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Cloning of Two Endoglucanase Genes of *Xanthomonas campestris* pv. *campestris*: Analysis of the Role of the Major Endoglucanase in Pathogenesis

Clare L. Gough, J. Maxwell Dow, Christine E. Barber, and Michael J. Daniels

John Innes Institute, Colney Lane, Norwich, NR4 7UH, U.K.
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A genomic library of *Xanthomonas campestris* pv. *campestris* was mobilized into the noncellulolytic *Xanthomonas campestris* pv. *translucens*, and transconjugants were screened for endoglucanase activity on carboxymethylcellulose. pIJ3081 and pIJ3082, two positive clones whose inserts shared no homology, were identified. pIJ3082 encoded an endoglucanase activity that had the same pH optimum as the major endoglucanase of *X. c.* pv. *campestris*, and like the major endoglucanase of *X. c.* pv. *campestris*, was mainly extracellular. Conversely, pIJ3081 directed the synthesis of an endoglucanase with a different pH optimum that was largely intracellular in *X. c.* pv. *translucens*. Viscometric and reducing sugar analysis confirmed that the enzyme activities encoded by pIJ3081 and pIJ3082 and the major enzyme activity of *X. c.* pv. *campestris* were all endoglucanases. The major endoglucanase activity of *X. c.* pv. *campestris* was purified and shown to be a protein of 53 kD. This protein was absent from culture filtrates of endoglucanase-minus mutants made by marker exchange of endoglucanase-minus Tn*5* insertions in pIJ3082, implying that pIJ3082 contains the gene for the major endoglucanase of *X. c.* pv. *campestris*. Subcloning and Tn*5* mutagenesis located the gene to a 1.5-kb region of pIJ3082 DNA. Several different pathogenicity tests with these endoglucanase-minus mutants showed that the major endoglucanase of *X. c.* pv. *campestris* plays a minor role in the early stages of pathogenicity of *X. c.* pv. *campestris* on turnip and radish.

Additional keywords: marker-exchange mutagenesis, pathogenicity testing, *Brassica*.

The study of the mechanism of bacterial phytopathogenicity has been greatly facilitated by the use of modern genetic techniques. These techniques have allowed the role of suspected pathogenicity factors to be rigorously tested and have also led to the cloning of a number of pathogenicity genes whose precise functions then remain to be determined. Our work on *Xanthomonas campestris* pv. *campestris*, the causal agent of black rot of crucifers (Williams 1980), has used both of these approaches (Daniels *et al.* 1984b; Turner *et al.* 1985; Barrère *et al.* 1986; Tang *et al.* 1987).

The extracellular enzymes produced by phytopathogenic bacteria may have important roles in disease by facilitating plant tissue maceration. *X. c.* pv. *campestris* produces a number of such enzymes, including endoglucanase, polygalacturonate lyase, amylase, and protease. Previous work in our laboratory has shown that the synthesis and secretion of these enzymes as a whole are essential for pathogenesis. The genes controlling these processes have been described (Daniels *et al.* 1984b, Dow *et al.* 1987), but mutations in these genes have pleiotropic effects, so that the roles of individual enzymes in pathogenesis could not be assessed. We have begun to examine the roles of these enzymes by the construction of mutants, each one specifically defective in a single enzyme activity. This has been achieved by Tn*5* mutagenesis of the cloned structural genes, followed by marker-exchange of mutations into the bacterial genome, and by using this approach it has been shown that protease is not critically important in the pathogenicity of *X. c.* pv. *campestris* (Tang *et al.* 1987).

Present address of the last three authors: Sainsbury Laboratory, John Innes Institute, Colney Lane, Norwich, NR4 7UH, U.K.

Address correspondence to M. J. Daniels.

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In this paper we describe the cloning of two endoglucanase genes of *X. c.* pv. *campestris* and demonstrate that one of these is the structural gene for the major extracellular endoglucanase of *X. c.* pv. *campestris*. By using Tn*5* mutagenesis and marker-exchange techniques, endoglucanase-minus mutants of *X. c.* pv. *campestris* were made and used in plant tests to assess the contribution of the extracellular endoglucanase to the pathogenicity of *X. c.* pv. *campestris*.

MATERIALS AND METHODS

Bacteria and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Media and growth conditions. Growth media, conditions of incubation, genetic techniques, and antibiotic selection have been described previously (Daniels *et al.* 1984a; Turner *et al.* 1984, 1985).

