Effects of a Virulence Locus from *Xanthomonas campestris* 528^T on Pathovar Status and Ability to Elicit Blight Symptoms on Crucifers

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ABSTRACT

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All *Xanthomonas campestris* pv. *campestris* strains cause systemic black vein and rot of crucifers; and some strains, such as Xcc528^T, additionally cause blight symptoms. By contrast, *Xanthomonas campestris* pv. *armoraciae* strain Xca417 causes a nonsystemic leaf spot of crucifers. Total DNA libraries of Xcc528 and Xca417 were constructed, and the Xcc528 library clones were individually transferred by conjugation into Xca417, and vice versa. No effects on pathogenicity of Xcc528 were observed by any of the clones from Xca417. However, a 5.4-kb DNA fragment from Xcc528 significantly altered the phenotype elicited by Xca417 when inoculated onto cabbage. Plasmid pJC41, carrying the 5.4-kb Xcc528 DNA fragment, conferred to Xca417 the ability to cause blight symptoms in cabbage. A vascular hypersensitive response, characteristic of wild type Xca417, was also observed on crucifers inoculated with Xca417/pJC41 transconjugants. This hypersensitive response appeared to be tissue specific and localized to the vascular region, but the response was insufficient to stop the spread of blight symptoms induced by these transconjugants. Southern hybridization analyses of 16 different *Xanthomonas* species and pathovars revealed that only *X. c. campestris* strains capable of causing blight symptoms hybridized with pJC41. The gene(s) conferring blight symptoms may therefore be unique to *X. campestris* strains capable of causing blight on crucifers. Since Xca417 causes only a leaf spot on crucifers, and since pJC41 conferred distinctive symptoms associated with *X. c. campestris* strains, the 5.4-kb fragment affected the apparent pathovar status of the of the recipient strain.

Additional keywords: systemic movement.

Members of the genus *Xanthomonas* are all plant-associated and collectively attack a wide range of hosts. However, individual strains are limited in host range and are grouped into pathovars based primarily on the host plants from which they were first isolated. The pathovar (short for pathogenic variant) designation has no standing in bacterial nomenclature but is used to indicate pathogenic information (10). Since pathogenic information is based primarily on a host response phenotype, and very little on intrinsic properties of the bacterium, pathovar names may or may not reflect phylogenetic relatedness. Phylogenetic analyses of several different economically important xanthomonads reveal a generally clonal population structure in which clonality is observed within, but not among, pathovars (15,16). That is, *Xanthomonas campestris* pathovars may contain one or more clonal groups, but clonal groups have not been observed to contain multiple pathovars. A clonal population structure is typical of asexually reproducing pathogenic species, and is thought to be due to 1) low rates of recombination and 2) selective host amplification of strains with specific virulence factors (32). In this hypothesis, virulence genes with high selective value on a given host are thought to cause strains to be clonally amplified in direct proportion to the distribution and quantity of the host. If so, clonal pathogen groups, and therefore some pathovars, might differ from one another by only one or a few virulence genes that have a high selective value on specific crop genotypes.

At least three general types of cloned microbial virulence genes may be distinguished: those needed for virulence on any plant, those that affect host range, and those that affect disease symptoms (12). Within the Pseudomonadaceae, the *hrp* genes appear to be common to all pathogenic strains and are required for pathogenicity and virulence on any host (41). Genes required for host range on specific plant genera and species have been cloned from *Pseudomonas solanacearum* (27), *P. syringae* (30), *Xanthomonas campestris* pv. *translucens* (37), and *X. campestris* pv. *citrumelo* (23). Genes required for specific disease symptoms include *Pseudomonas* toxin genes (19) and the *Xanthomonas* host-specific pathogenicity genes *pthA* (35,36) and *avrb6* (42). Pathovar status can be determined either by genes that determine host range or by those that determine distinct disease phenotypes; both types of genes should have a high selective value to the pathogen on its hosts and may help determine a clonal population structure.

