Plant Pathol. J. 17(6) : 336-341 (2001)

The Plant Pathology Journal

Phage Typing and Lysotype Distribution of *Xanthomonas axonopodis* pv. *citri*, the Causal Agent of Citrus Bacterial Canker in Korea

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(Received on November 16, 2001)

The distribution of citrusphages and phage types of *Xanthomonas axonopodis* pv. *citri* was investigated in Korea. Forty-eight strains of the bacterial pathogen and 28 bacteriophage strains were isolated from citrus leaves showing the citrus canker symptom. Only a single bacteriophage group, named CPK, was identified based on their aggressiveness to the bacterial pathogen. The bacterial strains were differentiated into two lysotypes based on their sensitivity to CPK. Lysotype I, which was sensitive to CPK, was more predominant (96%), while only 4% belonged to lysotype II, which was resistant to CPK. Among the 13 xanthomonads including lysotype A and lysotype B of *X. axonopodis* pv. *citri*, CPKs were only aggressive to BC 83 (=Xc 62) strain of *X. axonopodis* pv. *citri* reported as lysotype A. Thus, bacterial pathogens and citrusphages related to citrus plants mainly distributed in Korea were presumed as lysotype A of *X. axonopodis* pv. *citri*, and lysotype A-infecting CP_1, respectively.

Keywords : Korea, lysotype, phage, *Xanthomonas axonopodis* pv. *citri*.

Xanthomonas axonopodis pv. *citri* is endemic in Jeju island, Korea where citrus plants are grown. Host of the bacterium includes a wide variety of *Citrus* spp. and its relatives in the family Rutaceae. Symptoms of the citrus canker include erumpent and corky lesions on all aerial parts of mature citrus trees including leaves, stems, and fruits (Schoulties et al., 1987). The disease causes reduction of photosynthetic leaf area, defoliation, depreciation of fruit quality, and fruit drop leading to serious economic losses (Schoulties et al., 1987).

Bacteriophages (phages) specific to a particular species or subspecific group of bacteria have been used to help identify plant bacterial pathogens (Billing, 1963 and 1970; Cupples, 1984; Dye et al., 1964; Stolp and Starr, 1964; Thornberry et al., 1949). The reason for its prevailing use is that the technique is more rapid, simple and effective than conventional time-consuming procedure. Phage lysis zones usually become visible within 18-24 h of incubation such as spot inoculation of phage solution. The phage technique has been used extensively for studying epidemiology of human pathogens (Anderson and Williams, 1956) and the occurrence and distribution of lysotypes of plant pathogenic bacteria (Goto, 1965; Gross et al., 1991; Hayward, 1964; Kauffman and Pantulu, 1972; Liew and Alverez, 1981; Obata, 1974; Sutton and Wallen, 1967; Wakimoto, 1967).

Three phages highly specific to bacterial pathogens related to citrus plants namely, CP_1, CP_2, (Wakimoto, 1967), and CP_3 (Goto et al., 1980) have been described previously. Wakimoto (1967) observed two groups of phages and three lysotypes of *X. axonopodis* pv. *citri* in Japan. Each lysotype is lysed by its virulent phage: lysotype A is sensitive to CP_1 and resistant to CP_2; lysotype B shows reverse characteristics; and lysotype C is resistant to both CP_1 and CP_2. Goto and Starr (1972) found that virulent citrusphages are highly specific to *X. axonopodis* pv. *citri* strains among other strains of xanthomonads tested.

To obtain primary information for epidemiologic studies and detection of *X. axonopodis* pv. *citri*, distribution of virulent phages and lysotypes of the bacterial pathogens in Jeju island, Korea was investigated in this study.

