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Differentiation of citrus bacterial canker strains in Korea by host range, rep-PCR fingerprinting and 16S rDNA analysis

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Abstract New *Xanthomonas* isolates causing citrus bacterial canker in Korea were differentiated primarily on the basis of host range by comparison with reference strains. The new isolates were pathogenic to *Citrus sinensis, C. paradisi, C. limon* and *C. unshiu* and formed crater-like canker on the plants; this indicated that they were *X. axonopodis* (*X.a.*) pv. *citri* A types. Molecular characterization using rep-PCR fingerprinting and 16S rDNA sequence analysis was also performed. Cluster analysis by combining the band patterns of ERIC-, BOX- and REP-PCR clearly separated one group including only *X. a.* pv. *citrumelo* and the other group including *X. a.* pv. *citri* and *X. a.* pv. *aurantifolii* strains. There was a clear separation between *X. a.* pv. *citri* Asiatic types and *X. a.* pv. *aurantifolii* B, C types in the second group. Partial sequence analysis of 16S rDNA revealed that all strains of *X. a.* pv. *aurantifolii* B and C type, and *X. a.* pv. *citrumelo* formed a distinct cluster with a similarity of 99%. Our results indicate that the isolates causing citrus canker in Korea belong to the A type of *X. a.* pv. *citri*.

Keywords Citri · Pathotype · *Xanthomonas axonopodis*

Several pathotypes have been recognized within the genus *Xanthomonas* causing citrus bacterial canker (CBC) primarily distinguished by their geographical origin and host range in addition to certain genotypic characteristics. The most commonly widespread group of *X. a.* pv. *citri* strains is the main Asiatic group A, with a host range on all citrus varieties. Two groups of strains affecting only Mexican lime and Alemow have recently been identified and designated as A* and A^w (Cubero and Graham 2002, 2004; Verniere et al. 1998). Two other canker types, which are known as *X. a.* pv. *aurantifolii* B and C were reported in South America. Pathotype B has a restricted host range including lemon and Mexican lime, whereas pathotype C is restricted to Mexican lime (Stall and Civerolo 1993).

Several techniques such as RFLP analysis, genomic fingerprinting, and rDNA analysis, have been reported for the identification of the bacterial strains and pathotype assignation. REP-, ERIC-, and BOX-PCR

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were also used to generate genomic fingerprints of a variety of *Xanthomonas* isolates and to identify pathovars and strains that were previously not distinguishable by other classification methods (Cubero and Graham 2002; Louws et al. 1994, 1995; Opgenorth et al. 1996). In this paper, we used primarily host range test for the differentiation of Korean isolates and then rep-PCR and 16s rRNA analysis was performed. These methods allowed us to evaluate the diversity of *Xanthomonas* strains collected from symptomatic citrus plants in Korea and to relate them to a worldwide collection.

Diseased samples of citrus plants were collected from 1997 to 2005 in the southern part of Korea including Jeju island, which is the major producing area. The *Xanthomonas* strains of which the pathotype was already known were obtained as reference strain from several institutes as described in Table 1. One hundred and twelve isolates of the pathogen were newly isolated from symptomatic leaf and fruit samples, and compared with reference pathotypes. We could not recover any *Xanthomonas* isolates from other regions excepting Jeju island. The colonies were mucoid, convex, and yellow on nutrient agar plates as other xanthomonads. After confirmation of pathogenicity, the isolates were identified to species level by analysis of fatty acid composition and carbon source utilization using MIDI and Biolog, respectively.

Leaf assays on four cultivars of *Citrus* hosts such as *C. sinensis* (sweetorange), *C. paradisi* (grapefruit), *C. limon* (lemon), and *C. aurantifolii* (Mexican lime) allow rapid and accurate evaluation of bacterial strains

