Detection of Viable *Xanthomonas fragariae* Cells in Strawberry Using Propidium Monoazide and Long-Amplicon Quantitative PCR

Hehe Wang¹ and William W. Turechek^{2,†}

¹ Department of Agricultural and Environmental Sciences, Clemson University, Blackville, SC

² U.S. Horticultural Research Laboratory, U.S. Department of Agriculture—Agricultural Research Service, Fort Pierce, FL

Abstract

Xanthomonas fragariae causes angular leaf spot in strawberry. The pathogen's association with its host tissue is thought to be a condition for its survival. Consequently, transmission of the pathogen to field production sites occurs almost exclusively through the movement of contaminated planting stock. The aim of this study was to develop a propidium monoazide (PMA)-quantitative PCR (qPCR) protocol for specific detection of viable *X. fragariae* cells. The qPCR procedure was developed for two different primer pairs: one producing a long amplicon (863 bp) and the other a short amplicon (61 bp). Both pairs were tested on mixtures of viable and heat-killed bacteria cells, bacteria-spiked strawberry petiole samples, and petioles collected from symptomatic, inoculated plants. The results showed that long-amplicon PMA-qPCR enabled specific and sensitive detection of *X. fragariae* with a detection limit of 10^3 CFU/ml,

and it significantly improved PMA efficiency in differentiating viable from dead bacterial cells relative to short-amplicon PMA-qPCR. Based on the delta threshold cycle (C_t) values (i.e., the difference in C_t values between PMA-treated and nontreated samples), the long-amplicon PMA-qPCR was able to suppress the detection of dead *X. fragariae* cells 1.9- to 3.1-fold across all petiole samples tested. The quantification results from PMA-qPCR for mixtures of viable and dead cells were highly correlated with the predicted bacterial concentrations in a linear relationship ($R^2 = 0.981$). This assay can be useful for identifying inoculum sources in the strawberry production cycle, which may lead to improved disease management strategies.

Keywords: fruit, pathogen detection, prokaryotes, small fruits

Angular leaf spot (ALS) of strawberry, caused by the bacterium *Xanthomonas fragariae*, is an important disease in both nursery and field production of strawberry (López et al. 2006; Maas et al. 1995; Smith et al. 1992). Symptoms of ALS are most evident on leaves and calyxes as discrete angular lesions that grow and coalesce with age, leading to discolored tissue that eventually shrivels and dies. On the calyx, this symptom is referred to as “black cap” and can reduce fruit marketability. The pathogen is transmitted to new plantings mainly through infected or contaminated planting stock (Wang et al. 2018). Secondary spread in the field occurs largely through splashing water and with production practices that require plant handling, such as de-blossoming (Gigot et al. 2017). Currently, there are no strawberry cultivars that are immune to disease and no effective chemical treatments are registered for commercial use on strawberry.

ALS affects the commercial value of the crop in several ways. For fruit producers, unsightly infections of the calyx (black cap) reduce marketable fruit yield when infection is severe. In nursery production, where ALS typically has its most significant impact, losses occur when plant batches are rejected by inspectors as a result of violations of quarantine regulations. Phytosanitary measures enforced

by the European and Mediterranean Plant Protection Organization dictate that planting material be derived from certified, *X. fragariae*-free mother plants, and that production sites be documented free from ALS for the past five growing seasons (López et al. 2006; Smith et al. 1992). The California strawberry nursery industry estimates annual production losses attributable to ALS in the range of 5 to 8%, valued between approximately \$700,000 and \$1,100,000, with individual nurseries suffering losses between 2 and 30%.

ALS has been a perennial threat to both nursery and fruit producers for the past 2 decades. The bacterium seems firmly embedded in nursery production, so disease expression in the field and the resulting severity of an epidemic is largely tied to the incidence of infected nursery stock and the favorability of environmental conditions. However, because symptoms on nursery stock are often cryptic, our ability to diagnose infected stock to predict the risk of an epidemic is reliant on good diagnostic tools. The current diagnostic tools for *X. fragariae* are mainly DNA based, such as PCR, quantitative PCR (qPCR), and loop-mediated isothermal amplification (Gétaz et al. 2017; Moltmann and Zimmermann 2005; Ogenorth et al. 1996; Pooler et al. 1996; Roberts et al. 1996; Turechek and Peres 2009; Vandroemme et al. 2008; Wang and Turechek 2016; Weller et al. 2007; Zimmermann et al. 2004). Although many of these tools are very sensitive for detection of *X. fragariae* DNA, they lack the capability to differentiate whether the DNA originated from viable or dead cells; the DNA from dead bacterial cells can be amplified in PCR reactions. Traditional dilution plating can be used to selectively cultivate viable cells, but this technique is not well suited for early detection of *X. fragariae* from asymptomatic strawberry tissue because these slow-growing bacteria are easily overgrown by other bacterial contaminants. A detection tool that allows accurate quantification of viable *X. fragariae* is needed.

In recent years, the use of DNA-intercalating dyes in PCR protocols has become a popular method for quantification of viable cells, sometimes referred to as viability PCR. Propidium monoazide (PMA) and ethidium monoazide (EMA) are two such DNA-intercalating dyes that can permeate dead cells with a compromised membrane and covalently bind to DNA upon photoactivation (Nocker et al. 2006; Nogva et al. 2003). The dye cross-linking serves to interfere and prevent DNA amplification in downstream PCR

[†]Corresponding author: W. W. Turechek; william.turechek@ars.usda.gov

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reactions. Of the two dyes, PMA has been shown to have higher specificity in selectively permeating only dead cells, resulting in a robust detection assay and thus making PMA the popular choice for viability PCR (Fittipaldi et al. 2012; Flekna et al. 2007; Nocker et al. 2006; Seinige and Krschek 2014).

