Pathogenic Interactions Between *Xanthomonas axonopodis* pv. *citri* and Cultivars of Pummelo (*Citrus grandis*)

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ABSTRACT

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The aggressiveness of strains of *Xanthomonas axonopodis* pv. *citri* on seven *Citrus* species, including *Citrus sinensis* (navel orange), *C. paradisi* (grapefruit), *C. unshiu* (Satsuma mandarin), *C. junos* (Yuzu), *C. aurantifolia* ('Mexican' lime), *C. tachibana* (Tachibana), and *C. grandis* (pummelo: 'Otachibana', 'Banpeiyu', and 'Anseikan'), were assessed by comparing lesion expansion and growth in planta, using a prick inoculation method. The existence of two groups distinct in aggressiveness was demonstrated on the pummelo cultivars, whereas the remaining species tested were uniformly susceptible. The two groups of strains were dis-

tinct in lesion expansion and growth in planta; however, both caused canker lesions on the 'Otachibana' pummelo. The sensitivity of the bacterial strains to phages Cp1 and Cp2 was associated with differences in aggressiveness. Namely, all the strains sensitive to Cp2 but resistant to Cp1 were aggressive to 'Otachibana', whereas all the strains sensitive to Cp1 but resistant to Cp2 were weakly aggressive. When a repetitive sequence-based polymerase chain reaction amplification was carried out by enterobacterial repetitive intergeneric consensus (ERIC) sequences (ERIC1R and ERIC2) as the primers, these two groups were also distinguishable by the presence or absence of a 1.8-kb DNA fragment among otherwise identical fragments. The 1.8-kb fragment was amplified only from the strains aggressive to *C. grandis*.

Asiatic citrus canker, which is caused by *Xanthomonas axonopodis* pv. *citri* (synonym *X. campestris* pv. *citri* pathotype A) (27), is one of the biggest problems in citrus production worldwide (23). *X. axonopodis* pv. *citri* has a broad host range among members of the Rutaceae, although differences in susceptibility exist among *Citrus* spp. (22). Little is known, however, about differences in pathogenicity, including aggressiveness within this pathovar. Several restriction fragment length polymorphism (RFLP) analyses for *X. axonopodis* pv. *citri* have indicated that, with few exceptions, these bacteria share a similar genetic background (5,9,23). This genetic uniformity seems to explain the uniformity of their aggressiveness on different host species of the Rutaceae (23).

Recently, strains of *X. axonopodis* pv. *citri* have been isolated from 'Mexican' lime (*Citrus aurantifolia* Swingle) trees in Southwest Asia that are phenotypically distinct from other strains of this pathovar (28). The Southwest Asian strains elicited typical canker lesions on 'Mexican' lime but not on other *Citrus* spp., such as grapefruit (*C. paradisi* Macf.) (28). DNA analyses could not distinguish the Southwest Asian strains from other strains of *X. axonopodis* pv. *citri* (28). However, the Southwest Asian strains were distinct, because they had no affinity for polyclonal antiserum prepared against a typical strain of *X. axonopodis* pv. *citri* (28).

Several studies have reported distinct phenotypes among Japanese strains of *X. axonopodis* pv. *citri* (6,7,30). They differ in sensitivity to the bacterial phages Cp1 and Cp2 (17), and only strains sensitive to Cp1 are able to oxidize mannitol (6,30). Japanese strains of *X. axonopodis* pv. *citri* resemble typical strains of *X. axonopodis* pv. *citri* because of their affinity to a polyclonal antiserum prepared against the pathogen and because of the typical

canker symptoms they produce on *Citrus* spp. (7). Because Japanese strains have been identified as *X. axonopodis* pv. *citri*, it would be reasonable to expect to find no differences in aggressiveness among the strains. The *Citrus* spp. used for pathogenicity tests in previous studies, however, were restricted to the main species planted in Japan. In order to breed resistant cultivars and eventually control this disease, we should investigate differences in pathogenicity among the strains by testing them against a wider variety of species.

