Bot. Bull. Acad. Sin. (2000) **41:** 113-120

A DNA probe for identification of *Xanthomonas campestris* pv. *campestris*, the causal organism of black rot of crucifers in Taiwan

Hsin-Der Shih[1], Yuan-Chuen Lin[1], Hsiou-Chen Huang[2], Kuo-Ching Tzeng[1], and Shih-Tien Hsu[1,3]

[1]*Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan, Republic of China*

[2]*Graduate Institute of Agricultural Biotechnology, National Chung Hsing University, Taichung, Taiwan, Republic of China*

(Received April 7, 1998; Accepted September 7, 1999)

Abstract. A DNA probe was developed for identification of strains of *Xanthomonas campestris* pv. *campestris* from Taiwan. *Eco*RI restriction fragments of total DNA from *X. campestris* pv. *campestris* strain Xcc70 were cloned into pBluescript II KS and transformed into *Escherichia coli* DH10B. The recombinant plasmid DNAs from clones randomly selected were labeled with digoxigenin and screened for their specificity for *X. campestris* pv. *campestris*. In Southern hybridization, one of the clones (pXcc70-8) hybridized to several *Eco*RI-digested fragments of total DNA from only strains of *X. campestris* pv. *campestris*. Digestion of the insert DNA of the clone pXcc70-8 with *Eco*RI yielded fragments of 2.7, 1.6 and 0.6 kb. When a subclone containing the 0.6 kb fragment was used as a probe (Xcc70-8-l), it hybridized with all 51 strains of *X. campestris* pv. *campestris* and all seven strains of *X. campestris* pv. *armoraciae*, but not with the 60 strains of other bacteria tested. This probe, however, distinguishably detected a single fragment of 0.7 kb in strains of *X. campestris* pv. *campestris* and a single fragment of 2.5 kb in strains of *X. campestris* pv. *armoraciae* when their total DNAs were digested with *Kpn*I. The detection limits of the probe Xcc70-8-l for the amount of DNA was 25 pg and for the number of cells was about 6×10^4 CFU in dot blot assays. The colony and dot blot hybridizations with the probe were used to detect *X. campestris* pv. *campestris* in extracts of infected leaves of cabbage and seeds of several crucifers. The results indicate that the DNA probe can be used to detect *X. campestris* pv. *campestris* in plant tissues but probably not in seeds. The probe could be a useful tool for rapid identification of the pathogen in epidemiological studies in Taiwan.

Keywords: Crucifers (*Brassica* spp.); Detection; DNA probe; Identification; *Xanthomonas campestris* pv. *campestris*.

Introduction

Black rot of crucifers caused by *Xanthomonas campestris* pv. *campestri*s (Pammel) Dowson is a destructive disease of worldwide importance (Williams, 1980). In Taiwan, black rot affects many cruciferous crops and can be observed at all growing seasons, particularly in most cabbage and cauliflower fields. The black rot pathogen is seed-borne (Walker and Tisdale, 1920; Williams, 1980; Schaad, 1982). Seed testing to ensure absence of the pathogen and seed treatments to eradicate the pathogen are important measures for preventing the occurrence of the disease (Williams, 1980). The success of these measures depends on an efficient and reliable method to detect the pathogen in seeds and to verify the efficacy of seed treatments. The pathogen also survives in infected crop debris, infested cruciferous weeds, and infected plant residues in soil (Alvarez and Cho, 1978; Schaad and White, 1974b; Schaad and Dianese, 1981). The importance of these sources of inoculum in the annual recurrence of the disease in Taiwan remains to be investigated. This kind of ecological study also requires an effective method to detect the pathogen in fields.

Selective media and serology are the commonly used methods for the detection of *X. campestris* pv. *campestris* in seed, plant, or soil samples (Chun and Alvarez, 1983; Franken, 1992; Fukui et al., 1994; Schaad and White, 1974a; Schaad and Donaldson, 1980). The detection efficiency of selective media varies greatly with the source of samples. Most of the available selective media are not efficient for detection of the pathogen in seeds and soils under Taiwan conditions (Huang and Hsu, 1987). Furthermore, the identity of the suspect colonies on the media needs to be confirmed either by the pathogenicity test, which is time-consuming, or by serology. Serological assays are useful in the confirmation test for the isolated colonies (Schaad, 1979) and for direct detection of the pathogen in seeds (Franken, 1992; Schaad and Donaldson, 1980), infected leaves (Alvarez and Lou, 1985), and soil (Domen and Alvarez, 1978). Serology with monoclonal antibodies is particularly specific for the identification or differentiation of strains of *X. campestris* pv. *campestris* (Alvarez et al., 1985) and has been used suc-

[3]Corresponding author. Fax: 886-4-2877585; E-mail: sthsu@mail.nchu.edu.tw

cessfully in monitoring particular strains of the bacterium in fields in the epidemiological study (Yuen et al., 1987). Recently, DNA-based probes have offered another approach to the rapid and specific identification of plant pathogenic bacteria (Rasmussen and Reeves, 1992). A polymerase chain reaction (PCR) technique using primers designed from the sequence of a cloned DNA fragment specific to *X. campestris* pv. *campestris* has been developed for detection of the pathogen in crucifer seeds (Aline and Gabrielson, 1993). In this paper, we describe the development of a cloned DNA probe useful in Taiwan for identification and detection of *X. campestris* pv. *campestris* in plant tissues.