Table 1 *Xanthomonas* isolates and reference strains used in this study

Type	Name	Origin	Source
X. a. pv. *citri* A	CFBP2859	Brazil	CIRAD
	JH410-1	China	CIRAD
	C43, CFBP1814	France	CIRAD
	CFBP2900	Japan	CIRAD
	JJ238-3, JK4-3	Korea	CIRAD
	CFBP2525	New Zealand	CIRAD
	JK148-2	Philippines	CIRAD
	M9, A-5246, M5, A-5208	USA	DPI
X. a. pv. *citri* A*	IR01, IR02, IR03, IR04	Iran	PPDSI
	JF90-2	Oman	CIRAD
	JK2-10	Saudi Arabia	CIRAD
	A-1609	USA	DPI
X. a. pv. *citri* A^w	A-2032	USA	DPI
X. a. pv. *aurantifolii* B	CFBP2868, CFBP2903	Argentina	CIRAD
X. a. pv. *aurantifolii* C	CFBP2866	Brazil	CIRAD
X. a. pv. *citrumelo* (CBS)	XC05-252, A-1902, A-1887	USA	DPI
Korean isolates	SL-0870, 0874, 4021, 4024, 4026, 4028, 4029, 4034, 4036, 4040, 4041, 4042, 4043, 4044, 4045, 4046, 4047, 4049, 4050, 4052, 4054, 4056, 4057, 4059, 4060, 4062, 4063, 4064, 4066, 4067, 4068, 4070, 4071, 4072, 4073, 4090, 4091, 4093, 4095, 4096, 4098, 4099, 4100, 4468, 4469, 4474, 4477, 4478, 4483, 4484, 4492, 4493, 4500, 4501, 4510, 4511 4516, 4517, 4518, 4519, 4520, 4525, 4526, 4528, 4529, 4530, 4537, 4538, 4539, 4547, 4548, 4553, 4554, 4556, 4558, 4560, 4562, 4564, 4566, 4567, 4568, 4915, 4916, 4917, 4918, 4920, 4922, 4925, 4926, 4928, 4931, 4932, 4933, 4934, 4935, 4937, 4943, 4945, 4946, 4947, 4949, 4950, 4952, 4953, 4954, 4955, 4956, 4958, 4990,4993, 4995	Korea	PPD
	KACC10443	Korea	KACC

CIRAD The Agricultural Research Centre for International Development; *DPI* Division of Plant Industry; Florida, USA; *PPD* Plant Pathology Division, NIAST, Korea; *PPDSI* Plant Pests and Diseases Research Institute, Iran; *KACC* Korean Agricultural Culture Collection

(Brunings and Gabriel 2003). In this experiment, freshly grown cultures of bacterial isolates were diluted to approximately 10^5 cfu ml^{-1} using phosphate buffer saline. Bacterial suspensions were infiltrated into three to five immature leaves of *C. sinensis, C. paradisi, C. limon* and *C. unshiu* (mandarin). All inoculated plants were kept in a greenhouse with an average temperature of 26°C and examined for canker symptoms for 1 month.

The *X. a.* pv. *citri* isolates were pathogenic producing cankers on all of the tested plants, but the A* and A^w reference strains were non-pathogenic on all of the tested plants, confirming that strains of these types have a narrow host range (Table 2). *X. a.* pv. *aurantifolii* B group was weakly virulent on the tested varieties except mandarin and *X. a.* pv. *aurantifolii* C caused HR on grapefruit, lemon, and sweetorange. Lesions generally developed about a week after inoculation of leaves, and the eruptive callus formation of *X. a.* pv. *citri* was readily discerned from the flat lesion types of *X. a.* pv. *citrumelo.* The HR of *X. a*. pv. *aurantifolii* C also clearly differentiated from the disease symptoms of *X. a*. pv. *aurantifolii* B. The citrus bacterial spot (CBS) pathogens were moderately virulent on all of the tested plants without producing canker. All of the new isolates produced canker and showed severe pathogenicity to all plants, indicating that they belonged to *X. a.* pv. *citri* A.

PCR based on repetitive sequences (rep-PCR) has been used effectively for analysis of several species of bacteria (Louws et al. 1999), and for strain identification (Cubero and Graham 2002; Louws et al. 1994, 1995; Opgenorth et al. 1996). The *Xanthomonas* isolates in this experiment were analyzed by rep-PCR as described below and compared with reference strains.

Total genomic DNA was extracted as described by Shiotani et al. (2000) with small modification. *Xanthomonas* isolates were cultured on peptone sucrose agar (1% polypeptone, 1% sucrose and 0.2% L-glutarmate) or LB media, and the cells were harvested. Bacterial cells were washed twice with 5 M NaCl and suspended in 600 ul of Tris-EDTA (TE) buffer (pH 8.0; Sambrook et al. 1989) containing SDS and proteinase K at a final concentration of 2% and 250 ug ml^{-1}, respectively. After incubation at 37°C for 1 h, 100 ul of 5 M NaCl and 80 ul of cetyltrimethylammoniumbromide (CTAB)–NaCl solution (10% hexadecyltrimethyl ammonium bromide in 0.7 M NaCl) was added and incubated at 65°C for 10 min. DNA was extracted using phenol chloroform isoamyl alcohol (25:24:1) and used as the templates. BOX–PCR reaction was carried out in 25 µl mixtures containing 1× Taq buffer, 2 mM $MgCl_2$, 4 uM primer BOX1R (Louws et al. 1994), each deoxynucleoside triphosphate (dNTP) at a concentration of 1.25 mM, and 2 U of EX-Taq polymerase (Takara); the amplification conditions consisted of 94°C for 1 min, 52°C for 1 min, and 65°C for 8 min for 30 cycles plus an initial step of 94°C for 7 min and a

