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Design and development of a DNA microarray for rapid identification of multiple European quarantine phytopathogenic bacteria

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Abstract The European and Mediterranean Plant Protection Organization (EPPO) lists for quarantine status 26 phytopathogenic bacteria which pose serious economic threats to agricultural and natural ecosystems. A prototype diagnostic DNA microarray was developed for the rapid and simple identification of 22 of these quarantine bacteria. The microarray has 38 probes targeted to the 16S rDNA and the house-keeping genes *rpoB, groEL* and *ftsZ*. The 16S rDNA probes were selected according to a multiple-probe concept taking into account the hierarchical structure of phytobacterial systematics. Hybridisation with Cy3-labelled PCR products of corresponding genes enabled differentiation of the quarantine bacteria down to the species and subspecies level.

Keywords Plant inspection · Bacterial diagnostics · Quarantine pathogen · Microarray

Introduction

The European and Mediterranean Plant Protection Organization (EPPO) list of phytopathogenic quarantine bacteria comprises 26 species, subspecies, and pathovars that cause serious economic losses worldwide and pose a significant threat to agricultural and natural ecosystems in Europe and surrounding regions (http://www.eppo.org/). Plant protection decisions to implement quarantine control measures against accidental or intentional introductions require rapid and reliable identification of these pathogens without unduly obstructing trade in healthy plant material (Fletcher et al. 2006). Available methods for pathogen detection include combinations of culture plating, serology, plant bioassays, and for many, but not all, specific PCR assays. A drawback of these methods is their selectivity for individual pathogens necessitating the processing of plant samples with a suite of methods to detect more than one pathogen potentially present in a consignment. Access to a single method capable of simultaneous detection of a range of pathogens would circumvent these difficulties. Furthermore, a multiplex method would also facilitate interception of unexpected pathogens in plant consignments.

Microarrays are one of the most promising tools for simultaneous detection of a wide-range of organisms in a single assay (Lievens and Thomma 2005; Ward et al. 2004). Oligonucleotide or DNA microarrays are a relatively recent technology development originally designed in the 1990s for transcriptomic applications in functional genomics and genetic analysis (Stoughton 2005). The versatility of the microarray platform has since been exploited for microbial genotyping (Kim et al. 2004; Vora et al. 2005), environmental diversity analysis (Zhou 2003) and most recently for diagnostics (Tobler et al. 2006; Wang et al. 2002). The appeal of diagnostic DNA microarrays rests in their potential for rapid, multiplex detection of theoretically hundreds of

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microorganisms and in the minimisation of required work steps, typically a single PCR reaction followed by hybridisation and reading. Depending on the choice of probes spotted onto a microarray the level of discrimination can extend to genus, species and deeper taxonomic levels (i.e., subspecies, pathovar) (Bodrossy and Sessitsch 2004). In the past few years, agricultural applications of microarrays have been developed for the detection and identification of plant pathogenic viruses (Deyong et al. 2005), fungi (Lievens et al. 2005), phytoplasmas (Nicolaisen and Bertaccini 2007) and bacteria (Fessehaie et al. 2003). The majority of these are targeted towards a specific host plant and include primarily endemic pathogens (Boonham et al. 2003; Lievens et al. 2003; Sholberg et al. 2005). No microarray is currently available that has a focus on multiple quarantine pathogens critical for phytosanitary inspection applications. Here we present the design and development of a robust DNA microarray that combines ease of use and rapid identification of most of the EPPO-listed quarantine bacteria to their genus, species and in some cases subspecies levels.

Materials and methods

Strains

The bacterial strains used in this study are listed in Table 1. Strains were routinely grown on YPGA (yeast 7 g, bacto peptone 7 g, glucose 7 g, agar 15 g l^{-1}) and LB (5 g NaCl, 5 g yeast, 10 g tryptone, 15 g agar l^{-1}) agar media with incubation at 28°C.

DNA extraction

Whole cell lysates were used for PCR. For lysate preparation one loop from a fresh colony was diluted in 300 µl double-distilled water (ddH_2O) and boiled at 95°C for 30 min. After centrifugation at 12,000 rpm for 5 min, 250 µl of the supernatant was transferred into a new microcentrifuge tube and diluted 1:10 with ddH_2O.

Primer design

DNA sequences of *rpoB* (RNA polymerase beta subunit), *gltA* (citrate synthase), *ftsZ* (cell division protein) and *groEL* (heat shock protein) of *Xanthomonas*, *Pseudomonas*, and *Erwinia* were obtained from the NCBI database and aligned using the SEQUENCHER software (v. 4.6, Gene Codes Corp., Ann Arbor, USA). Primers were designed within the conserved part of the sequences and found to successfully amplify products for *rpoB*, *ftsZ, gltA*, and *groEL*. An additional *groEL* set of primers was designed for the Gram-positive bacteria on the EPPO list (Table 2).

PCR reaction and sequencing procedure

All PCRs were performed in a total volume of 20 µl containing 1 µl bacterial lysate, 0.2 µM of each primer and 10 µl of HotStarTaq MasterMix (Qiagen, Hombrechtikon, Switzerland). The initial denaturation step (94°C, 15 min) was followed by 10 cycles of 30 s at 94°C; 15 s at 47°C; 1.5 min at 72°C, and an additional 35 cycles of 30 s at 94°C; 15 s at 56°C; 1.5 min at 72°C. Five microliters of the PCR amplification products were separated on 1.2% agarose gels to confirm amplification. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) following the protocol of the manufacturer. The DNA concentration was measured with a NanoDrop spectrophometer (Witec AG, Littau, Switzerland) and linear amplification on both strands was performed using 25–30 ng of purified DNA. Each sequencing reaction (8 µl) consisted of 2 µl Dye 3.1, Terminator Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Zweigniederlassung Rotkreuz) and 0.5 µM of one of the primers (Table 2). The sequences used for probe generation were deposited in the GenBank database accession numbers FJ971127 to FJ971172.

