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Extracellular Proteases from *Xanthomonas campestris* pv. Campestris, the Black Rot Pathogen

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Two proteases (PRT1 and PRT2) were fractionated from culture supernatants of wild-type *Xanthomonas campestris* pv. campestris by cation-exchange chromatography on SP-5PW. Inhibitor experiments showed that PRT 1 was a serine protease which required calcium ions for activity or stability or both and that PRT 2 was a zinc-requiring metalloprotease. PRT 1 and PRT 2 showed different patterns of degradation of β-casein. The two proteases comprised almost all of the extracellular proteolytic activity of the wild type. A protease-deficient mutant which lacked both PRT 1 and PRT 2 showed considerable loss of virulence in pathogenicity tests when bacteria were introduced into mature turnip leaves through cut vein endings. This suggests that PRT 1 and PRT 2 have a role in black rot pathogenesis.

Xanthomonas campestris pv. campestris Pammel (Dowson) is the causal agent of the black rot disease of cruciferous plants (15). Studies of the molecular biology of pathogenicity of this bacterium have suggested a role for extracellular enzymes in the disease process. Mutants pleiotropically defective in the synthesis or export of cellulase, polygalacturonate lyase, protease, and amylase are nonpathogenic in all plant tests used (2, 5). More recently, mutants specifically deficient in particular extracellular enzyme activities have been derived by marker exchange mutagenesis with Tn*5*, using cloned structural genes for the enzymes (4, 7, 13). In particular, mutants deficient in protease activity have been derived by using a clone (pIJ3070) carrying a protease structural gene (13). These mutants are as virulent as the wild type in a number of plant tests on turnip (13). However, more recently we have observed that the mutants were considerably less virulent in tests in which the bacteria were introduced into mature turnip leaves through vein endings. This finding has prompted a study of the extracellular proteolytic activity of *X. campestris* pv. campestris. In this paper, we report the characterization of two major proteases from *X. campestris* pv. campestris, their absence from the protease-deficient mutant, and the reduced virulence of this mutant in leaf-nicking tests.

MATERIALS AND METHODS

Growth of bacteria. *X. campestris* pv. campestris strains used in this work were as follows: 8004, a rifampin-resistant mutant of a wild-type isolate (1); 516-9, a protease-deficient mutant generated by marker exchange mutagenesis, using cloned DNA from *X. campestris* pv. campestris (pIJ3070) which carries a protease structural gene expressed in *Escherichia coli* (13); 516-60, a protease-positive mutant generated by marker exchange of a Tn*5* insertion in pIJ3070 which did not cause a loss of protease activity in *E. coli* (13).

The bacteria were grown in peptone-yeast extract-glycerol (NYGB) medium or minimal (MMX) medium with 0.5% skimmed milk (1, 13) to induce the protease activity for 24 h at 28°C. Turnip leaf cell walls were prepared from leaf tissue (with the mid-rib removed) by the procedure described by Hahn et al. (8) for soybean stems. Cell walls were added to MMX medium to a final concentration of 5 mg/ml to induce protease activity.

Concentration of culture supernatants. Culture filtrates were obtained by centrifugation and were concentrated by addition of ammonium sulfate to 65% saturation. The precipitate which formed, which contained over 90% of the total proteolytic activity, was suspended in 10 mM Tris hydrochloride (pH 8) before dialysis overnight against the same buffer. The concentration factor was 10- to 20-fold.

High-performance liquid ion-exchange chromatography. Before fractionation by ion-exchange chromatography, concentrated culture filtrates were dialyzed overnight against distilled water (rather than Tris hydrochloride buffer) before being adjusted to pH 5 by the addition of 1 M sodium acetate buffer (pH 5) to a final concentration of 20 mM immediately prior to chromatography. High-performance liquid ion-exchange chromatography was on an SP-5PW column connected to a Gilson high-performance liquid chromatography system. The column was equilibrated with 20 mM acetate (pH 5), and all of the proteolytic activity bound to the column at this pH. After application of the sample, the column was washed with 20 mM acetate buffer (pH 5) before elution with a gradient of NaCl in 20 mM acetate buffer (pH 5) at a flow rate of 1 ml/min. Fractions of 1 ml were collected.

