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Improved PCR for identification of members of the genus *Xanthomonas*

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Abstract A PCR-based system was developed to reliably and robustly identify group I and II members of the genus *Xanthomonas*. Primer sets developed from three gene targets namely *fyuA*, ITS and *gumD* were evaluated in the study. Primer sets were evaluated using DNA extracted from 45 *Xanthomonas* strains representing 25 species broadly covering the genus. Fifteen non-*Xanthomonas* strains of plant-associated bacteria including phylogenetically closely related species *Stenotrophomonas maltophilia* and *Xylella fastidiosa* were also tested. The primers targeting *fyuA* amplified DNA from all xanthomonads except *X. theicola*, while the ITS primers amplified a DNA fragment of 254 bp in all 45 *Xanthomonas* strains; whereas no amplification was observed for non-xanthomonads. The *gumD* primers allowed efficient amplification of DNA in 38 out of 39 isolates from Group II, whereas no or very weak amplification occurred with DNA from Group I members. Internal controls of primers targeting bacterial 16S rDNA or plant 26S mitochondrial rDNA were successfully applied in multiplex PCRs for testing bacterial cultures or plant tissue, respectively. The findings give us a PCR based approach that can reliably and effectively differentiate xanthomonads from non-xanthomonads as well as separating the strains belonging to the two described groups of the genus *Xanthomonas*. The study thus offers valuable tools for disease surveillance and management. It can effectively be applied in rapid assessment of new disease occurrences, for which no specific detection tools could be in place.

Keywords *Xanthomonas* · Identification · ITS · gumD · TonB dependent receptor · Multiplex PCR

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Introduction

The genus *Xanthomonas* contains more than 100 plant-pathogenic members and is phylogenetically placed in the family *Xanthomonadaceae*. This family contains more than 10 other genera of bacteria found in different and often extreme environments. The genus *Xylella* is the most closely related plant associated bacteria to *Xanthomonas* in the *Xanthomonadaceae* family

followed by *Stenotrophomonas* (Schaad et al. 2001; Swings and Civerolo 1993). A third genus, *Pseudoxanthomonas* is also closely related to *Xanthomonas* but no plant associations of this genus have so far been recorded (Finkmann et al. 2000; Hayward et al. 2010). *Xanthomonas* spp. are reported to cause a range of symptoms in plants including wilt, necrosis, leaf spots, leaf streaks, stripes, blights, cankers and gummosis on leaves, fruits or stems of monocotyledonous and dicotyledonous plants (Bradbury 1986; Hayward 1993; Leyns et al. 1984; Swings and Civerolo 1993). Members of the genus *Xanthomonas* have different physiological properties but often produce a characteristic extracellular polysaccharide (EPS)—xanthan, and a membrane-bound, brominated, aryl-polyene, yellow pigment (xanthomonadin) (Chun et al. 1997), involved in pathogenicity, virulence and epiphytic survival (Aslam et al. 2008; Chou et al. 1997; Dow and Daniels 1994; Dunger et al. 2007; Jenkins and Starr 1982; Katzen et al. 1996, 1998; Poplawsky and Chun 1998; Katzen et al. 1998).

Several PCR protocols have been published for specific detection and identification of individual *Xanthomonas* species (Palacio-Bielsa et al. 2009). When analyzing newly emerging diseases for which specific diagnostic protocols have not yet been developed or when surveying a disease complex involving more than one species of xanthomonads, it can be very useful to apply a tool detecting and correctly identifying all members of the genus at the same time (Mbega 2011; Mbega et al. 2012a, b). PCR protocols amplifying DNA from a large number of *Xanthomonas* species have been published. These protocols are based on 16S rDNA (Maes 1993) and on the hrp gene cluster of *Xanthomonas campestris* pv. *vesicatoria* (Leite et al. 1994), respectively. In our studies, these two protocols when tested against a wide range of xanthomonads and non-xanthomonads did not provide a fully adequate genus-specific identification of the strains. Recently, a semi-selective medium for xanthomonads, Xan-D, was described (Lee et al. 2009) taking advantage of the gene *estA,* involved in Tween 80 hydrolysis and being conserved across the genus. The protocol designed by Lee et al. 2009 combines the use a semi-selective medium and a PCR specifically detecting *estA* gene from xanthomonads (Lee et al. 2009).

We intended to develop a fast molecular-based genus specific PCR, that is also independent of bacterial isolation and cultivation. In addition, we intended to include internal controls for PCR allowing quality control of general PCR amplification when applied to either pure bacterial DNA or to crude DNA extracted from plant tissue. These internal controls were obtained by multiplexing with primers targeting either bacterial 16S rDNA or plant mitochondrial 26S rDNA as recently demonstrated for a single species (Adriko et al. 2012).

We exploited the possibility of developing a new genus specific PCR by testing three genes: i) the gene encoding the TonB-dependent receptor involved in signal transmission (Koebnik 2005), which has been used for multilocus analysis of species of the *Xanthomonas* genus (Young et al. 2008), ii) the ribosomal ITS sequence known as a classical phylogenetic target sequence strongly represented in sequence databases (Hauben et al. 1997), and iii) *gumD*, a gene known to be characteristic of *Xanthomonas* species by its involvement in biosynthesis of the extracellular polysaccharide, xanthan (Katzen et al. 1996, 1998; Kim et al. 2009).

The 16S ribosomal DNA and multilocus sequence analyses have been widely used for studying phylogenetic diversity in microbial communities (Hauben et al. 1997; Lane et al. 1985; Vos et al. 2012; Amann et al. 1995; Young et al. 2008, 2010; Almeida et al. 2010). More recently genome-based phylogeny of the genus *Xanthomonas* with species representing the major lineages within the group have been reported (Rodriguez et al. 2012; Studholme et al. 2011). Our selection of *Xanthomonas* strains for tests were based on previous phylogenetic studies carried out using 16S ribosomal DNA sequences (Hauben et al. 1997) and multilocus sequence analysis (Young et al. 2008). These tools have divided *Xanthomonas* species into two main groups; Group I including the species *X. albilineans, X. hyacinthi, X. theicola, X. sacchari* and *X. translucens*, and Group II consisting of *X. arboricola, X. axonopodis, X. bromi, X. campestris, X. cassavae, X. codiaei, X. cucurbitae, X. fragariae, X. hortorum, X. melonis, X. oryzae, X. pisi, X. populi, X. vasicola* and *X. vesicatoria*. We also included recently identified *Xanthomonas* strains isolated from tomato (Mbega et al. 2012a, b). For the development of the genus specific PCR, we selected 45 strains of 25 different species of *Xanthomonas* from groups I and II as well as 15 bacterial species from other genera of plant associated bacteria including members of the closely related *Xyllela* and *Stenotrophomonas* genera (Rodriguez et al. 2012; Simões et al. 2007; Young et al. 2008).

