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Isolation of a Gene Cluster from *Xanthomonas campestris* pv. *vesicatoria* that Determines Pathogenicity and the Hypersensitive Response on Pepper and Tomato

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Nonpathogenic mutants of *Xanthomonas campestris* pv. *vesicatoria* that had lost the ability to induce a hypersensitive response in resistant host plants and in nonhosts were complemented by clones from a wild-type library. Analysis of transposon insertion mutants defined a 25-kilobase region of the genome of *X. c.* pv. *vesicatoria*, which is necessary for the interaction of the bacterium with the plant. The genes in this region were designated *hrp* genes. Complementation studies revealed that the *hrp* region is organized into at least six different complementation groups. Southern blot experiments showed that DNA sequences homologous to a 23-kilobase region from the *hrp* cluster were present in other pathovars of *X. campestris*. In some pathovars homology was high enough to permit marker gene exchange or complementation of nonpathogenic mutants by *X. c.* pv. *vesicatoria* sequences. Cross-hybridization between the *hrp* sequences of *X. c.* pv. *vesicatoria* and genomic DNA of different species of *Pseudomonas* was not observed.

Additional keywords: bacterial spot, disease resistance.

Bacterial spot disease of pepper and tomato is caused by *Xanthomonas campestris* pv. *vesicatoria*, a gram-negative bacterium. After infection of a plant with *X. c.* pv. *vesicatoria*, two different types of reactions can be observed depending on the susceptibility of the particular pepper or tomato cultivar. If the plant is susceptible, the infection gives rise to water-soaked lesions (compatible interaction). In a resistant plant a hypersensitive response (HR; incompatible interaction) is induced. The HR is a local defense reaction accompanied by a rapid necrosis of the infected tissue (Klement and Goodman 1967). Such an incompatible interaction requires the presence of a resistance locus in the particular cultivar of pepper or tomato and a corresponding avirulence locus in the particular race of the pathogen (Minsavage *et al.* 1990). The HR is not only elicited on resistant cultivars of the susceptible host species but also on nonhost plants, such as tobacco (Klement 1982).

The natural isolates of *X. c.* pv. *vesicatoria* can be classified into three groups based on their host specificity for either pepper or tomato or both plant species (Minsavage *et al.* 1990). Several avirulence genes from *X. c.* pv. *vesicatoria* have been isolated previously and studied on the molecular level (Swanson *et al.* 1988; Ronald and Staskawicz 1988; Whalen *et al.* 1988; Bonas *et al.* 1989; Minsavage *et al.* 1990). The analysis of these avirulence genes indicated that they are necessary but not sufficient for the incompatible interaction with the resistant plant. For different species of *Pseudomonas*, it has been shown that the induction of the HR as well as the induction of pathogenicity are controlled by *hrp* (*h*ypersensitive *r*eaction and *p*athogenicity; Niepold *et al.* 1985; Lindgren *et al.* 1986; Boucher *et al.* 1987; Huang *et al.* 1988, 1990) genes.

In this study we describe the identification and isolation of a large chromosomal region from *X. c.* pv. *vesicatoria* that controls pathogenicity and, in the presence of appropriate avirulence genes, the HR on pepper and tomato. Genetic analysis revealed that this region contains a cluster of *hrp* genes. We tested the ability of the cloned *X. c.* pv. *vesicatoria hrp* region to complement *hrp* mutations in *X. c.* pv. *vesicatoria* and other pathovars of *Xanthomonas* and screened for cross-hybridization of *hrp* sequences.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *Escherichia coli* were cultivated in Luria-Bertani medium (Miller 1972). All strains of *X. campestris* were grown in either nutrient or NYG broth (Daniels *et al.* 1984a). For culture on solid medium, 1.5% agar was added. Strains of *Pseudomonas* were cultivated on King's B (KB; King *et al.* 1954) medium. Antibiotics were added to the media at the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 10 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 50 μg/ml; tetracycline, 10 μg/ml; and rifampicin, 100 μg/ml.

Plasmids were introduced into *E. coli* by transformation and into *Xanthomonas* by conjugation using pRK2013 as a helper plasmid in triparental matings (Figurski and Helinski 1979; Ditta *et al.* 1980).

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Plant material, plant inoculations, and bacterial *in planta* growth curves. Descriptions of pepper cultivars ECW, ECW-10R, and ECW-30R have been given by Minsavage *et al.* (1990). Cultivars ECW-10R and ECW-30R carry the dominant resistance genes *Bs1* and *Bs3*, respectively. Other cultivars that were used are as follows: tomato cultivar Bonny Best, tobacco cultivar Bottom Special, bean cultivar Bush Lake Blue, radish cultivar Red Globe, grapefruit cultivar Duncan, and cowpea cultivar Blackeye. *Philodendron oxycardum* was also used as a host plant.

The leaves of plants were inoculated with bacterial suspensions as described previously (Staskawicz *et al.* 1984; Swanson *et al.* 1988) by infiltrating the bacteria into the intercellular spaces using a plastic syringe. In all plants young, fully expanded leaves were inoculated. The concentration of the inoculum was approximately 10^8 colony-forming units (cfu) per milliliter in 1 mM $MgCl_2$ unless otherwise stated. Reactions were scored over a period of several days.

For the measurement of bacterial growth *in planta*, leaves were infiltrated with 10^4 to 10^5 cfu/ml. After various incubation times, 1-cm^2 leaf disks were cut out and macerated in 1 mM $MgCl_2$, and cell counts were determined by plating appropriate dilutions.

