# Absence of Cell Wall Polysaccharide Degradation by Erwinia amylovora

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## ABSTRACT

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The composition of the cell wall polysaccharides prepared from *Erwinia amylovora*-infected and noninfected *Cotoneaster pannosa* and *Malus sylvestris* shoot and immature *Pyrus communis* fruit tissues was determined by gas-liquid chromatographic, wet chemical, and enzymatic techniques. Infected and comparable noninfected tissues did not differ significantly in the percentage composition of arabinose, xylose, glucose, galactose, mannose, rhamnose, galacturonic acid, or cellulose. No pectolytic, cellulolytic, or xylolytic enzyme activity was detected in extracts of infected shoots or in ooze produced during infection of pear fruits. No pectolytic enzymes were detected in the filtrates of ten liquid media in which the pathogen had grown. Maceration of infected pear fruit tissue was not observed. Potato tuber and pear fruit disks were not macerated when treated with the supernatant liquid of bacterial ooze or extracts of infected tissues. This work indicates that cell wall polysaccharide degradation is absent during the development of the fire blight disease.

Cell wall degradation by pathogen-produced enzymes has been considered a universal phenomenon by several workers (1). Despite much speculation on the physiology of pathogenesis, little is known concerning the biochemical mechanisms of fire blight infection and disease development (7). Once infection by *Erwinia amylovora* (Burr.) Winslow et al. is initiated, the bacteria migrate through host tissues as 'zoogloea' as described by Nixon (16). During early stages of infection, the bacteria are intercellular; but at later stages, lysigenous cavities are formed and the pathogen is found within cells indicating degradation of the cell wall. Miller (14) reported larger intercellular spaces in infected tissue and localized openings in the cell walls that he attributed to enzyme action. However, neither Miller (14) nor Pierstorff (17) found indications of tissue maceration. More recently Lewis and Goodman (13) observed large lysigenous cavities in apple shoots within six days after inoculation.

Dye (6) found no culture of *E. amylovora* that liquified calcium pectate, disintegrated potato tuber or carrot root slices, or exhibited cellulolytic activity when grown on carboxymethyl cellulose in stab-inoculated tubes. His results confirmed earlier studies that utilized the inability of *E. amylovora* to hydrolyze cellulose and pectic substances as taxonomic criteria (4).

Thus, an apparent dilemma exists. Histological studies of *E. amylovora*-infected tissue suggest that cell wall degrading enzymes are present. In vitro taxonomical studies concerned with the utilization and degradation of isolated polysaccharides suggest that *E. amylovora* is not capable of producing either pectolytic or cellulolytic enzymes. The objective of the present work was to resolve this apparent conflict. A preliminary report of this work has been published (18).

## MATERIALS AND METHODS

**Production of infected plant material.**—Parent plants of the fire blight-susceptible ornamental *Cotoneaster pannosa* Franch., (19) were obtained from Aldridge Nursery, Inc. (Von Ormy, TX). Plants were grown from rooted cuttings in a greenhouse at 23 ± 3 C. Shoots were inoculated 3-6 mm below the apex with a suspension of *E. amylovora* injected with a hypodermic syringe. Inoculum was grown in nutrient broth in shake culture for 18-24 hours at 25 C. The particular isolate used (strain 2-73) was derived from a single colony isolated in 1971 from a naturally infected *Malus sylvestris* 'Rhode Island Greening' tree in Wayne County, New York. After inoculation, plants were maintained in the greenhouse at 23 ± 1 C. Shoot tips were harvested 9 days after inoculation following a dark period (30 hours) to reduce the starch content. Thirty shoots with lesions extending 4-10 cm from the tip were removed at the lesion margin and

then cut into two pieces of equal length; the lower (older) portion is referred to as the shoot base; the upper (younger) portion is referred to as the shoot tip. By similar techniques, comparable noninfected shoots that had been injected with water and maintained similarly were harvested. Shortly after harvesting, cutting, and weighing, shoots were frozen at −20 C.

Shoots of *Malus sylvestris* 'Idared' were collected from a severely blighted commercial orchard in Wayne County, New York, in July. Noninfected shoots comparable in age and size to blighted shoots were harvested at the same time.

Immature (2-3 cm diameter) longitudinally-sliced fruits of *Pyrus communis* 'Bartlett' were inoculated (after surface disinfestation) with a broth suspension of *E. amylovora*. Fruits were incubated at 24 ± 3 C for 3-4 days on sterile moist paper towels in covered glass dishes.

**Preparation and analysis of cell wall polysaccharides.**—Cell wall polysaccharides were prepared by the method of English et al. (9). Frozen shoots were ground in liquid nitrogen in a mortar, homogenized in potassium phosphate buffer, and then filtered on a fritted glass filter funnel. The residue was washed with the buffer and distilled water before it was blended in and washed with chloroform-methanol (1:1, v/v) at 4 C. The preparation was washed finally with acetone and dried.

