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Robust Detection and Identification of Multiple Oomycetes and Fungi in Environmental Samples by Using a Novel Cleavable Padlock Probe-Based Ligation Detection Assay▽†‡

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Simultaneous detection and identification of multiple pathogenic microorganisms in complex environmental samples are required in numerous diagnostic fields. Here, we describe the development of a novel, background-free ligation detection (LD) system using a single compound detector probe per target. The detector probes used, referred to as padlock probes (PLPs), are long oligonucleotides containing asymmetric target complementary regions at both their 5′ and 3′ ends which confer extremely specific target detection. Probes also incorporate a desthiobiotin moiety and an internal endonuclease IV cleavage site. DNA samples are PCR amplified, and the resulting products serve as potential targets for PLP ligation. Upon perfect target hybridization, the PLPs are circularized via enzymatic ligation, captured, and cleaved, allowing only the originally ligated PLPs to be visualized on a universal microarray. Unlike previous procedures, the probes themselves are not amplified, thereby allowing a simple PLP cleavage to yield a background-free assay. We designed and tested nine PLPs targeting several oomycetes and fungi. All of the probes specifically detected their corresponding targets and provided perfect discrimination against closely related nontarget organisms, yielding an assay sensitivity of 1 pg genomic DNA and a dynamic detection range of 10^4. A practical demonstration with samples collected from horticultural water circulation systems was performed to test the robustness of the newly developed multiplex assay. This novel LD system enables highly specific detection and identification of multiple pathogens over a wide range of target concentrations and should be easily adaptable to a variety of applications in environmental microbiology.

Clinical diagnostics and disease management strategies increasingly require fast and accurate methods for the detection and identification of multiple pathogenic microorganisms from complex samples. Conventional techniques used to detect and identify pathogenic microorganisms have typically relied upon culture-based morphological approaches. Unfortunately, these methods are often time-consuming, laborious, and restricted to those microorganisms that can be cultured routinely.

Several recently developed molecular techniques, such as conventional and real-time PCRs, circumvent some of these drawbacks. PCR-based detection mechanisms are sensitive, accurate, and relatively fast and allow the detection of difficult-to-culture microorganisms. This last aspect is of considerable importance, given the fact that the majority of the microorganisms present in environmental samples still elude conventional cultivation efforts (1, 21). Although PCR-based methods for microbial identification and detection offer several advantages over conventional microbiological approaches, they still often have serious limitations. The attainable level of multiplexing is relatively low and is typically restricted to the detection of only a few target pathogens per assay (15, 40). Adding multiple specific primer pairs to a single reaction mixture can result in undesired amplification products (16), and for TaqMan PCR, the attainable level of multiplexing is low due to the limited number of fluorescent probes (8, 34, 41). Reliable detection and identification of several pathogens in a single sample, therefore, requires separate reaction mixtures, making large-scale screening of samples more laborious, time-consuming, and expensive. To increase efficiency and reduce expenses, it is desirable to develop simple and rapid multiplex assays that can specifically detect and identify several pathogens simultaneously.

DNA microarray- and macroarray-based technologies offer the possibility of adding a highly multiplexed aspect to PCR-based pathogen detection and identification (9, 37, 54, 58). Array-based pathogen detection strategies typically involve PCR amplification of universal phylogenetic target genes (e.g., 16S, 18S, and 23S rRNA genes) or a number of microorganism-specific genetic markers (10, 19, 58) or random amplification of genomic DNA (gDNA) fragments (54). The combination of nucleic acid amplification strategies with array-based

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detection has resulted in the development of sensitive, high-throughput microbial diagnostic microarrays (MDMs) (4, 38, 50, 60). Although several array-based detection technologies have been realized to detect pathogens, only a minority of these methods can discriminate target pathogens from closely related nontarget organisms, which may differ from the target organisms by only a single nucleotide in the probe-binding region (36). Designing sufficiently discriminating oligonucleotide detectors for arrays, however, is relatively complicated, requiring extensive hybridization specificity testing. Moreover, the oligonucleotide detectors spotted onto the microarray are target organism specific, making it necessary to redesign microarrays if accommodation of additional probes is required for the detection of new targets.

DNA ligase requires a double-stranded match to allow ligation, facilitating the development of ligation-based systems to discriminate point mutations (29). This feature of ligation detection (LD) has led to the development of several strategies for genotyping single-nucleotide polymorphisms (SNPs) and detecting pathogens (6, 7, 11, 46). However, current LD assays require two adjacent detection oligonucleotide probes with the same melting temperature (T_m) for each target sequence, although the use of intramolecular ligation, as in padlock probe (PLP) technologies, has been demonstrated to hold clear advantages (44, 45, 51). PLPs are long oligonucleotides, ~100 bases long, containing target complementary arms at both termini of the probe. In the assay developed in this study, the target complementary arms are connected via a compound linker sequence containing spacer sequences, a thymine-linked desthiobiotin moiety for specific capture and release (25, 53), deoxyuracil nucleotides for probe cleavage, and a unique sequence identifier, the so-called ZipCode, for standardized microarray hybridization (18) (Fig. 1A). The unimolecular nature of the PLP allows asymmetric target complementary arm design, whereby a long 5′ arm serves as an anchor sequence and the short 3′ arm, with a low T_m, facilitates extremely specific target detection (17, 51, 53). Microarray-based PLP technology was previously shown to provide reliable detection of multiple pathogenic microorganisms, but PCR amplification of residual, unligated PLPs resulted in significant background signals, thereby complicating data analysis and decreasing the overall dynamic range of reliable detection (3, 51).

