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A Plant-Inducible Gene of *Xanthomonas campestris* pv. campestris Encodes an Exocellular Component Required for Growth in the Host and Hypersensitivity on Nonhosts

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Using Tn*4431*, a transposon that allows transcriptional fusions to a promoterless luciferase (*lux*) operon, we have isolated a nonpathogenic mutant of *Xanthomonas campestris* pv. campestris, i.e., JS111, that does not incite any of the black rot symptoms on all tested cruciferous host plants (J. J. Shaw, L. G. Settles, and C. I. Kado, Mol. Plant Microbe Interact. 1:39–45, 1988). In the study reported here, we determined that in contrast to the wild-type strain, JS111 is unable to induce a hypersensitive necrotic response on nonhost plants such as datura, tomato, and cucumber, suggesting that JS111 is a nonpathogenic, nonhypersensitive Hrp mutant. JS111 displayed culture growth rates, exopolysaccharide production, and protease, pectate lysase, cellulase, amylase, and phosphatase activities comparable to those of the wild-type strain. However, the growth of JS111 in host leaves was markedly attenuated. Coinoculation of JS111 with the wild-type strain in cauliflower or radish leaves rescued the growth deficiency of the mutant to normal levels. The locus mutated in JS111 was cloned and named *hrpXc*, and transcriptional and genetic complementation analyses of the *hrpXc* locus were conducted. The regulation of *hrpXc* expression was also investigated in vitro and in planta, using fusions to a *lux* or chloramphenicol acetyltransferase reporter gene. The *hrpXc* gene was found to be strongly induced in radish leaves. This is the first report and analysis of a *hrp* locus from a *Xanthomonas* species.

In recent years, the application of molecular genetic approaches to plant pathogenic bacteria has led to the identification and isolation of a number of genes involved in the interaction between the bacterium and the plant. These genes can be tentatively classified into four groups relative to the phenotype they confer on the bacteria. (i) *hrp* (hypersensitive response and pathogenicity) genes are required for pathogenicity on hosts and for the induction of a hypersensitive response (HR) on nonhost plants (1, 2, 23). (ii) *dsp* (disease-specific) genes are necessary for pathogenicity but do not affect HR induction on nonhosts (6). (iii) *avr* (avirulence) genes confer race- or cultivar-specific interactions. The avirulence genes are dominant genes and restrict the host range of the bacteria to the cultivars that contain a complementary resistance gene(s) (36, 37). (iv) Of considerable importance are the *hsv* (host-specific virulence) genes that have been isolated from bacteria with wide host ranges and are required for pathogenicity on only some of the host plants (13, 14, 28; D. Gabriel, ASM News **52**:19–25, 1986). *hsv* genes can extend the host range when introduced in narrow-host-range strains (25, 39).

In this paper, we report the identification and characterization of a *hrp* gene from *Xanthomonas campestris* pv. campestris, the causal agent of the black rot of crucifers, a worldwide and economically important plant disease (40). Hydathodes are the principal natural mode of entry in the plant, although secondary entry sites can develop in the roots or in wounds (5). The invasion of *X. campestris* pv. campestris in crucifers is usually confined to the xylem (3), where it grows and eventually causes the distinct blackening of leaf veins, followed by the drying and death of panels of leaf tissue. Soft rots and water soaking symptoms induced by *X. campestris* pv. campestris are not observed in the field. However, these atypical symptoms can be experimentally obtained by infiltration of the bacteria in young seedlings or detached host leaves (34).

The mechanism by which *X. campestris* pv. campestris incites black rot is not known. Mutants that lack degradative enzymes and extracellular polysaccharides (EPS) were nonpathogenic (9), whereas mutants defective only in cellulase, protease, polygalacturonate lyase, or EPS production were still able to cause black rot (8, 11, 31, 35, 38). Therefore, the exact roles of degradative enzymes and of EPS production in the disease cycle are still unclear. It has been proposed that degradative enzymes may play a role when *X. campestris* pv. campestris is growing saprophytically on dead plant tissue (11).

Using Tn*4431*, a transposon that allows transcriptional fusions to a promoterless luciferase (*lux*) operon, we previously isolated a nonpathogenic mutant of *X. campestris* pv. campestris, JS111, that does not incite any of the black rot symptoms on all tested host plants (35). The locus mutated in JS111 was cloned and named *hrpXc*. In this paper, evidence is presented that JS111 is a Hrp mutant and that this mutant can be complemented exocellularly in planta by coinoculation with the wild-type strain. These results suggest that a diffusable factor is needed for pathogenic invasiveness into host tissues.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used are listed in Table 1. The spontaneous rifampin-resistant (Rm^r) mutants were constructed as previously described (33).