The components of the hemicellulosic and pectic substances of the cell wall preparations were determined by the gas-liquid chromatographic method of Jones and Albersheim (11). Cell wall polysaccharides were hydrolyzed by treatment with trifluoracetic acid and a mixture of polysaccharide-degrading enzymes. A dual column, temperature programmable gas-liquid chromatograph equipped with dual flame-ionization detectors was used for analysis of the resulting sugar alcohol derivatives.

The cellulose content of the isolated cell walls was determined by an adaptation of Updegraff's (20) method. Noncellulosic components were removed with hot acetic and nitric acids. The residue was treated with sulphuric acid and the glucose liberated was estimated with anthrone reagent.

All cell wall polysaccharide analyses were conducted in duplicate. The total experiment on cotoneaster was done three times and the experiment on pear was done twice.

**Assays for polysaccharide-degrading enzymes.**—Polysaccharide-degrading enzyme activity was sought in extracts of infected cotoneaster and apple shoots, infected pear fruits, in culture filtrates, and in the bacterial ooze produced during infection of immature pear fruits. Cotoneaster shoots were inoculated as above, but were incubated for 4 days in a chamber at 23 ± 1 C with continuous mist and a 14-hour photoperiod. Freshly harvested shoots were homogenized in water or 40 mM Tris-HCl buffer pH 7.0 (2.5 ml/gm of shoot). The homogenate was strained through cheese cloth, and the filtrate was centrifuged at 25,000 *g* for 1 hour at 4 C. The supernatant liquids were tested directly or first dialyzed overnight against distilled water. Infected pear halves were rinsed in sterile distilled water and then extracted in the same manner as shoot tissue.

Ooze produced on pear fruit surfaces was collected by vacuum, diluted 1:5 with water, and centrifuged at 35,000 *g* for 1 hour at 4 C. Cell-free ooze was either tested directly or first dialyzed. Cultures of *E. amylovora* were grown at 25 ± 2 C for 24 hours with shaking. Five different basal media, containing 0.5% (w/v) pectin or sodium polypectate as the major carbon source, were used. Culture filtrates were treated in a similar manner to ooze. Preparations to be tested for polysaccharide-degrading enzyme activity were incubated with model substrates at 30 ± 1 C for up to 2 hours. After incubation, aliquots of the reaction mixtures were tested for reducing equivalent content by the method of Somogyi as modified by Nelson (15). Preparations were also tested for enzyme activity by the "cup-plate" assay (5). Autoclaved (121 C, 15 minutes) preparations served as controls in enzyme assays. The following substrates were tested in both assays: birch (*Betula alba* ) xylan, sodium polypectate (Sunkist Growers, Ontario, CA), pectin N.F., and carboxymethyl cellulose (Type 7 MP, Hercules Powder Co., Wilmington, DE). Assays were conducted at pH 5, 6, 7, and 8 using acetate, phosphate, or Tris-HCl buffers at final concentrations of 40 mM. In assays for pectic enzyme activity, substrates were prepared both with and without additional calcium chloride (final concentration 10 mM) to favor trans-eliminase and hydrolase activity, respectively. Pectin methyl esterase activity was tested by Kertesz's (12) method with pectin N. F. as substrate at pH 5, 6, 7, and 8.

The ability of infected tissue extracts and cell-free ooze to macerate pear tuber disks (1 mm thick × 8 mm diameter) was tested. Eight disks of each tissue were incubated in 8 ml of each extract. During and after incubation for up to 20 hours, disks were examined visually and scraped with a blunt instrument to determine if maceration had occurred.

## RESULTS

The carbohydrate compositions of cell walls prepared from *E. amylovora*-infected and comparable noninfected tissues are presented in Table 1. Differences in monosaccharide, uronic acid, and cellulose content of infected vs. noninfected tissues were small for all three hosts. In the three experiments on cotoneaster, the coefficient of variation was generally less than 10% for all components. There was a highly significant correlation ($r = 0.999$, $P = 0.001$) between the percent of individual carbohydrate components in infected vs. noninfected tissues in the cotoneaster shoot tip, shoot base, and the apple shoot cell wall preparations.

Noninfected cotoneaster shoot base cell wall preparations apparently contained more xylose and cellulose but less arabinose, galacturonic acid, and galactose than shoot tip cell wall preparations. These differences likely reflect difference in tissue age. A higher proportion of cellulose-rich secondary cell wall would be expected in shoot bases relative to shoot tips.

The composition of infected pear fruit slices also was virtually unchanged relative to noninfected slices (Table 1). Gas-liquid chromatographic analysis indicated the major uronic acid component of cell wall preparations was galacturonic acid. Subsequent analysis of cell wall hydrolysates with uronate dehydrogenase (3) indicated that the uronic acid content of infected and noninfected preparations was virtually identical.

