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Development of species-, strain- and antibiotic biosynthesis-specific quantitative PCR assays for *Pantoea agglomerans* as tools for biocontrol monitoring

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ABSTRACT

Pantoea agglomerans is a cosmopolitan plant epiphytic bacterium that includes some of the most effective biological antagonists against the fire blight pathogen *Erwinia amylovora*, a major threat to pome fruit production worldwide. Strain E325 is commercially available as Bloomtime Biological™ in the USA and Canada. New quantitative PCR (qPCR) assays were developed for species- and strain -specific detection in the environment, and for detection of indigenous strains carrying the biocontrol antibacterial peptide biosynthesis gene *paaA*. The qPCR assays were highly specific, efficient and sensitive, detecting fewer than three cells per reaction or 700 colony forming units per flower, respectively. The qPCR assays were tested on field samples, giving first indications to the incidence of *P. agglomerans* E325 related strains, total *P. agglomerans* and pantocin A producing bacteria in commercial orchards. These assays will facilitate monitoring the environmental behavior of biocontrol *P. agglomerans* after orchard application for disease protection, proprietary strain-tracking, and streamlined screening for discovery of new biocontrol strains.

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1. Introduction

Pantoea agglomerans is a Gram-negative bacterium that belongs to the family of Enterobacteriaceae. It exists primarily as epiphyte on plant surfaces (Lindow and Brandl, 2003) but can also be found in aquatic environments, soil or sediments (Brown and Leff, 1996; Francis et al., 2000). *P. agglomerans* has been used as biological control agent against fungal post-harvest diseases (Bonaterra et al., 2005; Nunes et al., 2002) as well as bacterial diseases, such as basal kernel blight of barley (Braun-Kiewnick et al., 2000), and most predominantly against fire blight on pome fruits. In fact, several strains of the genus *Pantoea* (e.g., *P. agglomerans* strains E325 and P10c, and *Pantoea vagans* C9-1) provided highly effective control of the fire blight bacterium *Erwinia amylovora* in field studies (Johnson et al., 2004; Pusey, 2002; Stockwell et al., 2010). They were developed into plant protection products, have gone through registration processes by national regulatory authorities and are now commercially available alternatives and/or complements to antibiotic use in USA and Canada (Bloomtime Biological™, BlightBan C9-1™) and New Zealand (BlossomBless™).

Mechanisms by which *Pantoea* strains suppress plant diseases include nutritional competition and preemptive exclusion (Braun et al., 1998; Smits et al., 2011; Stockwell et al., 2010; Wilson and Lindow, 1994) and a variety of antibacterial organic acids and peptide antibiotics (Kamber et al., 2012; Pusey et al., 2011; Stockwell et al., 2002; Vanneste et al., 1992; Wodzinski et al., 1994; Wright et al., 2001). Pantocin A, a histidine-reversible tripeptide antibiotic, is perhaps the best characterized antibiotic involved in *E. amylovora* inhibition (Ishimaru et al., 1988; Jin et al., 2003a, 2003b) and is thus far known to be produced by only a few strains (i.e., *P. agglomerans* Eh318, *P. agglomerans* P10c, *P. vagans* C9-1, *Pantoea* sp. Eh252 and *Pantoea brenneri* LMG 5343 (Rezzonico et al., 2009)) that show high sequence identity between their biosynthetic genes (Vanneste et al., 2008).

Proprietary protection and registration of a biological control agent like *P. agglomerans* E325 need the development of quantitative, specific strain detection methods, since many biological control agents belong to species that are common inhabitants of plants. Therefore, strain-level detection methods are needed as “tracking devices” in order to better understand the ecology and biology of the introduced organism and to estimate its environmental impact on natural microbial communities (Gullino et al., 1995; Montesinos, 2003; Schena et al., 2004). Methods for species level detection are also needed to evaluate impact of introduced strains on indigenous species in the same habitat and/or on closely related/cohabitating bacteria. Finally, methods targeted to specific traits such as pantocin A production will provide data on incidence of strains with natural

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biocontrol potential in a certain environment and for novel strain selection.

