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Cloning and Expression in *Escherichia coli* of Pectinase Genes of *Erwinia carotovora* subsp. *carotovora*†

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Genes coding for an endo-pectate lyase, an exo-pectate lyase, and an endopolygalacturonase of *Erwinia carotovora* subsp. *carotovora* Ecc71 were cloned in *Escherichia coli* HB101, using the cosmid pHC79. The products of the cloned pectinase genes paralleled their counterparts in strain Ecc71 in isoelectric mobility, mode of substrate degradation, and ability to macerate potato tuber tissue.

The soft-rot-causing *Erwinia* spp. produce several classes of pectinases (see references 8 and 11 for review), some of which have been reported to be involved in plant tissue maceration (2, 6, 8, 17, 19). In addition, most soft-rot *Erwinia* spp. produce multiple isozymes of pectate lyase (PL) (1, 17), and as a result, little progress has been made in determining the role of each class of pectinase in disease development. Conventional in vitro genetic and mutagenic techniques have proved inefficient for construction of *Erwinia* strains defective in individual classes of pectinases, owing in part to the masking of the loss of one pectinase by the continued expression of remaining pectinases (7). To circumvent this difficulty in mutant selection and to facilitate the study of individual pectinase genes in and out of their natural background, a genomic library of *Erwinia carotovora* subsp. *carotovora* 71 (22), hereafter Ecc71, was constructed. In this study, we compared the properties of pectolytic enzymes produced by *Escherichia coli* carrying hybrid cosmids with the pectolytic enzymes produced by Ecc71.

The wild-type strain Ecc71 produces at least five classes of pectinases, depending on substrate and growth conditions. Cells grown in minimal salts medium (4) with citrus pectin as the sole carbon source secreted three distinct classes of pectinases separable by nonequilibrium isoelectric focusing (Fig. 1). Based on catalytic properties and mode of substrate cleavage, these pectinases were classified in the following manner: exo-PL (PLI), endo-PL (PLII), and endopolygalacturonase (endo-PG). The remaining pectinases produced by Ecc71, pectin methylesterase (unpublished data) and pectin lyase (20), will not be discussed in this report.

The genomic library of Ecc71 was constructed as described by Collins (9), utilizing the *Sal*I site of pHC79. Lambda-packaged hybrid cosmids were absorbed to *E. coli* HB101, in which ampicillin-resistant (Amp^r), tetracycline-sensitive (Tet^s) transductants were detected at a frequency of $1.5 \times 10^4/\mu g$ of chromosomal DNA. Assuming there is the same degree of similarity between the chromosomes of *E. coli* and *E. carotovora* as reported for *E. coli* and *Erwinia chrysanthemi* (5, 14, 18), we estimated that a library of 1,000 transductants would represent the complete genome of Ecc71. This was substantiated by the detection of several hybrid cosmid clones complementing strain HB101 for each of the following markers: *pro*, *leu*, *thi*, *lac*, *gal*, *xyl*, and *recA*. *Sal*I digests of representative hybrid cosmids from each complementation class revealed a DNA insert size ranging from 10 to 43 kilobases, with a mean of 30 kilobases.

Putative pectinase clones were scored on polygalacturonate-yeast extract agar (6) by the development of a precipitate or clearing around the colony or both after flooding of the medium with 6 N HCl. Of the 1,000 Amp^r Tet^s transductants screened, 30 were positive for pectinase on polygalacturonate-yeast extract agar. Each of these was subsequently assayed for intracellular and extracellular PL and PG as described previously (6, 10). Of these, 6 were found to produce PL and 24 were found to produce PG. We did not detect the production of more than one class of pectinase by any of the pectolytic *E. coli* clones under these experimental conditions. The aberrant high frequency of PG-producing clones is likely due to the fact that the PG gene occurs in a region of DNA which lacks internal *Sal*I sites.

