

Pathogen profile

Xanthomonas citri: breaking the surface

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SUMMARY

Taxonomy: Bacteria; Proteobacteria, Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae, Xanthomonas.

Microbiological properties: Gram-negative, obligately aerobic, straight rods, motile by a single polar flagellum, yellow pigment.

Related species: *X. campestris*, *X. axonopodis*, *X. oryzae*, *X. albilineans*.

Host range: Affects Rutaceous plants, primarily *Citrus* spp., *Fortunella* spp., and *Poncirus* spp., world-wide. Quarantined pathogen in many countries. Economically important hosts are cultivated orange, grapefruit, lime, lemon, pomelo and citrus rootstock.

Disease symptoms: On leaves, first appearance is as oily looking, 2–10 mm, similarly sized, circular spots, usually on the abaxial surface. On leaves, stems, thorns and fruit, circular lesions become raised and blister-like, growing into white or yellow spongy pustules. These pustules then darken and thicken into a light tan to brown corky canker, which is rough to the touch. On stems, pustules may coalesce to split the epidermis along the stem length, and occasionally girdling of young stems may occur. Older lesions on leaves and fruit tend to have more elevated margins and are at times surrounded by a yellow chlorotic halo (that may disappear) and a sunken centre. Sunken craters are especially noticeable on fruit, but the lesions do not penetrate far into the rind. Defoliation and premature abscission of affected fruit occurs on heavily infected trees.

Useful websites: <<http://www.biotech.ufl.edu/PlantContainment/canker.htm>>; <<http://cancer.lbi.ic.unicamp.br/xanthomonas/>>

INTRODUCTION TO XANTHOMONAS: A PLANT/MICROBE SPECIFICITY SMORGASBORD

Of all phytopathogenic bacteria, members of the genus *Xanthomonas* exhibit the highest level by far of both host range

and race specificity. Unlike *Rhizobium*, *Agrobacterium*, *Pseudomonas*, *Ralstonia* and *Erwinia*, members of the genus *Xanthomonas* are always plant-associated (although not always pathogenic); none are free-living or soil-borne and nearly all growth is endophytic. Although the genus itself has a very wide host range that includes at least 68 plant families and more than 240 genera (Hayward, 1993), any given strain is limited to a very narrow range of hosts, often a group of genera within one plant family or a group of species within one genus. This makes *Xanthomonas* an ideal genus for the study of plant/microbe specificity.

Some operational definitions and terminology used in discussing the genetics of host/pathogen specificity may be needed to clarify meaning in discussing the genetics of the various citrus canker causing xanthomonads. By 'host range' is meant the plant species or group of species that may serve as hosts for the pathogen. In *Xanthomonas*, host range is usually indicated by the pathovar status. Within the genus *Xanthomonas*, there are over 140 different pathovars (Hayward, 1993). Thus, *X. campestris* pv. *malvacearum* as a group has a host range on malvaceous species such as cotton. However, the cotton blight strains within pathovar *malvacearum* cannot attack hibiscus (a malvaceous species), nor can the hibiscus leaf spotting strains within pathovar *malvacearum* attack cotton (Lazo *et al.*, 1987). Pathovar status with xanthomonads is therefore only a rough and imperfect indication of host range. Pathovar groups can be further subdivided into races.

Host range and race specificity are not the same. By 'race specificity' is meant the cultivars within a host species that a given race within a pathovar can attack. Thus, *X. campestris* pv. *malvacearum* (cotton blight) race 1 strains can attack a given set of cotton lines, each of which differs by at least one resistance (*R*) gene. Race 2 strains of the same pathovar can attack a different set of cotton lines, and so on. Race specificity is determined by microbial avirulence (*avr*) genes (Leach and White, 1996). Microbial *avr* genes may be thought of as encoding 'bullets' that strike highly specific 'targets' encoded by host *R* genes. When an *R* gene encoded 'target' is struck by a cognate microbial *avr* gene encoded 'bullet', then an otherwise pathogenic or 'compatible' interaction becomes incompatible: the host is called 'resistant'

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and the pathogen is called 'avirulent' on that particular host line. On other host lines, without the cognate, 'gene-for-gene' *R* gene, the pathogen is called 'virulent'. Of course, plant breeders look for, and deliberately select for *R* genes, rendering a pathogen avirulent on the selected breeding lines. Pathogenicity and virulence are not the same. Making the pathogen avirulent on a particular host line does not make the pathogen non-pathogenic. It retains pathogenicity—and is called virulent—on any line without the selected *R* gene. For a more detailed discussion of these concepts and definitions, see Gabriel (2001a).

In any host where there has been resistance breeding against a given *Xanthomonas* pathovar, races have emerged as variants within the pathovar, usually by mutation or loss of specific *avr* genes. By contrast with cotton blight disease, there has been no breeding of citrus for resistance against canker disease, and consequently there are no races of the pathogen. However, this does not mean that the citrus canker pathogens do not carry *avr* genes; indeed, as discussed below, the first fully sequenced strain has no shortage of *avr* genes and an understanding of their function, mutability and mobility may be highly relevant to future control strategies. Transgenic technologies and cloned *R* genes from other plants are becoming available and offer hopeful alternative citrus canker controls to the current practice of eradication by burning infected and exposed trees.

The term 'avirulence' is an operational term, not a description of biochemical function. The term obscures the likely functional purpose, which is to condition pathogenicity, but in a highly host specific way. A key discovery in understanding *avr* function was the elucidation of the type III secretion (T3S) machine, encoded in plant pathogens by hypersensitivity response and pathogenicity (*hrp*) genes (for reviews, see Alfano and Collmer, 1996, 1997; Cornelis and VanGijsegem, 2000; He, 1998). This secretion machine is somewhat misnamed; in both animal and plant pathogens, it is a close contact-dependent (Marenda *et al.*, 1998), generalized eukaryotic cell injection device, used to literally inject highly adapted pathogenicity effector proteins into both host cells and non-host cells (Kubori *et al.*, 1998; Silhavy, 1997; Jin and He, 2001). To extend the metaphor, the *hrp* genes form the T3S gun that fires host-specific effector bullets through the plant cell wall, but only when the gun barrel is pressed through or at least tightly against the plant cell wall. These host-specific effector proteins then elicit the diverse programmed phenotypes of the various different pathogenicity responses in the hosts to which they are specifically adapted. However, the genes encoding these effectors are often located on mobilizable plasmids and so can transfer horizontally between strains of different pathovars and species. Depending upon the plant/pathogen context, these same effectors can also elicit the plant hypersensitive response (HR) in hosts and non-hosts alike.

One of the most salient facts about the T3S system is that it is highly indiscriminate, and will inject whatever effector proteins

are available, even including some from animal pathogens (for example, see Anderson *et al.*, 1999; Rossier *et al.*, 1999). Plant cells need not be host cells to activate the system, since the ability to inject T3S effectors into non-host plant cells provides the basis for the non-host HR (Klement, 1963), which has long been used as a general indicator of pathogenicity. The demonstrated practical utility of the non-host HR test for pathogenicity implies that with few exceptions, intimate contact, attachment and injection are not (very) host specific. Host range is therefore not determined by attachment and the T3S machine *per se*, but is instead determined in part by the effector proteins that are delivered into plant cells. The effectors can be either Avr or Pth proteins, depending on context, and it is the effectors that exhibit the high level of host specificity, not the T3S system. To stretch the metaphor further, the T3S machine gun is highly indiscriminate when it comes to ordnance; it will fire whatever effectors are available into plant cells, regardless of whether the plant cell is host or non-host.

