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Transposon Tn*4431* Mutagenesis of *Xanthomonas campestris* pv. *campestris*: Characterization of a Nonpathogenic Mutant and Cloning of a Locus for Pathogenicity

Joe J. Shaw, Lynette G. Settles, and Clarence I. Kado

Department of Plant Pathology, University of California, Davis CA 95616, U.S.A.
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Mutants of *Xanthomonas campestris* pv. *campestris*, the causal agent of black rot of crucifers, were isolated by transposon mutagenesis with Tn*4431*, which was carried on the suicide vector pSa325. This novel transposon was constructed from a derivative of Tn*1721* and contains a promoterless luciferase (*lux*) operon of *Vibrio fischeri*, which serves as an unique reporter gene, and a gene that confers tetracycline resistance. Tn*4431* facilitates the *in vivo* construction of transcriptional fusions between the *lux* operon and bacterial promoters. A nonpathogenic mutant, JS111, was isolated that was able to grow in the excised leaves of cauliflower (like the parent strain) but was unable to grow in attached leaves (unlike the parent strain). JS111 was complemented to full pathogenicity with a clone from a cosmid genomic library of the parent strain. The complementing locus was identified in a 2.1-kb genomic DNA fragment.

Additional keywords: black rot, cauliflower, luciferase, *lux* operon, transposon Tn*4431*

Black rot of crucifers, a worldwide and economically important plant disease (Williams 1980), is caused by *Xanthomonas campestris* pv. *campestris* (Russel 1898; Young *et al.* 1978). Unlike most *Xanthomonas* diseases, black rot is a vascular wilt, sometimes affecting plants in a one-sided fashion reminiscent of fungal wilts (Smith 1911; Wallis *et al.* 1973). The nature of wilt induction by *X. c.* pv. *campestris* is not understood, but the elaboration of extracellular polysaccharides has been suggested to be involved (Sutton and Williams 1970a, 1970b).

The mechanism by which black rot is induced by *X. c.* pv. *campestris* is not understood. Proteolytic and pectolytic enzymes produced and secreted by *X. c.* pv. *campestris* were demonstrated to be involved in the destruction of plant tissue (Daniels *et al.* 1984a, 1984b). However, these same enzymes are produced by a number of bacteria associated with plants, including purely saprophytic bacteria, which are incapable of causing disease on crucifers (Collmer *et al.* 1982; Perombelon 1982), indicating that the elaboration of degradative enzymes alone is insufficient to cause black rot. Moreover, these enzymes were implicated in connection with symptoms not characteristic of black rot (i.e., tissue masceration, water soaking, and seedling collapse).

Mutants of *X. c.* pv. *campestris* defective in extracellular polysaccharide production have been complemented and the respective genes have been cloned recently (Harding *et al.* 1987), but the role of these genes in pathogenesis remains uncertain. Certain *X. c.* pv. *campestris* DNA promoters of unknown function have been cloned and shown to be induced when bacteria are infiltrated into the mesophyll of turnip leaves (Osbourn *et al.* 1987), but their role in pathogenesis is undefined. Tn*5* and Tn*7* transposon mutagenesis of *X. c.* pv. *campestris* has been reported (Turner *et al.* 1984) but was deemed unsuitable for experimental work because of low transposition frequency, instability, or preferential insertion sites.

To obviate the unsuitability of Tn*5* and Tn*7*, we have constructed a novel transposon, Tn*4431*, for use in the genetic analysis of pathogenicity in *X. c.* pv. *campestris*. Tn*4431* is not a Tn*5* or Tn*7* derivative but is derived from Tn*1721*. It contains the promoterless luciferase (*lux*) operon of *Vibrio fischeri* and a tetracycline resistance gene. The *lux* "cassette" has been oriented so that transcriptional fusions between the *lux* operon and bacterial gene promoters are facilitated when the transposon inserts into the bacterial chromosome. Tn*4431* was constructed to take advantage of bacterial bioluminescence that has been demonstrated to be a powerful tool in the study of plant-bacterial associations, providing *in planta* data about the location of bacteria during the ongoing process of infection and movement in the host (Shaw and Kado 1986) and their gene expression (Rogowsky *et al.* 1987). Tn*4431* is carried on pSa325 (Zaitlin 1985), a "suicide" vector enabling efficient transposition into recipient cells. Part of this work was presented at the Third International Symposium on the Molecular Genetics of Plant-Microbe Interactions in Montreal, Canada (1986).

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* HB101 (Boyer and Roulland-Dussoix 1969) and DH1 (Hanahan 1983), hosts for all hybrid plasmids, were grown on Luria-Bertani (LB) medium (Miller 1972) at 30° C. These bacteria are resistant to streptomycin (Sm) and nalidixic acid (Nd), respectively, and were routinely cultured on these antibiotics. *E. coli* RR1 (Bolivar *et al.* 1977) was used as a plasmid host in experiments designed to allow homologous DNA recombination between plasmids. For genetic selection, the following antibiotics (Sigma) were used: kanamycin (Km) 15 μg/ml, ampicillin (Ap) 50 μg/ml, tetracycline (Tc) 10 μg/ml, spectinomycin (Sp) 40 μg/ml, Sm 40 μg/ml, Nd 30

Present address of first author: Department of Plant Pathology, University of California, Riverside CA 92521.

