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An *Xanthomonas citri* Pathogenicity Gene, *pthA*, Pleiotropically Encodes Gratuitous Avirulence on Nonhosts

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The pathogenicity gene, *pthA*, of *Xanthomonas citri* is required to elicit symptoms of Asiatic citrus canker disease; introduction of *pthA* into *Xanthomonas* strains that are mildly pathogenic or opportunistic on citrus confers the ability to induce cankers on citrus (S. Swarup, R. De Feyter, R. H. Brlansky, and D. W. Gabriel, Phytopathology 81:802-809, 1991). The structure and the function of *pthA* in other xanthomonads and in *X. citri* were further investigated. When *pthA* was introduced into strains of *X. phaseoli* and *X. campestris* pv. *malvacearum* (neither pathogenic to citrus), the transconjugants remained nonpathogenic to citrus and elicited a hypersensitive response (HR) on their respective hosts, bean and cotton. In *X. c.* pv. *malvacearum*, *pthA* conferred cultivar-specific avirulence. Structurally, *pthA* is highly similar to *avrBs3* and *avrBsP* from *X. c.* pv. *vesicatoria* and to *avrB4*, *avrb6*, *avrb7*, *avrBIn*, *avrB101*, and *avrB102* from *X. c.* pv. *malvacearum*. Surprisingly, marker-exchanged *pthA*::Tn*5-gusA* mutant B21.2 of *X. citri* specifically lost the ability to induce the nonhost HR on bean, but retained the ability to induce the nonhost HR on cotton. The loss of the ability of B21.2 to elicit an HR on bean was restored by introduction of cloned *pthA*, indicating that the genetics of the nonhost HR may be the same as that found in homologous interactions involving specific *avr* genes. In contrast with expectations of homologous HR reactions, however, elimination of *pthA* function (resulting in loss of HR) did not result in water-soaking or even moderate levels of growth *in planta* of *X. citri* on bean; the nonhost HR, therefore, may not be responsible for the "resistance" of bean to *X. citri* and may not limit the host range of *X. citri* on bean. The pleiotropic avirulence function of *pthA* and the heterologous HR of bean to *X. citri* are both evidently gratuitous.

Additional keywords: gene-for-gene, host specificity, normosensitive response

The function and extent of the role(s) avirulence (*avr*) genes play in plant-associated microbes have been a subject of some speculation (Gabriel and Rolfe 1990; Keen 1990). In practical terms, *avr* genes determine race specificity by limiting the range of host cultivars and occasionally host species and genera that a pathogenic strain may attack. Race- and cultivar-specific interactions can usually be shown to require the presence of specific resistance (*R*) genes in the host; these negative (incompatible) interactions are termed gene-for-gene interactions. Gene-for-gene interactions confer incompatibility and therefore must be superimposed on a basic ability to parasitize (Ellingboe 1976). Gene-for-gene interactions involving bacterial plant pathogens are generally associated with a plant defense or hypersensitive response (HR).

Plant defense responses are also observed when pathogens are inoculated onto nonhosts. Single cloned avirulence genes isolated from a pathogen of one host species can cause an otherwise virulent pathogen of another host species to become avirulent on its own host (Kobayashi *et al.* 1989; Whalen *et al.* 1988). These and other data led to the suggestion that avirulence genes may also determine host range above the race level (e.g., refer to Keen 1990; Keen and Staskawicz 1988). Alternatively, it has been argued that the role of avirulence genes in nonhost incompatibility is generally gratuitous and that positive-functioning genes play the major role in determining host range at the microbial pathovar or species level and higher (Gabriel 1989). According to this argument, positive-acting virulence genes, such as the host-specific nodulation (*hsn*) genes of *Rhizobium* spp. (Djordjevic *et al.* 1987; Martinez *et al.* 1990) and the host-specific virulence (*hsv*) genes of *Xanthomonas* spp. (Waney *et al.* 1991) and *Pseudomonas* spp. (Ma *et al.* 1988; Salch and Shaw 1988) may play the predominant roles in determining host range at the species level and higher.

Inactivation of known avirulence genes has not resulted in the loss of the nonhost HR, possibly because of the virtually limitless numbers of nonhosts that could be tested. Also, a large number of *avr* genes may be involved in the many nonhost HR reactions, and elimination of any one *avr* gene would not affect the epistatic effects of other *avr* genes. In one case, which likely involved an *avr* gene, both chemical- and transposon-induced mutants of *Erwinia rubrifaciens* Wilson, Zeitoun and Fredrickson, which had lost ability to induce the nonhost HR on tobacco and yet were as pathogenic to walnut (the normal host) as the wild type (Azad and Kado 1984), were obtained. The transposon mutants likely affected a single locus, indicating that some nonhost HR reactions are under the control of single *avr* genes in the pathogen. Although there was no obvious pathogenicity of the resulting *E. rubrifaciens* mutants on tobacco, asymptomatic growth in tobacco was not determined.

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The cloning and characterization of pathogenicity locus *pthA* of *X. citri* ex Hasse on citrus were previously reported (Swarup *et al.* 1991). Here, we report on the further characterization of *pthA* and show that it pleiotropically functions in *X. citri* to elicit the nonhost HR of bean cv. Calif. Lt. Red; functions as an avirulence gene in *X. phaseoli* ex Smith and in *X. campestris* pv. *malvacearum* (Smith) Dye; is physically similar to other avirulence genes of *Xanthomonas* spp.; and when it is inactivated in *X. citri*, the nonhost HR is eliminated on bean, but growth *in planta* is not substantially affected, and host range is not thereby extended.

MATERIALS AND METHODS

Bacterial strains, plasmids, and general techniques. The sources and characteristics of all bacterial strains and plasmids used in this study are presented in Table 1, except for the strains used exclusively in Figure 1 and the plasmids used exclusively in Figure 2 (referenced in the legends of the respective figures). *Escherichia coli* (Migula) Castellani and Chalmers DH5α was the cloning host for all double-stranded plasmids used in this work; these strains were cultured on Luria-Bertani (LB) medium at 37° C (Miller 1972). All *Xanthomonas* spp. strains were cultured on PYGM (peptone-yeast extract-glycerol-MOPS) medium at 30° C (De Feyter *et al.* 1990). Antibiotics were used at concentrations as described (Swarup *et al.* 1991). To transfer all wide host range plasmids from *E. coli* DH5α to various Spc^r *Xanthomonas* strains, we performed triparental matings by using pRK2013 or pRK2073 as helper plasmids as described (Swarup *et al.* 1991). Plasmids were isolated by alkaline lysis from *E. coli* (Sambrook *et al.* 1989) and *Xanthomonas* (De Feyter and Gabriel 1991a) and purified by CsCl-ethidium bromide gradient fractionation when required (Sambrook *et al.* 1989).

