Comparison of Pectic Enzymes Produced by Erwinia chrysanthemi, Erwinia carotovora subsp. carotovora, and Erwinia carotovora subsp. atroseptica

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Erwinia spp. that cause soft-rot diseases in plants produce a variety of extracellular pectic enzymes. To assess the correlation between patterns of pectic enzyme production and taxonomic classification, we compared the enzymes from representative strains. Supernatants obtained from polygalacturonate-grown cultures of nine strains of Erwinia chrysanthemi, three strains of E. carotovora subsp. carotovora, and three strains of E. carotovora subsp. atroseptica were concentrated and subjected to ultrathin-layer polyacrylamide gel isoelectric focusing. Pectate lyase, polygalacturonase, and exo-poly-α-d-galacturonosidase activities were visualized by staining diagnostically buffered pectate-agarose overlays with ruthenium red after incubation of the overlays with the isoelectric focusing gels. The isoelectric focusing profiles of pectate lyase and polygalacturonase were nearly identical for strains of E. carotovora subsp. carotovora and E. carotovora subsp. atroseptica, showing three pectate lyase isozymes with isoelectric points higher than 8.7 and a polygalacturonase with pI of ca. 10.2. Isoelectric focusing profiles of the E. chrysanthemi pectic enzymes were substantially different. Although there was considerable intraspecific heterogeneity, all strains produced at least four isozymes of pectate lyase, which could be divided into three groups: basic (pI, ca. 9.0 to 10.0), slightly basic (pI, ca. 7.0 to 8.5), and acidic (pI, ca. 4.0 to 5.0). Several strains of E. chrysanthemi also produced a single form of exo-poly-α-d-galacturonosidase (pI, ca. 8.0). The pectic enzyme isoelectric profiles corroborate previous taxonomic studies of soft-rot Erwinia spp., showing the similarity of E. carotovora subsp. carotovora and E. carotovora subsp. atroseptica and the distinctness of E. chrysanthemi.

Erwinia chrysanthemi, E. carotovora subsp. carotovora, and E. carotovora subsp. atroseptica cause soft rots and other diseases in plants and are capable of producing massive amounts of pectolytic enzymes. These two characteristics distinguish them from other members of the enterobacterial genus Erwinia. During the historical development of Erwinia taxonomy it was suggested that the pectolytic Erwinia spp. belong in a separate genus, Pectobacterium (36), that E. chrysanthemi be classified as a subspecies of E. carotovora (11), and that E. carotovora be divided into the subspecies carotovora and atroseptica (11). The current inclusion of pectolytic species in the genus Erwinia is supported by the existence of species that are taxonomically intermediate between them and the nonpectolytic-type species, E. amylovora (18). The separate-species status of E. chrysanthemi and E. carotovora is supported by differences in DNA homology (3, 26), serological relationships (37), and physiological traits such as acid production from trehalose, sensitivity to erythromycin, and the presence of lecinthinase activity (18).

The current recognition of two subspecies of E. carotovora is somewhat more controversial, being based on differences in four phenotypic characteristics: maximum growth temperature, production of reducing substances from sucrose, and acid production from maltose and α-methylglucoside (18). Serological studies indicate that E. carotovora subsp. atroseptica (the potato blackleg pathogen) represents a homogeneous group of strains that fall predominantly into a single serogroup, whereas E. carotovora subsp. carotovora is composed of 16 serogroups (7). The precise relationship of the two subspecies is not clear.

E. chrysanthemi has also been divided into six subdivisions or pathovars on the basis of the host of origin and limited physiological differences among strains (8, 10, 18). The biological significance of these subdivisions is presently unclear, since the reactions of a set of test plants to inoculation with E. chrysanthemi strains do not correlate reliably with subdivision designations (9).

