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Phenotypic and Genetic Characterization of Novel Strains of *Xanthomonas axonopodis* pv. *citri* Which Induce Atypical Symptoms on Citrus Leaves in Taiwan

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ABSTRACT

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A total of 46 strains of *Xanthomonas axonopodis* pv. *citri* isolated from Taiwan were examined for their pathogenicity on leaves of Mexican lime (*Citrus aurantifolia*), grapefruit (*C. paradisi*), Liucheng (*C. sinensis*) and lemon (*C. limon*). These strains were grouped into three types (A, A^f and A^r) based on symptoms induced on leaves of the four citrus species. There were 43 (93.5%) strains belonging to type A which induced typical erumpent canker lesions with watersoaked margin on leaves of all four citrus species. Whereas strains in the other two types A^f and A^r caused atypical symptoms on citrus leaves. Strain XW47 in type A^f which induced typical erumpent canker lesions with watersoaked margin on Mexican lime, but induced flat necrotic lesions with watersoaked margin on grapefruit, Liucheng, and lemon; Strains XW16 and XW121 in type A^r induced restricted and raised corky lesions with no watersoaked margin on leaves of all the four citrus species. Based on physiological, biochemical and genetic characterizations including NaCl tolerance, hydrolysis of gelatin, oxidation of carbon sources, polymerase chain reactions with primers specific to *X. axonopodis* pv. *citri* (*Xac*), rep-PCR and DNA sequence of leucine-responsive regulatory protein (*lrp*), strains in types A^f and A^r were identified as *Xac*. These two types of atypical symptoms-inducing *Xac* strains could be differentiated from two atypical *Xac* strains A* and A^w isolated from southwest Asia and Florida by *lrp* sequence assay and amplified DNA profiles of PCR with primer pairs pthAP7/pthAR2, 2/3, 4/7 or ERIC1R/ ERIC2. They appeared to be novel strains of *Xac*. We designated these two types of atypical symptoms-inducing strains as *Xac*-A^f type and *Xac*-A^r type, respectively.

Key words: citrus canker, leucine-responsive regulatory protein (*lrp*) gene, pectolytic enzymes, *pthA* gene, rep-PCR, *Xanthomonas axonopodis* pv. *citri*, *Xanthomonas campestris* pv. *citri*,

INTRODUCTION

Bacterial canker is one of the most destructive diseases of citrus. The disease is caused by *Xanthomonas campestris* pv. *citri (Xcc)* and leads to defoliation, and premature fruit drop of citrus plant [26, 29]. The strains of the bacterium have been grouped into pathotypes A, B, C, D and E based on host range, serology, phage typing and geographical distribution [3, 4, 11, 29]. Strains in pathotype A (Asiatic form) have a broad host range among members of the Rutaceae, and are widely distributed and endemic in Asia [3, 28]. Strains in pathotype B primarily affect lemon (*Citrus limon*), but also affect some other *Citrus* species. They are found only in Argentina, Uruguay, and Paraguay [3]. Strains in pathotype C infect only Mexican lime (*Citrus aurantifolia*) and have been isolated in Brazil [3, 27]. Strains in pathotype D cause lesions on leaves and twigs but not on fruit of Mexican lime, and they are mainly distributed in Mexico [30]; however, the disease is now believed to be caused by *Alternaria limicola* [23]. Strains in *Xcc* pathotypes A, B and C induce raised canker lesions on citrus plants. However, Strains in pathotype E have been found in Florida, induce flat necrotic lesions with watersoaked margins on citrus species [12, 28]. The disease caused by the pathotype E strain is known as citrus bacterial spot [28]. These pathotypes have been reclassified and named as *X. axonopodis* pv. citri (pathotype A), *X. axonopodis* pv. *aurantifolii* (pathotypes B and C), and *X. axonopodis* pv. *citrumelo* (pathotype E) [34]. The typical canker lesion caused by *X. axonopodis* pv. *citri* (*Xac*) is erumpent, with watersoaked, or oily tan or brown colored margin, often surrounded by a chlorotic halo. However, several distinct phenotypes of *Xac* have been reported [20, 31, 37]. Strains of *Xac* designated as type A* isolated from Mexican lime in southwest Asia elicit typical canker lesions on Mexican lime and induce flat watersoaked lesions or blister-like lesions on grapefruit and other citrus species [37]. Strains designated as type A^w from key /Mexican lime in Florida induce canker lesions on key lime, but flat necrotic lesions on grapefruit and other citrus species [31]. In contrast to strains in pathotype A, both of A* and A^w strains are primarily limited in host range to Mexican lime, whereas pathotype A has a wide host range. An atypical strain *Xac* RK has been discovered in southern Iran. It induces severe symptom on Mexican lime, however it is only weakly virulent on grapefruit and sweet orange [20].

The pathological variants of *Xcc* strain have been characterized or differentiated by physiological and biochemical assays [36, 38], serological approaches [1, 4], phage typing [37], protein profiles [35], fatty acid analysis [13, 35], DNA-DNA hybridization [35], restriction-fragment length polymorphism [13, 14], plasmid DNA fingerprints [24], and specific primer pairs [5, 31]. In addition, Southern hybridization with a *pth*A probe reveals distinct profiles among strains in pathotype A, pathotype B, pathotype C, and pathotype E [33]. And rep-PCR with BOX and ERIC primer pairs has been used to separate pathotypes of *Xcc* or to differentiate strains in the same pathotype. The rep-PCR technique also allows to evaluate the diversity of *Xac* in certain geographic areas of the world [5]. Moreover, the differences in sequence of the leucine-responsive regulatory protein (*lrp*) gene generated by *J-lrp3* or *J-lrp6b* and *J-lrp5* primers are useful to distinguish the wild-host-range strains of *Xac* pathotype A, the narrow-host-range strains of *Xac* type A^w and A*, and pathotype B, C and E strains into different subgroups [6]. In Taiwan, bacterial canker of citrus was first reported by Okabe in 1932 [21]. It is commonly observed on grapefruit, sweet orange and lemon [43]. Physiological and biochemical characteristics of citrus canker bacteria have been well studied by Wu *et al.* [41]. However, the variation of pathogenicity among strains of citrus canker bacteria has not been thoroughly studied yet [40]. In this study, we examined the pathogenicity of randomly selected 46 strains of *Xac* from Taiwan on leaves of Mexican lime, grapefruit, Liucheng, and lemon. We found two types of atypical symptoms-inducing strains and further characterized these strains based on their physiological, biochemical and genetic characteristics. The results revealed that these atypical symptom-inducing strains are novel strains of *Xac*.

