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Importance of *opgH*$_{Xcv}$ of *Xanthomonas campestris* pv. *vesicatoria* in Host-Parasite Interactions

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Tn*5* insertion mutants of *Xanthomonas campestris* pv. *vesicatoria* were inoculated into tomato and screened for reduced virulence. One mutant exhibited reduced aggressiveness and attenuated growth in planta. Southern blot analyses indicated that the mutant carried a single Tn*5* insertion not associated with previously cloned pathogenicity-related genes of *X. campestris* pv. *vesicatoria*. The wild-type phenotype of this mutant was restored by one recombinant plasmid (pOPG361) selected from a genomic library of *X. campestris* pv. *vesicatoria* 91-118. Tn*3-gus* insertion mutagenesis and sequence analyses of a subclone of pOPG361 identified a 1,929-bp open reading frame (ORF) essential for complementation of the mutants. The predicted protein encoded by this ORF was highly homologous to the previously reported pathogenicity-related HrpM protein of *Pseudomonas syringae* pv. *syringae* and OpgH of *Erwinia chrysanthemi*. Based on homology, the new locus was designated *opgH*$_{Xcv}$. Manipulation of the osmotic potential in the intercellular spaces of tomato leaves by addition of mannitol at low concentrations (25 to 50 mM) compensates for the *opgH*$_{Xcv}$ mutation.

Xanthomonas campestris pv. *vesicatoria* (Doidge) causes bacterial spot disease on tomato (*Lycopersicon esculentum* Mill.) and pepper (*Capsicum annuum* L.). Although several genetically and phenotypically distinct groups can be identified among strains of this diverse pathogen (Jones and Stall 1998), all share a common functional *hrp* gene cluster with associated regulatory genes required for both pathogenicity on susceptible host plants and induction of the hypersensitive response (HR) on resistant host and nonhost plants (Lindgren 1997). Genes within the *hrp* region are responsible for the type III secretion system (TTSS) of virulence "effector" proteins (Noël et al. 2002; Rossier et al. 1999) associated with growth of the bacteria within susceptible host tissue and the occurrence of disease. In addition to these essential pathogenicity-related genes, the avirulence gene *avrBs2* has been shown to modify virulence of this pathogen (Gassmann et al. 2000). Recent focus on the conservation of virulence determinants and pathogenicity systems shared by bacterial pathogens of plants and mammals has led to the identification of several new genetic loci involved in the disease process (Mahajan-Miklos et al. 1999; Rossier et al. 1999). Among these are genes involved in the synthesis of periplasmic glucans.

Oligosaccharides, as common constituents of the periplasm of gram-negative bacteria (Amemura and Cabrera-Crespo 1986; Bohin 2000; Schulman and Kennedy 1979), play a role in maintenance of cell equilibrium and osmotic adaptation (Miller et al. 1986; Stock et al. 1977). Four families of periplasmic glucans have been described among bacteria in and related to the family *Enterobacteriaceae* based on variable substitutions along either a linear or branched backbone of linked glucose units (Bohin 2000; Page et al. 2001). These membrane-derived oligosaccharides (MDO) are synthesized under strict osmotic regulation in *Escherichia coli* (Kennedy 1982; Lacroix et al. 1989; Loubens et al. 1993) with increased levels of production in media of low osmolarity. MDO synthesis, strongly stimulated in media of very low osmolarity, results in an increase in the osmolarity of the periplasm in order to maintain an appropriate osmotic gradient across the cytoplasmic membrane and a minimum threshold level of intracellular K^+ for normal enzyme function (Higgins et al. 1987). Periplasmic glucans analogous to MDO have been shown to play an important role in bacterium-plant interactions of the symbionts *Bradyrhizobium japonicum* (Bhagwat et al. 1999) and *Sinorhizobium meliloti* (Geremia et al. 1987) and of several pathogens including *Agrobacterium tumefaciens* (Puvanesarajah et al. 2001), *Pseudomonas syringae* (Mills and Mukhopadhyay 1990; Mukhopadhyay et al. 1988; Talaga et al. 1994), and *Erwinia chrysanthemi* (Cogez et al. 2001; Page et al. 2001).

Mutations within the *opgGH* operon of strains of *E. chrysanthemi* defective in osmoregulated periplasmic glucan synthesis resulted in a pleiotropic phenotype accompanied by a loss of virulence (Page et al. 2001). Mutant strains were unable to grow in planta. A similar loss of virulence and growth ability in planta was noted for an osmoregulated periplasmic glucan biosynthesis (OPG) mutant (*hrpM*) of *P. syringae* pv. *syringae* (Mukhopadhyay et al. 1988). The *hrpM* mutant also failed to elicit a nonhost HR when inoculated on tobacco. A transposon insertion mutant (36A4) in a gene homologous to *hrpM* was identified in the opportunistic pathogen *P. aeruginosa* PA14, based on altered virulence (Mahajan-Miklos et al. 1999). Interestingly, mutant 36A4 exhibited severely reduced pathogenicity in both plant (*Arabidopsis thaliana*) and animal (mouse and nematode *Caenorhabditis elegans*) model systems tested.

In this paper, we describe the cloning and molecular analysis of a genetic locus in *X. campestris* pv. *vesicatoria* translationally homologous to *hrpM* of *P. syringae* and *opgH* of *E. chrysanthemi*. We provide evidence that mutation of this homologue attenuates virulence and HR and is affected by alteration of the osmotic potential in the periplasmic spaces.

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Nucleotide sequence data reported here are available in the DDBJ/EMBL/GenBank databases under accession number AY248747.

RESULTS

Characterization of Tn*5* mutants.

We performed an *X. campestris* pv. *vesicatoria* mutagenesis screen to identify mutants with altered pathogenicity or virulence. Tn*5* mutants (3,000) were individually inoculated into tomato leaves, and disease symptoms were monitored for two weeks. Eleven mutants of *X. campestris* pv. *vesicatoria* 75-3 were selected as variants from the wild-type control, based on symptom phenotype. The mutants were purified for reinoculation tests and further characterized by physiological tests, population dynamics, and genetic complementation analyses.

Several mutants recovered were found to be auxotrophic (no growth on minimal M9 medium) or *hrp* mutants (i.e., were unable to multiply in planta and were complemented back to virulence by previously cloned *hrp* genes) and were excluded from further testing. One of the mutants (designated 75-3::Tn*5*-16, Table 1) was selected for further study because of its ability to multiply in planta (tomato) while exhibiting reduced symptoms to a level intermediate between that of the wild-type strain and *hrp* mutant 75-3::Tn*5*-1 (Fig. 1). Similar results were found when growth of 75-3 and 75-3::Tn*5*-16 were compared in the susceptible pepper cv. Early Calwonder (ECW) (data not shown). Growth rate of 75-3::Tn*5*-16 in nutrient broth and on nutrient agar medium was identical to that of the wild-type strain 75-3.

Cosmid clones (28) containing the Tn*5* insertion region were obtained from a genomic library of 75-3::Tn*5*-16 by selection for the kanamycin resistance conferred by the transposon. Several of these clones were used to generate homologous mutants in *X. campestris* pv. *vesicatoria* 91-118 (tomato race 3) by marker exchange. The 91-118 marker-exchange mutants exhibited the same altered virulence phenotype as the original 75-3::Tn*5*-16 mutant, confirming that this phenotype was attributed to the Tn*5* insertion in the cloned region. Tn*5* insertion and marker exchange mutations were confirmed by Southern hybridization analyses using labeled Tn*5* DNA as a probe (data not shown). One marker-exchange mutant (designated 91-118::Tn*5*-16) was used as a recipient in a preliminary cosmid library complementation screen.