Both E. chrysanthemi and E. carotovora possess the ability to produce high amounts of pectate lyase (PL; EC 4.2.2.2), which cleaves internal glycosidic linkages in polygalacturonate by β-elimination. It is likely that the enzyme has a role in pathogenesis for several reasons: (i) the purified enzyme can cause tissue maceration and cell death (1, 25, 33), (ii) recombinant Escherichia coli clones producing E. chrysanthemi PL possess at least a limited ability to macerate plant tissues (5, 14), and (iii) E. chrysanthemi mutants deficient in the production of extracellular PL are unable to cause maceration (4). Scattered evidence based on isoelectric focusing (IEF) has suggested that both E. chrysanthemi and E. carotovora produce multiple isozymes of PL (2, 12, 20, 28, 29, 34). Molecular cloning has revealed the existence of multiple pel genes encoding PL in both species (5, 14–16, 30, 35, 38). Biochemical studies with selected strains have also documented extracellular exo-poly-α-β-galacturonosidase (exoPG; EC 3.2.1.82) production in E. chrysanthemi (6) and polygalacturonase (PG; EC 3.2.1.15) production in E. carotovora (27). Both enzymes attack polygalacturonate hydrolytically, exoPG releases digalacturonate from the nonreducing end of the polymer; PG attacks internal linkages, generating a series of

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oligogalacturonates. A gene encoding PG has been cloned into *Escherichia coli* (17, 38).

Analysis of the growing body of information on the biochemistry and genetics of pectic enzyme production and its relationship to the taxonomy and pathogenicity of the soft-rot *Erwinia* spp. is complicated by the use of several different strains in the studies. For example, it is unclear whether *E. chrysanthemi* EC16, which has attracted considerable genetic research, is similar to strain CUCPB630 in its production of exoPG rather than PG (4, 6). It is similarly unclear whether PG production is a universal property of *E. carotovora*, or even whether all *E. chrysanthemi* strains produce multiple isozymes of PL.

We have addressed the problem of diversity in the production of PL, PG, and exoPG in *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* by directly comparing pectate-degrading enzyme profiles. Culture supernatants of strains from the six subdivisions of *E. chrysanthemi* and representative strains of *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* were resolved by IEF and activity stained with a pectate-agarose overlay. The resulting profiles were compared with each other to determine the variability of PL and PG for both *E. chrysanthemi* and *E. carotovora* and to determine the correlation between *E. chrysanthemi* pectolytic profiles and subdivision designations.

**MATERIALS AND METHODS**

**Bacterial strains.** Nine strains of *E. chrysanthemi* were used. These comprised all of the six pathovar subdivisions based on the host of origin and physiological properties (Table 1) as proposed by Dickey (8, 9) and Dickey and Victoria (10). In addition, three strains of both *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* were used (Table 2). *E. chrysanthemi* strains were obtained from R. S. Dickey (Cornell University) and A. K. Chatterjee (Kansas State University). Strains of both subspecies of *E. carotovora* were obtained from H. E. Moline (USDA, ARS, Beltsville Agricultural Research Center).

**Bacterial growth and extracellular enzyme preparation.** *E. chrysanthemi* strains were grown to late-stationary phase in LB broth (19) supplemented with 0.5% polygalacturonic acid (product P21750 of Pfaltz and Bauer). The extracellular supernatant, obtained after centrifugation at 8,000 × g, was concentrated 50-fold and equilibrated with 10 mM Tris hydrochloride (pH 7.5) by ultrafiltration. Enzyme samples from *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* strains were prepared exactly as described above (see Fig. 2) or prepared from cultures grown to late stationary phase in Zucke and Hankin medium (39) supplemented with 0.5% polygalacturonic acid (Fig. 1; see Fig. 4 and 5). In the latter case, the culture supernatants obtained after centrifugation were saturated to 90% with ammonium sulfate and allowed to stand for 16 h at 4°C. The mixture was centrifuged at 7,000 × g, and the pellet was suspended in 10 mM Tris hydrochloride (pH 7.5) and dialyzed extensively in dialysis tubing against the same buffer. The dialysis tubing was handled carefully because cellulases excreted by these bacteria slowly degrade it. The extracellular supernatant was concentrated approximately 100-fold. Results identical to those presented in this paper were obtained by preparing *E. chrysanthemi* CUCPB 1237 enzyme samples from supernatants of cultures grown on Zucke and Hankin-polygalacturonic acid medium and precipitated with ammonium sulfate as described previously (5, 31).

