Differentiation of Erwinia carotovora subsp. atroseptica and carotovora by Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

Seo, Sang-Tae
Laboratory of Plant Pathology, Division of Plant Pathology and Pesticide Science, Department of Applied Genetics and Pest Management, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Furuya, Naruto
Laboratory of Plant Pathology, Division of Plant Pathology and Pesticide Science, Department of Applied Genetics and Pest Management, Faculty of Agriculture, Kyushu University

Takeshita, Minoru
Laboratory of Plant Pathology, Division of Plant Pathology and Pesticide Science, Department of Applied Genetics and Pest Management, Faculty of Agriculture, Kyushu University

Takanami, Yoichi
Laboratory of Plant Pathology, Division of Plant Pathology and Pesticide Science, Department of Applied Genetics and Pest Management, Faculty of Agriculture, Kyushu University

https://doi.org/10.5109/24452

出版情報：九州大学大学院農学研究院紀要. 47 (1), pp.1-6, 2002-10-30. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **47** (1), 1–6 (2002)

Differentiation of *Erwinia carotovora* subsp. *atroseptica* and *carotovora* by Enterobacterial Repetitive Intergenic Consensus (ERIC)–PCR

Sang-Tae SEO*, Naruto FURUYA, Minoru TAKESHITA and Yoichi TAKANAMI

Laboratory of Plant Pathology, Division of Plant Pathology and Pesticide Science, Department of Applied Genetics and Pest Management, Faculty of Agriculture, Kyushu University, Fukuoka 812–8581, Japan
(*Received June 17, 2002 and accepted July 12, 2002*)

The biochemical and genetic characteristics of 11 *Erwinia carotovora* strains from potato were compared with those of reference strains, *E. carotovora* subsp. *atroseptica* (Eca) and *E. carotovora* subsp. *carotovora* (Ecc). On the basis of 26 phenotypic characters, four of the 11 strains showed intermediate characters between Eca and Ecc, and the rest of the strains were identified as typical members of Ecc. Current methods for differentiation of Eca and Ecc are both imprecise and time consuming. We have used enterobacterial repetitive intergenic consensus (ERIC)−PCR to differentiate the bacteria. Similarity of ERIC−PCR patterns within the Eca strains was very high, whereas within the Ecc group extensive genetic diversity was found. ERIC−PCR patterns of biochemically intermediate strains showed low similarity to those of Eca strains. An ERIC−PCR fragment from Eca type strain was isolated, and it was used as probe for Southern hybridization. The probe hybridized only with Eca strains. One reference strain (MAFF 301296) originally described as Ecc, was identified as Eca by ERIC−PCR analysis, and this result was confirmed by biochemical tests. ERIC−PCR analysis for differentiation of Eca and Ecc provides a relatively fast and sensitive alternative to routinely applied biochemical tests.

INTRODUCTION

Erwinia carotovora (Jones) Bergey *et al.* is the causal agent of bacterial soft rot in many different plant species, including potatoes (*Solanum tuberosum* L.). Under temperate conditions, soft rot of potato crops is primarily caused by two *E. carotovora* subspecies. While *E. carotovora* subsp. *atroseptica* (Eca) is specifically associated with potato, *E. carotovora* subsp. *carotovora* (Ecc) is widespread and has a wide host range besides potato (Perombelon and Kelman, 1980). Thus, differentiation of the two subspecies is important for potato growers in making adequate crop rotation choices when they face a field contamination problem.

Differences were reported between Eca and Ecc strains regarding their pathogenicity and biochemical characteristics (Lelliot and Dickey, 1984; Smith and Bartz, 1990). Biochemical tests are accepted as a standard for differentiation of Eca and Ecc (De Boer *et al.*, 1978, 1987; Dye, 1969). However, the biochemical tests are lengthy and costly.

* Laboratory of Plant Pathology, Division of Plant Pathology and Pesticide Science, Department of Applied Genetics and Pest Management, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Moreover, *E. carotovora* strains that are intermediate between Eca and Ecc in biochemical properties were also found (Tanii, 1984; Thomson *et al.*, 1981).