Cloning of the $opgH_{Xcv}$ locus.

Cosmid clones (500) from a genomic library of the wild-type *X. campestris* pv. *vesicatoria* 91-118 were individually conjugated into mutant 91-118::Tn*5*-16 to recover one or more nonmutated genes essential for complementation of this mutant phenotype. After inoculations of transconjugants into tomato, one clone (designated pOPG361) was identified that restored 91-118::Tn*5*-16 to the wild-type phenotype. Subcloning of pOPG361 localized the active locus to a 3.1-kbp *Bam*HI-*Eco*RI DNA fragment obtained on a clone designated pOPG9. To confirm that the complementing gene was novel, we used labeled pOPG9 DNA to probe *X. campestris* pv. *vesicatoria* genomic DNA in Southern hybridization analysis. Labeled DNA of pOPG9 did not hybridize with DNA of the previously cloned *hrp*, *hrpXv*, *hrpG*, or *avrBs2* genes of *X. campestris* pv. *vesicatoria* (data not shown), suggesting that pOPG9 encoded a novel gene required for plant pathogenesis.

Table 1. Bacterial strains, plasmid vectors, and plasmid constructs used in this study

Designation	Relevant characteristics	Source or reference
X. campestris pv. *vesicatoria*		
75-3	Tomato race 1, pepper race 2, Rif^r	This study
91-118,91-118*	Tomato race 3, Rif^r	This study
XV157	Pepper race 6	This study
85*,85*Δ*hrcV*	Pepper strains	Wengelnik et al. 1999
75-3::Tn*5*-16	75-3 Tn*5* $opgH_{Xcv}$ mutant	This study
75-3::Tn*5*-1	75-3 Tn*5* *hrp* mutant	This study
91-118::Tn*5*-16	Marker exchange $opgH_{Xcv}$ mutants	This study
91-118Tn*3*-12		
85*::Tn*3*-6		
91-118Δ$opgH_{Xcv}$7	$opgH_{Xcv}$ deletion mutants	This study
91-118*Δ$opgH_{Xcv}$2		
Escherichia coli		
DH5α	F$^-$*recA*ϕ80d*lac*ZΔM15	BRL, Gaithersburg, MD, U.S.A.
C2110	Nal^r*polA*$^-$	Stachel et al. 1985
HB101	F$^-$*recA*	Maniatis et al. 1982
SM10	C600*recA*[*chr*::RP4-2-Tc::Mu]	Simon et al. 1983
DH5αλPIR	Host for pOK1	Huguet et al. 1998
Plasmids		
pLAFR3	Tc^r*rlx*$^+$RK2 replicon	Staskawicz et al., 1987
pBluescript II KS +/–	Phagemid sequencing vector, Ap^r	Stratagene, La Jolla, CA, U.S.A.
pSUP1011	PACYC184::Tn*5*ColE1, Cm^{r}Nm^r, Mob$^+$, suicide plasmid	Simon et al. 1983
pOK1	Suicide vector	Huguet et al. 1998
pRK2073	Sp^r Tra$^+$, helper plasmid	Daniels et al. 1984
pHoKmGUS	Km^{r}Ap^r*tnpA*$^-$, Tn3-*uidA* fusion	B. Staskawicz[z]
pSShe	Cm^r*tnpA*	Stachel et al. 1985
pXV9	*X. campestris* pv. *vesicatoria hrp* clone	Bonas et al. 1991
p81538	*X. campestris* pv. *vesicatoria avrBs2* clone	Swords et al. 1996
pSX2	*X. campestris* pv. *vesicatoria hrpXv* clone	Wengelnik and Bonas 1996
pSG72	*X. campestris* pv. *vesicatoria hrpG* clone	Wengelnik et al. 1996
pOPG361	91-118 pLAFR3 cosmid library clone (27-kbp *X. campestris* pv. *vesicatoria* DNA insert)	This study
pOPG9	3.1-kbp *Eco*R1-*Bam*HI subclone of pOPG361 in pLAFR3	This study
pBOPG9	pBluescript containing the insert from pOBG9 for sequencing	This study
pT3-5::249	*avrXv3*::*uidA* fusion	Astua-Monge et al. 2000
pPG1	*hrpG::uidA* fusion	Wengelnik et al. 1996
pL3::GUS	pLAFR3 *lacZ::uidA* fusion	B. Staskawicz
pDD62	NPT II marker	Mudgett et al. 2000

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DNA sequence analysis.

The 3.1-kbp *Bam*HI-*Eco*RI DNA insert fragment of pOPG9 was moved into pBluescript II K/S (designated pBOPG9), and the nucleotide sequence was obtained. A single 1,929-bp open reading frame (ORF) coding for a 643-amino acid protein with a predicted molecular mass of 70 kDa was identified. A BLASTP 2.0.6 GenBank search identified a significant homology match with the previously sequenced OPG proteins of *Xanthomonas axonopodis* pv. citri 306 (96% identical), *Xanthomonas campestris* pv. campestris 3515 (90% identical), *Xylella fastidiosa* 9a5c (77% identical, 85% similar), MdoH of *Vibrio cholerae* group O1 N16961 (42% identical, 59% similar), the pathogenicity-related HrpM protein of *Pseudomonas syringae* pv. *syringae* (39% identical, 53% similar), and OpgH of *E. chrysanthemi* (36% identical, 51% similar). Based on the homology data, the new locus was designated $opgH_{Xcv}$. Sequence data was submitted to the DDBJ/EMBL/GenBank nucleotide sequence data library under accession number AY248747.

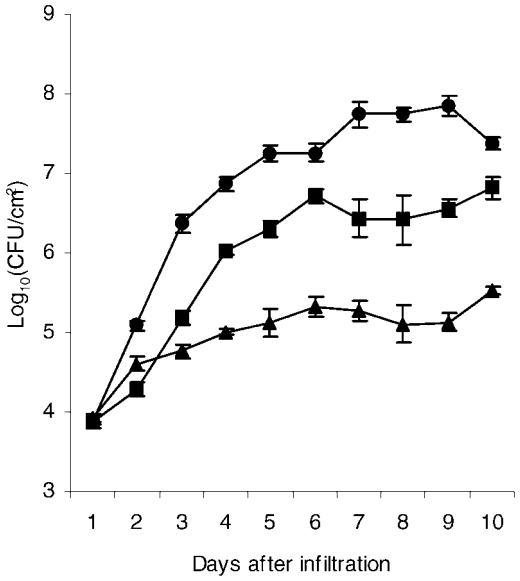

Fig. 1. Growth of *Xanthomonas campestris* pv. *vesicatoria* 75-3 (wild-type, ●), $opgH_{Xcv}$ Tn*5* mutant 75-3::Tn*5*-16 (■), and *hrp* Tn*5* mutant 75-3::Tn*5*-1 (▲) in tomato cv. Bonny Best.

Generation of *X. campestris* pv. *vesicatoria* mutant derivatives.

