Genotyping of Erwinia carotovora subsp. carotovora Strains from Asia Based on recA Gene Restriction Fragment Length Polymorphisms

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Genotyping of *Erwinia carotovora* subsp. *carotovora* Strains from Asia Based on *rec*A Gene Restriction Fragment Length Polymorphisms

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Genetic diversity of a collection of 92 strains of *Erwinia carotovora* subsp. *carotovora* (Ecc) isolated from various host plants in Asian countries (Japan, Korea and Thailand) was assessed by means of PCR-RFLP of recombinase A gene (*rec*A). Ten RFLP groups were obtained on the basis of restriction analysis of *rec*A gene fragments with four restriction endonucleases (*Hind* III, *Alu* I, *Dde* I and *Tas* I). Most of Asian strains (71 out of 92 strains) belonged to RFLP groups 1, 2 and 3. When the RFLP groups were compared with the phenotypic groups and hosts of origin, there were found some relationships. RFLP groups 2 and 3 coincided with phenotypic groups A and B, respectively. RFLP group 4 contained only isolates from mulberry trees, indicating that the mulberry strains are in a distinct group in Ecc. The results of this study will facilitate further understanding of the population structure of Ecc in Asia.

INTRODUCTION

Erwinia carotovora subsp. *carotovora* (Ecc) is a member of the pathogenic enterobacteria causing soft rot of a wide range of plants in different parts of the world (Perombelon and Kelman, 1980). Ecc is a complex unit in which strains are diverse at various levels. The diversity within the population may result from several factors such as genetic change, host plants, migration from other geographic areas, etc.

Analysis of genetic diversity is important for understanding the distribution of the strains. Several studies of taxonomy and diversity of Ecc strains have previously been undertaken using molecular techniques, such as PCR-RFLP (Darrasse *et al.*, 1994), AFLP (Avrova *et al.*, 2002), RAPD (Hadas *et al.*, 2001; Maki-Valkama and Karjalainen, 1994) and 16S rDNA analyses (Hauben *et al.*, 1998). They have confirmed the heterogeneity of the Ecc by the various techniques. However, most of the studies concerning genetic diversity of the pathogen have not included Asian strains, and little is known about genetic diversity of Ecc strains in Asian areas.

Recombinase A (RecA) is a multifunctional protein involved in homologous recombination, DNA repair and the SOS response (Eisen, 1995). Waleron *et al.* (2002) reported

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the usefulness of the *rec*A PCR-RFLP for genotyping of Ecc, but Asian strains were not included in their work. Therefore, we have also tried to assess the diversity of Ecc strains obtained from Asian areas by means of PCR-RFLP of the *rec*A gene, and have compared the results of the genotyping with previously determined phenotypic groups (Seo *et al.*, 2002), hosts and geographic origins.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study are listed in Table 1. All the strains were routinely cultured on MGY agar (mannitol 10.0 g, L-glutamic acid 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 (Keane *et al.*, 1970).

PCR-RFLP of the *rec*A gene

For performing PCR, total DNA was extracted according to the method of Sambrook *et al.* (1989). PCR was performed in a thermal cycler (Astec, Japan) by using the primers previously described (Waleron *et al.*, 2002). Amplification was performed in a reaction mixture of 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 primer, and 1 μl of a 50 ng/ml solution of purified DNA. The mixture was overlaid with 50 μl of mineral oil. PCR reactions were performed under the following conditions; 95 °C for 3 min for the first cycle, 35 cycles of 94 °C for 1 min, 51 °C for 1 min and 72 °C for 2 min, and a final cycle of 72 °C for 5 min. The amplified DNA fragments were digested with the following restriction endonucleases (Fermentas, Lithuania): *Hin*d III, *Dde* I, *Alu* I and *Tas* I, according to the manufacturer's instructions. The reaction products were analyzed by agarose (2%, w/v) gel electrophoresis in 1× TBE buffer and visualized by staining with ethidium bromide.

