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Detection of *Pectobacterium chrysanthemi* Using Specific PCR Primers Designed from the 16S-23S rRNA Intergenic Spacer Region

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The 16S-23S rRNA intergenic spacer regions (ISRs) were sequenced and analyzed to design specific primer for identification of *Pectobacterium chrysanthemi*. Two types ISRs, large and small ISRs, were identified from three strains (ATCC 11663, KACC 10163 and KACC 10165) of *P. chrysanthemi* and *Pectobacterium carotovorum* subsp. *carotovorum* ATCC 15713. Large ISRs contained transfer RNA-Ile ($tRNA^{Ile}$) and $tRNA^{Ala}$, and small ISRs contained $tRNA^{Glu}$. Size of the small ISRs of *P. chrysanthemi* ranged on 354-356 bp, while it was 451 bp in small ISR of *P. carotovorum* subsp. *carotovorum* ATCC 15713. From hypervariable region of small ISRs, species-specific primer for *P. chrysanthemi* with 20 bp length (CHPG) was designed from hypervariable region of small ISRs, which was used as forward primer to detect *P. chrysanthemi* strains with R23-1R as reverse primer. The primer set CHPG/R23-1R produced PCR product of about 260bp size (CHSF) only from *P. chrysanthemi* strains, not from other *Pectobacterium* spp. and *Erwinia* spp. Direct PCR from bacterial cell without extracting DNA successfully amplified a specific fragment, CHSF, from *P. chrysanthemi* ATCC 11663. The limit of PCR detection was 1×10^2 cfu/ml.

Keywords : 16S-23S rRNA intergenic spacer region (ISR), PCR detection, *Pectobacterium chrysanthemi.*

Amplification of the specific target region of the bacterial genome by polymerase chain reaction (PCR) is widely used for detection and identification purposes (Chun et al., 1999; Henson and French, 1993). The ability to amplify a specific genome region from the target bacteria offer advantages over conventional cultural and phenotypic methods: simplicity, rapidity and sensitivity. The method has been applied to detect a variety of micrological agents directly in environmental samples as well as on genomic DNA and growth medium (Picard et al., 1992; Tsai and Olson, 1992). Especially, gram-negative bacteria are easily identifiable from cell and colony by PCR without the preparation of genomic DNA.

The structures of 16S-23S rRNA intergenic spacer regions (ISRs) of bacteria were very heterogeneous (Garcia-Martinez et al., 1996; Chun et al., 1999). As well as the number, type and length of ISRs may be variable on the level of genus and species, the length and sequence variations among strains were observed in ISRs. The hypervariability of struture of ISRs in bacterial genomes has been used for the species identification, strain typing and phylogenetic analysis.

Pectobacterium chrysanthemi (former *Erwinia chrysanthemi*) was originally isolated from chrysanthemum by Burkholder et al. (1953). Along with *Pectobacterium carotovorum*, it is representative pectolytic phytopathogen causing soft-rot, stunting, and wilting on a wide range of plants in different parts of the world (Lelliott, 1974; Dickey, 1979; Korean Society of Plant Pathology, 1998). Furthermore, this species were demonstrated to be heterogeneous on the basis of physiological, biochemical, and serological properties (Lelliott, 1974; Yakrus and Schaad, 1978; Dickey, 1979). The identification and differentiation of *P. chrysanthemi* from other pectolytic bacteria has relied mainly on the biochemical and pathogenic properties.

In this study, the ISRs from *P. chrysanthemi* and *P. carotovorum* were sequenced and analyzed to seek specific nucleotide sequences for *P. chrysanthemi*. The specific primer set designed for *P. chrysanthemi* was applied to detect *P. chrysanthemi* directly from cell.

Materials and Methods

Organisms and culture conditions. The bacterial strains used in this study were listed on Table 1. All bacterial strains were grown overnight on nutrient agar medium (DIFCO) at 28°C.