DNA techniques. Plasmid DNA was isolated either by a rapid boiling method (Holmes and Quigley 1981) or by an alkaline method (Kieser 1984) for physical analysis; or by a rapid alkaline method (Birnboim and Doly 1979) for transformation. Plasmid transfer by transformation and triparental mating, Tn*5* mutagenesis, and marker-exchange have all been described (Daniels *et al.* 1984b; Turner *et al.* 1984, 1985). The procedures of DNA ligation, Southern blotting, nick translation, and hybridization were essentially as described by Maniatis *et al.* (1982).

Endoglucanase detection and assay. Bacterial colonies producing endoglucanase were detected on plates

containing carboxymethlycellulose (CMC) by Congo red staining. Colonies were grown on nutrient plates (NYGA for *X. c.* pv. *campestris*, Daniels *et al.* 1984a; LA for *E.coli*, Miller 1972), which were supplemented with appropriate antibiotics and 0.5% (w/v) CMC (low viscosity, Sigma Chemical Co., St. Louis, MO). After incubation, plates were stained with 0.1% Congo red for 30 min, rinsed with water, and washed twice with 1 M NaCl. Endoglucanase-positive colonies were surrounded by a pale yellow zone of clearing against a red background. Relative levels of endoglucanase activity were measured by a radial diffusion assay into CMC-containing agar plates (20 ml of 1.5% agar, 0.2% CMC in 50 mM phosphate buffer at different pH values) as described by Wood (1981). Cytoplasmic and periplasmic fractions were obtained as described by Dow *et al.* (1987).

Viscometric and reducing sugar assays. The mode of action of the endoglucanases was investigated by a study of the relationship between the reduction in viscosity of a CMC solution and reducing sugar release measured by the Nelson-Somogyi method (Nelson 1944). For these assays, the enzyme fractions were 0–80% saturated ammonium sulphate fractions of culture filtrates or cell lysates as appropriate. The reducing sugar release accompanying a 50% reduction in viscosity was determined graphically.

Purification of endoglucanase. Endoglucanase was purified by fast protein liquid chromatography (FPLC) from culture filtrates of strain 516-9, which is a protease-deficient Tn*5* mutant of strain 8004 of *X. c.* pv. *campestris* (Tang *et al.* 1987). This strain was used to minimize proteolytic degradation of endoglucanase during purification. Before FPLC, endoglucanase activities were partially purified on SP-Sephadex at pH 5.0. The 30–65% saturated ammonium sulphate fraction of the culture filtrate of an overnight culture of strain 516-9 was dialysed overnight against 20 mM acetate buffer pH 5.0 at 4° C. The dialysate was applied to a column of SP-Sephadex (1.6 × 6.0 cm), equilibrated with 20 mM acetate, pH 5.0. All the activity bound to the column. The column was washed with buffer, and the activity was then eluted with 20 mM acetate, pH 5.0, containing 0.2 M NaCl. This eluate was dialysed overnight against 20 mM acetate buffer, pH 4.3, at 4° C. FPLC used a mono S column equilibrated with 0.02 M acetate buffer, pH 4.3. Samples (2–5 ml) were applied, and the column was then washed with this buffer. Elution was achieved with a gradient of 0–0.2 M NaCl in 20 mM acetate buffer, pH 4.3. Endoglucanase activity eluted as a single peak at approximately 0.08 M NaCl.

Other analytical methods. Protease and polygalacturonate lyase activities were assayed as described by Daniels *et al.* (1984b). Sodium dodecyl sulfate (SDS)-PAGE was performed on 11% separation gels, essentially as described by Laemmli (1970).

Plant tests. Pathogenicity tests on turnips (*Brassica campestris*) with needle-inoculated seedlings and infiltrated leaves were as described by Daniels *et al.* (1984a). Inoculation by seed-soaking was done by soaking turnip and radish seeds (*Brassica napus*) in bacteria before transferring the seeds to agar medium in Replidishes. Seeds were surface sterilized, rinsed thoroughly in sterile water, and left to soak at room temperature for 5 hr in bacteria at a concentration of 10^8 cells per milliliter. The bacteria were then drained away from the seeds, and the seeds sown as described by Daniels at al. (1984a), except that propagators were incubated at 15° C for the first 24 hr. Bacterial growth rate in infected seedlings was determined as described by Tang *et al.* (1987). Inoculation of the leaf margin of mature turnip leaves was done by cutting a small notch in the end of veins while the leaves were submerged in a bacterial suspension of 10^8 cells per milliliter. Measurement of the spread of bacteria within the leaf after inoculation by this method was made by removing a series of contiguous 6-mm-diameter leaf disks and plating out homogenates of these onto selective media.