Probe selection

Based on the alignments of the partial *rpoB, ftsZ* and *groEL* sequences, probes were selected manually such that species-specific bases were positioned at the centre of each selected probe. As thermodynamic properties cannot be accurately predicted for microarray probes (Pfunder and Frey 2005; Pozhitkov et al. 2006) these were not used as a selection criterion. Eighteen 20-mer 16S rDNA probes were selected from the ARB Probe Selection Database (ARB probe library v. 1.2). Selected probes had a GC content of 50–70% and a melting temperature of 60–66°C.

Each probe contained a 15 T spacer and an amino-linker at the 3′end to improve hybridisation behaviour

Table 1 Strains and DNA used in this study

Strains		Source [a]
2429	*Burkholderia caryophylli*	INRA
2404	*Clavibacter michiganensis* subsp. *insidiosus*	INRA
4999	*Clavibacter michiganensis* subsp. *michiganensis*	INRA
ACW503	*Clavibacter michiganensis* subsp. *michiganensis*	ACW (1982)
2049	*Clavibacter michiganensis* subsp. *sepedonicus*	INRA
ACW143	*Clavibacter michiganensis* subsp. *sepedonicus*	NCPPB 4110
3418	*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*	INRA
ACW214	*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*	K. Rudolph, Göttingen
2048	*Dickeya chrysanthemi*	INRA
V2/60	*Erwinia amylovora*	ACW
3517	*Pantoea stewartii* subsp. *stewartii*	INRA
1573	*Pseudomonas syringae* pv. *persicae*	INRA
ACW52	*Pseudomonas syringae* pv. *pisi*	Z. Klement, Ungarn
2047	*Ralstonia solanacearum* biovar 1	INRA
ACW435	*Ralstonia solanacearum* biovar IV	A.C. Hayward
1159	*Xanthomonas arboricola* pv. *corylina*	INRA
6771	*Xanthomonas arboricola* pv. *fragariae*	INRA
3894	*Xanthomonas arboricola* pv. *pruni*	INRA
2005	*Xanthomonas arboricola* pv. *pruni*	ACW
2525	*Xanthomonas axonopodis* pv. *citri*	INRA
3133	*Xanthomonas axonopodis* pv. *dieffenbachiae*	INRA
6546	*Xanthomonas axonopodis* pv. *phaseoli*	INRA
2534	*Xanthomonas axonopodis* pv. *phaseoli*	INRA
6817	*Xanthomonas axonopodis* pv. *vesicatoria*	INRA
6821	*Xanthomonas axonopodis* pv. *vesicatoria*	INRA
2157	*Xanthomonas fragariae*	INRA
2054	*Xanthomonas translucens* pv. *translucens*	INRA
2532	*Xanthomonas oryzae* pv. *oryzae*	INRA
2286	*Xanthomonas oryzae* pv. *oryzicola*	INRA
4645	*Xanthomonas vesicatoria*	INRA
2537	*Xanthomonas vesicatoria*	INRA
119298	*Xylophilus ampelinus*	INRA
209898	*Xylophilus ampelinus*	INRA
DNA	*Escherichia coli*	Sigma

[a] *INRA* (L'institute national de la recherché agronomique), *DSMZ* (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig), *ACW* (Agroscope Changins-Wädenswil), *ART* (Agroscope Reckenholz-Tänikon)

(Pfunder and Frey 2005) and to allow attachment of the probes to the aldehyde-coated glass slides (Genetix, Hampshire, UK). The spotting solution contained 25 μM each of the 3′-15-T- amino terminated probes and 1x Genetix Microarray Spotting Solution. The probes were spotted onto aldehyde-coated glass slides using solid pins with a QArrayMini automated spotter (Genetix) with spot diameter 200 μm and 1.1 mm between the spot centres. Each slide included two replicates of each block (only one is shown in the figures). One block included two adjacent replicates of each probe as well as Cy3-labelled amino-linked probe as a spotting control and orientation aid. Each probe was stamped three times. No inconsistencies could be observed between replicate spots. Spotting was conducted at 55% relative humidity. After spotting, the slides were post-processed according to the manufacturers protocol (Genetix).

Table 2 Primers used in this study [a]

Primer	Sequence	Reference
fD1	5′-AGAGTTTGATCCTGGCTCAG-3′	Weisburg et al. (1991)
rP1	5′-ACGGTTACCTTGTTACGACTT -3′	Weisburg et al. (1991)
rpoBF	5′-TCGGYCTGATCAACTCCCT-3′	this study
rpoBR	5′-TGATGCAGGTGTTCTGGT-3′	this study
ftszF	5′- TCATCACCRCHGGYATGGG-3′	this study
ftsZR	5′-CGTCKGCGAAGTCGACGTT-3′	this study
groELFneg	5′-GCGKGCYTTCTTYTCYTTCA -3′	this study
groELRneg	5′-GCTTCGGYGAYCGYCGYAA -3′	this study
groELFpos	5′- CCTTGATGTTCGAGATCTTC-3′	this study
groELRpos	5′-CGGCATCGAGAAGGCCGT-3′	this study