DNA preparation and PCR amplification of the ISRs. Chromosomal DNAs were isolated by the method of Ausubel et al. (1987), except that the lysates were extracted twice with chloroform to remove residual phenol. The ISRs were amplified by using the following primers: R16-1 (5'-CTTGTACACACCGCCCGTCA-3') and R23-1R (5'-GGTACTTAGATGTTTCAGTT-

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Table 1. Bacterial strains used in this study and GenBank accession numbers of ISRs

Speices or subspecies	Strains[a]	GenBank Accession No.	
		Small ISR	Large ISR
Pectobacterium chrysanthemi	ATCC 11663	AF232681	AF234287
	KACC 10163	AF232682	AF234285
	KACC 10165	AF232683	AF234286
	LMG 2469, LMG 2484(t1), LMG 2484(t2), LMG 2495, LMG 2516, LMG 2521, LMG2531, LMG 2923, LMG 10521	–	–
P. carotovorum subsp. *carotovorum*	ATCC 15713	AF232684	AF234279
	KACC 10342, KACC 10345, KACC 10371, KACC 10401, KACC 10421	–	–
P. carotovorum subsp. *betavasculorum*	ATCC 43762	–	–
P. carotovorum subsp. *wasabiae*	ATCC 43316	–	–
Erwinia cypripedii	ATCC 29267	–	–
Erwinia rhapontici	ATCC 29283	–	–
Pantoea ananas	ATCC 33244	–	–
Pantoea agglomerans	ATCC 33243	–	–
Brenneria quercina	LMG 2724	–	–
Brenneria salicis	ATCC 15712	–	–
Escherichia coli	DH5α	–	–

[a] ATCC, American Type Culture Collection, Rockville, Md., USA; LMG, Laboratorium voor Microbiologie, Gent, Belgium; KACC, Korean Agricultural Culture Collection, RDA, Suwon, Korea.

C-3') redesigned from the primer set of Nakagawa et al. (1994). Each PCR mixture (50 μl) contained primers (each at a concentration of 20 pmol), a mixture of deoxynucleotide triphosphates (Promega Co., Southampton, England) (each at a concentration of 200 μM), *Taq* polymerase buffer, chromosomal DNA (ca. 50 ng), and *Taq* polymerase (2.5 Unit) (Promega Co.) and 1 drop of mineral oil was added to each of the reaction mixtures. DNA thermal cycler (Perkin-Elmer Co., Norwalk, Conn.) used for PCR amplification was programmed as follows: (i) an initial extensive denaturation step consisting of 94°C for 5 min; (ii) 35 cycles, with each cycle consisting of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and (iii) a final extension step consisting of 72°C for 10 min.

Cloning and sequencing of ISRs and design of specific primer, CHPG. The fragments of purified ISRs were ligated into pGEM-T easy vector (Promega Co.). Purified plasmids were automatically sequenced on an Applied Biosytems 377 sequencer. Primers R16-1 and R23-3R were used for the sequencing of ISRs. For PCR detection of *P. chrysanthemi*, oligonucleotide primer (CHPG) specific for *P. chrysanthemi* was designed from small ISR of *P. chrysanthemi* strains.

Sensitivity of PCR detection of *P. chrysanthemi*. Colonies of *P. chrysanthemi* ATCC 11663 were taken from NA plates and suspended in water. The colony forming unit (CFU) was determined by plating the suspensions onto NA media. For PCR reaction, CFUs were serially adjusted to establish concentration ranges of 1-3 to 1×10^5 cfu/ml. PCR using specific primer set CHPG/R23-1R was conducted with the same mixture above mentioned. In case of direct PCR from cell, colony picked by toothpick was used instead of genomic DNA. PCR condition was as follows: (i) an initial extensive denaturation step consisting of 94°C for 5 min; (ii) 30 cycles, with each cycle consisting of 94°C for 1 min, 60-65°C for 30 sec, and 72°C for 30 sec; and (iii) a final extension step consisting of 72°C for 10 min. PCR products were electrophoresed on 1.2% agarose gel and visualized by staining with ethidium bromide.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the GenBank data library under the accession numbers shown in Table 1.

Results

Electrophoresis of PCR products for ISRs of *P. chrysanthemi* strains and *P. carotovorum* subsp. *carotovorum* formed multibands (data not shown). We eluted as many bands as possible, and cloned into pGEM-T easy vector. The ISRs of *P. chrysanthemi* and *P. carotovorum* subsp. *carotovorum* were differentiated into two types, large and small ISRs, based on sequence analysis. The small ISRs of two species were encoding a transfer RNA genes (tRNA), $tRNA^{Glu}$ (Fig. 1). The small ISRs for three strains (ATCC 11663, KACC 10163 and KACC 10165) of *P. chrysanthemi* (GenBank accession No. AF 232681, AF 232682 and AF 232683, respectively) were 354-356bp in length while small ISR of *P. carotovorum* subsp. *carotovorum* ATCC 15713 (GenBank accession No. AF 232684) were 451bp in length. The sequence homologies of small ISRs among three strains of *P. chrysanthemi* were 90.1-94.6%, but they showed 48.3-48.6% of sequence homologies to ISR of *P.*

