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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.

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 × 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.