A Pathogenicity Locus from *Xanthomonas citri* Enables Strains from Several Pathovars of *X. campestris* to Elicit Cankerlike Lesions on Citrus

Sanjay Swarup, Robert De Feyter, Ronald H. Brlansky, and Dean W. Gabriel

First, second, and fourth authors: Plant Pathology Department, University of Florida, Gainesville 32611. Third author: University of Florida, Institute of Food and Agricultural Sciences, Citrus Education and Research Center, Lake Alfred 33850. Current address of second author: Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra ACT 2601.

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ABSTRACT

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A virulence enhancement approach was used to clone a pathogenicity (*pth*) locus from a highly virulent pathogen by assaying the library in a second, less virulent strain that was compatible with the same host. A genomic library of the virulent Asiatic canker pathogen *Xanthomonas citri* was conjugally transferred to the opportunistic pathogen, *X. campestris citrumelo,* and the transconjugants were screened on *Citrus paradisi* 'Duncan' (grapefruit) leaves. Transconjugants able to induce host cell proliferation and raised, Asiatic cankerlike lesions were identified, and clone pSS10.35 was found to carry the gene(s) responsible. This clone was transferred to other *Xanthomonas* strains, including two that are weakly pathogenic to citrus in greenhouse tests (members of *X. c. alfalfae* and *X. c. cyamopsidis*) and two that are avirulent on citrus (*X. phaseoli* and *X. c. malvacearum*). Transconjugants of the two weakly pathogenic *Xanthomonas* strains induced cankerlike lesions when inoculated on citrus; these same strains became avirulent on their homologous host plants. Transconjugants of *X. phaseoli* and *X. c. malvacearum* strains remained unaltered in phenotype on citrus. A 3.7-kb region of pSS10.35 carrying the *pthA* locus was identified by subcloning and Tn*5-gusA* mutagenesis. Marker-exchange mutagenesis of *X. citri* using Tn*5-gusA* insertions in the 3.7-kb region resulted in a complete loss of virulence (disease symptoms and growth in planta) on citrus and loss of the hypersensitive response on heterologous hosts (i.e., an Hrp^- phenotype). The Hrp^- phenotype, but not growth in planta, of the marker-exchanged mutants was restored by subclones of pSS10.35 containing the 3.7-kb region.

Additional keywords: citrus canker, host range, virulence enhancement.

Xanthomonas citri ex Hasse (17) is the causal agent of Asiatic citrus canker disease. The host range of the pathogen includes a wide variety of *Citrus* spp. and relatives in Rutaceae. Untreated infestations can result in defoliation and premature fruit drop leading to serious economic losses (40). Symptoms of Asiatic citrus canker disease include erumpent, corky lesions on all aerial parts of mature citrus trees including fruits, leaves, and stems (40). *X. campestris citrumelo* Gabriel pv. Nov. (17) is the causal agent of citrus bacterial spot. These pathogens produce only mild or opportunistic leaf spot infections on juvenile citrus foliage (19). *X. c. citrumelo* strains are a heterogeneous group, genetically and pathologically related to other heterogeneous pathovars, including *X. c. alfalfae* (16,21), *X. c. cyamopsidis* (17), *X. c. fici,* and *X. c. maculifoliigardeniae* (20).

Various approaches to cloning pathogenicity determinants in *Xanthomonas* spp. have been employed in the past, including rapid in vitro plate assays (49) and complementation of mutations affecting pathogenicity using in planta assays (51). Another successful method involves identifying plant-inducible promoters in "promoter-probe" vectors, and later identifying any pathogenicity genes that may be transcribed from the promoters (36,46). A strategy to cloning virulence genes that has been attempted without success was that of trying to increase the host range of a strain from one *Xanthomonas* pathovar by adding DNA fragments from a strain of a different pathovar having a nonoverlapping host range (38). We hypothesized that a strategy similar to the latter approach might work, provided that the recipient strain was at least mildly compatible with the host plant of the donor and that there were easily scorable differences in virulence or disease phenotypes. The fact that citrus can serve as a host for both a *Xanthomonas* species that causes severe disease and one that causes only mild or opportunistic infections led us to attempt the "virulence enhancement" approach. The approach is based on the hypothesis that the disease phenotype induced by a pathogen like *X. citri* can be attributed to gene functions that induce pathogenic reactions in plants (i.e., pathogenicity genes), in addition to those required for growth in planta (i.e., parasitism

genes) (13). Such pathogenicity genes from highly virulent strains should be either absent or nonfunctional in milder pathogens compatible on the same host.

We report here the cloning of a DNA fragment carrying a pathogenicity (*pth*) gene locus that is required by *X. citri* to induce the plant symptoms associated with Asiatic citrus canker. The locus was identified by screening an *X. citri* library in *X. c. citrumelo* for virulence enhancement of the milder pathogen on citrus. Preliminary results of this study have been reported (47,48).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. Strains of *Escherichia coli, Xanthomonas* spp., and plasmids used in this study are listed in Table 1 along with their relevant characteristics and sources or references. *Xanthomonas* spp. were cultured on PYGM medium at 30 C, as described previously (17). *E. coli* strains, unless otherwise stated, were grown in Luria-Bertani (LB) medium at 37 C (33). Antibiotics were used at the following final concentrations (in μg/ml): chloramphenicol (Cm), 35; kanamycin (Km), 25; nalidixic acid (Nal), 100; spectinomycin (Sp), 35; streptomycin (Sm), 100; tetracycline (Tc), 15; gentamicin (Gm), 1.5 for *E. coli* and 3 for *Xanthomonas* spp.

Genetic and bacteriological techniques. *Xanthomonas citri* 3213^T genomic DNA, partially digested with *Sau*3AI, was size-fractionated on a sucrose density gradient and the 20- to 25-kb fraction ligated to *Bam*HI linearized pUFR027 DNA. Competent *E. coli* DH5α cells were transformed and plated on selective medium containing Bluo-gal (halogenated indolyl-β-D-galactoside, Bethesda Research Laboratories, Gaithersburg, MD) and IPTG (isopropylthio-β-galactoside, Bethesda Research Laboratories) according to Sambrook et al (37). Eighteen white colonies from the clone bank were randomly chosen, their plasmid DNAs were extracted by alkaline lysis (2), and the restriction profiles of each were analyzed to determine the average insert size. The *X. citri* 3213 clone bank was introduced into *X. c. citrumelo* 3048 using *E. coli* HB101 (pRK2013) as a helper strain in triparental matings (11). Unless otherwise stated, the same conjugation method was used to transfer other clones into various *Xanthomonas* strains used in the study.

