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Identification of *Xanthomonas fragariae*, *Xanthomonas axonopodis* pv. phaseoli, and *Xanthomonas fuscans* subsp. *fuscans* with Novel Markers and Using a Dot Blot Platform Coupled with Automatic Data Analysis▽†

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Phytosanitary regulations and the provision of plant health certificates still rely mainly on long and laborious culture-based methods of diagnosis, which are frequently inconclusive. DNA-based methods of detection can circumvent many of the limitations of currently used screening methods, allowing a fast and accurate monitoring of samples. The genus *Xanthomonas* includes 13 phytopathogenic quarantine organisms for which improved methods of diagnosis are needed. In this work, we propose 21 new *Xanthomonas*-specific molecular markers, within loci coding for *Xanthomonas*-specific protein domains, useful for DNA-based methods of identification of xanthomonads. The specificity of these markers was assessed by a dot blot hybridization array using 23 non-*Xanthomonas* species, mostly soil dwelling and/or phytopathogens for the same host plants. In addition, the validation of these markers on 15 *Xanthomonas* spp. suggested species-specific hybridization patterns, which allowed discrimination among the different *Xanthomonas* species. Having in mind that DNA-based methods of diagnosis are particularly hampered for unsequenced species, namely, *Xanthomonas fragariae*, *Xanthomonas axonopodis* pv. phaseoli, and *Xanthomonas fuscans* subsp. *fuscans*, for which comparative genomics tools to search for DNA signatures are not yet applicable, emphasis was given to the selection of informative markers able to identify *X. fragariae*, *X. axonopodis* pv. phaseoli, and *X. fuscans* subsp. *fuscans* strains. In order to avoid inconsistencies due to operator-dependent interpretation of dot blot data, an image-processing algorithm was developed to analyze automatically the dot blot patterns. Ultimately, the proposed markers and the dot blot platform, coupled with automatic data analyses, have the potential to foster a thorough monitoring of phytopathogenic xanthomonads.

Xanthomonas is a genus of *Gammaproteobacteria* that includes numerous phytopathogenic species, each characterized by a narrow host range. However, as a whole, the genus members are able to infect a broad range of plants, distributed over 124 monocotyledonous and 268 dicotyledonous plant species (15). The nomenclature of this complex genus is still under debate, and the taxonomic rank of many previously described pathovars has been revised (28, 41, 48). At the moment, the European and Mediterranean Plant Protection Organization (EPPO) recommends that 13 members of the genus *Xanthomonas* be considered quarantine pests. Therefore, reliable, fast, and technically and commercially accessible screening methods of detection and identification are needed to allow the survey of a large number of samples. This would ensure the phytosanitary certification of plants, prevent the spread of contaminated plant material, and facilitate the implementation of timely phytosanitation and quarantine measures (4).

The current certified methods of bacterial detection rely mainly on culture-based approaches and plant bioassays (35). While these methods allow for a presumptive identification, they lack resolution of detection to the species or pathovar level, are often exceedingly time-consuming and costly for routine usage in quarantine procedures, or require specific biocontainment facilities, such as greenhouses or growth chambers (17). To circumvent these limitations, molecularly based detection methodologies have been proposed as more accurate and efficient alternatives. Particularly, DNA-based detection methods, some of which have already been validated in ring tests, have had their potential acknowledged for application in routine surveys (18, 25, 43).

The selection of DNA signatures, i.e., taxon-specific markers with discriminatory resolution for the target organism(s), and the optimization of a sensitive and suitable detection technique

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(PCR or hybridization based) are key premises for the development of a specific and reliable DNA-based method of bacterial detection and identification. For identification of xanthomonads, the selection of DNA signatures has been made mostly within specific regions of functional genes (7, 11, 14, 27). However, apart from the low number of markers provided by these approaches, these loci are frequently characterized by a low infrageneric resolution. Furthermore, the identification of other genes coding for specific functional traits is dependent on a comprehensive knowledge of bacterial metabolism, including the bacterium-specific infection mechanisms, such as virulence factors. This extensive knowledge, required to search for new markers, is poor or missing for most phytopathogenic bacteria. Other approaches for selection of DNA signatures have been described for *Xanthomonas* species, based on random specific regions discovered through repetitive sequence-based PCR (rep-PCR) (23), randomly amplified polymorphic DNA (RAPD)-PCR (12, 21), or subtractive hybridization (13, 34). Even though such approaches potentially allow the design of primers and probes for poorly characterized organisms, they require an extensive and laborious specificity validation (16, 44). Furthermore, the number of specific markers obtained with such approaches is low, and their genomic stability or intraspecific variability is generally not known (20).

Presently, the more than 1,200 fully sequenced bacterial genomes and the overall genomic information available in public databases allow access to a large spectrum of bacterial taxa and genomic information facilitating the selection of DNA signatures to any sequenced target bacteria, using the increasingly resourceful bioinformatics applications (2). However, these workflows are dependent on comparative genomics and thus are mainly restricted to fully sequenced organisms, which undermines their utility concerning unsequenced bacterial species. Therefore, new strategies are required to select markers for unsequenced phytopathogenic species. To date, most of the EPPO-recommended quarantine *Xanthomonas* species do not have a fully sequenced representative, among which are *Xanthomonas fragariae*, *Xanthomonas axonopodis* pv. phaseoli, and *Xanthomonas fuscans* subsp. *fuscans*, phytopathogens responsible for considerable losses in the agricultural production of strawberry and bean plants. *X. fuscans* subsp. *fuscans* strains are responsible for disease symptoms on bean plants identical to the common bacterial blight caused by *X. axonopodis* pv. phaseoli (24) and, until recently, were considered to be a variety of *X. axonopodis* pv. phaseoli (*X. axonopodis* pv. phaseoli variant fuscans). Although both the EPPO and the International Seed Testing Association (ISTA) still do not take into account this updated nomenclature (10, 35), the work of Schaad et al. (33) and subsequent research (1, 28) have helped to establish the taxonomic distinctiveness of *X. fuscans* subsp. *fuscans*.

The use of disease-free propagating material is considered the best control method for these phytopathogens, as chemical treatment of infected plants and use of resistant cultivars are considered secondary disease management procedures (35), which emphasizes the importance of developing rapid and effective detection methods. For *X. fragariae*, a few loci were identified as suitable for the design of species-specific primers: three RAPD-specific regions (29) and within the *hrp* (31) and *gyrB* (45) genes. Further research has mainly been focused in technological improvements of PCR-based detection methods, using the mentioned DNA regions (36, 39, 40, 49). In the case of *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans*, DNA-based detection methods are limited to conventional PCR-based methodologies (5, 38), with the classical methods remaining the standard detection procedures.