Here, we describe the development, testing, and implementation of a novel, background-free, LD-dependent strategy in which multiple PLPs are ligated on fragmented, PCR-preamplified DNA sequences. The target complementary regions recognize adjacent sequences on the target DNA, and ligation occurs only if the end nucleotides perfectly match their target, resulting in a circular molecule (Fig. 1B). Next, the probes are captured with streptavidin-coupled magnetic beads, allowing separation from the rest of the sample. Subsequently, the washed probes are eluted from the beads and cut at the internal polydeoxyuracil probe region by enzymatic cleavage. Thus, the desthiobiotin moiety needed for fluorescent labeling of unligated PLPs is removed, while the ligated probes are linearized (Fig. 1B). Finally, the sample is hybridized on a universal complementary ZipCode (cZipCode) microarray (18) and visualized via fluorescent labeling of the desthiobiotin moiety (Fig. 1B).

In this paper, we report the development and application of cleavable PLPs combined with LD for the simultaneous, background-free detection and identification of multiple plant pathogens in environmental samples. The specificity, sensitivity, and dynamic range of detection of the developed assay were determined by using nine target-specific PLPs, and the robustness of the assay was evaluated by using samples collected from hydroponic horticultural water recirculation systems.

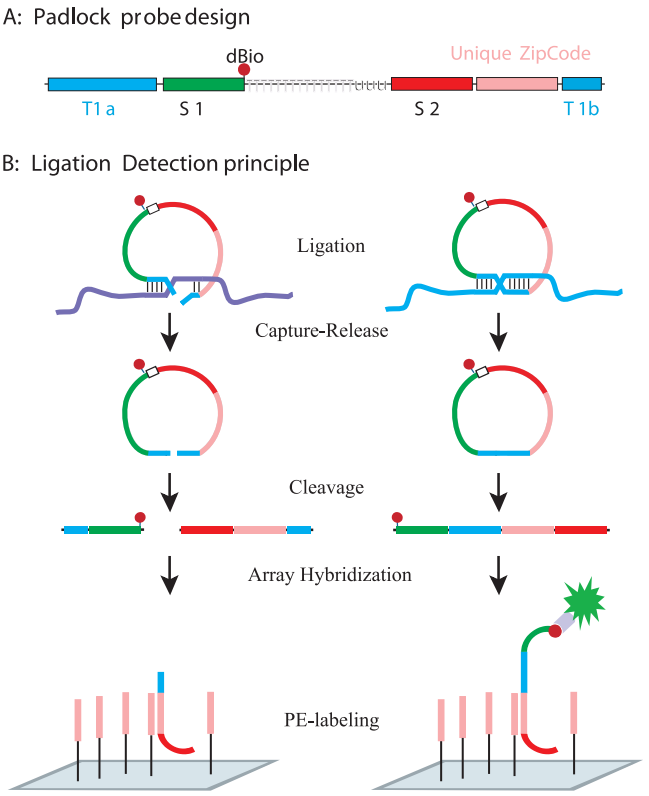

FIG. 1. Schematic overview of the novel single-molecule LD system. (A) PLP design. T1a and T1b are asymmetric target complementary regions. Each PLP contains a unique ZipCode sequence for universal array hybridization, two spacer sequences (S1 and S2), a desthiobiotin moiety (dBio) for probe capture, a polyoligo(dT) linker sequence, and a polydeoxyuracil sequence for probe cleavage. (B) Multiple target-specific PLPs are ligated to PCR-preamplified DNA samples. T1a and T1b bind to adjacent sequences of the target, and in the case of a perfect match, the probe is circularized by enzymatic ligation. The PLPs are reversibly captured and washed via the desthiobiotin moiety with magnetic streptavidin-coated beads. Next, the washed probes are cleaved at the polydeoxyuracil sequences with UNG and endonuclease enzymes. The sample containing the cleaved PLPs is hybridized on a universal microarray. Finally, only the hybridized PLPs that were originally ligated can be labeled and visualized with streptavidin *R*-PE by using the desthiobiotin moiety.

MATERIALS AND METHODS

Nucleic acids used in this study. Microorganisms were procured from the culture collection of Plant Research International B.V. (Table 1). Environmental test samples were derived from several Dutch hydroponic horticultural systems. These were designed to provide an examination of detection capabilities in complex environmental samples of practical relevance. To create samples with known plant pathogens, fungal and oomycete spores (10^3 to 10^6 spores, depending on the pathogen and horticultural system) were used to spike recirculation water systems. At least 4 weeks after pathogen inoculation, 250 ml of each

TABLE 1. Isolates of plant pathogenic species and subgroups used in this study

Phylum	Order	Species	Isolate
Oomycota	*Peronosporales*	*Phytophthora cactorum*	PRI-18.1
		Phytophthora nicotianae	PRI-28.6
		Pythium ultimum	N2001/5
		Pythium aphanidermatum	89
		Pythium undulatum	CBS 157.64
Basidiomycota	*Ceratobasidales*	*Rhizoctonia solani* AG 2-2	IIIB 02-337 IRS
Ascomycota	*Hypocreales*	*Fusarium oxysporum* f. sp. *radicis-lycopersici*	364N2
		Fusarium solani	T05
		Myrothecium roridum	PRI-15.2
		Myrothecium verrucaria	CBS 189.46
	Phyllacorale	*Verticillium dahliae*	809.97
		Verticillium alboatrum	40.1

recirculation water sample was collected and filtered and the filters were stored at −80°C prior to analysis. All gDNA extractions from cultured microorganisms, as well as filters, were performed with the Puregene gDNA isolation kit (Gentra-Biozym) according to the manufacturer's instructions.

For assay development and optimization, ligation targets were generated by performing PCR preamplification with various amounts of gDNA from cultured microorganisms as the PCR template. Ligation targets for the assay validation experiments were prepared with 10 ng gDNA extracted from the environmental samples as the PCR template. Depending on the experiment (see below), preamplification reactions were performed in the presence or absence of 0.1 aM internal amplification control (IAC) oligonucleotide (5′ TCCGTAGGTGAAC CTGCGGCGGATCGTTACAAGGGTCTCCAACTACGTCTAGCGCATAG ACCACGTATCGAAGCTAGGTGCATATCAATAAGCGGAGGA 3′). Specific PCR amplification of the internal transcribed spacer (ITS) regions of the fungal and oomycetal rRNA operons was achieved with the ITS1 and ITS4 primers (56). For each PCR, an initial 10-min incubation at 95°C was followed by 40 cycles consisting of 30 s at 95°C, 30 s at 54°C, and 72°C for 60 s. After the final cycle, samples were incubated at 72°C for 10 min and then cooled to 4°C.