Detection methods for biocontrol agents are required, but their availability is restricted. A relative quantification method specific for *P. agglomerans* CPA-2 based on SCAR markers (Nunes et al., 2008) was found to be impractical for high-throughput environmental sampling, as it requires selective plating and a quantitative method that includes testing a larger number of colonies per condition. Although qPCR has proven to be the most effective molecular tool for rapid and sensitive detection and quantification of plant pathogens, its use for tracking biocontrol agents has been limited. For instance, Pujol et al. (2006) used qPCR to assess the environmental fate of *Pseudomonas fluorescens* EPS62e at the strain level once it was introduced to the apple phyllosphere. Diagnostic qPCR methods were developed for Stewart's wilt pathogen *Pantoea stewartii* on corn (Wensing et al., 2010) as well as for several pathogenic and epiphytic *Erwinia* spp. that occur in orchards (Wensing et al., 2012), but these methods were not adapted to quantify population densities. Another qPCR method designed for *P. agglomerans* based on the *gnd* gene (Lehman, 2007) cannot distinguish *P. agglomerans* from *P. vagans* strains. Specific methods are currently not available for other *Pantoea* strains.

The objective of this study was to develop specific, sensitive real-time PCR assays for quantitative detection of the commercial biocontrol *P. agglomerans* strain E325, indigenous *P. agglomerans*, and strains that can produce the biological trait pantocin A. A genomics approach was taken to identify gene sequences suitable for strain-specific, species-specific and biological trait-specific qPCR development. In addition to validation of methods using several *P. agglomerans* and related strains as well as other bacteria found predominantly in the apple environment, methods were also validated using orchard samples. To our knowledge, this is the first report on using qPCR assays for the quantitative detection of *P. agglomerans*. Especially the species-specific detection method will therefore provide a suitable tool to detect and track *P. agglomerans* in systems other than the apple phyllosphere.

2. Materials and methods

2.1. Development of qPCR assays

Primers and probes (Table 1) for E325 specific and pantocin A biosynthesis gene *paaA* specific detection were designed using the program Primer3 included in the Universal ProbeLibrary Assay Design Center (Roche Applied Science, Mannheim, Germany). For the E325 specific region LNA probe #106 and for *paaA* LNA probe #137 were selected. Both probes are very specific small locked nucleic acid probes that contain FAM as reporter dye at the 5′ end and a dark hole quencher at the 3′ end. For the autoinducer gene *pagR*, the primer and probe set was designed using the Primer Express software (PE Applied Biosystems, Foster City, MA). The probe *pagR2* carried a VIC reporter dye at the 5′ end and a minor-groove-binding non-fluorescent quencher (MGBNFQ) at the 3′ end (PE Applied Biosystems).

Real-time PCR was performed in final reaction volumes of 20 µl containing 4 µl of extracted DNA, 300–900 nM of each primer, 100–200 nM of probe (depending on the primer/probe combination optimized for each gene target (Table 1)), 2× TaqMan® Environmental PCR Master Mix (Applied Biosystems Europe BV, Zug, Switzerland) for duplex reactions, or 2× TaqMan® Universal PCR Master Mix (Applied Biosystems Europe BV) for singleplex reactions, respectively. PCR reactions were performed on an Applied Biosystems 7500 FAST Real-Time PCR instrument (Applied Biosystems Europe BV). The baseline was set automatically, and the fluorescence threshold manually at a predetermined value based on validation data (0.25 using E325 probe, 0.2 using *pagR2*, and 0.05 using *paaA*). Cycling conditions were: 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Except when mentioned otherwise, all PCR reactions were conducted in triplicate.

2.2. Specificity

Specificity of qPCR assays was assessed with extracted DNA from 94 bacteria from world-wide collections (Supplemental Table S1) containing 27 *P. agglomerans* strains, 28 related *Pantoea* species, and 39 strains from other genera isolated from or commonly applied as biocontrol agents to rosaceous plants. Bacteria were grown on half-concentration tryptic soy agar (15 g l^{-1} Tryptic Soy Broth; 18 g l^{-1} agar) at 25 °C for 24 h. For DNA extraction from pure cultures, bacterial colonies were resuspended in 10 mM phosphate buffer saline (PBS) to concentrations of $1–2 \times 10^8$ CFU ml^{-1}, thermo-lysed at 95 °C for 15 min, and 500 µl aliquots centrifuged at 12,000 ×*g* for 10 min. Pellets were resuspended in 300 µl lysis buffer (BioSprint 96 Plant DNA extraction kit; QIAGEN AG, Hombrechtikon, Switzerland), shaken (800 ×*g*, 65 °C, 20 min), and centrifuged at 6000 ×*g* for 5 min. Aliquots of 200 µl were transferred to 96-well microplates in the Qiagen BioSprint workstation, and purified using magnetic beads following manufacturer's instructions. Bacterial DNA was resuspended in 100 µl Milli-Q ultrapure water (Millipore AG, Zug, Switzerland) and stored at −20 °C until used for qPCR. In each qPCR run, a negative control without DNA and a positive control of E325 (for strain-specific detection) or Eh318 (for pantocin A detection), respectively, were included. All reactions were performed in duplicate.