MATERIALS AND METHODS

Bacterial strains and culture condition

The bacterial strains used in this study are listed (Table 1). All strains were stored in a YPD broth (yeast extract 7 g/L, bactopeptone 7 g/L, dextrose 7 g/L, pH 7.2) [36] containing 20 % glycerol at -80℃. When required, each bacterial strain was cultured on YPDA plates at 30℃ for 3 days.

Pathogenicity test

Citrus plants (*Citrus paradisi*, grapefruit; *C. aurantifolia*, Mexican lime; *C. sinensis*, Liucheng; *C. limon*, lemon) grown in pots under greenhouse condition were used for examination of pathogenicity of *X. axonopodis* pv. *citri* strains. Citrus plants inoculated with various *Xanthomonas* strains were moved into a growth chamber in which the relative humidity was between 65-90% and temperature was at 30℃ during the light period (12 hr) and 25℃ in the dark period (12 hr).

Bacterial cells grown overnight in YPD broth were

Table 1. Bacterial strains used in this study

Taxon	Strains	Host	Origin	Source or reference
X. axonopodis pv. *citri*	XW16	*C. sinensis* cv. Valencia	Taiwan	(41)
	XW19	*Poncirus trifoliate* × *C. sinensis*	Taiwan	(41)
	XW23	*C. sinensis* cv. Liucheng	Taiwan	(41)
	XW26	*C. reshni*	Taiwan	(41)
	XW31	*C. sinensis* cv. Liucheng	Taiwan	(41)
	XW32	*C. sinensis* cv. Liucheng	Taiwan	(41)
	XW33	*C. sinensis* cv. Liucheng	Taiwan	(41)
	XW37	*C. sinensis cv*. Malta egg-orange	Taiwan	(41)
	XW38	*C. sinensis cv*. Pineapple	Taiwan	(41)
	XW45	*C. sinensis cv*. Valencia	Taiwan	(41)
	XW46	*C. aurantifolia*	Taiwan	(41)
	XW47	*C. sinensis* cv. Valencia	Taiwan	(41)
	XW55	*C. sinensis* cv. Liucheng	Taiwan	(41)
	XW77	*C. sinensis* cv. Liucheng	Taiwan	(43)
	XW78	*C. sinensis* cv. Liucheng	Taiwan	(43)
	XW84	*C. paradisi*	Taiwan	(41)
	XW85	*C. paradisi*	Taiwan	(43)
	XW86	*C. tankan*	Taiwan	(41)
	XW87	*C. tankan*	Taiwan	(41)
	XW88	*C. sinensis* cv. Liucheng	Taiwan	(43)
	XW89	*C. sinensis* cv. Liucheng	Taiwan	(41)
	XW90	*C. tankan*	Taiwan	(41)
	XW92	*C. grandis* cv. Sour orange	Taiwan	(41)
	XW95	*C. sinensis* cv. Liucheng	Taiwan	(41)
	XW96	*C. lemon*	Taiwan	(41)
	XW97	*C. paradisi*	Taiwan	(41)
	XW100	*C. sinensis* cv. Liucheng	Taiwan	(41)
	XW105	*C. limonia*	Taiwan	(41)
	XW108	*C. grandis* cv. Sour orange	Taiwan	(41)
	XW121	*C. sinensis* cv. Liucheng	Taiwan	(41)
	XL1	*C. sinensis* cv. Liucheng	Taiwan	This study
	XL2	*C. sinensis* cv. Liucheng	Taiwan	This study
	XL3	*C. paradisi*	Taiwan	This study
	XL4	*C. lemon*	Taiwan	This study
	XL5	*C. paradisi*	Taiwan	This study
	XL6	*C. sinensis* cv. Liucheng	Taiwan	This study
	XL7	*C. paradisi*	Taiwan	This study
	XL8	*C. paradisi*	Taiwan	This study
	XL9	*C. sinensis* cv. Liucheng	Taiwan	This study
	XL10	*C. lemon*	Taiwan	This study
	XC77	*C. paradisi*	Taiwan	This study
	XC78	*C. grandis*	Taiwan	This study
	XC84	*C. sinensis* cv. Liucheng	Taiwan	This study
	XC85	*C. sinensis* cv. Liucheng	Taiwan	This study
	XC86	*C. sinensis* cv. Liucheng	Taiwan	This study
	XC87	*C. sinensis* cv. Liucheng	Taiwan	This study
	2863		New Zealand	(42)
	2865		Brazil	(42)
X. axonopodis pv. *citri* (A*)	XC205		Florida	(5)
	XC322		USA	
X. axonopodis pv. aurantifolii (C)	XC70		Brazil	(42)
X. axonopodis pv. citrumelo (E)	F2		Florida USA	(42)
X. axonopodis pv. vesicatoria	XVT40	*Lycopersicon esculentum*	Taiwan	This study
X. axonopodis pv. dieffenbachiae	H2	*Anthrium andraeanum*	Taiwan	This study
X. campestris pv. campestris	XCC70	*Brassica oleracea*	Taiwan	This study

harvested by centrifugation and resuspended in sterile distilled water to a concentration of approximately 10^8 colony-forming units (CFU)/ml. Prior to inoculation, six wounds per 1-cm^2 on new fully expanded citrus leaves were made with a standard 26-gauge needle. An aliquot (20 μl) of bacterial suspension was dropped onto each of six wounds on leaves, and the inoculum drops were wiped off with sterile cotton just after inoculation. Symptoms were examined visually or with dissection microscope.