**Ultrathin-layer polyacrylamide gel IEF of concentrated extracellular supernatants.** Procedures for IEF have been described elsewhere (31). Briefly, ultrathin (0.35 mm) 5.0% polyacrylamide gels containing broad-range and high-pH ampholytes were cast on gel support film for acrylamide (Bio-Rad Laboratories). The electrode wicks were soaked in 0.04 M aspartic acid (anode) and 0.5 M NaOH supplemented with the high-pH ampholytes (cathode). Gels were pre-electrofocused at 2.0 W for 30 min, and then various amounts (1 to 20 μl) of concentrated extracellular supernatant were applied at the anodic portion of the gel. The specific amount of supernatant depended upon the level of PL activity in each sample as determined empirically by activity staining (see below) a test IEF gel. Subsequent electrofocusing was at 6.0 W with a 2,100-V maximum for 30 to 60 min. The minimum electrofocusing time required for protein banding was determined by applying the chromophore cytochrome c to the IEF gel and noting when it banded in the cathodic region. This did not constitute equilibrium IEF, but it did provide the best conditions for resolving isozymes and comparing isoelectric profiles. All electrophoresis was performed at 6°C in a N₂ environment on an LKB 2117 Multiphor apparatus modified with adjustable electrodes.

**Detection of pectolytic activity in gels following IEF.** Ultrathin (0.35 mm) pectate-agarose gels were cast on gel support film for agarose (Bio-Rad). Gels contained 0.1% polygalacturonic acid, 1.0% agarose (Bethesda Research Laboratories), and either 50 mM Tris hydrochloride (pH 8.5)–1.5 mM CaCl₂ for detection of PL, 50 mM potassium phosphate (pH 6.0)–5.0 mM EDTA for detection of hydrolytic pectic enzymes (optimum for exoPG) from *E. chrysanthemi* (6), or 50 mM sodium acetate (pH 5.5)–5.0 mM

### Table 1. *E. chrysanthemi* strain abbreviations, subdivisions, number, and hosts of origin

<table>
<thead>
<tr>
<th>Strain abbreviation</th>
<th>Subdivision</th>
<th>No.</th>
<th>Host of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ech 6</td>
<td>VI</td>
<td>PDDCC 2349</td>
<td>Plantain</td>
</tr>
<tr>
<td>Ech 1</td>
<td>I</td>
<td>EC 183</td>
<td>Diefenbachia</td>
</tr>
<tr>
<td>Ech 4A</td>
<td>IV</td>
<td>NCPPB 2543</td>
<td>Corn</td>
</tr>
<tr>
<td>Ech 4B</td>
<td>IV</td>
<td>113-2</td>
<td>Corn</td>
</tr>
<tr>
<td>Ech 2A</td>
<td>II</td>
<td>NCPPB 1849</td>
<td>Parthenium</td>
</tr>
<tr>
<td>Ech 2B</td>
<td>II</td>
<td>NCPPB 1861</td>
<td>Parthenium</td>
</tr>
<tr>
<td>Ech 3A</td>
<td>III</td>
<td>EC 16</td>
<td>Chrysanthemum</td>
</tr>
<tr>
<td>Ech 3B</td>
<td>III</td>
<td>NCPPB 427</td>
<td>Chrysanthemum</td>
</tr>
<tr>
<td>Ech 5</td>
<td>V</td>
<td>CUCPB 1237</td>
<td>Carnation</td>
</tr>
</tbody>
</table>

* As defined by Dickey (8, 9) and Dickey and Victoria (10).

* PDDCC, Plant Diseases Division Culture Collection, Auckland, New Zealand; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, England; CUCPB, Cornell University Collection of Phytopathogenic Bacteria, Ithaca, N.Y.

