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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 topoismerase 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 $\mu$l 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 $\mu$l reaction mixture containing 68.5 $\mu$l deionized water, 10 x reaction buffer (100 mM Tris–HCl, pH 9.0, 500 mM KCl and 1% Triton X–100) 10 $\mu$l, 25 mM $MgCl_2$ 10 $\mu$l, 5 mM dNTPs 4 $\mu$l, DNA solution 5 $\mu$l, each primer 1 $\mu$l and *Taq* DNA polymerase 0.5 $\mu$l, and was overlaid with 50 $\mu$l 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 cyles 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 vissually 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 |
|---|---|---|
| *P. syringae* | | |
| pv. *syringae* | ATCC 19310$^T$ | ATCC |
| pv. *oryzae* | MAFF 301538 | NIAR |
| pv. *tabaci* | PA-28 | KTES |
| pv. *tabaci* | Ku-7102 | AKU |
| pv. *lachrymans* | NIAS 1319 | NIAS |
| pv. *lachrymans* | NIAS 1321 | 〃 |
| pv. *mori* | P-23 | 〃 |
| *pv. pisi* | MAFF 301211 | NIAR |
| pv. *pisi* | MAFF 301213 | 〃 |
| pv. *theae* | MAFF 750001 | 〃 |
| pv. *coronafaciens* | MAFF 301314 | 〃 |
| pv. *phaseolicola* | MAFF 301616 | 〃 |
| pv. *phaseolicola* | MAFF 301716 | 〃 |
| pv. *japonica* | MAFF 301163 | 〃 |
| pv. *atropurpurea* | MAFF 301307 | 〃 |
| pv. *striafaciens* | P-71 | NIAS |
| pv. *myricae* | MAFF 301464 | NIAR |
| pv. *tomato* | MAFF 301593 | 〃 |
| pv. *glycinea* | KN-28 | NIAS |
| *X. campestris* | | |
| pv. *citri* | 12-5 | AKU |
| pv. *citri* | 32-5 | 〃 |
| pv. *citri* | Ku-7501 | 〃 |
| pv. *citri* | C 1 | 〃 |
| pv. *citri* | C 2 | 〃 |
| pv. *campestris* | ATCC 33913$^T$ | ATCC |
| pv. *campestris* | I | TUA |
| pv. *hyacinthi* | X 1-4-1 | NIAS |
| pv. *translucens* | X 1-11-2 | 〃 |
| pv. *glycines* | S-9 | AKU |
| pv. *glycines* | S-10 | 〃 |
| pv. *vitians* | I | NIAS |
| pv. *vitians* | II | 〃 |
| pv. *pisi* | I | 〃 |
| pv. *pisi* | II | 〃 |
| pv. *pisi* | III | 〃 |
| pv. *physalidicola* | 2 | 〃 |
| pv. *phaseoli* | 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.

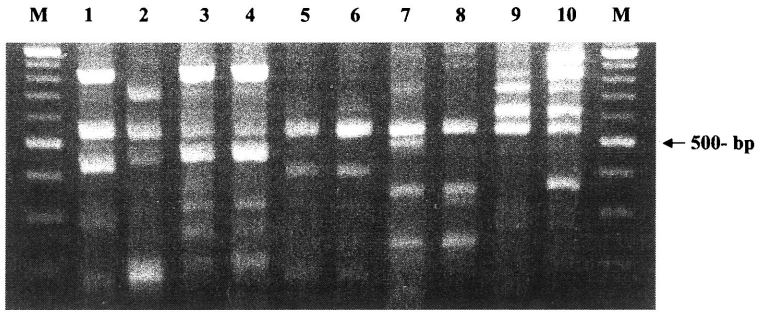


**Fig. 1a.** Electrophoretic patterns of PCR-amplified topoisomerase genes of *P. syringae* pathovars and strains.
M: Molecular marker (100-bp ladder); 1: *P. syr.* pv. *syringae* ATCC 19310$^T$; 2: pv. *tomato* MAFF 301593; 3: pv. *phaseolicola* MAFF 301716; 4: pv. *phaseolicola* MAFF 301616; 5: pv. *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.

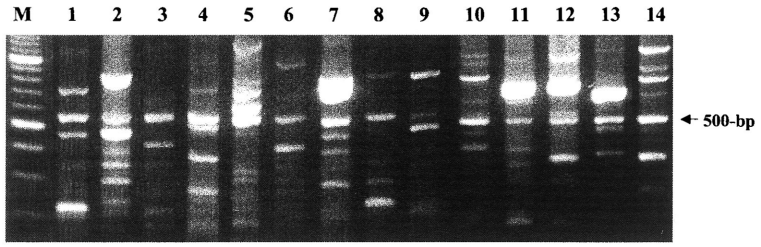


**Fig. 1b.** Electrophoretic patterns of PCR-amplified topoisomerase genes of *P. syringae* pathovars and strains.
M: Molecular marker (100-bp ladder); 1: pv. *tomato* MAFF 301593; 2: pv. *phaseolicola* MAFF 301716; 3: pv. *pisi* MAFF 301211; 4: pv. *tabaci* PA-28; 5: pv. *lachrymans* NIAS 1319; 6: pv. *straifaciens* 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.

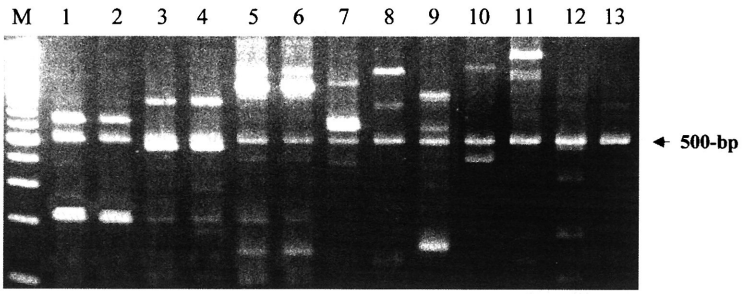


**Fig. 2.** Electrophoretic patterns of PCR-amplified topoisomerase genes of *Xanthomonas* pathovars and strains.
Lanes M: Molecular marker (100-bp ladder), 1: *X. campestris* pv. *citri* 32-5, 2: pv. *citri* Ku 7501, 3: pv. *pisi* II, 4: pv. pisi III, 5: pv. *vitians* I, 6: pv. *vitians* II, 7: pv. *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. *pisi*, 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. *pisi* and two strains of *X. c.* pv. *vitians* showed homogenous patterns within the respective pathovars. 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 reproductive 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.

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