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ORIGINAL ARTICLE

Development and evaluation of a real-time PCR assay targeting chromosomal DNA of *Erwinia amylovora*

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fire blight, MGB, relative quantification, specific sensitive detection.

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Abstract

Aims: To develop and evaluate a new and reliable real-time PCR detection protocol on chromosomal DNA of the contagious plant pathogenic bacterium *Erwinia amylovora*, the causal agent of fire blight.

Methods and Results: A Taqman® minor-groove-binder real-time PCR assay targeting a hypothetical protein coding gene of *Erw. amylovora* has been developed. Colony PCR of 113 bacterial strains from different taxa was performed to prove specificity. Serial decimal dilutions of *Erw. amylovora* showed a consistent detection sensitivity of 2 bacterial units per μl. All strains of *Erw. amylovora* could be identified, and there were no cross-reactions with matrices or other bacteria also testing naturally contaminated samples.

Conclusions: Rapid, reliable and sensitive detection of *Erw. amylovora* is important to avoid the spread of the disease within orchards, and the distribution by contaminated plant material or vectors carrying the pathogen. The selected conserved target gene allows relative quantitative detection of *Erw. amylovora* from different sources and host taxa. The newly developed protocol also enables the detection of recently found natural strains that lack the species-specific plasmid pEA29, which was so far widely used as target for detection and identification of this plant pathogen by PCR.

Significance and Impact of the Study: This study demonstrates that the newly developed and evaluated real-time assay can specifically be used for identifying all known strains of the EU quarantine plant pathogen *Erw. amylovora*. Low concentrations of the bacteria can be detected and relatively quantified using a different target area than other real-time PCRs designed so far.

Introduction

Erwinia amylovora (Burrill) Winslow *et al.* is a plant pathogenic bacterium causing fire blight on host species mainly within the subfamily *Pomoideae*. Because of its infection dynamics and impact on fruit production, it is classified as a regulated organism in the European Union (EU Council Directive 2000/29, Annex designation: II/A2). It is believed that it was introduced into Europe in the 1950s from North America and has been spreading around the subcontinent on ornamental and crop plants (Crosse *et al.* 1960; van der Zwet 2006) causing economic losses in orchards (Bonn and van der Zwet 2000). The first detection of fire blight in Austria was in the western part of the country in the Vorarlberg province in 1993; in spite of monitoring and strict eradication measures, it has constantly spread from west to the very east of the country within 10 years (Keck 2004). There are also records from Africa, Asia, Oceania and Central America (information available on the EPPO website: http://www.eppo.org. Accessed 31 March 2010).

Often fire blight outbreaks can be linked to the introduction of latent infected plant material (Jock *et al.* 2002; Donat *et al.* 2007). Testing of latent infected plant parts is particularly difficult as very low concentrations of the pathogen may be below detection limits of commonly used serological methods (Llop *et al.* 2000). Generally isolation and enrichment of *Erw. amylovora* on plates is more sensitive but, in samples from asymptomatic plants,

often inhibited by overgrowth with accompanying and/or antagonistic micro-organisms (EPPO 2004).

Several molecular detection methods have been published, which offer an alternative to morphological plating and/or serological testing, mainly targeting the *Erw. amylovora Pst*I fragment of the plasmid pEA29 (Bereswill *et al.* 1992; McManus and Jones 1995; Llop *et al.* 2000; Stöger *et al.* 2006). This nontransmissible plasmid has been described as common to *Erw. amylovora* strains (Falkenstein *et al.* 1989; Laurent *et al.* 1989), with certain traits on the physiology and metabolism of the bacterium. Because of the specificity and the potential role of the pEA29 plasmid in the virulence performance of the bacterium, only a few molecular detection methods have been designed on the chromosomal DNA of *Erw. amylovora* (Bereswill *et al.* 1995; Taylor *et al.* 2001; Obradovic *et al.* 2007). Some genetic variations in the pEA29 plasmid of different strains have been described (Lecomte *et al.* 1997; Kim and Geider 1999; McGhee and Jones 2000; Barionovi *et al.* 2006; Halupecki *et al.* 2006; Rico *et al.* 2008). Even the absence of the pEA29 plasmid in some natural isolated strains showing similar virulence levels could be proved (Llop *et al.* 2006; Mohammadi *et al.* 2009). However, real-time PCR (qPCR) assays on the pEa29 plasmid for the quantification of detected *Erw. amylovora* bacteria are available (Salm and Geider 2004; De Bellis *et al.* 2007). Considering the variability or even the absence of the pEA29 plasmid in some naturally isolated strains, it is evident that new pathogenicity genes for detection of *Erw. amylovora* have been targeted. A recent publication describes the levansucrase gene as a target for a quantitative detection of *Erw. amylovora* (Lehman *et al.* 2008). Several different bacteria species have been described to possess levansucrase genes with certain homologies to *Erw. amylovora* (Hettwer *et al.* 1995; Arrieta *et al.* 2004), imposing a certain probability of cross-reactions when targeting these genes. Two new chromosomal qPCR detection methods, which have been published targeting *ams* genes that are involved in the amylovoran synthesis in *Erw. amylovora* (Mohammadi *et al.* 2009; Pirc *et al.* 2009). The exopolysaccharide amylovoran is known as a pathogenicity factor (Menggad and Laurent 1998), and the expression of the genes involved is induced by conserved regulatory proteins which were first identified in *Escherichia coli* as regulators of the capsule synthesis (Oh and Beer 2005). Interspecific pathogenicity gene exchange in bacteria without losing the functionality of the gene could be shown experimentally on exopolysaccharide-deficient mutants of *Pantoea stewartii* and *Erw. amylovora* (Bernhard *et al.* 1996).