Table 2 Pathogenicity of reference strains and Korean isolates of *Xanthomonas* on different host plants

CBC group	Strain	Host				Canker
		GF	Lemon	SO	MD	
X. a. pv. *citri* A	CFBP2900, CFBP2859, JK148–2	+++[a]	+++	+++	+++	Y
	JJ238–3, JK4–3, M9, 5246, M5, A-5208, CFBP1814, CFBP2525, JH410–1	+++	+++	+++	++	Y
	C43	+	+	+	+	Y (small)
X. a. pv. *citri* A*	IR01, IR02, IR03, IR04, JF90–2, JK2–10, 1609	−	−	−	−	N
X. a. pv. *citri* A^w	A-2032	HR	−	−	−	N
X. a. pv. *aurantifolii* B	CFBP2868, CFBP2903	+	++	+	−	N
X. a. pv. *aurantifolii* C	CFBP2866	HR	HR	HR	v	N
X. a. pv. *citrumelo* (CBS)	XC05-252, 1902, 1887	++	+	++	+	N
Korean isolates	Total 112 strains (Table 1)	+++	+++	+++	+++	Y

GF Grapefruit (*C. paradisi*), lemon (*C. limon*), *SO* sweetorange (*C. sinensis*), *MD* Mandarin (*C. unshui*), *HR* hypersensitive reaction, − no pathogenicity, + weak, ++ moderate, +++ severe pathogenicity, *v* variable, *N* no canker

[a] Pathogenicity was recorded 6 weeks after inoculation.

final step of 72°C for 10 min. ERIC-PCR was carried out in 25 µl with 2 uM primer ERIC1R and ERIC2 (Louws et al. 1994) and the amplification conditions were the same as those used for the BOX-PCR excepting the extension at 50°C. REP-PCR was also carried out in 25 µl with 50 uM primer of REP1R-I and REP2-I (Louws et al. 1994), and the extension temperature was 45°C. The PCR products were analyzed by 1.5% agarose gel electrophoresis in 1× TBE buffer at 50 V.

The PCR bands were compared based on the presence or absence of fragments at a specific position, and similarity coefficients for pairs of isolates were calculated with the programme NTSYS, version 2.1 (Rohlf 2000) and clustered with UPGMA method (un-weighted pair group method with arithmetic mean) to determine the genetic relationship among bacterial isolates. Most bands were present in all tested strains, but there were differences in the intensity of some amplified fragments as well as in the occurrence of several polymorphic bands. When the data were analyzed by combining each fingerprinting results of the BOX–, REP– and ERIC–PCR, two groups were clearly separated with 50% similarity. One group included only *X. a.* pv. *citrumelo*, and the other group included *X. a.* pv. *citri* and *X. a.* pv. *aurantifolii* strains (Fig. 1). There was also a clear separation between X. *a.* pv. *citri* A types and *X. a.* pv. *aurantifolii* B, C strains in the second group. Cubero and Graham (2002, 2004) reported that A* and A^w types are closely related to type A strains but affect only Mexican lime and Alemow. Our results of rep-PCR also indicated that A* and A^w types were close to A types, but they were excluded from A types in this combined analysis. Trindade et al. (2005) argued that dendrograms obtained with REP, ERIC