Among the best known strains of *X. campestris* are those included in pathovar *campestris,* which cause a serious disease on crucifers worldwide known as black rot (40). *X. campestris* pv. *armoraciae* is the causal agent of a leaf spot disease on the same hosts (3,28,39). Both pathogens are often found on the same plant in natural field infestations (2), but nothing is known of genetic differences between these two groups of pathogens that results in such distinct disease symptoms. *X. campestris* pv. *campestris* strains colonize the plant vascular system and are capable of systemic movement in planta. By contrast, *X. c. armoraciae* strains are nonsystemic (mesophyllic) pathogens that cause leaf lesions limited by the veins. A tissue-specific hypersensitive response is observed around the vascular tissue of crucifers inoculated with *X. c. armoraciae* strains (22), suggesting that some type of avirulence factor might act in a negative fashion to elicit the vascular hypersensitive response (VHR) and thereby limit movement of these strains. Alternatively, *X. c. campestris* strains may possess a pathogenicity factor(s) required for movement and/or disease that is missing in *X. c. armoraciae* strains. The purpose of this study was to attempt to test these two alternative hypotheses. Since both pathogens have the same host range, an avirulence gene present in *X. c. armoraciae* might be cloned in the usual fashion by screening a library of that strain in an *X. c. campestris* strain. Alternatively, if a pathogenicity gene were present in Xcc, it might be cloned using the "virulence enhancement" method of Swarup et al (35), by screening an *X. c. campestris* library in an *X. c. armoraciae* strain.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strain		
Escherichia coli		
DH5α	*f*80d*lac*ZΔM15 Δ(*lacZYA-argF*)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_{K-}, m_{K+}) *sup*E44 l *thi*-1 *gyr*A96 *rel*A1	Gibco-BRL
HB101	*sup*E44 *hsd*S20($r_{B-}m_{B-}$) *rec*A13 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20 *xyl*-5 *mtl*-1	(4)
ED8767	*sup*E44 *supF58 hsdS3* ($r_{B-}m_{B-}$) *recA56 galK2 galT22 metB1*	(29)
Xanthomonas campestris pv. *campestris*		
Xcc528^T	Type strain of *X. campestris*, causes blight and black rot of crucifers; ATCC 33913). Isolated in the United Kingdom	(1)
Xcc528R	Spontaneous Rif^r derivative of Xcc528	This study
G2-12	Hawaiian blight and black rot strain	(1)
G2-17	Hawaiian blight and black rot strain	(1)
H-M	Wisconsin black rot strain	(1)
PHW-117	Wisconsin black rot strain	(1)
XC-114	North Carolina black rot strain	(1)
X3	Florida black rot strain	(1)
GAC-137	Hawaiian black rot strain	(1)
A249	Hawaiian black rot strain	(1)
GAC-17	Hawaiian black rot strain	(1)
GAC-20	Hawaiian black rot strain	(1)
G3-38A	Hawaiian black rot strain	(1)
X. campestris pv. *armoraciae*		
Xca417	Causes leaf spot of crucifers. Isolated from East Asia	(1)
Xca417R	Spontaneous Rif^r derivative of Xca417	This study
Xca417RK	Xca417R (chr::Tn*5-gusA*), Km^r, Tc^r, Rif^r	This study
A342	Hawaiian leaf spot strain	(1)
G3-27	Hawaiian leaf spot strain	(1)
756	East Asia leaf spot strain	(1)
XLS2	North Carolina leaf spot strain	(1)
XLS6	North Carolina leaf spot strain	(1)
XLS10	North Carolina leaf spot strain	(1)
X. campestris pv. *alfalfae*		
KX-1	Leaf spot of alfalfa	(36)
L-142	Leaf spot of alfalfa	(36)
X. campestris pv. *aurantifolii*		
Xc69	False citrus canker, Cancrosis B, ATCC 51301	(36)
Xc70	Lime canker, Cancrosis C, ATCC 51302	(36)
X. campestris pv. *citrumelo*		
3048^R	Citrus bacterial spot, holopathotype strain, ATCC 49120	(36)
4600	Citrus bacterial spot	(36)
X. campestris pv. *cyamopsidis*		
13D5	Leaf spot of guar	(36)
X002	Leaf spot of guar	(36)
X. campestris pv. *dieffenbachiae*		
2032	Leaf spot of dieffenbachia	(36)
084-729	Leaf spot of dieffenbachia	(36)
X. campestris pv. *glycines*		
ATCC 17915	Leaf spot of soybean	(36)
1717	Leaf spot of soybean	(36)
X. campestris pv. *malvacearum*		
XcmN	Angular leaf spot of cotton	(36)
XcmH	Angular leaf spot of cotton	(36)
X. campestris pv. *malvacearum-hibiscus*		
X-10	Leaf spot of hibiscus	(36)
X. campestris pv. *pisi*		
XP1	Leaf spot of pea	(36)
X. campestris pv. *translucens*		
82-1	Black chaff of cereals	(36)
216.2	Black chaff of cereals	(36)
X. campestris pv. *vesicatoria*		
82-23	Leaf spot of pepper	(36)
X. campestris pv. *vignicola*		
A81-331	Leaf spot of grape	(36)
Xv19	Leaf spot of grape	(36)
X. citri		
3213^T	Asiatic citrus canker, Cancrosis A, type strain, ATCC 49118	(36)
3210	Asiatic citrus canker, Cancrosis A	(36)
X. phaseoli		
G-27	Common bean blight, type strain, ATCC 49119	(36)
XP-JL	Common bean blight	
Plasmid		
pUFR004	ColEl, Mob$^+$, mob(P), Cm^r	(7)
pUFR043	IncW, Mob$^+$, LacZα, Gm^r, Nm^r, Cos	(6)
pUFR047	IncW, Mob$^+$, LacZα, Ap^r, Gm^r	(8)
pRK2013	Tra$^+$, Mob$^+$, ColEl replicon, Km^r, helper plasmid	(9)
pRK2073	pRK2013 derivative, Km^s (npt::Tn7), Sp^r, helper plasmid	(26)
pJC6.4	35.7-kb insert from Xcc528 in pUFR043, Gm^r, Km^r carries black vein/blight activity	This study
pJC41	5.4-kb *Hin*dIII fragment of pJC64.H6 in pUFR047, Ap^r, Gm^r carries black vein/blight activity	This study

