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Xanthomonas campestris Contains a Cluster of *hrp* Genes Related to the Larger *hrp* Cluster of *Pseudomonas solanacearum*

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All *Xanthomonas campestris* pathovars tested contain DNA which hybridizes to the large *hrp* gene cluster of *Pseudomonas solanacearum* (C. A. Boucher, F. Van Gijsegem, P. A. Barberis, M. Arlat, and C. Zischek, J. Bacteriol. 169:5626-5632, 1987). Clones carrying these sequences were isolated from genomic libraries of *X. campestris* pvs. *campestris* and *vitians*. Mutagenesis of the corresponding genomic regions of both pathovars gave strains defective in both pathogenicity and hypersensitive response induction. *X. c.* pv. *campestris* contained a *hrp* gene cluster covering about 25 kb, which was homologous and colinear over a continuous 19-kb DNA region with the *P. solanacearum hrp* cluster. Cross-complementation showed that *X. c.* pv. *vitians* and *X. c.* pv. *campestris hrp* sequences are functionally interchangeable, but the source of the *hrp* genes did not determine the compatibility-incompatibility of the host-pathogen interaction. One *X. c.* pv. *campestris* Hrp$^-$ mutant was "complemented" by specific subclones of the *P. solanacearum hrp* cluster, suggesting the existence of some functional homology between the clusters of the two species. Expression of *hrp* genes (studied by *lacZ* fusions) was repressed in rich medium, and in minimal medium the level of expression depended on the carbon source supplied to the cells. Transcription of *hrp* genes was not regulated by genes that control the synthesis of extracellular enzymes, which are required for pathogenicity. In addition *X. campestris* Hrp$^-$ mutants produced wild-type levels of these extracellular enzyme activities. These results suggest the existence of two independent sets of pathogenicity genes that are regulated differently.

Additional keywords: lettuce, pepper, turnip.

The hypersensitive response (HR) of higher plants is a defense reaction characterized by the rapid, localized necrosis of plant tissues, which is specifically induced by pathogenic microorganisms (Klement 1982). This reaction occurs in so-called incompatible interactions involving pathogens and either nonhost or resistant plants. It has been suggested that factors of the pathogen inducing HR in nonhosts might also contribute to pathogenicity in susceptible hosts (Klement 1982). This hypothesis has been confirmed for several phytopathogenic bacteria. Molecular genetic studies have revealed a class of pathogenicty genes, designated *hrp* genes, which are essential both for the development of disease symptoms on susceptible plants and for the elicitation of HR on nonhost or resistant plants in *Pseudomonas syringae* van Hall (Anderson and Mills 1985; Lindgren *et al.* 1986; Huang *et al.* 1988), *Pseudomonas solanacearum* (Smith) Smith (Boucher *et al.*, 1987, Huang *et al.* 1990), *Erwinia amylovora* (Burrill) Winslow *et al.* (Beer *et al.* 1991; Barny *et al.* 1990), and *Xanthomonas campestris* (Pammel) Dowson (Bonas *et al.* 1991; Kamoun and Kado 1990). The apparent dual role of *hrp* genes suggests that plants have developed resistance mechanisms based on recognition of bacterial determinants that control pathogenicity. The identification of these determinants and of the biochemical functions encoded by *hrp* genes could give insight into one of the basic defense mechanisms of plants.

Most of the *hrp* genes are organized in a large cluster in all the bacteria studied so far. The *hrp* clusters are conserved among *P. solanacearum* strains (Boucher *et al.* 1988) and among *P. syringae* pathovars (Lindgren *et al.* 1988), but no homology was found between the *hrp* clusters of these two species (Boucher *et al.* 1987). Recently Beer *et al.* (1991) have reported the existence of homology between the *hrp* clusters of *E. amylovora* and *P. syringae*, suggesting that these two species share some core pathogenicity functions. Similarly in our previous work, we established that the large *hrp* cluster of *P. solanacearum* that covers about 23 kb possesses homologous sequences in the genome of all pathovars tested of *X. campestris* (Boucher *et al.* 1987).

In this paper, we report the cloning of these homologous DNA sequences from two *X. campestris* pathovars, *X. c.* pv. *campestris* (Pammel) Dowson and *X. c.* pv. *vitians* (Brown) Dye, the causal agents of black rot of crucifers and leaf spot of lettuce, respectively, and we show that they carry related *hrp* genes. In *X. c.* pv. *campestris* these *hrp* genes form a cluster of approximately 25 kb. This *hrp* cluster is homologous and colinear over 19 kb with the *hrp* cluster of *P. solanacearum*. Functional complementation was detected between some of the *hrp* sequences of the two pathogens. We have also established that the loss of pathogenicity of *X. campestris* Hrp$^-$ mutants is not due to a failure to produce extracellular enzymes known to be required for pathogenicity. Finally, data concerning the expression of *X. c.* pv. *campestris hrp* genes *ex planta* are given.

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MATERIALS AND METHODS

Bacteria, plasmids, phage, and transposon. The bacterial strains, plasmids, phage, and transposon used in this work are listed in Table 1.

Growth media and conditions and genetic techniques. Growth media, conditions of incubation, and antibiotic selection have been described previously (Collinge *et al.* 1987; Daniels *et al.* 1984a; Turner *et al.* 1984, 1985). Plasmid transfer and other genetic techniques were described by Turner *et al.* (1984). Marker exchange followed the procedure of Turner *et al.* (1985), except that for *X. c.* pv. *vitians* the plasmid used to displace the pLAFR3 derivative, pIJ3228 carrying the Ω cassette, was pIJ3011, a spontaneous spectinomycin susceptible (Sp^s) derivative of pPH1JI (Sawczyc *et al.* 1989). Marker exchange was verified by tetracycline (Tc) sensitivity and by Southern analysis (Turner *et al.* 1985).

DNA manipulation. Plasmid purification, restriction endonuclease mapping, gel electrophoresis, and DNA ligation were performed by standard procedures (Maniatis *et al.* 1982). DNA restriction fragments were isolated from agarose gels using a Geneclean Kit (Bio 101, La Jolla, CA). Southern transfers and colony lifts were performed with Biodyne (Pall, Glen Cove, NY) membranes as recommended by the manufacturer. High-stringency hybridization was performed at 42° C in 5× SSC (1× SSC = 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0)-50% formamide, followed by three washes in 2× SSC-0.1% sodium dodecyl sulfate (SDS) at room temperature and two washes in 0.1× SSC-0.1% SDS at 50° C. Low-stringency hybridization was performed at 37° C in 5× SSC-50% formamide, followed by three washes in 2× SSC-0.1% SDS at room temperature.

