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Genetic diversity in Thai strains of *Erwinia carotovora* subsp. *carotovora* based on ERIC profiles and RFLP of a *pel* gene

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Molecular fingerprinting techniques of enterobacterial repetitive intergenic consensus (ERIC) and restriction fragment length polymorphism (RFLP) of a *pel* gene were applied to determine genetic diversity in Thai strains of *Erwinia carotovora* subsp. *carotovora*. ERIC fingerprinting revealed a greater genotypic diversity with 10 PCR groups in 22 Thai strains. When PCR for the *pel* gene was carried out using the four subspecies *atroseptica*, *carotovora*, *betavasculorum* and *wasabiae*, no PCR products were obtained in the case of *betavasculorum*. RFLP analysis with *Sau*3A was undertaken for the amplified fragments. Digestion patterns of 22 Thai strains were divided into two groups. ERIC group 1 and RFLP group 2 were composed of biovar D that were isolated from chinease cabbage, coriander and sweet pepper. The genetic diversity of *E. carotovora* subsp. *carotovora* appears to be related to their host plants and biovars in considering ERIC and RFLP data. These results indicate that ERIC and RFLP analyses will be useful as benchmarks for genetic characterization of *E. carotovora* at subspecies and infraspecific level.

INTRODUCTION

Three soft rot erwinias, *Erwinia carotovora* subsp. *atroseptica* (Eca), *E. carotovora* subsp. *carotovora* (Ecc) and *E. chrysanthemi* (Ech) are pathogenic to many economically important crops, including potatoes, causing blackleg in the field and soft rot of tubers in storage (Perombelon and Kelman, 1980). Ecc and Ech are associated with soft rot diseases on many important horticultural and agricultural crops, whereas Eca is largely restricted to potato diseases (Smid *et al.*, 1995). Ecc strains have a wide distribution in both temperate and tropical zones, showing wider host range than those of Eca and Ech (Perombelon and Kelman, 1980). It is important to improve our understanding of the diversity of Ecc for development of the control measures of the diseases.

Variability among soft rot erwinias has been demonstrated by various phenotypic and genotypic assessments including physiological tests, cellular fatty acid profiles, DNA homologies, 16S ribosomal DNA sequence analysis, and protein profiles (Alarcon *et al.*, 1995; Chatterjee *et al.*, 1995; Smith and Bartz, 1990). More recently, enterobacterial repetitive intergenic consensus (ERIC) sequences have been used to obtain strain-specific fingerprints that can differentiate bacterial strains below the level of subspecies (Louws *et al.*, 1994; Versalovic *et al.*, 1991). Furthermore, several *pel* genes which are involved in soft rot diseases were sequenced and three families (*BC*, *ADE* and *Y*

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family) were identified on the basis of sequence homology (Darrasse *et al.*, 1994).

The aim of this study was to characterize strains of Ecc isolated from various plants in Thailand by using ERIC and RFLP of a *pelY* analyses. The strains used were previously grouped into several pathovars and biovars by physiological, biochemical and pathological characterization (Karnjanarat *et al.*, 1987).

MATERIALS AND METHODS

Bacterial strains and culture conditions.

Twenty–two Thai strains of *Erwinia carotovora* subsp. *carotovora* listed in Table 1 and four type strains of *E. carotovora* subsp. *carotovora* ATCC15713^T, *E. carotovora* subsp. *atroseptica* ATCC 33260^T, *E. carotovora* subsp. *betavasculorum* ATCC 43762^T, and *E. carotovora* subsp. *wasabiae* ATCC 43316^T were used in this study. All bacteria were stored in skim milk (10% final concentration) at −70 °C. Each bacterial strain was cultured on MGY (mannitol 10.0 g, L–glutamic aicd 2.0 g, KH_2PO_4 0.5 g, NaCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, yeast extract 0.25 g, agar 15.0 g, distilled water 1 liter, pH 7.0) at 28 °C for 2 days.

