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Erwinia amylovora loop-mediated isothermal amplification (LAMP) assay for rapid pathogen detection and on-site diagnosis of fire blight

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ABSTRACT

Several molecular methods have been developed for the detection of *Erwinia amylovora*, the causal agent of fire blight in pear and apple, but none are truly applicable for on-site use in the field. We developed a fast, reliable and field applicable detection method using a novel target on the *E. amylovora* chromosome that we identified by applying a comparative genomic pipeline. The target coding sequences (CDSs) are both uniquely specific for and all-inclusive of *E. amylovora* genotypes. This avoids potential false negatives that can occur with most commonly used methods based on amplification of plasmid gene targets, which can vary among strains. Loop-mediated isothermal AMPlification (LAMP) with OptiGene Genie II chemistry and instrumentation proved to be an exceptionally rapid (under 15 min) and robust method for detecting *E. amylovora* in orchards, as well as simple to use in the plant diagnostic laboratory. Comparative validation results using plant samples from inoculated greenhouse trials and from natural field infections (of regional and temporal diverse origin) showed that our LAMP had an equivalent or greater performance regarding sensitivity, specificity, speed and simplicity than real-time PCR (TaqMan), other LAMP assays, immunoassays and plating, demonstrating its utility for routine testing.

1. Introduction

Erwinia amylovora is a Gram-negative enterobacterium first described in the USA in the 1790s (Bonn and Zwet, 2000). It infects more than 200 host species in the *Rosaceae* family causing fire blight on economically important cultivars of the subfamily Spiraeoideae (van der Zwet and Keil, 1979). Introduced to Europe in the 1950s, it is due to its devastating effects mainly on apple and pear orchards, listed as a regulated quarantine organism in the EPPO region (Anonymous, 2004), as well as in Australia, Japan and the USA (Calvin and Krissoff, 1998; Roberts et al., 1998). Several studies have shown that the greatest genetic diversity occurs in North America on *Rubus* and Spiraeoideae host plants (Braun and Hildebrand, 2005; McManus and Jones, 1995). Within Europe, the diversity was shown to be much lower (Rezzonico et al., 2011; Smits et al., 2011a). However, further effort using whole genome sequencing or genotyping of informative DNA markers is required in order to deliver more data and to infer migratory and evolutionary histories of *E. amylovora*.

Natural dispersal of *E. amylovora* occurs by insects or rain splashes on a local scale. Over long distances, *E. amylovora* can be transmitted by trade host plants which are latently infected or have undetectable cankers (Anonymous, 2004). The way the disease has spread in Europe indicates that aerosols may have played a role in the spread of the pathogen over long distances (McManus and Jones, 1994). The symptoms caused by *E. amylovora* consist of wilting of leaves and shoots with a color change from green to brown or even black depending on the host. Bacterial ooze and cankers can be recognized on shoots of heavily infected plants which will not survive the infection (Thomson, 2000). The course of the infection is strongly dependent on climate and weather causing severe fire blight seasons and years with low infections and tree losses (Johnson and Stockwell, 1998). Control measurements consist of eradication of infected plants and therefore substantial economic damage or treatment with antibiotic compounds to prevent infection. Antibiotics though, have unknown effects on the development of resistance (McGhee et al., 2011) and potentially show unwanted side effects on bees and other pollinators. Several research projects also focus on the development of antimicrobial peptides (Güell et al., 2012), phage therapy (Born et al., 2011; Müller et al., 2011) or bacterial antagonists of *E. amylovora* (Chen et al., 2009; Halgren et al., 2011; Paternoster et al., 2010; Pusey et al., 2011; Smits et al., 2011b). In the long-term,

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there is hope and anticipation that breeding of resistant apple cultivars will contribute to integrated control of this disease (Jensen et al., 2012; Khan et al., 2007; Le Roux et al., 2010).

Implementation of phytosanitary control measures can be drastic and relies upon accurate detection and diagnosis, preferably with quantitative measurement of pathogen inoculum presence. A number of detection methods have been published in recent years that are based upon semi-selective culture plating (Kritzman et al., 2003), serology (Braun-Kiewnick et al., 2011), PCR (Powney et al., 2011), and real-time PCR based on TaqMan chemistry (Gottsberger, 2010; Pirc et al., 2009). A new molecular technology, LAMP (Moradi et al., 2012; Temple and Johnson, 2011), is revolutionizing clinical diagnostics and gaining attention for improving plant pathogen detection and diagnostics (Harper et al., 2010; Rigano et al., 2010; Tomlinson et al., 2010). LAMP has a number of intrinsic advantages over PCR: it is generally faster, more specific, simpler to learn and interpret, and more adaptable to on-site implementation for field diagnostics (Fang et al., 2010a, 2010b). We have recently shown that integration with unique, stable reaction chemistry and with Genie II (OptiGene, Horsham, UK), a new inexpensive, small, easy to use and portable isothermal real-time detection device (Bühlmann et al., 2013), elaborates these advantages of LAMP and has been found in a recent EPPO and Q-Detect survey to garner greater acceptance by phytosanitary officers and diagnosticians (unpublished, www.qdetect.org/3_events/meeting_detail.php?ID=7&cat=2).