Standard recombinant DNA procedures (37) were followed for various cloning and restriction mapping experiments. Southern blot analyses (43) were performed using Genescreen Plus nylon membranes (NEN Research Products, Boston, MA) according to the manufacturer's recommendations. The membranes were treated and probed with [^{32}P]dCTP radiolabeled plasmid DNA as described previously (28). For gene expression studies using the Tn*5-gusA* promoter/probe transposon (42), β-glucuronidase assays were performed as described by Jefferson (23).

Fragments from pUFR027 (Nm^r) were subcloned in pUFR042 (Nm^{r}Gm^r) or in pUFR044 (Gm^r). pUFR049 (Cm^{r}Sm^r) was constructed by inserting a 1.0-kb incW fragment from pUFR034 (10) into an RSF1010-derived replicon.

Transposon mutagenesis. Transposon mutagenesis was carried out in a three-step process. The target clone (p35KX15) (Gm^r) was used to transform competent *E. coli* C600-387 cells, which harbor the transposon Tn*5-gusA* on the chromosome. Tn*5-gusA* produces transcriptional fusions (42) on insertion in one orientation in a transcriptionally active gene.

Approximately 250 independent transformants, resistant to Km, Tc, and Gm, served as donors in triparental matings using *E. coli* HB101/pUFR035 (Sm^{r}Cm^r) as the recipient and ED8767/pRK2073 (Sp^r) as the helper strain. Mid-log phase cultures of the recipient and helper strains (mixed 1:1) were spot-inoculated (10–20 μl per spot) on LB agar plates. After the excess liquid was absorbed by the agar, transformants (donors) were transferred onto and mixed with the individual spots of recipient and helper using sterile toothpicks. After incubation of these patch-mating plates at 37 C for 4–6 h, cells from each patch were transferred to LB plates containing Sm, Cm, Km, Tc, and Gm using sterile toothpicks (i.e., HB101/[pUFR035 + p35KX15::Tn*5-gusA*]).

In the final step, pUFR035 was eliminated by conjugally transferring p35KX15::Tn*5-gusA* derivatives into an *E. coli* strain (C2110 Nal^r) carrying a *polA* mutation. Recipient *E. coli* C2110 and helper ED8767/pRK2073 strains were spot-inoculated and donor cells subsequently patched on them as before. Trans-

TABLE 1. Bacterial strains, plasmids, and transposons used in this study

Strain, plasmid, or transposon	Relevant characteristics	Source or reference
Escherichia coli		
DH5α	*supE44*Δ *lacU169*(ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
HB101	*supE44 hsdS20*($r_B^-m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	5
ED8767	*supE44 supF58 hsdS3*($r_B^-m_B^-$) *recA56 galK2 galT22 metB1*	34
C600-387	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21 hflA150* [chr::Tn*5-gusA* (Km^{r}Tc^r)]	This study
C2110	*polA* Nal^r	44
Xanthomonas		
KX-1	*X. campestris* pv. *alfalfae*, Sp^r	28
3048^H	*X. c. citrumelo*, Sp^r	17
13D5	*X. c. cyamopsidis*, Sp^r	28
HSp	*X. c. malvacearum*, Sp^r	15
G27^T	*X. phaseoli*, Sp^r	17
3213^T	*X. citri*, Sp^r	17
B21.2	*X. citri* 3213^T (*pthA*::Tn*5-gusA*), Sp^{r}Km^{r}Tc^r, marker-exchanged mutant of 3213	This study
Plasmids		
pUC119	ColE1, M13 lg, Ap^r	52
pUFR027	IncW, Nm^r, Mob$^+$, *mob*P, *lacZ*α^+, Par$^+$	10
pUFR034	IncW, Nm^r, Mob$^+$, *lacZ*α^+, Par$^+$, cosmid	10
pUFR035	ColE1 replicon, Cm^r, Sm^r, Mob$^-$, *lacZ*α^-	10
pUFR042	pUFR027 derivative, Nm^r,Gm^r	De Feyter and Gabriel, *unpublished*
pUFR044	pUFR042 derivative, Gm^r	This study
pUFR049	RSF1010 replicon, Cm^r Sm^r, IncW$^+$, displacement vector	This study
pSS10.35	20.1-kb genomic DNA of *X. citri* 3213 in pUFR027, Nm^r	This study
pSS35KS	5.4-kb *Kpn*I-*Sal*I fragment of pSS10.35 in pUC119, Ap^r	This study
pSS35KBg	7.7-kb *Kpn*I-*Bgl*II fragment of pSS10.35 in pUFR042, Nm^r,Gm^r	This study
p35KX15	16.8-kb *Kpn*I-*Xho*I fragment of pSS10.35 in pUFR044, Gm^r	This study
pRK2013	ColE1, Km^r, Tra$^+$, helper plasmid	12
pRK2073	pRK2013 derivative, *npt*::Tn*7*, Km^s, Sp^r, Tra$^+$, helper plasmid	29
Transposon		
Tn*5-gusA*	Tn*5-uidA1*, Km^{r}Tc^r, forms transcriptional fusions	42

[a]Superscript characters indicate the following: H = Holopathotype strain; T = Type strain, r = resistant; s = sensitive; + = functional; − = nonfunctional.

conjugants were selected on plates containing Nal, Km, Tc, and Gm. Two colonies were retained per each initial mating, making a total of ~500 Tn*5-gusA* insertional derivatives. The site and orientation of the inserted transposons were determined by restriction analyses.

The mutagenized clones were introduced into various *Xanthomonas* strains (Sp^r) by triparental matings using individual *E. coli* C2110/p35KX15::Tn*5-gusA* ($Nal^rKm^rTc^rGm^r$) clones as donors and HB101/pRK2013 (Km^r) as the helper strain. Conjugations were harvested on selective plates containing Sp, Km, Tc, and Gm.

