Full Length Research Paper

Genetic diversity of Iranian potato soft rot bacteria based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

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Bacterial soft rot diseases caused by Pectobacterium-Dickeya complex are the most important and yield losses diseases of potato crop worldwide. Loss due to these diseases in some years/fields under Iran condition is huge and destructive. To screen and characterize the causal agents, thirty bacterial soft rot isolates including 10 authentic pectolytic strains were investigated. Based on biochemical and physiological tests, the Iranian strains were identified as either Pectobacterium carotovorum subsp. carotovorum or P. carotovorum subsp. wasabiae. Sequence analysis of recA gene revealed that the strains closely related to Pectobacterium spp. To assess the genomic diversity, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was performed. PCR amplification of the recA gene followed by RFLP revealed 14 distinct RFLP groups. Here also, the same results were obtained and all 19 Iranian strains were assigned as either Pectobacterium carotovorum subsp. carotovorum or P. carotovorum subsp. wasabiae.

Key words: Genetic diversity, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), recA gene, Pectobacterium spp.

INTRODUCTION

Soft rot bacteria are global pathogens and amongst the most prevalent and destructive bacterial diseases that affect potato, particularly during storage and transport. The most commercially important of the soft rot Erwinas are Erwinia chrysanthemi, Erwinia carotovora subsp. carotovora and Erwinia carotovora subsp. atroseptica, which cause diseases of potato and other commercially important crops (Toth et al., 2001; Czajkowski et al., 2009; Laurila et al., 2008; De Haan et al., 2008; Diallo et al., 2009; Johnson et al., 2011). These three species in the Pectobacterium-Dickeya disease complex are responsible for soft rot, aerial stem rot and blackleg of potatoes. They have a wide host range, and most succulent plants are host to at least one (sub) species of the bacteria (Collmer and Keen, 1986). All known soft rot bacteria, including Pectobacterium carotovorum subsp. carotovorum, Pectobacterium betavasculorum, Pectobacterium atrosepticum, Pectobacterium wasabiae, Pectobacterium carotovorum subsp. oederferum and Dickeya spp., have been isolated from several different potato growing areas of Iran (Bahar and Danesh, 1986; Ferydoni, 1994; Marefat and Ghasemi, 2000; Soheyl-Moghadam et al., 2004; Zohour-Paralak et al., 2006; Firuz et al., 2006; Baghaee-Ravari et al., 2010; Tavasoli et al., 2011).

Abbreviations: RFLP, Restriction fragment length polymorphism; AFLP, amplified fragment length polymorphism; RecA, recombinase A; NA, nutrient agar.

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The variation among these bacteria has been studied with different methods (Laurila et al., 2009) such as PCR amplification and sequencing, RFLP (restriction fragment length polymorphism) of the 16S gene and the 16S-23S rDNA intergenic spacer (Toth et al., 2001; Fessehaie et al., 2002). RFLP of recA gene fragments (Waleron et al., 2002) and AFLP fingerprinting (Avrova et al., 2002).

PCR-RFLP analysis of a recA gene fragment (Waleron et al., 2002) was found to be a suitable method for identifying the species and subspecies of Erwinia. PCR amplification and restriction digestion of the amplified recA fragments by four endonucleases (Alul, HinfI, TaqI and TruI) was able to differentiate the 177 strains studied into 57 RFLP groups. They also separated E. carotovora from Erwinia chrysanthemi successfully.

RecA (recombinase A) is a multifunctional protein involved in homologous recombination, DNA repair and the SOS response. It is thought to be universally present in prokaryotic and eukaryotic cells as it shows a high degree of sequence conservation. RecA protein and recA gene sequence comparisons have been used speculate about phylogenetic relationships among genera and species. The recA gene has also been used in the typing of Acinetobacter spp., genotyping of bacteria belonging to the former Erwinia genus, and identification of Mycobacterium species and the Burkholderia cepacia complex (Waleron et al., 2002). In that study, the major diversity was found with RFLP patterns produced by TaqI digestion and not by HinfI.

