrus, marker integration mutagenesis was carried out. Southern blot analysis showed that *pthC* had been interrupted in CIM1 (*pthC*::pUFR004). CIM1 was unable to cause typical canker symptom in lime, but elicited an HR in grapefruit that was as strong as the HR elicited by the wild type strain C340. CIM1 was fully complemented by pZit45 (*pthA*), pQY96 (*pthB*) and pQYC1.1 (*pthC*) to elicit a wild type response in lime (data not shown).

None of the *pthA* Homologs from Group A Strain 3213 Increased the Host Range of Group A* Strain 270 to Include Grapefruit

All four *pthA* homologs from group A strain 3213 were isolated and cloned from the 3213 library by colony hybridization with an internal fragment of *pthA*: pAW20.2, pAW20.4, pAW20.7 and pAW20.11 carry *pthA, pthA1, pthA2* and *pthA3,* respectively (Figure 4-5). None of these clones complemented B21.2. When these clones were conjugated into the A* strain Xc270, none extended the host range of the strain to include Duncan grapefruit. As with Xc270, all three transconjugants elicited cankers in Mexican lime. When pZit45, which carries *pthA* from 3213 and complements B21.2 (Swarup et al., 1992), and pAW20.2 were introduced into Xc270, they similarly did not extend the host range of Xc270 to include grapefruit (Figure 4-5).

Sequence Analysis of *pthA* Homologs from All Known *X. citri* Groups

The DNA sequences of all 13 available *pthA* homologs were analyzed and the predicted amino acid sequences were found to be >75% identical (Table 4-3). With the notable exception of Apl3, all seven other PthA homologs within *X. citri* pv citri group A (PthA, PthA1, PthA2, PthA3, PthA4, Apl1 and Apl2) were more closely related to each other (> 92% identical), than the active PthA homologs from all *X. citri* groups (PthA, PthB, PthC, PthAW and PthA*) which were >97% identical (Figure 4-6). Comparative analysis of the 34 aa direct repeat regions of all thirteen genes revealed three primary regions of variation within each repeat, at positions 3 and 4 (region 1), positions 11-13 (region 2) and positions 30-32 (region 3) (Figure 4-7). In region 1, no particular set of amino acids was universally conserved among any of the repeats of active *pthA* homologs. However, in regions 2 and 3, and only in repeat number 17 in each gene,

N(12)G(13) in region 2 and Q(31)A(32) in region 3 were correlated with active pathogenicity gene function.

Discussion

Southern hybridization analyses of a limited number of *X. citri* strains revealed a common 3.4 kb *Bam*HI band shared by all strains examined in all five described groups of strains; all group A strains tested carried four hybridizing fragments, while all other strains examined carried only two. Among the 13 sequenced and functionally tested *pthA* homologs, including three tested by others [Apl1, Apl2 and Apl3; (Kanamori and Tsuyumu, 1998)] and the ten tested in this study, only the 3.4 kb fragment appeared to encode the active pathogenicity gene that is required for elicitation of citrus canker. This includes genes *pthA**, *pthAW, pthB* and *pthC* from the A*, A^w, B and C strains, respectively, as well as *pthA*. All five of these genes were found to be fully isofunctional, and capable of eliciting the typical canker phenotype in grapefruit in B21.2, even though the source A*, A^w and C strains were unable to elicit the canker phenotype in grapefruit. Furthermore, *pthA*, pthAW* and *pthC* did not elicit an avirulence phenotype of any type in B21.2, despite being members of an *avr* gene family, and despite the avirulence of the respective source strains in grapefruit. Indeed, the *pthC* knockout mutation in CIM1 eliminated pathogenicity in lime, but did not affect the HR in grapefruit, which remained as strong as that elicited by the wild type. The HR elicited by the wild type C group strain C340 is therefore independent of *pthC*. These results suggest that the A*, A^w and C strains likely carry yet to be identified *avr* genes that prevent compatible phenotypes from developing in grapefruit.

The C strain C340 and A* strain Xc270 fragments that hybridized with *pthA* did not complement B21.2 to pathogenicity in lime or grapefruit. The sequenced Xc270

homolog that failed to complement, *pthA*-2,* carried 15.5 repeats and appeared to have intact promoter, a SD region and an open reading frame. This gene was 97% identical to PthA2 and Apl2 (Table 4-3) and carried the same number of repeats. All three of these genes appear intact and yet also appear non-functional in terms of pathogenicity or avirulence. Although the C340 homolog (on pQYC2.1) that did not complement B21.2 was not sequenced, restriction enzyme analysis (not shown) of the 20 kb insert indicated that the promoter region was intact, making this homolog unlikely to be responsible for avirulence in grapefruit.

The other three group A 3213 *pthA* homologs did not complement B21.2 and also appeared to be non-functional, confirming and extending the work of Kanamori and Tsuyumu (Kanamori and Tsuyumu, 1998) on group A strain L-9. However, the fact that all wide host range group A strains examined carry two additional *pthA* homologs that are not present in the more narrow host range B, C, A* and A^w strains suggests a potential role in determining host range. Indeed, Ponciano et al (2003) reported that *apl1*, a *pthA* homolog that is functionally equivalent to *pthA* but found in a different group A strain, suppressed tobacco defense response and HR. However, when *pthA* or any of its 3213 homologs (*pthA1, pthA2 or pthA3*) were transferred into Xc270, no increase in host range of A* strain Xc270 to include grapefruit was observed (Figure 4-5). Although, additional *pthA* homologs in a given *X. citri* strain may contribute marginally to pathogenicity (Kanamori and Tsuyumu, 1998), the primary value of multiple copies of the gene family in a given strain may be to facilitate recombination and the potential for rapid adaptation to new hosts (Gabriel, 1999a; Yang and Gabriel, 1995).

All *pthA* homologs that are required for citrus canker disease from all five known *X. citri* groups carried exactly 17.5 repeats. All other homologs, even those nearly identical to *pthA* (e.g., from *X. citri* pv citri group A) were not required for canker and had a different number of repeats. Interestingly, deletion mutants of various repeats and numbers of repeats in *pthA* can result in a gene that confers a weak canker phenotype in citrus to B21.2 (Yang and Gabriel, 1995). In that study, however, repeat numbers 1-5 and 16,17 were not affected in deletion derivatives capable of conferring canker. This indicates that while the total number of repeats may be important, the number of repeats may be less important than the relative location of the specific repeats within the gene. Surprisingly, sequence variation among these active *pthA* genes (PthA, PthAW, PthA*, PthB and PthC) was greater than variation among the *pthA* homologs within the A group. Even the nonfunctional homologs were closer to the active genes within the A group than to active homologs from B and C groups.

The relatively high level of variation within the active homologs from different phylogenetic groups allowed the possibility of identifying amino acids within the 34 aa direct repeat that might be critical for pathogenic specificity in citrus. Three somewhat variable regions were found in each of the repeats, at amino acid positions 3 and 4, 11-13, 30-32. The aligned repeat regions of all active genes revealed that only amino acids N(12)G(13) in the second and Q(31)A(32) in the third variable regions of the 17th repeat were conserved. No such conservation of identical amino acids was found in any other repeat (Figure 4-6). Interestingly, only the 17th repeat of the South American group B and C strains show a sequence identity to Asiatic strains in the third variable region. Q(31),A(32) is not seen in any other PthB repeat and in only two other PthC repeats,

which favor E(31)Q(32) at that position. In addition, the deletion mutants evaluated by Yang and Gabriel (Yang and Gabriel, 1995) never affected the 17th repeat. These results suggest that the 17th repeat may be critical for pathogenicity of *X. citri.*

Table 4-1. Strains and plasmids used in this study

Strain or plasmid	Relevant Characteristics	Reference or source
Escherichia coli		
DH5α	F^-, *end*A1, *hsd*R17($r_k^-m_k^-$), *sup*E44, *thi*-1, *rec*A1	Gibco-BRL
Xanthomonas citri		
3213	Group A, wild type	Gabriel et al. 1989
3213Sp	Spontaneous Sp^r derivative 3213, Sp^r	Gabriel et al. 1989
B21.2	*pth*A::Tn*5*-*gus*A, marker exchanged mutant of 3213Sp, Sp^rKn^r	Swarup et al. 1991
B69	Group B, wild type	Stall et al. 1982
B69Sp	Spontaneous Sp^r derivative of B69, Sp^r	
C340	Group C, wild type	Stall et al. 1982
Xc205	Group A*, wild type	Verniere et al. 1989
Xc205	Spontaneous Rif^r derivative of Xc205, Rif^r	This study
Xc270	Group A*, wild type	Verniere et al. 1989
Xc270Rif	Spontaneous Rif^r derivative of Xc270, Rif^r	This study
Xc280	Group A*, wild type	Verniere et al. 1989
Xc290	Group A*, wild type	Verniere et al. 1989
Xc322	Group A*, wild type	Verniere et al. 1989
Xc406	Group A*, wild type	Verniere et al. 1989
X0053	Group A^w, wild type	Sun et al. 2004
X0053Rif	Spontaneous Rif^r derivative of X0053, Rif^r	This study
BIM2	*pthB*::pUFR004, marker integrated mutant of B69Sp	El-Yacoobi, 2005
CIM1	*pthC*::pUFR004, marker integrated mutant of C340	This study
Plasmids		
pRK2013	ColE1, Km^r,Tra^+, helper plasmid	Figurski and Helinski 1979
pRK2073	pRK2013 derivative, npt::Tn7, Km^sSp^r, Tra^+, helper plasmid	Leong et al. 1982
pUC119	ColE1, M13 lg, Ap^r, *lacZ*α^+	Vieira and Messing, 1987
pUFR004	ColE1, Mob^+, Cm^r, $lacZ^+$	De Feyter et al. 1990
pUFR043	IncW, Mob^+, *lacZ*α^+, Gm^r, Nm^r, *cos*, shuttle vector	De Feyter and Gabriel, 1991
pUFR047	IncW, Mob^+, *lacZ*α^+, Par^+, Gm^rAp^r	De Feyter et al. 1993

Table 4-1. Continued.