In this paper, we describe differential aggressiveness among Japanese strains of *X. axonopodis* pv. *citri* on pummelo (*C. grandis* Osbeck) cultivars; in addition, we discuss a specific DNA fragment found in aggressive strains amplified with repetitive sequence-based polymerase chain reaction (rep-PCR), a method that has been developed to examine relationships among bacterial species, including the xanthomonads (16,26). A possible relationship between differential aggressiveness and phage sensitivity among *X. axonopodis* pv. *citri* strains is also discussed.

MATERIALS AND METHODS

Bacterial strains. Strains of *X. axonopodis* pv. *citri* were isolated from lesions on the leaves of 10 *Citrus* spp. and 6 hybrids from 27 groves at different locations in Japan (Table 1). They were serologically identified as *X. axonopodis* pv. *citri* by an agglutination test, using a rabbit antiserum prepared by previously described methods (19), namely, by injecting a heat-treated cell suspension of bacterial strain 9011 of *X. axonopodis* pv. *citri*. After identification, cultures of the strains were suspended in a skim milk medium (10% skim milk and 1% sodium L-glutamate) and maintained at -80°C. Strains KC5, KC11, KC12, KC16, KC19, KC21, and KC34 were randomly selected and used in pathogenicity tests with seven *Citrus* spp. (described below) to find those species on which differences in aggressiveness could be observed. The reference strains were NCPPB409 of *X. axonopodis*

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pv. *citri* and XC148 of *X. axonopodis* pv. *aurantifolii* (27) (synonym *X. campestris* pv. *aurantifolii*), which is causal agent of citrus canker B (23).

Plant materials. In this study, seven *Citrus* spp. were used: *C. sinensis* Osbeck (navel orange), *C. paradisi* (grapefruit), *C. unshiu* Marc. (Satsuma mandarin), *C. aurantifolia* ('Mexican' lime), *C. junos* Sieb. ex Tanaka (Yuzu), *C. tachibana* Tanaka (Tachibana), and *C. grandis* (pummelo 'Otachibana', 'Banpeiyu', and 'Anseikan'). Scions of these had been grafted onto potted 2-year-old rootstocks of *C. jambhiri* Lush (Rough lemon).

Differences in lesion expansion. The aggressiveness of each strain of *X. axonopodis* pv. *citri* was evaluated after inoculation into leaves of different *Citrus* spp. (7,8,15). Bacterial strains were grown on Wakimoto's potato semisynthetic medium with 1.5% agar (PSA medium) (30) at 28°C for 1 day. Bacteria were suspended in 0.85% NaCl at a concentration of 1×10^8 cells per ml,

and approximately 3 µl of the suspension was placed in each of six spots on an attached mature leaf of each citrus plant. Immediately after pricking the leaves through the suspension with an insect pin (0.5-mm diameter), the inoculum drops were wiped off with sterile cotton. Four leaves of each citrus plant were used for each strain, and the assays were repeated three times. The results of one test are reported. The inoculated plants were grown in a greenhouse in which the temperature was 28°C during the day and 25°C at night. The diameters of the circular lesions, including cork tissue and water-soaked margin, were measured 40 days after inoculation. Differences in lesion expansion among strains for each host were evaluated by analysis of the Tukey-Kramer honestly significant difference (HSD) test.

