

ORIGINAL ARTICLE

Comparative genomics-guided loop-mediated isothermal amplification for characterization of *Pseudomonas syringae* pv. phaseolicolaX. Li¹, J. Nie¹, L. Ward¹, M. Madani¹, T. Hsiang², Y. Zhao³ and S.H. De Boer¹¹ Canadian Food Inspection Agency, Charlottetown Laboratory, Charlottetown, PE, Canada² Department of Environmental Biology, University of Guelph, Guelph, ON, Canada³ Department of Crop Sciences, University of Illinois, Urbana, IL, USA**Keywords**

characterization, comparative genomics, detection, loop-mediated isothermal amplification, *Pseudomonas syringae* pv. phaseolicola.

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Abstract

Aims: To design and evaluate a loop-mediated isothermal amplification (LAMP) protocol by combining comparative genomics and bioinformatics for characterization of *Pseudomonas syringae* pv. phaseolicola (PSP), the causal agent of halo blight disease of bean (*Phaseolus vulgaris* L.).

Methods and Results: Genomic sequences of *Pseudomonas syringae* pathovars, *P. fluorescens* and *P. aeruginosa* were analysed using multiple sequence alignment. A pathovar-specific region encoding pathogenicity-related secondary metabolites in the PSP genome was targeted for developing a LAMP assay. The final assay targeted a polyketide synthase gene, and readily differentiated PSP strains from other *Pseudomonas syringae* pathovars and other *Pseudomonas* species, as well as other plant pathogenic bacteria, e.g. species of *Pectobacterium*, *Erwinia* and *Pantoea*.

Conclusion: A LAMP assay has been developed for rapid and specific characterization and identification of PSP from other pathovars of *P. syringae* and other plant-associated bacteria.

Significance and Impact of the Study: This paper describes an approach combining a bioinformatic data mining strategy and comparative genomics with the LAMP technology for characterization and identification of a plant pathogenic bacterium. The LAMP assay could serve as a rapid protocol for microbial identification and detection with significant applications in agriculture and environmental sciences.

Introduction

With the rapid development of DNA sequencing and analytical techniques, genomic sequence data of prokaryotes are accumulating at a very rapid pace. As of October 2008, there are 873 complete and published genome sequences, as well as 2025 prokaryotic genomes in various stages of sequencing (<http://www.genomesonline.org/>). In the NCBI GenBank database, there are as many as 1386 prokaryotic genomes listed with complete or nearly completed sequence data (<http://www.ncbi.nlm.nih.gov/>). The enormous amount

of sequence data that has become available in recent years requires careful and extensive analysis to tease out segments best suited for diagnostic and identification protocols.

Moreover, enormous strides have been made in technologies for the detection of micro-organisms over the last two decades. There is great potential for the application of such technologies for detection and identification of micro-organisms for food safety, as well as for the protection of animal and plant resources. One new technology that is particularly interesting is loop-mediated isothermal amplification (LAMP) that employs a

strand-displacement DNA polymerase and four to six primers targeting specific DNA regions with predesigned secondary structures (Notomi *et al.* 2000).

The LAMP reaction is initiated by hybridization of one set of primers, the inner primers, to specific sites on the target DNA followed by extension in the presence of a DNA polymerase (Fig. 1a). A second set of primers, the outer primers, complementary to specific priming sites just outside the inner primer binding sites, initiates synthesis of new complementary strands that displace the DNA strands initiated from the inner primers, resulting in the release of those strands. The displacement reaction, which does require the use of a strand displacement polymerase, precludes the need for temperature cycling as required in conventional PCR. The first amplification products form stem-loop structures at each end as a result of the unique design of the inner primers, and are autoprimed, driving the amplification of massive amounts of DNA. The LAMP reaction can be further accelerated using a third set of primers targeting the loops of the first DNA amplicons (Fig. 1a). The addition of loop primers

not only accelerates the reaction but also improves the specificity because they require transcription of the correct starting material (Nagamine *et al.* 2002; Kubota *et al.* 2008). The amplified end product is a mixture of large amounts of stem-loop DNA strands with manifold inverted repeats of the target, exhibiting a cauliflower-like structure with multiple loops. LAMP-amplified DNA forms a ladder of multiple bands in gel electrophoresis and can also be visualized directly in the amplification tube by adding SYBR Green I, a fluorescent DNA-binding dye, and observing under UV light. Alternatively, successful LAMP reactions can be visually confirmed by the observation of magnesium pyrophosphate precipitation (Mori *et al.* 2001).