Media and antibiotics. *Escherichia coli* was routinely grown in LB medium or LB 1.5% agar plates (29) at 37°C; *X. campestris* pathovars were grown in medium 523 broth or 523 1.5% agar plates or on minimal medium 925 1.5% agar plates (17) at 29°C. The antibiotics used for *E. coli* were

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TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli HB101		H. W. Boyer
X. campestris pv. campestris		
84-81	Wild-type isolate from wild mustard	R. Campbell
2D520	Spontaneous Rm^r mutant of 84-81	35
JS111	Rm^r Tc^r Hrp^-; Tn*4431* mutant of 2D520	35
2D520TL	Rm^r Km^r Ap^r; 2D520 containing one copy of pUCD607 integrated in the chromosome	R. McElhany
2D540	Wild-type isolate from wild radish	This study
2D540R	Spontaneous Rm^r mutant of 2D540	This study
X. campestris pv. translucens		
10D5	Wild-type isolate from wheat	M. P. Starr
10D5R	Spontaneous Rm^r mutant of 10D5	This study
X. campestris pv. vesicatoria		
6D53	Wild-type isolate	K. Kimble
6D53R	Spontaneous Rm^r mutant of 6D53	This study
Plasmids		
pUCD615	*lux* promoter probe; *ori*pSa *ori*pBR322 Ap^r Km^r (Gm^r)	32
pUCD206B	*cat* promoter probe; *ori*pSa *ori*pBR327 Ap^r Km^r (Nm^r)	4
pTZ18R	Ap^r	U.S. Biochemical Corp.[b]
pUCD607	*tet-lux* fusion; *ori*pSa *ori*pBR322 Ap^r Km^r (Gm^r)	33
pUCD656	pUCD615-based cosmid containing *hrpXc*, from 2D520 gene bank	35
pUCD2550	pTZ18R::pUCD656 11.0-kb *Eco*RI fragment	This study
pUCD2554	pUCD206B::pUCD2550 4.0-kb *Bam*HI-*Bgl*II fragment	This study
pUCD2556	pUCD206B::pUCD2550 0.6-kb *Bam*HI fragment	This study
pUCD2552	pUCD615::pUCD2550 4.0-kb *Bam*HI-*Bgl*II fragment	This study
pUCD2559	pUCD615::pUCD2550 0.6-kb *Bam*HI fragment	This study
pUCD682	pUCD2::*hrpXc* 4.4-kb *Xho*I-*Bgl*II fragment	35
pUCD685	*Sac*II deletion of pUCD682	35
pUCD645	pUCD615-based cosmid with Tc^r, from JS111 gene bank	35
pUCD646	*Bam*HI subclone of pUCD646 with Tc^r	J. Shaw

[a] Km(Gm) and Km(Nm) designate kanamycin resistance genes that also encode resistance to gentamicin and neomycin, respectively; Rm, Tc, and Ap designate rifampin, tetracycline, and ampicillin resistance genes, respectively; *ori* and a plasmid name indicate the source of the origin of replication.
[b] Cleveland, Ohio.

kanamycin (20 μg/ml), chloramphenicol (20 μg/ml), and ampicillin (50 μg/ml); the antibiotics used for *X. campestris* were rifampin (50 μg/ml), kanamycin (20 μg/ml), and tetracycline (5 μg/ml).

Enzymes and chemicals. Enzymes were obtained from New England BioLabs, Inc. (Beverly, Mass.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used as recommended by the manufacturer. Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

DNA manipulations. DNA manipulations were performed as described by Maniatis et al. (26). DNA fragments were isolated from agarose gels, using a Geneclean kit (Bio 101, La Jolla, Calif.).

Electroporation of *X. campestris* pv. campestris. We have developed the following DNA transformation procedure by electroporation for *X. campestris* pv. campestris. Bacteria are grown on medium 523 1.5% agar plates overnight, suspended in 9 ml of sterile distilled H_2O to an optical density at 600 nm of 1.0, and pelleted by centrifugation for 5 min at 15,000 × *g* at 4°C. Cells were resuspended in 1 ml of H_2O and washed again twice in the same volume. The final pellet was resuspended in 50 μl of H_2O, from which 40 μl was thoroughly mixed with 1 to 2 μl (approximately 50 to 100 ng) of plasmid DNA prepared from minipreparations. The mixture was introduced in the electroporation chamber and pulsed at 2.5 kV cm^{-1} with a pulse duration of 40 ms, using a Gene-Pulser (Bio-Rad Laboratories, Richmond, Calif.) (10). A 1-ml amount of SOB medium (29) was immediately added to the cells, and then the mixture was incubated with shaking for 2 h at 30°C before plating. Transformation frequencies as high as 10^6 transformants per μg of DNA were obtained for *X. campestris* pv. campestris 2D520X and derivative strains.

Enzymatic assays. Assays for pectate lyase activity were conducted on sodium polypectate medium (11, 19), extracellular protease activity was assayed on skimmed milk agar (6), cellulase activity was determined on carboxymethyl cellulose medium (11), amylase production was assayed on starch medium (41), and alkaline phosphatase activity was detected by adding 40 μg of 5-bromo-4-chloro-3-indolyl phosphate per ml to the medium (27). Maceration of potato tuber slices and detached plant leaves was determined as previously described (35). The chloramphenicol acetyltransferase (CAT) assay was performed as reported earlier (18).