[a] For the amplification of hybridisation products, primers had a Cy3 modification at their 5′ end

Microarray hybridisation

Target sequences for hybridisation were labelled using 5'-CY3-labelled forward and reverse primers in the PCR reaction (Table 2). The labelled PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). The hybridisation solution contained 750–1,000 ng of the purified product either in Denhardt buffer (60 µl 20x SSC, 4 µl 50X Denhardt buffer (Sigma, Buchs, Switzerland), 2 µl 10% SDS) or 1 x Genhyb buffer (Genetix, UK) in a total volume of 200 µl. The Denhardt hybridisation solution was denaturated for 5 min at 95°C, then snap-cooled on ice for 3 min and kept on ice until hybridisation. When Genhyb buffer was used, the DNA was denaturated for 5 min, cooled on ice and buffer added afterwards. Hybridisation was performed for 3 h at 34°C, followed by three washes under low stringency at 37°C (6 min in 2xSSC and 0.2% SDS, 2 min in 0.2XSSC and 0.2%SDS, and 2 min in 0.075XSSC) on a Lucidea SlidePro (GE, Healthcare Europe GmbH, Otelfingen, Switzerland).

After hybridisation slides were analysed on a GenePix Personal 4100 A microarray scanner (Axon Instruments, USA) at a wavelength of 532 nm with PMT (photomultiplier tube) at 1,000. The average fluorescence of the double-spotted probes was calculated at 10 nm resolution. The sum of the fluorescence of all probes present on a microarray was estimated (=100%) and the percentage of a single probe calculated. Spotting controls (SC) were not taken into consideration. The average fluorescence of two microarrays x2 when hybridised with buffer only (negative control) was considered as a negative result and data smaller than this value were discarded. Cross-hybridisation of a heterologous probe was defined at a fluorescence level above 50% of the fluorescence value of a homologous probe.

Results

16S rDNA-selection of probes and hybridisation results

For the purpose of this work strains of listed EPPO bacteria were classified according to their current systematics (Janse 2005) and a multiple probe concept taking into account the hierarchical structure was chosen. The bacterial strains tested in development and assessment of the quarantine microarray belong to the families *Microbacteriaceae* (*Clavibacter* and *Curtobacterium* strains), *Burkholderiaceae* (*Burkholderia* and *Ralstonia* strains), *Enterobacteriaceae* (*Dickeya*, *Erwinia* and *Pantoea* strains), *Pseudomonadaceae* (*Pseudomonas* strain), and *Xanthomonadaceae*(*Xanthomonas* strains) (Fig. 1). Using the ARB probe selection library, eighteen 20-mer 16S rDNA probes were chosen covering the family, genus and/or species level of the EPPO bacteria (positive control: Eubacteria, negative control: Archaebacteria). Selected 16S rDNA probes (Table 3) targeted the order *Xanthomonadales* and the families *Enterobacteriaceae*, *Pseudomonadaceae* and *Microbacteriacea.* At the genus level 16S rDNA probes were selected for *Xanthomonas*, *Burkholderia, Ralstonia, Xylella* and *Curtobacterium*. For *Xanthomonas fragariae, Burkholderia caryophylli*, *R. solanacearum*, *Dickeya chrysanthemi*, *Erwinia amylovora*, *Clavibacter michiganensis,* and *Xylophilus ampelinus* the ARB Library offered 16S rDNA probes at the species level and at the subspecies level for