2.3. Orchard sampling and quantitative detection

A total of 108 apple flower samples from three commercial orchards were collected during full bloom in 2009. Samples consisted of 50 flowers each. Flowers collected were about three days old with partially dehisced anthers. Flowers with petals removed were collected in plastic bags and kept on ice. Sample processing was conducted the next day by adding 50 ml of AEB buffer (AgriStrip Extraction buffer B; Bioreba AG, Reinach, Switzerland) to bags, brief shaking and sonication for 1 min (37 kHz). Suspensions were transferred to sterile 50 ml centrifuge tubes and stored at −20 °C until DNA extraction.

Table 1
Primers and TaqMan probes designed for quantitative PCR detection of *Pantoea agglomerans* strain E325 (E325), *P. agglomerans* species (*pagR2*), and pantocin A producers (*paaA*) LNA Probes are from the Roche Universal Probe Library.

Targets (probe name)	Primer/probe	Sequence (5′-3′)	T_m (°C)	Conc.[a] (nM)	Amplicon
Pantoea agglomerans strain E325 (E325)	E325_F	GGA TCG CAT CTC ATC AGG TT	64.0	300	60 bp
	E325_R	GCC TGT AGC CCG GTT TAT GTG	64.3	300	
	LNA probe #106	–	–	100	
pantocin A producers (*paaA*)	Pantocin_F	TAT CTT TGG CCG CAT CAA CT	64.4	900	60 bp
	Pantocin_R	GAC AGG TGT TGT ATC GCA CAG	63.2	900	
	LNA probe #137	–	–	200	
P. agglomerans as species (*pagR2*)	PagRrt2_F	ACG GTG CGT TCC GCA ATA	60.0	900	60 bp
	PagRrt2_R	GGC GCC GGG AAA ACA TAC	60.0	900	
	pagR2 probe	5′-VIC-AAG TTG CGG TCA TTT TAT-3′ MGBNFQ	71.0	200	

[a] Final concentration of primer or probe in the assay.

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Extraction and purification of bacterial DNA was done using 500 µl aliquots of flower suspensions and the BioSprint 96 Plant DNA extraction kit following manufacturer instructions. DNA was resuspended in 100 µl Milli-Q ultrapure water and stored at −20 °C until used for qPCR.

For quantification, standard curves of *P. agglomerans* strains E325 (for strain-specific detection) or Eh318, respectively, (for pantocin A specific detection) were prepared by adding a range of bacterial cell concentrations (from 7×10^6 to 7×10^1 CFU ml^{-1}) to flower extracts. These flower extracts were obtained from 30 uninoculated apple flowers that had been placed without petals in 30 ml of 10 mM PBS, vortexed briefly and sonicated for 60 s. DNA extraction was done as described above using the BioSprint 96 Plant DNA extraction kit. The number of bacteria in orchard samples was quantified against these standard curves. The slope (k) of the linear regression line between logarithmic values of CFU counts (x-axis) and C_T values (y-axis) was used to calculate the amplification efficiency, $E=(10^{[-1/k]})-1$, where a value of one corresponds to 100% amplification efficiency (Pfaffl, 2001).

3. Results

3.1. Specific qPCR primer design

Three primer sets with their respective probes (Table 1) were designed against sequence targets for *P. agglomerans* species-level detection (*pagR*), biocontrol strain-level detection (E325), and pantocin A producing strains (*paaA*).

Comparative analysis of the draft genome sequence of *P. agglomerans* E325 (Smits and Duffy, unpublished) against the genome of *P. vagans* C9-1 (Smits et al., 2011, 2010) using MAUVE (Darling et al., 2004) yielded several regions that were specific for *P. agglomerans* E325. BlastN analysis against the draft genome of *P. agglomerans* ATCC 27155^T (Smits and Duffy, unpublished) and against the NCBI database narrowed the selection to only few regions. One *P. agglomerans* E325-specific region was identified as an approximately 3 kb genomic island encoding largely hypothetical proteins (Fig. 1). A primer/probe combination was designed to an intergenic region within this genomic island.