TABLE 1. Polysaccharide components detected in cell wall preparations of *Erwinia amylovora*-infected and comparable noninfected host tissues[a]

| | *Cotoneaster pannosa*[b] | | | | *Malus sylvestris* | | *Pyrus communis* | |
|---|---|---|---|---|---|---|---|---|
| | Shoot tips | | Shoot bases | | Shoots | | Immature fruit | |
| | Noninfected | Infected | Noninfected | Infected | Noninfected | Infected | Noninfected | Infected |
| Arabinose | 6.8 | 6.2 | 4.8 | 4.7 | 6.5 | 5.7 | 4.0 | 5.0 |
| Xylose | 8.2 | 6.6 | 12.8 | 12.3 | 14.3 | 14.2 | 21.3 | 21.7 |
| Galactose | 3.5 | 3.6 | 2.7 | 2.4 | 3.0 | 2.4 | 1.6 | 1.7 |
| Glucose | 1.8 | 1.8 | 1.4 | 1.2 | 1.7 | 1.3 | 1.1 | 1.0 |
| Mannose | 1.6 | 1.9 | 1.2 | 1.2 | 0.7 | 0.8 | trace | trace |
| Rhamnose | 0.6 | 0.5 | 0.6 | 0.6 | 0.4 | 0.5 | trace | trace |
| Galacturonic acid | 9.6 | 9.8 | 8.2 | 8.1 | 6.4 | 6.6 | 12.1 | 9.0 |
| Glucuronic acid | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | trace | trace |
| Cellulose | 26.3 | 25.3 | 30.7 | 33.4 | 30.2 | 31.5 | 29.0 | 30.5 |
| Carbohydrate | 59.3 | 56.0 | 63.7 | 64.8 | 63.2 | 63.0 | 69.1 | 68.9 |

[a]Values presented are the mean weight percent of individual components detected in cell wall preparations.
[b]Cotoneaster shoots were cut into two pieces of equal length. The upper (younger) portion is the shoot tip; the lower (older) is the shoot base.

When cell-free ooze produced on pears and extracts of infected cotoneaster shoots, apple shoots, and pear slices were examined, no pectolytic, cellulolytic, or xylolytic enzyme activity was detected by either the reducing equivalent or "cup-plate" assay. Cell-free ooze and extracts of the above tissues did not macerate immature pear fruit or potato tuber disks when incubated for up to 24 hours. We could demonstrate no pectic enzyme activity in any of the culture filtrates tested.

## DISCUSSION

Cell wall polysaccharides prepared from *E. amylovora*-infected host tissues contained substantiately the same proportions of monomeric components as similar preparations made from noninfected tissues. Statistical analysis of the cotoneaster data revealed no differences in composition significant at greater than $P = 0.2$. The coefficients of variation among the individual carbohydrate determinations were low (less than 10% except in two cases). This suggests that the analyses of apple shoot tissue, which were not repeated because naturally-infected material was available only once, are also valid. Had significant cell wall polysaccharide degradation occurred, significant differences in the composition of the cell wall preparations would have been expected. If all cell wall polysaccharide components were equally degraded in proportion to their occurrence, differences in cell wall yields based on fresh and/or dry tissue weight would be expected. Cell wall yields from infected and comparable noninfected tissues were virtually identical. If one carbohydrate component was selectively removed from the cell walls, differences in the ratio of the amounts of that moiety relative to others in the two types of tissue would be expected. Manipulation of the data presented in Table 1 indicated that there were only small differences in the ratios of components in infected vs. noninfected tissues.

Where degradation of cell wall polysaccharides has been reported as significant, infected tissue contained much less of the degraded polysaccharide constituent than comparable noninfected tissue. Preparations of cell walls of bean (*Phaseolus vulgaris*) hypocotyls, infected by *Rhizoctonia solani* and *Sclerotium rolfsii,* contained less than 25% and 4%, respectively, of the amount of galacturonic acid found in preparations from comparable noninfected hypocotyls (2).

All analyses indicated that the fire blight pathogen did not produce, in vivo or in vitro, detectable levels of any of the three most prevalent classes of polysaccharide-degrading enzymes; pectinases, cellulases, or xylases. The substrates of these enzymes together constituted more than 75% of the polysaccharides detected in the cell wall preparations from the three host species analyzed. Neither maceration of immature infected pear fruit slices nor maceration of pear fruit or potato tuber disks incubated with extracts of infected cotoneaster or apple shoots was observed. Maceration of such tissues would have been indicative of cell wall degradation.

The principal constituent of bacterium-free ooze is an ethanol-precipitable polysaccharide that contains about 75% galactose, 16% uronic acid, 7% glucose, and 3% mannose (8). Dilute solutions of the polysaccharide induce rapid wilting of shoots of apple and other hosts of *E. amylovora* (8, 10, and Sjulin and Beer, *unpublished*). We found these same monomeric constituents in cell wall preparations of the three hosts analyzed. Lack of significant reduction of these constituents in preparations from infected vs. noninfected tissues indicates that the ooze polysaccharide is not synthesized at the expense of host cell wall polysaccharides.

The disintegration of suscept cell walls observed in several histological studies (14, 16) may have been due to mechanical rupture caused by bacterial masses or may have been so slight, relative to the total amount of tissue, that the several techniques used in the present studies were insufficiently sensitive to detect it. The absence of cell wall degradation in fire blighted-tissues indicates that this mechanism, important in many other pathogen-suscept combinations (1), is of little or no importance in pathogenesis by *Erwinia amylovora*.

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