PMA-PCR assays have been developed for a wide variety of food-borne pathogens and environmental microorganisms, including bacteria, protozoa, viruses, and fungi (Brescia et al. 2009; Fittipaldi et al. 2010, 2012; Vesper et al. 2008; Yang et al. 2011) and, more recently, for several plant pathogens (Al-Daoud et al. 2017; Crespo-Sempere et al. 2013; Han et al. 2018; Hu et al. 2013; Temple et al. 2013; Tian et al. 2016; Vilanova et al. 2017; Wang and Turechek 2015). PMA efficacy varies based on the species of target organism, the sensitivity and specificity of the assay, and the size of the PCR DNA target. In some cases, PMA-PCR assays with smaller amplicons (<300 bp) showed incomplete suppression of PCR signals when a large quantity of dead cells was present, resulting in a higher false-positive rate (Banihashemi et al. 2012; Fittipaldi et al. 2012; Pacholewicz et al. 2013; Schnetzinger et al. 2013; Seinige et al. 2014). In contrast, larger PCR amplicons (400 to 1,500 bp) are believed to increase the chance of PMA binding to target DNA sequences and significantly increase the efficiency of PMA-qPCR in differentiating viable and dead cells (Banihashemi et al. 2012; Contreras et al. 2011; Luo et al. 2009; Martin et al. 2013; Schnetzinger et al. 2013).

The aim of this study was to develop a PMA-qPCR assay for selective identification of viable *X. fragariae*. *X. fragariae* suspensions of viable and dead cells, as well as mixtures of the two, bacteria-spiked strawberry petioles, and inoculated strawberry plants, were used for assay development and evaluation. Primers that produce large amplicons were developed and compared with current qPCR primers to determine whether the sensitivity of PMA-qPCR could be improved with larger amplicons. The assay was tested on four different strains of *X. fragariae*.

Materials and Methods

Sample preparation for viable and dead *X. fragariae* cell suspensions. *X. fragariae* strain Xf100 was the primary strain used in developing the PMA-qPCR assay, and three additional strains (Xf128, Balm, and Xf1425) were used to evaluate the assay. Strains Xf100 and Xf1425 were collected from Florida around 1992/1993; Xf128 was collected from Quebec, Canada, in 1993; and Balm was collected by Turechek in Balm, Florida, in 2013. To prepare viable cell suspensions, *X. fragariae* isolates were grown on sucrose-peptone agar (SPA) for 3 to 7 days at room temperature, then a single colony (approximately 2 mm in size) was transferred to sucrose-peptone broth in a flask to grow overnight on an orbital table shaker (Hayward 1960). The bacterial suspensions were centrifuged at $3,000 \times g$ for 3 min, resuspended in 1× phosphate buffered saline (PBS), and adjusted to an optical density (OD_{620}) of 0.1, which is equivalent to approximately 10^8 CFU/ml (Hazel and Civerolo 1980; Wang and Turechek 2016). Samples were further diluted into desired concentrations as required per the experimental design discussed below. To confirm bacterial densities, serial dilutions were prepared on SPA, the dilution yielding 100 to 300 colonies after 4 days of growth was identified, and the number of colonies from that dilution was multiplied by the dilution factor to obtain the bacterial density of the original suspension. To prepare dead cell suspensions, heat was the method chosen to kill cells because it has been shown to be effective for killing *X. fragariae* in strawberry planting stock (Turechek and Peres 2009). Specifically, a 4-h heat treatment at 44 to 46°C is simultaneously effective at killing bacterial cells while minimally damaging the plants. In the absence of plants, however, a shorter incubation period at a higher temperature was used in this study to quickly generate dead bacterial cells. Briefly, for each sample, an approximately 10-ml aliquot of the viable cell suspension was transferred to a 15-ml tube, incubated in a water bath at 90°C for 5 min, and then set on the laboratory bench to cool to room temperature. Aliquots of the heat-killed *X. fragariae* suspension (10 µl) were plated on SPA plates to confirm kill.

For development and testing of the PMA-qPCR procedure on bacterial cells, 500 µl of each bacterial cell sample—viable cells or dead cells at different concentrations, as well as the mixture of viable and dead cells in different ratios—was collected in 2-ml clear microcentrifuge tubes to proceed with PMA treatment (described below).

Sample preparation for bacteria-spiked strawberry tissue suspension. Remnant petioles are the main tissue used for pathogen testing in bare-root planting stock because they can harbor numerous pathogens. Moreover, collecting, processing, and testing petioles is a simple, less destructive, and economically more sensible process than collecting and testing entire plants or crown tissue (Turechek and Peres 2009; Wang et al. 2018). Petiole tissue from the strawberry cultivar Festival was used to develop the PMA-qPCR assay. The sample preparation procedure from Wang and Turechek (2016) was used. Briefly, approximately 1 g of healthy petiole tissue was cut with a clean razor blade into approximately 2-mm² pieces and added to 10 ml of 1× PBS containing different concentrations of bacteria (10^3 to 10^7 CFU/ml) in each 50-ml sterilized Erlenmeyer flask. Flasks were covered with aluminum foil and placed on an orbital shaker overnight. At 21 h after incubation, 500 µl of the suspension was collected per sample in 2-ml clear microcentrifuge tubes and processed for PMA treatment and DNA extraction before testing by qPCR (described below). PBS containing petiole tissue without added bacteria served as the negative control.

PMA treatment. The PMA treatment procedure was modified from the protocol of Nkuiopou-Kenfack et al. (2013). PMAxx (Biotium, Fremont, CA) was diluted with 20% dimethylsulfoxide into 1.7 mM of working solution, which was then added to each sample. The optimal PMAxx concentration was found to be 10 and 50 µM for the pure bacterial suspension and the bacteria-spiked or inoculated petiole samples, respectively (*data not shown*). The samples were mixed thoroughly with PMAxx in the dark within a cardboard box by gently flipping and shaking the box, and they were then incubated in an oven at 35°C for 30 min. Samples were mixed every 10 min during incubation. After incubation, samples were exposed to blue light (wavelength: 465 to 475 nm) for 20 min in a PMA-Lite LED Photolysis Device (Biotium, Hayward, CA) before centrifugation at $20,000 \times g$ for 5 min. The supernatant was removed and the pellets were used for DNA extraction (described below).