μg/ml, and chloramphenicol (Cm) 30 μg/ml. Broth was supplemented with 1.5% Bacto agar (Difco) when plates were used. *X. c.* pv. *campestris* 2D520, which is fully pathogenic and resistant to rifampicin (Rm) was routinely cultured at 30°C on medium 523 (Kado *et al.* 1972) supplemented with 1.5% Bacto agar and Rm (50 μg/ml). Plasmid pRU675 containing the transposon Tn*1731*-Ba152, a derivative of Tn*1721* (Rogowsky 1985), was provided by R. Schmidt. Plasmids pRK2013 (Figurski and Helinski 1979), pUCD320 (Shaw and Kado 1986), pSa (Tait *et al.* 1982, Ireland 1983), pUCD615 (Rogowsky *et al.* 1987), pUCD2 (Close *et al.* 1984), and PSa325 (Zaitlin 1985) are described elsewhere.

Phenotypic characterizations. Assays for pectate lyase activity were performed on Na-polypectate medium (Keen *et al.* 1984) and extracellular protease activity was assayed on milk agar (Daniels *et al.* 1984b). The production of precipitable extracellular polysaccharides was assayed by use of hexadecyl trimethyl ammonium bromide (HTAB) essentially as described by Close *et al.* (1987). The ability to mascerate potato tuber slices was as described earlier for enteric soft rot organisms (Kelman and Dickey 1980). Bioluminescence determinations were performed visually, photographically, and electronically as reported previously (Shaw *et al.* 1987).

Conjugations. Triparental matings were conducted with overnight cultures as follows: 1-ml portions of bacteria in early stationary phase (10^9 cells per milliliter) were pelleted by centrifugation (15,600×*g*, 1 min), rinsed twice in LB, and resuspended to their original volumes in LB. Equal amounts (200 μl) of donor, mobilizing strain HB101 (pRK2013), and recipient were mixed and 100 μl was placed on sterile 0.2-μ filters on LB plates. The filters were placed at 30° C for 48 hr, and the bacteria were streaked onto appropriate selective media. Biparental matings were conducted similarly except that HB101 (pRK2013) was omitted.

Construction of Tn*4431*. A 7.5-kb *Bam*HI fragment of pUCD320, containing the promoterless *lux* operon (Fig. 1), was inserted into the unique *Bam*HI site of pRU675 to produce the plasmid pUCD620. The new transposon, Tn*4431*, was transposed to the plasmid pSa325 by transformation of pUCD620 into HB101 (pSa325) and subsequent mating with DHI and selection for Nd^r, Cm^r, and Tc^r. An Sm sensitive clone was analyzed and determined to contain pUCD623.

Transposition mutagenesis. The original transposon, Tn*1721*, transposes best at 30°C at a frequency of 10^{-6} to 10^{-7} depending on the strain used. All tranpositions were performed as follows: the donor strain HB101 (pUCD623) was allowed to conjugate with *X. c.* pv. *campestris* on filters, and after 48 hr the mixture was spread onto 523 agar supplemented with Rm and Tc. Colonies appearing were restreaked several times, then checked for light production.

Plant hosts and inoculation procedures. Cauliflower (*Brassica oleracea* L. var. *botrytis* L. cv. Early Super Snowball, Park Seed Co., Greenwood, SC) was used as the host plant in all pathogenicity assays. Seeds were germinated in the greenhouse and transferred to individual pots (5-in. diameter) before the development of true leaves (10–14 days). At transplantation the plants were supplemented with fertilizer (10-10-10, NPK). Inoculations were made when the plants were at the four to six leaf stage, on leaves three, four, and five (counting acropetally).

Wound inoculations were made by jabbing a sterile needle into bacteria on plates or in buffer (0.7% NaCl, 0.115% K_2HPO_4, 0.02% KH_2PO_4, 0.02% KCl) (5×10^7 colony forming units [cfu] per milliliter) and then pricking the petiole of a cauliflower leaf in three places. Alternatively, bacteria in buffer were inoculated into wounds by injecting approximately 10 μl into petioles with a sterile syringe and needle (27 gauge). Leaf infiltration assays were conducted essentially as described by Daniels *et al.* (1984a) on both attached and detached leaves.

DNA manipulations. *E. coli* cells were made competent, stored, and transformed according to the method of Close and Rodriguez (1983). Small amounts of plasmid DNA were isolated from *E. coli* and *X. c.* pv. *campestris* 2D520 by the Froman method (Tait *et al.* 1982) or the method of Kado and Liu (1981). In the latter case, the DNA was prepared for enzymatic digestion or use in transformations by further extraction with phenol and chloroform (1:1, v/v) and precipitating and washing with ethanol and resuspending the pellet in sterile H_2O. DNA endonucleases and T4 ligase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). All enzymatic reactions were performed essentially as described by Maniatis *et al.* (1982) except for preparation of radiolabeled DNA.