Pathogenicity assays. Plant pathogenicity assays of *X. campestris* pv. campestris strains were conducted on cauliflower (*Brassica oleracea* L. var. *botrytis* cv. 'Early Super Snowball'; Park Seed Co.) and white radish (*Raphanus sativus* cv. 'White Icicle'; Burpee Co.) by whole plant wound inoculation (34). Pathogenic strains were scored as those producing black rot symptoms regardless of atypical symptoms. The assay for detached leaves was performed by infiltrating the petiole of white radish leaves with 500 μl of H_2O containing 10^7 cells of the strain(s) tested. Subsequently, the leaves were wrapped with plastic film and incubated at 23°C. Leaf blots were obtained by exposing the leaves to an X-ray film for 3 h at different times of incubation.

In planta growth. In planta growth was determined in cauliflower leaves as previously described (35). A 1:1 (cell/cell) mixture of two strains was used for the coinoculation experiments. The number of bacteria per leaf for each strain was determined by serially diluting macerated leaves in PM buffer (10 mM potassium phosphate [pH 7.2], 1 mM $MgCl_2$) and plating on medium 523 agar plates supplemented with the appropriate antibiotics. In the coinoculation experiments, JS111 populations were determined on rifampin and tetracycline plates, whereas differential enumeration (rifampin plates-rifampin and tetracycline plates) was used to estimate the number of wild-type derivatives. Statistical analysis of the data was performed by using Duncan's multiple-range test (24).

Hypersensitivity assays. Hypersensitivity tests were performed on the leaves of the following nonhost plants: tomato (*Lycopersicon esculentum* cv. 'Watters PF'; Harris Moran Seeds), jimson weed (*Datura stramonium*, UCD cultivation), and cucumber (*Cucumis sativus* cv. 'Strait Eight'; Northrup King Co.). A bacterial solution in sterile distilled water (10^8 cells per ml) was infiltrated in leaf panels as described by Klement et al. (20). HR was scored as a brown necrosis occurring in all of the infiltrated area 2 to 4 days after inoculation.

Plant induction Lux assay. Strains 2D520TL and JS111 were grown overnight on medium 523 agar plates from which cells were used to inoculate radish leaves by wounding the petiole (34). At times 0, 2, 10, and 24 h after inoculation, sections of the leaves were cut and emitted light was measured with a photon detector (Beckman Instruments, Inc., Fullerton, Calif.) (33). Bacterial populations were estimated after each leaf section was macerated in PM buffer, serially diluted, and plated on 523 agar plates supplemented with rifampin and kanamycin for 2D520TL or with rifampin and tetracycline for JS111. Light production by 2D520TL and JS111 ex planta was determined by inoculating 30 ml of 523 medium in Nephelo flasks to an initial optical density at 600 nm of 0.01 and monitoring both luminescence and growth at different times of incubation (32).

RESULTS

Phenotypic characterization of JS111. Tn*4431* mutagenesis of *X. campestris* pv. campestris 2D520 generated a nonpathogenic mutant, JS111, that does not incite any of the black rot symptoms on any of the cruciferous host plant tested, i.e., cauliflower, radish, cabbage, turnip, and *Arabidopsis thaliana* (S. Kamoun and C. I. Kado, unpublished data). We previously noted that both the wild type and JS111 produced similar amounts of exopolysaccharides and degradative enzymes and displayed similar growth rates when grown ex planta in the laboratory (35). Protease, pectate lyase, cellulase, amylase, and phosphatase activities along with maceration of potato tuber slices and detached cauliflower and radish leaves were identical between JS111 and its parental strain 2D520 (Table 2). These results indicate that the nonpathogenic mutant JS111 is distinct from the previously described pathogenicity mutants of *X. campestris* pv. campestris (6, 7).

Interaction of JS111 with nonhost plants. To determine whether JS111 is a Hrp or a Dsp mutant, the interaction of *X. campestris* pv. campestris 2D520 and JS111 with nonhost plants such as *D. stramonium* was investigated. When infiltrated in the leaves of *D. stramonium*, 2D520 induced a dark necrosis typical of an HR after 2 to 4 days (20). In contrast with the wild-type strain, JS111 was unable to cause

TABLE 2. Degradative enzyme production by the wild-type and JS111 strains

Substrate	Enzyme(s) assayed	Production[a]	
		Wild type	JS111
Skimmed milk	Protease	+	+
Sodium polypectate	Pectate lyase	+	+
Carboxymethyl cellulose	Cellulase	+	+
Starch	Amylase	+	+
X-phosphate	Phosphatase	+	+
Potato tuber	Various	SR	SR
Detached cauliflower or radish leaves	Various	SR	SR

[a] +, Enzymatic activity was consistently detected; SR, soft rotting of the plant tissue tested was observed. The enzyme(s) responsible for the soft rotting was not determined.

an HR when infiltrated in *D. stramonium* leaves (Fig. 1). Similar results were obtained with two other nonhost plants, tomato and cucumber. Since JS111 is nonpathogenic on host plants and unable to induce an HR on nonhost plants, it is a new member of the Hrp mutant family (23).