DNA extraction. DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) based on the manufacturer's protocol for Gram-negative bacteria with the following modifications. Specifically, the pellets obtained from each sample (as described in the section above) were resuspended in a tissue lysis (ATL) buffer and proteinase K and incubated at 56°C for 30 min for cell lysis. Samples were then centrifuged at $20,000 \times g$ for 5 min and the supernatant was removed with a pipette before proceeding to the next step. DNA was eluted in 200 µl of an elution (AE) buffer and 1 µl of DNA was used as template in qPCR reactions (described below).

Primer design and long-amplicon qPCR reactions. The 241-long primers (forward: 5'-GCGTTGAACAAATCCCATCT-3'; reverse: 5'-CTGGCTATGAAGTTGCGACA-3'; 863-bp amplicon) were designed using Primer 3 (<http://bioinfo.ut.ee/primer3/>) based on the genomic sequence that encompass RAPD marker 241 (550 bp), which is one of the most widely used markers established for *X. fragariae* detection (Pooler et al. 1996; Turechek et al. 2008; Vandroemme et al. 2008). The q241 primers and probe set (61-bp amplicon; Turechek et al. 2008) targeting the same genomic region were compared with 241-long primers in the development of the PMA-qPCR assay.

Long-amplicon qPCR reaction. For reactions containing the 241-long primers, the 20-µl SYBR Green PCR reaction contained 0.2 µM of forward and reverse primers and 1× KAPA SYBR FAST qPCR master mix (KAPA Biosystems, Wilmington, MA). The SYBR reactions were run at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 63°C for 20 s, and 72°C for 60 s, and a melting curve analysis from 65 to 95°C with an increment of 0.2°C every 5 s. qPCR reactions were conducted in a CFX96 TOUCH Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The nontemplate control

was included in each run. Each sample was run in two technical replicates.

Short-amplicon qPCR reaction. For reactions containing the original q241 primers (Turechek et al. 2008), the PCR reaction contained 0.36 μ M of forward and reverse primers, 0.17 μ M of the probe, and 1 $\times$ master mix from the DyNAmo Flash Probe qPCR Kit (Thermo Fisher Scientific, Pittsburgh, PA). The qPCR reactions were run at 95°C for 7 min, followed by 40 cycles of 95°C for 10 s and 56°C for 30 s. qPCR reactions were conducted in a CFX96 TOUCH Real-Time PCR Detection System (Bio-Rad). The nontemplate control was included in each run. Each sample was run in two technical replicates.

Amplification specificity and efficiency of 241-long primers. The specificity of the 241-long primers was first tested in silico by NCBI nucleotide BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Then the qPCR reactions were tested with the set of DNA from an isolate collection used in the study of Wang and Turechek (2016), which included 40 *X. fragariae* isolates, 17 isolates of other *Xanthomonas* species, 29 isolates of different strawberry pathogens, 15 unclassified bacterial and fungal isolates from healthy strawberry tissues, and five *Pseudomonas* isolates from other hosts (Table 1). To ensure DNA quality, each bacterial isolate was also tested with universal 16s primers (1237F-1492R; Turner et al. 1999), and the fungal isolates were tested with universal internal transcribed spacer (ITS) primers (ITS1-ITS4; White et al. 1990). There were three technical replicates for each isolate tested.

To determine the amplification efficiency (E), a 10 $\times$ dilution series of purified DNA from Xf100 (10³ to 10⁸ CFU/ml) was used to build the standard curves in qPCR. PCR efficiency was calculated using the equation:

$$(1 + E) = 10^{(-1/\text{slope})}$$

The experiment was repeated three times, with two biological replicates per experiment.

PMA-qPCR detection of viable and dead *X. fragariae* cells.
Pure bacterial culture. To determine the effect of PMA on PCR amplification of viable *X. fragariae* and its ability to suppress amplification of dead *X. fragariae* cells, viable- and dead-cell suspensions of Xf100 in 1 $\times$ PBS were prepared at 10⁸, 10⁷, and 1 $\times$ 10⁴ CFU/ml. Then, 500- μ l samples of each viable cell and dead cell were treated separately with the control (0 μ M) or 10 μ M of PMAxx added as described above. DNA was extracted as described above and the samples were tested by qPCR with both 241-long primers and q241 primers as described above. The qPCR results from these two primer sets were compared to examine the effect of amplicon size on PMA-qPCR using the statistical analysis described below. The experiment was repeated three times, with two biological replicates per treatment combination per experiment.

Bacteria-spiked strawberry petiole samples. Similarly, to determine the effects of PMA on PCR amplification of viable *X. fragariae* and its ability to suppress amplification of dead *X. fragariae* in bacteria-spiked petiole samples, viable and dead Xf100 cells were mixed separately with 1 g of healthy strawberry petiole sections in 1 $\times$ PBS at 10⁷, 10⁶, 10⁴, and 1 $\times$ 10³ CFU/ml. Compared with the pure-bacteria suspensions described above, a 10 $\times$ lower range of bacterial concentrations was chosen to spike the strawberry petiole suspensions because of the lower detection limit reported for *X. fragariae* in bacteria-spiked strawberry tissue suspensions relative to pure bacterial suspensions (presumably a consequence of the preparation procedure; Wang and Turechek 2016). Healthy petiole tissue in PBS alone was included in each experiment as a negative control. Samples (500 μ l each) were collected at 21 h after incubation and treated with PMAxx (0 or 50 μ M) as described above. DNA was extracted and samples were tested by qPCR with both 241-long primers and q241 primers as described above. There were two replicates per treatment combination and the experiment was conducted three times.