Construction of genomic libraries. Total cellular *X. c.* pv. *campestris* DNA was extracted as follows: 250-ml overnight cultures were pelleted (10,000×*g* 10 min) and rinsed in 50 ml of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5), repelleted, and resuspended in 50 ml of TE. Five milliliters of pronase (50 mg/ml) was added and 1 ml of RNase (5 mg/ml). Twenty milliliters of Na-lauryl sarcosyl (5% in TE, w/v) was gently mixed in, and the cells were placed at 37°C for 1 hr. The lysate was extracted three times with a mixture of phenol and chloroform (1:1, v/v) and once with chloroform alone. The chromosomal DNA was recovered from the lysate by CsCl gradient centrifugation as described by Maniatis *et al.* (1982). Chromosomal DNA was partially digested with Sau3A restriction endonuclease, and fragments 25–35 kb in size were recovered by sucrose density gradient centrifugation (Maniatis *et al.* 1982). The partially digested chromosomal DNA was then ligated into the *Bam*HI site of the cosmid vector pUCD615 and packaged using a lamda DNA packaging kit (Amersham). Transfections were performed according to manufacturers' specifications. Two libraries were constructed; the first one was made from the chromosomal DNA of the transposon mutant JS111 and the second from the chromosomal DNA of *X. c.* pv. *campestris* 2D520. Serial dilution and plating of the genomic library revealed that approximately 1,000 separate phage particles had infected HB101 with cosmid DNA from JS111, whereas 10,000 had infected HB101 with cosmid DNA from *X. c.* pv. *campestris* 2D520. Ten independent clones were selected containing DNA from *X. c.* pv. *campestris* 2D520, and their DNA digestion pattern indicated that all 10 contained substantially different inserts with an average size of 27 kb.

Southern blots and colony hybridization. DNA fragments were separated by electrophoresis in 0.7% agarose gels (Sea-Kem) and transferred to Zeta-Probe membranes (Bio-Rad Laboratories, Richmond, CA) by blotting according to manufacturers' instructions. Colony/Plaque Screen membranes (New England Nuclear, Boston, MA) were used for colony hybridization of *E. coli* and *X. c.* pv. *campestris* 2D520. DNA was radiolabeled with [α-^{32}P]deoxycytosine triphosphate by the oligolabeling procedure (Feinberg and Vogelstein 1984). Oligolabeling reagents were from Pharmacia Inc. (Piscataway, NJ) and used according to manufacturers' instructions.

RESULTS

Transposon mutagenesis. Several hundred separate matings were performed between *E. coli* HB101 (pUCD623) (Fig. 1) and *X. c.* pv. *campestris* 2D520. All Tc^r exconjugants emitted photometrically detectable light indicating the presence of the transposon, in contrast to *X. c.* pv. *campestris* 2D520, which is dark and emits no detectable light. A wide range of light-producing mutants were observed, the brightest of which emitted about 15,000 times as much light as the dimmest (Table 1). Twenty randomly selected exconjugants were sensitive to Ap and Cm, demonstrating the loss of the delivery vector (pSa325). Furthermore, colony hybridization of 18 other exconjugants demonstrated that the bacteria had acquired DNA homologous to the transposon and had not retained portions of the plasmid vector pSa325 (data not shown).

Tn*4431* mutagenesis produced a number of phenotypic variants, although most of the transposon mutants (approximately 95%) were normal with respect to the visual and biochemical tests (Table 1) and none were detected that were unable to degrade sodium polypectate or that lacked extracellular protease activity or that could not mascerate potato tuber slices. The most commonly altered phenotype was growth rate.

Three phenotypes, 1) slow growth, 2) nonmucoid colony (dry), and 3) auxotrophy, were associated with attenuated virulence. Thus, while these mutants were pathogenic, i.e., could cause black rot, they did so at less than 50% of the inoculation sites: this is in contrast to the parent strain that produces disease at virtually all inoculation sites (Table 1). The dry mutants were found to be deficient in the production of extracellular polysaccharides as judged by HTAB precipitation. A single mutant was recovered that produced white colonies instead of the usual yellow and this mutant was fully virulent.

A nonpathogenic mutant, JS111, was recovered that resembled the parental strain in most respects but was unable to cause black rot on cauliflower (Table 1). In 150 separate wound inoculations, JS111 was never observed to cause vein blackening or leaf chlorosis or necrosis of leaf panels when inoculated onto plants, symptoms characteristic of black rot. Localized tissue necrosis did not occur when JS111 was infiltrated into the mesophyll of leaves still attached to plants, in contrast to the parent strain 2D520. However, when infiltrated into the mesophyll tissue of detached leaves, extensive soft rot was observed that also occurred when the parent strain was similarly inoculated.

Complementation of JS111. A Tc^r clone was selected from the cosmid library of JS111 chromosomal DNA. The 16-kb cosmid insert that contained part of Tn*4431* (7.5 kb) and flanking JS111 DNA sequences (8.5 kb) was used to probe the gene bank of the parental strain 2D520. Twelve colonies were recovered that hybridized specifically to the probe. All 12 clones were mated into JS111 and the exconjugants were inoculated onto cauliflower. Three of the exconjugants were able to cause vein blackening and leaf necrosis on cauliflower although black rot did not result at all inoculation sites. Thus, each of the three cosmid clones (pUCD648, pUCD652, and pUCD656) restored the ability to cause black rot to JS111, but with reduced virulence (Fig. 2).