Exocellular complementation of JS111. JS111 showed very attenuated growth in cauliflower leaves and was never able to grow to more than 2.5×10^6 CFU per leaf, in contrast with the wild-type strain, 2D520, which reached a population level about 1,000 times higher than that of the mutant 8 days after inoculation (Fig. 2A). Interestingly, when a 1:1 (cell/cell) mixture of the wild type and JS111 was inoculated on cauliflower, the two strains displayed very similar growth curves. Equal populations of the wild type and JS111 were

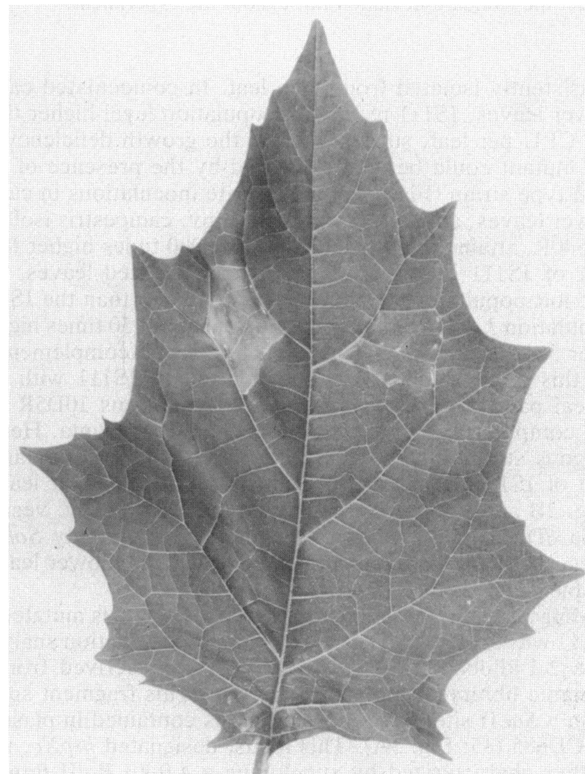

FIG. 1. HR of datura 4 days after inoculation with *X. campestris* pv. campestris 2D520 (left) and JS111 (right).

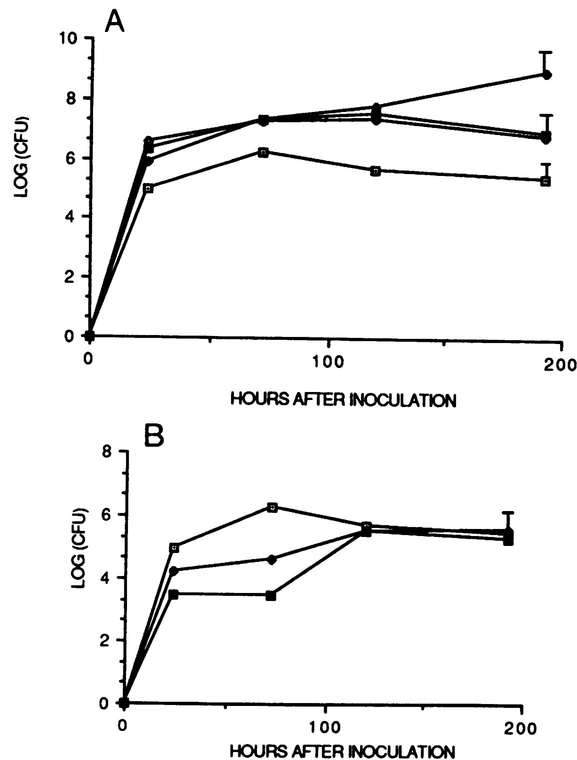

FIG. 2. Time course of the growth of *X. campestris* pv. campestris strains in separate and mixed inoculations in cauliflower leaves. (A) 2D520 (◆), JS111 (⊡), and mixed inoculations (2D520 [◇] and JS111 [■]). (B) 10D5R (◆) JS111 (⊡), mixed inoculations (JS111 [■]). Each point represents the average of three to six replicas. Bars reflect the standard deviation throughout the experiment.

consistently isolated from each leaf. In coinoculated cauliflower leaves, JS111 reached a population level higher than 10^7 CFU per leaf, suggesting that the growth deficiency of the mutant could be complemented by the presence of the wild-type strain (Fig. 2A). In separate inoculations in cauliflower leaves, another *X. campestris* pv. campestris isolate, 2D540R, attained a population level 1,000 times higher than that of JS111 in only 5 days. In coinoculated leaves, the 2D540R population was only 10 times higher than the JS111 population 3 days after inoculation and about 50 times higher after 5 days, suggesting that JS111 was also complemented by this strain (Table 3). Coinoculation of JS111 with the cereal pathogen *X. campestris* pv. translucens 10D5R did not complement the growth of the mutant in planta. Heterologous strain 10D5R reached a population size similar to that of JS111 5 days after inoculation in cauliflower leaves (Fig. 2B and Table 3). Similarly, *X. campestris* pv. vesicatoria 6D53R, a pathogen of members of the family *Solanaceae*, also did not complement JS111 in cauliflower leaves (Table 3).