Table 1. Bacterial strains, phage, and plasmids used in this study

Strain, phage, or plasmid	Relevant characteristics	Reference
Escherichia coli		
DH5$\propto$	F$^-$, *endA1*, *hsdR17* ($r_k^- m_k^+$), *supE44*, *thi-1*, *recA1*, *gyrA*, *relA1*, ϕ*80dlacZ* ΔM15, Δ(*lacZYA-argF*)U169	Gibco-BRL, Gaithersburg, MD
DH5αF'IQ	F', λ^-, *endA1*, *hsdR17* ($r_{k7}^- m_k^+$), *supE44*, *thi-1*, *recA1*, *gyrA*, *relA1*, ϕ*80dlacZ* ΔM15, Δ(*lacZYA-argF*)U169/F' proAB$^+$, lacI^qZΔM15, *zzf*::Tn5 (Km^r)	Gibco-BRL
Xanthomonas citri		
3213^T	ATCC 49118; virulent citrus canker type strain	Gabriel *et al.* 1989
3213Sp	Spc^r derivative of 3213^T	Swarup *et al.* 1991
B21.2	Spc^r, avirulent derivative of 3213Sp (*pthA*::Tn5-*gusA*)	Swarup *et al.* 1991
X. phaseoli		
$G27^T$	ATCC 49119, virulent bean blight type strain	Gabriel *et al.* 1989
G27Sp	Spc^r derivative of $G27^T$	Swarup *et al.* 1991
X. campestris pv. *alfalfae*		
KX-1Sp	Spc^r derivative of KX-1, isolated from alfalfa	Swarup *et al.* 1991
X. campestris pv. *citrumelo*		
3048^H	ATCC 49120, citrus leaf spot pathotype strain	Gabriel *et al.* 1989
3048Sp	Spc^r derivative of 3048^H	Swarup *et al.* 1991
X. campestris pv. *cyamopsidis*		
13D5Sp	Spc^r derivative of 13D5, isolated from guar	Swarup *et al.* 1991
X. campestris pv. *malvacearum*		
XcmN	Widely virulent cotton blight strain	Gabriel *et al.* 1986
Xcm1003	Spc^r Rif^r derivative of XcmN, a widely virulent cotton blight strain	De Feyter and Gabriel 1991a
Plasmids		
pUFR027	IncW, Nm^r, Mob$^+$, *mob*(P), *lacZ*α,$^+$, Par$^+$	De Feyter *et al.* 1990
pUFR042	IncW, Nm^r, Gm^r, Mob$^+$, *mob*(P), *lacZ*α,$^+$, Par$^+$ *cos*	De Feyter *et al.* 1990
pUFR047	8.6 kb; IncW, Ap^r, Gm^r, Mob$^+$, *mob*(P), *lacZ*α, Par$^+$	De Feyter and Gabriel, unpublished
pSS10.35	20.1-kb DNA fragment from *X. citri* 3213 in pUFR027, PthA$^+$	Swarup *et al.* 1991
pSS35KBg	7.7-kb *Kpn*I-*Bgl*II DNA fragment pSS10.35 in pUFR042, PthA$^+$	Swarup *et al.* 1991
pSS35BP3	16.5-kb *Bam*HI fragments from pSS10.35 in pUFR042, PthA$^-$	This study
pSS35BD	3.6-kb *Bam*HI deletion derivative of pSS10.35, PthA$^-$	This study
pZit34	4.5-kb subclone from pSS35KBg in pUFR047, PthA$^+$	This study
pZit45	4.5-kb subclone from pSS35KBg in pUFR047, PthA$^+$	This study
pRK2073	ColE1, *npt*::Tn7, Km^s, Sp^r, Tra$^+$, helper plasmid	Leong *et al.* 1982
pUC119	ColE1, Ap^r, *lacZ*α^+	Vieira and Messing 1987
pZit34.119	4.5-kb subclone from pZit34 in pUC119	This study
pZit45.119	4.5-kb subclone from pZit45 in pUC119	This study
pZit45BB	700-bp *Bam*HI-*Bal*I subclone from pZit45 in pUC119	This study
Phage		
R408	Stable, interference-resistant helper phage	Stratagene, La Jolla, CA

Xanthomonas total DNA was prepared as described by Gabriel and De Feyter (1992). Restriction digests were carried out as recommended by the manufacturers. We performed Southern blots by using nylon membranes as described (Lazo *et al.* 1987). Probes were made from double-stranded plasmids labeled with ^{32}P-dCTP by primer extension (Feinberg and Vogelstein 1983) of random primers (Fig. 2) or the "universal" M13 forward sequencing primer (Fig. 1).

Localization and subcloning of *pthA* from pSS10.35. Gene *pthA* was previously localized to a 3.7-kb region of pSS10.35 (Swarup *et al.* 1991). To further localize the gene(s) responsible for the PthA$^+$ pleiotropic phenotypes, seven additional Tn*5*::*gusA* inserts affecting *pthA* activity were mapped (Swarup *et al.* 1991) and physically oriented by restriction fragment size analyses. Assays for β-glucuronidase (GUS) activity were performed after *X. c.* pv. *citrumelo* Gabriel 3048Sp cells were grown with various pSS35KX15::Tn*5*-*gusA* plasmids in PYGM broth as described (Swarup *et al.* 1991). Additional subclones and deletion derivatives of pSS10.35 and pSS35KBg were generated for further localization. These subclones were tested in 3048Sp for ability to induce cankerlike lesions on grapefruit leaves (*Citrus paradisi* Macfady 'Duncan') and for ability to induce an HR on bean leaves (*Phaseolus vulgaris* L. 'California Light Red') as described (Swarup *et al.* 1991). Subclone pSS35BP3 was selected from a group of plasmids that resulted from a *Bam*HI partial digest of pSS10.35 ligated in pUFR042. Subclone pSS35BP3 carries two *Bam*HI fragments in the same contiguous orientations as cloned in pSS10.35, without evident rearrangements. Deletion derivative pSS35BD has lost the two *Bam*HI fragments cloned in pSS35BP3 and consists of a 3.6-kb *Sau*3A-*Bam*HI to *Bam*HI junction fragment cloned in pUFR027. Together pSS35BP3 and pSS35BD contain the entire 20.1-kb insert of pSS10.35.

