MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 3, No. 5, pp. 280-285, 1990

Identification and DNA Sequence of a Pathogenicity Gene of *Xanthomonas campestris* pv. *campestris*

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Received 9 November 1989. Accepted 20 March 1990.

A region of *Xanthomonas campestris* pv. *campestris* DNA containing at least two pathogenicity genes was identified. Mutants in one gene were clearly reduced in pathogenicity while mutants in the other were only moderately reduced. Both classes of mutants were prototrophic and motile, and had wild-type levels of extracellular enzymes and extracellular polysaccharide. They also grew *in vitro* and *in planta* at the same rate as the wild type. Experiments involving one of the clear pathogenicity mutants indicated that the recovery of mutant cells from turnip seedlings 24 hr after inoculation was lower than for the wild type. This may be due to cell death as a result of action by some preformed or induced plant factor. From DNA sequencing an open reading frame was identified that encompassed the site of the mutations giving a clear reduction in pathogenicity. The predicted protein sequence had no homology with other proteins in the computer data base.

Additional keywords: *Brassica*, promoter.

In a previous study (Osbourn *et al.* 1987), we reported the identification of DNA fragments from *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson having enhanced promoter activity *in planta*. Recent work has been directed toward determining whether the genes associated with these plant-inducible promoters are involved in pathogenicity. This was achieved by testing the effects on pathogenicity of Tn*5*-induced mutations in DNA in and around two of these promoters; these mutations were generated by marker exchange mutagenesis (Turner *et al.* 1985). Marker exchange mutants in DNA immediately downstream from one of these promoters (cloned as pIJ3109, Osbourn *et al.* 1987) had delayed symptom expression in tests for determining pathogenicity; this was likely to be due to their reduced growth rate *in vitro* (Osbourn *et al.* 1988).

In this study, we describe the effect of introducing mutations into DNA in and around a second plant-inducible promoter fragment (cloned as pIJ3107, Osbourn *et al.* 1987). We could not detect any phenotypic changes arising from the insertion of Tn*5* into DNA within and in close proximity to this promoter; however, mutations in a region of DNA more than 3 kilobases (kb) away from the promoter did result in a reduction in pathogenicity. These mutations cannot be under the direct control of the plant-inducible promoter, and so the identification of this pathogenicity region is likely to have been fortuitous.

Here we describe in detail the phenotype of the pathogenicity mutants and show that they retain the properties of the wild-type strain in a range of tests for factors which are likely to be important for pathogenesis (such as production of extracellular polysaccharide and plant cell wall degrading enzymes). The relevant region of DNA was sequenced, and from a knowledge of the G + C content and codon usage of *X. c.* pv. *campestris* DNA, it was possible to identify the most likely potential open reading frame (ORF) in this region. However, the predicted amino acid sequence was not homologous with any other peptide in the computer data base.

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Nucleotide and/or amino acid sequence data is to be submitted to GenBank as accession number JO3684.

MATERIALS AND METHODS

Microbiological methods. Plasmids and bacterial strains used in this study are listed in Table 1. Standard media, antibiotic concentrations, and cultural and mating procedures are described by Turner *et al.* (1984, 1985). Antibiotic concentrations for pIJ3100 and its derivatives are given in Osbourn *et al.* (1987). Turner *et al.* (1985) give details of transposon mutagenesis and marker exchange of Tn*5* insertions into the *X. c.* pv. *campestris* genome; the site of insertion was verified for marker exchange mutants 12 and 29 by DNA hybridization analysis.

DNA analysis. Plasmid DNA was isolated using either the rapid-boiling method (Holmes and Quigley 1981) or the alkaline treatment method (Birnboim and Doly 1979). Chromosomal DNA was extracted using the method of Daniels *et al.* (1984a). Digestion of DNA with restriction enzymes and cloning procedures followed the methods of Maniatis *et al.* (1982), as did the procedures of Southern blotting, nick translation, and hybridization. For sequencing, DNA was cloned into the pBluescript KS+ and KS− M13 vectors (Stratagene, La Jolla, CA), and nested deletions were generated using exonuclease III (Henikoff 1984). Preparation of single-stranded templates followed instructions given by Stratagene, and these were sequenced using Sequenase (United States Biochemical Corporation, Cleveland, OH) as directed by the supplier.

Assays for determining pathogenicity. Turnip cultivar Just Right was used in all except the seed-soaking assays.

