Evidence that Polygalacturonase Is a Virulence Determinant in
Erwinia carotovora

SHAU-PING LEI, HUN-CHI LIN, LAUREL HEFFERNAN, AND GARY WILCOX*

Department of Microbiology and Molecular Biology Institute, University of California, Los Angeles, California 90024

Received 13 May 1985/Accepted 14 August 1985

Polygalacturonase (PG) was purified from Erwinia carotovora EC. A hybrid cosmid, pSH711, that encodes PG activity but not pectate lyase activity was identified from an E. carotovora genomic library by an immunological screening method. A cell extract of Escherichia coli cells containing pSH711 was able to produce plant tissue maceration when spotted on carrot, potato, or turnip slices. In addition, the E. coli strain containing this plasmid was able to macerate carrot, potato, and turnip slices. Our results suggest that PG plays an important role in soft-rot disease.

The pectolytic enterobacteria Erwinia carotovora and Erwinia chrysanthemi are the most important members of the soft-rotting Erwinia species (18). They synthesize and excrete pectic enzymes, cellulases, proteases, and phospholipases (1). The pectic enzymes are thought to be a major factor in the production of soft-rot disease in plants (2). Pectic substances make up the major component of the middle lamella and a portion of the primary plant cell wall. Pectate lyase (polygalacturonic acid trans-eliminase; PL) and polygalacturonase (PG) are pectic enzymes which depolymerize the same substrate, polygalacturonic acid, but use different mechanisms (16). PL is able to cleave polygalacturonic acid by a trans-elimination type of reaction, the monomer products of which are 4-deoxy-5-oxo-D-glucurionate, whereas PG hydrolyzes polygalacturonic acid to yield monomers of D-galacturonic acid.

An avirulent mutant of E. chrysanthemi EC16 that had a reduced level of PL activity but retained the same level of PG activity has been isolated (5). Therefore, it was concluded that PL was the only important pectic enzyme involved in causing soft-rot disease in E. chrysanthemi. Because polygalacturonic acid is a substrate for both PG and PL, we wanted to determine whether PG also digests the polygalacturonic acid in the plant cell wall and acts as a virulence determinant in E. carotovora. Therefore, we purified PG from E. carotovora, cloned and expressed the E. carotovora gene coding for PG in Escherichia coli, and then tested the ability of the E. coli strain expressing PG to macerate plant tissue.

MATERIALS AND METHODS

Strains and plasmids. Bacterial strains used were E. coli HB101 (proA2 ara-l4 leu galK2 zyl-5 lacY1 mtl-l rpsL20 hsdS20 supE44 recA13) (3) and E. carotovora EC (obtained from A. K. Chatterjee, Kansas State University, Manhattan) and EC1000 (11). The cosmid used in this study was pJBB (8).

Chemicals and media. Minimal salts casein hydrolysate medium was previously described (11). Tryptone-yeast extract broth (4) was used for the routine cultivation of bacterial strains. Polygalacturonic acid, purchased from Sigma Chemical Co. (catalog no. P 3889), was added to the media at a final concentration of 0.5% (wt/vol). Ampicillin, purchased from Bristol Laboratories, was added to the media when required at a concentration of 50 μg/ml. Restriction enzymes were from Bethesda Research Laboratories, Inc. [α-32P]dATP was purchased from Amersham Corp.

PG assay. The method for determining PG activity was described previously (11). Briefly, an appropriate amount of purified PG or cell extract was added to Eppendorf tubes containing 0.05 M sodium acetate (pH 5.2), 0.1 M NaCl, and 0.5% polygalacturonic acid, and the tubes were incubated at 30°C for 30 min. After the reaction was stopped, thiobarbituric acid reagent was added to the tubes, and they were heated at 100°C for 30 min. The activity was determined from the increase in the absorbance (optical density) at 515 nm (OD515). One unit of PG was defined as an increase of 1 OD515 unit in 1 h at 30°C.

PL assay. An appropriate amount of purified PL protein was added to 1.5-ml Eppendorf tubes containing 1 ml of reaction buffer (0.05 M Tris hydrochloride [pH 8.5], 1 mM CaCl2, 0.25% polygalacturonic acid), and the tubes were incubated at 30°C for 30 min (11). The reaction was stopped by heating the samples at 100°C for 5 min. The activity was determined from the increase in the absorbance at 235 nm (OD235) (14). One unit of PL was defined as an increase of 1 OD235 unit in 30 min at 30°C.

Purification of PG. E. carotovora EC was grown at 30°C in minimal casein hydrolysate broth supplemented with polygalacturonic acid. Cells in late log phase (OD600, 1.0) were harvested by centrifugation. The supernatant was diluted with 3 volumes of distilled water and applied to a CM-52 ion-exchange column (Whatman, Inc.) equilibrated with 10 mM phosphate buffer (pH 7.5). Proteins were eluted with a gradient of 0 to 0.3 N NaCl in 10 mM phosphate buffer (pH 7.5). Fractions containing PG activity were saved.

