Duplex Real-Time Polymerase Chain Reaction Reveals Competition Between *Erwinia amylovora* and *E. pyrifoliae* on Pear Blossoms

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ABSTRACT

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Erwinia amylovora and *E. pyrifoliae* are the causative agents of fire blight and Asian pear blight, respectively. The pathogens are closely related, with overlapping host ranges. Data are unavailable on the current distribution of *E. pyrifoliae* and on the interaction between the two species when they are present together on the same host. In this study, a duplex real-time polymerase chain reaction (PCR) protocol was developed to monitor the population dynamics of *E. amylovora* and *E. pyrifoliae* on the surface of Bartlett pear blossoms. Bacterial cells washed from blossoms were used directly as the PCR template without DNA

extraction. Primers and a probe based on the *E. amylovora* levansucrase gene detected all *E. amylovora* strains. All *E. pyrifoliae* strains, including the Japanese *Erwinia* strains previously described as *E. amylovora*, were detected with a primer and probe combination based on the *E. pyrifoliae* *hrpW* gene. Disease development and severity were not significantly different in blossoms inoculated with individual *Erwinia* species or with a mixture of the two species. However, *E. amylovora* grew to greater population sizes than did *E. pyrifoliae* in both single species inoculations and in mixtures, suggesting that *E. amylovora* has a greater competitive fitness on Bartlett pear blossoms than *E. pyrifoliae*.

Additional keywords: bioassay, microbial population dynamics, multiplex detection, quantitative PCR, virulence.

Erwinia amylovora is well known as the causative agent of fire blight, a necrotic disease affecting species in the family Rosaceae. Fire blight was first described on apple trees in 1780 in the north-eastern United States. The disease has since spread throughout much of North America, to New Zealand, England, most of Europe, and the Middle East (6). *E. amylovora* has a significant economic impact on commercial apple and pear crops. In the United States alone, fire blight is estimated to cost about \$100 million annually in crop loss and management costs (31). The annual disease cycle begins when *E. amylovora* is present in the orchard, usually in the margins of overwintering cankers from previous year's infections, and colonizes open blossoms in the spring (41). Under favorable temperature and moisture conditions, the *E. amylovora* population will grow and initiate new infections via natural openings in the blossom. Subsequent intercellular and intravascular growth of the pathogen leads to necrosis which can spread rapidly throughout the tree (41). Chemical and biological control methods are available to prevent initial infections, but once a tree is infected, the spread of the bacteria can only be stopped by cutting out infected tissue.

E. pyrifoliae Kim et al. (17), was isolated in the 1990s from Asian pear trees in South Korea that showed symptoms very similar to fire blight. The isolated organism had several morphological and biochemical characteristics in common with *E. amylovora* (34), and its 16S rRNA gene shared 99% homology with the *E. amylovora* 16S rRNA gene (17). However, DNA:DNA hybridization data and the sequences of the 16S-23S intergenic transcribed spacer regions revealed the *E. pyrifoliae* isolates to be a

single species, distinct from *E. amylovora* (17). Additional molecular evidence for the distinction between *E. pyrifoliae* and *E. amylovora* has also been presented (14,27,28). To date, *E. pyrifoliae* has only been isolated from Asian pear trees and has not been reported since the localized outbreaks in the 1990s (18).

Extensive work has been done to genetically characterize *E. pyrifoliae*, but except for some small-scale pathogenicity tests on apple and pear seedlings and immature pear fruit, the in vivo behavior of this organism has not been studied. Despite the genetic differences on which their classification is based, and differences in certain pathogenicity factors (20), both *E. amylovora* and *E. pyrifoliae* are pathogenic on both Asian and European pear varieties (17,19). An understanding of how the two species interact when present together on an open blossom may shed light on their current distribution and on the potential for future spread of each organism.

Here, we describe the development of a duplex real-time polymerase chain reaction (PCR) protocol that permits simultaneous quantification of *E. pyrifoliae* and *E. amylovora* directly from plant samples. This approach is rapid and quantitative, and thus has advantages over previously described methods for differentiating *E. amylovora* and *E. pyrifoliae* using conventional PCR and gel electrophoresis (19). We then used this system to study the population dynamics of these two pathogens when present individually or together on the surface of Bartlett pear blossoms. In particular, we wanted to evaluate the potential for this tool to facilitate in situ studies of ecological relationships such as inter-species competition between bacteria in their natural environment.