Molecular analysis of the *hrpXc* locus. The locus mutated in JS111 was cloned and localized by complementation analysis to a 2.1-kilobase (kb) sector of a cosmid derived from a genomic library of the parental strain. This fragment spans from a *Sac*II site to a *Xho*I site and is contained in plasmid pUCD685 (35; Fig. 3A). This locus, designated *hrpXc*, was further characterized by subcloning a 4.0-kb *Bgl*II-*Bam*HI and a 0.6-kb *Bam*HI fragment from pUCD2550 into the *Bam*HI site of the broad-host-range promoter probe vector

TABLE 3. Growth of the different strains in separate and mixed inoculations in cauliflower leaves

Strain[a]	No. of bacteria/leaf[b] at given time after inoculation	
	72 h	120 h
2D520	4.0×10^7 A	2.1×10^8 A
JS111	2.3×10^6 C	1.3×10^6 BC
2D520 I	3.6×10^7 AB	5.7×10^7 A
JS111 I	2.8×10^7 AB	4.2×10^7 A
2D540R	2.1×10^8 AB	2.0×10^9 A
2D540R II	3.7×10^6 BC	1.2×10^8 A
JS111 II	3.2×10^5 C	2.5×10^6 B
6D53R III	3.4×10^4 D	3.8×10^5 CD
JS111 III	2.3×10^6 C	4.2×10^5 BCD
10D5R IV	5.3×10^3 E	5.1×10^5 BCD
JS111 IV	2.0×10^5 C	9.6×10^5 BC

[a] Strains followed by the same roman numeral were paired for the mixed-inoculation experiments.

[b] For each time point, values followed by the same letter did not differ significantly ($P = 0.05$) by Duncan's multiple-range test.

pUCD206B (4), yielding pUCD2554 and pUCD2556, respectively. These two plasmids were electroporated into JS111, and both JS111(pUCD2554) and JS111(pUCD2556) remained nonpathogenic, suggesting that the *Bam*HI site is located inside the *hrpXc* gene (Fig. 3A).

To determine the direction of transcription of *hrpXc*, *E. coli* and *X. campestris* pv. campestris strains containing pUCD2554 and pUCD2556 with bidirectional fusions of *hrpXc* to the promoterless *cat* gene of pUCD206B were assayed for CAT activity. No CAT activity was detected from *E. coli* HB101(pUCD2554). However, HB101(pUCD2556) showed CAT activity of about 10 nmol/min per mg (Fig. 3B). JS111 or 2D520 containing these two plasmids failed to produce any CAT activity, suggesting that the *hrpXc-cat* fusion is not expressed in *X. campestris* pv. campestris under these conditions. Subsequently, the 4.0-kb *Bgl*II-*Bam*HI and the 0.6-kb *Bam*HI fragments from pUCD2550 were also cloned into the *Bam*HI site of pUCD615 (32), yielding two plasmids, pUCD2552 and pUCD2559, respectively, that contain bidirectional fusions of the *hrpXc* gene to a promoterless *lux* operon. Light emission was detected only from HB101(pUCD2559) and 2D520(pUCD2559). Thus, the direction of transcription of the *hrpXc* locus is likely to occur from right to left, and the *hrpXc* promoter is probably located in the 0.6-kb *Bam*HI-*Eco*RI fragment (Fig. 3B).

The orientation of Tn*4431* in JS111 was determined by restriction enzyme analysis of pUCD645 and pUCD646. pUCD645 is a Tc^r cosmid isolated from a gene bank of JS111 (35). Subcloning of pUCD645 localized the Tc^r gene to a 17-kb *Bam*HI fragment contained in pUCD646. Further restriction enzyme mapping of pUCD646 showed that the promoterless *lux* operon of Tn*4431* is inserted in the same orientation as the *hrpXc* locus. Thus, JS111 contains a *hrpXc-lux* fusion, and expression of the *hrpXc* promoter could be assayed simply by measuring the luminescence of JS111 (Fig. 3B).

Analysis of *hrpXc* expression. To evaluate the level of expression of the *hrpXc* gene in rich media and in the plant, light emission from JS111 (*hrpXc-lux*) was compared with that from 2D520TL (*tet-lux*). 2D520TL contains one copy of plasmid pUCD607 (33) stably integrated in the chromosome and thus carries one copy of the *lux* operon fused to the constitutive promoter of a *tet* gene. In the first experiment, the bioluminescences of 2D520TL and JS111 were moni-