Materials and Methods

Bacterial Strains

The bacterial strains used in this study are listed in Table 1. All strains of *X. campestris* pv. *campestris* were isolated from various cruciferous plants in Taiwan except for strain NCPPB 528 (type strain of the pathovar), which was originated from the United Kingdom and was obtained from the Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan. Xanthomonads were subcultured on potato dextrose agar (PDA, Difco), fluorescent pseudomonads were subcultured on King's B medium (King et al., 1954), *Ralstonia solanacearum* (formerly *Pseudomonas solanacearum*) was subcultured on TZC medium (Kelman, 1954), *Escherichia coli* was subcultured on Luria-Bertani (LB) agar medium (Sambrook et al., 1989), and other bacteria were subcultured on nutrient agar (Difco Laboratories, Detroit, MI). All cultures were incubated at 28~30°C except for *E. coli* which was grown at 37°C. All strains were stored in LB broth containing 15% glycerol at -70°C.

Isolation of Total DNA from Bacteria

Total DNA was prepared by a modification of the method of Lazo et al. (1987). Each of the bacterial cultures was grown in 40 ml of LB broth at 28°C overnight and harvested by centrifugation at 8,000 *g* for 10 min. The cell pellet was washed in 4 ml of STE buffer (100 mM NaC1, 10 mM Tris-HC1, 1 mM EDTA, pH 8.0) and resuspended in 4 ml of STE buffer. The suspension was then added with a sodium dodecyl sulfate (SDS) solution at a final concentration of 1%, incubated at 65°C for 1.5 h, and cooled to room temperature. Subsequently, a proteinase K solution was added to a final concentration of 100 µg per ml, and the mixture was incubated at 37°C for more than 4 h. After addition of 10 ml of STE buffer, the mixture was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, phenol equilibrated to pH 8 with 100 mM Tris) and twice with an equal volume of chloroform-isoamyl alcohol (24:1). All of the phenol or chloroform extractions were done gently and mixed well until emulsifying, and emulsified suspensions were separated by centrifugation at 12,000 *g* for 15 min. The supernatant containing DNA was transferred to a clean tube and precipitated by adding two volumes of ice- cold 95% ethanol, mixing gently, and incubating at -20°C for 30 min. The DNA was spooled out of the precipitation mixture with a sterile glass bar, rinsed with ice- cold 70% ethanol, and then air-dried. The DNA was resuspended in TE buffer (10 mM Tris, 1 mM disodium EDTA) containing 10 µg/ml of DNase-free ribonuclease A.

Recombinant DNA Manipulations

Restriction enzyme digestion, preparation of plasmid DNA using alkaline extraction method, DNA ligation, agarose gel electrophoresis, bacterial transformation ($CaCl_2$ procedure), and cloning of DNA fragments were performed as described by Sambrook et al. (1989). Strain Xcc70 of *X. campestris* pv. *campestris* was used for constructing a genomic library. Total DNA of Xcc70 was digested with *Eco*RI and ligated into the vector pBluescript II KS (Stratagene, USA). The ligation mixture was subsequently transformed into competent cells of *E. coli* DH10B. DNA fragments used in subcloning were separated by electrophoresis through a 0.8% agarose gel and then purified by electroelution from excised gel pieces in an Elutrap apparatus (Schleicher & Schuell, USA).

DNA Hybridizations

The recombinant plasmid DNAs and cloned DNAs for use as probes were labeled with digoxigenin-11-dUTP (DIG-dUTP) using the random primed method according to the manufacturer's instructions (Boehringer Mannheim, Germany). Dot and Southern blots were made on Hybond N filters (Amersham International, Buckinghamshire, England) as recommended by the manufacturer. Generally, 10 µg of DNA was added to each dot in dot blots performed with Bio-dot apparatus (Bio-Rad, USA) and 4 µg of DNA was added to each lane in Southern blots. DNA hybridization procedures and immunodetection of hybridization signals were performed according to the user's guide provided by the manufacturer (Boehringer Mannheim, Germany). Prehybridization and hybridization were conducted in hybridization buffer (5X SSC, 0.1% N-lauroyl sarcosine, 0.02% SDS, 1% blocking reagent) at 55°C for at least 5 h and at 68°C for at least 10 h, respectively. After hybridization, the filters were washed twice (10 min each) in 2X SSC containing 0.1% SDS at room temperature and twice (15 min each) in 0.1X SSC containing 0.1% SDS at 68°C. The hybridization signals were detected using the DIG luminescent detection kit (Boehringer Mannheim, Germany). The substrate for anti-DIG- conjugant alkaline phosphatase was Lumingen PPD (4-methoxy-4-[3-phosphate-phenyl]-spiro [1,2-dioxtane-3, 2'-adamantane] disodium salt).

Specificity and Sensitivity of Cloned DNA Probes

The specificity of the probes was determined by Southern blot hybridization. Total DNA (4.5 µg) from each of the bacteria listed in Table 1 was digested with *Eco*RI. In addition, the total DNAs of *X. campestris* pv. *campestris* and *X. campestris* pv. *armoraciae* were also digested with *Kpn*I. The DNA fragments separated by electrophoresis

Table 1. Strains of bacteria used in this study.