Chemical, transposon, and marker exchange mutageneses. Nonpathogenic mutants of *Xanthomonas* were obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) as described by Daniels *et al.* (1984a). Treated cells were plated on a minimal medium as described by Starr (1946). Therefore, only prototrophic bacteria were tested for pathogenicity on the respective host plants.

For random insertion mutagenesis, clones of *X. c.* pv. *vesicatoria* DNA in pLAFR3 were mutagenized in *E. coli* using λ::Tn*5* (Ruvkun and Ausubel 1981) or Tn*3-gus* (B.

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Source or reference
Xanthomonas campestris		
pv. *alfalfae* KS		D. L. Stuteville[b]
pv. *armoraciae* XA		R. E. Stall
pv. *begoniae* 077-3382		R. E. Stall
pv. *campestris* 33913		ATCC[c]
pv. *citri* 50E		R. E. Stall
pv. *dieffenbachiae* 729		R. E. Stall
pv. *glycines* 202		R. E. Stall
pv. *malvacearum* G-34		M. Essenberg[d]
pv. *oryzae* 50909		DSM[e]
pv. *phaseoli* 85-6		R. E. Stall
pv. *translucens* 82-1		R. E. Stall
pv. *vignicola* 81-30		R. E. Stall
pv. *vitians* 164		R. E. Stall
T55	Opportunistic	R. E. Stall
X. c. pv. *vesicatoria*		
71-21	Pepper race 1; HR on ECW-30R; carries *avrBs3*	Minsavage *et al.* 1990
82-8	Pepper race 1; HR on ECW-30R; carries *avrBs3*	Minsavage *et al.* 1990
81-23	Tomato-pepper race 2; HR on ECW-10R; carries *avrBs1*	Minsavage *et al.* 1990
85-10	Tomato-pepper race 2; HR on ECW-10R; carries *avrBs1*	Bonas *et al.* 1989
75-3	Tomato race 1; HR on pepper	Minsavage *et al.* 1990
Pseudomonas		
P. solanacearum 131	Pathogenic on tomato	R. E. Stall
P. syringae		
pv. *glycinea* 80-2		R. E. Stall
pv. *phaseolicola* 83-1		R. E. Stall
pv. *tomato* PT2		R. E. Stall
Agrobacterium tumefaciens		
Strain C58		F. de Bruijn[f]
Escherichia coli		
DH5α	F⁻ *recA* φ*80dlacZ* Δ*M15*	Bethesda Research Laboratories[g]
C2110	Nal[r] *polA*	E. Nester[h]
HB101	F⁻ *recA*	Boyer and Roulland-Dussoix 1969
Rhizobium		
WBM13	Nodulates *Sesbania punctata*	F. de Bruijn
R. meliloti		
1021	Nodulates alfalfa	F. de Bruijn
R. loti		
N2P2037	Nodulates *Lotus corniculatus*	F. de Bruijn
Azorhizobium caulinodans		
ORS571	Nodulates *S. rostrata*	F. de Bruijn
Plasmids		
pLAFR3	Tc[r] *rlx*⁺ RK2 replicon	Staskawicz *et al.* 1987
pRK2013	Km[r] TraRK2⁺ Mob⁺ ColE1 replicon	Figurski and Helinski 1979
pTn*3*-gus	Km[r] Ap[r] *tnpA*⁻; Tn*3*HoHo derivative containing promoterless β-glucuronidase gene	B. Staskawicz
pXV2	pLAFR3 clone from *X. c.* pv. *vesicatoria* 75-3	This study
pXV9	pLAFR3 clone from *X. c.* pv. *vesicatoria* 75-3	This study

[a] HR, hypersensitive response; Nal, nalidixic acid; Tc, tetracycline; Km, kanamycin; Ap, ampicillin; and [r], resistant.
[b] Kansas State University, Manhattan.
[c] American Type Culture Collection, Rockville, MD.
[d] Oklahoma State University, Stillwater, OK.
[e] Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German culture collection), Braunschweig, Federal Republic of Germany.
[f] Max-Planck-Institut für Züchtungsforschung, Köln, Federal Republic of Germany.
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Staskawicz, unpublished) as described by Bonas *et al.* (1989). The insertions were mapped by restriction enzyme analysis. For marker gene replacement, mutant plasmids were mobilized from *E. coli* into *Xanthomonas* by triparental mating. The transconjugants were cycled more than 10 times on NYG plates containing rifampicin and kanamycin to maintain selection for the presence of the transposon. Subsequent plating on NYG agar containing 4 mM D-cycloserine enriched for tetracycline-sensitive colonies (Tu *et al.* 1989). All Tn*3-gus* and selected Tn*5* marker exchange mutants were analyzed in Southern blot hybridization experiments using the wild-type *hrp* region and the transposon as probes. The marker exchange mutants were tested for pathogenicity and induction of the HR on appropriate plant cultivars.

Physiological tests. The ability of *X. c.* pv. *vesicatoria* to utilize arabinose, cellobiose, galactose, glucose, mannose, rhamnose, sucrose, and trehalose was tested by adding the carbohydrate to Dye's medium C at 0.5% w/v concentrations (Dye 1962). Likewise, the utilization of acetate, benzoate, citrate, lactate, propionate, succinate, and tartrate was tested. The hydrolysis of esculin, Tween 80, and starch as well as clearing of milk agar were determined by methods published in Schaad (1988). Cellulolytic and pectolytic activities were determined by the pitting of gels containing carboxymethylcellulose and sodium polypectate, respectively (Schaad 1988). The effect of osmotic pressure on growth was determined on nutrient agar media containing 1.0, 0.5, 0.25, or 0.13 M glucose, KCl, mannose, or NaCl. In addition, growth curves were obtained by measuring light absorbance of cultures in different concentrations of KB (1×, 0.1×, and 0.01×) at 600 nm. Bacterial fatty acids were derivatized to their methyl esters in a four-step extraction procedure, and the methyl esters were separated by gas chromatography as described by L. Miller and T. Berger in an article on the identification of bacteria by gas chromatography of whole cell fatty acids, which was published in Hewlett-Packard (Palo Alto, CA) Gas Chromatography Application Note 228-38 (1985).