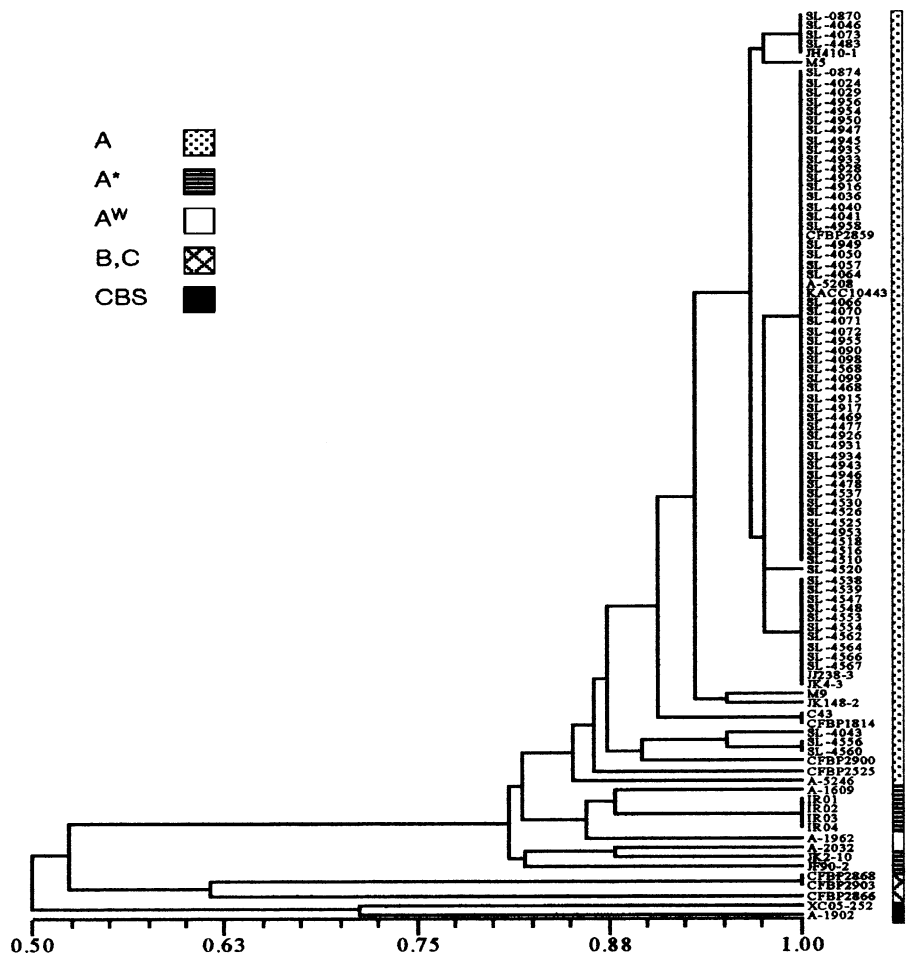

Fig. 1 Dendrogram showing relationships among *Xanthomonas* strains causing CBC based on combined analysis of ERIC–, BOX– and REP–PCR results. The lines indicate the CBC group as follows: A, *X. axonopodis* pv. *citri* A; A*, *X. a.* pv. *citri* A*; A^w, *X. a.* pv. *citri* A^w, B; C, *X. a.* pv. *aurantifolii* strains; and CBS, *X. a.* pv. *citrumelo*

and BOX primers showed an overall similar pattern of separation when compared to the combined analysis without showing data. In our study the differentiation capacity was significantly increased when the ERIC–, REP– and BOX–PCR results were combined.

Sequence analysis of 16S rDNA gene has been applied to bacterial identification and strain discrimination within species (Louws et al. 1999). We amplified 16S rDNA using primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′; Lane 1991) to investigate genetic relatedness of strains. The amplification reaction was performed in a 50 µl solution containing 50 ng of template DNA, 5 pmol of both primers, 0.2 mM of each dNTP with 1.5 mM $MgCl_2$, and 1 U of E-Taq DNA polymerase (SolGent Ltd., Korea). Each reaction mixture was run for 30 cycles using a 9,700 DNA thermal cycler (Perkin–Elmer, Norwalk, CN, USA) with the following temperature profiles: denaturation at 95°C for 20 s, annealing at 50°C for 40 s, and extension at 72°C for 1 min and 30 s plus an initial step of 95°C for 15 min and a final step of 72°C for 5 min. The 16S rDNA PCR products were purified using the SolGent PCR purification kit and sequenced at the DNA sequencing laboratory of SolGent Ltd. (Korea) using the BigDye terminator sequencing kit and an ABI PRISM 3730xl DNA analyzer (Applied Biosystems, Norwalk, CN, USA). The resultant 16S rDNA sequences were aligned using the fingerprinting II informatix^{TM} software (Bio-Rad, Hercules, CA, USA). Gaps were excluded, and the final alignment used for phylogenetic analysis consisted of 880 bp ranging from 1,017 to 1,896 nucleotides corresponding with the position in *X. a.* pv. *citri* 306 strain (NCBI accession number, AE012039). A phylogenetic tree was constructed by using UPGMA method.