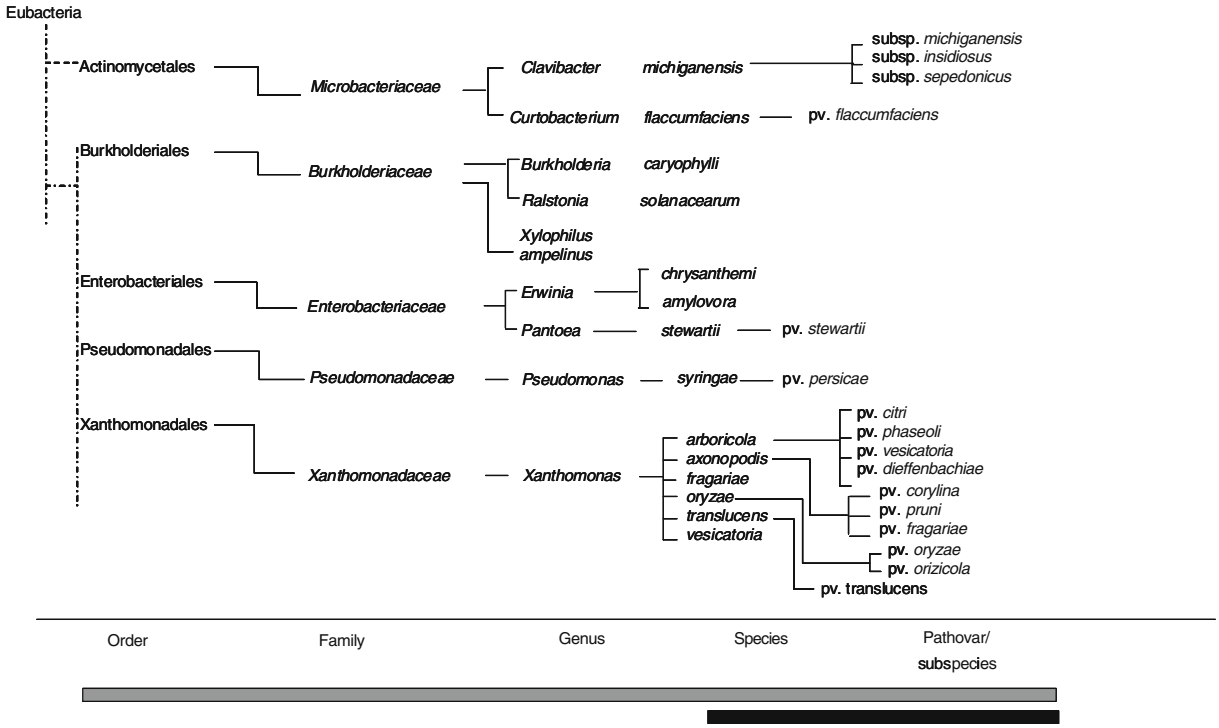

Fig. 1 Systematics of the EPPO quarantine bacteria. The grey bar indicates taxonomic levels resolved using the 16S rDNA probes, while the black bar indicates taxonomic levels resolved using housekeeping gene probes

Pantoea stewartii subsp. *stewartii* (Fig. 1). A 16S rDNA probe specific for *Clavibacter michiganensis* subsp. *sepedonicus* (Arahal et al. 2004) was also included. The selected probes were twice double-spotted in a row and their specificity tested. For this purpose 750 ng purified and Cy3-labelled 16S rDNA PCR fragments were hybridised onto the microarray. In most cases the Eubacteria probe (Eub) did not hybridise, which might in part be due to the multiple probe concept and the resulting competition of Cy3-labelled 16S rDNA product.

Hybridisation results for the 16S rDNA probes confirmed the general robustness of the 16S rDNA probes for discrimination at the level of genus (e.g., *Curtobacterium*, *Xanthomonas*) or species (e.g., *C. michiganenesis*, *R. solanacearum*). Significant cross-hybridisation occurred between the *Erwinia* probe (Erw, which was not included in further analyses) and *Xanthomonas* strains, and between the Cy3-labelled 16S rDNA of *E. amylovora* and *P. stewartii* subsp. *stewartii* (Table 4). The *Burkholderia* 16S rDNA probe (Burk) showed no hybridisation. In contrast the *Ralstonia* 16S rDNA probe (Ral) was positive with *R. solanacearum* (R.so), *B. caryophylli* (B.ca) and *X. ampelinus* (Xy.am). Sequence alignments of *R. solanacearum*, *X. ampelinus*, and *B. caryophylli* 16S rDNA revealed the Ral-probe to be identical within the first 18 (in case of *B. caryophylli*) or 17 bases (*X. ampelinus*) which resulted under our conditions in a positive signal. As all these strains belong to the *Burkholderiales* (Fig. 1), the 16S-Burk probe behaved as an order-level marker on the microarray.

As to be expected, the hybridisation results for the 16S rDNA order and family probes for *Xanthomonas oryzae* pv. *oryzae, Xanthomonas arboricola* pv. *corylina*, *Xanthomonas axonopodis* pv. *vesicatoria*, and *Xanthomonas axonopodis* pv. *phaseoli* revealed an identical pattern. *Xanthomonas fragariae* showed an additional signal due to the hybridisation of its species-specific 16S rDNA probe (Table 5). Control hybridisations with the 16S rDNA of *Escherichia coli* led to a signal with only the *Enterobacteriacea* probe.

Housekeeping genes — primer selection and PCR amplification

The 16S rDNA probes did not enable discrimination of most *Xanthomonas, Clavibacter* or *Pseudomonas*