Materials and methods

Bacterial isolates and strains

Forty five *Xanthomonas* strains of 25 different species covering the strains reported in the phylogenetic studies of the genus *Xanthomonas* (Gurtler and Stanisich 1996; Parkinson et al. 2007, 2009; Simões et al. 2007; Young et al. 2008) were used in the analyses, together with fifteen non-*Xanthomonas* bacterial strains and five bacterial strains isolated from tomato seeds (Table 1). The majority of strains were obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB), UK, while the other sources included the American Type Culture Collection (ATCC), USA and Plant Research International (IPO), Netherlands. The *X. translucens* pv. *undulosa* B498 isolate was kindly provided by Dr. Norman W. Schaad, and *X. axonopodis* pv. *phaseoli* No. 17, from the Danish Seed Health Centre (DSHC), Denmark. Among the tested strains five bacterial isolates recovered from tomato seed samples collected from Tanzania were included in the study. These isolates were previously identified based on results from pathogenicity testing, Biolog identification and 16S rRNA sequencing (Mbega et al. 2012a, b). Tomato isolates 73 and 167 identified as *X. gardneri* and *X. arboricola* pv. *poinsettiicola*, respectively (Mbega et al. 2012a, b) were used as *Xanthomonas* positive controls while isolates 27, 36 and 38 identified as *Stenotrophomonas* spp. (Mbega et al. 2012a, b) served as negative controls. Cultures of the reference strains were kept on Protect Bacterial Preservers (Technical Service Consultants Ltd, Heywood, UK) at −80 °C and transferred to Nutrient Agar (NA) media for 48 h at 27 °C for use in plant inoculations and DNA extractions.

Plant inoculation, sampling and DNA extraction

Cabbage (*Brassica oleracea* L. var. *capitata* sub.var. *alba* L.) and rice (*Oryza sativa* L.) plants inoculated with strains of *Xanthomonas* were used to provide diseased plant samples for PCR testing. Cabbage variety Copenhagen market, and rice cv. MTL579 plants were artificially inoculated with the phytopathogenic *Xanthomonas* reference strains *Xanthomonas campestris* pv. *campestris* NCPPB 3207 and *Xanthomonas oryzae* pv. *oryzae* NCPPB 3002, respectively. Host plants treated with sterile distilled water were used as negative controls. Bacterial growth was scraped from the NA plates and suspended in 10 ml of sterile distilled water to produce a turbid suspension (10^8 CFU/ml) with OD600 of 0.01 determined using a Nanodrop (ND-1000 Spectrophotometer, NanoDrop Technologies, Inc.). Leaf veins of cabbage plants at the 2–3 leaf stage, were pricked with sterile insect pins previously dipped in inoculum suspension of *Xanthomonas campestris* pv. *campestris* NCPPB 3207. Inoculation of 21 days-old rice plants was conducted by clipping the tips of 25–30 leaves/isolate while still immersed in the cell suspension of *Xanthomonas oryzae* pv. *oryzae* NCPPB 3002. The plants were kept under highly humid conditions at 26–28 °C with 12 h day-light cycle and observed for the development of disease symptoms. Un-inoculated and symptomatic inoculated samples from cabbage and rice plants were taken at 7 and 10 days post-inoculation, respectively for DNA extraction. DNA was extracted using the QIAGEN DNeasy Plant Mini Kit (Qiagen). Extracted DNA was quantified using a Nanodrop ND1000 spectrophotometer (Nanodrop Technologies) and stored at 4 °C until use.

DNA extraction from bacterial cultures

DNA was extracted from the test bacterial strains presented in Table 1. Starting material for DNA extraction consisted of scraping the surface of 48 h-old pure cultures grown on NA. Extraction of DNA was conducted using the Qiagen DNeasy Blood and Tissue kit (Qiagen) and performed according to the manufacturer's instructions. Extracted DNA samples were stored at 4 °C until use.

Primer design and PCR reactions

Development of primers involved multiple sequence alignment and identification through BioEdit (Hall 1999) of conserved consensus regions in genes common among xanthomonads, including those in the xanthomonadin synthesis pathway, extracellular polysaccharide (EPS) synthesis pathway and the DNA polymerase gene. Other primers were developed from the TonB dependent receptor gene (*fyuA*) and internal transcribed spacer (ITS) region due to abundant *Xanthomonas* sequences of these in the NCBI GenBank. The Primer3 Software (Rozen and Skaletsky 2000) was used for developing quality primers for DNA amplification. The designed primers from the various gene targets were screened using DNA from xanthomonads and non-xanthomonads to test them for robustness and specificity with respect to detection of *Xanthomonas* spp. Out of

Table 1 Bacterial strains used in the study

Bacterial species	Bacterial Strain	Host
Xanthomonas		
Xanthomonas albilineans	NCPPB 1830	Sugarcane
X. arboricola pv. *celebensis*	NCPPB 1832	Banana
X. arboricola pv. *poinsettiicola*	Tomato isolate 167	Tomato
X. axonopodis	NCPPB 457	Axonopus
X. a. pv. *aurantifolii*	NCPPB 4377	Citrus
X. a. pv. *cyamopsidis*	NCPPB 637	Guar Gum
X. a. pv. *glycines*	NCPPB 1124	Soy bean
X. a. pv. *manihotis*	NCPPB 2965	Cassava
X. a. pv. *phaseoli*	DSHC No 17	Beans
X. a. pv. *vasculorum*	NCPPB 206	Maize
X. a. pv. *vignicola*	NCPPB 555	Cowpea
X. a. pv. *vignicola*	NCPPB 638	Cowpea
X. bromi	NCPPB 4343	Rescue Brome grass
X. campestris var *aberrans*	NCPPB 2986	Cabbage
X. c. var *armoraciae*	NCPPB 1930	Horseradish
X. c. pv. *barbareae*	NCPPB 983	Garden Yellow rocket
X. c. pv. *campestris*	NCPPB 528	Cabbage
X. c. pv. *carotae*	NCPPB 3440	Carrot
X. codiaei	NCPPB 3443	Freijo cordia-wood
X. c. pv. *incanae*	NCPPB 937	Stock plant
X. c. pv. *musacearum*	NCPPB 4387	Banana
X. c. pv. *raphani*	NCPPB 1946	Radish
X. cassavae	NCPPB 101	Cassava
X. citri subsp. *citri*	NCPPB 410	Orange
X. citri subsp. *malvacearum*	NCPPB 210	Cotton
X. cucurbitae	NCPPB 2597	Squash
X. euvesicatoria	NCPPB 2968	Pepper
X. fragariae	NCPPB 2949	Strawberry
X. fuscans subsp. *fuscans*	IPO 482	Bean
X. gardneri	NCPPB 881	Tomato
X. gardneri	Tomato isolate 73	Tomato
X. hortorum pv. *pelargonii*	NCPPB 305	Geranium
X. hyacinthi	NCPPB 205	Hyacinth
X. melonis	NCPPB 3434	Melon
X. oryzae pv. *oryzae*	NCPPB 3002	Rice
X. o. pv. *oryzicola*	NCPPB 1151	Rice
X. perforans	NCPPB 4321	Tomato
X. pisi	NCPPB 762	Pea
X. pruni	NCPPB 416	Plum
X. pruni	NCPPB 3155	Plum
X. sesame	NCPPB 631	Sesame
X. theicola	NCPPB 4353	Tea
X. translucens pv. *translucens*	NCPPB 2389	Barley
X. t. pv. *undulosa*	B498	Wheat
X. sacchari	NCPPB 4341	Sugarcane
X. vasicola pv. *holcicola*	NCPPB 2417	Sorghum
X. vesicatoria	NCPPB 422	Tomato
Non-*Xanthomonas*		
Acidovorax avenae subsp. *avenae*	NCPPB 1011	Maize
A. a. subsp. *citrulli*	ATCC 29625	Melon
Burkholderia glumae	NCPPB 2391	Rice
Clavibacter michiganensis subsp. *michiganensis*	IPO 542	Tomato
Dickeya dadantii	NCPPB 3090	Rice
Pantoea agglomerans	NCPPB 2971	Wisteria
Pectobacterium carotovorum subsp. *carotovorum*	NCPPB 1280	Potato
Pseudomonas corrugata	NCPPB 2445	Potato
P. savastanoi pv. *phaseolicola*	NCPPB 1321	Bean
P. syringae subsp. *syringae*	NCPPB 1417	Rice
P. s. subsp. *tomato*	NCPPB 269	Tomato
Ralstonia solanacearum	NCPPB 2315	Banana
Stenotrophomonas maltophilia	NCPPB 1974	NG
Stenotrophomonas maltophilia	ATCC 13637	NG
Stenotrophomonas sp.	Tomato isolate 27	Tomato
Stenotrophomonas sp.	Tomato isolate 36	Tomato
Stenotrophomonas sp.	Tomato isolate 38	Tomato
Xylella fastidiosa	ATCC 700964	Grape