Molecular genetic techniques. Standard molecular genetic techniques were used (Ausubel *et al.* 1987; Maniatis *et al.* 1982) unless otherwise stated.

Total genomic DNA was isolated from bacterial cells grown in liquid culture using the CTAB method (Ausubel *et al.* 1987). Transfer to Nytrane membranes (Schleicher & Schüll, Dassel, Federal Republic of Germany) was as described previously (Bonas *et al.* 1989). Hybridization probes were labeled with ^{32}P by the random priming method (Feinberg and Vogelstein 1983). Hybridizations were performed at 65° C for 20 hr in 3× SSPE (1× SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% sodium dodecyl sulfate, and 0.05% salmon sperm DNA. The filters were washed at 68° C in 2× SSPE containing 0.1% sodium dodecyl sulfate (10–15 min each).

RESULTS

Isolation and identification of a DNA region involved in pathogenicity of *X. c.* pv. *vesicatoria*. Strains 81-23 (race 2) and 82-8 (race 1) of *X. c.* pv. *vesicatoria* were mutagenized with NTG and tested on pepper cultivar ECW for loss of pathogenicity. Eight nonpathogenic mutants of *X. c.* pv. *vesicatoria* 81-23 (of the first 329 tested) and six mutants of *X. c.* pv. *vesicatoria* 82-8 (of 1,000 tested) were isolated. These mutant bacteria not only failed to induce symptoms but also failed to grow to wild-type levels in the plant. In addition, mutants were unable to induce an HR on resistant pepper cultivars (ECW-10R and ECW-30R) and on tobacco plants. It was then established that the expression of the avirulence genes, *avrBs1* and *avrBs3*, which are located on conjugative plasmids (Swanson *et al.* 1988; Bonas *et al.* 1989), was not affected in the mutants. The former gene was still functionally active when it was conjugated from a nonpathogenic mutant of strain 81-23 into a pathogenic strain of race 1. The expression of *avrBs3* in nonpathogenic mutants of strain 82-8 was unaltered as determined by western blot analysis (data not shown).

Different physiological tests were performed to compare the mutants to wild-type cells as described in Materials and Methods. Fatty acid composition of whole cells analyzed by gas chromatography was unaltered. Growth rates in media containing different concentrations of sugars and salts to simulate osmotic conditions in the plant, or in 1×, 0.1×, and 0.01× KB, were similar for the mutant and wild-type cells. The mutants could not be distinguished from wild-type cells when tested for the ability to utilize different carbon sources (see Materials and Methods). Cellulolytic and pectolytic activities of the mutants were like that of the wild-type. Since no differences between wild-type and the nonpathogenic mutant strains could be found in these tests, we assumed that the mutants were only affected in genes necessary for pathogenicity.

To identify these genes, one of the nonpathogenic mutants of *X. c.* pv. *vesicatoria* 81-23, M103, was chosen for complementation with a genomic library from wild-type *X. c.* pv. *vesicatoria* 75-3. The construction of this library in pLAFR3 and mobilization of clones into *X. c.* pv. *vesicatoria* were described previously (Minsavage *et al.* 1990). Of 1,000 transconjugants tested, 13 different cosmid clones restored the ability to grow *in planta* and to cause water-soaked lesions on leaves of ECW and an HR on ECW-10R. These clones were then transferred into all other NTG-induced mutants of *X. c.* pv. *vesicatoria* 81-23 and *X. c.* pv. *vesicatoria* 82-8. They restored pathogenicity in all but three mutant strains. Since these three mutants could not be complemented by any cosmid from the genomic library, they might carry mutations in more than one region in the genome. Restriction enzyme analysis and Southern hybridization experiments showed that the original 13 cosmid clones carried overlapping inserts which were 25 to 29 kilobases (kb) long. Two clones, designated pXV2 and pXV9, were chosen for further analysis. The insert of plasmid pXV9, which complemented all of the NTG mutants except for the three mutant strains mentioned above, contained a region common to all clones, flanked on one side by several kilobases of additional sequences. Plasmid pXV2 also carried this common segment with additional sequences on the other side. Restriction enzyme maps of pXV2 and pXV9 are shown in Figure 1.