LAMP technology has been applied in medical laboratories for rapid detection of human pathogenic bacteria, such as *Mycobacterium tuberculosis* (Iwamoto *et al.* 2003) and methicillin-resistant *Staphylococcus aureus* in blood samples (Misawa *et al.* 2007). However, only one report on its application has been published for plant pathogenic bacteria (Kubota *et al.* 2008).

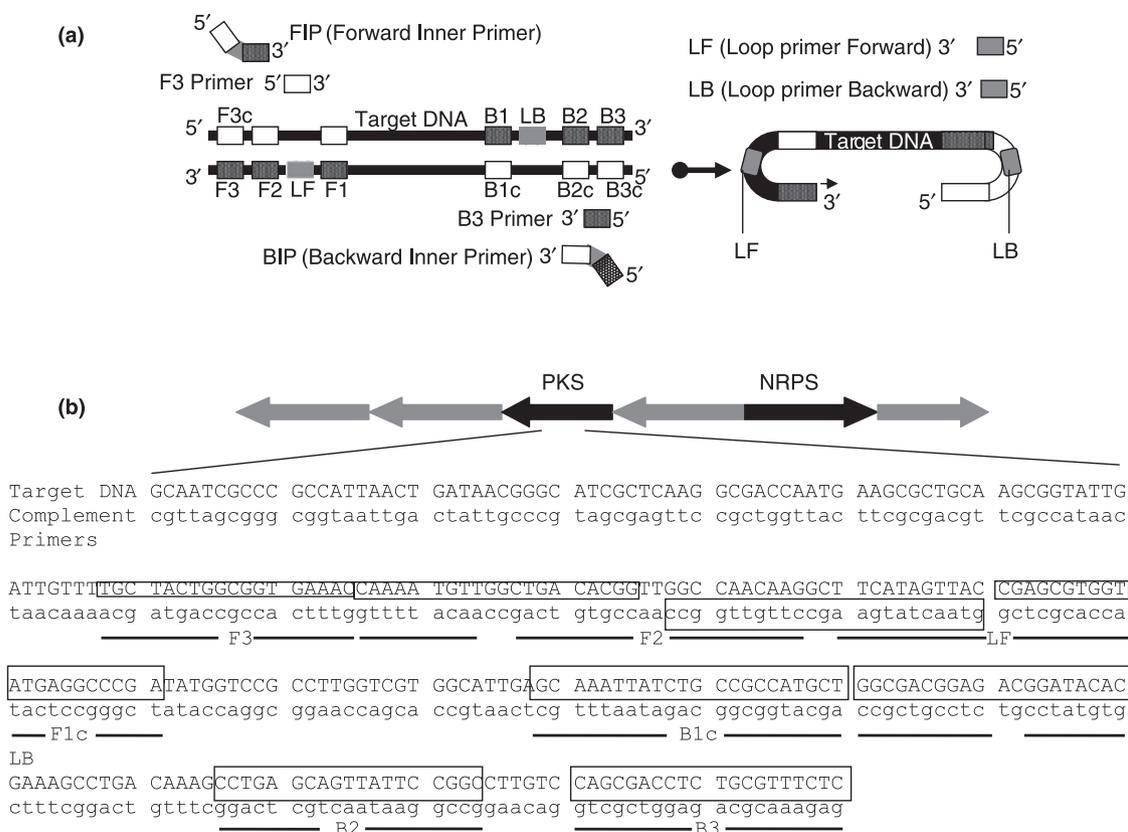


Figure 1 (a) Schematic illustration of the LAMP primers used in this study. Primer sequences are listed in the text. (b) Nucleotide sequences of the putative PKS region of *Pseudomonas syringae* pv. *phaseolicola* used for designing the LAMP primer sets. Recognition sequences of the primers are highlighted in boxes.