Strain or plasmid	Relevant Characteristics	Reference or source
pUFR053	IncW, Gm^r,Cm^r, Mob^+, mob(P), *lacZ*α^+, Par^+	El-Yacoobi, 2005
pUFR071	IncW, Mob^+, Cm^r, Gm^r, $lacZ^+$, Par^+	Castaneda, 2005
pYD9.3	*pthA* in pUC118, Ap^r	Duan et al. 1999
pZit45	4.5Kb fragment containing *pth*A from 3213 cloned in pUFR47, Ap^r	Swarup et al. 1992
pAB2.1	*Eco*RI/*Hind*III fragment of pZit45, containing *pthA,* in pLAFR3	This study
pAB18.1	*Eco*RI/*Hind*III fragment of pYD9.3, containing *pthA,* in pUFR47	This study
pQY93.3	23 kb *Eco*RI fragment containing *pthB* in pUFR53	This study
pQY22.1	4.3 kb *Eco*RI fragment containing $pthB_0$ (non-functional) in pUFR53	This study
pQY99.3	8.8 Kb *Sal*I fragment containing *pthB* from B69 was cloned in pUC119	This study
pQY96	14 kb *Hind*III fragment containing *pthB* cloned in pUFR53	This study
pQY103.5	5 Kb *Sal*I fragment containing *pth*C from C340 cloned in pUC119	This study
pQYC1.1	6 kb *Sal*I fragment containing *pthC* cloned in pUFR47	This study
pQYC2.1	20 kb *Sal*I fragment containing $pthC_0$ (non-functional) cloned in pUFR47	This study
pAW5.1- 5.8	5Kb *Eco*RI-*Kpn*I fragment containing *pthAW* from X0053 cloned in pUFR47	This study
pAW12.1-12.2	22 kb *Eco*RI/*Hind*III fragment containing *pthA** from A* group strain Xc270 cloned in pUFR71	This study
pAW12.3	6 kb *Eco*RI/*Hind*III fragment containing *pthA*-2* from A* group strain Xc270 cloned in pUFR71	This study
pAW20.2	36 kb *Mbo*I fragment containing *pthA* from 3213 cloned in pUFR43	This study
pAW20.4	17 kb *Mbo*I fragment containing *pthA1* homolog from 3213 cloned in pUFR43	This study
pAW20.7	32 kb *Mbo*I fragment containing *pthA2* homolog from 3213 cloned in pUFR43	This study
pAW20.11	40 kb *Mbo*I fragment containing *pthA3* homolog from 3213 cloned in pUFR43	This study

Table 4-2. Phenotypic responses of *X. citri* strains in 2 citrus hosts

	Mexican Lime		**Grapefruit**	
Strains/Plasmid	**Low**[a]	**High**[b]	**Low**	**High**
3213	+[c]	+	+	+
B21.2	**0**[d]	**0**	**0**	**0**
B21.2/pZit45(*pthA)*	+	+	+	+
B21.2/pQY96(*pthB*)	+	+	+	+
B21.2/pQYC1.1(*pthC*)	+	+	+	+
B21.2/pAW5.2(*pthAW*)	+	+	+	+
B21.2/pAW12.1(*pthA)**	+	+	+	+
B21.2/pAW12.3(*pthA*2*)	**0**	**0**	**0**	**0**

a=10^4-10^5cfu/ml, b=10^8-10^9cfu/ml, c= canker, d= no canker,

Table 4-3. Amino acid sequence identity between pathogenicity genes from *X. citri* strains

	PthA	**PthA4**	**Apl1**	**PthAW**	**PthA***	**PthA*-2**	**PthA1**	**PthA2**	**PthA3**	**Apl2**	**Apl3**	**PthB**	**PthC**
PthA	100	100	100	99	98	92	95	93	92	94	84	87	87
PthA4		100	100	99	98	92	95	93	92	94	84	87	87
Apl1			100	99	98	92	95	93	92	94	84	87	87
PthAW				100	97	92	95	93	92	93	84	87	87
PthA*					100	92	95	93	92	93	84	87	87
PthA*-2						100	95	97	97	97	79	82	83
PthA1							100	95	95	95	81	84	85
PthA2								100	98	99	79	82	82
PthA3									100	98	79	82	82
Apl2										100	80	82	82
Apl3											100	75	75
PthB												100	98
PthC													100

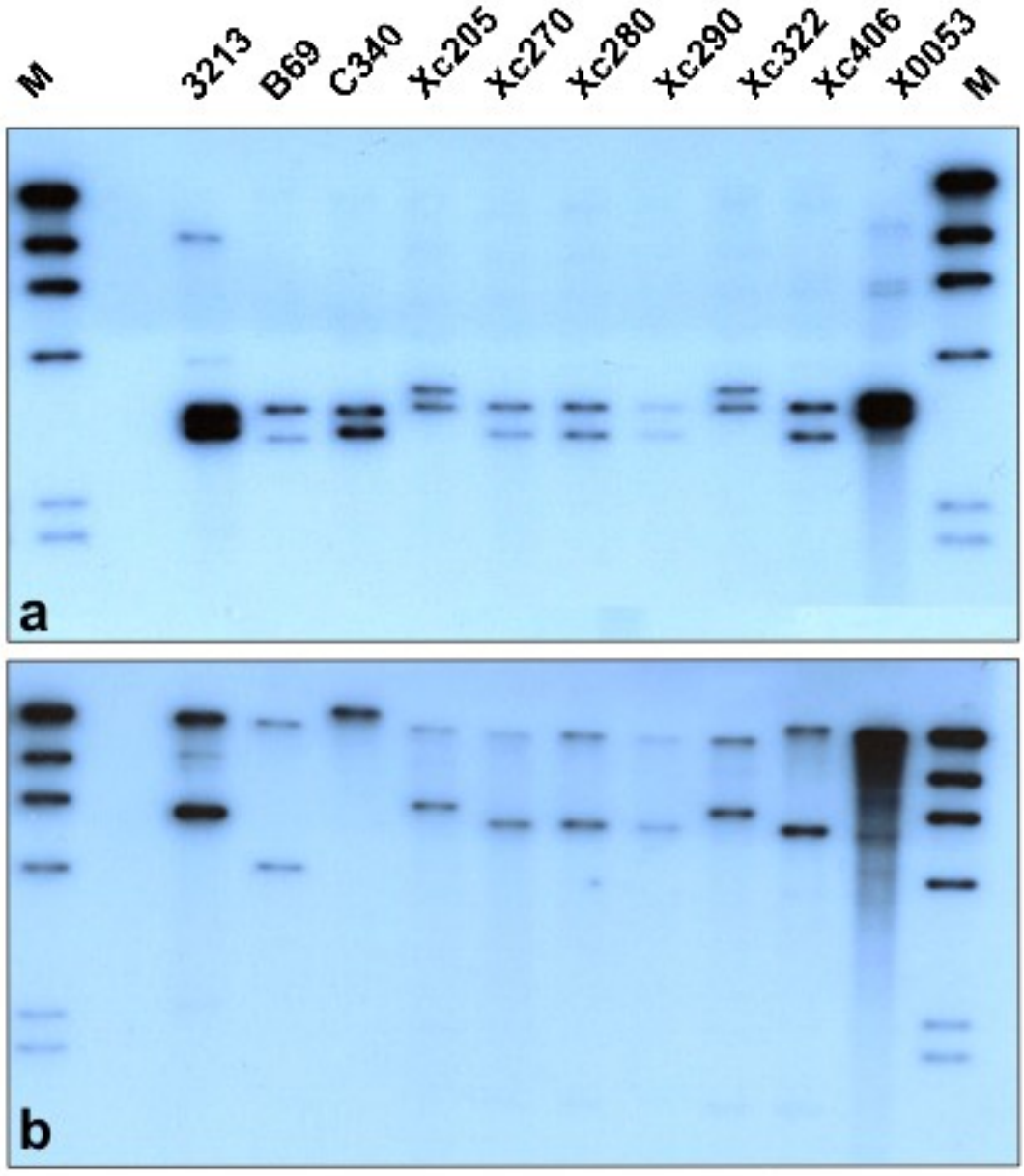


Figure 4-1. Southern Hybridization analysis of *X. citri* strains hybridized with the *Bam*HI internal fragment of pthA. A). *Bam*HI restriction digested genomic DNA from *X. citri* strains. B). *Eco*RI restriction digested genomic DNA.