The present study describes the genetic diversity of Iranian potato soft rot bacteria based on recA gene polymorphisms and sequencing analysis.

MATERIALS AND METHODS

Bacterial strains and media

Bacterial strains used in this study are listed in Table 1. Nineteen bacterial cultures isolated from different potato-producing provinces were obtained from J. Razmi, Islamic Azad University, Science and Research Branch. A single isolate isolated from corn crop (corn-Abyek) was received from Iranian Plant Protection Research Institute.

The type strains were provided by Patricia van der Zouwen (International Plant Research of Netherlands) for Dickeya dianthicola 980, Dickeya dianthicola 2114(T), E. carotovora subsp. atroseptica 161 and 1007, P. carotovorum subsp. carotovorum 1955 and 1949, and Dickeya solani 2222.

Furthermore, Dickeya chrysanthemi DSM4610 and Pectobacterium atrosepticum SCR1043 were provided by Dr. Minna Pirhonen (Helsinki University, Finland). All strains were maintained in deep frozen cultures in a medium containing 10% skimmed milk supplemented with 15% glycerol at -80°C (Seo et al., 2003).

Isolation and PCR amplification of genomic DNA

The genomic DNA of strains was extracted using the alkaline lysis method (Rademaker et al., 1997). The bacterial strains were cultured on NA (nutrient agar) medium at 27°C for 24 h. 100 µl of 0.05 M NaOH was added to 10 µl of cell suspension (10^1 to 10^7 bacteria) and incubated at 95°C for 15 min. The bacterial suspensions were centrifuged for 2 min at 14,000 rpm. 1 µl from each culture supernatant was used per PCR reaction.

The oligonucleotide primers were designed based on the sequence of recA in Erwinia species (Waleron et al., 2002). The forward and reverse primer sequences were 5’-GGTAAAGGGTCT-ATCATGGG-3’ and 5’-CTTCCACATATAATTGGA-3’, respectively. These sequences were adopted from scientific sources and synthesized by Eurofins MWG Operon Corporation, Germany. The concentration of each primer was adjusted to 10 pmol/µl by ddH2O and stored at -20°C.

DNA amplification was done according to a conventional method described by Waleron et al. (2002). The PCR reaction mix was in a final reaction volume of 50 µl and contained 5 µl 10X reaction buffer (Fermentas), 1.25 µl MgCl2 (1.5 mM), 0.5 µl each of dATP, dCTP, dGTP and dTTP (10 mM), 1 µl each primer (10 pmol), 1 to 1.5 µl DNA template and 1.5 U Taq DNA polymerase.

The reaction involved initial denaturation (95°C, 3 min), followed by 32 cycles of denaturation (94°C, 1 min), annealing (47°C, 1 min) and extension (72°C, 2 min), with a final extension (72°C, 5 min). The amplified products were electrophoretically separated in a 1.5% (w/v) agarose gel at 70 V for 1 h in TBE buffer and visualized with UV light after staining in a solution of ethidium bromide (0.5 µg/ml).

Restriction fragment length analysis

The amplified DNA fragments were digested separately with four restriction endonucleases (Alul, HinfI, TaqI and TruI). 2.5 U of each restriction enzyme was used for digestions, which were incubated overnight at the temperature recommended by the manufacturer (Fermentas). The restriction patterns were compared after electrophoresis on a 1.5% (w/v) agarose gel at 70 V for 1.5 h in TBE buffer followed by ethidium bromide staining (0.5 µg/ml) and visualized with UV light.

Data analysis

The band profiles were analyzed by NTSYSpc Ver. 2.2 software. The unweighted pair-group method of averages (UPGMA) and DICE correlation coefficient within NTSYSpc Ver. 2.2 were then used to construct dendrograms from the similarity matrices. Dice’s coefficient produced a high correlation coefficient and the most adequate genetic divergence as compared to other correlations.