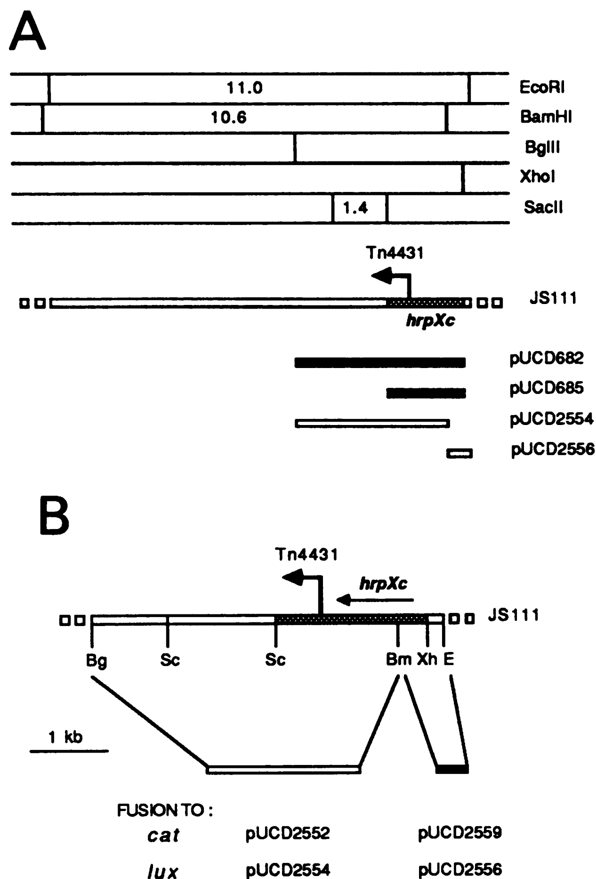

FIG. 3. Molecular analysis of the *hrpXc* locus. (A) Restriction map and complementation analysis of *hrpXc*. The restriction sites of the enzymes *Eco*RI, *Bam*HI, *Bgl*II, *Xho*I, and *Sac*II are shown. Numbers represent the estimated size of each fragment in kilobases. The location and orientation (relative to the promoterless *lux* operon) of Tn*4431* in the mutant JS111 are shown by the arrow. The different subclones constructed are shown as boxes that represent the size and location of the insert. Filled boxes represent complementing clones; open boxes represent noncomplementing clones. (B) Transcriptional analysis of *hrpXc*. The restriction map and location of Tn*4431* are represented as described above. The fragments fused to either *cat* or *lux* cassettes are represented by the boxes. Filled boxes represent constructs with either CAT or LUX activity; open boxes represent constructs that do not show any activity. The deduced size of the *hrpXc* gene is represented by the shaded area; and orientation is shown by the arrow.

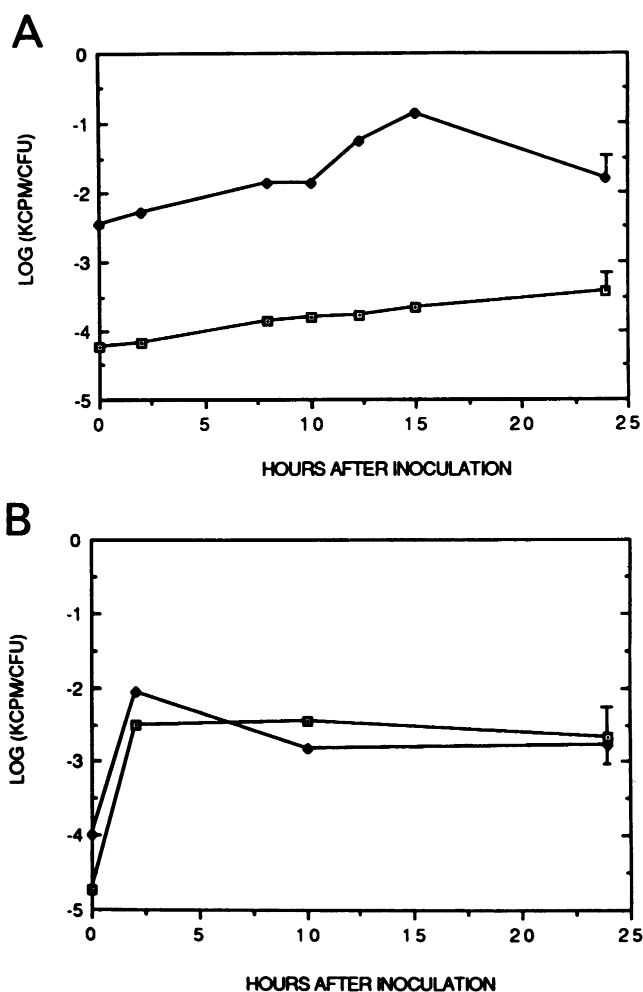

FIG. 4. Analysis of *hrpXc* and *tet* expression by using *lux* fusions in medium 523 (A) and in radish leaves (B). Symbols: ⊡, light emission from JS111 (*hrpXc-lux*); ◆, light emission from 2D520TL (*tet-lux*). Each point represents the average of three (A) or four (B) replicas. Bars reflect the standard deviation throughout the experiment.

tored in rich medium 523. 2D520TL reached light production levels as high as 10^{-1} kcpm/CFU, whereas JS111 only produced levels close to the background (about 10^{-4} kcpm/CFU) (Fig. 4A). A similar difference in the expression of the *hrpXc* and *tet* promoters was observed in the minimal medium 925 (data not shown). In a second experiment, both strains were separately inoculated into attached radish leaves, and light production from leaf sections was measured at different times and equilibrated to the number of bacteria. In this case and as early as 2 h after inoculation, 2D520TL and JS111 showed similar levels of bioluminescence varying around 2×10^{-3} kcpm/CFU (Fig. 4B). These results suggest that the *hrpXc* promoter is strongly induced and expressed at a constitutive level in radish leaves.

