Evaluation of Likelihood of Co-Occurrence of *Erwinia amylovora* with Mature Fruit of Winter Pear

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Accepted for publication 30 April 2007.

ABSTRACT

Temple, T. N., Stockwell, V. O., Pusey, P. L., and Johnson, K. B. 2007. Evaluation of likelihood of co-occurrence of *Erwinia amylovora* with mature fruit of winter pear. *Phytopathology* 97:1263-1273.

Phytosanitary concerns about fire blight prohibit export of U.S.-grown pears to some countries without this disease. To examine these concerns, we evaluated the potential for co-occurrence of *Erwinia amylovora* with mature, symptomless winter pear fruit by inoculation experiments and by survey of commercial orchards. Immature pear and apple fruit were inoculated in orchards with *E. amylovora* strain 153N as resuspended lyophilized cells or as ooze from diseased tissues. Regardless of inoculum source, population size of Ea153N on fruit declined by an order of magnitude every 3 to 4 days during the first 2 weeks after inoculation; at 56 days after inoculation, Ea153N was not detected, except on 1 of 450 fruit with 4 colony forming units (CFU). After inoculation of flowers, calyx-end survival of Ea153N on pear and apple fruit declined from high populations at petal fall to a few cells at harvest, with no detection of the pathogen after a 7-week cold storage. Migration of Ea153N into symp-

tomless pear fruit from diseased branches was evaluated by enrichment assay and nested polymerase chain reaction of internal fruit core tissues; these assays failed to detect the pathogen in healthy fruit from diseased trees. At harvest, *E. amylovora* could not be detected on 5,599 of 5,600 fruit of d'Anjou pear sampled from commercial orchards in major production areas of the Pacific Northwest; one fruit yielded 32 CFU of the pathogen. Postharvest, mature pear fruit contaminated with Ea153N and subsequently wounded required a dose of >10,000 cells at the wound site to allow for persistence of the pathogen through a 7-week-cold storage. We conclude that epiphytic *E. amylovora* shows similar survival characteristics on both pear and apple fruit, this pathogen is not an endophyte within mature symptomless pear fruit, its presence is exceptionally rare on commercially produced fruit, and that epiphytic survival of *E. amylovora* through a postharvest chilling period is unlikely given the unrealistically high population size required for persistence.

Additional keywords: exotic pest quarantine, phyllosphere microbiology, risk assessment.

Fire blight, caused by the bacterium *Erwinia amylovora*, is an economically important disease of pear (*Pyrus communis* L.) and apple (*Malus pumila* P. Mill.) in many parts of the world (6). The pathogen is native to North America, but in the last century was introduced to Europe, the Middle East, and New Zealand. Retrospective analysis of these introductions (6), as well as experimental evidence (7,21,37,38), have implicated pathogen-infested nursery stock and/or scion wood as the likely pathways of introduction. For countries in which *E. amylovora* is not present, importation of plants and scions from countries with fire blight is prohibited by phytosanitary regulation (1,41). In addition, some countries also prohibit importation of mature, symptomless pear and apple fruit, although fruit as a vehicle for long-range introduction of *E. amylovora* has never been documented.

In particular, the likelihood of apple fruit as a vehicle for dissemination of *E. amylovora* has been the subject of considerable research and risk assessment proceedings (12,16,29,30,35,36,39,41–43). In a recent World Trade Organization dispute resolution proceeding (41), a panel of judges, after reviewing scientific literature and expert testimony, concluded that there is no evidence that mature, symptomless apple fruit can be infected or infested endophytically by *E. amylovora*, and that epiphytic infestations of this pathogen on apple fruit are rare and limited to those harvested on or very close to blighted trees. Moreover, even though epiphytic infestations are present occasionally, there is no

evidence that fruit can serve as a pathway for the dissemination of the fire blight pathogen (41). Thus, as also concluded earlier in a risk assessment (29), the likelihood that the dissemination pathway can be completed by commercial trade of mature, symptomless apple fruit is considered negligible (41).

In contrast to apple, the consideration of pear fruit as a dissemination vehicle for *E. amylovora* has received little research effort (8,24). On average, pear cultivars are more susceptible to fire blight than cultivars of apple (6), a difference that in countries with fire blight, limits commercial production of pear to drier climates. Furthermore, perhaps because of greater susceptibility, immature pear fruit (1 to 3 months old) are routinely used as the bioassay material of choice to verify the pathogenicity of *E. amylovora*, regardless of the host of origin (1,5). In a moist chamber, immature pear fruit develop symptoms of fire blight after a few days, and ooze of the pathogen is visible after 5 to 7 days (5). Mature pear fruit, in contrast, show resistance to inoculation with *E. amylovora* (10,44), and decay of fruit by this pathogen is not recognized as a postharvest problem by the pear industry. Nonetheless, by implication and anecdotal reporting, healthy pear fruit have been assumed to have a comparatively greater likelihood of harboring populations of *E. amylovora* than fruit of apple (41).

The purpose of this study was to address questions concerned with the likelihood of co-occurrence of *E. amylovora* with mature, symptomless fruit of winter pear. The study was conducted within the context of the commercial fruit production system in the Pacific Northwest region of the United States. The semi-arid pear production areas within the region produce 2.5×10^7 kg of fresh market winter pears annually, of which 20 to 25% is exported to markets outside of the country. The primary cultivars are

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'd'Anjou' (80%) and 'Bosc' (20%), both of which require a post-harvest chilling period of 4 to 6 weeks for the fruit to ripen properly (9). We developed four questions for detailed examination: (i) does *E. amylovora* survive as an epiphyte on pear fruit surfaces?; (ii) are commercial fruit produced within the region contaminated with *E. amylovora*?; (iii) does *E. amylovora* have a potential to reside endophytically within fruit produced on diseased trees?; (iv) and does the pathogen have the ability to survive on winter pear fruit through the required postharvest chilling period? These data are discussed in relation to a risk assessment model developed for apple fruit from the same production area (29); most experiments reported in this study involved apple fruit as a comparative control.

MATERIALS AND METHODS

Bacterial strains. Strains of *E. amylovora* and of *Pantoea agglomerans* were used in field and laboratory experiments. *E. amylovora* strain Ea153, originally isolated from a fire blight canker on an apple tree in Milton-Freewater, OR, shows virulence and aggressiveness characteristics typical of North American strains of *E. amylovora* (19). Ea153N is a spontaneous, nalidixic acid-resistant (100 µg/ml) selection of Ea153 with characteristics similar to the parental strain; Ea153N has been used as a representative pathogen strain in numerous field studies (19,27,28,32,33). Ea153HrpL⁻ is an avirulent *hrpL* mutant of Ea153 obtained using marker-exchange mutagenesis methods with donor strains provided by S. Beer (Cornell University, Ithaca, NY) (40); Ea153HrpL⁻ is resistant to kanamycin (100 µg/ml). *P. agglomerans* strain PaC9-1S is a spontaneous streptomycin (100 µg/ml) and rifampicin (100 µg/ml) resistant selection of PaC9-1, with characteristics similar to the original strain (18); this strain is an excellent colonist of pear and apple flowers (20) and has been used to suppress floral infection by *E. amylovora* (19,27,28,32). Unless otherwise noted, inoculum of these strains consisted of lyophilized cells prepared at a titre of 1×10^{11} to 1×10^{12} colony forming units (CFU)/g (19); these preparations were produced once and stored at -50°C with subsamples withdrawn for use in experiments.