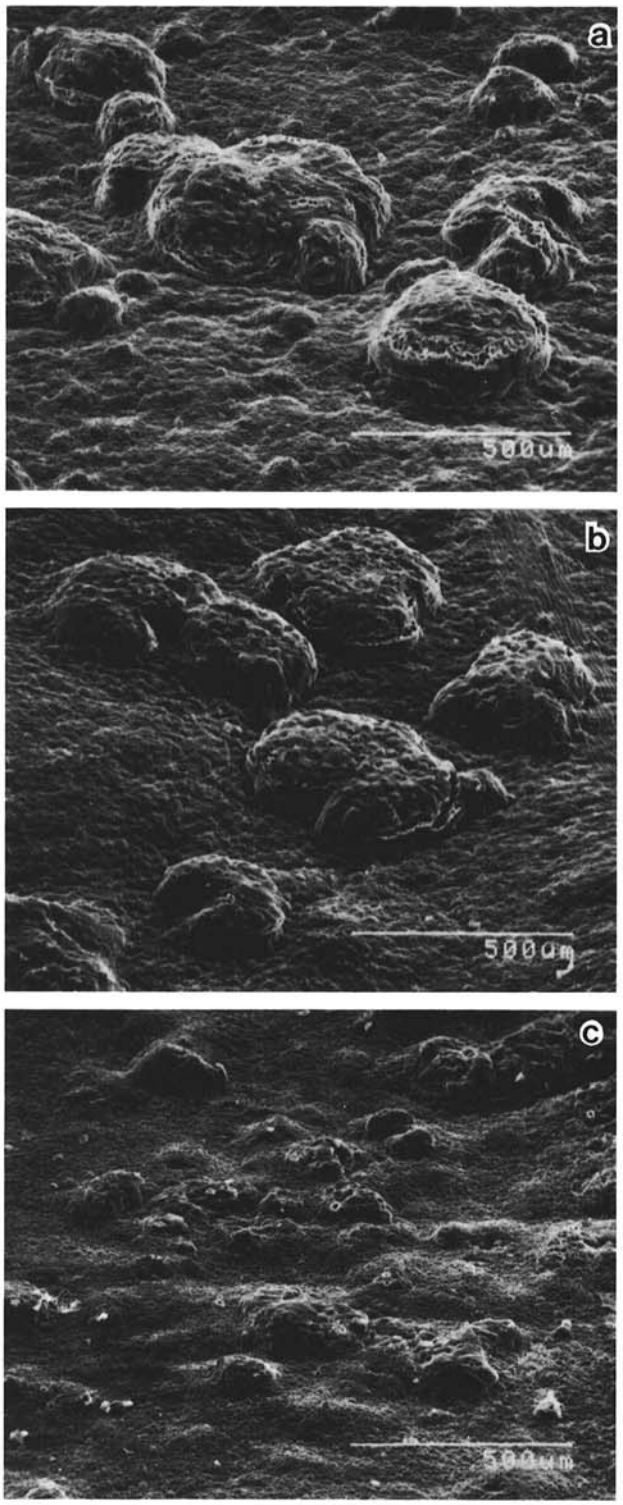

Fig. 1. Scanning electron micrographs of lesions induced on *Citrus paradisi* 'Duncan' leaves 17 days after spray inoculations. **A,** *Xanthomonas citri* 3213^T; **B,** *X. c.* pv. *citrumelo* 3048/pSS10.35; **C,** *X. c.* pv. *citrumelo* 3048.

Marker-exchange mutagenesis. Marker-exchange mutagenesis of wild-type *X. citri* 3213 (Sp^r) was accomplished by mobilizing the IncW displacement vector pUFR049 (Cm^rSm^r) with pRK2013 into transconjugants harboring p35KX15::Tn*5-gusA* ($Gm^rKm^rTc^r$) derivatives. Transconjugants were selected on plates containing Sp, Km, Tc, and Cm. Approximately 50 transconjugants from each mating were screened for Gm^s. The $Sp^rKm^rTc^rGm^s$ clones then were grown on medium lacking Cm to allow for segregation and loss of pUFR049, and Cm^s colonies retained. These marker-exchanged mutants were maintained on plates containing Sp, Km, and Tc.

Pathogenicity screening and testing. All plants were grown under natural light in quarantine greenhouse facilities equipped with HEPA air filters at the Division of Plant Industry, Florida Department of Agriculture, Gainesville. Temperatures in these