A number of other methods have been used to differentiate the subspecies, but all have limitations. The high serological heterogeneity and cross-reactivity between the subspecies have limited the use of serology (De Boer *et al.*, 1987). Fatty acid profiling has been used to differentiate the subspecies but has been of only limited success (De Boer and Sasser, 1986). DNA-DNA hybridization is accurate but time-consuming and unsuitable for routine use, especially when large numbers of strains are involved (Vandamme *et al.*, 1996).

Recently, rep-PCR (ERIC, REP and BOX) technique for subspecific differentiation of some pathovars of *Pseudomonas syringae* and *Xanthomonas* species has been reported (Louws *et al.*, 1994). Enterobacterial repetitive intergenic consensus (ERIC) sequences, which are short repetitive DNA sequences with highly conserved central inverted repeats, are dispersed throughout the genomes of diverse bacterial species. The objectives of this study were to determine whether ERIC-PCR genomic fingerprinting could be used to differentiate the two *Erwinia* subspecies (Eca and Ecc). Furthermore, the result is compared with that obtained using biochemical tests.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this work are listed in Table 1. All the strains were stored at -70 °C in 10% skim milk and cultured on MGY agar (mannitol 10.0 g, L-glutamic

Table 1. Strains used in this experiment

Species	Strain designation[a)]	Plant host	Geographic origin
E. carotovora subspecies	493-3	*Solanum tuberosum* L.	Thailand
	493-5	〃	〃
	3/95	〃	〃
	6/95	〃	Korea
	11/96	〃	〃
	14/96	〃	〃
	3/97	〃	〃
	1/94	〃	〃
	1/96	〃	〃
	Sr79-33-3	〃	Japan
E. carotovora subsp. *atroseptica*	ATCC 33260^T	〃	United Kingdom
	LMG 6693	〃	Sweden
	MAFF 301614	〃	Japan
E. carotovora subsp. *carotovora*	ATCC 15713^T	〃	Denmark
	MAFF 301296	〃	Japan

[a)] ATCC, American Type Culture Collection, Rockville, Md, USA; LMG, Laboratorium voor Microbiologie, Gent., Belgium; MAFF, Ministry of Agriculture, Forestry and Fisheries Gene Bank, Japan

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).

Biochemical tests

Twenty-six physiological and biochemical tests were performed according to the authentic methods reported by various researchers (Dye, 1969; Schaad, 1980; Lelliot and Dickey, 1984). Tests included: growth at 36 °C; anaerobic growth; ability to cause soft rot in potato; presence of oxidase; production of yellow pigment on YDC; mucoid growth; urease activity; H_2S production; tolerance to KCN and 5% NaCl; production of reducing substances from sucrose; casein hydrolysis; indole production; acid production from carbohydrates utilized as a sole source of carbon; and utilization of various organic compounds.

ERIC-PCR and Southern hybridization

PCR was performed in a thermal cycler (Astec, Japan) by using primers, ERIC2 and ERIC1R (Versalovic *et al.*, 1991). Total DNA for PCR was extracted according to the method of Sambrook *et al.* (1989). 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 primer, and 1 μl of a 50 ng/ml solution of purified DNA. PCR reactions were performed under the following conditions; 94 °C for 4 min for the first cycle, 30 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, and a final cycle of 72 °C for 10 min. Aliquots were analyzed on 1.5% agarose gels and the PCR products were visualized by staining with ethidium bromide.

The DNA fragments amplified with primers ERIC2 and ERIC1R were transferred from the gel onto nylon membranes (Hybond™ NT, Amersham Pharmacia Biotech, UK). An unique band detected in Eca strains was extracted and used as a DNA probe. The probe was labeled with peroxidase and hybridized DNA was detected by ECL gene detection system (Amersham Pharmacia Biotech, UK). Hybridization was performed as recommended by the supplier at 42 °C overnight. The membranes were washed twice in primary washing buffer (6 M Urea, 0.4% SDS, 0.5×SSC) at 42 °C for 20 min and then twice in secondary washing buffer (2×SSC) at room temperature for 5 min before chemiluminescence detection.