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  1  ACCGAGTAGAAGCACCTGCGTGGTGTCCACACAGATTGTCTGATGAAAAG

 51  AAAGAGTCAAAAGCGTCTTGCGAAGCTGACAAGCGATGTCCCCTTCGTCT

101  AGAGGCCCAGGACACCGCCCTTTCACGGCGGTAACAGGGGTTCGAATCCC
                          tRNA(Glu)
151  CTAGGGACGCCAATATGACTGACGGTGGGTGAAAGGCACGGTCAACGCTA

201  ACCTAAAACTGATTAGAGATAGTCAGGTTTATGTTATCTGCTCTTTAACA

251  ATCCGGAACAAGCTGAATAATTTGAAACGACGGCATGCGAATGCGAATTC
                                             CHPG -->
301  ATGTGTTGTTCGAGTCTCTCAAAATACTTGCCTCCCGAAACACTTTCGGG

351  TTGTGA
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Fig. 1. Nucleotide sequence of small 16S-23S intergenic spacer region of *P. chrysanthemi* ATCC 11663 (GenBank No. AF232681). Small ISR comprises tRNA^{Glu} (underlined) and CHPG (arrow).

Species (Strain)	GenBank accession No.	Nucleotide sequence
		G-YG AATTCRTRTG TTGTTCG: CHPG
P. chrysanthemi (ATCC 11663)	AF 232681	GCGAATG-CG AATTCATGTG TTGTTCGAGT
P. chrysanthemi (KACC 10163)	AF 232682	GTGAATG-CG AATTCGTATG TTGTTCG-GT
P. chrysanthemi (KACC 10165)	AF 232683	GTGAATG-CG AATTCGTATG TTGTTCGAGT
P. carotovorum (ATCC 15713)	AF 232684	GTGAGTGATG CATCAGTCTG TCAAT-GAGT

Fig. 2. Nucleotide sequence comparison of *P. chrysanthemi* strains and *P. carotovorum* subsp. *carotovorum* ATCC 15713 on primer region.

carotovorum subsp. *carotovorum* ATCC 15713.

The sequences of ISRs for *P. chrysanthemi* strains and *P. carotovorum* subsp. *carotovorum* were aligned to design species-specific primer for *P. chrysanthemi* (Fig. 2). CHPG, 20 bp in size, was made on comparison of small ISRs of three *P. chrysanthemi* strains, and used as forward primer for PCR amplification (Fig. 2). It located from 293 bp to 312bp of GenBank accession No. AF232681. Due to a little sequence variations among *P. chrysanthemi* strains, CHPG included code letters Y (C or T) and R(A or G) (Fig. 2).

PCR using primer set, CHPG/R23-1R, amplified about 260 bp fragment (CHSF), which comprised partial ISR and 23S rRNA gene. CHSF was produced only from *P. chrysanthemi* strains collected from a variety of regions and hosts, but not from other *Pectobacterium* spp. and *Erwinia* spp (Fig. 3A, B). Cell-based PCR without DNA extraction successfully amplified the specific band (CHSF) from *P. chrysanthemi* ATCC 11663, and the detection limit was 1×10^2 cfu/ml (Fig. 4).

Fig. 4. Detection sensitivity of primer set, CHPG and R23-1R, from different number of cell of *P. chrysanthemi* ATCC 11663. M: 1 kb ladder; Lane 1: colony, 2: 1×10^5 cfu/ml, 3: 1×10^4 cfu/ml, 4: 1×10^3 cfu/ml, 5: 1×10^2 cfu/ml, 6: 1×10^1 cfu/ml, 7: water.