Tn*5*-B20 mutagenesis of pIJ3225. Phage λ573::Tn*5*-B20 lysates were prepared from *Escherichia coli* C600 as described by Silhavy *et al.* (1984). The plasmid pIJ3225 was mutagenized by infecting exponentially growing *E. coli* S17-1(pIJ3225) cells with the phage lysate at a multiplicity of infection of 0.5–1. After incubation for 2 hr at 37° C, cells were washed twice and plated on L-SmTcKm (streptomycin, tetracycline, kanamycin), which allowed growth of cells in which Tn*5*-B20 had transposed. To select for mutants in which the transposon had integrated into the plasmid, these colonies were suspended and pooled in L broth. After dilution in fresh broth and growth, they were mated with

Table 1. Bacteria, plasmids, phage and transposon used in this study

Designation	Relevant characteristics[a]	Reference or source
Bacteria		
Escherichia coli		
S17-1	F^-, λ^-, *recA*, *hsdR*, RP4-2 (Tc::Mu) (Km::Tn7)	Simon *et al.* 1989
C2110 $Nal^r Rif^r$	*polA1 rha his* Nal^r Rif^r	Leong *et al.* 1980
C600	*thr*, *leu*, *rpsl*, *hsdR*	Maniatis *et al.* 1982
Xanthomonas campestris pv. vitians		
9001	Rif^r	Collinge *et al.* 1987
9010	9001::Ω, delta *hrp*, Sp^r, Sm^r, contains pIJ3011	This work
Xanthomonas campestris pv. campestris		
8004	Rif^r	Turner *et al.* 1984
8258	Sm^r, Cm^r, contains pPH1JI	Turner *et al.* 1985
8237	*rpf* derivative of 8004, protease-minus, polygalacturonate lyase-minus, endoglucanase-minus amylase-minus; non-pathogenic	Tang *et al.* 1991
8288	*xps* derivative of 8004, unable to secrete protease, endoglucanase, polygalacturonate lyase, amylase; non-pathogenic	Dow *et al.* 1987
516-9	8004::Tn*5*, protease-minus	Tang *et al.* 1987
8409	8004::Tn*5*, endoglucanase-minus	Gough *et al.* 1988
Plasmids		
pLAFR1	IncP1, Tc^r, cos^+, Tra^-, Mob^+	Friedman *et al.* 1982
pLAFR3	IncP1, Tc^r, cos^+, Tra^-, Mob^+	Staskawicz *et al.* 1987
pVir2	*Pseudomonas solanacearum* DNA cloned in pLAFR3	Boucher *et al.* 1987
pGMI1731	pVir2 with partial deletion of the original insert	C. Boucher
pGMI737	pVir2 with partial deletion of the original insert	Boucher *et al.* 1987
pVir2-1	pVir2 with partial deletion of the original insert	This work
pAFE8	*P. solanacearum* DNA cloned in pLAFR3	Arlat *et al.* 1990
pIJ3011	IncP1, Sp^s, Gm^r, Cm^r, Tra^+, Mob^+	Sawczyc *et al.* 1989
pIJ3020	*Xanthomonas campestris* pv. *campestris* DNA cloned in pLAFR1	Daniels *et al.* 1984b
pIJ3079	*X. c. campestris* DNA cloned in pLAFR3	Tang *et al.* 1990
pIJ3220	*X. c. vitians* DNA cloned in pLAFR3	This work
pIJ3221	*X. c. vitians* DNA cloned in pLAFR3	This work
pIJ3228	pIJ3220 with a partial deletion of the original insert and the insertion of the Ω cassette, Sp^r, Sm^r	This work
pIJ3222–pIJ3227	*X. c. campestris* DNA cloned in pLAFR1	This work
Phage		
λ 573	*b221* (*att*$^-$, *int*$^-$), red$^-$, *O*am, *P*am, cI857	N. Kleckner
Transposon		
Tn*5*-B20	Tn*5* carrying a promotorless *lacZ* gene cloned into IS*50*L	Simon *et al.* 1989

[a] Abbreviations: Cm, chloramphenicol; Gm, gentamycin; Km, kanamycin; Nal, nalidixic acid; Rif, rifampicin; Sm, streptomycin; Sp., spectinomycin; Tc, tetracycline.

E. coli C2110 Nal^{r}Rif^r (nalidixic acid resistant, rifampicin resistant). Selection on L-NalKmTc gave colonies carrying pIJ3225::Tn*5*-B20.

Pathogenicity tests. Pathogenicity of *X. c.* pv. *campestris* strains was tested on turnip (*Brassica campestris* 'Just Right') by inoculation of leaf margins of mature turnip leaves as described by Gough *et al.* (1988), by infiltration of mature leaves, or by needle-inoculation of seedlings as described by Daniels *et al.* (1984a). In this latter test, disease progression was assessed as described by Osbourn *et al.* (1990). Mutants that rotted more than 80% of the seedlings during the test were considered pathogenic; mutants that rotted less than 20% of the inoculated seedlings and that did not induce any visible symptoms on at least 75% of the remaining seedlings after 7 days of incubation were considered nonpathogenic. Mutants that induced intermediate phenotypes on seedlings were considered attenuated.

Pathogenicity of *X. c.* pv. *vitians* derivatives was tested on lettuce plants (varieties Buttercrunch and Winter Density). Suspensions of bacteria at 10^7 and 10^8 cells ml^{-1} were infiltrated into the lamina of fully expanded leaves of 3- to 6-wk-old plants. After inoculation, plants were kept at 28° C in sealed plastic bags to maintain high humidity for 4–5 days. Under these conditions the pathogenic strain 9001 induced blackening and rotting of infiltrated tissues.

HR tests. HR-inducing ability of *X. c.* pv. *campestris* strains was tested on pepper cultivar Early Calwonder-10R (ECW-10R) (Minsavage *et al.* 1990). Suspensions of bacteria at 10^7 or 10^8 cells ml^{-1} in water were infiltrated into the intercellular spaces of mature leaves. The wild type strain induced yellow-green confluent necrosis that appeared within 24 hr and did not spread beyond the inoculated area.

HR-inducing ability of *X. c.* pv. *vitians* strains was tested by infiltrating turnip leaves (cv Just Right) as previously described (Conrads-Strauch *et al.* 1990).

β-galactosidase activity assay. Screening of the different Tn*5*-B20 fusions for expression was performed in rich medium (NYGB, Turner *et al.* 1984) and in MME minimal medium (K_2HPO_4, 10.5 g L^{-1}; KH_2PO_4, 4.5 g L^{-1}; $(NH_4)_2SO_4$, 1 g L^{-1}; Difco Casamino acids, 0,15 g L^{-1}; and 1 mM $MgSO_4$) supplemented with sucrose (20 mM). *X. c.* pv. *campestris* mutants containing Tn*5*-B20 insertions were grown to midexponential phase in NYGB at 28° C, washed twice with sterile water, and resuspended at 3×10^8 cfu ml^{-1} in MME + sucrose or NYGB. Cultures were shaken at 28° C for 14 hr and then assayed for β-galactosidase activity as described below. For NYGB-grown cells, the culture was also assayed for β-galactosidase activity at 8×10^8 cfu ml^{-1}.