One diagnostic trait common to all of *P. agglomerans* strains that was identified was presence of the chromosomally encoded autoinducer gene *pagR* (Chalupowicz et al., 2008; Rezzonico et al., 2009)(Fig. 1). The *P. agglomerans*-specific primers were designed on the basis of 28 *pagR* gene sequences from different *P. agglomerans* strains (Rezzonico et al., 2009) and excluded *pagR* sequences from four strains (Eh252, P6WAL, ACW55802, C9-1) that are only loosely associated to *P. agglomerans* based on the *gyrB* tree (Rezzonico et al., 2009). Pantocin A is the best described antibiotic involved in biocontrol against *E. amylovora* (Ishimaru et al., 1988; Jin et al., 2003a, 2003b). Genetic data from three isolates (*P. agglomerans* Eh318, *P. vagans* C9-1 and *Pantoea* sp. Eh252) showed high sequence identity between the biosynthetic genes (Vanneste et al., 2008), which are located on a 28 kb genomic island (Rezzonico et al., 2009; Smits et al., 2011). A primer/probe set was designed based on the *paaA* gene of *P. vagans* C9-1 (Fig. 1)(Smits et al., 2011), as the encoded PaaA protein is unique in GenBank.

Table 2
Performance characteristics of qPCR assays using standard curves based on dilutions of *P. agglomerans* E325 and Eh318 cells, respectively, mixed with flower extracts ($n=10$ runs).

qPCR assay	Dynamic range[a] (CFU per flower)		Linear regression[b]			Limit of detection (LOD)[c]		
	Low limit	High limit	Slope (k)	R^2	E	CFU per flower	Average C_T	C_T SD
E325	10^3	10^7	−3.269	0.995	1.029	700	38.3	0.47
pagR2	10^3	10^7	−3.263	0.997	1.032	690	39.8	0.62
paaA	10^3	10^7	−3.637	0.997	0.889	660	39.3	1.37

[a] The range of concentrations for which C_T values were in linear relationship with logarithms of concentrations (determined by exploring slope values across sections of C_T values × $\log_{10}$ CFU number).
[b] Linear regression of all positive samples in a plot of C_T values against logarithmic number of *P. agglomerans* cells: k = slope of the linear regression line, R^2 = average square regression coefficient; E = efficiency of amplification.
[c] LOD = limit of detection, for the purpose of this study defined as concentration at which at least one of the parallel reactions was positive; i.e., detecting fewer than three CFU per reaction.

3.2. Sensitivity and specificity of qPCR

Using standard curves based on dilutions of E325 and Eh318 cells, respectively, mixed with flower extracts, a good linearity was observed over a 4-log range (from 10^3 to 10^7 CFU per flower; Table 2). All qPCR assays were highly efficient ($E=0.9$–1.0), detecting fewer than three CFU per reaction (corresponding to 7×10^2 CFU per flower). Linear regression analyses demonstrated slope values of −3.3 to −3.6 with regression coefficients of $R^2>0.99$ (Table 2). These results are similar to qPCR performance and correlation with plating for monitoring the environmental fate of the biocontrol agent *P. fluorescens* EPS62e (Pujol et al., 2006).

To assess specificity, a large collection of *Pantoea* spp. (Table 3), closely related *Erwinia* spp. and other non-*Pantoea* biocontrol strains (Table 4) were tested using the qPCR assays. The increase in fluorescence crossing the threshold value at a specific cycle indicated the positive amplification of the targeted gene. A standard qPCR containing about 10 ng DNA per PCR (approximately 10^6 CFU per reaction), successfully amplified the gene targets with C_T values ranging from 20 (for E325 and *paaA*) to 25 (for *pagR2*).