Three pectinase-producing *E. coli* clones, AC501, AC502, and AC503, each representing one of the three classes of

TABLE 1. Ability of various pectinases to depolymerize sodium polypectate

Strain	Pectinase	Source of pectinase	Assay conditions[a]	Viscosity index[b]
Ecc71	PLII	Electrofocused	pH 8.5, Ca^{2+}	2,100
Ecc71	PLI	Electrofocused	pH 8.5, Ca^{2+}	664
Ecc71	PG	Electrofocused	pH 5.2, EDTA	3,125
AC501	PL	Cell lysate	pH 8.5, Ca^{2+}	2,300
AC502	PL	Cell lysate	pH 8.5, Ca^{2+}	361
AC503	PG	Cell lysate	pH 5.2, EDTA	2,857
W2	PL	Cell lysate	pH 8.5, NaCl	277

[a] Isoelectric-focused enzyme or cell lysate (1 ml) was combined with 5 ml of 0.6% sodium polypectate in either 0.12 M Tris hydrochloride (pH 8.5) or 0.1 M acetate buffer (pH 5.2) supplemented with 0.25 M NaCl, 25 mM EDTA, or 0.4 mM $CaCl_2$, as indicated. The reaction mixtures were incubated at 30°C for set time intervals, after which the reaction was terminated by the addition of 1 ml of Nelson copper reagent (15). The decrease in viscosity due to the depolymerization of sodium polypectate was measured in an Ostwald-Fenske 200 viscometer at 21°C.

[b] The viscosity index is the reciprocal of the time, in minutes, multiplied by 10^3, required for one unit of enzyme to cause a 50% reduction in viscosity (3). One unit of enzyme is that amount required to generate 1 μmol of either unsaturated digalacturonate (for PL) or galacturonate (for PG) from polygalacturonate per min per ml at 30°C.

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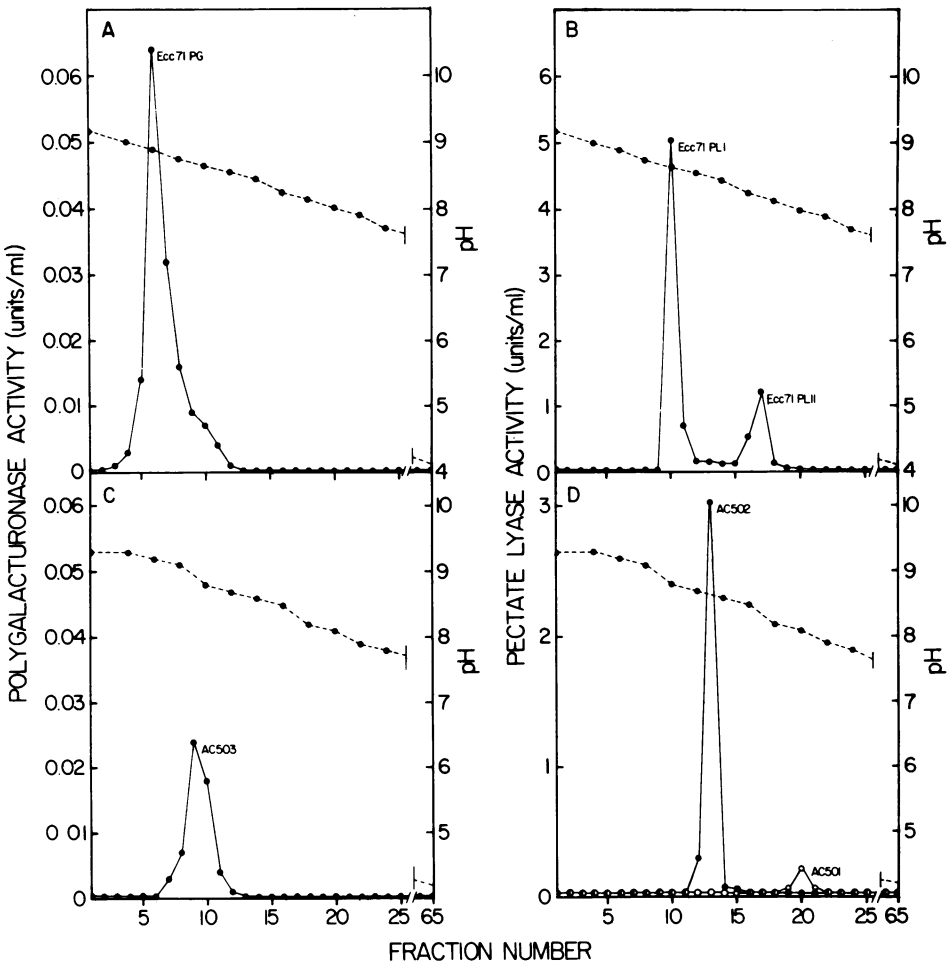