In our research program on improving the identification and detection of plant pathogenic bacteria we thought to utilize recently available genomic data to select DNA targets by which subspecific pathovars of plant pathogenic bacteria could be easily and accurately identified using the LAMP DNA-amplification strategy. To test this strategy we selected a fluorescent plant pathogenic species, *Pseudomonas syringae*, clustering with rRNA-similarity group I of the genus *Pseudomonas* (Palleroni 1984). This species encompasses more than 50 pathovars, i.e. subspecific taxons differentiated on the basis of their pathogenicity to specific plant species (Young *et al.* 1996). One of the pathovars is the seed-borne pathogen, *Pseudomonas syringae* pv. phaseolicola (PSP), the causal agent of halo blight disease of beans (*Phaseolus vulgaris* L.). The disease is characterized by symptoms of water-soaked lesions surrounded by chlorotic halos caused by the release of the nonhost-specific toxin, phaseolotoxin (Mitchell and Bielecki 1977). Like other *P. syringae* pathovars, PSP cannot be readily distinguished by routine microbiological methods from many other pseudomonad pathovars because of overall similarity, despite their different pathogenicity profiles. Specific pathovars of quarantine and/or regulatory significance often need to be distinguished from other pathovars of *P. syringae* and saprophytic pseudomonads that are not of regulatory concern. In the import and export trade, rapid methods for detection and identification of bacteria would be useful to determine whether an agricultural product meets the phytosanitary standard for export or import.

In this study, we made use of currently available genomic data on plant pathogenic pseudomonads in various international databases. We explored whether computationally intensive comparative genomic approaches can be used to identify gene regions as candidates useful for characterization and differentiation of plant pathogenic bacteria at the pathovar level (Brukner *et al.* 2007). Candidate gene regions, especially the regions encoding secondary metabolites which likely play a role in pathogenicity, virulence, and other disease factors, could be very useful for developing pathovar-specific LAMP primer sets. The objective of this study was to determine as proof-of-principle that specific and rapid detection methods can be developed by combining comparative genomics and LAMP technology, with PSP as a model system. The new LAMP technology may be especially useful for rapid identification and testing that can possibly be adapted for on-site testing methods and so allow a far greater amount of testing to be done for international trade and also to gather data on incidence and distribution of pathogens in plant disease outbreaks.

Materials and methods

Bacterial cultures

Bacterial strains used in this study are listed in Table 1. All strains were stored in 15% glycerol solution at -20°C . Bacteria were routinely grown in Tryptone Soya Agar and King's B medium at 28°C .

Conventional characterization

Identity of bacterial strains was confirmed by conventional microbiological procedures using the LOPAT scheme (Lelliot *et al.* 1966) for differentiation of fluorescent pseudomonads and other bacteria. The LOPAT scheme, comprised of Levan production on sucrose medium, Oxidase reaction, Pectolytic activity on potato tuber slices or pectate gel, Arginine dihydrolase activity, and hypersensitivity reaction on Tobacco leaves, is described in Braun-Kiewnick and Sands (2001).

Genome sequence alignment and target identification

The genome data of PSP (GenBank accession CP000058.1), *P. syringae* pv. *syringae* (GenBank accession CP000075.1), *P. syringae* pv. *tomato* (GenBank accession AE016853.1), *P. fluorescens* (GenBank accession CP000076) and *P. aeruginosa* (GenBank accession AE004091) have been published previously (Stover *et al.* 2000; Buell *et al.* 2003; Feil *et al.* 2005; Joardar *et al.* 2005; Paulsen *et al.* 2005), and were downloaded from GenBank.

The concatenated chromosomal sequences of each of the three plant pathogenic bacteria, together with *P. fluorescens* and *P. aeruginosa*, were aligned using Mauve 2.0.0 (Darling *et al.* 2004) with multiple alignment of conserved genomic sequences of whole genomes. The genome sequence of PSP was used as the reference against which the sequences of *P. s.* pv. *syringae*, *P. s.* pv. *tomato*, *P. fluorescens* and *P. aeruginosa* were aligned and compared. Mauve identifies and aligns regions of locally collinear blocks (LCBs) with modest computational requirements without compromising the alignment quality (Choudhary *et al.* 2007). Each LCB, at the minimum weight of 45, was identified as a homologous region of sequence shared by two or more of the genomes under study, and did not contain any rearrangements of homologous sequences. Target regions were manually identified and confirmed through BLAST search for absence of matches to other bacteria in the GenBank database.