Bacterial growth in planta. The growth in planta of an aggressive and a weakly aggressive strain of *X. axonopodis* pv. *citri* were observed. An aggressive strain, KC16, and a weakly aggres-

Fig. 1. Lesion expansion for eight strains of *Xanthomonas axonopodis* pv. *citri* on citrus species, A, navel orange; B, grapefruit; C, Satsuma mandarin; D, 'Mexican' lime; E, Yuzu; F, Tachibana; G, 'Otachibana'; H, 'Banpeiyu'; and I, 'Anseikan'. Each bar represents the mean diameter of 24 lesions from each strain 40 days after inoculation by pricking the attached leaves of each species. Bars with different letters differ significantly at $\alpha = 0.01$ according to the Tukey-Kramer honestly significant difference test.

sive strain, KC19, were randomly selected for use in the experiment. Strain XC148 of *X. axonopodis* pv. *aurantifolli* was used for comparison. The bacteria suspended in 0.85% NaCl were inoculated into mature attached leaves of *C. grandis* ('Otachibana') and *C. sinensis* (navel orange) by pricking as described above or by infiltrating with a syringe at a concentration of 1×10^5 cells per ml. The inoculated plants were grown in a greenhouse in which the temperature was controlled as described above. The bacterial populations in the lesions at 0, 1, 2, 3, 4, 8, and 16 days after inoculation were determined by removing the lesion with a cork-borer. Internal populations of leaves inoculated by infiltrating

were determined at 0, 5, 10, 15, and 20 days after inoculation by harvesting a leaf disk (0.52 cm^2) from a randomly chosen site. The removed tissue was macerated in 0.85% NaCl, and the suspension was plated on a semiselective XCSM medium (18) composed of 1% starch, 0.2% polypeptone, 0.1% monopotassium phosphate, 0.02% magnesium chloride, 0.05% calcium nitrate, 0.1% disodium hydrogenphosphate dibasic, and 1.5% agar in distilled water. After autoclaving, methylgreen, cycloheximide, chloramphenicol, and fradiomycin sulfate were added to final concentrations of 10, 100, 1, and 1 $\mu\text{g/ml}$, respectively.

Symptoms elicited by *X. axonopodis* pv. *citri* strains in *C. grandis*. Cultures of KC16 and KC19 strains grown on PSA medium were suspended in 0.85% NaCl at a concentration of 1×10^8 cells per ml. A syringe was used to inject a bacterial suspension of each strain into the intercellular spaces of mature and immature attached leaves of *C. grandis* 'Otachibana'. Reference strain XC148 of *X. axonopodis* pv. *aurantifolli* also was used in this experiment.

Phage sensitivity. Phages Cp1 and Cp2 were provided by the Moji Plant Protection Station, Fukuoka, Japan. The drop method described by Wakimoto (30) was used for the phage test. The bacterial strains were grown on PSA medium at 28°C for 1 day and were suspended in sterile distilled water at a concentration of 1×10^8 cells per ml. A mixture of 2 ml of the bacterial suspension and 5 ml of PSA medium that was melted and kept at 55°C , was plated. Each phage suspension at a titer of 1×10^9 plaque forming units per ml was spotted on the medium, and the plates were incubated at 28°C for 15 h. A zone of clearing or plaques indicated sensitivity to the phage.

Extraction of genomic DNA. A modified procedure described by Ausubel et al. (1) was used to extract total genomic DNA. Bacteria were grown in 2 ml of YP medium (0.3% yeast extract

Fig. 2. Lesions on attached mature leaves of pummelo 'Otachibana' inoculated with strains KC16 and KC19 of *Xanthomonas axonopodis* pv. *citri* and strain XC148 of *X. axonopodis* pv. *aurantifolli*. **A and B,** Lesions developed 40 days after inoculation by pin-pricking with **A**, the aggressive strain KC16 and **B**, the weakly aggressive strain KC19. Strain KC16 caused more extensive corked tissue and a larger water-soaked margin than KC19. **C,** Lesions photographed 10 days after inoculation by infiltration with bacterial suspensions of the indicated strains (KC16, KC19, and XC148). All the strains elicited canker symptoms 5 days after inoculation of the cultivar.