MATERIALS AND METHODS

Bacterial strains and media. Strains of *E. amylovora*, *E. pyrifoliae*, Japanese *Erwinia* sp., *Pantoea agglomerans* (formerly

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Erwinia herbicola), *E. carotovora*, and *Escherichia coli* were grown on Difco nutrient agar. *Pseudomonas* strains were grown on NBY agar (37). All strains were incubated at 28°C, except for *E. coli*, which was incubated at 37°C. Bacteria used in these experiments are described in Table 1. All listed strains were used for specificity testing of real-time PCR probes and primers. *E. amylovora* Ea6-4 and *E. pyrifoliae* Ep1/96 were used for competition assays.

Real-time PCR. *E. amylovora*- and *E. pyrifoliae*-specific primers and TaqMan probes were designed based on the levansucrase gene (GenBank X75079) and the *hrpW* gene (GenBank AY237642), respectively, by using web-based software provided by Integrated DNA Technologies (Coralville, IA). Candidate oligonucleotide designs were analyzed to ensure compatibility in a duplex reaction. Probe and primer sequences are listed in Table

2. All primers and probes were synthesized by Integrated DNA Technologies.

Reactions were run in 25- μ l volumes using 1 $\times$ ThermoPol PCR buffer (New England Biolabs, Ipswich, MA), 0.2 mM each dTTP, dCTP, dATP, and dGTP, 2 mM MgCl₂, 0.1 mM each probe, 0.2 mM each primer, and 1.5 U of *Taq* polymerase (New England Biolabs). Two microliters of the processed sample was used as the template in each reaction. Reactions were run in a Stratagene Mx4000 Multiplex Quantitative PCR system (La Jolla, CA) under the following conditions: 95°C for 5 min; 40 cycles of 95°C for 10 s and 60°C for 16 s, with two endpoint fluorescence readings during each amplification segment. Negative suspension buffer, and master mix-only controls were also included in each reaction cycle. Specificity tests were conducted in duplex reactions using the bacteria listed in Table 1, singly and in mixtures.

Optimization of real-time PCR for quantitative analysis.

Singleplex and duplex standard curves were constructed to allow quantification of each target organism. Fresh overnight plate cultures of *E. amylovora* Ea6-4 and *E. pyrifoliae* Ep1/96 were aseptically scraped from the plate surface and suspended in 0.01 M sodium phosphate buffer, pH 6.8, to a concentration of at least 1 $\times$ 10⁹ CFU/ml (OD₆₀₀ = 0.6) using a Beckman DU 640 spectrophotometer. The concentration of each suspension was calibrated by plating 100 μ l of serial dilutions in phosphate buffer and counting single colonies after 2 days. Templates for real-time PCR standard curves were prepared by serially diluting cell suspensions in clean blossom wash, either individually or as a mixture of both species. Standard curve construction was repeated using new bacterial cultures. PCRs were run in triplicate.

Competition assays. Bartlett pear shoots bearing dormant buds were harvested in late winter. Shoots were forced to bloom by placing them in water at 23°C in a well-lit room. Newly opened blossoms with yellow, undehiscent anthers were harvested by hand and individually placed into scintillation vials such that the peduncle extended through a hole drilled in the lid and into the sterile tap water contained in the vial.

Suspensions of *E. amylovora* and *E. pyrifoliae* were prepared by aseptically scraping cells from fresh overnight cultures into 0.01 M sodium phosphate buffer, pH 6.8. Suspensions were adjusted to 1 $\times$ 10⁹ CFU/ml, stored on ice, and further diluted in sodium phosphate buffer as needed immediately prior to application.

Ten microliters of a bacterial suspension was inoculated directly onto the hypanthium. Treatments were as follows: 0.01 M sodium phosphate buffer, pH 6.8 (negative control); 1 $\times$ 10⁶ CFU of either *E. amylovora* or *E. pyrifoliae* per blossom (positive control for virulence); 1 $\times$ 10⁴ CFU of *E. amylovora* or *E. pyrifoliae* per blossom or a 1:1 mixture of both for a total bacterial count of 1 $\times$ 10⁴ CFU/blossom; and 1 $\times$ 10² CFU of *E. amylovora* or *E. pyrifoliae* per blossom or a 1:1 mixture of both for a total bacterial count of 1 $\times$ 10² CFU/blossom. Each treatment was applied to 20 blossoms. Following inoculation, the blossoms were loosely sealed in large plastic bins containing about 500 ml of water to maintain a high relative humidity and incubated at 23°C.