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *Escherichia coli* were grown in Luria-Bertani (LB) medium (31) at 37 C. Strains of *Xanthomonas* were grown in peptone-yeast extract-glycerol-MOPS medium (PYGM) at 30 C (7). For culture on solid medium, agar was added at 15 g/L. Antibiotics were used at the following final concentrations: ampicillin (Ap), 25 mg/L; kanamycin (Km), 20 mg/L; gentamycin (Gm), 3 mg/L; and rifampicin (Rif), 75 mg/L. Single colonies of *X. c. campestris* 528^T (Xcc528, syn. ATCC 33913) and *X. c. armoraciae* strain 417 (Xca417) were isolated and tested for pathogenicity. Spontaneous Rif-resistant (Rif^r) mutants of these strains were obtained by spreading bacterial cells at 10^9 cfu/ml on PYGM containing rifampicin at 50 μg/ml. Spontaneous mutants were selected after incubation for 3 days at 30 C and were restreaked on PYGM plates containing rifampicin at 75 μg/ml. Strain identity was verified in comparisons with controls by restriction fragment length polymorphism (RFLP) analyses of total DNA digested with *Eco*RI and separated on 0.65% agarose gels stained with ethidium bromide (14).

Recombinant DNA techniques. Plasmids were isolated from *E. coli* by alkaline lysis methods (31). Restriction enzyme digestion, alkaline phosphatase treatment, DNA ligation, and random priming reactions were performed as recommended by the manufacturers. Southern hybridizations were performed using nylon membranes as described (24). Otherwise, standard recombinant DNA procedures were used (31).

Construction of DNA libraries and subcloning. Total DNA of Xca417 and Xcc528 were prepared as described (14) and purified by cesium chloride-ethidium bromide density centrifugation. The DNA was then subjected to *Sau*3A partial digestion, and the resulting fragments were size-fractionated by sucrose gradient centrifugation. A 10.3-kb cosmid cloning vector, pUFR043 (6), was prepared for construction of the library with two enzymes (*Eco*RI and *Sal*I) as described (31) and dephosphorylated to prevent self-concatamerization. The vector fragments were then cut with *Bam*HI and ligated to *Xanthomonas* DNA fragments in the size range of 30–50 kb. The recombinant linear fragments were packaged into λ phage heads with GIGAPACK Gold packaging mix (Stratagene, La Jolla, CA) and introduced into *E. coli* strain HB101 via transfection, according to the manufacturer's instructions. Individual clones were selected on LB agar medium containing gentamycin and neomycin, and stored in LB broth with 14% glycerol in microtiter dishes at −70 C. Subclones of the inserts from pUFR043 were made in pUFR047.

Bacterial conjugation. DNA transfer between *E. coli* donor (HB101/pUFR043/inserts) and *Xanthomonas* recipients (Xca417R and Xcc528R) was accomplished by triparental transconjugation as described (35), using a narrow host range plasmid, pRK2013, as the helper. Transconjugants were selected on PYGM agar plates containing the appropriate antibiotics.

Plant pathogenicity assays. *Brassica oleracea* var. *capitata* cultivar "636" (Known You Seeds Distribution [S.E.A.] PTE Company, LTD., 110 Owen Road, Singapore 0821) or locally available nonhybrid cultivars were used as susceptible hosts for Xca417 and Xcc528 in all plant seedling assays. Plants were grown either in a greenhouse or in a growth chamber under 16 h of artificial light at 25 C. Several different pathogenicity assays were used. Initial library screens were performed using an excised-cotyledon assay exactly as described (1). Briefly, 7-day-old seedlings grown in soil were cut at the base of one cotyledon without injuring the growing stem, using a sterilized razor. The wounded region was immediately inoculated with single colonies from overnight cultures grown on agar plates, using sterile toothpicks. For older plant assays, 4- to 5-wk-old plants grown in individual pots in growth chambers or in the greenhouse were inoculated by spraying the leaves with 106 cfu/ml from a mist sprayer or by wounding the leaves. For inoculation by the spray method, plants were held at 100% humidity for 24 h after inoculation. For wound inoculation at the leaf edge, leaves were cut 1.5–2.0 cm deep from the edges at a 45° angle relative to the main leaf vein with scissors that were flame sterilized and dipped into 10^6 cfu/ml broth culture prior to each inoculation. For wound inoculation at the midvein, a sterilized toothpick dipped into cells adjusted to about 10^6 cfu/ml from an overnight liquid culture was used to stab the midvein at the base of the leaf.

In planta movement assays. Suicide vector pRK600 (33) was used to transfer Tn*5-gusA* from *E. coli* strain 387 to Xca417R. Selection was for resistance to kanamycin and rifampicin and for dark blue colonies on PYGM plates supplemented with X-glucuronic acid (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, Molecular Probes, Inc., Eugene, OR) at 40 μl/ml, indicating strong transcription of the *gusA* reporter gene. Twenty-five dark blue colonies were purified by restreaking and reevaluated by adding 10 μl of fresh overnight cultures to 0.1 ml of 0.1 M phosphate buffer, pH 7.0, containing X-glucuronic acid at 40 μl/ml. GUS activity was indicated by a blue color developing within 24 h; comparison of all 25 colonies in a microtiter dish allowed selection of several strains exhibiting the strongest GUS activity. These strains were evaluated on minimal media for growth characteristics relative to the parental strain, and in plant inoculations for ability to elicit leaf spot symptoms. Strain Xca417RK was fully pathogenic and prototrophic and was used to evaluate in planta movement.