FIG. 1. Separation of pectinases produced by *E. carotovora* subsp. *carotovora* Ecc71 and *E. coli* strains AC501, AC502, and AC503 by nonequilibrium isoelectric focusing. Cells of strain Ecc71 were grown in 100 ml of minimal salts medium (4) containing 0.5% citrus pectin at 30°C. After 16 h of incubation, the culture fluid was cleared by centrifugation, dialyzed against 20 volumes of 10 mM acetate buffer (pH 5.2) for 6 h, concentrated 100-fold by lyophilization, and dialyzed against 10^3 volumes of 10 mM acetate buffer (pH 5.2) for 4 h. Cell sonicates of *E. coli* strains were clarified by centrifugation and used directly. Isoelectric focusing was done in tube gels (16, 20) with pH 3 to 10 Isolytes (Isolab Inc., Akron, Ohio). (A) PG activity of strain Ecc71; (B) PL activities of strain Ecc71; (C) PG activity of *E. coli* AC503; (D) PL activities of *E. coli* strains AC502 (●———●) and AC501 (○———○). ●– – –●, pH.

pectinases produced by Ecc71, were further characterized. When a crude enzyme preparation from each of these *E. coli* strains was subjected to nonequilibrium isoelectric focusing, one activity peak was detected in each gel at a pH which coincided with the pH of an activity peak in a nonequilibrium isoelectric focusing gel of Ecc71 culture supernatant (Fig. 1). Although this is not an equilibrium system, proteins are still separated by charge, indicating similar isoelectric mobilities between individual cloned pectinases produced in *E. coli* and individual pectinases produced in Ecc71.

By measuring the rate of reduction in viscosity of sodium polypectate by enzymatic degradation, it was possible to classify Ecc71 PLI and AC502 PL as exo-PLs, Ecc71 PLII and AC501 PL as endo-PLs, and Ecc71 PG and AC503 PG as endo-PGs (Table 1). *Erwinia* sp. strain W2 (12), which produces only an exo-PL, was used as a control in viscometric assays. This classification was further supported by thin-layer chromatography of the products generated from the degradation of polygalacturonate by the pectinases produced by the *E. coli* strains. The products formed by AC502 were unsaturated dimer and trimer; by AC501, unsaturated dimer to multimer; and by AC503, saturated dimer to multimer (Table 2). In addition to in vitro assays, potato tuber maceration experiments, although not quantitative, did demonstrate that both crude pectinase preparations from the *E. coli* strains and electrofocused pectinases from Ecc71

TABLE 2. Chromatographic mobilities of products formed from the degradation of sodium polypectate by cloned pectinases from *E. carotovora* subsp. *carotovora*[a]

E. coli strain	Pectinase	Reaction conditions[b]	R_{gal} values[c]
AC501	PL	pH 8.5, Ca^{2+}	0.14, 0.24, 0.40, 0.58, 0.81
AC502	PL	pH 8.5, Ca^{2+}	0.76, 0.98
AC503	PG	pH 7.0, EDTA	0.24, 0.37, 0.54, 0.73

[a] Ascending thin-layer chromatography was done on an Eastman 13255 cellulose chromatogram sheet for 8 h at room temperature in *n*-butanol–water–acetic acid (5:3:2, vol/vol). Chromatograms were developed with bromophenol blue.