Recovery of bacteria from sampled fruit. In all field experiments, fruit were sampled individually into resealable plastic bags (20 × 20 cm) (100 µm thickness, Saket Co., Azusa, CA) and transported to the laboratory in insulated chests with ice. The resealable plastic bags also were the vessel in which the fruit were washed for bacterial isolation. Depending on the fruit size and time of season, 1 to 50 ml of sterile, 10 mM phosphate buffer (pH 7) was added to each bag, followed by hand massage of the fruit sample (10 s) and sonication in a bath-style sonicator (2 min, Branson 2000, Danbury, CT). A total of 20 to 250 µl of the wash suspension and up to two serial 100-fold dilutions were spread onto the surface of Miller Schroth (MS) medium with sorbitol as the carbon source for isolation of *E. amylovora* (24) and on Pseudomonas agar F (PAF, Difco Laboratories, Detroit, MI) for recovery of PaC9-1S; these media were amended with cycloheximide (50 µg/ml) and, if necessary, the antibiotic selective for each individual strain (antibiotics and rates given above). The detection limit for the dilution plating assay was 100 to 5,000 CFU per fruit, depending on wash buffer volume. For samples with expected populations near or lower than 100 CFU per fruit, the wash suspension was passed through a 250-ml filter sterilization apparatus (Millipore, Billerica, MA) fitted with a 0.2-µm glass fiber filter membrane (Supor-200, 45 mm diameter, Pall Life Sciences, Ann Arbor, MI) (30). Membranes with the wash filtrate were incubated on the surface of the MS or PAF medium. The detection limit of the filtration assay has been reported previously as 19 CFU (30); this limit was reevaluated for purposes of this study. For both recovery methods, CFU characteristic to each species were counted after 3 to 7 days incubation at room tem-

perature (20 to 24°C). Sixty percent of the 19,200 fruit washings performed in this study required both recovery methods.

Survival of *E. amylovora* on fruit in orchards. During the summers of 2004 and 2005, epiphytic survival experiments were established in experimental pear (*Pyrus communis* cv. Bosc, 37 years old) and apple orchards (*Malus pumila* cv. Gala, 5 years old) located near Corvallis, OR, and in an experimental pear orchard (cv. d'Anjou, 31 years old) located near Medford, OR. Ea153 (2004 only) and Ea153N were inoculated as lyophilized cells resuspended in distilled water at 1×10^7 CFU per ml; inoculum was sprayed onto fruit to runoff with a hand-held, adjustable trigger sprayer (0.5 liter capacity). In addition, fresh ooze containing 10^8 to 10^9 CFU of Ea153N was applied directly onto the calyx end of developing fruit with a no. 4 camel hair paint brush modified such that only two hairs remained. For this purpose, ooze inoculum was produced in the laboratory by stab inoculating Ea153N into detached, 1- to 2-cm diameter, immature pear fruit (cv. Bartlett) followed by incubation at room temperature for 5 to 7 days in crisper boxes lined with moist paper. Each year, the experiment was initiated in early May when calyx ends are oriented upward, and repeated in July, when calyx ends are oriented down. Each experiment was arranged as a randomized block design with three, single-tree replicates per treatment. Fruit that received inoculum of *E. amylovora* (50 per tree) were tagged with plastic ribbons or paint markings on the stem. Five fruit from each tree were sampled at 1 h, and 3, 7, 14, 35, and 56 days after inoculation.

To determine where bacteria were most likely to be detected on developing fruit, additional inoculations were performed with Ea153N during the July inoculations. Thirty fruit were sampled from trees at 2 h and 1, 2, and 3 days after inoculation. In the laboratory, the stem (including attached flesh), a 2 × 2 cm peel of skin (tissue from neck, body, or base of fruit), and calyx of each fruit were excised with a flamed knife, weighed, and washed in phosphate buffer. The wash of each subsample was dilution plated and filter assayed on MS. Also, an estimate of the population size of indigenous bacteria was obtained by dilution plating the fruit washes onto PAF.

Bacterial survival on fruit calyxes. In 2005 and 2006, flowers in the d'Anjou (Medford) and Bosc pear and Gala apple (Corvallis) orchards were inoculated at full bloom with Ea153N, Ea153HrpL⁻, and PaC9-1S. The nonpathogenic strains were applied to near runoff at a concentration of 1×10^7 CFU per ml; the inoculum concentration of Ea153N was reduced to 1×10^5 CFU per ml to limit infection. Ea153N was co-sprayed with fluorescent, yellow-green, carboxylate microspheres (1×10^7 per ml, 1-µm-diameter Fluoresbrite YG, Polyscience Inc., Warrington, PA), which, when applied to open flowers, adhere and remain detectable on the calyx of developing fruit through summer (33). Inocula were applied to near runoff (2 to 3 liters per tree) with a backpack sprayer equipped with a hand wand. To further limit the incidence of infection on trees treated with Ea153N, the bacteriostatic antibiotic oxytetracycline (0.2 g/liter a.i., Mycoshield 17%, NuFarm Americas Inc., Burr Ridge, IL) was over-sprayed once (2005) or twice (2006) at 3 to 6 days after inoculation (32); also, beginning in May, diseased blossom clusters were pruned from trees during weekly visual inspections. The experiment was arranged in a randomized block design with three (2005) or five (2006) single tree replicates of each bacterial strain.

Initial establishment of the bacterial strains was evaluated on eight flowers sampled from each replicate tree at 8 days after inoculation. These flowers were washed individually in 1 ml of sterile phosphate buffer and dilution plated (19). Similarly, five immature fruit per replicate tree were sampled and assayed for bacterial recovery at 70 to 90 days after inoculation (late June to early July). At harvest (late August), 270 symptomless fruit were bulk harvested from each treatment and subdivided into three groups: no further treatment, a dump tank disinfestation treatment

(described below), and dump tank plus a 6-week-storage treatment at 2°C. For all postharvest samples, the calyx region of each fruit was excised with a flamed knife and then washed and filter assayed. After washing, sampled flowers and calyxes from mature fruit of the Ea153N treatment were stored at 2°C until they could be examined with the aid of a microscope (Leica DMRB, 485-nm excitation filter, 520-nm emission filter, Hamburg, Germany) for the presence of the fluorescent microspheres.

Surveys for *E. amylovora* on fruit from commercial orchards. During 2003, 2004, and 2005, fruit from a total of 56 pear orchards of cv. d'Anjou located in the Rogue (OR), Hood River (OR), Yakima (WA), Wenatchee (WA), and Okanogan (WA) valleys of the Pacific Northwest were sampled within 1 week of commercial harvest. At the time of sampling, the orchards were visually inspected for symptoms of fire blight and categorized as apparently symptomless or with fire blight in the orchard or adjacent cultivar block. Four healthy fruit were sampled individually from each of 25 haphazardly selected trees within each orchard. Fruit were stored at 2°C until washed and filter assayed for bacterial detection (0 to 2 weeks) (30). Fruit maturity was monitored with a hand-held penetrometer (model FT327, Facchini, Alfonsine, Italy), which averaged 6 kg at the time of processing. Once every 50 processed fruit, resuspended Ea153N (10 to 50 CFU per ml) was filter assayed as a positive control.

The colony morphology of bacterial isolates on MS from some commercial fruit was similar to that expected for colonies of *E. amylovora*. When observed, these isolates were cultured on PAF, CCT (17), and CG (11) to compare colony morphology to *E. amylovora*. In addition, the ability of recovered isolates to grow on nutrient agar (Difco Laboratories) at 37°C was determined; *E. amylovora* does not grow at this temperature. Isolates were evaluated for ability to induce fire blight after stab inoculation into immature Bartlett pear (5) and to induce the hypersensitive response after infiltration ($\approx 10^8$ CFU per ml) into tobacco leaves (40). Isolates were tested for oxidase, urease, and phenylalanine deaminase and catalase activity following standard methods (26). In addition, for a subset of 179 isolates with one or more test results similar to *E. amylovora*, a nested polymerase chain reaction (23) for the ubiquitous plasmid of *E. amylovora*, pEA29, and gas chromatography, fatty acid methylsterase (GC-FAME) were used to characterize isolates (34). Finally, for three isolates, partial sequence of 16S rDNA (3) was obtained and compared in GenBank or CLUSTAL to known bacterial species.