Samples from inoculated strawberry plants. The PMA-qPCR was also evaluated with petiole samples from strawberry plants (cultivar Festival) spray inoculated with *X. fragariae* strain Xf100 (10⁸ CFU/ml). Three different samples of whole strawberry petioles (1 g per

sample) were collected at three different time points (2, 3, and 4 weeks after inoculation [wai]) and processed as described above. At 21 h after incubation, one aliquot (500 μ l) from each petiole sample was directly treated with PMAxx (0 or 50 μ M) as described above, whereas another aliquot was heat treated at 56°C for 60 min before the same PMA treatment. DNA was extracted and samples were tested with qPCR with 241-long primers and q241 primers as described above. The *X. fragariae* cells in these petiole samples were not able to be quantified by colony count, since other bacterial contaminants from the petiole tissues quickly overgrew the slow-growing target pathogen, but the heat treatment of pure *X. fragariae* cells at 56°C for 15 min or more has been shown to prevent bacterial growth on SPA plates (Turechek and Peres 2009).

Statistical analysis. The three dependent variables—the qPCR threshold cycle (C_t) values from samples treated with PMA

Table 1. Bacterial and fungal isolates used for testing the amplification specificity of the 241-long primers and their corresponding mean C_t values from quantitative PCR (qPCR) with the 241-long primers, and universal 16s and internal transcribed spacer (ITS) primers

Species/isolate	Isolates (n)	Host	C _t ^x		
			241-long	16s ^y	ITS ^z
<i>Alternaria</i> spp.	1	Strawberry	N/A	–	18.41
<i>Botrytis cinerea</i>	3	Strawberry	N/A	–	22.24
<i>Cercospora</i> spp.	1	Strawberry	N/A	–	19.43
<i>Colletotrichum acutatum</i>	1	Strawberry	38.47	–	18.02
<i>Fusarium oxysporum</i>	1	Strawberry	N/A	–	22.70
<i>Gnomonia</i> spp.	2	Strawberry	N/A	–	25.69
<i>Hainesia lythri</i>	1	Strawberry	N/A	–	17.72
<i>Macrophomina phaseoli</i>	2	Strawberry	N/A	–	24.35
<i>Pestalotiopsis</i> spp.	2	Strawberry	N/A	–	21.73
<i>Phomopsis obscurans</i>	3	Strawberry	39.20	–	25.26
<i>Phytophthora cactorum</i>	3	Strawberry	38.47	–	24.35
<i>Pseudomonas cichorii</i>	1	Tomato	N/A	12.60	–
<i>Pseudomonas putida</i>	1	Tomato	37.92	17.17	–
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	1	Bean	38.98	13.47	–
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	1	Tomato	38.97	16.42	–
<i>Pseudomonas viridiflava</i>	1	Tomato	N/A	12.04	–
<i>Pseudomonas</i> spp.	4	Strawberry	38.13	16.22	–
<i>Pseudomonas syringae</i>	1	Strawberry	N/A	14.36	–
<i>Pythium irregulare</i>	1	Strawberry	39.22	–	18.46
<i>Verticillium dahliae</i>	3	Strawberry	37.92	–	20.43
<i>Xanthomonas albilineans</i>	1	Sugar cane	N/A	14.64	–
<i>X. alfalfae</i>	1	Citrus	N/A	13.00	–
<i>X. arboricola-pruni</i>	1	Peach	39.82	13.58	–
<i>X. axonopodis</i> pv. <i>dieffenbachiae</i>	1	Anthurium	38.85	15.43	–
<i>X. campestris</i> pv. <i>citri</i>	1	Citrus	35.61	12.91	–
<i>X. campestris</i> pv. <i>campestris</i>	1	Cabbage	N/A	16.17	–
<i>X. campestris</i> pv. <i>euvesicatoria</i> R4	1	Pepper	37.66	15.98	–
<i>X. codiae</i>	1	Croton	N/A	18.95	–
<i>X. fragariae</i>	40	Strawberry	14.73	15.00	–
<i>X. fuscans</i>	1	Bean	38.23	13.14	–
<i>X. hortorum</i> pv. <i>pelargonii</i>	1	Geranium	38.52	16.48	–
<i>X. hyacinthi</i>	1	Hyacinthus	38.59	19.28	–
<i>X. perforans</i> T4	1	Tomato	38.80	20.56	–
<i>X. pisi</i>	1	Pea	38.92	18.53	–
<i>Xanthomonas</i> spp.	1	Unknown	37.22	12.00	–
<i>X. translucens</i>	1	Wheat	N/A	18.41	–
<i>X. vesicatoria</i>	1	Tomato	39.33	18.06	–
<i>X. euvesicatoria</i>	1	Pepper	N/A	17.46	–

^x Means based on three technical replicates per isolate and over the number of isolates tested where applicable. Dashes indicate that no qPCR reaction was conducted. N/A = no amplification.

^y Universal 16s primers 1237F-1492R (Turner et al. 1999) for bacteria only.

^z Universal ITS primers ITS1- ITS4 (White et al. 1990) for fungi only.

(C_{L_PMA}), without PMA ($C_{L_No\ PMA}$), and their difference ($\Delta C_t = C_{L_PMA} - C_{L_No\ PMA}$)—were analyzed individually for viable and dead bacterial cell suspensions in a general linear mixed model with an identity link function and normal error distribution using SAS version 9.4 (SAS Institute Inc., Cary, NC). Amplicon size, cell status, and bacterial concentration served as main effects in the first two experiments, whereas amplicon, heat treatment, and sampling time served as main effects for the final experiment with inoculated plants. For all experiments, the main effects as well as their interactions were treated as fixed effects, and replication and experimental run (for the first two experiments) were treated as random effects. Means of fixed effects were compared using the lsmeans/lines statement.

Evaluation of the PMA-qPCR protocol across *X. fragariae* strains using mixtures of viable and dead cells. To test the robustness of the assay, pure bacterial suspensions of four *X. fragariae* strains (Xf100, Xf128, BALM, and Xf1425) were tested. For each strain, viable and dead cell suspensions were prepared at 10^7 CFU/ml and 500- μ l aliquots of viable/dead cell mixtures were prepared in the following ratios: 0:1, 1:999, 1:9, and 1:0. Each sample was treated with PMAxx (0 or 10 μ M) as described above. There were two samples for each treatment and the experiment was conducted two times. DNA was extracted and tested by qPCR with 241-long primers as described above.