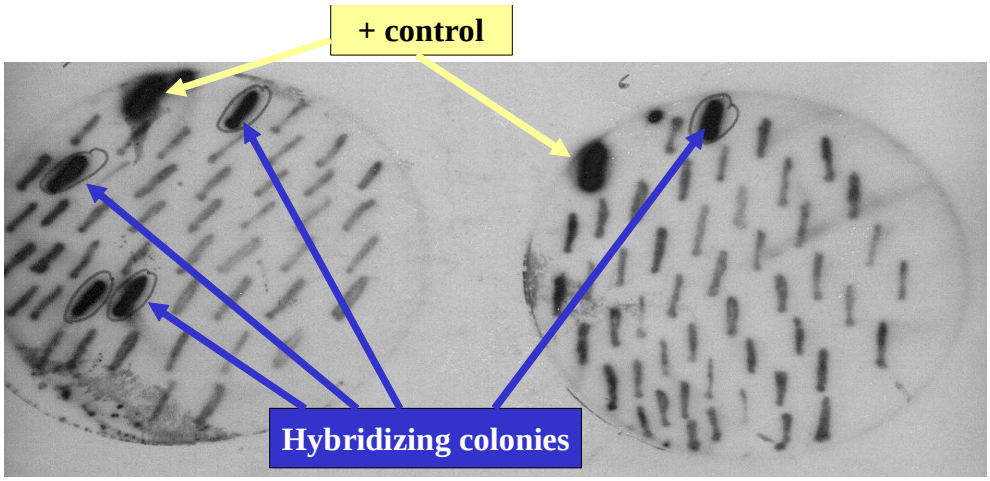


Figure 4-2. Colony Hybridization of *E. coli* with cloned X0053 A^w plasmid DNA fragments using ^{32}P-labeled *pthA*. pZit45 (*pthA*) was used as a positive control.

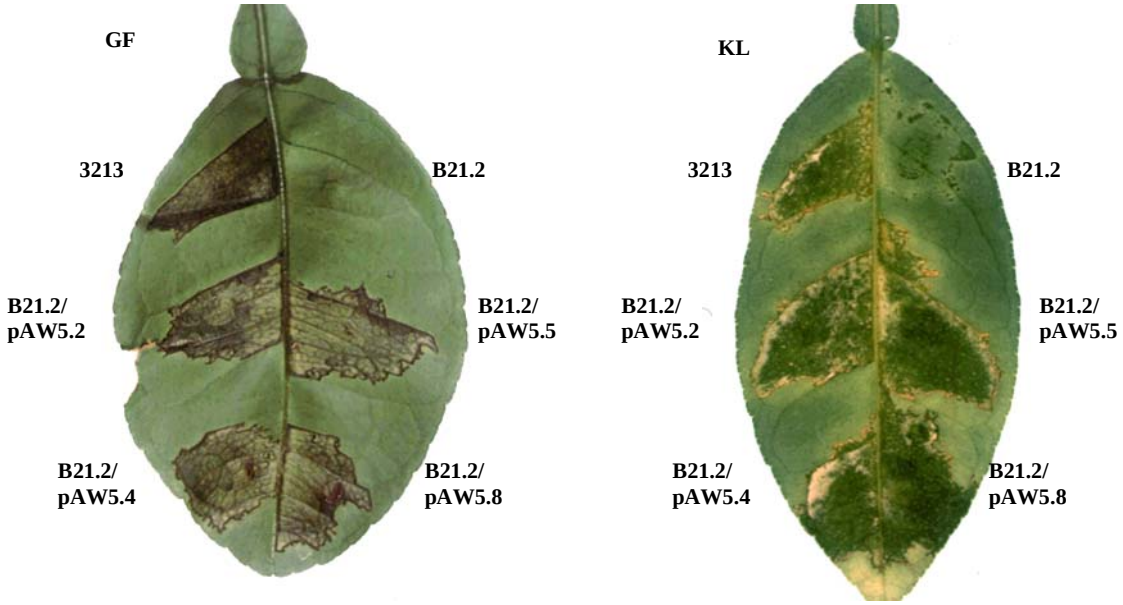


Figure 4-3. Complementation of A strain knockout B21.2 (*pthA*::Tn5) with *pthA* homologs from A^w strain X0053 in citrus. pAW5.2, pAW5.4, pAW5.5 and pAW5.8 carry fragments that hybridized with *pthA* in grapefruit (left) and Key lime (right).

Figure 4-4. Complementation of A strain knockout B21.2 (*pthA*::Tn*5*) with *pthA* homologs in citrus. *pthAW* (pAW5.2), *pthA** (pAW12.1) and *pthA*-2* (pAW12.3) in B21.2 and *pthA* (3213) in grapefruit (left) and Key lime (right).

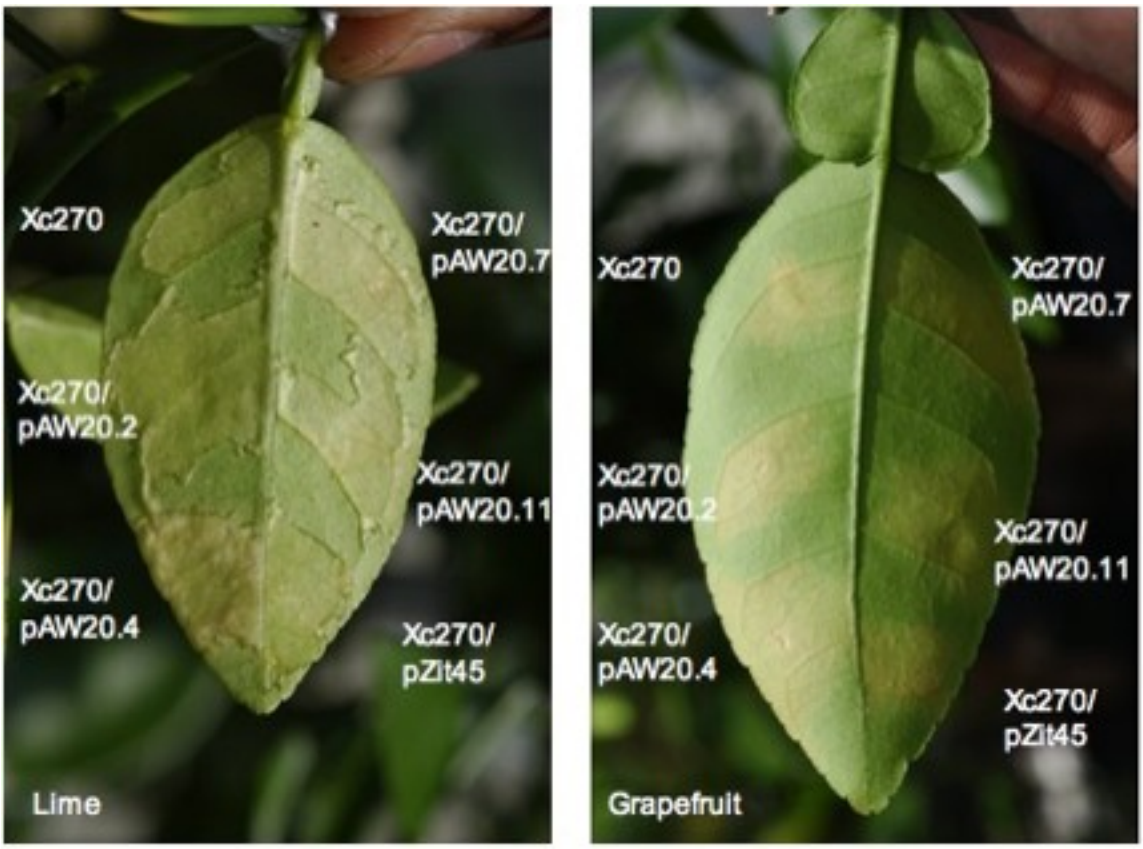


Figure 4-5. Analysis of *pthA* and its three homologs in A* strain Xc270. Key lime (left) and grapefruit (right)

