

Evaluation of Mature Apple Fruit from Washington State for the Presence of *Erwinia amylovora*

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ABSTRACT

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Erwinia amylovora was not detected in core tissues or aqueous sonicates from 1,555 mature, symptomless fruit harvested from blighted trees of seven apple cultivars grown at five locations in Washington State. Strains typical of Enterobacteriaceae on selective media (Miller-Schroth medium alone or replica-plated onto the crystal violet medium of Ishimaru and Klos) were characterized by phenotypic and pathogenicity tests as belonging in the *E. herbicola*, *Enterobacter agglomerans*, *Escherichia adecarboxylata* complex. *E. amylovora* was not detected in either year. Detection sensitivities were about 20 and 30 bacterial cells per fruit for external and internal assays, respectively. None of the strains from either year were pathogenic on apple seedlings. Thus, healthy, symptomless apples produced in Washington State are unlikely to harbor detectable populations of *E. amylovora*.

Additional keywords: plant quarantine, postharvest

Fruit boxes piled near the first recognized infection locus of fire blight in England have been implicated as the original source of inoculum from which the disease was introduced into England, despite a lack of physical evidence that these boxes harbored *Erwinia amylovora* (Burr.) Winslow et al (30). Mature apple fruit have not been shown to play a role in long-distance dispersal of the fire blight bacterium, *E. amylovora*, although Anderson (1), Goodman (9), and McLarty (20) have suggested that fruit could be responsible for long-distance spread of the bacterium. Anderson (1) isolated virulent cultures of *E. amylovora* from inoculated green pears after storage for up to 7 mo in a refrigerator and suggested that infected, mummified fruit may serve as inoculum reservoirs in the absence of cankers. Goodman (9) isolated virulent *E. amylovora* from beneath the skin of naturally infected Jonathan apples that were still soft and moist (not mummified) and suggested these fruit may serve as inoculum reservoirs in the absence of holdover cankers. However, he did not

report isolation of virulent *E. amylovora* from these fruit the following spring. McLarty (20) demonstrated survival of *E. amylovora* at the wound site in mature fruit for up to 5 mo after inoculation. These results do not, however, provide evidence for the ability or probability that mature fruit may serve as dispersal agents of *E. amylovora*, as the conditions of inoculation may not reflect the conditions under which *E. amylovora* would have to infect or persist in healthy fruit tissue in situ. Under more realistic conditions, McLarty (21) later showed that none of several hundred bacterial strains isolated from mature fruit harvested from badly blighted trees were *E. amylovora* or were capable of producing disease symptoms in pathogenicity tests.

Schroth et al (25) believe that fruit are of questionable importance in the fire blight disease cycle. The Great Britain Ministry of Agriculture, Fisheries and Food also maintains that spread of the disease by transport of infected fruit is unlikely (2). The purpose of this study was to determine if *E. amylovora* is present on or in mature apple fruit harvested from blighted trees grown in Washington State and to evaluate whether such fruit poses a phytosanitary risk to foreign countries importing Washington apples.

MATERIALS AND METHODS

Location of blighted orchards and sampling of fruit. Apple orchards at Prosser, Yakima, and Bray's Landing (about 40 km north of Wenatchee),

Washington, with fire blight symptoms were located through contacts with local growers, extension agents, and scientists. Shoots with typical fire blight symptoms and signs were cut at each location from several apple trees from which mature fruit were later harvested. Ooze from blighted shoots was streaked onto the selective medium of Miller and Schroth (MS) (22), which was used for all isolations. The medium was prepared with sorbitol as the carbon source and was modified by addition of 0.167 g/L of cobalt dichloride (28). Identity of all strains from blighted tissue as *E. amylovora* was confirmed by standard physiological and biochemical tests (8,17). These strains were also tested for pathogenicity by inoculation of surface-disinfested immature pear fruit. Fruit were punctured with a dissecting needle laden with cells grown for 24 hr at 27 C on nutrient yeast-extract agar (NYA; 8.0 g nutrient broth, 4.0 g yeast extract, 20.0 g agar, and 1,000 ml water, pH 6.9). Inoculated fruit were incubated in a moist chamber for 3-5 days at 27 C. In 1988, additional sampling sites in Toppenish (blight present) and Wenatchee (no blight present) were selected (Table 1).