TABLE 1. Bacterial strains used in this study

Isolate	Host	Origin	Source or reference
<i>E. amylovora</i>			
Ea6-4	<i>Pyrus</i> sp.	Canada	(10)
Ea1/79	<i>Cotoneaster</i> sp.	Germany	(8)
Ea321	<i>Crataegus oxyacantha</i>	France	CFBP1367 ^u
IH3-1	<i>Raphiolepis indica</i>	USA	(12)
IL5	<i>Rubus</i> sp.	USA	(29)
CA11	<i>Malus</i> sp.	USA	(7)
UTR12 ^v	<i>Malus</i> sp.	USA	(39)
Leb B66 ^v	<i>Malus</i> sp.	Lebanon	(35)
IVIA1614-2a ^v	<i>Crataegus</i> sp.	Spain	(24)
<i>E. pyrifoliae</i>			
Ep1/96	<i>Pyrus pyrifolia</i>	Korea	(17)
Ep2/97	<i>Pyrus pyrifolia</i>	Korea	(15)
Japanese <i>Erwinia</i> strains			
Ejp556	<i>Pyrus pyrifolia</i>	Japan	(18)
Ejp557	<i>Pyrus pyrifolia</i>	Japan	(18)
Ejp617	<i>Pyrus pyrifolia</i>	Japan	(18)
<i>P. agglomerans</i>			
Eh21-5	<i>Pyrus</i> sp.	Canada	A. M. Svircev ^w
C9-1	<i>Malus</i> sp.	USA	(13) ^x
E325	<i>Malus</i> sp.	USA	(33) ^x
<i>E. carotovora</i> Ecc26			D. Cupples ^y
<i>E. coli</i> DH-5 α			A. J. Castle ^z
<i>P. fluorescens</i> A506			(22) ^x
<i>P. syringae</i> pv. <i>populans</i> 4404			A. M. Svircev ^y

^u Collection Française de Bactéries Phytopathogènes.

^v Strains do not contain the pEA29 plasmid.

^w Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, Vineland Station, Canada.

^x *Pantoea agglomerans* C9-1 and *Pseudomonas fluorescens* A506 are the active ingredients in commercial bacterial antagonists produced under the BlightBan7 label by Nufarm Americas Inc. (Burr Ridge, IL) for the control of fire blight. *P. agglomerans* E325 is the active ingredient in Bloomtime FD, a commercial bacterial antagonist produced by Northwest Agricultural Products (Pasco, WA) for similar use.

^y Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, London, Canada.

^z Department of Biological Sciences, Brock University, St. Catharines, Canada.

TABLE 2. Sequences, product sizes, and targets of the forward primer (F), the reverse primer (R), and the TaqMan7 probe (P) used for duplex detection and quantification of *Erwinia amylovora* (Ea) and *E. pyrifoliae* (Ep)

Oligonucleotide	Sequence (5' to 3')	Position ^y	Product size
Ea-lscF	CGCTAACAGCAGATCGCA	345–362	105 bp
Ea-lscR	AAATACGCGCAGACCAT	449–432	
Ea-lscP	(CY5)CTGATAATCCGCAATTCAGGATG(IAbRQ) ^z	366–389	
Ep-hrpwF	CGCTAACCCGACTGTGCT	756–773	77 bp
Ep-hrpwR	TGAAGTTTGCCCTTTGC	832–815	
Ep-hrpwP	(FAM)ATGACACCATCATCGTAAAGGCGG(BHQ-1) ^z	776–799	

^y Binding positions are based on GenBank files X75079 (*lsc*) and AY237642 (*hrpW*).

^z IAbRQ and BHQ-1 are quencher molecules available from the manufacturer.

Ten blossoms from each treatment were assessed after 3 days, and another 10 blossoms after 5 days. Each blossom was removed from the scintillation vial using sterile forceps. Disease symptoms were scored using a severity index (0, no necrosis; 1, necrosis on the stigma and hypanthium; 2, necrosis visible on the underside of the sepals, but not extending into the ovary; 3, necrosis extends into the ovary, no farther than the widest point; 4, necrosis extends to the base of the ovary; 5, necrosis extends into the peduncle). Every second blossom was further processed in order to quantify surface bacterial populations (described below). The experiment was conducted three times using suspensions prepared from independent bacterial cultures.