Plasmid pJC41 was transferred into Xca417RK by conjugation, and the transconjugants were stab inoculated into the petioles of three to four equivalently sized (12–15 cm long) leaves of 5- to 7-wk-old cabbage plants. Xcc528R and Xca417RK were each inoculated into three to four leaves of equivalent size on separate plants as controls. Each set of inoculated plants constituted one experiment. On each sampling date, the inoculated leaves were removed from the plant and sectioned into three even pieces by slices perpendicular to the stem, resulting in three 4- to 5-cm-long leaf sections. The first leaf section included the petiole inoculation zone; the edge of the second leaf section closest to the inoculation site was at least 3 cm from that site; and the edge of the third leaf section closest to the inoculation site was at least 7 cm from that site. Each entire leaf section was macerated using sterile mortars and pestles in sterile tap water, and the suspensions from each section were serially diluted and plated on PYGM medium supplemented with appropriate antibiotics. The population size of each introduced strain was determined for each leaf section at 0, 2, and 4 days after inoculation, when no black vein or blight symptoms were apparent. These experiments were performed seven times. In one additional experiment, bacterial population sizes were determined for three separate leaves 2 wk after inoculation, when blight symptoms were readily apparent.

For histochemical localization of GUS activity, cabbage leaves infected with Xca417RK or Xca417RK/pJC41 were immersed overnight in 50 ml of GUS staining buffer (50 mM $NaPO_4$, pH 7.0; 1% Triton X-100; 10 mM EDTA; and 25 mg of X-glucuronic acid) (21), and the chlorophyll in the leaves was cleared with 70% ethanol as described by Kamoun et al (22).

RESULTS

Construction of genomic libraries. Two genomic DNA libraries were constructed, one from Xcc528 and the other from Xca417. The Xcc528 and Xca417 libraries consisted of 572 and 528 cosmid clones, respectively. Based on the restriction profiles of 18 randomly selected clones from each of the libraries, the insert DNAs were randomly cloned fragments, with an average size of 34 kb for the Xcc528 library and 35 kb for the the Xca417 library. Each of the libraries appeared to be fully representative of the two genomes with 99% probability (5).

Initial library screens. In an attempt to detect pathogenicity or avirulence genes, all clones from the Xcc528 DNA library were individually transferred by conjugation into Xca417R, and all clones from the Xca417 library were similarly transferred into Xcc528R. The resulting transconjugants were individually screened on plants using the excised-cotyledon assay. Plants inocu-

lated with transconjugants of Xcc528R carrying cloned Xca417 DNA all exhibited similar symptoms, characteristic of Xcc528R: yellowing on the first pair of true leaves approximately 6 days postinoculation, followed by blight symptoms about 1 wk later. The entire Xca417 library was screened, and in no case was a reproducibly different phenotype conferred by a DNA library clone in these assays.

Plants inoculated with most transconjugants of Xca417R carrying cloned Xcc528 DNA exhibited similar symptoms, characteristic of Xca417R: necrotic stem girdling appeared at the inoculation site; true leaves developed poorly; and the affected plants collapsed about 4 days after inoculation. The severity of stem girdling was attenuated with increased seedling age, and no girdling was observed when Xca417R was inoculated on seedlings with a pair or more of developed true leaves. The entire Xcc528 library was screened, and three clones were recovered that partially aborted the girdling and lodging symptoms typical of seedlings inoculated with Xca417R and conferred symptoms more typical of those elicited by the Xcc528R transconjugants. Clone pJC6.4 exhibited the strongest girdling-suppression activity in Xca417R and was selected for subcloning and further evaluation. The 35.7-kb insert in pJC6.4 was mapped, and fragments were subcloned in pUFR047 using *Eco*RI and *Hin*dIII. Overlapping subclones were conjugally transferred into Xca417R, and the resulting transconjugants were assayed on cabbage seedlings. The symptom alteration activity was localized to a 5.3-kb *Hin*dIII fragment on pJC41; this subclone was used in all subsequent assays. The restriction map and activity assays of selected fragments are shown in Figure 1.

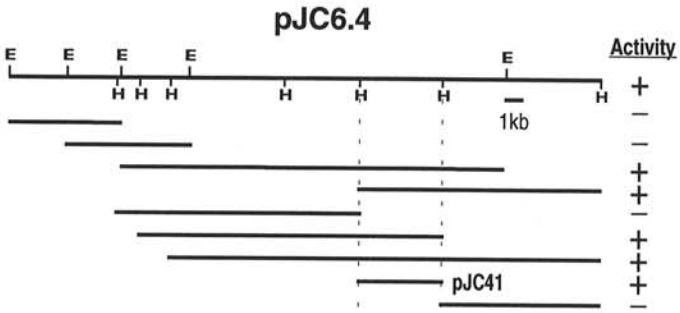

Fig. 1. Restriction map of the pJC6.4 insert and localization of the region conferring blight symptoms. Activity was assayed using cabbage seedling assays as described in the text. E, *Eco*RI; H, *Hin*dIII.