Bacterium	Strain	Host	Location	Source[a]
Burkholderia caryophylli	NCPPB2151	Carnation	U. S. A.	2
	PCAR1, 6	Carnation	Nantou, Yunlin	1
B. glumae	PG1	Rice	Taichung	1
Erwinia chrysanthemi	HAS11, 12	Welsh onion	Changhua	1
E. carotovora subsp. carotovora	SP45	Potato	Kaohsiung	1
	Cel-2	Celery	Taichung	1
Escherichia coli	ATCC11775			2
Pseudomonas cichorii	PC13-1, PC6	Celery	Taichung	1
P. syringae pv. *syringae*	Pss64	Wheat	U. S. A.	7
P. putida	YLFP1, 3	Cabbage	Tainan	1
	YLFP37	Mustard	Taoyuan	1
P. fluorescens	YLFP98	Radish	Taichung	1
Ralstonia solanacearum	PS63	Pepper	Nantou	1
	PS95, PSS4	Tomato	Taipei, Tainan	1
Xanthomonas campestris				
pv. *campestris*	Xcc4, 70, 77,78, 79, 94	Cabbage	Changhua	1
	Xcc7, 45	Cabbage	Hualien, Ilan	1
	Xcc34, 90, 91, 92, 93	Cauliflower	Changhua	1
	Xcc40	Kohlrabi	Taichung	1
	Xcc61, 111	Cabbage	Tainan, Taitung	1
	Xcc104, 105, 112, 113, 114	Cabbage	Tainan	3
	Xcc62, 68, 99	Cabbage	Taichung	1
	Xcc107	Cabbage	Taichung	3
	Xcc71, 72, 73, 74	Cabbage	Hsinchu	1
	Xcc75, 76	Broccoli	Kaohsiung	3
	Xcc80, 81, 82, 83, 84, 85, 87	Cabbage	Nantou	1
	Xcc108, 109, 110	Cabbage	Nantou	3
	Xcc86	Mustard	Yunlin	1
	Xcc88	Cabbage	Nantou	1
	Xcc89	Broccoli	Nantou	1
	Xcc95, 102, 115	Cauliflower	Tainan	3
	NCPPB528	Brussels sprouts	U. K.	2
	Xcc101	Kohlrabi	Tainan	3
	Xcc103	Broccoli	Tainan	3
	Xcc106	Cabbage	Kaohsiung	3
pv. *armoraciae*	417, 756	Cabbage seeds	East Asia	4
	A342	Broccoli	U. S. A.	4
	G3-27, XLS2, XLS6, XLS10	Cabbage	U. S. A.	4
pv. *begoniae*	ICMP194	Tuberous begonia	New Zealand	2
pv. *citri*	XW23, 32	Orange (Liucheng)	Chiayi, Kaohsiung	5
	XW27	Orange (Wentan)	Chiayi	5
	XW35	Orange (Jaffa)	Hsinchu	5
	XW47	Orange (Valencia)	Hualien	5
pv. *dieffenbachiae*	A044, 049, 058	Anthurium	Tainan	8
pv. *glycines*	XPS1, 3	Soybean	Yunlin	1
pv. *mangiferaeindicae*	3, 4, 12, 30	Mango	Tainan	7
pv. *phaseoli*	XP1, 4	Bean	Taichung	1
pv. *pruni*	3, 4, 5	Peach	Taichung	7
pv. *uppalii*	NCPPB586	*Ipomoea muricata*	India	2
	J-3, T-12-1	*I. nil*	Chiayi, Taichung	1
pv. *vesicatoria*	XV7, 12	Tomato	Taitung, Yunlin	1
	XV30, 48	Tomato	Tainan, Changhua	1
	XV42, 51	Pepper	Pingtung, Hualien	1
X. albilineans	ICMP196	Sugarcane	Fiji	2
X. fragariae	NCPPB1469	Strawberry	U. S. A.	2
X. oryzae pv. *oryzae*	21, 84	Rice	Taichung, Nantou	6
	117	Rice	Pingtung	6
Unidentified bacteria	Sb1, 3, 4	Cabbage leaves	Taichung	1
	Sb2	Cabbage leaves	Nantou	1
	Sb5, 6, 7	Cabbage seeds	Taichung	1

[a]1: Phytobacteriology laboratory, Department of Plant Pathology, National Chung Hsing University (NCHU), Taichung, Taiwan; 2: Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan; 3: J. F. Wang, Asian Vegetable Research and Development Center, Tainan, Taiwan; 4: A. M. Alvarez, Department of Plant Pathology, University of Hawaii, Honolulu, U. S. A.; 5: W. C. Wu, NCHU; 6: S. P. Y. Hsieh, NCHU; 7: H. S. Huang, NCHU; and 8: S. H. Hseu, Taiwan Agricultural Research Institute, Taichung, Taiwan.

through the agarose gel were denatured and transferred to Hybond N filters. The filters were then hybridized with the DIG-labeled DNA probe as described above.

The dot blot hybridization was used for probe sensitivity assays. Total DNA and cells of *X. campestris* pv. *campestris* Xcc70 cultured in LB broth were both two-fold serially diluted and spotted on Hybond N filters. Each spot contained the amount of total DNA ranging from 0.8 ng to 6.25 pg or the amount of cells ranging from 1.0×10^6 to 3.1×10^4 colony-forming units (CFU). The filters were then hybridized with the DIG-labeled DNA probe.

Detection of X. campestris pv. campestris in Leaves and Seeds

Direct detection of the pathogen in leaves and seeds was performed by dot blot hybridization. Leaves of cabbage with black rot-like lesions were collected from fields in Taichung. About 0.1 g of leaf lesions was placed in an Eppendorf tube and macerated in 100 µl sterile distilled water. Aliquots (5 µl) of the resulting suspension were spotted on the Hybond N filter. For seed detection, seven crucifer seed samples (three of cabbage and one each of Chinese kale, mustard, Chinese cabbage, and cauliflower) obtained from the Known-you Seed Company were assayed. Each seed sample (0.1 g) was ground in 0.25 ml of phosphate-buffered saline containing 0.1% Tween 80 in a mortar, and 5 µl of the resulting suspension was spotted on Hybond N filters. The blots for leaf and seed samples were then hybridized with the DIG-labeled DNA probe as previously described.