Fruit from seven cultivars were harvested from three (1987) or five (1988) study sites (Table 1). Symptomless fruit were picked from spurs that were as nearly adjacent to blight symptoms as possible, i.e., from spurs where one or more blossoms were blighted earlier in the year, from spurs directly adjacent to an infected shoot, or from branches where symptoms were present. In 1987, fruit were picked directly into new 1-bu boxes and stored at 0 C without further handling or treatment until assayed. Assays for the presence of *E. amylovora* began on 17 November 1987 and terminated on 21 January 1988. In 1988, fruit were picked into individual plastic bags to prevent possible cross-contamination, then treated as in 1987. Assays in 1988 began on 27 September and ended on 27 October. The fruit maintained their color and firmness to the touch throughout the study period.

Assay for external populations of *E. amylovora*. In 1987, a modification of Dueck's technique (6) was used to detect *E. amylovora* in sonication water

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(sonicate) of 755 fruit. Individual fruit were coded for number and cultivar and sonicated for 1 min in about 250 ml of sterile deionized water in a 600-ml beaker suspended in a 150W Bransonic 3200 ultrasonic cleaner (Bransonic Ultrasonics Corp., Parrott Drive, Shelton, CT). Fruit were removed from the sonicate, shaken off into the sonicate to remove adhering droplets, and set aside. Sonicates were passed through a 250-ml filter sterilization unit (Nalge Co., Rochester, NY) fitted with a 47-mm-diameter, 0.2- μ m pore-size cellulose triacetate membrane filter (GA-8 Supor, Gelman Sciences, Inc., Ann Arbor, MI). Pressure (0.35 kg/cm²) was applied to the upper chamber with sterile nitrogen. Each membrane was then removed and inverted onto the surface of MS. After 15 min, the membranes were removed and placed onto a second plate. Plates were incubated at 27 C for 3–5 days and observed for the presence of reddish orange to orange colonies typical of *Erwinia* spp. on MS (10,11). Colonies whose appearance most closely resembled that of *E. amylovora* on either or both of the plates were streaked onto MS plates for isolation of single colonies. Individual colonies from these plates were subcultured onto NYA and lyophilized in sterile aqueous nonfat dry milk (7.0%, w/v).

In 1988, 800 fruit were assayed as in 1987, with minor modifications. Fruit were washed by stirring for 1 min prior to sonication, then sonicated as described. Polycarbonate membranes were used instead of cellulose triacetate and were placed on MS plates without inverting and incubated without being placed on a second plate. Samples (0.1 ml) of the sonication buffer were plated in triplicate onto MS so individual colonies could be evaluated if growth on membranes was confluent. Representative colonies were selected from MS plates for phenotypic characterization and pathogenicity studies, and, in addition, all colonies on each MS isolation plate were replica-plated onto the differential medium (CCT) of Ishimaru and Klos (13) to further evaluate the identity of the bacteria. These plates were incubated 48 hr at 26 C,

then observed for characteristic pulvinate, striate colonies.

Assay for internal *E. amylovora*. In 1987, after being sampled for external *E. amylovora*, fruit were surface-disinfested by immersion and sonication for 5 min in 2,600 ppm NaOCl and 1,000 ppm dodecylbenzene sulfonic acid, pH 6.0. Fruit were then thoroughly washed with running sterile deionized water and placed inside a sterile vertical laminar flow hood to dry. Core and cortex tissues, including the stem if present, and the entire calyx were removed by passing an ethanol-flamed cork borer through the vertical axis of each fruit. Excised cores were aseptically sliced into radial sections 0.5–1.0 cm thick, placed into 125-ml Erlenmeyer flasks containing 30 ml of nutrient yeast-extract broth, and shaken for 20–24 hr at 27 C and 200 rpm in an environmental shaker (Lab-Line Instruments, Inc., Melrose Park, IL). This treatment partially macerated the core tissues. Next, 0.1-ml samples were spread by glass rod onto each of three 60-mm-diameter MS plates per fruit. Plates were incubated as previously described and observed for the presence of reddish orange to orange colonies. With a few exceptions, when reddish orange to orange colonies developed, confluent bacterial lawns grew on isolation plates. Bacteria from plates with such growth were streaked for isolation onto MS plates and incubated 3–4 days at 27 C. The morphology of the isolated colonies on these plates was generally uniform within each sample, but nonpigmented colonies were occasionally observed. However, growth rate, hue, and mucoid appearance of the colonies varied among samples. Because some of the *E. amylovora* strains obtained from blighted shoot tissue did not closely conform to the appearance of *E. amylovora* on MS as described by Miller and Schroth (22), and because the appearance of *E. herbicola* (Lohnis) Dye is similar to that of *E. amylovora* on MS (24), isolated, reddish orange to orange pigmented colonies representative of those on each plate were picked off, cultured for 24 hr on NYA at 27 C, and lyophilized.