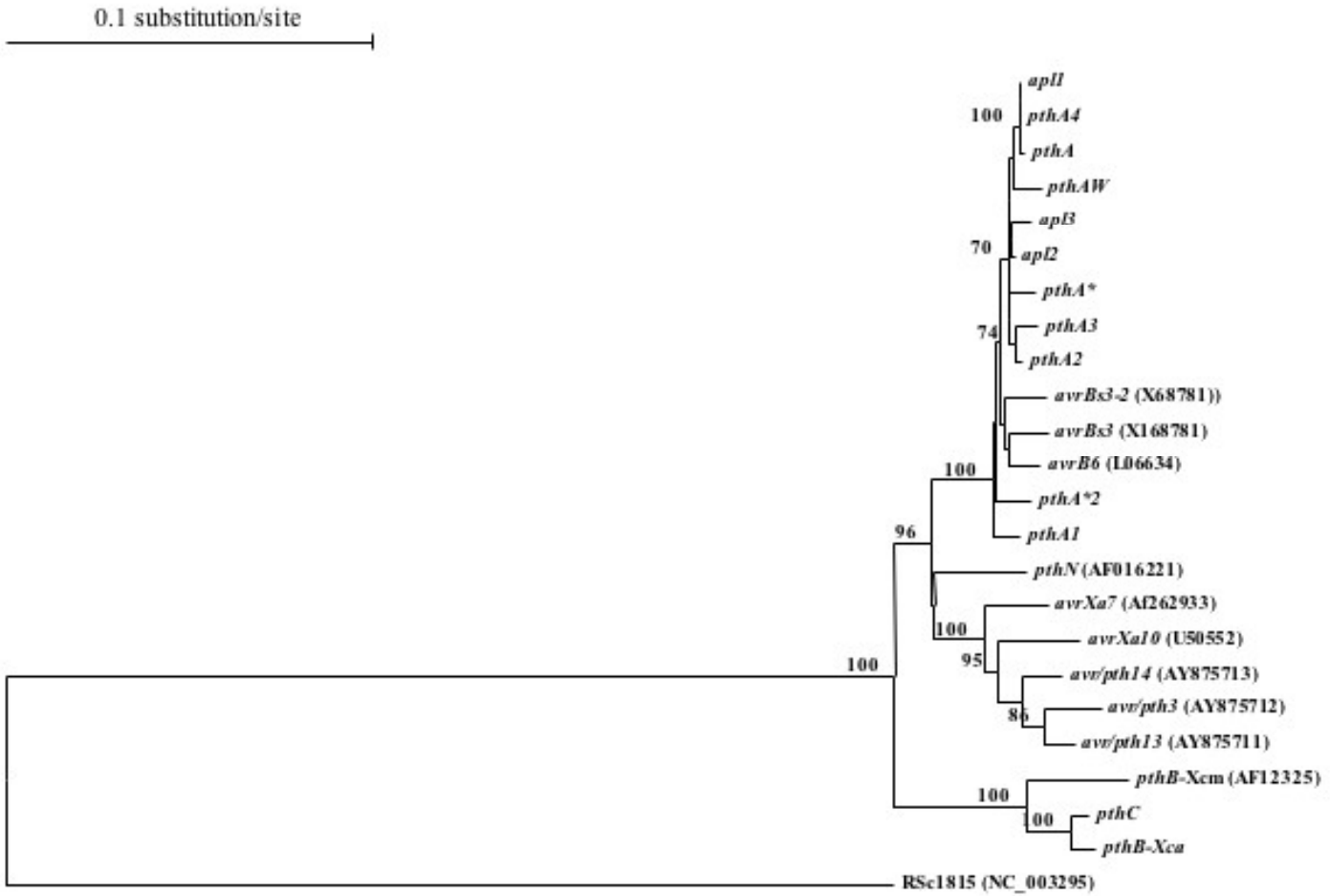


Figure 4-6. Neighbor-joining dengogram depicting phylogenetic relastionship based on pairwise comparison of neucleotide sequences of members of *avrBs3/pthA* genes from different species and pathovars of *Xanthomonas*. Numbers at the nodes represent bootstrap values (based on 100 replicates). GenBank Accesions numbers are presented to the right of the gene for genes not mentioned in material and methods.

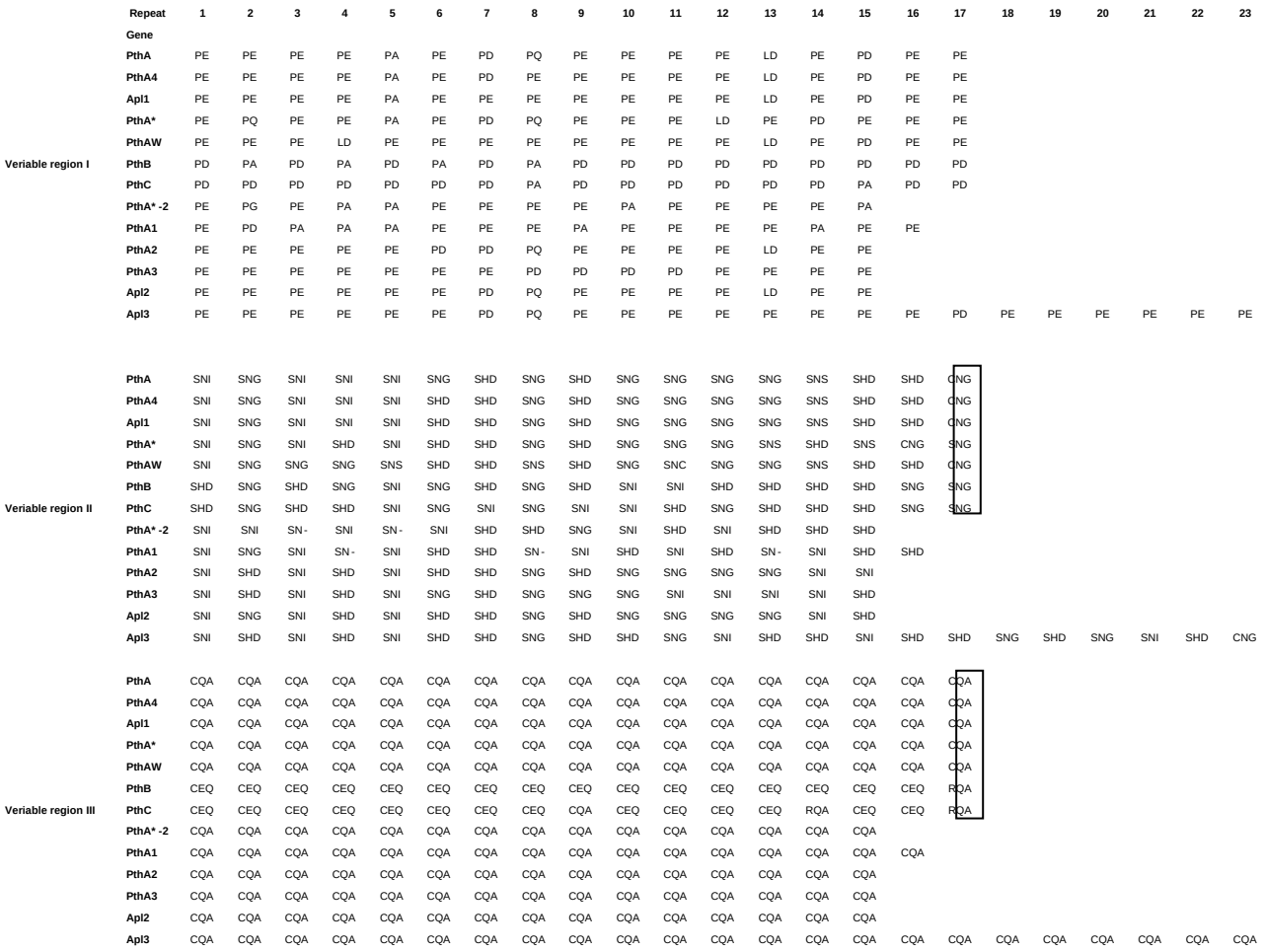


Figure 4-7. Sequence alignment of the predicted amino acids encoded in the main variable portion of the repeat region of all 13 *pthA* homologs. Boxed areas indicate the regions in the 17th repeat that are conserved among all *pthA* homologs with experimental evidence of active pathogenic function.

CHAPTER 5
SUMMARY AND CONCLUSION

The main objective of this dissertation was to study host range determination factors among all described *Xanthomonas citri* groups that are known world-wide. Five variant groups of *X. citri* have been described in the literature, and all are known from field observations to differ in host range and/or pathogenicity. In this study, all known groups were studied together in lime, grapefruit and sweet orange. All groups were readily distinguished by inoculation of only two host differentials, lime and grapefruit. The *in planta* growth of strains from two different groups that did not elicit an obvious defense response in grapefruit was found to be poor. This indicated that either these strains carry negative acting (avirulence) factors that limited growth in grapefruit, or that they are missing positive acting (pathogenicity) factors that are present in strains from groups that can attack grapefruit. The lack of a grapefruit defense response that is typical of bacterial infections limited by avirulence factors led to an attempt to identify positive pathogenicity factors.

A DNA library of an *X. citri* strain able to attack grapefruit was moved into one of the strains unable to attack grapefruit in an attempt to identify one or more positive acting host range factors. Despite using a DNA library that theoretically covered the wide host range *X. citri* genome with 99% probability, no pathogenicity factors were found, despite multiple screens of all library clones. It is possible that *in planta* growth requires multiple effectors, and that no individual cosmid would carry enough factors to reveal a strong difference. Another possibility is that Xc270 may carry *avr* genes that function in

grapefruit and prevent Xc270 from growing. Avirulence is usually epistatic over virulence and therefore a screen for positive factors would fail if this were the case.

In addition to the DNA library screen, particular attention was paid to the *pthA* homologs from all five *X. citri* strain groups, since *pthA* is known to be required by at least three strain groups for citrus canker disease. In this study, *pthA* was demonstrated to be required by the remaining strain groups. The fact that all wide host range group A strains examined carried two additional *pthA* homologs that were not present in the narrow host range B, C, A* and A^w strain groups suggested a potential role for these additional homologs in determining host range. However, when *pthA* or any of its group A homologs (*pthA1*, *pthA2* or *pthA3*) were transferred into a narrow host range group (A*) strain, no increase in host range to include grapefruit was observed.

Three new *pthA* homologs were cloned, isolated and sequenced from strains of group A* (*pthA** and *pthA*-2*) and A^w (*pthAW*) and functionally compared with *pthA* homologs previously isolated from strains of the three remaining groups: A (*pthA*), B (*pthB*) and C (*pthC*). *pthA**, *pthAW*, *pthB* and *pthC* were found to be fully isofunctional with *pthA*, and capable of eliciting the typical canker phenotype in grapefruit in complementation tests using an *X. citri* group A *pthA*- mutant strain (B21.2), even though the source A*, A^w and C strains were unable to elicit the canker phenotype in grapefruit. Furthermore, *pthA*, pthAW , pthA** and *pthC* did not elicit an avirulence phenotype of any type in B21.2, despite the fact that *pthA* homologs are all members of an avirulence gene family, and despite the avirulence of the respective source strains in grapefruit.

DNA sequence comparisons of the three new *pthA* homologs cloned, sequenced and characterized in this study with ten previously sequenced *pthA* homologs revealed

that all functional *pthA* homologs (i.e., those that are required for citrus canker disease in their respective strains) from all five known *X. citri* groups carried exactly 17.5, 102bp direct tandem repeats. All other homologs that are not functional for citrus canker pathogenicity carried a different number of repeats.

Phylogenetic comparisons of the DNA and predicted protein sequences of the thirteen available *pthA* homologs revealed the same phylogenetic distinctions that are found by more general phylogenetic studies. In addition, comparisons of the five functional *pthA* homologs from each group against those that were nonfunctional revealed that amino acids N(12)G(13) in the second and Q(31)A(32) in the third variable regions of the 17th direct tandem repeat were only conserved in functional genes. These results suggest that the 17th repeat plays a critical role in citrus canker pathogenicity and may help explain the origination of new citrus canker strains.