Partial sequence analysis of 16S rDNA revealed high levels of similarity among the *Xanthomonas* strains studied. A dendrogram based on pairwise

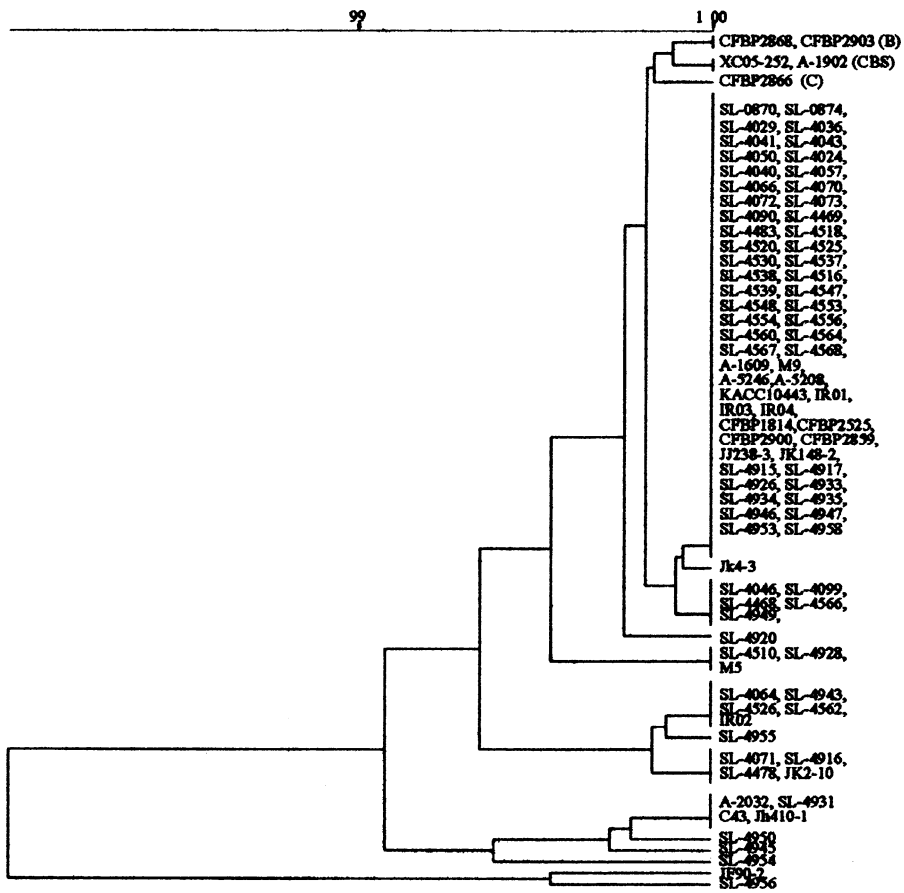

Fig. 2 Phylogenetic tree based on 16S rDNA sequences of the *Xanthomonas* strains causing CBC. The tree was constructed by using the fingerprinting II informatix software with UPGMA method

comparisons of all the strains showed diverse clusters (Fig. 2). Interestingly, according to the phylogenetic analysis, all of the strains of *X. a.* pv. *aurantifolii* B, C type and *X. a.* pv. *citrumelo* formed a distinct cluster with 99% similarity. The 16S gene was considered unsuitable for discriminating and identifying closely related strains, because of the high levels of sequence similarity in this region (Fox et al. 1992). In spite of those limits, the sequence corresponding to the 16S rDNA has been used to study relationships among *Xanthomonas* species. In this experiment the sequence was not enough to clearly discriminate the *Xanthomonas* pathotypes, though the *X. a.* pv. *aurantifolii* B, C type and *X. a.* pv. *citrumelo* was differentiated from the Asiatic pathotypes. The 16S rDNA analysis also could not discriminate *X. a.* pv. *citri* A* and A^w types from *X. a.* pv. *citri* A types, which were reliably discriminated in combined analysis of rep-PCR results.

Overall, this paper supports that rep-PCR fingerprinting can be an important tool for identifying and monitoring the diversity of the CBC pathogens. Moreover the differentiation capacity increased significantly if the band polymorphisms were analyzed together, and this is the first report of a combined analysis for the *Xanthomonas* strains. These results strongly indicate that only *X. a.* pv. *citri* A type is present in Korea.

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