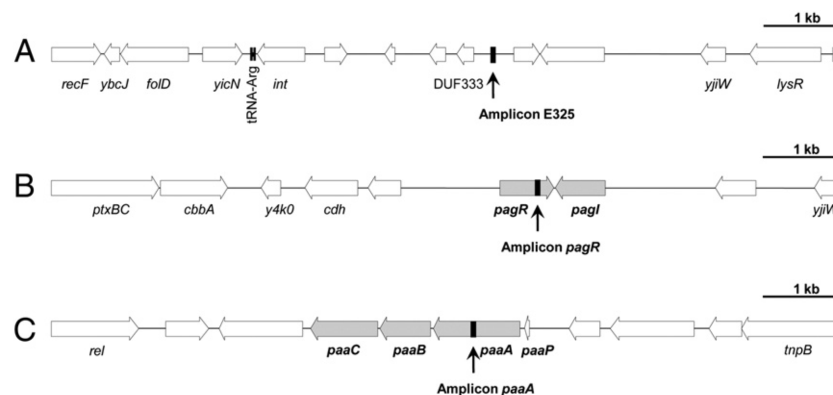

Fig. 1. Genetic maps of amplicon location for design of (A) *Pantoea agglomerans* E325 strain-specific, (B) species-specific, and (C) pantocin A specific quantitative PCR assays.

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Table 3
Results of the qPCR specificity assays for *P. agglomerans* and other *Pantoea* species from worldwide collections.

Species	Strain	qPCR results		
		E325	*pagR2*	*paaA*
Pantoea agglomerans	Eh318	(+)[a]	+	+
	E325	+	+	–
	P10c	–	+	+
	Eh 1087	–	+	–
	Eh 239	–	+	–
	Eh 454	–	+	–
	Eh 460	–	+	–
	EPS125	–	+	–
	ATCC 27987	–	–	–
	ATCC 27155^T	–	+	–
	CIP A181	–	+	–
	EM21cb	–	+	–
	EM22cb	–	+	–
	VA21971	–	+	–
	CIP 82.100	–	+	–
	LMG 2557	–	+	–
	LMG 2595	–	+	–
	LMG 2941	–	+	–
	P1SAA	–	+	–
	P2SAA	–	+	–
	P3SAA	–	+	–
	P4SAH	–	+	–
	P5WAM	(+)	+	–
	P7NSW	–	+	–
	P9QLB	–	+	–
P.agglomerans pv. *gypsophilae*	ATCC 43348	–	+	–
	CFBP 4342	–	+	–
Pantoea sp.	Eh252	–	–	+
Pantoea sp.	P6WAL	–	–	–
Pantoea ananatis	ATCC 27995	–	–	–
	ATCC 27996	–	–	–
	LMG 5342	–	–	–
	LMG 20103	–	–	–
	LMG 2665^T	–	–	–
	LMG 2676	–	–	–
Pantoea conspicua	EM17cb	–	(+)	+
Pantoea dispersa	LMG 2770	–	–	–
	LMG 2603^T	–	–	–
	LMG 2605	–	–	–
	CIP 102701	–	–	–
Pantoea brenneri	ATCC 29001	–	–	–
	LMG 5343^T	–	–	+
Pantoea septica	LMG 5345	–	–	–
Pantoea anthophila	EM13cb	–	–	–
Pantoea stewartii subsp. *indologenes*	CFBP 3614^T	–	–	–
P. stewartii subsp. *stewartii*	CFBP 3517^T	–	–	+
Pantoea vagans	C9-1	–	–	+
	C9-1W	–	–	+
	LMG 24196	–	–	–
	LMG 24199^T	–	–	–
Pantoea sp.	P8SAA	–	(+)	–
Pantoea sp.	P10QLC	–	(+)	–
Pantoea sp.	EPS 486	–	–	–
Pantoea sp.	EPS 595	–	(+)	–

[a] (+) indicates a weak positive fluorescence signal at higher C_T values (>30).

3.3. Strain-specific qPCR

Using the E325 specific probe, no increase in fluorescence above the threshold was recorded for 92 of the tested bacterial strains including closely related *P. agglomerans* and unrelated strains ($n=94$; Table 3, Table 4), resulting in a specificity of 98% (percentage of strains correctly identified) for strain-specific detection. However, *P. agglomerans* strains Eh318 and P5W5 gave a random fluorescent signal at higher C_T values of 31 and 35, respectively (Table 3), which is closer to the detection limit at C_T 38.3 (Table 2) than to the positive detection signal at around $C_T=20$ for the positive control at the same DNA concentration. This indicates that these strains may contain a highly similar, but not identical target site for the E325 probe, having possibly mismatches in the primer or probe regions that cause the qPCR reaction to be less efficient.

Table 4
Results of the qPCR assay specificity tests with species other than *Pantoea*.