Table 1. Bacteria and plasmids

	Genotype or relevant characteristics	Source or reference
Bacteria		
X. c. pv. *campestris*		
8004	*rif*-14	Turner *et al.* 1984
8258	Sm^R, contains pPH1JI	Turner *et al.* 1985
516-9	8004::Tn*5*, protease-minus	Tang *et al.* 1987
8409	8004::Tn*5*, endoglucanase-minus	This work
8410	8004::Tn*5*, endoglucanase-plus	This work
X. c. pv. *translucens*		
XT02	Sp^R	Atkins *et al.* 1987
E. coli		
ED8767	*rec*A	Murray *et al.* 1977
JM107	Δ(*lac-pro*AB) (F′,*tra*D36, *pro*AB, *lac*I^{q}Z M15)	Yanisch-Perron *et al.* 1985
PCT800	As ED8767, but::Tn*5*	Turner *et al.* 1985
HB101	*rec*A13,*rps*L	Boyer and Roulland-Dussoix 1969
Plasmids		
pLAFR1	Tc^R	Friedman *et al.* 1982
pLAFR3	Tc^R	Staskawicz *et al.* 1987
pIJ3080	*X. c.* pv. *campestris* DNA cloned in pLAFR1	This work
pIJ3081	*X. c.* pv. *campestris* DNA cloned in pLAFR3	This work
pIJ3082	*X. c.* pv. *campestris* DNA cloned in pLAFR3	This work
pIJ3083	Subclone of pIJ3082	This work
pIJ3084	Subclone of pIJ3083	This work
pRK2013	Tra^+,Mob^+,Km^R,colE1 replicon	Figurski and Helinski 1979
pRK2073	pRK2013 Sp^R::Tn*7*	Leong *et al.* 1982
pPH1JI	Tra^+,Mob^+,GM^R,Sp^R,Sm^R,Cm^R Inc P replicon	Beringer *et al.* 1978

RESULTS

Isolation of endoglucanase-encoding clones. No positive clones were identified when about 1,000 transconjugants from a genomic library of *X. c.* pv. *campestris* DNA made in pLAFR3 and maintained in the *E. coli* host strain JM107 (Tang *et al.* 1987) were screened for endoglucanase activity on CMC-containing agar plates. However, when a pool of this library was mated into a noncellulolytic pathovar of *Xanthomonas campestris*, *X. c.* pv. *translucens*, two positive colonies were detected. The cosmids isolated from these were designated pIJ3081 and pIJ3082. Another positive recombinant cosmid, pIJ3080, was isolated when a pLAFR1 library of *X. c.* pv. *campestris* DNA (Daniels *et al.*

1984b) was similarly screened. DNA hybridization studies showed that pIJ3082 shared no homology with pIJ3080 or pIJ3081. pIJ3080 and pIJ3081 did share homology and by restriction fragment analysis were found to be overlapping clones containing 16 kb of common DNA. pIJ3081 was chosen for further study.

Comparison of the endoglucanases encoded by cloned DNA with the major endoglucanase of *X. c.* pv. *campestris*. Endoglucanase activity produced by wild-type *X. c.* pv. *campestris* was shown to be mainly extracellular (Table 2), confirming previous work by Dow *et al.* (1987). SDS-PAGE revealed that culture filtrates of *X. c.* pv. *campestris* contain predominantly one major protein band of 53 kD (Fig. 1). A minor band of 43 kD was present in culture filtrates of 8004, but not of strain 516-9, which has considerably reduced protease activity (Fig. 1). The endoglucanase from strain 516-9 eluted as a single peak on FPLC and showed a major band of 53 kD with a number of minor bands. During partial purification on SP-Sephadex, a minor band of 43 kD appeared (Fig. 1). This may have been a proteolytic degradation product of the 53-kD protein. Degradation was much more pronounced when attempts were made to purify the 53-kD protein from cultures of 8004.

To determine whether pIJ3081 or pIJ3082 coded for the major endoglucanase of *X. c.* pv. *campestris*, the properties of the cloned activities were compared with those of the major endoglucanase of *X. c.* pv. *campestris*. Analysis of the cellular distribution of the endoglucanase activities of *X. c.* pv. *translucens*/pIJ3081 and *X. c.* pv. *translucens*/pIJ3082 showed that the pIJ3082-encoded enzyme was almost exclusively extracellular, whereas the enzyme encoded by pIJ3081 was almost exclusively intracellular (Table 2).