APPENDIX A
SEQUENCE OF *pthC*

DNA sequence of *pthC:*

atggatcccattcgtccgcgcacgtcaagtcctgcccacgaacttttggccggaccccagccggatagggttcagccg
cagccgactgcagatcgtgggggggctccgcctgctggcagccccctggatggcttgcccgctcgacggacgatgtcccgaa
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Predicted amino acid sequence of *pthC*:

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KQALETVQRLLPVLCEQHGLTPDQVVAIASNGGGKQALETVQRLLPVLCEQHGLTPDQVVAIASH

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TPDQASLHAFADALERELDAPSPIDQAGQALASSSRKRSRSESSVTGSFAQQAVEVRVPEQRDALH

LPPLSWGVKRPRTRIGGGLPDPGTPMDADLAASSTVMWEQDADPFAGAADDFPAFNEEEMAWL

MELFPQ

APPENDIX B
SEQUENCE OF *pthAW*

DNA sequence of *pthAW*:

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Predicted amino acid sequence of PthAW*:*

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SGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQA

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SAQQSFEVRVPEQRDALHLPLSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVM

REQDEDPFAGAADDFPAFNEEELAWLMELLPQ

APPENDIX C
SEQUENCE OF pthA*

DNA sequence of *pthA

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Predicted amino acid sequence of PthA*

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APPENDIX D
SEQUENCE OF *pthA*-2*

DNA sequence of *pthA*-2*

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gcaggggcagcggatgatttcccggcattcaacgaagaggagctcgcatggttgatggagctattgcctcagtga

Predicted amino acid sequence of PthA*-2

MDPIRSRTPSPARELLPGPQPDRVQPTADRGVSPPAGGPLDGLPARRTMSRT
RLPSPPAPLPAFSAGSFSDLLRQFDPSLFNTSLFDSLPPFGAHHTEAATGEWDEVQ
SGLRAADAPPPTMRVAVTAARPPRAKPAPRRRAAQPSDASPAAQVDLRTLGYSQ
QQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQHPAALGTVAVKYQDMIA
ALPEATHEAIVGVGKQWSGARALEALLTVAGELRGPPLQLDTGQLLKIAKRGGV
TAVEAVHAWRNALTGAPLNLTPEQVVAIASNIGGKQALETVQRLLPVLCQAHGL
HPGQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGKQALETV
QRLLPVLCQAHGLTPAQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPAQVVA
IASNGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLC
QAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGK
QALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLT
PAQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETV
QRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAI
ASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVL
CQAHGLTPAQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGG
RPALESIVAQLSRPDPALAALTNDHLVALACLGGRPALDAVKKGLPHAPALIKRT
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RRVGVTELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFAD
SLERDLDAPSPTHEGDQRRASSRKRSRSDRAVTGPSAQQSFEVRVPEQRDALHLP
LSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVMREQDEDPFAGAADDFPAFNE
EELAWLMELLPQ

APPENDIX E

ALIGNMENT OF PATHOGENECITY GENES FROM *X. citri* STRAINS

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                  1                                                  50
      PthA      (1) MDPIRSRTPSPARELLPGPQPDGVQP--TADRGVSPPAGGPLDGLPARRT
     PthA4      (1) MDPIRSRTPSPARELLPGPQPDGVQP--TADRGVSPPAGGPLDGLPARRT
      Apl1      (1) MDPIRSRTPSPARELLPGPQPDGVQP--TADRGVSPPAGGPLDGLPARRT
     PthAW      (1) MDPIRSRTPSPARELLPGPQPDRVQP--TADRGVSPPAGGPLDGLPARRT
     PthA*      (1) MDPIRSRTPSPARELLPGPQPDGVQP--TADRGVSPPAGGPLDGLPARRT
   PthA*-2      (1) MDPIRSRTPSPARELLPGPQPDRVQP--TADRGVSPPAGGPLDGLPARRT
     PthA1      (1) MDPIRSRTPSPARELLPGPQPDGVQP--TADRGVSPPAGGPLDGLPARRT
     PthA2      (1) MDPIRSRTPSPARELLPGPQPDGVQP--TADRGVSPPAGGPLDGLPARRT
     PthA3      (1) MDPIRSRTPSPARELLPGPQPDGVQP--TADRGVSPPAGGPLDGLPARRT
      Apl3      (1) MDPIRSRTPSPARELLPGPQPDGVQP--TADRGVSPPAGGPLDGLPARRT
      Apl2      (1) MDPIRSRTPSPARELLPGPQPDGVQP--TADRGVSPPAGGPLDGLPARRT
      PthB      (1) MDPIRPRTSSPAHELLAGPQPDRVQPQPTADRGGAPPAGSPLDGLPARRT
      PthC      (1) MDPIRPRTSSPAHELLAGPQPDRVQPQPTADRGGAPPAGSPLDGLPARRT
 Consensus      (1) MDPIRSRTPSPARELLPGPQPDGVQP  TADRGVSPPAGGPLDGLPARRT

                  51                                                 100
      PthA     (49) MSRTRLPSPPAPSPAFSAGSFSDLLRQFDPSLFNTSLFDSLPPFGAHHTE
     PthA4     (49) MSRTRLPSPPAPSPAFSAGSFSDLLRQFDPSLFNTSLFDSLPPFGAHHTE
      Apl1     (49) MSRTRLPSPPAPSPAFSAGSFSDLLRQFDPSLFNTSLFDSLPPFGAHHTE
     PthAW     (49) MSRTRLPSPPAPSPAFSAGSFSDLLRQFDPSLFNTSLFDSLPPFGAHHTE
     PthA*     (49) MSRTRLPSPPAPSPAFSAGSFSDLLRQFDPSLFNTSLFDSLPPFGAHHTE
   PthA*-2     (49) MSRTRLPSPPAPLPAFSAGSFSDLLRQFDPSLFNTSLFDSLPPFGAHHTE
     PthA1     (49) MSRTRLPSPPAPSPAFSAGSFSDLLRQFDPSLFNTSLFDSLPPFGAHHTE
     PthA2     (49) MSRTRLPSPPAPSPAFSAGSFSDLLRQFDPSLFNTSLFDSLPPFGAHHTE
     PthA3     (49) ISRTRLPSPPAPSPAFSAGSFSDLLRQFDPSLFNTSLFDSLPPFGAHHTE
      Apl3     (49) MSRTRLPSPPAPSPAFSAGSFSDLLRQFDPSLFNTSLFDSLPPFGAHHTE
      Apl2     (49) MSRTRLPSPPAPSPAFSAGSFSDLLRQFDPSLFNTSLFDSLPPFGAHHTE
      PthB     (51) MSRTRLPSPPAPLPAFSAGSFSDLLCQFDPLLLDTLLFDSMSAFGAPHTE
      PthC     (51) MSRTRLPSPPAPLPAFSAGSFSDLLCQFDPLLLDTLLFDSMSAFGAPHTE
 Consensus     (51) MSRTRLPSPPAPSPAFSAGSFSDLLRQFDPSLFNTSLFDSLPPFGAHHTE

                  101                                                150
      PthA     (99) AATGEWDEVQSGLRAADAPPPTMRVAVTAARPPRAKPAPRRRAAQPSDAS
     PthA4     (99) AATGEWDEVQSGLRAADAPPPTMRVAVTAARPPRAKPAPRRRAAQPSDAS
      Apl1     (99) AATGEWDEVQSGLRAADAPPPTMRVAVTAARPPRAKPAPRRRAAQPSDAS
     PthAW     (99) AATGEWDEVQSGLRAADAPPPTMRVAVTAARPPRAKPAPRRRAAQPSDAS
     PthA*     (99) AATGEWDEVQSGLRAADAPPPTMRVAVTAARPPRAKPAPRRRAAQPSDAS
   PthA*-2     (99) AATGEWDEVQSGLRAADAPPPTMRVAVTAARPPRAKPAPRRRAAQPSDAS
     PthA1     (99) AATGEWDEVQSGLRAADAPPPTMRVAVTAARPPRAKPAPRRRAAQPSDAS
     PthA2     (99) AATGEWDEVQSGLRAADAPPPTMRVAVTAARPPRAKPAPRRRAAQPSDAS
     PthA3     (99) AATGEWDEVQSGLRAADAPPPTMRVAVTAARPPRAKPAPRRRAAQPSDAS
      Apl3     (99) AATGEWDEVQSGLRAADAPPPTMRVAVTAARPPRAKPAPRRRAAQPSDAS
      Apl2     (99) AATGEWDEVQSGLRAADAPPPTMRVAVTAARPPRAKPAPRRRAAQPSDAS
      PthB    (101) AAPGEADEVQSGLRAVDDPHPTVHVAVTAARPPRAKPAPRRRAAHTSDAS
      PthC    (101) AAPGEADEVQSGLRAVDDPHPTVHVAVTAARPPRAKPAPRRRAAHTSDAS
 Consensus    (101) AATGEWDEVQSGLRAADAPPPTMRVAVTAARPPRAKPAPRRRAAQPSDAS
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                151                                                   200
     PthA   (149) PAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQ
    PthA4   (149) PAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQ
     Apl1   (149) PAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQ
    PthAW   (149) PAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQ
    PthA*   (149) PAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQ
  PthA*-2   (149) PAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQ
    PthA1   (149) PAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQ
    PthA2   (149) PAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQ
    PthA3   (149) PAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQ
     Apl3   (149) PAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQ
     Apl2   (149) PAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQ
     PthB   (151) PAGQVDLCTLGYSQQQQDEIKPKARATVAQHHQALMGHGFTRAHIVALSQ
     PthC   (151) PAGQVDLCTLGYSQQQQDEIKPKARATVAQHHQALMGHGFTRAHIVALSQ
Consensus   (151) PAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQ

                201                                                   250
     PthA   (199) HPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGEL
    PthA4   (199) HPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGEL
     Apl1   (199) HPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGEL
    PthAW   (199) HPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGEL
    PthA*   (199) HPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGEL
  PthA*-2   (199) HPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGEL
    PthA1   (199) HPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGEL
    PthA2   (199) HPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGEL
    PthA3   (199) HPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGEL
     Apl3   (199) HPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGEL
     Apl2   (199) HPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGEL
     PthB   (201) HPAALGTVAVKYQAMIAALPEATHEDIVGVGKQWSGARALEALLTVSGEL
     PthC   (201) HPAALGTVAVKYQAMIAALPEATHEDIVGVGKQWSGARALEALLTVSGEL
Consensus   (201) HPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGEL

                251                                                   300
     PthA   (249) RGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPEQVVAIA
    PthA4   (249) RGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPEQVVAIA
     Apl1   (249) RGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPEQVVAIA
    PthAW   (249) RGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPEQVVAIA
    PthA*   (249) RGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPEQVVAIA
  PthA*-2   (249) RGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPEQVVAIA
    PthA1   (249) RGPPLQLDTGQLLKIAKRGGVTAVEAVYAWRNALTGAPLNLTPEQVVAIA
    PthA2   (249) RGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPEQVVAIA
    PthA3   (249) RGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPEQVVAIA
     Apl3   (249) RGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPEQVVAIA
     Apl2   (249) RGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPEQVVAIA
     PthB   (251) RGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPDQVVAIA
     PthC   (251) RGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPDQVVAIA
Consensus   (251) RGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPEQVVAIA
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                301                                                        350
     PthA   (299) SNIGGK-------------------------------------------------
    PthA4   (299) SNIGGK-------------------------------------------------
     Apl1   (299) SNIGGK-------------------------------------------------
    PthAW   (299) SNIGGK-------------------------------------------------
     PthA*  (299) SNIGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLL
   PthA*-2  (299) SNIGGK-------------------------------------------------
     PthA1  (299) SNIGGK-------------------------------------------------
     PthA2  (299) SNIGGK-------------------------------------------------
     PthA3  (299) SNIGGK-------------------------------------------------
     Apl3   (299) SNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLL
     Apl2   (299) SNIGGK-------------------------------------------------
     PthB   (301) SHDGGK-------------------------------------------------
     PthC   (301) SHDGGK-------------------------------------------------
Consensus   (301) SNIGGK

                351                                                        400
     PthA   (305) ------------------------QALETVQRLLPVLCQAHGLTPEQVVA
    PthA4   (305) ------------------------QALETVQRLLPVLCQAHGLTPEQVVA
     Apl1   (305) ------------------------QALETVQRLLPVLCQAHGLTPEQVVA
    PthAW   (305) ------------------------QALETVQALLPVLCQAHGLTPEQVVA
     PthA*  (349) PVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVA
   PthA*-2  (305) ------------------------QALETVQRLLPVLCQAHGLHPGQVVA
     PthA1  (305) ------------------------QALETVQRLLPVLCQAHGLTPDQVVA
     PthA2  (305) ------------------------QALETVQALLPVLCQAHGLTPEQVVA
     PthA3  (305) ------------------------QALETVQALLPVLCQAHGLTPEQVVA
     Apl3   (349) PVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVA
     Apl2   (305) ------------------------QALETVQRLLPVLCQAHGLTPEQVVA
     PthB   (307) ------------------------QALETVQRLLPVLCEQHGLTPAQVVA
     PthC   (307) ------------------------QALETVQRLLPVLCEQHGLTPDQVVA
Consensus   (351)                          QALETVQRLLPVLCQAHGLTPEQVVA

                401                                                        450
     PthA   (331) IASNGG-KQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQR
    PthA4   (331) IASNGG-KQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQR
     Apl1   (331) IASNGG-KQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQR
    PthAW   (331) IASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQR
     PthA*  (399) IASHDGGKQALETVQRLLPVLCQAHGLTPAQVVAIASNIGGKQALETVQR
   PthA*-2  (331) IASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASN-GGKQALETVQR
     PthA1  (331) IASNGG-KQALETVQRLLPVLCQAHGLTPAQVVAIASNIGGKQALETVQR
     PthA2  (331) IASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQR
     PthA3  (331) IASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQR
     Apl3   (399) IASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQR
     Apl2   (331) IASNGG-KQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQR
     PthB   (333) IASNGGGKQALETVQQLLPVLCEQHGLTPDQVVAIASHDGGKQALETVQR
     PthC   (333) IASNGGGKQALETVQRLLPVLCEQHGLTPDQVVAIASHDGGKQALETVQR
Consensus   (401) IASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQR
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                451                                                 500
     PthA   (380) LLPVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPAQV
    PthA4   (380) LLPVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPAQV
     Apl1   (380) LLPVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPAQV
    PthAW   (381) LLPVLCQAHGLTLDQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQV
     PthA*  (449) LLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPDQV
   PthA*-2  (380) LLPVLCQAHGLTPAQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPAQV
    PthA1   (380) LLPVLCQAHGLTPAQVVAIASN-GGKQALETVQRLLPVLCQAHGLTPAQV
    PthA2   (381) LLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQV
    PthA3   (381) LLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQV
     Apl3   (449) LLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPDQV
     Apl2   (380) LLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQV
     PthB   (383) LLPVLCEQHGLTPAQVVAIASNGGGKQALETVQQLLPVLCEQHGLTPDQV
     PthC   (383) LLPVLCEQHGLTPDQVVAIASHDGGKQALETVQRLLPVLCEQHGLTPDQV
Consensus   (451) LLPVLCQAHGLTPEQVVAIASN GGKQALETVQRLLPVLCQAHGLTPDQV

                501                                                 550
     PthA   (430) VAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETV
    PthA4   (430) VAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETV
     Apl1   (430) VAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETV
    PthAW   (431) VAIASNSGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETV
     PthA*  (499) VAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETV
   PthA*-2  (430) VAIASN-GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETV
    PthA1   (429) VAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETV
    PthA2   (431) VAIASNIGGKQALETVQRLLPVLCQAHGLTPDQVVAIASHDGGKQALETV
    PthA3   (431) VAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETV
     Apl3   (499) VAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETV
     Apl2   (430) VAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETV
     PthB   (433) VAIASNIGGKQALETVQRLLPVLCEQHGLTPAQVVAIASNGGGKQALETV
     PthC   (433) VAIASNIGGKQALETVQRLLPVLCEQHGLTPDQVVAIASNGGGKQALETV
Consensus   (501) VAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASN GGKQALETV

                551                                                 600
     PthA   (480) QRLLPVLCQAHGLTPDQVVAIASHDGGK--------------------
    PthA4   (480) QRLLPVLCQAHGLTPDQVVAIASHDGGK--------------------
     Apl1   (480) QRLLPVLCQAHGLTPEQVVAIASHDGGK--------------------
    PthAW   (481) QRLLPVLCQAHGLTPEQVVAIASHDGGK--------------------
     PthA*  (549) QRLLPVLCQAHGLTPEQVVAIASHDGGK--------------------
   PthA*-2  (479) QRLLPVLCQAHGLTPEQVVAIASHDGGK--------------------
    PthA1   (479) QRLLPVLCQAHGLTPEQVVAIASHDGGK--------------------
    PthA2   (481) QRLLPVLCQAHGLTPDQVVAIASHDGGK--------------------
    PthA3   (481) QRLLPVLCQAHGLTPEQVVAIASHDGGK--------------------
     Apl3   (549) QRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPE
     Apl2   (480) QRLLPVLCQAHGLTPDQVVAIASHDGGK--------------------
     PthB   (483) QQLLPVLCEQHGLTPDQVVAIASHDGGK--------------------
     PthC   (483) QRLLPVLCEQHGLTPDQVVAIASNIGGK--------------------
Consensus   (551) QRLLPVLCQAHGLTPEQVVAIASHDGGK
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                601                                                 650
     PthA   (508) ------------------------------------------------QALET
    PthA4   (508) ------------------------------------------------QALET
     Apl1   (508) ------------------------------------------------QALET
    PthAW   (509) ------------------------------------------------QALET
     PthA*  (577) ------------------------------------------------QALET
   PthA*-2  (507) ------------------------------------------------QALET
     PthA1  (507) ------------------------------------------------QALET
     PthA2  (509) ------------------------------------------------QALET
     PthA3  (509) ------------------------------------------------QALET
     Apl3   (599) QVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGKQALET
     Apl2   (508) ------------------------------------------------QALET
     PthB   (511) ------------------------------------------------QALET
     PthC   (511) ------------------------------------------------QALET
Consensus   (601)                                                QALET