A great many T3S dependent Avr proteins have been identified, probably because the resulting HR phenotype is so readily assayed, even on non-hosts. By contrast, only a relatively few T3S dependent Pth proteins have been identified and shown to be necessary to condition pathogenicity on a given host. The largest number of *avr* genes cloned to date from any phytopathogenic microbe are from the genus *Xanthomonas* (Gabriel, 1999a). A single citrus canker strain is the source of three functional *pthA* genes (Kanamori and Tsuyumu, 1998; Swarup *et al.*, 1991) but the recently completed citrus canker genomic sequence reveals an even larger number of putative *avr* genes (see below). Why do pathogens carry *avr* genes—even pathogens such as *X. citri*, which lack races—and why so many? Nearly all *avr* genes examined to date are functionally T3S dependent, indicating that they should have a function in support of pathogenicity. Yet most *avr* genes are not evidently needed for pathogenicity on hosts. Since *avr* genes can horizontally transfer into strains adapted to different hosts, they may often be out-of-context pathogenicity genes that encode proteins that are sometimes detected by the non-host plant cell as bad news and result in an HR. For a more detailed discussion of why pathogens carry *avr* genes, refer to Gabriel (1999b).

Host range is not (always) exclusively determined by *avr/pth* genes. Host range can also be determined, in part, by the composition of the lipopolysaccharide (LPS) found as a unique structural feature of Gram-negative bacterial outer membranes. The LPS may serve as a structural permeability barrier against toxic plant defence compounds. A gene affecting the LPS structure in *X. campestris* pv. *citrumelo* (leaf spot of citrus), is essential for pathogenicity and growth in citrus hosts, but not in common bean hosts (Kingsley *et al.*, 1993). There are undoubtedly other factors that also influence host range.

Table 1 Relative pathogenicity of all known *X. citri* strain groups on four Citrus species.

Canker group	<i>C. sinensis</i> (Sweet orange)	<i>C. paradisi</i> (Grapefruit)	<i>C. limon</i> (Lemon)	<i>C. aurantifolii</i> (Mexican lime)
<i>X. citri</i> pv. citri A	+++	++++	+++	++++
<i>X. citri</i> pv. citri A*	–	–	–	++++
<i>X. citri</i> pv. citri A ^W	–	HR	–	++++
<i>X. citri</i> pv. aurantifolii B	+	+	+++	++++ (white)
<i>X. citri</i> pv. aurantifolii C	HR	HR	HR	++++

+ weak canker; ++++ strong canker; – no symptoms; HR, hypersensitive response.

INTRODUCTION TO CITRUS CANKER DISEASE: MOLECULAR AND DESCRIPTIVE BIOLOGY

Citrus canker disease is caused by two groups of phylogenetically distinct *Xanthomonas* strains, discussed in the taxonomy section below. The two phylogenetically distinct groups cause identical symptoms, implying a common pathogenicity mechanism. Naturally occurring variation among these strains includes differences in host range that do not seem to involve an HR or active plant defence, although citrus plants can exhibit an HR, and some citrus canker strains elicit an HR on some citrus species (Table 1). There are no races of citrus canker, probably because the conventional breeding of citrus trees for resistance is all but impossible.

All cultivars of citrus are susceptible to canker. Grapefruit, Mexican lime and lemon are highly susceptible. Sour orange and sweet orange are moderately susceptible. Mandarins, on the other hand, are termed moderately resistant in the literature (for example, see Gottwald *et al.*, 2002). It deserves emphasis that literature reports of the field resistance of some varieties of citrus does not appear to be due to active physiological resistance. In extensive comparisons of 54 citrus species, cultivars and relatives, no significant level of resistance was detected when the citrus was inoculated with *X. citri* by wounding (Gottwald *et al.*, 1993). The resistance observed in field situations has instead been correlated with the size and number of stomatal openings available to allow entry of the canker bacteria into the plant by wind-blown rain (Goto, 1969; McLean and Lee, 1922). In addition, the observed field resistance appears to be correlated with a relative lack of 'aggressive' growth habits of some citrus species, such as frequent and large foliage flushing. It is well known that tender, immature, freshly flushed citrus leaves and stems are much more susceptible to canker than mature citrus (Stall *et al.*, 1982). Therefore, a citrus scion grafted on to an 'aggressive' rootstock, such as Volka Mariana, is much more susceptible to canker than that same scion grafted on to a less 'aggressive' rootstock, such as Swingle. The lack of effective physiological resistance to canker in citrus makes the likelihood of finding any sort of canker resistance gene in citrus unlikely, because all cultivars of citrus are highly susceptible in artificial inoculations.



Fig. 1 Typical, naturally occurring citrus canker lesions from a relatively heavy infestation, causing premature leaf and fruit abscission.

Citrus canker disease is characterized by the formation of circular, water soaked lesions that become raised and blister-like, growing into white or yellow spongy pustules that then darken and thicken into a light tan to brown corky canker which is rough to the touch. On heavily infected trees, citrus canker causes defoliation and premature fruit drop; an essential diagnostic symptom is citrus tissue hyperplasia (Fig. 1; Gabriel, 2001b). The disease is one of the most economically damaging diseases affecting citrus world-wide, and is subject to strictly enforced quarantine and eradication laws in the USA and in many other countries. In Florida, a diagnosis of citrus canker results in regulatory actions that force the immediate removal and destruction of infected trees and also all ('exposed') citrus trees within a 1900 ft. radius of the infected tree (an area of *c.* 0.4 square miles). For a recent comprehensive review on the epidemiology of citrus canker disease with particular reference to the continuing Florida eradication efforts, refer to Gottwald *et al.* (2002).

X. citri enters its hosts naturally by rain splash directly through stomata or by way of wounds; there is no evident epiphytic growth stage. Disease initiation by T3S requires tight attachment to host mesophyll cells, either by way of *hrp* pili (He, 1998) or by way of type IV pili (see below). Type IV pili are known to be essential in *Ralstonia* pathogenicity (Kang *et al.*, 2002), and are significant because of their ability to attach to a variety of surfaces and retract (Skerker and Berg, 2001). Tight attachment of *X. campestris*

pv. malvacearum to cotton cell walls is observed within 48 h of inoculation (Al-Mousawi *et al.*, 1982). Although equivalent ultrastructural studies have not been published for *X. citri*, *X. citri* is closely related to *X. campestris* pv. malvacearum by DNA-DNA hybridization (Egel *et al.*, 1991), and such tight attachment is also likely for *X. citri*. *X. citri* induces cell divisions in citrus within 72 h of inoculation (Lawson *et al.*, 1989), presumably by injecting the cell division signalling molecule PthA (discussed in detail below), by way of T3S. The evidence to support this presumption is as follows: (i) mutations of either *hrp* genes (El Yacoubi *et al.*, 2001; Yang and Gabriel, 1995a) or of *pthA* in *X. citri* abolish canker pathogenicity (Swarup *et al.*, 1991), (ii) *E. coli* carrying both a *hrp* cluster and a *pthA* homologue causes canker-like symptoms on citrus (Kanamori and Tsuyumu, 1998), and (iii) when *pthA* is expressed by itself in citrus cells, visible cankers are formed within 10–14 days (Duan *et al.*, 1999). In wild-type *X. citri* infections, large pustules or cankers are formed on leaves, stems and fruit as the pathogen induces cell enlargement (hypertrophy) and division (hyperplasia) among contacted host cells (Lawson *et al.*, 1989; Swarup *et al.*, 1991; Fig. 1).