In 1988, internal fruit tissues were

assayed as in 1987, but core tissues were suspended in 30 ml of 0.05 M phosphate buffer, pH 6.4, and shaken at 200 rpm for 1 hr; 0.1-ml samples were then plated in triplicate onto MS. Any MS plates with colonies were replica-plated onto CCT as described for external assays.

Determination of assay sensitivities. Sensitivity of the sonication/membrane filtration isolation technique was evaluated with inoculated fruit. The fruit were surface-disinfested as described, then rinsed thoroughly in sterile deionized water. Individual apples were inoculated with either 1 or 5 μ l of serially diluted suspensions of *E. amylovora* 88-38. Immediately after the drops of inoculum dried (<5 min), the fruit were sampled as described above. There were three single fruit replicates per inoculum concentration. The procedure was repeated once with similar results.

The sensitivity of the internal assay was evaluated by spiking studies. Apple cores were removed as described above and placed in 30 ml of buffer. Then, 1-ml samples of serially diluted *E. amylovora* suspensions were added to each flask and the contents analyzed as described above. Samples (0.1 ml) from each flask were plated in triplicate onto CCT and incubated 48 hr at 26 C. Counts of inoculum plated directly from flasks on NYA plates were used as the basis for the sensitivity of recovery.

Identification of strains. Strains isolated during the assays were phenotypically characterized by the tests of Smibert and Krieg (27) and Dye (8): gram reaction; aerobiosis on Liefson O/F agar; requirement for growth factors; presence of mucoid growth on NYA with 5% sucrose; nitrate reduction; presence or absence of yellow pigment on NYA; growth at 36 C; presence of oxidase, catalase, phenylalanine deaminase, and urease; and production of acetoin, indole, and H₂S from cysteine, gas from glucose, and reducing compounds from sucrose. Atypical strains from *E. herbicola* and a random selection of typical strains were further tested for: production of acid and/or gas from glucose, lactose, and adonitol; production of H₂S from triple sugar iron (TSI) agar; methyl red; citrate utilization; presence of lysine and ornithine decarboxylase; lactose/sucrose and glucose fermentation on TSI agar; and gas on TSI agar.

Pathogenicity tests. In a modification of the technique of Ritchie and Klos (23), radicles of germinated apple seedlings, secured between moistened paper toweling with pins so only the cotyledons and epicotyls protruded, were placed in polypropylene bins covered with perforated polyethylene film and held in growth chambers at 26 C and 16-hr photoperiod. After the first true leaves began to expand, a tuberculin syringe containing an aqueous cell suspension

Table 1. Orchard locations, cultivars, harvest dates, and number of fruit assayed

Location	Cultivar	Harvest date		No. of fruit assayed	
		1987	1988	1987	1988
Prosser	Super Jonathan	27 August	8 September	151	100
	Jonagold	27 August	8 September	112	100
Yakima	Jonathan	3 September	9 September	45	100
	Golden Delicious	3 September	9 September	110	100
	Starking	3 September	...	75	...
Bray's Landing	Golden Delicious	4 September	13 September	112	100
	Red Chief	4 September	...	150	...
Toppenish	Super Jonathan	...	9 September	...	100
	Jonagold	...	9 September	...	100
Wenatchee	Red Delicious	...	26 September	...	100

(about 10^9 cfu/ml) was inserted completely through the stem. Inoculum was introduced into the wound as the needle was withdrawn from the stem. In this manner, four seedlings were inoculated with each strain. Inoculum had been prepared from 24-hr cultures grown at 26 C on NYA and suspended in sterile water. For controls, seedlings were inoculated with known strains of *E. amylovora* isolated from blighted shoot tissue or with sterile deionized water. The inoculated seedlings were incubated in the growth chamber for 5 days, then examined for signs and symptoms.