In this work, a comprehensive screening of *Xanthomonas*-specific molecular markers was validated by PCR and dot blot hybridization, in order to select and validate markers for the unsequenced xanthomonads *X. fragariae*, *X. axonopodis* pv. phaseoli, and *X. fuscans* subsp. *fuscans*. In addition, a dot blot platform coupled with automatic image analysis software was optimized to allow the fast detection of these bacteria over a large number of isolates.

MATERIALS AND METHODS

Selection of *Xanthomonas*-specific protein domains. Identification of *Xanthomonas*-specific protein domains was carried out using the "Compare Genomes" feature of the Pfam online database (release 22.0) (9), as previously described (42). Six fully sequenced *Xanthomonas* strains and 16 soil-dwelling or phytopathogenic strains were compared: *Xanthomonas axonopodis* pv. citri strain 306, *Xanthomonas campestris* pv. campestris strain 8004, *X. campestris* pv. campestris strain ATCC 33913, *X. campestris* pv. vesicatoria strain 85-10, *Xanthomonas oryzae* pv. oryzae KACC 10331, *Xanthomonas oryzae* pv. oryzae MAFF 311018, *Agrobacterium tumefaciens* Cereon, Aster yellows witches' broom phytoplasma, *Chromobacterium violaceum*, *Frankia* sp., *Nocardia farcinica*, *Pseudomonas fluorescens* Pf-5, *Pseudomonas fluorescens* PfO-1, *Pseudomonas putida*, *Pseudomonas syringae* pv. phaseolicola, *Pseudomonas syringae* pv. syringae, *Pseudomonas syringae* pv. tomato, *Ralstonia solanacearum*, *Rhizobium etli*, *Rhizobium loti*, *Rhizobium meliloti*, and *Xylella fastidiosa* 9a5c. The Pfam analysis allowed filtering out the protein domains that were not exclusive to *Xanthomonas*, and the nucleotide sequences coding for the remaining proteins' domains (a total of 48), i.e., xanthomonad specific, were retrieved. These sequences were checked for specificity using the BLAST (blastn) utility (3), and 21 were further chosen for experimental validation (Table 1).

Bacterial strains, culture conditions, and DNA extraction. The bacterial strains used in this study are listed in Table S1 in the supplemental material. *Xanthomonas* spp. and *Stenotrophomonas maltophilia* were cultured in YGC medium (glucose, 10 g liter^{-1}; yeast extract, 5 g liter^{-1}; $CaCO_3$, 30 g liter^{-1}; agar, 15 g liter^{-1}) at 28°C, except for *X. fragariae*, which was cultured in YPGA medium (yeast extract, 5 g liter^{-1}; Bacto peptone, 5 g liter^{-1}; glucose, 10 g liter^{-1}; agar, 15 g liter^{-1}) at 20°C. Non-*Xanthomonas* strains were cultured in nutrient agar (beef extract, 1 g liter^{-1}; yeast extract, 2 g liter^{-1}; peptone, 5 g liter^{-1}; NaCl, 5 g liter^{-1}; KH_2PO_4, 0.45 g liter^{-1}; $Na_2HPO_4 \cdot 12H_2O$, 2.39 g liter^{-1}; agar, 15 g liter^{-1}), except for *Xylella fastidiosa*, which was cultured in BCYE medium (46). DNA was extracted from pure bacterial cultures using the EZNA bacterial DNA purification kit (Omega Bio-Tek, Norcross, GA), following the manufacturer's instructions, and quantified by NanoDrop (Thermo Scientific, Wilmington, DE). *Escherichia coli* was cultured on Luria-Bertani medium at 37°C. Standard *E. coli* manipulation and *in vitro* DNA manipulations were carried out as described by Sambrook and Russell (32).

Primers and PCR validation. Primer pairs (see Table S2 in the supplemental material) were designed for each of the 21 selected loci, using the Vector NTI software (Invitrogen, Carlsbad, CA). In order to allow PCR assays to be performed using identical reaction conditions, all primer pairs were chosen in order to have a predicted amplicon size of 150 to 350 bp and a calculated optimal annealing temperature of around 60°C. Primer pairs were designed having as the template the sequence of *X. axonopodis* pv. citri strain 306 for primers XA1F/R to XA5F/R; *Xanthomonas campestris* pv. campestris strain 8004 for primers XC1F/R to XC12F/R, and *Xanthomonas oryzae* pv. oryzae MAFF 311018 for primers XO1F/R to XO4F/R (see Table S2 in the supplemental material). Moreover, based on a BLAST analysis of all predicted amplicons, primers were designed to anneal to the sites of each locus that showed higher specificity toward *Xanthomonas*. Most of the predicted amplicons exhibited specificity to *Xanthomonas*, as shown by the high E values obtained with non-*Xanthomonas* strains. Markers XC1, XC2, XC4, XC8, XC9, and XC10 revealed similarity with the member of the *Xanthomonadaceae S. maltophilia*, and the lowest E value was obtained for marker XC9 (E-value, 2e−51). Concerning marker XC12, the best BLAST hit for non-*Xanthomonas* was with the

TABLE 1. *Xanthomonas*-specific protein domains selected for molecular marker design[a]

Protein domain	Locus	No. of domains (no. of proteins) in[b]:						Corresponding marker
		X. axonopodis pv. citri strain 306	*X. campestris* pv. campestris		*X. campestris* pv. vesicatoria strain 85-10	*X. oryzae* pv. oryzae		
			Strain 8004	ATCC 33913		KACC 10331	MAFF 311018	
NAGLU	XAC0709	1 (1)	0	0	0	1 (1)	2 (2)	XA1
Peptidase_M35	XAC2763	1 (1)	0	0	1 (1)	0	0	XA2
DUF239	XAC3314	1 (1)	0	0	1 (1)	0	0	XA3
TFR_dimer	XAC3611	1 (1)	0	0	0	0	0	XA4
Sigma70_ECF	XAC4128	1 (1)	0	0	1 (1)	0	0	XA5
DUF938	XC_0087	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	0	XC1
DUF1105	XC_0608	1 (1)	1 (1)	1 (1)	1 (1)	0	0	XC2
Glyco_hydro_12	XC_0783	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	XC3
DUF819	XC_1392	1 (1)	1 (1)	1 (1)	1 (1)	0	0	XC4
CelD_N	XC_1727	1 (1)	1 (1)	1 (1)	1 (1)	0	0	XC5
Avidin	XC_2088	0	1 (1)	0	0	0	1 (1)	XC6
DUF1130	XC_2584	1 (1)	1 (1)	1 (1)	1 (1)	0	0	XC7
3-HAO	XC_2679	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	XC8
PLA1	XC_2818	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	XC9
Peptidase_M2	XC_3130	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	XC10
Glyco_hydro_39	XC_4065	1 (1)	1 (1)	1 (1)	0	0	0	XC11
Glyco_hydro_67C	XC_4193	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	XC12
LEA_4	XOO_0116	0	0	0	0	1 (1)	1 (1)	XO1
NTase_sub_bind	XOO_3261	0	0	0	0	1 (1)	1 (1)	XO2
CBM_6	XOO_3566	1 (1)	0	0	1 (1)	1 (1)	1 (1)	XO3
BsuBI_PstI_RE	XOO_3728	0	0	0	0	0	1 (1)	XO4

[a] The corresponding gene identifier (locus) and distribution of each domain, across the six analyzed *Xanthomonas* proteomes, are shown.