Materials and Methods

Bacterial isolation. Sources and relevant information on the bacterial strains used in this study are listed in Table 1. The bacteria were isolated from various citrus plants showing bacterial citrus canker. The marginal regions of diseased parts were used for bacterial isolation. The surface-sterilized 2×3 mm sections were incubated on peptone sucrose agar (PSA: 10 g peptone, 10 g sucrose, 1 g sodium glutamate, 15 g agar in 1 liter of D. W., pH 7.0) at

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Table 1. Strains of *Xanthomonas axonopodis* pv. *citri* and their related phages, plants, locations and years of isolation[a]

Bacterium and phage	Lab strain no.	Previous name	Plant	Location	Year of isolation
Xanthomonas axonopodis pv. *citri*	BC 1	XCK9211	*Citrus reticulata* cv. *unshu*	Shihung, Kyungki Prov.	1992
	BC 3	XCK9317	*C. limon*	Shinrae, NamJeju Gun, Jeju Prov.	1993
	BC 4	XCK9348	*C. natsudaidai*	Namwon, Namjenu Gun, Jeju Prov.	1993
	BC 5	XCK9349	*C. natsudaidai*	Sogwipo City, Jeju Prov.	1993
	BC 6	XCK9350	*C. reticulata* cv. *unshu*	NamJeju Gun, Jeju Prov.	1993
	BC 7	XCK9351	*C. natsudaidai*	NamJeju Gun, Jeju Prov.	1993
	BC 8	XCK9352	*C. reticulata* cv. *unshu*	NamJeju Gun, Jeju Prov.	1993
	BC 9	XCK9353	*C. reticulata* cv. *unshu*	NamJeju Gun, Jeju Prov.	1993
	BC 10	XCK9354	*C. reticulata* cv. *unshu*	NamJeju Gun, Jeju Prov.	1993
	BC 11	XCK9355	*C. reticulata* cv. *unshu*	NamJeju Gun, Jeju Prov.	1993
	BC 12	XCK9356	*C. reticulata* cv. *unshu*	NamJeju Gun, Jeju Prov.	1993
	BC 13	XCK9359	*C. natsudaidai*	Sogwipo City, Jeju Prov.	1993
	BC 15	XCK9360B	*C. natsudaidai*	Dosoon, Sogwipo City, Jeju Prov.	1993
	BC 16	XCK9361A	*C. reticulata* cv. *unshu*	Hagwi, PukJeju Gun, Jeju Prov.	1993
	BC 17	XCK9361B	*C. reticulata* cv. *unshu*	Hagwi, PukJeju Gun, Jeju Prov.	1993
	BC 18	XCK9362A	*C. limon*	Shinrae, NamJeju Gun, Jeju prov.	1993
	BC 20	XCK9363	*C. reticulata* cv. *unshu*	Youngpyung, Jeju City, Jeju Prov.	1993
	BC 21	XCK9364A	*C. natsudaidai*	Hawon, Sogwipo City, Jeju prov.	1993
	BC 25	XCK9365	*C. natsudaidai*	Seoho, Sogwipo City, Jeju Prov.	1993
	BC 27	XCK9366B	*C. grandis*	Donghung, NamJeju Gun, Jeju Prov.	1993
	BC 28	XCK9367	*C. grandis*	Topyung, Sogwipo City, Jeju Prov.	1993
