Detection of Xanthomonas campestris pv. citri by PCR
Using Primers from the Spacer Region between
the 16S and 23S rRNA Genes

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Abstract

Polymerase chain reaction (PCR) primers targeting genomic DNA of Xanthomonas campestris pv. citri, a causal agent of citrus bacterial canker, were designed for a rapid, sensitive and specific detection of the bacterium. The intergenic spacer region of approximately 500 bp between the 16S and 23S rRNA genes from X. c. pv. citri, X. c. pv. glycines, X. c. pv. alfalfae, X. c. pv. physalidicola, X. c. pv. pisi, X. c. pv. pruni, X. c. pv. cucurbitae and X. c. pv. vesicatoria were sequenced. Subsequently, a pair of PCR primers, XCF (5' AGGCCGGTATGCGAAAGTCCCATCA-3') and XCR (5' CAAGTTGCCTCGGAGCT-ATC-3'), were designed on the basis of the sequence data of X. c. pv. citri, because these sequences differed from those of the other pathovars except X. c. pv. glycines. Although a fragment of 424 bp was specifically amplified for all 36 isolates of X. c. pv. citri having different origins and for one isolate of X. c. pv. glycines, no PCR products were obtained from the other pathovars. To confirm specific detection of X. c. pv. citri, 81 bacteria isolated from citrus leaf surfaces were examined by PCR and pathogenicity tests. Consequently, PCR products were obtained from only nine pathogenic strains belonging to X. c. pv. citri, but no products were detected from the other non-pathogenic bacteria, indicating that X. c. pv. glycines is not an inhabitant of citrus orchard. The detectable limit of PCR amplification was 30 cfu per ml, which was 10 times more sensitive than the leaf infiltration technique which had been considered the most sensitive. PCR using the specific primers designed in this study, was useful for rapid identification and detection of X. c. pv. citri from citrus samples within 6 hr of sample collection.

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Key words: PCR, specific detection, spacer region, Xanthomonas campestris pv. citri.

INTRODUCTION

Citrus bacterial canker disease, caused by Xanthomonas campestris pv. citri, is a serious disease of leaves, fruit and shoots in Japan. Furthermore, the disease has become a barrier to the export of satsuma mandarin (C. unshiu Marc.) into the United States of America. Because the pathogen is a target of international quarantine efforts, the development of rapid, reliable procedures for detecting the pathogen has been a priority. Although the phage technique\(^1\), enzyme-linked immunosorbent assay (ELISA)\(^2\), leaf infiltration technique\(^3\) and a method of inoculation into a detached leaf\(^4\) have been applied for detecting the pathogen, the detectable limit by the phage technique\(^5\) and ELISA\(^6\) is \(10^3\) to \(10^4\) cfu/ml, whereas that of the other methods\(^5,6\) is \(10^2\) cfu/ml with a detectable period of 7 to 14 days after. In addition, a number of problems with the rapidity, sensitivity and specificity of the detection of the bacterial pathogens have been unsolved.

Recently, polymerase chain reactions (PCR)\(^7,8\) have been developed to detect plant pathogenic bacteria, because of its rapidity, sensitivity and specificity. Several assays have also been developed to detect X. c. pv. citri by PCR amplification of plasmid DNA\(^9\). However, the plasmid is easily cured, frequently mutates within the internal sequence and is not present in all pathogens. Therefore, more reliable methods are needed to detect all the citrus canker pathogens.

In this paper, spacer regions between the 16S and 23S rRNA genes (16S-23S spacer) of X. c. pv. citri on the specific oligonucleotide sequences were screened for use as primer sites in PCR-based detection.

MATERIALS AND METHODS

Bacterial strains Bacterial cultures used for DNA sequencing of X. c. pv. citri (MAFF 311134), X. c. pv. glycines (MAFF 301462), X. c. pv. alfalfae (MAFF

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The plate count procedure on PSA medium. In addition, each concentration of bacteria was determined by a standard saline and diluted 10 times in a 10-fold series. The template was then visualized with ethidium bromide staining of the products were separated by electrophoresis in a 2% agar, 15.0g; distilled water, 1000ml; pH 7.0; PSA medium in follows) at 28°C for 48hr. Template DNAs for PCR were extracted from cells by using InstaGene DNA purification matrix (Bio-Rad Laboratories, U.S.A.) according to the supplier's instructions.

**DNA extraction** Bacteria were grown in Wakimoto's potato decoction semisynthetic medium (decocion from potato 300 g; Ca(NO₃)₂.4H₂O, 0.5 g; Na₂HPO₄.12H₂O, 2.0 g; peptone, 5.0 g; sucrose, 15.0 g; agar, 15.0 g; distilled water, 1000 ml; pH 7.0; PSA medium in follows) at 28°C for 48 hr. Template DNAs for PCR were extracted from cells by using InstaGene DNA purification matrix (Bio-Rad Laboratories, U.S.A.) according to the supplier's instructions.