DNA sequencing and sequence analysis

The PCR products were purified by the British Gene Service Company, and the same primers were used for sequencing and PCR, and had the following sequences: forward primer: 5’-GGTAAAGGGTCTATCATGGG-3’; reverse primer: 5’-CTTCCACATATAATTGGA-3’.

The PCR products of 11 selected isolates from different RFLP groups were sequenced by Geneservice, England, for further characterization. Multiple sequence alignments were determined using the ClustalX software. The sequences were then compared with the databases available at the National Centre for Biotechnology Information (NCBI).

RESULTS

PCR-RFLP analysis

The specific primers were designed to bind within recA
directed amplification of fragment of about 730 bp from all 30 bacterial strains during PCR analysis (Figure 1).

RFLP of recA gene fragments digested by TasI resulted in 13 distinct banding patterns; digestion with HinII resulted in 4 different banding patterns. Thus, these enzymes yielded the most and least diversity in their RFLP patterns, respectively. AluI and TruII produced six and nine RFLP patterns, respectively (Figure 2). In total, all strains were divided into 14 groups (Figure 3). The P. carotovorum subsp. carotovorum strains were distributed in six different RFLP groups (groups 1, 3, 5, 7, 6 and 10) whereas P. carotovorum subsp. wasabiae strains were scattered in just three RFLP groups (groups 2, 4 and 8).

The two strains of P. carotovorum subsp. carotovorum 88-1 and 128A and two isolates of P. carotovorum subsp. wasabiae 31A and 58B were placed in groups 3 and 8 with the highest degree of similarity about 100%. The Dickeya Dianthicola 2114 and 980 were clustered in group 11. The two strains of P. carotovorum subsp. carotovorum 1949 and 1955 were placed in the same group with similarity above 90%.

The DNA sequences obtained for 11 selected strains were compared with available sequences deposited in GenBank (http://www.ncbi.nlm.nih). Most of these strains were highly similar (>96%) with GenBank sequences. Seven isolates, numbers 6, 11, 12, 14, 17, 20 and 38, were similar to P. carotovorum subsp. carotovorum; corn isolate CA was similar to Dickeya sp.; and three isolates, numbers 33, 10 and 41, were similar to Pectobacterium carotovorum subsp. wasabiae. The determined sequences of recA of the above-mentioned strains were deposited in NCBI under the accession numbers HQ424862.1 to HQ424871.1 and HM854829.1 (Figure 4).

DISCUSSION

A huge amount of seed potatoes has been imported from...
Figure 1. Agarose electrophoresis of PCR-amplified DNA from several strains. Lane/bacterial strains: lane 2, 83; lane 3, 117A; lane 5, 88-1; lane 6, 128A; lane 7, 74B; lane 8, 31A; lane 9, CA; lane 10, 58B; lane 11, 104B; lane 12, 116B; lane 14, 87D; lane 33, 120B; lane 36, 117B; lane 38, 84; lane 41, 110A. M: 1-kb DNA ladder.

Figure 2. Restriction analysis of recA gene fragments amplified by PCR. RFLP patterns were obtained after digestion of PCR products with AluI (A), TruI (B), TasI (C) and Hinfl (D). Lane/bacterial strains: lane 16, 112A; lane 17, 45A; lane 18, 17B; lane 19, 108A; lane 20, 123D; lane 21, 2114; lane 22, 1949; lane 23, 161; lane 24, 2222; lane 25, 980; lane 26, 1007; lane 27, 1955; lane 28, DSM4610; lane 29, SCR1043; lane 30. M: 1-kb DNA ladder.
neighboring and overseas countries with reports of increasing incidence and dispersal of important bacterial potato diseases in the main potato growing areas. However, only a few studies are available concerning the genetic diversity of these pathogens in Iran (Marefat, 2009; Baghaee-Ravari et al., 2010; Tavasoli et al., 2011). In most cases, the phenotypic characteristics of soft rot bacteria do not match with the traits published in diagnostics key tables. This indicates that a considerable diversity exists among Iranian isolates.