This study focuses on a chromosomal gene sequence coding for a hypothetical protein (systematic i.d. 'AMY1267') first identified in the *Erw. amylovora* strain Ea273. The full genome data of this strain is available on the Sanger Institute homepage (http://www.sanger.ac.uk/Projects/E_amylovora/ Accessed 19 July 2010). The selected gene sequence was compared with the sequences of the US National Center for Biotechnology Information database (NCBI), and no similar identities were found.

The aim of this study was to choose a conserved chromosomal gene as a target for designing a highly specific and sensitive quantitative molecular detection assay for *Erw. amylovora*. This new assay should be suitable for testing latent infected plant material, even with strains that lack the pEA29 plasmid or which have variances in the so far identified pathogenicity genes.

Materials and methods

Bacterial strains and media

All bacterial strains used in this study (Table 1) were grown on King's B agar (Pseudomonas-agar F; Merck, Darmstadt, Germany) at 26°C for 24–36 h. DNA was either purified from these strains with phenol–chloroform–isoamyl alcohol and precipitated with isopropyl alcohol and sodium acetate, or the bacteria were kept in phosphate buffer for 20 min at 95°C and shock-frozen at −20°C for 30 min.

Real-time PCR

Based on a partial sequence of the hypothetical protein 'AMY1267' of the *Erw. amylovora* strain Ea273, a downstream hpEaF (5′-CCGTGGAGACCGATCTTTTA-3′) and an upstream primer hpEaR (5′-AAGTTTCTCCGCCCTACGAT-3′) were designed. Part of this chromosomal target area was already described as specific to *Erw. amylovora* using random amplified polymorphic DNA PCR with subsequent molecular cloning and sequencing (Obradovic *et al.* 2007). The specificity of the new primers annealing sites on the selected gene was tested *in silico* by aligning the primers and probe sequences with data of *Erwinia* spp. from the NCBI database and *in vitro* using Power SybrGreen PCR Master Mix (Applied Biosystems, Vienna, Austria). After the *in vitro* specificity tests with related and out-group strains (see Table 1), a FAM Taqman® minor-groove-binder (MGB) nonfluorescent quencher probe hpEaP (5′-TCGTCGAATGCTGCCTCTCT-3′) was desig-ned. Primers were synthesized by Eurofins MWG (Ebersberg, Germany) and the Taqman®-MGB probe by Applied Biosystems. For the assay, a Taqman® Universal PCR Master Mix (AmpErase, uracil-*N*-glycosylase; Applied Biosystems) was used. Reactions were run in 20 μl volume using 0·5 mmol l^{-1} primers and 0·05 mmol l^{-1} probe and 1 μl template. Reactions were run on an Eppendorf Realplex^4 Mastercycler

Table 1 Bacterial strains used and qualitative results of the new qPCR (this study)