PLP design. The PLP target complementary regions were engineered according to previously described design criteria (51) and connected by a 75-nucleotide compound linker sequence. The linker sequence contained a 20-nucleotide PLP-specific sequence (ZipCode) for array hybridization (Table 2), two spacer sequences (S1 and S2), a 15-dT sequence, and a polydeoxyuracil cleavage region. All ZipCodes have equal T_ms to allow standardized array hybridization conditions. The ZipCodes were chosen from the GeneFlex TagArray set (Affymetrix) so as to minimize PLP probe secondary structures and to optimize array hybridization conditions. Potential for secondary structures and ZipCode specificity was examined with Visual OMP 6.0 software (DNA Software Inc.). The prediction parameters were set to match ligation ([monovalent ions] = 0.025 M, $[Mg^{2+}]$ = 0.01 M, T = 65°C, [probe] = 20 nM) and array hybridization conditions (1× tetramethylammonium chloride, T = 55°C). When necessary, PLP arm sequences were adjusted to avoid strong secondary structures that might interfere with efficient ligation, as described previously (51).

A thymine-linked desthiobiotin was introduced for specific capture and release with streptavidin-coated magnetic beads (see below). The PLPs listed in Table 2 (for the entire probe sequence, see Fig. S1 in the supplemental material), and all of the other oligonucleotides used in this study, were synthesized by Eurogentec SA.

Ligation. PCR-amplified ITS products (see above) were used as templates for ligation. Prior to ligation, the PCR-amplified ITS products were fragmented by digestion with EcoRI, HindIII, and BamHI (New England BioLabs, Inc.) for 15 min at 37°C. Cycled ligation was performed in a 10-μl reaction mixture containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1% Igepal, 0.01 mM rATP, 1 mM dithiothreitol, 20 ng sonicated salmon sperm DNA, and 4 U *Pfu* DNA ligase (Stratagene). For multiplex detection, the optimized concentration of the individual PLPs was 20 nM. Reaction mixtures were prepared on ice and rapidly transferred into a thermal cycler. Before ligation, samples were denatured at 95°C for 5 min. The samples were subsequently subjected to 20 cycles of 30 s at 95°C and 5 min at 64°C. After the final cycle, the reaction mixture was immediately cooled to 4°C.

Probe capture, elution, and cleavage. After ligation, 30 μl distilled water was added to each reaction mixture and the samples were denatured at 95°C for 10 min, followed by rapid transfer onto ice. Capturing of the desthiobiotin moiety of the PLPs was performed in 80 μl of a capturing mixture containing 1 M NaCl, 5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.1 M NaOH, and 20 μg magnetic MyOne streptavidin C1 Dynabeads (Dynal Biotech ASA) with rotation at 4°C for 1 h. Subsequently, samples were centrifuged at 2,000 × *g* for 10 s, the Dynabeads were collected and separated from the sample via application of a magnetic field, and the Dynabeads were washed with 100 μl of a washing solution containing 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl. Consequently, gDNA and preamplicons were washed away. The Dynabeads were resuspended in 10 μl distilled water and incubated at 95°C for 10 min, allowing elution of the PLPs from the

TABLE 2. Target complementary regions and unique ZipCode sequences of PLPs

Targeted species/ group	Target complementary sequence (5′–3′)[a]		ZipCode sequence (5′–3′)
	5′	3′	
Phytophthora spp.	TATCTAGTTAAAAGCA **GA**GACTTTCGTC	CTGCTGAAAGTTGC	TACGAACGTCTTAGCACTCC
P. nicotianae	TAGTAGTCTTTTTTTCTTTTAAACCCATTCCTTAAT	GCTTCGGCCTGATT	TCCCGAATGACAAGGCACGA
R. solani AG 2-2	TCTGCCTCACAGGTTCACAGGTGTGTGTGG	TTCCCGTCCATGTC	GAGTTCCCGTGCGTTAGATC
P. ultimum	CGAAAAAACGAACGCAACCATG **T**GAGACACTT	CGACAGAT TCTCGAT	TCGCCGTTGGTCTGTATGCA
P. aphanidermatum	ATGTTCTGTGCTCTCTTT**T**GGGAGGG	TGAACCGTTGAAATC	GTTGACCGTTAGTTATGCGA
F. oxysporum	GCGAGTCCCAACACCAAGCTGTGCTTG	GGAACGCGAATTAAC	CTGGTGCATGTACTCGACTG
F. solani	CAAATAAAT TAAAACTTTCAACAA**T**GGATCTCTTGG	TTTTCTGAGTAAACAAG	GTACTACATTCGTGCGATGG
V. dahliae	TTTATACCAACGATACTTCTGAGTG TT	CATCAGTCTCTCTG	ATCTGGATCAACGTCGCGCT
M. roridum	CGG**T**GGTGGCCATGCCGT AAAACACC	ACTCGCATTGGAGCT	CATCCAGCTCAACGTATCCA
IAC	AGCGCATAGACCACGTATCGA	TCTCCAACTACGTCT	GTCTCGTCTTCGTGAGTGCA

[a] Boldface letters indicate polymorphism within the target group. Nucleotides or gaps owing to deletions used to discriminate from most similar nontarget sequences are underlined (for the entire probe sequences, see Fig. S1 in the supplemental material).