	BC 33	XCK9369A	*C. natsudaidai*	Shinrae, NamJeju Gun, Jeju Prov.	1993
	BC 39	XCK9370	*C. natsudaidai*	Sangrae 2, Sogwipo City, Jeju Prov.	1993
	BC 40	XCK9371	*C. natsudaidai*	Changchun, PukJeju Gun, Jeju Prov.	1993
	BC 41	XCK9372	*C. reticulata* cv. *unshu*	Walrang, Hanrim, PukJeju Gun, Jeju Prov.	1993
	BC 42	XCK9373A	*C. reticulata* cv. *unshu*	Sogwipo City, Jeju Prov.	1993
	BC 44	XCK9374	*C. reticulata* cv. *unshu*	Shinrae, NamJeju Gun, Jeju Prov.	1993
	BC 45	XCK9375	*C. ovodea*	Odung, Jeju City, Jeju Prov.	1993
	BC 46	XCK9376	*C. reticulata* cv. *unshu*	Odung, Jeju City, Jeju Prov.	1993
	BC 47	XCK9377	*C. reticulata* cv. *unshu*	Kumduk, PukJeju Gun, Jeju Prov.	1993
	BC 48	XCK9378	*C. reticulata* cv. *unshu*	Donghung, NamJeju Gun, Jeju Prov.	1993
	BC 49	XCK9379A	*C. reticulata* cv. *unshu*	Shinrae, NamJeju Gun, Jeju Prov.	1993
	BC 50	XCK9380D	*C. natsudaidai*	Shinrae, NamJeju Gun, Jeju Prov.	1993
	BC 51	XCK9380F	*C. reticulata* cv. *aoshima*	Shinrae, NamJeju Gun, Jeju Prov.	1993
	BC 52	XCK9381	*C. reticulata* cv. *unshu*	Oeisan, Chochun, PukJeju Gun, Jeju Prov.	1993
	BC 53	XCK9382	*C. reticulata* cv. *unshu*	Hachun, Pyosun, NamJeju Gun, Jeju Prov.	1993
	BC 56	XCK9382C	*C. reticulata* cv. *unshu*	Hachun, Pyosun, NamJeju Gun, Jeju Prov.	1993
	BC 57	XCK9383	*C. reticulata* cv. *unshu*	Seohul, Chochun, PukJeju Gun, Jeju Prov.	1993
	BC 58	XCK9384	*C. reticulata* cv. *unshu*	Nansan, Sungsan, NamJeju Gun, Jeju Prov.	1993
	BC 59	XCK9385	*C. reticulata* cv. *unshu* × *C. sinensis*	Pyosun, NamJeju Gun, Jeju Prov.	1993
	BC 60	XCK9386	*C. reticulata* cv. *unshu*	Susan, Sunsan, NamJeju Gun, Jeju Prov.	1993
	BC 61	XCK9387	*C. natsudaidai*	Odung, Jeju City, Jeju Prov.	1993
	BC 62	XCK9388	*C. hassaku*	Taehung 1, Namwon, NamJeju Gun, Jeju Prov.	1993
	BC 63	XCK9389A	*C. reticulata* cv. *unshu*	Taehung 1, Namwon, NamJeju Gun, Jeju Prov.	1993
	BC 64	XCK9389B	*C. reticulata* cv. *unshu*	Taehung 1, Namwon, NamJeju Gun, Jeju Prov.	1993
	BC 65	XCK9389C	*C. reticulata* cv. *unshu*	Taehung 1, Namwon, NamJeju Gun, Jeju Prov.	1993
	BC 66	XCK9389D	*C. reticulata* cv. *unshu*	Taehung 1, Namwon, NamJeju Gun, Jeju Prov.	1993
	BC 67	XCK9389	*C. reticulata* cv. *unshu*	Taehung 1, Namwon, NamJeju Gun, Jeju Prov.	1993