NCPPB National Collection of Plant Pathogenic Bacteria, England; *ATCC* American Type Culture Collection, USA; *IPO* Plant Research International, Netherlands; *X. translucens* pv. *undulosa* B498 isolate provided by Dr. Norman W. Schaad, USA; *DSHC* Danish Seed Health Centre, Denmark; Tomato isolates 27, 36, 38, 73 and 167 were isolated from tomato seed samples collected from Tanzania; *NG* not given

the several developed and tested primers, three proved to be very robust and specific for *Xanthomonas* diagnosis including the X-gumD primers from *gumD* gene in the EPS (xanthan) synthesis pathway, X-fyuA primers

targeting the Ton B dependent receptor (*fyuA*) sequence and the X-ITS primers from the ITS region. Primer combinations were used to ensure robustness as each modified primer catered for a nucleotide difference in the primer binding site among the xanthomonads as shown in Fig. 1. For internal controls in the PCR tests conducted with DNA from pure bacterial cultures or diseased plant material, we developed multiplex PCRs consisting of *Xanthomonas* primers plus primers targeting either bacterial 16S rDNA or plant mitochondrial 26S rDNA. The primers used in the PCR reactions and their concentrations are shown in Tables 2 and 3. To compare the performance of the presently developed approach with published *Xanthomonas* genus PCR protocols, we replicated the procedures of Leite et al. (1994) and Maes (1993) using the developed RS 21 and R22 and 16S rDNA primers, respectively. There was some variation in results for the RS 21, R 22 primers obtained when using different thermocyclers. For these primers PCR reactions were therefore conducted in a PT-100-60 thermocycler (MJ Research, Watertown, Mass., USA) used in reported studies (Leite et al. 1994).

Each 25 μl PCR reaction with the different primer sets contained 5X PCR GoTaq flexi buffer (Promega, Madison, Wis., USA), 200 μM of each deoxynucleotide triphosphate (Promega) and 1.25U of GoTaq DNA polymerase (Promega, Madison, Wis.), as well as either 20 ng or 50 ng of plant or bacterial DNA template, respectively. The concentration of $MgCl_2$ (Promega, Madison, Wis., USA) varied with the different PCRs (Table 3). Detailed PCR conditions are shown in Table 3.

The PCR reactions were performed in Eppendorf Mastercycler Gradient PCR machine (Eppendorf, Hamburg, Germany).

The amplified PCR products were separated by horizontal gel electrophoresis in 1.5 % agarose gels in 0.5x TBE (Tris-borate EDTA) buffer at 50 V/cm for 45 min. The pre-stained gel with ethidium bromide (0.5 μg/ml) was then visualized under UV transilluminator and photographed with Instant Black and White Professional Film (Fujifilm).

Results

There was improved specific detection of xanthomonads in pure cultures with X-ITS and X-fyuA primers

When the previously published RS 21 and RS 22 primers (Leite et al. 1994) were tested in PCR reactions, they did not amplify DNA from 12 of the tested *Xanthomonas* strains and gave two bands around the 1,075 bp size in *X.*

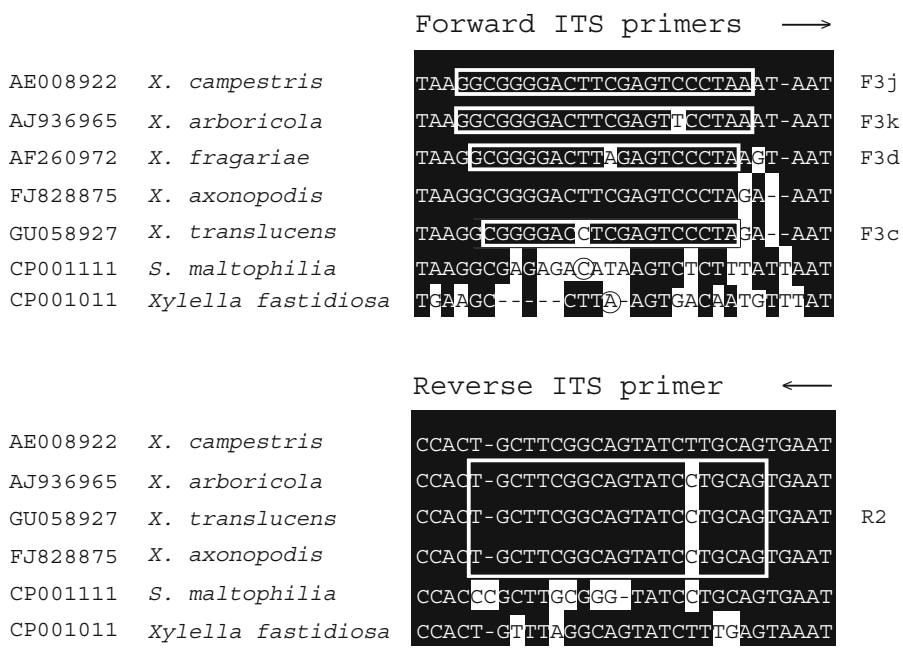

Fig. 1 The primer binding sites for the X-ITS primers based on multiple NCBI sequence alignment of some *Xanthomonas* and genetically closely related Non-*Xanthomonas* bacteria

Table 2 Primer sets used in the PCR reactions

Primers	Primer sequence	Product size (target amplified)
X-fyuA	X-fyuA Fw3 (5′GCCGGTGGACTACGATTGGAATTA3′); X-fyuA Fw3c (5′GCCGGTGGACTACGACTGGAATTA3′) X-fyuA Rv3 (5′CGGTGGCGAACAGGCTCA3′) X-fyuA Rv5 (5′GTCGCGGCGCCACTTCA3′)	239 bp (*Xanthomonas*)
X-ITS	X-ITS-F3j (5′GGCGGGGACTTCGAGTCCCTAA3′) X-ITS-F3k (5′GGCGGGGACTTCGAGTTCCTAA3′) X-ITS-F3c (5′CGGGGACCTCGAGTCCCTA3′) X-ITS-F3d (5′GCGGGGACTTAGAGTCCCTA3′) X-ITS-R2 (5′CTGCAGGATACTGCCGAAGCA3′)	254 bp (*Xanthomonas*)
X-gumD	X-gumD F7 (5′GGCCGCGAGTTCTACATGTTCAA3′) X-gumD R7 (5′CACGATGATGCGGATATCCAGCCACAA3′)	402 bp (*Xanthomonas*)
P16S	P16SF1 (5′GCCAGCAGCCGCGGTAATAC3′) P16SR2 (5′GCGCTCGTTGCGGGACTTA3′)	596 bp (Bacteria)
	P16S Fw3 (5′CGTGGGGAGCGAACAGGATTA3′) P16S Rv3 (5′CTTGACGGGCGGTGTGTACAA3′)	641 bp (Bacteria)
M26S	M26SF4 (5′ACCAGGGGGTAGCGACTGTTTATT3′) M26SR3; (5′CCCCAGGATGTGATGAGTCGACAT3′)	754 bp (Plant)

vesicatoria (NCPPB 422) (Fig. 2a, Table 4). On the other hand, the 16S rDNA primers (Maes 1993) gave positive DNA amplification in all the xanthomonads but also gave products of the delineated 480 bp in *Ralstonia solanacearum* (NCPPB 2315), *Stenotrophomonas maltophilia* (NCPPB 1974, ATCC13637) and *Xylella fastidiosa* (ATCC 700964) (Fig. 2b, Table 4). The developed X-fyuA primers targeting the gene *fyuA* effectively amplified a 239 bp fragment in all xanthomonads with the exception of *Xanthomonas theicola*. The X-ITS primers amplified a DNA fragment of 254 bp from pure cultures in all the tested *Xanthomonas* strains. None of the primers X-fyuA and X-ITS resulted in amplification of the non-xanthomonads (Fig. 2c and d, Table 4). The results revealed that these primers are quite specific and suitable for differentiation of xanthomonads from non-xanthomonads.