DNA from pSS35KBg was digested with *Kpn*I and *Hin*dIII to excise the PthA$^+$ active fragment (Fig. 3). We treated the fragment with a combination of *Bal*31 (titrated to yield 3.5- to 5.0-kb fragments) and S1 exonucleases (Sambrook *et al.* 1989) to generate 20 deletion derivatives and to delimit the functional boundaries of *pthA*. The exonuclease-treated fragments were modified to blunt ends by using the Klenow fragment, and the inserts were ligated to *Sma*I-linearized pUFR047. Plasmid pUFR047 is an unpublished 8.6-kb, Gm^{r}Ap^r derivative of pUFR042 (De Feyter and Gabriel 1991a). Deletion derivatives were subcloned in *E. coli* DH5α, analyzed by restriction

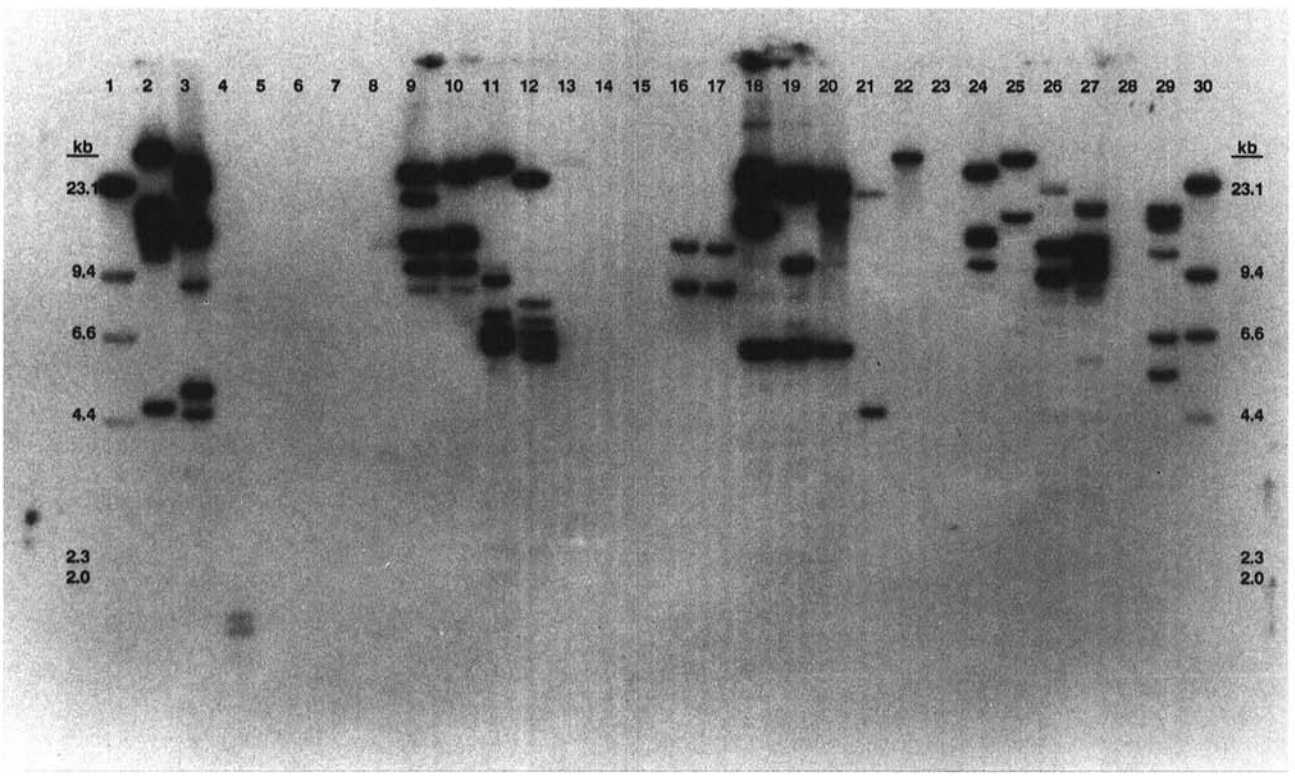

Fig. 1. Southern hybridization of total DNA from various xanthomonads with a *pthA* internal fragment. Total DNA, digested with *Eco*RI, was separated by size, blotted onto nylon, and probed with pZit45BB insert of DNA as described. 1 and 30, λ cut with *Hin*dIII; 2, XcmN; 3, XcmH; 4, *Xanthomonas campestris* pv. *malvacearum*-hibiscus X-10 (Lazo and Gabriel 1987); 5 and 6, *X. c.* pv. *dieffenbachiae* 2032 (provided by B. Stall, University of Florida, Gainesville) and 084-729 (Lazo and Gabriel 1987); 7 and 8, *X. c.* pv. *campestris* X-3 (provided by J. Hunter, Cornell University, Geneva) and ATCC 33913^T; 9 and 10, *X. c.* pv. *vignicola* A81-331 and Xv19 (Lazo and Gabriel 1987); 11 and 12, *X. c.* pv. *glycines* ATCC 17915 and 1717 (Lazo and Gabriel 1987); 13, *X. c.* pv. *vesicatoria* 82-23 (Lazo and Gabriel 1987); 14 and 15, *X. c.* pv. *citrumelo* 4600 (Gabriel *et al.* 1988) and 3048^H (ATCC 49120) (Gabriel *et al.* 1989); 16 and 17, *X. c.* pv. *alfalfae* KX-1 (Lazo *et al.* 1987) and L-142 (Gabriel *et al.* 1988); 18–20, *X. citri* 3210 (Gabriel *et al.* 1988), 3213^T (ATCC 49118) (Gabriel *et al.* 1989), and 59 (Gabriel *et al.* 1988); 21 and 22, *X. c.* pv. *aurantifolii* 69 and 70 (Gabriel *et al.* 1989); 23, *X. c.* pv. *pisi* XP1 (Lazo and Gabriel 1987); 24 and 25, *X. phaseoli* XP-JL (Lazo *et al.* 1987) and G-27^T (ATCC 49119) (Gabriel *et al.* 1989); 26 and 27, *X. c.* pv. *cyamopsidis* 13D5 and X002 (Lazo *et al.* 1987); 28 and 29, *X. c.* pv. *translucens* 82-1 (Lazo and Gabriel 1987) and 216.2 (Waney *et al.* 1991).

