MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 6, pp. 623-627, 1991

Research Notes

Xanthomonas campestris pv. *translucens* Genes Determining Host-Specific Virulence and General Virulence on Cereals Identified by Tn*5-gusA* Insertion Mutagenesis

V. R. Waney, M. T. Kingsley, and D. W. Gabriel

Plant Pathology Department, University of Florida, Gainesville 32611 U.S.A.
Received 4 February 1991. Accepted 27 June 1991.

Transposon mutagenesis was used to obtain mutations affecting general and host-specific virulence in *Xanthomonas campestris* pv. *translucens* strain Xct-216.2. This strain causes bacterial leaf streak on barley, wheat, rye, oats, and triticale. Transposon Tn*5-gusA* inserted randomly into Xct-216.2 at a frequency of 10^{-7}–10^{-8} per recipient after conjugal transfer from *Escherichia coli*. Three thousand Tn*5-gusA* insertional derivatives were inoculated on the five hosts. Prototrophic mutants affected in virulence (growth and symptom induction) on one of the host species, and not the others (host-specific virulence minus phenotype, or Hsv$^-$) were identified at frequencies of 0.07% on barley (HsvB$^-$), 0.04% on wheat (HsvW$^-$), 0.11% on rye (HsvR$^-$), 0.07% on oats (HsvO$^-$), and 0.07% on triticale (HsvT$^-$). These mutants were not affected in their hypersensitive response on cotton, a nonhost. Prototrophic mutants affected in virulence on all hosts and failing to induce a nonhost hypersensitive response (Hrp$^-$ phenotype) were obtained at a frequency of 0.42%. Cosmid clones complementing one Hrp$^-$ mutant, and the HsvW$^-$ and HsvB$^-$ mutants were recovered from an Xct-216.2 wild type genomic library using the cloned Tn*5*::Xct-216.2 flanking DNA regions. The Xct-216.2 *hrp* clone hybridized to the *hrp* locus from *Pseudomonas solanacearum* strain GMI1000 cloned in pVir2 (C. A. Boucher, F. VanGijsegem, P. A. Barberis, M. Arlat, and C. Zischek, J. Bacteriol. 169:5626-5632). There was no evidence of avirulence or cultivar specificity of the *hsv* genes isolated from Xct-216.2 on 17 barley and 14 wheat cultivars tested. These *hsv* loci evidently represent a class of virulence genes distinct from *hrp* and *avr* loci, superimposed on a basic ability to parasitize.

Additional keywords: host-parasite interaction, host range, host specificity.

Xanthomonas campestris pv. *translucens* (Jones *et al.*) Dye causes bacterial leaf streak on cereals and other Gramineae, and the range of hosts attacked by any one strain may vary considerably (Cunfer and Scolari 1982; Smith 1917). Loci that are required for virulence on specific hosts have been cloned from *Rhizobium* and *Pseudomonas* (for reviews see Djordjevic *et al.* 1987; Gabriel 1989; Gabriel and Rolfe 1990). In *X. c.* pv. *translucens* host range specificity is known to require an unknown number of virulence genes (Mellano and Cooksey 1988), however, the genes involved were not identified or cloned. Loci that are required for virulence on all hosts (*hrp*, or general virulence) have been cloned from several species of Pseudomonadaceae (for review see Willis *et al.* 1991). These genes are not host-specific, and their inactivation leads to loss of virulence on all hosts and loss of the nonhost hypersensitive response (HR). We report here the use of transposon Tn*5-gusA* insertion mutagenesis to identify and clone loci from *X. c.* pv. *translucens* encoding general virulence (including *hrp*) and host-specific virulence (*hsv*). Inactivation of these *hsv* genes narrowed the host range by one plant genus, did not affect virulence on the other plant genera tested, and did not affect the nonhost HR.

Florida Agricultural Experiment Station journal series R01333.