The X-gumD primers categorized xanthomonads into two groups

PCR assays using the X-gumD primers on DNA of the various bacterial strains appeared to be efficient in differentiating *Xanthomonas* strains of the two established phylogenetic groups (Hauben et al. 1997; Young et al. 2008). The primers allowed efficient DNA amplification of a 402 bp product in 38 out of 39 *Xanthomonas* strains of group II (represented by *X. campestris*) and only a weak amplification of DNA of *X. fragariae* (NCPPB 2949). No amplification of DNA was observed from strains of xanthomonads in group I (represented by *X. albilineans*) (Fig. 1e, Table 4) except for a very weak band observed with *X. sacchari* NCPPB 4341 (Table 4). The DNA from the non-xanthomonad strains was not amplified by the X-gumD primers (Fig. 2e, Table 4). This observation implies that DNA efficiently amplified by the X-gumD primers are from members of group II xanthomonads, while the group I *Xanthomonas* isolates give a very weak or no DNA amplification.

The 16S prokaryotic rDNA primers provided internal control in multiplex PCR with X-gumD and X-ITS primers in the screening of pure bacterial cultures

In multiplex PCR with P16S primers used as internal controls, double bands corresponding to the amplification of X-ITS (254 bp) and the 16S ribosomal DNA (596 bp) were observed in DNA from xanthomonads resulting from amplification of both the prokaryote and *Xanthomonas* DNA targets, while only the P16S rDNA sequence was amplified in the genomic DNA of the non-xanthomonads (Fig. 3b, Table 4). In multiplex PCR assay of the X-gumD with P16S primers, double bands were obtained with the members of group II and *X. sacchari* (NCPPB 4341), corresponding to the amplification of the *gumD* gene (402 bp) and the 16S ribosomal DNA (641 bp) (Table 4). In the rest of the group I xanthomonads and non-xanthomonads, only the band corresponding to the 16S ribosomal DNA gene was observed (Fig. 3a, Table 4). Multiplex PCR

Table 3 PCR conditions for the different primer sets used in the tests

PCR	Primers	Primer concentration	$MgCl_2$	Initial denaturation		Cycles	Denaturation		Annealing		Extension		Final extension	
				Temp	Time		Temp	Time	Temp	Time	Temp	Time	Temp	Time
X-fyuA	X-fyuA Fw3	12.5 pmol	1.5 mM	95 °C	3mins	30	95 °C	20s	66 °C	12 s	72 °C	10s	72 °C	3 min
	X-fyuA Fw3c	12.5 pmol												
	X-fyuA Rv3	12.5 pmol												
	X-fyuA Rv5	12.5 pmol												
X-ITS	X-ITS Fw3j	12.5 pmol	2 mM	95 °C	3mins	29	94 °C	15 s	69 °C	10s	72 °C	10s	72 °C	3 min
	X-ITS Fw3k	2.1 pmol												
	X-ITS Fw3c	8.3 pmol												
	X-ITS Fw3d	2.1 pmol												
	X-ITS Rv2	25 pmol												
X-ITS+P16S	X-ITS Fw3j	12.5 pmol	2 mM	95 °C	3mins	29	94 °C	15 s	69 °C	10s	72 °C	10s	72 °C	3 min
	X-ITS Fw3k	2.1 pmol												
	X-ITS Fw3c	8.3 pmol												
	X-ITS Fw3d	2.1 pmol												
	X-ITS Rv2	25 pmol												
	P16S Fw1	5 pmol												
	P16S Rv2	5 pmol												
X-ITS+M26S	X-ITS Fw3j	1.2 pmol	2 mM	95 °C	3mins	36	94 °C	20s	68 °C	15 s	72 °C	15 s	72 °C	3 min
	X-ITS Fw3k	0.4 pmol												
	X-ITS Fw3c	0.8 pmol												
	X-ITS Fw3d	0.4 pmol												
	X-ITS Rv2	2.4 pmol												
	M26S Fw4	2.4 pmol												
	M26S Rv3	2.4 pmol												
X-gumD	X-gumD Fw7	25 pmol	1.5 mM	95 °C	3mins	30	95 °C	20s	66 °C	15 s	72 °C	15 s	72 °C	3 min
	X-gumD Rv7	25 pmol												
X-gumD+P16S	X-gumD Fw7	25 pmol	1.5 mM	95 °C	3mins	30	95 °C	20s	66 °C	15 s	72 °C	15 s	72 °C	3 min
	X-gumD Rv7	25 pmol												
	P16S Fw3	3.75 pmol												
	P16S Rv3	3.75 pmol												
X-gumD+M26S	X-gumD Fw7	25 pmol	1.5 mM	95 °C	3mins	40	95 °C	20s	65 °C	15 s	72 °C	30s	72 °C	3 min
	X-gumD Rv7	25 pmol												
	M26S Fw4	10 pmol												
	M26S Rw3	10 pmol												