Mutations of *pthA* cause: (i) a loss of ability to cause hyperplastic cankers, (ii) loss of ability to cause water soaking and (iii) a significant loss of the pathogen's ability to grow *in planta* (Swarup *et al.*, 1991). The loss of ability to grow *in planta* indicates that *pthA* may be necessary for nutrient release from citrus (refer to '*in silico* research speculations', below). The loss of water soaking is interesting because water soaking is caused by many, if not most, xanthomonads, including those without members of the *avrBs3/pthA* gene family, such as *X. campestris* pv. citrumelo, which causes water soaked leaf spots of juvenile citrus (Gabriel *et al.*, 1989; Swarup *et al.*, 1991). Water soaking by *X. citri* is one of the first macroscopic symptoms observed about 4 days after inoculation, coincident with the loss of free intercellular space due to cell swelling and cell division. This brings the cell walls much closer together, to the point of contact. Such a dramatic loss of free space provides a potential mechanism for increased water uptake from the xylem through capillary action (Popham *et al.*, 1993). If the remaining intercellular spaces are filled with bacteria producing xanthan gum, this water will be trapped as the highly hygroscopic xanthan gum hydrates and swells. About 8 days after inoculation, the epidermis is ruptured, allowing bacterial egress to the plant surface (Fig. 1D), where bacteria are readily available for rain splash and repeated infection cycles. PthA is also responsible for necrosis of the abaxial epidermal cell layer of citrus immediately adjacent to the infected area (Duan *et al.*, 1999), which obviously favours pathogen egress by destroying the integrity of the epidermal layer.

Other pathogenicity genes are likely to be turned on as cell numbers increase, since 'quorum sensing' genes have been found in *X. citri* that in *X. campestris* are associated with the transcriptional regulation of pathogenicity genes (see '*in silico* research

speculations', below). Most interesting is the fact that xanthan gum genes are up-regulated by quorum sensing (Tang *et al.*, 1991). Maximum *X. citri* bacterial cell counts are coincident with epidermal rupture (Swarup *et al.*, 1991). Leaf wetting causes additional hydration of the overproduced xanthan and egress of large numbers of *X. citri* bacteria from infected leaf surfaces (Yang *et al.*, 1994). Wind-blown rain and mechanical transmission complete the life cycle.

Two phylogenetically distinct groups that cause identical canker symptoms

There are two phylogenetically distinct groups of xanthomonads that cause citrus canker disease (Gabriel, 2001b). Each group contains subgroups that are differentiated primarily on the basis of host range (Table 1). The first phylogenetically distinct group is the Asiatic group, named *Xanthomonas citri* ex Hasse (Gabriel *et al.*, 1989; synonyms: *X. campestris* pv. citri Dye pathotype A and *X. axonopodis* pv. citri Vauterin). The second phylogenetically distinct group is the South American group, named *X. campestris* pv. aurantifolii Gabriel (Gabriel *et al.*, 1989; syn = *X. campestris* pv. citri Dye and *X. axonopodis* pv. aurantifolii Vauterin). Different names for quarantined pathogens that cause an identical disease are confusing, and the confusion is made worse by the fact that the different quarantined citrus canker pathogens are currently lumped into species (*X. campestris* or *X. axonopodis*) that are primarily comprised of non-quarantined pathogens. A change in nomenclature is needed, retaining *X. citri* ex Hasse as a species that includes only citrus canker (hyperplasia-causing) pathogens.

Currently, the only taxonomic distinction between *Xanthomonas* pathogens that are subject to quarantine and those that are not is an infrasubspecific ranking—their 'pathovar' designation. Yet infrasubspecific ranks have no official standing in bacterial nomenclature (Staley and Krieg, 1984) and are often abused by the common practice of placing strains in pathovars '... solely on the basis of the reported host-plant-from-which-isolated' (Starr, 1983). Since any xanthomonad found on citrus, including opportunistic ones, can legitimately be called '*X. campestris* pv. citri' or '*X. axonopodis* pv. citri', regulatory agencies can and have taken costly action which later proved unwarranted (refer Gabriel, 2001b). Because of the need for a name for canker-causing strains that has nomenclatural standing and that reflects a quarantined pathogen's status, we use *X. citri* (Gabriel *et al.*, 1989) to refer to any citrus canker (hyperplasia) causing *Xanthomonas* strain. The name is validly published and on the 'Approved Lists of Bacterial Names' (Euzéby, 1999; Skerman *et al.*, 1989). We use *X. citri* pv. citri to refer to the Asiatic groups and *X. citri* pv. aurantifolii to refer to the South American groups.

By far the most commonly widespread group of *X. citri* pv. citri strains is the main Asiatic group A, with a host range on all citrus

varieties. Much less commonly found is the Asiatic A* group from south-west Asia (Verniere *et al.*, 1998). Most recently a new Asiatic strain, designated A^W, was found in one location in Florida (Sun *et al.*, 2000). Both the A* group and A^W strains are limited in host range to Mexican lime; the reason for this limitation is unknown. *X. citri* pv. *aurantifolii* is comprised of at least two groups of strains, both evidently originating in S. America. Symptoms caused by this pathovar are identical to those caused by *X. citri* pv. *citri*, but all strains of this pathovar are somewhat limited in host range: the *X. citri* pv. *aurantifolii* B group strains are primarily found on lemon and lime and the C group strains are limited to Mexican lime; again, the reason for the limitations are unknown. The host range of the various groups is summarized in Table 1 (format adapted from Stall and Seymour, 1983).

The canker pathogenicity determinant, PthA, is citrus specific and required for canker

A gene for pathogenicity, *pthA*, is necessary for *X. citri* pv. *citri* A to cause citrus canker disease, and the insertion of *pthA* into other xanthomonads confers an ability to elicit cankers on citrus (Swarup *et al.*, 1991). *pthA* is required for the production of necrotic cankers by any strain of *Xanthomonas* that causes canker on citrus, even those xanthomonads that are phylogenetically and taxonomically distinct. Significantly, *pthA* or a

homologue is present in every xanthomonad that causes citrus canker disease (Cubero and Graham, 2002; Swarup *et al.*, 1992). Multiple alleles are also always present in all strains causing canker disease and are never present in xanthomonads isolated from citrus that do not cause canker, which provides a useful diagnostic tool. Three *pthA* alleles, in addition to *pthA*, are found in all *X. citri* pv. *citri* group A strains examined; two of the alleles are slightly functional to elicit canker on citrus, and one appears to be non-functional (Kanamori and Tsuyumu, 1998; Swarup *et al.*, 1992).

Three fully functional *pthA* homologues have been cloned from *X. citri* pv. *aurantifolii*: *pthB* and *pthC* from groups B and C, respectively (Gabriel lab, unpublished), and *pthW* from *X. citri* pv. *citri* A^W (Al-Saadi and Gabriel, 2002). The *pthA*, *pthB*, *pthC* and *pthW* homologues are fully isofunctional and they are absolutely required for the xanthomonad that carries them to cause hyperplastic canker symptoms. Although *pthC* and *pthW* are from two different strains that are restricted to lime, when each is moved into an *X. citri* pv. *citri* A strain that is deficient for *pthA*, both complemented the strain for full pathogenicity on all citrus types. Therefore, *pthC* and *pthW* do not limit the host range of their respective strains to lime.

