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Molecular Cloning of Pectinase Genes from *Erwinia carotovora* Subspecies *carotovora* (Strain SCRI193)*

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Abstract

Several members of the genus *Erwinia* are phytopathogenic causing soft rot, black leg or stem rot of various plants. The effects on the plant are probably due to the concerted action of extracellular enzymes, including pectinases, cellulases and proteases. As part of our study of the secretion process in Gram negative bacteria we have cloned five structural genes for various pectinases from *Erwinia carotovora*. These have been preliminarily characterized and include genes encoding the three main pectolytic enzymes produced under laboratory conditions: two pectate lyases and a polygalacturonase. The other two enzymes are also pectate lyases and appear to have minor activities in this strain.

Keywords: *Erwinia carotovora*, molecular cloning, pectate lyase, polygalacturonase

Abbreviations: spp, species; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulphate; Kb, Kilobase pairs

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Introduction

Erwinia carotovora subspecies *carotovora* (*Ecc*) is an aetiological agent of soft rot in stored crops such as potato and carrot. The rotting of potato tubers can be a significant economic problem for some companies who may store potatoes up to 10 months after harvesting prior to processing. It has been known for sometime that factors such as temperature, pO_2, and humidity are important in predisposing the potato tuber to *Ecc*-mediated soft rot. However, it is not always easy, or economical, to control such factors sufficiently on a large scale in order to prevent rot. Consequently attention has turned towards a direct, detailed analysis of the major determinants of soft rot biodeterioration, namely extracellular macerating enzymes. *Erwinia* spp. make a range of pectinolytic, cellulolytic and proteolytic enzymes which are exported into the extracellular medium. This feature of *Erwinia* makes it doubly interesting to study because it is not only a phytopathogen, but also an excellent model system for the investigation of protein secretion. As part of our analysis of the determinants of *Ecc* pathogenicity, and the mechanism of protein export we have attempted to define the spectrum of pectolytic enzyme activities in one strain of *Ecc* (SCRI193). This strain has been chosen because it is amenable to a range of genetic techniques, including transformation (Hinton et al., 1985) and transposon mutagenesis (Salmond et al., 1986), thereby allowing a molecular analysis of all of the features we are investigating. Our initial aim was to identify a variety of enzyme activities in culture supernatants and then to clone (in *Escherichia coli*) the structural genes for such enzymes. The long term aim is to analyze the structure of each enzyme and the mechanisms involved in the export process.

1. Materials and Methods

Bacterial strains

Erwinia carotovora subspecies *carotovora* SCRI193 (Hinton et al., 1985), *Erwinia chrysanthemi* B374 (National Collection of Plant Pathogenic Bacteria 898). *Escherichia coli* NM522 ([lac-pro]$^{\Delta}$hsdΔ5:F' lacZΔM151acI^{Q} (Gough and Murray, 1983). *Erwinia* strains were grown at 30°C in Luria broth supplemented with 0.5% sodium polygalacturonate in order to induce the synthesis of pectinases. *E. coli* strains were grown at 37°C in Luria broth with 100μg/ml ampicillin when appropriate.

Preparation of DNA from Erwinia carotovora

A 20 ml culture of *Ecc* SCRI193 was harvested and resuspended in 2 ml of 0.15 M NaCl, 0.1 M EDTA pH 8 containing 2 mg/ml lysozyme, taken through two cycles of freezing and thawing in a dry ice/methanol bath and incubated at 37°C for 15 min. The cells were lysed by adding 2 ml of 1% SDS, 0.1 M NaCl, 0.1 M Tris HCl pH 9. 10μl of protease K (10 mg/ml) were added and the incubation continued for 2 hr. The lysate was gently extracted several times with an equal volume of phenol followed by chloroform. DNA was then spooled onto a glass rod after the addition of an equal volume of absolute ethanol, blotted dry on tissues and rehydrated in 0.5 ml 1 mM EDTA, 10 mM Tris HCl pH 7.5

Construction of genomic libraries

Chromosomal DNA was digested to completion with *Bam*HI, *Eco*RI and *Hind*III and ligated to correspondingly digested pUC8 treated with calf intestinal alkaline phosphatase. The same methods were used to construct an *Hind*III library in pBR322. Ligated DNAs were transformed into competent NM522 and plated onto M9 media supplemented with 0.5% glycerol, 0.1% yeast extract and 0.5% sodium polygalacturonate containing 100μg/ml ampicillin (PGA plates). Transformants were replicated onto Luria agar plates (containing 100μg/ml ampicillin and 0.5% glucose). Pectinase-positive clones were revealed by the appearance of a white halo after flooding the transformation plates with 7.5% cupric acetate. Potential clones were then rescued from the replicates and plasmid DNA prepared by the rapid isolation procedure of Holmes and Quigley (1981).