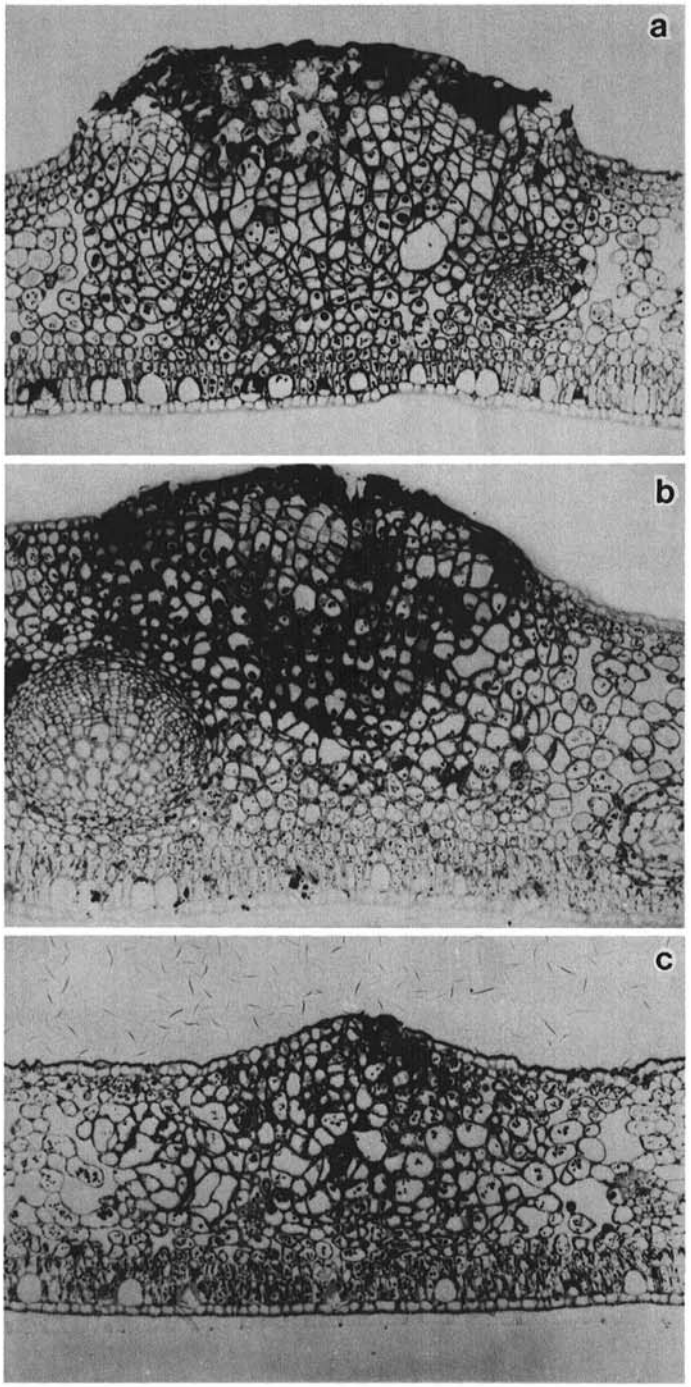

Fig. 2. Histopathology of *Citrus paradisi* leaves 17 days after spray inoculations (60× magnification). **A,** *Xanthomonas citri* 3213^T; **B,** *X. c.* pv. *citrumelo* 3048/pSS10.35; **C,** *X. c.* pv. *citrumelo* 3048.

greenhouses normally range from 28 to 35 C, with 50–100% relative humidity. All inoculations were carried out under BL-3P level containment in these quarantine greenhouses (refer to Federal Register Vol. 52, No. 154, pp. 29,800-29,814, 11 August 1987).

Over 500 transconjugant *X. c. citrumelo* 3048 strains were screened in duplicate inoculations for virulence enhancement on *C. paradisi* 'Duncan' (grapefruit) leaves. Transconjugants were picked from PYGM agar plates that had been incubated at 30 C for approximately 60 h. Colonies were resuspended in sterile tap water and inoculated through the abaxial surface of immature Duncan grapefruit leaves by pressure infiltration using blunt-ended syringes (17). Although bacterial concentrations in the inocula were not determined in these preliminary screenings, care was taken to pick approximately the same amount of cells from the PYGM agar plates. Symptoms were recorded 8–12 days later.

In retests of putative virulence-enhancing clones, transconjugants and control strains were grown to late log phase, and the concentrations of all inocula were adjusted to 10^8 cfu/ml using a spectrophotometer. Grapefruit leaves were inoculated as before. Inoculations of fully expanded, mature leaves of *Cyamopsis tetragonoloba* USDA PI 215590 cv. 13643 and *Phaseolus vulgaris* 'California Light Red' (Agway Corporation, Beanplant, NY) were performed by pressure infiltration as described previously (17).

In planta growth kinetics. Bacterial suspensions, adjusted to $\sim 10^5$ cfu/ml in sterile tap water, were pressure-infiltrated into fully expanded, yet immature, Duncan grapefruit leaves. These leaves were all of similar size and thickness. Leaf tissue disk punches (8 mm diameter) were removed at 0, 1, 5, 10, and 15 days after inoculation from nonwounded leaf zones, macerated in 1 ml of sterile tap water, and appropriate dilutions plated to obtain single colonies on media containing appropriate antibiotics. Growth of *X. citri* 3213, B21.2, and B21.2/pSS35KBg in planta was determined by counting only those colonies which carried all appropriate antibiotic markers. Populations were expressed as log cfu/ml of sterile tap water used for grinding leaf tissue.

Stability of plasmids in planta was determined by plating samples from the above leaf extracts on PYGM (Sp) plates. One hundred randomly chosen colonies from each time point were screened for antibiotic resistance markers on the plasmid. Plasmid loss over time was expressed as percent *Xanthomonas* cells expressing the plasmid encoded antibiotic resistance(s).

Scanning electron and light microscopy. Bacterial suspensions of $\sim 10^8$ cfu/ml in sterile tap water were sprayed on young Duncan grapefruit leaves using a Crown Spra-tool aerosol sprayer (no. 8011 power pack, Crown Industrial Products Co., Helbron, IL). Leaf disks (8 mm diameter) from infected areas were removed 7, 9, 12, 17, and 21 days after inoculation using sterile cork borers, and fixed at 4 C overnight in 2% glutaraldehyde solution made in aqueous 0.066 M phosphate buffer (pH 6.8). The disks were washed and stored in the aqueous 0.066 M phosphate buffer before thin sectioning and microscopy. Sample processing and microscopy was as described by Brlansky et al (7).

RESULTS

***X. citri* gene library.** Based on DNA restriction profiles of plasmids from 18 arbitrarily chosen clones, the *X. citri* strain 3213 genomic DNA library appeared to contain random inserts averaging 22.5 kb in size. The genomic library consisting of 1,344 clones was maintained in *E. coli* strain DH5α. (A fully representative library carrying inserts averaging 22.5 kb in size requires about 700 clones [37].) The plasmids transferred at an average frequency of 5.5×10^{-5} per recipient into *X. c. citrumelo* 3048.

Pathogenicity phenotypes of pSS10.35. Five hundred independent transconjugants of *X. c. citrumelo* 3048 containing recombinant plasmids that contained *X. citri* 3213 DNA were inoculated onto Duncan grapefruit leaves at $\sim 10^8$ cfu/ml. One transconjugant out of the 500 tested (pSS10.35, 20.0 kb in size) was found to induce raised, cankerlike lesions on grapefruit leaves after repeated inoculations. Plasmid pSS10.35 was again mated from *E. coli* DH5α into *X. c. citrumelo* 3048 and retested on grapefruit leaves, with the same results. To demonstrate that pSS10.35 carried a *trans*-acting pathogenicity gene(s), plasmid DNA was extracted from *X. c. citrumelo* 3048 transconjugants isolated from inoculated citrus leaves, reintroduced into *E. coli* DH5α cells by transformation, and again mated into *X. c. citrumelo* 3048. The resulting transconjugants also induced cankerlike lesions when inoculated onto citrus leaves. Based on restriction enzyme digestions at the beginning and end of these manipulations, pSS10.35 appeared to be unaltered. Inoculations of citrus with low cell numbers (10^5 cfu/ml) of *X. c. citrumelo* 3048/pSS10.35 gave results comparable to those obtained at high cell numbers, except that low inoculation concentrations gave rise to well-separated, individual, raised lesions.