Assays to test the activity of different carbon sources and nutrients on the expression of A2, B2, or F8 fusions were performed in MME containing supplements as in Figure 4, and used at 20 mM except for the following: glycerol, 40 mM, pyruvate, 40 mM, Peptone (Oxoid, 10 g L^{-1}), Casamino acids (Difco, 5 g L^{-1}), yeast extract (Beta-

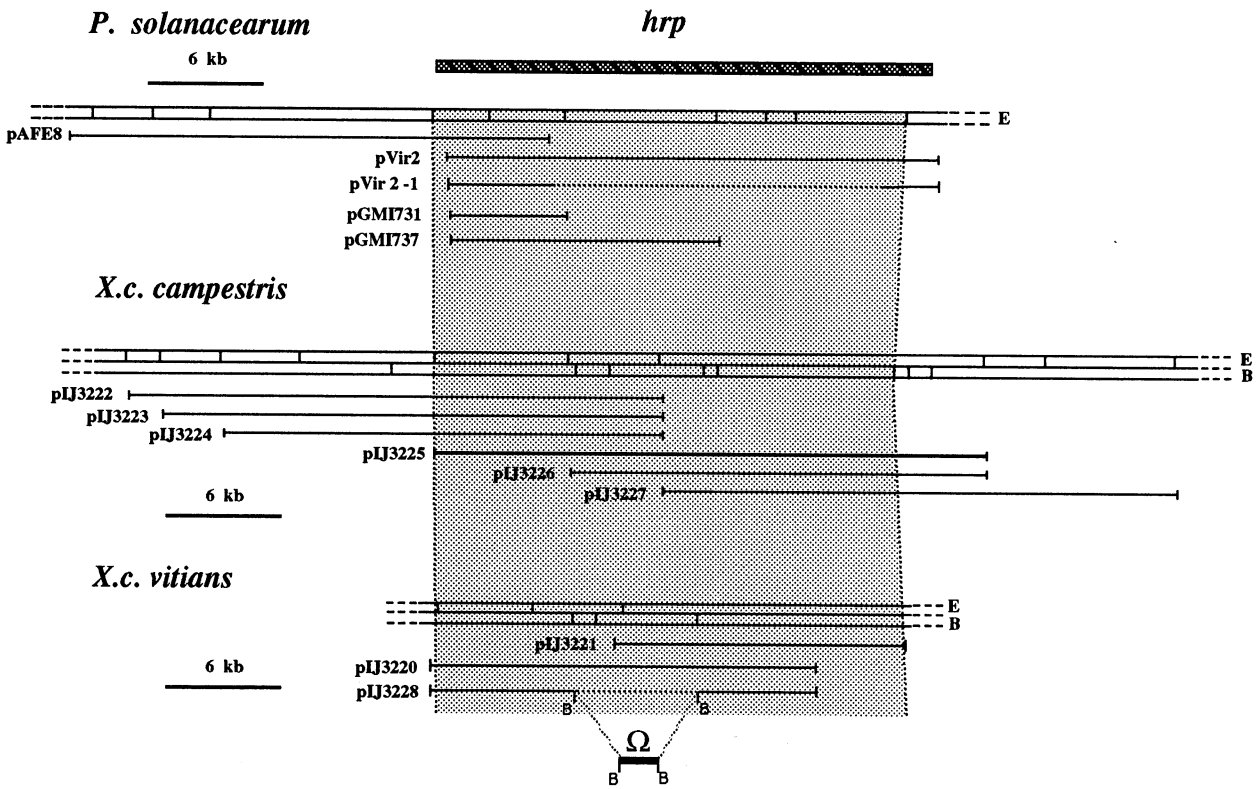

Fig. 1. Physical maps of the *hrp* region of *Pseudomonas solanacearum* and the related regions of *Xanthomonas campestris* pv. *campestris* and *X. c.* pv. *vitians*, and the domains of homology between these regions. E and B are sites for *Eco*RI and *Bam*HI, respectively. The hatched bar shows the extent of the *hrp* gene cluster of *P. solanacearum*. The bars below each physical map represent the insert DNA. For pVir2-1 the dotted line indicates the deleted portion. For pIJ3228 the internal *Bam*HI fragments were replaced with the Ω cassette as shown. The grey zone shows the DNA regions of the three pathogens that cross-hybridized under low stringency conditions.

lab, 6 g L^{-1}).

β-galactosidase assays were carried out as described by Miller (1972) following the modifications of Boivin *et al.* (1990).

Detection of extracellular enzyme activity in cell-free liquid culture medium. Relative activities of endoglucanase, amylase, and protease were measured by diffusion assays into substrate-containing agar plates (Dow *et al.* 1987; Tang *et al.* 1987). Polygalacturonate lyase (PGL) was assayed spectrophotometrically using bacteria that had been grown with polygalacturonate to induce the enzyme (Dow *et al.* 1987).

RESULTS

Cloning and physical study of *X. c.* pv. *campestris* and *X. c.* pv. *vitians* DNA sequences hybridizing with the *hrp* gene cluster of *P. solanacearum*. A pLAFR3 library of *X. c.* pv. *vitians* 9001 DNA (Roberts *et al.* 1987) was screened under low stringency conditions with subcloned DNA fragments from cosmid pVir2, which carries a large portion of the *hrp* cluster of *P. solanacearum* strain GMI1000 (Fig. 1; Boucher *et al.* 1987). Two cosmids, pIJ3220 and pIJ3221, carrying overlapping DNA fragments that showed strong hybridization under low stringency conditions with parts of pVir2 (Fig. 1) were identified. The orientation of *X. c.* pv. *vitians* sequences presented in Figure 1 was deduced as described below.

Subcloned DNA fragments from pIJ3220 were in turn used to screen a previously constructed pLAFR1 library of *X. c.* pv. *campestris* 8004 DNA (Daniels *et al.* 1984b). Six cosmids with overlapping inserts were identified (Fig. 1). The relative orientation of the inserts shown in Figure 1 was deduced from experiments presented below. Hybridization experiments using these six cosmids as probes against *Eco*RI or *Bam*HI digested genomic DNA of strain 8004 showed that the inserts were colinear with genomic DNA (data not shown).