Table 3 Probes used in this study

Name	Sequence (5′-3′)	Detected taxon
16S rDNA (ARB probe selection)		
Eub	TGCTGCCTCCCGTAGGAG-TTTTTTTTTTTTTTT	Eubacteria
Archae	CCCCCGCCAATTCCTTTAAG-TTTTTTTTTTTTTTT	Archaebacteria
X.-es	CAGTTCGCATCGTTTAGGGC-TTTTTTTTTTTTTTT	*Xanthomonadales*
X.-as	AAGCCCGAAGGTCCTCCGCT-TTTTTTTTTTTTTTTT	*Xanthomonas*
X.fra	GAGCAAGCTCTTACCGTGCT-TTTTTTTTTTTTTTT	*X. fragariae*
Entceae	TCCAGTAGTTATCCCCCTCC-TTTTTTTTTTTTTTT	*Enterobacteriaceae*
E.ch	AGAGAAGCCTGCAGAGATGC-TTTTTTTTTTTTTTT	*Erwinia chrysanthemi*
E.am	TCCACGGAATTCTGCAGAGA-TTTTTTTTTTTTTTT	*Erwinia amylovora*
P.stst	GGAGGTCCCCCACTTTGGTC-TTTTTTTTTTTTTTT	*Pantoea stewartii* subsp. *stewartii*
Bur	TCCTTAGTTGCTACGCAAGA-TTTTTTTTTTTTTTT	*Burkholderia*
B.ca	GAAAGAGAACCGTCGCACAG-TTTTTTTTTTTTTTTT	*B. caryophylli*
Pseu	TAATCCGACCTAGGCTCATC-TTTTTTTTTTTTTTT	*Pseudomonadaceae*
Micro	GGCCCAGAGATCTGCCTTC-TTTTTTTTTTTTTTT	*Microbacteriacea*
Cu	GCGCTAGATGTAGGGACCTT-TTTTTTTTTTTTTTT	*Curtobacterium*
C.m	AAGCTCTGACATCACCGTTC-TTTTTTTTTTTTTTT	*Clavibacter michiganensis*
C.ms	AAACGTGCAGAGATGTGCGC-TTTTTTTTTTTTTTT	*C. michiganensis* subsp. *sepedonicus*
Ral	CACACGTCATACAATGGTGC-TTTTTTTTTTTTTTT	*Ralstonia*
R.so	AAGCTACCCCCGCTGCCGTT-TTTTTTTTTTTTTTT	*R. solanacearum*
Xy.am	GGTACCGTCATGAGCTCTTT-TTTTTTTTTTTTTTT	*Xylophilus ampelinus*
Houskeeping genes (this study)		
ftsZ (5′-3′)		
f-X.or	GGCTCAACTCTTCGATGCCC-TTTTTTTTTTTTTTT	*X. oryzae*
f-X.ve	GACGACCGGTGCCGCACCGG-TTTTTTTTTTTTTTT	*X. vesicatoria*
***rpoB* (5′-3′)**		
r-X.tr	GGTGATCTGGCCGTCCACGA-TTTTTTTTTTTTTTT	*X. translucens*
r-X.trtr	GGTCAGGCGGCTCTTGGAAT-TTTTTTTTTTTTTTT	*X. translucens* pv. *translucens*
r-X.or	ATCGACGCCGCTCGTATCGT-TTTTTTTTTTTTTTT	*X. oryzae*
r-X.oror	GGCGGTTTCAACAGCGATTC-TTTTTTTTTTTTTTT	*X. oryzae* pv. *oryzae*
r-P.sy	CGTCGATCAGCTCTTGCTTGT-TTTTTTTTTTTTTTT	*P. syringae*
r-P.syper	CTGAAAGAAACACGATTTCG-TTTTTTTTTTTTTTT	*P. syringae* pv. *persicae*
r-X.ve	GTTGGCGTCATCGTGTTCCT-TTTTTTTTTTTTTTT	*X. vesicatoria*
r-X.ve/fra	AGAA**A**CCGTTGGTGGGTACC-TTTTTTTTTTTTTTT	*X. vesicatoria/fragariae*
r-X.arb	TCAATGCCTTGCG**G**GGTGGC-TTTTTTTTTTTTTTT	*X. arboricola*
***groEL* (5′-3′)gram negative strains**		
g-X.arb1	TCCTTGATCGTGGCCTTCTC-TTTTTTTTTTTTTTT	*X. arboricola*
g-X.arb2	ACGCGGGCTTCGATCGTGGC-TTTTTTTTTTTTTTT	*X. arboricola*
g-X.axo/vesi	TCCTTGATGGTCGCCTTCTG-TTTTTTTTTTTTTTT	*X. axonopodis/vesicatoria*
***groEL* (5′-3′)gram positive strains**		
g-C.mm/s2	CGATGGTGG<u>A</u>GTCGCCGGCG-TTTTTTTTTTTTTTT	*C. michiganensis* subsp. *michiganensis/ sepedonicus*
g-C.mi2	CGATGGTGG<u>C</u>GTCGCCGGCG-TTTTTTTTTTTTTTT	*C. michiganensis* subsp. *insidiosus*
g-C.mm3	CTCCTCCTT<u>G</u>GTCTCGATCT-TTTTTTTTTTTTTTT	*C. michiganensis* subsp. *michiganensis*
g-C.ms3	CTCCTCCTT<u>C</u>GTCTCGATCT-TTTTTTTTTTTTTTT	*C. michiganensis* subsp. *sepedonicus*
g-C.mi4	ACTTGAGCTCCTCAGTGACG-TTTTTTTTTTTTTTT	*C. michiganensis* subsp. *insidiosus*
g-Cu.f	TCGCGTTGGCGAGGAGCTGG-TTTTTTTTTTTTTTT	*Curtobacterium flaccumfaciens*

Bases in bold indicate inserted sequence mismatches

Underline indicates single-base differences in probes

Table 4 Hybridisation results using Cy3-labelled 16S rDNA PCR products

Name	B.cary	R.sol	Xy.amp	P.sype	E.am	P.stst	X.oror	X.arco	X.axpha	X.axve	C.mm	C.ff
Eub				0.09								
Archae												
H_2O												
Pseudo				0.336								
Xales							0.113	0.1	0.143	0.144		
Xan							0.22	0.107	0.107	0.1		
Xylella												
Xylella												
Mic											0.051	0.045
Ent					0.258	0.331						
Clami											0.042	
Paststt					0.108	0.0955						
Cffff												0.427
Erw				0.062	0.048		0.099	0.094	0.14	0.144		
Eram					0.048							
Burk												
Ral	0.206	0.241	0.361									
Bcary	0.251											
Ralso		0.239										
Xylamp			0.094									
H_2O												

Corresponding probes were twice-spotted in one microarray block. The average fluorescence of a double-spotted probe was calculated in percentage, given that the total fluorescence of all probes was 100%. The average fluorescence of two microarrays hybridised with buffer only (negative control) x2 was considered as a negative result and data smaller than this value were erased. Specific probes; Cross–hybridisation; False–negative

strains at the species or subspecies level. Therefore, several candidate housekeeping genes with the potential for such differentiation (Stackebrandt et al. 2002) were analysed and additional diagnostic probes designed.