Total endoglucanase activities expressed by *X. c.* pv. *translucens*/pIJ3081 and *X. c.* pv. *translucens*/pIJ3082 were 20% and 37%, respectively, of strain 8004. Further comparisons of the enzymes encoded by pIJ3081 and pIJ3082 were performed by using cell lysate and supernatant fractions of *X. c.* pv. *translucens*/pIJ3081 and *X. c.* pv. *translucens*/pIJ3082, respectively.

Differences were seen in the pH activity profiles of the two enzyme activities. The pIJ3082-encoded enzyme had a pH optimum of 6, with 14% of the maximum activity at pH 5. In contrast, the pIJ3081-encoded enzyme had a pH optimum of 7, with 14% of maximum activity at pH 6, but no activity at pH 5. Endoglucanase activity from culture filtrates of 8004 had a pH optimum of 6, with 5% of the maximum activity at pH 5.

Viscometric and reducing sugar measurements showed that a 50% reduction in viscosity was accompanied by the breakage of less than 0.2% of the glycosyl bonds in CMC for all the enzyme preparations, confirming an endoglycolytic action for all of the enzymes.

The overall properties of the activity encoded by pIJ3082 are thus similar to those of the extracellular activity of 8004. These activities were compared more closely by purification of the pIJ3082-encoded enzyme. The major band in culture filtrates of *X. c.* pv. *translucens*/pIJ3082 was a protein of 43 kD (Fig. 1). This was absent from culture filtrates of *X. c.* pv. *translucens*, which showed no obvious bands. The endoglucanase activity of *X. c.* pv. *translucens*/pIJ3082 was associated with the 43-kD protein and showed very similar FPLC characteristics to that of the culture filtrate of *X. c.* pv. *campestris*. This lack of correspondence of the molecular weights of the cloned gene product in *X. c.* pv.

Table 2. Percentage distribution of endoglucanase activity in cultures of strains of *Xanthomonas campestris*

Strain	Cytoplasm	Periplasm	Medium
8004	5	4	91
X. c. pv. *translucens*/ pIJ3081	93	0	7
X. c. pv. *translucens*/ pIJ3082	5	1	94