Dynabeads. Samples were then rapidly transferred onto ice, and the empty magnetic streptavidin beads were removed via application of a magnetic field, leaving the washed PLPs in the solution. Next, 10 μl of a cleavage mixture (10 U uracil-*N*-glycosylase [UNG; Applied Biosystems], 10 U endonuclease IV [New England BioLabs], 2× NEBuffer 3 [New England BioLabs], 2× bovine serum albumin) was added to each reaction mixture and the samples were incubated at 37°C for 1.5 h. Subsequently, 2 μl of 1.1 M NaOH was added and samples were incubated at 95°C for 10 min. Finally, 8 μl of 0.5 M Tris buffer (pH 6.8) was added to neutralize the solution.

MB assay. In order to determine the capture-and-release efficiency of the PLPs, a molecular beacon (MB) complementary to the PLP was designed with Visual OMP 6.0 software (DNA Software Inc.). Samples containing a dilution series of PLPs were captured and eluted as described above. Next, the fluorescence of 50 nM MB in each sample was monitored in a solution containing 1× PCR buffer with $MgCl_2$ (Roche Diagnostics) as a function of temperature. The measurements were carried out with an ABI Prism 7700 sequence detector (Applied Biosystems). After 15 min of equilibration at 80°C, the temperature of the samples was reduced from 80°C to 15°C in steps of 1°C lasting for 5 min each. Fluorescence was measured for the last 30 s of each step. The MB melting curves of three parallel samples were measured, and the results were averaged. At 30°C, the melting curves of all of the samples had reached the plateau phase, and the corresponding fluorescence levels were used for target input determination.

Microarray preparation and hybridization. The cZipCode oligonucleotides carrying a C_{12} linker and a 5′ NH_2 group were synthesized and spotted onto Nexterion MPX-E16 epoxy-coated slides by Isogen B.V. according to the manufacturer's instructions (Schott Nexterion). Before hybridization, the arrays were washed and blocked according to the manufacturer's instructions. The cleaved ligation samples were heated for 10 min at 99°C and then cooled rapidly on ice. The array hybridization mixtures were made up of 15 μl of sample and 1 μl of 0.1 μM Cy5-labeled corner oligonucleotide in 1× tetramethylammonium chloride in a final volume of 48 μl. Sixteen-well silicon structures (Schott Nexterion) were attached to the arrays to create 16 separate subarray chambers. After adding 40 μl of the array hybridization mixture to each well, the chambers were sealed and the arrays were hybridized at 55°C overnight in high humidity. The array was then washed once at 55°C for 5 min in preheated 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate and twice for an additional 1 min at room temperature in 0.1× SSC–0.1% sodium dodecyl sulfate and in TNT (0.1 M Tris-HCl [pH 7.6], 0.15 M NaCl, 0.05% Tween 20), respectively. Next, the array was incubated in blocking solution (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl, 0.5% blocking reagent [Perkin-Elmer]) for 10 min at high humidity at room temperature and then washed for 1 min in TNT. After the addition of 20 μl of staining solution (15 μg/ml streptavidin *R*-phycoerythrin [PE; Qiagen] in 20 μl of blocking solution) to each well, the array was incubated in the dark at room temperature for 15 min. The silicon structures were then removed, and the slides were washed three times for 1 min in TNT and twice for 1 min in 0.1× SSC, respectively. Finally, the slides were dried by spinning at 250 × *g* for 3 min.

Analysis of microarray data. Microarrays were analyzed with a confocal ScanArray 4000 laser scanning system (Packard GSI Lumonics) containing a GreNe 543-nm laser for PE and a HeNe 633-nm laser for Cy5 fluorescence measurement. Laser power was fixed at 70% for both lasers, while the photomultiplier tube power ranged from 50 to 70%, depending on the signal intensity. Fluorescence intensities were quantified by using QuantArray1 (Packard GSI Lumonics), and the mean signal minus the mean local background (mean PE-B) was used. The absolute signal intensity was defined as the mean PE-B minus the assay background. The assay background for each subarray was defined as the mean PE-B + 2 standard deviations (SDs) of the fluorescence of the unused cZipCodes. The cZipCode oligonucleotides were spotted in threefold triplicates (nine parallels) or twofold quadruplicates (eight parallels), depending on the array batch used. After exclusion of the outliers (mean PE-B ± 2 SDs), signals were averaged for the probes, and SDs were calculated.

Real-time PCR. Amplification of targets in the biological samples was monitored in real time with the 7500 Real-Time PCR system (Applied Biosystems). TaqMan PCR was performed with 1× TaqMan universal PCR Master Mix (Applied Biosystems) containing AmpliTaq Gold DNA polymerase, deoxynucleoside triphosphates with dUTP, passive reference 1, optimized buffer components, 0.12 μl UNG (Applied Biosystems), 10 ng DNA extract, 300 nM of each primer, and 100 nM of the corresponding TaqMan probe. SYBR green PCR was performed with 1× SYBR green PCR Master Mix (Applied Biosystems) containing SYBR green I dye, AmpliTaq Gold DNA polymerase, deoxynucleoside triphosphates with dUTP, passive reference 1, optimized buffer components, 0.12 μl UNG (Applied Biosystems), 10 ng DNA extract, and 300 nM of each primer. The reaction mixtures were subjected to initial incubation at 50°C for 2 min, followed by 10 min denaturation at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. Three different TaqMan probes targeting *Phytophthora* spp. (28), *P. aphanidermatum* (forward primer, 5′-CGTGAACCGTTGAAATC ATG-3′; reverse primer, 5′-TACATCGGCAGACTACAATT-3′; TaqMan probe, 5′-FAM-TTC**G**TT**C**AG**C**CC**T**CCC-BHQ-3′ [locked nucleic acids are in bold]), and *V. dahliae* (forward primer, 5′-CCGGTCCATCAGTCTCTCTG-3′; reverse primer, 5′-ACTCCGATGCGAGCTGTAAC-3′; TaqMan probe 5′-FAM-CGG**G**TC**C**GC**C**AC**T**GC-BHQ-3′ [locked nucleic acids are in bold]) and a SYBR green assay-based primer pair targeting *F. oxysporum* spp. (35) were used for comparative assay validation.