Assay for resident *E. amylovora* within mature fruit. In 2004 and 2005, the potential for *E. amylovora* to reside inside symptomless fruit was evaluated in experimental d'Anjou pear orchards located near Medford, OR, and Orondo, WA. At full bloom, flower clusters near terminal ends of two branches on each of five trees were misted with Ea153N prepared at 1×10^9 CFU per ml; five additional trees were misted with water to serve as controls. Inoculated branches were enclosed in fiberglass mesh screen bags to limit insect visitation; these bags remained through the summer. At harvest, locations of 20 symptomless fruit were mapped on each tree and sampled individually. The fruit were filter assayed immediately for the external incidence of *E. amylovora* and then placed into cold storage (2°C) for 6 weeks.

After storage, the fruit were surface disinfested in 0.6% NaOCl for 5 min followed by removal of the stem and calyx regions with a flamed knife. Cut fruit were surface disinfested a second time for 1 min in 0.6% NaOCl, rinsed with distilled water, and then cored with a flamed no. 9 cork borer. Cores were placed individually into resealable plastic bags with 5 ml of sterile 10 mM phosphate buffer (pH 7) and gently macerated with a rubber-coated hammer. Two 1-ml subsamples of each macerated core were frozen at -80°C in 10% (vol/vol) glycerol. Nutrient yeast extract broth (30 ml) was added to the remaining macerate solution. Macerate and broth were incubated at room temperature for 30 h

on a rotary shaker at 200 rpm. After incubation, three 0.25-ml samples of each core macerate were spread on MS medium.

Macerated core subsamples were subjected to DNA extraction and a nested PCR for detection of pEA29 of *E. amylovora* (23). Five microliters of core tissue was mixed with 5 μ l of Gene-Releaser (BioVentures, Inc., Murfreesboro, TN) in 0.2-ml PCR strip tubes. After the first PCR, a 1- μ l subsample of the reaction mixture was used as template for the second PCR. PCR products were visualized after electrophoresis on 1.5% agarose gel. Purified pEA29 (50 ng/ μ l) (4) and macerated pear mixed with Ea153N to a final concentration of 1×10^1 CFU/ml were used as positive controls.

Survival of *E. amylovora* on stored fruit. A suspension of fresh Ea153N cells scraped from agar cultures was prepared at 5×10^7 CFU/ml and sprayed with a hand pump sprayer to runoff onto 240 fruit. Fruit were allowed to air dry for 2 h. Inoculated fruit were divided into three treatments: ambient, defined as storage on cardboard trays in a box bagged with plastic and placed inside an incubator set at 20°C; refrigerated regular atmosphere (21% O₂, 0.1% CO₂); or refrigerated controlled atmosphere (1.5% O₂, 1% CO₂). Refrigerated chambers were set at 0°C with a relative humidity of 86 to 90%. Fruit that received refrigerated treatments were divided among 14 fabricated chambers (each 0.14 m³) with atmospheres established and maintained by a computerized monitoring system (Technical Consulting Services, Chelan, WA). Use of multiple chambers allowed for samples to be taken over time without disturbing the atmospheres around fruit stored for longer periods. Population sizes of Ea153N on 10 fruit per treatment were determined by dilution plating and filter assay at 0, 1, 3, 5, 7, 14, 28, and 56 days inoculation. The experiment was performed twice.

In 2004, 2005, and 2006, 300 healthy, mature pear fruit (cv. d'Anjou) from Medford, OR, and from Wenatchee, WA (2005 and 2006 only), and 300 apple fruit (cv. Braeburn) from Corvallis, OR, and from Wenatchee, WA (cv. Gala 2005, cv. Braeburn 2006), were bulk harvested and transported to the laboratory. In the lab, fruit were surface disinfested in 0.6% NaOCl, rinsed in sterile water, and air dried. After drying, a 10- μ l drop that contained resuspended, lyophilized cells of Ea153N was placed onto a marked position on the side of each fruit. Inoculum concentrations were 0, 10, 100, and 1,000 cells per drop in 2004, and 0, 1,000, and 10,000 cells per drop in 2005 and 2006. Each dose was applied to three replications of 30 fruit (90 fruit per inoculum dose per cultivar). Once the drop of inoculum was air dry, a 1- $\times$ 3-mm-deep skin puncture was introduced at the site of inoculation with a sterile, 4D steel finishing nail secured through a wood block. After wounding, fruit were incubated at room temperature for 24 h followed by a dump tank immersion in 1.5% sodium ortho-phenylphenate (SOPP, Columbia Technical Assoc., Wenatchee, WA) for pear (and apple in 2006) or 0.006% NaOCl (100 ppm of chlorine) for apple (and pear in 2006) and then placed in a 0 to 3°C cold room for up to 49 days. SOPP and NaOCl are standard disinfectants used in commercial flotation systems for pear and apple, respectively. Fruit were assayed for Ea153N on day 0 (pre- and post-dump tank), 7, 14, 28, and 49. In 2005 and 2006, additional inoculated fruit were removed from cold storage after 49 days and incubated at room temperature for up to an additional 30 days. At each sampling time, tissue immediately surrounding the wound site (1 cm diameter $\times$ 5 mm in depth) was cut from 15 fruit per inoculum dose. Tissue samples were macerated with a sterile glass rod in 4 ml of sterile phosphate buffer, filter assayed, and incubated on MS medium.

Weather data. Orchard weather conditions were obtained from automated weather stations located within experimental orchards near Corvallis and Medford, OR, and Wenatchee, WA. These data are available online (AGRIMET and METAR weather networks, Integrated Plant Protection Center, Oregon State University).

Data summary. For all experiments, the incidence of recovery of the inoculated bacterial strains was determined for each replicate of sampled fruit (or flowers) on each sample date. Replicate means for population size of a strain were determined similarly, but only for fruit (or flowers) on which a strain was detected (33). In the computation of means, a $\log_{10}$ transformation of bacterial population size on an individual fruit was performed prior to averaging; also, in those cases where counts of recovered CFU were considered very low ($\leq 10^3$ CFU), means of untransformed counts of CFU were determined. Survival of *E. amylovora* and *P. agglomerans* as epiphytes on fruit and fruit calyxes, as measured by incidence of recovery and mean population size, were plotted in temporal arrays as a function of days after inoculation.

Analysis of variance (ANOVA) was performed on several data sets. Inoculum type and host cultivar were compared in the epiphytic survival experiments with two-factor ANOVA models. Similarly, the effects of bacterial strain and host cultivar were determined in the calyx-end survival experiments. Effect of storage atmosphere was evaluated in the postharvest experiments; and effect of inoculum dose and cultivar was compared in the post-harvest wounding experiment. Relative area under the incidence of recovery curve (A_{inc}) was the principle variable evaluated by ANOVA. This variable (in essence, a weighted average of recovery of a bacterial strain over the observed time period) was calculated as

$$A_{inc} = \left[\sum_{i=2}^n (y_i + y_{i-1}) / 2 \times (t_i - t_{i-1}) \right] / (t_n - t_1)$$

where y and t are the incidence of recovery of a bacterial strain and day from inoculation, respectively, for the i th sample date, and n is the total number of sample dates during the bloom period. Treatment effects were evaluated by the F test, and means were separated using Fisher's protected least significant difference at $P = 0.01$.