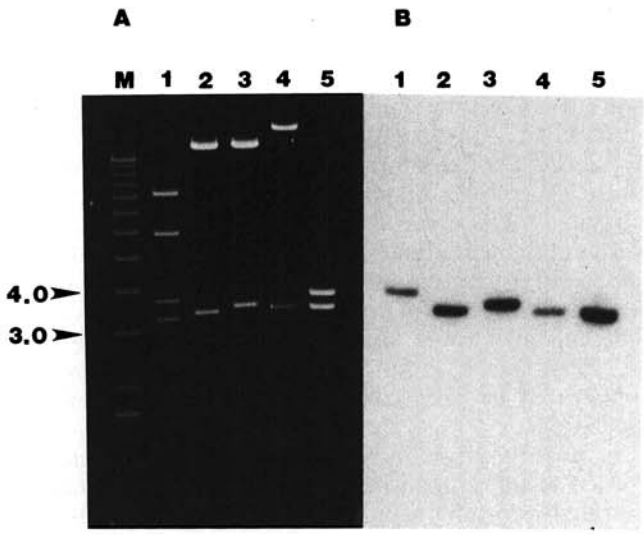

Fig. 2. Southern hybridization of various *Xanthomonas avr* genes with *pthA*. DNA was transferred to nylon membranes and probed with ^{32}P-labeled pZit45.119. M, Molecular weight marker (1-kb ladder); 1–3, *Bam*HI-digested DNA of pUFR117, pUFR122, and pUFR120 containing *avrBln*, *avrB101*, and *avrb7* respectively, from XcmH (De Feyter and Gabriel 1991a); 4, *Bam*HI-digested DNA of pL3XV1-6 containing *avrBs3* from *X. campestris* pv. *vesicatoria* (Bonas *et al.* 1989); and 5, *Bam*HI-digested DNA of pZit45 containing *pthA* from *X. citri*. **A,** Ethidium bromide stained gel. **B,** Autoradiograph of a Southern blot of the gel shown in A.

mapping, and conjugally transferred to 3048Sp for complementation tests on citrus as described (Swarup *et al.* 1991). Plasmids pZit34 and pZit45 carried the smallest (each 4.4 kb), independently derived inserts with full *pthA* activity. Plasmids pZit34 and pZit45 were also conjugally transferred to *X. citri* 3213 mutant strain B21.2 (*pthA*::Tn*5-gusA*) for complementation tests on grapefruit. Both plasmids were restriction-mapped in more detail and recloned in pUC119 for DNA sequence analyses; they yielded pZit34.119 and pZit45.119, respectively. Plasmid pZit45BB is a *Bam*HI to *Bal*I subclone of pZit45 and carries a 700-bp fragment, which includes the *Stu*I site of pZit45 and is cloned in pUC119.

DNA sequence analysis. The DNA inserts from pZit34 and pZit45 were directionally subcloned into pUC119 to yield pZit34.119 and pZit45.119, respectively. We generated sets of overlapping, deletion subclones in pUC119 by using DNase I exactly as described (Sambrook *et al.* 1989). We prepared single-stranded DNA from DH5αF'IQ cells by using R408 helper phage (Stratagene, La Jolla, CA) and a Stratagene modification (Strategies, 4(1):58, 1990) of the single-strand rescue technique of Vieira and Messing (1987). We determined the DNA sequence of the desired fragments by using the dideoxy nucleotide chain termination technique with the Amersham (Arlington Heights, IL) multiwell microtiter plate DNA sequencing system RPN1590 (T7 DNA polymerase, ^{35}S-dATP and the

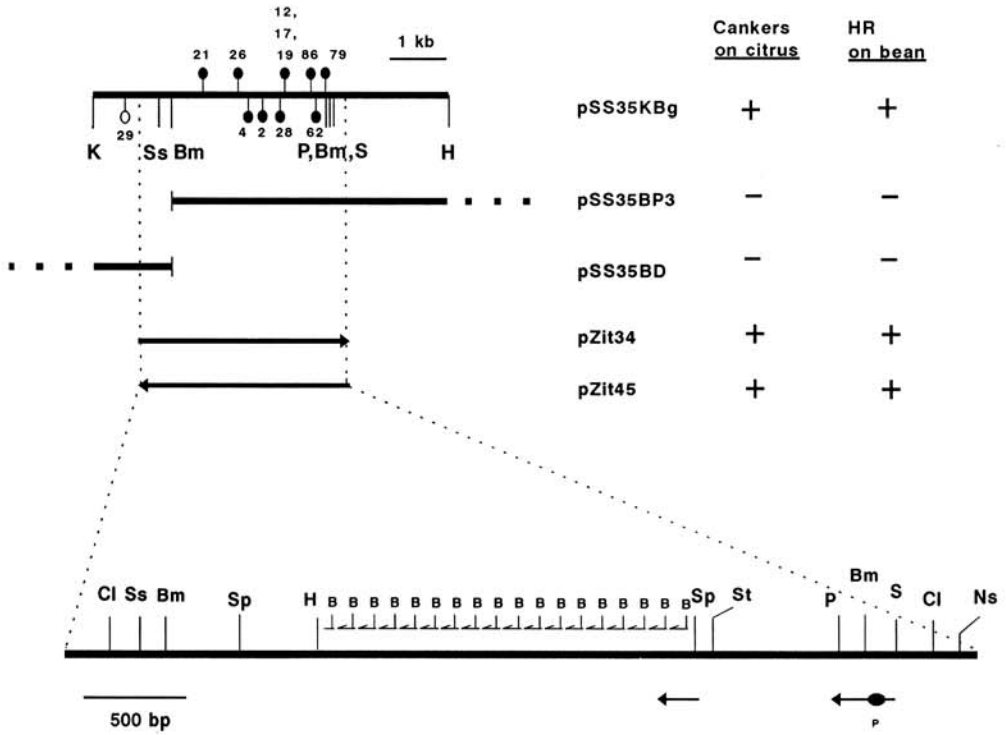

Fig. 3. Structure and localization of *pthA* on pSS35KBg were determined by activity assays of the indicated clones in 3048Sp on citrus and bean. The fragments shown are the subcloned inserts of the indicated plasmids as described in the text and in Table 1. Sites of specific Tn*5-gusA* insertions in pSS35KX15 were mapped, and their orientations were determined as indicated by the numbered circles. All circles above the line are oriented to create transcriptional fusions if promoter activity is left to right; none of these exhibited β-glucuronidase (GUS) activity. All circles below the line are oriented to create transcriptional fusions if promoter activity is right to left; all of these exhibited GUS activity on PYGM medium. Closed circles indicate inserts that abolished *pthA* activity (cankers on citrus and hypersensitive response [HR] on bean in 3048Sp). The open circle indicates no observed effect on *pthA*. Insertion number 21 was used to create the marker-exchanged mutant B21.2. (Swarup *et al.* 1991). The more detailed restriction map at the bottom of the figure is of pZit45. The direction of transcription of *pthA* is indicated from the promoter (P) region by the arrow; this arrow depicts the region sequenced in Figure 4A. The other arrow indicates the region sequenced in Figure 4B. Restriction sites are abbreviated as follows: B, *Bal*I; Bm, *Bam*HI; Bg, *Bgl*II; Cl, *Cla*I; H, *Hin*cII; K, *Kpn*I; Ns, *Nsi*I; P, *Pst*I; S, *Sal*I; Sp, *Sph*I; Ss, *Sst*I; St, *Stu*I.

"universal" forward sequencing primer). Additionally, an oligonucleotide primer of sequence TGCATGCATGG-CGCAATGCACT was synthesized (ICBR DNA Synthesis Core, University of Florida, Gainesville) and utilized for sequencing the fragment in Figure 4B.