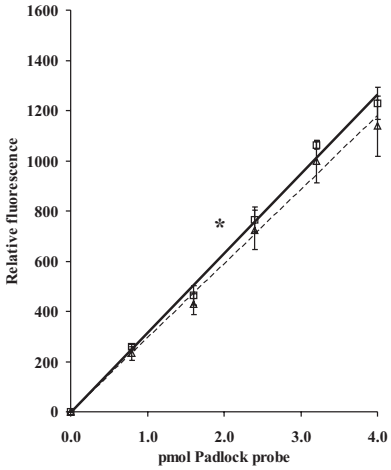

FIG. 2. Capture-and-release efficiency of the LD system. Dilution series of PLPs were captured and released as described in Materials and Methods ($n = 3$ for each treatment), and then MB melting curves were constructed. □ and solid line, uncaptured control samples ($r^2 = 0.995$); Δ and dashed line, captured and released samples ($r^2 = 0.993$); *, applied PLP concentration in the developed LD assay (2.0 pmol PLP). For both treatments at all PLP concentrations, $P > 0.05$ (*t* test).

RESULTS

Probe design and optimization. The designed probing system was experimentally tested with nine PLPs engineered to detect several economically important plant pathogens at different taxonomic levels (Tables 1 and 2). In contrast to previously designed methods, the PLPs used in this assay contain a desthiobiotin moiety between the spacer sequences for reversible PLP capture and a polydeoxyuracil nucleotide sequence for probe cleavage.

This novel PLP-based LD system was optimized and validated in several steps. In order to determine the capture-and-release efficiency of desthiobiotin-labeled probes, a dilution series of a desthiobiotin-labeled probe containing an internal MB hybridization site was captured, washed, and released. Next, MB melting curves were determined with these samples to visualize the amount of remaining probe after the capture-and-release procedure. As a control, MB melting curves were also determined with a dilution series of desthiobiotin-labeled probes that had not undergone the capture-and-release procedure. These experiments showed no significant differences in fluorescent signal between the reversibly captured PLPs and the control treatments at the PLP mixture concentration used ($P > 0.05$) (Fig. 2).

To test the cleavability of the probes, unligated PLPs were subjected to UNG and endonuclease IV treatment and subsequently hybridized on the array. Hybridization of uncleaved

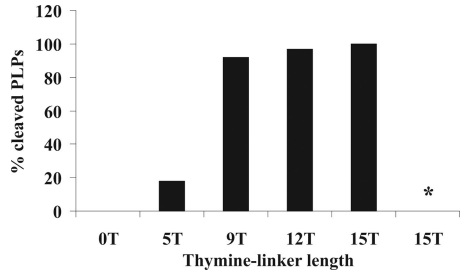

FIG. 3. Thymine (T) linker length-dependent probe cleavage. The percentage of cleaved probe was calculated relative to uncleaved control probe hybridizations. *, uncleaved control sample.

probes could be visualized by labeling the desthiobiotin moiety with a fluorescent label. The cleavage efficiency was highly sensitive to the position of the desthiobiotin moiety with respect to the polydeoxyuracil cleavage site. When placed in close proximity, the desthiobiotin moiety was found to interfere with the removal of uracil bases by UNG and endonuclease IV (Fig. 3). Therefore, oligo(dT) linkers of various lengths were introduced between the two modifications and tested for their effects on the efficiency of PLP cleavage. As shown in Fig. 3, a 15-dT linker sequence was required to provide quantitative (100%) cleavage of all of the probes. This design was adopted for all of the PLPs in subsequent experiments.

Assay optimization and characterization. To test the performance of the LD PLPs, the mixture of nine probes was ligated to various individual (Fig. 4A to C) and mixed (Fig. 4D to E) PCR amplicon targets. Without exception, targets were specifically detected and no signal was observed in the absence of target DNA (Fig. 4H).

To ensure specificity for the targeted pathogens, three nontarget organisms containing similar ligation regions (1, 2, and 7 mismatches compared to the perfect ligation sequences) for the PLP mixture were tested. No detectable fluorescent signals were observed in the presence of *P. undulatum* (with one nucleotide difference, the most similar nontarget of the *Phytophthora* species PLPs) and *V. alboatrum* (with two nucleotide differences, the most similar nontarget of the *V. dahliae* PLPs), even when an initial amount of 10 ng nontarget gDNA was added to the preamplification PCR mixture (Fig. 4F). LD in the presence of *P. cactorum* (with seven nucleotide differences, the most similar nontarget of the *P. nicotianae* PLPs) resulted in fluorescent signals for the *Phytophthora* species PLP, while the *P. nicotianae* PLP signal was completely absent (Fig. 4G). Therefore, the asymmetric PLPs could discriminate targets with a single nucleotide difference.

Applying the LD assay to a 10-fold dilution series of a multiplex target gDNA mixture containing all targets in equal ratios resulted in a detection sensitivity of 1 pg gDNA for each target (data not shown). In biological samples, however, targets are often present in highly unbalanced concentrations. To test the maximum dynamic range of the LD assay, a dilution series of target gDNAs was used to spike a background of 10 ng of nontarget ITS1-ITS4 gDNA before the preamplification reaction (Table 3). As shown in Table 3, the maximum dynamic range of the LD assay was $1{:}10^4$ and the presence of high concentrations of competing ITS1-ITS4 gDNA in the preamplification reaction mixture did not influence the final target detection sensitivity of 1 pg gDNA.