Clone pOPG9 was subjected to Tn*3*-*gus* mutagenesis and insertion mutants unable to complement mutant 91-118::Tn*5*-16 were used to generate marker exchange mutants in *X. campestris* pv. *vesicatoria* 91-118 and 85* (designated 91-118::Tn*3*-12 and 85*::Tn*3*-6, respectively). Strain 91-118::Tn*3*-12 was selected for use in population dynamics, electrolyte leakage, and greenhouse disease assessment tests. Strain 91-118::Tn*3*-12 was identical to the wild-type strain 91-118 in all biochemical and physiological test comparisons made, including salt sensitivity, starch hydrolysis, pectate degradation, bacteriocin production, phage sensitivity, and fatty acid profile. Strains 85* and 85*::Tn*3*-6 were generated for use in the AvrBs2 secretion and protein immunoblot experiments.

The importance of $opgH_{Xcv}$ was examined in more detail when more precise mutations of the gene were generated in *X. campestris* pv. *vesicatoria* strains. The $opgH_{Xcv}$ deletion plasmid constructed in the suicide vector pOK1 was used to generate genomic substitutions in *X. campestris* pv. *Vesicatoria* 91-118 and 91-118*. These suicide mutants (designated 91-118$\Delta opgH_{Xcv}$7 and 91-118*$\Delta opgH_{Xcv}$2, respectively) exhibited altered virulence as the original Tn*5* mutants and were fully complemented by pOPG9, confirming that the $opgH_{Xcv}$ mutation was nonpolar. The deletions in the strains were confirmed by Southern hybridization and polymerase chain reaction (PCR) before they were used in some plant assays.

Plant assays.

Wild-type *X. campestris* pv. *vesicatoria* 91-118 is pathogenic (virulent) to tomato cvs. FL 7060 and Bonny Best and induces a hypersensitive response (HR) on tomato cv. near-isogenic line (NIL) 216 and pepper cv. ECW (avirulent HR reaction due to the *X. campestris* pv. *vesicatoria avrXv3* gene). The parental *X. campestris* pv. *vesicatoria* 75-3 used in this paper was selected for virulence on pepper ECW (absence of *avrBsT* due to spontaneous loss of plasmid) before the Tn*5* mutagenesis experiment. Therefore, *X. campestris* pv. *vesicatoria* 75-3 is virulent on tomato cvs. FL 7060 and Bonny Best and pepper ECW. *X. campestris* pv. *vesicatoria* 75-3 induces an HR on pepper NIL ECW20R (avirulent HR reaction due to the *X. campestris* pv. *vesicatoria avrBs2* gene).

In repeated tests, *X. campestris* pv. *vesicatoria* $opgH_{Xcv}$ mutants consistently multiplied in tomato leaves to a level 1 to 1.5 log units lower than the complemented and wild-type

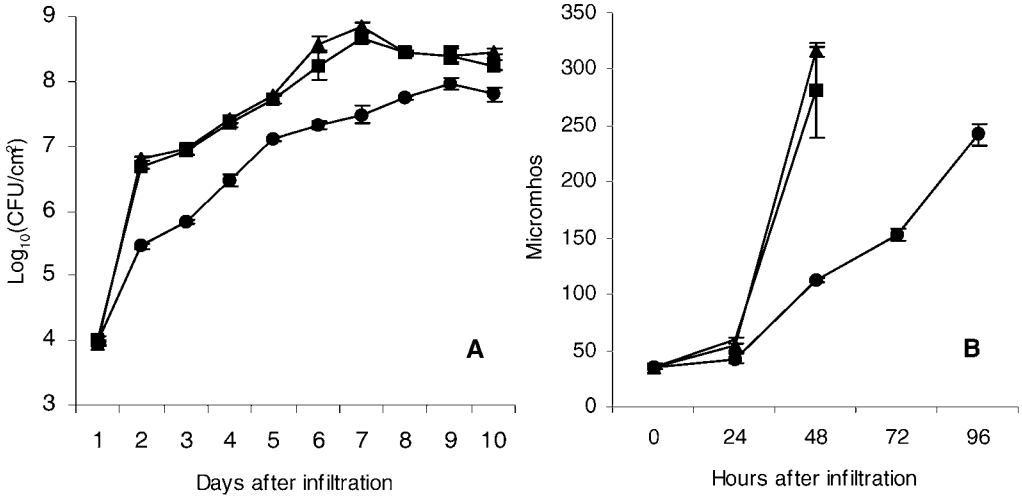

Fig. 2. A, Growth of *Xanthomonas campestris* pv. *vesicatoria* 91-118 (wild-type, ▲), $opgH_{Xcv}$ marker exchange mutant 91-118::Tn*3*-12 (●), and transconjugant 91-118::Tn*3*-12(pOPG9) (complement, ■) in susceptible tomato cv. FL 7060. **B,** Electrolyte leakage induced by the same strains in susceptible tomato cv. FL 7060.

strains (Fig. 2). Further evidence that both virulence and HR-inducing ability were affected in the $opgH_{Xcv}$ mutants was obtained in electrolyte leakage experiments. In electrolyte leakage tests with tomato and pepper, a delay in both the susceptible and HR reactions was caused by the $opgH_{Xcv}$ mutants when compared with the complemented and wild-type strains (Figs. 2 and 3). In these experiments, the HR caused by the avirulence gene *avrXv3* was delayed in both pepper ECW and tomato NIL 216 (data not shown). A delay was noted in the HR response of the mutant strains in pepper cv. NIL ECW20R plants induced by the interaction of *avrBs2* and the *Bs2* resistance gene. On ECW20R a significant reduction was noted for both growth of the $opgH_{Xcv}$ mutant and amount of electrolyte leakage caused by this strain, when compared with its complement (Fig. 3).

Pathogenicity of 91-118, 91-118::Tn*3*-12, and 91-118::Tn*3*-12(pOPG9) was also compared on dip-inoculated tomato plants incubated in the greenhouse. In these tests, disease severity ratings were made 14 to 21 days after inoculation. 91-118::Tn*3*-12 caused significantly less disease on both stems and leaves, when compared with the complement and the wild type (Table 2). When compared with the complement and wild type, lesion size for the mutant was reduced in both stems and leaflets (Fig. 4), although there was no significant difference in lesion numbers (Table 2). These plant phenotypes clearly revealed that the *X. campestris* pv. *vesicatoria* $opgH_{Xcv}$ mutants are less virulent in susceptible plants and are impaired in their ability to induce a defense response in resistant plants.

Effect of osmotic pressure.

Based on previous literature concerned with the osmoregulation of genes involved in periplasmic glucan biosynthesis, we tested to determine if the $opgH_{Xcv}$ mutant phenotype could be overcome by changing the osmotic conditions within inoculated tomato leaves. Tomato cuttings were incubated in water containing various concentrations of mannitol, a solute not metabolized by either the plant host or *X. campestris* pv. *vesicatoria* (data not shown), to alter the osmotic pressure (OP) of the tissue to a point where a functional $opgH_{Xcv}$ gene was nonessential for occurrence of a normal disease response. Results of preliminary tomato cutting inoculations indicated that the $opgH_{Xcv}$ mutant phenotype could be overcome by increasing the OP of the solution in which the cuttings were incubated. Partial complementation of the *X. campestris* pv. *vesicatoria* mutant 91-118::Tn*3*-12 was evident at a mannitol concentration of 25 mM, and the mutant became indistinguishable from the wild-type strain as the mannitol concentration was adjusted to 50 mM (data not shown). Similar results were obtained using mutant 91-118Δ$opgH_{Xcv}$7 in electrolyte leakage tests. Mutant and wild-type leakage curves became indistinguishable at a mannitol concentration of 50 mM, and growth of the mutants was also increased (Fig. 5). No significant release of electrolytes due to mannitol alone was detected in noninoculated leaflets (data not shown), although increase of mannitol concentrations to greater than 50 mM was not useful in our tests, due to damage to the leaf tissue caused by the solute over extended incubations.