Plant inoculations and *in planta* bacterial growth curves. All bean and cotton (*Gossypium hirsutum* L.) plants were grown under greenhouse conditions, transferred to growth chambers 3 days before inoculation, and maintained under conditions as described (Swarup *et al.* 1991). Cotton lines reported here as B2, B4, b6, b7, and BIn each appear to have different, single genes for resistance to bacterial blight of cotton, and all are congenic with line Acala 44 (Ac44) by backcrossing (De Feyter and Gabriel 1991a). Cultivar Ac44 has no known genes for bacterial blight resistance. Cotton line B1 was derived from Acala B1 (Hunter and Brinkerhoff 1961) by four backcrosses to Ac44 (R. De Feyter, M. Essenberg, and D. W. Gabriel, unpublished). Cotton line Acala B5 (Hunter and Brinkerhoff 1961) is known to carry at least four genes for resistance (McNally 1990); lines B5a (M. Essenberg and K. L. McNally, unpublished) and B5b (R. De Feyter and D. W. Gabriel, unpublished) were independent segregants of Acala B5, backcrossed to Ac44. Cotton line BIn3 was a segregant derived from Acala BIn (Hunter and Brinkerhoff 1961) by backcrossing to Ac44 and appears to have a single gene for resistance (R. De Feyter and D. W. Gabriel, unpublished).

All citrus plants were grown under greenhouse conditions and inoculated in quarantine greenhouse facilities at the Division of Plant Industry, Florida Department of Agriculture, Gainesville. All plant inoculations involving *X. citri* or *pthA* or derivatives of *pthA* were conducted at BL-3P level containment (refer to Federal Register 1987). For macroscopic symptom assays, we inoculated all plants by pressure infiltration of the abaxial leaf surface with the blunt end of a tuberculin syringe as described (Swarup *et al.* 1991; Gabriel *et al.* 1986). Bacterial suspensions were standardized in sterile tap water to 10^8 cfu/ml for these inoculations.

For growth curves in bean, bacterial suspensions were adjusted to 10^6 cfu/ml in sterile tap water and pressure-infiltrated into fully expanded abaxial leaf surfaces. Leaf sections of 1 cm^2 were taken from noninjured areas of the leaves with sterile cork borers and macerated in 1 ml of sterile tap water; various dilutions were plated on appropriate selective antibiotic-containing media. Viable counts were made after 3 days of incubations, and these were expressed as $\log_{10}$ cfu/cm^2 of inoculated leaf surface. At least three replications of each inoculation of each strain

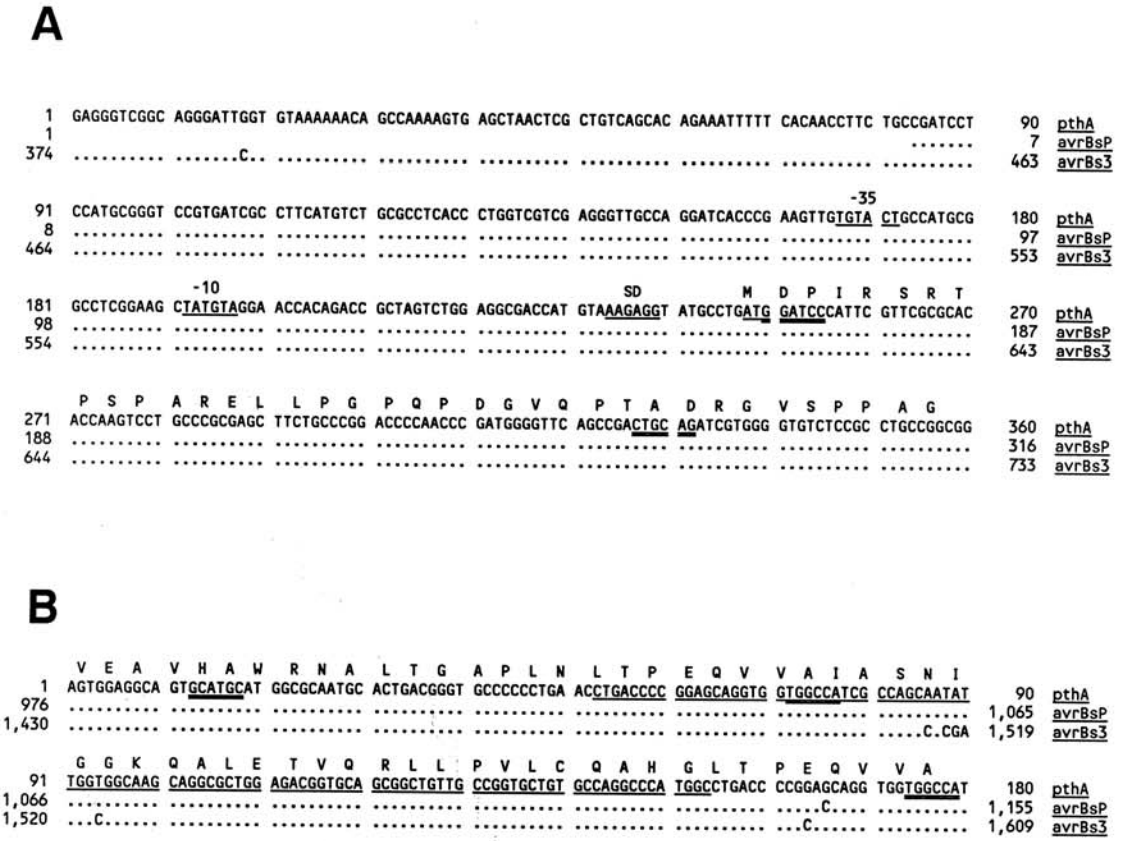

Fig. 4. Comparison of nucleotide sequences of two DNA fragments from pZit45, which has *pthA* activity, with nucleotide sequences of *avrBsP* and *avrBs3*. The nucleotide positions indicated for *avrBsP* and *avrBs3* are from the respective publications. **A,** Based on nearly perfect DNA sequence homology with the 5′ ends of *avrBsP* and *avrBs3* and the known direction of transcription of *pthA*, the predicted translational start site and downstream amino acid sequence of *pthA* are shown above the sequences. Sequences homologous to −35, −10, and Shine-Delgarno (SD) promoter regions are underlined. The *Bam*HI and *Pst*I sites are double-underlined. **B,** Based on nearly perfect DNA sequence homology with *avrBsP* and *avrBs3*, the predicted amino acid sequence of this fragment of *pthA* is shown; it includes the first 102-bp repeat of *pthA* (underlined). The *Sph*I site and two *Bal*I sites on this fragment are double-underlined.

on two to three plants were included in a single experiment. Each experiment was repeated at least three times.