Seedling assays. Assessment of pathogenicity to turnip seedlings was described by Daniels *et al.* (1984b). After incubation seedlings were scored for disease symptoms and assigned to one of three categories: no symptoms (0), localized brown lesion (+), and rotten (++). The numbers of seedlings in each category were then converted to percentage values, and the three values for each mutant were compared with the values for the wild type. Each set of mutant values was tested separately against the wild-type values using a contingency test to obtain a value for chi-squared, and hence a *P* value.

Multiplication of bacteria in turnip seedlings was assessed by inoculating with standardized bacterial suspensions in the usual way. At intervals of 1 to 3 days after inoculation, batches of five seedlings were harvested and ground in a glass homogenizer. The homogenate was made up to 1 ml with sterile distilled water and vortexed; serial dilutions were plated onto selective medium to enable numbers of viable bacteria per seedling to be determined. The homogenate was also plated onto NYGA (Turner *et al.* 1984) without antibiotics to ensure that no other micro-organisms were present in the seedlings.

The ability of the bacteria to spread within turnip seedlings was measured as follows. Seedlings were inoculated from liquid suspensions using the method of Daniels *et al.* (1984b), and groups of three seedlings per strain were sampled 1, 2, and 3 days afterward to determine how far the bacteria had migrated from the point of inoculation. This was assessed by cutting with a sterile scalpel across the stem at approximately 1-mm intervals both above and below the inoculation point and touching the cut stem ends onto nutrient agar containing rifampicin (100 μg/ml). After a 2-day incubation period at 32° C, bacterial colonies were visible when stem sections containing bacteria had been touched onto the agar. Stem sections from water-inoculated seedlings did not give rise to any bacterial colonies.

Infiltration of mature leaves. Inoculation by infiltration of bacterial suspensions through stomata was described by Osbourn *et al.* (1987). This method was also used to determine whether mutants still elicited a hypersensitive response on tobacco plants.

Inoculation of leaf margins. Inoculation of mature leaves by nicking the vein ends while submerging the leaves in inoculum was described by Gough *et al.* (1988), as was the subsequent measurement of the spread of bacteria within the leaf.

Seed soaking. Inoculation of the radish cultivar Sparkler by seed soaking also followed the method of Gough *et al.* (1988).

Detection of extracellular enzyme activity in cellfree liquid culture medium. Relative activities of endoglucanase, amylase, and protease were measured by diffusion assays into substrate-containing agar plates (Dow *et al.* 1987; Tang *et al.* 1987). Polygalacturonate lyase (PGL) was assayed spectrophotometrically using either polygalacturonic acid or a turnip cell wall preparation to induce the enzyme (Dow *et al.* 1987).

Measurement of PGL activity *in planta*. Cultivar Just Right turnip seedlings were inoculated as above. After incubation for 7 days, batches of five seedlings were homogenized together in 1 ml of sterile water, with duplicate batches for each strain of *X. c.* pv. *campestris*. Aliquots of the homogenate were serially diluted and inoculated onto NYGA (containing rifampicin, 100 μg/ml) for estimating cell numbers. The rest of the homogenate was centrifuged to pellet bacterial cells and plant debris, and 50-μl aliquots of the supernatant were assessed for PGL activity as above.

Measurements of growth rates in liquid culture. Cultures of *X. c.* pv. *campestris* were grown overnight in nutrient broth (NYGB, Turner *et al.* 1984) with shaking at 28° C. Aliquots of 2.5 ml were then subcultured into prewarmed 50-ml batches of NYGB and returned to the shaker. The optical density at 600 nm was measured at intervals.

Assessment of extracellular polysaccharide production. Strains of *X. c.* pv. *campestris* were spotted onto nutrient or minimal agar supplemented with 2% glucose, and colonies were assessed for slime production after 2–4 days of incubation at 32° C (Barrère *et al.* 1986).