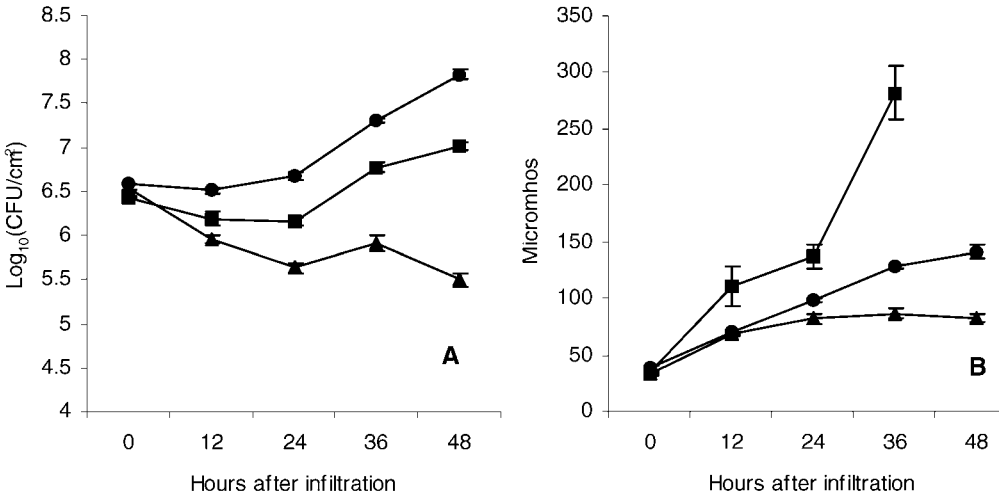

Fig. 3. A, Growth of *Xanthomonas campestris* pv. *vesicatoria* 91-118Δ$opgH_{Xcv}$7 (▲), 91-118Δ$opgH_{Xcv}$7 (pOPG9) (■), and XV157 (●) in resistant pepper cv. near-isogenic line (NIL) ECW20R. **B,** Electrolyte leakage induced by the same strains in pepper cv. NIL ECW20R. *X. campestris* pv. *vesicatoria* XV157 was included in this test as compatible (virulent) strain control.

Table 2. Comparison of lesion development and disease severity[x]

	Leaf lesion ratings[y]			Stem lesion ratings		
Strain[z]	**Number**	**Size**	**Severity**	**Number**	**Size**	**Severity**
ME	23.71 a	0.14 a	1.93 a	12.14 a	0.43 a	1.57 a
ME-C	23.00 a	0.28 b	2.98 b	15.71 a	1.25 b	3.57 b
WT	25.71 a	0.24 b	3.16 b	15.00 a	1.54 c	3.86 b

[x] On tomato cv. FL 7060 dip-inoculated with mutant, complemented, and wild-type tomato race 3 strains of *Xanthomonas campestris* pv. *vesicatoria* in the greenhouse.

[y] Lesion values are the average of one lateral leaflet or stem (for number per square cm), ten lesions (for size in mm), and three leaves (for severity) from every plant per treatment. Severity ratings were made on a scale of 0 to 5 with 0 = no lesions and 1 to 5 ranging from very few or small lesions to large expanded or coalesced lesions. Different letters following means in table indicate significant differences ($p = 0.05$) in Duncan's multiple range test.

[z] ME = marker exchange mutant 91-118::Tn*3*-12, ME-C = transconjugant 91-118::Tn*3*-12(pOPG9), and WT = *X. campestris* pv. *vesicatoria* tomato race 3 strain 91-118.

Estimates of the osmotic potentials for tissue sap collected from leaf cuttings incubated in the 25 and 50 mM mannitol solutions (–14.12 ± 0.01 and –14.99 ± 0.15 Bars, respectively) were higher than those of the sap from cuttings incubated in water (–12.71 ± 0.07 Bars) or leaves collected from an intact tomato plant (–13.09 ± 0.02 Bars).

Effect of *opgH*$_{Xcv}$ mutation on the expression of *avrXv3* and *hrpG*.

We hypothesized that the *X. campestris* pv. *vesicatoria* *opgH*$_{Xcv}$ mutants may induce a delayed HR response in planta if the expression or secretion of an avirulence protein is affected. To investigate this, we measured the expression of *avrXv3* in the *X. campestris* pv. *vesicatoria* mutants 75-3::Tn*5*-16, 91-118Δ*opgH*$_{Xcv}$7, and 91-118*Δ*opgH*$_{Xcv}$2 and compared it with that of the respective parental strain. Pepper leaves were inoculated with transconjugants carrying the reporter plasmid pT3-5::249 (*avrXv3::uidA*). The expression of *avrXv3* was measured 16 h postinoculation by quantifying β-glucuronidase (GUS) activity. The overall expression of *avrXv3* in the mutants was two- to threefold lower compared with their respective parental strains (Table 3). However, the levels of GUS activity were not significantly different when normalized for population growth differences between the strains. Similarly, no differences were found in the levels of constitutive expression of *uidA* in the wild-type and mutant strains (data not shown).

Interestingly, GUS activity and growth of strain 91-118*Δ*opgH*$_{Xcv}$2(pT3-5::249) possessing the *hrpG** mutation (mutation in the regulatory gene *hrpG* leads to constitutive expression of the Hrp TTSS) was similar to that in strain 91-118(pT3-5::249). This suggests that the *hrpG** mutation compensates for the mutation in *opgH*$_{Xcv}$. The *opgH*$_{Xcv}$ mutant phenotype was similarly compensated when 91-118*Δ*opgH*$_{Xcv}$2 and 91-118 strains were compared for HR timing and phenotype intensity in planta (data not shown). No difference was found in the levels of GUS activity for the pPG1 transconjugants of these two strains (Table 3).

Fig. 4. Leaflets of tomato cv. FL 7060 two weeks after dip-inoculation with *Xanthomonas campestris* pv. *vesicatoria* *opgH*$_{Xcv}$ mutant 91-118::Tn*3*-12 (left), transconjugant 91-118::Tn*3*-12(pOPG9) (center), and wild-type 91-118 (right).

Secretion of AvrBs2.

We next tested if the protein expression and secretion of chromosomally encoded *avrBs2* was affected by mutations in *opgH*$_{Xcv}$. Considering that secretion from wild-type *X. campestris* pv. *vesicatoria* cells is difficult to detect, we used *X. campestris* pv. *vesicatoria* 85*, which contains a chromosomal copy of *avrBs2* and the *hrpG** mutation. An *opgH*$_{Xcv}$ mutation was introduced into *X. campestris* pv. *vesicatoria* 85* by marker exchange, creating the strain 85*::Tn*3*-6. We also used *X. campestris* pv. *vesicatoria* 85*Δ*hrcV*, a secretion defective strain, as a control to test the *hrp*-dependent secretion of