RESULTS AND DISCUSSION

We determined the phenotypic characteristics of all the strains listed in Table 1. Eleven strains from potato were positive in the following phenotypic characters: anaerobic growth, ability to cause soft rot of potato, H_2S production, tolerance to KCN, utilization of polygalacturonate, acid production from: lactose, D(+)cellobiose, inositol, mellibiose and levulose; and were all negative for the presence of oxidase, production of yellow pigment on YDC, mucoid growth, urease activity and utilization of malonate. Bacteriological characteristics useful for distinguishing Eca from Ecc are considered to be the ability to grow at 36 °C, and to product acids from α-methyl glucoside and reducing substances from sucrose (De Boer *et al.*, 1978). Two strains (1/94 and 1/96) were nega-

Table 2. Bacteriological characteristics of Eca and Ecc strains

Characteristic	Strains of Eca[a]				Strains of Ecc											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Anaerobic growth	+[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Potato soft rot	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Yellow pigment on YDC	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mucoid growth	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Growth at 36 °C	−	−	−	−	+	+	+	+	+	+	+	+	+	−	−	+
Urease	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H_2S production	+	N	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KCN	+	N	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5% NaCl	+	N	+	+	+	+	+	+	+	+	+	+	+	−	−	+
Reducing substance from sucrose	+	N	+	+	−	−	−	−	+	−	+	−	−	−	−	−
Casein hydrolysis	+	N	−	−	+	−	−	−	+	+	−	+	+	+	+	−
Indole production	−	N	−	−	−	+	+	+	−	+	−	−	−	−	−	−
Acid production from																
Palationse	+	N	+	+	w	−	−	−	+	−	+	−	−	−	−	−
Sorbitol	−	N	−	−	−	−	−	−	+	−	+	−	−	−	−	−
α-methyl glucoside	+	N	+	+	−	−	−	−	+	−	−	−	−	−	−	−
Lactose	+	N	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D(+)Cellobiose	+	N	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	+	N	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D(+)Trehalose	+	N	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Mellibiose	+	N	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D(+)Arabitol	−	N	−	−	−	−	−	−	+	−	+	−	−	−	−	−
Levulose	+	N	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	N	−	−	w	−	−	−	−	−	+	−	−	−	−	−
Utilization of																
Polygalacturonate	−	N	−	−	+	+	+	+	+	+	+	+	+	+	+	+
Malonate	−	N	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] 1, ATCC 33260^T; 2, LMG 6693; 3, MAFF 301296; 4, MAFF 301614; 5, ATCC 15713^T; 6, 493–1; 7, 493–3; 8, 493–5; 9, 3/95; 10, 6/95; 11, 11/96; 12, 14/96; 13, 3/97; 14, 1/94; 15, 1/96; 16, Sr79–33–3

[b] +, positive reaction; −, negative reaction; w, weakly positive reaction; N, not tested

tive in the test of growth at 36 °C and two strains (3/95 and 11/96) were positive in the tests of reducing substances from sucrose (Table 2). These results indicate that the tests for growth at 36 °C and production of reducing substance from sucrose appeared to be unreliable for differentiation of the two subspecies. MAFF 301296 used as Ecc reference strain was unable to grow at 36 °C and was positive in the tests of acid production from α –methyl glucoside and reducing substances from sucrose, which are typical characters of Eca.