Table 1. Plasmids and bacterial strains used in this study

Designation	Relevant characteristics[a]	Reference
Plasmids		
pIJ3100	Sm^r Cm^s IncQ replicon (promoter probe plasmid)	Osbourn *et al.* 1987
pIJ3107	pIJ3100 containing a plant-inducible *Xanthomonas campestris* pv. *campestris* promoter	Osbourn *et al.* 1987
pLAFR3	Tc^r Tra^- Mob^+ RK2 replicon	Staskawicz *et al.* 1987
pIJ3121	pLAFR3 clone containing *X. c.* pv. *campestris* DNA obtained by probing the library with PIJ3107	Osbourn *et al.* 1987
pIJ3122	Subclone of pIJ3121	This study
pBluescript KS+/− M13	Ap^r $LacZ^+$ M13IG ColE1 replicon	Stratagene[b]
Escherichia coli		
ED8767	RecA *met*	Murray *et al.* 1977
X. c. pv. *campestris*		
8004	Rif^r	Turner *et al.* 1984
8004-zzz	8004::Tn*5*-zzz, produced by marker exchange from pIJ3122::Tn*5*-zzz	This study

[a] Sm, streptomycin; Cm, chloramphenicol; Tc, tetracycline; Ap, ampicillin; Rif, rifampicin; r, resistant; and s, sensitive.
[b] Stratagene, La Jolla, CA.

RESULTS

Subcloning and mutagenesis of *X. c.* pv. *campestris* DNA in and around the plant-inducible promoter. A pLAFR3 clone (pIJ3121) with homology to the plant-inducible promoter in pIJ3107 (insert size 0.75 kb) has already been identified (Osbourn *et al.* 1987). DNA of this clone was digested with the restriction enzyme *Eco*RI to give a total of seven fragments, and the one 6.2-kb fragment that still hybridized to pIJ3107 was extracted from agarose using Geneclean (Bio-101, La Jolla) and ligated with pLAFR3 DNA which had been digested with *Eco*RI and treated with alkaline phosphatase. After transformation into strain

ED8767 of *Escherichia coli* (Migula) Castellani and Chalmers, a subclone with the appropriate restriction fragment was identified. This subclone was designated pIJ3122.

pIJ3122 was mutagenized with Tn*5*, and individual transposon insertions were located within the *X. c.* pv. *campestris* DNA by mapping with restriction enzymes. Southern blots of digested DNA from clones with differently positioned Tn*5* insertions were probed with the plasmid containing the plant-inducible promoter (pIJ3107) to locate the promoter within pIJ3122. The approximate positions in pIJ3122 of the promoter and of the Tn*5*-induced mutations are given in Figure 1. Mutations 12 and 29 were indistinguishable by restriction enzyme mapping, and they may be siblings. Selected Tn*5* insertions were introduced into the wild-type *X. c.* pv. *campestris* (8004) genome by the marker exchange procedure. The orientation of the promoter relative to its orientation in the promoter probe plasmid was determined by hybridization of similar Southern blots with restriction fragments containing the proximal and distal portions of the promoter, respectively; this enabled the direction of the promoter activity in pIJ3122 to be determined (Fig. 1).

Phenotype of marker exchange mutants. Assessment of pathogenicity of some of the marker exchange mutants in the pIJ3122 region to turnip seedlings is presented in Table 2. Tn*5*-induced mutations in and within 3 kb downstream from the promoter region (mutations 5, 21, 17, 8, and 20) had no effect on pathogenicity; the disease levels for mutants at these positions were not significantly different from that of the wild type at the 5% level. However, mutations approximately 3 kb away from the promoter resulted in a moderate reduction in pathogenicity (for example, mutant 34), while marker exchange mutants more than 4 kb away (mutants 12 and 29) were clearly less pathogenic than the wild-type strain (8004) (Table 2, Fig. 1). The disease levels for both moderately and clearly reduced mutants were all significantly different from that of the wild type at the 0.1% level, and the clearly reduced mutants were both significantly less pathogenic than the moderately reduced mutants at either the 0.1 or 1% level (Table 2). All mutants listed in Table 2 were tested again with essentially the same outcome. In further experiments, mutants 12 and 29 (both tested an additional five times) and mutant 34 (tested twice more) were always significantly less pathogenic than the wild type at the 0.1% level, while mutants 12 and 29 were significantly less pathogenic than mutant 34 at the 0.1% level in the two experiments where all three mutants were tested together.

Pathogenicity mutants 12, 29, and 34 were all able to spread through turnip seedlings at rates similar to that of the wild type. These mutants retained the wild-type ability to elicit a hypersensitive response when inoculated into tobacco leaves (other mutants were not tested). In addition, mutant 29 was tested in a range of other qualitative assays for determining pathogenicity that involved inoculation of mature turnip leaves at wound sites made at the leaf margins, infiltration of intracellular spaces with bacterial suspensions, or inoculation by soaking in bacterial suspensions. Each test was conducted at least twice. The mutant was reduced in pathogenicity in all of these tests (data not shown).