RESULTS AND DISCUSSION

All products obtained after PCR amplification with the primers designed to be complementary to the *E. carotovora rec*A gene were of the expected size (about 730 base pairs in length). The PCR products were digested with four restriction enzymes (*Hin*d III, *Dde* I, *Alu* I and *Tas* I) for RFLP analysis. Each of *Hin*d III and *Alu* I gave its own one restriction pattern, while each of *Dde* I and *Tas* I gave four RFLP patterns. The obtained PCR-RFLP patterns showed that there are ten RFLP groups. Representative RFLP patterns and the groups associated with the respective strains are given in Figure 1 and Table 1, respectively. Most of the strains (71 out of 92 strains) tested belong to RFLP group 1, 2 and 3. Waleron *et al.* (2002) reported that 57 Ecc strains isolated from 13 host plants in mainly European countries were divided into 18 different RFLP groups by analysis of the *rec*A gene. Thus, Asian strains isolated from 35 host plants are more homogeneous than the European strains.

We previously reported that Asian Ecc strains were composed of two groups, A (typical Ecc) and B (atypical Ecc), on the basis of 26 phenotypic characteristics (Seo *et*

Table 1. Origin, phenotypic groups, PCR–RFLP patterns and RFLP groups of *E. carotovora* subsp. *carotovora* strains used in this experiment

Strain[a]	Host	Geographic origin and year isolated	Phenotypic group[b]	PCR–RFLP patterns[c]				RFLP group[d]
				Hind III	*Dde* I	*Alu* I	*Tas* I	
N7101	Sweet pepper	Japan, 1971	B	1	1	1	1	1
N7109	Cauliflower	Japan, 1971	B	1	1	1	1	1
N7116	Cabbage	Japan, 1971	B	1	1	1	1	1
N7129	Radish	Japan, 1971	A	1	1	1	1	1
N7135	Tomato	Japan, 1971	B	1	1	1	1	1
Sr79–33–3	Potato	Japan, 1979	A	1	1	1	1	1
1B	Shallot	Japan, 1982	A	1	1	1	1	1
645ar	Chinese cabbage	Japan, 1960	A	1	1	1	1	1
K1	Radish	Japan, 1985	A	1	1	1	1	1
K2	Radish	Japan, 1985	A	1	1	1	1	1
MAFF 106567	Cucumber	Japan, 1985	A	1	1	1	1	1
MAFF 301049	Eggplant	Japan, 1948	A	1	1	1	1	1
MAFF 301282	Melon	Japan, 1976	A	1	1	1	1	1
MAFF 301394	Cabbage	Japan, 1971	B	1	1	1	1	1
MAFF 301404	Parsley	Japan, 1973	A	1	1	1	1	1
MAFF 301917	Cauliflower	Japan, 1971	A	1	1	1	1	1
MAFF 311115	Calla	Japan, 1994	A	1	1	1	1	1
MAFF 810035	Lettuce	Japan, 1980	A	1	1	1	1	1
MAFF 810020	Mulberry	Japan, 1980	–	1	1	1	1	1
MAFF 810030	Mulberry	Japan, 1980	–	1	1	1	1	1
Ecc3/95	Potato	Korea, 1995	B	1	1	1	1	1
Ecc1/96	Chinese cabbage	Korea, 1996	A	1	1	1	1	1
Ecc3/96	Chinese cabbage	Korea, 1996	A	1	1	1	1	1
Ecc4/96	Chinese cabbage	Korea, 1996	A	1	1	1	1	1
Ecc6/96	Wasabi	Korea, 1996	A	1	1	1	1	1
Ecc8/96	Crisphead	Korea, 1996	A	1	1	1	1	1
Ecc9/96	Radish	Korea, 1996	A	1	1	1	1	1
Ecc13/96	Pumpkin	Korea, 1996	A	1	1	1	1	1
Ecc3/97	Potato	Korea, 1997	A	1	1	1	1	1
Ecc1/98	Cactus	Korea, 1998	A	1	1	1	1	1
014–2	Cauliflower	Thailand, 1980	A	1	3	1	1	2
014–9	Cauliflower	Thailand, 1980	A	1	3	1	1	2
435–2	Lettuce	Thailand, 1982	A	1	3	1	1	2
435–6	Lettuce	Thailand, 1982	A	1	3	1	1	2
485–5	Cabbage	Thailand, 1982	A	1	3	1	1	2
489–4	Cabbage	Thailand, 1982	A	1	3	1	1	2
489–5	Cabbage	Thailand, 1982	A	1	3	1	1	2
493–1	Potato	Thailand, 1982	A	1	3	1	1	2
493–3	Potato	Thailand, 1982	A	1	3	1	1	2
493–5	Potato	Thailand, 1982	A	1	3	1	1	2
Ku7514	Watermelon	Japan, 1975	B	1	3	1	1	2
EH8504	Cucumber	Japan, 1985	A	1	3	1	1	2
EH8510	Cucumber	Japan, 1985	A	1	3	1	1	2
EH8514	Cucumber	Japan, 1985	A	1	3	1	1	2
EH8519	Cucumber	Japan, 1985	A	1	3	1	1	2
MAFF 301891	Sweet pepper	Japan, 1971	A	1	3	1	1	2
Ecc1/95	Wasabi	Korea, 1995	A	1	3	1	1	2
Ecc4/95	Chinese cabbage	Korea, 1995	A	1	3	1	1	2
Ecc5/95	Chinese cabbage	Korea, 1995	A	1	3	1	1	2
Ecc6/95	Potato	Korea, 1995	A	1	3	1	1	2
Ecc1/97	Pepper	Korea, 1997	A	1	3	1	1	2
N7127	Carrot	Japan, 1971	B	0	1	0	1	3
N7128	Celery	Japan, 1971	B	0	1	0	1	3