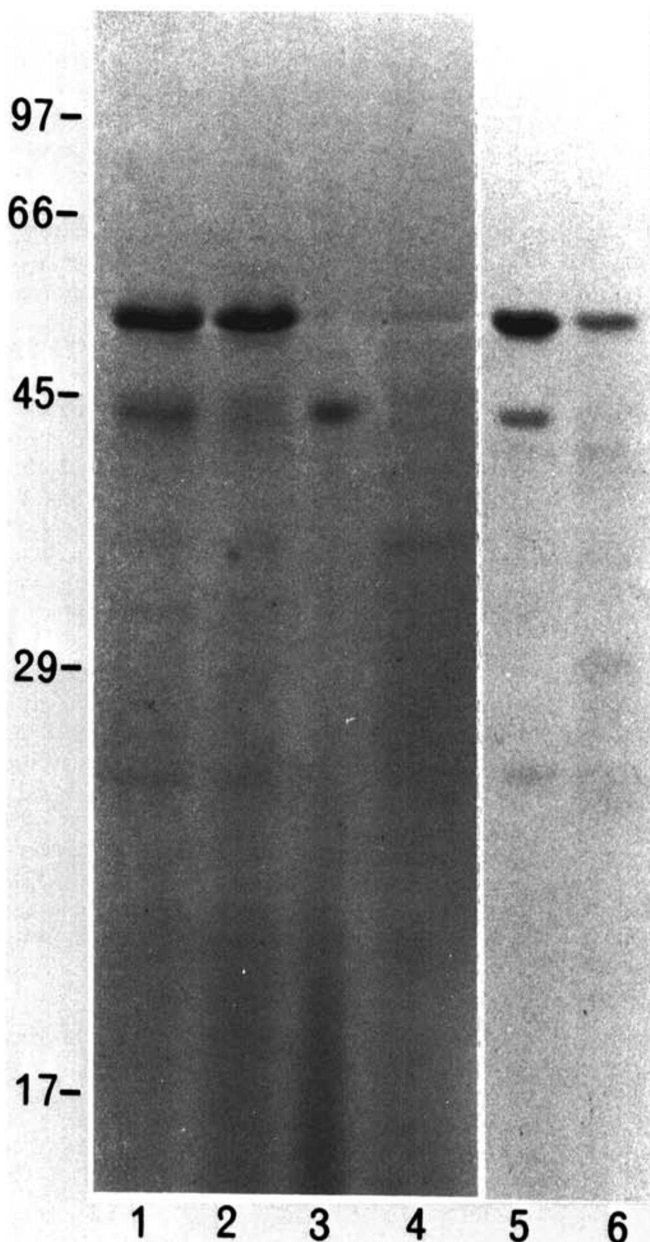

Fig. 1. SDS-PAGE of endoglucanase-containing fractions. The gel was stained with Coomassie blue. Lanes 1–4 are 0–80% saturated ammonium sulphate fractions of culture filtrates of *Xanthomonas campestris*. Lane 1, 8004 (wild-type *X. c.* pv. *campestris*); lane 2, 516-9 (protease-deficient mutant of *X. c.* pv. *campestris*); lane 3, *X. c.* pv. *translucens*/pIJ3082; lane 4, 8409 (endoglucanase-minus marker-exchange mutant of *X. c.* pv. *campestris*). Lane 5, SP-Sephadex-purified endoglucanase from 516-9; lane 6, fast protein liquid chromatography-purified endoglucanase from 516-9.

translucens with the major endoglucanase of *X. c.* pv. *campestris* may be due to the much higher proteolytic activity present in cultures of *X. c.* pv. *translucens* compared with *X. c.* pv. *campestris*.

Subcloning and Tn*5* mutagenesis of pIJ3082. pIJ3082 was subcloned by complete *Bam*HI digestion, followed by religation. A resulting positive subclone, designated pIJ3083, contained as insert 7-kb DNA from one end of the original insert and 1.5 kb from the other end, but was missing the central 16 kb of pIJ3082. Endoglucanase activity of *X. c.* pv. *translucens*/pIJ3083 was comparable to that of *X. c.* pv. *translucens*/pIJ3082, and the cellular distributions of the enzymes were identical in the two strains.

pIJ3083 was mutagenised with Tn*5*, essentially as described by Turner *et al.* (1985). Twenty-two individual insertions were transferred by mating from *E. coli* ED8767 into *X. c.* pv. *translucens* and subcultured onto CMC-containing agar plates. Twelve insertions were endoglucanase-positive, giving endoglucanase phenotypes similar to *X. c.* pv. *translucens*/pIJ3083; two (designated pIJ3083::Tn*5* 6 and pIJ3083::Tn*5* 89) had levels of activity between three and seven times higher than *X. c.* pv. *translucens*/pIJ3083, and eight were completely negative. The negative Tn*5* insertions all map to a 1-kb region of pIJ3083 flanked by two normally positive insertions 2.1 kb apart (Fig. 2).

A subclone was made from pIJ3083::Tn*5* 6 by using one of the *Hin*dIII sites in the Tn*5* insertion. This was done by complete *Hin*dIII digestion and religation, so that the resulting subclone, pIJ3084, contained as insert 1.8 kb of *X. c.* pv. *campestris* DNA from pIJ3083 and 1.2 kb of Tn*5* terminal repeat (Fig. 2). *X. c.* pv. *translucens*/pIJ3084 showed enzyme levels very similar to those of *X. c.* pv. *translucens*/pIJ3083. All the negative Tn*5* insertions of pIJ3083 map in the area subcloned in pIJ3084.

Construction and characterization of endoglucanase-minus mutants. Eight Tn*5* insertions were introduced into the wild-type *X. c.* pv. *campestris* genome by marker-exchange as described by Turner *et al.* (1985). These were three endoglucanase-negative insertions (pIJ3083::Tn*5* 91, 94, and 95), three endoglucanase-positive insertions (pIJ3083::Tn*5* 1, 14, and 67), and two endoglucanase-enhanced insertions (pIJ3083::Tn*5* 6 and 89) (Fig. 2). Supernatant and cell lysate fractions of all marker-exchange products were tested for endoglucanase activity at pH 5 and pH 8 by using CMC-containing agar plates. The marker-exchange products of pIJ3083::Tn*5* 1, 6, 14, 67, and 89 had intracellular and extracellular endoglucanase activities similar to the wild type at both pH 5 and pH 8. No enzyme activity could be detected for supernatant and cell lysate fractions of the marker-exchange products of pIJ3083::Tn*5* 91, 94, and 95 at pH 5, but at pH 8 these strains showed limited activity that was almost exclusively intracellular, indicating that these mutants were defective only in the pIJ3082-encoded activity.

Analysis of extracellular proteins of the endoglucanase-minus mutants by SDS-PAGE showed that the 53-kD protein, which represents the main extracellular endoglucanase activity of the wild type, was absent (Fig. 1). A number of minor proteins were unchanged, but the 43-kD protein, which is always seen in supernatants of 8004, was absent, providing more evidence that this protein is a degradation product of the 53-kD protein.