Transposon Tn*5-gusA* insertion mutagenesis. Low transposition frequencies and/or instability of Tn*5* have been reported for *X. c.* pv. *campestris* (Turner *et al.* 1984) and for *X. c.* pv. *translucens* (Mellano and Cooksey 1988). Suicide vector pRK600 (Sharma and Signer 1990) was used to transfer Tn*5-gusA* from *E. coli* strain 387 to *X. c.* pv. *translucens* strain Xct-216.2 (a spectinomycin-resistant derivative of Xct-216) (Cunfer and Scolari 1982) by conjugation at moderate frequencies of 10^{-7}–10^{-8} per recipient. Randomness of the Tn*5-gusA* insertion was determined by Southern analysis of total genomic DNA digested with *Eco*RI and hybridized with pRK600 as a probe. The resulting autoradiograph showed 1) no hybridization of the parental strain to the Tn*5* probe; 2) two bands hybridizing per kanamycin-resistant exconjugant (Tn*5-gusA* contains a unique *Eco*RI site); and 3) different sizes of *Eco*RI fragments containing the transposon, indicating random, single insertions of the Tn*5-gusA* into the Xct-216.2 genome (data not shown).

Three thousand one hundred independent kanamycin-resistant exconjugants were obtained; 0.5% were white mutants that had lost all or most yellow pigments. One thousand exconjugants were screened for auxotrophic requirements as previously described (Gabriel *et al.* 1989). The frequency of amino acid auxotrophs recovered was 8.3% (83/1,000), 0.6% (6/1,000) required threonine, 0.1% (1/1,000) tryptophan, and 0.1% lysine. Another 0.4% (4/1,000) was identified as other than amino acid auxotrophs.

Plant symptom assays. Strains of *X. c.* pv. *translucens* may induce symptoms on homologous hosts ranging from watersoaking followed by necrosis to no symptoms at all (Cunfer and Scolari 1982). Under growth chamber conditions, Xct-216.2 caused watersoaking on 17 different cultivars of barley (*Hordeum vulgare* L.), 14 different cultivars of wheat (*Triticum aestivum* L.), two cultivars each of rye (*Secale cerealis* L.), oats (*Avena sativa* L.), and triticale (× *triticosecale* Wittm.). There was no indication of cultivar specificity. Screening of the prototrophic Tn*5-gusA* Xct-216.2 derivatives was performed as described in Figure 1. Prototrophic insertional derivatives that were affected in virulence on one host and not on the other four hosts were recovered at frequencies of: 2/2,848 (0.07%) on barley, 1/2,848 (0.04%) on wheat, 2/2,848 (0.07%) on oats, 3/2,848 (0.11%) on rye, and 2/2,848 (0.07%) on triticale. Twelve prototrophic insertional derivatives (0.42%) that were affected in virulence on all five homologous hosts and failed to induce an HR on cotton, a nonhost, (Hrp^-) were obtained; this frequency is comparable to the frequency of the Hrp phenotypic class previously reported in *P. syringae* (Lindgren *et al.* 1986). As illustrated in Table 1 and Figure 1, all possible Hsv^- mutant classes (affected on each individual host genus), as well as Hrp^- mutants (affecting all hosts and nonhosts tested), were recovered.