Fig. 2. Symptoms caused by Xca417R/pJC41 on a cabbage leaf. Blight and black vein symptoms were observed at the leaf margin 7 days after stab inoculation of the leaf stem in the center of the leaf. A 2.5-cm necrotic lesion formed along the midvein, downstream from the inoculation site, indicated by the arrow. The inset is a ×2 magnification of the region enclosed in the black rectangle, and the photo in the inset was taken 24 h later, with blight symptoms fully developed.

Pathogenicity tests on 5- to 7-wk-old plants. After wound inoculation of 10- to 15-cm cabbage leaves using scissors, Xca417R symptoms appeared as narrow, V-shaped, water-soaked lesions limited by leaf veins along the cut edges approximately 4 days after inoculation. Using the same inoculation method, Xcc528R symptoms became visible after 10 days as yellowish blight lesions, usually with black veins, freely expanding from the cut edges. Clone pJC41 conferred a blight (1) phenotype to Xca417R (not shown).

To determine if pJC41 also conferred systemic movement to Xca417R, stab inoculations of the leaf stems and petioles, and spray inoculations of 5- to 7-wk-old plants were also performed. In the stab inoculation tests, Xca417R induced a VHR observed as a necrotic lesion limited around the point of inoculation within 24 h. Xcc528R exhibited blight symptoms with blackened leaf veins that started to appear along the nearby leaf edges approxi-

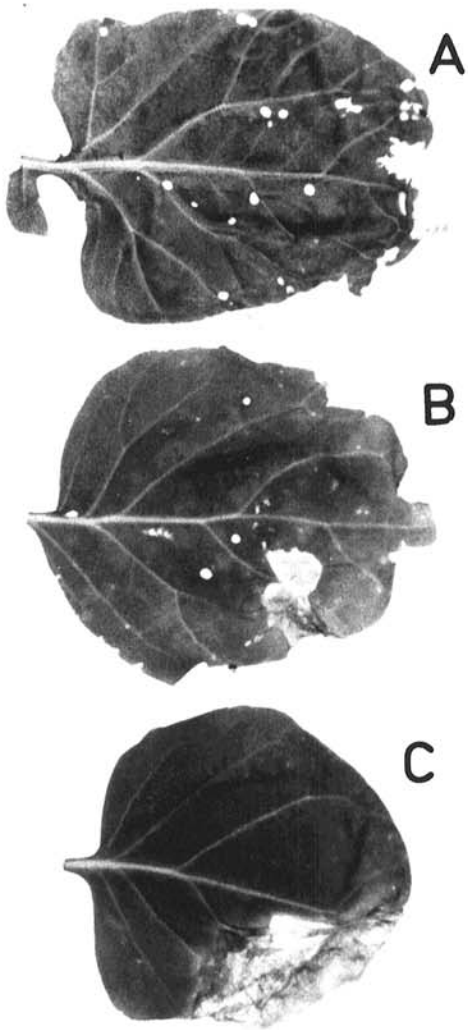

Fig. 3. Symptoms caused by Xca417R, Xca417R/pJC41, and Xcc528 on cabbage leaves following spray inoculation. **A,** Xca417R, 5 days after inoculation; **B,** Xca417R/pJC41, 14 days after inoculation (the leaf spots appeared 5 days after inoculation); **C,** Xcc528, 14 days after inoculation.

mately 10 days after stab inoculation. Plasmid pJC41 conferred to Xca417R the ability to induce blight and black vein symptoms similar to those induced by Xcc528R, but the symptoms were not as extensive. These black vein and blight symptoms appeared several centimeters distant from the point of inoculation, indicating possible movement of the transconjugant cells (Fig. 2). Interestingly, Xca417R/pJC41 also exhibited symptoms typical of Xca417R; a limited necrotic lesion (VHR) was visible around the point of inoculation. In spray inoculation tests, Xca417R and Xca417R/pJC41 both appeared to enter through stomata (Fig. 3). The Xca417R lesions were a typical leaf spot that appeared 4–5 days after inoculation, while some Xca417R/pJC41 lesions exhibited blight symptoms that appeared approximately 2 wk after inoculation, and which were never observed with Xca417R. The blight symptoms caused by Xca417R/pJC41 were similar to those caused by Xcc528, except that black veins did not develop, whereas a VHR did. Also, Xcc528 always produced larger lesions, usually with black veins, and appeared to enter at the hydathodes.