IAC. It is often observed that DNA extracted from environmental samples contains PCR-inhibiting compounds (55), which may lead to false negatives. In order to monitor PCR inhibition during the preamplification reaction, an IAC was developed. This IAC consisted of a single-stranded oligonucleotide containing a random nonsense sequence flanked by ITS1-ITS4 primer regions, which was added to each preamplification reaction mixture. An IAC PLP containing ligation arms targeting the reverse complement of the IAC oligonucleotide was added to each ligation mixture. In the case of PCR inhibition, the complementary strand of the IAC oligonucleotide will not be generated. Therefore, the target of the IAC

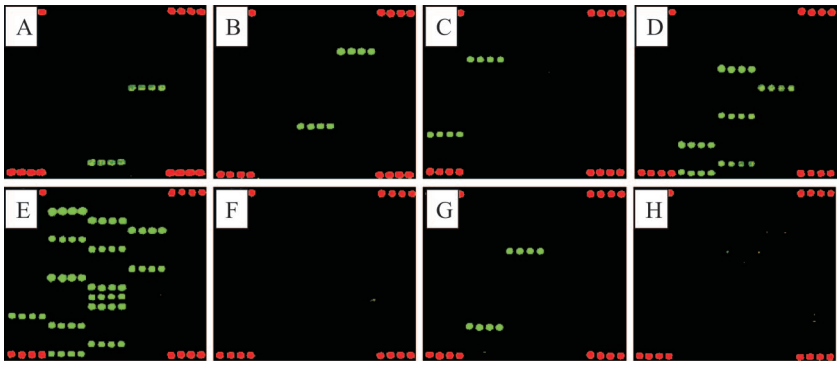

FIG. 4. Detection of genomic DNAs corresponding to individual pathogen samples (A to C), mixed-pathogen samples (D and E), targets with closely related nontarget sequences (F and G), and a no-target control (H). The targets analyzed were as follows: (A) *V. dahliae*, 100 pg; (B) *M. roridum*, 100 pg; (C) *F. oxysporum*, 100 pg; (D) *P. nicotianae*, 100 pg, and *V. dahliae*, 100 pg; (E) *V. dahliae*, 100 pg, *M. roridum*, 100 pg, *F. oxysporum*, 100 pg, *P. nicotianae*, 100 pg, *P. aphanidermatum*, 100 pg, and *R. solani* AG 2-2, 100 pg; (F) *P. undulatum*, 10 ng gDNA in the preamplification reaction mixture (closest nontarget of *Phytophthora* species PLP; one nucleotide difference compared to perfect ligation sequence), and *V. alboatrum*, 10 ng gDNA in the preamplification reaction mixture (closest nontarget of the *V. dahliae* PLP; two nucleotide differences); (G) *P. cactorum*, 10 ng gDNA in the preamplification reaction mixture (closest nontarget of *P. nicotianae* PLP; seven nucleotide differences); (H) no-target control. Green fluorescent spots represent PE-labeled PLPs. Red fluorescent spots represent Cy5-labeled hybridization control oligonucleotides. Similar data were obtained with all of the other PLPs in the presence of the corresponding targets. For the array spotting pattern, see Fig. S2 in the supplemental material.

TABLE 3. Dynamic range of detection shown by analyses of DNAs from several microorganisms mixed in different ratios

PLP	Fluorescence intensity[a]															
	F. oxysporum (10 ng) vs *P. nicotianae* at:				*P. nicotianae* (10 ng) vs *V. dahliae* at:				*V. dahliae* (10 ng) vs *P. ultimum* at:				*F. oxysporum* (10 ng) vs *R. solani* AG 2-2 at:			
	100 pg ($1:10^2$)	10 pg ($1:10^3$)	1 pg ($1:10^4$)	100 fg ($1:10^5$)	100 pg ($1:10^2$)	10 pg ($1:10^3$)	1 pg ($1:10^4$)	100 fg ($1:10^5$)	100 pg ($1:10^2$)	10 pg ($1:10^3$)	1 pg ($1:10^4$)	100 fg ($1:10^5$)	100 pg ($1:10^2$)	10 pg ($1:10^3$)	1 pg ($1:10^4$)	100 fg ($1:10^5$)
F. oxysporum	94.3	101	97.8	100	—	—	—	—	—	—	—	—	95.1	88.3	91.3	88.7
V. dahliae	—	—	—	—	72.2	68.9	32.4	—	104.6	101.4	99.2	90				
Phytophthora spp.	98.4	100	6.1	—	100.1	109	110.9	115.2								
P. nicotianae	80.5	79.2	10.6	—	91.2	102.5	103.3	103.4								
P. ultimum									86.7	81.3	6.3	—	—	—	—	—
R. solani AG 2-2									—	—	—	—	83.5	80.5	5.4	—
Other PLPs	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

[a] gDNAs of different targets were mixed in several ratios, ranging from $1:10^2$ to $1:10^5$, and analyzed by the LD system. Data represent fluorescence intensities of two quadruplicate assays. The fluorescent signals for each probe were calculated relative to the maximum possible fluorescent signal of the corresponding PLP. —, not detected. No fluorescent PLP signal was observed in the no-target control samples. Separate experiments with different targets yielded similar data and the same detection sensitivity. Other PLPs for *F. oxysporum* versus *P. nicotianae* and *P. nicotianae* versus *V. dahliae*: *P. ultimum*, *M. roridum*, *P. aphanidermatum*, *F. solani*, and *R. solani* AG 2-2. Other PLPs for *V. dahliae* versus *P. ultimum* and *F. oxysporum* versus *R. solani* AG 2-2: *Phytophthora* spp., *P. nicotianae*, *M. roridum*, *P. aphanidermatum*, and *F. solani*.

PLP will not be present during the ligation reaction and, consequently, no IAC PLP signal will be observed on the array.