Assessment of bacterial numbers on blossoms. After disease severity was assessed, the petals and peduncle were removed and the remaining tissue (ovary, hypanthium, stamens, stigma) was placed in a sterile 1.5-ml microcentrifuge tube. One milliliter of Direct Plant Extraction Buffer (Agdia Inc., Elkhart, IN) was added to each tube. Tubes were mixed by vortexing briefly, placed in a water bath sonicator such that only the cap was not immersed, and sonicated for 3 min to dislodge bacterial cells. Plant tissue was removed with a sterile toothpick and discarded. Bacterial cells were concentrated 10-fold by centrifuging the blossom wash at $13,000 \times g$ for 5 min, decanting the supernatant, and resuspending the pellet in 100 μ l of DiPEB. This processed sample was used directly as the template for real-time PCR. This procedure was also conducted using uninoculated blossoms, and the resulting clean blossom wash was used as a negative control.

The DiPEB buffer used in this study has been licensed by Agriculture and Agri-Food Canada to Agdia Incorporated (Elkhart, IN). Until such time as the buffer becomes commercially available, small quantities can be made available to academic researchers for the purposes of repeating these results.

Statistical analysis. Regression analysis of standard curve data was conducted in SigmaPlot, version 8 (SPSS, Inc., Chicago, IL). All other data were analyzed using SAS (Statistical Analysis Systems 8.2; SAS Institute, Cary, NC). Disease severity index ratings from the competition assay were converted to a percent scale based on the actual surface area of the hypanthium and ovary that corresponds to the extent of visible necrosis that was associated with each disease rating (0 = 0%; 1 = 20%; 2 = 48%; 3 = 61%; 4 = 82%; and 5 = 100%). Population data were logarithmically transformed (base 10) in order to produce normally distributed data suitable for further analysis. If a given pathogen species was not detected on a blossom, the population size was assigned a value of 2 CFU in order to avoid undefined log functions or ratios of logs. Disease severity data and transformed population data were analyzed with the general linear model (PROC GLM) using the Tukey-Kramer mean separation test for multiple comparisons.

To assess whether competition occurred between *E. amylovora* and *E. pyrifoliae*, their growth on independent blossoms was compared with their growth on coinoculated blossoms. Each blossom inoculated with only *E. amylovora* was paired randomly with a blossom from the same replicate that had been inoculated with only *E. pyrifoliae*. The "relative growth performance" of each species was expressed as $\log(\text{species 1 alone})/[\log(\text{species 1 alone}) + \log(\text{species 2 alone})]$, or $\log(\text{species 1 in mixture})/[\log(\text{species 1 in mixture}) + \log(\text{species 2 in mixture})]$.

For each species, relative growth performance alone was compared with relative growth performance in a mixture using a *t* test with a Satterthwaite adjustment for unequal variances.

There was no significant difference between replicated experiments ($P > 0.05$); therefore, data from multiple replications were pooled. When data were not normally distributed ($P < 0.05$ in a Kolmogorov-Smirnov test), the results of parametric tests and nonparametric tests were compared. In no case was the departure from normality sufficient to invalidate the parametric tests. All mean values are given as mean $\pm 95\%$ confidence limits.

Specificity and sensitivity of duplex real-time PCR. The specificity of each set of probes and primers was tested using all bacterial strains and species listed in Table 1. The levansucrase gene (*lsc*) was selected as the amplification target for *E. amylovora* because levansucrase activity, which mediates the extracellular breakdown of sucrose into glucose and levan, has been observed in *E. amylovora* but not in *E. pyrifoliae* (9,11,18). Specific quantification of *E. pyrifoliae* was achieved with primers and a probe that amplify and detect a 77-bp region of the *hrpW* gene. This gene is part of the *hrp* cluster, which is present on the chromosome of both *E. amylovora* and *E. pyrifoliae*, but which shows sufficient interspecies variation to permit reliable differentiation of *E. pyrifoliae* from *E. amylovora*. Using the Ea-lsc primers and probe, all *E. amylovora* strains were positive regardless of the presence or absence of the pEA29 plasmid. No cross reactions were observed with *E. pyrifoliae* strains or any other species. The *hrpW* probe and primers detected all *E. pyrifoliae* strains as well as the Japanese *Erwinia* strains. This combination did not detect any *E. amylovora* strain or any other species. No change in cycle threshold (C_T) values was observed in the presence of non-target species.