Not only is *pthA* essential for citrus canker disease, but when expressed—by itself—inside host cells, it is also sufficient for the diagnostic symptoms of citrus canker disease: hypertrophy, hyperplasia and cell death (Duan *et al.*, 1999; refer Fig. 2B). The

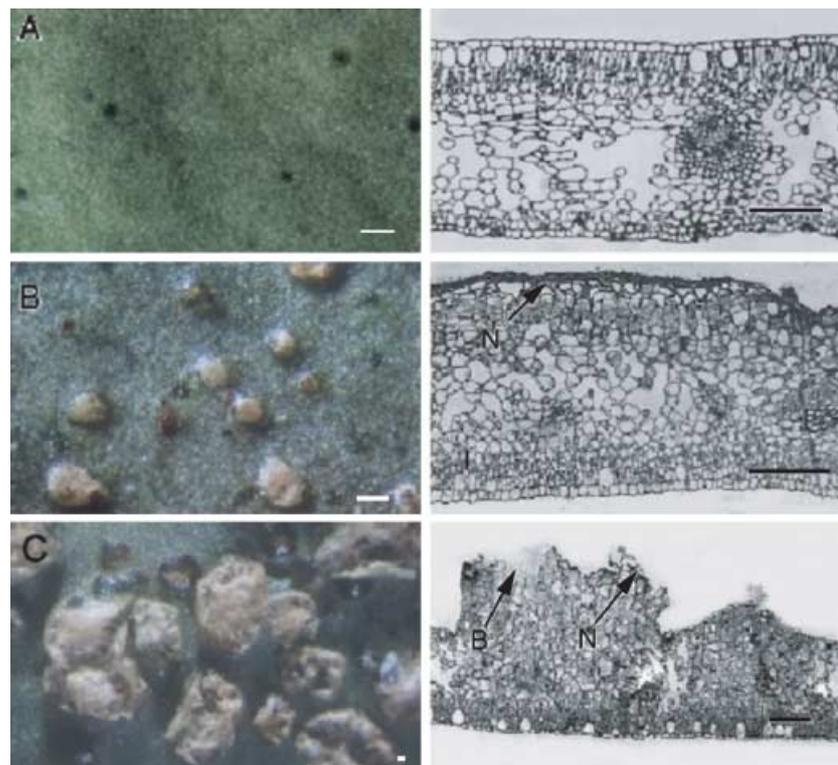


Fig. 2 Comparisons of canker symptoms on the citrus leaf surface and thin-sections through leaves. (A) Normal citrus leaf. (B) citrus leaf following particle bombardment with *pthA* under control of a plant promoter. (C) citrus leaf following artificial inoculation with *X. citri*. Note the loss of free space in the spongy mesophyll region, the necrosis (N) of the abaxial epidermal layer, and *X. citri* bacteria (B) oozing from the artificial inoculation. (Figure excerpted from Duan *et al.*, 1999.)

transient expression of *pthA* in citrus leaves induced small cankers on leaf surfaces 10–14 days after particle bombardment (Fig. 2B) or *Agrobacterium* inoculation (Duan *et al.*, 1999). Cell division and cell enlargement were caused when *pthA* was delivered to citrus cells by either method, and the symptoms elicited were qualitatively typical of the symptoms induced by control *X. citri* inoculations, although *X. citri* induced much larger lesions (Fig. 2C). A progressive development of necrosis in the abaxial epidermal tissue layer was also observed in these transient expression assays, similar to control inoculations using *X. citri*, but delayed and greatly reduced (Fig. 2B,C). The induction of necrosis by *pthA* alone strongly indicates that *pthA* causes programmed cell death (for reviews, see Greenberg, 1997; Gilchrist, 1998) in citrus. Furthermore, rupture of the citrus epidermis was observed when *pthA* was delivered by *A. tumefaciens*, but it was greatly reduced compared to *X. citri* controls (Duan *et al.*, 1999).

PthA can also cause canker when delivered by *E. coli*. A gene that encodes a predicted amino acid sequence identical to *PthA*, *aplI*, was isolated and cloned from a Japanese strain of *X. citri* pv. *citri* A (Kanamori and Tsuyumu, 1998). *E. coli* carrying the *hrp* cluster from *P. syringae* pv. *syringae* together with *aplI* caused citrus-canker like symptoms on *Citrus natsudaidai* (Kanamori and Tsuyumu, 1998).

The pathogenic symptoms directly elicited by *pthA* expression in citrus cells are citrus-specific. In transient expression assays of *pthA* in tobacco, bean, poplar and cotton, no canker phenotype was observed. Instead, a rapid plant defence reaction known as a hypersensitive response (HR) phenotype is observed (Duan *et al.*, 1999). These observations are consistent with published results of transferring *pthA* into xanthomonads that are pathogenic to plant hosts other than citrus; when placed in bean-specific or cotton-specific xanthomonads which are then inoculated on their respective hosts, *pthA* causes an HR (Swarup *et al.*, 1992).

The host-specific pathology elicited by *pthA* genes probably helps to condition host range primarily by affecting dispersal. For example, *pthA*, *pthB*, *pthC* and *pthW* are essential for their source strains to induce hyperplastic cankers on citrus. The formation of cankers ruptures the citrus epidermis and releases abundant bacteria to the leaf surface (Swarup *et al.*, 1991). Similarly, other members of the *avrBs3/pthA* gene family are responsible for the increased release of bacteria to the leaf surface. For example, *avrb6* is important for *X. campestris* pv. *malvacearum* dispersal, since *avrb6* confers an ability to release 240-fold more bacteria to the leaf surface than would otherwise be released (Yang *et al.*, 1994). The most obvious visual pathogenic phenotype associated with *avrb6* is water soaking and necrosis, not hyperplasia. In fact, at least seven out of 10 *avr/pth* genes tested from a single *X. campestris* pv. *malvacearum* strain contribute additively and synergistically to water soaking; when seven of these *avr/pth* genes are destroyed in a single strain the mutant strain is nearly asymptomatic and 1600-fold less bacteria are

released to the leaf surface (Yang *et al.*, 1996). Water soaking in the case of cotton blight may be due to cellular collapse and also perhaps hypertrophy (in a manner similar to that elicited by *AvrBs3* in susceptible hosts; Marois *et al.*, 2002) and thus bringing about a loss of cellular free space and increased water uptake from the xylem through capillary action (Popham *et al.*, 1993). In the case of cotton blight and perhaps other blights as well, the lack of water soaking is not evidently important for growth *in planta*. In the case of citrus canker disease, the importance of water soaking to growth *in planta* is unknown. However, since both citrus canker disease and bacterial blight of cotton are primarily spread by rain splash, the presence of larger numbers of these bacteria on the host surface undoubtedly contributes to pathogen dispersal in both cases.

The host-specific pathology elicited by *pthA* on citrus also appears to help to directly condition host range by affecting growth on citrus. However, and by contrast with a single knockout mutation of *pthA*, the multiple knockout mutations that make *X. campestris* pv. *malvacearum* asymptomatic have no apparent effect on growth *in planta* (Yang *et al.*, 1994, 1996). Perhaps not enough *avr/pth* genes were destroyed in these experiments to see a reduction in growth, since multiple *avr/pth* genes remained in the mutant strains. However, we speculate that blight-causing strains of *Xanthomonas* obtain their nutrition by a different mechanism from canker-causing strains of *Xanthomonas*.

pthA is a member of the *avrBs3/pthA* gene family. Members of this large gene family are widely distributed in the genus *Xanthomonas*, although not all xanthomonads carry members of the family. Currently, at least 27 members have been cloned (for reviews, see Gabriel, 1999a; Leach and White, 1996). DNA sequence analyses reveal an unusually high level of sequence conservation among family members. The predicted peptides of full length members always include over 12, 34-amino acid, leucine rich, direct tandem repeats. The repeat region has been shown to be important for pathogenicity/avirulence specificity (Yang *et al.*, 1994). When the regions encoding the leucine rich repeats of *avrBs3/pthA* family members are swapped, the resulting chimeric genes exhibit the phenotypes expected of the gene that was the source of the repeat region (Yang *et al.*, 1994). Novel specificities are readily generated via intragenic recombination of *pthA* and its homologues, which may explain the rapid adaptation of members of this gene family to new hosts following random horizontal transfer of the genes (Yang and Gabriel, 1995b). The gene family also encodes two distinctly eukaryotic features: three functional nuclear localization sequences (Gabriel, 1997; Szurek *et al.*, 2002; Yang and Gabriel, 1995a) and a functional eukaryotic transcriptional activation domain (Zhu *et al.*, 1998). All members are also flanked by terminal inverted repeats that suggest that the genes can transpose (De Feyter *et al.*, 1993).