The homology between the *hrp* sequences of *P. solanacearum* and the corresponding region of *X. c.* pv. *campestris* is summarized in Figure 1. Hybridization under low stringency conditions of *Eco*RI or *Bam*HI digests of these six cosmids with pVir2 as probe defined the restriction fragments of these cosmids carrying DNA sequences homologous to pVir2. The *X. c.* pv. *campestris* fragments homologous to the pVir2 probe formed a continuous region of about 25 kb that corresponded to the pIJ3225 insert (Fig. 1). The same Southern transfers were rehybridized under low stringency conditions with a probe corresponding to pAFE8, which overlaps pVir2 and carries the leftmost *hrp* sequences and the adjacent genomic sequences of *P. solanacearum* (Arlat *et al.* 1990) (Fig. 1). We found that pIJ3226 and pIJ3227 did not hybridize with this latter probe and in the other cosmids only one *Eco*RI fragment, common to all the clones, was detectable. This unique *Eco*RI fragment is the leftmost *Eco*RI fragment carried by pIJ3225 (Fig. 1). These hybridization results allowed us to orient the *X. c.* pv. *campestris* genomic region defined by the six cosmids relative to the *P. solanacearum* region defined by pVir2 and pAFE8 (Fig. 1). They also suggested that the *P. solanacearum* sequences adjacent to the left border of the *hrp* cluster do not have homologues in the *X. c.* pv. *campestris* region studied (Fig. 1). To confirm this, we hybridized *Eco*RI and *Eco*RI–*Bam*HI digests of pVir2 and pAFE8 with each of the six *X. c.* pv. *campestris* clones. All the *Eco*RI restriction fragments of pVir2 carried DNA sequences homologous to the six *X. c.* pv. *campestris* clones (Fig. 1). However only the right part of pAFE8, which carries *hrp* sequences, gave hybridization (Fig. 1). The pattern of hybridization obtained with each probe also confirmed the orientation of the two genomic regions previously deduced.

pVir2 and pAFE8 (*P. solanacearum*) and pIJ3225 and pIJ3222 (*X. c.* pv. *campestris*) were subcloned (data not shown) and the subclones were digested with different restriction enzymes to generate a set of subfragments that were isolated after agarose gel electrophoresis. Twelve independent subfragments ranging from 1.5 to 7.5 kb covering the genomic region defined by pIJ3225 and pIJ3222 (Fig. 2) and 21 different subfragments ranging from 0.5 to 7.5 kb covering the genomic region defined by pVir2 and pAFE8 were isolated (Fig. 2). The latter subfragments were electrophoresed, transferred to membranes, and hybridized under high stringency conditions with probes prepared from each of the 12 *X. c.* pv. *campestris* subfragments (Fig. 2). A region of pIJ3225 encompassing about 19 kb hybridized in a colinear manner with a region of about 18 kb of the *hrp* cluster of *P. solanacearum*. The data also confirmed the relative orientation of the two regions. The left and central parts of the inserts carried by pVir2 and pIJ3225 gave hybridization but the right parts did not. It is however interesting to note that the previous low stringency hybridization experiments suggested relatedness between pIJ3225 and the rightmost *Eco*RI fragments of pVir2 (Fig. 1). Either weak overall homology exists between these two regions or the homology extends only over a very small stretch of DNA. Finally, as expected, no hybridization was detected between subfragments specific to pIJ3222 and pAFE8.

Hybridization between the two *X. c.* pv. *vitians* clones and the six *X. c.* pv. *campestris* clones allowed us to orientate and place the *X. c.* pv. *vitians* DNA relative to the corresponding regions of *X. c.* pv. *campestris* and *P. solanacearum* (Fig. 1).

Mutagenesis of *X. c.* pv. *vitians* DNA sequences: Hrp phenotype. pIJ3220 was used to generate a localized insertion-deletion mutant of *X. c.* pv. *vitians*. The cosmid was first digested with *Bam*HI and the internal fragments were discarded and replaced with the Ω fragment (Prentki and Krisch 1984) previously restricted with *Bam*HI (Fig. 1). The Ω-containing cosmid, designated pIJ3228 was transferred into the wild-type strain 9001 of *X. c.* pv. *vitians*. The insertion-deletion mutation was then marker-exchanged into the *X. c.* pv. *vitians* genome.

The insertion-deletion mutant, designated 9010, was unable to induce disease symptoms on lettuce or to elicit HR in turnip leaves. The Hrp^- phenotype of mutant 9010 showed that the DNA sequences of *X. c.* pv. *vitians* deleted or interrupted by the Ω fragment insertion encode *hrp* functions.

Tn*5*-B20 mutagenesis of *X. c.* pv. *campestris* DNA sequences. pIJ3225 was mutagenized in *E. coli* with Tn*5*-

B20, which contains a *lacZ* reporter gene. Thirty-three insertions distributed through the insert were obtained.

Each Tn*5*-B20 insertion was marker-exchanged into *X. c.* pv. *campestris* 8004. The position and orientation of these marker-exchange insertions are shown in Figure 3.

All mutants were tested for pathogenicity to turnip seedlings and mature leaves, and HR-inducing ability was tested in pepper leaves. The results of these experiments are summarized in Figure 3 and show that pIJ3225 carries a region of approximately 25 kb that is required both for pathogenicity and HR-inducing ability. The Tn*5*-B20 insertions that produce an altered phenotype on plants are divided into three groups separated by two regions of about 2 and 4 kb, which do not appear to control either pathogenicity on turnip or HR-inducing ability on pepper. The leftmost group was defined as a $Hrp^{+/-}$ group, because these mutants induced delayed and partial HR on pepper plants and were attenuated on turnip seedlings, rather than nonpathogenic like the Hrp^{-} mutants in the other groups.

Cross-complementation experiments between *X. c.* pv. *vitians* and *X. c.* pv. *campestris hrp* sequences. pIJ3225 carrying *X. c.* pv. *campestris* DNA was introduced into the *X. c.* pv. *vitians* mutant 9010. The resulting strain produced disease symptoms on lettuce and induced a partial and delayed HR on turnip.

Similarly pIJ3221 carrying *X. c.* pv. *vitians* DNA was introduced into Hrp^{-} mutants of *X. c.* pv. *campestris*, carrying Tn*5*-B20 insertions G2, A2, B6, B3, A3, and B2 (hereafter, the Hrp^{-} mutants of *X. c.* pv. *campestris* will be designated by the letters Xch followed by the number of the insertion they carry, i.e., XchG2, XchA2, XchB6, XchB3, XchA3, and XchB2). The cosmid restored the ability to induce HR on pepper to mutants XchB6, XchB3, and XchA3, but not to mutants XchG2, XchA2, and XchB2. These results were expected as pIJ3221 carries DNA sequences homologous to the sequences altered by insertions B6, B3, and A3, but does not contain sequences homologous to the sequences mutated by insertions G2 and A2. For mutant XchB2, our hybridization data are not accurate enough to determine whether pIJ3221 carries DNA sequences homologous to the sequences altered by insertion B2, and therefore we cannot determine whether the inability to complement this mutation is due to the absence of homologous sequences in pIJ3221 or to other reasons.