Enzyme assays. Proteolytic activity was measured by two methods. Method i was a radial diffusion assay in agar plates containing skimmed milk as substrate. Plates contained 0.5% (wt/vol) skimmed milk, 2% (wt/vol) agar, and 50 mM Tris hydrochloride (pH 8) in a total volume of 20 ml. Wells 6 mm in diameter were cut in the plate with a cork borer, and 30 μl of enzyme solution was applied. Zones of clearing around the well due to the degradation of the substrate were measured after 24 h of incubation at 37°C. One unit of activity caused a zone of clearing 18 mm in diameter. Method ii was an estimation of the degree and pattern of degradation of β-casein at 37°C. Assays contained 50 μl of enzyme, 250 μg of β-casein, and 50 mM Tris hydrochloride (pH 8) in a total volume of 100 μl. The substrate and degradation products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 14% gels as

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described by Hill and Gasson (9). After staining of the gels with Coomassie blue, the degree of substrate degradation was assessed by densitometric scanning at 626 nm on a Joyce-Loebel Chromoscan 3. The relative activities of PRT 1 and PRT 2 under different conditions of pH, after inhibition, and after interaction with metal ions were determined from standard curves of the degree of degradation against the amount of protease in the assay.

To assess the role of different divalent metal ions in the activity of the proteases, the preparations were treated with 2 mM EDTA for 30 min before being dialyzed against 10 mM Tris hydrochloride (pH 8) for 90 min at 4°C. Divalent metal ions (as chloride salts) were then added to a final concentration of 2.5 mM before the activity of the protease was assessed by the β-casein digestion method. Longer dialysis gave protease preparations that could not be reactivated by addition of divalent metal ions. PRT 2 was inhibited with 1,10-phenanthroline and reactivated with zinc ions as follows: 0.5 ml of PRT 2 (11.6 U/ml) was mixed with an equal volume of 10 mM 1,10-phenanthroline and incubated for 30 min at 25°C. Controls received an equal volume of water. To 49-μl samples of the inhibited enzyme were added zinc ions in 1 μl to a final concentration of 0.1, 0.2, 0.4, 0.5, and 1.0 mM. After a further 30 min at 25°C, the activity of each sample was assayed by the β-casein digestion method, using a 10-min incubation.

Gel permeation chromatography. The molecular weights of the proteases were determined by gel filtration on a column (1.5 by 48.5 cm) of Sephadex G-75 at 4°C. The flow rate was 1 ml/min and 1-ml fractions were collected. The column was equilibrated and eluted with 0.1 M Tris (pH 8) containing 5 mM $CaCl_2$. Molecular weight markers were cytochrome *c*, ovalbumin, bovine serum albumin, and blue dextran.

Virulence testing. Bacteria were introduced into the mature leaves of 4- to 5-week-old turnip plants (variety Just Right) through the cut vein ends. Turnip leaf vein endings were clipped out while the leaves were immersed in the appropriate bacterial suspension; then the leaves (still on the plant) were placed in a plastic bag for 7 to 13 days before scoring. The symptoms were rated according to the following scale: 0, no visible effect; 1, chlorosis only around nick; 2, chlorosis extending from nick site; 3, blackened leaf veins, death, and drying of tissue within the chlorotic area; 4, extensive vein blackening, death, and drying of tissue.

RESULTS

Induction of protease activity. The effect of growth in different media on the levels of extracellular proteolytic activity of *X. campestris* pv. campestris wild-type strain 8004 was as follows. No detectable (<0.06 U/ml) protease activity was seen in either minimal medium (MMX) or minimal medium supplemented with Casamino Acids (1.5 mg/ml). Considerable induction of protease activity, 4.6 and 1.6 U/ml, respectively, occurred in MMX medium supplemented with 0.5% skimmed milk and in NYGB medium, and these culture supernatants were used as sources of the proteases described in this paper. In MMX medium with leaf cell walls (5 mg/ml), proteolytic activity was 2.5 U/ml. (Data given are the means of two determinations which differed from the mean by <10%.)