Enzyme assays

Pectate lyase (PL) activity was assayed by monitoring the increase in absorbance at 235 nm in reaction mixtures containing sodium polygalacturonate or pectin (Bulmers Type 104, 70% methylated). Assays contained 2.4 mg/ml substrate in 50 mM Tris HCl pH 7.6 and were carried out at room temperature. Polygalacturonase (PG) activity was assayed by measuring the production of reducing groups using dinitrosalicylic acid (Rombouts and Pilnik, 1980). Reaction mixtures contained 2.4 mg/ml substrate in 40 mM sodium phosphate buffer pH 7, 1 mM EDTA and were incubated at 30°C. β-lactamase was assayed by monitoring the increase in absorbance at 490 nm in reaction mixtures containing the chromogenic β-lactam nitrocefin (O'Callaghan et al., 1972) in 10 mM sodium phosphate buffer pH 7 and were carried out at room temperature.

Electrophoresis of pectinases

Culture supernatants (concentrated if necessary using Amicon CF25 ultrafiltration cones) were analyzed by isoelectric focusing followed by blotting to a pectate-agarose overlay and staining with ruthenium red essentially according to the method of Ried and Collmer (1985).

2. Results and Discussion

Isolation of clones carrying pectinase genes

Clones were classified according to the size of the insert DNA. Four classes were obtained, two from the *Bam*HI bank (plasmids B1 and B6), one form the *Eco*RI bank (E4) and one from the *Hin*dIII bank (H2). Plasmid DNA from representatives of each class was then reintroduced into NM522 in order to confirm that the plasmids carried pectinase genes. Interestingly three distinct types of halo were distinguishable on PGA plates, with clones B6 and H2 producing similar patterns. Another class of *Hin*dIII insert, H5, was obtained from a clone bank in pBR322. The plasmids were then characterized further by restriction site mapping (Fig. 1). Only two of the cloned fragments carried overlapping DNA; the 2.2 kilobase (kb) *Bam*HI insert carried by B6 was found within the 8.2kb *Hin*dIII insert carried by H2, and indeed these two clones gave similar phenotypes on PGA plates. The 8.7kb *Bam*HI fragment carried by B1, the 5.7kb *Eco*RI fragment in E4 and the 7kb *Hin*dIII fragment in H5 all appear to be different. All of the *Bam*HI and *Hin*dIII inserts contain several *Eco*RI sites (not shown) and genes carried on these fragments would not therefore be expected to be isolated from an *Eco*RI bank. However, H5 was not isolated from the *Hin*dIII bank in pUC8 or the *Bam*HI bank even though it does not contain any internal *Bam*HI sites suggesting that the banks were not representative of the whole genome.

Characterization of pectinases produced by the clones

Pectic enzymes are classified into two main types: de-esterifying enzymes such as pectin methylesterase which removes methoxyl groups from pectin to produce polygalacturonate and methanol, and a range of depolymerizing enzymes. This latter class can be further divided into those which break the glycosidic linkages in pectin by hydrolysis e.g. polygalacturonase, and those which cleave by β-elimination, the lyases. *Ecc* produces all three of these pectinases when grown in Luria broth containing 0.5% PGA, with the level of pectate lyases increasing approximately twenty-fold when PGA is added. The screening procedure used will identify clones producing both polygalacturonases and pectate lyases but not pectin methylesterase. All of the strains