pthA is the first member of the *avrBs3/pthA* gene family to be recognized as being essential for pathogenicity on a host and was cloned by screening for pathogenicity, not avirulence (Swarup *et al.*, 1991). Most of the members of this gene family were first isolated as *avr* genes, and without evidence of *pth* function. The exceptions are all the members involved in inducing citrus canker disease (*pthA*, *pthB*, *pthC* and *pthW*) and two involved in cotton blight (*pthN* and *pthN2*; Chakrabarty *et al.*, 1997). The fact that *pthA* is a member of the largest family of *avr* genes reported in any microbe to date (not just *Xanthomonas*) is important because there should exist plant *R* genes that would be effective against these *avr* genes. *pthA* is unusual in that it does not cause avirulence on any citrus variety. In fact, all *X. citri* pv. *citri* strains that cause canker carry three other *pthA* homologues, and none of these cause avirulence on any citrus variety tested, either. However, all citrus canker causing strains carry *pthA* alleles that are essential for pathogenicity on hosts and yet, when placed into other xanthomonads and inoculated on to other plants, each gene also functions for avirulence and triggers an HR. For example, many cotton *R* genes react with *pthA* in a gene-for-gene manner, indicating that *R* genes from plants other than citrus may provide useful transgenic resistance in citrus (Swarup *et al.*, 1992). Finding *R* genes that react with a required pathogenicity gene such as *pthA* and its functional alleles may make the *R* gene last longer in field use.

Additional potential pathogenicity determinants: mining the *X. citri* genome

Besides the *hrp* genes and *pthA*, there are a few reports of other genes and/or pathogenicity factors reportedly required or suspected to be required by *X. citri* to cause disease. For example, auxotrophic mutants of *X. citri* that require phosphoglucose isomerase (Tung and Kuo, 1999), tryptophan (Tsuyumu *et al.*, 1996) or glutamic acid (Tung and Kuo, 2000) are reduced in pathogenicity on citrus. Xanthomonadin, the unique yellow *Xanthomonas* pigment, has been found to be important in protecting *X. citri* from the effects of ultraviolet light (Tsuyumu *et al.*, 1996). Random Tn5 mutagenesis has produced additional mutants affected in pathogenicity (Tung and Kuo, 2000), but the mutants were not characterized. As mentioned above, the LPS has been shown to play a role in citrus pathogenicity; mutants of *opsX* are unable to grow in citrus (Kingsley *et al.*, 1993). An *X. citri* gene encoding a protein 90% identical to *OpsX* is also found in *X. citri* and is likely to be similarly essential for pathogenicity and growth in citrus.

The recent publication of the complete *X. citri* pv. *citri* A strain 306 and *X. campestris* pv. *campestris* strain ATCC33913 genomes (da Silva *et al.*, 2002) should accelerate the incomplete functional genomics research on *X. citri* outlined above by providing a comparative roadmap. Indeed, comparative genomic analyses indicate

a number of interesting research directions (Van Sluys *et al.*, 2002). Following are speculations of future research directions in *X. citri* functional genomics, based on our analyses of the newly revealed *X. citri* genome.

In silico research speculations based on the *X. citri* strain 306 genome.

Overview of *X. citri* gene regulation

All pathogens utilize environmental sensing mechanisms. Even upon first entering the plant, phytopathogenic bacteria do not immediately turn on all the pathogenicity genes. *X. citri* presumably determines it has entered a plant, and possibly a citrus host, by using some of its many available two-component transcriptional regulators to up-regulate some pathogenicity genes, possibly involving motility and attachment. After a period of limited endophytic growth, presumably due to poor nutrient availability, *X. citri* attaches to the cell wall and uses its T3S system to turn on additional pathogenicity genes in a contact-dependent manner (Pettersson *et al.*, 1996) and then secretes and/or injects an unknown number of pathogenicity effectors, including its Avr, Pop and Pth proteins. The resulting host cell divisions presumably loosen and thin cell walls, provide additional xylem sap by capillary action as discussed above, and some host cell death is induced, all possibly providing nutrition.

At least some pathogens multiply until they have reached a particular concentration and then turn on pathogenicity factors by way of a quorum sensing mechanism (Bassler, 1999). In *X. campestris*, quorum sensing is accomplished by the production of a diffusible signal factor (DSF) produced by two Rpf proteins, RpfB and RpfF (Barber *et al.*, 1997). When the DSF concentration reaches a particular threshold, other Rpf proteins are thought to respond to activate the transcription of pathogenicity factors (Slater *et al.*, 2000). Seven Rpf encoding genes, including *rpfB* and *rpfF*, are found in *X. citri*, indicating that quorum sensing plays a role in canker pathogenicity, possibly by up-regulating cellulases, proteases and pectinases (da Silva *et al.*, 2002), and also xanthan gum production (Tang *et al.*, 1991), thereby assisting in egress of the pathogen from the canker lesion.

Nutrition

The genome of *X. citri* does not have pectin esterases. There are, however, three pectate lyases, six cellulases, five xylanases and an endoglucanase. The endoglucanase, BcsZ (gi|22001634), belongs in family 8 of the glycosyl-hydrolases which hydrolyse 1,4- β -D-glucosidic linkages in cellulose. In addition, there is a permease which imports degraded pectin products in a hydrogen-transport coupled fashion into the bacterial cell. *X. citri* has fewer cell wall degrading enzymes than *Xanthomonas campestris* pv. *campestris*, the causal agent of black rot and blight of crucifers, and it has been suggested that this is part of the genetic basis for

the difference in symptoms caused by the two species (da Silva *et al.*, 2002; Van Sluys *et al.*, 2002). Cell wall degrading enzymes are exported and secreted by the general secretory (type II secretion) system, and are known pathogenicity factors in *X. campestris* pv. *campestris* (Daniels *et al.*, 1988). *X. campestris* pv. *campestris* enters its host through hydathodes or wounds and spreads systemically in the xylem, causing rotting and even blight symptoms (Alvarez *et al.*, 1994) whereas *X. citri* does not become systemic and does not cause rotting or blight. If *X. citri* fails to inject PthA inside the plant cell, the bacteria simply do not multiply well in citrus (Swarup *et al.*, 1991). This indicates that *X. citri* growth requires a host response induced by PthA, perhaps endogenous loosening of the cell wall in preparation for cell division, hyperplasia to draw in xylem sap as discussed above, programmed cell death (Greenberg, 1993) or all three, to provide its nutrition. We speculate that *X. citri* is much more dependent for nutrition upon the host responses induced by PthA than its more necrotrophic, blight inducing cousins are dependent upon their AvrBs3/PthA family members.