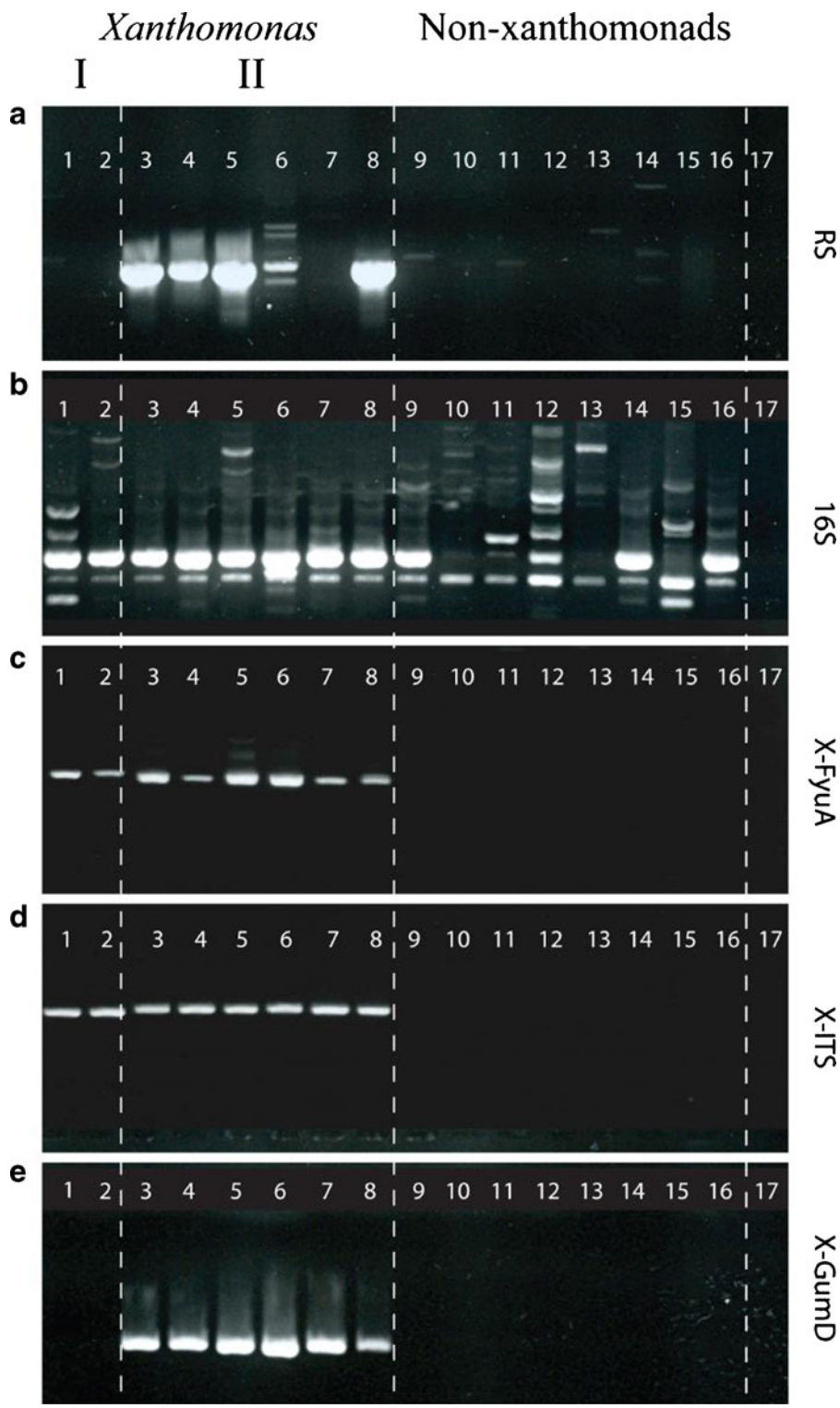

Fig. 2 DNA amplification from cultures of xanthomonads Groups I and II and non-xanthomonads using RS (**a**), 16S rRNA (**b**), X-FyuA (**c**), X-ITS (**d**), and X-gumD primers (**e**). Samples are *Xanthomonas albilineans* NCPPB 1830 (lane 1), *X. hyacinthi* NCPPB 205 (lane 2), *X. codiaei* NCPPB 3443 (lane 3), *X. cassava* NCPPB 101 (lane 4), *X. oryzae* pv. *oryzae* NCPPB 3002 (lane 5), *X. vesicatoria* NCPPB 422 (lane 6), *X. gardneri* tomato isolate 73 (lane 7), *X. arboricola* pv *poisentiicola* tomato isolate 167 (lane 8), *Stenotrophomonas maltophilia* ATCC 13637 (lane 9), *Burkholderia glumae* NCPPB 2391 (lane 10), *Pseudomonas syringae* pv. *tomato* NCPPB 269 (lane 11), *Xylella fastidiosa* ATCC 700964 (lane 12), *Acidovorax avenae* subsp. *avenae* NCPPB 1011 (lane 13), tomato isolate 27 (lane 14), tomato isolate 36 (lane 15), tomato isolate 38 (lane 16) and water (lane 17)

showing double bands of similar intensity of X-gumD and P16S amplifications generally distinguished *Xanthomonas* strains of group II from the members of group I (Fig. 3a).

Table 4 Single and multiplex PCR assays with the developed X-gumD, X-fyuA and X-ITS primers and previously developed RS21/22 and X.16SrDNA primers in the differentiation of *Xanthomonas* groups I and II from other genera of plant associated bacteria

Bacterial species/strains	*Xanthomonas* Group	Singlex PCR Primers					Multiplex PCR primers	
		RS21, RS22	16S rRNA, X.16SrDNA	X-fyuA	X-ITS	X-gumD	X-ITS/ P16S	X-gumD/ P16S
Xanthomonas albilineans NCPPB 1830	I	–	+	+	+	–	+/+	–/+
X. hyacinthi NCPPB 205	I	–	+	+	+	–	+/+	–/+
X. sacchari NCPPB 4341	I	–	+	+	+	vw+	+/+	vw+/+
X. theicola NCPPB 4353	I	–	+	–	+	–	+/+	–/+
X. translucens pv. *translucens* NCPPB 2389	I	–	+	+	+	–	+/+	–/+
X. t. pv. *undulosa* B498	I	–	+	+	+	–	+/+	–/+
X. arboricola pv. *celebensis* NCPPB 1832	II	+	+	+	+	+	+/+	+/+
X. arboricola pv. *poinsettiicola* tomato isolate 167	II	+	+	+	+	+	+/+	+/+
X. axonopodis NCPPB 457	II	+	+	+	+	+	+/+	+/+
X. a. pv. *aurantifolii* NCPPB 4377	II	+	+	+	+	+	+/+	+/+
X. a. pv. *cyamopsidis* NCPPB 637	II	+	+	+	+	+	+/+	+/+
X. a. pv. *glycines* NCPPB 1124	II	–	+	+	+	+	+/+	+/+
X. a. pv. *manihotis* NCPPB 2965	II	+	+	+	+	+	+/+	+/+
X. a. pv. *phaseoli* No 17	II	+	+	+	+	+	+/+	+/+
X. a. pv. *vasculorum* NCPPB 206	II	+	+	+	+	+	+/+	+/+
X. a. pv. *vignicola* NCPPB 555	II	+	+	+	+	+	+/+	+/+
X. a. pv. *vignicola* NCPPB 638	II	+	+	+	+	+	+/+	+/+
X. bromi NCPPB 4343	II	+	+	+	+	+	+/+	+/+
X. campestris var *aberrans* NCPPB 2986	II	+	+	+	+	+	+/+	+/+
X. c. var *armoraciae* NCPPB 1930	II	+	+	+	+	+	+/+	+/+
X. c. pv. *barbareae* NCPPB 983	II	+	+	+	+	+	+/+	+/+
X. c. pv. *campestris* NCPPB 528	II	+	+	+	+	+	+/+	+/+
X. c. pv. *carotae* NCPPB 3440	II	–	+	+	+	+	+/+	+/+
X. c. pv. *incanae* NCPPB 937	II	+	+	+	+	+	+/+	+/+
X. c. pv. *musacearum* NCPPB 4387	II	+	+	+	+	+	+/+	+/+
X. c. pv. *raphani* NCPPB 1946	II	+	+	+	+	+	+/+	+/+
X. cassavae NCPPB 101	II	+	+	+	+	+	+/+	+/+
X. citri subsp. *citri* NCPPB 410	II	+	+	+	+	+	+/+	+/+
X. citri subsp. *malvacearum* NCPPB 210	II	+	+	+	+	+	+/+	+/+
X. codiaei NCPPB 3443	II	+	+	+	+	+	+/+	+/+
X. cucurbitae NCPPB 2597	II	–	+	+	+	+	+/+	+/+
X. euvesicatoria NCPPB 2968	II	+	+	+	+	+	+/+	+/+
X. fragariae NCPPB 2949	II	+	+	+	+	w+	+/+	w+/+
X. fuscans subsp. *fuscans* IPO 482	II	+	+	+	+	+	+/+	+/+
X. gardneri NCPPB 881	II	+	+	+	+	+	+/+	+/+
X. gardneri tomato isolate 73	II	–	+	+	+	+	+/+	+/+
X. hortorum pv. *pelargonii* NCPPB 305	II	+	+	+	+	+	+/+	+/+
X. melonis NCPPB 3434	II	–	+	+	+	+	+/+	+/+
X. oryzae pv. *oryzae* NCPPB 3002	II	+	+	+	+	+	+/+	+/+
X. o. pv. *oryzicola* NCPPB 1151	II	+	+	+	+	+	+/+	+/+