Growth *in planta*. Three mutants and the wild-type strain 2D520 were selected for study. JS153 is a Tn*4431* induced mutant that is fully pathogenic, JS111 is a Tn*4431* induced mutant that is nonpathogenic and JS111 (pUCD652) is

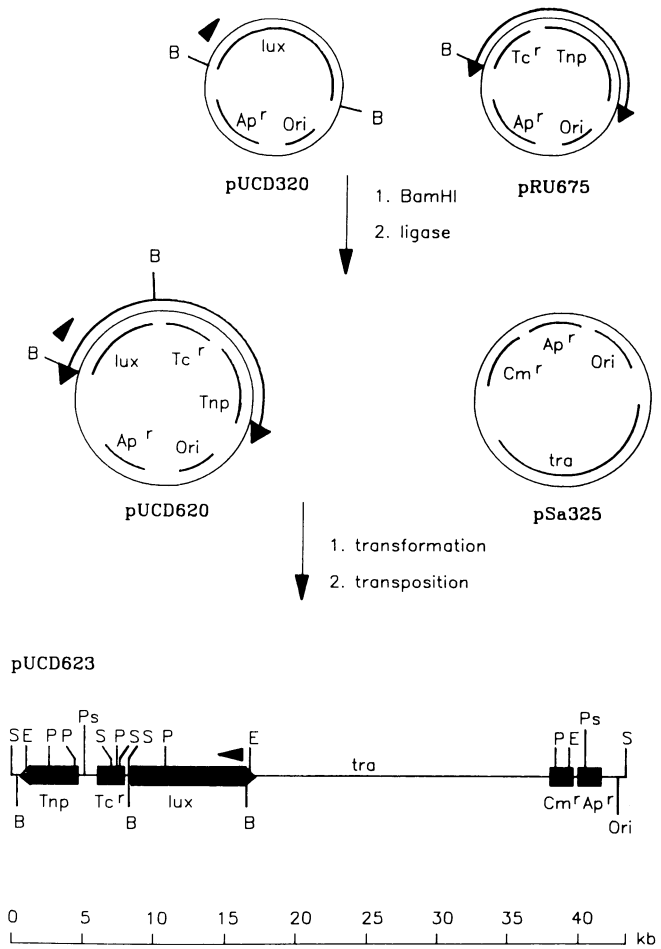

Fig. 1. The 7.5-kb *Bam*HI fragment of pUCD320 that contains the *lux* operon was transferred into the unique *Bam*HI site of Tn*1731*-Ba152 that resides on pRU675 and making the plasmid pUCD60. Transposition of Tn*4431* from pUCD620 to PSa325 resulted in the plasmid pUCD623. *Eco*RI sites are located within at both ends of the transposon (15 base pairs from the end) within the inverted terminal repeats. The *lux* operon was inserted at a *Bam*HI site that is 50 bases inside one end of the transposon. The small arrow defines the direction of transcription in the *lux* operon. Tnp indicates the genes from Tn*1721* that are required for transposition and Ori denotes the Col E1 origin of DNA replication. Restriction sites are as follows: B (*Bam*HI), E (*Eco*RI), P (*Pvu*II), Ps (*Pst*I), S (*Sal*I). Circular plasmids are not to scale.

Table 1. Tn*4431* induced mutations in *Xanthomonas campestris* pv. *campestris*

Mutant	Colony phenotype[a]	Growth rate	Prototrophic growth	Pathogenic ability	Relative bioluminescence
2D520 (parent)	+	+	+	+	0
JS4	+	slow	+	attenuated	1
JS5	+	+	−	attenuated	45
JS20	+	slow	+	attenuated	178
JS111	+	+	+	nonpathogen	414
JS128	+	+	+	+	5,040
JS153	+	+	+	+	403
JS156	dry	+	+	attenuated	72
JS167	dry	+	+	attenuated	21
JS241	+	slow	+	attenuated	3,850
JS252	+	slow	+	attenuated	651
JS255	+	slow	+	attenuated	113
JS260	white	+	+	+	175
JS414	+	+	+	+	15,100

[a] A positive sign indicates that the response was the same as the parent strain, 2D520. Slow refers to colonies that have doubling times greater than 4 hr; the doubling time of the parent strain is 2.3 hr.

complemented with the wild-type allele in pUCD652. Bacteria in buffer (5×10^7 cfu/ml) were inoculated via syringe into the petioles of the third and fourth leaves of young cauliflower plants. At intervals the leaf laminae were harvested and extracted for bacteria. The leaf macerate was serially diluted and plated to determine the number of bacteria in the leaf. Within 24 hr all four strains were recoverable in the leaf blade (several centimeters from the inoculation site) in approximately equal numbers (Fig 3). However, JS111 was not recovered in increasing numbers at later sampling times, in contrast to 2D520 and JS153, which increased more than 1,000-fold, and JS111 (pUCD652), which increased 100-fold (Fig. 3). However, all strains grew well *in planta* when infiltrated into the mesophyll of detached leaves, growing to greater than 10^9 bacteria per leaf over a 1-wk period.