Fluorescence signals were regularly obtained from standardized samples containing as few as 20 CFU of either *E. amylovora* or *E. pyrifoliae* in a 25- μ l reaction. This detection threshold occurred at an approximate C_T value of 33. Reaction efficiency, as calculated by the Stratagene Mx4000 software, was greater than 95%.

Duplex standard curves for *E. amylovora* and *E. pyrifoliae* are shown in Figure 1. Concentrations higher than 5×10^6 CFU per 25- μ l reaction were not tested. For *E. amylovora*, the relationship between C_T and initial quantity was linear across a 4.5-log range. For *E. pyrifoliae*, the relationship between C_T and initial quantity was linear across a 5-log range. While detection, indicated by a fluorescence signal by 33 cycles, was reliable and for initial pathogen quantities above 20 CFU per 25- μ l reaction, quantification of both pathogens was highly repeatable only for initial quantities greater than approximately 100 CFU per reaction. The standard curves for both species are essentially identical; the slight differences between slope and intercept values were within the range of variation between replicate real-time PCR runs.

Standard curves were prepared from cells diluted in buffer that had been sonicated with uninoculated blossoms according to the sampling procedure used for the competition assays. The curves therefore correspond directly to initial cell numbers, and account for the presence of any PCR inhibitors that may be present in the

Fig. 1. Duplex standard curves for the quantitative detection of *Erwinia amylovora* and *E. pyrifoliae*. Known amounts of each pathogen were diluted in clean blossom wash. Quantification of *E. amylovora* (solid line) is given by $y = 38.4 - 3.48\log(x)$, $R^2 = 0.975$. Quantification of *E. pyrifoliae* (dotted line) is given by $y = 37.0 - 3.48\log(x)$, $R^2 = 0.973$.

experimental samples. Since blossom infection occurs through the stigma and hypanthium, petals and peduncles were removed from the blossom, and bacteria were collected from the surfaces of the remaining structures. Removal of petals and peduncles improved the sensitivity of bacterial quantification (data not shown), presumably because it reduced the total volume of plant tissue, and thus the concentration of plant-derived PCR inhibitors. DNA extraction was not performed as part of sample preparation. The components of the wash buffer, along with the 5 min initial denaturation cycle at 95°C, were sufficient to make the bacterial target DNA accessible for amplification.

There is no substantial difference between the standard curves produced by singleplex and duplex reactions (data not shown). For a given target species, duplex detection of a given cell concentration occurred about 0.5 C_T later than if the same sample was tested in a singleplex reaction. However, this difference is within the range of variation between replicate runs. Sensitivity did not differ between duplex and singleplex reactions.

Virulence of *E. amylovora* and *E. pyrifoliae*. Disease severity in blossoms treated with either *E. amylovora* or *E. pyrifoliae*

TABLE 3. The effect of initial inoculum size on disease severity in pear blossoms

Time frame	Initial inoculum size (CFU/blossom)	Percent disease (mean ±95% CL) ^z
Across entire experiment	0	12.5 ± 8.1 a
	1 × 10 ²	38.8 ± 8.1 b
	1 × 10 ⁴	48.2 ± 8.2 b
	1 × 10 ⁶	64.4 ± 8.2 c
3 days elapsed since inoculation	0	5.0 ± 9.8 a
	1 × 10 ²	13.4 ± 9.8 ab
	1 × 10 ⁴	23.3 ± 9.9 b
	1 × 10 ⁶	46.0 ± 9.8 c
5 days elapsed since inoculation	0	20.0 ± 13.1 a
	1 × 10 ²	64.2 ± 13.1 b
	1 × 10 ⁴	73.1 ± 13.3 b
	1 × 10 ⁶	82.8 ± 13.3 b

^z CL, confidence limit. Disease severity is expressed as percent surface area that is visibly necrotic. Values are the means of two experiments, each with 10 blossoms per treatment. Within each time frame, means with the same letter are not significantly different (*P* < 0.05).