Table 4 (continued)

Bacterial species/strains	*Xanthomonas* Group	Singlex PCR Primers					Multiplex PCR primers	
		RS21, RS22	16S rRNA, X.16SrDNA	X-fyuA	X-ITS	X-gumD	X-ITS/ P16S	X-gumD/ P16S
X. perforans NCPPB 4321	II	+	+	+	+	+	+/+	+/+
X. pisi NCPPB 762	II	–	+	+	+	+	+/+	+/+
X. pruni NCPPB 416	II	w+*	+	+	+	+	+/+	+/+
X. pruni NCPPB 3155	II	w+*	+	+	+	+	+/+	+/+
X. sesame NCPPB 631	II	+	+	+	+	+	+/+	+/+
X. vasicola pv. *holcicola* NCPPB 2417	II	+	+	+	+	+	+/+	+/+
X. vesicatoria NCPPB 422	II	w+*	+	+	+	+	+/+	+/+
Acidovorax avenae subsp. *avenae* NCPPB 1011	NX	–	–	–	–	–	–/+	–/+
A. a. subsp. *citrulli* ATCC 29625	NX	–	–	–	–	–	–/+	–/+
Burkholderia glumae 2391	NX	–	–		–	–	–/+	–/+
Clavibacter michiganensis subsp. *michiganensis* IPO 542	NX	–	–	–	–	–	–/+	–/+
Dickeya dadantii NCPPB 3090	NX	–	–	–*	–	–	–/+	–/+
Pantoea agglomerans NCPPB 2971	NX	–	–	–	–	–	–/+	–/+
Pectobacterium carotovorum subsp. *carotovorum* NCPPB 1280	NX	–	–	–	–	–	–/+	–/+
Pseudomonas corrugata NCPPB 2445	NX	–	–	–	–	–	–/+	–/+
P. savastanoi pv. *phaseolicola* NCPPB 1321	NX	–	–	–*	–	–	–/+	–/+
P. syringae pv. *syringae* NCPPB 1417	NX	–	–	–*	–	–	–/+	–/+
P. syringae pv. *tomato* NCPPB 269	NX	–	–	–	–	–	–/+	–/+
Ralstonia solanacearum NCPPB 2315	NX	–	+	–	–	–	–/+	–/+
Stenotrophomonas maltophilia NCPPB 1974	NX	–	+	–	–	–	–/+	–/+
Stenotrophomonas maltophilia ATCC13637	NX	–	+	–	–	–	–/+	–/+
Stenotrophomonas sp. tomato isolate 27	NX	–	+	–	–	–	–/+	–/+
Stenotrophomonas sp. tomato isolate 36-	NX	–	–	–	–	–	–/+	–/+
Stenotrophomonas sp. tomato isolate 38	NX	–	+	–	–	–	–/+	–/+
Xylella fastidiosa ATCC 700964	NX	–	+*	–	–	–	–/+	–/+

Symbols: I, group I *Xanthomonas*, II, group II *Xanthomonas* and NX the Non-*Xanthomonas* bacteria; + and—indicate species DNA amplification or no amplification, respectively; w+ and vw+ are weak and very weak reactions, respectively; +* indicates expected band size plus bands of other size, –* is amplification of fragments other than expected target size; +/+ is positive *Xanthomonas* target and 16S rDNA amplification, and −/+ is no *Xanthomonas* target amplification but positive 16S rDNA amplification

The X-gumD and X-ITS multiplex PCRs were effective in detection of xanthomonads infected plants

The M26S primers targeted a DNA fragment encoding mitochondrial ribosomal RNA highly conserved in plants and was previously used successfully in multiplex PCR (Adriko *et al*. 2012). The X-ITS and X-gumD primers were multiplexed with M26S primers and evaluated with un-inoculated (healthy) and inoculated rice and cabbage plants to test the ability to discriminate infected and non-infected plants with co-amplification of plant DNA as an internal control. In the multiplex PCR amplification of purified plant DNA, double bands corresponding to *Xanthomonas* target and plant mitochondrial rDNA were observed for DNA extracted from inoculated plant tissue and only a single 754 bp band corresponding to the expected plant mitochondrial DNA fragment was observed for DNA extracted from un-inoculated healthy plants (Fig. 4a and B). The discrimination of infected and non-infected

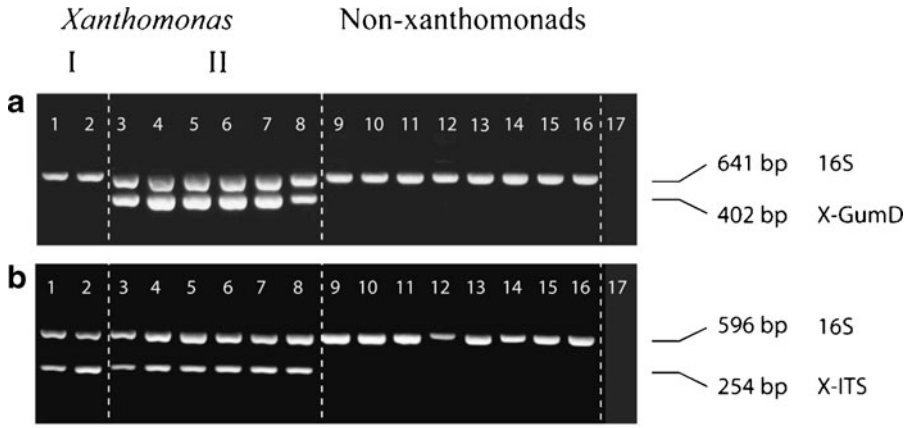

Fig. 3 Multiplex PCR amplification of DNA from pure cultures of xanthomonads (groups I and II) and non-xanthomonads by X-gumD (**a**) and X-ITS (**b**) primers. Samples are *Xanthomonas albilineans* NCPPB 1830 (lane 1), *X. hyacinthi* NCPPB 205 (lane 2), *X. codiaei* NCPPB 3443 (lane 3), *X. cassava* NCPPB 101 (lane 4), *X. oryzae* pv. *oryzae* NCPPB 3002 (lane 5), *X. vesicatoria* NCPPB 422 (lane 6), tomato isolate 73 (lane 7) and tomato isolate 167 (lane 8) identified as *Xanthomonas*, *Stenotrophomonas maltophilia* ATCC 13637 (lane 9), *Burkholderia glumae* NCPPB 2391 (lane 10), *Pseudomonas syringae* pv. *tomato* NCPPB 269 (lane 11), *Xylella fastidiosa* ATCC 700964 (lane 12), *Acidovorax avenae* subsp. *avenae* NCPPB 1010 (lane 13), *Stenotrophomonas* spp.: tomato isolates 27 (lane 14), 36 (lane 15), and 38 (lane 16) and water control (lane 17)

plant tissue and co-amplification of plant DNA thereby proved to be successful.