Inhibitor studies on culture supernatants. The contribution of different classes of protease to the total extracellular proteolytic activity of wild-type *X. campestris* pv. campestris was assessed in preliminary experiments from the effects of different inhibitors on the proteolytic activity of concentrated dialyzed culture supernatants from NYGB-grown cultures (Table 1). Phenylmethylsulfonyl fluoride (PMSF), an inhibitor of serine proteases, caused a 70% reduction of activity in 1 mM concentration, whereas activity was almost entirely abolished by 1 mM EDTA. These results were not changed with increasing concentrations of PMSF. 1,10-Phenanthroline, which is believed to be a specific chelator for Zn^{2+} (8), caused a partial inhibition of activity. Apart from *N*-tosyl-L-phenylalanine chloromethyl ketone, an inhibitor of chymotrypsin, all other inhibitors were ineffective. The PMSF-insensitive fraction could be inhibited by EDTA but not by *N*-tosyl-L-phenylalanine chloromethyl ketone (data not shown). The inhibition by EDTA and PMSF together with the absence of an effect of iodoacetic acid and mercuric chloride (inhibitors of cysteine proteases) suggest that serine proteases and metalloproteases, but not cysteine proteases, make a significant contribution to the extracellular proteolytic activity of *X. campestris* pv. campestris in NYGB cultures.

TABLE 1. Effect of various inhibitors on activity of proteases from *X. campestris* pv. campestris

Treatment[a]	% Activity remaining		
	Total dialyzed culture supernatant	PRT 1	PRT 2
None	100	100	100
PMSF, 1 mM, 1 h	30	0	100
TPCK, 1 mM, 1 h	85	65	100
TLCK, 10 mM, 1 h	100	ND[b]	ND
Iodoacetate, 2 mM, 1 h	100	ND	ND
Mercuric chloride, 10 mM, 1 h	100	ND	ND
EDTA, 1 mM, 1 h	<5	0	0
1,10-Phenanthroline, 5 mM, 1 h	80	100	0
Benzamidine, 10 mM, 1 h	100	ND	ND

[a] Enzyme preparations were pretreated at 25°C with inhibitors under the conditions specified before assay by the radial diffusion or β-casein degradation method. Data are derived from averages of three determinations which differed from the average by <5%. TPCK, *N*-Tosyl-L-phenylalanine chloromethyl ketone; TLCK, *Nα-p*-tosyl-L-lysine chloromethyl ketone.

[b] ND, Not determined.

Fractionation and characterization of culture supernatant proteases. Ion-exchange chromatography on SP-5PW revealed two peaks of proteolytic activity: a sharp peak (PRT 1) eluting at 0.1 M NaCl and a broad peak (PRT 2) eluting between 0.25 and 0.45 M NaCl (Fig. 1). PRT 1 and PRT 2 differed in their pattern of cleavage of β-casein (Fig. 2); PRT 1 produced simultaneously a number of high-molecular-weight products rather than the prevalent high-molecular-weight product seen with PRT 2. This suggests that the enzymes have different peptide bond specificities. All fractions in the broad peak designated PRT 2 produced the same pattern of β-casein degradation. Both proteases had pH optima of about 8 (Fig. 3). The same chromatographic and β-casein cleavage results were obtained from cultures grown in either NYGB medium or MMX medium containing skimmed milk.

The effects of the various inhibitors on the activity of PRT 1 and PRT 2 were assessed by both the radial diffusion assay and the β-casein digestion method. The complete inhibition of PRT 1 by PMSF and EDTA (Table 1) suggests that this enzyme is a serine protease which also requires divalent metal ions for activity or stability or both. After treatment with 2 mM EDTA and dialysis as described in Materials and Methods, addition of 2.5 mM Ca^{2+} or Mg^{2+} restored PRT 1 activity to 19 and 8%, respectively, of an untreated control; Mn^{2+} and Zn^{2+} ions at the same concentration were not

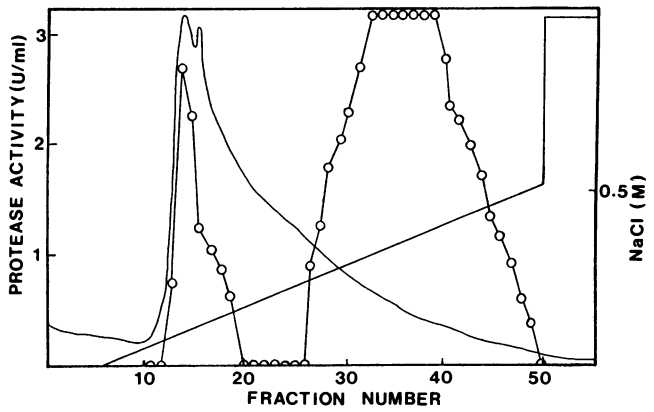

FIG. 1. High-performance liquid ion-exchange chromatography on SP-5PW of the 0 to 65% ammonium sulfate fraction of the culture filtrate. Symbols: ○, protease activity; —, A_{254}. The flow rate was 1 ml/min and 1-ml fractions were collected. Protease activities were measured by the radial diffusion assay. Fractions 13 to 19 were pooled as PRT 1; fractions 28 to 45 were pooled as PRT 2.