Attachment

Bacteria can attach to host cells with special proteins called adhesins, or specialized organelles called pili (Lee and Schneewind, 2001). *X. citri* has four gene clusters and two separately located genes that are predicted to be involved in type IV pilus biosynthesis and regulation. Two genes encoding proteins called fimbrillins, FimA, gj|21243966 and gj|21243967 (85% similar in predicted amino acid sequence), are located within one of the clusters, and a gene designated *pilA* elsewhere in the genome is similar to type II pilin (PilE) from *Neisseria meningitidis*. The sequence of the two *fimA* genes is similar to PilA from other bacteria and they are located in a cluster of genes containing other type IV pilus genes *pilB*, *pilC*, *pilD*, *pilR* and *pilS*. The gene products of *pilS* and *pilR* are homologous to a two-component sensor protein and its corresponding regulatory protein, respectively, and control the expression of *pilA* (Hobbs *et al.*, 1993; Wu and Kaiser, 1997). The major subunit of the type IV pilin is first exported by the general secretory pathway (GSP). It has a short, basic N-terminal signal sequence, unique to type IV pilus proteins. PilD is a specific leader sequence peptidase that removes the signal sequence of PilA and other type IV pilus biosynthesis proteins, and methylates the new N-terminus (Finlay and Falkow, 1997; Russel, 1998). Mature, translocated pilin polymerizes at the plasma membrane, and the pilus is pushed through the central cavity of the outer membrane secretin (Parge *et al.*, 1995; Russel, 1998). Interestingly, there are two genes encoding proteins similar to PilA in *X. citri*. *Pseudomonas stutzeri* has type IV pili that are required for DNA uptake and natural transformation, and two genes encoding proteins similar to PilA (74% similar at the amino acid level) have opposite effects on natural genetic transformation (Graupner and Wackernagel, 2001).

The pilus biogenesis machinery and assembly is highly conserved in bacteria (Hultgren *et al.*, 1993). Their assembly genes are similar to type II secretion genes, but the N-terminal signal sequences are different (Russel, 1998). Type IV pili (also called fimbriae) have been proposed to attach bacterial pathogens to the host cell wall (Farinha *et al.*, 1994; Kang *et al.*, 2002) and retract (Skerker and Berg, 2001; Wall and Kaiser, 1999), pulling the bacterium closer to the host cell (Wall and Kaiser, 1999). Type IV pili have been shown to be important for the virulence of *Ralstonia solanacearum* (Kang *et al.*, 2002) and *Pseudomonas aeruginosa* (Hahn, 1997). However, and as discussed above, the ability of *X. citri* to provoke a non-host HR (Swarup *et al.*, 1992) indicates that plant cell wall attachment is not (very) host specific.

Just upstream of the *xps* cluster (see below), *X. citri* has two genes with a similarity to *xadA* from *X. oryzae* pv. *oryzae*. XadA is a non-fimbrial, adhesin-like outer membrane protein which is required for the virulence of *X. oryzae* pv. *oryzae* (Ray *et al.*, 2002). Proposed to be a cell wall surface anchor protein, XadA belongs to a family of proteins that are more similar at their C-terminus (which forms an outer membrane anchor domain) than at their signal sequences (Conserved Domain Database; Marchler-Bauer *et al.*, 2002). Some non-fimbrial adhesins are autotransporters (type V secretion): they are exported across the bacterial inner membrane by the general secretory pathway, and then secrete themselves across the outer membrane (Henderson and Nataro, 2001; Henderson *et al.*, 2000). One of the *xadA* genes encodes a protein (gj|21244271) that is missing the unusually long, highly conserved N-terminal signal sequence that is typical of autotransporters (Henderson *et al.*, 2000) and is therefore not likely to be a functional XadA homologue. The other XadA (gj|2124427) has a conserved autotransporter N-terminal sequence and it therefore may be involved in tight adhesion to plant cell walls, and could potentially have a function in bacterial virulence. Two *yapH* genes (similar to *yapH* from *Yersinia pestis*) are predicted to encode proteins similar to XadA, but both lack the typical N-terminal signal sequence of autotransporters.

Type II (general) secretion (T2S)

Type II secretion systems (recently reviewed in Sandkvist, 2001) are common in Gram-negative bacteria, but not ubiquitous (Finlay and Falkow, 1997). Secretion occurs in two steps (Finlay and Falkow, 1997; Lee and Schneewind, 2001): the Sec machinery exports substrates with a signal peptide across the inner membrane of Gram-negative bacteria and the type II secretion genes secrete them across the outer membrane. Bacterial T2S systems have been shown to secrete diverse molecules such as cellulases, pectate lyases, toxins, proteases and alkaline phosphatases (Russel, 1998).

There are two T2S clusters in the *X. citri* genome associated with two rearrangements of the otherwise highly co-linear

genomes of *X. citri* and *X. campestris* pv. *campestris* (da Silva *et al.*, 2002). The first one (the *xcs* cluster) consists of one transcriptional unit of 13 open reading frames based on the presence of transcriptional terminators as predicted by the GeSTer algorithm (Unniraman *et al.*, 2001, 2002). Twelve genes are *xcsC* through *xcsN*, and one (downstream of *xcsN*) is similar to a TonB-dependent receptor gene. Consistent with the idea that this T2S cluster has been transferred horizontally, it has a higher ($\approx 68\%$) G + C content than the surrounding DNA region ($\approx 65\%$). The second T2S system (the *xps* cluster) contains two transcriptional units, one consists of the *xpsE* and *xpsF* genes and the other of 10 genes: *xpsG* through *xpsN*, *xpsD* and a conserved hypothetical gene, similar to glycosyltransferase genes. Part of this cluster has a G + C content as low as 47.5%.

Type III secretion (T3S)

Many plant and animal pathogenic bacteria have a T3S system consisting of more than 20 proteins which together function to inject pathogenicity factors directly into host cells (Büttner and Bonas, 2002; Hueck, 1998). The T3S genes of plant pathogens are called *hrp* (hypersensitive response and pathogenicity) and are required both for pathogenicity on hosts and elicitation of the HR on hosts and non-hosts (Fenselau and Bonas, 1995; Roine *et al.*, 1997). Nine of these genes are highly conserved between the T3S systems of plant and animal pathogens and have been renamed *hrc* (*h*rp conserved) as proposed by Bogdanove *et al.* (1996). In fact, the *X. campestris* pv. *vesicatoria* Hrp system can secrete at least some heterologous type III secreted proteins from both plant and animal pathogens (Rossier *et al.*, 1999).

Expression of the T3S system can be contact-dependent (Ginocchio *et al.*, 1994; Ménard *et al.*, 1994; Pettersson *et al.*, 1996; Rosqvist *et al.*, 1994; Watarai *et al.*, 1995), but Daefler (1999) suggests that, at least in the case of *Salmonella*, T3S may not be contact-dependent. The induction of *Ralstonia* T3S system genes was shown to be dependent on HrpB, a sensor of contact between the pathogen and the host plant cell (Aldon *et al.*, 2000). Moreover, as mentioned above, an adhesin-like protein XadA proposed to be a cell wall surface anchor protein is required for the virulence of *X. oryzae* pv. *oryzae* (Ray *et al.*, 2002). In addition, type IV pili, which are also proposed to mediate close contact between the pathogen and the host cell, are required for the virulence of *Ralstonia solanacearum* (Kang *et al.*, 2002). Close contact with plant host cells appears necessary for at least some plant pathogens. Clearly, PthA appears to be delivered into the plant cell (Duan *et al.*, 1999; Yang and Gabriel, 1995a), a process that is likely to involve close contact.

The *hrp* genes are proposed to encode proteins that form a *hrp* pilus (Roine *et al.*, 1997). The *X. citri* *hrp* cluster is part of a pathogenicity 'island' in the main chromosome, as indicated by the following features (Hacker and Kaper, 2000): (i) it spans more than 23 kb; (ii) it encodes a system that secretes pathogenicity

factors into host cells; (iii) it is always associated with pathogenic species of *Xanthomonas*; (iv) it has regions with a different G + C content than the rest of the *X. citri* genome; (v) it carries mobile genetic elements (transposases), and (vi) it represents an unstable region of DNA since there are differences between the *X. citri* T3S system and the closely related T3S system cluster from *X. campestris* pv. *vesicatoria*, which is also part of a pathogenicity island (Büttner and Bonas, 2002; Noël *et al.*, 2002). The genes of the *hrp* cluster are induced *in planta* and controlled by the *hrp* regulatory proteins HrpG and HrpX (Wengelnik and Bonas, 1996; Wengelnik *et al.*, 1996, 1999). For some HrpX-regulated genes involved in the T3S system, a PIP (plant-inducible promoter) box has been identified (Fenselau and Bonas, 1995), although there are genes with PIP boxes that are not regulated by HrpX, and there are genes whose expression is under control of HrpG and HrpX that do not have a PIP box (Büttner and Bonas, 2002).