alone was modeled in terms of (i) pathogen identity, (ii) initial inoculum size, and (iii) time elapsed since inoculation. Across the experiment as a whole, initial inoculum size and time elapsed since inoculation were significant predictors of disease severity (*P* < 0.0001 for both). There was also a significant interaction between these two factors (*P* = 0.0075). Specifically, initial inoculum size showed a significant (*P* < 0.0001), but qualitatively different, effect on disease severity at each of the two sampling times. Table 3 shows the results of a virulence assessment of *E. amylovora* and *E. pyrifoliae* on Bartlett pear blossoms. Blossoms showed no disease symptoms at the time of inoculation. In general, mean disease severity increased with increasing inoculum. Three days after inoculation, blossoms inoculated with 1 × 10⁶ CFU of either pathogen showed significantly more severe symptoms than blossoms that had received a smaller inoculum (*P* < 0.01). The differences between blossoms treated with 1 × 10⁴ CFU, 1 × 10² CFU, and 0 CFU were less clear, as the difference between the 0 and 1 × 10⁴ treatments was just significant at the 5% level (*P* = 0.0496). By day 5, however, the only significant difference in disease severity was between inoculated and uninoculated blossoms (*P* < 0.0001).

There was no significant effect of pathogen identity on disease severity across the experiment as a whole (*P* = 0.5182), or within any level of initial inoculum size at either sampling time (all *P* > 0.4100).

Effect of initial pathogen ratio on disease severity. Figure 2 shows the overall effect of initial pathogen ratio on disease development, both 3 and 5 days after inoculation. Regardless of the time of observation, within each inoculum level, there was no significant difference between the severity of symptoms in blossoms treated with a 1:1 mixture of both pathogens and the severity of symptoms in blossoms treated with the same total amount of either species alone.

Relative population sizes of *E. amylovora* and *E. pyrifoliae*. Bartlett pear blossoms were inoculated with *E. amylovora*, *E. pyrifoliae*, or a 1:1 mixture of the two. Real-time PCR was used to determine the population sizes of both species after 3 days and again after 5 days. The logarithmically transformed population sizes were modeled in terms of (i) pathogen identity, (ii) whether the blossom had been inoculated with a single species or co-inoculated with both, (iii) initial inoculum size, and (iv) time elapsed since inoculation. There was no difference in population sizes between days 3 and 5 (*P* = 0.5491) or among different levels of initial inoculum (*P* = 0.3414). Pathogen identity was sig-

Fig. 2. Effect of initial pathogen ratio on disease severity in blossoms inoculated with *Erwinia amylovora* only, *E. pyrifoliae* only, or a 1:1 mixture of both. Percent disease is shown as mean + 95% confidence limit for **A**, 3 days after inoculation and **B**, 5 days after inoculation. On each day, means with the same letter are not significantly different (*P* < 0.05).

nificant ($P < 0.0001$), as was mixture ($P = 0.0003$), and there was a significant interaction between these two factors ($P = 0.0021$).

Figure 3A shows the mean population sizes of *E. amylovora* and *E. pyrifoliae* on the pear blossoms, when present alone or together. In blossoms inoculated with only one of the two pathogen species, *E. amylovora* populations grew to a greater size than did *E. pyrifoliae* populations ($P = 0.0002$). *E. amylovora* populations were not significantly smaller on blossoms inoculated with both species than they were when *E. pyrifoliae* was absent ($P = 0.6688$). However, *E. pyrifoliae* population sizes were significantly smaller in the presence of *E. amylovora* than when they grew alone on the blossoms. This difference is more clearly demonstrated by the relative growth performance index (Fig. 3B), where the growth of a species in either situation was expressed in relation to the total growth of both species. The relative contribution of *E. amylovora* to total bacterial growth was significantly greater ($P = 0.0001$), and the relative contribution of *E. pyrifoliae* was significantly less ($P = 0.0001$), on blossoms inoculated with both species than on blossoms inoculated with a single species.