Physiological and biochemical characterization

Physiological and biochemical characteristics used to differentiate pathovars of *X. axonopodis* associated with citrus plants were performed to characterize the *X. axonopodis* pv. *citri* strains isolated in Taiwan. Salt tolerance was tested by growing bacteria on YPDA plates containing 3 % NaCl [37]. Hydrolysis of gelatin was performed as described in Lelliott *et al.* [18]. Oxidation of carbon sources was carried out on Biolog® GN microplates (Biolog Inc., Hayward, CA) as described by Verniere *et al.* [38]. Pectolytic activity was tested on CVP medium [7] and on Hildebrand's medium with pH values at 5.0, 7.0 and 8.5 [16].

Total DNA extraction

Total DNAs of xanthomonad strains were extracted using the method described by Sambrook *et al.* [25]. PCR was performed with a thermocycler (GeneAmp PCR System 2400, Perkin-Elmer, CT)

Identification of *Xanthomonas* strains with specific primer pairs

Primer pairs 2/3 and 4/7 designed from sequence of plasmid of the *X. axonopodis* pv. *citri* [15], and primer pairs *J-pth1/J-pth2, J-RXg/J-RXc2* and XCF/XCR designed based on sequences of nuclear localization signal in *pthA* [5], rDNA [5] and 16S-23S spacer gene of *X. axonopodis* pv. *citri* [19], respectively, were used to identify or differentiate the *Xac* strains tested in this study.

REP- and ERIC-PCR analyses

The genetic diversity among the *Xac* strains was analyzed by REP- and ERIC-PCR. The REP-PCR was carried out as described by Versalovic *et al.* [39] with some modifications. A 25 μl reaction mixture contained 150 ng template DNA, 1 ×*Taq* buffer, 3 mM $MgCl_2$, 1.25 μM each of primers REP1-1 and REP2-1, 250 μM each of deoxynucleoside triphosphates and 0.8 U Taq polymerase (DyNAzyme™ II DNA Polymerase, Finnzymes Oy Inc. Finland). The amplification condition consisted of 94℃ for 1 min, 44℃ for 1 min, and 65℃ for 8 min for 30 cycles plus an initial step of 95℃ for 7 min and a final step of 65℃ for 15 min. The ERIC-PCR was carried out with primer pair ERIC1R/ERIC2 in a 25 μl reaction mixtures in which the other ingredients were the same as that for REP-PCR; the amplification condition consisted of 94℃ for 1 min, 52℃ for 1 min, and 65℃ for 8 min for 30 cycles plus an initial step of 95℃ for 7 min and a final step of 65℃ for 15 min. DNA products of the REP- and ERIC-PCR were analyzed by 2% agarose gel electrophoresis in 0.5 ×TAE buffer at 90V for 4 hours and were stained with ethidium bromide. REP- and ERIC-PCR fingerprint profiles were converted to binary form (0 = absence ; 1 = presence), and Dice coefficient was calculated to determine the similarity among the bacterial strains tested [9].

Sequence analysis of *lrp* gene

Sequence analysis of *lrp* gene of *Xac* strains was performed as described by Cubero and Graham [6]. Primer pair ERIC1R/ERIC2 was used to amplify a 375 bp product which is part of the *lrp* gene of *Xac* and primer pair *J-lrp3/J-lrp5* was used to amplify a DNA fragment about 600 bp including the full length of the *lrp* gene of *Xac*. The amplified 600 bp fragment from each of the tested strains was further purified by Viogene Gel-M™ Gel Extraction system (Viogene Corporation, Taiwan) and cloned into pCR2.1-TOPO cloning vector (Invitrogen, CA) for sequencing. *lrp* sequence of each bacterial strain was compared with those available in the GenBank database at the National Center for Biotechnology Information (NCBI) network service (http://www.ncbi.nlm.nih.gov) for *Xanthomonas* strains causing citrus bacterial canker. Distance matrices for *lrp* sequences of xanthomonads were calculated from alignments with GENDIST in PHYLIP software package version 3.6a [17], and phylogenetic relationships were established by the neighbor-joining method. The repeatability of the branching points obtained was estimated using the SEQBOOT program for bootstrap resampling (1000 bootstrap reiterations) of the multiple sequence alignment. The NEIGHBOR unrooted tree was generated with the program TREEVIEW [22].

Pectolytic genes detection

Primers used to detect pectolytic genes in *Xanthomonas* species are listed (Table 4). Primers pehF and pehR were designed based on sequence of the *peh-1* gene (accession number NC 003919) encoding for an endopolygalacturonase; primers pglF and pglR were designed based on sequence of the *pglA* gene (accession number NC 003919) encoding for a polygalacturonase; primers pel1F and pel1R were designed based on sequence of one *pel* gene (accession number NC 003919) encoding

for a pectate lyase; primers pel2F and pel2R were designed based on sequence of a second *pel* gene (accession number NC 003919) encoding for a degenerated pectate lyase; primers pel3F and pel3R were designed based on sequence of the *pelB* gene (accession number NC 003919) encoding for a pectate lyase II[8]. Polymerase chain reaction for pectolytic genes was performed in a 50-μl mixtures containing 150 ng template DNA, 1× Taq buffer, each primer at a concentration of 1 μM, each deoxynucleoside triphosphates at a concentration of 300 μM, 1.5 U of Taq plus DNA polymerase (BioBasic Inc. Canada) with proof-reading function, and dimethyl sulfoxide (DMSO) 2.5 μl. The amplification condition consisted of 95℃ for 70 sec, 65℃ for 1min, and 72℃ for 2 min for 30 cycles plus an initial step of 95℃ for 5 min and a final step of 72℃ for 10 min. The PCR products were visualized under UV light after electrophoresis in 1% agarose gels and stained with ethidium bromide.