To verify that the Xca417R/pJC41 symptoms were not the result of contamination with Xcc528 or another *X. c. campestris* strain, Rif^r xanthomonads were reisolated from the inoculated leaf tissue margin shown in Figures 2 and 3 (exhibiting blight symptoms). Approximately half of the colonies were Rif^r, Gm^r and half were Rif^r, Gm^s, indicating loss of the pJC41 plasmid. Based on an average initial inoculation level of 10^3 cfu and a final achieved concentration of 10^8 cfu/cm^2, the rate of plasmid loss was about 50% in 16 generations of growth in planta. The RFLP analyses of DNA from nine Rif^r, Gm^s and nine Rif^r, Gm^r xanthomonads reisolated from the inoculated leaves confirmed identity of the strains as Xca417R or Xca417R/pJC41.

In planta movement of Xca417R/pJC41 in advance of symptoms. To determine whether pJC41 carried a gene(s) conferring movement in leaves to Xca417R, 5- to 7-wk-old cabbage leaves were inoculated at the base of the petiole with Xca417RK, Xcc528R, and Xca417RK(chr::Tn*5-gusA*)/pJC41 cells. In seven experiments, inoculations consistently provided about 10^3 cfu in the petiole, as assayed by destructive sampling of leaves following inoculations with each strain (Table 2). Movement of Xcc528R to the middle and ends of inoculated leaves was unpredictable in advance of symptoms but occurred in about half of the leaves sampled in these experiments by day 4. In all cases, whenever Xcc528R moved to the middle portion of the leaf, from 10^3 to 10^6 cfu were also found in the distal end of the leaves 4 days after inoculation. Xca417RK cells were also found in the middle portion of about half of all leaves inoculated by day 4, but at about 10-fold lower total numbers (about 10^3–10^4). These results indicated that both *X. c. campestris* and *X. c. armoraciae* cells moved in the transpiration stream to the middle portion of some leaves as a result of the artificial inoculation technique. However, in repeated tests with Xca417RK, no lesions of any type developed outside of the necrotic inoculation zone extending about 1 cm along the midvein in leaves inoculated with Xca417RK, and no symptoms of any type developed in the middle portion of any leaves, despite the occurrence of Xca417RK cells in the central stems of about half of the inoculated leaves. By contrast with Xcc528R, neither Xca417RK nor Xca417RK/pJC41 cells were detected in the distal portions of any inoculated leaves, nor were any symptoms detected in the distal portions of leaves inoculated at the base of the petiole with these strains.

Similarly to Xca417RK, Xca417RK/pJC41 cells (10^1–10^5) were found in the middle sections of three out of seven inoculated leaves, 4 days after inoculation. In no case were the transconjugant cells found in the distal leaf sections. However, the transconjugants elicited a VHR in secondary xylem vessels and blight symptoms in leaf tissue adjacent to the secondary xylem vessels in leaves inoculated with the Xca417RK/pJC41 transconjugant. Shown in Figure 4 is a typical example of the spreading of Xca417RK/pJC41 cells and the beginning of blight symptoms, 7 days after inoculation. The leaf in Figure 4 was stained with GUS, revealing that Xca417RK/pJC41 cells had traveled 6 cm up the midvein from the point of inoculation and then escaped the midvein and traveled in secondary xylem vessels, causing blight symptoms in the mesophyl. GUS activity was most intense along the midvein but was also observed along secondary veins. GUS-stained leaves

TABLE 2. Limited movement of Xca417RK/pJC41 transconjugants in leaves. Cabbage leaves were inoculated at the base of the leaf petiole with Xcc528R, Xca417RK, or Xca417RK/pJC41 as indicated, and the entire leaf was destructively sampled at 0, 2, or 4 days after inoculation

		Cell count (cfu)[b]								
		Day 0			Day 2			Day 4		
Strain	Experiment[a]	Prox.	Mid.	Dist.	Prox.	Mid.	Dist.	Prox.	Mid.	Dist.
Xca417RK	1	10^3	0	0	10^4	0	0	10^5	10^3	0
	2	10^3	0	0	10^4	0	0	10^6	0	0
	3	10^3	0	0	10^4	0	0	10^4	0	0
	4	10^3	0	0	10^4	0	0	10^5	10^4	0
	5	10^3	0	0	...[c]	...	...	10^4	0	0
	6	10^3	0	0	...	...	...	10^5	10^4	0
	7	10^3	0	0	...	...	...	10^5	0	0
Xcc528R	1	10^3	0	0	10^4	10^5	0	10^6	10^5	10^3
	2	10^2	0	0	10^4	0	0	10^5	10^6	10^6
	3	10^3	0	0	10^4	0	0	10^6	0	0
	4	10^3	0	0	10^4	0	0	10^4	0	0
	5	10^3	0	0	...	...	...	10^4	0	0
	6	10^2	0	0	...	...	...	10^4	0	0
	7	10^3	0	0	...	...	...	10^4	10^5	10^4
Xca417RK/pJC41	1	10^3	0	0	10^4	10^2	0	10^5	10^3	0
	2	10^3	0	0	10^5	0	0	10^6	10^4	0
	3	10^3	0	0	10^4	0	0	10^6	0	0
	4	10^3	0	0	10^4	0	0	10^5	10^5	0
	5	10^3	0	0	...	...	...	10^5	0	0
	6	10^3	0	0	...	...	...	10^5	0	0
	7	10^3	0	0	...	...	...	10^5	0	0

[a] Dilutions of each macerated leaf section were sampled four times each on PYGM plates containing rifamycin, and cfu counts were averaged. To check for plasmid stability, leaves inoculated with Xca417RK/pJC41 were also plated on PYGM medium containing rifamycin and gentamycin, and cfu counts were averaged. There were no significant differences in cell counts taken on these two antibiotic media, indicating no significant loss of plasmid pJC41 by day 4.