IAC performance was tested under several different reaction conditions. A preamplification reaction of the IAC oligonucleotide resulted in the detection of an IAC PLP signal (see Fig. S3a in the supplemental material). In the absence of a polymerase in the preamplification reaction mixture, no IAC PLP signal was observed (see Fig. S3b in the supplemental material), indicating that the IAC PLP could not be circularized in the absence of the complementary strand of the IAC oligonucleotide. The presence of 10 ng competing *V. dahliae* target gDNA in the preamplification reaction mixture did not influence IAC PLP detection (see Fig. S3c in the supplemental material). Additionally, the detection sensitivity of 1 pg target gDNA in a $1:10^4$ ratio background DNA remained unaltered (see Fig. S3d in the supplemental material). To test the influence of PCR-inhibiting compounds on IAC PLP performance, 50 pg *F. oxysporum* gDNA was used to spike 10 ng of a DNA extract previously observed to fully inhibit PCR amplification. In the presence of the inhibiting DNA extract, no *F. oxysporum* and IAC PLP signals were detected on the array (see Fig. S3e in the supplemental material). Diluting the inhibiting DNA extract by a factor of 50 after spiking restored the detection of the IAC and *F. oxysporum* PLPs (see Fig. S3f in the supplemental material). Thus, in the presence of spiked target DNA in a background of nontarget DNA, the target is clearly detected when the IAC shows amplification as well. These results indicate that the IAC is a suitable tool to reduce false negatives caused by the presence of PCR-inhibiting compounds.

Assay validation. The robustness of the LD assay was demonstrated with biological samples recovered from recirculation water from several Dutch horticultural systems which had been inoculated previously with various plant pathogens. At least 4 weeks after pathogen inoculation, recirculation water samples were collected and filtered, DNA was extracted from the filters, and the resulting samples were subjected to the developed LD procedure. The data generated with the LD assay were compared with data obtained by TaqMan PCR detection for a selected range of targets for which TaqMan probes were available (*Phytophthora* spp., *P. aphanidermatum*, and *V. dahliae*) and data obtained by SYBR green-based PCR detection for *F. oxysporum* spp. (35).

As shown in Table 4, the detected PLP signals corresponded to samples spiked with pathogens, while the nonspiked control samples were negative. Moreover, all of the observed LD assay results were supported by the real-time PCR data. Positive real-time PCR data always corresponded to positive LD assay signals, and negative real-time PCR data always corresponded to negative LD assay signals. Thus, the newly developed multiplex LD procedure and real-time PCR showed complete congruence when applied to environmental samples typical of a practical application scenario.

DISCUSSION

In this study, we demonstrated a specific, background-free, cleavable PLP-based LD assay which uses universal cZipCode hybridization arrays for the simultaneous detection and identification of several economical important plant pathogens.

Unlike conventional LD assays that require two detection oligonucleotide probes with the same T_m for each target, PLP-based LD has the advantage of asymmetric target arm design within a single probe molecule. Besides highly specific target detection, the low T_m of the 3′ arm also reduces the chance of cross-ligation between PLPs. The most obvious strategy to distinguish residual linear PLPs from circularized PLPs would be exonuclease treatment. Although effective removal of linear probes by exonuclease treatment has been reported (22), the remaining linear PLPs resulted in significant background signals (not shown), as previously observed in PLP-based diagnostic assays (3, 51). Thus, to incorporate the high SNP specificity provided by the asymmetric PLPs into an LD assay, probes were modified such that ligated probes could be discriminated from unligated, linear probes upon array hybridization. The PLPs were designed with a desthiobiotin moiety, providing a means of reversible PLP capture and a polydeoxyuracil nucleotide sequence for probe cleavage. A simple enzymatic reaction cleaves the polydeoxyuracil region, thereby linearizing circularized PLPs and cutting the unligated probe into two fragments, thereby separating the ZipCode region for ar-

TABLE 4. LD assay validation with environmental samples

Cultivated crop	Spiked pathogen(s)[a]	LD PLP signal	Real-time PCR signal
Spathiphyllum	*M. roridum*, *P. nicotianae*	IAC PLP, *Phytophthora* spp., *P. nicotianae*	*Phytophthora* spp.
Spathiphyllum	*P. nicotianae*	IAC PLP, *Phytophthora* spp., *P. nicotianae*	*Phytophthora* spp.
Spathiphyllum		IAC PLP	
Rosa	*V. dahliae*	IAC PLP, *V. dahliae*	*V. dahliae*
Rosa	*Phytophthora* spp.	IAC PLP	
Rosa		IAC PLP	
S. lycopersicum	*F. oxysporum* f. sp. *radicis-lycopersici*, *P. nicotianae*	IAC PLP, *Phytophthora* spp., *P. nicotianae*	*Phytophthora* spp.
S. lycopersicum	*F. oxysporum* f. sp. *radicis-lycopersici*, *P. ultimum*	IAC PLP, *F. oxysporum*, *P. ultimum*	*F. oxysporum*
S. lycopersicum	*F. oxysporum* f. sp. *radicis-lycopersici*, *P. nicotianae*	IAC PLP, *F. oxysporum*	*F. oxysporum*
S. lycopersicum	*F. oxysporum* f. sp. *radicis-lycopersici*	IAC PLP, *F. oxysporum*	*F. oxysporum*
S. lycopersicum	*P. nicotianae*	IAC PLP, *Phytophthora* spp., *P. nicotianae*	*Phytophthora* spp.
S. lycopersicum		IAC PLP	
Cucumis sativus	*P. aphanidermatum*	IAC PLP, *P. aphanidermatum*	*P. aphanidermatum*
Cucumis sativus	*P. aphanidermatum*	IAC PLP, *P. aphanidermatum*	*P. aphanidermatum*
Cucumis sativus		IAC PLP	
Capsicum annuum	*P. capsici*	IAC PLP, *Phytophthora* spp.	*Phytophthora* spp.
Capsicum annuum	*P. capsici*	IAC PLP, *Phytophthora* spp.	*Phytophthora* spp.
Capsicum annuum		IAC PLP	

[a] The indicated pathogens were used to spike recirculation water from several Dutch horticultural systems. After sampling, DNA was extracted and subjected to the developed LD procedure containing all 10 of the PLPs described in Table 2. All DNA samples were also subjected to real-time PCR analyses with *Phytophthora* species, *P. aphanidermatum*, and *V. dahliae* TaqMan probes and an *F. oxysporum* SYBR green-based assay.

ray hybridization from the desthiobiotin moiety needed for fluorescent labeling (Fig. 1).