[b] The numbers shown represent the number of domains, with the number of proteins in which the domain is present shown in parentheses.

Brassicaceae pathogen *Hyaloperonospora parasitica* (E value, 8e−71) (see Table S2 in the supplemental material).

Three different annealing temperatures were tested (57, 59, and 61°C) in order to optimize the PCR conditions for each primer pair. The PCR mastermix contained 1× reaction buffer IV (ABgene, Epsom, United Kingdom), 0.2 mM each deoxynucleoside triphosphate (dNTP; Fermentas, Ontario, Canada), 1.5 mM $MgCl_2$, 0.2 μM each primer, and 1 U of Simple Red DNA polymerase (ABgene). Twenty-five nanograms of genomic DNA was used as the template, and the PCR conditions were as follows: an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 57, 59, or 61°C, and 30 s at 72°C, with a final extension step of 10 min at 72°C. Amplicons were extracted and purified from agarose gels stained with ethidium bromide (Bio-Rad, Hercules, CA), using the GFX PCR and gel band purification kit (GE Healthcare, Buckinghamshire, United Kingdom). Purified amplicons were cloned in pGEM-T Easy vector (Promega, Madison, WI), according to the manufacturer's instructions, and their identity was confirmed by sequencing (STAB Genomica, Portugal).

Dot blot hybridization assays. For dot blot assays, 100 ng of heat-denatured DNA from each bacteria was spotted into a nylon membrane using a Bio-Dot apparatus (Bio-Rad). DNA probes were obtained from purified PCR amplicons labeled with digoxigenin (DIG), using the DIG-High Prime labeling kit (Roche, Basel, Switzerland) and following the manufacturer's instructions. Hybridization was carried out overnight at 68°C, with a final probe concentration of 100 ng ml^{-1}. Washing and detection steps were carried out according to the DIG system recommendations (Roche). DIG-labeled nucleic acids were detected by chemiluminescence using X-ray films (GE Healthcare) or a Molecular Imager ChemiDoc system (Bio-Rad).

Dot blot analysis using an image-processing algorithm. In order to ensure an unbiased and automated analysis of the dot blot assays, an algorithm was developed to process the images obtained (22). Briefly, this MATLAB-based algorithm, available upon request, allows the automated rotation of the obtained dot blot images and adjustment of all dots to a user-defined grid. The software then calculates the probability of each dot being a positive (ON) result, using as references the positive and negative controls present in each membrane (6a). To achieve a proper quantification of signal intensities and ON probability, the exposure time of the Chemidoc system was adjusted so that all dots were below pixel saturation. Each probability value was calculated based on the analysis of four independent dots. By doing so, the variation of dot intensities due to different membrane positioning and/or to different hybridization assays was taken into account.

Nucleotide sequence accession number. DNA sequences have been deposited in the NCBI database under accession no. HQ315628 to HQ315642.

RESULTS

Selection of *Xanthomonas*-specific protein domains. The "Compare Genomes" feature of Pfam was used to directly compare all the protein domains from the deduced proteomes of the selected microorganisms. On average, 1,700 different protein domains are present in each *Xanthomonas* proteome, with around 1,500 domains being shared by the six fully sequenced strains considered in this study. All of the unspecific domains, present in at least one of the 16 nonxanthomonads were filtered out, leaving 48 protein domains exclusive to at least one of the six *Xanthomonas* species used for this *in silico* screening. After the Pfam comparison, the nucleotide coding region for each specific protein domain region was retrieved, and a BLAST analysis was carried out, aiming to widen the specificity assessment to the full universe of genomic sequences deposited in the NCBI database. Twenty-one out of the 48 loci, corresponding to the xanthomonad-specific protein domains, showed low similarity toward any other genus and were selected for PCR and hybridization validation. The occurrence of the selected loci was not uniform among *Xanthomonas* strains, ranging from domains present in only one strain (TFR_dimer and Bsu_PstI_RE) to domains present in all strains (Glyco_hydro_12, 3-HAO, PLA1, Peptidase_M2, and Glyco_hydro_67C) (Table 1).

PCR validation of markers with the nonsequenced *X. fragariae* and *X. axonopodis* pv. phaseoli. PCRs were initially performed with DNA extracted from *Xanthomonas* strains corresponding to the three template genome sequences used for

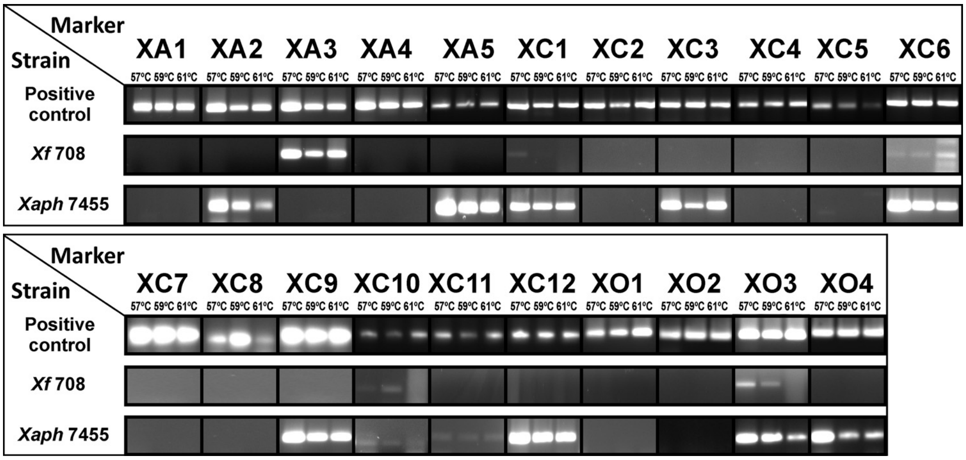

FIG. 1. PCR analysis results using the selected primer pairs. The template DNA used as positive control was *Xanthomonas axonopodis* pv. citri LMG 9322 for markers XA1 to XA5, *Xanthomonas campestris* pv. campestris LMG 568 for markers XC1 to XC12, and *Xanthomonas oryzae* pv. oryzae LMG 5047 for markers XO1 to XO4. *Xf* 708, *X. fragariae* LMG 708; *Xaph* 7455, *X. axonopodis* pv. phaseoli LMG 7455.

primer design. As expected, positive amplification was obtained for markers XA1 to XA5 with DNA from *X. axonopodis* pv. citri LMG 9322, markers XC1 to XC12 with *X. campestris* pv. campestris LMG 568, and markers XO1 to XO4 with *Xanthomonas oryzae* pv. oryzae LMG 5047 (Fig. 1). Moreover, amplification was obtained for the three annealing temperatures assayed, and the amplicons' identity was confirmed by sequencing.