[b] In each experiment, a different leaf was sectioned on a given day into three equal longitudinal parts: the proximal (prox.) portion contained the inoculation zone; the middle (mid.) was at least 3 cm from the inoculation zone; and the distal (dist.) portion was at least 7 cm from the inoculation zone. A different leaf was used on each sampling date.

[c] Not determined.

inoculated in the same manner with Xca417RK cells never exhibited blight, black veins, or GUS staining outside the midvein; and no GUS activity was visually detected beyond 2 cm of the inoculation zone along the midvein (not shown). Taken together, these results indicated that pJC41 conferred to *X. c. armoraciae* the ability to break out of the midvein area and to cause blight, but did not confer ability to move extensively or systemically in leaves.

In other experiments where symptoms were allowed to develop, a few leaves inoculated in the petiole with Xca417RK/pJC41 developed blight symptoms in the distal sections of the leaves. When the distal regions of these leaves were examined for the presence of *Xanthomonas* cells, significant numbers of Xca417RK/pJ41 (Gm^r) cells were found (10^3–10^4 cfu), but the vast majority (10^7 cfu) of the cells found were Xca417RK (Gm^s), evidently cured of the plasmid. The fact that significant numbers of the transconjugants appeared to have lost pJC41 indicated that part of the limitation on movement may be due to plasmid instability.

Nonconservation of 5-kb DNA fragment from Xcc528 on pJC41. Conservation of the cloned 5-kb DNA insert from Xcc528 among *X. c. campestris* strains and among other xanthomonads was examined. Plasmid pJC41 was used as a probe against a Southern blot of total DNA extracted from all 46 *Xanthomonas* strains listed in Table 1. While the three blight strains tested (Xcc528, G2-12, and G2-17) hybridized strongly to the probe under high-stringency hybridization and wash conditions, no other strain tested hybridized even faintly to the probe (autoradiographs not shown). Even when the hybridizations were repeated under low-moderate stringeny, only the three blight strains tested (Xcc528, G2-12, and G2-17) (1) hybridized to the probe (autoradiographs not shown).

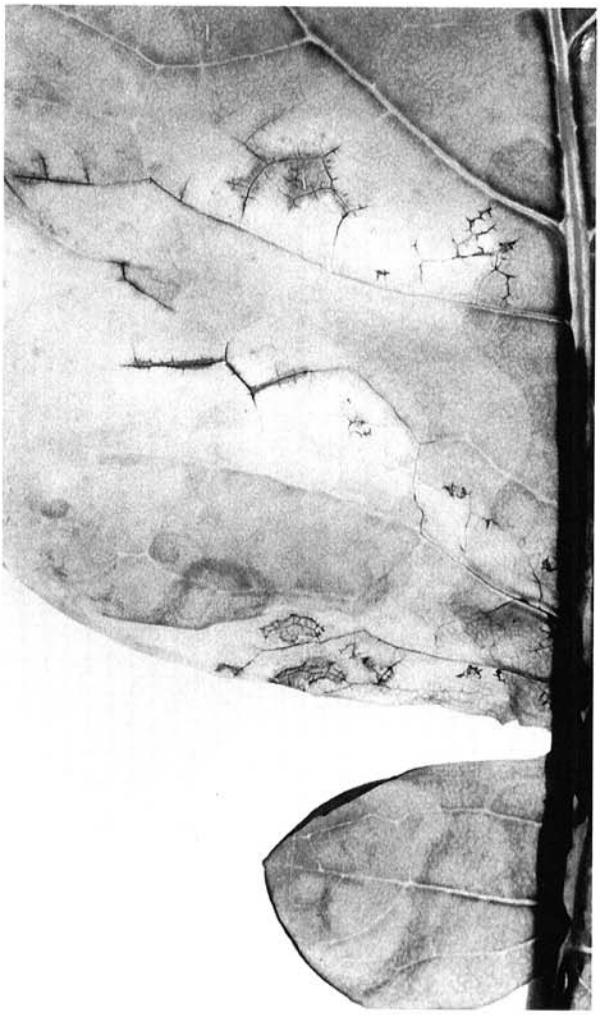

Fig. 4. Symptoms caused by Xca417RK/pJC41 on a cabbage leaf following stab inoculation at the base of the leaf (inoculation zone off the bottom edge of the photo). The leaf was stained with GUS 7 days after inoculation and partially cleared with ethanol. Intensely dark areas along the midvein and secondary xylem vessels are GUS-stained.