Cross-complementation between *X. c.* pv. *campestris* and *P. solanacearum hrp* clusters. pVir2 was introduced into the same set of *X. c.* pv. *campestris* Hrp^{-} mutants. For each mutant three transconjugant colonies were picked and infiltrated into pepper leaves. None of the transconjugants obtained with mutants XchA2, XchB6, XchB3, XchA3, and XchB2 were able to induce any reaction on pepper. However, one of the transconjugants from XchG2 induced a partial and delayed HR. The plasmid harboured by the HR-inducing transconjugant, designated pVir2-1, carried a spontaneous deletion (Fig. 1). This deletion removes the central part of the pVir2 insert but does not affect the DNA sequences homologous to the region surrounding the G2 insertion. Two other XchG2 transconjugants that remained HR-negative harboured pVir2, which was apparently unchanged.

Two derivatives of pVir2, pGMI731, and pGMI737, (which carry inserts from the left part of the *hrp* cluster [Fig. 1], including the region homologous to the G2 region)

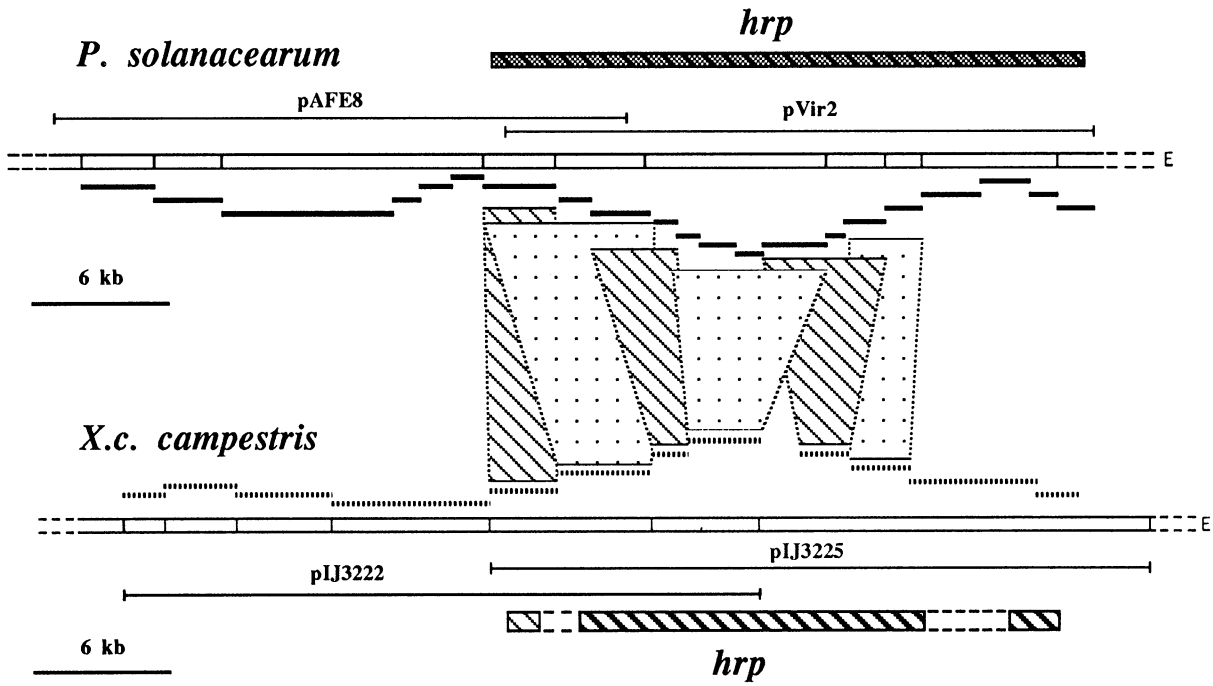

Fig. 2. Structural homology between the *hrp* region of *Pseudomonas solanacearum* and the related region of *Xanthomonas campestris* pv. *campestris*. The dotted lines above the *X. c.* pv. *campestris* restriction map represent the subfragments of this region used as probes in hybridization experiments against subfragments of the *P. solanacearum* region, which are shown by the bars below the *P. solanacearum* map. The shaded zones show the *P. solanacearum* subfragments that were detected with each *X. c.* pv. *campestris* probe under high stringency conditions. The hatched bars represent the *hrp* gene clusters of *P. solanacearum* and *X. c.* pv. *campestris* (see Fig. 3). Abbreviations: E, *Eco*RI; B, *Bam*HI.

were also introduced into XchG2. Three transconjugants colonies containing each plasmid were isolated and tested in pepper leaves. The three pGMI731-containing colonies elicited a delayed and partial HR, whereas the transconjugants harbouring pGMI737 did not induce any reaction. Mutants XchA2, XchB6, and XchA3 carrying pGMI731 and pGMI737 were unable to induce any reaction in pepper leaves.

XchG2 carrying pGMI73l, pGMI737, pVir2, or pVir2-1 was cultured nonselectively in MME supplemented with sucrose, and the loss of the Tc resistance marker carried by the plasmids was measured. The larger plasmids pVir2 and pGMI737 were slightly less stable than their shorter counterparts pVir2-1 and pGMI73l (data not shown). However the difference in stability did not seem to be enough to explain the differences in complementing ability.

Expression of *X. c.* pv. *campestris hrp* genes. *X. c.* pv. *campestris* 8004 is devoid of β-galactosidase activity (Tang *et al.* 1991); transcriptional fusions to *lacZ* induced by Tn*5*-B20 insertions were therefore used to monitor the expression of *hrp* genes.

After growth of each mutant in MME + sucrose, about 50% of the strains did not show a significant level of β-glactosidase (<10 Miller units, Miller 1972) (Fig. 3), presumably because of insertion in the wrong orientation or in a nontranscribed region. For the other strains, the level varied from 100 to 750 units, probably reflecting the activity of a transcription unit.

Mutants XchA2 and XchB2 (Lac^+ in MME + sucrose), and XchF8 (Lac^-) were used to test the effect of various carbon sources and nutrient supplements on the expression of *hrp* genes. No significant activity was detected with XchF8, whatever carbon source was used. However, the β-galactosidase activity varied 37.5-fold for mutant XchA2 (Fig. 4) and 18-fold for XchB2 (data not shown), according to the carbon source. Generally the activity for XchA2 was higher than that for XchB2. The relative effect of each carbon source was very similar for both mutants. There was no obvious correlation between the β-galactosidase activity induced and the ability of the carbon sources to support vigorous growth of the bacteria (data not shown).

β-galactosidase activity was also measured after growth in minimal medium containing sucrose in addition to each of the 18 carbon sources or supplements (Fig. 4). Peptone, yeast extract, and casamino acids strongly repressed the expression of the *hrp* genes. With the exception of glutamate, glycerol, and fructose, all the substances had significant repressive properties.