All 21 primer pairs were then evaluated with DNA from the pathovar reference strain of *X. axonopodis* pv. phaseoli (LMG 7455) and type strain of *X. fragariae* (LMG 708), using the same PCR conditions. The results showed that for *X. fragariae* LMG 708, positive amplification was only consistently achieved with markers XA3, XC6, XC10, and XO3, along with a faint amplification with marker XC1. However, for *X. axonopodis* pv. phaseoli LMG 7455, a larger number of markers (XA2, XA5, XC1, XC3, XC6, XC9, XC10, XC11, XC12, XO3, and XO4) provided consistent positive amplification under the tested PCR conditions (Fig. 1). The PCR amplicons were sequenced, and high similarity was verified with the template *Xanthomonas* strains used for primer design (query coverage higher than 98% and E value lower than 1e−60), therefore demonstrating the presence of the selected regions in *X. fragariae* and *X. axonopodis* pv. phaseoli.

Dot blot specificity analysis. In order to access the specificity of the selected markers, 13 quarantine *Xanthomonas* strains and 23 non-*Xanthomonas* strains were analyzed by dot blot hybridization. The PCR products, obtained using template DNA from *X. fragariae* and *X. axonopodis* pv. phaseoli, were labeled with digoxigenin and used as *X. fragariae*- and *X. axonopodis* pv. phaseoli-specific probes. The tested probes were XA3, XC6, XC10, and XO3 obtained from *X. fragariae* LMG 708 and XA2, XA5, XC1, XC3, XC9, XC11, XC12, and XO4 obtained from *X. axonopodis* pv. phaseoli LMG 7455.

Concerning the two target bacteria, seven probes provided positive hybridization signals with *X. fragariae*, while XA3 was the only marker negative for *X. axonopodis* pv. phaseoli (Fig. 2). Probes XA2 and XC3 provided positive hybridization signals with *X. fragariae* LMG 708, although no PCR amplification was observed.

When assessing 13 quarantine *Xanthomonas* strains, the hybridization results vary from markers present in all tested strains (XC1 and XC10) to markers present only in four strains (XO4). When the full array of 12 probes was considered, the strain-specific hybridization profile allowed us to distinguish the different *Xanthomonas* strains, with the exception of *Xanthomonas arboricola* pv. corylina LMG 689, *X. arboricola* pv. pruni LMG 852, *Xanthomonas axonopodis* pv. dieffenbachiae LMG 695, and *Xanthomonas vesicatoria* LMG 911, which shared the same hybridization pattern (Fig. 2).

To dismiss unspecific hybridization to other bacteria and further confirm the unequivocal specificity of the selected probes toward *Xanthomonas*, 23 nonxanthomonads, comprising other phytopathogens or bacteria with matching hosts or habitats, were assayed by dot blotting (data not shown). Results confirmed the probes' specificity for *Xanthomonas*, with exception of probe XC9, for which hybridization signals were obtained with the closely related member of the *Xanthomonadaceae Stenotrophomonas maltophilia* LMG 958, while for *Xylella fastidiosa* LMG 17159, another phytopathogenic member of the *Xanthomonadaceae*, no hybridization was observed (Fig. 2).

Identification of *X. fragariae*, *X. axonopodis* pv. phaseoli, and *X. fuscans* subsp. *fuscans* strains by dot blot hybridization with automatic analysis. The exploratory dot blot used for marker validation enabled the choice of a combination of three markers (XA3, XA5, and XO4) that provided unique hybridization patterns for *X. fragariae* and *X. axonopodis* pv. phaseoli in comparison with the other 13 *Xanthomonas* species tested. Marker XC1, a broad-spectrum marker present in all tested *Xanthomonas* species, was also included in this validation as a horizontal marker for xanthomonads.

To avoid operator-dependent analyses of the dot blot results, a key limitation to implement this hybridization technique in routine phytodiagnostics assays, an image analysis algorithm was developed. Essentially, this algorithm allows the determination of the variation in dot intensities among exper-

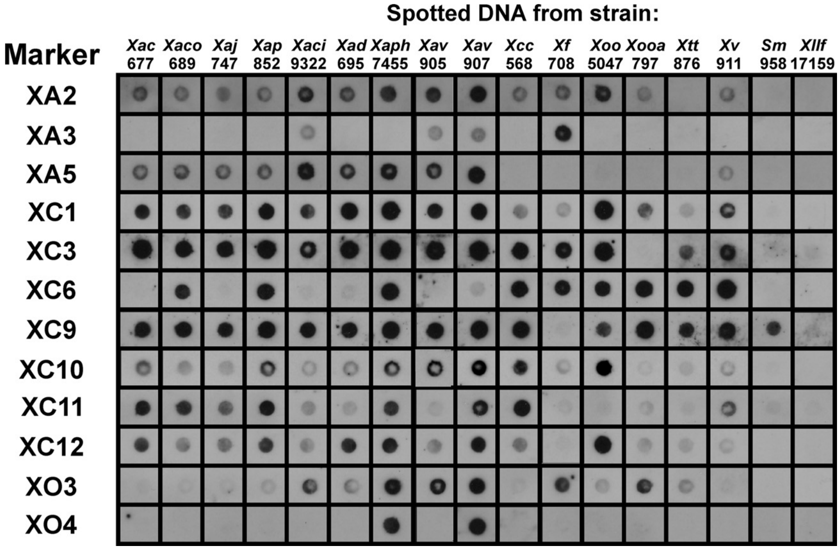

FIG. 2. Dot blot analysis of digoxigenin-labeled probes using DNA from a collection of phytopathogenic *Xanthomonas* strains and the closely related *Xanthomonadaceae Stenotrophomonas maltophilia* LMG 958 and *Xylella fastidiosa* LMG 17159. Strain abbreviations are defined in Table 2. Markers XA3, XC6, XC10, and XO3 were obtained with DNA template from *X. fragariae* LMG 708, and markers XA2, XA5, XC1, XC3, XC9, XC11, XC12, and XO4 were obtained with DNA template from *Xanthomonas axonopodis* pv. phaseoli LMG 7455.

imental replicates and, therefore, allows us to evaluate the reliability of the hybridization patterns. Furthermore, as the algorithm outputs a probability value, with each dot being a positive hybridization signal based on the measured intensity of the pixels, it was possible to calculate the variation among the signals obtained for each strain and marker tested (6a, 22).