Strains belonging to both *X. c. alfalfae* and *X. c. cyamopsidis* are compatible with citrus and cause mild water-soaked lesions on the foliage under greenhouse conditions (16). Introduction of pSS10.35 into strains of these pathovars followed by inoculation of grapefruit leaves resulted in corky, erumpent lesions similar to those obtained with *X. c. citrumelo* 3048/pSS10.35. When pSS10.35 was introduced into *X. phaseoli* and *X. c. malvacearum* strains (neither is able to grow on citrus), no visible symptoms were obtained. Even at high inoculum cell densities ($\sim 10^8$ cfu/ml), the transconjugants of these incompatible strains containing pSS10.35 were unable to incite any disease symptoms on citrus or multiply in planta, indicating that pSS10.35 does not carry genes capable of extending the host range of these strains to

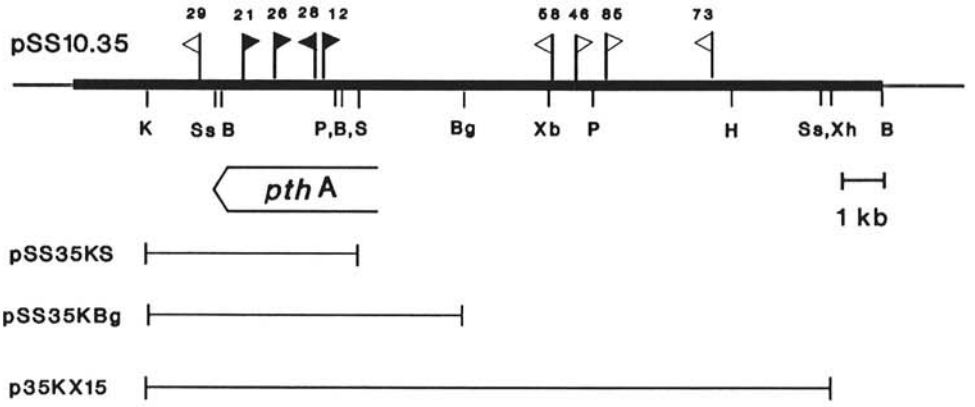

Fig. 3. Molecular characterization of pSS10.35 and of transcription of *pth*A as determined by subclones and Tn*5-gusA* insertions. Subclones pSS35KS, pSS35KBg, and p35KX15 are shown below the partial restriction map of pSS10.35. Sites and orientation of Tn*5-gusA* insertions are shown above the restriction map. Arrowheads indicate the direction of *gusA*1 transcription, solid arrows indicate insertions leading to loss of gene function. Numbers indicate some of the insertional derivatives characterized. B = *Bam*HI; Bg = *Bgl*II; H = *Hin*dIII; K = *Kpn*I; P = *Pst*I; S = *Sal*I; Ss = *Sst*I; Xb = *Xba*I; Xh = *Xho*III.

include citrus.

Introduction of pSS10.35 into *X. c. citrumelo* 3048 changed the reaction of the strain on bean (inoculated at 10^8 cfu/ml) from water-soaking to a hypersensitive response (HR), indicative of avirulence (*avr*) gene function on bean. Similarly, introduction of pSS10.35 into *X. c. cyamopsidis* 13D5 changed the reaction of the strain on guar (inoculated at 10^8 cfu/ml) from water-soaking to little or no symptom development, again indicative of *avr* gene function on guar.

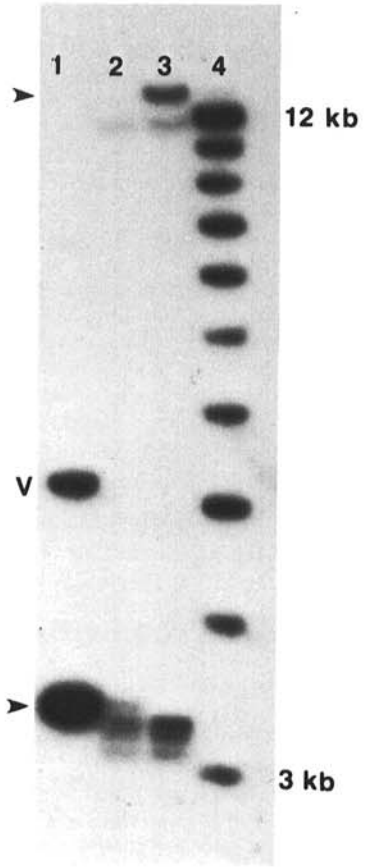

Fig. 4. Southern hybridization showing marker-exchange mutagenesis of *pthA* in *Xanthomonas citri*. Lane 1, *Bam*HI digested pSS35KS DNA; Lane 2, *Bam*HI digested genomic DNA of *X. citri* 3213^T; Lane 3, *X. citri* B21.2 (*pthA*::Tn*5*-*gusA* mutant derivative of 3213); Southern blot probed with 3.7-kb *Sst*I-*Sal*I fragment of pSS35KS carrying *pthA*; Lane 4, ^{32}P-labeled molecular weight markers (1 kb multimers from 3 to 12 kb shown). V = vector plus pSS35KS junction fragments to the *Bam*HI sites. Arrows indicate Tn*5*-*gusA*-affected fragments.

Surface morphology and histology of lesions caused on *C. paradisi* 'Duncan' leaves. Lesions induced by transconjugants of *X. c. alfalfae, X. c. citrumelo,* and *X. c. cyamopsidis* with and without pSS10.35 on citrus were compared with those caused by *X. citri* in both light and scanning electron microscopy studies. *X. c. citrumelo* 3048 induced relatively flat-surfaced lesions that were easily distinguished from the pustules elicited by *X. citri* 3213 (6) or *X. c. citrumelo* 3048/pSS10.35 strains. Lesions induced by *X. c. citrumelo* 3048/pSS10.35 were indistinguishable from those induced by *X. citri* 3213 in external surface morphology (Fig. 1) and histology (Fig. 2). Host cell proliferation to the extent of rupturing the epidermal layer seemed to be of the same magnitude in both cases. Additionally, hypertrophy of the spongy mesophyll rather than hyperplasia seemed to be the main cause of pustule formation in both cases. *X. citri* 3213 and *X. c. citrumelo* 3048/pSS10.35 induced lesions characterized by a raised epidermis that ruptures, exposing the upper palisade tissue, whereas in lesions induced by *X. c. citrumelo* 3048, the epidermal cells seemed intact and no such rupture was observed (Fig. 2).