Recently reported LAMP methods for *E. amylovora* (Moradi et al., 2012; Temple and Johnson, 2011) have intrinsic limitations warranting improvements to enable detection of all *E. amylovora* strains including those lacking the plasmid pEA29 (Llop et al., 2006). Improvements were made to eliminate false positive reactions that the already existing methods deliver and to add better validation data to the *E. amylovora* LAMP assay. Although gene targets on plasmids providing pathogenicity to otherwise harmless bacteria are an elegant way to bypass the labor intensive search for suitable targets in the chromosome, such a strategy can lead to false negative results in bacteria that have lost the plasmid but still elicit pathogenicity as shown previously (Llop et al., 2006). In this study, several published genomes as well as unpublished draft genomes were used to apply a comparative genomic strategy in order to find suitable gene targets for specific detection of *E. amylovora*. Such a comparative genomic approach has advantages over other methods (Gottsberger, 2010) due to the complete genomic information used which leads to a lower probability of misidentification. A LAMP assay was developed using the sequence information identified. Following validation by comparison with standard techniques, the LAMP assay was shown to have similar performance characteristics.

2. Material and methods

2.1. Bacterial strains and culture conditions

A geographically and genetically representative collections (Rezzonico et al., 2012, 2011) of *E. amylovora* isolates ($n=25$), related *Erwinia* species ($n=16$) and other bacteria expected to co-occur on host plants ($n=11$) (Table 1) were used. These bacterial strains were obtained from several sources including culture collections, gifts from other laboratories, or our own field samples and originated from areas in Europe, North America, New Zealand, and the Mediterranean region. These strains were isolated from various cultivars of apple and pear, as well as other fire blight host species within the *Rosaceae* (Table 1). Strains were grown overnight (16–18 h) at 28 °C on Luria Bertani (LB) agar plates prior to boiling in sterile H_2O at 99 °C for 20 min.

2.2. Comparative genomics and primer design

A total of 12 *E. amylovora* complete and draft genome sequences and a further 12 genomes from bacterial species expected to co-occur on host plants of *E. amylovora* such as other *Erwinia* or *Pantoea* species (Table 2) were used for the *in silico* analysis. We employed a comparative genomic pipeline using the program EDGAR (Efficient Database framework for comparative Genome Analyses using BLAST score Ratios) version 1.2 (Blom et al., 2009) and the stand-alone BLASTN version 2.2.22 (Altschul et al., 1990). The remaining sequences were filtered using web BLAST, selecting against sequences with partial hits on full NCBI nt database. The true singleton CDSs were screened for possible LAMP primers using the program LAMPdesigner version 1.02 (Premier Biosoft International, Palo Alto, CA, USA).

2.3. Primer validation specificity and sensitivity

All designed primers were synthesized at Microsynth AG (Balgach, Switzerland). The primer sets for the two most specific targets identified above were ordered as full LAMP sets and tested in triplicate for specificity and sensitivity with boiled cells and plant extracts spiked with *E. amylovora* DNA. Additionally, the performance of the newly developed LAMP assay was tested together with other LAMP assays (Moradi et al., 2012; Temple and Johnson, 2011) to compare specificity and speed. LAMP was performed in 12 µl reactions on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) at 65 °C for 30 min or on Genie II (OptiGene) under the same conditions. A melting curve was established after the amplification by measuring fluorescence while cooling from 95 to 70 °C to detect the amplification product specific annealing temperature T_a. For the LAMP reaction, Isothermal MMX (OptiGene) was used in a 1× concentration and a reaction volume of 12 µl. The following primer concentrations were used: 0.16 µM outer primers, 1.6 µM inner primers and 0.8 µM loop primers. Fluorescence was detected in real time on the FAM channel with no reference dye. Sensitivity was tested on a dilution series of boiled cells of *E. amylovora* CFBP 1430, ranging from 1.2×10^9 CFU/ml to 1.2×10^2 CFU/ml in different matrices. Visual determination of positive reactions was achieved by adding 1 µl of Quant-IT™ Pico Green® Reagent (Invitrogen, Carlsbad, CA, USA).

2.4. Performance comparison

The performance of the newly developed LAMP assay was compared to standard plating on LB media, immunoassays using the Ea AgriStrip®, a lateral flow immunoassay using polyclonal antibodies developed by injecting heat-killed *E. amylovora* cells into rabbits (Braun-Kiewnick et al., 2011) and a real-time PCR assay developed (Pirc et al., 2009). All real-time PCR reactions were run using TaqMan environmental master mix 2.0 (ABI) and performed on the 7500 Fast Real-Time PCR System.