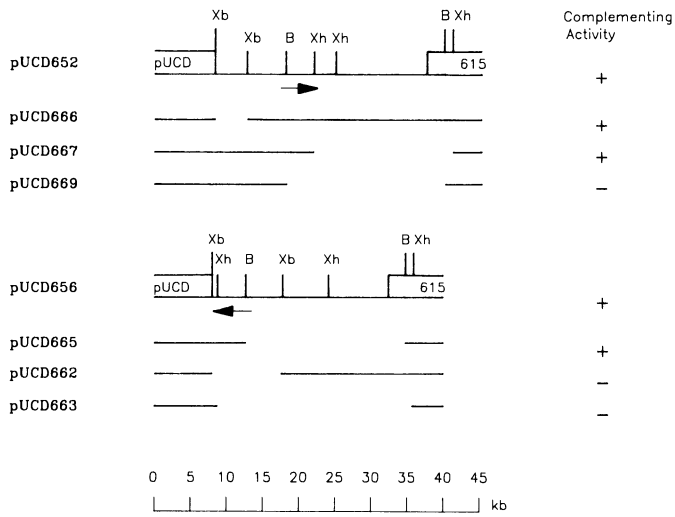

Fig. 2. Plasmids pUCD652 and pUCD656 both complement JS111 and contain overlapping fragments of DNA in opposite orientations. The deletion derivatives are shown directly beneath each plasmid. Plasmids pUCD662 and pUCD666 are *Xba*I deletions, pUCD663 and pUCD667 are *Xho*I deletions, pUCD665 and pUCD669 are *Bgl*II deletions. The arrow indicates the 4.4-kb fragment of DNA that is common to all cosmids that complement JS111 (i.e., pUCD652, pUCD666, pUCD667, pUCD656, and pUCD665). Plasmids pUCD652 and pUCD666 are incompletely mapped with respect to *Bgl*II. Restriction sites are as follows: B (*Bgl*II), Xb (*Xba*I), Xh (*Xho*I).

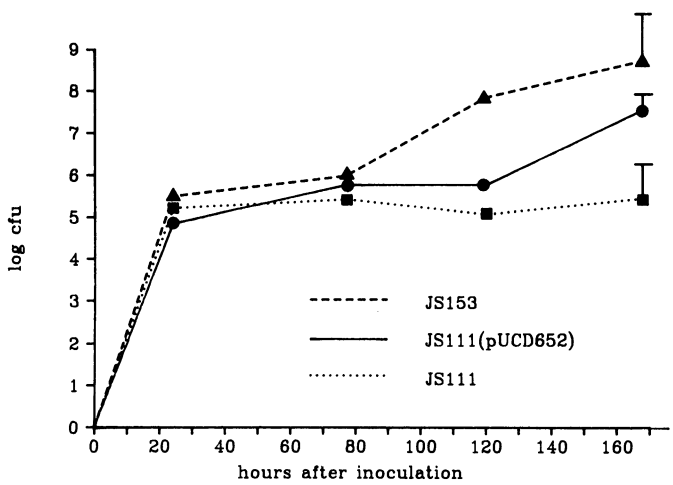

Fig. 3. Growth *in planta*. Tn*4431* induced mutant JS153 causes strong black rot in cauliflower; JS111 is nonpathogenic and JS111 (pUCD652) is complemented with the wild-type allele in pUCD652. Bacteria were inoculated into cauliflower petioles from agar plates. At the indicated times, leaves were harvested (no petiole), mascerated, serially diluted, and plated. Each point represents the average value from three or four separate leaves and the standard deviation bars at the last point are representative of the variation throughout the experiment. No bacteria were recovered at zero time. The growth of 2D520 superimposes that of JS153 and therefore was not included in the figure.

Analysis of complementing plasmids. Electrophoretic analysis of DNA restriction fragments revealed that pUCD648 and pUCD656 are very similar plasmids, whereas pUCD652 is substantially different. Plasmids pUCD652 and pUCD656 were selected for further study and were found to contain inserts that overlapped by approximately 10 kb (Fig. 2). Also, these inserts were found to be cloned in opposite orientation in the cosmid vector, pUCD615. Several deletion derivatives of pUCD652 and pUCD656 (Fig. 2) were constructed and mated into *X. c.* pv. *campestris* 2D520. A 4.4-kb fragment of DNA, which complemented JS111, was identified that carried all of the activity associated with the larger cosmid inserts.

This 4.4-kb fragment of DNA was subcloned in both orientations into the *Sal*I site of pUCD2 as an *Xho*I fragment, creating the plasmids pUCD681 and pUCD682 (Fig. 4). Two deletions were made in pUCD682 resulting in the plasmids pUCD684 and pUCD685 (Fig. 4). To allow biparental matings, recombination between the pUCD681, pUCD682, pUCD684, and pUCD685 and pSa was allowed to occur. The recombinants were designated pUCD689, pUCD690, pUCD695, and pUCD696. As a control, the recombinant plasmid pUCD692 (pSa::pUCD2) was constructed. The five cointegrate, self-transmissible plasmids were introduced into JS111, which was then assayed for pathogenicity. By this analysis a 2.1-kb fragment of DNA (carried on pUCD695) was identified that complemented JS111. As before, the complementation was partial because black rot was observed at less than 50% of all inoculation sites.