The same leaf and seed samples were also used in colony hybridization tests for rapid identification of the pathogen in the samples. After the samples were macerated, l0-fold serial dilutions were made, and 50 µl of appropriate dilutions were plated on PDA for leaf samples and on PDA containing 80 mg/ml of cycloheximide for seed samples. Colonies appearing on the plates after incubation at 30°C for 3 days were blotted on Hybond N filters, irradiated in a microwave oven, denatured with 0.5 M NaOH/I.5 M NaC1, and neutralized with 1 M Tris-HC1 (pH 7.2)/3 M NaC1 according to the procedure of Datta et al. (1988). After neutralization, the filters were air-dried, UV cross-linked, and hybridized with the DIG labeled DNA probe as previously described. Colonies randomly selected from the original plates were tested for their pathogenicity on leaves of cabbage plants grown in pots in a greenhouse at 25~35°C by the notched leaf inoculation method (Alvarez et al., 1994).

Results

Selection of the DNA Probe

Approximately 1000 transformant clones were obtained in the cloning of *Eco*RI-digested total DNA of *X. campestris* pv. *campestris* strain Xcc70. Recombinant plasmids of randomly selected 10 clones were isolated, labeled with digoxigenin and used as probes. These probes hybridized to total DNA of five strains of *X. campestris* pv. *campestris* tested, but also to that of some other pathovars of *X. campestris* in dot blots (data not shown). However, in Southern hybridization analyses, one of the 10 probes, pXcc70-8 (containing a 4.9 kb insert DNA) hybridized to three DNA fragments that were only observed in *Eco*RI-digested total DNA from strains of *X. campestris* pv. *campestris*, but not in that from strains of other bacteria initially screened (Figure 1). This clone (pXcc70-8) was therefore selected for subcloning. Digestion of the insert DNA of the clone pXcc70-8 with *Eco*RI yielded fragments of 2.7, 1.6 and 0.6 kb. The 0.6 kb fragment was cloned into pBluescript II KS and then transformed into *E. coli* DH10B. The cloned 0.6 kb *Eco*RI fragment, designated probe Xcc70-8-1, was further evaluated for its specificity against *X. campestris* pv. *campestris*.

Specificity and Sensitivity of Probe Xcc 70-8-1

The specificity of the probe Xcc70-8-1 labeled with digoxigenin was tested by the Southern hybridization with *Eco*RI-digested total DNA from various bacteria. All of the 51 strains of *X. campestris* pv. *campestris* and the seven strains of *X. campestris* pv. *armoraciae* (Table 1) gave a positive hybridization signal, whereas the 60 strains of other bacteria including other pathovars of *X. campestris*, *Xanthomonas* spp., distantly related bacteria, and unidentified saprophytic bacteria from cabbage leaves and seeds (Table 1) were all negative (representative results are shown in Figure 2). The probe hybridized to a single DNA fragment of 0.6 kb in all strains of *X.*

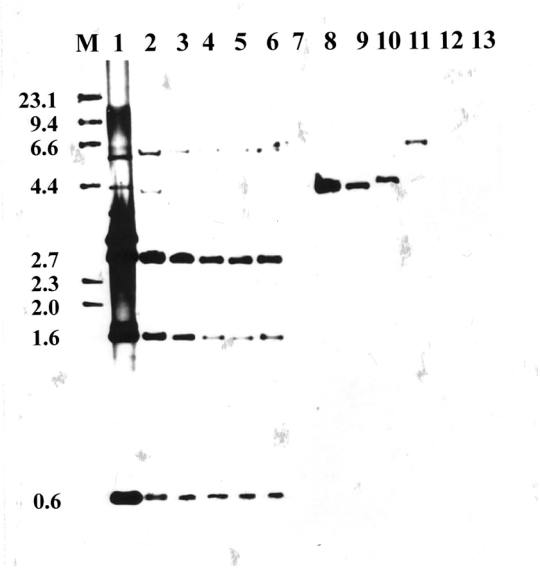

Figure 1. Southern hybridization of *Eco*RI-digested bacterial total DNAs probed with the digoxigenin-labeled clone pXcc70-8 selected from a genomic library of *Xanthomonas campestris* pv. *campestris*. Lane M, *Hin*dIII-digested lambda marker (the sizes in kb shown in the margin); lane 1, plasmid pXcc70-8 DNA; lanes 2-6, *X. campestris* pv. *campestris* strains Xcc70, Xcc78, Xccl02, Xccl03 and Xccl04; lane 7, unidentified bacterium Sbl; lanes 8-9, *X. campestris* pv. *vesicatoria* strains XV48 and XV7; lane 10, *X. campestris* pv. *citri* XW47; lane 11, *X. oryzae* pv. *oryzae* 21; lane 12, *Erwinia carotovora* subsp. *carotovora* SP45; lane 13, *Ralstonia solanacearum* PSS4.