Since JS111 is strongly bioluminescent in the plant, it should be possible to monitor growth of the bacteria in the leaf simply by visualizing light production. Thus, detached radish leaves were inoculated with either JS111 or a 1:1 mix of JS111 and the wild type. The light produced by JS111 was visualized by exposing the leaves to an X-ray film at different times of incubation. The leaf blots obtained clearly indicated that the growth and invasiveness of JS111 in the xylem of radish leaves were greatly enhanced by coinoculation with the wild type (Fig. 5). These data confirm the exocellular complementation of JS111 by the wild-type strain earlier reported in attached cauliflower leaves (Fig. 2A) and clearly show that the *hrpXc* promoter is strongly expressed in radish xylem.

DISCUSSION

JS111 is a Hrp mutant. JS111 was shown to be similar to the wild-type strain in growth rate in rich or minimal medium, EPS production, production of degradative enzymes tested, and the ability to macerate potato tuber slices and detached host leaves. However, JS111 was unable to cause an HR on several nonhost plants, displayed attenuated

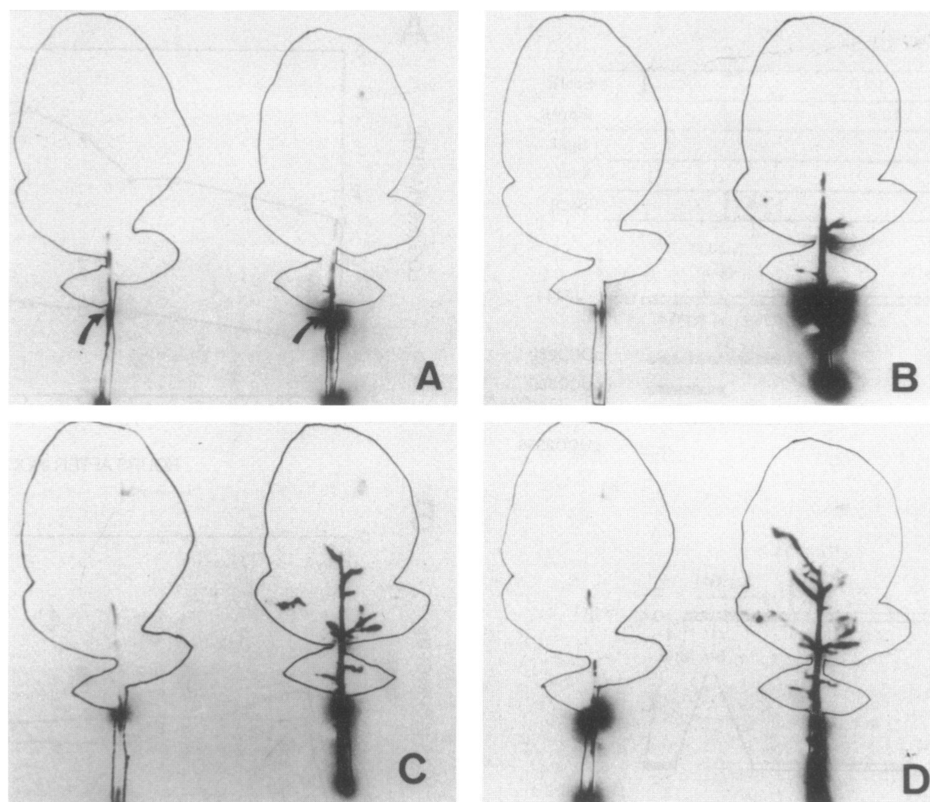

FIG. 5. Light leaf blots of detached radish leaves inoculated with JS111 (left) or a 1:1 (cell/cell) mixture of JS111 and 2D520 (right) determined at 1 (A), 4 (B), 5 (C), and 6 (D) days after inoculation at the position shown by the arrows. Light emission reflects metabolically active JS111.

growth in host leaves, and could not induce any black rot disease symptoms on any of the cruciferous hosts tested.