For each mutant producing a significant activity in MME (Fig. 3), β-galactosidase activity was measured following growth in rich medium. No significant activity (<10 units) was detected during exponential growth. However all the mutants produced significant activity (20–70 units) when they reached stationary phase, although the level was in all cases at least sevenfold lower than that measured in MME + sucrose (data not shown).

Hrp functions and production of extracellular enzymes. *X. c.* pv. *campestris* 8004 produces polysaccharide (EPS) and a range of extracellular enzymes including protease (Prt), polygalacturonate lyase (Pgl), endoglucanase (Egl) and amylase (Aml) activities. Prt and EPS are important for pathogenicity (Dow *et al.* 1990; Tang 1989; Tang *et al.* 1987).

All the *X. c.* pv. *campestris* Hrp^- or $Hrp^{+/-}$ mutants produced wild-type levels of each extracellular enzyme after growth on NYGB or MME + sucrose, and colony appearance indicated normal EPS production. Similarly the *X. c.* pv. *vitians* Hrp^- mutant produced wild-type levels of Prt and Egl (the parent strain does not produce Pgl or Aml). Previously isolated Prt^- or Egl^- mutants of *X. c.* pv. *campestris* (516-9 and 8409 respectively) as well as mutant 8288 unable to secrete extracellular enzymes elicited on pepper a HR identical with that induced by the wild-type strain.

The *X. c.* pv. *campestris rpf* mutant 8237 (Tang *et al.* 1991) and strain 8004 (pIJ3079) (Tang *et al.* 1990) are unable to induce disease and produce lower amounts of extracellular enzymes and EPS. However both strains induced

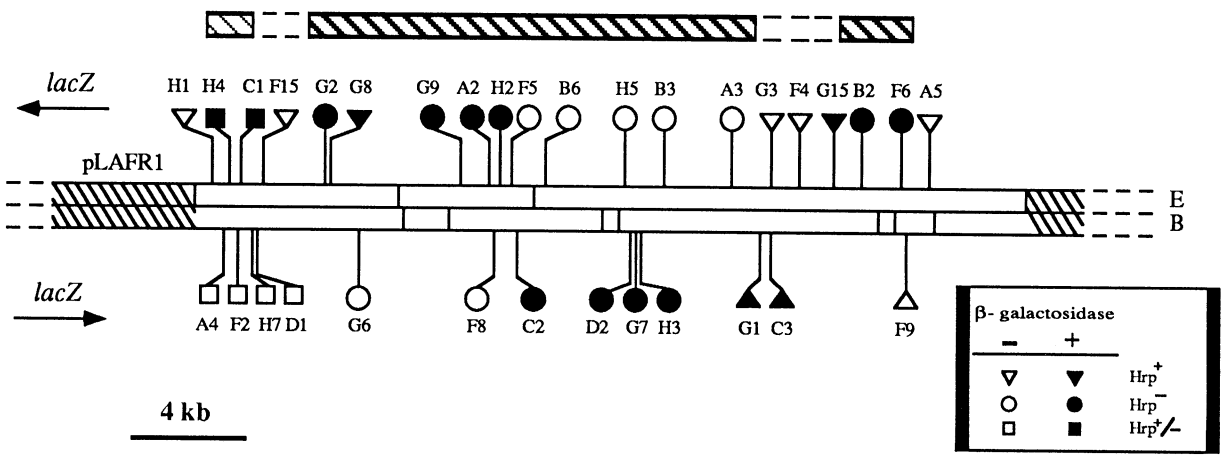

Fig. 3. Locations and orientations of Tn*5*-B20 insertions in pIJ3225 insert. The vertical bars show the position of insertions marker-exchanged into the *Xanthomonas campestris* pv. *campestris* genome. Circles show insertions that caused an Hrp^- phenotype. Squares represent insertions that gave a $Hrp^{+/-}$ phenotype, whereas triangles show insertions that did not affect the phenotype on plants. Filled symbols represent insertions that produced significant β-galactosidase activity in MME + sucrose, whereas open symbols correspond to silent insertions. The arrows indicate the transcription orientation of the *lacZ* gene. Abbreviation: E, *Eco*RI; B, *Bam*HI.

normal HR in pepper leaves. pIJ3225 carrying insertions A2 or B2 was introduced into both the wild-type strain 8004 and into the *rpf* mutant 8237. No significant difference in β-galactosidase activity ascribable to the *rpf* mutation was observed after growth of the cells in NYGB or in MME + sucrose, glucose, or pyruvate (data not shown). Also, introduction of pIJ3079 into mutants XchA2 and XchB2 did not affect β-galactosidase production after growth of the cells in the same media.

DISCUSSION

We have used *P. solanacearum hrp* genes to clone homologous sequences of *X. c.* pv. *campestris* and *X. c.* pv. *vitians* and shown by mutation that the latter carry *hrp* genes. In *X. c.* pv. *campestris* the *hrp* genes form a cluster covering approximately 25 kb, which is organized into three regions controlling HR-inducing ability and pathogenicity, separated by small domains wherein mutations have a Hrp^+ phenotype. The relatedness to the genes of *P. solanacearum* extends over 19 kb. There was no homology between the sequences flanking the *hrp* clusters of the two species, which are not considered to be closely related (De Vos *et al.* 1985). It is possible that the *hrp* clusters of both pathogens have evolved separately from a common origin and that the selective pressure was higher for the *hrp* sequences than for the surrounding sequences, or alternatively horizontal transmission may have taken place more recently. Restriction fragment length polymorphism studies on 52 different strains of *P. solanacearum* suggested that the *hrp* sequences have coevolved with the rest of the genome (Boucher *et al.* 1988). It will be interesting to undertake similar studies for *X. campestris* pathovars.

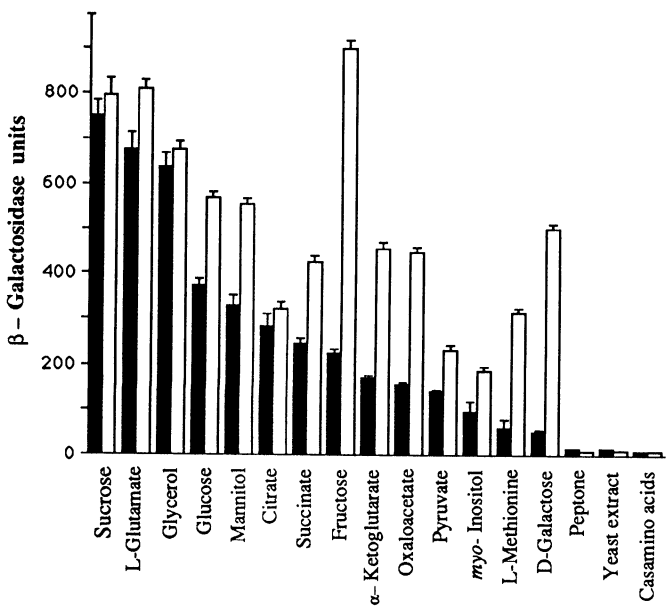

Fig. 4. Transcriptional activity of the *hrp* gene(s) mutated by insertion A2 in *Xanthomonas campestris* pv. *campestris* grown in MME containing various supplements. β-galactosidase activity is normalized for cell concentration (units as given by Miller 1972; values are means ±1 standard deviation for five independent experiments). Solid bars, supplement only; open bars, supplement with additional 20 mM sucrose.