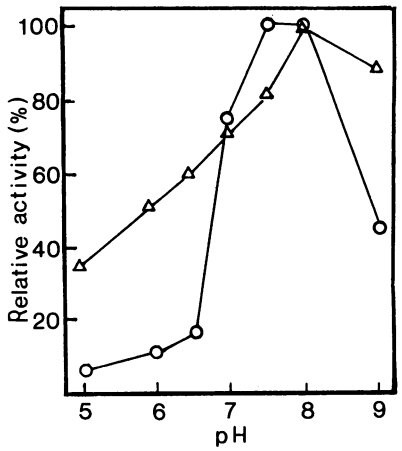

FIG. 3. Activity of PRT 1 (△) and PRT 2 (○) as a function of pH. Activities are expressed as a percentage of the maximal activity (at pH 8 for both enzymes) and were measured by the β-casein digestion method. The buffers used were 50 mM acetate for pH 5, 50 mM phosphate for pH 6, 6.5, and 7, and 50 mM Tris for pH 7.5, 8, and 9. The data given are the mean of two determinations which differed from the mean by <5%.

effective. PRT 1 was completely inhibited by 2 mM EGTA, suggesting that Ca^{2+} rather than Mg^{2+} ions are the active ions in the native enzyme. No inhibition of PRT 1 activity was seen with 1,10-phenanthroline at concentrations up to 5 mM. The partial inhibition of PRT 1 by the chymotrypsin inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone but not the trypsin inhibition *Nα-p*-tosyl-L-lysine chloromethyl ketone (Table 1) suggests that the enzyme may have some preference for hydrophobic residues rather than positively charged residues adjacent to the peptide bond to be cleaved. The molecular weight of PRT 1 was 36,000 as estimated by size exclusion chromatography on Sephadex G-75.

The inhibition of PRT 2 by EDTA alone suggests that this enzyme is a metalloprotease. PRT 2 was not inhibited by 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] but was completely inhibited by 5 mM 1,10-phenanthroline (Table 1). This inhibition took place even in the presence of 5 mM Ca^{2+}, Mn^{2+}, or Mg^{2+}. The activity of the 1,10-phenanthroline-inhibited enzyme could be partially restored by addition of Zn^{2+}; 1 mM Zn^{2+} restored activity to 48% of an untreated control (Fig. 4), although addition of 0.1 or 1 mM Zn^{2+} had no effect on the activity of the native enzyme (data not shown). These results suggest that Zn^{2+} ions are required for PRT 2 activity. After treatment of the enzyme with 2 mM EDTA and dialysis, addition of 2.5 mM Ca^{2+}, Mn^{2+}, or Mg^{2+} ions restored PRT 2 activity to 13, 4, and 11%, respectively, of the untreated enzyme. These results may suggest that other divalent metal ions are required for PRT 2 activity or stability or both. The molecular weight of PRT 2 was estimated to be 25,000 from size exclusion chromatography.

The ratio of PRT 1/PRT 2 activity determined by ion-exchange chromatography is less than would be expected from the initial studies on the concentrated culture filtrate. This is probably due to the reduced stability of PRT 1 compared with PRT 2. There was insufficient protease

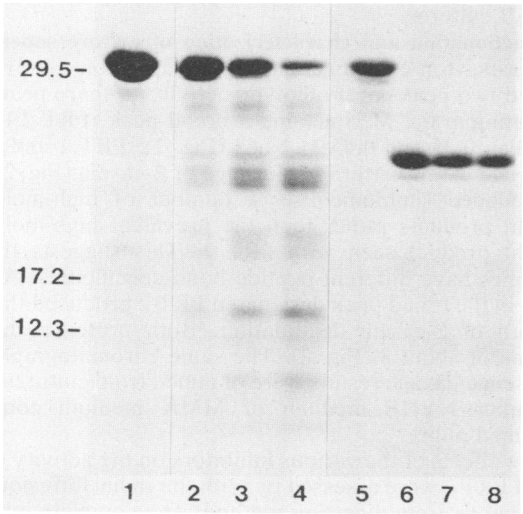

FIG. 2. Patterns of degradation of β-casein by PRT 1 and PRT 2 revealed by polyacrylamide gel electrophoresis and Coomassie blue staining. Assays were done as described in Materials and Methods. Lanes 1 and 5, β-casein standard; lanes 2, 3, and 4, effect of incubation of β-casein with 0.06 U of PRT 1 for 10, 30, and 60 min; lanes 6, 7, and 8, effect of incubation of β-casein with 0.17 U of PRT 2 for 10, 30, and 60 min. Molecular mass standards (10^3) are given on the left.