Statistical analysis. The qPCR C_t values of samples treated with (C_{L_PMA}) and without PMA ($C_{L_No\ PMA}$) were analyzed separately in a general linear mixed model using Minitab version 18. Bacterial strain was treated as a fixed effect, and replication and its interaction

with strain were treated as random effects. Linear regression was used to predict the log-transformed concentrations of viable bacteria as a function of C_t values from PMA-qPCR (the 100% dead cells were excluded from the analysis).

Results

Amplification specificity and efficiency of 241-long primers.

None of the qPCR reactions with 241-long primers amplified any nontarget bacterial or fungal isolates within 37 cycles, with the exception of *X. campestris* pv. *citri*, which amplified at a C_t of 35.61. However, all isolates of *X. fragariae* (approximately 10^8 CFU/ml) amplified within 15 cycles (Table 1). With the 10 $\times$ dilution series from purified DNA, qPCR with 241-long primers detected 100% of the samples with bacterial concentrations that ranged from 10^4 to 10^8 CFU/ml ($C_t = 14.81$ to 28.62), from which the PCR efficiency was calculated as 95.1%. The detection rate (samples with $C_t < 35$) decreased to 66.7% when only 10^3 CFU/ml of *X. fragariae* was present.

PMA-qPCR detection of viable and dead *X. fragariae* cells.

Pure bacterial culture. PMA-qPCR was tested for its ability to detect and differentiate viable and dead cells in pure *X. fragariae* suspensions at 10^4 , 10^7 , and 10^8 CFU/ml with both short- and long-amplicon primer pairs (Fig. 1A, all dashed lines with gray symbols are generally expected to be 0; Tables 2 and 3). Compared with short-amplicon PMA-qPCR, less DNA was amplified from dead cells with long-amplicon PMA-qPCR as evidenced by the mostly larger C_t values associated with PMA-treated dead cells and larger ΔC_t values (Table 3). With the 241-long primers, PMA treatment was able to suppress amplification of dead bacterial cells at 10^4 and 10^7 CFU/ml concentrations with C_t values > 38 while having minimal impact on viable cell amplification (approximately 1.62 increase in C_t values compared with the “no PMA” treatments) (Table 3). However, when the bacterial cell concentration reached 10^8 CFU/ml, an amplification signal that was equivalent to a low concentration of cells (approximately 10^4 CFU/ml, 0.01%) appeared in the PMA-treated dead cells ($C_t = 30.43$; Fig. 1A; Table 3).

The mixed model analyses showed that most independent variables and their interactions significantly affected the C_t values for each of the three dependent variables (i.e., the C_t values for reactions with and without PMA, and the ΔC_t value) (Table 2). The exceptions were “amplicon” in the qPCR reaction with PMA and all interaction terms with amplicon in the qPCR without PMA. qPCR reactions without PMA responded proportionally with regard to “cell status” or “concentration” with both primer sets (amplicon size), and the interactions of both variables with “amplicon” were statistically insignificant. This was not the case when PMA was added to the reaction. Despite amplicon’s small main effect (i.e., small F value), the interactions of amplicon with cell status and concentration were significant as a result of the more complex (nonlinear) relationship between these variables.

The mean separation applied to the three-way interaction showed significant differences in the C_t values in both the PMA- and no-PMA-treated reactions for nearly all combinations of amplicon size, cell viability status, and *X. fragariae* concentration (Table 3). Looking at ΔC_t , a slight increase was consistently observed among the viable cells treated with PMA. Nonetheless, ΔC_t values were statistically equivalent for the viable cells for nearly all combinations of amplicon size and *X. fragariae* concentration, indicating that the addition of PMA had little effect on the qPCR reaction of viable cells. The one exception was the comparison between ΔC_t values of the long-amplicon reaction at 10^8 CFU/ml versus the short amplicon at 10^7 CFU/ml (Table 3).

Bacteria-spiked strawberry samples. Similar results were obtained with the bacteria-spiked strawberry petiole samples, in which samples were spiked with suspensions of viable or dead cells at concentrations of 10^3 , 10^4 , 10^6 , or 10^7 CFU/ml (Fig. 1B; Tables 2 and 4). For all concentrations tested, ΔC_t values were significantly larger for dead cell versus viable cell suspensions and ranged between 5.28 and 17.48 for dead cells and 1.20 and 1.90 for viable cells (Table 4). In contrast, ΔC_t values ranged between only 2.00 and 6.49 for dead

Fig. 1. Amplification curves for long-amplicon propidium monoazide (PMA)-quantitative PCR designed in this study. **A**, Pure cell culture of viable or dead cells (10^4 , 10^7 , and 10^8 CFU/ml) and **B**, bacteria-spiked strawberry petiole samples spiked with viable or dead *Xanthomonas fragariae* cells (10^3 , 10^4 , 10^6 , and 10^7 CFU/ml). Samples were treated with (broken line) and without (solid line) PMA. The nontemplate control was also included as a negative control but is not visible on the figure because it maintains a y-axis value of “0” along the entire x-axis.

cells and 0.35 and 1.02 for viable cells for short-amplicon PMA-qPCR. Amplification was not well suppressed for the dead cell population in the petiole suspensions spiked with 10^6 and 10^7 CFU/ml dead bacteria.

For the mixed model analyses, the independent variables and most of their interactions significantly affected the C_t values for each of the three dependent variables (Table 2). The exceptions, as with the pure cell reactions, were all interaction terms with amplicon in the qPCR without PMA. For both the short- and long-amplicon reactions, this lack of interaction can be seen in the evaluation of the three-way interaction by noting the same stepwise pattern of increase in C_t values (and their significance) between the two amplicon sizes under the “no PMA” column (Table 4). As with the pure cell reactions, the mean separation test applied to the three-way interaction showed significant differences in the C_t values in both the PMA and no-PMA-treated reactions for nearly all combinations of amplicon size, cell viability status, and *X. fragariae* concentration (Table 4). For viable cell suspensions, the delta C_t values fell into the last two groups (i.e., F and G) for all combinations of amplicon size and *X. fragariae* concentration, indicating that the addition of PMA had little effect on the qPCR reaction of viable cells in the range of concentrations tested. Although it was not statistically significant and consistent with the pure cell reactions, a slight increase was always noted among the viable cells treated with PMA.