Assays for protease and polygalacturonate lyase showed that both the levels and distributions of these enzyme activities were the same as those of the wild type.

Assessment of the role of endoglucanase in pathogenesis. Pathogenicity tests were performed on an endoglucanase-minus mutant of *X. c.* pv. *campestris*, 8409 (the marker-exchange product of pIJ3083::Tn*5* 95); an endoglucanase-positive mutant of *X. c.* pv. *campestris*, 8410 (the marker-exchange product of pIJ3083::Tn*5* 1); and the wild type, 8004. In each of the tests, the rates of growth and symptom production of 8410 were very similar to those of 8004.

When these strains were inoculated into turnip seedlings at inoculum levels ranging from 10^3–10^5 bacteria per seedling, the progression of visible disease symptoms caused by 8409 was not as rapid as that caused by 8004. The difference was most marked 3 days after inoculation, when seedlings inoculated with 8409 showed only about 50% of the disease rating of 8004-inoculated seedlings. By day 6, the difference was not so great (Fig. 3). Although the development of rotting caused by 8409 was slow compared with 8004, the physical appearance of the rotted tissue was similar. Below 10^3 bacteria per seedling, the symptoms caused by both 8004 and 8409 were very slow to develop. There were no differences between 8004 and 8409 in either their growth rates or their final population sizes *in planta* (Fig. 3). When 8409 was extracted from seedlings to measure

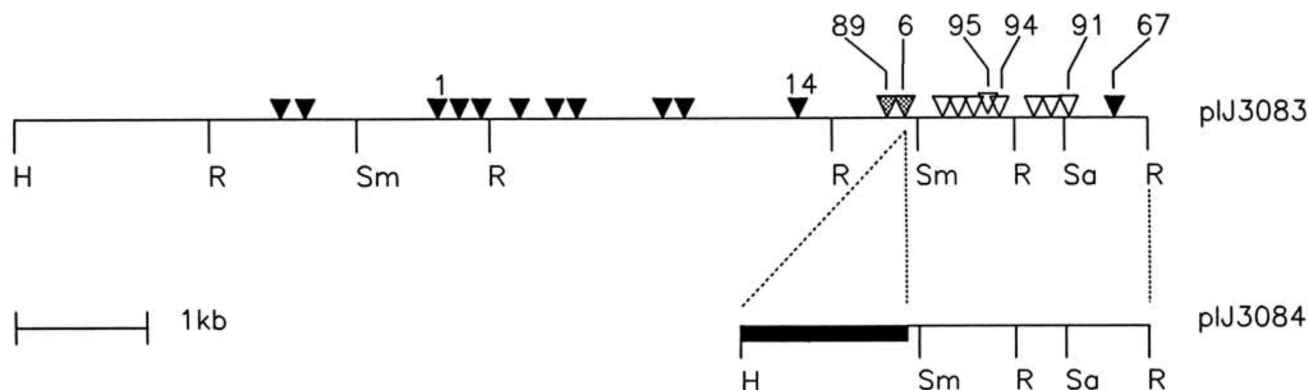

Fig. 2. Tn*5* map of pIJ3083, showing the area of pIJ3083 contained in the subclone pIJ3084. The solid part of pIJ3084 represents part of the terminal repeat of Tn*5*. The triangles represent the endoglucanase phenotypes of the Tn*5* insertions when they were introduced into *X. c.* pv. *translucens*: filled = positive, open = negative, and stippled = enhanced. Restriction enzyme sites are indicated: H = *Hin*dIII, R = *Eco*RI, Sm = *Sma*I, and Sa = *Sal*I. Numbered Tn*5* insertions are those which were marker-exchanged into the genome of *X. c.* pv. *campestris*.

its growth rate, the number of colonies on plates containing rifampicin and kanamycin was not significantly different from the number on plates containing only rifampicin, indicating that the Tn*5* insertions in the marker-exchange mutants are stable *in planta*. Similar patterns in the behavior of 8409 and 8004 were seen in needle-inoculated radish seedlings.