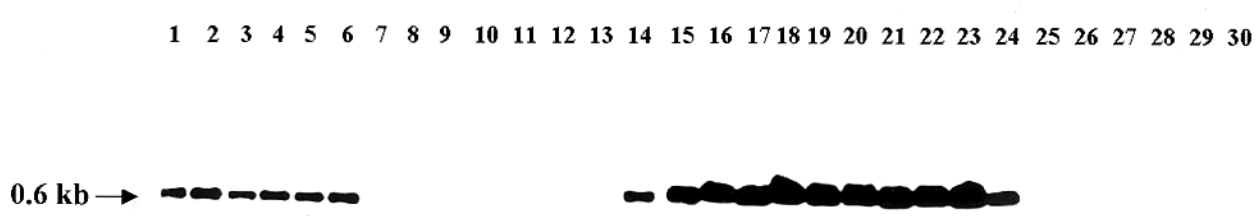

Figure 2. Southern blots of *Eco*RI-digested total DNAs from various bacteria hybridized with the digoxigenin-labeled probe Xcc70-8-1 containing a 0.6 kb insert DNA of *Xanthomonas campestris* pv. *campestris*. Lanes 1-6, *X. campestris* pv. *campestris* strains Xcc70, NCPPB528, Xccl0l, Xccl02, Xccl03, and Xccl04; lanes 7-8, *X. campestris* pv. *vesicatoria* strains XV7 and XV51; lane 9, *X. oryzae* pv. *oryzae* 21; lane 10, *X. campestris* pv. *citri* XW47; lane 11, *X. campestris* pv. *mangiferaeindicae* 30; lane 12, *Erwinia carotovora* subsp. *carotovora* SP45; lane 13, *Ralstonia solanacearum* PS95; lane 14, *X. campestris* pv. *campestris* Xcc88; lane 15, plasmid pXcc70-8-1 DNA; lanes 16-22, *X. campestris* pv. *campestris* strains Xccl07, Xccl08, Xccl09, Xccll0, Xcc71, Xcc72 and Xcc99; lanes 23-24, *X. campestris* pv. *armoraciae* strains 417 and XLS10; lane 25, *X. campestris* pv. *begoniae* ICMP194; lane 26, *X. campestris* pv. *uppalii* NCPPB586; lane 27, *X. albilineans* ICMP196; lane 28, *Burkholderia caryophylli* NCPPB2151; lane 29, *Ralstonia solanacearum* PSS4; lane 30, *Pseudomonas putida* YLFPI. The size (in kb) is shown in the margin.

campestris pv. *campestris* and *X. campestris* pv. *armoraciae* tested. However, when total DNAs of these two pathovars were digested with *Kpn*I, the probe detected a single 0.7 kb fragment in all strains of *X. campestris* pv. *campestris* and a single 2.5 kb fragment in all strains of *X. campestris* pv. *armoraciae* tested (Figure 3). The sensitivity of the probe was determined by dot blot to a dilution series of total DNA and cells of strain Xcc70. The detection limits were 25 pg of DNA and 6.2×10^4 CFU of cells.

Detection of X. campestris pv. campestris in Infected Leaves and Seeds of Crucifers

The hybridization signals were observed on the Xcc70-8-l-probed dot blots from extracts of infected cabbage leaves and also from extracts of two (Chinese kale and Chinese cabbage) of the seven seed samples tested. Yellow, mucoid colonies could be isolated on the medium from the extracts of the hybridization positive samples, and these colonies were identified as *X. campestris* pv. *campestris* by the pathogenicity test.

Several types of colonies were formed on the media plated with the suspensions of macerated tissues of leaves and seeds. In colony hybridization probed with Xcc70-8-1, the signals were observed on some colonies formed from the cabbage leaves (Figure 4) and also from the two (Chinese kale and Chinese cabbage) of seven seed samples. These hybridization positive colonies produced black rot symptoms when inoculated into cabbage leaves, confirming that they were *X. campestris* pv. *campestris*, whereas those hybridization negative colonies were not pathogenic to cabbage.

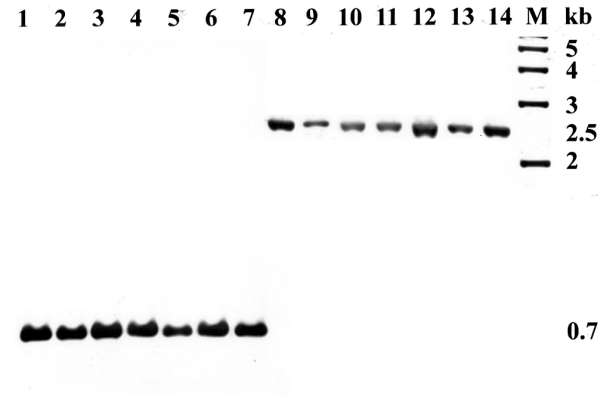

Figure 3. Southern blots of *Kpn*I-digested total DNAs from *Xanthomonas campestris* pv. *campestris* and *X. campestris* pv. *armoraciae* hybridized with the digoxigenin-labeled probe Xcc70-8-1 containing a 0.6 kb insert DNA of *X. campestris* pv. *campestris*. Lanes 1-7, *X. campestris* pv. *campestris* strains Xcc34, Xcc70, Xcc88, Xcc92, Xccl04, Xccl05 and Xccl06; lanes 8-14, *X. campestris* pv. *armoraciae* strains 417, A342, G3-27, 756, XLS2, XLS6 and XLSI0; lane M, 1 kb marker (Stratagene, USA). The sizes (in kb) are shown in the margin.

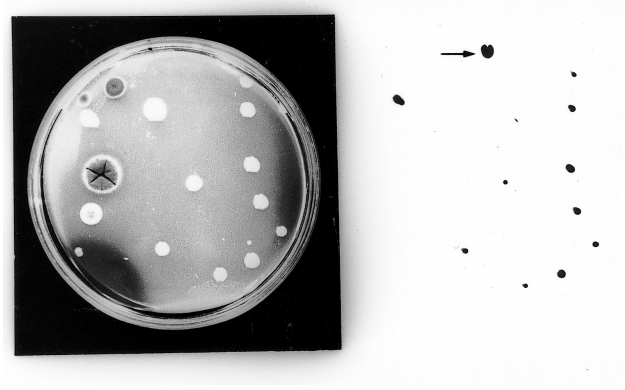

Figure 4. Colony hybridization analysis of the bacterial colonies formed on a potato dextrose agar plate spread with the extract of the black rot-infected cabbage leaves. A, plate before the colonies were blotted; B, hybridization of the colonies blotted on a Hybond N filter with the digoxigenin-labeled probe Xcc70-8-1. Hybridization signals were observed only for colonies of *Xanthomonas campestris* pv. *campestris*. The Xcc-70-8-1 plasmid DNA was blotted in the arrow place as CK.