                651                                                 700
     PthA   (513) VQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTP
    PthA4   (513) VQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLTP
     Apl1   (513) VQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLTP
    PthAW   (514) VQRLLPVLCQAHGLTPEQVVAIASNSGGKQALETVQRLLPVLCQAHGLTP
     PthA*  (582) VQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLTP
   PthA*-2  (512) VQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTP
     PthA1  (512) VQRLLPVLCQAHGLTPEQVVAIASN-GGKQALETVQRLLPVLCQAHGLTP
     PthA2  (514) VQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTP
     PthA3  (514) VQRLLPVLCQAHGLTPDQVVAIASNGGGKQALETVQRLLPVLCQAHGLTP
     Apl3   (649) VQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLCQAHGLTP
     Apl2   (513) VQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTP
     PthB   (516) VQRLLPVLCEQHGLTPAQVVAIASNGGGKQALKTVQQLLPVLCEQHGLTP
     PthC   (516) VQRLLPVLCEQHGLTPAQVVAIASNGGGKQALETVQQLLPVLCEQHGLTP
Consensus   (651) VQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLTP

                701                                                 750
     PthA   (563) EQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQAL
    PthA4   (563) EQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQAL
     Apl1   (563) EQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQAL
    PthAW   (564) EQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQAL
     PthA*  (632) EQVVAIASNGGGKQALETVQRLLPVLCQAHGLTLDQVVAIASNGGGKQAL
   PthA*-2  (562) EQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPAQVVAIASNIGGKQAL
     PthA1  (561) AQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQAL
     PthA2  (564) EQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQAL
     PthA3  (564) DQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPDQVVAIASNGGGKQAL
     Apl3   (699) EQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQAL
     Apl2   (563) EQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQAL
     PthB   (566) DQVVAIASHDGGKQALETVQRLLPVLCEQHGLTPDQVVAIASNIGGKQAL
     PthC   (566) DQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPDQVVAIASNIGGKQAL
Consensus   (701) EQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQAL
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                      751                                              800
     PthA    (613) ETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQRLLPVLCQAHGL
    PthA4    (613) ETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQRLLPVLCQAHGL
     Apl1    (613) ETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQRLLPVLCQAHGL
    PthAW    (614) ETVQRLLPVLCQAHGLTPEQVVAIASNCGGKQALETVQRLLPVLCQAHGL
    PthA*    (682) ETVQRLLPVLCQAHGLTPEQVVAIASNSGGKQALETVQRLLPVLCQAHGL
  PthA*-2    (612) ETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGL
    PthA1    (611) ETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLCQAHGL
    PthA2    (614) ETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQRLLPVLCQAHGL
    PthA3    (614) ETVQRLLPVLCQAHGLTPDQVVAIASNIGGKQALETVQRLLPVLCQAHGL
     Apl3    (749) ETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLCQAHGL
     Apl2    (613) ETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQRLLPVLCQAHGL
     PthB    (616) ETVQRLLPVLCEQHGLTPDQVVAIASNIGGKQALETVQRLLPVLCEQHGL
     PthC    (616) ETVQRLLPVLCEQHGLTPDQVVAIASHDGGKQALETVQRLLPVLCEQHGL
Consensus    (751) ETVQRLLPVLCQAHGLTPEQVVAIASN GGKQALETVQRLLPVLCQAHGL

                      801                                              850
     PthA    (663) TPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLTLDQVVAIASNGGGKQ
    PthA4    (663) TPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLTLDQVVAIASNGGGKQ
     Apl1    (663) TPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLTLDQVVAIASNGGGKQ
    PthAW    (664) TPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLTLDQVVAIASNGGGKQ
    PthA*    (732) TPDQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNSGGKQ
  PthA*-2    (662) TPEQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQ
    PthA1    (661) TPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASN-GGKQ
    PthA2    (664) TPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLTLDQVVAIASNGGGKQ
    PthA3    (664) TPEQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQ
     Apl3    (799) TPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPDQVVAIASHDGGKQ
     Apl2    (663) TPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLTLDQVVAIASNGGGKQ
     PthB    (666) TPDQVVAIASHDGGKQALETVQRLLPVLCEQHGLTPDQVVAIASHDGGKQ
     PthC    (666) TPDQVVAIASNGGGKQALETVQRLLPVLCEQHGLTPDQVVAIASHDGGKQ
Consensus    (801) TPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPDQVVAIASN GGKQ

                      851                                              900
     PthA    (713) ALETVQRLLPVLCQAHGLTPEQVVAIASNSGGKQALETVQRLLPVLCQAH
    PthA4    (713) ALETVQRLLPVLCQAHGLTPEQVVAIASNSGGKQALETVQRLLPVLCQAH
     Apl1    (713) ALETVQRLLPVLCQAHGLTPEQVVAIASNSGGKQALETVQRLLPVLCQAH
    PthAW    (714) ALETVQRLLPVLCQAHGLTPEQVVAIASNSGGKQALETVQRLLPVLCQAH
    PthA*    (782) ALETVQRLLPVLCQAHGLTPEQVVAIACNGGGKQALETVQRLLPVLCQAH
  PthA*-2    (712) ALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAH
    PthA1    (710) ALETVQRLLPVLCQAHGLTPAQVVAIASNIGGKQALETVQRLLPVLCQAH
    PthA2    (714) ALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLCQAH
    PthA3    (714) ALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLCQAH
     Apl3    (849) ALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQRLLPVLCQAH
     Apl2    (713) ALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLCQAH
     PthB    (716) ALETVQRLLPVLCEQHGLTPDQVVAIASHDGGKQALETVQRLLPVLCEQH
     PthC    (716) ALETVQRLLPVLCEQHGLTPDQVVAIASHDGGKQALETVQRLLPVLRQAH
Consensus    (851) ALETVQRLLPVLCQAHGLTPEQVVAIASN GGKQALETVQRLLPVLCQAH
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                 901                                                 950
     PthA   (763) GLTPDQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIAS----
    PthA4   (763) GLTPDQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIAS----
     Apl1   (763) GLTPDQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIAS----
    PthAW   (764) GLTPDQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIAS----
     PthA*  (832) G-------------------------------------------------
   PthA*-2  (762) G-------------------------------------------------
     PthA1  (760) GLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHG---------------
     PthA2  (764) G-------------------------------------------------
     PthA3  (764) G-------------------------------------------------
     Apl3   (899) GLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGG
     Apl2   (763) G-------------------------------------------------
     PthB   (766) GLTPDQVVAIASHDGGKQALETVQRLLPVLCEQHGLTPDQVVAIAS----
     PthC   (766) GLTPAQVVAIASHDGGKQALETVQRLLPVLCEQHGLTPDQVVAIAS----
Consensus   (901) GLTPDQVVAIASHDGGKQALETVQRLLPVLC  HGLTPEQVVAIAS

                 951                                                1000
     PthA   (809) -------------------------------------------------
    PthA4   (809) -------------------------------------------------
     Apl1   (809) -------------------------------------------------
    PthAW   (810) -------------------------------------------------
     PthA*  (833) -------------------------------------------------
   PthA*-2  (763) -------------------------------------------------
     PthA1  (795) -------------------------------------------------
     PthA2  (765) -------------------------------------------------
     PthA3  (765) -------------------------------------------------
     Apl3   (949) KQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQRLLPVLCQ
     Apl2   (764) -------------------------------------------------
     PthB   (812) -------------------------------------------------
     PthC   (812) -------------------------------------------------
Consensus   (951)

                 1001                                               1050
     PthA   (809) --------------HDGGKQALETVQRLLPVLCQAHGLTPEQVVAIACNG
    PthA4   (809) --------------HDGGKQALETVQRLLPVLCQAHGLTPEQVVAIACNG
     Apl1   (809) --------------HDGGKQALETVQRLLPVLCQAHGLTPEQVVAIACNG
    PthAW   (810) --------------HDGGKQALETVQRLLPVLCQAHGLTPEQVVAIACNG
     PthA*  (833) ------------------------------------LTPEQVVAIASNG
   PthA*-2  (763) ------------------------------------LTPAQVVAIASHD
     PthA1  (795) ------------------------------------LTPEQVVAIASHD
     PthA2  (765) ------------------------------------LTPEQVVAIASNI
     PthA3  (765) ------------------------------------LTPEQVVAIASHD
     Apl3   (999) AHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIACNG
     Apl2   (764) ------------------------------------LTPEQVVAIASHD
     PthB   (812) --------------NGGGKQALETVQRLLPVLCEQHGLTPDQVVAIASNG
     PthC   (812) --------------NGGGKQALETVQRLLPVLCEQHGLTPDQVVAIASNG
Consensus  (1001)                GGKQALETVQRLLPVLC  HGLTPEQVVAIASNG
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                1051                                                1100
     PthA   (845) GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGRPALESIVAQLSRP
    PthA4   (845) GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGRPALESIVAQLSRP
     Apl1   (845) GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGRPALESIVAQLSRP
    PthAW   (846) GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGRPALESIVAQLSRP
    PthA*   (846) GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGRPALESIVAQLSRP
  PthA*-2   (776) GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGRPALESIVAQLSRP
    PthA1   (808) GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGRPALESIVAQLSRP
    PthA2   (778) GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGRPALESIVAQLSRP
    PthA3   (778) GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGRPALESIVAQLSRP
     Apl3  (1049) GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGRPALESIVAQLSRP
     Apl2   (777) GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGRPALESIVAQLSRP
     PthB   (848) GGKQALETVQRLLPVLRQAHGLTPAQVVAIASNGGGRPALESIFAQLSRP
     PthC   (848) GGKQALETVQRLLPVLRQAHGLTPAQVVAIASNGGGRPALESIFAQLSRP
Consensus  (1051) GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGRPALESIVAQLSRP

                1101                                                1150
     PthA   (895) DPALAALTNDHLVALACLGGRPALDAVKKGLPHAPALIKRTNRRIPERTS
    PthA4   (895) DPALAALTNDHLVALACLGGRPALDAVKKGLPHAPALIKRTNRRIPERTS
     Apl1   (895) DPALAALTNDHLVALACLGGRPALDAVKKGLPHAPALIKRTNRRIPERTS
    PthAW   (896) DPALAALTNDHLVALACLGGRPALDAVKKGLPHAPALIKRTNRRIPERTS
    PthA*   (896) DPALAALTNDHLVALACLGGRPALDAVKKGLPHAPALIKRTNRRIPERTS
  PthA*-2   (826) DPALAALTNDHLVALACLGGRPALDAVKKGLPHAPALIKRTNRRIPERTS
    PthA1   (858) DPALAALTNDHLVALACLGGRPALDAVKKGLPHAPALIKRTNRRIPERTS
    PthA2   (828) DPALAALTNDHLVALACLGGRPALDAVKKGLPHAPALIKRTNRRIPERTS
    PthA3   (828) DPALAALTNDHLVALACLGGRPALDAVKKGLPHAPALIKRTNRRIPERTS
     Apl3  (1099) DPALAALTNDHLVALACLGGRPALDAVKKGLPHAPALIKRTNRRIPERTS
     Apl2   (827) DPALAALTNDHLVALACLGGRPALDAVKKGLPHAPALIKRTNRRIPERTS
     PthB   (898) DQALAALTNDHLVALACLGGRPALEAVKKGLPHAPTLIKRTNRRLPERTS
     PthC   (898) DQALAALTNDHLVALACLGGRPALEAVKKGLPHAPTLIKRTNRRLPERTS
Consensus  (1101) DPALAALTNDHLVALACLGGRPALDAVKKGLPHAPALIKRTNRRIPERTS

                1151                                                1200
     PthA   (945) HRVADHAQVVRVLGFFQCHSHPAQAFDDAMTQFGMSRHGLLQLFRRVGVT
    PthA4   (945) HRVADHAQVVRVLGFFQCHSHPAQAFDDAMTQFGMSRHGLLQLFRRVGVT
     Apl1   (945) HRVADHAQVVRVLGFFQCHSHPAQAFDDAMTQFGMSRHGLLQLFRRVGVT
    PthAW   (946) HRVADHAQVVRVLGFFQCHSHPAQAFDDAMTQFGMSRHGLLQLFRRVGVT
    PthA*   (946) HRVADHAQVVRVLGFFQCHSHPAQAFDDAMMQFGMSRHGLLQLFRRVGVT
  PthA*-2   (876) HRVADHAQVVRVLGFFQCHSHPAQAFDDAMTQFGMSRHGLLQLFRRVGVT
    PthA1   (908) HRVADHAQVVRVLGFFQCHSHPAQAFDDAMTQFGMSRHGLLQLFRRVGVT
    PthA2   (878) HRVADHAQVVRVLGFFQCHSHPAQAFDDAMTQFGMSRHGLLQLFRRVGVT
    PthA3   (878) HRVADHAQVVRVLGFFQCHSHPAQAFDDAMTQFGMSRHGLLQLFRRVGVT
     Apl3  (1149) HRVADHAQVVRVLGFFQCHSHPAQAFDDAMTQFGMSRHGLLQLFRRVGVT
     Apl2   (877) HRVADHAQVVRVLGFFQCHSHPAQAFDDAMTQFGMSRHGLLQLFRRVGVT
     PthB   (948) HRVADHAQVARVLGFFQCHSHPAQAFDEAMTQFGMSRHGLLQLFRRVGVT
     PthC   (948) HRVADHAQVARVLGFFQCHSHPAQAFDEAMTQFGMSRHGLLQLFRRVGVT
Consensus  (1151) HRVADHAQVVRVLGFFQCHSHPAQAFDDAMTQFGMSRHGLLQLFRRVGVT
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                  1201                                                 1250
     PthA   (995) ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSL
    PthA4   (995) ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSL
     Apl1   (995) ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSL
    PthAW   (996) ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSL
    PthA*   (996) ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSL
  PthA*-2   (926) ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSL
    PthA1   (958) ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSL
    PthA2   (928) ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSL
    PthA3   (928) ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSL
     Apl3  (1199) ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSL
     Apl2   (927) ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSL
     PthB   (998) ELEARGGTLPPAPQRWHRILQASGMKRAEPSGASAQTPDQASLHAFADAL
     PthC   (998) ELEARGGTLPPAPQRWHRILQASGMKRAEPSGASAQTPDQASLHAFADAL
Consensus  (1201) ELEARSGTLPPASQRWDRILQASGMKRAKPSPTSTQTPDQASLHAFADSL

                  1251                                                 1300
     PthA  (1045) ERDLDAPSPTHEGDQRRASS-RKRSRSDRAVTGPSAQQSFEVRAPEQRDA
    PthA4  (1045) ERDLDAPSPTHEGDQRRASS-RKRSRSDRAVTGPSAQQSFEVRVPEQRDA
     Apl1  (1045) ERDLDAPSPTHEGDQRRASS-RKRSRSDRAVTGPSAQQSFEVRAPEQRDA
    PthAW  (1046) ERDLDAPSPTHEGDQRRASS-RKRSRSDRAVTGPSAQQSFEVRVPEQRDA
    PthA*  (1046) ERDLDAPSPTHEGDQRRASS-RKRSRSDRAVTGPSAQQSFEVRVPEQRDA
  PthA*-2   (976) ERDLDAPSPTHEGDQRRASS-RKRSRSDRAVTGPSAQQSFEVRVPEQRDA
    PthA1  (1008) ERDLDAPSPTHEGDQRRASS-RKRSRSDRAVTGPSAQQSFEVRVPEQRDA
    PthA2   (978) ERDLDAPSPMHEGDQTRASS-RKRSRSDRAVTGPSAQQSFEVRVPEQRDA
    PthA3   (978) ERDLDAPSPTHEGDQRRASS-RKRSRSDRAVTGPSAQQSFEVRVPEQRDA
     Apl3  (1249) ERDLDAPSPTHEGDQRRASS-RKRSRSDRAVTGPSAQQSFEVRAPEQRDA
     Apl2   (977) ERDLDAPSPTHEGDQRRASS-RKRSRSDRAVTGPSAQQSFEVRAPEQRDA
     PthB  (1048) ERELDAPSPIDQAGQALASSSRKRSRSESSVTGSFAQQAVEVRVPEQRDA
     PthC  (1048) ERELDAPSPIDQAGQALASSSRKRSRSESSVTGSFAQQAVEVRVPEQRDA
Consensus  (1251) ERDLDAPSPTHEGDQRRASS RKRSRSDRAVTGPSAQQSFEVRVPEQRDA

                  1301                                                 1350
     PthA  (1094) LHLP-LSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVMREQDEDPFAGA
    PthA4  (1094) LHLP-LSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVMREQDEDPFAGA
     Apl1  (1094) LHLP-LSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVMREQDEDPFAGA
    PthAW  (1095) LHLP-LSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVMREQDEDPFAGA
    PthA*  (1095) LHLP-LSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVMREQDEDPFAGA
  PthA*-2  (1025) LHLP-LSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVMREQDEDPFAGA
    PthA1  (1057) LHLP-LSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVMREQDEDPFAGA
    PthA2  (1027) LHLP-LSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVMREQDEDPFAGA
    PthA3  (1027) LHLP-LSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVMREQDEDPFAGA
     Apl3  (1298) LHLP-LSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVMREQDEDPFAGA
     Apl2  (1026) LHLP-LSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVMREQDEDPFAGA
     PthB  (1098) LHLPPLSWGVKRPRTRIGGGLPDPGTPMDADLAASSTVMWEQDADPFAGA
     PthC  (1098) LHLPPLSWGVKRPRTRIGGGLPDPGTPMDADLAASSTVMWEQDADPFAGA
Consensus  (1301) LHLP LSWRVKRPRTSIGGGLPDPGTPTAADLAASSTVMREQDEDPFAGA
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                  1351             1371
      PthA  (1143) ADDFPAFNEEELAWLMELLPQ
     PthA4  (1143) ADDFPAFNEEELAWLMELLPQ
      Apl1  (1143) ADDFPAFNEEELAWLMELLPQ
     PthAW  (1144) ADDFPAFNEEELAWLMELLPQ
     PthA*  (1144) ADDFPAFNEEELAWLMELLPQ
   PthA*-2  (1074) ADDFPAFNEEELAWLMELLPQ
     PthA1  (1106) ADDFPAFNEEELAWLMELLPQ
     PthA2  (1076) ADDFPAFNEEELAWLMELLPQ
     PthA3  (1076) ADDFPAFNEEELAWLMELLPQ
      Apl3  (1347) ADDFPAFNEEELAWLMELLPQ
      Apl2  (1075) ADDFPAFNEEELAWLMELLPQ
      PthB  (1148) ADDFPAFNEEEMAWLMELFPQ
      PthC  (1148) ADDFPAFNEEEMAWLMELFPQ
Consensus  (1351) ADDFPAFNEEELAWLMELLPQ
```

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BIOGRAPHICAL SKETCH

Abdulwahid Al-Saadi was born January 25th, 1971, in Mombasa, Kenya. His family moved back to Oman in 1978. He obtained his primary and secondary education in Oman. He earned his Bachelor of Science degree in agriculture at Sultan Qaboos University (S.Q.U) in Oman in October of 1995. After graduating S.Q.U. he worked for the Diwan of Royal court. In 1997 he got a scholarship by the Diwan of Royal Court to pursue the Doctor of Philosophy degree in the field of plant molecular and cellular biology at the University of Florida. After graduating from University of Florida, Abdulwahid will go back to Oman and continue working for the Diwan of Royal Court.