The selected candidate genes were *rpoB* (RNA polymerase beta subunit), *gltA* (citrate synthase), *ftsZ* (cell division protein) and *groEL* (heat shock protein). In order to generate primers suitable for the amplification of these housekeeping genes in the EPPO bacteria, corresponding DNA sequences of related bacteria (*Burkholderia*, *Xanthomonas*, *Pseudomonas*, *Erwinia*) were obtained from NCBI (National Centre for Biological Information). For the Gram-positive *Clavibacter* and *Curtobacter* bacteria, only *groEL* (AY837553.1, AY263152.1) sequences were available for use. The selected sequences were aligned and used to design degenerate PCR primers (Table 2). Using cell lysates of characterised strains (Table 1) of quarantine bacteria PCR amplification of fragments of the chosen housekeeping genes was successfully performed and the nucleotide sequence of the respective products (600 bp *rpoB*, 350 bp *ftsZ,* 500 bp *gltA*, 350 bp *groEL*) obtained.

Housekeeping genes-probe design

For probe design, the resulting sequences of above and corresponding genes of *X. oryzae* pv. *oryzae*

Table 5 Hybridisation results using Cy3-labelled house keeping genes

PCR product probe	16S-C.ms	16S-E.ch	16S-X.fra	rpoB-X.fr	rpoB-X.oror	rpoB-X.orori	rpoB-X.trtr	rpoB-X.arph	rpoB-X.vesi	rpoB-P.sype	ftsz-X.oror	ftsz-X.vesi	groEL-X.arpr	groEl-X.axdi	groEL-X.fr	groEl-C.ff	groEL-C.mm	groEL-C.mm	groEL-C.ms	groEL-C.mi
16S-Eub	0.197	0.093	0.153											0.052						
16S-Archae																				
16S-Micro																				
16S-C.m	0.368																			
16S-Cu																				
16S-C.ms	0.147			0.047																
16S-Ral																				
16S-B.cary																				
16S-Xy.amp																				
16S-R.sol																				
16S-X.-es																				
16S-X.-as			0.263																	
16S-X.fra			0.511																	
16S-Pseu				0.141																
16S-Entceae		0.710									0.122									
16S-E.am																				
16S-E.ch		0.080																		
16S-P.stst																				
r-X.ve									0.868											
r-X.tr							0.628													
r-X.trtr							0.351													
r-X.ve/fra				0.536											0.046					
r-X.or					0.929	0.738														
r-X.oror											0.145									
r-P.ay										0.668										
r-P.syper																				
r-X.ar								0.954												
ft-X.or											0.592	0.779								
ft-X.ve												0.777								
g-C.mi2															0.070		0.058	0.049	0.050	0.375
g-C.ms3																			0.429	
g-C.mm3																	0.738	0.480		
g-C.mm/ms3																	0.161	0.390	0.463	0.335
g-C.mi4																				0.226
g-Cf																0.892				
g-X.ax/ve															0.047					
g-X.arb1													0.341							
g-X.arb2													0.606							

Hybridisation results using purified Cy3-labelled *rpoB*, *ftsZ* or *groEL* PCR products. Corresponding probes were twice-spotted. Data were corrected to the total luminescence. The average fluorescence of a double-spotted probe was calculated in percentage, given that the total fluorescence of all probes was 100%. The average fluorescence of two microarrays hybridised with buffer only (negative control) x2 was considered as a negative result and data smaller than this value were erased. Specific probes; Cross–hybridisation; False-negative

KACC10331(NC_006834), *X. oryzae* pv. *oryzae* MAFF 311018 (NC_007705), *X. campestris* pv. *vesicatoria* 85-10 (NC_007508), *X. axonopodis* pv. *citri* 306 (NC_003919) were aligned. For the design of *Pseudomonas syringae*, probes only *rpoB* gene sequences (present in the NCBI database AJ973033 to AJ973084) were used. Microarray probes were selected manually, preferably with at least two species-specific bases positioned in the centre of each selected gene probe.

No probes were designed for *gltA* as the partial sequences did not reveal promising sequence differences between the species. The *ftsZ* probes were designed for *X. oryzae* (f-X.or) and *X. vesicatoria* (f-X.ve) (Table 3). *rpoB* probes were generated for *X. arboricola* strains (r-X.arb) and *X. vesicatoria* (r-X.ve) strains. An additional *rpoB* probe was generated for the detection of both *X. fragariae* and *Xanthomonas vesicatoria* strains (r-X.ve/fra). Our r-X.arb and r-X.ve/fra probes differed by only one bp from sequences of other *Xanthomonas* species. Preliminary hybridisation tests revealed that single-base mismatches were often insufficient for a distinct hybridisation result under the given conditions (data not shown). Thus, to improve discrimination power, an additional mismatch was inserted into the r-X.arb (position 14) and r-X.ve/fra probes (position 5). For *Xanthomonas translucens* pv. *translucens*, *X. oryzae* pv. *oryzae* and *P. syringae* pv. *persicae* two *rpoB* probes were designed. A probe was designed for the identification of each species (r-X.tr, r-X.or, r-P.sy), and another for each pathovar strain (r-X.trtr, r-X.oror, r-P.syper). Two *groEL* probes (g-X.arb1, X.arb2) were generated for *X. arboricola* strains and one probe (g-X.ve/ax) for both *X. vesicatoria* and *X. axonopodis* strains. No additional *rpoB*, *ftsZ*, *gltA* probes could be designed for *X. axonopodis* because suitable gene samples were not conserved among the analysed pathovar strains *phaseoli*, *vesicatoria*, *citri* and *dieffenbachiae*.