Furthermore, a pairwise distance matrix between selected pseudomonad genomes was generated using GRIMM (Genome rearrangement algorithms) (Pevzner and Tesler 2003) with *Pectobacterium atrosepticum* as an

Table 1 Bacterial strains used in this study and their biochemical characteristics and reaction in the LAMP assay

Scientific name of the bacteria (culture collection no.)	LOPAT Scheme						LAMP Reaction	Code used in Fig. 4
	King's B medium	Levan Production	Oxidase activity	Pectolytic activity	Arginine dihydrogenase activity	Hypersensitive reaction in tobacco		
<i>P. s. pv. phaseolicola</i> (LMG 2245)	+	+	-	-	-	+	+	4
<i>P. s. pv. phaseolicola</i> (LMG 5186)	+	+	-	-	-	+	+	5
<i>P. s. pv. phaseolicola</i> (HRI 1449A)	+	+	-	-	-	+	+	20
<i>P. s. pv. coronafaciens</i> (LMG 2330)	+	+	-	-	-	+	-	1
<i>P. s. pv. glycinea</i> (LMG 5066)	+	+	-	-	-	+	-	2
<i>P. s. pv. glycinea</i> (LMG 5144)	+	+	-	-	-	+	-	3
<i>P. s. pv. savastanoi</i> (0693-10)	+	±	-	-	-	-	-	19
<i>P. s. pv. glycinea</i> (PG4180)	+	+	-	-	-	+	-	16
<i>P. s. pv. atrofaciens</i> (LMG 5000)	+	+	-	-	-	+	-	6
<i>P. s. pv. atrofaciens</i> (LMG 5001)	+	+	-	-	-	+	-	7
<i>P. s. pv. lachrymans</i> (LMG 2204)	+	+	-	-	-	+	-	8
<i>P. s. pv. lachrymans</i> (LMG 2205)	+	+	-	-	-	+	-	9
<i>P. s. pv. lachrymans</i> (1188-1 377)	+	+	-	-	-	+	-	21
<i>P. s. pv. pisi</i> (LMG 5009)	+	+	-	-	-	+	-	10
<i>P. s. pv. pisi</i> (LMG 5079)	+	+	-	-	-	+	-	11
<i>P. s. pv. syringae</i> (LMG 1247)	+	+	-	-	-	+	-	12
<i>P. s. pv. syringae</i> (LMG 2231)	+	+	-	-	-	+	-	13
<i>P. s. pv. syringae</i> (B728 a)	+	-	-	-	-	+	-	17
<i>P. s. pv. cannabina</i> (ATCC 13436)	+	+	-	-	-	-	-	14
<i>P. s. pv. tomato</i> (DC3000)	+	+	-	-	-	+	-	15
<i>P. s. pv. maculicola</i> (ES4326)	+	+	-	-	-	+	-	18
<i>P. s. pv. tabaci</i> (0791-14)	+	+	-	-	-	+	-	22
<i>P. s. pv. morsprunorum</i> (PC782)	+	+	-	-	-	+	-	23
<i>P. fluorescens</i> (GSPB1714)	+	+	+	+	+	-	-	24
<i>P. marginalis</i> pv. <i>marginalis</i> (Bulb 6-1)	+	-	+	+	+	+	-	N
<i>Pectob. atropeticum</i> (Eca 1043)	-	-	-	+	N/A	-	-	N
<i>Pectob. atropeticum</i> (Eca 1044)	-	-	-	+	N/A	-	-	N
<i>Pectob. atropeticum</i> (ATCC 2386 ^T)	-	-	-	+	N/A	-	-	N
<i>Pectob. carotovorum</i> subsp. <i>brasiliensis</i> (Ecb 212)	-	-	-	+	N/A	+	-	N
<i>Pectob.c.</i> subsp. <i>brasiliensis</i> (Ecb 8)	-	-	-	-	N/A	+	-	N
<i>Pectob.c.</i> subsp. <i>carotovorum</i> (Ecc 2404 ^T)	-	-	-	+	N/A	-	-	N
<i>E. chrysanthemi</i> (Ech 573)	-	-	-	+	N/A	-	-	N
<i>E. chrysanthemi</i> (Ech 2804 ^T)	-	-	-	+	N/A	-	-	N

T: type culture; N: data not included in Fig. 4; N/A: data not available; LMG: BCCM/LMG, Laboratorium voor Microbiologie, Universiteit Gent (UGent), K.L. Ledeganckstraat, Belgium. ATCC: American Type Culture Collection (ATCC), Manassas, USA.

outgroup. A phylogenetic tree was re-constructed using Phylip (Felsenstein 2005) as described.