RESULTS AND DISCUSSION

We detected *E. amylovora* in sonicates from inoculated fruit when as few as 19 cfu (average of two trials) were applied (Table 2). The sensitivity of the sonication-membrane filtration detection technique exceeds that reported by Lin et al (18) for an immunofluorescent assay using monoclonal antibodies. The demonstrated level of sensitivity of the isolation technique used supports our view that the lack of recovery of *E. amylovora* from fruit is not an artifact of isolation technique. Recovery of *E. amylovora* from spiked core samples incubated as usual for study samples showed a similar level of sensitivity (Table 2).

Isolated outbreaks of fire blight in apples in several apple-growing regions of Washington State occurred in 1987 and 1988. The severity of the disease varied by location, cultivar, and year. Resistant cultivars (e.g., Red and Golden Delicious) developed blossom and occasionally shoot blight that remained limited to the current year's growth. In contrast, susceptible cultivars (Jonathan or Jonathan parentage) grown in two locations—Prosser in 1987 and Yakima in 1988—developed extensive blossom and shoot blight that progressed into scaffold limbs and the trunk; some trees had to be removed. Only at Toppenish (Table 1) was blighted tissue pruned during the growing season.

Although readily isolated from diseased tissues in the spring, *E. amylovora* could not be detected in either aqueous sonicates of intact fruit or excised core tissues of any of the 1,555 fruit assayed. In 1987, orange to reddish orange colonies typical of the Enterobacteriaceae on MS were isolated from 82 of the 755 fruit assayed; 36 and 58 fruit were positive for Enterobacteriaceae from core tissues and sonicates from intact fruit, respectively, and 12 fruit were positive from both. In 1988, similar colonies were isolated from 189 of 800 fruit; 38 and 168 fruit were positive from core tissues and sonicates from intact fruit, respectively, and 17 fruit yielded colonies from both.

The use of MS alone during the 1987 study might have introduced a negative

bias for detection of *E. amylovora* similar to that discussed by Beer and Oppenorth (3). They reported some disadvantages of using either MS or crystal violet-sucrose (CG) selective media exclusively. Many strains on MS that appeared typical of *E. amylovora* were not pathogenic and did not form craters on CG, and when CG was used alone, some rapidly growing colonies overgrew the media before cratering could occur (3). In our study, growth on MS plates from internal assays in 1987 was often confluent because of the 24-hr enrichment in nutrient broth. However, no *E. amylovora* was detected when samples of the growth were streaked on plates of MS. In 1988, the shorter incubation time and incubation of core pieces in buffer rather than nutrient broth led to isolation of fewer bacteria, and fewer fruit had populations that produced characteristic colonies on MS. Also, the total number of saprophytic bacteria isolated was reduced, but again, *E. amylovora* was not detected. In 1987, the plating of membrane filters onto MS

restricted isolation primarily to enteric bacteria, and only a small percentage of the total bacteria isolated were phenotypically characterized and tested for pathogenicity. In 1988, however, replica plating onto CCT allowed evaluation of all colonies that developed on MS plates.

None of the strains isolated in 1987 and 1988 and characterized by phenotypic tests (yellow pigment, requirement for growth factors, growth at 36 C, nitrate reduction, H_2S produced from cysteine, and presence of phenylalanine deaminase) were *E. amylovora*. In 1987, all but one strain were gram-negative, facultatively anaerobic rods, which places them in the Enterobacteriaceae. Fifty-seven strains had the phenotypic characteristics of *E. herbicola*. Of the remaining strains, 26 were *Enterobacter agglomerans* (Beijerinck) Ewing and Fife, 2 were *Escherichia adecarboxylata* Leclerc, 3 were *Yersinia* spp., and 1 was an obligate aerobe that was not characterized. Five of 94 strains were lost during lyophilization and could not be characterized. Of 47 strains characterized