For the Gram-positive *Curtobacterium* and *Clavibacter* strains *groEL* probes were designed for the differentiation at species (*Curtobacterium flaccumfaciens*) or subspecies level (*C. michiganensis* subsp. *sepedonicus, C. michiganensis* subsp. *michiganensis, C. michiganensis* subsp. *insidiosus*). The *groEL* sequence contained only single-base mismatches between subspecies of *C. michiganensis*. Therefore, two probes were designed that differed by only one base each (Table 3). One probe was specific for a single subspecies and the second was specific for either of the remaining two subspecies. Thus, even when the single-base difference did not lead to a distinct hybridisation result, the difference in fluorescence intensity enabled the discrimination of the strains to the subspecies level.

Housekeeping genes-hybridisation results

The probes r-X.trtr, r-X.oror, r-P.syper and r-X.ve/fra, did not hybridise at all or only weakly with the matching target sequences. In contrast, f-X.or, f-X.ve, r-X.arb, r-X.ve, r-X.tr, r-X.or, r-P.sy, g-Xarb1, g-X.arb2 differentiated the strains at the species level (Table 5). The *groEL* probes with only a single base discriminated between the subspecies *C. michiganensis* subsp. *michiganensis* and subsp. *sepedonicus*. However, due to cross-hybridisation (g-Cmi2) and weak positive reaction (Cmi4) *C. michiganensis* subsp. *insidiosus* identification was less definitive. The negative hybridisation results of some probes were most likely due to probe-specific thermodynamic properties or secondary structures.

Microarray hybridisation using various strains

The microarray was tested using strains from additional strain collections (Table 1, ACW214, ACW143, ACW435, ACW52) or isolated from infected plant samples analysed at ACW (ACW2005, ACW503) using serological methods and bioassays. Cell lysates of these strains defined as *X. arboricola* pv. *pruni* (2005), *C. flaccumfaciens* pv. *flaccumfaciens* (ACW214), *C. michiganensis* subsp. *michiganensis* (ACW503), *C. michiganensis* subsp. *sepedonicus* (ACW143), *R. solanacearum* (ACW435) *P. syringae* pv. *pisi* (ACW52), and *E. amylovora* were prepared and Cy3-labelled 16S rDNA, *groEL*, and *rpoB* (for Gram-negative strains) PCR reactions performed. The 1 μg of 16S rDNA and 750 ng of each *groEL* and *rpoB*-purified PCR products were mixed with GenHYB buffer. Hybridisation was performed with all three PCR products of the Gram-negative bacteria regardless of whether or not a corresponding probe was present on the microarray (e.g., only 16S rDNA probes are present for *E. amylovora*) in order to expand cross-hybridisation analyses. Hybridisation results enabled the identification of *C. flaccumfaciens, R. solanacearum*, *P. syringae* pv. *pisi* and the *X. arboricola* pv.

Eub	Archae	Ral	SC	X.-es	X.-as	Pseu	Entceae
Micro	Cm	B.car	Xy.am	X.fra	H_2O	SC	E.am
Cu	Cms	R. sol	r-X.or	r-X.vesi	r-X.tr	r-P.sy	P.stst
H2O	SC	H_2O	r-X.oror	r-X.ve/fra	r-X.trtr	r-P.syp	E.ch
g-C.mm/s_2	Cmi4	SC	f-X.or	f-X.vesi	r-X.arb	H_2O	H_2O
g-C.mi2	H_2O	H_2O	H_2O	g-X.ax/ve	g-X.arb1	H_2O	H_2O
g-C.ms_3	Cmm_3	H_2O	H_2O	H_2O	g-X.arb2	H_2O	H_2O
g-Cu.f	SC	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O

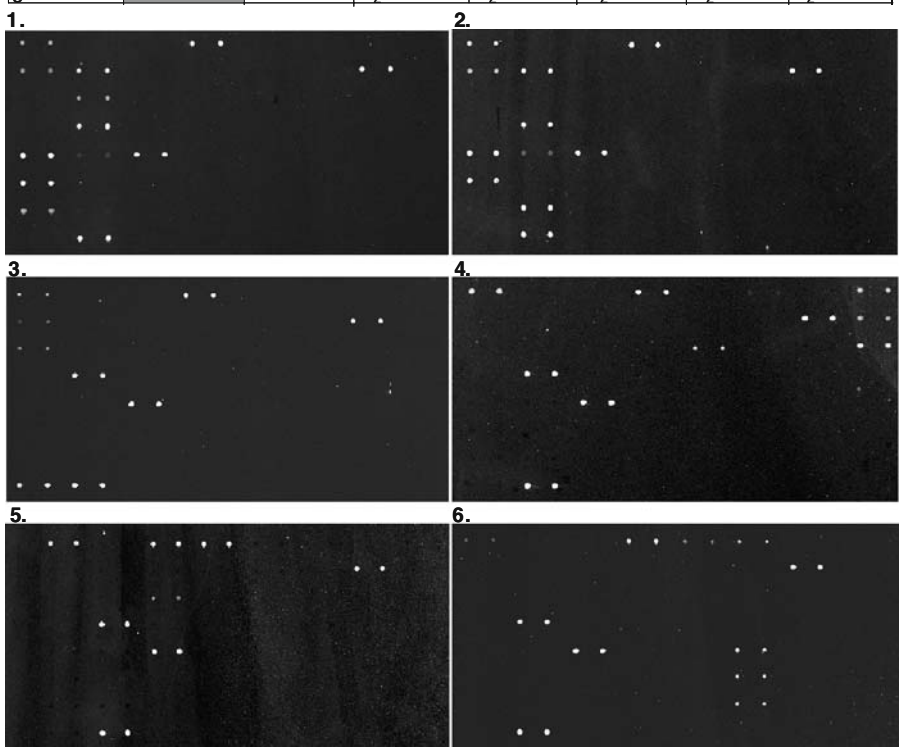

Fig. 2 Hybridisation of the quarantine microarray using Cy3-labelled PCR products of 16S rDNA, *groEL*, and *rpoB* (for Gram-negative bacteria). The spotting pattern of the microarray is as follows (*1) Clavibacter michiganensis* subsp. *sepedonicus* (ACW143); (2) *C. michiganensis* subsp. *michiganensis* (ACW503); (3) *C. flaccumfaciens* subsp. *flaccumfaciens* (ACW214); (4.) *Erwinia amylovora;* (5) *Ralstonia solanacearum* (ACW435); (6) *Xanthomonas arboricola* pv. *pruni* (2005). SC indicates the spotting control