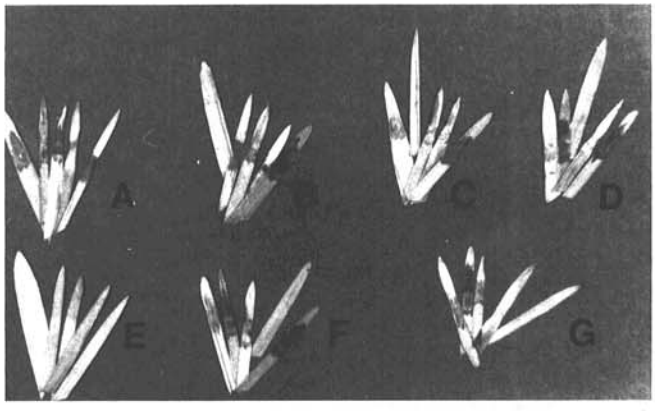

Fig. 1. Symptom induction on barley (cv. Robust), wheat (cv. FL 301), rye (cv. FL 401), oats (cv. FL 501), and triticale (cv. FL 201), syringe inoculated with *Xanthomonas campestris* pv. *translucens* and selected Tn*5-gusA* derivatives (see Table 1). Each of the five hosts is arranged in a cluster in the order given above. Xct-216.2 (wild type) is inoculated on the first cluster of hosts in the top row at the far left cluster (**A**); followed by Tn*5-gusA* mutant derivatives T2 ($HsvB^-$) (**B**); T18 ($HsvW^-$) (**C**); and T3 ($HsvR^-$) (**D**). In the bottom row, clusters inoculated with Tn*5-gusA* mutants T21 (Hrp^-) (**E**); T4 ($HsvO^-$) (**F**); and T12 ($HsvT^-$) (**G**) are shown. All strains were inoculated at high (at least 10^7 cfu/ml) cell densities. The nonhost hypersensitive response was tested on cotton (*Gossypium hirsutum* 'Acala 44') using turbid, 10^8–10^9 cfu/ml of bacterial suspensions (not shown). Plants were kept under growth chamber conditions (20–24° C, fluorescent lights, 24-h photoperiod, 26% relative humidity) for four days following inoculation. Individual mutants shown in the figure were screened at least five times. For transposon insertion mutagenesis, Xct-216.2 was grown in TYM broth (Gabriel *et al.* 1989) to late log phase, washed in sterile tap water, resuspended in one-tenth volume of sterile tap water, and 200 μl of suspension was spotted on TY plates. After excess liquid was absorbed, 100 μl of stationary phase donor cells were overlaid on the recipient spots and the plates were incubated at 28° C. The cells were then plated on TYM supplemented with 25 μg/ml of kanamycin and 50 μg/ml of spectinomycin to select exconjugants.

To investigate whether we have found a *hrp* region in *X. c.* pv. *translucens* corresponding to that found in *P. solanacearum*, we hybridized the transposon-flanking DNA region of Xct-216.2 DNA from Hrp mutant T21 with pVir2, a plasmid containing *hrp* loci from *P. solanacearum* strain GMI1000 (Boucher *et al.* 1987). Plasmid pVir2 hybridized strongly to this DNA (data not shown). This DNA fragment from T21 was also used to probe an Xct-216.2 genomic library (N = 1,390; average insert size: 26 kb) cloned in cosmid vector pUFR043 (De Feyter and Gabriel, 1991), resulting in the recovery of pUFT900. Plasmid pUFT900 fully complemented mutant strain T21 (Hrp^-), restoring virulence on all hosts. We conclude that we have cloned at least part of a locus from *X. c.* pv. *translucens* that corresponds to the *hrp* locus from *P. solanacearum* GMI1000. The *hrp* locus from *X. c.* pv. *translucens* may be more related to the *P. solanacearum hrp* locus than to the *hrp* loci from other xanthomonads. *X. c.* pv. *translucens* and *P. solanacearum* total DNA hybridized only weakly to a *hrp* locus cloned from *X. c.* pv. *vesicatoria*, while total DNA from other pathovars of *X. campestris* hybridized strongly (Bonas *et al.* 1991).

Recovery of wild type DNA fragments, complementation, and growth *in planta*. Growth *in planta* of Tn*5-gusA* mutant T2 ($HsvB^-$) was affected on barley, and not on wheat (Fig. 2B-f, 2D-f). Similarly, growth of mutant T18 ($HsvW^-$) was affected on wheat and not on barley (Fig. 2B-d, 2D-d). Cosmid clones pUFT200 and pUFT300 were obtained from the Xct-216.2 genomic library by colony hybridization as described in Figure 3. When pUFT200 and pUFT300 were transferred by conjugation to T18 and T2, these clones restored the respective mutants to phenotypic virulence and growth *in planta* on the appropriate host. The cosmids did not cross-hybridize and did not cross-complement. Subclones pUFT210 and pUFT211 (both derived from pUFT200), and pUFT316 and pUFT317 (both derived from pUFT300) contained the complementation activity of the larger cosmids (data not shown). Both pUFT210 and pUFT316 were extracted from T18 and T2 exconjugants, transformed into *E. coli*, and reintroduced into T18 and T2 respectively; virulence was again observed, and no DNA rearrangements were found. Confirmation that the cloned DNA fragments on the complementing

Table 1. Symptom induction on barley (cv. Robust), wheat (cv. FL 301), rye (cv. FL 401), oats (cv. FL 501), and triticale (cv. FL 201) and on a nonhost cotton (cv. Acala 44) four days after syringe inoculation with *Xanthomonas campestris* pv. *translucens* strain Xct-216.2 and Tn*5-gusA* mutant derivatives T2, T3, T4, T11, T12, T18, T21, and T22 (for phenotype see Figure 1)[a]