RESULTS

Localization of *pthA*. Gene *pthA* was previously localized to a 3.7-kb region of pSS10.35 that was contained within pSS35KBg; all 14 Tn*5-gusA* insertions in the region had lost *pthA* activity, but only a few were precisely mapped (Swarup *et al.* 1991). In this study, all 14 Tn*5-gusA* inserts were localized to a 3.4-kb *Bam*HI fragment, and 11 of these were placed on the map of pSS35KBg shown in Figure 3. Subclone pSS35BP3, which carried the entire 3.4-kb *Bam*HI fragment affected by the 14 transposon inserts plus a contiguous 13.1-kb region of pSS10.35 (Swarup *et al.* 1991) as indicated in Figure 3, conferred no *pthA* activity to 3048Sp. Deletion derivative pSS35BD, which carries the remaining 3.6 kb of insert from pSS10.35, also conferred no *pthA* activity to 3048Sp. These observations indicated that *pthA* activity required a minimum uninterrupted stretch of 3.4 kb of DNA, from the left *Bam*HI site of pSS35KBg to Tn*5-gusA* insert 79—essentially the entire *Bam*HI fragment. Of the 20 *Bal*31 deletion derivatives of pSS35KBg tested, pZit34 and pZit45 carried the smallest inserts (each 4.4 kb) with full *pthA* activity in 3048Sp. Plasmids pZit34 and pZit45 carried independently derived inserts in opposite orientations. When transferred to mutant strain B21.2 (*X. citri* 3213, *pthA*::Tn*5-gusA*) and inoculated on grapefruit, pZit34 and pZit45 fully complemented the mutation.

Effects of *pthA* on homologous interactions involving bean and cotton. When introduced into *X. c.* pv. *citrumelo* strain 3048Sp, pZit45 conferred the ability to elicit a strong HR on bean leaves 48 hr after inoculation at 10^8 cfu/ml. When introduced into *X. phaseoli* strain G27Sp, pZit45 conferred the ability to elicit a slightly stronger HR on bean leaves 48 hr after inoculation than the HR observed with 3048Sp and pZit45. (The same strains without pZit45 are compatible on bean with obvious water-soaking [Swarup *et al.* 1991].) The effect of *pthA* on the ability of 3048Sp and G27Sp to grow in bean plants was monitored. As expected, a significant decrease in growth was seen when *pthA* (in pSS35KBg) was present in either strain, and the effect on G27Sp was more pronounced in the first 48 hr after inoculation than the effect on 3048Sp (Fig. 5A,B). Within 5 days of inoculation, transconjugants of both strains were at approximately 10-fold lower concentrations *in planta* than their respective wild-type strains.

When introduced into *X. c.* pv. *malvacearum* strain Xcm1003, pZit45 conferred cultivar-specific avirulence on a set of nine different congenic resistant lines of *G. hirsutum* (cotton). As with *X. c.* pv. *malvacearum avr* genes (Gabriel *et al.* 1986; De Feyter and Gabriel 1991a), the specificity conferred by *pthA* strongly suggested *R* gene-for-*avr*-genes (plural) interactions (Table 2). The specificity conferred by *pthA* to Xcm1003 was different from that of all previously cloned *X. c.* pv. *malvacearum avr* genes.

Effects of *pthA* on nonhost (heterologous) interactions involving bean and cotton. In inoculations of the congenic cotton lines with 10^8 cfu/ml of wild-type *X. citri* 3213, a strong HR was observed on all lines, including Acala 44. A somewhat stronger HR was induced on bean within

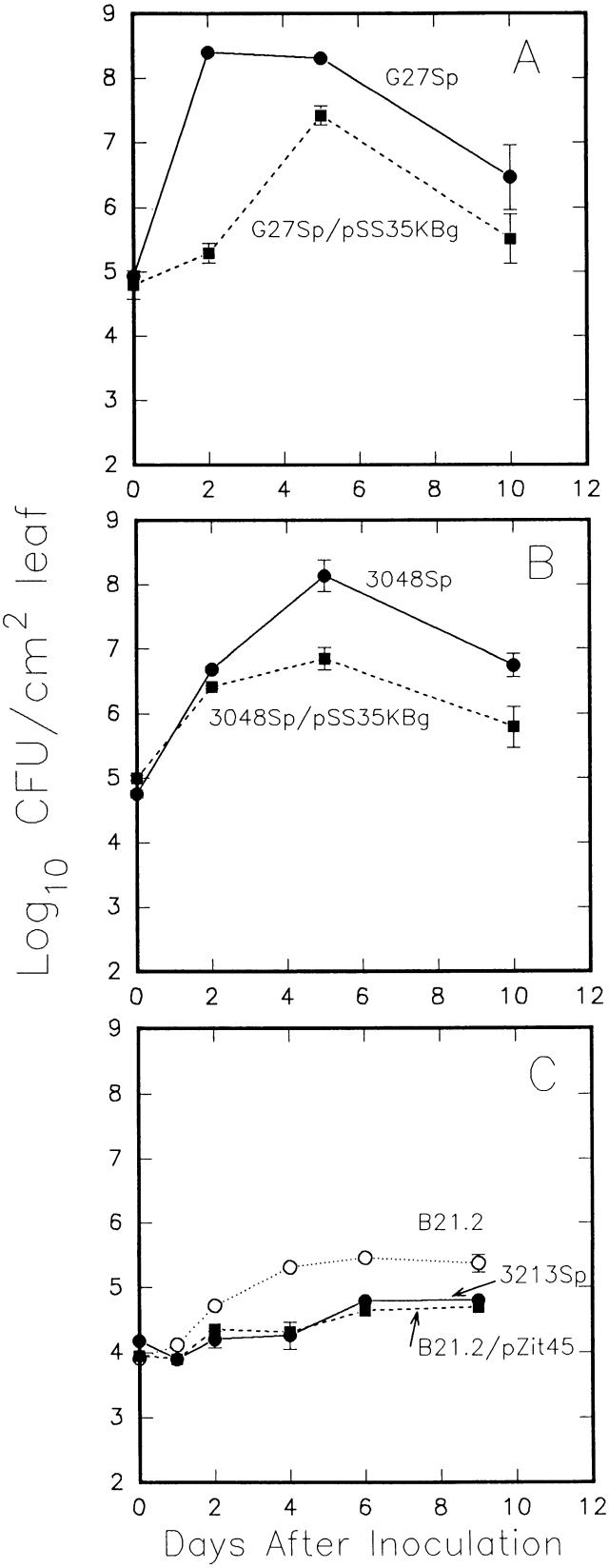

Fig. 5. Growth of *Xanthomonas* strains on bean over time, with and without *pthA*. **A,** *X. phaseoli* G27 and G27-pSS35KBg containing *pthA*; **B,** *X. c.* pv. *citrumelo* 3048 and 3048-pSS35KBg containing *pthA*; **C,** *X. citri* 3213, B21.2 *pthA*$^-$, and B21.2-pZit45 containing *pthA*. Vertical bars show the standard error at each sampling time. Data shown are from a single representative experiment.