**DNA sequencing** Sequences of the 16S-23S spacer regions were determined by using the method described in the previous paper. Primers for sequencing were designed on the basis of a database search of published sequences of 16S and 23S rRNA genes of xanthomonads in GenBank, with sequences as follows: Space FB-2 (5'–TTTCACACACGAGAAACGCTATGAGCGGTTGAACTGTCAACAAGGTCACGCCC-3') and Space R2 (5'–GGCCAGGTTTTCCAGCTACAGCATCGCCTCTGACTGCCAAGGCATC-3').

**Selection of PCR primers for specific amplification** Sequence data for the 16S-23S spacer region for the eight pathovars of X. campestris were manually aligned. Primers were designed based on the sequences which differed between X. c. pv. citri and the other pathovars. PCR amplification of the target sequence was performed using a model 2400 DNA thermal cycler (Perkin-Elmer Cetus, U.S.A.) with 20 μl (total volume) reaction mixture containing Taq polymerase by NIPPON GENE, Japan, 2.5 mM dNTP Mixture, 20 pmol of each primer, 1 μl of template, 0.5 μl of 10% skim milk and 5 units of Taq polymerase (NIPPON GENE, Japan). The reaction mixtures were initially denatured at 95°C for 2.5 min, followed by 32 cycles consisting of denaturation at 95°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 2 min, and an additional extension step consisting of 72°C for 10 min. After PCR amplification, 10 μl of the products were separated by electrophoresis in a 2% agarose gel in TAE buffer. The amplified DNA fragment was then visualized with ethidium bromide staining and trans-illumination.

The lower sensitivity limit of PCR amplification was determined by testing dilution series of bacterial culture suspension. Loopfuls of bacterial strain 94040 of X. c. pv. citri were suspended in 10 ml of a sterile physiological saline and diluted 10 times in a 10-fold series. The concentration of bacteria was determined by a standard plate count procedure on PSA medium. In addition, each dilution was also tested with the leaf infiltration technique which has been used for detecting the hypersensitive reaction in plants. The DNA template for PCR was prepared from the remaining 1 ml of each dilution by using 100 μl InstaGene. To determine the sensitivity of PCR detection, 5 μl of the template was used per total reaction mixture (20 μl).

**PCR for detection of X. c. pv. citri in citrus leaf** Bacterial DNA in citrus leaves was extracted using the following method. A piece (1–2 cm) of leaf was suspended in 1 ml of sterile physiological saline and vigorously shaken for 10 min. After removing the leaf piece, the suspension was then centrifuged at 14,000 × g for 3 min. The DNA template for PCR was extracted from the resulting pellet with 100 μl InstaGene, and 5 μl of this template was then used for PCR.

**Pathogenicity tests** Bacterial cultures were grown in PSA medium for 48 hr at 28°C. The cells were suspended in sterile physiological saline at 10⁶ cfu/ml. For each strain, three citrus leaves (cv. Natsudaidai) were inoculated by the leaf infiltration technique. This method was successful for identification of the pathogen.

**RESULTS**

**Sequence data and accession numbers** Spacer regions of 16S-23S of eight pathovars of X. campestris: citri, alfalfae, cucurbitae, glycines, physalidicola, pisi, pruni, vesicatoria, were determined. No base differences were observed among sequences of spacer regions of six strains (MAFF 311134, 94001, 94040, 94048, 94119 and N1) of X. c. pv. citri. Among the spacer regions of the eight pathovars, sequences were highly homologous (more than 95%), especially between pv. citri and pv. glycines which were differentiated by only 1bp. In these regions the genes for alanine-tRNA (tRNAalan), and isoleucine-tRNA (tRNAlle) were highly homologous (more than 95%), especially between pv. citri and pv. glycines which were differentiated by only 1bp. In these regions the genes for alanine-tRNA (tRNA dense) and isoleucine-tRNA (tRNA iso) were contained (Fig. 1).

The sequences of the strains investigated in this study have been deposited in the DDBJ (DNA Data Base of Japan, Mishima, Japan) under the following accession numbers: AB004282 for MAFF 301090, AB004283 for MAFF 311127, AB004284 for MAFF 301081, AB004285 for MAFF 301294, AB004286 for MAFF 301206, AB004287 for MAFF 301421, AB004288 for MAFF 301462, AB004289 for MAFF 301294.