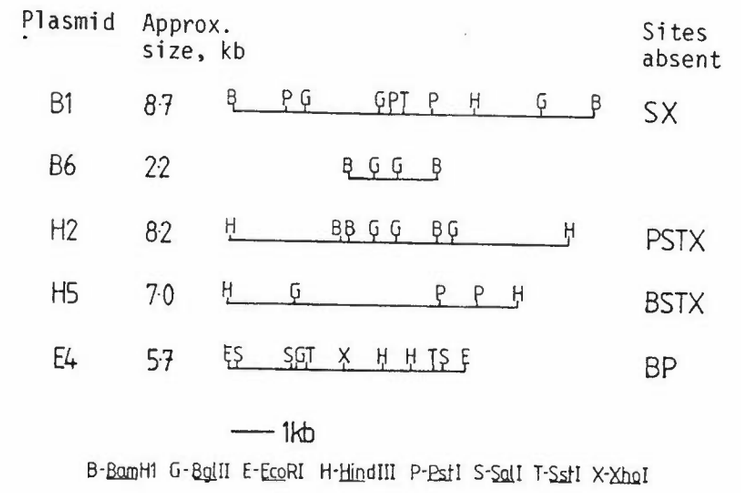

Figure 1. Physical maps of inserts carrying pectinase genes.

grew poorly even on plates supplemented with 0.5% glucose (added to ensure catabolite repression of *pel* promoters and of the *lac* promoter in pUC8). This is perhaps not surprising considering the high copy number of pUC8. For this reason cultures were always grown up from fresh transformation plates to avoid mutation or loss of the insert DNA. A second consequence of this high copy number was that pectinase activity could always be detected in the culture medium after overnight growth; supernatants also contained significant amounts of β-lactamase activity (recognized as a periplasmic marker). This suggests that the activity in the medium was not due to secretion but was probably due to nonspecific leakage from the periplasm resulting from the large amount of enzyme synthesized. This observation has also been reported for pectinase genes cloned in *E. coli* from *Erwinia chrysanthemi* (*Ech*) (Collmer et al., 1985) and *Ecc* (Zinc and Chatterjee, 1985).

Strains carrying the plasmid E4 did not produce any lyase activity (in supernatants or sonicates) but did elicit reducing groups from PGA in the presence of EDTA (Table 1), indicative of polygalacturonase. Strains carrying the plasmids B1, B6, H2 and H5 all produced lyases (Table 1). However the enzyme activities fell into at least two groups with respect to their activity on pectin or their response to the addition of 1.5 mM $CaCl_2$. The enzyme(s) produced from B1 demonstrated a ten-fold stimulation of activity when calcium was added to PGA, and sixty-fold on pectin compared to PGA, while

Table 1. Pectinase activities of *Ecc* SCRI193 and strains of *E. coli* carrying various pectinase clones.

Strain	Pectate Lyase PGA/50 mM no add.	Tris pH 8.6 1.5 mM $CaCl_2$	Pectin/50 mM no add.	Tris pH 8.6 1.5 mM $CaCl_2$	% Activity in supernatant PL	β-lactamase
Ecc	0.96[1]	–	–	–	94	
B1	0.15	1.85	7.17	–	34	50
B6	0.1				6	25
E4	Nil					
H2	0.69	0.66	1.02	0.9	29	54
H5	1.45	1.34	2.12	nd[2]	50	–

[1] ODU_{235}/min/ml
[2] Rapid initial rate which rapidly decayed

Strain	Polygalacturonase PGA/40 mM PO_4 pH 7 1 mM EDTA	% Activity in supernatant PG	β-lactamase
Ecc	0.026[1]		
E4	0.166	52	48

[1] ODU_{540}/min/ml

H2 (B6) and H5 showed little response. However, all of the enzymes were completely inhibited by the addition of EDTA indicating that they all have a requirement for divalent cations as would be expected for lyases (Rombouts and Pilnik, 1980). We have not investigated this aspect any further nor have we established whether these enzymes are endo- or exo-pectinases.

The enzymes were then characterized further by isoelectric focusing followed by blotting with an agarose-substrate gel in order to detect pectinase activities (not shown). Supernatants from *Ecc* SCRI193 grown in the presence of 0.5% PGA contain two major pectate lyases with approximate pI's of 9.1 and 9.3 (and migrating with a similar pI to PL's *d* and *e* of *Ech* B374 (van Gijsegm et al., 1985) used for comparison) and a single polygalacturonase with an approximate pI of 9.3. Minor activities can sometimes be observed if the blotting time is extended, however there were no major activities corresponding to PL's *b* and *c* of *Ech* B374. The clone E4 was confirmed to encode the polygalacturonase by this method. Interestingly the two major