Fig. 3. Distribution of *Xanthomonas axonopodis* pv. *citri* strains by lesion size on attached leaves of **A**, pummelo cv. Otachibana and **B**, navel orange 40 days after inoculation by pin-pricking. The 42 strains shown are listed in Table 1.

and 0.5% polypeptone) in 15-ml test tubes at 28°C for 20 h on a shaker (150 rpm). Bacterial cells were washed twice with 2 M NaCl and suspended in 600 µl of Tris-EDTA (TE) buffer (pH 8.0) (21) containing sodium dodecyl sulfate (SDS) and proteinase K at a final concentration of 2% and 250 µg/ml, respectively. After incubation at 37°C for 1 h, 100 µl of 5 M NaCl was added to the solution, which was mixed thoroughly. Eighty microliters of cetyltrimethylammoniumbromide (CTAB)-NaCl solution (10% hexadecyltrimethyl ammonium bromide in 0.7 M NaCl) was added to the solution, which was mixed thoroughly and incubated at 65°C for 10 min. DNA was extracted using chloroform isoamyl alcohol (24:1) and phenol chloroform isoamyl alcohol (25:24:1) precipitated by the addition of 0.6 vol of isopropanol, and washed with 70% ethanol. Pelleted DNA was dried in a vacuum and dissolved in 100 µl of TE buffer (pH 8.0).

Oligonucleotide primers and polymerase chain reaction condition. The reaction mixture and amplification conditions for the repetitive sequence-based polymerase chain reaction (rep-PCR) were those described by Louws et al. (16). The primers

enterobacterial repetitive intergeneric consensus (ERIC)1R [5'-ATGTAAGCTCCTGGGGATTCAA-3'] and ERIC2 [5'-AAG-TAAGTGACTGGGGTGAGCG-3'] (16) were purchased from Sawady Technology, Tokyo, Japan. Each 25-µl PCR reaction buffer contained 50 pmol of each primer, 50 ng of template total DNA, 1.25 mM each of four dNTPs, 2 units *AmpliTaq* polymerase (Perkin-Elmer/Cetus, Foster City, CA), and 10% dimethyl sulfoxide (DMSO) (vol/vol) (14). The PCR amplification was carried out by a DNA thermal cycler (Model 480, Perkin-Elmer/Cetus). The PCR mixture was overlaid with 25 µl of mineral oil during amplification. The initial denaturation was at 95°C for 7 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 65°C for 8 min, with a final extension at 65°C for 15 min and a soak at 4°C (16).

An 8-µl portion of the amplified PCR product was subjected to electrophoresis at 4°C on a 1.5% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer (21) for 3 h at 100 V, stained with ethidium bromide, and photographed on a UV transilluminator with Polaroid film (Type 667, Polaroid Corp., Cambridge, MA).