Strains	Family	Species	Host plant/matrix	Geographical origin	Year of isolation	qPCR results
	Enterobacteriaceae					
AGES295/93*		*Erwinia amylovora*	*Cotoneaster salicifolius*	Austria/Vorarlberg	1993	+
AGES674/94†			*Pyrus communis*	Austria/Vorarlberg	1994	+
AGES273/98			*Malus domestica*	Austria/Vorarlberg	1998	+
AGES329/98			*Cotoneaster* sp.	Austria/Vorarlberg	1998	+
AGES511/98			*Pyrus communis*	Austria/Salzburg	1998	+
AGES963/99			*Sorbus aucuparia*	Austria/Styria	1999	+
AGES3723/00			*Sorbus aucuparia*	Austria/Burgenland	2000	+
AGES1851/01			*Sorbus aria*	Austria/Vorarlberg	2001	+
AGES2642/01			*Sorbus torminalis*	Austria/Vorarlberg	2001	+
AGESWeide1‡			*Salix* sp.	Austria	2001	+
AGES3218/02			*Malus domestica*	Austria/Lower Austria	2002	+
AGES3331/02			*Pyrus communis*	Austria/Lower Austria	2002	+
AGES3956/02			*Malus domestica*	Austria/Upper Austria	2002	+
AGES3962/02			*Pyrus communis*	Austria/Lower Austria	2002	+
AGES3975/02			*Cotoneaster* sp.	Austria/Upper Austria	2002	+
AGES1041/03			*Pyrus communis*	Austria/Lower Austria	2003	+
AGES1141/03			*Malus domestica*	Austria/Styria	2003	+
AGES1144/03			*Acer palmatum*	Austria/Styria	2003	+
AGES1168/03			*Pyrus communis*	Austria/Lower Austria	2003	+
AGES1170/03			*Pyrus communis*	Austria	2003	+
AGES1196/03			*Malus domestica*	Austria/Carinthia	2003	+
AGES1672/03			*Prunus domestica*	Austria/Styria	2003	+
AGES374/03			*Cydonia oblonga*	Austria/Styria	2003	+
AGES376/03			*Malus domestica*	Austria/Styria	2003	+
AGES377/03			*Malus domestica*	Austria/Styria	2003	+
AGES378/03			*Malus domestica*	Austria/Styria	2003	+
AGES2663/03			*Pyracantha* sp.	Austria/Burgenland	2003	+
AGES408/05§			*Cydonia oblonga*	Austria/Styria	2005	+
AGES351/05§			*Sorbus* sp.	Austria/Lower Austria	2005	+
AGES769/05			*Cydonia oblonga*	Austria/Burgenland	2005	+
AGES384/07			*Chaenomeles* sp.	Austria/Tirol	2007	+
AGES3156/07			*Fragaria* sp.	Austria	2007	+
AGES3374/08			*Malus domestica*	Austria/Vorarlberg	2008	+
AGES392/09			*Malus domestica*	Austria/Tirol	2009	+
AGES568/09			*Pyrus communis*	Austria/Carinthia	2009	+
AGES602/09			*Pyrus communis*	Austria/Tirol	2009	+
AGES603/09			*Pyrus communis*	Austria/Carinthia	2009	+
CFBP1232¶			*Pyrus communis*	UK	1959	+
CFBP1399			*Malus sylvestris*	Denmark	1972	+
CFBP1430			*Crataegus oxyacantha*	France	1972	+
CFBP2150			*Rubus* sp.	USA		+
CFBP2151			*Rubus* sp.	USA		+
CFBP2582			*Pyrus communis*	Irland	1986	+
CFBP2584			*Cotoneaster* sp.	Irland	1986	+
CFBP3042			*Pyrus calleryana*	UK	1964	+
CFBP3049			*Malus domestica*	Canada		+
CFBP3794			*Rosa* sp.	Iran		+
DSM17948			*Malus domestica*	Germany	1979	+
Ea91/R2			*Pyrus communis*	Germany	1995	+
Ea40			*Malus domestica*	USA		+
Ea458**			*Malus domestica*	Greece		+
Ea450††			*Pyrus amygdaliformis*	Greece	1994	+
ERA45/85‡‡			*Pyrus* sp.	Greece	1994	+
GCCM909			*Pyrus communis*	Greece		+

Table 1 (*Continued*)