Insertion mutagenesis of the pathogenicity region. To

ascertain the role of the identified DNA region in pathogenicity, the plasmids pXV2 and pXV9 were mutagenized with Tn*5* and Tn*3-gus*. The insertions were mapped by restriction enzyme analysis. Seventy-five different Tn*5* insertions generated in pXV9 were introduced into the genome of the tomato strain *X. c.* pv. *vesicatoria* 75-3 by marker gene exchange (Fig. 2B). Likewise, 32 different Tn*3-gus* insertions (in pXV2 or pXV9) were introduced into the genome of pepper strain *X. c.* pv. *vesicatoria* 85-10 (Fig. 2C). This strain was chosen because of higher conjugation rates compared to *X. c.* pv. *vesicatoria* 81-23. The positions of the insertions in the genome were verified by Southern hybridization for each of the Tn*3-gus* and for selected Tn*5* mutants. All mutants were scored for their infection phenotypes on tomato and pepper. As shown in Figure 2 (B and C), a number of insertions near the borders of this 30-kb genomic region had no effect on pathogenicity. However, insertions distributed over a region of approximately 25 kb eliminated both pathogenicity and the ability to induce the HR. Insertions mapping to a region of 4 kb within the 25-kb segment had no phenotypic effect. When plasmid pXV9 was transferred into a number of nonpathogenic insertion mutants, it restored wild-type pathogenicity in each case. A large number of these insertion mutants were also tested for HR induction on nonhost plants, for example, tobacco, bean, and cowpea. Except for Tn*3-gus* insertion 75, which induced a very delayed HR on tobacco compared to the wild type, in no case did a nonpathogenic mutant induce an HR. Since both pathogenicity and the ability to induce the HR were affected by the mutations, the genomic region defined by the mutations is a *hrp* region (Lindgren *et al.* 1986).

Identification of complementation groups. The genetic organization of the *hrp* region was determined by complementation analysis of the Tn*3-gus* and several Tn*5* mutants. Transposon-induced nonpathogenic *X. c.* pv. *vesicatoria* mutants were conjugated with different *E. coli* strains harboring pXV2::Tn*3-gus* or pXV9::Tn*3-gus* in which the transposon insertions were in a region flanking the site of insertion in the recipient. The transconjugants were tested on pepper cultivars ECW and ECW-10R for symptom production. The results showed that the *hrp*

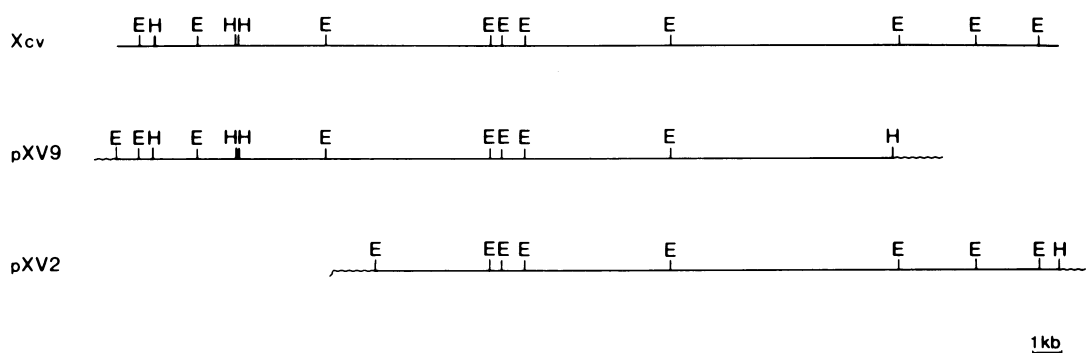

Fig. 1. Restriction map of the DNA region containing *hrp* genes. Plasmid clones pXV9 and pXV2 are compared. They contain *Sau*3A fragments cloned into the *Bam*HI site of pLAFR3. The inserts are flanked by an *Eco*RI (E; left) site and a *Hin*dIII (H; right) site originating from the vector. The wavy lines represent vector sequences. The resulting map of the corresponding genomic region in *Xanthomonas campestris* pv. *vesicatoria* (Xcv; approximately 30 kilobases [kb]) is shown in the upper line.

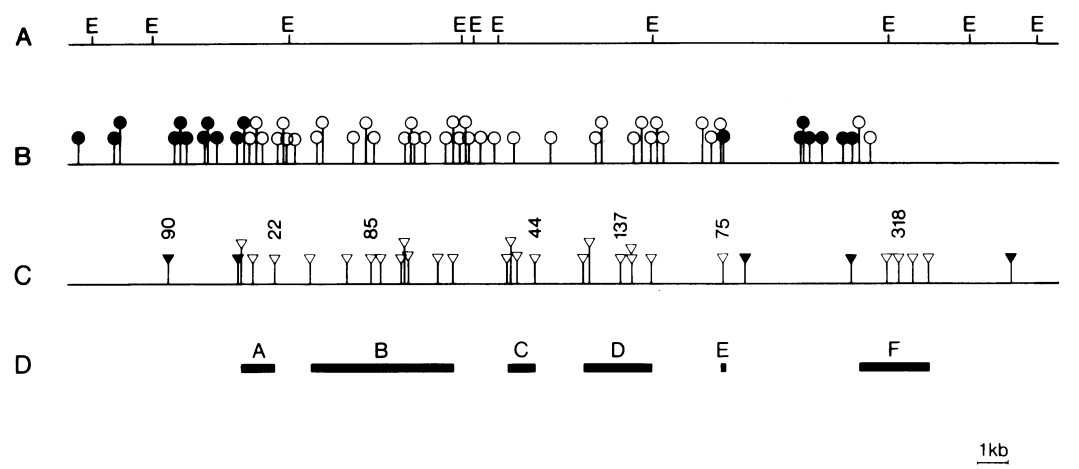

Fig. 2. Transposon insertions within the *hrp* region of *Xanthomonas campestris* pv. *vesicatoria* and complementation analysis. The restriction map of the genomic region is shown in **A** (E = *Eco*RI). The sites of Tn*5* insertions into the genome are shown in **B**, and the sites of Tn*3-gus* insertions are shown in **C**. Filled-in symbols represent the wild-type reaction of the insertion mutants in plant inoculation tests; open symbols indicate sites of insertions resulting in loss of pathogenicity and induction of the hypersensitive response. The numbers above the triangles indicate the Tn*3-gus* insertions tested for growth *in planta* (see Fig. 3). **D**, Complementation analysis of Tn*3-gus* and selected Tn*5* insertions revealed the presence and position of six complementation groups, *hrpA* to *hrpF*. The preliminary length of each complementation group is indicated by the black bars.

region is organized into at least six different complementation groups designated *hrpA* through *hrpF* (Fig. 2D). The complementation groups *hrpA* to *hrpE* span a region of 16 kb in which all of the insertion mutations caused a Hrp$^-$ phenotype. The *hrpE* complementation group was defined by only two insertions mapping at nearly the same position (Fig. 2). A complementation group spanning 3 kb (*hrpF*) was located at the right border of the *hrp* region. Insertions in an approximately 4-kb region between *hrpE* and *hrpF* had no detectable phenotypic effect.