The rate of increase in cell numbers of pathogenicity mutants in turnip seedlings did not appear to differ from that of the wild type over a period of 3 days after inoculation (data not shown). However in studies involving pathogenicity mutant 29, the total number of cells recovered from the seedlings 24 hr after inoculation was always lower than for the wild type. When compared to the number of wild-type cells recovered, the mutant cells were only 11.1% ± 4.3% of this value (an average value from three separate experiments). Similar trends were apparent for pathogenicity mutants 12 and 34, but further experiments are needed to establish the significance of these observations. There were no differences between the wild type and mutants in the number of colony forming units in the inoculum prior to introduction into seedlings. No differences in the level of growth were observed when the wild type and mutant 29 were inoculated into minimal medium broth. Also, incubation of these two strains with a plant extract made from homogenized turnip seedlings did not reveal any differential killing or inhibition of growth of the mutant.

All marker exchange mutants in the pIJ3122 region were prototrophic, had wild-type growth rates in nutrient broth,

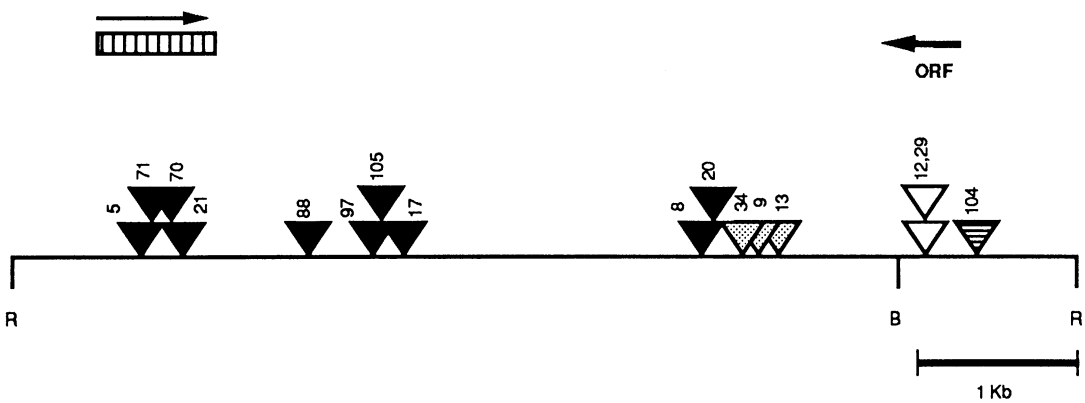

Fig. 1. Physical map of pIJ3122, the region of *Xanthomonas* DNA showing homology with the plant-inducible *Xanthomonas* promoter fragment from pIJ3107 (Osbourn *et al.* 1987). The position of the promoter is shown by the striped rectangle, and the direction in which it functions is indicated by an arrow. Tn*5* insertions that have a clear or partial effect on pathogenicity, or no effect at all, when introduced into the wild-type genome by recombination are represented by open, stippled, or solid triangles, respectively. A marker exchange mutant was not obtained for Tn*5* insertion 104 (striped triangle). The position and direction of the pathogenicity gene open reading frame (ORF) are indicated by the solid arrow.

and were motile (observed by microscopy). They also produced wild-type levels of the extracellular enzymes endoglucanase, amylase, protease, and PGL, as well as extracellular polysaccharide. Mutants 12, 29, 34, and 17 were tested for their ability to synthesize and export PGL *in planta*, and all had activities similar to that of wild-type strain 8004 (data not given). This experiment was repeated and the same results were obtained.

Location of the pathogenicity gene ORF and its promoter. A double-stranded DNA sequence was obtained for the region of DNA progressing inward from the right side of pIJ3122 to 300 nucleotides beyond the *Bam*HI site (Fig. 2). An ORF either 459 or 504 nucleotides long (depending on the position of the initiation codon) was identified that spanned the *Bam*HI site and the site(s) of insertion of mutations 12 and 29 (Fig. 1). Analysis of the sequence using the Frame program (Bibb *et al.* 1984) was consistent with the presence of an ORF in this region. The direction of the ORF was from right to left as shown in Figure 1, that is, it was transcribed toward the plant-inducible promoter, and so could not be under its control. The region of pIJ3122 in which mutations gave rise to a partial reduction in pathogenicity lay outside this ORF.