The *P. solanacearum hrp* cluster is located on a megaplasmid (Boucher *et al.* 1986). Attempts to detect megaplasmids or other indigenous plasmids in *X. c.* pv. *campestris* 8004 have failed, and we therefore assume that the *hrp* cluster is located on the chromosome.

The *hrp* sequences of *X. c.* pv. *campestris* and *X. c.* pv. *vitians* cross-hybridize, and each can complement mutations in the other for the induction of disease and elicitation of HR, suggesting that these two *hrp* regions are highly conserved. It is noteworthy that the compatibility or incompatibility of a particular pathovar-plant interaction is not determined by the source of the *hrp* genes used to complement the mutation. Thus other factors (perhaps avirulence genes) must determine the choice between disease and HR. It is likely that all pathovars of *X. campestris* carry a *hrp* cluster analogous to that of *X. c.* pv. *campestris*, and studies are under way to determine if the clusters we have identified are related to the *hrp* cluster of *X. c.* pv. *vesicatoria* isolated by Bonas *et al.* (1991) or to the *hrp* locus identified by Kamoun and Kado (1990).

One *X. c.* pv. *campestris* mutant, XchG2, could be partially complemented by certain subclones carrying part of the *P. solanacearum hrp* region, whereas other subclones or the whole region could not complement the mutation. The explanation of this is not known.

Transcription of *X. c.* pv. *campestris hrp* genes is regulated in response to the nutritional status of the bacterium, being repressed in rich medium like *hrp* genes of *P. solanacearum* (Arlat *et al.* 1990), *P. syringae* pv. *glycinea* (Huynh *et al.* 1989), *P. s. phaseolicola* (Rahme *et al.* 1991) and *E. amylovora* (Beer *et al.* 1991). Expression of the *hrp* genes depended on the carbon source supplied. Sucrose, glutamate, and glycerol gave the highest levels of expression, followed by tricarboxylic acid (TCA) cycle intermediates, whereas casamino acids, yeast extract, and peptone strongly repressed transcription. It is interesting to compare our data with the results obtained by Huynh *et al.* (1989) on transcription of the *P. s. glycinea avrB* gene, which is itself controlled by *hrp* genes. The general pattern of nutritional regulation of *avrB* expression is similar to that of *X. c.* pv. *campestris hrp* genes. There are some differences in the relative effects of different carbon sources that may reflect differences in metabolic pathways in the two bacteria. In *Pseudomonas* species synthesis of carbohydrate utilization enzymes and transport systems is subject to catabolite repression by TCA cycle intermediates (Lessie and Phibbs, 1984) but this occurs only to a slight extent in *X. c.* pv. *campestris* (Whitfield *et al.* 1982). In *Pseudomonas aeruginosa* glycerol derepresses TCA cycle enzymes (Hamlin *et al.* 1967), whereas it has the opposite effect in *X. c.* pv. *campestris* (Whitfield *et al.* 1982). Huynh *et al.* (1989) suggested that sucrose, which is abundant in leaf tissues, contributes to induction of *avrB* and *hrp* genes during infection. It is likely that the regulation of these classes of pathogenicity gene helps the bacterium to exploit its host more efficiently.

hrp genes are not involved in regulation of production or export of extracellular enzymes and EPS. Thus, there are at least two independent sets of essential pathogenicity functions, one involved in the coordinated production of degradative extracellular enzymes and another which in-

cludes the *hrp* genes. The contribution of each to the infection process is not yet clear.

We have established that two different unrelated phytopathogenic bacteria, which induce different classes of disease and have different host specificities, carry similar *hrp* gene clusters. This implies that these two pathogens employ some common strategies and mechanisms to attack plants, and also suggests that some of the plant mechanisms involved in HR induction in reponse to these two pathogens are similar.

Although no homology (by hybridization or complementation) has yet been found between the *P. solanacearum-X. campestris hrp* cluster and the *hrp* clusters of other phytopathogenic bacteria, it will be interesting to determine if the different clusters share some common functions.

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LITERATURE CITED

Anderson, D. M., and Mills, D. 1985. The use of transposon mutagenesis in the isolation of nutritional and virulence mutants in two pathovars of *Pseudomonas syringae*. Phytopathology 75:104-108.

Arlat, M., Barberis, P., Trigalet, A., and Boucher, C. 1990. Organization and expression of *hrp* genes in *Pseudomonas solanacearum*. Pages 419-424 in: Proc. Int. Conf. Plant Pathogenic Bact., 7th. Z. Klement, ed. Akadémiai Kiadó, Budapest.

Barny, M. A., Guinebretière, M. H., Marcais, B., Coissac, E., Paulin, J. P., and Laurent, J. 1990. Cloning of a large gene cluster involved in *Erwinia amylovora* CFBP1430 virulence. Mol. Microbiol. 4:777-786.

Beer, S. V., Bauer, D. W., Jiang, X. H., Laby, R. J., Sneath, B. J., Wei, Z. M., Wilcox, D. A., and Zumoff, C. H. 1991. The *hrp* gene cluster of *Erwinia amylovora*. Pages 53-60 in: Proc. Int. Symp. Mol. Genet. Plant-Microbe Interact. 5th. H. Hennecke and D. P. Verma, eds. Kluwer Academic Publisher, Dordrecht.

Boivin, C., Camut, S., Malpica, C. A., Truchet, G., and Rosenberg, C. 1990. *Rhizobium meliloti* genes encoding catabolism of trigonelline are induced under symbiotic conditions. Plant Cell 2:1157-1170.

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-Microbe Interact. 4:81-88.

Boucher, C. A., Barberis, P. A., and Arlat, M. 1988. Acridine orange selects for deletion of *hrp* genes in all races of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 1:282-288.

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.

Boucher, C., Martinel, A., Barberis, P., Alloing, G., and Zischek, C. 1986. Virulence genes are carried by a megaplasmid of the plant pathogen *Pseudomonas solanacearum*. Mol. Gen. Genet. 205:270-275.

Collinge, D. B., Milligan, D. E., Dow, J. M., Scofield, G., and Daniels M. J. 1987. Gene expression in *Brassica campestris* showing a hypersensitive response to the incompatible pathogen *Xanthomonas campestris* pv. *vitians*. Plant Mol. Biol. 8:405-414.