2.5. Bacterial DNA isolation from artificially inoculated and naturally infected plant samples

To test the applicability of the developed LAMP assay for field samples it was validated on 90 samples. The samples were obtained from inoculating the apple cultivars Enterprise, Gala, Heimenhofer, and Liberty by spraying blossoms with an inoculum solution of *E. amylovora* FAW 610 at a concentration of 1×10^6 CFU/ml. At 3 week post inoculation, 0.1 g samples of leaves and/or twigs were collected in 1 ml PBS buffer. The samples were tested with an Ea AgriStrip®, (lateral flow based immunoassay) Bioreba AG (Reinach, Switzerland), and 100 µl were plated on Luria Bertani (LB) agar plates. To perform the LAMP assay or real-time PCR, bacterial DNA was isolated using the Biosprint 96 Plant Kit (Qiagen AG, Hilden, Germany). All real-time PCR was performed as described in Pirc et al. (2009). Briefly, 1 µl of purified DNA was mixed with 1× TaqMan environmental master mix 2.0 (Qiagen). Primers and probes were used at concentrations of 900 nM and 400 nM, respectively. The reactions were performed on an ABI PRISM® 7500 Sequence Detection

Table 1
Bacterial strains used for specificity testing in LAMP and TaqMan PCR.

Species	Strain[a]	Origin	Host plant	LAMP[b]	Temple LAMP[b]	Moradi LAMP[b]
Erwinia amylovora	AFRS 2	USA	*Malus* sp.	+	+	+
E. amylovora	MO-35	USA	*Malus* sp.	+	+	+
E. amylovora	MO-E-101b	USA	*Malus* sp.	+	+	+
E. amylovora	SLAPL-3	USA	*Malus* sp.	+	+	+
E. amylovora	Ea7-96r	Canada	*Rubus* sp.	+	+	+
E. amylovora	Ea6-96r	Canada	*Rubus* sp.	+	+	+
E. amylovora	ACW 56400	Switzerland	*Pyrus* sp.	+	+	+
E. amylovora	ATCC 49946	USA	*Malus* sp.	+	+	+
E. amylovora	Ea153	USA	*Malus* sp.	+	+	+
E. amylovora	01SFR-BO	Italy	*Sorbus* sp.	+	+	+
E. amylovora	JL1185	USA	*Pyrus* sp.	+	+	+
E. amylovora	CFBP 1430	France	*Crataegus* sp.	+	+	+
E. amylovora	Ea1/79	Germany	*Malus* sp.	+	+	+
E. amylovora	UPN527	Spain	*Malus* sp.	+	–	+
E. amylovora	Ea110	USA	*Malus* sp.	+	+	+
E. amylovora	CFBP 3792	USA	*Prunus* sp.	+	+	+
E. amylovora	UTRJ2	USA	*Malus* sp.	+	+	+
E. amylovora	LebA3	Lebanon	*Malus* sp.	+	+	+
E. amylovora	ATCC BAA-2158	USA	*Rubus* sp.	+	+	+
E. amylovora	IH3-1	USA	*Rhaphiolepis* sp.	+	+	+
E. amylovora	OR25	USA	*Pyrus* sp.	+	+	+
E. amylovora	LA036	USA	*Malus* sp.	+	+	+
E. amylovora	JL1168	USA	*Pyrus* sp.	+	+	+
E. amylovora	Ea644	USA	*Rubus* sp.	+	+	+
E. amylovora	Ea646	USA	*Rubus* sp.	+	+	+
Erwinia aphidicola	CFBP 6829^T	Japan	*Acyrthosiphon* sp.	–	–	–
Erwinia billingae	LMG 2613^T	UK	*Pyrus* sp.	–	–	–
Erwinia lupinicola	CU3299^T	N/A	N/A	–	–	–
Erwinia oleae	CFBP 6632^T	Spain	*Olea* sp.	–	–	–
Erwinia papayae	CFBP 5189^T	Martinique	*Carica* sp.	–	–	–
Erwinia persicina	CFBP 3622^T	Japan	*Lycopersicon* sp.	–	–	–
Erwinia piriflorinigrans	CFBP 5888^T	Spain	*Pyrus* sp.	–	–	–
Erwinia psidii	CFBP 3627^T	Brasil	*Psidium* sp.	–	–	–
Erwinia pyrifoliae	DSM 12163^T	South Korea	*Pyrus* sp.	–	–	+
E. pyrifoliae	CFBP 4174	South Korea	*Pyrus* sp.	–	–	+
Erwinia rhapontici	ACW 41072	Switzerland	*Malus* sp.	–	–	–
E. rhapontici	ACW 44286	Switzerland	*Malus* sp.	–	–	–
E. rhapontici	CFBP 3163^T	UK	*Rheum* sp.	–	–	–
Erwinia tasmaniensis	LMG 25318^T	Australia	*Malus* sp.	–	–	–
Erwinia toletana	LMG 24162^T	Spain	*Olea* sp.	–	–	–
Erwinia tracheiphila	LMG 5021	USA	*Cucumis* sp.	–	–	–
Agrobacterium tumefaciens	C58	USA	*Prunus* sp.	–	–	–
Brenneria rubrifaciens	LMG 2709^T	USA	*Juglans* sp.	–	–	–
Dickeya dadantii	CFBP 2048^T	USA	*Chrysanthemum* sp.	–	–	–
Enterobacter cancerogenus	DSM 17580^T	Czech Republic	*Populus* sp.	–	–	–
Lonsdalea quercina subsp. *quercina*	NCPPB 1852^T	USA	*Quercus* sp.	–	–	–
Pantoea agglomerans	ATCC 27155 ^{T}	Zambia	Knee wound	–	–	–
Pantoea vagans	C9-1	USA	*Malus* sp.	–	–	–
Pectobacterium cacticida	NCPP B3849^T	USA	*Carnegiea* sp.	–	–	–
Pseudomonas syringae pv. *persicae*	CFBP 1573	France	*Prunus* sp.	–	–	–
Stenotrophomonas maltophila	CFBP 3035^T	USA	Cancer patient	–	–	–
Xanthomonas albilineans	DSM 3583^T	Fiji	*Saccharum* sp.	–	–	–