Design and evaluation of primers and the LAMP protocol

To design pathovar-specific LAMP DNA oligonucleotide primers, an approximately 29.2 Kb region of the PSP genome sequence (CP000058.1 numbering 5174641-5203841) was initially selected based on multiple genome sequence alignment (Fig. 2b). LAMP primer sets for efficient stem-

loop formation were designed to target a putative polyketide synthase (PKS) gene (CP000058.1 numbering 5183490-5186910) using PRIMEREXPLORER V4 software program (http://primerexplorer.jp/e/v4_manual/index.html). The primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Figure 1 illustrates the locations of various primer sets within the putative PKS region of PSP. The backward inner primer (BIP) for the region consisted of B1 [22 nucleotides (nt)] (5'GCAAATTATCTGCCGCCATGCT3'), an AAAA linker and B2 (19 nt) (5'GCCGGAATAACTGCTCAGG3'); and



Figure 2 Mauve representation of local collinear blocks (LCBs) between concatenated chromosomal sequences of three plant pathogenic pseudomonads. (a) Sequence of the forward strand of *P. s. pv. phaseolicola* 1448A genome as the reference against which the sequences of *P. s. pv. syringae* and *P. s. pv. tomato* were aligned and compared. LCBs placed under the horizontal bars represent the reverse complement of the reference DNA sequence. (b) Mauve display of the zoomed window on pathovar specific regions (grey arrows) of interest for selection of LAMP primers. PKS, Polyketide synthase; NRPS, Nonribosomal peptide synthetase.

the forward inner primer (FIP) consisted of F1c (20 nt) (5'TCGGGCTCATACCAGCTC3'), an AAAA linker, and a complementary sequence of F2c (20 nt) (5'CAA-AATGTTGGCTGACACGG3'). The outer primers B3 (18 nt) and F3 (18 nt) were 5'GAAACGCAGAGGTCG-CTG3' and 5'TGCTACTGGCGGTGAAAC3', respectively. Loop primer forward (LF) (20 nt) (5'ACTATGAAGCCT-TGTTGGCC3') and loop primer backward (LB) (20 nt) (5'GGCGACGGAGACGGATACAC3') were designed and synthesized to increase the amplification efficiency (Fig. 1b).

The LAMP protocol was optimized by evaluating the effect of changing the *Bst* enzyme (New England Biolabs) concentration (0, 2, 4 and 8 Units), Mg concentration (0, 2, 4, 6, 8 and 10 mmol l⁻¹), Betaine (Sigma) concentra-

tion (0, 0.5, 1, 1.5, 2 and 2.5 mol l⁻¹), and the time for DNA amplification reaction (40–90 min) as described by Yeh *et al.* (2005).

After evaluation, the LAMP reaction was performed at the optimal conditions in a final volume of 25 µl consisting of 0.8 µmol l⁻¹ each of FIP and BIP, 0.2 µmol l⁻¹ each of F3 and B3, 0.4 µmol l⁻¹ each of LF and LB, 1 mol l⁻¹ Betaine, 400 µmol l⁻¹ each of dNTPs, 4 mmol l⁻¹ MgSO₄, 1X *Bst* buffer (New England Biolabs), and 8 U of *Bst* DNA polymerase large fragment (New England Biolabs) and 200 ng genomic DNA, followed by incubation at 65°C for 50 min and heating at 80°C for 10 min to terminate the reaction.

Aliquots of 2–5 µl of LAMP product were electrophoresed in 1% agarose gels (0.5 × TBE) containing ethidium

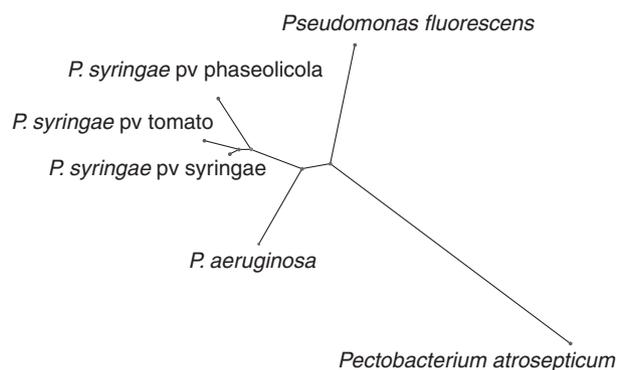


Figure 3 Pairwise distance tree of genomes from selected pseudomonad species and pathovars. The tree was re-generated using Phylip (Felsenstein 2005) from the distance matrix data generated by genome rearrangement algorithms (GRIMM) (Pevzner and Tesler 2003). *Pectobacterium atrosepticum* is used as the outgroup.

bromide. Remaining products were mixed with SYBR Green I for visualization under UV light.