DISCUSSION

There is currently a need for simple, sensitive methods of studying the in situ growth and interactions of microbial populations. *E. amylovora*, the causative agent of fire blight, is the focus of intensive research aimed at understanding and inhibiting its epiphytic and endophytic growth phases. *E. pyrifoliae* is very closely related to *E. amylovora* and causes a similar disease on Asian pear trees. In this study, a duplex real-time PCR method was developed that allows simultaneous identification and quantification of *E. amylovora* and *E. pyrifoliae* directly from blossom surfaces, independent of their cultivability. The application of this technique to an in situ competition assay then revealed that *E. amylovora* had a greater competitive fitness than *E. pyrifoliae* on Bartlett pear blossoms. This is the first time that the interactions of these two close relatives have been studied.

A supplemental outcome of this study was the production of new evidence about the relationships among *E. amylovora*, *E. pyrifoliae*, and *Erwinia* strains from Japan. The latter were isolated from Asian pear trees on Hokkaido. Initial studies suggested that these were very similar to *E. amylovora* (1), which agreed with a 1981 Japanese report. However, the subsequent identification of *E. pyrifoliae* as a distinct species from *E. amylovora*, despite many shared morphological, metabolic, and genetic simi-

larities, has prompted a reassessment of that conclusion. The detection of the Japanese *Erwinia* isolates with the primers and probe designed for *E. pyrifoliae* in this study is further evidence that these isolates should not be considered variants of *E. amylovora*, but likely belong to *E. pyrifoliae*. This is supported by data from other studies, which indicate that these isolates are not identical to *E. amylovora*, and, in fact, resemble *E. pyrifoliae* more closely than *E. amylovora* (14,15,18,26).

Most previous PCR methods for *E. amylovora* detection have been based on the pEA29 plasmid (3,23,30,36); of the others (2,25,38), only one was truly species-specific and targets a known chromosomal region (2). A chromosomal target was chosen for this study because of the emergence of *E. amylovora* strains in nature that lack pEA29 (24). For both *E. amylovora* and *E. pyrifoliae* detection, fluorescence signals were regularly obtained from standards containing as few as 20 CFU per 25- μ l reaction ($C_T = 33$) and quantification was very reliable for cell concentrations as low as 100 CFU per 25- μ l reaction ($C_T = 30$). For the purposes of qualitative detection of these species on blossoms, samples yielding a C_T value of 30 or less would be considered positive for the respective pathogen, while samples yielding a C_T value between 30 and 35 may warrant retesting to confirm the presence of the target. During the population monitoring experiments described in this study, C_T values less than 26 were very rarely obtained. Despite the fact that the primers and probes described here target single-copy chromosomal genes, the sensitivity of the method developed in this study is comparable to that achieved in a pEA29-based real-time PCR assay for *E. amylovora* alone (36).

The blossom bioassay used for these experiments was based on the fact that primary infection by *E. amylovora* occurs through the reproductive organs (41). In blossoms treated with a single pathogen species, there was no apparent difference between the virulence of *E. amylovora* and *E. pyrifoliae* on Bartlett pear blossoms, despite the absence of levan production by *E. pyrifoliae* (18), and the association between levan-deficiency and reduced virulence in *E. amylovora* (9). No more than 100 cells of either pathogen were needed to cause fire blight symptoms in a single blossom, and there was no difference in the progression of symptoms resulting from inoculation with a given amount of either pathogen. However, the mean population size of *E. pyrifoliae* on these blossom surfaces was significantly smaller than the mean *E. amylovora* population size, which suggests that *E. pyrifoliae* does not grow as well as *E. amylovora* on Bartlett pear blossoms. This difference was not sufficient to hinder the infection process, which may

Fig. 3. **A**, Population sizes of *Erwinia amylovora* and *E. pyrifoliae* on pear blossoms inoculated with each species alone or with a 1:1 mixture of both species. Populations are given as mean + 95% confidence limits. Means with the same letter are not significantly different ($P < 0.05$). **B**, Relative growth performance of *E. amylovora* and *E. pyrifoliae* when growing on Bartlett pear blossoms alone or in a mixture. Values are mean + 95% confidence limits. Data from both sample times and all initial inoculum levels (excluding uninoculated controls) were pooled since neither factor significantly affected population size.

indicate that infection following inoculation of the hypanthium is associated with a threshold population size smaller than 4.7×10^4 CFU/blossom, which was the mean population of *E. pyrifoliae* in the absence of *E. amylovora*. Population sizes larger than 1×10^5 CFU/blossoms have previously been associated with *E. amylovora* infection of pear blossoms (16,40). Those studies involved spray-inoculated blossoms where pathogen growth on the stigma would also be relevant to disease development, since bacteria colonizing the stigma tend to be washed down into the hypanthium prior to colonization. The slightly lower threshold population suggested by our study may be a real difference due to inoculation method, or may simply be a consequence of the different analytical methods used in each study.