Plasmid stability. Because the various plasmids had only partially restored the virulence of JS111, we wanted to assess the replication properties and stability of pSa and its

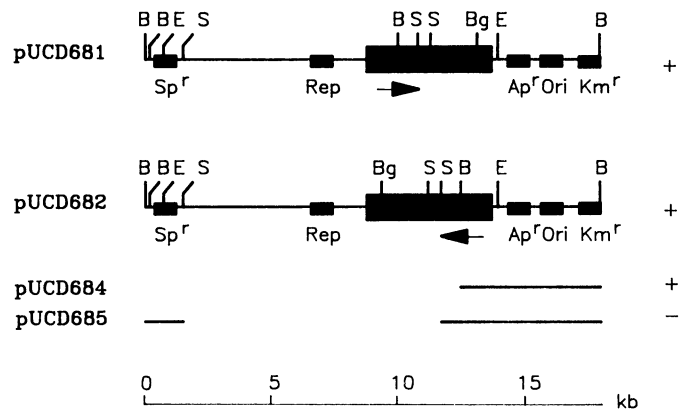

Fig. 4. pUCD681 and pUCD682. An *Xho*I DNA fragment, containing the 4.4-kb region from pUCD665 (depicted by arrow in Fig. 2) that complemented JS111, was cloned into the *Sal*I site of pUCD2 (Close *et al.* 1984) in both orientations, resulting in the plasmids pUCD681 and pUCD682. The two deletion derivatives of pUCD682 are shown directly beneath as pUCD684 and pUCD685. pUCD685 is an *Sst*II deletion and pUCD684 is a *Bst*EII deletion. The large rectangle denotes the DNA cloned from pUCD665 and was placed in opposite orientations in pUCD2. Cointegrate forms of the plasmids with pSa were mated into JS111 and assayed for complementation. pUCD681, pUCD682 and pUCD684 were all able to restore JS111 to pathogenicity. pUCD684 contains only 2.1 kb of *X. c.* pv. *campestris* DNA. Rep indicates the pSa origin of DNA replication and Ori denotes the Col E1 origin, restriction sites are as follows: B (*Bst*EII), Bg (*BGl*II), E (*Eco*RI), S (*Sst*II).

cosmid derivative (pUCD615) in *X. c.* pv. *campestris*. Unlike pSa, pUCD615 was not seen in visual analysis of miniscreen DNA in gel electrophoresis. However, pUCD615 was recovered infrequently when miniscreen DNA of *X. c.* pv. *campestris* was used to transform *E. coli*. The recovery by transformation was approximately 5% of that obtained for pSa.

X. c. pv. *campestris* 2D520 containing pUCD615 was inoculated into plants and recovered at intervals. Within 2 wk, up to half of the bacteria had lost the plasmid as judged by phenotypic selection on antibiotics. Similar results were obtained when bacteria were cultured in the absence of antibiotic selection in 523 medium. These results suggest that the pUCD615 replicates in *X. c.* pv. *campestris* but that it is unstable.

The plasmid pSa was found to be entirely stable in culture with or without antibiotic selection, but both it and the cointegrate plasmids (pUCD689, pUCD690, pUCD692, pUCD695, and pUCD696) were rapidly lost when *X. c.* pv. *campestris* was inoculated into plants. After only 20 generations of *in planta* growth, more than 95% of the bacteria had lost pSa as judged by selection on antibiotics, and similar results were obtained for the cointegrate plasmids.

Loss of Tn*4431* by homologous recombination. Southern blot analysis of JS111 DNA showed that Tn*4431* interrupted a 10.5-kb *Eco*RI/*Xba*I fragment of DNA at a point 1.6 kb from one end (Fig. 5, lanes a and c). JS111 (pUCD666) (Fig. 2) contained the uninterrupted 10.5-kb DNA fragment (plasmid borne), as well as the two transposon-generated fragments of DNA (8.9 and 1.6 kb) (Fig. 4, lane d).

JS111 (pUCD666) was inoculated into plants and bacteria were recovered from the plant after 14 days. Unlike previous experiments in which many of the recovered colonies were Km^s, only 3% (of 200 colonies) were Km^s and Ap^s indicating that only a few bacteria had lost the plasmid markers. When further tested, these colonies were also found to be Tc^s and Lux^-, demonstrating that the phenotypic markers characteristic of the transposon had been lost as well as the plasmid (pUCD666). One strain, JS111C, was picked for further study. Southern blot analysis indicated that JS111C had lost both the transposon and pUCD666 and that the interrupted fragment of DNA had been restored to its original size (Fig. 5, lane b). When reinoculated back into plants, these bacteria were fully pathogenic and fully virulent, indistinguishable from *X. c.* pv. *campestris* 2D520. In a subsequent test, where the inoculated plants were quarantined to prevent any possibility of cross inoculation, a similar strain was recovered, i.e., Tc^s, Km^s, Ap^s, Lux^-, and fully virulent.

DISCUSSION

Based on the types of mutants generated, Tn*4431* appears to insert into a wide variety of regions of the chromosome of *X. c.* pv. *campestris*. This is corroborated by the diverse bioluminescent abilities of the mutants, ranging continuously from virtually undetectable to very bright. It is interesting to note that all transposon mutants were detectably bioluminescent. This is in accord with previous observations in *E. coli* and *V. fischeri*; the presence of the *lux* operon can always be detected by the production of light if sensitive photomultipliers are used (Shaw *et al.* 1987). This is presumed to be due to low level, read-through transcription that occurs throughout the bacterial chromosome; that it can be detected at all is due to the unique nature of bioluminescence.