Growth of marker exchange mutants *in planta*. Seven different Tn*3-gus* mutants of *X. c.* pv. *vesicatoria* 85-10 were inoculated into pepper to monitor their growth in the plant (Fig. 3). Tn*3-gus* insertion 90, which was still pathogenic, showed wild-type growth. All of the nonpathogenic mutants representing the different complementation groups (Fig. 2C) failed to reach densities above 10^5 cfu per 1-cm^2 leaf disk. The growth curves determined in these experiments resemble those obtained with the NTG mutants described above (data not shown) and are reminiscent of the behavior of saprophytic bacteria *in planta*. To test whether nonpathogenic mutants can produce symptoms when present at higher densities in the plant leaf, several insertion mutants were inoculated into pepper at cell densities of 10^8 to 10^{10} cfu/ml. In no case were symptoms observed in plants.

Homology of the *hrp* region of *X. c.* pv. *vesicatoria* to DNA of other *X. campestris* pathovars. Total genomic DNA of different races of *X. c.* pv. *vesicatoria* and of different pathovars of *X. campestris* was digested with *Eco*RI, separated on agarose gels, blotted, and hybridized with a 23-kb *Hin*dIII fragment of plasmid pXV9 containing almost the entire *hrp* region (see Fig. 1). The hybridizing fragments in DNA of *X. c.* pv. *vesicatoria* strains isolated in Florida (Fig. 4, lanes 1–3) correspond in length and number to the *Eco*RI fragments predicted from the restriction maps of clones pXV2 and pXV9 (Fig. 1). Signals corresponding to the small *Eco*RI fragments (0.5 and 0.8 kb) present in clone pXV9 were only visible after longer exposures of the autoradiograph. *X. c.* pv. *vesicatoria* strains pathogenic on pepper or tomato showed identical hybridization patterns (Fig. 4, lanes 1–3) as did a plasmid-free strain of *X. c.* pv. *vesicatoria* (data not shown). Likewise, no restriction fragment length polymorphisms were detectable in this region when *Bam*HI fragments were analyzed. This was also true for several other strains of *X. c.* pv. *vesicatoria* that were originally isolated from diseased plants in different geographic areas (G. V. Minsavage and U. Bonas, unpublished data). The results of these experiments also showed that the insert of clone pXV9 is colinear with the corresponding genomic region in *X. c.* pv. *vesicatoria*.

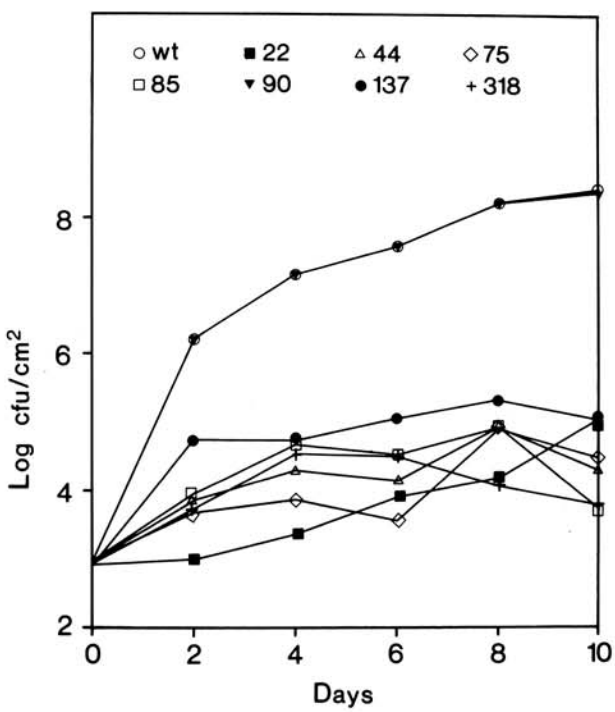

Fig. 3. Bacterial multiplication in pepper leaves. Wild-type strain 85-10 (wt) and seven different Tn*3-gus* insertion mutants of strain 85-10 were inoculated into pepper cultivar ECW. The positions of the insertions are indicated in Figure 2C. Values represent the mean from three samples; the results are from one of several independent experiments.