Pathogenicity (*pthA*) gene analysis

Primer pair pthAP7/pthAR2 was designed based on *pthA* gene cluster sequence (accession number U28802) in GenBank by using PC/GENE software version 6.85 (Intelli Genetics, Inc., CA). Polymerase chain reaction for *pthA* gene was performed in a 50-μl mixtures containing 150 ng template DNA, 1× Taq buffer, 1 μM primer pthAP7, 1 μM primer pthAR2, each deoxynucleoside triphosphate at a concentration of 300 μM, 1U of Taq plus DNA polymerase, and 5 μl DMSO. The amplification condition consisted of 94℃ for 1 min, 63℃ for 1 min, and 72℃ for 5 min for 35 cycles plus an initial step of 94℃ for 10 min and a final step of 72℃ for 10 min. For Southern blot analysis, the PCR products were separated by electrophoresis in 1% agarose gel, transferred to nylon membrane (Zeta-Probe® Blotting Membranes, Bio-Rad Laboratories, CA), and hybridized with a biotin-labeled pthAP7, a PCR product amplified from total DNA of XW19 strain with primer pair pthAP7/pthAR2.

RESULTS

Pathogenicity test

Based on the symptoms induced on leaves of the four *Citrus* species plants, the 46 strains of *Xac* tested were grouped into A, A^f and A^r three types (Table 2). Among them, there were 43 (93.5%) strains in the A type which induced typical erumpent canker lesions on leaves of the four *Citrus* species tested. Strains of the other two types induced atypical-symptoms on the citrus leaves. In the A^f type, strain XW47 induced typical erumpent canker lesions with watersoaked margins, and surrounded with light chlorotic haloes as that induced by strain XW19, a representative strain of type A, on leaves of Mexican lime (Table 2, Fig. 1A and 1B). However, XW47 induced flat necrotic lesions with watersoaked margins and light chlorotic haloes on the leaves of grapefruit (Fig. 1E), Liucheng and lemon (Table 2). The size of lesions induced by XW47 on leaves of four citrus species was not significantly different from that induced by XW19. The lesion size was in a range from 1.8 to 2.5 mm in diameter 24 days after inoculation and the lesions could continue to expand until 40-50 days later. In the A^r type, strains XW16 and XW121 induced restricted and raised corky lesions with no watersoaked margins and light chlorotic haloes on leaves of the four *Citrus* species (Table 2, Fig. 1C and 1F). The size of lesions induced by XW121 and XW16 were remarkably smaller than those induced by XW19 or XW47. The lesion size induced by A^r type strains was in a range of 1.0 to 1.6 mm in diameter 24 days after inoculation. The lesions induced by XW16 or XW121 did not expand further.

Physiological and biochemical characterization

Type A^f strain XW47 and type A^r strains XW16 and XW121 were able to grow in the presence of 3% NaCl, possessed hydrolysis ability of gelatin and were positive in utilization of L-fucose, D-galactose and alaninamide in the Biolog® GN plate. However, type A strain XW19 and type A^f strain XW47 could utilize N-acetyl-D-glucosamine, acetic acid, malonic acid and propionic acid but type A^r strains XW16 and XW121 did not or weakly utilize these four carbon sources (Table 3). Strains XW19, XW47, XC2863 and XC2865 were positive for pectolytic activity

Table 2. Symptoms induced by 46 strains of *X. axonopodis* pv. *citri* strains on four *Citrus* species

Type	No. Strains	*C. aurantifolii* (Mexican lime)	*C. paradisi* (Grapefruit)	*C. sinensis* (Liucheng)	*C. limon* (Lemon)
A	43	canker[1]	canker	canker	canker
A^f	1 (XW47)	canker	FW	FW	FW
A^r	2 (XW16, XW121)	RC	RC	RC	RC

[1] Canker: Typical erumpent canker lesion with watersoaked margin and light chlorotic halo; FW: Flat necrotic lesion with watersoaked margin and light chlorotic halo; RC: Restricted, raised corky lesion without watersoaked margin and light chlorotic halo

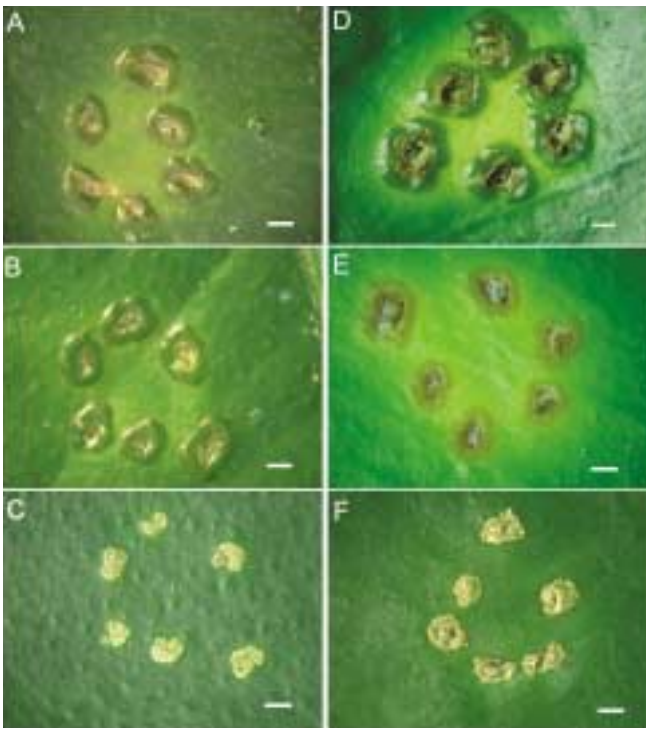

Fig. 1. Symptoms induced on leaves of Mexican lime (A, B, C) and grapefruit (D, E, F) by strains of *X. axonopodis* pv. *citri* 24 days after inoculation. A and D: Typical erumpent canker lesions with watersoaked margin and light chlorotic halo induced by type A strain XW19; B: Typical erumpent canker lesions with watersoaked margin and light chlorotic halo induced by type A^f strain XW47; E: Flat necrosis lesions with watersoaked margin and light chlorotic halo induced by type A^f strain XW47; C and F: Restricted and raised corky lesions with no watersoaked margin and light chlorotic halo induced by type A^r strain XW121. (Bars = 1 mm)

on CVP medium and Hildebrand's medium at pH 7.0 and pH 8.5 (Table 3), whereas strains XW16 and XW121 did not have any pectolytic activity on the CVP medium and Hildebrand's medium at various pH values (Table 3).