	Hosts					Nonhost
Strain	Barley	Wheat	Rye	Oats	Triticale	Cotton
Xct-216.2	+	+	+	+	+	HR
T21	−	−	−	−	−	−
T2	−	+	+	+	+	HR
T18	+	−	+	+	+	HR
T3	+	+	−	+	+	HR
T4	+	+	+	−	+	HR
T12	+	+	+	+	−	HR
T11	+	−	+	+	−	HR
T22	−	+	+	−	+	HR

[a] + = watersoaking; − = no symptoms; HR = hypersensitive response.

plasmids (refer to Fig. 3) corresponded to the Tn*5-gusA* insertion sites in the mutants T18 and T2 is provided by Southern hybridization (Fig. 4). Complemented mutants T18/pUFT210 and T2/pUFT316 reached similar cell densities *in planta* as Xct-216.2 (Fig. 2B-e and 2D-g). The Hrp$^-$ mutant T21 grew poorly in both wheat and barley (Fig. 2A-b and 2B-b), and no symptoms developed.

The two *hsv* loci, *hsvB* and *hsvW*, were necessary for virulence on either barley or wheat respectively, and appear to be superimposed on a basic ability to parasitize. These

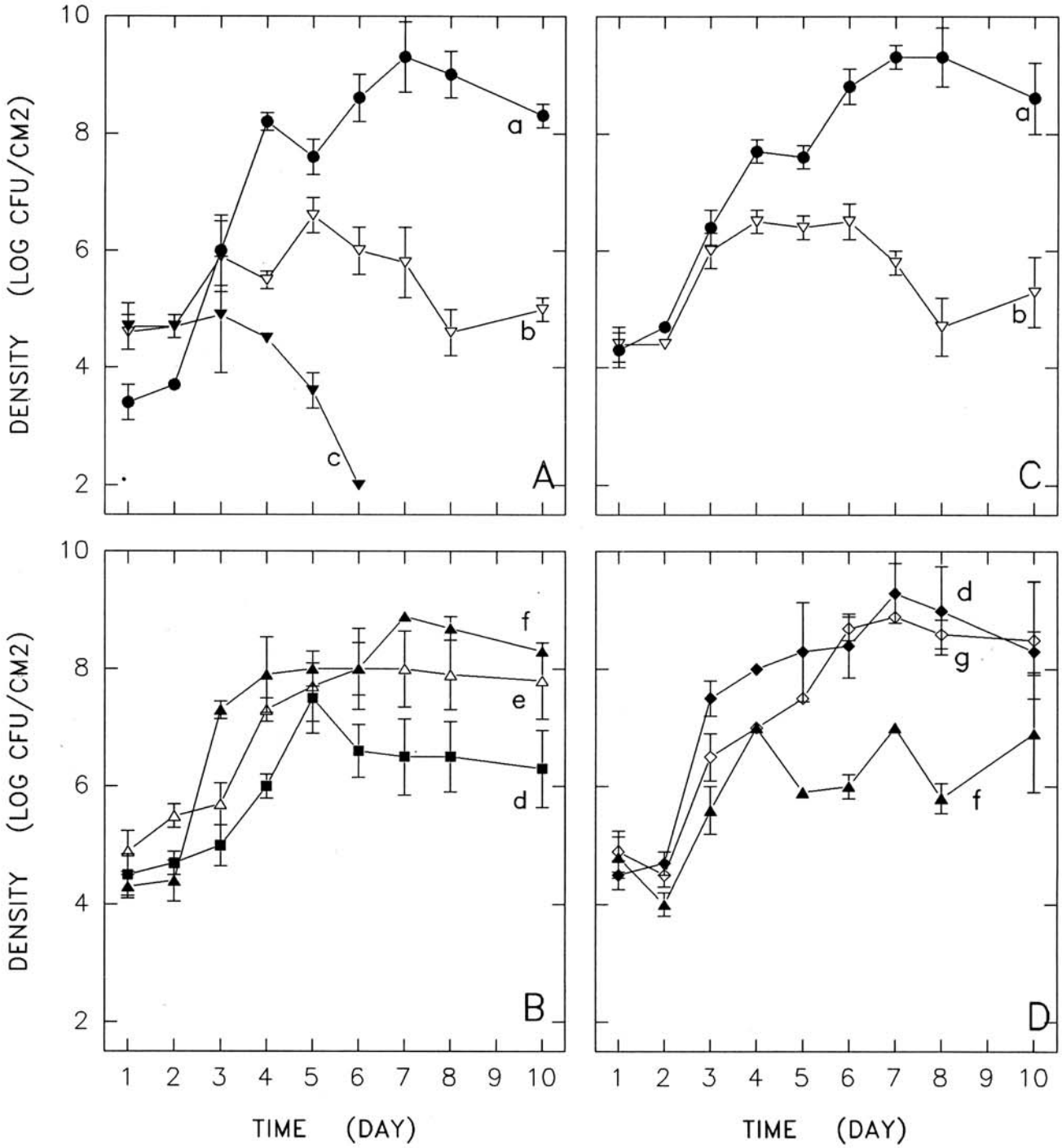

Fig. 2. Growth *in planta* assays on barley and wheat were performed using broth-grown cultures of wild type Xct-216.2 and mutant derivatives T21 (Hrp$^-$), T2 (HsvB$^-$), and T18 (HsvW$^-$). The heterologous pathogen *Xanthomonas campestris* pv. *malvacearum* strain H (Gabriel *et al.