The position of the desthiobiotin moiety with respect to the polydeoxyuracil cleavage region affected the efficiency of the enzymatic cleavage reaction. Introducing a stretch of 15 thymine bases, in order to increase the physical distance between the cleavage region and the desthiobiotin moiety, provided a cleavage efficiency of 100%. Because, in contrast to previously developed PLP-based detection assays, no PLP amplification is required (meaning that no background signals can arise from amplification of remaining unligated PLPs), the demonstrated 100% cleavage efficiency resulted in a completely background-free detection assay.

Previous studies have reported decreased array hybridization specificity (36) and sensitivity (54) in the presence of an excess of DNA amplicons. In our assay, DNA amplicons that could possibly interfere with array hybridization specificity and sensitivity were removed by the incorporation of streptavidin-desthiobiotin-mediated PLP purification after the ligation reaction. The rationale of using desthiobiotin instead of biotin is the approximately 1,000-fold lower affinity for streptavidin (20, 25), which permits subsequent release of the PLPs (26, 53). The fluorescent signals of the reversibly captured PLP samples were not significantly different from the control treatments, indicating that this procedure did not compromise the final assay sensitivity.

The developed system was tested with mixtures of gDNA extracted from cultured organisms. All targets were appropriately detected, and no signals were observed in the absence of target DNA. Furthermore, ligation reactions performed in the presence of high levels of nontarget organisms with very similar ligation target regions did not result in detectable fluorescent signals, demonstrating the highly specific SNP detection abilities of the LD assay. The observed SNP specificity is of prime importance since organisms that are closely related to target pathogens, possessing only one or a few mismatches in the probe target sites, may have ecologies highly disparate from that of the target pathogen itself.

In environmental samples, the ratios of target organisms may vary widely, often making detection of the less abundant organisms problematic. For reliable disease screening, however, detection and identification of all targets present are of the utmost importance, especially when samples are analyzed for, e.g., quarantine organisms. We demonstrated that the PLP-based LD assay could identify and detect several organisms at a ratio of $1{:}10^4$ (0.01% relative abundance). The final assay sensitivity of 1 pg gDNA was of the same order of magnitude as the sensitivity of the tested TaqMan probes in this study (data not shown) and previously published multiplex TaqMan assays (33, 48, 52, 61). To ensure even more reliable sample analyses, an IAC was developed to control for false negatives as a consequence of potential PCR inhibition by compounds coextracted with the DNA from environmental samples. We demonstrated that the absence of an IAC PLP signal was indicative of PCR-inhibiting compounds, allowing one to distinguish between the true absence of the target and PCR inhibition. The use of such an IAC greatly reduces the risk of false negatives.

The applicability of the PLP-based LD assay was demonstrated by analyzing recirculation water samples from horticultural systems previously spiked with various pathogens. Real-time PCR and LD assay data fully matched the targets

compared. It is interesting that negative results in both assays were sometimes obtained with samples spiked with pathogens. Although it could not be ruled out completely that negative results can occur in the presence of low levels of pathogen in these systems, the convergence of both methods and the efficiency of the IAC strongly suggest that the spiked pathogens had not established themselves in the systems or were confined to locations beyond the scope of the sampling procedures.

The novel LD system demonstrated here is currently limited to the detection of eukaryotic organisms due to our reliance on eukaryote-specific primers. Ideally, one would wish to amplify all potential targets by either multiplex PCR or whole-genome amplification. However, preamplification attempts with multiple primer pairs in a single PCR and ϕ29 DNA polymerase-based whole-genome amplification have not yet yielded the dynamic range of detection required for diagnostic assays, especially when low target numbers are present in a high background of competing target DNA (data not shown).

Recently, the use of MDMs for the detection of pathogenic microorganisms in environmental samples has increased significantly (38, 50). Platforms enabling very high probe densities (>10,000 probes) have also become powerful tools in analyses addressing the overall structure of complex microbial communities (13, 57–59), but their high cost and low flexibility, in combination with the complex hybridization patterns and resulting data analysis, somewhat limit their application for some fast microbial diagnostics (38). The true SNP level detection of the LD system presented here precludes the need for multiple probe pairs for target detection, although inclusion of multiple probes per target in any assay would, of course, serve to increase robustness further. Reliable pathogen detection has also been achieved with MDMs with low and intermediate probe densities, often with reported relative abundance sensitivity thresholds of 0.1 to 5% (2, 5, 12, 14, 24, 27, 30–32, 39, 42, 43, 47, 49). However, because the oligonucleotide detectors that are spotted onto the microarrays are target organism specific, these assays often lack the flexibility necessary for rapid probe adaptation to, e.g., newly emerging pathogens and changing diagnostic requirements. The newly developed PLP-based LD assay, with its flexibility and background-free sensitivity, therefore represents an approach that is highly complementary to various MDM methodologies.

The system described in this paper utilizes the extremely specific target detection capabilities provided by asymmetric PLP design and enables routine screening of complex environmental samples. Because the oligonucleotide detectors on the microarrays are not target organism specific, the target range of the PLP-based LD assay can easily be adapted for use across a wide range of applications. For instance, an adaption of this LD assay is currently being used to identify several closely related *Pectobacterium* species in diseased potato tubers (M. Sławiak et al., unpublished data). The universal cZipCode-based microarray approach should allow the development of highly multiplexed assays (22, 23) (www.affymetrix.com) since, in contrast to other methods, the PLPs are not amplified, making background issues essentially irrelevant to the scaling-up process. Thus, scaling up of such LD systems is only dependent on the identification of suitable probes, since the detection of each specific target remains essentially independent. Although we envision that specific target numbers will not exceed the number of available ZipCodes (± 3,000) in the immediate future, there is no barrier to the engineering of additional ZipCode sequences if necessary. The LD assay described here should serve as a model for the routine detection and identification of a wide range of organisms in diverse biological systems where background-free multiplex target detection is required.

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