Table 2. Recovery of *Erwinia amylovora* from inoculated apple fruit surfaces and spiked core samples^a

Fruit surface (\log_{10} cfu)		Core tissue (\log_{10} cfu/ml)	
Applied	Recovered	Applied	Recovered
3.28	2.08	3.72	3.35
2.98	1.75	2.72	2.53
2.28	1.03	1.72	1.26
1.98	0.54	0.72	1.00
1.28	-0.30	-0.27	0

^aFruit were sonicated in buffer for 1 min, the resulting suspension was filtered through polycarbonate membranes, and the membranes were plated on MS and incubated for 3-5 days at 27 C. Core tissue was removed from surface-disinfested fruit and added to 30 ml of 0.05 M phosphate buffer, the buffer was shaken at 200 rpm for 1 hr at 26 C, and 0.1-ml samples were plated in triplicate onto CCT. All values represent averages of two trials, three fruit per trial per inoculum density.

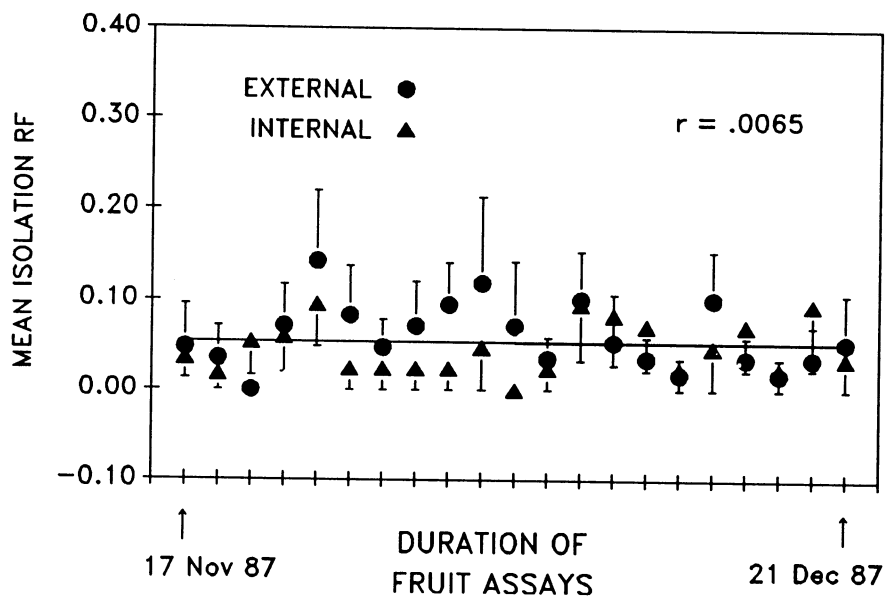


Fig. 1. Isolation of *Erwinia*-like bacteria in 1987 from internal and external apple fruit assays. Each point represents the number of strains per the number of fruit assayed of cultivars and locations pooled at each assay date. One-way error bars represent the standard error of the mean. The first-order regression line ($r = 0.0065$) was calculated using data from both internal and external isolation frequencies of pooled cultivars and locations for each date.

in 1988, 31 were *E. herbicola*, 13 were *Enterobacter agglomerans*, 2 were fluorescent pseudomonads, and 1 was an obligate aerobe. *E. herbicola*, *Enterobacter agglomerans*, and *Escherichia adecarboxylata* have been placed in synonymy with each other by different authors and are currently treated as a species complex (4).

All strains of *E. amylovora* obtained from blighted shoot tissues were virulent. Copious drops of bacterial ooze appeared on the surface of inoculated pears, and typical signs and symptoms of fire blight (rust-colored to dark brown necrotic lesions, droplets of cream- to orange-colored bacterial ooze at the inoculation site, and epinasty of the epicotyl) were observed on the inoculated apple seedlings. Seedlings inoculated with water or the unknown strains from fruit did not become diseased.