Table 1. Continued

Bacterium and Phage	Lab strain no.	Previous name	Plant	Location	Year of isolation
Phage	P1		*C. natsudaidai*	Sogwipo City, Jeju Prov.	1993
	P2		*C. natsudaidai*	Sogwipo City, Jeju Prov.	1993
	P3		*C. grandis*	Gukwang, NamJeju Gun, Jeju Prov.	1993
	P5		*C. reticulata* cv. *unshu*	NamJeju Gun, Jeju Prov.	1993
	P6		*C. natsudaidai*	Sogwipo City, Jeju Prov.	1993
	P7		*C. reticulata* cv. *unshu*	NamJeju Gun, Jeju Prov.	1993
	P8		*C. natsudaidai*	Namwon, Namjenu Gun, Jeju Prov.	1993
	P10		*C. natsudaidai*	NamJeju Gun, Jeju Prov.	1993
	P12		*C. iyo*	Sogwipo City, Jeju Prov.	1993
	P14		*C. sinensis*	Namjeju Gun, Cheju Prov.	1993
	P15		*C. natsudaidai*	Dosoon, Sogwipo City, Jeju Prov.	1993
	P18		*C. reticulata* cv. *unshu*	Hoesu, Chungmun, Sogwipo City, Jeju Prov.	1993
	P19		*C. limon*	Shinrae, NamJeju Gun, Jeju prov.	1993
	P25		*C. natsudaidai*	Hawon, Sogwipo City, Jeju prov.	1993
	P26		*C. sinensis*	Shinrae, NamJeju Gun, Jeju Prov.	1993
	P29		*C. reticulata* cv. *unshu*	Musu, Hanrim, PukJeju Gun, Jeju Prov.	1993
	P34		*C. reticulata* cv. *unshu*	Odung, Jeju City, Jeju Prov.	1993
	P40		*C. sinensis*	Donghung, NamJeju Gun, Jeju Prov.	1993
	P42		*C. grandis*	Topyung, Sogwipo City, Jeju Prov.	1993
	P44		*C. natsudaidai*	Sangrae 2, Sogwipo City, Jeju Prov.	1993
	P47		*C. natsudaidai*	Changchun, PukJeju Gun, Jeju Prov.	1993
	P48		*C. reticulata* cv. *unshu*	Walrang, Hanrim, PukJeju Gun, Jeju Prov.	1993
	P50		*Poncirus* Raf. × *C. sinensis*	Changsu, Aewal, PukJeju Gun, Jeju Prov.	1993
	P57		*C. reticulata* cv. *unshu*	Kwangyoung, Aewal, PukJeju Gun, Jeju Prov.	1993
	P58		*C. reticulata* cv. *unshu*	Shinrae, NamJeju Gun, Jeju Prov.	1993
	P60A		*C. sinensis*	Shinrae, Namjeju Gun, Jeju Prov.	1993
	P60B		*C. reticulata* cv. *unshu*	Oeisan, Chochun, PukJeju Gun, Jeju Prov.	1993
	P60C		*C. sinensis*	Shinrae, NamJeju Gun, Jeju Prov.	1993

27°C for 72 h. After incubation, the pale yellow bacteria growing on the plate were purified through three successive cultures. The bacterial isolates were stored in -70°C in 20% glycerol for further research.

Pathogenicity tests. Pathogenicity of the bacterial strains were tested on the fresh leaves of *Citrus reticulata* cv. Unshu cultivated in 20-cm-diameter pots in a greenhouse for 4 weeks. The bacterial cells grown in PSA at 27°C for 24 h were suspended in 0.01 M sterile phosphate buffered saline (PBS), pH 7.0, and centrifuged at 10,000 rpm for 10 minutes. The pellets were re-suspended in the PBS prior to inoculation. Twenty (20) microliters of the suspended bacterial cells (about 1×10^8 colony forming units/ml) were inoculated onto the leaves of plants within 20 days after budding by using spot inoculation method. The plants incubated in a controlled growth chamber (RH 100%) at 27°C for 48 h in the dark were transferred to a greenhouse (27±3°C). Symptoms were recorded 3 weeks after inoculation. The bacteria were re-isolated from the diseased tissue as described above.

Isolation of phages. A total of 156 putative phage solutions were prepared from the diseased parts of citrus plants in Jeju island from March to September 1993. Double-layer method was used to isolate the phage, with the hard agar (1.5% agar) forming the basal layer, and the soft agar (0.6% of agar) forming the upper overlay. Ten to twenty of diseased tissues collected from citrus leaves were macerated with 1 ml of sterile distilled water in a sterilized mortar. To eliminate contaminated microorganisms and tissue debris, the suspensions were centrifuged by 12,000 rpm for 10 min at 4°C. Twenty (20) microliters of the supernatant was dropped on the surface of the solidified upper layer seeded with *X. axonopodis* pv. *citri* (BC 1). Plaques formed at the point of each drop after about 20 h incubation were transferred into 3 ml of PS broth containing about 1×10^8 cells/ml of *X. axonopodis* pv. *citri* BC 1 and multiplied by incubation at 200 rpm at 25°C for 20 h. For single plaque isolation of phage, supernatants containing the multiplied phages were diluted in PS broth and incubated as above. For pure culture of phage, the single plaque isolation was repeated three times.