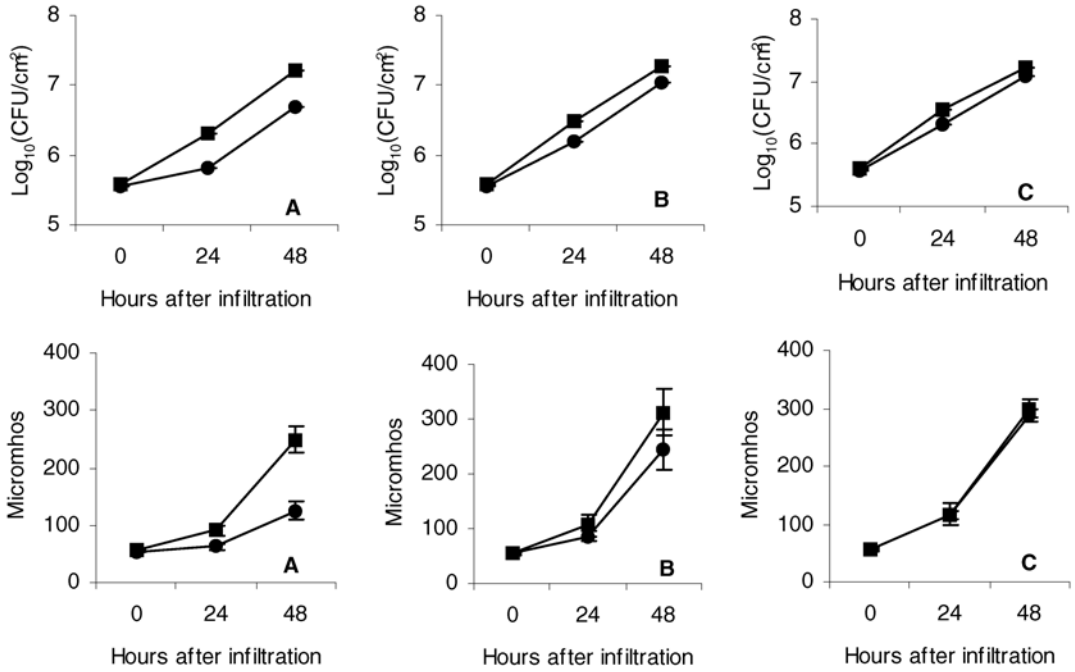

Fig. 5. Growth of bacteria and electrolyte leakage in cuttings of tomato cv. Bonny Best incubated in **A,** sterile deionized water or deionized water containing mannitol **B,** at 25 mM and **C,** 50 mM at 25°C. Cuttings were inoculated with *Xanthomonas campestris* pv. *vesicatoria* 91-118Δ*opgH*$_{Xcv}$7 (●) and 91-118Δ*opgH*$_{Xcv}$7(pOPG9) (■).

AvrBs2. Conditions previously established to assay *X. campestris* pv. *vesicatoria* protein secretion were used to detect AvrBs2 secretion. Immunoblot analysis showed that AvrBs2 protein was similarly expressed in cellular lysates from *X. campestris* pv. *vesicatoria* 85*, 85*Δ*hrcV*, and 85*::Tn*3*-6 (Fig. 6) grown in pH 7 (noninducing) and pH 5.4 (secretion inducing) media. Moreover, secretion of AvrBs2 in vitro was not impaired in *X. campestris* pv. *vesicatoria* 85*::Tn*3*-6. In fact, slightly more AvrBs2 protein was secreted in the *opgHXcv* mutant strain. Increased AvrBs2 secretion could be triggered in strains tested when an increasing concentration of mannitol (5 or 25 mM) was added to the pH 5.4 inducing media (Fig. 6). We confirmed that AvrBs2 protein in the concentrated culture fluids was not due to cell lysis by reprobing the immunoblots with antisera for NPT II, a cytoplasmic protein encoded by pDD62 carried by these strains (data not shown). These experiments indicate that the $opgH_{Xcv}$ mutation in the *X. campestris* pv. *vesicatoria* 85* background does not significantly reduce AvrBs2 synthesis or in vitro secretion. It also suggests that osmotically stabilized *X. campestris* pv. *vesicatoria* cells secrete type III effector proteins more efficiently.

Total protein profiles.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of strains 91-118 and 91-118Δ$opgH_{Xcv}$7 grown in media containing NaCl or mannitol were compared to evaluate the effects of solute and concentration on protein synthesis. Addition of NaCl at 150 or 300 mM to the nutrient broth caused a change in profiles of both the wild-type and mutant strain. Loss of a large protein band (approximately 90 kDa) and a shift in the relative production of two smaller proteins near 26 kDa was noted in the profiles for both strains (Fig. 7). Addition of mannitol to the broth resulted in no observable profile changes for either strain.

DISCUSSION

Our experiments implicate the involvement of another genetic locus in *Xanthomonas campestris* pv. *vesicatoria*, unrelated to previously identified pathogenicity-related genes, in both the virulent and avirulent host reactions of this pathogen. Mutations in this locus, designated $opgH_{Xcv}$, resulted in attenuation of growth of *X. campestris* pv. *vesicatoria* in susceptible host tissue and a significant reduction in disease severity. A delay in the avirulent HR phenotype was also noted.

The predicted *X. campestris* pv. *vesicatoria* $OpgH_{Xcv}$ protein encoded by this locus showed homology to the HrpM protein, which was originally associated with a nonpathogenic mutant phenotype in *Pseudomonas syringae* pv. *syringae* (Mukhopadhyay et al. 1988). The *hrpM* locus is conserved in many pathovars of *P. syringae* and HrpM is thought to be functionally homologous to MdoH of the *Escherichia coli* MdoGH operon (Loubens et al. 1993). MdoH is essential for the synthesis of MDO. MDO are soluble periplasmic glucans found in various other gram-negative bacteria and have been shown to play a role in bacteria-plant interactions in *Agrobacterium* and *Rhizobium* species (Bhagwat et al. 1999; Geremia et al. 1987; Puvanesarajah et al. 1985). A report concerned with the *opgGH* (OPG) operon of *E. chrysanthemi* (Page et al. 2001) indicated that loss of virulence attributed to mutations in this region were also associated with a pleiotropic phenotype. It is interesting to note that *X. campestris* pv. *vesicatoria* mutants in the $opgH_{Xcv}$ locus were identical to the wild-type strains in terms of all physiological and biochemical tests.

The phenotypes of *X. campestris* pv. *vesicatoria* $opgH_{Xcv}$ mutants generated in this study resembled those reported recently in *P. aeruginosa* PA14. A transposon insertion mutant (36A4) in a gene homologous to *hrpM* exhibited severely reduced pathogenicity in both plant and animal model systems (Mahajan-Miklos et al. 1999). A reduction in virulence in this case, as compared with a complete loss of pathogenicity as reported for *Pseudomonas* and *Erwinia* spp., may indicate the presence of at least two different closely related systems involved in periplasmic glucan production. Analysis of sequence data of *X. campestris* pv. *vesicatoria* clone pBOPG9 indicated that no other genes related to the *hrpM* or *opgGH* operons are in close proximity to $opgH_{Xcv}$. Partial reduction of virulence in *X. campestris* pv. *vesicatoria* may indicate that other genes involved in the periplasmic glucan production may be expressed from other loci in the genome of this pathogen. A search of the genome sequences available for *X. axonopodis* pv. *citri* and *X. campestris* pv. *campestris* indicated the presence a second genetic locus homologous to *mdoG* (*X. campestris* pv. *vesicatoria* $opgG_{Xcv}$ homolog) located separate from *mdoH*. Recent findings with *X. axonopodis* pv. *citri* in vitro expression profiling have shown that the *mdoG* and *mdoH* genes in this organism are independently upregulated or repressed, respectively, in the *hrp*-inducing medium XVM2

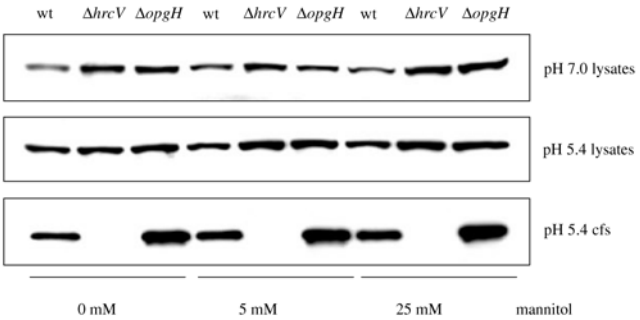

Fig. 6. Expression and secretion of AvrBs2 by *Xanthomonas campestris* pv. *vesicatoria*. Immunoblot analysis of total cell lysate proteins and cell-free supernatants (cfs) of strains 85* (wild type), 85*Δ*hrcV* (Δ*hrcV*), and 85*$opgH_{Xcv}$ mutant 85*::Tn*3*-6 (Δ*opgH*) under secreting (pH 5.4) and nonsecreting (pH 7.0) conditions.