RESULTS

Evaluation of bacterial recovery methods. With the dilution plating method, the lower limit for reliable detection of bacterial CFU occurred in the range of 1×10^2 to 1×10^3 CFU per fruit or per ml depending on the volume of the wash buffer; below this range, the filter assay method provided superior results (Table 1). Within their respective range, dilution plating of cells dried onto and washed from fruit surfaces had a ratio of recovered cells to estimated inoculum density that averaged 55%, but this ratio dropped to 5% for the filter assay method (Table 1). In contrast, for cells resuspended into fruit macerate, the ratio of recovered cells to estimated inoculum density averaged 83 and 71% for the dilution plating and filter assay methods, respectively. At the lowest inoculum density applied to fruit surfaces, the ratio of recovered cells to estimated inoculum density was 5% for 43 CFU

of *E. amylovora* inoculated onto a pear fruit surface and then allowed to dry. This sensitivity and efficiency of recovery is similar to a previously reported detection limit for the filter assay method (30).

Survival of *E. amylovora* on fruit in orchards. On the day of inoculation, *E. amylovora* strains Ea153N and Ea153 were recovered from 100% of fruit from all cultivars in all experiments ($n = 450$) (Fig. 1A to C). Average initial population sizes were 4.3×10^5 CFU for resuspended cells sprayed onto fruit surfaces, and 1.6×10^7 CFU per fruit for cells produced in ooze and brushed onto fruit calyxes (Fig. 1D to F). By 7 days after inoculation, measurable populations of the pathogen on fruit had decreased by two to three log units (90 to 99%) regardless of cultivar or strain treatment. Incidence of recovery also declined rapidly after inoculation, with the pathogen being detected on approximately 40% of the fruit at 7 days after inoculation, declining to <5% fruit at 5 weeks after inoculation. At 56 days after inoculation, *E. amylovora* was not detected on any fruit of d'Anjou pear or Gala apple ($n = 150$ fruit per cultivar); however, one ooze-inoculated fruit of Bosc pear yielded 4 CFU (May 2004 inoculation). At no time were symptoms of fire blight apparent on any of the 90 trees that received pathogen inoculum; the pathogen was not detected on control fruit treated with water.

Extinction curves for *E. amylovora* on fruit as a function of time showed similar shapes among cultivar and inoculum treatment, but with consistently higher incidence of recovery of the pathogen obtained for cells applied in ooze compared with cells resuspended in water, and for cells recovered from rougher-skinned Bosc pear as opposed to smooth-skinned d'Anjou pear or Gala apple (Fig. 1). ANOVA of relative area under incidence of recovery curve confirmed these observations with A_{inc} values for cells in ooze being significantly greater than resuspended cells in all experiments (P values ranged from <0.0001 to 0.0045), and A_{inc} for Bosc pear being significantly greater than the other cultivars in both of the 2004 experiments ($P < 0.0001$). In 2004, A_{inc} for Ea153 and Ea153N resulted in similar values (0.148 ± 0.033 [standard error] and 0.156 ± 0.042 , respectively), and consequently, the wild-type strain, Ea153, was not included in the 2005 experiment. Within a season, the differences in A_{inc} values for the same treatment between the early and late summer experiments were small (<10%), but A_{inc} values measured in 2004 were twice those measured in 2005.

Isolation from specific areas of selected pear fruit ($n = 570$) influenced recovery of the pathogen. After the day zero sample, Ea153N was not recovered from peel tissue or stem tissue, but other bacteria continued to be recovered from stem tissues (but not peel) at population sizes between 1×10^2 and 1×10^3 CFU/g. For calyx ends of pear fruit, Ea153N was detected on all sample dates (i.e., 0, 1, and 3 days after inoculation) at incidences ranging from 60 (day 0) to 10% (day 3), and at populations up to 1×10^5 CFU/g on day 0 and 1×10^3 CFU/g on day 3 (data not shown).

TABLE 1. Recovery of *Erwinia amylovora* strain Ea153N from inoculated pear fruit surfaces and macerates

Recovery method	Fruit surface ^a			Fruit macerate ^b		
	Inoculum dose (CFU fruit ⁻¹)	Recovered (CFU fruit ⁻¹)	Ratio of recovery	Inoculum dose (CFU ml ⁻¹)	Recovered (CFU ml ⁻¹)	Ratio of recovery
Dilution plate	13,000 $\pm$ 1,100 ^c	5,700 $\pm$ 6,000	0.44	16,000 $\pm$ 8,800	14,000 $\pm$ 5,200	0.87
Dilution plate	500 $\pm$ 210	330 $\pm$ 100	0.66	620 $\pm$ 96	490 $\pm$ 71	0.79
Filter assay	500 $\pm$ 210	25 $\pm$ 29	0.05	—	—	—
Filter assay	43 $\pm$ 27	2 $\pm$ 2	0.05	58 $\pm$ 7	41 $\pm$ 20	0.71

^a Fruit of pear cv. Bartlett were inoculated with a 10- μ l drop containing the indicated number of colony forming units (CFU) per fruit. The area of inoculation was estimated to be 0.1% of the fruit surface. Inoculum consisted of resuspended freeze-dried Ea153N, which was allowed to dry for 2 h after placement on the fruit. Fruit were washed in 30 ml of phosphate buffer (10 mM, pH 7.0), sonicated for 2 min, followed by dilution plating and/or filter assay onto Miller Schroth medium.

^b Core tissue from surface-disinfested fruits was removed with a no. 9 cork borer, added to 30 ml of sterile phosphate buffer (10 mM, pH 7.0), and then macerated. Resuspended freeze-dried Ea153N was added to the macerate to obtain the indicated number of CFU per milliliter. Resulting macerate was dilution plated and/or filter assayed onto Miller Schroth medium.

^c Each mean (and standard deviation) was computed from eight replicates. Data represent combined results of two independent experiments.

Bacterial survival on fruit calyxes. For orchard experiments conducted in 2005 and 2006, pear flowers sprayed with Ea153N, Ea153HrpL⁻, and PaC9-1S yielded incidences of recovery at 8 days after inoculation that averaged 72, 95, and 89%, respectively (Fig. 2A and B). Similarly, incidences of recovery of Ea153N, Ea153HrpL⁻, and PaC9-1S from sampled flowers of Gala apple were 47, 92, and 93%, respectively (Fig. 2C). Initial population sizes of the inoculated strains on flowers from which the respective bacteria were detected ranged from 1.0×10^2 to 1.8×10^5 CFU per flower, with generally similar means obtained among the strains and cultivars (Fig. 2D to F). For flowers treated with virulent Ea153N, the development of fire blight on inoculated clusters in 2005 occurred at relatively low incidence in d'Anjou pear (<10%) but at high incidence in Bosc pear and Gala apple (>60%); in contrast, in 2006, incidences of diseased blossom clusters were high for d'Anjou and Bosc pear (> 80%) and low for Gala apple (< 20%). Diseased blossom clusters were removed as detected.

Temporal patterns of strain survival in association with calyx ends of healthy, developing fruit were similar among the 2005 and 2006 seasons (Fig. 2A to F). The nonpathogenic epiphyte, *P. agglomerans* strain C9-1S, persisted through to harvest (≈ 150 days after inoculation) in populations averaging $>1 \times 10^4$ CFU per calyx on most inoculated pear and apple fruit. Conversely, incidence and population size of virulent Ea153N and avirulent Ea153HrpL⁻ declined progressively through the summer. In 2005, neither strain of *E. amylovora* could be detected on symptomless fruit sampled at harvest, whereas in 2006, these strains persisted on 7% of pear fruit but could not be detected on apple (Fig. 2); when recovered on pear, the population size averaged 7 CFU per

fruit. In contrast to *E. amylovora*, fluorescent microspheres applied with the inoculum were detected on 80 to 100% of flowers and fruit sampled at midsummer. For the harvest, post-dump tank and post-cold storage sampling times, the incidence of microspheres on calyxes averaged 76, 81, and 73% for d'Anjou pear; 72, 82, and 79% for Bosc pear; and 85, 94, and 88% for Gala apple, respectively ($n = 90$ for each fruit type on each sample date).