Discussion

Development of specific DNA probes for identification and detection has been reported for a number of plant pathogenic bacteria (Rasmussen and Reeves, 1992; Hartung, 1992; Darrasse et al., 1994). Some of these DNA probes are developed by screening randomly cloned fragments of total genomic or plasmid DNA for their specificity (Gilbertson et al., 1989; Johansen et al., 1989; Thompson et al., 1989; Manulis et al., 1991; Hartung, 1992). In this study, the same approach was used to obtain a DNA probe specific for *X. campestris* pv. *campestris*. Unlike some pathovars of *X. campestris*, strains of *X. campestris* pv. *campestris* are variable in plasmid content (Lazo and Gabriel, 1987). Therefore, we used the total genomic DNA from a strain (Xcc70) of *X. campestris* pv. *campestris* to construct a genomic library from which a recombinant clone (pXcc70-8) containing partly specific insert DNA was identified. When the recombinant plasmid of this clone, pXcc70-8, was used to probe Southern blots of genomic digests from strains of *X. campestris* pv. *campestris*, restriction fragment length polymorphism was evident within these strains. This result agrees with the reports of Alvarez et al. (1994) and Chiu et al. (1996), who showed that strains of *X. campestris* pv. *campestris* are genetically heterogeneous based on RFLP analysis of total DNA. However, the hybridization pattern of *Eco*RI-digested total DNA probed with pXcc70-8 revealed a 0.6 kb fragment that was observed in strains of *X. campestris* pv. *campestris* but not in strains of other bacteria initially tested. A subclone (pXcc70-8-1) containing this 0.6 kb fragment was subsequently proved to be specific for *X. campestris* pv. *campestris*, except for *X. campestris* pv. *armoraciae*. Further tests showed that this probe distinguished between the *X. campestris* pv. *campestris* and pv. *armoraciae* by revealing a single DNA fragment of different size detected in their total DNAs digested with *Kpn*I. Because the DNA probe was tested against strains of *X. campestris* pv. *campestris* only from Taiwan except one strain, the specificity of the probe to strains from other geographical regions remains to be determined. However, for use in Taiwan, the probe should be useful for rapid identification of *X. campestris* pv. *campestris*.

The probe Xcc70-8-1 developed in this study identified and distinguished *X. campestris* pv. *campestris* colonies from colonies of other bacteria on the agar media plated with extracts of infected leaves using the colony hybridization assay and detected the *X. campestris* pv. *campestris* directly in infected leaves using the dot blot hybridization method. In colony hybridization tests, colonies that showed hybridization signals were confirmed to be *X. campestris* pv. *campestris* as determined by the pathogenicity test. Likewise, in the dot blot assays, *X. campestris* pv. *campestris* was isolated from the hybridization positive samples. These results indicate that the hybridization procedures can be used for detection or confirmation of the presence of *X. campestris* pv. *campestris* in plant tissues. Although *X. campestris* pv. *campestris* in infected plant tissues usually have populations high enough to be readily recovered on selective media, the DNA probe assay is more rapid and specific. Furthermore, the DNA probe is feasible for assaying large numbers of samples in a relatively short time.

Serological tests have been used for rapid identification and detection of *X. campestris* pv. *campestris*. However, polyclonal antibodies against strains of *X. campestris* pv. *campestris* often cross-react with one or a few other pathovars of *X. campestris* (Alvarez and Lou, 1985; Franken et a1., 1992; Schaad, 1978; Thaveechai and Schaad, 1984, 1986). For example, strains of *X. campestris* pv. *vesicatoria* often react with the polyclonal antibodies of *X. campestris* pv. *campestris* (Alvarez and Lou, 1985; Franken et al., 1992; Schaad, 1978; Thaveechai and Schaad, 1984, 1986). *Xanthomonas campestris* pv. *vesicatoria* is a widely distributed pathogen causing bacterial spot disease on tomato and pepper in Taiwan (Hartman et al., 1990). The chance of introducing this bacterial spot pathogen to the fields of cruciferous crops in Taiwan exists because of the planting multiple crops within a relatively small area and the spread of the pathogen by wind-driven rain. Therefore, differentiation between *X. campestris* pv. *campestris* and pv. *vesicatoria* would still be desired in studies involving, for example, the recovery of *X. campestris* pv. *campestris* from field samples that might be contaminated with *X. campestris* pv. *vesicatoria*. Our results showed that the probe Xcc70-8-l did not hybridize with any of the six strains of *X. campestris* pv. *vesicatoria* tested and can be used for their differentiation. Monoclonal antibodies produced against *X. campestris* pv. *campestris* are highly specific although strains of *X. campestris* pv. *armoraciae* may possess some of the same antigenic determinants (Alvarez et al., 1985, 1994). Our results also showed that DNAs from strains of *X. campestris* pv. *armoraciae* hybridized to the probe Xcc70-8-l. These two pathovars are considered to be very closely related (Alvarez et al., 1994). However, pv. *campestris* could be distinguished from pv. *armoraciae* by this probe when the total DNAs were digested with *Kpn*I. The seven strains of *X. campestris* pv. *armoraciae* used in our DNA hybridization tests represented strains that also reacted with monoclonal antibodies generated to *X. campestris* pv. *campestris* (Alvarez et al., 1994). Thus, our DNA probe offers an advantage over serological methods for specific identification or differentiation of these two pathovars. The ability of the DNA probe to rapidly distinguish *X. campestris* pv. *campestris* from pv. *armoraciae* is valuable because these two pathovars infect crucifers, and the pathogens recovered from cruciferous plant and seed samples previously were reliably differentiated only by time-consuming pathogenicity tests (Alvarez et al., 1994).

A diagnostic tool's effectiveness depends largely on its sensitivity in addition to its specificity. The sensitivity of the probe Xcc70-8-l for detecting *X. campestris* pv. *campestris* was about 10^4 CFU, similar to that of the enzyme-linked immunosorbent assay method (Alvarez and Lou, 1985), but not as sensitive as either the immunofluorescence method (Domen and Alvarez, 1978) or the miniplate ELISA enrichment method (Arias et al., 1996).