### Table 2. *E. carotovora* subsp. *atroseptica* (Eca) and *E. carotovora* subsp. *carotovora* (Ecc) strain abbreviations, numbers, and hosts of origin

<table>
<thead>
<tr>
<th>Strain abbreviation</th>
<th>No.</th>
<th>Host of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eca A</td>
<td>E18</td>
<td>Potato tuber</td>
</tr>
<tr>
<td>Eca B</td>
<td>E22S</td>
<td>Potato stalk</td>
</tr>
<tr>
<td>Ecc A</td>
<td>ATCC 33260</td>
<td>Potato tuber</td>
</tr>
<tr>
<td>Ecc B</td>
<td>ATCC 15713</td>
<td>Potato tuber</td>
</tr>
<tr>
<td>Ecc C</td>
<td>E31</td>
<td>Iris leaf</td>
</tr>
</tbody>
</table>

* ATCC type strain.
EDTA for detection of hydrolytic pectic enzymes (optimum for PG) from *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* (27). The casting apparatus was heated to 50°C before the 95°C pectate-agarose mixture was pipetted. The cooled pectate-agarose overlay was pressed against the IEF gel and incubated at room temperature for various times (5 to 30 min) depending upon the desired sensitivity. Prior to activity staining for PG in samples from *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora*, the IEF gel was washed for 5 min in 50 mM sodium acetate (pH 5.3) and then incubated with the pectate agarose overlay. The agarose overlay was then placed in 0.05% (wt/vol) ruthenium red (Sigma Chemical Co.) for 20 min, rinsed in water, and preserved by drying. Ruthenium red stains pectate and leaves a clear zone where the pectate has been enzymatically degraded.

**Analysis of hydrolytic pectic enzyme reaction products.**

Pectolytic fragments produced by the action of PG or exoPG on polygalacturonic acid were analyzed by cellulose chromatography (38). Concentrated supernatants from representative strains were incubated for 16 h at 30°C in a solution consisting of 0.25% polygalacturonic acid, 0.1 M NaCl, 0.05 M sodium acetate (pH 5.5), and 5.0 mM EDTA. Portions (20 μl) were applied to a thin-layer cellulose sheet (Kodak 13255) and developed for 4 h with an n-butanol-water-acetic acid (5:3:2, vol/vol) solvent system. Pectic fragments were visualized by immersion of the chromatogram in 0.05% bromophenol blue-1 mM Tris hydrochloride (pH 7.5)–95% ethanol. The chromatogram was then air dried. Areas containing fragments appeared yellow on a blue background.

**RESULTS**

**PL isozyme profiles.** Concentrated supernatants from *E. chrysanthemi*, *E. carotovora* subsp. *atroseptica*, and *E. carotovora* subsp. *carotovora* cultures were subjected to IEF and assayed for PL activity by using a pectate-agarose overlay buffered to specifically detect this enzyme (Fig. 1). All *E. chrysanthemi* strains exhibited multiple isozymes of PL that could be classified into three groups as first described by Pupillo et al. (28): basic (pH >8.5), neutral (pH 7.0 to 8.5), and acidic (pH < 7.0). (The acidic isozyme previously shown to be produced by strain Ech 5 [5] does not appear on this figure.) The 8-min incubation with the overlay demonstrated that some of the basic bands were doublets (lane Ech 5, basic isozymes). Additional bands were detected when the same IEF gel was incubated for 20 min with another pectate-agarose overlay.

*E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* cultures were incubated for 8 min with one overlay (left panel) and then for 20 min with another overlay (right panel). An explanation of strain abbreviations is given in Tables 1 and 2. The positions of the pl markers (carbonic anhydrase, horse myoglobin minor and major bands, whale myoglobin minor and major bands, and cytochrome c; FMC Corp.) are shown.

**FIG. 1.** IEF profiles of extracellular PL isozymes produced by strains of *E. chrysanthemi* (Ech strains), *E. carotovora* subsp. *atroseptica* (Eca strains), and *E. carotovora* subsp. *carotovora* (Ecc strain). PL isozymes in concentrated culture supernatant samples were activity stained with a pectate agarose overlay following IEF. The IEF gel was incubated for 8 min with one overlay (left panel) and then for 20 min with another overlay (right panel). An explanation of strain abbreviations is given in Tables 1 and 2. The positions of the pl markers (carbonic anhydrase, horse myoglobin minor and major bands, whale myoglobin minor and major bands, and cytochrome c; FMC Corp.) are shown.
subsp. carotovora strains also exhibited multiple isozymes of PL (Fig. 2). These strains, however, produced fewer isozymes, there was less variation in the profiles between strains, and the isozymes all had alkaline isoelectric points. The last point is best seen in Fig. 1, in which E. chrysanthemi, E. carotovora subsp. atroseptica, and E. carotovora subsp. carotovora are directly compared.