[b] Reaction mixtures contained 0.3 ml of enzyme and 0.1 ml of 1% polygalacturonate in either 50 mM Tris hydrochloride (pH 8.5)–1.0 mM $CaCl_2$ or 40 mM phosphate buffer (pH 7.0)–1.0 mM EDTA. Reaction mixtures were incubated at 30°C for 16 h.

[c] R_{gal} values are the ratio of product migration to the distance migrated by a D-galacturonic acid standard.

TABLE 3. Pectinase activities of *E. carotovora* subsp. *carotovora* Ecc71, three *E. coli* HB101 cosmid clones (AC503, AC502, AC501), and *E. coli* HB101 containing pHC79

Strain[a]	Enzyme[b]	Total activity[c]	% of total activity in[d]:		
			Culture fluid	Periplasmic fraction	Cell lysate
Ecc71	PL	0.29	34	37	29
	PG	0.30	6	34	60
AC503	PL	Nil			
	PG	0.26	13	69	13
AC502	PL	0.05	41	55	4
	PG	Nil			
AC501	PL	0.09	11	84	5
	PG	Nil			
HB101(pHC79)	PL	Nil			
	PG	Nil			

[a] Strains AC503, AC502, and AC501 are *E. coli* HB101 carrying pHC79 with Ecc71 DNA inserts of 38, 42, and 36 kilobases, respectively.

[b] PL and PG activities were assayed as described previously (6, 10).

[c] Total activity = specific activity (micromoles of unsaturated digalacturonate [for PL] or galacturonate [for PG] generated from polygalacturonate per minute per milligram of protein) per milliliter of culture.

[d] Cultures were grown in L broth at 33°C for 24 h; cosmid-containing cultures were supplemented with ampicillin (75 μg/ml). Cells were concentrated 10-fold, and spheroplasts were prepared by the technique of Witholt et al. (21). Spheroplasts were lysed by ultrasonication. Culture fluids were used directly.

caused maceration of tuber tissue. This indicates that these individual pectinases can macerate tuber tissue independently and that the products of the cloned genes retained activity on plant tissue.

Comparison of the total pectinase activities of cosmid clones with pectinase activities of Ecc71 presents an interesting difference. With regard to PG activity, strain AC503 produced approximately the same level of activity as Ecc71 when Ecc71 was grown under noninduced conditions (Table 3). The relatively high level of PG activity in AC503 is likely due to the presence of more than one copy of the hybrid cosmid and to the derepressed state of the PG gene. This is supported by separate studies (unpublished) which indicate that the PG gene is naturally derepressed in Ecc71. On the other hand, with regard to PL activity, strains AC501 and AC503 produced only a fraction of the activity produced by Ecc71 when Ecc71 was grown under noninduced conditions (Table 3). In Ecc71, PL genes appear to be under stringent regulation (unpublished data), and as a result, when cloned in *E. coli* they may be repressed. In each case, however, all three cloned pectinases were localized chiefly in the periplasmic space of *E. coli* with various amounts occurring in the culture fluid; this is similar to observations made for *E. coli* strains carrying *E. chrysanthemi* PL genes (13).

On the basis of these data, we conclude that Ecc71 when grown in the presence of pectin produces three pectinases which can be differentiated by pI and mode of substrate degradation. Moreover, the structural genes for these three pectinases are also represented in the genomic library constructed from Ecc71. The possibility remains, however, that additional pectinases exist, undetected by the techniques we have used. Such an inconsistency would likely be revealed by a more detailed analysis of this system. Aside from this, cloning of these pectinase genes in *E. coli* now makes it possible to conduct site-directed mutagenesis in Ecc71 and to assess the roles of each enzyme in the development of soft-rot disease.

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