**Primer design and specificity** The sequence alignment of the 16S-23S spacer regions of X. c. pv. citri, X. c. pv. alfalfae, X. c. pv. cucurbitae, X. c. pv. glycines, X. c. pv. physalidicola, X. c. pv. pisi, X. c. pv. pruni and X. c. pv. vesicatoria, is shown in Fig. 1. On the basis of the sequence data, a pair of PCR primers, XCF (5'–AGGCCGGTATGCGAAAGTCCCATCGCCTCTGACTGCCAAGGCATC-3') and XCR (5'–CAAGTTGCCTCGGAGCTATCGCCTCTGACTGCCAAGGCATC-3') was designed for specific amplification. A fragment of 424 bp was amplified for all 36 strains of X. c. pv. citri having different origins and for X. c. pv. glycines, by PCR using XCF and XCR primers, whereas no PCR
Fig. 1. Sequence alignment of the 16S-23S spacer regions X. campestris pathovars. Row 1, X. c. pv. citri; row 2, X. c. pv. glycines; row 3, X. c. pv. alfalfae; row 4, X. c. pv. physalidicola; row 5, X. c. pv. pisi; row 6, X. c. pv. pruni; row 7, X. c. pv. cucurbitae; row 8, X. c. pv. vesicatoria. The underlined part of the sequence is tRNA^Ala. The double-underlined sequence is tRNAlle. Dash (−), alignment gaps and base deletions; dot (·), identity with sequence of X. c. pv. citri. The shaded and single underlined regions are PCR primer sites.
products were obtained from the other bacteria (Fig. 2). PCR products were obtained from only nine pathogenic strains belonging to X. c. pv. citri out of the 81 bacteria isolated from citrus leaf surfaces. No PCR products were detected from the other non-pathogenic bacteria. These results showed that our primers served to detect the pathogens of citrus canker. Our primers could detect 30 cfu per ml, which was 10 times more sensitive than the leaf infiltration technique in comparative tests (Table 2).

**DISCUSSION**

The eubacterial intergenic 16S–23S spacer region has been considered an ideal region for developing specific PCR primers that can differentiate bacteria, because it contains extensive sequence variations\(^1\). Recently, specific primers for detecting plantpathogenic bacteria have been developed\(^10,15\).

Four pathotypes of X. c. pv. citri, A,B,C,D, have been described on the basis of the host range, geographic distribution, serology, phage typing, plasmid analysis and restriction fragment length polymorphisms (RFLP) of the bacterial strains\(^2,3,6,8\). In this study, however, pathotype A was used because it is the only pathotype in Japan on the basis of RFLP analysis\(^13\).

Complete sequencing of the 16S–23S spacer region of plant pathogenic X. c. pathovars showed that all six strains of X. c. pv. citri from diverse geographical regions in Japan share the same sequence, which coincides with previous results of RFLP analysis of this pathogens\(^13\). The degree of similarity between X. c. pv. citri and other pathovars, alfalfa, cucurbitae, glycines, physalisidico, pisi, pruni, vesicatoria, is more than 95%; the difference in sequence between citri and glycines was only 1 bp (Fig. 1). These results suggest that the spacer regions within X. campestris pathovars are highly conserved, especially within the Japanese isolates of X. c. pv. citri used in this study, and that the sequence differences in this region can be used for differentiating X. c. pv. citri from other pathovars.

At first, PCR primers were designed on the basis of sequences which differed between X. c. pv. citri and the other pathovars (including X. c. pv. glycines), but the primers did not work well (data not shown). We then selected the sequences of X. c. pv. citri which differed from the other pathovars except X. c. pv. glycines, namely, X. c. pv. citri and X. c. pv. glycines are identical for these sequences, and designed XCF and XCR. By the
PCR using XCF and XCR, a 424 bp fragment was amplified in all 36 strains of *X. c. pv. citri* and 1 strain of *X. c. pv. glycines*. No PCR products were obtained from the other bacteria (Table 1, Fig. 2).

To confirm the existence of *X. c. pv. glycines* in citrus orchard, 81 bacteria isolated from citrus leaf surfaces were examined by PCR and pathogenicity tests. Consequently, PCR products were obtained from only nine pathogenic strains of *X. c. pv. citri*, and no PCR products were obtained from other non-pathogenic bacteria (Table 1). *X. c. pv. glycines* is not considered to be an inhabitant of citrus leaves. These results indicate that the PCR primers, XCF and XCR, could be used as specific primers to detect *X. c. pv. citri* on citrus trees. In comparing PCR with the leaf infiltration technique, detection limit of PCR was greater (Table 2). PCR also had an excellent detection time. Thus, PCR method has greater advantages in detecting *X. c. pv. citri* than conventional ones\(^{2,5,9,11}\).

Another advantage of the PCR detection test based on the sequences of spacer region is that the method does not depend on the presence of a plasmid. Other PCR tests for *X. c. pv. citri* using plasmid sequences as primers\(^7\) failed to detect pathogens that lacked the plasmid, because of curing and frequent mutation in the plasmid.

PCR methods using the specific primers designed in this study were also be useful for the rapid identification and detection of *X. c. pv. citri* in citrus leaf samples. This method may also aid epidemiological studies of *X. c. pv.
c. pv. citri, because pathogens in citrus leaf samples can be detected within approximately 6 hr after sample collection by the PCR method.

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Literature cited