[a] Culture collections are abbreviated as CFBP (Collection Française de Bactéries associées aux Plantes), DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen), LMG (Collection of the Laboratorium voor Microbiologie en Microbiele Genetica), NCPPB (National Collection of Plant Pathogenic Bacteria). Superscripts ^{T} following strain names indicate the type strain of a species.
[b] LAMP using primer set EAMY3196; Temple LAMP using primers from (Temple and Johnson, 2011); Moradi LAMP using primers from (Moradi et al., 2012).

Systems using universal cycling conditions, 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.

2.6. Field samples

The developed assay was validated on 100 field samples from all over Switzerland. Infected trees of diverse host species and locations were sampled by collecting 0.1 g of infected plant material and grinding it in 1 ml 1× PBS buffer prior to testing it with Ea AgriStrip® (Braun-Kiewnick et al., 2011), plating on LB media, and the LAMP method described above. The results were compared with each other in order to gain information on sensitivity and specificity of the assays.

3. Results

3.1. Comparative genomics and LAMP primer design

The first step in identifying singleton CDS was to identify the core genome, the set of genes shared by all analyzed genomes, of *E. amylovora*. Analysis using EDGAR yielded a set of 2935 CDS. This core genome was then used in BLAST analysis against a broader set of genomes (Table 2) to return a set of 129 singleton CDS. CDS annotated by automatic pipelines as hypothetical proteins were excluded. This provided a remaining set of 48 CDSs that were analyzed with BLASTN and BLASTX (Table S1) against the full NCBI database, with exclusion of the *E. amylovora* genomes. The CDS were rated according

Table 2
Erwinia and other genomes used for comparative genomic analysis.

Species	Strain	Plasmids	Reference
Erwinia amylovora	CFBP 1430	pEA29	Smits et al. (2010b)
E. amylovora	ATCC 49946	pEA29, pEA72	Sebaihia et al. (2010)
E. amylovora	MR-1	pEA29	Mann et al. (2012)
E. amylovora	01SFR-BO	pEA29	Mann et al. (in press)
E. amylovora	ACW 56400	pEA29, pEI70	(Llop et al., 2011)
E. amylovora	CFBP 1232^T	pEA29	Mann et al. (in press)
E. amylovora	ATCC BAA-2158	pEA29, pEAR5.2, pEAR4.3	Powney et al. (2011)
E. amylovora	Ea266	pEA29	Mann et al. (in press)
E. amylovora	Ea356	pEA29	Mann et al. (in press)
E. amylovora	Ea495	pEA29, pEA30	Mann et al. (in press)
E. amylovora	Ea644	pEA29	Mann et al. (2012)
E. amylovora	UPN527	–	Mann et al. (in press)
Erwinia billingae	Eb661	pEb102, pEb170	Kube et al. (2010)
Erwinia pyrifoliae	Ep1-96	pEp03, pEp05, pEp26, pEp36	Kube et al. (2010)
E. pyrifoliae	DSM 12163^T	pEp2.6, pEp03, pEp05, pEp36	Smits et al. (2010a)
Erwinia sp.	Ejp617	pJe01, pJe02, pJe03, pJe04, pJe05	Park et al. (2011)
Erwinia tasmaniensis	Et1-99	pEt09, pEt35, pEt45, pEt46, pEt49	Kube et al. (2008)
Pantoea ananatis	LMG 20103	–	De Maayer et al. (2010)
P. ananatis	AJ 13355	pPa320	Hara et al. (2012)
P. ananatis	LMG 5342	pPANA01	De Maayer et al. (2012)
Pantoea agglomerans	E325	pPag1, pPag4, pPag3	Unpublished
Pantoea sp.	At-9b	pA-t9b01, pA-t9b02, pA-t9b03, pA-t9b04, pA-t9b05	Unpublished
Pantoea vagans	C9-1	pPag1, pPag2, pPag3	Smits et al. (2010c)