Discussion

Bacterial diseases, including those caused by members of the genus *Xanthomonas*, are commonly reported in new geographical areas, as revealed by new disease reports of British Society for Plant Pathology (http://www.ndrs.org.uk/) and Plant Disease Notes of the American Phytopathological Society (http://apsjournals.apsnet.org/loi/pdis). These are often considered emerging diseases in the tropics. This situation thus calls for tools that can readily be used to detect and identify and thus support an effective management of such diseases. Early and accurate detection and identification of new cases of *Xanthomonas* diseases as provided by molecular based methods (López et al. 2003; Louws et al. 1999; Narayanasamy 2011) will aid to speed up their management. Detection of pathogens in both symptomatic and asymptomatic plant tissues is of importance in certification programs for both domestic and international plant quarantine.

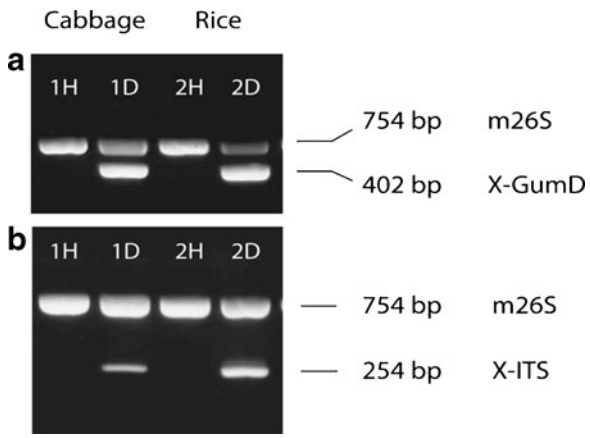

Fig. 4 Multiplex PCR amplification of xanthomonads in plant tissue where X-gumD (**a**) and X-ITS (**b**) primers were multiplexed with plant M26S primers. The lanes are healthy cabbage (1H), cabbage inoculated with *X. campestris* pv. *campestris* NCPPB 3207 (1D), healthy rice (2H), and rice inoculated with *X. oryzae* pv. *oryzae* NCPPB 3002 (2D)

In the present study, a genus-specific *Xanthomonas* PCR was developed due to the large number of pathogens represented in this important genus that affect several monocot and dicot crop species. PCR tests with primers designed from the TonB Dependant Receptor (X-fyuA), 16S–23S ITS region (X-ITS) and EPS (xanthan) synthesis pathway gene *gumD* (X-gumD) proved to be effective and robust in the detection of the members of the genus *Xanthomonas*. These three gene targets were very specific and quite effective in detecting a larger number of xanthomonads than previously

published PCR-based *Xanthomonas* diagnostic tools without giving false positive results among non-xanthomonads. Under the conditions described, the tested tools were more robust in application to xanthomonads when compared to the RS 21, and R 22 primers (Leite et al. 1994). The primers developed in this study also had the advantages of reducing false positive results as compared to the 16S rRNA primers (Maes 1993), which in our tests gave rise to PCR products of similar size as expected for xanthomonads when strains of *Ralstonia solanacearum*, *Stenotrophomonas maltophilia* and *Xylella fastidiosa* were tested.

The use of internal controls in a PCR increased reliability of results ensuring quality check especially with regard to negative test results. It ensured that the lack of amplification was not due to the absence of DNA or presence of PCR inhibitors but rather that the targeted DNA fragment being absent in the unamplified sample. In our tests, the presence of an internal control of the PCR clearly distinguished between *Xanthomonas* strains, non-*Xanthomonas* strains and the water control. It also distinguished between, diseased, healthy plants and the water control. The use of this internal control is of particular value when working with plant hosts possessing PCR inhibiting compounds. Multiplex PCR with internal controls in the diagnosis of *Xanthomonas* species have previously been reported (Berg et al. 2005; Glick et al. 2002; Robène-Soustrade et al. 2010).

The *gumD* based PCR appeared to be a potential tool for the differentiation of the described xanthomonads groups I and II (Hauben et al. 1997; Young et al. 2008). As expected the X-gumD PCR did not detect or gave very weak amplification with xanthomonads from group I consisting of *X. albilineans*, *X. hyacinthi*, *X. theicola*, *X. sacchari* and *X. translucens*, while 38 out 39 strains of group II were effectively amplified. No *gum* genes have been reported in the fully sequenced genome *X. albilineans*, a representative of Group I. Studholme et al. 2011, reported that the *gum* cluster was probably present in the common ancestor of *Xylella, Stenotrophomonas* and *Xanthomonas* and subsequently lost by *X. albilineans* and *Stenotrophomonas* but retained in *X. sacchari* and the members of Group II.

In conclusion, the PCR tools developed in the present study contribute to the improved detection and identification of members of the genus *Xanthomonas* and both from pure cultures and directly from infected plant material.

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References

Adriko, J., Aritua, V., Mortensen, C. N., Tushemereirwe, W. K., Kubiriba, J., & Lund, O. S. (2012). Multiplex PCR for specific and robust detection of *Xanthomonas campestris* pv. *musacearum* in pure culture and infected plant material. *Plant Pathology, 61*, 489–497.

Almeida, N. F., Yan, S., Cai, R., Clarke, C. R., Morris, C. E., Schaad, N. W., et al. (2010). PAMDB, a multilocus sequence typing and analysis database and website for plant-associated microbes. *Phytopathology, 100*, 208–215.

Amann, R. I., Ludwig, W., & Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiology Reviews, 59*, 143–169.

Aslam, N. S., Newman, M.-A., Erbs, G., Morrissey, L. K., Chincilla, D., Boller, T., et al. (2008). Bacterial polysaccharides suppress induced innate immunity by calcium chelation. *Current Biology, 18*, 1078–1083.

Berg, T., Tesoriero, L., & Hailstones, D. L. (2005). PCR-based detection of *Xanthomonas campestris* pathovars in *Brassica* seed. *Plant Pathology, 54*, 416–427.

Bradbury, J. F. (1986). *Xanthomonas Dowson 1939* (pp. 198–260). Slough: CAB International Mycological Institute.

Chou, F. L., Chou, H. C., Lin, Y. S., Yang, B. Y., Lin, N. T., Weng, S. F., et al. (1997). The *Xanthomonas campestris gumD* gene required for synthesis of xanthan gum is involved in normal pigmentation and virulence in causing black rot. *Biochemical and Biophysical Research Communications, 233*, 265–269.

Chun, W., Cui, J., & Poplawsky, A. R. (1997). Purification, characterization and biological role of a pheromone produced by *Xanthomonas campestris* pv. *campestris*. *Physiological and Molecular Plant Pathology, 51*, 1–14.

Dow, J. M., Daniels, M. J. (1994) Pathogenicity determinants and global regulation of pathogenicity in *Xanthomonas campestris* pv. *campestris* (pp. 29–41). Springer, Berlin, Germany.

Dunger, G., Relling, V. M., Tondo, L. M., Barreras, M., Ielpi, L., Orellano, G. E., et al. (2007). Xanthan is not essential for pathogenicity in citrus canker but contributes to *Xanthomonas* epiphytic survival. *Microbiology, 188*, 127–135.

Finkmann, W., Alterndorf, K., Stackebrandt, E., & Lipski, A. (2000). Characterization of N2O-producing *Xanthomonas*-like isolates from biofilters as *Stenotrophomonas nitritireducens* sp. nov., *Luteimonas mephitis* gen. nov., sp. Nov. and *Pseudoxanthomonas broegbernensis* gen. nov., sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 50*, 273–282.

Glick, D. L., Coffey, C. M., & Sulzinski, M. A. (2002). Simultaneous PCR detection of the two major bacterial

pathogens of Geranium. *Journal of Phytopathology, 150*, 54–59.

Gurtler, V., & Stanisich, V. A. (1996). New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. *Microbiology, 142*, 3–16.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symposium Series, 41*, 95–98.