Preparation of material for identification of the PG clone. The preparation of the genomic library and the procedures for antibody preparation, radioimmunoscreening, and immunoblot analysis were previously described (11).

Preparation of cell extracts. E. carotovora EC1000 containing pJBB and E. coli HB101 containing pJBB or pSH711 were grown at 30 and 37°C, respectively, in 10 ml of minimal salts casein hydrolysate broth supplemented with polygalacturonic acid and ampicillin. Cells in the late log phase of growth were harvested by centrifugation. The cell pellet was suspended in 0.5 ml of 30 mM Tris hydrochloride (pH 8.0)–10 mM EDTA and sonicated three times for 10 s at 30-s intervals with a sonicator (Heat System Ultrasones; model W-220F) at a power setting of 6. After sonication, the sample

* Corresponding author.
was centrifuged for 15 min in an Eppendorf microfuge at 4°C. The supernatant was collected and used as a cell extract. The periplasmic and cytoplasmic fractions were prepared as previously described (11).

DNA hybridization. Plasmid pSH711 was digested with various restriction enzymes, and the fragments were separated on a 1% agarose gel. The DNA was then blotted to nitrocellulose paper as described by Smith and Summers (17). The prehybridization was performed in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) and 1× Denhardt solution (13) at 65°C. The hybridization was done under moderately stringent conditions with 30% formamide–10 mM Tris (pH 7.4)–1 M NaCl in 10× Denhardt solution (19). Plasmid pSH2111 (11) that codes for the 44,000- and 41,000-dalton PL proteins and plasmid pJB8 were nick translated separately (15) and used as DNA hybridization probes.

Plant tissue maceration assay. Carrot, potato, or turnip slices were spotted with E. coli HB101 containing pSH711, with cell extracts, or with purified PG and incubated at 30°C. When anaerobic conditions were desired, the slices were incubated in an anaerobic jar. Sliced potatoes were rinsed with sterile 1% NaCl to prevent browning before they were placed in sterile petri dishes and spotted with protein or cells. Cells were washed once with 0.03 M Tris hydrochloride–10 mM EDTA (pH 8.0) before they were spotted on the sliced vegetables.

RESULTS

Purification and characterization of PG. PG was purified from the culture supernatant of E. carotovora as described in Materials and Methods. It eluted from the column at a NaCl concentration of approximately 0.17 M, whereas PL eluted at an NaCl concentration of approximately 0.13 M. PG (3 mg) was isolated from 2 liters of E. carotovora culture supernatant; the protein was greater than 95% pure as judged by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Purified PG was assayed for PL activity, and none was found. The molecular weight of the protein was estimated to be 43,000 by SDS-polyacrylamide gel electrophoresis (Fig. 1). The size of PG was very close to that of the PL proteins (44,000 and 41,000 daltons) (11). Antibody raised against PG cross-reacted with the PLs but with approximately one-fifth of the affinity the antibodies had for PG (Fig. 2). Antibody raised against the PLs cross-reacted with PG but with approximately one-fifth of the affinity the antibodies had for PL (Fig. 2).

Immunoscreening of the genomic library. The antibody raised against PG was used to screen the cosmid library as previously described (11). A positive clone was identified, and subsequent restriction analysis showed that it contained an insert of approximately 8 kilobases. This plasmid was designated pSH711. Cell extracts prepared from E. coli HB101 harboring plasmid pSH711 contained functional PG as measured by the PG enzyme assay. No PL activity was detected (data not shown). The cell extracts were subjected to immunoblot analysis by using an anti-PG antibody, and a single protein band of the same size as the purified PG was revealed (Fig. 3). In an identical experiment with anti-PL antibody, no protein band corresponding to the known size of any of the PL proteins was identified.

Enzyme location in E. coli cells. The location of PG in the E. coli cell was determined by preparing different cell fractions as described in Materials and Methods. The amount of PG located in the culture supernatant and periplasmic and cytoplasmic spaces was determined by immunoblot analysis. Approximately 80% of the PG was observed in the periplasmic region; PG was also detected in the culture supernatant (Fig. 4).

Restriction map analysis of pSH711. Because antibody against PG can recognize PL and antibody against PL can recognize PG, these proteins have at least some homology. We took advantage of this observation and used the cloned PL genes (11) as a DNA probe to localize the PG gene on plasmid pSH711. pSH2111 (11) and vector pJB8 were nick translated and used to probe pSH711. The result of DNA

FIG. 1. SDS-polyacrylamide gel electrophoresis of purified PG. Left: PLs (1.5 μg) purified from E. carotovora EC culture supernatant. Center: PG (1 μg) purified from E. carotovora EC culture supernatant. Right: Protein molecular weight size standards. Protein samples were prepared as described in Materials and Methods. Each sample was mixed with loading buffer, heated at 100°C for 5 min, and loaded on a 15.9% SDS-polyacrylamide (acrylamide:bis, 25:1) gel. The gel was stained with Coomassie blue.