Conrads-Strauch, J., Dow, J. M., Milligan, D. E., Parra, R., and Daniels, M. J. 1990. Induction of hydrolytic enzymes in *Brassca campestris* in response to pathovars of *Xanthomonas campestris*. Plant Physiol. 93:238-243.

Daniels, M. J., Barber, C. E., Turner, P. C., Cleary, W. J., and Sawczyc, M. K. 1984a. Isolation of mutants of *Xanthomonas campestris* pv. *campestris* showing altered pathogenicity. J. Gen. Microbiol. 130:2447-2455.

Daniels, M. J., Barber, C. E., Turner, P. C., Sawczyc, M. K., Byrde, R. J. W., and Fielding, A. H. 1984b. Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad host range cosmid pLAFR1. EMBO J. 3:3323-3328.

De Vos, P., Goor, M., Gillis, M., and De Ley. 1985. Ribosomal ribonucleic acid cistron similarities of phytopathogenic *Pseudomonas* species. Int. J. Syst. Bacteriol. 35:169-184.

Dow, J. M., Clarke, B. R., Milligan, D. E., Tang, J.-L., and Daniels M. J. 1990. Extracellular proteases from *Xanthomonas campestris* pv. *campestris*, the black rot pathogen. Appl. Environ. Microbiol. 56:2994-2998.

Dow, J. M., Scofield, G., Trafford, K., Turner, P. C., and Daniels M. J. 1987. A gene cluster in *Xanthomonas campestris* pv. *campestris* required for pathogenicity controls the excretion of polygalacturonate lyase and other enzymes. Physiol. Mol. Plant Pathol. 31:261-271.

Friedman, A. M., Long, S. R., Brown, S. E., Buikema, W. J., and Ausubel, F. M. 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene 18:289-296.

Gough, C. L., Dow, J. M., Barber, C. E., and Daniels, M. J. 1988. Cloning of two endoglucanase genes of *Xanthomonas campestris* pv. *campestris*: Analysis of the role of the major endoglucanase in pathogenicity. Mol. Plant-Microbe Interact. 1:275-281.

Hamlin, B. T., Ng, F. M. -W., and Dawes, E. A. 1967. Regulation of enzymes of glucose metabolism in *Pseudomonas aeruginosa* by citrate. Pages 211-231 in Microbial physiology and continuous culture. London: H.M.S.O.

Huang, H. -C., Shuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel I., Hutcheson, S. W., and Collmer, A. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit hypersensitive response in tobacco plants. J. Bacteriol. 170:4748-4756.

Huang, Y., Xu, P., and Sequeira, L. 1990. A second cluster of genes that specify pathogenicity and host response in *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 3:48-53.

Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.

Kamoun, S., and Kado, C. I. 1990. A plant inducible gene of *Xanthomonas campestris* pv. *campestris* encodes an exocellular component required for growth in the host and hypersensitivity on nonhosts. J. Bacteriol. 172:5165-5172.

Klement, S. 1982. Hypersensitivity. Pages 149-177 in: Phytopathogenic Prokaryotes. Vol. 2. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.

Leong, S., Ditta, G., and Helinski, D. 1982. Heme biosynthesis in *Rhizobium*. J. Biol. Chem. 257:8724-8730.

Lessie, T. G., and Phibbs, P. V. 1984. Alternative pathways of carbohydrate utilization in Pseudomonads. Ann. Rev. Microbiol. 38:359-387.

Lindgren, P. B., Panopoulos, N. J., Staskawicz, B. J., and Dahlbeck, D. 1988. Genes required for pathogenicity and hypersensitivity are conserved and interchangeable among pathovars of *Pseudomonas syringae*. Mol. Gen. Genet. 21:499-506.

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

Maniatis, T., Fritsch, E., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Miller, J. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Minsavage, G. V., Dahlbeck, D., Whalen, M. C., Kearney, B., Bonas, U., Staskawicz, B. J., and Stall, R. E. 1990. Gene-for-gene relationships specifying disease resistance in *Xanthomonas campestris* pv. *vesicatoria*-pepper interactions. Mol. Plant-Microbe Interact. 3:41-47.

Osbourn, A. E., Clarke B. R., and Daniels, M. J. 1990. Identification and DNA sequence of a pathogenicity gene of *Xanthomonas campestris* pv. *campestris*. Mol. Plant-Microbe Interact. 3:280-285.

Prentki, P., and Krisch, H. M. 1984. In vitro insertional mutagenesis

with a selectable DNA fragment. Gene 29:303-313.
Rahme, L. G., Mindrinos, M. N., and Panopuolos, N. J. 1991. Genetic and transcriptional organization of the *hrp* cluster of *Pseudomonas syringae* pv. *phaseolicola*. J. Bacteriol. 173:575-586.
Roberts, I. N., Dow, J. M., Lum K. -Y., Scofield, G., Barber C. E., and Daniels M. J. 1987. Antiserum against *Xanthomonas* phytopathogen inhibits host-pathogen interaction in seedlings of *Brassica campestris*. FEMS Microbiol. Lett. 44:383-387.
Sawczyc, M. K., Barber, C. E., and Daniels, M. J. 1989. The role in pathogenicity of some related genes in *Xanthomonas campestris* pv. *campestris* and *translucens*: A shuttle strategy for cloning genes required for pathogenicity. Mol. Plant-Microbe Interact. 2:249-255.
Silhavy, T. J., Berman, M. L., and Enquist, L. W. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Simon, R., Quandt, J., and Klipp, W. 1989. New derivatives of transposon Tn*5* suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. Gene 80:161-169.
Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:5789-5794.
Tang, J. -L., Liu, Y. -N, Barber, C. E., Dow, J. M., Wootton, J. C., and Daniels, M. J. 1991. Genetic and molecular analysis of a cluster of *rpf* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. 226:409-417.
Tang, J. -L., Gough, C. L., and Daniels, M. J. 1990. Cloning of genes involved in negative regulation of production of extracellular enzymes and polysaccharide of *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. 222:157-160.
Tang, J. -L. 1989. Aspects of extracellular enzyme production by *Xanthomonas campestris*. Ph.D. thesis, University of East Anglia, Norwich, UK.
Tang, J. -L., Gough, C. L., Barber, C. E., Dow, J. M., and Daniels, M. J. 1987. Molecular cloning of protease gene(s) from *Xanthomonas campestris* pv. *campestris*: Expression in *Escherichia coli* and role in pathogenicity. Mol. Gen. Genet. 210:443-448.
Turner, P., Barber, C., and Daniels, M. 1984. Behaviour of the transposons Tn*5* and Tn*7* in *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. 195:101-107.
Turner, P., Barber, C., and Daniels, M. 1985. Evidence for clustered pathogenicity genes in *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. 199:338-343.
Whitfield, C., Sutherland, I. W., and Cripps, R. E. 1982. Glucose metabolism in *Xanthomonas campestris*. J. Gen. Microbiol. 128:981-985.