* 1986) was included as a negative control. Each culture, washed and diluted to 10^3–10^5 cfu/ml, was pressure-infiltrated at the middle of the first leaf of either barley or wheat. Samples represent 1 cm^2 of tissue taken from the top of a leaf and macerated in 1 ml of sterile tap water. Appropriate dilutions were plated. Cell counts are the average of two experiments of three replicates each. **A** and **B,** wheat. **C** and **D,** barley. a) *Xanthomonas campestris* pv. *translucens* strain Xct-216.2 (wild type); b) T21 (Hrp$^-$); c) *X. c.* pv. *malvacearum* strain H; d) T18 (HsvW$^-$); e) T18/pUFT210; f) T2 (HsvB$^-$); g) T2/pUFT316. Bars represent standard error.

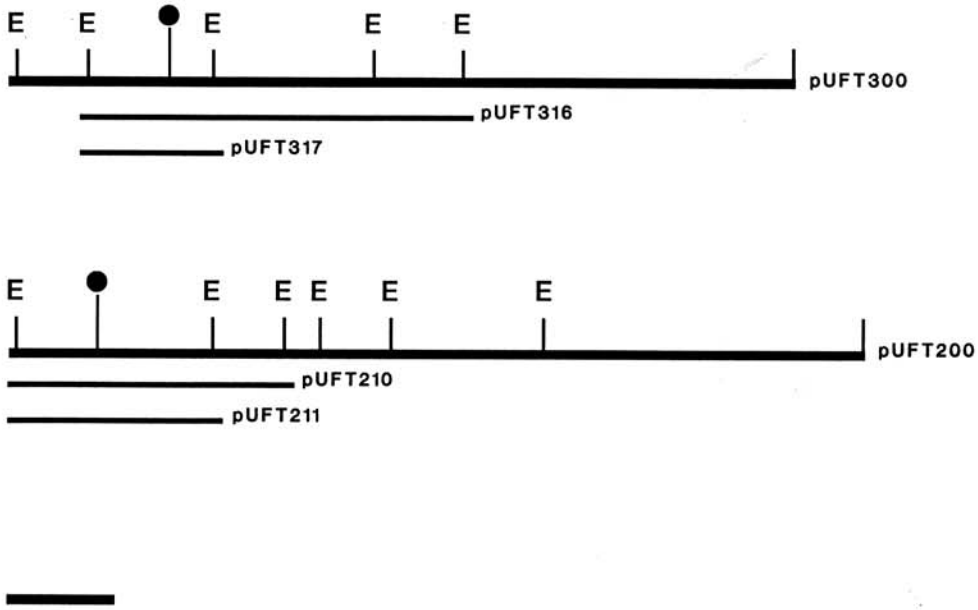

Fig. 3. Restriction maps of cosmid clones pUFT300 (35 kb) and pUFT200 (38 kb), carrying *hsvB* and *hsvW* loci, respectively. Plasmids pUFT316, pUFT317, pUFT210, and pUFT211 carry the indicated fragments subcloned in pUFR047, an unpublished Gm^RAp^R derivative of pUFR027 (De Feyter *et al.* 1990); the original Tn*5-gusA* insertion in Xct-216.2 is added to the figure. Both cosmid clones were obtained by colony hybridization (Maniatis *et al.* 1982) using the Xct-216.2 DNA region flanking the Tn*5-gusA* insertion sites of the Hsv^- mutants as probes. The *Xanthomonas campestris* pv. *translucens hrp* containing cosmid clone pUFT900 was obtained as described for pUFT300 and pUFT200. E = *Eco*R1.