Table 3. Expression of *avrXv3* and *hrpG* in pepper leaves[w]

X. campestris pv. *vesicatoria* strain	Fluorescence units[x]	Cell population[y]	GUS activity[z]
91-118Δ$opgH_{Xcv}$7(pT3-5::249)	0.032 ± 0.001	5.80 ± 0.08	5.3 ± 0.9
91-118(pT3-5::249)	0.072 ± 0.005	6.16 ± 0.14	5.0 ± 0.4
91-118*Δ$opgH_{Xcv}$2(pT3-5::249)	0.114 ± 0.012	6.40 ± 0.04	4.7 ± 0.8
91-118*(pT3-5::249)	0.200 ± 0.013	6.54 ± 0.01	5.7 ± 0.3
91-118Δ$opgH_{Xcv}$7(pPG1)	0.072 ± 0.001	6.49 ± 0.04	2.9 ± 0.2
91-118(pPG1)	0.072 ± 0.002	6.58 ± 0.05	2.6 ± 0.2

[w] Measured 16 h after inoculation with *Xanthomonas campestris* pv. *vesicatoria* transconjugants carrying pT3-5::249 or pPG1, respectively.

[x] Average change in fluorescence units per minute.

[y] Average log CFU per square cm of inoculated leaf area.

[z] Average level of β-glucuronidase (GUS) activity normalized for cell numbers and leaf area sampled, reported as U/10^9 CFU.

(Astua-Monge et al. 2003; and G. Astua-Monge, *personal communication*).

Synthesis of periplasmic glucans in *E. coli* and *P. syringae* has been demonstrated to be inversely correlated to the osmolarity of the growth medium of the cells (Talaga et al. 1994) and implicates a possible role for these compounds in the physiological ecology of these bacteria. Our experiments concerned with adjustment of OP of tomato cuttings provide evidence that $opgH_{Xcv}$ is somehow involved in osmolarity phenomena in association with disease development caused by *X. campestris* pv. *vesicatoria*. Since cell membranes are impermeable to mannitol, an increase in the OP of the substrate surrounding the plant cells would result in a decrease in the internal turgor pressure of the cells (Langridge 1958) and could affect function of the *hrp* secretory system essential for normal disease development. Our experiments on expression of *avrXv3* and secretion of AvrBs2 indicated that the effect of the $opgH_{Xcv}$ mutation (i.e. reduced virulence and delayed HR) may be due to altered environmental sensing and gene induction rather than a defect in the *hrp* secretory mechanism. However, the effect of this gene on translocation of effector or pathogenicity-related proteins via the TTSS requires further, more careful analysis beyond the scope of this study.

An interesting observation was the interaction between the $opgH_{Xcv}$ and *hrpG* in combined mutants. The *hrpG** mutation was able to overcome the mutant phenotype caused by deletion of $opgH_{Xcv}$ in *X. campestris* pv. *vesicatoria* 91-118, although the effect of the mutation was still evident in strain 91-118*Δ$opgH_{Xcv}$2 as a reduction in the intensity of the *avrXv3*-induced HR compared with that of strain 91-118*. The exact relationship between *hrpG* and $opgH_{Xcv}$ is of particular interest because, although the *hrpG** mutation can compensate for the $opgH_{Xcv}$ mutation, expression of this gene was not identified as one of the genes controlled by the *hrpG* regulon in cDNA-amplification fragment length polymorphism analysis (Noël et al. 2001). However, HrpG belongs to the subgroup of response regulator proteins of enteric bacterial two-component systems that includes OmpR (Wengelnik et al. 1996). Extensive studies on the EnvZ ('sensor') and OmpR ('regulator') pair of regulatory proteins in *Salmonella typhimurium* have demonstrated their sensitivity to osmolarity and subsequent effects on OmpC and OmpF porin protein expression and virulence of this pathogen (Graeme-Cook et al. 1989). Differential synthesis of outer membrane proteins of *Escherichia coli* was also shown to be caused by changes in the osmotic strength of growth media supplemented with various carbohydrate solutes, including mannitol (Kawaji et al. 1979). In our in vitro experiments, addition of high concentrations of NaCl to the growth medium resulted in altered protein synthesis that was not observed when mannitol was added as solute at the same concentrations. These results suggest that the solutes were perceived differently by the *X. campestris* pv. *vesicatoria* strains. The complementation effect of adding mannitol to our plant inoculations could be due either to a shift in bacterial protein synthesis not detected under our SDS-PAGE conditions, alteration of protein function, or other unknown factors critical in the pathogen-host interaction (in planta).

Further experiments are needed to assess the relationship of osmotic potential on disease development caused by the $opgH_{Xcv}$ mutant and wild-type *X. campestris* pv. *vesicatoria* strains. Localization of other essential pathogenicity-related genes needs to be investigated by further sequential mutation experiments. Specifically, the importance of mutation of the *X. campestris* pv. *vesicatoria* $opgG_{Xcv}$ alone and in combination with mutation of $opgH_{Xcv}$ would be a particular interest in future experiments.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions.

Strains of *X. campestris* pv. *vesicatoria* were grown at 28°C in Difco nutrient broth (Becton, Dickinson and Company,