Homologous DNA sequences were detected in *X. c.* pv. *alfalfae*, *X. c.* pv. *armoraciae*, *X. c.* pv. *begoniae*, *X. c.* pv. *campestris*, *X. c.* pv. *citri*, *X. c.* pv. *dieffenbachiae*, *X. c.* pv. *glycines*, *X. c.* pv. *malvacearum*, *X. c.* pv. *oryzae*, *X. c.* pv. *phaseoli*, *X. c.* pv. *translucens*, and *X. c.* pv. *vignicola* (Fig. 4, lanes 5–16). An opportunistic *Xanthomonas* strain, T55, which was isolated from a black nightshade plant but was not pathogenic on this plant or on pepper or tomato, was identified as *X. campestris* by fatty acid profiles and physiological tests (Gitaitis *et al.* 1987). DNA of strain T55 did not hybridize at all to the 23-kb *Hin*dIII fragment from pXV9 (Fig. 4, lane 4), to the flanking 2.7-kb *Eco*RI fragment, or to sequences at the very left end of pXV9. However, strong hybridization was found when the rightmost 2-kb *Eco*RI fragment from pXV2 (see Fig. 1; data not shown) was used as a probe. Therefore, we believe that strain T55 lacks a large portion of the pathogenicity region. Since strain T55 was not complemented to pathogenicity by clones of the genomic library of *X. c.* pv. *vesicatoria* 75-3, a region much larger than 25 kb is presumably missing.

DNA of *P. solanacearum* showed only very weak homology to the *X. c.* pv. *vesicatoria hrp* sequences even though a threefold larger amount of DNA was loaded (Fig. 4, lane 17). DNA of strains of *P. s.* pv. *glycinea*, *P. s.* pv. *phaseolicola*, and *P. s.* pv. *tomato* as well as strains of *Rhizobium* and *Agrobacterium tumefaciens* (Fig. 4, lane 18) did not hybridize to the *hrp* region of *X. c.* pv. *vesicatoria* (data not shown).

The *hrp* genes and the pathogenicity functions of different *X. campestris* pathovars seem to be homologous. A strain of the bean pathogen *X. c.* pv. *phaseoli* as well as the black rot-inducing pathogen *X. c.* pv. *campestris* were mutagenized with NTG, and colonies were tested for loss of pathogenicity on bean and radish, respectively. Five nonpathogenic mutants of *X. c.* pv. *phaseoli* and two mutants of *X. c.* pv. *campestris* were tested for complementation by each of the 13 *X. c.* pv. *vesicatoria hrp* clones. Four of the five mutants of *X. c.* pv. *phaseoli* but neither of the two mutants of *X. c.* pv. *campestris*

were complemented. Thus, homologous functions are present in *X. c.* pv. *phaseoli* and *X. c.* pv. *vesicatoria*, whereas the NTG mutants in *X. c.* pv. *campestris* carry mutations in a different genomic region, in multiple loci, or in nonhomologous functions.

To further explore if there are common *hrp* functions in these and other pathovars of *X. campestris* that showed DNA hybridization to the *hrp* region of *X. c.* pv. *vesicatoria* (Fig. 4), we attempted to introduce Tn*5* insertions by marker gene exchange. Several exchanges were successful in strains of *X. c.* pv. *citri*, *X. c.* pv. *dieffenbachiae*, and *X. c.* pv. *vignicola* and were verified by Southern hybridization. The Tn*5* insertions that were exchanged from the *hrp* region of *X. c.* pv. *vesicatoria* into these pathovars resulted in loss of pathogenicity on their host plants grapefruit, *P. oxycardum*, and cowpea, respectively, and loss of the ability to induce the HR in the nonhosts pepper and tobacco. In *X. c.* pv. *citri* and *X. c.* pv. *vignicola* three different Tn*5* insertions were obtained, and in *X. c.* pv. *dieffenbachiae* two Tn*5* insertions were obtained. The positions of the insertions mapped in *hrpA*, *hrpB*, and *hrpC* of *X. c.* pv. *vesicatoria*, respectively. Since only a small number of exchange mutants were obtained, it is possible that there are also common functions in the region carrying *hrpD* to *hrpF*. The plasmid pXV9 was transferred into the insertion mutants to test for complementation to the wild type. Only mutants of *X. c.* pv. *vignicola* were fully restored. No mutants of *X. c.* pv. *armoraciae*, *X. c.* pv. *phaseoli*, and *X. c.* pv. *vitians* were isolated although transconjugants were obtained. We attempted several times to mutate a strain of *X. c.* pv. *phaseoli* without success. This result was surprising, since nonpathogenic NTG mutants were complemented by the *hrp* sequences of *X. c.* pv. *vesicatoria* (see above).

DISCUSSION

Mutants of *X. c.* pv. *vesicatoria* defective for growth in susceptible plants could be restored to wild-type growth by complementation with plasmids harboring a genomic region of about 30 kb. A correlation between bacterial growth and symptom production has been observed in other systems (e.g. Lindgren *et al.* 1986; Huang *et al.* 1988). Previously, genes from *X. c.* pv. *campestris* that conferred altered pathogenicity phenotypes have been isolated (Daniels *et al.* 1988). Since the mutants described here were not only affected in their interaction with the susceptible host plant but also in induction of an HR on resistant host cultivars or on nonhost plants, the genes localized in the isolated DNA region were designated *hrp* genes.