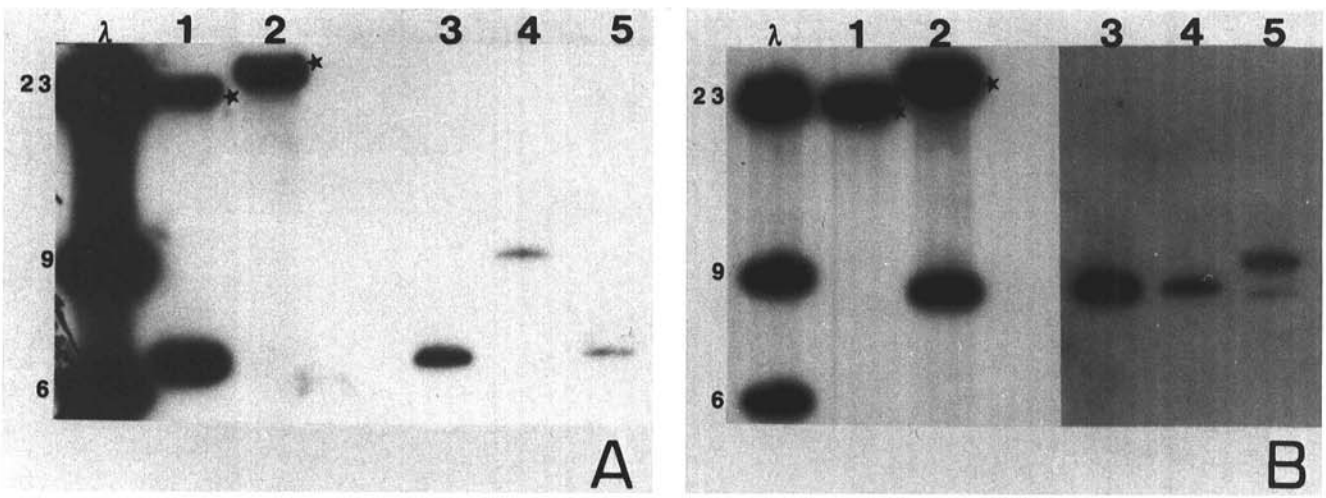

Fig. 4. Hybridization patterns of total genomic DNA of *Xanthomonas campestris* pv. *translucens* strain Xct-216.2 (lane 3), T2 (lane 4), and T18 (lane 5), and cosmid clones pUFT300 (lane 1), and pUFT200 (lane 2), probed with **A,** pUFT317, and **B,** pUFT211 (composite figure made out of two different autoradiographic exposure times). All DNA was digested with *Eco*R1; the Tn*5-gusA* contains a unique *Eco*R1 site. Note the band shift in A in lane 4 (T2), and in B in lane 5 (T18), relative to the band in lane 3 (Xct-216.2). The molecular weight marker λ*Hin*dIII is partially shown; no other bands appear on both autoradiographs. * = Vector bands.

genes were functionally distinct from *hrp* genes; they were not involved in the nonhost HR, and they were not required for virulence on genera other than the one indicated. Other *hsv* loci could be inactivated for each plant species in the Xct-216.2 host range, indicating that separate *hsv* genes are necessary for virulence of Xct-216.2 on individual host genera. The *hsv* genes reported were distinct from avirulence (*avr*) genes; they were positive-acting, and there was no evidence found of cultivar specificity on any of the 14 wheat and 17 barley cultivars from various countries. The *X. c.* pv. *translucens hsv* loci and other loci of similar function from *P. syringae* (Salch and Shaw 1988) and from *P. solanacearum* (Ma *et al.* 1988) may represent a distinct class of virulence genes that may be largely responsible for host range at the plant genus and perhaps species level. The *hsv* loci may thus be functionally analogous to the host-specific nodulation (*hsn*) genes of *Rhizobium*.