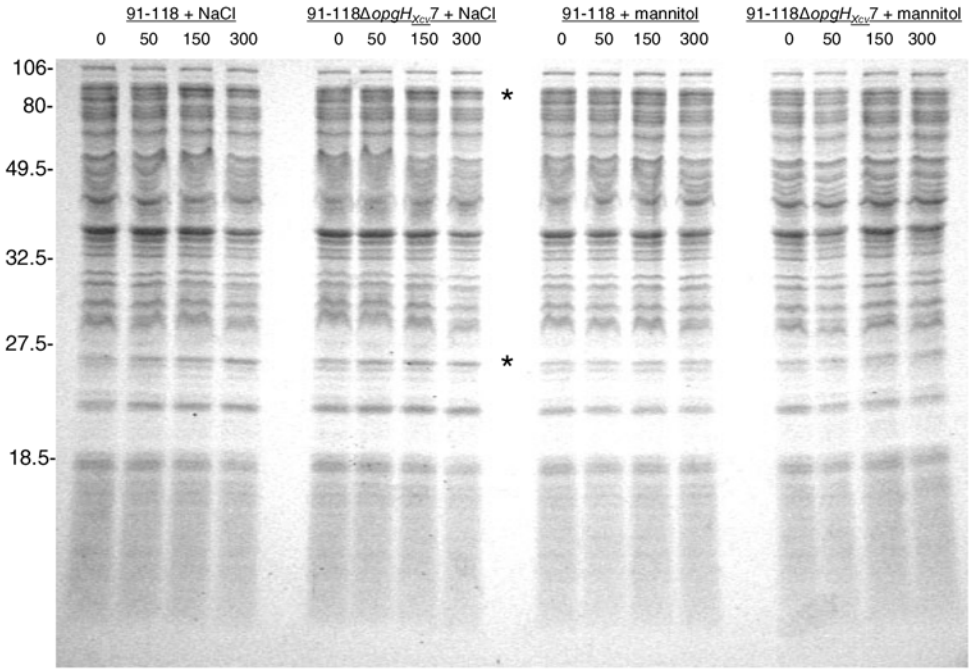

Fig. 7. Effect of osmolarity of growth medium on sodium dodecyl sulfate-polyacrylamide gel electrophoresis total protein profiles of *Xanthomonas campestris* pv. *vesicatoria* 91-118 and 91-118Δ$opgH_{Xcv}$7. Bacteria were grown in nutrient broth or nutrient broth containing NaCl or mannitol at 50, 150, or 300 mM final concentration. Regions of profile variations are noted by asterisks (*). Migration and size of protein molecular mass standards are indicated in kDa.

Sparks, MD, U.S.A.) or M9 medium (Maniatis et al. 1989). *Escherichia coli* was grown at 37°C in Luria-Bertani (LB) broth (Maniatis et al. 1989). Solid media were obtained by adding BD Bacto agar to broth at 15 g/liter. Triparental conjugations were performed on nutrient yeast extract glycerol agar (Daniels et al. 1984).

Antibiotics were used at the following concentrations (µg/ml): rifamycin SV, 100; tetracycline, 12.5; ampicillin, 100; spectinomycin, 50; chloramphenicol, 34; nalidixic acid, 50; and kanamycin, 25 for plasmids and 50 for genomic mutants.

Plant material and plant inoculations.

Seeds of tomato (*Lycopersicon esculentum*) cvs. Bonny Best, FL 7060 and its NIL 216 containing the *Xv3* resistance gene, and pepper (*Capsicum annuum*) cv. ECW and its NIL ECW20R containing the *Bs2* resistance gene were planted in Plugmix (W. R. Grace & Co., Cambridge, MA, U.S.A.). After 2 weeks, the emerged seedlings were transferred to Metromix 300 (W. R. Grace & Co.) in 10-cm plastic pots. Seedlings were grown in the greenhouse at temperatures ranging from 25 to 35°C (night and day, respectively).

Bacterial cultures for plant inoculations were grown in nutrient broth for 18 h at 28°C with shaking (100 rpm). Cells were pelleted by centrifugation (4,000 × *g*, 15 min) and resuspended in sterile tap water. Bacterial suspensions were standardized to an optical density at 600 nm (OD_{600}) = 0.3 (3×10^8 CFU/ml) with a Spectronic 20 spectrophotometer (Spectronic-UNICAM, Rochester, NY, U.S.A.) and subsequently diluted in sterile tap water to appropriate cell densities for individual experiments.

Transposon mutagenesis.

Transposon Tn*5* (Rothstein et al. 1981) was introduced into *X. campestris* pv. *vesicatoria* 75-3 (tomato race 1) by conjugation with *Escherichia coli* SM10 carrying the suicide plasmid pSUP1011. In order to identify mutants affected in pathogenicity and virulence, tap water suspensions of 3,000 transposon mutants at a concentration of 3×10^8 CFU/ml were individually inoculated into leaflets on four- to five-week old tomato plants (cv. Bonny Best) by infiltration, using a syringe fitted with a hypodermic needle as previously described (Hibberd et al. 1987). Inoculated plants were maintained in the greenhouse and were observed for symptom development for up to two weeks.

Mutants selected from the preliminary inoculation tests were further tested for auxotrophy by growth on minimal M9 medium. These strains were also subjected to several biochemical and physiological tests according to standard protocols to assure they were *X. campestris* pv. *vesicatoria* (Schaad et al. 2001). Substrate utilization was tested with Biolog GN2 plates, according to the manufacturer's instructions (Biolog, Hayward, CA, U.S.A.). The mutants altered in pathogenicity were also tested for complementation using previously cloned *hrp* genes (Bonas et al. 1991).

Recombinant DNA techniques.

Standard procedures were performed for genomic (Ausubel et al. 1992) and plasmid (Maniatis et al. 1989) DNA extraction. Restriction enzymes, T4 DNA ligase, and Taq DNA polymerase (Promega Corp., Madison, WI, U.S.A.) were used, according to the manufacturer's recommendations. PCR were performed in a DNA thermocycler (M. J. Research, Watertown, MA, U.S.A.). For Southern hybridizations, DNA probes were labeled and detected using the Genius nonradioactive DNA labeling and detection kit (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.). Target DNA was immobilized on Nytran membranes (Shleicher and Schuell Inc., Keene, NH, U.S.A.).

Total genomic libraries of *X. campestris* pv. *vesicatoria* wild-type and mutant strains were constructed in *Escherichia coli* using the cosmid vector pLAFR3, as previously described (Minsavage et al. 1990; Staskawicz et al. 1987). Individual clones were mobilized into *X. campestris* pv. *vesicatoria* recipient strains by triparental mating, as previously described (Ditta et al. 1980; Figurski and Helinski 1979).

Insertion mutagenesis and marker exchange.

Insertion mutagenesis of pLAFR3 clones was performed using Tn*3-gus*, as previously described (Bonas et al. 1989). *Escherichia coli* HB101(pHoKmGus, pSShe) was transformed with the plasmid to be mutated. Transformants were then mated with *Escherichia coli* C2110, using pRK2073 as a helper plasmid. Transconjugants containing insertionally mutated plasmids were selected by plating onto LB media containing nalidixic acid, tetracycline, and kanamycin. Insertional derivatives were analyzed by restriction enzyme digestion profiles.

Select clones were used to generate marker exchange mutants in *X. campestris* pv. *vesicatoria* wild-type strains. Derivative plasmids were triparentally conjugated into *X. campestris* pv. *vesicatoria* recipient strains for mutation. Transconjugants were transferred daily for at least 10 days on nutrient agar plates containing kanamycin. At the end of the cycling period, the bacterial cells from each mating were dilution-plated onto nutrient agar plates containing kanamycin and NaH_2PO_4 (10 g/liter) to enrich for tetracycline-sensitive colonies. Confirmation of marker exchange was accomplished by Southern hybridization. In addition, marker exchange strains were tested for pleiotropic mutations, using established techniques (Schaad et al. 2001).

DNA sequence analysis.

Sequencing of pBluescript clone pBOPG9 was initiated using vector forward and reverse primers at the ICBR sequencing facility (University of Florida, Gainesville, FL, U.S.A.) with the Applied Biosystems model 373 system (Foster City, CA, U.S.A.). To complete sequencing of both strands of DNA, custom primers were synthesized at the ICBR facility with an Applied Biosystems model 394 DNA synthesizer. The computer program SeqAid II version 3.81 was used to analyze nucleotide sequence data and predicted protein products. A search for nucleotide and amino acid sequence homology was conducted with the BLAST 2.0 algorithm (Altschul et al. 1997).

Generation of deletion mutants.