The probe Xcc70-8-l may not be sensitive enough to detect *X. campestris* pv. *campestris* with populations normally present in alternative weed hosts, soils, and seeds. Although a small sample size (5 µl taken from the extract of 25 seeds ground in 250 µl buffer) was taken, *X. campestris* pv. *campestris* was detected in two of the seven seed samples tested by the DNA hybridization assay used in the present study. Therefore, these two seed samples were likely to have been naturally infested with unusually high numbers of the pathogen. In seed assays, a DNA probe should also detect strains of *X. campestris* pv. *campestris* from different geographical regions because the seed-borne pathogen could be imported from different regions. The specificity of our DNA probe to non-local strains has not been determined. Therefore, unless specificity and sensitivity can be satisfied, the DNA probe in its present form is not well-suited for seed assays. Its best use would be in providing a useful tool for rapid identification of *X. campestris* pv. *campestris* in epidemiological studies in Taiwan. An improvement in detection sensitivity may be achieved by the development of an amplification technique such as PCR using a primer set which can be designed from the sequence of the cloned DNA fragment (Saiki et al., 1988). Such developments are currently underway.

Acknowledgments. This study was supported by Grants NSC83-0409-B005-029 and NSC84-2321-B005-070 from the National Science Council, Republic of China.

Literature Cited

Aline Jr., R. and R.L. Gabrielson. 1993. PCR detection of *Xanthomonas campestris* pathovar *campestris*. Abstr. 6th Int. Cong. Plant Pathol., Montreal, Canada, pp. 46.

Alvarez, A.M. and J.J. Cho. 1978. Black rot of cabbage in Hawaii: Inoculum source and disease incidence. Phytopathology **68:** 1456-1459.

Alvarez, A.M. and K. Lou. 1985. Rapid identification of *Xanthomonas campestris* pv. *campestris* by ELISA. Plant Dis. **69:** 1082-1086.

Alvarez, A.M., A.A. Benedict, and C.Y. Mizumoto. 1985. Identification of xanthomonads and grouping of strains of *Xanthomonas campestris* pv. *campestris* with monoclonal antibodies. Phytopathology **75:** 722-728.

Alvarez, A.M., A.A. Benedict, C.Y. Mizumoto, J.E. Hunter, and D.W. Gabriel. 1994. Serological, pathological, and genetic diversity among strains of *Xanthomonas campestris* infecting crucifers. Phytopathology **84:** 1449-1457.

Arias, R.S., G. Mochizuki, R. Fukui, and A.M. Alvarez. 1996. Most probable number method to enumerate a bioluminescent *Xanthomonas campestris* pv. *campestris* in soil. Soil Biol. Biochem. **28:** 1725-1728.

Chiu, S.P., C.M. Chang, M.Y. Chen, and Y.A. Lee. 1996. Genetic diversity among strains of *Xanthomonas campestris* pv. *campestris* in Taiwan. (Abstr.) Plant Pathol. Bull. **5:** 211-212.

Chun, W.W.C. and A.M. Alvarez. 1983. A starch-methionine medium for isolation of *Xanthomonas campestris* pv. *campestris* from plant debris in soil. Plant Dis. **67:** 632-635.

Darrasse A., A. Kotoujansky, and Y. Bertheau. 1994. Isolation by genomic substraction of DNA probes specific for *Erwinia carotovora* subsp. *atroseptica*. Appl. Environ. Microbiol. **60:** 289-306.

Datta, A.R., B.A. Wentz, D. Shook, and M.W. Trucksess. 1988. Synthetic oligodeoxyribonucleotide probes for detection of *Listeria monocytogenes*. Appl. Environ. Microbiol. **54:** 2933-2937.

Domen, H.Y. and A.M. Alvarez. 1978. Detection of *Xanthomonas campestris* in soil using a direct immunofluorescent technique. *In* Proc. 4th Int. Conf. Plant Pathog. Bact., Angers, France, pp. 301-305.

Franken, A.A.J.M. 1992. Comparison of immunofluorescence microscopy and dilution-plating for the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds. Neth. J. Plant Pathol. **98:** 169-178.

Franken, A.A.J.M., J.F. Zilverentant, P.M. Boonekamp, and A. Schots. 1992. Specificity of polyclonal and monoclonal antibodies for the identification of *Xanthomonas campestris* pv. *campestris*. Neth. J. Plant Pathol. **98:** 81-94.

Fukui, R., R. Arias, and R. Alvarez. 1994. Efficacy of four semiselective media for recovery of *Xanthomonas campestris* pv. *campestris* from tropical soils. J. Appl. Bacteriol. **77:** 534-540.

Gilbertson, R.L., D.P. Maxwell, D.J. Hagedom, and S.A. Leong. 1989. Development and application of a plasmid DNA probe for detection of bacteria causing common bacterial blight of bean. Phytopathology **79:** 518-525.

Hartman, G.L., C.H. Yang, and J.F. Wang. 1990. Races of *Xanthomonas campestris* pv. *vesicatoria* in Taiwan and an inoculation technique to evaluate host resistance. Plant Prot. Bull. **32:** 112-124.

Hartung, J.S. 1992. Plasmid-based hybridization probes for detection and identification of *Xanthomonas campestris* pv. *citri*. Plant Dis. **76:** 889-893.

Huang, T.C. and S.T. Hsu. 1987. Efficiency of various selective media for detection of *Xanthomonas campestris* pv. *campestris* in Taiwan. Plant Prot. Bull. **29:** 203-215.

Johansen, I.E., O.F. Rasmussen, and M. Heide. 1989. Specific identification of *Clavibacter michiganense* subsp. *sepedonicum* by DNA-hybridization probes. Phytopathology **79:** 1019-1023.

Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology **44:** 693-695.

King, E.O., M.K. Ward, and D.E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. **44:** 301-307.

Lazo, G.R. and D.W. Gabriel. 1987. Conservation of plasmid DNA sequences and pathovar identification of strains of *Xanthomonas campestris*. Phytopathology **77:** 448-453.

Lazo, G.R., R. Roffey, and D.W. Gabriel. 1987. Pathovars of *Xanthomonas campestris* are distinguishable by restriction fragment-length polymorphism. Int. J. Syst. Bacteriol. **37:** 214-221.

Manulis, S., Y. Gafni, E. Clark, D. Zutra, Y. Ophir, and I. Barash. 1991. Identification of a plasmid DNA probe for detection of strains of *Erwinia herbicola* pathogenic on *Gypsophila paniculata*. Phytopathology **81:** 54-57.

Rasmussen, O.F. and J.C. Reeves. 1992. DNA probes for the detection of plant pathogenic bacteria. J. Biotechnol. **25:** 203-220.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G. T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. Science **239:** 487-491.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schaad, N.W. 1978. Use of direct and indirect immunofluorescence tests for identification of *Xanthomonas campestris*. Phytopathology **68:** 249-252.

Schaad, N.W. 1979. Serological identification of plant pathogenic bacteria. Ann. Rev. Phytopathol. **17:** 123-147.

Schaad, N.W. 1982. Detection of seedborne bacterial plant pathogens. Plant Dis. **66:** 885-890.

Schaad, N.W. and J.C. Dianese. 1981. Cruciferous weeds as sources of inoculum of *Xanthomonas campestris* in black rot of crucifers. Phytopathology **71:** 1215-1220.

Schaad, N.W. and R.C. Donaldson. 1980. Comparison of two methods for detection of *Xanthomonas campestris* in infected crucifer seeds. Seed Sci. Technol. **8:** 383-391.

Schaad, N.W. and W.C. White. 1974a. A selective medium for soil isolation and enumeration of *Xanthomonas campestris*. Phytopathology **64:** 876-880.

Schaad, N.W. and W.C. White. 1974b. Survival of *Xanthomonas campestris* in soil. Phytopathology **64:** 1518-1520.

Thaveechai, N. and N.W. Schaad. 1984. Comparison of different immunogen preparations for serological identification of *Xanthomonas campestris* pv. *campestris*. Phytopathology **74:** 1065-1070.

Thaveechai, N. and N.W. Schaad. 1986. Serological and electrophoretic analysis of a membrane protein extract of *Xanthomonas campestris* pv. *campestris* from Thailand. Phytopathology **76:** 139-147.

Thompson, E., J.V. Leary, and W.W.C. Chun. 1989. Specific detection of *Clavibacter michiganense* subsp. *michiganense* by a homologous DNA probe. Phytopathology **79:** 311-314.

Walker, J.C. and W.B. Tisdale. 1920. Observations on seed transmission of the cabbage black rot organism. Phytopathology **10:** 175-177.

Williams, P.H. 1980. Black rot: A continuing threat to world crucifer. Plant Dis. **64:** 736-742.

Yuen, G.Y., A.M. Alvarez, A.A. Benedict, and K.J. Trotter. 1987. Use of monoclonal antibodies to monitor the dissemination of *Xanthomonas campestris* pv. *campestris*. Phytopathology **77:** 366-370.

鑑定台灣十字花科蔬菜黑腐病菌之核酸探針

石信德[1] 林元春[1] 黃秀珍[2] 曾國欽[1] 徐世典[1]

[1]國立中興大學植物病理學系

[2]國立中興大學農業生物科技學研究所

十字花科蔬菜黑腐病菌 (*Xanthomonas campestris* pv. *campestris*) Xcc70 菌株之全 DNA 經 *Eco*RI 切割後，選殖於質體 pBluescript II KS 內，再轉形至大腸桿菌 DHl0B 中。由此隨機選取之選殖株，將其重組質體以 digoxigenin 標識後作為探針，用於篩選其對鑑定黑腐病菌台灣菌株之專一性。在南方雜配分析上，其中一個選殖株 (pXcc70-8) 僅能與黑腐病菌全 DNA 以 *Eco*RI 切割後之數個片段產生雜配訊號。此選殖株內之黑腐病菌嵌入 DNA 片段以 *Eco*RI 切割後含有 2.7，1.6 及 0.6 kb 三個片段，將其中之 0.6 kb 片段藉由次選殖後所製備的探針 (Xcc70-8-1) 與黑腐病菌所有 51 個菌株及蔬菜細菌性葉斑病菌 (*X. campestris* pv. *armoraciae*) 7 個菌株均有雜配訊號，而與其他細菌之 60 個供試菌株，則無任何雜配反應。當黑腐病菌及細菌性葉斑病菌的全 DNA 以 *Kpn*I 切割後，此探針在所測試的 51 個黑腐病菌株，均可雜合出單一條 0.7 kb 片段，而在所測試的 7 個葉斑病菌株，則可雜合出單一條 2.5 kb 片段。此探針 Xcc70-8-1 能偵測黑腐病菌 DNA 量及細胞數之最低限度分別為 25 pg 及約 6 x l0^4 CFU。利用菌落及點漬雜配法偵測罹病甘藍葉片及數種十字花科蔬菜種子磨碎液中的黑腐病菌，結果顯示此DNA探針可用於偵測植物組織內的黑腐病菌，但較不適用於種子之偵測。在台灣黑腐病流行病學研究上，此探針可作為快速及正確鑑定黑腐病菌之有用工具。

關鍵詞：十字花科蔬菜；黑腐病菌；核酸探針；偵測；鑑定。