Strains	Family	Species	Host plant/matrix	Geographical origin	Year of isolation	qPCR results
Ea12			*Cotoneaster* sp.	Hungary	1998	+
Ea17			*Cotoneaster* sp.	Hungary	1998	+
115-22			*Cydonia oblonga*	Bulgaria	1989	+
7/74			*Cotoneaster bullatus*	Germany		+
IVIA1509-B			*Malus domestica*	Spain	1995	+
IVIA1578-3			*Crataegus* sp.	Spain	1996	+
IVIA1596§§			*Pyrus communis*	Spain	1996	+
IVIA1598-3				Spain		+
IVIA1614-2§§			*Crataegus* sp.	Spain	1996	+
IVIA1892-1			*Pyrus communis*	Spain	1998	+
IVIA1951-8			*Pyracantha* sp.	Spain	1998	+
ICMP13415			*Malus domestica*	Hungary	1996	+
NCPPB2291			*Rubus idaeus*	USA		+
NCPPB2292			*Rubus idaeus*	USA	1949	+
NCPPB595¶¶			*Pyrus communis*	UK	1958	+
OMPBO1204.1/94			*Pyracantha* sp.	Italy	1994	+
OMPBO691.2/95			*Pyrus communis*	Italy	1995	+
OMPBO1185			*Malus domestica*	Italy		+
CFBP4171		*Erwinia pyrifoliae*	*Pyrus pyrifolius*	South Korea	1996	–
GEKB		*Enterobacter amnigenus*	Soil	Austria	2003	–
VII/3			Ammonia reducing product		2001	–
E. coli		*Escherichia coli*				–
AHN2		*Leclercia adecarboxylata*	Soil	Hungary	2003	–
AHN8			Soil	Hungary	2003	–
AHN9			Soil	Hungary	2003	–
CFBP1255		*Pantoea agglomerans*	*Sorbus* sp.	USA		–
GEKH			Soil	Austria	2003	–
R95-91						–
MB0838		*Pantoea* sp.	Air sample	Austria	2008	–
AGES-Ungarn		*Pantoea stewartii* ssp. *stewartii*	*Zea mays*	Brazil	1999	–
CFBP1719			*Zea mays*	USA		–
CFBP3167¶			*Zea mays* var. *rugosa*	USA	1970	–
CFBP3614¶		*Pantoea stewartii* ssp. *indologenes*	*Setaria italica*	India	1970	–
CFBP3394		*Pantoea stewartii* ssp. *stewartii*	Coleoptera	USA	1954	–
DSM30170		*Pectobacterium carotovorum* ssp. *carotovorum*	*Solanum tuberosum*			–
5.1		*Pragia fontium*	Soil	France	2002	–
TFI-08		*Raoultella terrigena*	Tree Fertilizer Injector	the Netherlands	2002	–
BR780/01B		*Serratia marcescens*	Soil	Austria/Carinthia	2001	–
	Pseudomonadaceae					
CFBP2431¶		*Pseudomonas corrugata*	*Solanum lycopersicum*	UK	1972	–
CFBP2351		*Pseudomonas syringae* pv. *morsprunorum*	*Prunus domestica*	USA		–
CFBP1573		*Pseudomonas syringae* pv. *persicae*	*Prunus persica*	France		–
CFBP1392¶		*Pseudomonas syringae* pv. *syringae*	*Syringa vulgaris*	UK	1950	–
	Xanthomonadaceae					
LMG849¶		*Xanthomonas axonopodis* pv. *poinsettiicola*	*Euphorbia pulcherrima*	India	1950	–
CFBP2157¶		*Xanthomonas fragariae*	*Fragaria* sp.	USA	1960	–
DSM50861¶		*Xanthomonas vesicatoria*	*Solanum lycopersicum*	New Zealand	1955	–
DSM10026		*Xylella fastidiosa*	*Vitis* sp.	USA		–
	Sphingomonadaceae					
AGES-NR08		*Novosphingobium resinovorum*	*Euphorbia pulcherrima*	Austria	2007	–
	Rhizobiaceae					
DSM6583		*Agrobacterium vitis*	*Vitis vinifera*	Greece		–

Table 1 (*Continued*)

Strains	Family	Species	Host plant/matrix	Geographical origin	Year of isolation	qPCR results
DSM30205¶		*Agrobacterium tumefaciens*	*Malus* sp.			–
	Microbacteriaceae					
AGES-CMS		*Clavibacter michiganensis* ssp. *sepedonicus*	*Solanum tuberosum*			–
	Burkholderiaceae					
CFBP4823		*Ralstonia solanacearum*	*Solanum tuberosum*	Egypt		–
CFBP4812			*Solanum tuberosum*	France	1996	–
CFBP4813			*Solanum lycopersicum*	France	1996	–
	Bacillaceae					
NCTC10341¶		*Bacillus licheniformis*				–
NCTC10334¶		*Bacillus coagulans*				–
NCTC2599¶		*Bacillus cereus*				–
NCTC10315		*Bacillus subtilis* ssp. *subtilis*				–
	Staphylococcaceae					
ATCC65389		*Staphylococcus aureus*				–
	Micrococcaceae					
ATCC10240		*Micrococcus luteus*	Air sample			–