Based on DNA sequence analyses, custom primers were designed to facilitate a clean in-frame deletion of the $opgH_{Xcv}$ ORF from pBOPG9. Briefly, divergent primers opgL (5′-GGTTCTTCGAACATCAGGGCTGCCCGTCG-3′) and opgR (5′-GGTCCTTCGAATGATCTGAGTGTGCGTCG-3′) were used to amplify pBOPG9 and the up- and downstream regions of DNA flanking $opgH_{Xcv}$, while simultaneously adding a unique *Csp*45I restriction site (underlined in the primer sequences), used to recircularize the construct. The deleted region was excised from the pBluescript clone with the flanking polylinker restriction sites *Apa*I and *Bam*HI, for ligation into the suicide vector pOK1 linearized with the same two enzymes. The mutated sequence was introduced into the genome of *X. campestris* pv. *vesicatoria* by homologous recombination in two steps, as previously described (Huguet et al. 1998). Southern analysis and sequencing of PCR products were used to confirm that the correct deletion had occurred.

Plant assays.

To study growth of bacteria in planta, sterile tap water suspensions containing 3×10^5 CFU/ml of the wild-type, mutant,

or complemented *X. campestris* pv. *vesicatoria* strains were infiltrated into leaflets of tomato cvs. FL 7060 or Bonny Best. The inoculated plants were incubated in a growth room at 25 to 28°C for 10 days. Leaflets were sampled daily for bacterial populations of each strain by maceration of 1-cm^2 samples of inoculated area in 1 ml of sterile tap water, followed by standard dilution-plating onto plates of nutrient agar. Plates were incubated at 28°C for 3 days, and colonies were counted to calculate the CFU per square cm of leaf tissue for each sample. Bacterial populations were also determined at 12-h intervals in the incompatible pepper host NIL ECW20R after infiltration of leaves with suspensions of bacteria at 3×10^8 CFU/ml.

To quantitatively assess the progress of disease and the amount of tissue damage caused after inoculation by different strains, electrolyte leakage tests were run. In these experiments, sterile tap water suspensions of bacteria adjusted to 3×10^8 CFU/ml were infiltrated into leaflets of tomato cvs. FL 7060 and NIL 216 or leaves of pepper cvs. ECW and NIL ECW20R. Inoculated plants were maintained in a growth room at 25 to 28°C. At selected times, 3-cm^2 samples of inoculated area were carefully transferred to 3 ml of deionized water in 16 × 100 mm test tubes. A zero time electrolyte reading was recorded for each sample, using a Model 31 conductivity bridge (YSI Instrument Co., Inc., Yellow Springs, OH, U.S.A.). The samples were placed under vacuum for 1 min, shaken for 1 h at 28°C, and then vortexed briefly before a second (final) electrolyte reading was made. Leakage was reported as the difference in μmhos between the two readings for each sample.

Disease assessments for *X. campestris* pv. *vesicatoria* strains were made based on leaf and stem ratings compiled from three separate greenhouse inoculation tests. In each test, seven plants were inoculated with each strain by dipping young plants (four-true-leaves stage) into sterile tap water suspensions containing 0.025% Silwet L-77 (Loveland Industries, Inc., Greeley, CO, U.S.A.) and 3×10^7 CFU of bacteria per ml for 15 s. Plants were maintained in the greenhouse during the evaluation period.

To test for the possible role of $opgH_{Xcv}$ in osmotic stability of *X. campestris* pv. *vesicatoria*, tomato leaf cuttings were inoculated, incubated in a series of mannitol solutions, and observed for disease development. Leaflets of tomato cv. FL 7060 on intact plants were inoculated by infiltration with *X. campestris* pv. *vesicatoria* mutant or wild-type strains at a concentration of 3×10^7 CFU/ml. After the infiltrated areas had dried completely (1 h), the petioles were detached from the plants while submerged in a tub of deionized water, then immediately transferred to individual test tubes (28 × 200 mm) containing either deionized water or deionized water containing mannitol (SigmaUltra D-Mannitol, Sigma Chemical Co., St. Louis) at final concentrations of 25, 50, 100, 150, 200, or 250 mM. The tubes containing the cuttings were incubated in a growth room at 25°C with 12-h-light day and were rated daily for symptom development, using a 0 to 4 scale representing 0 to 100% of tissue collapse in the inoculated area.

Disease progress was also assessed on detached leaves inoculated at 3×10^8 CFU/ml, by analysis of electrolyte leakage at 25°C. Mannitol concentrations in the incubation solutions were adjusted to 25 or 50 mM for these tests. Leakage from tomato tissue due to the addition of mannitol alone was determined in noninoculated leaf cuttings. In addition, growth of the bacteria within leaflets of cuttings incubated in water or mannitol solutions was determined as above.

Estimation of the osmotic potentials for plant tissues used in the mannitol experiments was obtained from measurements on sap collected by centrifugation (4,000 × *g*, 30 min) from frozen and quick-thawed tomato leaflets. Measurements were made using a SC-10A thermocouple psychrometer sample changer and NT-3 nanovoltmeter thermometer (Decagon Devices, Inc. Pullman, WA, U.S.A.) calibrated against NaCl solutions of known water potentials.

GUS reporter assay.

Activity of GUS expressed in *X. campestris* pv. *vesicatoria* transconjugants carrying either pT3-5::249 (*avrXv3*::*uidA*) or pPG1 (*hrpG::uidA*) grown in pepper ECW was measured as previously described (Astua-Monge et al. 2000). Induction of GUS activity from transconjugants of the parental background strains were compared with their respective $opgH_{Xcv}$ deletion mutants 16 h after infiltration of pepper leaves at 3×10^8 CFU/ml. *X. campestris* pv. *vesicatoria* transconjugants carrying pL3::GUS were inoculated as controls for constitutive expression of *uidA*.

Secretion assay and protein immunoblot analysis.

AvrBs2 protein expression and Hrp TTSS secretion was analyzed for *X. campestris* pv. *vesicatoria* 85*, 85* Δ*hrcV*, and 85* $\Delta opgH_{Xcv}$, as previously described (Mudgett et al. 2000; Rossier et al. 1999). AvrBs2 secretion was tested in pH 5.4 minimal medium A containing 0, 5, or 25 mM mannitol. Protein samples were boiled for 5 min and then analyzed in an 8% gel by SDS-PAGE. For immunoblot analysis, proteins were transferred from gels to nitropure nitrocellulose (GE Osmonics Labstore, Minnetonka, MN U.S.A.) by electroblotting at 0.3 amps for 1 h in transfer buffer containing 10 mM 3-[cyclohexylamino]-1-propane sulfonic acid, pH 11, 10% (vol/vol) methanol. AvrBs2 was detected by using rabbit polyclonal antisera (Gassmann et al. 2000) at a dilution of 1:2,000 in 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween 20 buffer containing 5% nonfat milk, followed by horseradish peroxidase-conjugated secondary antibodies and chemiluminescence.

Protein analysis.

Bacteria were grown in nutrient broth, or nutrient broth containing filter-sterilized mannitol or NaCl at 50, 150, or 300 mM final concentration. Total cell protein for electrophoresis was prepared from 1.2 ml of exponentially growing cells (OD_{600} = 0.5) by resuspending cell pellets, washed once in sterile deionized water, in 200 μl of sample buffer containing 2-mercaptoethanol (Laemmli 1970). The samples were boiled for 5 min, debris was removed by centrifugation for 5 min in a microcentrifuge, and 30-μl samples were loaded into the wells of a 10% SDS polyacrylamide gel for electrophoresis (Maniatis et al. 1989). Proteins were stained with Coomassie brilliant blue R-250.

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