Production of hydrolytic pectic enzymes. Concentrated extracellular supernatants were subjected to IEF and assayed for PG and exoPG activity by using appropriately buffered pectate-agarose overlays. The buffer systems were designed to inhibit PL (by chelating essential divalent cations with EDTA) and to maximize the activity of the previously characterized E. carotovora PG and E. chrysanthemi exoPG. Figure 3 shows the production of a single species of exoPG by E. chrysanthemi strains that were originally isolated from dicot hosts (Ech 2A, Ech 2B, Ech 3A, Ech 3B, and Ech 5; Table 1), but not from strains isolated from monocot hosts (Ech 1, Ech 6, Ech 4A, and Ech 4B). The IEF gel from which this profile was obtained was first briefly incubated with a PL-detecting pectate-agarose overlay and then incubated for a longer period with an exoPG-detecting overlay. The PL profile of the IEF gel (not shown) revealed that no major PL bands corresponded to the exoPG bands and that equivalent amounts of PL from each strain were applied to the gel. The production of exoPG by only certain strains of E. chrysanthemi is thus not an artifact of differences in sample load. The PG produced by E. carotovora subsp. atroseptica and E. carotovora subsp. carotovora (Fig. 4) is quite distinct from those produced by E. chrysanthemi strains. A single band of activity was present in the extremely alkaline region, with a pl value that is identical for all strains.

Determination of the action pattern of the hydrolytic pectic enzymes. The action patterns of the hydrolytic pectin enzymes (endo-cleaving PG or exo-cleaving exoPG) produced by the well-studied strain E. chrysanthemi EC 16 and the ATCC type strains for E. carotovora subsp. atroseptica (ATCC 33260) and E. carotovora subsp. carotovora (ATCC 15713) (Tables 1 and 2) were determined by analyzing reaction products by thin-layer chromatography. The E. chrysanthemi strain cleaved the polymer in an exo-fashion and generated only dimers (Fig. 5, lanes 3 through 7), as determined from Rf values previously reported (38) and from comparisons with the monomer standard (lanes 1, 8, and 19). E. carotovora subsp. atroseptica and E. carotovora subsp. carotovora generated a series of oligomers (lanes 9 through 13 and 14 through 18, respectively), indicating that the polymer was cleaved in an endo or random fashion.

**FIG. 4.** IEF profiles of extracellular PG produced by strains of E. carotovora subsp. atroseptica (Eca strains) and E. carotovora subsp. carotovora (Ecc strains). PG isoenzymes in concentrated culture supernatant samples were activity stained following IEF by using a pectate-agarose overlay that specifically inhibited PL activity and enhanced PG activity. An explanation of the strain abbreviations is given in Table 2.

**DISCUSSION**

By directly comparing the extracellular pectic enzyme complexes of representative strains of E. chrysanthemi, E. carotovora subsp. carotovora, and E. carotovora subsp. atroseptica, we have documented fundamental, consistent differences between the enzyme systems of the two species. The E. chrysanthemi strains that were studied represent the six subdivisions or pathovars within that taxon. The E. carotovora strains were previously used in studies on the taxonomic utility of acidic ribosomal protein analysis (23, 24) and contain the ATCC type strains for E. carotovora subsp. atroseptica and E. carotovora subsp. carotovora.

The data presented here further support the taxonomic distinction between E. chrysanthemi and E. carotovora. Pectic enzyme IEF profiles may provide a useful means of identifying these bacteria that is an alternative to the presently used analysis of culture and physiological properties.
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(18) or two-dimensional polyacrylamide gel electrophoresis patterns of acidic ribosomal proteins (23, 24). IEF of pectic enzymes has the advantage of providing, in a single step, characteristic profiles of an attribute that appears central to pathogenicity in the soft rot erwinias. The test appears unambiguous because of the distinctness of the patterns produced by the two species.