A 1-kb fragment extending from the internal *X. c.* pv. *campestris* *Bam*HI site in the pathogenicity gene rightward in Figure 1 to a *Bam*HI site just inside the vector polylinker was cloned into the promoter probe plasmid pIJ3100 (Osbourn *et al.* 1987) to test for transcriptional activity through the pathogenicity gene. No activity was detected for either orientation *in vitro* or in seedlings using the methods of Osbourn *et al.* (1987), suggesting that the promoter for the pathogenicity gene was not present in pIJ3122. To test DNA further upstream from this gene, an overlapping 5.2-kb *Bam*HI fragment (extending rightward in Fig. 1 from the same internal *Bam*HI site to an *X. c.* pv. *campestris* *Bam*HI site 4.2 kb outside pIJ3122) was subcloned from the original cosmid library clone pIJ3121 into the promoter probe plasmid and tested for activity. The fragment had strong constitutive promoter activity when *X. c.* pv. *campestris* transconjugants harboring the constructs were tested on chloramphenicol gradient plates (Bryson and Szybalski 1952), conferring resistance to more than 30 μg of chloramphenicol per milliliter of agar (the wild type was sensitive to less than 5 μg/ml). This activity was only evident when the fragment was orientated such that the direction of the pathogenicity gene ORF was in accordance with that of the reporter gene. No significant level of promoter activity was detected in *E. coli* regardless of the orientation of the fragment.

The extent of the ORF. The DNA sequence of the pathogenicity gene ORF (extending upstream to the site of Tn*5* insertion 104) is given in Figure 2. There are two possible sites for initiation of translation. The first of these is the ATG triplet at nucleotide 177 (Fig. 2). This does not have a consensus ribosome binding site (Stormo *et al.* 1982). However the start codon is overlapped by the triplet TAA giving a sequence TAATG, which may be the termination codon for a preceding gene. Thus, the requirement for a ribosome binding site may be circumvented

Table 2. Pathogenicity of some of the Tn*5* marker exchange products obtained for the pIJ3122 region[a]

Strain	% of seedlings showing symptoms[b]			Effect of mutation on pathogenicity
	0	+	++	
Wild type (8004)	0.0	16.7	83.4	—
8004-5	0.0	18.6	81.4	No reduction
8004-21	0.0	16.9	83.0	No reduction
8004-17	0.0	17.6	82.4	No reduction
8004-8	0.0	29.2	70.8	No reduction
8004-20	0.0	15.4	84.6	No reduction
8004-34	5.9	45.1	49.0	Partial reduction[c]
8004-9	0.0	46.9	53.1	Partial reduction[c]
8004-13	2.4	38.1	59.5	Partial reduction[c]
8004-12	22.7	50.0	27.3	Clear reduction[c]
8004-29	21.6	31.4	47.1	Clear reduction[c]

[a]The marker exchange mutants are listed in the order in which their corresponding Tn*5* insertions map (from left to right) in Figure 1.

[b]Approximately 50 seedlings per strain were tested. Symptoms were assessed on a 0 to ++ scale as follows: 0, no symptoms; +, localized brown lesion; and ++, rotten. (Percentage values do not always add up to exactly 100 due to rounding up.)

[c]Indicates deviation at $P < 0.1\%$ from the null hypothesis that the mutant and wild-type reactions are the same. In addition, mutant 12 was significantly less pathogenic than mutants 34, 9, and 13 at $P < 0.1\%$; mutant 29 was significantly less pathogenic than mutants 9 and 13 at $P < 0.1\%$, and than mutant 34 at $P < 1.0\%$.