Discussion

The 16S and 23S rRNA intergenic spacer region (ISR) has been considered an ideal site for developing specific PCR primers that can differentiate bacteria because of its exten-

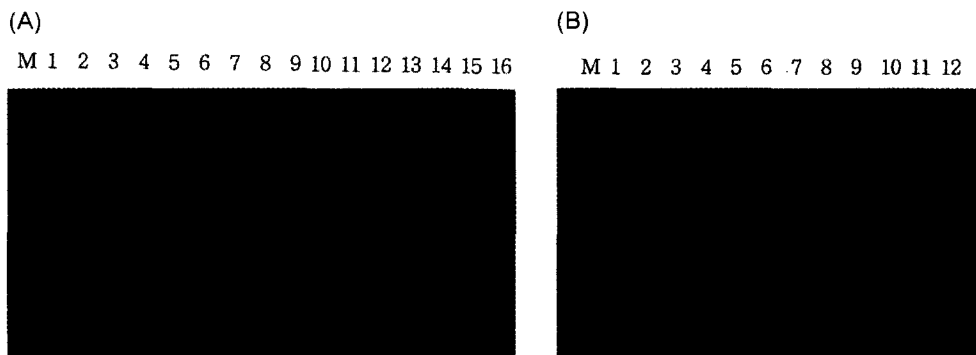

Fig. 3. PCR specificity of primer set, CHPG and R23-1R, for *P. chrysanthemi* strains; (A) PCR products from various species. M: 1 kb ladder, Lane 1: *P. chrysanthemi* ATCC 11663, 2-9: *P. carotovorum* strains, 10: *Erwinia cypripedii*, 11: *Erwinia rhapontici*, 12: *Pantoea ananas*, 13: *Pantoea agglomerans*, 14: *Brenneria quercina*, 15: *Brenneria salicis*, 16: *Escherichia coli*, (B) PCR products from *P. chrysanthemi* strains.

sive sequence variation (Barry et al., 1991). Among closely related bacteria, high sequence variation in ISRs is considered to be the result of low selective pressure to maintain conserved sequences, in contrast to sequences of genes that have important functional roles such as 16S and 23S rRNA genes. Thus, the spacer region separating 16S and 23S rRNA genes has been used for developing PCR primers for detections of other bacteria (Chun et al., 1999).

Recently, Nassar et al. (1996) developed a method for PCR detection and characterization of *P. chrysanthemi* based on *pel* genes. However, it is problematic in that PCR detection using the *pel* genes was carried out on high annealing temperature (72°C), and the detection ability for *P. chrysanthemi* were compared with *E. coli* which did not produce pectate lyase, the product of *pel* genes, not *Pectobacterium* and *Erwinia* spp. (Nassar et al., 1996).

According to the sequence analysis of ISRs, multiple ribosomal RNA gene clusters (rrn clusters) of *Pectobacterium* species could be differentiated into two types, large ISRs and small ISRs, on the basis of size of ISRs and types of transfer RNA within ISRs. *P. chrysanthemi* contained high sequence variation from *P. carotovora* both on large and small ISRs. The type strain (ATCC 11663) of *P. chrysanthemi* showed sequence homologies of 48.4% and 48.6 % to that (ATCC 15713) of *P. carotovorum* subsp. *carotovorum* on large and small ISRs, respectively. These high sequence variations of ISRs between *P. chrysanthemi* and *P. carotovorum* subsp. *carotovorum* were unexpected when considering high sequence homology level (96.2%) among 16S rRNA genes of *P. chrysanthemi* and *P. carotovorum* subspecies (Kwon et al., 1997).

Using high sequence variation of ISRs between two species, we designed two primers from the variation region of large and small ISRs for specific PCR detection of *P. chrysanthemi*. Although CHPG designed from small ISR produced about 260 bp fragment specific for *P. chrysanthemi* strains, the other primer (CHPI) designed from the large ISR produced bands of various size from strains of *P. chrysanthemi* and formed faint bands from other *Pectobacterium* species (data not shown). The non-specificity of CHPI primer was supposed to be generated from mis-alignments of ISRs, which resulted from the high sequence variation of ISRs among *Pectobacterium* species.

PCR using the CHPG and R23-1R was shown to be specific for *P. chrysanthemi* strains on relatively high stringent condition of 63-65°C annealing temperature. When the annealing temperature was lowered below 60°C, other *Pectobacterium* species also formed faint bands (data not shown). However, the specificity of CHPG and R23-1R was thought to be high as compared with annealing temperature (72°C) of PCR detection using *pel* genes.

In PCR using colony, the various components of cell will be obstacles for PCR amplification of target gene. Actually, cell-based PCR did not amplified 260 bp fragment specific for *P. chrysanthemi* strains on annealing temperature of above 65°C, of which temperature was applicable for 260 bp fragment amplification from genomic DNA of *P. chrysanthemi* strains. In case of colony PCR, 60-62°C of annealing temperature were optimum temperature that could obtain fragment specific for *P. chrysanthemi* without PCR band production of other *Pectobacterium* and *Erwinia* species.