Identification of *Xanthomonas* strains with specific primer pairs

Xac-specific primers 2/3, 4/7, *J-pth1/J-pth2*, *J-RXg/J-RXc2*, and XCF/XCR were used to identify strains of XW16, XW19, XW47 and XW121. Specific PCR products can be detected from all four strains as well as reference strains of *Xac* 2863 and 2865. No specific amplified product was detected from DNAs of *X. axonopodis* pv. *citrumelo* F2 with these primer pairs, but specific amplified products could be detected from DNAs of *X. axonopodis* pv. *aurantifolii* XC70 with primer pairs *J-pth1/J-pth2* and XCF/XCR, respectively (Table 3).

REP- and ERIC-PCR analyses

The similarity coefficient of each atypical symptoms-inducing strain was 0.9-1.0 to *Xac* reference strain 2863 based on REP- and ERIC-PCR analyses. These strains could be clearly differentiated from *X. axonopodis* pv. *aurantifolii* C pathotype strain XC70 and *X. axonopodis* pv. *citrumelo* E pathotype strain F2 by REP and ERIC-PCR (Table 3).

Leucine-responsive regulatory protein (*lrp*) gene sequence analysis

ERIC-PCR analysis showed the same fingerprints among the strains of *X. axonopodis* pv. *citri* including types A, A^f and A^r strains and two *Xac* reference strains 2863 and 2865. All of these strains possessed a 375-bp DNA fragment containing a partial *lrp* gene (Fig. 2).

Leucine-responsive regulatory protein (*lrp*) gene from each of strains XW19, XW47, XW16 and XW121 was cloned and sequenced. A dendrogram based on pairwise comparison of *lrp* sequences of *Xac* strains available in GenBank showed that strains of XW19, XW47, XW16 and XW121 were grouped into the same cluster with typical *Xac* A pathotype strains. Whereas, *Xac*- A* or A^w strains were grouped into another cluster (Fig. 3).

Pectolytic genes detection

Five pectolytic genes were detected in DNAs from *Xac* strains XW19, XW47, XW16, XW121, 2863, 2865 and *Xac*-A*strains XC205 and XC322 using primer pairs pehF/pehR, pglF/pglR, pel1F/pel1R, pel2F/pel2R and pel3F/pel3R, respectively. The PCR products of 1.4, 1.7, 1.2, 1.3 and 1.1 kb specific for each pectolytic gene were amplified, respectively. In addition, *pglA* gene was also detected in DNAs from *X. axonopodis* pv. *aurantifolii* (strain XC70) and *X. axonopodis* pv. *vesicatoria* (strain XVT40) by PCR with primer pair pglF/pglR. Whereas, utilization of primer pair pel3F/pel3R could amplify 1.1 kb PCR product specific for *pelB* gene from DNAs of *X. axonopodis* pv. *vesicatoria* (strain XVT40) and *X. campestris* pv. *campestris* (strain XCC70) (Table 5).

Pathogenicity (*pthA*) gene analysis

A 3.8kb DNA product containing a full length of *pthA* gene of *Xac* strain was amplified with primer pair pthAP7/pthAR2 from each of *Xac* strains XW19, XW47, XW16, XW121, 2863 and 2865 and *Xac*-A* strains XC205 and XC322 but not from *X. axonopodis* pv. *aurantifolii* pathotype C strain XC70 and *X. axonopodis* pv. *citrumelo* pathotype E strain F2. The amplified profiles showed that three additional DNA products of 0.84kb,

Table 3. Physiological, biochemical and genetic characteristics of *X. axonopodis* pv. *citri* type A^f strain XW47, and type A^r strains XW16 and XW121

Item	XW47	XW16	XW121	XW19[5]	2863	2865	XC70	F2
Growth on NaCl (3%)	+[6]	+	+	+	+	+	—	+
Hydrolysis of gelatin	+	+	+	+	+	+	—	+
Oxidation of carbon sources[1]								
D-Galactose	+	+	+	+	+	+	+	+
Alaninamide	+	+	+	+	+	+	—	+
L-Fucose	+	+	+	+	+	+	+	—
N-Acetyl-D-glucosamine	+	(±)	(±)	+	+	+	+	—
Acetic acid	+	(±)	(±)	+	+	+	—	+
Malonic acid	+	(±)	(±)	+	+	+	+	+
Propionic acid	+	(±)	(±)	+	+	+	+	+
Pectolytic activity on								
CVP medium[2]	+	—	—	+	+	+	—	—
Hildebrand's medium (pH5.0)	—	—	—	—	—	—	—	—
Hildebrand's medium (pH7.0)	+	—	—	+	+	+	—	—
Hildebrand's medium (pH8.5)	+	—	—	+	+	+	—	—
PCR with primers[3]								
2/3	+	+	+	+	+	+	—	—
4/7	+	+	+	+	+	+	—	—
J-pth1/J-pth2	+	+	+	+	+	+	+	—
J-RXg/J-RXc2	+	+	+	+	+	+	—	NT[7]
XCF/XCR	+	+	+	+	+	+	+	—
rep-PCR (similarity coefficient)[4]								
primers REP1-1/REP2-1	1	0.9	1	1	1	1	0.59	0.67
primers ERIC1R/ERIC2	1	1	1	1	1	1	0.54	0.3

[1] Oxidation of carbon sources was done in the Biolog GN plates as described by Verniere *et al.*[38].

[2] Pectolytic activity was tested on CVP medium[7] and Hildebrand's medium[16].

[3] The primer pairs 2/3 and 4/7 are specific for DNA fragments for in the *X. axonopodis* pv. *citri* plasmid[15]; The primer pair *J-pth1/J-pth2* is specific for the nuclear localization signal in *pthA* of pv. *X. axonopodis* pv *citri*[5]; The primer pair *J-RXg/J-RXc2* is specific for rDNA of *X. axonopodis* pv. *citri*[5]; The primer pair XCF/XCR is specific for 16S-23S spacer gene of *X. axonopodis* pv. *citri*[19].