Table 1. Continued

Strain[a]	Host	Geographic origin and year isolated	Pheno-typic group[b]	PCR-RFLP patterns[c]				RFLP group[d]
				Hind III	*Dde* I	*Alu* I	*Tas* I	
N7131	Tomato	Japan, 1971	B	0	1	0	1	3
N7157	Chinese cabbage	Japan, 1971	B	0	1	0	1	3
S8488	Sunflower	Japan, 1984	B	0	1	0	1	3
190	Carrot	Japan, 1984	B	0	1	0	1	3
43	Cabbage	Japan, 1984	B	0	1	0	1	3
ar13	Chinese cabbage	Japan, 1960	B	0	1	0	1	3
20	Chinese cabbage	Japan, 1970	B	0	1	0	1	3
MAFF 301053	Radish	Japan, 1957	B	0	1	0	1	3
MAFF 301396	Carrot	Japan, 1971	B	0	1	0	1	3
MAFF 301399	Elephant's foot	Japan, 1971	B	0	1	0	1	3
MAFF 301905	Tobacco	Japan, 1971	B	0	1	0	1	3
MAFF 302773	Garlic	Japan, 1983	B	0	1	0	1	3
MAFF 810032	Mulberry	Japan, 1980	–	0	1	0	1	3
Ecc2/95	Chicory	Korea, 1995	B	0	1	0	1	3
Ecc5/96	Chinese cabbage	Korea, 1996	B	0	1	0	1	3
Ecc7/96	Onion	Korea, 1996	B	0	1	0	1	3
Ecc11/96	Potato	Korea, 1996	B	0	1	0	1	3
Ecc12/96	Cucumber	Korea, 1996	B	0	1	0	1	3
MAFF 301937	Mulberry	Japan, 1974	B	1	4	1	1	4
MAFF 810017	Mulberry	Japan, 1969	–	1	4	1	1	4
MAFF 810022	Mulberry	Japan, 1979	–	1	4	1	1	4
MAFF 810029	Mulberry	Japan, 1980	–	1	4	1	1	4
MAFF 810031	Mulberry	Japan, 1980	–	1	4	1	1	4
MAFF 810034	Mulberry	Japan, 1982	–	1	4	1	1	4
168-7	Chinese cabbage	Thailand, 1980	A	1	2	1	3	5
486-4	Sweet pepper	Thailand, 1982	A	1	2	1	3	5
486-5	Sweet pepper	Thailand, 1982	A	1	2	1	3	5
486-7	Sweet pepper	Thailand, 1982	A	1	2	1	3	5
486-8	Sweet pepper	Thailand, 1982	A	1	2	1	3	5
476-4	Bird chili	Thailand, 1982	A	1	2	1	1	6
476-7	Bird chili	Thailand, 1982	A	1	2	1	1	6
MAFF 301941	Ginger	Japan, 1974	A	1	2	1	1	6
131-1	Bell pepper	Thailand, 1980	A	1	2	1	2	7
479-2	Coriander	Thailand, 1982	A	1	2	1	2	7
Ecc2/97	Calla	Korea, 1997	A	1	2	1	2	7
473-1	Chinese cabbage	Thailand, 1982	A	0	2	0	1	8
475-1	Hot pepper	Thailand, 1982	A	0	2	0	1	8
Ecc2/96	Chinese cabbage	Korea, 1996	B	0	1	0	4	9
B1	Broccoli	Japan, 1985	A	1	1	1	2	10