TABLE 1. Strains of *Xanthomonas* spp. used in this study

Strain	Host plant	Origin ^a	Aggressiveness ^b	Phage sensitivity ^c	
				Cp1	Cp2
<i>X. axonopodis</i> pv. <i>citri</i>					
NCPBP409 ^d	<i>Citrus limon</i>	New Zealand	++	S	S
9011	<i>C. unshiu</i>	Matsubase, Kumamoto	++	R	S
KC2	<i>C. grandis</i>	Matsubase, Kumamoto	++	S	S
KC4	<i>C. limon</i>	Matsubase, Kumamoto	++	R	S
KC5	<i>C. hassaku</i>	Gamagori, Aichi	++	R	S
KC6	<i>C. jambihili</i>	Gamagori, Aichi	++	R	S
KC10	<i>C. tamurana</i>	Takaono, Kagoshima	++	R	S
KC11	(<i>C. nobilis</i> × <i>C. unshiu</i>) × <i>C. reticulata</i>	Nago, Okinawa	++	R	S
KC12	<i>C. reticulata</i> × <i>C. sinensis</i>	Nago, Okinawa	++	R	R
KC13	<i>C. sinensis</i> × <i>C. clementina</i>	Nago, Okinawa	++	R	S
KC15	(<i>C. nobilis</i> × <i>C. unshiu</i>) × <i>C. reticulata</i>	Kochi, Kochi	++	R	S
KC16	<i>C. natsudaaidai</i>	Tarumizu, Kagoshima	++	R	S
KC17	<i>C. unshiu</i>	Shima, Fukuoka	++	R	S
KC18	<i>C. unshiu</i>	Chita, Aichi	++	R	S
KC20	<i>C. natsudaaidai</i>	Inami, Wakayama	++	S	S
KC22	<i>C. tangerina</i> × <i>C. grandis</i>	Hirokawa, Wakayama	++	R	S
KC24	<i>C. sinensis</i> × <i>C. clementina</i>	Nago, Okinawa	++	R	S
KC25	<i>C. unshiu</i>	Nago, Okinawa	++	R	S
KC31	<i>C. unshiu</i>	Minamata, Kumamoto	++	R	S
KC36	<i>C. iyo</i>	Ikata, Ehime	++	R	S
KC37	<i>C. unshiu</i>	Kashima, Saga	++	R	S
KC38	<i>C. limon</i>	Akune, Kagoshima	++	R	S
KC1	<i>C. grandis</i>	Matsubase, Kumamoto	+	S	R
KC3	(<i>C. sinensis</i> × <i>C. unshiu</i>) × <i>C. reticulata</i>	Matsubase, Kumamoto	+	S	S
KC7	<i>C. unshiu</i>	Gamagori, Aichi	+	S	R
KC8	<i>C. shaerocarpa</i>	Tsukumi, Oita	+	S	R
KC9	<i>C. sinensis</i>	Tarumizu, Kagoshima	+	S	R
KC14	<i>C. unshiu</i>	Odawara, Kanagawa	+	S	R
KC19	<i>C. unshiu</i>	Tanabe, Wakayama	+	S	R
KC21	<i>C. natsudaaidai</i>	Tanabe, Wakayama	+	S	R
KC23	<i>C. limon</i>	Yoshida, Ehime	+	S	R
KC26	<i>C. unshiu</i>	Koga, Fukuoka	+	S	R
KC27	<i>C. sinensis</i>	Shingu, Fukuoka	+	S	R
KC28	<i>C. sinensis</i>	Matsuyama, Ehime	+	S	R
KC29	<i>C. iyo</i>	Tobe, Ehime	+	S	R
KC30	<i>C. sinensis</i>	Yuasa, Wakayama	+	S	R
KC32	<i>C. sinensis</i>	Hondo, Kumamoto	+	S	R
KC33	<i>C. iyo</i>	Ikata, Ehime	+	S	S
KC34	<i>C. natsudaaidai</i>	Seto, Ehime	+	S	S
KC35	<i>C. unshiu</i>	Yamamoto, Kagawa	+	S	R
KC39	(<i>C. sinensis</i> × <i>C. unshiu</i>) × <i>C. reticulata</i>	Nichinan, Miyazaki	+	S	R
KC40	<i>C. sinensis</i>	Tobe, Ehime	+	S	R
<i>X. axonopodis</i> pv. <i>aurantifolli</i>					
XC148 ^e	<i>C. limon</i>	Argentina	+	R	R

^a Except for NCPBP409 and XC148 all strains originated in Japan.

^b Aggressiveness in pathogenicity of each strain to *C. grandis* (cv. Otachibana); ++ = aggressive, + = weakly aggressive.

^c Sensitivity to phages Cp1 and Cp2; S = sensitive, R = resistant.

^d National Collection of Plant Pathogenic Bacteria, England.

^e The strain provided by J. S. Hartung.