Hauben, L., Vauterin, L., Swings, J., & Moore, E. R. B. (1997). Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *International Journal of Systematic Bacteriology, 47*, 328–335.

Hayward, A. C. (1993). *The hosts of Xanthomonas* (pp. 1–199). United Kingdom: Chapman and Hall, London.

Hayward, A. C., Fegan, N., Fegan, M., & Stirling, G. R. (2010). *Stenotrophomonas* and *Lysobacter*: ubiquitous plant-associated gamma-proteobacteria of developing significance in applied microbiology. *Journal of Applied Microbiology, 108*, 756–770.

Jenkins, C. L., & Starr, M. P. (1982). The brominated aryl-polyene (xanthomonadin) pigments of *Xanthomonas juglandis* protect against photobiological damage. *Current Microbiology, 7*, 323–326.

Katzen, F., Becker, A., Zorreguieta, A., Puhler, A., & Ielpi, L. (1996). Promoter analysis of the *Xanthomonas campestris* pv. *campestris gum* operon directing biosynthesis of the xanthan polysaccharide. *Journal of Bacteriology, 178*, 4313–4318.

Katzen, F., Ferreiro, D., Oddo, C., Ielmini, M. V., Becker, A., Puhler, A., et al. (1998). *Xanthomonas campestris* pv. *campestris* gum mutants: effects on xanthan biosynthesis and plant virulence. *Journal of Bacteriology, 180*, 1607–1617.

Kim, S.-Y., Kim, J.-G., Lee, B.-M., & Cho, J.-Y. (2009). Mutational analysis of the gum gene cluster required for xanthan biosynthesis in *Xanthomonas oryzae* pv. *oryzae*. *Biotechnology Letters, 31*, 265–270.

Koebnik, R. (2005). TonB-dependent trans-envelope signalling: the exception or the rule? *Trends in Microbiology, 13*, 343–347.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences, 82*, 6955–6959.

Lee, Y.-A., Sung, A.-N., Liu, T.-F., & Lee, Y.-S. (2009). Combination of chromogenic differential medium and *estA*-specific PCR for isolation and detection of phytopathogenic *Xanthomonas* spp. *Applied and Environmental Microbiology, 75*, 6831–6838.

Leite, P. R., Jr., Minsavage, V. G., Bonas, U., & Stall, E. R. (1994). Detection and identification of phytopathogenic *Xanthomonas* strains by amplification of DNA sequences related to the *hrp* genes of *Xanthomonas campestris* pv. *vesicatoria*. *Applied and Environmental Microbiology, 60*, 1068–1077.

Leyns, F., DeCleene, M., Swings, J. G., & Deley, J. (1984). The host range of the genus *Xanthomonas*. *Botanical Review, 50*, 308–356.

López, M. M., Bertolini, E., Olmos, A., Caruso, P., Gorris, T. M., Llop, P., et al. (2003). Innovative tools for detection of plant pathogenic viruses and bacteria. *International Microbiology, 6*, 233–243.

Louws, F. J., Rademaker, J. L. W., & de Bruijn, F. J. (1999). The three Ds of PCR-based genomic analysis of phytobacteria: diversity, detection, and disease diagnosis. *Annual Review of Phytopathology, 37*, 81–125.

Maes, M. (1993). Fast classification of plant-associated bacteria in the *Xanthomonas* genus. *FEMS Microbiology Letters, 113*, 161–166.

Mbega, R. E. (2011) Detection, characterization and control of *Xanthomonas* spp. causal agents of bacterial leaf spot of tomato in Tanzania. PhD Thesis, University of Copenhagen.

Mbega, E. R., Wulff, E. G., Mabagala, R. B., Adriko, J., Lund, O. S., & Mortensen, C. N. (2012a). Xanthomonads and other yellow-pigmented *Xanthomonas*-like bacteria associated with tomato seeds in Tanzania. *African Journal of Biotechnology, 11*, 14303–14312.

Mbega, R. E., Mabagala, R. B., Adriko, J., Lund, O. S., Wulff, E. G., & Mortensen, C. N. (2012b). Five species of xanthomonads associated with bacterial leaf spot symptoms in tomato from Tanzania. *Plant Disease, 96*, 760.

Narayanasamy, P. (2011). *Diagnosis of bacterial diseases of plants* (pp. 233–246). London: Springer.

Palacio-Bielsa, A., Cambra, M. A., & López, M. M. (2009). PCR detection and identification of plant-pathogenic bacteria: updated review of protocols (1989–2007). *Journal of Plant Pathology, 91*, 249–297.

Parkinson, N., Aritua, V., Heeney, J., Cowie, C., Bew, J., & Stead, D. (2007). Phylogenetic analysis of *Xanthomonas* species by comparison of partial *gyrase* B gene sequences. *International Journal of Systematic and Evolutionary Microbiology, 57*, 2881–2887.

Parkinson, N., Cowie, C., Heeney, J., & Stead, D. (2009). Phylogenetic structure of *Xanthomonas* determined by comparison of *gyrB* sequences. *International Journal of Systematic and Evolutionary Microbiology, 59*, 264–274.

Poplawsky, A. R., & Chun, W. (1998). *Xanthomonas campestris* pv. *campestris* requires a functional pigB for epiphytic survival and host infection. *Molecular Plant—Microbe Interactions, 11*, 466–475.

Robène-Soustrade, I., Legrand, D., Gagnevin, L., Chiroleu, F., Laurent, A., & Pruvost, O. (2010). Multiplex nested PCR for detection of *Xanthomonas axonopodis* pv. *allii* from onion seeds. *Applied and Environmental Microbiology, 76*, 2697–2703.

Rodriguez, L. M., Grajales, A., Arrieta-Ortiz, M. L., Salazar, C., Restrepo, S., & Bernal, A. (2012). Genome-based phylogeny of the genus *Xanthomonas*. *BMC Microbiology, 12*, 43.

Rozen, S., & Skaletsky, H. J. (2000). *Primer3 on the WWW for general users and for biologist programmers* (pp. 365–386). Totowa: Humana Press.

Schaad, W. N., Jones, J. B., & Lacy, H. G. (2001). *Xanthomonas* (p. 26). St Paul: APS Press.

Simões, H. N. T., Gonçalves, R. E., Rosato, B. Y., & Mehta, A. (2007). Differentiation of *Xanthomonas* species by PCR-RFLP of *rpf*B and *atp*D genes. *FEMS Microbiology Letters, 271*, 33–39.

Studholme, D. J., Wasukira, A., Paszkiewicz, K., Aritua, V., Thwaites, R., Smith, J., et al. (2011). Draft genome sequences of *Xanthomonas sacchari* and two banana-associated

xanthomonads reveal insights into the *Xanthomonas* group I clade. *Genes, 2*, 1050–1065.

Swings, J. G., & Civerolo, E. L. (1993). *Xanthomonas* (p. 399). London: Chapman and Hall.

Vos, M., Quince, C., Pijl, A., Hollander, M., & Kowalchuk, G. (2012). A comparison of rpoB and 16S rRNA as markers in pyrosequencing studies of bacterial diversity. *PLoS ONE, 7*, 1–8.

Young, J. M., Park, C. D., Shearman, H. M., & Fargier, E. (2008). A multilocus sequence analysis of the genus *Xanthomonas*. *Systematic and Applied Microbiology, 31*, 366–377.

Young, J. M., Wilkie, J. P., Park, D. C., & Watson, D. R. W. (2010). New Zealand strains of plant pathogenic bacteria classified by multi-locus sequence analysis; proposal of *Xanthomonas dyei* sp. nov. *Plant Pathology, 59*, 270–281.