[a] MAFF, Ministry of Agriculture, Forestry and Fisheries Gene Bank, Japan

[b] As described by Seo *et al.* (2002); –, not determined

[c] Numbers correspond to RFLP patterns shown in Fig. 1. Zero indicates the absence of restriction digestion by a given endonuclease

[d] Numbers of RFLP groups based on the combined PCR-RFLP patterns

al., 2002). When the *rec*A RFLP groups were compared with the two phenotypic groups, RFLP groups 2 and 3 were composed of phenotypic groups A and B, respectively, except one strain (Ku7514) (Table 1). These results showed a close relationship between genetic groups by the *rec*A RFLP analysis and phenotypic ones. However, no association

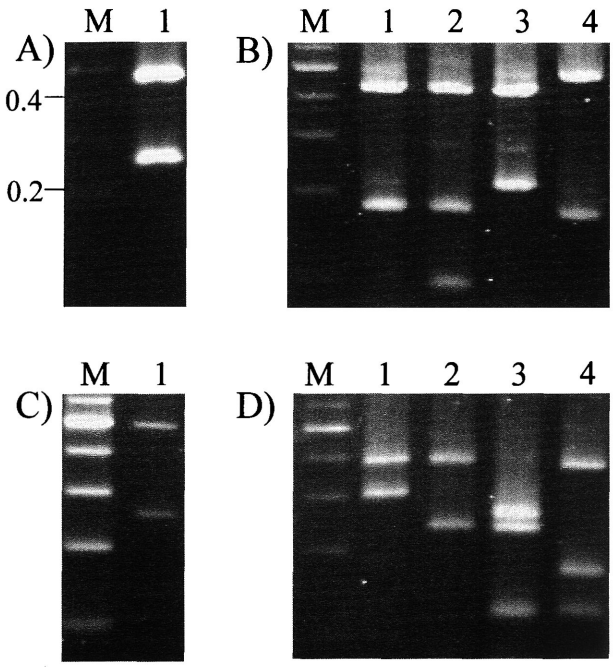

Fig. 1. RFLP analysis of the amplified fragments of *rec*A gene. The DNA products of *rec*A gene were digested with restriction enzyme *Hin*d Ⅲ (A), *Dde* I (B), *Alu* I (C) and *Tas* I (D), and separated on 2% agarose gels, stained with ethidium bromide, and photographed under UV illumination. Lane M, Molecular marker (kb). Lane numbers correspond to the PCR–RFLP patterns obtained for each restriction enzyme as shown in Table 1.

was found between the genetic groups and geographic origins of the strains.

RFLP profiles of the *rec*A gene of the strains were not strictly correlated to their hosts of origin. This result agrees with those by Waleron *et al.* (2002). RFLP group 4 consists of only six isolates from mulberry trees. Among the four endonucleases used in RFLP analysis, *Dde* I could differentiate the six mulberry strains from other Ecc strains.

To understand evolution and differentiation of the pathogen, further research is necessary. Combined with information already published for other parts of the world on the population structure of Ecc, a more comprehensive understanding of this pathogen is emerging.

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