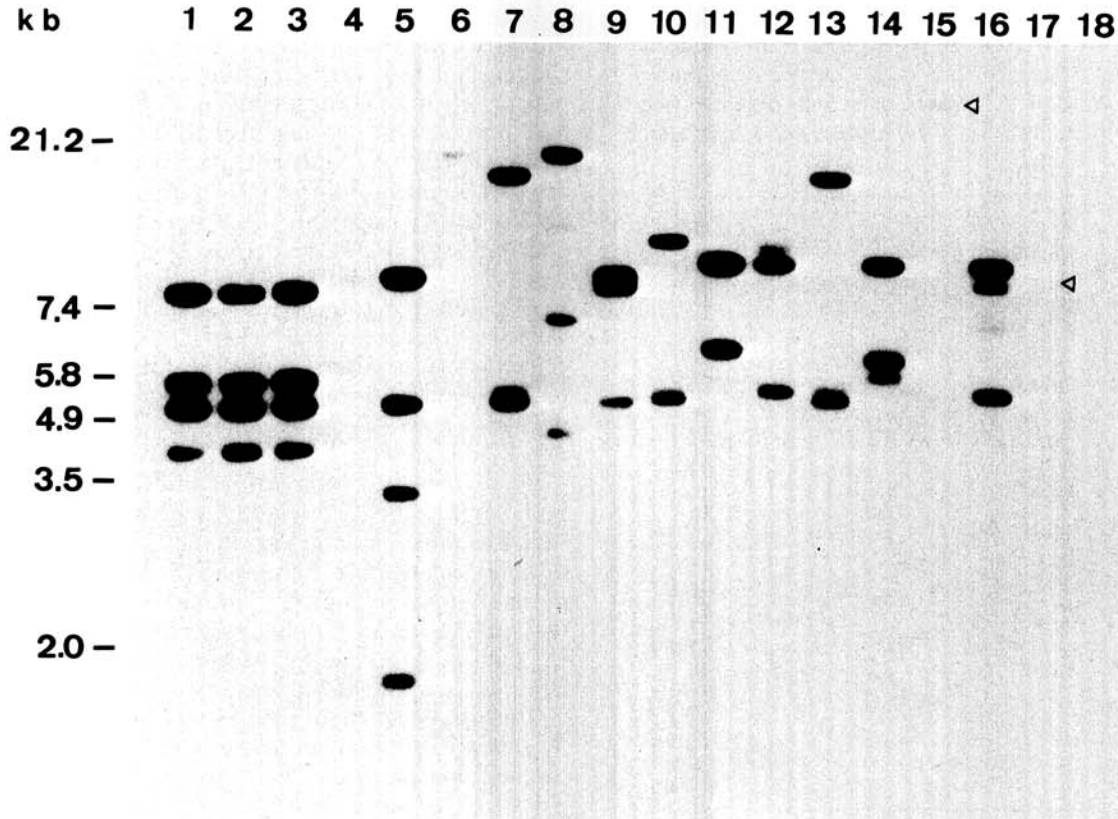

Fig. 4. Genomic Southern blot of total DNA isolated from different bacterial strains. Approximately 2 μg of *Eco*RI-digested DNA was loaded per lane (lanes 8, 16, and 18 contain 6 μg of DNA) and separated on a 0.7% agarose gel. The blot was hybridized with the 23-kilobase (kb) *Hin*dIII fragment of plasmid pXV9. Lanes 1–3, *Xanthomonas campestris* pv. *vesicatoria* 71-21 (1), 85-10 (2), and 75-3 (3). Lane 4, *X. campestris* T55; lanes 5–16, *X. c.* pv. *alfalfae* (5), *X. c.* pv. *armoraciae* (6), *X. c.* pv. *begoniae* (7), *X. c.* pv. *campestris* (8), *X. c.* pv. *citri* (9), *X. c.* pv. *dieffenbachiae* (10), *X. c.* pv. *glycines* (11), *X. c.* pv. *malvacearum* (12), *X. c.* pv. *oryzae* (13), *X. c.* pv. *phaseoli* (14), *X. c.* pv. *translucens* (15), and *X. c.* pv. *vignicola* (16). Lane 17 contains *Pseudomonas solanacearum*, and lane 18, *Agrobacterium tumefaciens*. The arrows mark the position of very weak bands in lanes 15 and 17.

This designation, first used by Lindgren *et al.* (1986), operationally defines a set of genes involved in the interaction with the plant. To our knowledge, this is the first example of the identification of *hrp* genes in a pathovar of *X. campestris*. *hrp* genes have been identified in *P. solanacearum* (Boucher *et al.* 1987; Huang *et al.* 1990) and in pathovars of *P. syringae* (Niepold *et al.* 1985; Lindgren *et al.* 1986; Huang *et al.* 1988). The *hrp* genes of *X. c.* pv. *vesicatoria* are probably located on the chromosome, as is the case in *P. syringae*, since a plasmid-free strain shows the same hybridization pattern when probed with a *hrp*-specific sequence (data not shown).

The *hrp* region appears to be highly conserved among different strains of *X. c.* pv. *vesicatoria* irrespective of their host specificity (pepper or tomato or both). The complementation of NTG mutants of *X. c.* pv. *vesicatoria* 82-8 (pepper) and *X. c.* pv. *vesicatoria* 81-23 (pepper and tomato) by sequences from tomato strain 75-3 suggests functional homology between the *hrp* region of pepper and that of the tomato strains of *X. c.* pv. *vesicatoria*. Moreover, loci of the different strains of *X. c.* pv. *vesicatoria* were interchangeable in marker exchange experiments. Furthermore, several strains of *X. c.* pv. *vesicatoria* isolated in Florida (Fig. 4) or in different geographical regions (Australia, Hawaii, and Taiwan) when analyzed by Southern hybridization (*Eco*RI and *Bam*HI digests) revealed no restriction fragment length polymorphisms in the *hrp* region (G. V. Minsavage and U. Bonas, unpublished results). The high degree of homology of the *hrp* region could indicate that the strains of *X. c.* pv. *vesicatoria* have evolved from a common ancestor and were spread from one country to another only recently. Also, the functions encoded in the *hrp* region might be under strong selection pressure. Sequence analysis of *hrp* regions from different strains could clarify this point.