Molecular characterization of pSS10.35 and localization of *pthA*. A partial restriction map of pSS10.35 (20 kb) is shown in Figure 3. The region required to elicit cankerlike lesions on citrus was localized by subcloning and transposon mutagenesis to span a 3.7-kb segment (Fig. 3). Transposon mutagenesis of a 16.8-kb subclone, p35KX15, was carried out to insertionally inactivate gene(s) involved in conferring the pathogenicity phenotypes on citrus, bean, and guar. Ninety-nine of 120 independent insertional derivatives carried Tn*5*-*gusA* in the 16.8-kb subcloned fragment (and not in the vector) were mated into *X. c. citrumelo* 3048 and screened on plants. Fourteen of these failed to induce cankerlike lesions on citrus and the avirulence phenotype on bean; the remaining 85 insertional derivatives induced cankerlike lesions on citrus and avirulence on bean. All 14 Tn*5*-*gusA* inserts were mapped to the 3.7-kb fragment; four of these are localized in Figure 3. When these 14 p35KX15::Tn*5*-*gusA* insertional derivatives were introduced into *X. c. cyamopsidis* strains, none of the insertional derivatives induced cankerlike lesions on citrus or reduced virulence on guar. This region has at least one gene, designated *pthA* (*path*ogenicity *A*), that appeared to operate in trans for virulence enhancement of strains compatible with citrus and for avirulence of the same strains on other homologous hosts. No subclone, nor any of the 99 insertional derivatives tested, separated the two phenotypes (virulence enhancement on citrus and avirulence on bean and guar).

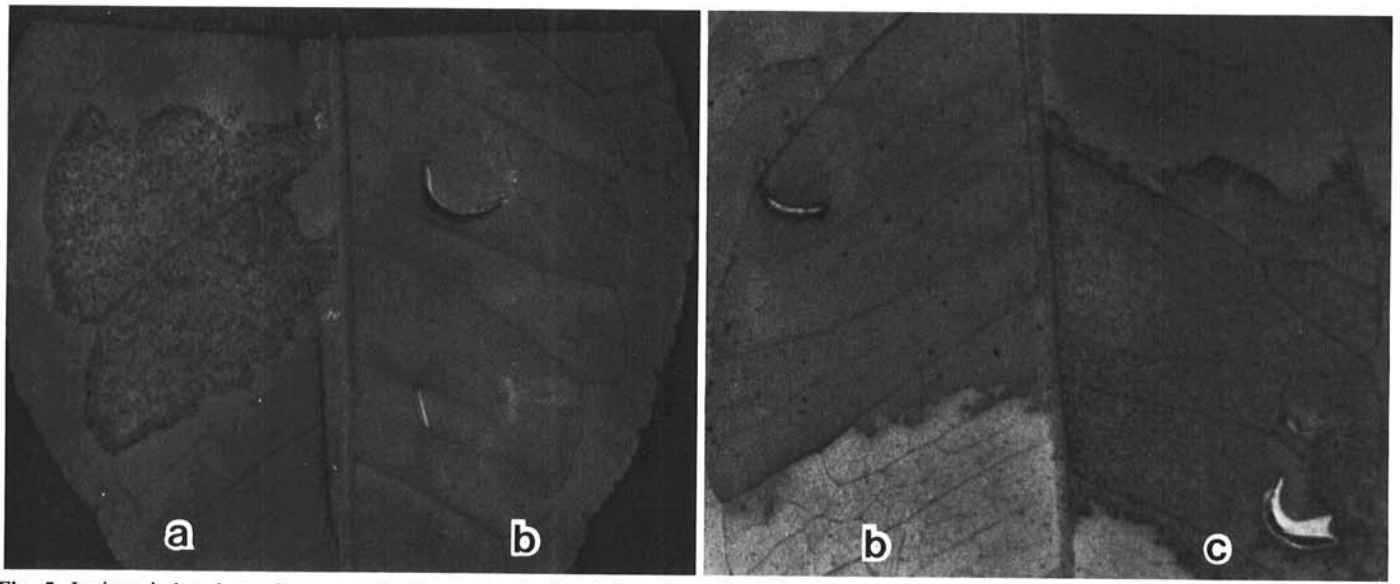

Fig. 5. Lesions induced on *Citrus paradisi* leaves 1 wk after infiltration with 10^8 cfu/ml of *Xanthomonas citri* 3213, marker-exchanged mutant B21.2, and complemented mutant. **A,** *X. citri* 3213^T; **B,** B21.2; **C,** B21.2/pSS35KBg.

Marker-exchange mutagenesis. In order to determine the role of the *pthA* locus in *X. citri* pathogenicity on citrus, marker-exchange mutagenesis was carried out. Transfer of displacement vector pUFR049 into *X. citri* 3213/p35KX15::Tn*5-gusA* aided the recovery of marker-exchanged mutants. The frequency of marker-exchange varied with clones, position of transposon insertion, and strains, but was generally in the range of 1–10% of the colonies recovered in the procedure. A Southern blot analysis and pathogenicity test of one representative marker-exchanged mutant, *X. citri* B21.2 (*pthA*::Tn*5-gusA*), are shown in Figures 4 and 5, respectively. In Figure 4, the band corresponding to the 3.5-kb *Bam*HI fragment of wild-type *pthA* is indicated by an arrow in both lanes 1 (pSS35KS) and 2 (*X. citri* 3213 total DNA). The heavier intensity of the band in lane 1 is due to overloading the DNA sample in lane 1 relative to the amount of the identical band loaded in lane 2. The multiple hybridizing bands in lanes 2 and 3 indicate the presence of at least four similarly sized, and one larger sized, *Bam*HI DNA fragments homologous with the 3.7-kb probe. Note the disappearance of the 3.5-kb *pthA* *Bam*HI fragment in lane 3 and the appearance of a new 12.9-kb band (Tn*5-gusA* is 9.4 kb in size and has no *Bam*HI sites). The blot shown in Figure 4 was rehybridized with radiolabeled Tn*5-gusA* as a probe and revealed homology only to the 12.9-kb band (rehybridized blot not shown), demonstrating insertion of Tn*5-gusA* by marker-exchange in the appropriate 3.5-kb (*pthA*) *Bam*HI fragment.