Storage time had no effect on relative isolation frequency of *E. amylovora*-like bacteria (Fig. 1). The date of assay was not correlated with the relative isolation frequency ($r = 0.0065$) of *E. amylovora*-like bacteria for either internal or external tissues. Thus, the period between harvest and initial assay (80–114 days in 1987 and 1–19 days in 1988) was not responsible for the absence of *E. amylovora* on isolation plates. In 1988, as in 1987, no *E. amylovora* was recovered from any fruit, even that harvested from severely blighted trees (Jonathan, Yakima). Thus, the interval between harvest and assay and duration of assays in 1987 were not responsible for the observed lack of detection of *E. amylovora*. Van der Zwet et al (31) were also unable to isolate *E. amylovora* from internal tissues of apples harvested from trees with fire blight in Ontario (Canada), West Virginia, or Washington. Although *E. amylovora* was isolated from some of the fruit from Utah (31), it does not appear to be a consistent resident on or in fruit tissues as suggested by Keil and van der Zwet (15,16).

Studies of epiphytic and endophytic *E. amylovora* on apple fruit in various parts of the world provide no consensus on the epiphytic occurrence of the bacterium on mature, symptomless apple fruit. Both Dueck (6) and McLarty (21) have shown *E. amylovora* to be absent from mature fruit harvested from blighted trees in Canada. Sholberg et al (26) isolated *E. amylovora* from all of a small number of apples harvested from trees directly adjacent to blighted pear trees. In Washington, pear trees can undergo a short spurt of vegetative growth after harvest, and if present, fire blight cankers can become active until growth ceases. A similar occurrence in the Okanagan Valley of British Columbia may have been the source of the *E. amylovora* detected on apple fruit at harvest. Van Buskirk et al (29) and van der Zwet and Van Buskirk (32)

reported isolation of *E. amylovora* from small, immature fruit in West Virginia but did not sample mature symptomless fruit. Although Hale et al (10) isolated *E. amylovora* from a small percentage of fruit at harvest from severely blighted trees in New Zealand, the number of fruit from which *E. amylovora* was isolated and presumably populations of *E. amylovora* on fruit declined dramatically near the end of the growing season. In the same study, they could not isolate *E. amylovora* from nonblighted or moderately blighted trees. Dueck and Morand (7) monitored epiphytic populations of *E. amylovora* on symptomless, susceptible apple and pear leaves and dormant buds in Canada. They, too, found that epiphytic *E. amylovora* populations varied during the growing season, and they were unable to isolate *E. amylovora* from leaves on their two final sampling dates during two growing seasons.

Because *E. herbicola* and other closely related species were consistently isolated, often in high numbers, from fruit harvested from the most severely blighted trees, we suggest that biotic factors such as naturally occurring biological control may explain the observed lack of recovery of *E. amylovora*. Strains of *E. herbicola* are well-documented antagonists of *E. amylovora*. Possible mechanisms of antagonism suggested include antibiosis (12,14,33), production of toxic aglucones by hydrolysis of arbutin or phloridzin (5,11), and preemptive site occupation by closely related microorganisms competing for niche space (19). Interactions between nonenteric bacteria and *E. amylovora* on fruit not detected by the media used may be significant as well. One or several of these mechanisms or other unidentified factors may have contributed to decreased recovery of *E. amylovora* late in the growing season observed in New Zealand (10) and Canada (7) and in the lack of isolation of *E. amylovora* from symptomless mature fruit in Washington.

High average temperatures and low relative humidities during the latter parts of the growing season in Washington State may also have contributed to the lack of recovery of *E. amylovora*. Average daytime high temperature for August 1987 and 1988 was 30.8 C. Average relative humidity during these months was low, ranging from about 12 to 27%. Although meteorological data were not recorded at the sample sites, the hot, dry weather throughout the state may have adversely affected survival of epiphytic populations of *E. amylovora* had they been present on fruit. Although historically improbable, humid conditions during the growing season in Washington State might produce different results than those of 1987 and 1988.

Further studies are required to eluci-

date the nature, scope, and effect of interactions between epiphytic populations of *E. amylovora* on fruit surfaces with other microorganisms and environmental variables. Mature, healthy fruit do not appear to be an ecologically suitable substrate for survival of epiphytic *E. amylovora*. Thus, healthy, mature apple fruit, even when harvested from blighted trees, are unlikely to harbor *E. amylovora* populations and therefore are unlikely to pose a phytosanitary risk to areas free from fire blight.

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