Samples from inoculated strawberry plants. The PMA-qPCR assay was also evaluated with petiole samples collected from spray-inoculated strawberry plants (Table 5). Over the three sampling periods (2 to 4 wai), the nonheat-treated petiole samples had C_t values from 19.27 to 17.29 and from 24.47 to 22.45 corresponding to bacterial concentrations between 5×10^6 and 2×10^7 CFU/ml based on their standard curves for both long- and short-amplicon qPCR, respectively. PMA treatment had a minimal effect on these nonheat-treated samples (delta C_t values < 0.5), indicating that most *X. fragariae* cells from inoculated petioles were viable. The heat-treated

samples yielded similar C_t values as nontreated samples without PMA treatment; however, when they were treated with PMA, their C_t values were 35.6 to 40 (delta C_t of 18.49 to 22.0) for long-amplicon qPCR and 29.29 to 33.65 (delta C_t of 7.0 to 9.72) for short-amplicon qPCR.

For the mixed model analyses, fewer significant effects were seen among the independent variables and their interactions. The main effect of heat significantly affected the C_t values for

Table 3. Effect of long- and short-amplicon propidium monoazide (PMA)-quantitative PCR (qPCR) on detection of viable and dead *Xanthomonas fragariae* (Xf) cells at different concentrations

Amplicon size ^y	Concentration (CFU/ml)	Cell status	C_t values ^z		
			No PMA	PMA	Delta C_t
Short	10^4	Dead	36.44 A	39.89 A	3.45 D
		Viable	33.26 B	33.93 C	0.66 EF
	10^7	Dead	24.83 E	32.03 D	7.20 C
		Viable	23.10 F	23.48 G	0.38 F
	10^8	Dead	21.32 G	28.83 F	7.51 C
		Viable	19.73 H	20.29 H	0.56 EF
Long	10^4	Dead	31.02 C	38.30 B	7.28 C
		Viable	28.69 D	30.11 EF	1.42 EF
	10^7	Dead	19.78 H	38.17 B	18.39 A
		Viable	17.96 I	19.58 H	1.62 EF
	10^8	Dead	16.33 J	30.43 E	14.10 B
		Viable	14.72 K	16.55 I	1.83 DE

^y Long-amplicon PMA-qPCR utilizes the 241-long primer pair developed in this study, producing an amplicon of 863 bp; short-amplicon PMA-qPCR utilizes the q241 primer pair developed in Turechek et al. (2008) and produces an amplicon of 61 bp.

^z The standard error of means was 0.4225, 0.4932, and 0.5556 for $C_{t_No\ PMA}$, C_{t_PMA} , and delta C_t , respectively. Values sharing the same letter are statistically equivalent.

Table 2. Type III tests of fixed effects from linear mixed model analyses examining the effects of amplicon size, cell status, bacterial cell concentration and their interactions on the dynamics of propidium monoazide (PMA)-quantitative PCR, as measured by the cycle threshold (C_t) values for both pure cell (“pure”), bacteria-spiked petiole (“spiked”), and inoculated plant (“inoculated”) sample preparations treated with or without PMA

Treatment ^y	Num <i>df</i>	No PMA ^z			PMA ^z			Delta C _t ^z		
		Den <i>df</i>	<i>F</i> value	Pr > <i>F</i>	Den <i>df</i>	<i>F</i> value	Pr > <i>F</i>	Den <i>df</i>	<i>F</i> value	Pr > <i>F</i>
Pure										
Amplicon	1	54.00	1,318.98	<0.0001	2.51	4.21	0.1503	2.85	78.21	0.0037
Status	1	54.00	217.11	<0.0001	2.38	690.67	0.0005	2.90	316.48	0.0005
Concentration	2	4.00	2,174.80	<0.0001	4.05	905.66	<0.0001	4.18	101.61	0.0003
A*S	1	54.00	0.80	0.3757	47.03	350.71	<0.0001	46.69	314.57	<0.0001
A*C	2	54.00	0.06	0.9424	47.03	50.14	<0.0001	46.69	43.77	<0.0001
S*C	2	54.00	6.75	0.0024	47.03	146.63	<0.0001	46.69	165.66	<0.0001
A*S*C	2	54.00	1.16	0.3203	47.03	52.45	<0.0001	46.69	34.42	<0.0001
Spiked										
Amplicon	1	3.10	643.87	0.0001	71.68	4.76	0.0325	3.23	249.76	0.0004
Status	1	3.70	176.02	0.0003	3.89	612.63	<0.0001	3.33	700.93	<0.0001
Concentration	3	68.48	1,985.74	<0.0001	71.68	396.39	<0.0001	65.44	59.16	<0.0001
A*S	1	68.48	3.27	0.0748	71.68	215.74	<0.0001	65.44	180.86	<0.0001
A*C	3	68.48	0.73	0.5374	71.68	15.85	<0.0001	65.44	17.09	<0.0001
S*C	3	68.48	4.07	0.0102	71.68	100.13	<0.0001	65.44	77.44	<0.0001
A*S*C	3	68.48	0.14	0.9383	71.68	18.73	<0.0001	65.44	17.17	<0.0001
Inoculated										
Amplicon	1	22.00	396.60	<0.0001	22.00	2.45	0.1317	24.00	129.27	<0.0001
Heat	1	22.00	4.63	0.0426	22.00	1,172.57	<0.0001	24.00	755.61	<0.0001
Wai	2	22.00	16.61	<0.0001	22.00	8.72	0.0016	24.00	0.16	0.8547
A*H	1	22.00	0.11	0.7411	22.00	210.28	<0.0001	24.00	121.18	<0.0001
A*W	2	22.00	0.82	0.4531	22.00	6.82	0.0050	24.00	3.02	0.0677
H*W	2	22.00	2.79	0.0833	22.00	0.22	0.8076	24.00	0.35	0.7081
A*H*W	2	22.00	1.05	0.3666	22.00	9.75	0.0009	24.00	4.31	0.0251

^y A = amplicon (long or short), S = cell status (viable or dead), C = cell concentration, H = heat treated (yes/no), and W = weeks after inoculation (2, 3, or 4). The asterisk symbolizes the interaction between the main factors denoted.