36 hr of inoculation with 10^8 cfu/ml of *X. citri* 3213. When the nonpathogenic *X. citri* mutant strain B21.2 (*pthA*::Tn*5-gusA*) was inoculated at these levels onto cotton, a strong HR was observed on all lines, including Acala 44. Surprisingly, however, neither the HR nor water-soaking symptoms were elicited on inoculation of bean with 10^8 cfu/ml of the mutant B21.2 (*pthA*::Tn*5-gusA*) strain, and no visible disease symptoms appeared on bean 14 days after these high level inoculations. Although B21.2 did not elicit the nonhost HR, it also failed to grow *in planta* beyond 5×10^5 cfu/cm^2, although its growth increased in bean to a level about a log higher than the wild-type strain 3213Sp (Fig. 5C).

Gene *pthA* is homologous to an *avr* gene family found in many xanthomonads. Plasmids pZit34 and pZit45 were further mapped by restriction endonuclease digestion and were found to be indistinguishable from *avrBs3* of *X. c.* pv. *vesicatoria* (Doidge) Dye (Bonas *et al.* 1989) from the *Bam*HI site (near *Pst*I) to the *Sst*I site for all enzymes tested (*Bam*HI, *Bal*I, *Hin*cII, *Pst*I, *Sph*I, *Sst*I, and *Stu*I; refer Fig. 3). Furthermore, similarity of size and of restriction sites was also found with DNA fragments carrying *avrB4*, *avrb6*, *avrb7*, *avrBIn3*, *avrB101*, and *avrB102* cloned from *X. c.* pv. *malvacearum* (De Feyter *et al.* 1991a). Southern hybridization revealed that pZit45.119 hybridized at high stringency to the *Bam*HI fragments from all these *avr* genes (Fig. 2, some data not shown). DNA sequencing of the *Sal*I to *Pst*I region of pZit45.119 revealed nearly perfect homology with the published sequences of *avrBs3* and *avrBsP* over the entire 360-bp region sequenced and includes putative transcriptional and translational start sites (Fig. 4A). With partial digestion of pZit45.119 DNA with *Bal*I, a consistently sized ladder with 17 multimeric steps of about 102 bp was observed (data not shown). DNA sequencing of a fragment, including the *Sph*I and first two *Bal*I sites near the *Stu*I site of *pthA*, revealed that this *Bal*I fragment was 102 bp in size and was homologous with the 102-bp tandem repeats found in *avrBs3* and *avrBsP* (Fig. 4B).

Plasmid pZit45BB contains the *Bam*HI to *Bal*I fragment near the 5′ end of *pthA* internal to the gene. When pZit45BB was used as a probe against *Eco*RI total DNA from various xanthomonads, fragments of a size similar to *pthA* were detected by hybridization in *X. phaseoli* and in *X. campestris* pvs. *alfalfae* (Riker, Jones and Davis) Dye, *aurantifolii* Gabriel, *cyamopsidis* (Patel, Dhande and Kulkarni) Dye, *glycines* (Nakano) Dye, *translucens* (Jones, Johnson and Reddy) Dye, and *vignicola* (Burkholder) Dye (Fig. 1).

DISCUSSION

Pathogenicity locus *pthA* is essential for the elicitation of Asiatic citrus canker symptoms by *X. citri* 3213^T (Swarup *et al.* 1991). A marker-exchange mutation of *pthA* (carrying Tn*5-gusA*) in *X. citri* 3213 totally abolished the pathogenicity of the resulting strain (B21.2) on citrus and adversely affected its growth *in planta*. Strain B21.2 also failed to elicit an HR on bean, a nonhost, but did elicit a normal HR on another nonhost, cotton. Judging from the host reaction on bean and citrus, one could consider *pthA* to be a *hrp* (Lindgren *et al.* 1986) gene. It has previously been pointed out that reliance on a single nonhost plant species for heterologous HR tests may lead to erroneous conclusions about virulence (Azad and Kado 1984). Our observations confirm this and point out the need for inoculating a number of nonhost plants in mutational analyses of genes that affect virulence. Gene *pthA* was clearly distinguished from a *hrp* gene that, on mutation, would lose the ability to induce HR on all nonhosts.

Screening of congenic cotton resistant lines with transconjugants of the virulent cotton pathogen *X. c.* pv. *malvacearum* XcmN containing *pthA* showed that *pthA* conferred cultivar-specific avirulence (Table 2). Gene *pthA* could have been recovered as a cultivar-specific *avr* gene by screening an *X. citri* library in XcmN. The cultivar specificity of *pthA* was different from that of all previously cloned *X. c.* pv. *malvacearum avr* genes tested in XcmN and suggests a gene-for-genes avirulence function of *pthA*, as indicated for other *X. c.* pv. *malvacearum avr* genes on cotton (refer to De Feyter and Gabriel 1991a). However, gene *pthA* is not known to function in *X. citri* for avirulence on citrus. No races of *X. citri* are known, and no genes governing resistance in citrus have been documented. In *X. citri*, *pthA* played an essential role in the elicitation of citrus canker symptoms (Swarup *et al.* 1991), which may be consistent with an avirulence function in other xanthomonads on their hosts. Both phenotypes—elicitation of cankers on citrus and elicitation of the HR on bean and cotton—are essentially host reponses to the presence of *pthA* in *Xanthomonas* strains. It is possible that both types of host response may involve similar biochemical induction pathways in the respective plants.

Physical characterization of *pthA* revealed several lines of evidence that *pthA* is a member of an *avr* gene family that is widespread in the genus *Xanthomonas*. First, the restriction map (Fig. 3) indicated remarkable similarity to *avrBs3* (Bonas *et al.* 1989) and *avrBsP* (Canteros *et al.* 1991) of *X. c.* pv. *vesicatoria* and to fragments carrying *avrB4*, *avrb6*, *avrb7*, *avrBIn3*, *avrB101*, and *avrB102* cloned from *X. c.* pv. *malvacearum* (De Feyter and Gabriel 1991a). Second, the density of the Tn*5-gusA* inserts in the 3.4-kb *Bam*HI fragment indicated that a single long gene was involved. Third, as with *avrBs3* and *avrBsP*, multiple *Bal*I fragments of about 102 bp form the central region of the gene. Fourth, Southern hybridization revealed that a fragment of DNA carrying *pthA*, pZit45.119, hybridized

Table 2. Specificity of *pthA* on cotton cv. Acala 44 and nine congenic resistant lines