Dot blots using the four markers (XA3, XA5, XC1, and XO4) mentioned above as probes against template DNA of all the bacterial species used in this study confirmed the previous qualitative validation (Fig. 2), strengthening the consistency of the obtained patterns (Fig. 3). Indeed, the computed probability values obtained with the automatic analyses of the dot blots show a high consistency (Table 2). Furthermore, all of the non-*Xanthomonas* strains presented low probability values, including the *Xanthomonadaceae S. maltophilia* and *Xylella fastidiosa*, further emphasizing the specificity of these four markers and the software's reliability to quantify the signals. The results obtained for marker XC1 showed a negligible probability for *Xanthomonas translucens* pv. translucens LMG 876, which displayed a positive hybridization signal in the previous dot blot analysis (Fig. 2). This result is likely due to the low signal intensity obtained for this strain when chemiluminescence was acquired by a ChemiDoc system below the saturation point of all pixels.

These markers were further validated on 27 *X. fragariae* strains, 13 *X. axonopodis* pv. phaseoli strains, and 4 *X. fuscans* subsp. *fuscans* strains (Fig. 4) to determine if the hybridization patterns were consistent between different strains of the target *Xanthomonas* species. All tested *X. fragariae* strains displayed maximum probability for marker XA3 (1 ± 0) and low probabilities for markers XA5 (≤0.18) and XO4 (≤0.04), while *X. axonopodis* pv. phaseoli strains had low probability for XA3 (≤0.21) and high probability for XA5 (≥0.75) (see Table S3 in the supplemental material). Furthermore, the standard deviation in the probability values was very low for all the strains tested using these probes. When analyzing the results for marker XO4, positive hybridization results were obtained for all of the tested *X. axonopodis* pv. phaseoli strains (≥0.99), with the exception of one strain—*X. axonopodis* pv. phaseoli CPBF 400 (see Table S3 in the supplemental material). Concerning the four *X. fuscans* subsp. *fuscans* strains, these presented low probability for XA3 (≤0.01) and high probability for XA5 (≥0.96), similarly to *X. axonopodis* pv. phaseoli strains. However, and unlike *X. axonopodis* pv. phaseoli strains, all *X. fuscans* subsp. *fuscans* strains presented very low values for XO4 (0.00). The genus-specific marker XC1 presented high probability values for all *X. axonopodis* pv. phaseoli strains (≥0.87, with the exception of *X. axonopodis* pv. phaseoli strain CPBF 399, which presents a probability of 0.41). Similarly, all tested *X. fuscans* subsp. *fuscans* strains present high probability values for this marker (≥0.95). The probability values obtained with marker XC1 for *X. fragariae* strains were unexpectedly highly variable (≥0.30 and ≤ 0.81), with the standard deviations of the results from different experiments being much higher than those obtained for any other probe (see Table S3 in the supplemental material). Although XC1 was considered a broad-spectrum marker, the fact that it was obtained from amplification of *X. axonopodis* pv. phaseoli LMG 7455, coupled with the use of high-stringency conditions of hybridization, might explain the lower consistency obtained for the different *X. fragariae* strains for this marker. Nevertheless, these values are undoubtedly higher than the values obtained for those considered negative signals (see Table S3 in the supplemental material).

DISCUSSION

According to the recommended detection standards from EPPO and ISTA, the current methods for the detection of *X. fragariae* and *X. axonopodis* pv. phaseoli are primarily based on

	1	2	3	4	5	6	7	8	9	10	11	12
a	C+	*Xac* 677	*Xaco* 689	*Xaj* 747	*Xap* 852	*Xaci* 9322	*Xad* 695	*Xaph 7455*	*Xav* 905	*Xcv* 907	*Xcc* 568	C+
b	*Xf* 708	*Xoo* 5047	*Xooa* 797	*Xtt* 876	*Xv* 911	*Cmm* 7333	*Ea* 2024	*Pa* 2386	*Pcc* 2404	*Pch* 2804	*Pf* Pf0-1	*Pp* 2440
c	*Psvg* 5066	*Psvp* 2245	*Psh* 5067	*Psm* 5071	*Pso* 10912	*Pss* 10604	*Pst* 5393	*Pst* 3000	*Rp* 5942	*Rs* 2299	*Rs* 2302	*Rs* 2306
d	C-	*Rs* 17138	*Rs* 17140	*Sm* 958	*Xllf* 17159	C-	C-	C-	C-	C-	C-	C-
e	C-	*Xac* 677	*Xaco* 689	*Xaj* 747	*Xap* 852	*Xaci* 9322	*Xad* 695	*Xaph 7455*	*Xav* 905	*Xcv* 907	*Xcc* 568	C-
f	*Xf* 708	*Xoo* 5047	*Xooa* 797	*Xtt* 876	*Xv* 911	*Cmm* 7333	*Ea* 2024	*Pa* 2386	*Pcc* 2404	*Pch* 2804	*Pf* Pf0-1	*Pp* 2440
g	*Psvg* 5066	*Psvp* 2245	*Psh* 5067	*Psm* 5071	*Pso* 10912	*Pss* 10604	*Pst* 5393	*Pst* 3000	*Rp* 5942	*Rs* 2299	*Rs* 2302	*Rs* 2306
h	C+	*Rs* 17138	*Rs* 17140	*Sm* 958	*Xllf* 17159	C-	C-	C-	C-	C-	C-	C+

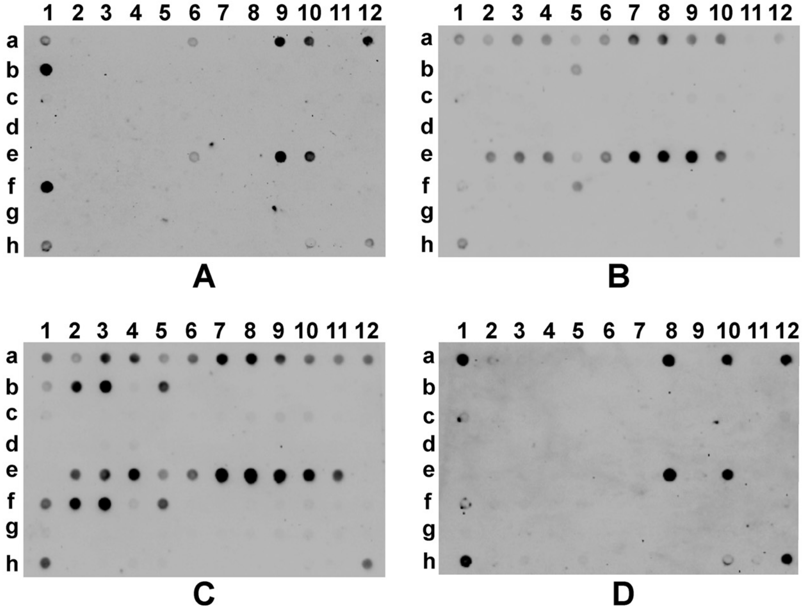

FIG. 3. Validation of dot blot hybridization patterns. Strain abbreviations are defined in Table 2. (A) Probe XA3; (B) probe XA5; (C) probe XC1; and (D) probe XO4. Tris-EDTA (TE) buffer was used for the negative controls (C−). A mixture of 6 ng of each purified PCR amplicon, corresponding to each of the digoxigenin-labeled probes, was used for the positive controls (C+). Probability values are detailed in Table 2.