It is logical to assume that the expression of type III effectors would be coordinately regulated with expression of the T3S system genes. In a screen performed by Guttman *et al.* (2002) for type III secreted *Pseudomonas syringae* effector proteins, 13 different *hop* (*h*rp outer protein) genes were identified, of which all but one had a *hrp* box (Innes *et al.*, 1993) in their promoters. The situation may be somewhat different in *Xanthomonas*. Although da Silva *et al.* (2002) found 20 potential PIP boxes in the *X. citri* genome, only a few of these indicated potential T3S effector proteins. Homologues of four *avr* genes (*avrBs2*, *avrXacE1*, *avrXacE2* and *avrXacE3*) and two *popC* family effector genes were also found. Interestingly, the *avrXacE2* homologue, one of the *popC* homologues, and all four members of the *avrBs3/pthA* gene family do not contain a PIP box (da Silva *et al.*, 2002). In fact, all the *avrBs3/pthA* gene family members examined to date are constitutively expressed and yet are known or thought to be delivered by T3S (Knoop *et al.*, 1991; Szurek *et al.*, 2002; Yang and Gabriel, 1995a).

Three mechanisms have been proposed to explain how the T3S system recognizes effectors for secretion. The first proposes that N-terminal signal sequences in the secreted protein are recognized by the T3S system and result in export of the effector (Miao and Miller, 2000; Mudgett *et al.*, 2000). However, type III secreted proteins lack a clearly defined signal sequence in their amino termini (Aldridge and Hughes, 2001). In the second mechanism, molecular chaperones bind effector proteins transiently, preventing them from folding incorrectly, and present them to the T3S system apparatus for subsequent secretion (Wattiau *et al.*, 1994; Wattiau *et al.*, 1996). A third mechanism was proposed by Anderson and Schneewind (2001) for the T3S of Yop proteins by *Yersinia*. They hypothesized that the secretion signal was encoded in the messenger RNA (mRNA) instead of in the amino acid sequence of the secreted protein. The three types of secretion signal are not mutually exclusive. For example, YopE appears to have two separate secretion signals in its amino acid

located in the main chromosome (da Silva *et al.*, 2002). T4S systems mediate the intercellular transfer of macromolecules (proteins or protein–DNA complexes) from Gram-negative bacteria to other bacteria or eukaryotic cells (Baron *et al.*, 2002; Christie, 2001). The prototype for T4S systems is the *Agrobacterium tumefaciens virB* cluster (Christie, 1997), of which *virB2* through *virB11* have all been shown to be essential for *Agrobacterium* virulence (Berger and Christie, 1994; Ward *et al.*, 1990). The adapted conjugation system is, as the name implies, required for the conjugational transfer of plasmids, including self-mobilizing plasmids, which carry the T4S transfer genes and an origin of transfer (*oriT* or mobilization site). Adapted conjugation systems can also mobilize other plasmids *in trans* if the plasmid carries an *oriT* site (Christie and Vogel, 2000; Winans *et al.*, 1996). A T4S system is also necessary for the secretion of pertussis toxin by *Bordetella pertussis* (Christie and Vogel, 2000). T4S systems also require a 'coupling factor' (Cabezón *et al.*, 1997; Moncalián *et al.*, 1999), a homologue of the *Agrobacterium* VirD4 protein.

The T4S system in the main chromosome of *X. citri* strain 306 is incomplete. There are three partial copies of the *virB6* gene, there is no *virB5* homologue, and most *virB* homologues that are present are incomplete. This partial *virB* cluster is surrounded by several transposases, indicating that this area may have been subject to more than one transposition event. This T4S cluster is not likely to encode a functional secretion system, since it lacks essential genes.

It has been suggested that the T4S system on plasmid pXAC64 is also incomplete, since it appears to lack *virB5*, *virB7* and *virD4* homologues (da Silva *et al.*, 2002). This conclusion experimental verification for the following reasons. First, the gene product of XACb0044 (gij21110908) on pXAC64 is similar to VirB5 and is already annotated in GENBANK as such. Second, a BLASTP (Altschul *et al.*, 1990) search with the predicted TrwB sequence from pXAC64 shows similarity with VirD4 and its homologues. TrwB functions as a coupling factor for T4S systems and *trwB* is a known *virD4* homologue (Gomis-Rüth *et al.*, 2002; Moncalián *et al.*, 1999). Third, the potential T4S cluster from pXAC64 is very similar in organization to the T4S cluster from plasmid pXcB, cloned from *X. citri* pv. *aurantifolii* strain B69 (Brunings *et al.*, 2001). pXcB is a self-mobilizing plasmid and knockout insertions

indicate that the T4S system on pXcB is functional (Yuan *et al.*, unpublished data). Finally, the T4S *virB* clusters on pXAC64 and pXcB are arranged co-linearly, there are two additional genes between *virB5* and *virB6* of both plasmids, and *virB7* is missing from in between *virB6* and *virB8* (Fig. 3). The organization is reminiscent of the *lvh* cluster from *Legionella pneumophila* (GENBANK accession no. Y19029; Segal *et al.*, 1999), where there also are two genes between the homologues of *virB5* (*lvhB5*) and *virB6* (*lvhB6*). Remarkably, the gene immediately downstream of *lvhB5* is a homologue of *virB7*, *lvhB7*.

We propose that the gene immediately downstream of *virB5* in pXAC64 is a *virB7* homologue, although it may not be a functional homologue. Most, but not all, T4S systems require a VirB7 homologue, and VirB7 homologues do not often share an extensive sequence similarity (Anderson *et al.*, 1996). VirB7 is a lipoprotein, of which the signal sequence is cleaved off and the resulting N-terminal cysteine residue is anchored to the outer membrane (Baron *et al.*, 1997; Fernandez *et al.*, 1996). It is proposed to form homodimers with itself and heterodimers with VirB9 in the periplasmic space of the bacterial membrane (Spudich *et al.*, 1996). Like the *virB7* gene in pXcB, the homologue in pXAC64 (XACb0043) has a second potential ATG start site downstream of the annotated start site (at position 39190 in pXAC64), resulting in a smaller predicted protein. In pXcB the gene would be predicted to encode a lipoprotein if transcription starts at the second ATG start site (Babu and Sankaran, 2002). However, the pXAC64 homologue may not encode a lipoprotein due to an apparent mis-sense mutation in the gene. This is illustrated in Fig. 4, where VirB7 from pXcB and the gene product of XACb0043 are aligned together with *L. pneumophila* LvhB7 and *A. tumefaciens* VirB7. The isoleucine in the gene product of XACb0043 destroys the canonical lipobox [LV][ASTV][ASG][C]. This could be the result of a mutation in a single nucleotide from either GTC (valine) or CTC (leucine) to ATC (isoleucine). At least four possibilities emerge: (i) if there is a sequencing error in the reported sequence at this point, the T4S system is likely complete, (ii) even without a sequencing error, XACb0043 may be functional in the T4S system, (iii) the T4S system in *X. citri* may function without a *virB7* homologue, and (4) the point mutation might render the entire *virB* cluster on pXAC64 non-functional.