Marker-exchange of *pthA*::Tn*5-gusA* in *X. citri* B21.2 resulted in a complete loss of pathogenic symptoms on Duncan grapefruit, even at 10^8 cfu/ml inoculation levels (Fig. 5). Furthermore, mutant B21.2 lost the ability to induce a heterologous HR on nonhost plants of *Phaseolus vulgaris* 'California Light Red' (data not shown). Complementation analysis of the *pthA*::Tn*5-gusA* mutation was carried out by introducing pSS35KBg (7.7 kb insert) into *X. citri* B21.2. Restoration of the phenotype to the wild-type response was observed on inoculated Duncan grapefruit leaves with B21.2/pSS35KBg at both high and low inoculum levels (Fig. 5). Restoration of the heterologous HR response was also observed.

The direction of transcription of *pthA* indicated in Figure 3 was determined by β-glucuronidase (GUS) assays of marker-exchanged mutants grown in planta and in PYGM broth. Expression of *pthA* was observed in B21.2 grown in planta and in broth. The 5′ end of *pthA* lies between the *Sal*I and *Bgl*II restriction sites, and the 3′ end lies between the *Sst*I and *Kpn*I sites, based on partial DNA sequence and subcloning analyses (47 and unpublished data). Transconjugants of *X. c. citrumelo* 3048 or *X. c. cyamopsidis* 13D5 strains containing all the subclones of pSS10.35 ending at either the *Sal*I site or the *Sst*I site (adjoining the *Bam*HI sites) failed to elicit cankerlike lesions when inoculated onto citrus. This indicated that the *pthA* region spanned (and was not delimited by) the *Sal*I and *Sst*I sites adjoining the *Bam*HI site.

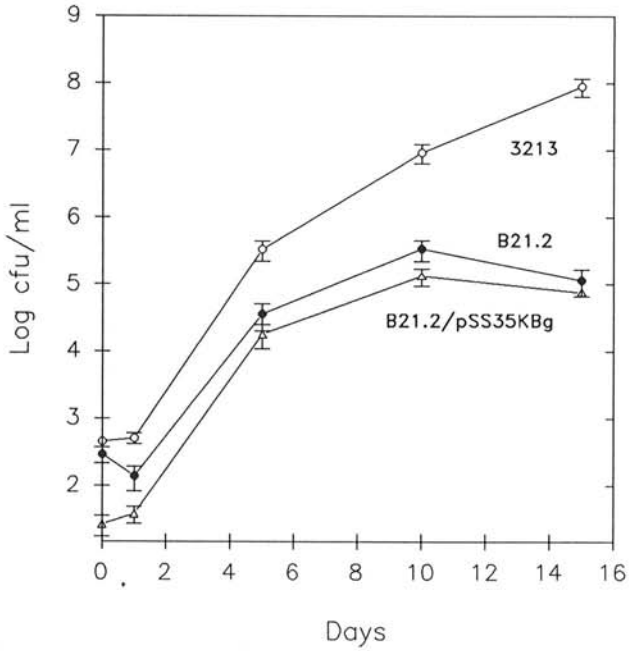

Fig. 6. Growth kinetics in planta of wild-type *Xanthomonas citri* 3213^T, marker-exchange mutant B21.2, and mutant B21.2 complemented by cloned wild-type DNA on pSS35KBg. **A,** *X. citri* 3213^T; **B,** B21.2; **C,** B21.2/pSS35KBg.

Growth kinetics in planta. Growth kinetics of *X. citri* 3213, *X. citri* B21.2 (*pthA*::Tn*5-gusA*), and *X. citri* B21.2/pSS35KBg were studied in Duncan grapefruit leaves. Results are presented in Figure 6. Growth of marker-exchange mutant strain B21.2 was reduced by two orders of magnitude after 15 days in planta as compared with the wild-type strain 3213. Tn*5-gusA* insertion in *pthA* also led to a rapid decline in the bacterial population as compared with the wild-type, which survived for a longer time in leaf tissue. Unexpectedly, growth kinetics of the complemented strain B21.2/pSS35KBg was found to be the same as that of B21.2 (Fig. 6), even though well-separated, raised, cankerlike lesions were observed. Percent retention of pSS35KBg over time was followed during the course of the in planta growth kinetics experiment. In several repetitions of the experiment, more than 80% of cells extracted from citrus leaf tissue had lost pSS35KBg within 24 h of inoculation, based on loss of the plasmid antibiotic markers.

DISCUSSION

Screening of a recombinant gene library of the highly virulent Asiatic canker pathogen *X. citri* 3213 in the mild pathogen *X. c. citrumelo* 3048 on citrus resulted in the isolation of a clone, pSS10.35, that conferred the ability to elicit cankerlike symptoms on citrus. This virulence enhancement on citrus was conferred to strains of two other pathovars tested (*X. c. alfalfae* and *X. c. cyamopsidis*) that are compatible with citrus, but was not conferred to strains of two other xanthomonads that are incompatible with citrus (*X. c. malvacearum* and *X. phaseoli*). There was no evidence that pSS10.35 extended the host range of the incompatible strains to citrus. In contrast to attempts to extend host range (38), the virulence enhancement approach requires that both the DNA donor and recipients are compatible with the same host. Nevertheless, the enhanced virulence of pSS10.35 was host-specific. When present in *X. c. alfalfae, X. c. citrumelo,* or *X. c. cyamopsidis,* pSS10.35 conferred enhanced virulence only to citrus, and avirulence in interactions with other homologous hosts.