^z Numerator (Num) and denominator (Den) degrees of freedom (*df*), the *F* statistic (*F*), and its associated probability (Pr > *F*) for separate analyses of samples treated with (PMA) or without (no PMA) PMA, and the cycle threshold differences (delta $C_t = C_{t_PMA} - C_{t_No\ PMA}$). Bold type indicates that model terms are not significant at *P* < 0.05.

each of the three dependent variables (Table 2). However, amplicon did not have a significant effect with PMA treatment and sampling time did not have a significant effect on delta C_t . The significance of the interaction terms varied across the three dependent variables, but like the other two experiments, the three-way interaction was significant for PMA treatment and delta C_t but not for the no-PMA treatment (Table 2).

Evaluation of the PMA-qPCR protocol across *X. fragariae* strains using mixtures of viable and dead cells. Bacterial strain had no significant effect on PMA-qPCR detection in the mixtures of viable and dead bacterial cells ($P = 0.814$ and 0.980 for C_t values from no PMA and PMA treatment, respectively). Therefore, the mean C_t values of the four *X. fragariae* strains are shown in

Table 4. Effect of long- and short-amplicon propidium monoazide (PMA)-quantitative PCR (qPCR) on detection of viable and dead *Xanthomonas fragariae* (Xf) cells at different concentrations in bacteria-spiked petiole samples

Amplicon size ^y	Concentration (CFU/ml)	Cell status	C_t values ^z		
			No PMA	PMA	Delta C_t
Short	10^3	Dead	37.28 A	39.28 A	2.00 F
		Viable	34.83 B	35.85 C	1.02 FG
	10^4	Dead	34.16 B	38.54 AB	4.38 E
		Viable	31.21 D	31.69 DE	0.48 G
	10^6	Dead	27.50 F	32.87 D	5.36 DE
		Viable	24.84 G	25.42 G	0.58 G
Long	10^7	Dead	24.59 G	31.07 E	6.49 D
		Viable	20.89 I	21.24 H	0.35 G
	10^3	Dead	33.20 C	38.49 AB	5.28 DE
		Viable	30.43 DE	32.33 DE	1.90 F
	10^4	Dead	29.97 E	38.61 AB	8.64 C
		Viable	26.62 F	27.97 F	1.35 FG
	10^6	Dead	23.78 H	38.06 AB	14.28 B
		Viable	20.32 I	21.81 H	1.48 FG
	10^7	Dead	20.16 I	37.64 B	17.48 A
		Viable	15.99 J	17.19 I	1.20 FG

^y Long-amplicon PMA-qPCR utilizes the 241-long primer pair developed in this study, producing an amplicon of 863 bp; short-amplicon PMA-qPCR utilizes the q241 primer pair developed in Turechek et al. (2008) and produces an amplicon of 61 bp.

^z The standard error of means was 0.3184, 0.5073, and 0.4719 for $C_{t_No\ PMA}$, C_{t_PMA} , and delta C_t , respectively. C_t = threshold cycle. Values sharing the same letter are statistically equivalent.

Table 5. Effect of long- and short-amplicon propidium monoazide (PMA)-quantitative PCR (qPCR) on detection of viable and dead *Xanthomonas fragariae* (Xf) cells in petiole samples from spray-inoculated strawberry plants

Amplicon size ^x	Sampling time (wai) ^y	Heat treatment	C_t values ^z		
			No PMA	PMA	Delta C_t
Short	2	Yes	23.93 AB	33.65 C	9.72 C
		No	24.47 A	24.48 F	0.01 E
	3	Yes	22.29 C	29.29 E	7.00 D
		No	24.13 AB	24.39 F	0.25 E
	4	Yes	22.92 BC	31.58 D	8.66 CD
		No	22.45 C	22.64 F	0.19 E
Long	2	Yes	19.18 DE	37.67 B	18.49 B
		No	19.27 D	19.66 G	0.39 E
	3	Yes	17.93 EF	40.00 A	22.07 A
		No	18.86 DE	18.96 G	0.09 E
	4	Yes	16.91 F	35.60 C	18.69 B
		No	17.29 F	17.79 G	0.50 E

^x Long-amplicon PMA-qPCR utilizes the 241-long primer pair developed in this study, producing an amplicon of 863 bp; short-amplicon PMA-qPCR utilizes the q241 primer pair developed in Turechek et al. (2008) and produces an amplicon of 61 bp.

^y wai = weeks after inoculation.

^z The standard error of means was 0.6906, 0.8954, and 0.8739 for $C_{t_No\ PMA}$, C_{t_PMA} , and delta C_t , respectively. C_t = threshold cycle. Values sharing the same letter are statistically equivalent.

Table 6. Regression analysis showed that the C_t values (x) from PMA-qPCR predicted 98.11% of the variability in viable bacterial concentrations ($x = \log[\text{concentration of viable bacteria}]$): $y = 12.154 - 0.264x$; $F = 2,388.33$; $df = 1, 47$; and $P = 0.000$.

Discussion

In this study, a molecular viability test was developed for specific detection of *X. fragariae*. PMAXx, an improved version of PMA, was used to treat the bacterial cells and inhibit PCR amplification of the DNA from dead cells by binding to the DNA after penetrating through the compromised cell membrane. PMAXx was chosen based on its superior performance compared with other chemicals tested in preliminary experiments, such as EMA, DNase I, and sodium deoxycholate (*data not shown*), which have been applied in viability tests for different bacteria (Flekna et al. 2007; Villarreal et al. 2013; Yang et al. 2011). The optimized PMA treatment contained 10 and 50 μM of PMAXx for pure bacterial suspensions and strawberry petiole samples, respectively. However, when PMAXx-treated samples were examined using a previously designed probe-based qPCR assay targeting a 61-bp region in *X. fragariae* (Turechek et al. 2008), partial detection of the dead cells was observed, especially with higher concentrations of dead cells (Tables 3, 4, and 5). Therefore, to improve the efficiency of PMA-qPCR, a long-amplicon qPCR assay was designed in this study to couple with the PMA treatment.