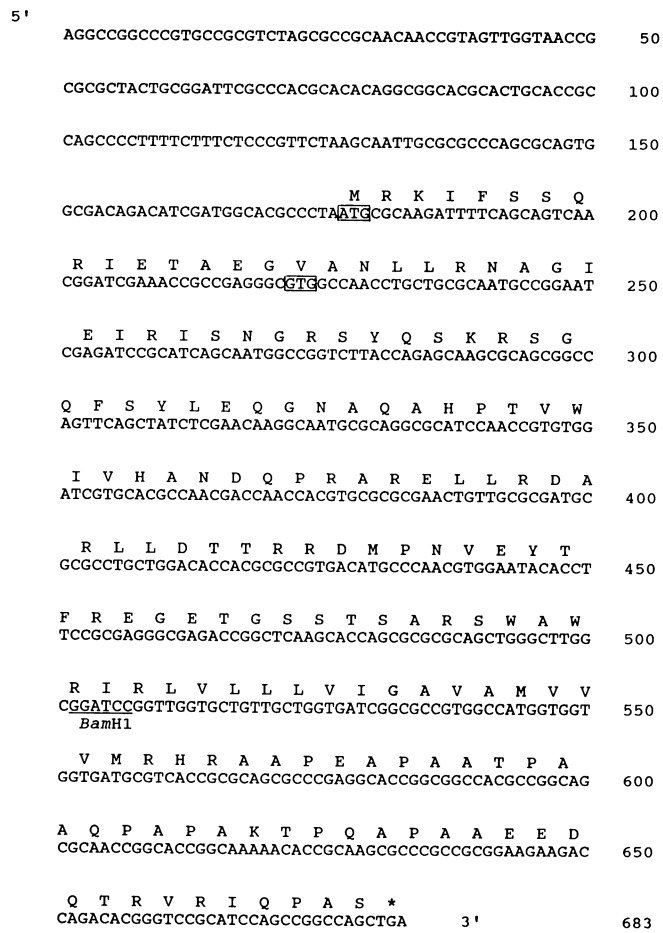

Fig. 2. DNA sequence and predicted amino acid sequence of the pathogenicity gene open reading frame. The two possible translational start codons are boxed, and the stop codon is indicated by an asterisk.

by translational coupling; a ribosome terminating translation of the preceding gene would be in a position to immediately initiate translation of mRNA of the pathogenicity gene (Oppenheim and Yanofsky 1980). Another possible initiation codon is the GTG triplet at nucleotide 222. GTG is a recognized start codon in *Streptomyces* (Hopwood *et al.* 1986), and its use may also be relatively common in *X. c.* pv. *campestris* DNA (which, like *Streptomyces*, is rich in guanine and cytosine). The GAGGG sequence extending from six to two nucleotides before the GTG codon may act as a ribosome binding site for initiation at this codon.

The ORF extends to a TGA termination codon at nucleotide 681 (Fig. 2). Thus, it is likely to be either 504 or 459 nucleotides long depending on whether it is initiated at the ATG or GTG codon. The predicted peptide would then be either 168 or 153 amino acids long; it was assumed to be 168 amino acids long for computer-aided protein comparison searches.

The properties of the predicted peptide. The protein is relatively small (21.6 or 19.6 kDa). Analysis of its properties using the PepPlot program of the University of Wisconsin Genetics Computer Group, Madison, package revealed that it would not have a hydrophobic leader sequence whether it was initiated at the ATG or GTG codon, and so is unlikely to be exported across the cell membrane. Nucleotides 510 to 555 code for the amino acid sequence LVLLLVIGAVAMVVVM toward the carboxy-terminal end of the protein. This sequence is extremely hydrophobic, giving a score of approximately 3 in a Kyte-Doolittle analysis (Kyte and Doolittle 1982), and may span or interact with the membrane. The protein has no other strongly hydrophobic domains. Immediately after the hydrophobic region there is a sequence of 25 amino acids (encoded by nucleotides 567–641) of which 12 are alanine and seven are proline. Searches of the PIR protein data base using the FastP algorithm (Lipman and Pearson 1985) failed to reveal any homology between this protein and other reported protein sequences.

DISCUSSION

We have identified a region of *X. c.* pv. *campestris* DNA cloned in the plasmid pIJ3122 that is required for pathogenicity to turnip seedlings and plants, and to germinating radish seedlings. Analysis of marker exchange mutants revealed two classes of mutations that significantly reduced pathogenicity. These mapped at distinct but physically close loci; those that were clearly reduced in pathogenicity (mutants 12 and 29) and those that were only moderately reduced (mutants 34, 9, and 13). Members of the former class of mutants were significantly less pathogenic than members of the latter. This region is apparently independent of the gene(s) controlled by a plant-inducible promoter also present in pIJ3122 (for which no role in pathogenicity could be found); introduction of Tn*5* into DNA between the site of mutations that affected pathogenicity and this promoter had no effect on pathogenicity. Also, the direction of the ORF in which the mutations were situated was contrary to that of the plant-inducible promoter.