For all experiments, A_{inc} values for *P. agglomerans* strain PaC9-1S were significantly greater ($P < 0.0001$) than corresponding values for Ea153N and Ea153HrpL⁻. After a 7-week cold storage period, an enrichment assay involving calyx ends of fruit placed in MS broth yielded growth of PaC9-1S from 100% of samples ($n = 13$), but this assay failed to detect Ea153N or Ea153HrpL⁻ on fruit ($n = 60$) from trees inoculated with these strains. Control samples ($n = 9$) spiked with 5 to 10 CFU of PaC9-1S, Ea153N, and Ea153HrpL⁻ all yielded a positive detection (data not shown).

Surveys for *E. amylovora* on fruit from commercial orchards. Of the 56 surveyed orchards, at the time of harvest, 15 (27%) had fire blight symptoms present in the orchard or in an adjacent block of a different cultivar; whereas the remaining 41 orchards (73%) were apparently disease free as determined by visual inspection. At least some bacteria were recovered on filter membranes of nearly all fruit washings, many of which were green-colored on MS medium and fluoresced when transferred to PAF medium, indicative of *Pseudomonas* spp. Orange to red colonies with resemblance to *E. amylovora* on MS medium were recovered from 15% of washed fruit; a subset of 179 orange/red colonies were selected and tested for identification (Table 2). Partial identification of the orange/red-colored isolates by fatty

Fig. 1. A to C, Incidence of recovery and **D to F,** mean size of detectable populations (expressed as $\log_{10}$ [CFU per fruit]) of *Erwinia amylovora* strains Ea153N and Ea153 on surfaces of pear and apple fruit after inoculation in orchards located near Medford ('d'Anjou' pear) and Corvallis, OR ('Bosc' pear and 'Gala' apple) during the summers of 2004 and 2005. Each panel depicts a summary of four experiments initiated in mid-May and early-July of both seasons. Inoculum was applied to fruit as resuspended freeze-dried cells prepared at 1×10^7 CFU/ml and sprayed to runoff (■ Ea153N, ▲ Ea153; strain Ea153 was applied in 2004 only), or cells in ooze (□ Ea153N) produced on diseased immature fruit of 'Bartlett' pear and applied with a small brush directly onto the calyx area of each fruit; the ooze applied to each fruit contained 10^8 to 10^9 CFU of Ea153N. For each sample time, five fruit were sampled from each of three replicate trees per experiment, washed individually, and dilution plated and/or filter assayed onto Miller Schroth medium. Vertical bars drawn through each point represent $\pm$ one standard deviation of the mean.

acid analysis (GC-FAME) most frequently yielded *Enterobacter* (8%), *Pantoea* (12%), and *Pseudomonas* spp. (8%), and a few nonpathogenic *Erwinia* spp. (2%) (*E. rhapontici* and *E. persicina*).

A total of 3 of 179 orange/red isolates produced a hypersensitive reaction (HR) in tobacco leaves, indicative of a type III secretion system. Partial sequences of the 16S rDNA of these isolates yielded (with >99% similarity in GenBank) *E. amylovora*, *E. persicina*, and *Citrobacter freundii*. The one isolate identified as *E. amylovora* by its 16S rDNA sequence also was positive for production of ooze on immature pear and for presence of pEA29; all other isolates were negative for these tests (Table 2). Consequently, 1 (0.02%) of 5,600 pear fruits sampled from commercial orchards yielded 32 CFU of *E. amylovora* (Table 3).

Assay for resident *E. amylovora* within mature fruit. In both seasons, the controlled inoculation of specific branches of d'Anjou pear with Ea153N resulted in two diseased branches on each of the experimental trees at each location ($n = 20$ diseased trees). These cankers expanded through the spring and summer, and measured 0.5 to 1.0 m at harvest. In September, 20 mature symptomless fruit on each replicate tree were mapped for proximity to a canker and harvested. Over both locations and years, external washings of fruit ($n = 400$) resulted in one isolation (0.25%) of 33 CFU of Ea153N (Wenatchee experiment, 2005); this fruit was located 5 cm from the proximal edge of canker. Positive isolation of Ea153N was confirmed by resistance to nalidixic acid at 100 $\mu\text{g/ml}$, and by assay for pEA29, tobacco HR, and ooze production in immature pear.

Ea153N was not detected in core tissues of sampled fruit ($n = 400$) in any experiment determined by either enrichment assay or nested PCR amplification of pEA29. Similarly, fruit ($n = 250$) from nondiseased trees failed to yield Ea153N. All control samples ($n = 30$) spiked with 1×10^1 to 1×10^5 CFU of Ea153N

resulted in positive detection of the pathogen with both assay methods.

Postharvest survival of *E. amylovora* on stored fruit. As in the orchard experiments, Ea153N was recovered from 100% of inoculated mature fruit sampled and assayed prior to assignment to one of three postharvest storage treatments (Fig. 3A); initial population sizes on the fruit averaged 1.3×10^6 CFU per fruit (Fig. 3B). Incidence of recovery of Ea153N over time was influenced strongly by storage condition, with the ambient treatment (20°C) showing a rapid decline in proportion of fruit with detectable pathogen populations (from 100 to 22%) over the first 14 days of storage; in contrast, the refrigerated controlled atmosphere (0°C, 1.5% O₂, 1% CO₂) treatment showed only a slight decline in the recovery incidence (from 100 to 96%) over the first 28 days of storage (Fig. 3), but an accelerated decline over the latter half of the storage period (Fig. 3A). For both experiments, ANOVA of A_{inc} values confirmed a significant effect of storage environment ($P < 0.0001$). Population sizes of Ea153N on stored fruit declined steadily over time with the rate of decline also affected by storage environment (Fig. 3B). These declines in population size averaged a log unit for every 2, 5, and 7 days of storage in the ambient, regular, and controlled atmosphere treatments, respectively (Fig. 3B). For all treatments, mean populations declined to $<1 \times 10^4$ CFU per fruit by 14 days of storage, and for the refrigerated treatments, the pathogen was undetectable on fruit ($n = 40$) stored for 56 days (for the ambient treatment, continuous ripening of fruit prevented sampling beyond 28 days).

Pathogen survival near wounds on stored fruit. The detection of Ea153N near small skin punctures on mature fruit was dependent on the inoculum dose. Of the doses evaluated in 2004 (water control, and 1×10^1 , 1×10^2 , and 1×10^3 CFU per wound site), only the highest dose resulted in pathogen recovery from the wound site after the dump tank treatment (Table 4). At

Fig. 2. A to C, Incidence of recovery and D to F, mean size of detectable populations (expressed as log₁₀[CFU per tissue]) of *Erwinia amylovora* strains Ea153N (■) and Ea153HrpL- (▲) and *Pantoea agglomerans* C9-1S (○) on floral (first sample only) and fruit calyx tissue after inoculation in orchards located near Medford ('d'Anjou' pear) and Corvallis, OR ('Bosc' pear and 'Gala' apple) during the summers of 2005 and 2006. Data from both seasons are combined in the figures. Inoculum (1×10^7 CFU/ml for Ea153HrpL- and C9-1S, and 1×10^5 CFU/ml for Ea153N) was misted onto flowers at full bloom. For each sample time, eight flowers or five fruit were sampled from each of three replicate trees per experiment, washed individually, and dilution plated and/or filter assayed onto Miller Schroth medium. For sample dates at harvest and at pre- and post-storage, 90 fruit per bacterial strain (bulk harvested for treated trees) were individually assayed in each season. Vertical bars drawn through each point represent $\pm$ one standard deviation of the mean.

this dose, Ea153N was detected on 27% of d'Anjou pear after 7 and 14 days of cold storage (0 to 2°C, regular atmosphere) and on 20% of Braeburn apple after 7 days storage; the pathogen was not detected on any fruit ($n = 480$) stored longer than 14 days. When detected, the mean recovered population size of Ea153N was $<1 \times 10^2$ CFU per fruit (Table 4). A dose of 1×10^3 CFU per wound site yielded similar results in the 2005 and 2006 experiments with no detection of the pathogen beyond 7 or 14 days from apple and pear, respectively.