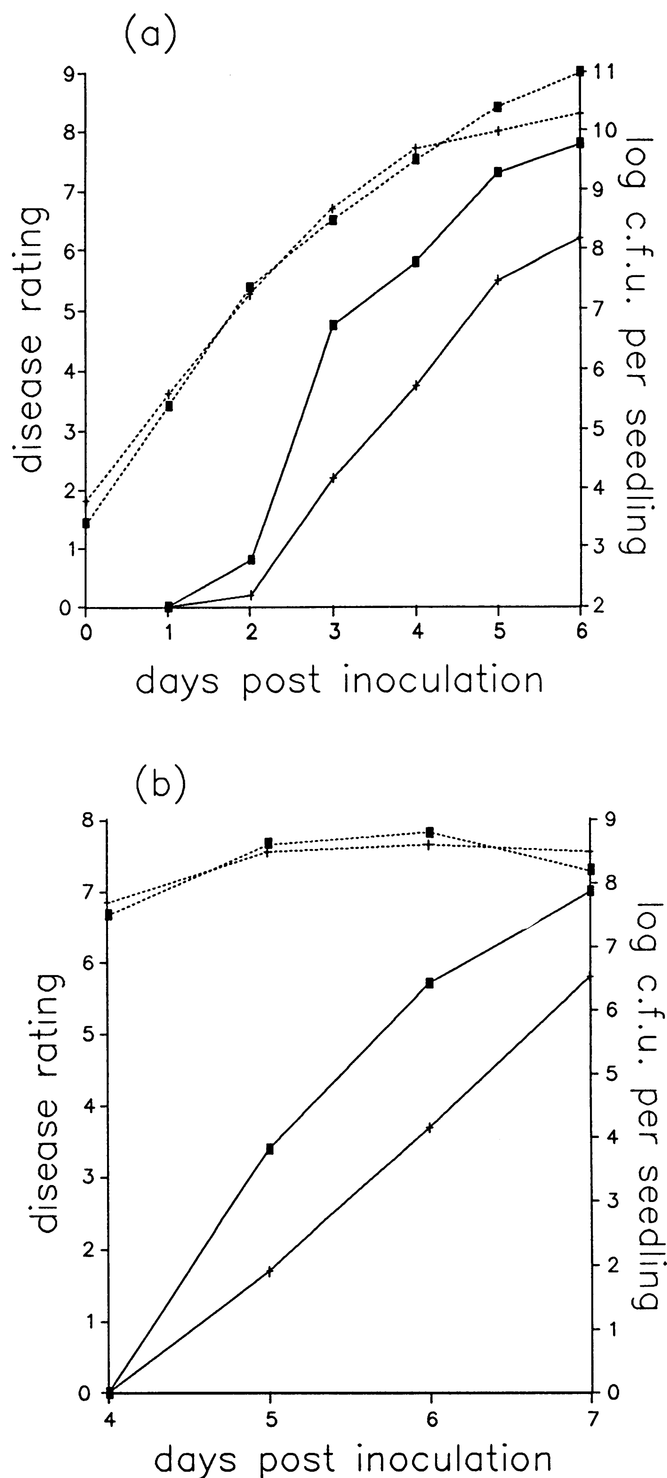

Fig. 3. Symptoms and growth of *X. c.* pv. *campestris* strains (**a**) in turnip seedlings after inoculation by needle; (**b**) in radish seedlings after inoculation by seed-soaking. The progression of disease symptoms was measured by using a disease rating index of 0–10, in which 10 represents 100% of seedlings showing full rotting symptoms. Symptom curves are shown as solid lines, and each graph point represents the average plant disease rating of about 50 seedlings. Growth curves are shown as dotted lines, and each graph point represents the average of five seedlings. ■ = 8004 (wild-type *X. c.* pv. *campestris*); + = 8409 (endoglucanase-minus mutant of *X. c.* pv. *campestris*).

Radish and turnip seedlings were inoculated with 8409, 8410, and 8004 by soaking the seeds in bacteria, as described in Materials and Methods. Control seeds were soaked in sterile water for 5 hr. The germination of these water-soaked seeds was 100%, and the resulting seedlings grew well without showing any visible signs of disease. About 80% of seeds soaked in bacteria germinated. Both 8004 and 8409 almost completely prevented growth of turnip seedlings soon after the soaked seeds had germinated. In contrast, about 80% of all germinated radish seeds that had previously been soaked in bacteria developed into apparently healthy seedlings. Five days after inoculation, the first visible signs of disease appeared. The first symptoms were rotted cotyledons and subsequently the rotting extended downwards along the stem of each seedling until eventually the whole stem was translucent. The development of this rotting was less rapid with 8409 compared with 8004 (Fig. 3). 8409 and 8004 were found to have similar growth rates in infected radish seedlings and reached similar final population sizes (Fig. 3).