Results

Genomic conservation in *Pseudomonas syringae* pathovars and related species

Comparison of the genomes of the three sequenced pathovars of *P. syringae* with two different species of *Pseudomonas*, *P. fluorescens* and *P. aeruginosa*, revealed that the chromosomes of *P. syringae* pathovars have a high degree of collinearity. In total, 183 LCBs were observed between concatenated chromosomal sequences of the three plant pathogenic fluorescent pseudomonads. Figure 2 displays the multiple alignments of conserved genomic sequences of PSP, *P. syringae* pv. syringae, and *P. syringae* pv. tomato. The sequence of the forward strand of PSP strain 1448A was used as the reference against which the sequences of *P. s.* pv. syringae and *P. s.* pv. tomato were aligned (Fig. 2a). LCBs placed under the horizontal bars represent the reverse complement of the reference DNA sequence and show that multiple rearrangement and inversion events exist in the chromosomes (Fig. 2a). Among the three pathovars, pv. phaseolicola shares more sequence similarity with pv. syringae, while pv. tomato is more similar to pv. syringae than pv. phaseolicola (Fig. 3). The identification of the chromosomal region with the most divergent DNA sequences among genomes of the different pathovars of *P. syringae* should facilitate pathovar differentiation. Furthermore, since *P. syringae* pathovars also shared some degree of genome sequence collinearity with the soil bacterium *P. fluorescens* and the opportunistic human pathogen, *P. aeruginosa*, analysis of their co-alignment was also important to dem-

onstrate the intrageneric relationship at the genome level among these fluorescent pseudomonads (Fig. 3).

Polyketide synthase and nonribosomal peptide synthetase

Through genomic comparison, a stretch of DNA (CP000058.1 numbering 5174641–5203841) of PSP was identified as a unique sequence with open reading frames (Fig. 2b). The fragment comprises multiple putative proteins of which many are of unknown function (PSPPH_4543–PSPPH_4546, PSPPH_4548, 4549). However, the presence of a putative polyketide synthase (PKS) gene (PSPPH_4547) and a nonribosomal peptide synthetase (NRPS) gene (PSPPH_4550) indicated that the region might be comprised of a gene cluster or clusters encoding secondary metabolism pathways possibly associated with pathogenicity. The putative PKS gene (PSPPH_4547) of PSP was selected as a target for development and evaluation of primers for the LAMP protocol.

The other target region in the chromosome of PSP potentially useful to develop specific LAMP primers covers a stretch (CP000058.1 numbering 2897910–2934690) of PSP genome, for which genes encoding periplasmic transporters and the type III secretion component proteins reside. However, many gene products in the region of 1485001–1685270 (CP000058.1) of PSP are involved in the type III secretion system with which the three *P. syringae* pathovars share a high degree of collinearity.

LAMP protocol and evaluation

Through a direct optimization process, the LAMP reactions were optimized by changing each key component of the reaction mixture (Yeh *et al.* 2005). The best results were repeatedly achieved using 8 U of *Bst* enzyme in a 25 μ l reaction with 4 mmol l⁻¹ of Mg and 1 mol l⁻¹ of betaine for 50 min at 65°C followed by deactivation at 80°C for 10 min. The LAMP assay evaluated in this study proved to be specific for differentiation of PSP from all other fluorescent pseudomonads tested (Table 1). The three authentic cultures of PSP tested were consistently differentiated from the other 23 pseudomonads, representing 12 different pathovars of *P. syringae*, two other pseudomonad species, and nine other plant pathogenic bacteria. Only the three strains of PSP showed positive reaction under standard LAMP reaction conditions (Table 1, Fig. 4).

The sensitivity of LAMP was 6.9×10^3 CFU ml⁻¹ when amplification was evaluated by gel electrophoresis (Fig. 5). The addition of the SYBR Green I dye to the