RESULTS

Distinct lesion expansion of *X. axonopodis* pv. *citri* strains on pummelo cultivars. To find *Citrus* spp. on which differences in aggressiveness of *X. axonopodis* pv. *citri* strains could be observed, eight strains of the pathogen were randomly selected and inoculated into attached mature leaves of seven *Citrus* spp. All strains caused water-soaked lesions at the inoculation sites within 5 days. On *C. sinensis* (navel orange), *C. paradisi* (grapefruit), *C. unshiu* (Satsuma mandarin), *C. aurantifolia* ('Mexican' lime), *C. junos* (Yuzu), and *C. tachibana* (Tachibana), no differences in lesion expansion among the bacterial strains were detected (Fig. 1A through F). However, different lesion expansion among the strains was observed on *C. grandis* cvs. (pummelos) Otachibana, Banpeiyu, and Anseikan. In particular, strains KC16, KC5, KC11, and KC12 uniformly produced larger lesions on the cultivars than KC21, KC1, KC19, and KC34 (Figs. 1G through I, 2A and B).

Testing an additional 32 strains of *X. axonopodis* pv. *citri* clearly demonstrated that at least two pathogenic (aggressive and weakly aggressive) groups infect 'Otachibana'. Namely, on 'Otachibana', the distribution of the lesions according to size was divided into two groups in contrast to a normal distribution of lesions observed on navel orange (Fig. 3A and B, Table 1).

In planta growth curves. In *C. grandis* 'Otachibana', populations of strains KC16 and KC19 increased similarly for the first 2 days after inoculation by pricking to approximately 1.5×10^5 CFU per lesion (Fig. 4A). After 2 days, however, the rates of multiplication differed. After 8 days, populations of both strains fell, although the KC19 numbers decreased more rapidly than those of KC16 (Fig. 4A). Differences in growth in 'Otachibana' between KC16 and KC19 were also confirmed after inoculation by infil-

tration (Fig. 5A). Populations of both strains continued to increase beyond 10 days after inoculation, in contrast to a decrease in the population on plants treated with inoculation by pricking (Fig. 4A). In navel orange (*C. sinensis*), populations of the two strains inoculated by pricking and infiltrating were equivalent throughout the experiment (Figs. 4B and 5B).

Reference strain XC148 of *X. axonopodis* pv. *aurantifolii* increased in both 'Otachibana' and navel orange until 3 days after inoculation by pricking (Fig. 4A and B). After 3 days, however, the population of this strain increased only slightly in both host (Fig. 4A and B). When the strain was inoculated by infiltrating, no bacterial cells were obtained from the inoculated leaves of either host beyond 5 days after inoculation (Fig. 5A and B).

Symptoms elicited after infiltration of bacterial suspension.

An aggressive and a weakly aggressive strain were infiltrated at high cell densities into 'Otachibana' to compare canker symptom development. Three days after inoculation, both strains KC16 and KC19 elicited water-soaking on a mature attached leaf of 'Otachibana'. An erumpent tissue reaction was observed 4 days after infiltration, not only with the suspension of strain KC16 but also with that of KC19 (Fig. 2C). Strain XC148 of *X. axonopodis* pv. *aurantifolii* was infiltrated into a mature leaf of 'Otachibana' and also elicited water-soaking and an erumpent tissue reaction 5 days after inoculation (Fig. 2C). The inoculation test with immature leaves from the plant showed the same results as those observed when mature leaves were used for all the strains tested (data not shown).

Phage sensitivity. All *X. axonopodis* pv. *citri* strains tested, except for strain KC12, were sensitive to the Cp1 or Cp2 phages (Table 1). Interestingly, all strains sensitive to Cp1 and resistant to Cp2 showed weak aggressiveness on 'Otachibana'. All strains

Fig. 4. Time course of bacterial growth in the leaves of citrus species **A**, pummelo 'Otachibana' and **B**, navel orange. An aggressive strain of *Xanthomonas axonopodis* pv. *citri* (KC16), a weakly aggressive strain (KC19) and *X. axonopodis* pv. *aurantifolii* strain XC148 were inoculated by pricking. Leaves were sampled for 16 days after inoculation. Values are means from three repetitions, and vertical bars represent the standard error at each sampling time.