The growth rates of all of the pathogenicity mutants in seedlings did not appear to differ from that of the wild type. The only detectable difference (apart from pathogenicity) between the wild type and pathogenicity mutant 29 was a reduction in the number of viable mutant cells reisolated from turnip seedlings 24 hr after inoculation. Mutants 12 and 34 also appeared to show this effect. This may be due to failure of a proportion of the mutant cells to adapt rapidly enough to the relatively low nutrient conditions within the seedling compared to that of nutrient broth for instance, resulting in cell death. However, in simulated experiments involving transfer of wild-type and mutant cells from rich medium to minimal medium, both types of cells survived and multiplied to equivalent levels. When wild-type and mutant cells were suspended in water, kept at room temperature for 24 hr, and then tested on agar for viability, there was no evidence to suggest that the pathogenicity mutants were more susceptible to osmotic shock than the wild type. Seedlings may have some preformed or induced defense mechanism that can be overcome by wild-type cells of *X. c.* pv. *campestris*, and this faculty may be deficient in the mutant cells. This "kill" factor would also have to be present in mature turnip plants and germinating radish seedlings, since the mutants are reduced in pathogenicity in tests involving all of these. The effect could not be reproduced by incubating cells of mutant 29 with turnip seedling extract, indicating that some intrinsic property of the intact seedling may be required.

DNA sequence information was obtained for the region encompassing the sites of insertion of mutations 12 and 29, and a short ORF spanning these insertions was identified. The absence of a Shine-Dalgarno sequence associated with the putative ATG translational start codon may suggest that initiation of translation of the ORF may be by translational coupling; this phenomenon has been described for a number of *E. coli* gene pairs, including *galK-galI* (Schümperli *et al.* 1982), *trpD-trpE* (Oppenheim and Yanofsky 1980), *trpB-trpA* (Aksoy *et al.* 1984; Das and Yanofsky 1984), and *ilvD-ilvA* (Harms *et al.* 1988), but has not yet been reported in *X. c.* pv. *campestris*. Alternatively, initiation of translation may be at a GTG codon. The predicted protein from this ORF is small (either 21.6 or 19.6 kDa) and has a strongly hydrophobic region comprised of 16 amino acids, suggesting that it may have a domain which either spans a membrane or is membrane-associated. No homology was found with any other protein in the data base. One notable region toward the carboxy-terminal end of the protein was very rich in proline and alanine. This feature resembles the "hinge" regions found in bacterial and fungal cellulases (Knowles *et al.* 1987). These consist predominantly of proline and usually serine or threonine and are situated near one end of the cellulase; their function is to separate domains of the protein.

Using a promoter probe plasmid, it was established that the promoter giving transcription through this ORF was not present on pIJ3122, but was situated on an overlapping 5.2-kb *Bam*HI fragment extending to the right of pIJ3122 as shown in Figure 1. It could thus be situated up to 4 kb from the ORF. The promoter was active at high levels in *X. c.* pv. *campestris in vitro*, but did not function at a detectable level in *E. coli*. The ORF is likely to be part

of a single transcriptional unit that may include a number of other genes which are also important for pathogenicity. This is supported by the observation that pIJ3122 was unable to completely complement mutants 12 and 29 (data not shown). From sequencing, it was determined that Tn*5* insertion 104 lies outside the pathogenicity gene ORF (and is upstream from it); disruption of transcription in this region evidently disrupts transcription through the ORF. Complementation studies with the partially reduced pathogenicity mutants 34, 9, and 13 have not been conducted, but it is possible that these mutations lie in part of the same transcriptional unit, distal to the pathogenicity gene ORF. More extensive mutagenesis and sequencing of DNA on either side of the ORF may give an insight into the function of the various genes in the transcriptional unit.

ACKNOWLEDGMENTS

We would like to thank Max Dow and other colleagues for useful discussion during the course of this work.

The Sainsbury Laboratory is supported by a grant from the Gatsby Charitable Foundation. This work was conducted under the provisions of license PHF 1185/8 (48) issued by the Ministry of Agriculture, Fisheries and Food under the Plant Pest (Great Britain) Order 1980.

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