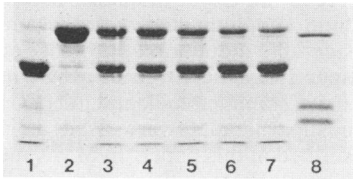

FIG. 4. Inhibition of PRT 2 by 1,10-phenanthroline and reactivation of the inhibited enzyme with zinc ions. PRT 2 was inhibited by 5 mM 1,10-phenanthroline and reactivated with different concentrations of zinc ions before assay as described in Materials and Methods. Lane 1, Uninhibited enzyme; lane 2, 1,10-phenanthroline-inhibited enzyme; lanes 3 to 7, inhibited enzyme reactivated with 0.1, 0.2, 0.4, 0.5, and 1.0 mM zinc ions; lane 8, 29.5-, 17.2-, and 12.3-kilodalton molecular mass standards.

protein associated with the active fractions of PRT 1 and PRT 2 derived from 2 liters of culture to allow further characterization.

Proteolytic activity in the marker exchange mutants. The extracellular protease activity from the marker exchange protease-deficient mutant 516-9 showed a pattern of degradation of β-casein that was different from both PRT 1 and PRT 2 (data not shown). In addition, the activity could not be inhibited by 5 mM EDTA, which inhibits both of the major proteases, and was only partially inhibited by 2 mM PMSF. This minor activity, which represented <5% of the total seen in the wild type, was not studied further. In contrast, the protease-positive marker exchange mutant 516-60 was indistinguishable from the wild type in the production of PRT 1 and PRT 2.

Protease induction by plant cell walls. A situation more closely related to the diseased plant could result from the use of turnip leaf proteins as inducers of *X. campestris* pv. campestris proteases. Turnip leaf cell walls were effective inducers of protease activity when added to MMX medium to a final concentration of 5 mg/ml (see above). The pattern of degradation of β-casein by the unfractionated culture filtrate under these conditions of induction was essentially the same as that seen with NYGB-grown cultures. This proteolytic activity was inhibited by 80% by 2 mM PMSF and by 15% by 5 mM 1,10-phenanthroline; the PMSF-insensitive and the 1,10-phenanthroline-insensitive activities showed the same pattern of degradation of β-casein as PRT 2 and PRT 1, respectively. Overall, these results suggest that PRT 1 and 2 are also the major proteases induced by the turnip cell walls.

Virulence of protease-deficient mutant. Previous work has shown that, when infiltrated into mature turnip leaves or inoculated into turnip seedlings, the protease-deficient mutant 516-9 showed little or no reduction in virulence compared with the wild type (13). However, when bacteria suspended at a concentration of 10^8 CFU/ml in NYGB were introduced into mature turnip leaves through cut vein endings, strain 516-9 showed a considerable reduction of symptom expression compared with the wild type or protease-positive strain 516-60 (Fig. 5). Differential responses between the strains were also observed when bacteria were washed and suspended in water at a range of concentrations from 10^4 to 10^8 CFU/ml before infiltration (Table 2). Inoculation with NYGB medium or water alone had no effect.