The dose of 1×10^4 CFU per wound site, evaluated in 2005 and 2006 experiments, increased persistence of the pathogen on fruit relative to a dose of 1×10^3 CFU per wound site, which was evaluated in all years of the study (Table 4). For pear, at the highest dose, populations of the pathogen between 1×10^2 and 1×10^3 CFU per wound site were detected on 13 to 37% of fruit at 49 days after inoculation; on apple, smaller populations (between 1×10^1 and 1×10^2 CFU per wound site) were detected on 13 to 20% of apple fruit at 28 days after inoculation. For both

fruit types, the incidence of detection of Ea153N was highest at the pre-dump tank assessment (except for pear in 2006 with NaOCl as the disinfectant) followed by consistently smaller rates of recovery as storage time progressed. In contrast, estimated population sizes for Ea153N in the vicinity of wound sites were consistently an order of magnitude higher after 7 days of cold storage compared with samples taken shortly after inoculation (pre-dump tank in Table 4). In pear, incubation of fruit at room temperature for 30 days after storage resulted in detection of Ea153N on 2 of 16 fruit in 2005 (average of 5 CFU per wound site), but no detection of the pathogen on similarly treated fruit in 2006.

DISCUSSION

Experiments and surveys performed in this study provided data useful toward understanding the potential for co-occurrence of *E. amylovora* with mature, winter pear fruit produced in the

TABLE 2. Assays performed to characterize 179 bacterial isolates recovered from commercial d'Anjou pear fruit^a that exhibited coloration similar to *Erwinia amylovora* grown on filter membranes on the surface of Miller Schroth medium

Valley location of sampled orchards	Sample date	Disease in orchard(s)	Number of fruit ^b	Number of positive Ea153N controls ^c	Number of fruit with <i>E. amylovora</i>
2003					
Hood River, OR	9/08	No	300	6/6	0
Yakima, WA	9/10	No	300	6/6	0
Rogue, OR	9/15	No	100	2/2	0
Wenatchee, WA	9/17	No	300	6/6	0
2004					
Hood River, OR	8/31	Yes	100	2/2	0
Hood River, OR	8/31	No	400	8/8	0
Wenatchee, WA	8/30	Yes	500	8/8	0
Wenatchee, WA	8/30	No	200	4/4	0
Rogue, OR	9/01	Yes	100	2/2	0
Rogue, OR	9/01	No	100	2/2	0
Yakima, WA	9/06	No	400	8/8	0
Okanogan, WA	9/06	No	300	6/6	0
2005					
Yakima, WA	9/05	No	300	6/6	0
Yakima, WA	9/05	No	300	6/6	0
Wenatchee, WA	9/07	Yes	500	10/10	1
Wenatchee, WA	9/07	No	300	6/6	0
Rogue, OR	9/09	Yes	100	2/2	0
Rogue, OR	9/09	No	100	2/2	0
Hood River, OR	9/12	Yes	200	4/4	0
Hood River, OR	9/12	No	100	2/2	0
Okanogan, WA	9/13	No	300	6/6	0
Hood River, OR	9/19	Yes	100	2/2	0
Hood River, OR	9/19	No	200	4/4	0

^a The wash from each fruit was passed through a 0.2 µm filter followed by incubation of the filter on Miller Schroth medium for 3 to 7 days.

^b In each orchard, four pear fruit were sampled from each of 25 haphazardly located trees for a total of 100 fruit per orchard.

^c Number of positive detections of Ea153N followed by number of control samples processed.

TABLE 3. Incidence of isolation of *Erwinia amylovora* from mature, symptomless d'Anjou pear fruit^a sampled from commercial orchards in Oregon and Washington in 2003, 2004, and 2005

Bacterial strain or unknown isolates	Cultural characteristics ^b				Enzymatic tests ^c				Pathogenicity tests ^d	
	Orange colony on MS	Fluorescent on PAF	Craters on CCT	Growth at 37°C	Oxidase	Urease	Catalase	Phenylalanine deaminase	HR	Ooze on fruit
Ea153N	Y ^e	N	Y	N	N	N	Y	N	Y	Y
PaC9-1	Y	N	N	Y	N	N	Y	N	N	N
Unknown isolates	100% (179) ^f	0% (0)	0.5% (1)	87% (156)	9% (16)	5% (9)	94% (168)	9% (17)	1.5% (32)	0.5% (1)

^a Isolates were recovered from surveys of commercial orchards in five pear production districts of Oregon and Washington in 2003, 2004, and 2005.

^b Phenotypic traits observed when a bacterial strain or isolate was grown on various media (orange/red colonies on Miller Schroth [MS], fluorescent pigment on Pseudomonas agar F [PAF], surface cratering of colonies on CCT (17), or growth on nutrient agar at 37°C).

^c Enzymatic reaction observed when bacterial strain or isolate was incubated with appropriate substrate.

^d Pathogenic reaction as indicated by development of a hypersensitive response (HR) when cells of the strain or isolate were infiltrated into tobacco leaf tissue or the production of ooze when cells were inoculated into immature fruit of cv. Bartlett pear.

^e Y represents a positive trait or response; N represents a negative trait or response. In the unknown isolates row, the percentage is the number of isolates yielding a positive response, Y, divided by 179.

^f Number of unknown isolates demonstrating a positive trait or response.

Pacific Northwest region of the United States. For countries that do not have fire blight, co-occurrence of the pathogen with the fruit is essential to the hypothesized disease introduction pathway on which phytosanitary restrictions to fruit importation are grounded. For apple, similar experiments, risk analyses, and regulatory proceedings have concluded that the likelihood of successful establishment of *E. amylovora* in a new geographic area via importation of commercial fruit is negligible (29,41,42). For this study, the use of established bacterial recovery methods and the inclusion of apple fruit as a control provide a link to previous efforts, which, we believe, can lead to a sharper analysis and discussion of the potential association of *E. amylovora* with healthy pear fruit, and the phytosanitary regulations pertaining to the export and import of this commodity.

The first experiments characterized the survival of *E. amylovora* on pear fruit surfaces in orchard environments and provided a baseline from which to judge the epiphytic potential of the

pathogen and its expected longevity when dispersed from diseased tissues onto healthy fruit. We mimicked natural dissemination by utilizing ooze of *E. amylovora* from diseased tissues and resuspended, freeze-dried cells of the pathogen as inocula, both of which have been reported to be comprised of cells that show superior survival in the environment relative to cells harvested fresh from artificial media (31,33). After deposition, the results depicted an organism in a survival phase, typified by declining incidence of detection and population size, as opposed to a growth phase, such as that observed when cells are inoculated onto floral stigmata (33,37). For both pear and apple, at 2 weeks after inoculation, the detectable population size of the pathogen declined by >99.99%, and after this point, the detection of the pathogen on fruit occurred infrequently (Fig. 1). Subsamples of fruit tissues revealed that surviving cells were associated with the calyx, and that the pathogen was not detected on the stem or skin. As Rosen (31) and Roberts et al. (29) speculated for cells in ooze, the poor survival of *E. amylovora* on plant surfaces may be due to fluctuating temperatures and relative humidity in the orchard environment. For a regional perspective, this first experiment also highlighted the climatic conditions typical for pear production. The region is semi-arid, and pear trees are rarely wet after the first of July. For example, for this study, rainfall averaged 23 (Corvallis), 6 (Medford), and 4 mm (Wenatchee) per month over the three summers, with June the wettest and July the driest month. Consequently, unlike fruit production areas in the eastern United States where the summer shoot blight phase of the disease occurs with regularity (13), fire blight in the Pacific Northwest is mostly a disease of the spring season, when active cankers coincide with significant rain events.