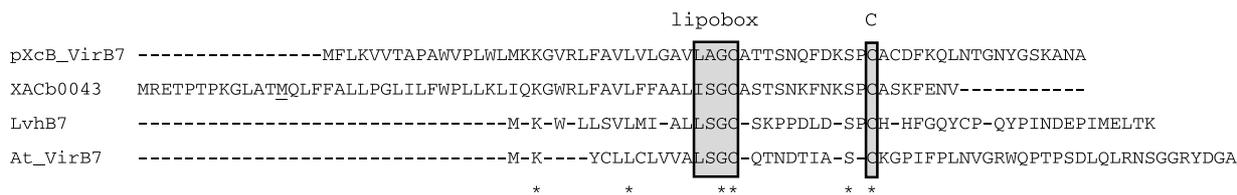


Fig. 4 Alignment of VirB7 from pXcB with the gene product of XACb0043 from pXAC64 are aligned together with *L. pneumophila* LvhB7 and *A. tumefaciens* VirB7. Conserved amino acid residues between the two gene products are indicated with *. The lipobox in VirB7 from pXcB and the corresponding region in the product of XACb0043 are shaded, as well as the conserved cysteine residue that is used for the formation of VirB7–VirB7 homodimers and VirB7–VirB9 heterodimers (Anderson *et al.*, 1996; Krall *et al.*, 2002; Spudich *et al.*, 1996). The underlined 'M' indicates the second potential translational start site in XACb0043.

The *virB* clusters from pXAC64 and pXcB both have an additional gene between *virB7* and *virB5* that do not resemble any other known T4S system genes. The genes are 33% identical and 45% similar to each other, but their function is unknown.

pXAC33 and pXAC64

X. citri pv. *citri* A strain 306 from Brazil has two native plasmids; pXAC33 is 33.7 kb in size, while pXAC64 is 64.9 kb (da Silva *et al.*, 2002; Fig. 3). Together, these two plasmids encode 55 potential genes of unknown function. The origins of replication for the two plasmids have not been determined. Both plasmids have two regions with partitioning-related genes. pXAC33 has one *repA* gene, while pXAC64 has two. Remarkably, the relative sizes and restriction endonuclease sites of these two plasmids from a Brazilian canker strain correspond almost perfectly to two plasmids previously characterized and reported from a Japanese canker strain (Tu *et al.*, 1989). The estimated sizes and positions of 59 restriction endonuclease fragments that were mapped on pXW45J and pXW45N from *X. citri* pv. *citri* A strain XAS4501 correspond precisely to the order and spacing of the same fragments on pXAC33 and pXAC64, respectively. The only exceptions are minor ones, involving very closely spaced markers.

pXW45N and pXW45J were found to carry two functional transposable elements, ISXc4 and ISXc5, respectively; these were functionally characterized by transposition in *E. coli*, and their locations mapped to on the native plasmids (Liu *et al.*, 1992; Tu *et al.*, 1989). The previously published sequence of ISXc5 (gi:1370597) is 99% identical over its entire length of 6938 bp to pXAC33 from positions 20 284 to 27 221, allowing a precise placement of ISXc5 on pXAC33. Although the sequence of ISXc4 was not published, it was reportedly homologous to ISXc5 at both ends, including the nearly identical 50 bp terminal inverted repeats (Tu *et al.*, 1989). Based on the terminal inverted repeats of ISXc5, ISXc4 appears to span positions 58 958–64 281 on pXAC64. The corresponding locations of these IS elements on pXAC64 and pXAC33 are indicated in Fig. 3.

These IS elements are of potential interest because the TnpR resolvase of ISXc5 represents a new subfamily of recombinases responsible for the resolution of co-integrates of class II transposable elements, such as Tn3 (Liu *et al.*, 1998). Intriguingly, pXAC64, which presumably carries a functional allele of *pthA* (*pthA4*), also has a *tnpA* transposase gene that is 99.9% similar to the *tnpA* of Tn5044, which is in the Tn3 subgroup of the Tn3 family of transposases (Kholodii *et al.*, 2000). Since all members of the *avrBs3/pthA* family have terminal inverted repeats that are similar to Tn3 transposable elements, it was hypothesized that these genes may transpose (De Feyter *et al.*, 1993). It should now be possible to test this hypothesis experimentally.

If the T4S system on pXAC64 is functional, the plasmid might be self-mobilizing, like pXcB from *X. citri* pv. *aurantifolii* B69. Self-mobilizing plasmids carry the transfer functions necessary to

transfer themselves and other plasmids with an origin of transfer (*oriT*). pXAC64 is very similar in organization to pXcB (Brunings *et al.*, 2001), which carries *pthB*, a functional homologue of *pthA*. If *pthA4* is isofunctional with *pthA* and *pthB*, and pXAC64 is self-mobilizing, then pXAC64 would be able to transfer itself into other xanthomonads resident on the same host, some of which may lack an ability to cause citrus canker. This scenario could explain the origin of the entire *X. citri* pv. *aurantifolii* group in South America. We hypothesize that a plasmid similar to pXAC64 or pXW45N with a functional T4S system (say, pXcB) transferred from an Asiatic citrus canker strain to a *Xanthomonas* strain resident on citrus in South America but incapable of causing canker prior to receiving the plasmid carrying the critical *pthA* homologue. If a transposase on the plasmid or in the recipient strain caused a transposition of the *pthA* gene, multiple copies would be created. The T4S system would be instrumental in initiating such a transfer.

Finally, it is possible that the T4S system in pXAC64 and similar plasmids might be directly involved in pathogenesis, since T4S systems are involved in the pathogenicity of several Gram-negative bacteria. T4S could, for example, secrete effector molecules in addition to those injected by T3S. Functional pathogenicity genes on self-mobilizing plasmids would form a readily transferable pathogenicity island. There is strong evidence that additional factors found on pXcB, in addition to *pthB*, may be involved in citrus canker pathogenicity (Brunings *et al.*, 2001).

CONCLUSION

X. citri pv. *citri* harbours a variety of demonstrated pathogenicity genes in its genome, including a required T3S cluster, four *pthA* homologues and *opsX*. It also harbours four putative *avr* genes not in the *avrBs3/pthA* gene family; these may or may not play a role in pathogenicity. *X. citri* is known to carry self-mobilizing plasmids such as pXcB, and possibly pXAC64 or pXW45N. Self-mobilizing plasmids could explain the origination of the South American citrus canker strains, *X. citri* pv. *aurantifolii*, since the transfer of an isofunctional allele of *pthA* to a citrus-compatible xanthomonad would enable the ability to grow better in citrus and to break the citrus epidermal layer and escape more efficiently. The complete genome sequence of strain 306 shows that numerous additional potential pathogenicity and regulatory genes are present in the genome, allowing some very testable hypotheses of gene function to be developed to provide a detailed accounting for the life cycle of the pathogen and its methods for disease elicitation. Some, like the T2S, T3S and T4S system genes appear to form pathogenicity islands within the genome. Circumstantial evidence points to the possibility that at least some *avrBs3/pthA* gene family members can transpose. The very structure of the host-specificity region in the *avrBs3/pthA*

gene family—102-bp direct repeats—facilitates recombination and therefore new specificities. Potential self-mobilizing plasmids carrying genes that might transpose and can easily rearrange are virtual mobile libraries of novel pathogenicity factors. This includes host range and race specificity determinants. These can transfer into *Xanthomonas* spp. that are adapted to the same host or perhaps other hosts. This system of ready-made mechanisms for change could explain why *Xanthomonas* spp. are so specifically adapted to such a wide variety of hosts, and cause new and emerging plant diseases on large scale agricultural monocultures.

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