The detection limit of colony PCR, 1×10^2 CFU on occasion of *P. chrysanthemi*, could be affected by several factors such as annealing temperature, G + C content of primers and copy number of target genes. Ribosomal RNA gene (rrn) clusters in eubacteria exist in multicopies, 1-11 rrn clusters (Chun et al., 1999). The rrn clusters of *Pectobacterium* were also reported to be multicopy genes (Kwon, 1997), and PCR using primer designed from rrn clusters could increase the sensitivity of PCR detection of *P. chrysanthemi*.

At present, a number of methods are being developed for detection of phytopathogenic bacteria from soils and hosts. Although we didn't try the detection of *P. chrysanthemi* from the samples such as soil and host, high sensitivity of detection of *P. chrysanthemi* would be useful tool for diagnosis of these pathogens.

References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidmann, J. G., Smith, J. A. and Struhl, K. 1987. *Current protocols in molecular biology.* Greene Publishing Associates and Wiley Interscience, New York, N. Y.

Burkholder, W. H., McFadden, L. A. and Dimock, A. W. 1953. A bacterial blight of chrysanthemums. *Phytopathology.* 53:522-526.

Barry, T., Colleran, G., Glennon, M., Dunican, L. K. and Gannon, F. 1991. The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. *PCR Methods Appl.* 1:51-56.

Chun, J. S., Huq, A. and Colwell, R. R. 1999. Analysis of 16S-23S rRNA intergenic spacer regions of *Vibrio cholerae* and *Vibrio mimicus. Appl. Environ. Microbiol.* 65:2202-2208.

Dickey, R. S. 1979. *Erwinia chrysanthemi*:a comparative study of phenotypic properties of strains from several hosts and other *Erwinia* species. *Phytopathology* 69:324-329.

Garcia-Martinez, J., Martinez-Murcia, A. J. and Rodriguez-Valera, F. 1996. Comparison of the small 16S to 23S intergenic spacer region (ISR) of the rRNA operons of some *Escherichia coli* strains of the ECOR collection and *E. coli* K-12. *J. Bacteriol.* 178:6374-6377.

Henson, J. M. and French, R. 1993. The polymerase chain reaction and plant disease diagnosis. *Annu. Rev. Phytopathol.* 31: 81-109.

Korean Society of palnt pathology. 1998. List of plant diseases in Korea. 3rd ed. Korean society of plant pathology, Suwon, Korea.

Kwon, S. W. 1997. Phylogenetic analysis of *Erwinia* spp. based on 16S rDNA sequences. Kyungpook National University. Thesis of Master Degree. pp. 27-28.

Kwon, S. W., Go, S. J., Kang, H. W., Ryu, J. C. and Jo, J. K. 1997. Phylogenetic analysis of *Erwinia* species based on 16S rRNA gene sequences. *Int. J. Syst. Bacteriol.* 47:1061-1067.

Lelliott, R. A. 1974. Description of the species of genus *Erwinia*. Pages 332-340 in R. E. Buchanan and N. E. Gibbons. eds. Bergey's Manual of the Determinative Bacteriology. 8th ed. The Williams and Wilkins Co., Baltimore. MD. 1,246 pp

Nakagawa, T., Shimada, M., Mukai, H., Asada, K., Kato, I., Fujino, K. and Sato, T. 1994. Detection of alcohol-tolerant Hiochi bacteria by PCR. *Appl. Environ. Microbiol.* 60:637-640.

Nassar, A., Darrasse, A., Lemattre, M., Kotoujansky, A., Dervin, C., Vedel, R. and Bertheau, Y. 1996. Characterization of *Erwinia chrysanthemi* by pectinolytic isozyme polymorphism and restriction fragment length polymorphism analysis of PCR-amplified fragments of *pel* genes. *Appl. Environ. Microbiol.* 62:2228-2235.

Picard, C., Ponsonnet, C., Paget, E., Nesme, X. and Simonet, P. 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Appl. Environ. Microbiol.* 58:2717-2722.

Tsai, Y. and Olson, B. H. 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. *Appl. Environ. Microbiol.* 58:754-757.

Yakrus, M. and Schaad, N. W. 1979. Serological relationships among strains of *Erwinia chrysanthemi*. *Phytopathology* 69: 517-522.