Table 1. Sources of Thai strains of *Erwinia carotovora* subsp. *carotovora* and their relevant characteristics

Strain	Source	Isolated from	Year isolated	Biovar[a)]	Pathovar[a)]	RFLP[b)]	ERIC[c)]
014-2	Bangkok	Cauliflower	1980	D	IV	1	8
014-9	〃	〃	〃	D	IV	1	8
131-1	〃	Bell pepper	〃	G	I	1	2
168-7	Lumpang	Chinese cabbage	〃	D	I	1	1
435-2	Bangkok	Lettuce	1982	D	I	1	7
435-6	〃	〃	〃	D	I	1	7
462-53-1	〃	Tomato	〃	E	I	1	4
473-1	〃	Chinese cabbage	〃	G	I	1	9
475-1	〃	Hot pepper	〃	D	I	1	9
476-4	〃	Bird chili	〃	G	I	1	10
476-7	〃	〃	〃	G	I	1	10
479-2	〃	Coriander	〃	D	I	2	3
485-5	〃	Cabbage	〃	E	I	1	5
486-4	〃	Sweet pepper	〃	D	I	2	1
486-5	〃	〃	〃	D	I	2	1
486-7	〃	〃	〃	D	I	2	1
486-8	〃	〃	〃	D	I	2	1
489-4	〃	Cabbage	〃	F	I	1	6
489-5	〃	〃	〃	F	I	1	6
493-1	〃	Potato	〃	F	I	1	6
493-3	〃	〃	〃	F	I	1	6
493-5	〃	〃	〃	F	I	1	6

[a)] Cited from the description of Karnjanarat *et al.*(1987)
[b)] RFLP, restriction fragment length polymorphism of a *pel* gene
[c)] ERIC, enterobacterial repetitive intergenic consensus

Molecular analyses.

Total DNA was extracted according to the method of Sambrook *et al.* (1989) and used for PCR.

ERIC primers, ERIC1R (5'–ATGTAAGCTCCTGGGGATTCAC–3') and ERIC2 (5'–AAG-TAAGTGACTGGGGTGAGCG–3'), are specific to the conserved repetitive sequences in bacteria (Mcmanus and Jones, 1995). Amplification was performed in a total volume of 50 μl containing 67 mM Tris–HCl (pH 8.8), 2.0 mM $MgCl_2$, 0.125 mM each of dATP, dCTP, dGTP and dTTP, 2.0 units of *Taq*DNA polymerase (TOYOBO, Japan), 50 pmol each of the ERIC primers, and 1 μl of a 50 ng/ml solution of purified DNA. PCR reactions were performed in a thermal cycler (Astec, Japan) under the following conditions: 94 °C for 4 min for the first cycle, 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, repeated for 30 cycles, and a final cycle of 72 °C for 10 min. Amplified DNA fragments (10 μl) were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

pelY primers, Y1 (5'–TTACCGGACGCCGAGCTGTGGCGT–3') and Y2 (5'–CAGGAA-GATGTCGTTATCGCGAGT–3'), are specific for *E. carotovora* (Helias *et al.*, 1998). Volume and content of the amplification reactions were as described above. Amplification were done in the thermal cycler under the following conditions: 35 cycles of 30 sec at 94 °C, 30 sec at 65 °C, and 45 sec at 72 °C. The amplification cycles were preceded by a denaturation step of 5 min at 95 °C and followed by an elongation step of 10 min at 72 °C. Initial analysis of PCR products was done by electrophoresis on 2% agarose gel. RFLP analysis of *pelY* gene was done as follows: The amplified DNA was ethanol precipitated and resuspended in 50 μl of 10 mM Tris–HCl (pH 8.0), 1 mM EDTA. Aliquots of the concentrated DNA were digested with *Sau*3A at 37 °C for 2 h in a 15 μl volume according to the manufacturer's recommendations. The samples were electrophoresed on 2.5% agarose gel and visualized by staining with ethidium bromide.

RESULTS AND DISCUSSION

PCR products from the genomic DNA of the all strains using primers ERIC1R and ERIC2 produced several bands between approximately 0.2 to 2.2 kb on the agarose gel (Fig. 1). The ERIC–PCR patterns generated from Ecc were found to be very different from those of the ATCC 33260^T (*E. carotovora* subsp. *atroseptica*), ATCC 43762^T (*E. carotovora* subsp. *betavasculorum*) and ATCC 43316^T (*E. carotovora* subsp. *wasabi-ae*). Various fingerprints observed were separated into 10 profiles in 22 Thai strains of Ecc (Fig. 1 and Table 1). These profiles correlated with the biovar and original host of the strains. For example, ERIC groups 1 and 6 were composed of biovar D and F, respectively. Toth *et al.* (1999) found only three groups in Eca using the ERIC–PCR method, suggesting that ERIC analysis is of limited value for differentiating Eca. This difference would be due to a lower level of polymorphism of Eca than that of Ecc.

