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## **Differentiation of Phytopathogenic *Pseudomonas* and *Xanthomonas* Pathovars and Strains by PCR Analysis for DNA Topoisomerase Genes**

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Differentiation of pathovars of *Pseudomonas syringae* and *Xanthomonas campestris* was conducted by analysis with polymerase chain reaction (PCR) of topoisomerase genes. Differences among the pathovars were observed on the migration patterns of the PCR products on agarose gel. Banding patterns of respective strains were pathovar specific with some exceptions. The technique is rapid, simple and reproductive to identify and classify phytopathogenic *Pseudomonas syringae* and *Xanthomonas campestris* at pathovar level, and it may be a useful diagnostic tool for these important plant pathogens.

### INTRODUCTION

The species *Pseudomonas syringae* and *Xanthomonas campestris* are subdivided at the infraspecific level into many pathovars. Since pathovars can not be readily distinguished by the ordinary physiological and biochemical methods (Dye, 1962; Palleroni, 1984; Van Zyl and Steyn, 1990), they are classified on the basis of their distinctive pathogenicity to one or more host plants. Unfortunately, identification based on the pathogenicity tests can be inconclusive and open to alternative interpretations (Lazo *et al.*, 1987; Gabriel *et al.*, 1989). Currently, serological tests (Benedict *et al.*, 1989, 1990), fatty acid profiling (Stead, 1992; Vauterin *et al.*, 1992), protein analysis (Van Zyl and Steyn, 1990; Vauterin *et al.*, 1991a, b; Li and Hayward, 1994) and nucleic acid based techniques (Lazo *et al.*, 1987; Alizadeh *et al.*, 1997; Hauben *et al.*, 1997; Manceau and Horvais, 1997) have been used to classify pathovars and strains of *Pseudomonas syringae* and *Xanthomonas campestris*. However, these techniques are often time-consuming, too expensive, or too insensitive for use in routine diagnosis. We have used PCR technique to identify rapidly and classify closely related pathogenic bacteria on the basis of genomic fingerprint approaches.

Eubacteria have two indispensable type II DNA topoisomerase, DNA gyrase encoded by *gyrB* and *gyrA* and topoisomerase IV encoded by *parE* and *parC*. These genes belong to a single family whose members span both eucaryotes and prokaryotes (Huang, 1996). The type II DNA topoisomerase gene sequences are easily obtainable from diverse sources and the high sequence conservation of this family of the genes throughout the evolution

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provides a rationale for design of universal primers used in the polymerase chain reaction in order to systematically generate a data set. We demonstrated the utility of the PCR technique with primers corresponding to *gyrB* gene sequences to generate specific DNA fingerprints and to classify and identify pathovars of phytopathogenic *P. syringae* and *X. campestris*.

## MATERIALS AND METHODS

### Bacterial strains

All the strains used are listed in Table 1. Strains of *Pseudomonas syringae* and *Xanthomonas campestris* were grown on King'B (King *et al.*, 1954) and YDC (Wilson, 1967) medium, respectively. Stock cultures were maintained at 4 °C.

### PCR-assay

Bacterial cultures were grown in 523 broth (Kado and Heskett, 1970) for 24 h at 30 °C. Bacterial cells were harvested in an Eppendorf tube by centrifugation at 5000 rpm for 5 min. About 5 mg bacterial cells was suspended homogeneously in 500 µl TE buffers (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and heated at 95 °C for 8 min. Centrifugation was carried out at 5000 rpm for 30 second and the supernatants were used for PCR.

The PCR primers GYRB-P (5' GTAGGTGTGTCGGTTGTGAA-3') and GYRB-PR (5'-TTTACGGCGGGTCATCTCAC-3') used in this experiment were designed from the conserved regions in the *gyrB* gene by comparing *P. putida* (Yamamoto and Harayama, 1995). PCR was carried out in a 100 µl reaction mixture containing 68.5 µl deionized water, 10 x reaction buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl and 1% Triton X-100) 10 µl, 25 mM MgCl<sub>2</sub> 10 µl, 5 mM dNTPs 4 µl, DNA solution 5 µl, each primer 1 µl and *Taq* DNA polymerase 0.5 µl, and was overlaid with 50 µl of mineral oil. The following PCR programme was used: 1 cycle 94 °C for 2 min, 45 °C for 3 min and 72 °C for 1 min; 30 cycles 93 °C for 1 min, 42 °C for 1 min and 72 °C for 3 min. Amplified DNA was subjected to electrophoresis in 2% agarose gel, stained with ethidium bromide, and photographed on a UV transilluminator. Fingerprints generated for different stains were compared visually.