[4] Similarity coefficient was calculated with the Dice coefficient (S=2a/2a+b+c)[9]; strain 2863 was used as a reference strain.

[5] XW19 is a representative strain of type A designated in this study; strains 2863 and 2865 were reference strains for *X. axonopodis* pv. *citri* (*X. campestris* pv. *citri* pathotype A); Strains XC70 and F2 were reference strains for *X. axonopodis* pv. *aurantifolii* (*X. campestris* pv. *citri* pathotype C) and *X. axonopodis* pv. citrumelo (*X. campestris* pv. *citri* pathotype E), respectively.

[6] +, positive reaction; —, negative reaction; (±), weakly positive or negative reaction.

[7] NT, not tested.

0.37kb and 0.21kb were amplified from DNAs of two *Xac*-A* strains XC205 and XC302. The *pthA* homologs were further confirmed by Southern hybridization with the pthAP7 probe (Fig. 4).

DISCUSSION

According to symptoms induced by *Xac* on leaves of four *Citrus* species, we found two types of atypical symptoms-inducing strains existed in Taiwan. Type A^f strain XW47 could induce typical canker lesions on leaves of Mexican lime, but it induced flat necrotic lesions on grapefruit, Liucheng and lemon. Symptoms induced by A^f strain XW47 on leaves of *Citrus* species were similar to those induced by *Xac*-A* type 2 strains[37] and *Xac*-A^w strains[31] from southwest Asia and Florida, respectively. Type A^r strains XW16 and XW121 could induce restricted and raised corky lesions with no watersoaked margin on leaves of all four *Citrus* species including Mexican lime, grapefruit, Liucheng and lemon. To our knowledge, there is no any other citrus canker bacterial strain which is pathologically similar to strains in type A^r.

In physiological and biochemical tests, the atypical symptoms-inducing strains of types A^f and A^r could grow on 3% NaCl, hydrolyze gelatin and utilize L-fucose, D-galactose and alaninamide. These strains shared typical *Xac* group profiles of assimilation of these three carbon sources as reported by Verniere *et al.*[38]. The study also showed that strain XC70, a reference strain of *X.*

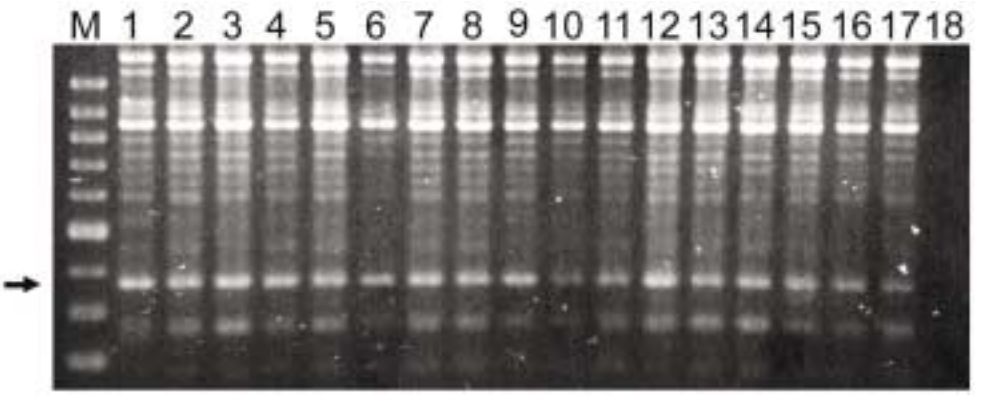

Fig. 2. Genomic fingerprints of *X. axonopodis* pv. *citri* strains generated from ERIC-PCR. Lanes 1-17: DNA templates from XW16, XW19, XW23, XW37, XW47, XW78, XW84, XW97, XW100, XW105, XW121, XL1, XL2, XL3, XT77, 2863 and 2865, respectively; lane 18: Negative control; lane M: Bio 100 DNA ladder (PROtech Technology, Taiwan). The arrow indicates a 375 bp product identified as part of the leucine-responsive regulatory protein (*lrp*) gene.

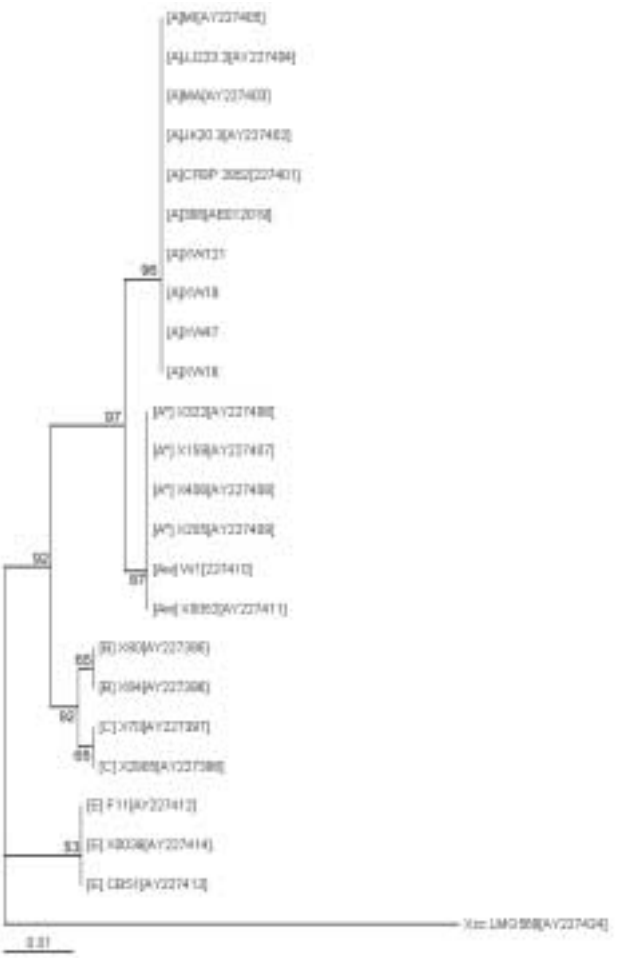

Fig. 3. The phylogenetic relationship of the *lrp* sequences of the xanthomonads. The tree was made with the strains of *X. axonopodis* pv. *citri* (A, A* and A^w), *X. axonopodis* pv. *aurantifolii* (B and C), *X. axonopodis* pv. *citrumelo* (E), and *X. campestris* pv. *campestris (Xcc)*. Bootstrap values (1000 replicates) are indicated at branch-points. Pathotype of strain, strain number and GenBank accession number are indicated.