Genus	Strain(s)	qPCR results		
		E325	*pagRI2*	*paaA*
Erwinia billingiae	23048B	–	(+)[a]	(+)[a]
	LMG 2613^T, 23050A, DAR72021, 38#14, BE 66	–	–	–
Erwinia amylovora	CFBP 1232^T, CFBP 1430, ATCC 49946, LA025, LA071, LA076, LA102, JL1168, JL1170	–	–	–
Erwinia pyrifoliae	DSMZ 12163^T, Ep 1/96	–	–	–
Erwinia tasmaniensis	Et1/99, Et2/99, Et4/99, BE57, BE65, DAR 61733, LA540	–	–	–
Erwinia piriflorinigrans	CFBP 5882, CFBP 5883, CFBP 5884, CFBP 5885, CFBP 5886, CFBP 5887	–	–	–
Tatumella citrea	LMG 23359	–	–	–
Tatumella punctata	LMG 22097	–	–	–
Pseudomonas syringae pv. *syringae*	NCPPB 281, FAW 460	–	–	–
Pseudomonas fluorescens	A506, CHA0	–	–	–
Bacillus subtilis	QST713, FZB24	–	–	–
Aureobasidium pullulans	CF10	–	–	–
Metschnikowia pulcherima	MSK1	–	–	–

[a] (+) indicates a weak positive fluorescence signal at higher C_T-values (>30).

3.4. Species-specific qPCR

The *pagR2* probe was positive for 24 of the 25 tested *P. agglomerans* strains (Table 3), indicating a high specificity (96%) for species-specific detection. Negative results were obtained only with *P. agglomerans* strain ATCC 27987, a result also obtained in the previous study (Rezzonico et al., 2009). Although this strain was genetically included in *P. agglomerans* based on *gyrB* sequencing and fAFLP, it was shown that this strain does not cluster to this species based on biochemical and MALDI-TOF-MS analysis (Rezzonico et al., 2010). Additionally, three of four strains of a new *Pantoea* sp. (P8SAA, P10QLC, and EPS 595 (Rezzonico et al., 2009, 2010), *Pantoea conspicua* EM17cb (Table 3) and *Erwinia billingiae* 23048B (Table 4) gave random fluorescent signals at higher C_T values (33–37), which are closer to the detection limit at $C_T=39$ (Table 2) than to the C_T around 25 of the positive control. Thus, it seems possible that these species contain only a slightly different *pagR2* sequence compared to *P. agglomerans*.

3.5. Pantocin A specific qPCR

For pantocin A specific detection, the developed assay was highly specific (96%), detecting all so far known pantocin A producers (*P. agglomerans* P10c, Eh318, *Pantoea* sp. Eh252, *P. vagans* C9-1, and *P. brenneri* LMG 5343^T; Table 3), but no unspecific increase in fluorescence above the threshold for any of the other tested strains ($n=94$; Table 3; Table 4). However, two other *Pantoea* species (*P. stewartii* subsp. *stewartii* CFBP 3517^T and *P. conspicua* EM17cb) were also positive for the pantocin A primer/probe set (C_T values of 28.3 and 28.5, respectively). These species were reported before as negative for the presence of the pantocin A genes based on a larger amplicon (Rezzonico et al., 2009), while it is not known whether these species can produce an antibacterial compound.

3.6. qPCR setup for application with field samples

A qPCR setup was chosen where two probes were detected simultaneously during a run, requiring two duplex qPCR runs for the targets envisaged during this study (Fig. 1, Table 1). A first run was done combining the E325 strain-specific probe in combination with the

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P. agglomerans species-specific probe (*pagR2*), the second combined the *paaA* specific probe with *pagR2*. This strategy was chosen to be able to estimate the numbers of E325 related bacteria within the total *P. agglomerans* population and of pantocin A producing bacteria within and beyond the genus *P. agglomerans*. It was observed that for both runs, the results for *pagR2* were comparable, allowing their comparison.

3.7. Incidence of P. agglomerans E325, indigenous P. agglomerans, and pantocin A producing bacteria in orchard bacterial communities

In commercial apple orchards, where *P. agglomerans* E325 was not previously introduced, two of 108 samples (1.7%) were positive with low bacterial cell densities (around 10^3 CFU per flower) in strain-specific qPCR, while in 39% of the tested flower samples (42 of 108) indigenous *P. agglomerans* could be detected with species-specific *pagR2* primers/probes. Here, the cell densities ranged between 10^2 and 5×10^4 CFU per flower. Pantocin A producing strains were found in 7.4% (8 of 108, range 10^2–10^4 CFU per flower) of the flower samples showing only a low incidence of pantocin A producing bacteria in the field. Four *paaA* positive samples (50%) were negative for *pagR2*, indicating the presence of pantocin A producing bacteria that do not belong to the species *P. agglomerans*. These may include other species such as the closely related biocontrol agent *P. vagans* C9-1, shown to contain a pantocin A genomic island (Smits et al., 2011).