## RESULTS AND DISCUSSION

Primers corresponding to DNA topoisomerase genes generated a specific genomic fingerprint for the strains of *Pseudomonas* and *Xanthomonas*. Differences among their pathovars were assessed visually on the basis of the migration patterns of PCR products on agarose gel electrophoresis. Banding patterns of the strains were pathovar specific in the most cases and homogeneity was observed among the strains within pathovar. Among the strains of *P. syringae*, distinct banding patterns were observed in *P. syringae* pv. *tomato*, pv. *lachrymans*, pv. *japonica*, pv. *myricae*, pv. *oryzae*, pv. *glycinea*, pv. *tabaci*, pv. *theae*, pv. *coronafaciens*, pv. *atropurpurea* and pv. *mori*. However, fingerprints generated for *P. syringae* pv. *syringae* and pv. *phaseolicola*; *P. syringae* pv. *pisi* and pv. *striafaciens* were identical (Figs. 1a, b)

PCR amplified products also generated unique genomic fingerprints for pathovars of *X. campestris* tested. The fingerprint patterns of the representative strains of *X.*

**Table 1.** List of *Pseudomonas* and *Xanthomonas* pathovars and strains.

Bacterial strain	Isolate	Source
<i>P. syringae</i>		
<i>pv. syringae</i>	ATCC 19310 <sup>T</sup>	ATCC
<i>pv. oryzae</i>	MAFF 301538	NIAR
<i>pv. tabaci</i>	PA-28	KTES
<i>pv. tabaci</i>	Ku-7102	AKU
<i>pv. lachrymans</i>	NIAS 1319	NIAS
<i>pv. lachrymans</i>	NIAS 1321	∕
<i>pv. mori</i>	P-23	∕
<i>pv. pisi</i>	MAFF 301211	NIAR
<i>pv. pisi</i>	MAFF 301213	∕
<i>pv. theae</i>	MAFF 750001	∕
<i>pv. coronafaciens</i>	MAFF 301314	∕
<i>pv. phaseolicola</i>	MAFF 301616	∕
<i>pv. phaseolicola</i>	MAFF 301716	∕
<i>pv. japonica</i>	MAFF 301163	∕
<i>pv. atropurpurea</i>	MAFF 301307	∕
<i>pv. strajfaciens</i>	P-71	NIAS
<i>pv. myricae</i>	MAFF 301464	NIAR
<i>pv. tomato</i>	MAFF 301593	∕
<i>pv. glycinea</i>	KN-28	NIAS
<i>X. campestris</i>		
<i>pv. citri</i>	12-5	AKU
<i>pv. citri</i>	32-5	∕
<i>pv. citri</i>	Ku-7501	∕
<i>pv. citri</i>	C 1	∕
<i>pv. citri</i>	C 2	∕
<i>pv. campestris</i>	ATCC 33913 <sup>T</sup>	ATCC
<i>pv. campestris</i>	I	TUA
<i>pv. hyacinthi</i>	X 1-4-1	NIAS
<i>pv. translucens</i>	X 1-11-2	∕
<i>pv. glycines</i>	S-9	AKU
<i>pv. glycines</i>	S-10	∕
<i>pv. vitians</i>	I	NIAS
<i>pv. vitians</i>	II	∕
<i>pv. pisi</i>	I	∕
<i>pv. pisi</i>	II	∕
<i>pv. pisi</i>	III	∕
<i>pv. physalidicola</i>	2	∕
<i>pv. phaseoli</i>	318-1	∕

ATCC: American Type Culture Collection, USA.

NIAR: National Institute of Agrobiological Resources, Tsukuba, Japan.

KTES: Kagoshima Tobacco Experiment Station, Japan.

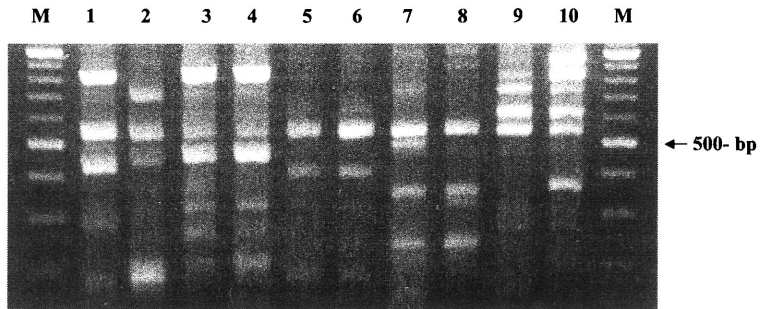
AKU: Faculty of Agriculture, Kyushu University, Fukuoka, Japan.

TUA: Tokyo University of Agriculture, Tokyo, Japan.