axonopodis pv. *aurantifollii* (C pathotype), could use L-fucose and D-galactose but not alaninamide, the results confirmed the carbon source utilization of *X. axonopodis* pv. *aurantifollii* (C pathotype) reported by Verniere *et al.*[38] (Table 3). In addition, we found type A^r strains XW16 and XW121 did not or weakly use N-acetyl-D-glucosamine, acetic acid, malonic acid and propionic acid. Utilization of these four carbon sources might be useful to identify or differentiate type A^r strains from strains in other pathotypes.

Similarity coefficients obtained from REP- and ERIC-PCR analyses grouped type A^f strain XW47, type A^r strains XW16 and XW121, and type A strain XW19 with the *Xac* reference strain 2863 into the same group. These strains were clearly differentiated from *X. axonopodis* pv. *aurantifolii* C pathotype strain XC70 and *X. axonopodis* pv. *citrumelo* E pathotype strain F2 (Table 3). ERIC-PCR analysis performed by Cubero and Graham[6] indicates that the primary difference between strains in *Xac* type A and strains in type A* and A^w is the absence of a 375 bp DNA fragment in A* and A^w strains. Our results of ERIC-PCR analysis showed that A^f and A^r possessed a 375 bp DNA fragment. Thus strain A^f and A^r could be differentiated from strains A* and A^w by ERIC-PCR analysis. Specific primers have been designed to identify or differentiate of *Xanthomonas* spp. associated with citrus plant[5,15,19]. In this study, strains in A^f and A^r could be identified as *Xac* with primer pairs 2/3, 4/7, *J-pth1/J-pth2*, *J-RXg/J-RXc2* and XCF/XCR. However, strains of A^w can not be detected with primer pairs 2/3[5] and 4/7[31]. Thus, A^f and A^r strains could be differentiated from A^w strains by these two primer pairs. In addition, a distinct DNA profile could be amplified from A* strains with primer pair pthAP7/pthAR2. Thus, the primer pair pthAP7/pthAR2 designed in this study could also be used to differentiate *Xac*-A* strains from types A, A^f or A^r strains.

Table 4. Primer pairs designed in this study

Primer	Sequence (5'-3')
pthAP7	5'-GTTGTGTACTGCCATGCGGCCTCGGA-3'
pthAR2	5'-GATCTGGTGACGTTGCAGTCGCCATC-3'
pehF	5'-CTCTCATATGAAACTGCTTACCGCTG-3'
pehR	5'-CCAGCGAGCTCACAGCGCGGGGAAG-3'
pglF	5'-GAAAACTCGAGTAACCGGAGAAAGACCTATGC-3'
pglR	5'-GAAAATCTAGAGATTCAAATCGGCGAATCCG-3'
pel1F	5'-GAAAACTCGAGGAACCCATTCGAGGGAGAGATC-3'
pel1R	5'-GAAAATCTAGAGCCAGCGGCAGTTACAGCTTGC-3'
pel2F	5'-GAAAAGAATTCGTTACTGCCTGCTATGGGGAGCGTG-3'
pel2R	5'-GAAAATCTAGACGTGTTTAGTGCGGCCCCGCAGAG-3'
pel3F	5'-GAAAACTCGAGGACTTCTTTGGGAGAGGCTACATGA-3'
pel3R	5'-GAAAATCTAGAGCGCGCGCCCGGTCACTCCGCCAGG-3'

Table 5. Specific DNA fragments amplified with each of primer pairs pehF/pehR, pglF/pglR, pel1F/pel1R, pel2F/pel2R and pel3F/pel3R for pectolytic genes in strains of *Xanthomonas* spp.

Taxon	Strain	pehF/ R[1]	pglF/ R	pel1F/ 1R	pel2F/ 2R	pel3F/ 3R
X. axonopodis pv. *citri* (A)	XW19, XW47, XW16, XW121,2863, 2865	+[2]	+	+	+	+
X. axonopodis pv. *citri* (A*)	XC205, XC322	+	+	+	+	+
X. axonopodis pv. *aurantifolii* (C)	XC70	−	+	−	−	−
X. axonopodis pv. *citrumelo* (E)	F2	−	−	−	−	−
X. axonopodis pv. *vesicatoria*	XVT40	−	+	−	−	+
X. axonopodis pv. *dieffenbachiae*	H2	−	−	−	−	−
X. campestris pv. *campestris*	XCC70	−	−	−	−	+

[1] Primer pair for the pectolytic genes *peh-1*, *pglA*, *pel*, *pel* and *pelB*, respectively.
[2] +, specific DNA fragment amplified; −, no specific DNA fragment amplified.

Fig. 4. Amplification profiles of DNAs from *X. axonopodis* strains by polymerase chain reaction with primer pair pthAP7/pthAR2 (A). Lanes 1-6: *X. axonopodis* pv. *citri* strains X16, XW19, XW47, XW121, 2863 and 2865, respectively; lanes 7 and 8: *X. axonopodis* pv. *citri* A*strains XC205 and XC322; lane 9: *X. axonopodis* pv. *aurantifolii* XC70; lane 10: *X. axonopodis* pv. *citrumelo* F2; lane 11: Negative control; no template DNA added; lane M: Gen-KB DNA ladder (Genemark Technology, Taiwan). (B) Southern blot of the gel of (A) probed with biotin-labeled *pth*A. The predicted 3.8 kb DNA fragment containg an entire length of *pth*A gene is marked with an arrow in the margin.