Plaque-forming supernatants were stored at 4°C for further study. The supernatants stored were inoculated on plates seeded with the strains of BC 45 and BC 67, which appeared to be resis-

tant to the citrus phage.

Phage typing. Spot tests were performed by using the double layer technique. Bacterial suspensions were prepared in 9 ml of PS soft agar (0.7 %), and poured over PSA basal medium. Twenty (20) μl of about 1×10^9 plaque forming unit/ml was dropped on the plates seeded with the bacterium. Sensitivity of bacteria to phages was recorded after incubation for 20 h at 25°C. All strains of *X. axonopodis* pv. *citri* were phage typed at least twice to verify the phage group.

Specificity of citrus phages. Spot tests were performed to test the specificity of the citrus phage isolated from 15 different *Xanthomonas* spp. listed in Table 2. Positive reactions were recorded after the plates were incubated as previously described.

Results and Discussion

Bacterial pathogenicity. Forty-eight strains of the bacterium were isolated from June to September in 1992 and 1993 in Jeju island and Kyung-gi Province (Table 1). The strains induced typical symptoms on *Citrus reticulata* cv. Unshu within 3 weeks (data not shown). The inoculated plants showed water-soaked symptoms within 48 h in the dark, and developed into dried, erumpent, and corky lesions with hollow in the greenhouse.

Phage specificity and phage typing. Phages have specificity to their host strains within pathovar related to citrus bacterial canker disease and different strains of xanthomonads (Civerolo and Fan, 1982; Goto and Starr, 1972). The 28 isolated phages designated to CPKs were highly specific to lysotype A of *X. axonopodis* pv. *citri* in the spot tests (Tables 2 and 3). Specificity of CPKs to strain differentiation of *X. axonopodis* pv. *citri* was consistent with a previous report that CP_1 infected the strain BC 83 (=Xc 62), lysotype A of *X. axonopodis* pv. *citri*, and did not infect the strain BC 82 (=Xc 61), lysotype B (Civerolo and Fan, 1982). Therefore, based on the host specificity of CPKs, it was presumed that the CPKs were CP_1 as reported by Wakimoto (1967). Phage distribution related to *X. axonopodis* pv. *citri* in Korea was different from that of Japan where there were two phages, CP_1 and CP_2 (Wakimoto, 1967). In addition, result of specificity of CPKs to 13 different xanthomonads tested was consistent with a previous report (Goto and Starr, 1972). *Xanthomonas axonopodis* pv. *glycine* which was not included in the study of Goto and Starr (1972) was not lysed by any CPKs.

The phages infected all of the Korean strains of *X. axonopodis* pv. *citri* except BC 45 and BC 67 (Table 3). Based

Table 2. Bacterial strains used in this study and specificity of citrus phages from Korea (CPKs) to different *Xanthomonas* spp.