DISCUSSION

X. c. campestris is genetically and pathologically heterogeneous (1,2,22,24,25,43). All *X. c. armoraciae* strains and incompatible isolates of *X. c. campestris* induce a localized VHR on crucifers (22), indicating the possibility of some sort of avirulence gene involvement in triggering the VHR, thereby limiting systemic movement. We did not detect the presence of such a gene in *X. c. armoraciae* that might limit systemic movement when transferred to Xcc528. This result was not entirely unexpected, since avirulence (*avr*) genes are known to determine race specificity; and despite speculation to the contrary, *avr* genes have not been shown to be involved in determining pathovar status (13,18).

Instead, a small DNA fragment was cloned from Xcc528 that conferred to an *X. c. armoraciae* strain symptoms of blight, a phenotype normally associated with *X. c. campestris* strains. Although the number of genes on the pJC41 clone and their role in Xcc528 are unknown, there is precedent for the ability of a single gene to alter apparent pathovar status. Gene *pthA* from *Xanthomonas citri* can alter the apparent pathovar status of *X. campestris* pv. *alfalfae* and *X. c. citrumelo* strains to cause hyperplastic cankers on citrus (35).

The idea that differences in pathovar status may be determined, at least in a few cases, by only one or a few virulence genes is relatively new and may affect the value or weight given to pathogenic information by phytopathologists (17). Some have taken the position that host specialization in *Xanthomonas* (as represented by pathovars) should determine species status (for discussion, refer to Starr [34]). Indeed, a number of journals, including this one, require a trinomial designation of strains (e.g., *X. c. campestris*) that appears to give the pathovar name species status, even though pathovar names have no standing in bacterial nomenclature. Nevertheless, strains which cause identical diseases on the same hosts may be phylogenetically quite distinct. For example, *X. phaseoli* and *X. campestris* pv. *fuscans* (16) cause an identical disease, common bean blight. Yet these two xanthomonads are only 20% homologous by DNA–DNA hybridizations (20). As another example, both *X. citri* and *X. campestris* pv. *aurantifolii* (16) cause an identical disease, citrus canker. Yet these two xanthomonads are only 60% similar by DNA–DNA hybridizations (11). (The phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA–DNA relatedness by hybridization [38]). The results of this study provide evidence that pathovar status may in some cases be determined (and altered) by a very few virulence genes, independent of phylogenetic background.

This is the first report of a blight factor isolated from a phytopathogenic bacterium. The excised-cotyledon assay procedure used in this study (1) allowed rapid screening for this function. The systemic pathogen, *X. c. campestris,* entered the plant vascular system of the seedlings through the basal cotyledon cut and moved up to the leaf veins, eventually eliciting black rot and blight symptoms. The mesophyllic pathogen, Xca417, lacking systemic functions, produced a localized infection, resulting in stem girdling, tissue maceration, and the consequent seedling collapse. Plasmids pJC6.4 and pJC41 repressed the stem girdling caused by Xca417RK, perhaps by allowing cells to escape containment in a localized region. Therefore, the ability to cause blight symptoms may contribute to systemic movement in planta. Ability to cause blight and some in planta movement was conferred by a 5-kb DNA fragment found only in blight-causing strains of Xcc but not present in *X. c. armoraciae* strains or non-blight-causing strains of Xcc. These results demonstrate that blight symptoms are the result of an add-on pathogenicity factor and indicate that systemic movement may be an add-on factor as well. Other factors, not encoded on pJC41, must be involved

in systemic movement. For example, the non-blight-causing *X. c. campestris* strains and *X. phaseoli* strains move systemically in their respective hosts, yet these strains did not hybridize even at low-moderate stringency to pJC41.

Plasmid pJC41 may be suitable for use as a DNA probe and for development of primers for use with the polymerase chain reaction (PCR). Since pJC41 carries a disease-specific pathogenicity factor, any primers or probes derived from such a factor may be specific for bacteria able to cause the specific disease symptom. Since pathovars are collections of strains that are able to cause specific disease symptoms, such disease-specific primers or probes may be pathovar-specific. In the present instance, pJC41 may be diagnostic for blight-causing strains of *X. c. campestris*. Since very few *X. c. campestris* strains were evaluated in this study, further evaluation of the diagnostic potential of pJC41 is needed. Theoretically, however, DNA probes and PCR primers derived from disease-specific pathogenicity genes should be highly reliable, since the target locus should be present in all strains capable of causing a given disease and absent in all strains unable to cause a given disease. By contrast, randomly derived DNA probes and primers examine regions of the genome which may mutate, rearrange, or be lost without affecting the disease-causing ability of the strain. Randomly derived primers and probes may therefore be expected to generate a higher level of false negative reactions than do disease-specific primers and probes.

Strains of different *Xanthomonas* species and pathovars differ by host range and/or elicit different disease symptoms if isolated from a common host. The cloning of host- and disease-specific factors from phytopathogenic bacteria is a first step toward understanding host range and pathogenicity at the molecular level. The virulence enhancement strategy used in this and the previous study of Swarup et al (35) allowed the identification of DNA fragments responsible (at least in part) for a pathogen's unique disease symptoms. Clones from a DNA library of a strain of one pathovar are assayed for their effect(s) in a strain of a different pathovar inoculated on a common compatible plant host. The virulence enhancement strategy may be generally useful for the purpose of rapidly isolating a number of disease-specific factors.

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