The calyx survival studies provided evidence that floral populations of *E. amylovora* can persist on pear and apple fruit for a period of time after bloom, but that persistence is characterized by small numbers of cells detected on fewer and fewer fruit as the summer progresses. Recovery of populations of *E. amylovora* occurred infrequently at harvest (12 of 360 sampled pear fruit with an average of 7 CFU per fruit), and in both 2005 and 2006, the pathogen was not detected on fruit at the end of a 7-week cold storage period. In contrast, populations of the nonpathogenic bacterial epiphyte *P. agglomerans* persisted in relatively high populations ($\approx 1 \times 10^4$ CFU per calyx) on most fruit through the summer and into the period of cold storage, indicating that this organism is better adapted to epiphytic survival than is the fire blight pathogen. Roberts et al. (30) also isolated *P. agglomerans* (syn. *Erwinia herbicola*) in high frequency from mature apple fruit sampled from commercial orchards in Washington State, and noted that persistence of this bacterium on fruit after harvest was not influenced by time in storage. As we reported in a previous study (33), fluorescent microspheres were a useful control in this experiment because they could be observed on the calyxes of 73 to 95% of fruit from which we attempted to isolate *E. amylovora*, indicating that a similar proportion of the flowers received an initial dose of the pathogen, and that lack of detection of the pathogen at harvest and during storage was not due to the flowers escaping the initial inoculations. For the Bosc pear and Gala apple inoculations in 2005, and for both pear inoculations in 2006, the inoculation with Ea153N (1×10^5 CFU per ml) resulted in high incidences of blossom blight (70 to 85% of blossom clusters became diseased), demonstrating the virulence of this strain. On healthy fruit, patterns of survival for Ea153N and the avirulent strain Ea153HrpL⁻ were similar, suggesting that the ability to survive epiphytically was not strongly associated with the ability to cause disease. Based on data from both seasons, there were no apparent differences in the patterns of calyx end survival on pear compared with those observed on apple. Like the conclusions made in regard to calyx-end survival on apple (16,29,30,36), there is a low probability that a floral, epiphytic population of *E. amylovora* can persist on calyxes of symptomless pear fruit through

Fig. 3. A, Incidence of recovery and B, mean size of detectable populations (expressed as $\log_{10}$ [CFU per fruit]) of *Erwinia amylovora* strain Ea153N on surfaces of 'd'Anjou' pear as influenced by postharvest storage condition. In the laboratory, mature fruit were inoculated to runoff with Ea153N (1×10^7 CFU/ml) and divided among three storage environments: ambient ($\square$, 20°C), and two refrigerated environments (0°C, 86 to 90% relative humidity) with regular ($\blacksquare$, 21% O₂, 0.1% CO₂) or controlled ($\blacktriangle$, 1.5% O₂, 1% CO₂) atmospheres. For each sample time, 10 fruit per storage treatment were sampled, washed individually, and dilution plated and/or filter assayed onto Miller Schroth medium. Data from two independent experiments are combined in the figure. Vertical bars drawn through each point represent $\pm$ one standard deviation of the mean.

the summer, but the size of the surviving populations is small, and from our data, unable to persist through a postharvest cold period.

The commercial orchard surveys were made in each of the major pear production areas of the Pacific Northwest, and within each area, we attempted to represent the orchard-to-orchard spectrum of fire blight incidence that occurred within a specific season. The disease was not observed in orchards labeled apparently without fire blight, whereas in orchards described as with fire blight, the incidence of disease ranged from a few blighted shoots in the entire orchard to several blighted branches per tree. Because the isolation or detection of *E. amylovora* from commercial fruit was rare (1 of 5,600 fruit), the hypothesis that the presence of fire blight in an orchard increases the likelihood of detection of the pathogen from fruit was not supported. Logically, disease in the orchard should increase this likelihood (13,16,39), and the one fruit from which we detected 32 CFU of the pathogen was from an orchard with blighted shoots on most trees. Notably, other bacteria were detected on nearly all samples (>99%), suggesting, at least for the production areas surveyed, that the relative epiphytic fitness of *E. amylovora* on fruit surfaces is of greater significance in understanding phytosanitary risk than is proximity to potential sources of inoculum. The conclusion derived from the results of this survey is essentially identical to a similar survey of apple fruit grown in the same region (30): nonpathogenic bacterial epiphytes can be isolated from the surfaces of most, if not all, commercially produced pear fruit sampled in the orchard at harvest, but the detection of epiphytic populations of the fire blight pathogen on these fruit is an exceptionally infrequent event.

The question as to whether *E. amylovora* can reside as a quiescent endophyte within mature symptomless apple fruit has been addressed in several studies (2,30,39,41,42). Its importance stems

from observations that endophytic cells of *E. amylovora* produced in diseased shoots and released into the vascular system can be a source of inoculum for infection of rootstocks and propagation materials (15,22,25,37). Fruit, however, are thought to be protected from cells moving in the vascular system because of the abscission zone formed between the branch spur and fruit pedicel (14,42). Our field trials, designed to measure the potential for endophytic infestations of mature, symptomless pear fruit, represented a moderate disease event, with the development and extension of two large cankers on the fruit-bearing branches of each of the experimental d'Anjou pear trees. Size of the expanding cankers was somewhat greater in Wenatchee, WA, compared with Medford, OR, and in Wenatchee, one fruit was found to harbor a small epiphytic population of the pathogen. *E. amylovora* was not detected inside of fruit at either location as measured by nested PCR or by incubation of core and cortex tissue in selective enrichment broth, even in fruit as close as 5 cm to the proximal edge of an expanding canker. Similar to our results, Roberts et al. (30) was unable to detect *E. amylovora* in core and cortex tissue of 1,555 mature symptomless fruit harvested from apple trees with oozing fire blight cankers. The panel of judges in a recent World Trade Organization trade dispute resolution hearing stated "there is not sufficient scientific evidence to conclude that mature symptomless apples would harbor endophytic populations of bacteria" (41,42), with Roberts et al. (29,30) and expert testimony cited as the primary sources of evidence. In contrast, Azegami et al. (2, reviewed in literature citation 42), in greenhouse studies, reported movement of *E. amylovora* into nearly mature apple fruit after artificial insertion of at least 1×10^5 CFU of the pathogen into spur tissue 17 to 30 days before harvest. To our knowledge, the detection of

TABLE 4. Effect cold storage^a on percentage of fruit with detectable populations of *Erwinia amylovora* strain Ea153N, and the mean recovered population (CFU) after small wounds^b were created near inoculation sites on mature, symptomless pear and apple fruit near harvest

Host ^c	Year	Inoculum dose	Dump tank disinfectant ^d	Pre-dump tank	Post-dump tank	Days in cold storage				Post-cold storage ^e
						7	14	28	49	
Pear	2004	1×10^3	SOPP	100% ^f	53%	27%	27%	0%	0%	n.d. ^g
Pear	2005	1×10^3	SOPP	96 ± 15	43 ± 43	50 ± 59	80 ± 78			
				20%	0%	0%	0%	0%	0%	0%
Pear	2006	1×10^4	SOPP	3 ± 6	23%	43%	47%	57%	13%	13%
				67%	10%	220 ± 143	204 ± 136	261 ± 145	90 ± 133	5 ± 1
Pear	2006	1×10^3	SOPP	43 ± 31	10 ± 12	10%	7%	0%	0%	0%
				34%	29 ± 18	3 ± 4	239 ± 237			
Pear	2006	1×10^4	SOPP	5 ± 7	47%	67%	47%	54%	37%	0%
				90%	137 ± 85	163 ± 165	1,376 ± 1,848	793 ± 859	263 ± 303	222 ± 294
Pear	2006	1×10^4	NaOCl	64%	30%	67%	47%	54%	27%	0%
				40 ± 53	101 ± 128	272 ± 351	317 ± 291	428 ± 555	230 ± 261	
Apple	2004	1×10^3	SOPP	100%	13%	20%	0%	0%	0%	n.d.
Apple	2005	1×10^3	SOPP	88 ± 4	1 ± 0	11 ± 6				
				13%	0%	0%	0%	0%	0%	0%
Apple	2006	1×10^4	NaOCl	4 ± 5	7%	3%	3%	0%	0%	0%
				50%	7%	3%	3%	0%	0%	0%
Apple	2006	1×10^3	NaOCl	70 ± 103	96 ± 1	115 ± 0	86 ± 0			
				47%	27%	20%	0%	0%	0%	0%
Apple	2006	1×10^4	NaOCl	6 ± 4	7 ± 4	29 ± 26				
				87%	27%	53%	13%	13%	0%	0%
Apple	2006	1×10^4	SOPP	18 ± 6	5 ± 6	153 ± 133	83 ± 21	13 ± 14		
				73%	20%	53%	27%	20%	0%	0%
Apple	2006	1×10^4	SOPP	18 ± 6	2 ± 2	232 ± 40	68 ± 10	31 ± 26		

^a Storage temperatures averaged 2 and 0°C in Corvallis and Wenatchee, respectively.