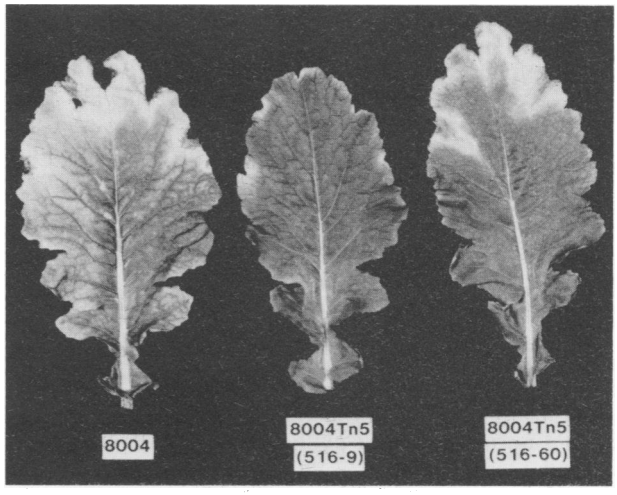

FIG. 5. Symptoms in mature turnip leaves 13 days after inoculation with strains of *X. campestris* pv. campestris through cut vein endings as outlined in Materials and Methods. Bacteria were suspended in NYGB medium at a concentration of 10^8 CFU/ml for inoculation. Strains were 8004, wild type; 516-9, protease-deficient Tn*5* mutant; and 516-60, protease-positive Tn*5* mutant.

DISCUSSION

Our results demonstrate that *X. campestris* pv. campestris produces two major extracellular proteases (PRT 1 and PRT 2) which belong to different protease classes and show different patterns of peptide bond cleavage. Multiple proteases with different patterns of peptide bond cleavage have also been reported in *X. campestris* pv. malvacearum (R. K. Gholson, C. Rodgers, and M. Pierce, Phytopathology **79:** 1199, 1989). The properties of PRT 1 are similar to those of the subtilisin family of serine proteases which also require a divalent metal ion (usually Ca^{2+}) for stability or activity or both (6). The properties of PRT 2 are similar to those of a neutral protease from *Aeromonas proteolytica* and two extracellular metalloproteases from *Erwinia chrysanthemi* which similarly require Zn^{2+} for activity and are stabilized by Ca^{2+} (3, 12).

The *X. campestris* pv. campestris clone (pIJ3070) used to generate the protease-deficient transposon insertion mutant carries a protease gene which is expressed in *E. coli* (13). Work parallel to that described here has shown that the encoded protease has amino acid sequence homology with the subtilisin family of serine proteases (10). Furthermore, the protease expressed in *E. coli* has the same pattern of degradation of β-casein as PRT 1 (13). These results strongly suggest that PRT 1 is the product of the cloned gene. It is not yet known why a single transposon insertion in the region of the *X. campestris* pv. campestris chromosome cloned in pIJ3070 leads to the loss of both PRT 1 and PRT 2 activities in mutant 516-9. Further studies on the genes cloned in pIJ3070, their organization, and precise mapping of the Tn*5* insertion will be required to establish the mechanism.

We do not know whether the proteases characterized here are also the major extracellular proteases expressed by *X. campestris* pv. campestris in planta. Experiments with plant cell walls as inducers suggest that this may be the case. Nevertheless, mutant 516-9 which we have now shown to

TABLE 2. Symptom expression in turnip leaves inoculated through vein endings with different strains of *X. campestris* pv. campestris

Inoculum level (CFU/ml)[a]	Avg disease severity rating[b]		
	8004	516-9	516-60
10^8	4.0	1.1[c]	4.0
10^7	4.0	1.2	4.0
10^6	3.6	0.5	3.5
10^5	3.0	0.4	3.1
10^4	1.6	0.3	1.5
Control	0		

[a] Bacteria were grown overnight in NYGB and then washed and suspended in water at the concentrations shown before inoculation as described in Materials and Methods. Control leaves were inoculated with water.

[b] The disease severity scale used is described in Materials and Methods. The average value is from a minimum of 60 inoculation sites distributed over at least six leaves on a minimum of four different plants. Symptoms were scored after 7 days.

[c] Average values for strain 516-9 are significantly different from those of strains 8004 and 516-60 at $P < 0.01$ for 10^4 CFU/ml and $P < 0.001$ for all other inoculum levels.

lack both PRT 1 and PRT 2 extracellular activities, shows reduced symptoms when introduced into mature turnip leaves through cut vein endings. This method of inoculation mimics natural black rot infection processes in which bacteria enter leaf veins through openings in the leaf margin called hydathodes (although in this case the hydathodes are removed). This appears to be a more stringent test for pathogenicity for *X. campestris* pv. campestris in turnip than tests in which bacteria are introduced into the leaf intercellular spaces or into seedlings (11). In these latter cases, no differences between the symptoms induced by the protease-deficient mutant and the wild type were seen (13).