to increasing percent identity of BLAST hits and the best 25 sequences were selected for further analysis. Among these 25 sequences, a cluster of seven CDS specific to *E. amylovora* was identified (Fig. 1) spanning 8578 bp. Automatic annotation predicts that this cluster could be involved in biosynthesis of an unknown compound. Although the relatively low G+C content ranging from 37 to 40% suggests acquisition by horizontal gene transfer, the occurrence of this cluster in all sequenced *E. amylovora* genomes makes it an ideal target to develop diagnostic markers. Thus, these 7 CDSs served as an input for primer design using the LAMPdesigner (Premier Biosoft International, Palo Alto, USA). Due to the low G+C only one primer set on EAMY_3195 was predicted (Table 3) and selected for further validation. EAMY_3195 is annotated as a *hisZ* gene by PSI-BLAST COG and InterPro. Thus it is superior to use as a diagnostic target compared to e.g. intergenic regions, hypothetical proteins or IS elements, for reasons such as stability in the genome.

3.2. Primer validation, analytical specificity and sensitivity

All 25 strains of *E. amylovora* gave positive signals with the primer set developed, whereas no signal was observed with all other bacterial strains tested (Table 1). A serial dilution of *E. amylovora* CFBP 1430 ranging from 1.2×10^9 CFU/ml to 1.2×10^2 CFU/ml was tested in triplicate to measure the detection limit as well as the speed and reproducibility of the reaction. The lowest amount of DNA consistently tested positive using the designed LAMP assay was 1.2×10^4 CFU/ml or the equivalent of 10 CFU/reaction since 1.2 µl of the serially diluted solutions had been used per 12 µl reaction (Fig. 2). However, this detection limit increased by an order of magnitude when used directly on samples with residual plant material of different tissues, probably due to inhibition by plant phenolic compounds (Fig. 3). The detection limit was thus the same as with real-time PCR assays under laboratory conditions (Pirc et al., 2009) but by one order of magnitude less sensitive when used under field conditions without DNA isolation. The speed of the LAMP reaction, enabling a positive detection in 15 min or less, much faster than the 90 min or more required to obtain a result using real-time PCR.

3.3. Performance comparison

The performance comparison on DNA isolated from inoculated apple plants revealed a good correlation between real-time PCR and LAMP (Fig. 4). When considered as qualitative data, 23% of the samples gave a contradictory result between LAMP and real-time. This may be attributable to LAMP failure to detect very low, and epidemiologically inconsequential, DNA concentrations in samples (Ruz et al., 2008). When considered as quantitative data, above bacterial concentrations of 10^5 CFU/ml, the correlation between LAMP and real-time PCR shows a R^2 value of 0.51. Statistical analysis (Grange and Lazlo, 1990) resulted in analytical specificity of 100% (Table 4). The lower analytical sensitivity of 71% reflects the higher sensitivity of the real-time PCR methods.

Compared to the immunoassay, all samples positive on the Ea AgriStrip® were also confirmed with LAMP, due to higher sensitivity, LAMP detected positive samples with bacterial loads below 10^5 CFU/ml. Three plant samples that were positive using classical plating with 10^2 CFU/ml *E. amylovora* were negative using LAMP, but 17% of the samples negative with plating were confirmed to be positive using LAMP. This agrees with reports that samples with a history of infection contain residual DNA and/or that bacteria entering a viable but non

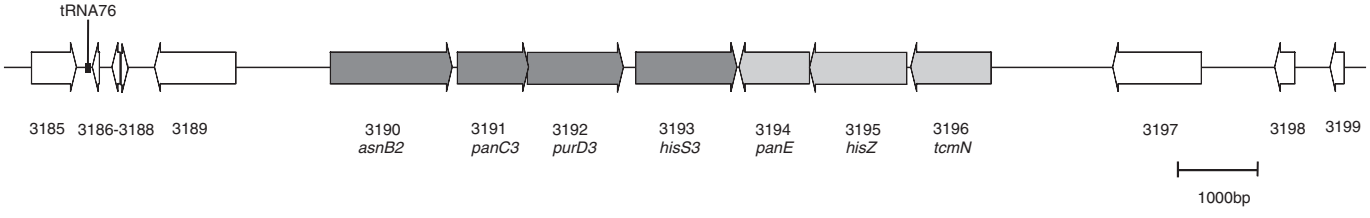

Fig. 1. Illustration of the genomic region selected as a chromosomal target for specific identification of *Erwinia amylovora*. Arrows indicate CDS sequences predicted and annotated in GenDB. Starting from EAMY_3190 on the left to EAMY_3196 on the right. All seven CDSs showed less than 15% to any other sequence else than *E. amylovora* genomes present in GenBank. The region is a putative biosynthetic pathway (Smits et al., 2010b).

Table 3
LAMP primers designed during this study.