Sequences of *lrp* gene have been used to characterize the relationship among strains in different pathovars of *Xanthomonas axonopodis* and other *Xanthomonas species*[6]. In this study, *lrp* gene of citrus canker bacterial strains was cloned and sequenced. Based on the *lrp* gene sequences, atypical symptoms-inducing strain XW47 in type A^f and strains XW16 and XW121 in type A^r were grouped into the same cluster with typical symptoms-inducing strain XW19 in type A and other *Xac* strains; whereas strains A* and A^w were grouped into another group. Thus, analysis of *lrp* gene sequence can be used to differentiate strains of types A^f and A^r existed in Taiwan from strains of A^w and A*.

Pectate lyase (Pel) and polygalacturonase (PG) play pathogenicity or virulence roles in both soft rot or nonsoft rot bacteria[2,10]. In this study, five pectolytic genes including three pectate lyase isozymes and two polygalacturonases were detected from DNAs of all the tested strains including strains in A, A^f and A^r types by PCR with specific primer pairs for each pectolytic genes. However, pectolytic activity was detected only from A and A^f strains on CVP medium and Hildebrand's medium but not from A^r strains XW16 and XW121 which induced restricted and raised corky lesions with no watersoaked margin on leaves of all the four *Citrus* species tested. It is likely that A^r strain might be defective in pectolytic

activity factors required for inducing typical symptoms. We have successfully cloned the genes of pectolytic enzyme by PCR with specific primer pairs from A type strain XW19. Transformation of the plasmid vector containing a pectate lyase gene from XW19 into cells of A^r strain XW121, the results show that pectolytic enzyme is involved in the formation of watersoaked margin around the lesions induced by *Xac* strain (data unpublished).

Although genome of *Xac* has been sequenced [8], it is still unclear how the gene product interacts with plants during the infection processes. *pthA* gene is a pathogenicity gene necessary for *Xac* to induce typical erumpent canker lesion. Transformation of *pthA* into cells of other xanthomonads confers the ability to induce canker-like lesion on citrus leaves to these species [32]. A 3.8 kb DNA fragment containing entire *pthA* gene was amplified from total DNA of type A strain XW19 with primer pair pthAP7/pthAR2. Transformation of the plasmid vector containing this 3.8 kb DNA fragment into cells of A^f strain XW47 could render it to induce typical canker lesion on grapefruit leaves. In addition, a *pthA*-homolog has been cloned from A^f strain XW47. Nucleotide sequence analyses showed that there were some point mutations in *pthA* gene of XW47 as compared to that of type A strain XW19. We have evidences showing that substitution of a single amino acid in PthA would alter symptoms formation on citrus leaves (data unpublished).

In the pathogenicity tests, three types of citrus canker bacterial strains were identified in Taiwan. Most (93.5%) of the tested strains are typical citrus canker bacteria (*Xac*-A type). We found new strains in types A^f and A^r which induce atypical symptoms on leaves of *Citrus* species tested. Based on physiological, biochemical and genetic analyses, strains in type A^f and A^r were characterized as *Xac*. And these two types of atypical symptoms-inducing strains could be differentiated from two atypical *Xac* strains A* and A^w existed in southwest Asia and Florida, respectively, by *lrp* gene sequence and PCR with specific primer pairs. Studies on interaction between A^f and A^r strains with citrus plants have been undertaken. The ecological roles of A^f and A^r strains are worthy for further studies.

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摘 要

林信成[1]、徐世典[1]、黃阿賢、曾國欽[1,3]. 2005. 台灣引起非典型病徵之柑橘潰瘍病菌 *Xanthomonas axonopodis* pv. *citri* 新特殊菌株之表現型及基因特性分析. 植病會刊 14:227-238. ([1] 台中市國立中興大學植物病理學系；[2] 嘉義市行政院農業委員會農業試驗所嘉義分所；[3] 聯絡作者，電子郵件：kctzeng@nchu.edu.tw；傳眞：+886-4-22854633)

將台灣分離之46 株柑橘潰瘍病菌 (*Xanthomonas campestris* pv. *citri*) 菌株，在墨西哥萊姆 (*Citrus aurantifolia*)、葡萄柚 (*C. paradisi*)、柳橙 (*C. sinensis*) 及檸檬 (*C. limon*) 等四種不同品種之柑橘植株葉片上，分別測試其病原性。依其在四種柑橘植株葉片上所呈現之病徵，可將其分成A、A^f 及 A^r 等三種類型之菌株，其中有43 (93.5%) 株菌株屬於 A 型菌株，可在測試之四種柑橘植株葉片上，引起裂開突起並具有水浸狀邊緣之典型潰瘍病斑。而其他菌株則分別屬於引起非典型病徵之 A^f 及 A^r 型菌株；其中 A^f 類型菌株 XW47，雖然在墨西哥萊姆上可引起裂開突起且具水浸狀邊緣之典型潰瘍病斑，然而在葡萄柚、柳橙與檸檬葉片上，則引起具水浸狀邊緣之扁平壞疽病斑；而 A^r 類型菌株 XW121 及 XW16，在測試之四種柑橘葉片上，則會引起不具水浸狀邊緣之木栓化突起小病斑。依據耐鹽性、明膠水解作用、碳素源利用、應用柑橘潰瘍病菌專一性引子對之聚合酵素連鎖反應、重複性序列聚合酵素連鎖反應 及 *lrp* 基因序列分析等生理、生化及基因特性分析，顯示 A^f 及 A^r 兩類型菌株係屬於 *Xanthomonas axonopodis* pv. *citri*。利用 *lrp*基因序列分析及 pthAP7/pthAR2、2/3、4/7 或 ERIC1R/ERIC2 等引子對之聚合酵素連鎖反應的DNA 圖譜，可將 A^f 及 A^r 菌株與西南亞及美國佛羅里達州所分離到，可引起非典型病徵之 A*與 A^w 菌株區分開。因此由台灣分離到可引起非典型病徵之 A^f 與 A^r 菌株，爲 *X. axonopodis* pv. *citri* 之新特殊菌株，並將其分別命名爲 *Xac*-A^f 及 *Xac*-A^r。

關鍵詞：柑橘潰瘍病、*lrp* 基因、果膠分解酵素、*pthA* 基因、重複性序列聚合酵素連鎖反應、柑橘潰瘍病菌