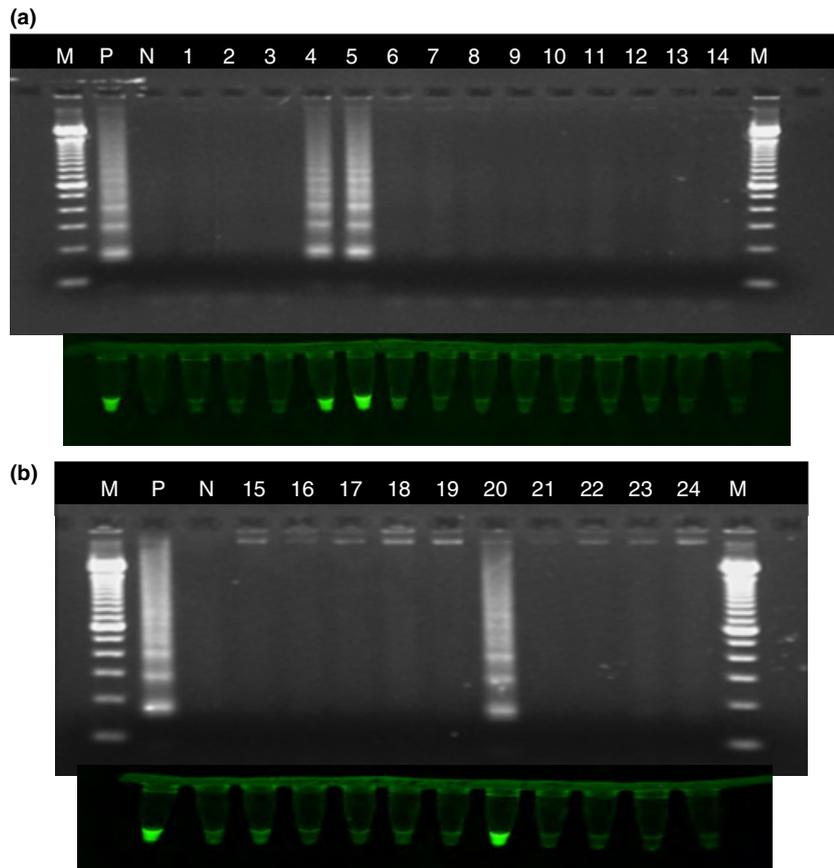


Figure 4 Agarose gels and LAMP reaction tubes illustrating the specificity of the LAMP assay for differentiating *Pseudomonas syringae* pv. phaseolicola DNA from DNA of other *P. syringae* pathovars and pseudomonad species (a and b). SYBR Green I stain added to LAMP reaction tubes post amplification and photographed under UV light. Lane numbers correspond to bacterial strains listed in Table 1, Lanes M, P, and N are 100 bp ladder, positive control and negative control, respectively.

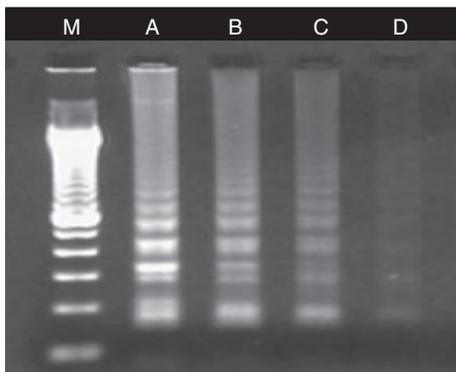


Figure 5 Agarose gel of LAMP reaction products amplified from serial dilutions of *P. syringae* pv. phaseolicola DNA. Lane M, 100 bp molecular weight ladder; lane A, DNA dilution representing 9.74×10^5 CFU ml⁻¹; lane B, 2.22×10^5 CFU ml⁻¹; lane C, 6.09×10^3 CFU ml⁻¹; lane D, 3.81×10^3 CFU ml⁻¹.

reaction mix after completion of the LAMP reaction enabled the test result to be visualized under UV light, and clearly differentiated the positive reaction from negative reactions of other closely related fluorescent pseudomonads, as well as several other plant pathogenic bacteria (Fig. 4).

Conventional bacterial characterization

All bacterial strains listed in Table 1 were subjected to the differentiation tests known as the LOPAT scheme (Lelliot *et al.* 1966) for confirmation purposes. Our results confirmed that differentiation of *P. syringae* pathovars was not feasible with currently available tests because of the variable reactions and lack of signature characteristics without further tedious experiments used for bacteriological characterization (Table 1). All *P. syringae* pathovars were positive for levan production except a strain of

P. syringae pv. *syringae* (B728a). *Pseudomonas savastanoi* pv. *savastanoi* did not produce the typical raised colonies unlike the rest of the *P. syringae* pathovars. All but two of the *P. syringae* strains caused a hypersensitive reaction on tobacco, while only a few of the other bacteria tested induced the reaction (Table 1).

Discussion

While whole genome sequence data for microbes, including plant pathogenic bacteria, are accumulating at an increasingly rapid pace, the science and practice of using the data to address agricultural problems is in its infancy. To date, there are limited publications about the host-specific virulence genes among *P. syringae* pathovars (Sarkar *et al.* 2006), and comparative genomics studies on this group of plant pathogenic bacteria are mainly concentrated on *P. s.* pv. *tomato* for detecting putative effector genes and defining their functionality (Petnicki-Ocwieja *et al.* 2002; Lan *et al.* 2006). It is still a challenge to effectively convert the huge amounts of sequence information into functional knowledge for practical application.