OMP BO, Osservatorio delle Malattie delle Piante, Bologna, Italy; CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France; AGES, Austrian Agency for Health and Food Safety, Vienna, Austria; DSM, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; LMG, Belgian Co-ordinated Collections of Micro-organisms (BCCM/LMG), Gent, Belgium; IVIA, Instituto Valenciano de Investigaciones Agrarias, Moncada, Spain; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; ICMP, International Collection of Microorganisms from Plants, Auckland, N.Z.; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK; ATCC, American Type Culture Collection, Manassas, USA; GCCM, Greek Coordinated Collections of Microorganisms, Athens, Greece; Ea12, 17, source M. Hevesi, Corvinus University of Budapest, Hungary; Ea91/R2, source K. Richter; Ea40, source unknown; ERA45/85, source P. Psallidas; Ea450, Ea458, source J. Tsiantos; 115-22, source S. Bobev; 7/74, source W. Zeller; all other strains, source M. Gorfer.

*CFBP6449.

†CFBP4175.

‡Occasional infection in the greenhouse.

§From latent infection.

¶Type strain.

**CFBP3466.

††CFBP3465.

‡‡CFBP3064.

§§Without pEA29 plasmid.

¶¶CFBP1196.

Epgradient S (Eppendorf, Hamburg, Germany) under following conditions: 50°C for 3 min (AmpErase activity), 95°C for 10 min; 50 cycles of 95°C 15 s and 60°C for 1 min and endpoint fluorescence readings in each cycle. The expected fragment size was calculated at 138 bp and was confirmed by electrophoresis on a 2% agarose gel and stained with ethidium bromide compared to a 100- bp ladder (Invitrogen, Carlsbad, CA) (data not shown). Specificity was again tested with bacterial suspensions and/or purified DNA from the strains indicated in Table 1, but also on natural infected samples by comparison with the conventional PCRs described in Bereswill *et al.* (1992) and Stöger *et al.* (2006).

The naturally infected samples were young branches, flowers, lignified plant parts and honey bees caught in areas with potential fire blight infections. The natural infected young branches were processed according to EPPO (2004) (for symptomatic material), and suspected colonies were boiled in phosphate buffer. Surface of flowers was washed with phosphate buffer (modified after Thomson *et al.* 1974), and suspensions were kept at 95°C for 15 min. For detection of *Erw. amylovora* in planta, lignified plant part was processed according to the protocol described by Stöger *et al.* (2006). Honey bees were macerated, and DNA obtained according to a protocol by Chomczynski and Sacchi (1987) modified for DNA extraction. For positive controls, samples were spiked with 10^6 colony-forming units (CFU) of the strain AGES 295/93.

Relative quantification of *Erwinia amylovora*

For the relative quantification of the bacteria, cell suspensions with defined concentrations were prepared from bacterial colonies on King's B agar, dissolved in water.

Three replicates of decimal dilutions were plated on King's B, and the optical density (OD) at 600 nm was determined and correlated after counting the bacterial colonies on the plates (data not shown). The OD of 0·1 corresponds to 2×10^8 CFU ml^{-1} of *Erw. amylovora*. Suspensions were adjusted to 2×10^8 CFU ml^{-1} boiled for 20 min at 95°C and shock-freezed at −20°C for 30 min. Seven decimal dilutions in three replicates were made from the stock suspensions and used as standards in each run. Linear regression curves were obtained by plotting cycle threshold (C_t) values of each reaction against logarithmic values of *Erw. amylovora* units.

Results

Specificity of the assay

After testing 113 bacterial strains from different taxa (see Table 1) and additional environmental samples (374 plant samples and 65 bee samples, see Table 2), the assay proved to be 100% specific to *Erw. amylovora*, independent of the host plant or the origin of the strain. There was no cross-reaction with any bacterium from another taxa or with the tested matrices. No significant similarity of the primers and probe with any nucleotide sequence in the NCBI database could be shown by *in silico* comparison (maximum query coverage 85%).