pruni strains to species level (Fig. 2). In the case of *E. amylovora*, cross-hybridisation occurred with the r-X. vesi probe, which was most likely due to the presence of the *rpoB/groEL* PCR products. As known from previous hybridisation results (Table 4) the 16S rDNA P.stst probe also reacted with *E. amylovora*. The hybridisation pattern of strains *C. michiganensis* subsp. *michiganensis* and *C. michiganensis* subsp. *sepedonicus* allowed the identification of the strains despite the g-Cmi2 cross-hybridisation.

Discussion

We report the development of a DNA microarray for simultaneous identification of multiple EPPO quarantine bacteria composed of 38 probes designed for 16S rDNA and housekeeping genes (*rpoB*, *groEL*, *ftsZ*). Our chip represents a prototype microarray identification system for a plant-inspection chip to aid in the simple detection of a wide range of quarantine bacteria in plant samples for presumptive diagnosis.

The 16S rDNA probes used on the microarray were selected from the ARB probe selection library choosing a multiple probe design to verify the identification of a given bacterium. As the ARB library is generated using a programme that designs probes out of large sequence databases (Ludwig et al. 2004), they are expected to react specifically at the order or species level for particular bacterial strains. The probes revealed a general robustness in the identification of the bacteria despite some cross-hybridisation (e.g., between the *Erwinia* probe and *Xanthomonas* strains or between the *E. amylovora* and the *P. stewartii* subsp. *stewartii* probe). As the 16S rDNA probes are not always sufficient for the discrimination at species/subspecies level probes derived from three housekeeping genes (*rpoB*, *groEL*, *ftsZ*) were added to the microarray in

order to enhance the discriminating power. These probes were generally capable of identifying the tested strains (e.g., *Xanthomonas* strains) to their species level. However, the analysed housekeeping genes were inadequate for detection of *X. axonopodis*-specific probes, mainly because of suitable probe sequences not being conserved among the sequenced *X. axonopodis* strains. Taking into account that multilocus sequence typing (MLST, Maiden et al. 1998) is by convention based on an average of seven genes (Scally et al. 2005), it is reasonable to increase the number of probes resulting from the analysis of additional housekeeping genes in future versions of the microarray. This will not only support the correct identification of the strains but also the resolution of the microarray.

Although the selected housekeeping probes were based on consensus sequences composed of a minimal set of sequences they were confirmed by strains which were not included in initial probe development (Table 1, ACW strains). Additional hybridisation experiments are still required to prove that the chosen probes are conserved within the species/subspecies. The quarantine bacterium microarray addresses the challenge to discriminate bacteria not only at their genus and species levels as environmental microarrays usually do, but also at the subspecies and pathovar levels required for plant inspection purposes. Our prototype quarantine microarray enabled the rapid identification of bacterial colonies at both the species and subspecies levels in several cases. In other cases, accurate discrimination to the pathovar level was not achieved to the exclusion of related pathovars (e.g., *P. syringae* pv. *persicae* shows the same pattern as *P. syringae* pv. *pisi*). However, when the host plant sample is considered, as is always the case in plant inspection, such cross-reactions are expected to pose an insignificant problem. For example, should a positive reaction with the probe for *P. syringae* pv. *persicae* (a stone fruit pathogen) be obtained from a peach sample, it is highly unlikely to be indicative of *P. syringae* pv. *pisi* (a pea pathogen) given the host plant material.

For a direct identification in plant material with a validated microarray, improvements have to be made to deal with the complex environmental microbial communities typical of agricultural habitats (Borneman et al. 1996). Additional pathovar-specific probes and hybridisation studies are clearly required since not only does the pathovar level have to be ascertained, but also cross-hybridisation with closely related pathovars must be excluded. For example, there are > 50 recognised pathovars of *P. syringae* alone (Hirano and Upper 2000) but only one *P. syringae* pv. *persicae* is on the EPPO quarantine list. It is known that horizontal gene transfer and genomic rearrangements play an important role in pathogenic differentiation. Thus, pathovar probes can originate from unique DNA fragments or random probes resulting in a specific pattern (Koh and Nou 2002; Park et al. 2005). Bacterial genome sequencing and comparative genomic advances assisted by bioinformatics innovations to analyse unwieldy data sets will yield rich sources for probe generation that can be incorporated in next-generation microarrays. In addition, sensitive and specific PCR-based techniques and subtractive hybridisation can be applied for identifying and designing pathovar-specific probes.

This study has demonstrated that DNA microarrays harbouring small numbers of selected probes are able to identify quarantine bacteria. The benefits of DNA microarray technology include the power to identify multiple taxa in a single step and the simplicity in design and use. Once validated, such chips would be optimal tools to prevent dissemination of quarantine phytopathogenic bacteria. DNA microarrays thus possess a great potential for use in diagnostic applications and merit further development.

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