pectate lyase activities appear to be encoded by a single clone B1. Although we have not yet shown that these enzymes are encoded by two genes we feel that this possibility is the most likely. This would be analogous to the situation in *Ech* where the genes for the two major PL's of this pI, *d* and *e* are linked (van Gijsegem et al., 1985; Reverchon et al., 1985). Furthermore Lei et al. (1985) have found three closely linked pectate lyase genes in a different strain of *Ecc*, (two of the lyases had pI's of 9.4 and the third a pI of 9.1). This suggests that the other clones (H2 and H5) must encode lyases with minor activities in *Ecc* or else further lyases with pI's around 9 as was found by Lei et al. (1985). The former seems to be the case for H2 which encodes a lyase with a pI of 8 (migrating between *b*, pI 7.8 and *c*, pI 8.2 of *Ech* B374). We have not yet identified the enzyme encoded by H5 in this system.

Conclusion

We have succeeded in isolating five pectinase genes from *Ecc* SCRI193: four pectate lyase genes and one polygalacturonase gene. We aim to determine the DNA sequence of these genes in order to search for potential domains of homology (or structural similarities) in the gene and protein sequences. Such homologies between the pectinases and cellulases and proteases might relate to common mechanisms of secretion for these enzymes as has been suggested by genetic studies (Andro et al., 1984; Thurn and Chatterjee, 1985; G.P.C. Salmond et al., pers. comm., 1986). This molecular approach together with the genetic approaches available will enable us to dissect the secretion process in this strain of *Erwinia carotovora*.

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REFERENCES

Andro, T., Chambost, J.-P., Kotoujansky, A., Cattaneo, J., Bertheau, Y., Barras, F., Van Gijsegm, F., and Coleno, A. 1984. Mutants of *Erwinia chrysanthemi* defective in secretion of pectinase and cellulase. *J. Bacteriol.* **160**, 1199–1203.

Collmer, A., Schoedel, C., Roeder, D.L., Ried, J.L., and Rissler, J.F. 1985. Molecular cloning in *Escherichia coli* of *Erwinia chrysanthemi* genes encoding multiple forms of pectate lyase. *J. Bacteriol.* **161**, 913–920.

Gough, J.A. and Murray, N.E. 1983. Sequence diversity among related genes for recognition of specific targets in DNA molecules. *J. Mol. Biol.* **166**, 1–19.

Hinton, J.C.D., Perombelon, M.C.M., and Salmond,G.P.C. 1985. Efficient transformation of *Erwinia carotovora* subsp. *carotovora* and *Erwinia carotovora* subsp. *atroseptica*. *J. Bacteriol.* **161**, 786–788.

Holmes, D.S. and Quigley, M. 1981. A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114**, 193–197.

Lei, S.-P., Lin, H.-C., Heffernan, L., and Wilcox, G. 1985. Cloning of the pectate lyase genes from *Erwinia carotovora* and their expression in *Escherichia coli*. *Gene* **35**, 63–70.

O'Callaghan, C.H., Morris, A., Kirby, S., and Shingler, A.H. 1972. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. *Antimicrob Agents Chemother.* **1**, 283–288.

Ried, J.L. and Collmer, A. 1985. Activity stain for rapid characterization of pectic enzymes in isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gels. *Appl. Environ. Microbiol.* **50**, 615–622.

Reverchon, S., Hugouvieux-Cotte-Pattat, N., and Robert-Baudouy,J. 1985. Cloning of genes encoding pectolytic enzymes from a genomic library of the phytopathogenic bacterium, *Erwinia chrysanthemi*. *Gene* **35**, 121–130.

Rombouts, F.M. and Pilnik, W. Pectic enzymes. In: *Microbial Enzymes and Bioconversions*. A.H. Rose, ed. (Economic Microbiology. Vol. 5). Academic Press, London, 1980, pp. 227–282.

Salmond, G.P.C., Hinton, J.C.D., Gill, D.R., and Perombelon, M.C.M. 1986. Transposon mutagenesis of *Erwinia*: Phage λ as a suicide vector. *Molec. Gen. Genet.* (in press).

Van Gijsegm, F., Toussaint, A., and Schoonejans, E. 1985. *In vivo* cloning of the pectate lyase and cellulase genes of *Erwinia chrysanthemi*. *EMBO J.* **4**, 787–792.

Zinc, R.T. and Chatterjee, A.K. 1985. Cloning and expression in *Escherichia coli* of pectinase genes of *Erwinia carotovora* subsp. *carotovora*. *Appl. Environ. Microbiol.* **49**, 714–717.