In our studies various degrees of homology between the *hrp* region of *X. c.* pv. *vesicatoria* and other pathovars of *X. campestris* became evident (Fig. 4). DNA from *X. c.* pv. *alfalfae*, *X. c.* pv. *begoniae*, *X. c.* pv. *citri*, *X. c.* pv. *dieffenbachiae*, *X. c.* pv. *glycines*, *X. c.* pv. *malvacearum*, *X. c.* pv. *oryzae*, *X. c.* pv. *phaseoli*, and *X. c.* pv. *vignicola* gave strong hybridization signals when probed with sequences from the *X. c.* pv. *vesicatoria hrp* region. Weaker signals were observed for *X. c.* pv. *campestris* and *X. c.* pv. *translucens*. Additional evidence suggests that in the strongly hybridizing pathovars the homology to *X. c.* pv. *vesicatoria* is quite high. First, the pathogenicity- and hypersensitivity-determining loci of *X. c.* pv. *vesicatoria* and *X. c.* pv. *phaseoli* are interchangeable as shown by complementation. Second, in some cases homology was high enough to permit recombination. Nonpathogenic mutants of *X. c.* pv. *citri*, *X. c.* pv. *dieffenbachiae*, and *X. c.* pv. *vignicola* were obtained by marker gene replacement with transposon-tagged alleles of the *hrp* region of *X. c.* pv. *vesicatoria*. The insertions mapped in *hrpA*, *hrpB*, and *hrpC*. When the insertion mutants were tested for complementation by pXV9, only mutants of *X. c.* pv. *vignicola* were fully restored. The difficulty in the evaluation of this result is that the extent of homology and the length of the segment actually exchanged in the mutants are not known. The results obtained indicate that homologous *hrp* functions may be present in these different pathovars. A similar observation was made by Lindgren *et al.* (1988) after introducing Tn*5* insertion derivatives of the *hrp* region of *P. s.* pv. *phaseolicola* into different pathovars of *P. syringae*. How is the host specificity of the different pathovars determined? So far the determinants of host species specificity are unknown. They could be positive (virulence genes) or negative (avirulence genes) functions. If such determinants really exist in the pathogen, the different pathovars might carry different alleles. It will be interesting to compare the nucleotide sequences of the *hrp* genes of various pathovars.

The *hrp* cluster of *X. c.* pv. *vesicatoria* is not homologous to DNA sequences of plant pathogenic strains of *Pseudomonas*. Although cross-hybridization of the *hrp* cluster of *P. solanacearum* with DNA of *X. c.* pv. *vesicatoria* has been reported (Boucher *et al.* 1987), we observed only a faint hybridization signal (four different strains tested). The discrepant results could be due to genetic variability between the strains tested. The lack of homology between the *hrp* region of *X. c.* pv. *vesicatoria* and sequences from different pathovars of *P. syringae* could be an indication that the *hrp* genes of these pathogens have evolved divergently or have independent origins. This notion is supported by the fact that the *hrp* region of *P. s.* pv. *phaseolicola* (Lindgren *et al.* 1986) failed to complement any of the nonpathogenic NTG mutants of *X. c.* pv. *vesicatoria*. Interestingly, the *hrp* genes from *P. s.* pv. *phaseolicola* also failed to hybridize to DNA from saprophytic or other pathogenic bacteria including *Xanthomonas* (Lindgren *et al.* 1988). Nevertheless, functional homologies cannot be ruled out.

The results of comparative Southern analysis in which a number of strains were probed with sequences from the *hrp* region of *X. c.* pv. *vesicatoria* could have interesting implications for classification and diagnostics. The fact that the hybridization patterns were different for each pathovar tested (one strain each) and that the pattern of different strains within *X. c.* pv. *vesicatoria* was conserved (see Fig. 4) suggests that *hrp* probes could be useful in the classification of *Xanthomonas* strains. However, more extensive hybridization experiments are needed to determine the feasibility of this approach. Since opportunistic strains of *X. campestris* lack homology to the *hrp* region (see Fig. 4; Stall and Minsavage 1990), *hrp* probes could be used in diagnostic tests to detect pathogenic strains of *Xanthomonas*, for example in seed material.

Insertion mutagenesis showed that the *hrp* genes are clustered in a region of approximately 25 kb. The right and left borders of the *hrp* region were defined by insertion mutants unaltered in pathogenicity. The region necessary for pathogenicity is interrupted by a 4-kb segment in which the two insertions obtained had no detectable phenotypic effect. Although no mutations mapping outside of the 25-kb region were found, it cannot be ruled out at present that additional genes elsewhere in the genome are involved in the interaction with the plant.

The *hrp* region of *X. c.* pv. *vesicatoria* contains at least six complementation groups (Fig. 2D). The complementation groups, which were designated *hrpA* through *hrpF* (Fig. 2D, from left), vary in length from less than 1 kb

to 5 kb. The length and number of complementation groups suggest the presence of several operons.

The function of the *hrp* genes in phytopathogenic bacteria remains elusive. Only for one of the *hrp* genes from *P. s.* pv. *phaseolicola* has a function been suggested. It was found to be homologous to regulatory genes of the *ntrC* family (Grimm and Panopoulos 1989). Both the so-called disease-specific genes, which encode extracellular enzymes or control their secretion (Daniels *et al.* 1984b; Dow *et al.* 1987), and the virulence genes, such as those of *Agrobacterium* (e.g. Stachel and Nester 1986), play a role in plant-pathogen interactions. Similar functions may be present in *X. c.* pv. *vesicatoria*. The isolation of *hrp* genes from *X. c.* pv. *vesicatoria* is the first step in attempting to understand their biochemical function.

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