Target	Primer [a]	Sequence (5′-3′)
EAMY_3195	F3	TCAAGATCGTGTGGCTATG
	B3	CTAAAAACCGGGGCAAAC
	FIP	ACGRTTCTACCCTTCCTGTCTACTTCTCTGGGGTTTCAGTC
	BIP	ATGTCACCTGATTCTACAGCCGCAATCATTCATGGTTCTGGAC
	loopF	ACATTAGCGGCCCGACCAA
	loopR	CTRTTAAGATGGCATGCAGA

[a] F3 indicates the forward outer primer, and R indicates the reverse outer primer for LAMP. FIP and BIP indicate inner LAMP primers while loopF and loopR are the forward and reverse loop primers.

culturable state remain detectable with molecular methods, including LAMP (Oliver, 2005).

3.4. Field samples

The comparison of LAMP with classical plating and immunoassays on field samples from naturally infected trees of different species and geographically diverse origin showed very promising results. One contradictory result where Ea AgriStrip® was positive but plating was negative could be confirmed negative by LAMP, suggesting that the Ea AgriStrip® result was a false positive. Furthermore, 20 additional samples that tested negative using Ea AgriStrip® were confirmed positive for *E. amylovora* using LAMP. Statistical evaluation (Table 5) results in a high analytical sensitivity and high likelihood ratio for negative results, but in a lower analytical specificity percentage due to the higher sensitivity of the LAMP method compared to the immunoassay. Compared to plating, LAMP failed to detect four samples with less than 10^2 CFU/ml on LB plates. Consistent with the results on inoculated greenhouse plants, approximately 15% of the samples showed a positive result with low bacterial concentration while being negative on plates indicating a history of infection with no viable bacteria left at the time of sampling. Overall, the LAMP technique outperformed the Ea AgriStrip® in terms of sensitivity and thus comprises the best method for testing directly in the field. The LAMP reaction under field conditions can either be run on portable fluorescent readers such as the Genie II (OptiGene) or on any heating device coupled with visual read out by adding DNA staining dye.

4. Discussion

The rise of the next-generation sequencing technology has made it relatively easy to generate vast amounts of DNA sequence information and to apply this information to diagnostic marker design (Li et al., 2009). The aim of this study was to apply the knowledge obtained from recent genomic sequencing efforts of *E. amylovora* to improve existing diagnostic techniques. The singleton CDS identified in this study cannot only be used as diagnostic markers but are also putative targets to study competition, fitness, and host specificity. In this study, a cluster of seven CDSs, unique to *E. amylovora*, was identified. The automatic annotation suggests involvement in biosynthesis of a small secondary metabolite of unknown structure. The fact that this cluster of genes occurs in and is limited to all sequenced *E. amylovora* strains studied so far suggests that it is a specific adaptation to the lifestyle of this pathogen that may play a role in infection dynamics, either by interaction with the host plant (Soto et al., 2009) or in bacterial competition (Hibbing et al., 2010). A second set of four CDS singletons EAMY_0446–EAMY_0450, part of a NRPS/PKS cluster (Smits et al., 2010b), was also identified. Part of a twelve CDS operon, it is linked to bacterial peptide antibiotic gramicidin S synthesis previously discovered in *Bacillus brevis* (Gause and Brazhnikova, 1944). However, further analyses including knock-out gene studies (Katashkina et al., 2009) combined with phenotyping using metabolic characterization techniques (Bochner, 2009) and mass spectrometry (Higgs et al., 2001) have to be applied to identify the product of these genes, which could potentially identify novel antibacterial compounds or virulence factors.

The performance of the LAMP assay developed in this study was evaluated. Because it is designed on a chromosomal target, it detects all known *E. amylovora* strains including those lacking the plasmid pEA29. Previous studies have shown that up to 6% of environmental *E. amylovora* strains in the USA lack this plasmid (Carey et al., 2011) and that such strains occur in different European countries (Llop et al., 2006). Such strains would represent false negatives by the LAMP assay developed previously (Temple and Johnson, 2011) and hence