All products obtained by PCR for *pelY* gene were of the expected size (about 430 bp) (Darrasse *et al.*, 1994; Helias *et al.*, 1998) except *E. carotovora* subsp. *betavasculorum* from which no fragment was amplified. An RFLP analysis by digesting the amplified fragments with *Sau*3A was undertaken on the collection of Ecc strains. Thai strains were separated into two groups on the basis of their RFLP patterns (Fig. 2 and Table 1). All of the biovar D strains isolated from coriander and sweet pepper were shown to belong to

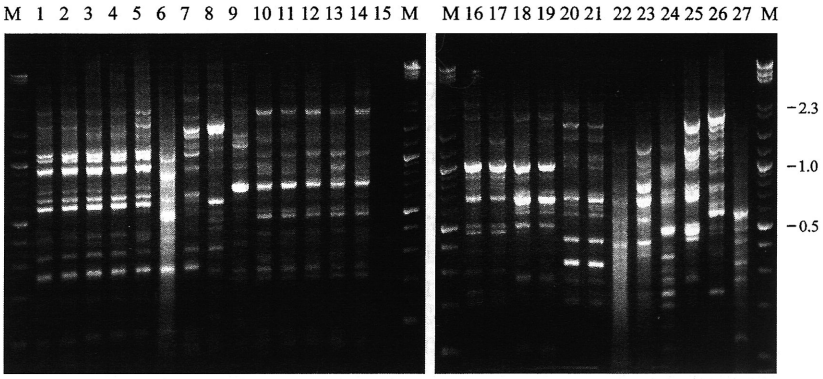

Fig. 1. ERIC–PCR patterns generated from purified DNA of *Erwinia carotovora*. PCR products were separated on a 2.0% agarose gel, stained with ethidium bromide, and photographed under UV illumination. Lanes labeled M were run with the DNA size standard (kb). Lane 1, 486–5; Lane 2, 486–7; Lane 3, 486–4; Lane 4, 486–8; Lane 5, 168–7, Lane 6,131–1; Lane 7, 479–2; Lane 8, 462–53–1; Lane 9, 485–5; Lane 10, 489–5; Lane 11, 493–3; Lane 12, 493–5; Lane 13, 489–4; Lane 14, 493–1; Lane 15, the same PCR mixture but lacking template DNA (control); Lane 16, 435–2; Lane 17, 435–6; Lane 18, 014–2; Lane 19, 014–9; Lane 20, 473–1; Lane 21, 475–1; Lane 22, 476–4; Lane 23, 476–7; Lane 24, ATCC15713^{T} (*E. carotovora* subsp. *carotovora*); Lane 25, ATCC33260^{T} (*E. carotovora* subsp. *atroseptica*); Lane 26, ATCC43762^{T} (*E. carotovora* subsp. *betavasculorum*); Lane 27, ATCC43316^{T} (*E. carotovora* subsp. *wasabiae*)

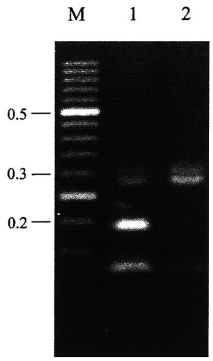

Fig. 2. RFLP analysis of the amplified fragments of *pelY* gene. The DNA products were digested with restriction enzyme *Sau*3A and separated on a 2.5% agarose gel, stained with ethidium bromide, and photographed under UV illumination. Lanes labeled M were run with the DNA size standard (kb). Lane 1, RFLP pattern 1; Lane 2, RFLP pattern 2

the same group. In general, strains of the same biovar from the same host showed similar RFLP patterns. Although the number of the samples used in this study was not adequate to draw definite conclusions, the results reported here would provide a direction for future genetics studies. Combining the ERIC–PCR and PCR–RFLP of a *pel* gene seems to offer a more reliable approach for assessing genetic diversity in *E. carotovora* strains.

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