Fig. 5. Time course of bacterial growth in leaves of citrus species **A**, pummelo 'Otachibana' and **B**, navel orange. An aggressive strain *Xanthomonas axonopodis* pv. *citri* KC16, a weakly aggressive strain KC19, and *X. axonopodis* pv. *aurantifolii* XC148 were inoculated into citrus leaves by infiltration at a concentration of 1×10^5 cells per ml. Leaves were sampled for 20 days after inoculation. Values are means from three repetitions, and vertical bars represent the standard error at each sampling time.

sensitive to Cp2 and resistant to Cp1, however, were aggressive. Strain KC12, which was resistant to both phages, was also aggressive on 'Otachibana' (Table 1). Thus, resistance to Cp1 may indicate aggressive pathogenicity on 'Otachibana'. Strains sensitive to both phages, however, included both aggressive and weakly aggressive strains (NCPPB409, KC2, and KC20 in the aggressive group and KC3, KC33, and KC34 in the weakly aggressive group) (Table 1).

Genotypic variation between groups of *X. axonopodis* pv. *citri* by rep-PCR. DNA fingerprints were generated from the total DNA extracted from 42 strains of *X. axonopodis* pv. *citri* (Table 1). Using primers corresponding to the ERIC sequences, PCR resulted in complex fingerprinting patterns consisting of 15 to 16 PCR products ranging in size from approximately 0.2 to 2.5 kb (Fig. 6; data not shown). Although the fingerprinting patterns were similar, they could be classified into two distinct genotypes based on the amplification of a 1.8-kb fragment by PCR (Fig. 6). This 1.8-kb PCR product was amplified only when the total DNA of the strains in the aggressive group was used as the template (Fig. 6). DNA fingerprints generated from the total DNA extracted from reference strain XC148 of *X. axonopodis* pv. *aurantifolli* were distinct from those of *X. axonopodis* pv. *citri* (Fig. 6).

DISCUSSION

Differential aggressiveness among *X. axonopodis* pv. *citri* strains was observed on *C. grandis* ('Otachibana'), based on analyses of bacterial growth in planta as well as lesion expansion after prick inoculation. The growth of an aggressive and a weakly

aggressive strain differed after inoculation by infiltration. Populations of both strains in 'Otachibana' continued to increase 10 days after inoculation by infiltration. Egel et al. (3) reported that infiltrating into the leaf mesophyll might be a means of delivering bacteria to many susceptible sites over a large area. These findings are in contrast to the observed decrease 8 days after inoculation by pricking. Bacteria inoculated by infiltration may have more room in the host to increase in number, compared with those inoculated by pricking.

'Otachibana' has been classified as an independent species, *C. otachibana*, by Tanaka (25). Nevertheless, this plant, grown in Japan, is distinct morphologically from that described by Tanaka. It is, however, identical with 'Tosa-buntan' of *C. grandis* (10,29). *C. grandis* has been distinguished from other members of the genus by numerical and biochemical analyses (2,11,20). The possible inclusion of 'Otachibana', 'Banpeiyu', and 'Anseikan' in the species *C. grandis* may imply that the differential aggressiveness observed is characteristic of the relationship between *X. axonopodis* pv. *citri* and this species.

Avirulence (*avr*)/pathogenicity (*pth*) genes have been identified in bacterial pathogens such as *Xanthomonas* spp. (4). *X. axonopodis* pv. *citri* has *pth* genes, belonging to the *avrBs3* family that are required for eliciting canker symptoms (4,13,24), but no *avr* genes that elicit a resistant reaction in any *Citrus* spp. have been reported. The existence of *pth* genes in *X. axonopodis* pv. *citri* might suggest their involvement in the specific interaction between pummelo cultivars and the pathogen. A weakly aggressive strain of *X. axonopodis* pv. *citri* inoculated into 'Otachibana' by infiltrating at high cell densities, however, did not elicit rapid necrosis corresponding to a hypersensitive reaction; instead, it produced canker symptoms that were identical to those produced by an aggressive strain. This result indicates that an *avr* gene may not be involved in the specific interaction observed in the present study. Instead, positive host range determinants such as the *hsv* genes for host-specific virulence that have been identified in *X. campestris* pv. *translucens* (31), may affect the specific interaction between pummelos and *X. axonopodis* pv. *citri*.