The ERIC–PCR of Eca and Ecc strains gave different ERIC banding patterns (Fig. 1A). There was only a limited variation in polymorphism found in the Eca strains by ERIC–PCR while there was a large variation within the Ecc group. This indicates that the two subspecies are different at the DNA level. Helias *et al.* (1998) reported that atypical

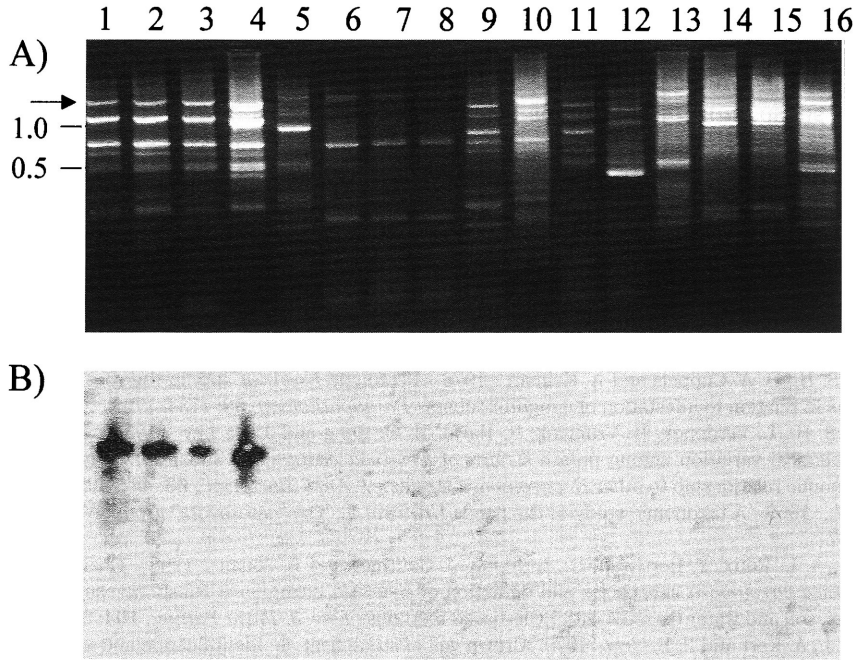

Fig. 1. A) Agarose gel electrophoresis of ERIC–PCR products generated from genomic DNA of *E. carotovora* subsp. *atroseptica* (Eca) and *carotovora* (Ecc). ERIC–PCR fragments were separated in a 1.5% agarose gel. 1, ATCC 33260^T; 2, LMG 6693; 3, MAFF 301296; 4, MAFF 301614; 5, ATCC 15713^T; 6, 493–1; 7, 493–3; 8, 493–5; 9, 3/95; 10, 6/95; 11, 11/96; 12, 14/96; 13, 3/97; 14, 1/94; 15, 1/96; 16, Sr79–33–3. B) Southern hybridization analysis of Eca and Ecc strains. ERIC sequences of Eca and Ecc strains were amplified with primers ERIC2 and ERIC1R, and the PCR products were blotted onto nylon filter after electrophoresis. The ERIC–PCR fragment from Eca group (arrow in Fig. 1A) was isolated and was used to probe the Southern blot.

strains, such as unable to grow at 36 °C, were genetically related to Ecc. In our study, ERIC–PCR patterns of two atypical Ecc strains (1/94 and 1/96), which were unable to grow at 36 °C, showed also low similarity to those of Eca strains (Fig. 1A). Moreover, the patterns of two atypical Ecc strains (3/95 and 11/96), which were positive in the tests of reducing substances from sucrose, showed similar results described above. ERIC–PCR patterns of MAFF 301296 strain that was originally described as Ecc, were similar to those of Eca strains. This result was confirmed by the biochemical tests. ERIC–PCR analysis clarified the status of strains with atypical biochemical features.

In order to test if Eca specific ERIC–PCR fragments were amplified from Ecc isolates, we purified an ERIC–PCR fragment from amplified DNA of Eca type strain (ATCC 33260^T), which produced a clear and unique band dissimilar to those found in amplified Ecc DNA. Purified DNA (arrow in Fig. 1A) was labeled with peroxidase to use as a probe

and the probe hybridized only with ERIC–PCR products of Eca isolates in Southern hybridization, indicating that the fragment are useful for PCR–based detection of Eca isolates (Fig. 2B).