serological techniques using polyclonal antibodies and on culture on selective medium, while the DNA-based methods are still largely underrepresented (10, 25). The frequent cross-reactions of the *X. fragariae* polyclonal antibodies, as highlighted by an assessment study of diagnostics methods for quarantine organisms carried out by several laboratories across Europe, known as DIAGPRO (25), and the need to develop culture-independent diagnostic standards to hasten the detection of these phytopathogens, particularly of the fastidious organism *X. fragariae*, underlined the importance of specific and reliable DNA-based methods of detection able to provide fast confirmatory diagnostics for *X. fragariae* and *X. axonopodis* pv. phaseoli. In this work, we propose several novel detection markers for xanthomonads in general, and for *X. fragariae*, *X. axonopodis* pv. phaseoli, and *X. fuscans* subsp. *fuscans* in particular, in order to increase diagnostic reliability and contribute to development of single-step DNA-based and culture-independent confirmatory identification of these phytopathogens.

At the moment, the limited number of specific primers described for *X. fragariae* (29, 31, 45), for *X. axonopodis* pv. phaseoli by Audy et al. (5) and other works that followed based on the same set of primers (19, 26, 30, 37), and for *X. fuscans* subsp. *fuscans* (38) have been hampering the implementation of DNA-based detection protocols as trustworthy alternatives to isolation in pure culture. Furthermore, the unavailability of complete genome sequences for most quarantine phytopatogenic *Xanthomonas* strains does not allow the direct selection of target-specific DNA signatures using comparative genomics bioinformatics tools. Therefore, to identify novel DNA-specific markers able to detect *X. fragariae*, *X. axonopodis* pv. phaseoli, and *X. fuscans* subsp. *fuscans* isolates, which are unsequenced bacteria, an indirect *in silico*-based approach previously described by us was used (42). Essentially, we hypothesized that some of the Pfam protein domains, present exclusively in the sequenced *Xanthomonas* strains, would also be present in the unsequenced target bacteria *X. fragariae*, *X. axonopodis* pv. phaseoli, and *X. fuscans* subsp. *fuscans*. Furthermore, having in mind that this marker selection methodology takes into account only putative functional regions, we increased the likelihood of these protein domain-encoding loci to be conserved across different species of the same genus.

From the comparison of the proteomes of *Xanthomonas* strains with several nontarget bacteria that share hosts or habitats, 48 protein domains were filtered as specific for the genus. The follow-up BLAST analysis of the selected protein domains and their respective encoding regions (i.e., putative markers) enabled confirmation of the specificity of 21 protein domains, emphasizing the importance of including a BLAST analysis as