The role of extracellular proteases in bacterial plant diseases has been little investigated by genetic methods. Protease-deficient mutants of *X. campestris* pv. oryzae and *X. campestris* pv. malvacearum have been derived by transposon and nitrosoguanidine mutagenesis, respectively (G.-W. Xu and C. F. Gonzalez, Phytopathology **79**:1210, 1989; Gholson et al., Phytopathology **79**:1199, 1989). These mutants show reduced disease symptoms and bacterial populations in their respective hosts (rice and cotton). The role of bacterial proteases in these different diseases may simply be nutritional, although it is possible that proteolysis of structural proteins in plant cell walls may be required to allow spread of the bacteria or to overcome some host defense reaction.

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LITERATURE CITED

1. **Daniels, M. J., C. E. Barber, P. C. Turner, W. G. Cleary, and M. K. Sawczyc.** 1984. Isolation of mutants of *Xanthomonas campestris* pv. *campestris* showing altered pathogenicity. J. Gen. Microbiol. **130**:2447–2455.
2. **Daniels, M. J., C. E. Barber, P. C. Turner, M. K. Sawczyc, R. J. W. Byrde, and A. H. Fielding.** 1984. Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad host range cosmid pLAFR1. EMBO J. **3**:3323–3328.
3. **Delepelaire, P., and C. Wandersman.** 1989. Protease secretion by *Erwinia chrysanthemi*. Proteases B and C are synthesised and secreted as zymogens without a signal peptide. J. Biol. Chem. **264**:9083–9089.
4. **Dow, J. M., D. E. Milligan, L. Jamieson, C. E. Barber, and M. J. Daniels.** 1989. Molecular cloning of a polygalacturonate lyase gene from *Xanthomonas campestris* pv. *campestris* and the role of the gene product in pathogenicity. Physiol. Mol. Plant Pathol. **35**:113–120.
5. **Dow, J. M., G. Scofield, K. Trafford, P. C. Turner, and M. J. Daniels.** 1987. A gene cluster in *Xanthomonas campestris* pv. *campestris* required for pathogenicity controls the excretion of polygalacturonate lyase and other enzymes. Physiol. Mol. Plant Pathol. **31**:261–271.
6. **Frommel, C., and C. Sander.** 1989. Thermitase, a thermostable subtilisin: comparison of predicted and experimental structures and the molecular cause of thermostability. Proteins Structure Function Genet. **5**:22–37.
7. **Gough, C. L., J. M. Dow, C. E. Barber, and M. J. Daniels.** 1988. Cloning of two endoglucanase genes from *Xanthomonas campestris* pv. *campestris*: role of the major enzyme in pathogenesis. Mol. Plant Microbe Int. **1**:275–281.
8. **Hahn, M. G., A. G. Darvill, and P. Albersheim.** 1981. Host-pathogen interactions. XIX. The endogenous elicitor, a fragment of a plant cell wall polysaccharide that elicits phytoalexin accumulation in soybeans. Plant Physiol. **68**:1161–1169.
9. **Hill, S. H. A., and M. J. Gasson.** 1986. A qualitative screening procedure for the detection of casein hydrolysis by bacteria, using sodium dodecylsulphate polyacrylamide gel electrophoresis. J. Dairy Res. **53**:625–629.
10. **Liu, Y. N., J. L. Tang, B. R. Clarke, J. M. Dow, and M. J. Daniels.** 1990. A multipurpose broad host range cloning vector and its use to characterise an extracellular protease gene of *Xanthomonas campestris* pathovar *campestris*. Mol. Gen. Genet. **220**:433–440.
11. **Shaw, J. J., and C. I. Kado.** 1987. Whole plant wound inoculation for consistent reproduction of black rot of crucifers. Phytopathology **78**:981–986.
12. **Spungin, A., and B. Blumberg.** 1989. *Streptomyces griseus* aminopeptidase is a calcium-activated zinc metalloprotein: purification and properties of the enzyme. Eur. J. Biochem. **183**:471–477.
13. **Tang, J. L., C. L. Gough, C. E. Barber, J. M. Dow, and M. J. Daniels.** 1987. Molecular cloning of protease gene(s) from *Xanthomonas campestris* pv. *campestris*: expression in *Escherichia coli* and role in pathogenicity. Mol. Gen. Genet. **210**:443–448.
14. **Wilkes, S. H., and J. M. Prescott.** 1976. *Aeromonas* neutral protease. Methods Enzymol. **45**:404–415.
15. **Williams, P. H.** 1980. Black rot: a continuing threat to world crucifers. Plant Dis. **64**:736–742.