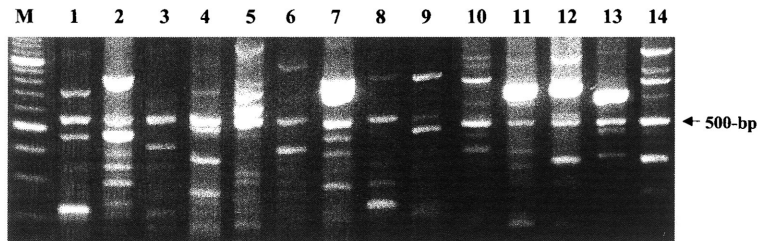
NIAS: National Institute of Agricultural Sciences, Tokyo, Japan.

KNAES: Kyushu National Agricultural Experiment Station, Kumamoto, Japan.

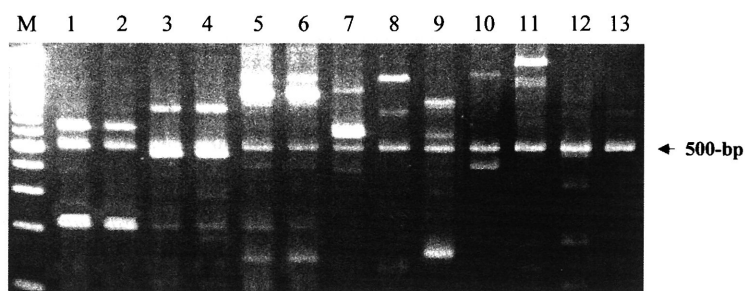
Type culture: Small T at the shoulder of isolate number indicates type culture.



**Fig. 1a.** Electrophoretic patterns of PCR-amplified topoisomerase genes of *P. syringae* pathovars and strains.  
 M: Molecular marker (100-bp ladder); 1: *P. syr. pv. syringae* ATCC 19310<sup>T</sup>; 2: *pv. tomato* MAFF 301593; 3: *pv. phaseolicola* MAFF 301716; 4: *pv. phaseolicola* MAFF 301616; 5: *pv. pisi* MAFF 301211; 6: *pv. pisi* MAFF 301213; 7: *pv. tabaci* PA-28; 8: *pv. tabaci* Ku-7102; 9: *pv. lachrymans* NIAS 1319; 10: *pv. lachrymans* NIAS 1321.



**Fig. 1b.** Electrophoretic patterns of PCR-amplified topoisomerase genes of *P. syringae* pathovars and strains.  
 M: Molecular marker (100-bp ladder); 1: *pv. tomato* MAFF 301593; 2: *pv. phaseolicola* MAFF 301716; 3: *pv. pisi* MAFF 301211; 4: *pv. tabaci* PA-28; 5: *pv. lachrymans* NIAS 1319; 6: *pv. strairfaciens* P 71; 7: *pv. oryzae* MAFF 301538; 8: *pv. glycinea* KN-28; 9: *pv. mori* P-23; 10: *pv. japonica* MAFF 301163; 11: *pv. coronafaciens* MAFF 301314; 12: *pv. atropurpurea* MAFF 301307; 13: *pv. myricae* MAFF 301464; 14: *pv. theae* MAFF 750001.



**Fig. 2.** Electrophoretic patterns of PCR-amplified topoisomerase genes of *Xanthomonas* pathogens and strains.

Lanes M: Molecular marker (100-bp ladder), 1: *X. campestris* pv. *citri* 32-5, 2: pv. *citri* Ku 7501, 3: pv. *lisi* II, 4: pv. *lisi* III, 5: pv. *vitians* I, 6: pv. *vitians* II, 7: pv. *hyacinthi* X-1-4-1, 8: pv. *physalidicola* 2, 9: pv. *pruni* 1-10-1, 10: pv. *phaseoli* 318-1, 11: pv. *translucens* X-1-11-2, 12: pv. *campestris* I, 13: pv. *glycines* S-10.

*campestris* pv. *citri*, pv. *lisi*, pv. *vitians*, pv. *hyacinthi*, pv. *physalidicola*, pv. *pruni*, pv. *phaseoli*, pv. *translucens*, pv. *campestris* and pv. *glycines* are shown in Fig. 2. Five strains of *X. c.* pv. *citri*, three strains of *X. c.* pv. *lisi* and two strains of *X. c.* pv. *vitians* showed homogenous patterns within the respective pathogens. Specific banding patterns were also observed for one strain of each *X. c.* pv. *hyacinthi*, pv. *physalidicola*, pv. *pruni*, pv. *phaseoli* and pv. *translucens*. However, those of strains of *X. c.* pv. *campestris* and *X. c.* pv. *glycines* were identical and not distinguishable each other (Fig. 2). The experiment was repeated 2 to 3 times and reproducible results for fingerprint profiles were obtained.