Xanthomonas campestris pv. *malvacearum* strains	Cotton cv. Acala 44 congenic resistant lines[a]									
	Ac44	**B1**	**B2**	**B4**	**B5a**	**B5b**	**b6**	**b7**	**BIn**	**BIn3**
Xcm1003/pUFR047	+	+	+	+	+	+	+	+	+	+
Xcm1003/pZit45 (*pthA*)	+*	−	−	+*	+−	−	+*	+*	+−	−

[a] + Indicates a compatible interaction, as indicated by a water-soaking lesion; +* is very similar to +, but indicates a slight reduction in water-soaking; − indicates an incompatible interaction, as indicated by a strong hypersensitive response (HR); +− indicates a weak hypersensitive response.

at high stringency to the *Bam*HI fragments from *avrBs3*, *avrB4*, *avrb6*, *avrb7*, *avrBIn3*, *avrB101*, and *avrB102* (Fig. 2, some data not shown). Fifth, the sequence of *pthA* is nearly identical to *avrBs3* from position 374 to at least position 733 of *avrBs3* and to *avrBsP* from position 1 to at least position 316; this region includes the putative transcriptional and translational start sites of *avrBs3* and *avrBsP* (Knoop *et al.* 1991; Canteros *et al.* 1991). Furthermore, the sequence of *pthA* fragment B in Figure 4B includes the canonical 102-bp repeated DNA contained in the *Bal*I fragments of *avrBs3* and *avrBsP*. Finally, Southern hybridization with a *pthA* internal fragment as a probe against total DNA from various xanthomonads revealed that multiple fragments of a size similar to *pthA* were detected by hybridization in *X. phaseoli* and in *X. c.* pvs. *alfalfae*, *aurantifolii*, *cyamopsidis*, *glycines*, *translucens*, and *vignicola* (Fig. 1). We conclude that *pthA* is a member of a family of *avr* genes that includes *avrBs3*, *avrBsP*, *avrB4*, *avrb6*, *avrb7*, *avrBIn3*, *avrB101*, *avrB102*, and many others widespread in the genus *Xanthomonas*.

According to genetic selection theories (Falconer 1989), it is expected that in the absence of a selective value, avirulence genes may eventually be lost from the pathogen population (Day 1974; Van der Plank 1968). However, certain avirulence genes in pathogen populations increase in frequency in the absence of the corresponding (gene-for-gene) resistance genes (Van der Plank 1975; Watson 1970). This phenomenon has been termed "stabilizing" selection and is based on a hypothetical pleiotropic selective value of *avr* genes (Crill 1977; Leonard and Czochor 1980; Parlevliet 1981). Genetic selection theories (Grant and Archer 1983) and segregation analyses (Bronson and Ellingboe 1986) have been used to test the hypothesis, but no evidence of selective value of the *avr* genes tested was found. Instead, both rapid increases and decreases in avirulence gene frequencies have been experimentally demonstrated to occur independently of host selection (Alexander *et al.* 1985). A likely explanation is that factors other than avirulence genes are ecologically selected, and the observed changes in avirulence gene frequencies are the result of linkage disequilibrium in clonally reproducing populations (Gabriel 1989). Only one *avr* gene, from *X. c.* pv. *vesicatoria* (*avrBs2*), has been reported to contribute pleiotropic fitness to a pathogen on its host (Kearney and Staskawicz 1990). Investigations in several laboratories have failed to produce evidence of a fitness value of other cloned *avr* genes. There are several host-specific nodulation (*hsn*) genes that are needed by specific *Rhizobium* strains to form nodules on their hosts and that behave in a formal genetic sense as *avr* genes when transferred to other species and biovars of *Rhizobium* (Debelle *et al.* 1988; Faucher *et al.* 1989; Lewis-Henderson and Djordjevic 1991). Gene *pthA* may be considered as a similar example, because it is needed by *X. citri* to form cankers on its host, and it behaves in a formal genetic sense as an *avr* gene when transferred to other species and pathovars of *Xanthomonas*. Although the *hsn* genes and *pthA* have clear selective value, it is unclear if they function for avirulence in their source strains (on hosts or nonhosts). Reports of any suggested or demonstrated selective value of *avr* genes that function as such in the source species (biovar or pathovar) are clearly exceptional.

There has always been some question as to the role of the nonhost HR in limiting host range, because hypersensitivity is known to be a defense-associated response in plants, and the nonhost HR is so general and common. The lack of evidence for a selective value of almost all *avr* genes, the lack of conservation of many *avr* genes within a microbial species or even pathovar, and the fact that most resistance genes are overcome or "defeated" by new pathogenic races led to the suggestion that most avirulence genes are gratuitous; they do not limit host range on nonhosts (Gabriel 1989). Race specificity is controlled by *avr* genes, which may limit the host range at higher than cultivar level, although usually within a plant family. For example, *avrBsT* prevents some strains of *X. c.* pv. *vesicatoria* (host range includes pepper) from attacking tested pepper cultivars (Minsavage *et al.* 1990). Similarly, *avrA* is thought to limit the host range of some *Pseudomonas solanacearum* (Smith) Smith strains (host range includes tobacco) from attacking tested tobacco cultivars (Carney and Denny 1990). Both of the examples given involve the role of *avr* genes in race specificity on known hosts, not nonhosts. These examples do not address if *avr* genes and the HR that they trigger are responsible for the relatively stable host range of microbes at higher than race level (e.g., pathovar and species level) when nonhosts are involved.

Elimination of the nonhost HR normally elicited by *X. citri* on bean did not expand the host range of *X. citri* beyond that already described. One could argue that other, non-HR-inducing *avr* genes that limit host range of *X. citri* on bean are present. There is no evidence for this, however, and this argument invokes a hypothetical *avr-R* gene interaction that results in no visible HR, yet a strong growth suppression does occur. Experimentally, the loss of a strong HR reaction due to *pthA* resulted in only a slight growth increase. One could also argue that *pthA* encodes a pleiotropic virulence function needed for *X. citri* to grow on bean. Gene *pthA* is not a *hrp* gene, however, and because *X. citri* does not have a host range on bean, a host-specific virulence function of *pthA* is unlikely. The simplest explanation is that *pthA* does not confer avirulence to *X. citri* on hosts or nonhosts. Even though *pthA* is a member of an *avr* gene family that is widespread in *Xanthomonas*, the only phenotype conferred by *pthA* that is associated with avirulence is the HR induced on nonhosts; in the case of bean at least, the nonhost HR appears inconsequential and does not limit host range on bean. We conclude that other, positive-acting factors, similar to the *hsv* genes of *X. c.* pvs. *citrumelo* (Kingsley and Gabriel 1991) and *translucens* (Waney *et al.* 1991), may be needed for host range on bean.

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