TABLE 2. Outputted probability values concerning the dot blot validation assays

Strain no.	Strain name	Species abbreviation in Fig. 1–4	Calculated ON probability[a]			
			XA3	XA5	XC1	XO4
1	*Xanthomonas arboricola* pv. celebensis LMG 677	*Xac*	0.04 ± 0.05	**0.86 ± 0.22**	**0.81 ± 0.22**	0.1 ± 0.13
2	*Xanthomonas arboricola* pv. corylina LMG 689	*Xaco*	**0.86 ± 0.12**	**1 ± 0**	**1 ± 0.01**	0 ± 0
3	*Xanthomonas arboricola* pv. juglandis LMG 747	*Xaj*	0.01 ± 0.01	**0.99 ± 0.02**	**0.97 ± 0.04**	0.02 ± 0.03
4	*Xanthomonas arboricola* pv. pruni LMG 852	*Xap*	0.01 ± 0.02	**1 ± 0**	**1 ± 0**	0 ± 0
5	*Xanthomonas axonopodis* pv. citri LMG 9322	*Xaci*	0.01 ± 0.01	**0.97 ± 0.06**	**0.92 ± 0.14**	0.01 ± 0.01
6	*Xanthomonas axonopodis* pv. diffenbachiae LMG 695	*Xad*	0.02 ± 0.03	**0.88 ± 0.13**	**0.87 ± 0.13**	0 ± 0
7	*Xanthomonas axonopodis* pv. phaseoli LMG 7455	*Xaph*	0 ± 0	**1 ± 0**	**1 ± 0**	**1 ± 0**
24	*Xanthomonas axonopodis* pv. vesicatoria LMG 905	*Xav*	**1 ± 0**	**1 ± 0**	**1 ± 0**	0 ± 0.01
25	*Xanthomonas campestris* pv. vesicatoria LMG 907	*Xcv*	**1 ± 0.01**	**1 ± 0**	**1 ± 0**	**0.99 ± 0.02**
26	*Xanthomonas campestris* pv. campestris LMG 568	*Xcc*	0.03 ± 0.03	0.04 ± 0.06	**0.84 ± 0.23**	0.01 ± 0.01
28	*Xanthomonas fragariae* 708	*Xf*	**1 ± 0**	0.4 ± 0.37	**0.8 ± 0.31**	0.27 ± 0.34
54	*Xanthomonas oryzae* pv. oryzae LMG 5047	*Xoo*	0.02 ± 0.01	0.08 ± 0.08	**1 ± 0**	0.05 ± 0.06
55	*Xanthomonas oryzae* pv. oryzicola LMG 797	*Xooa*	0.01 ± 0.02	0.06 ± 0.07	**1 ± 0**	0.01 ± 0.01
56	*Xanthomonas translucens* pv. translucens LMG 876	*Xtt*	0 ± 0.01	0.13 ± 0.17	0.14 ± 0.13	0 ± 0
57	*Xanthomonas vesicatoria* LMG 911	*Xv*	0 ± 0.01	**1 ± 0**	**0.98 ± 0.04**	0 ± 0
58	*Clavibacter michiganensis* subsp. *michiganensis* LMG 7333	*Cmm*	0 ± 0	0.02 ± 0.04	0.01 ± 0.01	0 ± 0
59	*Erwinia amylovora* LMG 2024	*Ea*	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.02	0 ± 0
60	*Pectobacterium atrosepticum* LMG 2386	*Pa*	0 ± 0	0 ± 0	0.01 ± 0.03	0 ± 0
61	*Pectobacterium carotovorum* subsp. *carotovorum* LMG 2404	*Pcc*	0.01 ± 0.01	0.01 ± 0.02	0.07 ± 0.05	0 ± 0
62	*Pectobacterium chrysanthemi* LMG 2804	*Pch*	0.02 ± 0.02	0.01 ± 0.01	0.06 ± 0.08	0.01 ± 0.01
63	*Pseudomonas fluorescens* Pf0-1	*Pf*	0.03 ± 0.02	0.01 ± 0.02	0.02 ± 0.02	0 ± 0.01
64	*Pseudomonas putida* KT 2440	*Pp*	0.07 ± 0.1	0 ± 0	0.02 ± 0.02	0.01 ± 0.03
65	*Pseudomonas savastanoi* pv. glycinea LMG 5066	*Psvg*	0.01 ± 0.02	0.03 ± 0.05	0.23 ± 0.35	0.1 ± 0.19
66	*Pseudomonas savastanoi* pv. phaseolicola LMG 2245	*Psvp*	0 ± 0.01	0.01 ± 0.02	0.07 ± 0.09	0 ± 0
67	*Pseudomonas syringae* pv. helianthi LMG 5067	*Psh*	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0 ± 0
68	*Pseudomonas syringae* pv. maculicola LMG 5071	*Psm*	0 ± 0	0.03 ± 0.03	0.01 ± 0.01	0 ± 0
69	*Pseudomonas syringae* pv. oryzae LMG 10912	*Pso*	0 ± 0	0.02 ± 0.04	0.05 ± 0.05	0 ± 0
70	*Pseudomonas syringae* pv. syringae DSM 10604	*Pss*	0 ± 0	0.01 ± 0.02	0.01 ± 0.01	0 ± 0
71	*Pseudomonas syringae* pv. tabaci LMG 5393	*Pstb*	0.04 ± 0.05	0.18 ± 0.2	0.09 ± 0.07	0.14 ± 0.14
72	*Pseudomonas syringae* pv. tomato DC 3000	*Pst*	0.01 ± 0.01	0.02 ± 0.04	0 ± 0	0.03 ± 0.05
73	*Ralstonia picketii* LMG 5942	*Rp*	0.04 ± 0.06	0.32 ± 0.1	0.13 ± 0.08	0 ± 0.01
74	*Ralstonia solanacearum* LMG 2299	*Rs*	0.06 ± 0.1	0.06 ± 0.05	0.07 ± 0.09	0 ± 0
75	*Ralstonia solanacearum* LMG 2302	*Rs*	0.05 ± 0.08	0.02 ± 0.04	0.01 ± 0.03	0 ± 0.01
76	*Ralstonia solanacearum* LMG 2306	*Rs*	0.1 ± 0.15	0.01 ± 0.02	0.01 ± 0.01	0.04 ± 0.05
77	*Ralstonia solanacearum* LMG 17138	*Rs*	0 ± 0.01	0.04 ± 0.08	0.01 ± 0.03	0 ± 0
78	*Ralstonia solanacearum* LMG 17140	*Rs*	0.01 ± 0.02	0.01 ± 0.02	0.06 ± 0.05	0 ± 0
79	*Stenotrophomonas maltophilia* LMG 958	*Sm*	0.01 ± 0.02	0.04 ± 0.06	0.06 ± 0.06	0 ± 0.01
80	*Xylella fastidiosa* LMG 17159	*Xllf*	0 ± 0.01	0.02 ± 0.04	0.04 ± 0.03	0.03 ± 0.05
7–19[b]	*Xanthomonas axonopodis* pv. phaseoli	*Xaph*	(0.000–0.094)	(0.873–1.000)	(0.855–0.990)	(0.831–1.000)
20	*Xanthomonas fuscans* subsp. *fuscans* CPBF 507	*Xff*	0.01 ± 0.01	**1 ± 0**	**0.95 ± 0.06**	0 ± 0.01
21	*Xanthomonas fuscans* subsp. *fuscans* CPBF 508	*Xff*	0 ± 0	**1 ± 0.01**	**0.98 ± 0.04**	0 ± 0
22	*Xanthomonas fuscans* subsp. *fuscans* CPBF 509	*Xff*	0.01 ± 0.01	**0.96 ± 0.07**	**0.96 ± 0.05**	0 ± 0
23	*Xanthomonas fuscans* subsp. *fuscans* CPBF 795	*Xff*	0 ± 0	**0.99 ± 0.02**	**1 ± 0**	0 ± 0
27–53[b]	*X. fragariae*	*Xf*	(1.000–1.000)	(0.026–0.088)	(0.450–0.630)	(0.006–0.018)

[a] The values shown represent the average probability ± standard deviation. The values considered as positive signals are highlighted in bold. The 99% confidence intervals for the mean probability values obtained with the *Xanthomonas axonopolis* pv. phaseoli (strains 7 to 19) and *X. fragariae* (strains 27 to 53) collections are displayed in parentheses and calculated according to the equation $\bar{x} + \bar{z}_{[1-(\alpha/2)]}(s/\sqrt{n})$, where $\bar{x}$ is the average probability value, z is the standard score, s is the standard deviation, and n is the number of replicates.

[b] Strain-specific probability values are detailed in Table S3 in the supplemental material.

a fine-tuning tool in any marker selection workflow (2). The distribution of the 21 selected protein domains among the sequenced *Xanthomonas* strains was not uniform, ranging from domains present in only one strain to domains common to all proteomes (Table 1). These data suggested the existence of a specific pattern of markers, determined by the presence or absence of each marker, for the different *Xanthomonas* species.

Using *X. fragariae*, *X. axonopodis* pv. phaseoli, and *X. fuscans* subsp. *fuscans* as the target bacteria, we analyzed the potential of the selected markers for detection of nonsequenced *Xanthomonas*. A preliminary PCR assay showed that eight markers were amplified with *X. axonopodis* pv. phaseoli LMG 7455, and one marker was amplified with *X. fragariae* LMG 708, while three markers were consistently amplified for both strains (Fig. 1). These 12 markers were used as probes in dot blot assays. Each probe provided positive hybridization with several *Xanthomonas* strains, and species-specific hybridization patterns were obtained, with the exception of four *Xanthomonas* strains that shared the same hybridization pattern (Fig. 2). Interestingly, although markers XA2 and XC3 gave positive hybridization for *X. fragariae*, the negative amplification obtained in the preliminary PCR assays for these markers (Fig. 1), suggests sequence mismatches at the primers' annealing sites, preventing amplification. These PCR false-negative results, which are predominantly frequent if the sequence differ-