Pleiotropic mutants of *X. campestris* pv. campestris classified as nonpathogenic have been reported by Daniels et al. (6, 7, 9). These mutants were deficient in either the synthesis or the export of protease, amylase, pectate lyase, and cellulase and also in the production of EPS. Surprisingly, mutants deficient only in protease, cellulase, polygalacturonate lyase, or EPS production are still able to induce black rot (8, 11, 31, 35, 38); thus, the role of degradative enzymes and EPS in black rot disease is still unclear and could be minor. It has been proposed that degradative enzymes could help the bacteria survive during saprophytic growth on dead plant tissue (11). The nonpathogenic mutant described in our studies clearly showed that both degradative enzymes and EPS are still produced at wild-type levels, suggesting that virulence factors other than these products are essential for black rot induction.

Hrp mutants of several plant pathogenic bacteria have been described (1, 2, 22, 23, 30). In *Pseudomonas solanacearum*, two *hrp* clusters have been identified (16); interestingly, one of these clusters contained in the cosmid pVir2 was found to hybridize to the DNA of several *X. campestris* pathovars, including *X. campestris* pv. campestris (2). Even though these studies suggested the presence of *hrp* genes in *Xanthomonas* species, the mutant described herein is the first reported xanthomonad Hrp mutant. However, no cross-hybridization was detected between pVir2 and *hrpXc* (H. Kamdar, S. Kamoun, and C. I. Kado, unpublished data).

JS111 requires an exocellular component for growth in the host. JS111 displays attenuated growth in the plant similarly to a number of Hrp mutants (23). However, the growth and invasiveness of the mutant were enhanced by coinoculation with the wild-type strain 2D520 in cauliflower and radish leaves, suggesting that 2D520 can supplement JS111 with an exocellular component required for growth in the host and pathogenicity (Fig. 2A and 5). 2D540R, another isolate of *X. campestris* pv. campestris, also complemented the growth of JS111, suggesting that the *hrpXc* locus could be conserved among different strains (data not shown). However, heterologous strains *X. campestris* pv. translucens 10D5R and *X. campestris* pv. vesicatoria 6D53R did not enhance or complement the growth of JS111 in cauliflower leaves (Fig. 2B and Table 3). It is unclear whether this lack of complementation is due to the absence of a functional homolog of the *hrpXc* gene in these pathovars or to the inability of these strains to attain high population levels in cauliflower.

Whether the HR is caused directly by a protein product or by a metabolite synthesized by the bacteria remains to be determined. A 60-kilodalton extracellular protein from *P. solanacearum* was recently shown to induce an HR on potato (15). However, fenthion, a small insecticidal molecule, was also shown to cause a necrosis on tomato cultivars containing the bacterial resistance gene *pto* (H. Laterrot, TGC Rep. **35**:6, 1985), and the product of the *avrD* gene of *P. solanacearum* pv. tomato is predicted to possess a catalytic activity involved in the synthesis of a specific low-molecular-weight HR elicitor (M. M. Stayton, S. J. Tamaki, and N. Keen, Phytopathology **79**:1144, 1989). The results of the coinoculation experiments conducted in this study suggest

that the *hrpXc* gene may encode an exocellular compound that is different from the major degradative enzymes and is required for both pathogenicity on crucifers and HR on nonhost plants. However, it is still unclear whether the same component is actually responsible for both phenotypes.

The *hrpXc* promoter is strongly induced in radish leaves. Complementation analysis of the *hrpXc* locus suggests that the size of this gene is between 1.0 and 2.1 kb. Transcriptional analysis showed that the orientation of *hrpXc* is identical to that of the promoterless *lux* operon in the Tn*4431* inserted in JS111. Thus, JS111 contains a chromosomal *hrpXc-lux* fusion that was used to study the regulation of the *hrpXc* promoter. In minimal or rich medium, the level of expression of the *hrpXc* promoter was about 2 to 3 orders of magnitude lower than that of the constitutive promoter *tet*, whereas in radish leaves the two promoters displayed similar activities, suggesting that the expression of *hrpXc* is highly induced when *X. campestris* pv. campestris is present in radish xylem (Fig. 4). Recently, a plant-inducible region (*hrpABCD*) was identified in the *hrp* cluster of *P. solanacearum* pv. phaseolicola by using an ice nucleation locus (*inaZ*) as a reporter gene (21). Expression of these genes appeared to be dependent on another locus, *hrpS*, whose predicted protein product shows strong homology to several procaryotic regulatory proteins (12). However, expression of the *hrpABCD* genes did not appear to require specific plant substances but rather seemed to be dependent on nutritional and physiological factors that the bacteria encounter in the apoplast (21). We are currently investigating whether the high level of expression of *hrpXc* in the plant is actually dependent on induction by a plant product or on some nutritional or physiological conditions specific to the xylem.

The use of a nondisruptive detection system, such as bioluminescence, in the study of plant-microbe interactions has many potential applications (33). In this study, for example, a *lux* system was successfully used to monitor the active invasiveness in radish leaves of an *X. campestris* pv. campestris nonpathogenic mutant under different conditions (Fig. 5) and also to determine the level and strength of expression of a *hrpXc* promoter in vitro and in radish xylem, a natural ecological niche for the bacteria (Fig. 4B). These results confirm our previous contention of the originality and usefulness of bioluminescence as a tool to study plant-microbe interactions.

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