Sensitivity of the assay

For the determination of the analytical sensitivity of the assay, bacterial suspensions of the *Erw. amylovora* strain AGES 295/93 were prepared. Serial decimal dilutions with three replicates down to 0·2 bacterial units per μl were prepared and tested with each run. The dilution with 2 bacterial units per μl was always amplified by the assay with cycle threshold (C_t) values of about 38. The dilution of 0·2 bacterial units per μl was inconsistently amplified. Furthermore, the sensitivity of the assay was compared with the conventional PCR published by Bereswill *et al.* (1992) and the qPCR published by Salm and Geider (2004) testing serial decimal dilutions (see Table 3). The new assay was at least ten times more sensitive than the other two PCR assays under tested conditions.

qPCR assay performance

The efficiency of the assay could be shown by analysing a standard curve with decimal serial dilutions of bacteria suspensions in three replicates. The slope was −3·405 and the efficiency (E) was 0·99 (99%) according to the equation $E = 10^{[-1/\text{slope}]} - 1$. The precision represented by the correlation coefficient of the standard curve (R-squared) was 0·996. The PCR amplification was 1·98 (compared to a theoretical maximum value of 2·0) demonstrating the high sensitivity of this assay. These values were generated by the Eppendorf realplex software, version 2.0.

Discussion

The validation of the newly developed assay presented in this study demonstrates a 100% specificity and consistent sensitivity down to the DNA equivalent of 2 bacterial units per reaction. A larger overlapping target area has already been described as specific to *Erw. amylovora*, however with different primer annealing sites (Obradovic *et al.* 2007).

The novelty of this Taqman®-MGB assay was the detection of all *Erw. amylovora* strains tested from a variety of geographical areas, isolation dates and host plants. All the *Erw. amylovora* strains tested in this study were isolated over the last 50 years and were readily detected by the new assay. This gives strong evidence for the highly conserved status of the selected genomic area, which has not been used as a target for a real-time PCR

Table 2 Positive detection of *Erwinia amylovora* in the same samples using different methods, in relation to the total number of environmental samples analysed

Samples	Total nr. of samples	Taqman qPCR (this study)	PCR Bereswill (1992)	Isolation EPPO (2004)*
Honey bees	65	19	5	–
Flowers	15	3	0	1
Symptomatic samples	225	88	73	88
Asymptomatic samples	134	15	2	7

*Suspected bacterial colonies were confirmed by PCR.

Table 3 Comparison of detection sensitivity between different PCR assays on bacterial suspensions of the *Erwinia amylovora* AGES 295/93 strain with serial decimal dilutions

Erw. amylovora (bacterial units) per μl	PCR Bereswill *et al.* (1992)	*SybrGreen* qPCR Salm and Geider (2004)	Taqman qPCR (this study)
200 000	+	+	+
20 000	+	+	+
2000	+	+	+
200	+	+	+
20	+*	+	+
2	–	+*	+
0·2	–	–	+*

*Inconsistent positive results.

so far. Sequencing of the amplicon of the AGES 295/93 strain showed 100% identity with sequences of the strains CFBP1430 (accession no. FN434113.1) and ATCC 49946 (accession no. FN666575.1); to date the only two fully sequenced strains of *Erw. amylovora* in the NCBI database. However, a conventional PCR with primer annealing sites in close vicinity of this new assay has also shown high sensitivity (Obradovic *et al.* 2007), but unsatisfactory specificity when tested on other *Erw. amylovora* strains (data not shown) than the strains described in Obradovic *et al.* (2007). The high specificity and sensitivity of the new assay designed, imposes that it is suitable for the specific detection and identification of all *Erw. amylovora* isolates. This includes strains lacking the pEA29 plasmid or strains showing possible variation on other less conserved genomic areas. Moreover, chromosomal real-time PCR methods targeting genes involved in pathogenicity of this pathogen (Mohammadi *et al.* 2009; Pirc *et al.* 2009) could readily be combined with this new developed assay, even increasing reliability of *Erw. amylovora* quantitative detection and confirmation in critical cases.

Taqman® DNA probes conjugated with MGB groups are more specific for single base mismatches increasing the specificity of the assay. With such probes, fluorescence quenching is more efficient and sensitivity of the assay is increased in comparison to probes with conventional quenchers (Kutyavin *et al.* 2000). The enhanced melting temperature of the hybridized MGB probes enables precise 5′-nuclease cleavage by the *Taq* polymerase during strand extension improving the fluorescence signal (Kutyavin *et al.* 2000). This is expected to increase the robustness and reliability of the assay compared to Taqman® assays without MGB probes when detecting low numbers of the target organism from natural samples. This is the first Taqman® real-time PCR assay for the detection of *Erw. amylovora* using MGB groups.

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