The pathogenicity of these three strains on mature turnip leaves was tested. Bacteria were infiltrated into the undersides of leaves at inoculum levels of 10^4, 10^5, 10^6, and 10^8 cells per milliliter. The rate and extent of development of chlorosis and rotting caused by 8409 were very similar to those caused by 8410 and 8004 at each of the inoculum levels used. When leaf margins were inoculated, the rate of development of symptoms was slightly slower on 8409-infected leaves compared with those infected with 8004: the development of full rotting took about 12 days instead of 10, and the *in planta* levels of 8409 were slightly lower than those of 8004. The ability of 8409 to spread away from the site of inoculation into the leaf was identical to that of 8004.

DISCUSSION

A number of criteria indicate that pIJ3082 carries the gene for the major extracellular endoglucanase of *X. c.* pv. *campestris*. This 53-kD protein was absent from culture filtrates of an endoglucanase-minus mutant of *X. c.* pv. *campestris* made by marker-exchange of a Tn*5* insertion in pIJ3082. The properties of the pIJ3082-encoded enzyme and the extracellular endoglucanase from *X. c.* pv. *campestris* were identical except for their molecular weights, but the lower molecular weight (43 kD) of the enzyme produced by pIJ3082 in *X. c.* pv. *translucens* is probably due to proteolytic cleavage. Several cases have been reported where suspected proteolysis of endoglucanases has resulted in enzymes that still retain activity (Knowles *et al.* 1987). Béguin *et al.* (1987) found that cloned endoglucanases of *Clostridium thermocellum* purified from *E. coli* were both smaller than the corresponding enzymes from the wild type and also smaller than was predicted from nucleotide sequences. An endoglucanase from *Cellulomonas fimi* still retains activity towards CMC after the N-terminal 76 amino acids are removed (Wong *et al.* 1986).

Preliminary data from nucleotide sequencing of pIJ3082 and N-terminal amino acid sequencing of endoglucanase purified from *X. c.* pv. *campestris* culture filtrates have

confirmed that pIJ3082 does contain the endoglucanase structural gene (unpublished data). The complete nucleotide sequence should indicate whether the Tn*5* insertions that give enhanced endoglucanase activity are in the coding or noncoding region of the gene.

The properties of the pIJ3081-encoded endoglucanase were markedly different from those of the extracellular endoglucanase activity of *X. c.* pv. *campestris*. As this extracellular activity was shown to reside in a single protein, the activity encoded by pIJ3081 probably represents an intracellular endoglucanase in *X. c.* pv. *campestris*, which will only make a minor contribution to the total endoglucanase activity of *X. c.* pv. *campestris* because this latter activity is largely extracellular.

Comparatively little is known about endoglucanases produced by phytopathogenic bacteria and the role of these enzymes in pathogenesis. Endoglucanase genes have been cloned from *Erwinia chrysanthemi* (Boyer *et al.* 1987) and *Pseudomonas solanacearum* (Roberts *et al.* 1988), but detailed investigation of the role of endoglucanase has only been performed with *P. solanacearum*. Roberts *et al.* (1988) found that the onset of disease symptoms caused by endoglucanase-minus mutants of *P. solanacearum* were delayed compared with the wild type. Our results similarly demonstrated that endoglucanase is necessary for normal disease symptom progression in *X. c.* pv. *campestris*. This was seen clearly in seedlings, but was less obvious in clipped leaf tests. The growth rate of the endoglucanase-minus mutant in seedlings, however, was the same as that of the wild type, suggesting that, at least in the early stages of infection, endoglucanase-minus bacteria can obtain sufficient nutrients for normal growth rates. Because these results show that the ability of *X. c.* pv. *campestris* to produce extracellular endoglucanase is unimportant to the growth of bacteria *in planta* and only of minor importance to the appearance of symptoms, overall this enzyme does not seem to be very important in pathogenesis. The intracellular endoglucanase of *X. c.* pv. *campestris* may play no role in pathogenesis, because marker-exchange mutants of pIJ3081 gave wild-type symptoms when tested (data not shown).

The evidence so far, which has come from work on endoglucanase (this work) and protease (Tang *et al.* 1987) concerning the role of extracellular enzymes in the pathogenicity of *X. c.* pv. *campestris*, has shown that these two enzymes play only relatively minor roles. An intriguing question is why *X. c.* pv. *campestris* should produce such large amounts of endoglucanase when this enzyme apparently has only a minor role in pathogenesis. The possibility exists that endoglucanase is important to *X. c.* pv. *campestris* when it is growing saprophytically on dead plant tissue.

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