FIG. 2. Immunoblot analysis of the purified proteins. (a) The antiserum raised by PG was used to detect 0.10 μg of purified PLs (left) and 0.02 μg of purified PG (right). (b) The antiserum raised by the PLs was used to detect 0.5 μg of purified PG (left) and 0.2 μg of purified PLs (right).
hybridization suggested that the location of the PG gene on pSH711 spanned the HindIII site between the two 1.9- and 2.2-kilobase HindIII fragments (Fig. 5). Subsequent subcloning confirmed that this region codes for PG activity. None of the individual HindIII fragments derived from pSH711 resulted in PG activity when subcloned in pBR322. However, a pBR322 subclone which contained both the 1.9- and 2.2-kilobase HindIII fragments coded for PG activity.

**Maceration test.** Five micrograms of purified PG or the PLs in a 20-μl volume of 10 mM phosphate buffer (pH 7.5) was separately spotted on carrot, potato, or turnip slices, respectively, to test its ability to macerate plant tissue. All three vegetables showed significant tissue maceration under aerobic conditions in 30 h (data not shown). Cells or cell extracts of *E. coli* HB101 containing pSH711 were spotted on vegetable slices. The carrots, potatoes (Fig. 6), or turnips were macerated within 30 h under aerobic conditions.

In a separate experiment with potato slices and purified PG or cell extracts of *E. coli* HB101 containing pSH711, maceration was observed under both aerobic and anaerobic conditions (data not shown). The slices incubated anaerobically showed more rapid maceration than the slices incubated aerobically.

**DISCUSSION**

PG was isolated and purified from *E. carotovora* EC to greater than 95% purity (Fig. 1). It has an apparent molecular weight of 43,000 as determined by SDS-polyacrylamide gel electrophoresis and induces plant tissue maceration when spotted on carrot, potato, or turnip slices. To demonstrate that the maceration caused by our purified PG protein was not due to any contamination of PL, the gene coding for PG was cloned and expressed in *E. coli*. Plasmid pSH711 that contains the PG gene was first identified by immunoscreening of a genomic library. No PL protein was identified from the same plasmid-containing *E. coli* strain. PG protein produced from the *E. coli* clone has the same apparent molecular weight as that produced by *E. carotovora* (Fig. 3).

*E. coli* cells producing PG were tested for their ability to macerate plant tissue. Both whole cells and cell extracts of strain HB101(pSH711) were able to macerate carrot, potato (Fig. 6), and turnip slices. Our results indicate that PG protein made in *E. coli* without any PL contamination can also macerate plant tissue, suggesting that PG alone can produce tissue maceration. A previous study showed that the PL genes cloned from the same *E. carotovora* strain
produced PL in *E. coli* that was capable of causing plant tissue maceration (11).

Soft-rot disease may be an anaerobic phenomenon (12). We used potato tuber slices incubated aerobically or anaerobically after being spotted with purified enzymes, whole cells, or cell extracts as a model system for soft-rot disease. The results from this model system suggest that both PL and PG are probably virulence determinants in *E. carotovora*.

The distribution of PG in *E. coli* cells is different from that of the PLs (11). Most of the PG made by the *E. coli* strain containing plasmid pSH711 was observed in the periplasmic space (Fig. 4), as was the case for the PLs (11). However, PG was detected in the culture fluid by the immunoblot analysis (Fig. 4), whereas the PLs were not (11). We do not know whether the PG that appeared in the culture supernatant was excreted by *E. coli* or whether it was released from dead cells. That PG appeared in the *E. coli* culture supernatant is consistent with the observation that *E. coli* HB101 containing pSH711 macerated potato slices. Thus, PG produced by the cloned gene appears to be a virulence factor in soft-rot disease.

The genes coding for PL and PG in *Erwinia* species probably constitute a gene family. Recent reports of the cloning of the PL genes from *E. chrysanthemi* (6, 7, 9, 10) showed that at least two PL genes are present in each strain. At least three PL genes are present in *E. carotovora* (11). The DNA sequences of the three PL genes in *E. carotovora* are highly homologous to each other (S. P. Lei and G. Wilcox, unpublished data). In this paper, we showed that sequences in or adjacent to the PG gene are homologous to the PL genes. Although the restriction map of plasmid pSH711 indicates that the PG gene does not share some restriction endonuclease sites with the three PL genes, the antibody cross-reactivity and DNA hybridization results suggest that there is some homology at the gene level.

The pectic depolymerases include many different enzymes (16). Different forms of pectic enzymes may exist in soft-rot bacteria, because plants may have different compositions of pectic substances in their cell walls. Multiple pectic enzymes may be necessary for the microorganism to degrade most rapidly the complex structure of different plant cell walls to obtain carbon and energy sources.

**ACKNOWLEDGMENTS**

We thank Lori Stoltzfu, Laura Cass, and Pat Clarke for comments on the manuscript. This research was supported by a grant from the U.S. Department of Agriculture (59-2065-1-1-1-745-0) and by a Public Health Service grant from the National Institute of General Medical Sciences (GM30491).

**LITERATURE CITED**


teriol. 159:825-831.