^b Fruit were inoculated with a 10-μl drop containing the indicated number (dose) of colony forming units (CFU). After the drops were dry, a 1 × 3 mm deep skin puncture was introduced at the site of inoculation with a sterile nail.

^c Pear experiments were conducted with cv. d'Anjou. Apple cv. Braeburn was used in Corvallis experiments and in Wenatchee in 2006; cv. Gala was used in Wenatchee in 2005.

^d SOPP = 1.5% sodium ortho-phenylphenate, and NaOCl = 100 ppm of sodium hypochloride are standard disinfectants used in commercial flotation systems for pear and apple, respectively. After inoculation, fruit were incubated at room temperature (20 to 22°C) for 24 h prior to dump tank immersion.

^e After cold storage, fruit were incubated at room temperature (20 to 22°C) for an additional 30 days before assay.

^f Percentage of 15 (2004 and post-storage samples in 2005 and 2006) or 30 fruit (2005 and 2006; 15 from each location) with detectable populations of Ea153N. Mean population size (CFU per fruit) and standard deviation are computed only for fruit on which Ea153N was detected.

^g Not determined.

endophytic populations of *E. amylovora* in mature, symptomless winter pear has not been demonstrated previously; fire blight lesions on the external surface of nearly mature fruit of summer pear cv. Bartlett, however, has been reported from Washington State (10,44).

Postharvest survival of *E. amylovora* on pear fruit was evaluated in two experiments, both with protocols that utilized concentrations of inoculum that were at least several orders of magnitude greater than observed on mature fruit harvested during the orchard studies. The first postharvest experiment was similar to the initial orchard experiment and was designed to evaluate potential for epiphytic survival of the pathogen on nonwounded fruit stored under different environmental conditions. As reported by Ceroni et al. (8), cold temperatures (0°C) prolonged pathogen survival relative to warm temperatures (20 to 22°C), and similarly, a cold, controlled atmosphere (1.5% O₂, 1% CO₂) prolonged storage relative to a cold, regular atmosphere (21% O₂, 0.1% CO₂). Nonetheless, as in the field, incidence of detection and population size declined with time, with no detection of the pathogen after 56 days, regardless of the storage environment. The observed rate of decline of population size of *E. amylovora* on pear fruit in cold storage (an order of magnitude every 6 to 8 days) was very similar to the rates of decline reported by Taylor and Hale (35), who measured postharvest survival of the fire blight pathogen on calyxes of stored apple. The second experiment, although realistic in its emulation of industrial fruit handling practices, also represented an improbable scenario in that a small wound (mimicking a stem puncture or other small abrasion caused during handling) was placed on fruit at a position that coincided with a concentration of pathogen cells confined within an area that represented approximately one thousandth of the total fruit surface. Our goal was to understand, in a dose response framework, how the reportedly greater susceptibility of pear fruit relative to apple would influence pathogen persistence in storage when intimately associated with wounded host cells. The results confirmed our expectation that wounded pear fruit provided a somewhat more conducive environment for persistence of *E. amylovora* compared with that of wounded apple fruit, but this result was dose-dependent, with the difference between the hosts only apparent at the highest concentration of pathogen inoculum (i.e., 1×10^4 CFU in close proximity to the wound site). For both pear and apple, the data also showed small but consistent increases in pathogen population size over the first 7 to 14 days of cold storage followed by declining incidence of detection and population size in the latter portion of the storage period. These increases in recovered population size during the early part of the storage period, however, were considerably smaller than that reported for growth of *E. amylovora* in a nutrient broth incubated at 2°C for 20 days (35). Macroscopically, both pear and apple fruit remained symptomless at the wound sites, although a very small, necrotic discoloration was apparent at the base of the wound of some fruit that were split with a knife. Pear fruit that received the high dose of Ea153N (1×10^4 CFU in the vicinity of the wound site) also were used to monitor the ability of the pathogen to grow on fruit after the 49-day cold storage period. Pears incubated at room temperature for 30 days post-cold storage showed a low incidence of detection with an estimated population size two orders of magnitude smaller (i.e., 5 CFU per fruit) than observed on fruit sampled at the end of the cold storage period (Table 4).

Collectively, the results of this study can be compared with a published risk model developed to assess the probability that the pathway to successful establishment of *E. amylovora* in a disease-free area can be completed by importation of commercial apple fruit (29). In that model, independent probabilities (P) were assigned to steps of the pathway: P₁, the fruit is infested with *E. amylovora*; P₂, *E. amylovora* survives storage; P₃, contaminated fruit is discarded near host; P₄, host is receptive; and P₅, *E. amylovora* is transferred to the new host and infection occurs.

Using these probabilities and an estimate of the potential number of fruit exported annually to the new area, Roberts et al. (29) found the likelihood of successful introduction of fire blight to a new area ranged from once every 11,000 to 38,000 years, depending on levels of phytosanitary precaution taken prior to export. In this likelihood estimate, their derived values for P₁, depending on phytosanitary scenario, ranged from 0.001 (one in a thousand fruit) to 0.035 (1 in 30 fruit), which are considerably higher than the 1 in 5,600 fruit that we obtained in our survey of commercial orchards. Similarly, P₂ was estimated to be 0.1 (1 in 10 fruit), which also was greater than we observed in the epiphytic and calyx survival experiments. We assume that the published values of P₃ and P₄ for pear would be the same as those estimated for apple, although in absolute terms, the potential number of pear fruit exported would be considerably less than apple (fresh pear production in the Pacific Northwest is one eighth that of apple). P₅, the probability that *E. amylovora* is transferred to a new host and infection occurs is largely dependent on how well this pathogen could increase its population size on mature discarded fruit to enable its transfer to a receptive host, most likely by visiting insects (29,37). We found that epiphytic survival of *E. amylovora* through a postharvest chilling period is expected to be an exceptionally rare event given the unrealistically high population size required for persistence. Moreover, after storage (and required fruit chilling), our data also indicated that mature pear fruit were an unsuitable substrate for continued reproduction of this pathogen. We conclude that the potential for successful establishment of *E. amylovora* into disease-free environments via the importation of fresh winter pear fruit is no greater than that predicted for apple; with both crops, the risk is extremely low.

ACKNOWLEDGMENTS

Research support was provided by the Northwest Horticultural Council, the Technical Assistance for Specialty Crops program of the USDA Foreign Agricultural Service, Fresh Pear Committee, Washington Tree Fruit Research Commission, Oregon State University Agricultural Experiment Station, and the USDA-Agricultural Research Service. Avirulent *E. amylovora* strain Ea153HrpL⁻ was field released under USDA APHIS permit 03-279-01r. We thank M. Willett and R. Roberts for critical suggestions to the manuscript.

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