Transposon mutagenesis and subcloning allowed localization of the pathogenicity/avirulence activities to a 3.7-kb fragment. All 14 Tn*5-gusA* inserts in this region abolished both virulence and avirulence phenotypes; all 85 Tn*5-gusA* inserts outside of the region left both phenotypes intact. A marker-exchange mutant of *X. citri* 3213^T, B21.2, did not elicit any cankerlike symptoms on citrus, even when inoculated at relatively high cell densities. We conclude that pSS10.35 carries at least one gene, *pthA*, that is necessary, but not sufficient, for citrus canker disease. We found no evidence for a second virulence (*hrp*) or avirulence (*avr*) locus on the 3.7-kb fragment, although complete DNA sequencing will be required to prove that the virulence enhancement and avirulence properties are pleiotropic effects of the Asiatic citrus canker pathogenicity gene, *pthA*.

The marker-exchange mutant *X. citri* B21.2 was greatly affected in both in planta growth and ability to induce a pathogenic reaction on citrus, as compared with the wild-type strain, *X. citri* 3213. Complementation of the disease-inducing ability, but not the reduced growth in planta of B21.2, was achieved with pSS35KBg, carrying the *pthA* locus. We were surprised that pSS35KBg did not even slightly restore growth in citrus (Fig. 6) despite the clear restoration of pathogenicity symptoms in B21.2 at high inoculation levels (Fig. 5). Even at low inoculation levels, B21.2/pSS35KBg elicited well-separated, raised, cankerlike lesions on citrus. The reduction of in planta growth of B21.2

caused by the Tn*5-gusA* insertion may be a secondary (perhaps polar) effect of the transposon. Alternatively, the reduction in growth may be a direct effect on *pthA* and growth in planta might be restored if pSS35KBg were stabilized. Although the vector pUFR042 is more than 95% stable in planta, pSS35KBg was lost rapidly in planta. The cause of the instability is not known.

Genes required for virulence but not involved in the heterologous HR (26) have been cloned from *X. c. campestris* (9,51), *Pseudomonas syringae tomato* (8), *P. s. pisi* (32), *P. solanacearum* (4), *P. s. syringae* (53), and others. Genes affecting both pathogenicity and the heterologous HR phenotypes (i.e., *hrp* genes [31]) have been cloned from a wide variety of gram-negative bacteria, including *P. solanacearum* (4,22), *P. s. syringae* (35), *P. s. phaseolicola* (31), *P. s. tomato* (8), *P. s. pisi* (32), *X. c. campestris* (24), *X. c. vesicatoria* (3), *X. c. citrumelo* (M. Kingsley and D. Gabriel, *unpublished*), *Erwinia amylovora* (1), and others. Based on hybridization and cross-complementation analyses, such genes appear to be highly conserved, even at the family level (4,30). The 3.7-kb *pthA* locus is not conserved at the genus level. Although *X. citri* 3213 carries at least four other DNA fragments homologous with the 3.5-kb *Bam*HI fragment of pSSKBg (Fig. 4), *X. c. citrumelo* 3048, *X. c. alfalfae* KX-1, and *X. c. cyamopsidis* 13D5 carried no homologous bands (based on unpublished hybridizations similar to those in Fig. 4). In these strains, which lack the *pthA* locus, *pthA* appeared to act as an add-on pathogenicity/avirulence factor.

The formation of cankers and rupturing of epidermal layers leading to oozing out of bacteria on the leaf surface (27) might be an important aid in the dispersal of *X. citri* strains. Strains of *X. c. citrumelo* rarely elicit lesions on citrus leading to rupture of leaf epidermis. As a consequence, they should not be able to emerge on the leaf surface in cell numbers comparable with those of *X. citri* strains. It is known that in field situations, *X. c. citrumelo* strains disperse very poorly on citrus compared with *X. citri* (18). Strains belonging to *X. citri* are narrow host-range pathogens but well adapted to many varieties of citrus. They occur worldwide wherever citrus is grown (40) and are highly clonal in their population structure (16,17,20). The genetic similarity of these pathogens implies the presence of virulence genes of high selective value on citrus. (For general discussions of the role of virulence factors on the population structures of microbial plant and animal pathogens, refer to 14 and 41, respectively.) Although we have not ruled out the possibility of more than one virulence gene on pSS10.35, at least one of them, *pthA*, may be partly responsible for the observed clonality of *X. citri* worldwide.

Virulence often has been argued to be a feature of a pathogen over and above its basic compatibility with a host (14,25). Pathogens with a broad host range might become preferentially and better adapted to one host plant by slow evolutionary selection or by horizontal gene transfer. Little is known about the actual frequencies of horizontal gene transfer among bacteria in natural environments (for reviews, see 45 and 50), except that it occurs (39). Since *X. citri* and *X. c. citrumelo* strains can occupy the same niche (e.g., citrus), there is an obvious potential for exchange of genetic material. Since the *pthA* locus is confined to a relatively small, 3.7-kb fragment, it could be readily transferred. If *pthA* conferred a selective advantage to *X. c. citrumelo,* its eventual transfer from *X. citri* might be predictable. Some factors affecting its transfer potential might be the potential frequency of transfer of the *pth* locus (by transformation, transduction, or conjugation), frequency of simultaneous colonization of citrus by both species, and reproductive advantage conferred by the locus on the recipient strain under normal field conditions. All of these factors are experimentally tractable and could lend insight into the origins of new pathogen epidemics.

The most fit combinations of host-specific virulence genes available in the genus *Xanthomonas* may not be present in any one strain at a given time. This may be particularly true if the host is a recently developed hybrid, such as the rootstock Swingle Citrumelo, a newly released hybrid of two plant genera (*Citrus paradisi* × *Poncirus trifoliata*). Strains of *X. c. citrumelo* seem to be broad host-range pathogens that are not well adapted to citrus and are not known to be highly virulent or well adapted to any plant species; even on Swingle Citrumelo they spread only on juvenile tissue (18). They never have been observed in other citrus-growing regions of the world. These strains may themselves be natural recombinants selected by the new citrus variety. Had there not been an aggressive program for citrus canker eradication in Florida (40), it is possible that the strains of *X. citri* and *X. c. citrumelo* found in Florida could have been simultaneously present on the same citrus hosts and allowed for an even more fit combination of virulence genes specific for citrus. It would be interesting to learn if *X. c. citrumelo* carrying *hsvA* is more virulent on citrus than *X. citri* 3213 in field situations. However, it seems unlikely that field studies can be undertaken in this case since the field release of such a strain would not likely gain the required state or federal regulatory agency approvals.

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