Aggressiveness of *X. axonopodis* pv. *aurantifolli* has been reported to be significantly less than that of strains from *X. axonopodis* pv. *citri* on several *Citrus* spp. (7). The reference strain of this bacterium used in our study also showed less bacterial growth in 'Otachibana' and navel orange than either an aggressive or a weakly aggressive strain of *X. axonopodis* pv. *citri*.

Differential aggressiveness in *X. axonopodis* pv. *citri* appears to be correlated with phage sensitivity. One of the mechanisms involved in phage sensitivity may be related to the differential aggressiveness. Nevertheless, the few exceptions that were observed with some of the strains suggests this might not be true. Differences in phage sensitivity appear to be correlated with the bacteria's ability to oxidize mannitol (6). Specifically, the strains sensitive to phage Cp1 oxidize and use mannitol as a sole carbohydrate source. This correlation was also observed in the present study (data not shown). These observations could be useful in ecological studies of the pathogen.

Phenotypically distinct strains of *X. axonopodis* pv. *citri* have been isolated in Southwest Asia (28). They were resistant to Cp1 and Cp2 phages (28). In contrast, most Japanese strains are sensitive to the phages, indicating that the Japanese strains are different from the Southwest Asian strains (17). In addition, the Southwest Asian strains elicit no or limited water-soaking on detached grapefruit leaves (28). Water-soaking and an erumpent tissue reaction, however, have been observed on this plant for all Japanese strains of the pathogen (data not shown). Therefore, the Japanese strains used in this study appear to be the causal agent of Asiatic citrus canker.

Rep-PCR with ERIC primers showed that the two groups (aggressive and weakly aggressive), without exception, can be identified based on the presence or absence of a 1.8-kb fragment.

Fig. 6. Agarose gel electrophoresis of repetitive sequence-based polymerase chain reaction (rep-PCR). Fingerprinting patterns obtained using bacterial genomic DNA and primers enterobacterial repetitive intergeneric consensus (ERIC)1R and ERIC2. Fingerprints of aggressive strains (KC12, KC11, KC5, and KC20) and of weakly aggressive strains (KC34, KC19, KC1, and KC21) of *Xanthomonas axonopodis* pv. *citri* are shown. A reference fingerprint of a *X. axonopodis* pv. *aurantifolli* strain (XC148) is also shown. Lane M contains DNA size markers; sizes are indicated in base pairs. The arrowhead indicates the characteristic 1.8-kb PCR product that was found only in the aggressive strains.

Amplified DNA fragments other than the 1.8-kb fragment were identical between the two groups. In addition, rep-PCR analysis with REP sequences (16) as the primer set also showed an identical fingerprint profile, without exception, among the strains in the two groups (data not shown). These results are consistent with observations in previous studies of genetic uniformity among *X. axonopodis* pv. *citri* strains (5,9,23). Therefore, the differential aggressiveness within *X. axonopodis* pv. *citri* might be associated with limited genomic variation (16). The ERIC sequences appear to be restricted to transcribed sequences of neighboring genes in *Escherichia coli* and *Salmonella typhimurium* (12). Although we have found no evidence that the 1.8-kb fragment carries a gene that is required for the specific interaction between pummelo cultivars and *X. axonopodis* pv. *citri*, investigating the sequences might contribute to a better understanding of the genetic mechanism of the interaction.

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