Tn*4431* causes transcriptional fusions between the *lux* genes and bacterial promoters. This feature is useful in the study of gene regulation *in planta* much the same way as the transposable element, Mini-Mu-Lux (Engebrecht *et al.* 1985), and work has been done to explore this alternative (Settles and Kado, unpublished data). Moreover, the transposon was useful in generating very brightly bioluminescent forms of *X. c.* pv. *campestris* that are visually detectable. These mutants have proven useful in observing the movement of bacteria in plants during black rot (Shaw and Kado 1987) and may be useful in following bacteria during the more cryptic phases of the disease, such as survival in seed and on alternate hosts.

X. c. pv. *campestris* mutants fell into three broad categories with respect to pathogenicity: 1) normal pathogenic abilities, 2) pathogenic but attenuated virulence, and 3) nonpathogenic. The first group was by far the largest and included very brightly bioluminescent isolates and a white mutant. From this result we infer that large portions of the genome of *X. c.* pv. *campestris* are not immediately required in order to cause black rot.

The second group (attenuated virulence) included about 5% of all mutants and these fell into three types: 1) auxotrophy, 2) slow growing mutants, and 3) mutants altered in the production of extracellular polysaccharides. These mutants were able to cause black rot but not at all inoculation sites. The slow growing mutants are presumed to have increased doubling times in the plant, and the reduced virulence may simply be a reflection of this slower growth. The uncharacterized auxotroph, JS5, grew normally on rich medium, but its reduced virulence may reflect a limiting supply of a critical nutrient as it grows in the host plant.

The correlation between reduced production of extracellular polysaccharides and reduced virulence has been reported (Corey and Starr 1957; Goto 1972), but it is

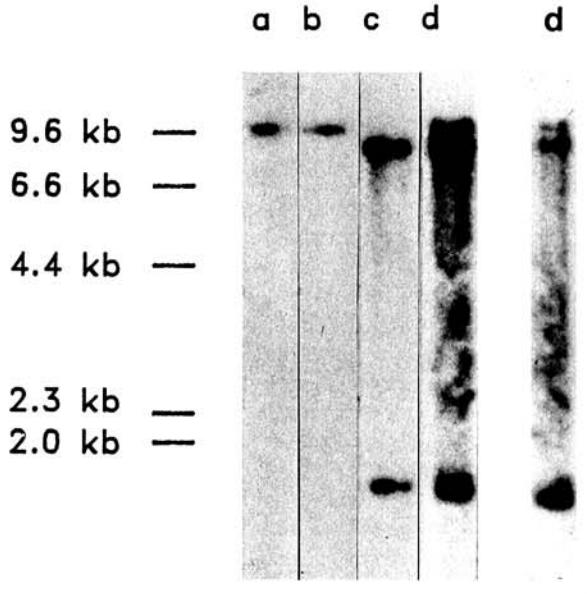

Fig. 5. Southern blot analysis. Genomic DNA was digested with *Eco*RI, *Xba*I, blotted and hybridized with the 4.4-kb fragment of DNA common to all complementing cosmids (Fig. 2). (a) *X. campestris* pv. *campestris* 2D520, (b) JS111C, (c) JS111, (d) JS111 (pUCD666). Lanes a and b each have a single 10.5-kb band, lane c has two bands (8.9 and 1.6), and lane d has three bands (10.5, 8.9, and 1.6).

unclear if the association is causal or not. Complementation of our mutants with recently cloned genes involved in xanthan production (Harding *et al.* 1987) might restore them to full polysaccharide production and virulence.

The most interesting mutant was the nonpathogen, JS111. This mutant was similar to *X. c.* pv. *campestris* 2D520 in most respects, including degrading Na-polypectate, producing extracellular proteases, elaborating extracellular polysaccharides, and displaying normal growth rates. In fact, both strains grew well in excised cauliflower leaves and caused extensive soft rot and water soaking similar to the observations we reported earlier for other *X. c.* pv. *campestris* isolates, *Erwinia carotovora* and *Pseudomonas fluorescens* (Shaw 1987). Most noteworthy was the dramatic difference when the bacteria were infiltrated into leaves still attached to plants: JS111 was unable to cause the death of the tissue in the infiltrated area. Although these effects are noteworthy, it should be pointed out that *X. c.* pv. *campestris* 2D520 cannot produce black rot when inoculated in this manner (i.e., infiltration) (Shaw 1987). Thus, bacteria are introduced into tissue they do not normally invade (i.e., mesophyll), and symptoms develop that are not typical of black rot. The relationship of these symptoms to black rot is not understood, if indeed there is one.

Other researchers have reported *X. c.* pv. *campestris* mutants that are attenuated or nonpathogenic and have cloned and characterized some of the genes involved (Daniels *et al.* 1984b). However, the pathogenicity assays involved included the use of very young plants, inoculating into tissues normally not affected (i.e., mesophyll parenchyma) by *X. c.* pv. *campestris*, which is generally restricted to the xylem (Russel 1898; Smith 1911; Wallis *et al.* 1979), and artificially high atmospheric humidity. Furthermore, unusual symptoms were reported in those studies (Daniels *et al.* 1984a, 1984b), including water soaking and tissue masceration, which are not symptoms of black rot but which can be caused by a number of different bacteria, *X. c.* pv. *campestris* included (Shaw 1987). In these cases there were no differences reported with respect to intact plants and harvested plant parts. Our mutant, JS111, efficiently mascerated detached cauliflower leaves (and potato tuber slices) when infiltrated into the mesophyll but did not affect attached leaves when inoculated similarly. Noteworthy is the fact that JS111 cannot cause black rot that is separate and distinct from tissue masceration and soft rot.