Lab strain	*Xanthomonas* spp.	Source strain no.[a]	Reaction[b]	Plant	Location	Source[c]	Remarks[c]
BC 83	*X. axonopodis* pv. *citri*	Xc 62	+	*Citrus reticulata*	Brazil	1	Sensitive to CP_1 and resistant to CP_2 (Civerolo and Fan, 1982)
BC 82	*X. a.* pv. *citri*	Xc 59	−	*C. reticulata*	Japan	1	Sensitive to CP_2 and resistant to CP_1 (Civerolo and Fan, 1982)
BC 122	*X. a.* pv. *aurantifolii*	XC-8	−	*C. limon*	Argentina	2	Pathotype B
BC 119	*X. a.* pv. *aurantifolii*	NCPPB 3654	−	*C. aurantifolia*	Brazil	2	Pathotype C
BC 164	*X. a.* pv. *aurantifolii*	Xc 90	−	*C. limon*	Mexico	1	Pathotype D (Hurtung and Civerolo, 1989)
BC 227	*X. a.* pv. *carotae*	ATCC 10547	−	*Daucus carota* var. *sativa*	USA	3	
BC192	*X. a.* pv. *diffenbachia*	XCD9301	−	*Anthurium* sp.	Korea	This study	
BC 74	*X. a.* pv. *glycine*	XCG 9301	−	*Glycine max*	Korea	This study	
BC 71	*X. a.* pv. *vesicatoria*	XCV 9301	−	*Lycoersicon lycopersicum*	Korea	This study	
BC 137	*X. arboricola* pv. *pruni*	XCP 9303	−	*Prunus persica*	Korea	This study	
BC 73	*X. campestris* pv. *campestris*	XCC1	−	*Brassica campestris* ssp. *perkinensis*	Korea	This study	
BC 234	*X. fragariae*	ATCC 33239^T	−	*Fragaria chiloensis* var. *ananassa*	USA	3	
BC 76	*X. oryzae* pv. *oryzae*	Xoo 170	−	*Oryza sativa*	Korea	This study	

[a] T, type culture.

[b] +, positive reaction, −, negative reaction. Using the twenty-eight (28) phages listed in Table 1, the reaction of each strain for the spot tests described in Materials and Methods was recorded at 20 h after incubation at 25°C.

[c] 1=Dr. J. S. Hartung, USDA, ARS, Beltsville, MD, USA; 2=Dr. Rui P. Leite Jr., Instituto Agrinomico, do Parana, Brazil; 3=The American Type Culture Collection.

Table 3. Lysotypes of *Xanthomonas axonopodis* pv. *citri* Korean strains and their percent distribution

Lysotype	Strain	Specificity	Percent distribution
I	BC 1, BC 3, BC 4, BC 5, BC 6, BC 7, BC 8, BC 9, BC 10, BC 11, BC 12, BC 13, BC 15, BC 16, BC 17, BC 18, BC 20, BC 21, BC 25, BC 27, BC 28, BC 33, BC 39, BC 40, BC 41, BC 42, BC 44, BC 46, BC 47, BC 48, BC 49, BC 50, BC 51, BC 52, BC 53, BC 56, BC 57, BC 58, BC 59, BC 60, BC 61, BC 62, BC 63, BC 64, BC 65, BC 66	+[a]	96
II	BC 45, BC 67	–	4

[a] + = positive reaction, – = negative reaction. Using the twenty-eight phages listed in Table 1, the reaction of each strain for the spot tests described in Materials and Methods was recorded at 20 h after incubation at 25°C.

on the lytic responses of Korean strains to the phages, the bacterial pathogens were differentiated into two lysotypes. The 45 strains tested (96%) were susceptible to CPKs, whereas, two strains, BC 45 and BC 67, appeared to be resistant (Table 3). These results showed that at least two different lysotypes were distributed in Korea. In this study, the susceptible strains were named as lysotype I, while the resistant ones were named lysotype II (Table 3). Lysotype I was presumed as lysotype A of Wakimoto (1967) based on the specificity of CPK. Lysotype II that was resistant to CPKs needs to be further characterized.

Phage typing is useful in epidemiological studies because it is a relatively simple and reliable technique for differentiating numerous strains of plant pathogenic bacterium. Information on the lysotypes and their phages in this study can be used for rapid identification and ecological studies of *X. axonopodis* pv. *citri* in Korea. With the typing scheme, it is now possible to study the dissemination of bacterial inocula using lysotypes as stable markers, or even to relate the occurrence and distribution of citrus canker to particular inoculum sources like infected plant materials. In addition, the phage technique may be useful in epidemiologic studies for the rapid detection of *X. axonopodis* pv. *citri* associated with symptomless tissue, like fruits and leaf surfaces.

Acknowledgments

We deeply thank Dr. J. S. Hartung, Fruit Lab., USDA/ARS, Beltsville MD, USA, and Dr. Leite Jr., Instituto Agrinomico, do Parana, Brazil, for their kind donation of bacterial strains.

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