4. Discussion

In this study we have developed and validated three new qPCR assays for *P. agglomerans* E325 strain-specific, *P. agglomerans* species-specific and pantocin A specific detection and quantification. Based on specificity testing, the assays proved to be highly specific (96–98%) for the target that they were designed for. The assays were also successfully used to determine the incidence of the targets in field samples.

Random fluorescent signals at higher C_T values for non-target strains has been reported for other bacterial detection assays (Lloyd-Jones et al., 2005; Pujol et al., 2006). It was considered as background no-template control and was not important for quantification if it remained outside the range used to generate the standard curve. Random fluorescent signals at higher C_T values were observed for all three primer sets, but they remained close to the detection limit. These incidences showed that there may be closely related target regions present in these strains, whose amplification efficiency is lower than for the true targets. The lower efficiencies can be caused by slight differences in the target sites for either primers or probes. Future studies with more strains of these species will have to clarify whether this is a common trait in these bacterial species or is restricted to only a few strains within the species.

The impact of the random fluorescent signals on the detection of the targets in field samples will be relatively marginal. The two strains that gave signals at high C_T values in the E325 strain-specific qPCR originated in the USA and New Zealand (Supplemental Table S1) and are so far not in use as active ingredients in any commercially available fire blight control product. With the exception of *Pantoea* sp. EPS 595, the strains with random amplification signals for *pagR2* qPCR originated in Australia or are clinical isolates. The two positive strains in the *paaA* qPCR are clinical isolates or were isolated from a non-fire blight host plant. For all cases, it is rather unlikely that they occur in European pome fruit orchards, since the amplification efficiency of these by-products is low. Therefore, it is relatively unlikely that these organisms might interfere with future *P. agglomerans* species-specific field monitoring studies.

In addition to the specificity tests using pure cultures, field samples were used to gain basal knowledge on the incidence of *P. agglomerans* E325, indigenous *P. agglomerans*, and pantocin A producing bacteria in orchard bacterial communities. Results confirmed specificity values reached with testing pure cultures. The *P. agglomerans* E325 specific gene target is intrinsically only present at very low levels within the total *P. agglomerans* population under field conditions and therefore presents a valuable tool to study the behavior of this biocontrol agent once it is introduced into the field. Indigenous *P. agglomerans*, which might be able to protect the flower stigmas from infection by *E. amylovora*, are present at a higher abundance on apple flowers, potentially giving the orchards an intrinsic biocontrol level. Many potential biological control agents belong to species that are common inhabitants of plants (Lindow and Brandl, 2003; Pusey et al., 2009). As for pantocin A producing strains results indicate only a low prevalence of strains with this biological trait in the field. However, results indicate as well the existence of pantocin A producing bacteria that do not belong to the species *P. agglomerans*. These may include other species such as the closely related biocontrol agent *P. vagans* C9-1, shown to contain a pantocin A genomic island (Smits et al., 2011).

To our knowledge, this is the first report on developing qPCR assays to detect and quantify *P. agglomerans* on the strain and species level as well as for one of its biological traits. This study provides tools that will facilitate investigation and monitoring of the environmental behavior (e.g., survival, dissemination) of strain-specific *P. agglomerans* E325 in field studies relevant to future registration of Bloomtime™ in Europe and will answer questions regarding the risk assessment including potential non-target impact on plants or indigenous bacterial communities in orchards (Berg et al., 2007). Furthermore, ecological studies on the establishment and multiplication of antagonists may answer questions regarding impact of formulation, application rate and timing to get sufficient efficacy against the pathogen. In addition, the species-specific probe can be used in any study relating to *Pantoea* species in general including phytopathological or medical/clinical applications, where correct identification of *P. agglomerans* is required (Rezzonico et al., 2012a, 2009, 2012b, 2010). Finally, we have developed an assay to investigate the prevalence of pantocin A producing bacteria in nature, which might offer a potential application to streamline selection of local, environmental adapted strains with higher biocontrol potential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2012.06.004.

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