There may be be two types of genes in *X. c.* pv. *campestris* that are important in plant-bacterial interactions: 1) genes that control general traits that are shared with other plant associated bacteria, including saprobes, and 2) genes that are required for the specific ability to grow in intact cauliflower leaves and cause black rot. Mutations in genes belonging to the first category might be responsible for some of the *X. c.* pv. *campestris* mentioned above that are altered in the ability to produce or secrete degradative enzymes. These enzymes might be of enormous value during the stages of the black rot disease cycle when the bacteria must survive without causing black rot, perhaps as seed contaminants, on alternate hosts, or in debris. The mutant reported here, JS111, probably belongs to the second class and is specifically deficient in the ability to grow in the host and cause black rot.

When inoculated into the petiole wounds, JS111 was recovered 24 hr later from the leaf laminae, but subsequent growth did not occur. Therefore it is interesting to know how the initial population of JS111 cells came to be in the leaf laminae. We think that the bacteria were initially moved by the transpiration stream from the point of inoculation and propose three facts to explain this: 1) the bacteria that were recovered did not further increase their numbers showing that growth did not occur (at least at later times); 2) the numbers recovered, while ultimately low, were initially quite high considering that the increase from zero must have occurred in less than 24 hr, showing that growth alone could not account for the phenomenon; and 3) it has been established that *X. c.* pv. *campestris* can move short distances in the transpiration stream of crucifers (Smith 1911).

Therefore, when JS111 was complemented with the DNA from pUCD652, both growth in the host and symptom induction returned, perhaps indicating either 1) that continued growth in the host is necessary for symptoms to become apparent or 2) that symptoms (e.g., tissue death) are a prerequisite for growth in the host.

The complementation that was observed with the 2.1-kb fragment of DNA was partial because JS111 was able to cause black rot symptoms but not at all inoculation sites. This could be due to several different reasons: 1) the vector (pUCD615 or pSa cointegrates) replicated poorly and as such did not provide a steady or stable dose of the necessary gene products, 2) the complementing fragment was incomplete and did not completely correspond to the transposon induced lesion, and 3) the complementing fragment works poorly in trans.

Plasmid pSa itself replicates stably in culture or in *X. c.* pv. *campestris* 2D520 and is not lost with or without antibiotic selection pressure. In contrast, both pSa and the cointegrate plasmids, although stable under selection in culture, were unstable *in planta*. This shared feature could be due to several reasons: 1) a previously reported instability associated with the Cm resistance gene of pSa (Ireland 1983), 2) *in planta* growth conditions (such as increased generation time) may contribute to plasmid loss in a general way, or 3) a specific pSa encoded function somehow encourages plasmid loss during growth in the host. At this time there is insufficient evidence to establish which, if any, of these possibilities is operative.

There is evidence that the cosmid vector, pUCD615, replicates poorly in *X. c.* pv. *campestris*. The plasmid is lost quickly in culture or in the plant, and the plasmid was not visible in agarose gels and was only infrequently recovered by transformation of *E. coli*. The basis of this replication and maintenance deficiency is not understood, as the cosmid pUCD615 is known to replicate well in *Agrobacterium* (Rogowsky *et al.* 1987). It may simply be that *X. c.* pv. *campestris* requires certain pSa encoded functions for normal plasmid maintenance that are not required in *Agrobacterium* or that the presence of the Col E1 origin of DNA replication interferes with normal plasmid maintenance. The latter explanation would also explain the results for pSa cointegrates (above) but not for pSa.

The second explanation for partial complementation (incomplete complementing fragment) is unlikely, because of the large size of the inserts in pUCD652 and pUCD656 and the 10-kb overlap. This, coupled with the small size identified by deletion analysis (2.1 kb), which complements as well as the large inserts, indicates that the transposon induced lesion has probably been completely cloned. Thus, while several possibilities exist, and cannot be formally ruled out at this time, we conclude that poor plasmid replication and maintenance probably explains the partial

complementation.

Complementation was complete, however, in the case of JS111C, which is fully virulent. This strain was derived from JS111 (pUCD666) and was apparently constructed by double recombination events between the plasmid and the bacterial chromosome. In effect, the mutated DNA from the chromosome was transferred to the plasmid and the cloned (wild type) plasmid DNA was reciprocally transferred to the chromosome. This demonstrates that the cloned DNA in pUCD666 corresponds to the region interrupted by Tn*4431*.

JS111 is an interesting mutant because it is normal in most respects (see above) but is absolutely nonpathogenic if assayed for black rot. It grows well on host tissue but only if excised from the plant. This observation could possibly be explained by a host defense mechanism that is operative in attached leaves and not in detached leaves. If so, JS111 may prove to be a useful tool in the further study of cauliflower disease defenses.

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