We have shown the potential for rapid identification of *E. carotovora* subsp. *atroseptica* and subsp. *carotovora* strains by ERIC–PCR analysis. Furthermore, we demonstrated a convenient method to generate a probe derived from an ERIC–PCR fragment useful for PCR–based detection of Eca strains.

REFERENCES

De Boer, S. H. and M. Sasser 1986 Differentiation of *Erwinia carotovora* ssp. *carotovora* and *E. carotovora* ssp. *atroseptica* on the basis of cellular fatty acid composition. *Can. J. Microbiol.*, **32**: 796–800

De Boer, S. H., D. A. Cuppels and A. Kelman 1978 Pectolytic *Erwinia* spp. in the root zone of potato plants in relation to infestation of daughter tubers. *Phytopathology*, **68**: 1784–1790

De Boer, S. H., L. Verdonck, H. Vruggink, R. Harju, H. O. Bang and J. De Ley 1987 Serological and biochemical variation among potato strains of *Erwinia carotovora* subsp. *atroseptica* and their taxonomic relationship to other *E. carotovora* strains. *J. Appl. Bacteriol.*, **63**: 487–495

Dye, D. W. 1969 A taxonomic study of the genus *Erwinia* II. The "carotovora" group. *N. Z. J. Sci.*, **12**: 81–97

Helias, V., A. L. Roux, Y. Bertheau, D. Andrivon, J. Gauthier and B. Jouan 1998 Characterization of *Erwinia carotovora* subspecies and detection of *Erwinia carotovora* subsp. *atroseptica* in potato plants, soil and water extracts with PCR–based methods. *Eur. J. Plant Pathol.*, **104**: 685–699

Keane, P. J., A. Kerr and P. B. New 1970 Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. *Aust. J. biol. Sci.*, **23**: 585–595

Lelliot, R. A. and R. S. Dickey 1984 Genus VII. *Erwinia* Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith 1920, 209[AL]. *In* "Bergey's manual of systematic bacteriology", Vol. 1, ed. by N. R. Krieg and J. G. Holt, Williams and Wilkins, Co., Baltimore, pp. 469–476

Louws, F. J., D. W. Fulbright, C. T. Stephens and F. J. de Bruijn 1994 Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Appl. Environ. Microbiol.*, **60**: 2286–2295

Perombelon, M. C. M., A. Kelman 1980 Ecology of the soft rot erwinias. *Ann. Rev. Phytopathol.*, **18**: 361–387

Sambrook, J., E. F. Fritsch and T. Maniatis 1989 *Molecular cloning*: a laboratory manual, 2[nd] edn. Cold Spring Harbor, N. Y. (U.S.A.)

Schaad, N. W. 1980 Initial identification of common genera. *In* "laboratory guide for identification of plant pathogenic bacteria", ed. by N. W. Schaad, Bact. Comm. Amer. Phytopath. Soc. Minn., pp. 1–14

Smith, C. and J. A. Bartz 1990 Variation in the pathogenicity and aggressiveness of strains of Ecc isolated from different hosts. *Plant Dis.*, **74**: 505–509

Tanii, A. 1984 Studies on the blackleg disease of potato in Hokkaido. Hokkaido Pref. Agr. Exp. Sta. **45**: 104.

Thomson, S. V., D. C. Hildebrand and M. N. Schroth 1981 Identification and nutritional differentiation of the erwinia sugar beet pathogen from members of *Erwinia carotovora* and *Erwinia chrysanthemi*. *Phytopathology*, **71**: 1037–1042

Vandamme, P., B. Pot, M. Gillis, P. De Vos, K. Kersters and J. Swings 1996 Polyphasic taxonomy, a consensus approach to bacterial systematics. *Micro–Biol. Rev.*, **60**: 407–438

Versalovic, J., T. Koeuth and J. R. Lupski 1991 Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucl. Acid Res.*, **19**: 6823–6831