To address this issue, the PCR-RFLP method was selected due to its high precision and reliability. PCR primers designed based on published recA gene sequences allowed the amplification of a DNA fragment of approximately 730 bp from each of the 30 strains tested. Four restriction enzymes (AluI, HinfI, TasI and TruI) were used, and from the resultant banding patterns, 14 different recA combined RFLP patterns (restriction groups) were defined (Figure 3).

The largest number of restriction fragment patterns for the recA gene fragments was obtained with P. carotovorum. On the other hand, RFLP groups 2, 4 and 8 (containing P. carotovorum subsp. wasabiae strains) clustered together with Pectobacterium spp. and thus no unique patterns for the latter strains (58B, 110A and 120B) were observed. This issue was resolved by sequencing analysis of P. carotovorum subsp. wasabiae strains.

According to results published by Waleron et al. (2002), PCR-RFLP analysis of the recA gene fragment is not only a useful tool for the identification of species and subspecies belonging to the former Erwinia genus, but also acts as an identification tool to differentiate strains of E. carotovora subsp. carotovora and E. chrysanthemi.

Using two different enzymes (DdeI and HindIII), Seo et al. (2002) were able to differentiate all tested Asian strains of E. carotovora subsp. carotovora into 10 distinct RFLP groups. Darrasse et al. (1994) developed a primer set that amplifies a fragment of the pelY family pectate lyases from Erwinia carotovora subspecies, with the exception of E. carotovora subsp. betavasculorum. Digestion of the amplified fragment with AluI, Haell, Sau3AI and HpaII resulted in 21 RFLP groups, a group just containing E. carotovora subsp. wasabiae, two groups possessing E. carotovora subsp. atroseptica, and
Figure 4. Phylogenetic tree showing the relationship of the plant pathogenic soft rot bacterial strains in Enterobacteriaceae. On the basis of recA sequence alignment, a phylogenetic tree was constructed using the neighbour-joining method. Stability of the tree was assessed by 1,000 bootstrap replications.

El Tassa et al. (2006) with application of the molecular marker of PCR-RFLP, digested the recA gene fragment in Erwinia carotovora subsp. brasiliensis strains with TasI and Hhal restriction enzymes. The restriction fragment length polymorphism (PCR-RFLP) analysis with TasI and Hhal enzymes generated seven and 12 patterns, respectively. Analysis of the combined results allowed the separation of 13 distinct groups and differentiation of P. carotovorum subsp. brasiliensis.

Here, the largest number of fragments was obtained following TasI rather than HinfI digestion. Our results confirm the efficacy of the recA PCR-RFLP method as a reliable and reproducible method for distinguishing subspecies of Pectobacterium and Dickeya. Among the tested isolates, P. carotovorum subsp. carotovorum was found to be the most dominant soft rot bacterium in Iranian potato field samples. The next most common was P. carotovorum subsp. wasabiae with three representatives (58B, 110A and 120B). The corn strain (CA) was identified as Dickeya sp. All these were confirmed by phenotypic, PCR-
RFLP and sequences analyses. Unlike the recent report from Iran (Baghaee-Ravari et al., 2010), no *P. atrosepticum* was detected within the screened potato strains from five main potato-growing regions. This may indicate that this species is rare and therefore has a negligible role in bacterial soft rot disease in Iranian potato fields.

**ACKNOWLEDGEMENTS**

The authors are thankful to Dr. Minna Pirhonen (Helsinki University, Finland) and Dr. Patricia van der Zouwen (Plant Research International, Netherlands) for their kind help in providing the standard strains. The authors wish to thank Dr. Michele Sata for her helpful comments and critical reading of manuscript.

**REFERENCES**