The long-amplicon qPCR (241-long, 863 bp) amplifies the flanking region of the probe-based qPCR for *X. fragariae* (q241, 61 bp), and both qPCR primer sets were designed based on the 241 RAPD marker, one of the most notable markers established for *X. fragariae* detection (Pooler et al. 1996; Turechek et al. 2008; Vandroemme et al. 2008). The 241-long qPCR primers efficiently amplified all 40 *X. fragariae* isolates tested and exhibited excellent specificity by failing to amplify any of the other bacteria and fungi tested within the acceptable C_t threshold, including some closely related *Xanthomonas* species and microbes associated with strawberry. With the amplicon length far exceeding a regular qPCR amplicon (<200 bp), the optimized 241-long qPCR protocol still allowed a PCR efficiency of 95.1% for accurate quantification of the *X. fragariae* DNA, with a detection limit of approximately 10^3 CFU/ml in a pure bacterial suspension. Compared with q241, the 241-long qPCR protocol provided higher detection sensitivity for viable cells in bacteria-spiked petiole samples (Table 4).

Use of a longer amplicon significantly increased the capability of PMA-qPCR in differentiating viable and dead cells. Examination of the delta C_t values between treatments with and without PMA showed that long-amplicon PMA-qPCR yielded values similar for viable cells but they were more than two-fold higher for dead cells than what was achieved when using the short-amplicon PMA-qPCR (Tables 3 and 4). The increased differentiating capacity of long-amplicon PMA-qPCR has been observed in several other bacterial systems. It is believed that with a longer amplicon, the likelihood of PMA binding along the target DNA sequence is high (Banihashemi et al. 2012; Contreras et al. 2011; Fittipaldi et al. 2012; Luo et al. 2010; Martin et al. 2013; Schnetzinger et al.

Table 6. Effect of long-amplicon propidium monoazide (PMA)-quantitative PCR (qPCR) on detecting viable *Xanthomonas fragariae* in different mixtures of viable and dead cells (10^7 CFU/ml) of four different strains^y

Ratio of live/dead cells	C_t ^z	
	No PMA	PMA
1:0	18.13 $\pm$ 0.06	19.55 $\pm$ 0.09
1:9	19.82 $\pm$ 0.12	23.36 $\pm$ 0.10
1:999	20.47 $\pm$ 0.15	30.70 $\pm$ 0.25
0:1	20.06 $\pm$ 0.13	37.54 $\pm$ 0.59

^y There was no significant strain effect ($P > 0.814$); thus, the means of threshold cycle (C_t) values for each treatment were obtained from the data of all four strains.

^z Long-amplicon PMA-qPCR utilizes the 241-long primer pair developed in this study, producing an amplicon of 863 bp.

2013). Another characteristic of PMA-qPCR is that there tends to be a slight increase in the C_t values of viable cells treated with PMA than those that have not been treated. This is likely due to the small proportion of dead cells naturally present in the viable cell suspension that have been removed from the reaction with the application of PMA. Similar levels of increase have been observed in other studies (Martin et al. 2013).

Long-amplicon PMA-qPCR suppressed the amplification of dead cell suspensions of *X. fragariae* to a $C_t > 37$, with or without petiole tissue, in concentrations ranging from 10^3 to 10^7 CFU/ml, and it allowed an accurate quantification of viable cells mixed with dead cells in different ratios across different *X. fragariae* strains ($R^2 = 0.981$; Tables 3, 4, 5, and 6). When concentrations of dead cells reached 10^8 CFU/ml in pure cell suspensions, PMA-qPCR with the long amplicon began to amplify a small percentage (0.01%) of the dead cells. Similar observations have been reported in many studies, and it was suggested that PMA effectiveness may be saturated with higher quantities of dead bacteria (Fittipaldi et al. 2012; Han et al. 2018; Varma et al. 2009). The dead cells in this study were generated by heat treatment, which was demonstrated to be effective at reducing ALS in strawberry nursery stock (Turechek and Peres 2009). Bacterial cells killed by alternative measures (e.g., UV light, antibiotic treatment) were not tested in this study.

Strawberry nursery stock is considered to be the major inoculum source for ALS. On cold-stored dormant plants, petiole tissue was the tissue most likely to harbor viable *X. fragariae* and was thus chosen as the strawberry tissue to be tested with long-amplicon PMA-qPCR (Turechek and Peres 2009; Wang et al. 2018). Indeed, petioles are routinely tested from suspect plants during the propagation process in efforts to prevent the proliferation and/or transmission of inoculum in clean nursery stock. One of the difficulties in applying PMA-qPCR is getting the PMA to contact the pathogen within plant tissues. The sample preparation procedure developed by Wang and Turechek (2016) helps to minimize this problem through the incubation step that releases bacterial cells from petiole sections into the PBS buffer, facilitating the access of PMA to these cells. Use of this procedure provided the same detection sensitivity as direct extraction of bacterial DNA from strawberry tissues with commercial kits, but it offers an economical alternative for testing larger quantities of tissue (Wang and Turechek 2016). With this sample preparation procedure, long-amplicon PMA-qPCR was able to differentiate viable and dead cells from 10^3 to 10^7 CFU/ml in the bacteria-spiked petiole suspensions and with petioles sampled 2, 3, and 4 weeks after inoculation with *X. fragariae*.

In summary, the long-amplicon PMA-qPCR assay developed in this study is the first molecular viability test specifically for *X. fragariae*. It allowed accurate quantification of the viable- and dead cell populations in cell suspensions up to 10^7 CFU/ml. This assay could serve in a certification protocol to satisfy quarantine regulations imposed on nursery stock destined to the European Union, or it can simply assist in the early detection of infected planting material or in the identification of inoculum sources in the strawberry production cycle.

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