Some necrosis was unexpectedly observed in the buffer-treated blossoms (Table 3, Fig. 2). Most blossoms showed no necrosis whatsoever, but four blossoms were completely necrotic by day 5, probably due to natural infestation by a necrotizing pathogen. Not all were tested using the real-time PCR assay, but surface populations of *E. amylovora* were not detected from those that were. This, combined with the random pairing of blossoms for statistical analysis, makes it very unlikely that the larger *E. amylovora* populations are the result of the pathogen being present prior to experimental inoculations.

In blossoms treated with a 1:1 mixture of both *E. amylovora* and *E. pyrifoliae*, disease severity was not significantly different than in blossoms inoculated with either species alone. Clearly the presence of both species together neither amplifies nor inhibits their pathogenicity. What is not clear from these results is whether the two species are contributing equally to the production of disease, or whether one species out-competes the other to become dominant. Real-time PCR data revealed that the growth of *E. amylovora* on Bartlett pear blossoms was unaffected by the presence of an equal number of *E. pyrifoliae* cells. In contrast, the growth of *E. pyrifoliae* was negatively affected by *E. amylovora* and the population size of *E. pyrifoliae* relative to *E. amylovora* was significantly smaller when both species were present together than when they grew alone. This observation suggests that *E. amylovora* has greater competitive fitness on Bartlett pear blossoms. It is not known whether this simply represents differential success in the utilization of available nutrients and other resources, or whether any specific antagonism is involved. Since sucrose is abundant in the nectaries of host blossoms (4), it has been suggested that the extracellular breakdown of sucrose by levansucrase may contribute to nutrient utilization or to osmotic adaptation by *E. amylovora* (11). In the case of nutrient utilization, it is intracellular sucrose metabolism by the *scr* regulon, and not extracellular levansucrase activity, that is required for pathogenicity (5), but this does not eliminate the possibility that levansucrase activity might play a role in the differential success of co-occurring *E. amylovora* and *E. pyrifoliae* populations.

It would be interesting to test whether the relative fitness of these pathogens is the same on blossoms of *Pyrus pyrifolia*, or whether *E. pyrifoliae* is better adapted to Asian pear blossoms than *E. amylovora*. *E. amylovora* and *E. pyrifoliae* are not currently known to coexist in nature, but they are very closely related and have partially overlapping host ranges. It is generally accepted that *E. amylovora* is native to North America (6), but little is known about the evolutionary relationship between *E. amylovora* and *E. pyrifoliae* beyond the substantial genomic similarity. An understanding of how they behave when they are present together on different host species could shed light on their current distribution or potential distribution in the future. In light of the rapid spread of modern plant pathogens, it is certainly possible that these species could be introduced to the same regions in the future. Their relative fitness will influence the outcome of such introductions. It may also be informative to look for differences in this competitive interaction in blossoms following stigma inoculation versus hypanthium inoculation.

One of the major advantages of the primers and probes developed in this study is that they are compatible in a duplex reaction, allowing simultaneous, independent quantification of both species. This method is transferable to a field situation and has been successfully used to monitor *E. amylovora* populations in experimental orchards as part of a fire blight biological control study (21). The omission of a DNA extraction step makes this technique attractive in terms of time- and cost-savings, especially for field studies, which tend to rapidly generate a large number of samples.

The potential for simultaneous quantification of several target species is not limited to *E. amylovora* and *E. pyrifoliae*. The methods described here are also applicable to simultaneous monitoring of a pathogen and biocontrol agent (21). The primers and probes developed in this study are species-specific. Strain-specific real-time PCR has been used to monitor the population of an applied fire blight biocontrol agent, *Pseudomonas fluorescens* EPS62e (32). The combination of species-specific and strain-specific primers and probes could be used to study the relative contribution of an applied biocontrol agent to the total population of that species. Ultimately, since all primers and probes must function at the same reaction temperature, the applicability of multiplex real-time PCR to phytopathology, in general, and biological control, in particular, will depend on the availability of sufficient sequence data for probe and primer design.

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