The data presented here suggest that the technique is useful to identify *P. syringae* and *X. campestris* at pathovar level, and this method will provide a rapid and precise way to identify closely related plant pathogenic bacteria.

## REFERENCES

- Alizadeh, A., M. Arlat, A. Sarrafi, C. A. Boucher and G. Barrault 1997 Restriction fragment length polymorphism analyses of Iranian strains of *Xanthomonas campestris* from cereals and grasses. *Plant Dis.*, **81**: 31-35.
- Benedict, A. A., A. M. Alvarez, J. Berestecky, W. Imanaka, C. Y. Mizumoto, L. W. Pollard, T. W. Mew and C. F. Gonzalez 1989 Pathovar specific monoclonal antibodies for *Xanthomonas campestris* pv. *oryzae* and for *Xanthomonas campestris* pv. *oryzicola*. *Phytopathology*, **79**: 322-328.
- Benedict, A. A., A. M. Alvarez and L. W. Pollard 1990 Pathovar specific antigens for *Xanthomonas campestris* pv. *begoniae* and *Xanthomonas campestris* pv. *pelargonii* detected with monoclonal antibodies. *Appl. Environ. Microbiol.*, **56**: 572-574.
- Dye, D. W. 1962 The inadequacy of the usual determinative tests for the identification of *Xanthomonas* spp. *N. Z. J. Sci.*, **5**: 393-416.
- Gabriel, D. W., M. T. Kingsley, G. E. Hunter and T. Gottwald 1989 Reinstatement of *Xanthomonas citri*

- (ex Hasse) and *X. phaseoli* (ex Smith) to species and reclassification of all *X. campestris* pv. *citri* strains. *Int. J. Syst. Bacteriol.*, **39**: 14–22.
- Hauben, L., L. Vauterin, J. Swings and E. R. B. Moore 1997 Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *Int. J. Syst. Bacteriol.*, **47**: 328–335.
- Huang, W. M. 1996 Bacterial diversity based on type II DNA topoisomerase genes. *Ann. Rev. Gen.* **30**: 79–107.
- Kado, C. I. and M. G. Heskett 1970 Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*. *Phytopathology*, **60**: 969–976.
- 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., 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.
- Li, X. and A. C. Hayward 1994 Bacterial whole cell protein profiles of the rRNA group II pseudomonads. *J. Appl. Bacteriol.*, **77**: 308–318.
- Manceau, C. and A. Horvias 1997 Assessment of genetic diversity among strains of *Pseudomonas syringae* by PCR–restriction fragment length polymorphism analysis of rRNA operons with special emphasis on *P. syringae* pv. *tomato*. *Appl. Environ. Microbiol.*, **63**: 498–505.
- Palleroni, N. J. 1984 Genus I *Pseudomonas* Mingula 1894. In Bergcy's Manual of Systematic Bacteriology. Vol. 1. Baltimore: Williams & Wilkins. pp.141–199.
- Stead, D. E. 1992 Grouping of plant–pathogenic and some other *Pseudomonas* spp. by using cellular fatty acid profiles. *Int. J. Syst. Bacteriol.*, **42**: 281–295.
- Van Zyl, E. and P. L. Steyn 1990 Differentiation of phytopathogenic *Pseudomonas* and *Xanthomonas* species and pathovars by numerical taxonomy and protein gel electrophoregrams. *Syst. Appl. Microbiol.*, **13**: 60–71.
- Vauterin, L., J. Swings and K. Kersters 1991a Grouping of *Xanthomonas campestris* pathovars by SDS–PAGE of proteins. *J. Gen. Microbiol.*, **137**: 1677–1687.
- Vauterin, L., P. Yang, B. Hoste, M. Vancanneyt, E. L. Civerolo, J. Swings and K. Kersters 1991b Differentiation of *Xanthomonas campestris* pv. *citri* strains by sodium dodecyl sulphate–polyacrylamide gel electrophoresis of proteins, fatty acid analysis, and DNA–DNA hybridization. *Int. J. Syst. Bacteriol.*, **41**: 535–542.
- Vauterin, L., P. Yang, B. Hoste, B. Pot, J. Swings and K. Kersters 1992 Taxonomy of xanthomonads from cereals and grasses based on SDS–PAGE of proteins, fatty acid analysis, and DNA–DNA hybridization. *J. Gen. Microbiol.*, **138**: 1467–1477.
- Wilson, E. E., F. M. Zeitoun and D. L. Fredrickson 1967 Bacterial phloem canker, a new disease of Persian walnut trees. *Phytopathology*, **57**: 618–621.
- Yamamoto, S. and S. Harayama 1995 PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl. Environ. Microbiol.*, **61**: 1104–1109.