	1	2	3	4	5	6	7	8	9	10	11	12
a	C+	*Xaph* 7455	*Xaph* 8014	*Xaph* 399	*Xaph* 400	*Xff* 507	*Xff* 508	*Xff* 509	*Xaph* 510	*Xaph* 511	*Xaph* 512	C+
b	*Xaph* 513	*Xaph* 514	*Xaph* 515	*Xaph* 516	*Xaph* 517	*Xaph* 644	*Xff* 795	*Xf* 708	*Xf* 706	*Xf* 405	*Xf* 463	*Xf* 466
c	*Xf* 468	*Xf* 609	*Xf* 610	*Xf* 613	*Xf* 618	*Xf* 621	*Xf* 823	*Xf* 828	*Xf* 863	*Xf* 864	*Xf* 865	*Xf* 867
d	C-	*Xf* 930	*Xf* 954	*Xf* 955	*Xf* 958	*Xf* 1044	*Xf* 1098	*Xf* 1099	*Xf* 1100	*Xf* 1101	*Xf* 1102	C-
e	C-	*Xaph* 7455	*Xaph* 8014	*Xaph* 399	*Xaph* 400	*Xff* 507	*Xff* 508	*Xff* 509	*Xaph* 510	*Xaph* 511	*Xaph* 512	C-
f	*Xaph* 513	*Xaph* 514	*Xaph* 515	*Xaph* 516	*Xaph* 517	*Xaph* 644	*Xff* 795	*Xf* 708	*Xf* 706	*Xf* 405	*Xf* 463	*Xf* 466
g	*Xf* 468	*Xf* 609	*Xf* 610	*Xf* 613	*Xf* 618	*Xf* 621	*Xf* 823	*Xf* 828	*Xf* 863	*Xf* 864	*Xf* 865	*Xf* 867
h	C+	*Xf* 930	*Xf* 954	*Xf* 955	*Xf* 958	*Xf* 1044	*Xf* 1098	*Xf* 1099	*Xf* 1100	*Xf* 1101	*Xf* 1102	C+

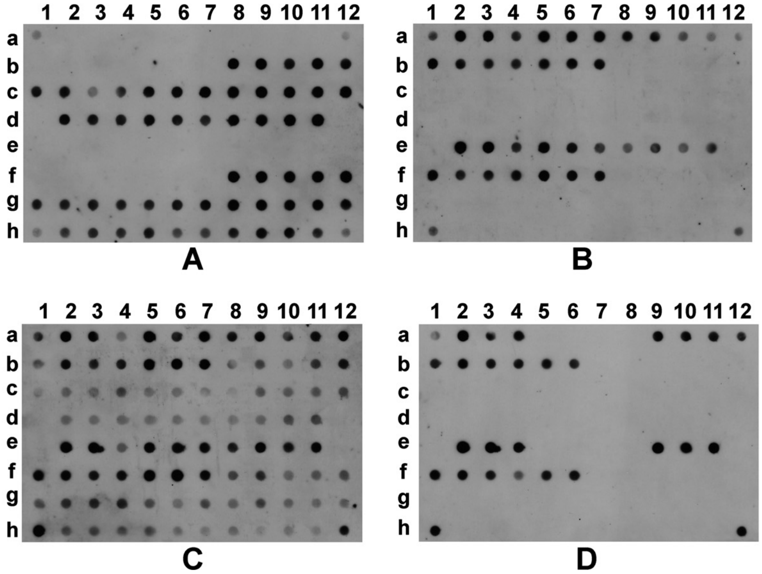

FIG. 4. Dot blot validation results with a collection of *Xanthomonas fragariae*, *Xanthomonas axonopodis* pv. phaseoli, and *Xanthomonas fuscans* subsp. *fuscans* strains. Strain abbreviations are defined in Table 2. (A) Probe XA3; (B) probe XA5; (C) probe XC1; and (D) probe XO4. TE buffer was used for the negative controls (C−). A mixture of 6 ng of each purified PCR amplicon, corresponding to each of the digoxigenin-labeled probes, was used for the positive controls (C+). Probability values are detailed in Table S3 in the supplemental material.

ences are located in the 3′-primer region (47), are a favorable argument for the implementation of hybridization detection methods over PCR-based methods.

The specificity of the probes toward *Xanthomonas* was further strengthened by the fact that no hybridization signal was obtained with the 21 non-*Xanthomonadacea* tested. The results obtained in the above-mentioned validation assays allowed identification of a combination of three probes (XA3, XA5, and XO4) able to distinguish specifically *X. fragariae*, *X. axonopodis* pv. phaseoli, and *X. fuscans* subsp. *fuscans* from the other tested *Xanthomonas* species. In fact, while probe XA3 was shown to be specific for *X. fragariae*, probe XA5 hybridized to all *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans* strains tested, but not to *X. fragariae*. To distinguish *X. axonopodis* pv. phaseoli from *X. fuscans* subsp. *fuscans*, a xanthomonad species symptomatically indistinguishable from *X. axonopodis* pv. phaseoli in infected plants and up to recently considered a subspecies of *X. axonopodis* pv. phaseoli (8, 33), probe XO4 hybridized to *X. axonopodis* pv. phaseoli, with exception of *X. axonopodis* pv. phaseoli strain CPBF400, but not to the *X. fuscans* subsp. *fuscans* strains used in this study, allowing a presumptive discrimination of these two species. Probe XC1, chosen as a xanthomonad-specific marker, hybridized to all *X. fragariae*, *X. axonopodis* pv. phaseoli, and *X. fuscans* subsp. *fuscans* strains tested and not to the closely related *Xanthomonadaceae S. maltophilia* and *Xylella fastidiosa*, confirming its usefulness as a genus-positive control.

Although the dot blot validation of these probes confirmed the consistency and specificity of the obtained hybridization profiles toward numerous strains of *X. fragariae*, *X. axonopodis* pv. phaseoli, and *X. fuscans* subsp. *fuscans*, the ambiguity inherent in operator-dependent analysis of dot blots' hybridization data is still a major weakness to the implementation of macroarrays for microbial detection assays and likely a reason why PCR-based protocols are generally favored. In order to overcome this limitation, we developed and optimized an innovative automated image analysis algorithm to ensure the numerical analysis of dot blot data (6a, 22). By converting each hybridization signal into probability values, the software enables comparisons of data from different independent experiments, which allows us to validate the data statistically.

Overall, this work proposes 21 novel markers useful for the identification of *Xanthomonas*, particularly for those species in which the number of markers for DNA-based methods of detection is limited. The proposed detection dot blots might complement the established PCR methods that do not possess the throughput of dot blotting, by narrowing down the samples for confirmatory PCR-based detection. It is further shown that dot blots coupled with automatic data analysis are convenient platforms for fast and easy screening of dozens of isolates simultaneously, contrary to microarrays that only allow the assay of a single isolate at a time and are economically unsustainable for routine phytosanitary analysis (6, 18). Most importantly, while the complex microarray data sets require extended expertise to interpret the results, the image-processing software developed here allows a reliable and user-friendly analysis of dot blot hybridization data, which ultimately might facilitate the use of macroarrays by plant diagnostic laboratories.

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