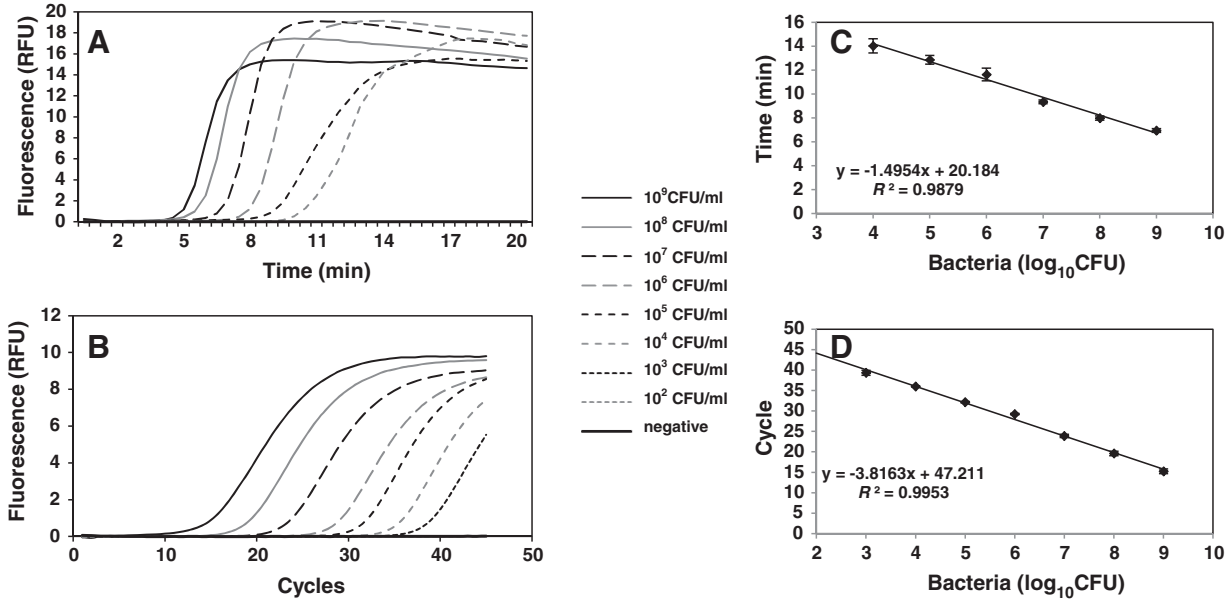

Fig. 2. Sensitivity of detection using DNA samples with the LAMP (A) or real-time PCR (B) assays. The x-axis values of (A) denote minutes in order to enable direct comparison of the methods for speed. The y-axis values differ in LAMP and real-time PCR graphs due to EvaGreen® dye used for fluorescence detection in LAMP and FAM dye used in real-time PCR. Over a range of six orders of magnitude the LAMP reaction shows a quantitative amplification signal. (C) and (D) present standard curves of LAMP and real-time PCR, showing R^2 value and standard error over three replicates as error bars.

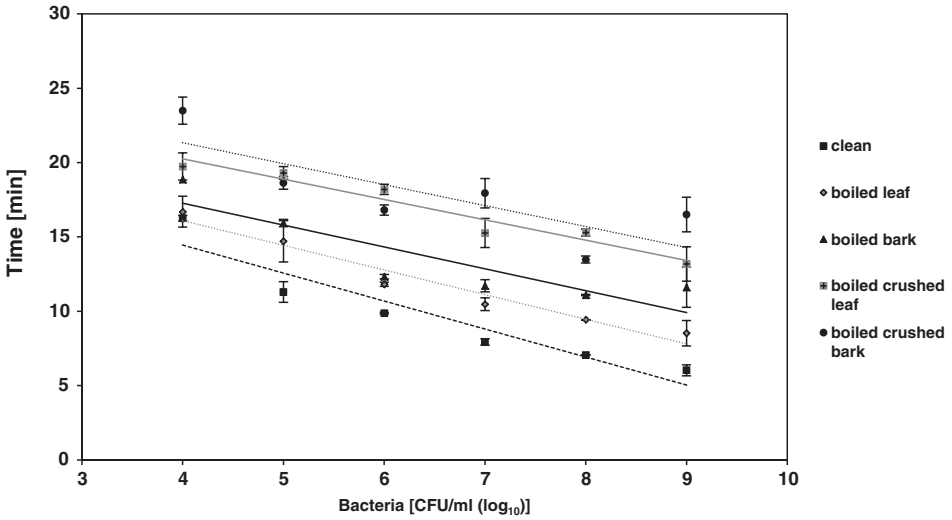

Fig. 3. Standard curves for the LAMP assay with different spiked matrices, isolated DNA, boiled apple leaves, boiled wooden tissue, boiled and crushed apple leaves, and boiled and crushed woody tissue. Standard error bars are given based on three replicates.

go undetected. Furthermore, the multi-copy nature of plasmids obscure quantification approaches such as real-time PCR and LAMP, since in comparison to chromosomal DNA the amounts of plasmid DNA is more variable.

The benefits of our new LAMP assay for *E. amylovora* extend beyond the chromosomal target feature. Due to the full genomic information used, it is highly specific producing no false positives, compared with existing LAMP methods for *E. amylovora* (Moradi et al., 2012; Temple and Johnson, 2011). Moreover, the use of two loop primers and the application of a novel strand displacement polymerase (OptiGene) double the speed of the reaction, enabling results in less than half an hour and thus a true point of care application of the assay if performed in conjunction with a portable device such as the Genie II (OptiGene).