Pectic enzyme IEF profiles do not appear to be useful for differentiating the two subspecies of E. carotovora, a distinction that continues to be controversial. Moline et al. (23, 24) suggested that the differences in two-dimensional polyacrylamide gel electrophoresis patterns of acidic ribosomal proteins warrant the separation of E. carotovora subsp. atroseptica and E. carotovora subsp. carotovora into two species. Mergaert et al. (22), on the other hand, found no significant differences between the two subspecies in a similarly recent numerical taxonomy study and argued for discontinuation of the differentiation of the two subspecies.

The PL isozyme profiles of E. chrysanthemi strains revealed interesting differences within an overall common pattern. All the strains tested produced basic, neutral (or slightly basic), and acidic isozymes, although the specific Pl values of those isozymes varied. Previous work with a limited number of strains had shown the production of all three isozyme groups (12, 28), but not for every strain or under all growth conditions. For example, Pupillo et al. (28) detected the production of an acidic PL isozyme in some strains only when they were grown on Dieffenbachia leaves.

The detection of an acidic isozyme in all the E. chrysanthemi strains we tested can be attributed to the extreme sensitivity of the pectate-agarose overlay when used in conjunction with ultrathin-layer IEF (31). It should be noted that the acidic isozyme band is not visible in the Ech 5 sample in Fig. 1, apparently because the sample was slightly underloaded relative to the others. The production of the acidic isozyme by Ech 5 has been documented (31).

A strong correlation was observed between E. chrysanthemi subdivision classification and PL isozyme profiles. Strains within a given subdivision produced very similar PL isozyme profiles. Two subdivisions, II and III (Fig. 1), were strikingly similar to each other in their PL isozyme profiles. A single species of exoPG could also be detected in IEF profiles, but, interestingly, only in subdivisions containing strains isolated from dicot hosts. Since there were equivalent amounts of PL in the culture supernatant samples assayed, it appears that the exoPG either is not produced or is regulated differently in the monocot strains. It is worth noting in this regard that monocot primary cell walls contain substantially less polygalacturonate than do dicot cell walls (21).

A gene encoding an exopolypgalacturonate lyase which releases unsaturated digalacturionate from pectic polymers was recently cloned into Escherichia coli from E. carotovora (38). Because of its mode of attack, such an enzyme would probably produce a minor band in the activity stain and would be indistinguishable from a PL band. We therefore cannot say whether exopolypgalacturonate lyase production is a widespread attribute of E. carotovora strains. It is also important to note that not all the bands appearing in these IEF profiles are direct gene products. A variety of posttranslational modifications can alter the isoelectric point of a protein, resulting in spuriously complex banding patterns in high-resolution IEF (13). Cloning experiments have revealed that some E. chrysanthemi strains contain at least five pel genes (15, 35). However, the PL isozyme profiles of subcloned pel gene products also reveal that a given pel gene can give rise to more than one band of activity (5).

The functional basis for the production of multiple PL isozymes by the soft-rot erwinias is unclear. Quantitative differences have been demonstrated in the ability of the isozymes to kill and macerate plant tissues, to reduce the viscosity of pectate solutions, and to be inactivated by host phenolic compounds (12, 29). These observations suggest that the isozymes have specialized activities. On the other hand, mutants deficient in two of the five E. chrysanthemi (Ech 5) pel genes are little altered in their ability to utilize pectate or macerate potato tuber tissues (32; D. L. Roeder and A. Collmer, in E. L. Civerolo, A. Collmer, R. E. Davis, and A. G. Gillaspie, ed., Proceedings of the Sixth International Conference on Plant Pathogenic Bacteria, in press). This observation suggests redundancy in the PL isozymes. The present demonstration that the production of multiple PL isozymes is a general feature among soft rot Erwinia strains invites further exploration of this phenomenon.

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LITERATURE CITED