In this study, we explored and utilized enormous amounts of available sequence data by comparative genomic analysis to identify a unique PKS gene of PSP that is discriminatory *in silico* against sequences in GenBank and potentially useful for specific characterization and identification of PSP using the LAMP technology. Pathovars of *P. syringae* are well known to produce secondary metabolites including polyketide and nonribosomal peptides closely associated with pathogenicity toward particular plant hosts (Donadio *et al.* 2007). Although there are no reports on the functionality of the PKS gene identified, the gene and the associated gene cluster remains an interesting target for future investigation with methods such as the gene knockout technique for exploring functional expression in the process of invasion, pathogenicity and transmission of the pathogen within and between host plants.

Presumably through selective pressures in interactions with specific host plants, genetic rearrangements such as random mutation, DNA duplication, gene loss, repeated inversions and translocation, and horizontal gene transfer take place in plant pathogenic bacteria (Choudhary *et al.* 2007). The comparative genomics data showed that these genetic rearrangements have appeared in multiple sites in the genomes of pseudomonads (Fig. 2). However, the LCBs observed, in general, were highly conserved among the three pathovars of *P. syringae*. Pathovars of *P. syringae* comparatively shared more DNA similarity than they did with the soil-borne bacterium, *P. fluorescens*, or the human pathogen, *P. aeruginosa* in the phylogenetic analy-

sis (Fig. 3). This coincides with earlier reports that *P. syringae*, *P. fluorescens* and *P. aeruginosa* are in separate intragenetic clusters based on their 16S rRNA, *gyrB* and *rpoD* sequences (Moore *et al.* 1996; Yamamoto *et al.* 2000).

LAMP technology, like PCR, is based on amplification of specific genomic fragments, but differs from PCR in several significant ways. LAMP technology as employed in this paper utilized 6 different primers in a single reaction. Since each of these primers needs to hybridize to target DNA, the LAMP procedure is even more specific than conventional or real-time PCR, which requires two primers and two primers plus one probe, respectively. In a 6-primer LAMP test, there are six opportunities for determining specificity. The LAMP test is also isothermal, which means that it can be carried out at one temperature, usually at 65°C, compared to the requirement for thermocycling of PCR methods. This feature allows LAMP tests to be carried out in unsophisticated laboratories to the extent that it could be done under field conditions.

An earlier report described the use of the LAMP protocol for detection of *Ralstonia solanacearum* (Kubota *et al.* 2008). Our objective also was to explore the value and possibilities of LAMP technology in diagnostic phytobacteriology, by developing such an assay for PSP. Serological and PCR methods were already available for PSP (Wong 1990; Schaad *et al.* 1995) but availability of the LAMP assay is anticipated to have greater potential applications in plant health programs. Selection of LAMP primers is a highly complex and complicated procedure, requiring a great deal of searching within genome sequences as well as sophisticated judgement and trial and error to find just the right sequences. Once a good primer set has been found and validated, the test is simple to carry out. LAMP assays can be done with a minimum of laboratory equipment and supplies. The test can be carried out in 1.5 ml plastic tubes, and amplified DNA yields are so high, that results can be easily read visually after staining. At present, results still need to be read with an UV light source, and are still often confirmed by gel electrophoresis, but simpler methods such as visualizing the turbidity of magnesium pyrophosphate precipitation with the naked eye are also available (Mori *et al.* 2001). With a LAMP assay, rapid identification and testing of micro-organisms can possibly be done on-site and allow a far greater amount of testing to be done on incidence and distribution of pathogens in disease outbreaks. To this end, we concentrated additional work on simplifying sample treatment and DNA extraction with promising progress (data not shown). Furthermore, the LAMP assay may be combined with miniature and microfluidic

systems in a high throughput format for hand-held and cost-effective devices being developed for clinical diagnosis (S. Hashsham, personal communication).

In summary, the concatenated chromosomal sequences of *P. syringae* pathovars were analysed in multiple alignments to search for unique and conserved sequences. By *in silico* analysis, genes encoding pathogenicity-related secondary metabolites of PSP were identified as being pathovar specific and targeted for developing a LAMP protocol with multiple primers selected from gene regions identified as discriminatory by comparative genomic analyses. PSP strains were readily discriminated from other *P. syringae* pathovars and species of *Pseudomonas* using the LAMP technology.

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