Validation of the developed LAMP assay on naturally infected field samples showed good correlation to existing methods and thus applicability of LAMP technology for monitoring infections in orchards and nurseries. This may help prevent long distance spread of the disease by commercial shipments of infected nursery material or fruits (Taylor et al., 2003) as well as local spread within single orchards by weather conditions or pollinators (Roberts et al., 1998; Roberts and Sawyer, 2008). Compared to existing point-of-care technologies, LAMP provides reliable detection almost as fast as immunoassays but increases the sensitivity by two orders of magnitude. Furthermore, it shows no cross reactivity to other *Erwinia* species. It offers true portability which is not the case for real-time PCR although some advances in technology have made it semi-portable (Holland and Kiechle, 2005). Compared to classical culturing methods, LAMP shows an increased count of bacterial

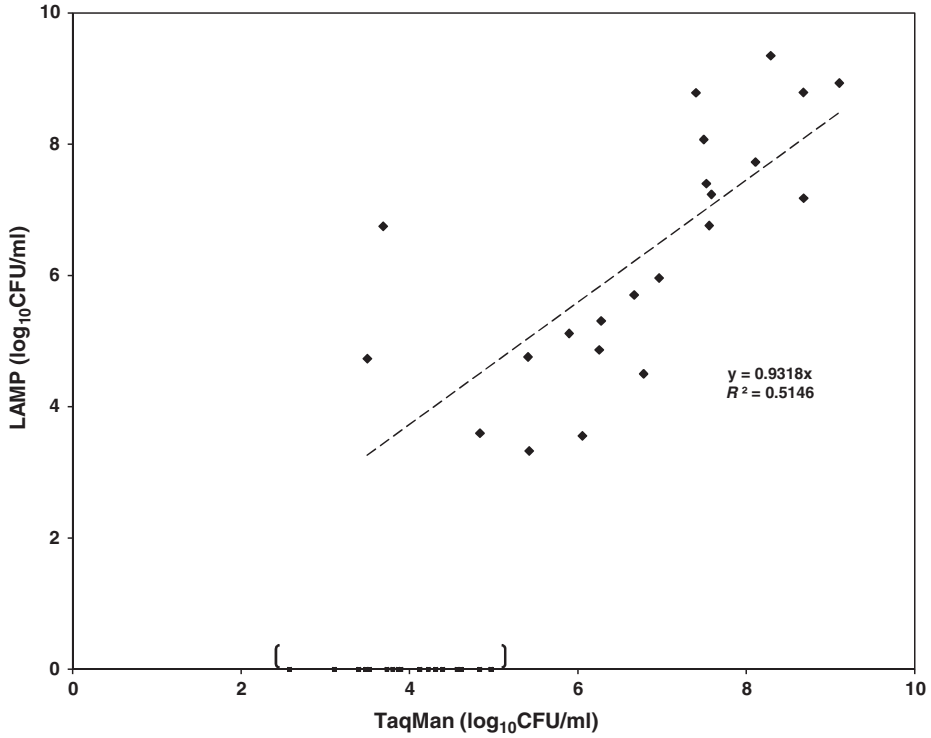

Fig. 4. Correlation between quantitative LAMP (y-axis) and real-time PCR (x-axis) determined using samples from inoculated greenhouse plants. Parentheses identify the positive but not quantifiable samples.

Table 4
Contingency table comparing TaqMan with LAMP for greenhouse samples of different apple cultivars inoculated with *E. amylovora*.

TaqMan				
LAMP		Positive	Negative	Total
	Positive	47	0	47
	Negative	19	11	30
	Total	66	11	77
Sensitivity (true positive rate) %	71.2			
Specificity (true negative rate) %	100.0			
Positive predictive value %	100.0			
Negative predictive value %	36.7			
False positive rate %	0.0			
False negative rate %	24.6			
Prevalence rate %	61.0			
Likelihood ratio for positive results	100.0			
Likelihood ratio for negative results	0.4			
Efficiency %	75.3			

Table 5
Contingency table comparing immunoassay (AgriStrip®) and LAMP for naturally infected field samples.

Agristrip®				
LAMP		Positive	Negative	Total
	Positive	53	15	68
	Negative	1	32	33
	Total	54	47	101
Sensitivity (true positive rate) %	98.15			
Specificity (true negative rate) %	68.09			
Positive predictive value %	77.94			
Negative predictive value %	96.97			
False positive rate %	14.85			
False negative rate %	0.01			
Prevalence rate %	52.48			
Likelihood ratio for positive results	3.03			
Likelihood ratio for negative results	0.02			
Efficiency %	84.16			

cells as PCR and real-time PCR do, most likely due to prevalence of viable but non-culturable bacteria (Ordax et al., 2009).

The challenges raised by bacterial infections such as *E. amylovora*, are best met by interdisciplinary approaches. Breeding of resistant cultivars will deliver best protection against infection but this approach takes some time and effort (Le Roux et al., 2010). Until such cultivars are available, disease in orchards have to be managed by using sophisticated computer models (Smith, 1998) and to be treated by antibiotics (McManus et al., 2002) or biocontrol agents (Stockwell et al., 2010) to prevent infection. To all these approaches a reliable monitoring of the infection status is required, which is best met by solid point-of-care molecular detection methods (King et al., 2010). Among the diverse detection methods that are available today, LAMP is probably closest to meeting this need since it offers true portability, is very cheap and is at a stage in development where, with minimal training, it may be implemented by phytosanitary inspectors. Well-designed LAMP assays can provide researchers and growers with reliable information on the infection status of their orchard and hence are a very promising new tool for sustainable crop protection.

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