ACKNOWLEDGMENTS

We thank B. M. Cunfer for the *Xanthomonas campestris* pv. *translucens* strains, and C. Boucher for pVir2 and strain GMI1000. We are also

grateful to R. D. Barnett, H. E. Bockelman, H. H. Luke, and D. C. Rasmusson for seed material, and B. Garagorry for technical assistance. R. De Feyter constructed two vectors used in this study and gave much appreciated comments and support. This work was supported by USDA 58-7B30-3-465.

LITERATURE CITED

Bonas, U., Schulte, R., Fenselau, S., Minsavage, G. V., Staskawicz, B. J., and Stall, R. E. 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol. Plant-Microb. Interact. 4:81-88.

Boucher, C. A., Van Gijsegem, F., Barberis, P. A., Arlat, M., and Zischek, C. 1987. *Pseudomonas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. J. Bacteriol. 169:5626-5632.

Cunfer, B. M., and Scolari B. L. 1982. *Xanthomonas campestris* pv. *translucens* on triticale and other small grains. Phytopathology 72:683-686.

De Feyter, R., and Gabriel, D. W. 1991. Use of cloned DNA methylase genes to increase the transfer frequency of foreign genes into *Xanthomonas campestris* pv. *malvacearum*. J. Bacteriol. 173:6421-6427.

De Feyter, R., Kado, C. I., and Gabriel, D. W. 1990. Small, stable shuttle vectors for use in *Xanthomonas*. Gene 88:65-72.

Djordjevic, M. A., Gabriel, D. W., and Rolfe, B. G., 1987. *Rhizobium*—The refined parasite of legumes. Annu. Rev. Phytopathol. 25:145-168.

Gabriel, D. W. 1989. The genetics of plant pathogen population structure and host-parasite specificity. Pages 343-379 in: Plant-Microbe Interaction III. T. Kosuge and E. W. Nester, eds. Macmillan Publishing Co., New York.

Gabriel, D. W., Burges, A., and Lazo, G. R. 1986. Gene-for-gene recognition of five cloned avirulence genes from *Xanthomonas campestris* pv. *malvacearum* by specific resistance genes in cotton. Proc. Natl. Acad. Sci. USA 83:6415-6419.

Gabriel, D. W., Kingsley, M. T., Hunter, J. E., and Gottwald, T. R. 1989. Reinstatement of *Xanthomonas citri* (ex Hasse) and *X. phaseoli* (ex Smith) and reclassification of all *X. campestris* pv. *citri* strains. Int. J. Syst. Bacteriol. 39:14-22.

Gabriel, D. W., and Rolfe, B. G. 1990. Working models of specific recognition in plant-microbe interactions. Annu. Rev. Phytopathol. 28:365-391.

Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168:512-522.

Ma, Q. -S., Chang, M. -F., Tang, J. -L., Feng, J. -X., Fan, M. -J., Han, B., and Liu, T. 1988. Identification of DNA sequences involved in host specificity in the pathogenesis of *Pseudomonas solanacearum* strain T2005. Mol. Plant-Microb. Interact. 1:169-174.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

Mellano, V. J., and Cooksey, D. A. 1988. Development of host range mutants of *Xanthomonas campestris* pv. *translucens*. Appl. Environ. Microbiol. 54:884-889.

Salch, Y. P., and Shaw, P. D. 1988. Isolation and characterization of pathogenicity genes of *Pseudomonas syringae* pv. *tabaci*. J. Bacteriol. 170:2584-2591.

Sharma, S. B., and Signer, E. R. 1990. Temporal and spatial regulation of the symbiotic genes of *Rhizobium meliloti in planta* revealed by transposon Tn*5-gusA*. Genes Dev. 4:344-356.

Smith, E. F. 1917. A new disease of wheat. J. Agric. Res. 10:51-53.

Turner, P., Barber, C., and Daniels, M. 1984. Behaviour of transposon Tn*5* and Tn*7* in *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. 195:101-107.

Willis, D. K., Rich, J. J., and Hrabak, E. M. 1991. *hrp* genes of phytopathogenic bacteria. Mol. Plant-Microb. Interact. 4:132-138.