Plant Pathogenic Bacteria, Versailles (France), June 9-12, 1992
Ed. INRA, Paris 1994 (Les Colloques, n°66)

Application of the polymerase chain reaction (PCR) for enhanced detection of *Xanthomonas campestris* pv. *citri*

J.S. HARTUNG, J.F. DANIEL* and O.P. PRUVOST**

USDA-ARS-Plant Sciences Institute, Beltsville, MD

* *ORSTOM, Montpellier, France*

** *CIRAD/IRFA, La Réunion, France*

ABSTRACT Plasmid pFL62.42 contains a 4.2 Kb *Bam* HI fragment cloned from plasmid DNA of strain XC62 of *Xanthomonas campestris* pv. *citri* (*Xcc*) and is a highly sensitive and specific hybridization probe for *Xcc*. Plasmid pFL1 contains a 562 base pair internal *Eco* RI fragment from pFL62.42 and gives slightly more specific results. Both plasmids detected 44/44 pathotype A strains of *Xcc* originally isolated in 15 countries. Nearly all strains of pathotypes B and C were also detected with these probes. Neither probe reacted with strains of *X. campestris* isolated from symptomless *Citrus* nor with 56 strains of *X. campestris* associated with citrus bacterial spot disease (CBS) in Florida citrus nurseries nor with bacteria from other genera. The sensitivity in dot blot assays using chemiluminescent detection was approximately 2-7 ng DNA/spot. Probe pFL1 specifically detected Asiatic strains of *Xcc* in extracts of leaf lesions. Nucleotide sequence information from pFL1 was used to design primers in order to apply the polymerase chain reaction (PCR) for the detection of *X. c. citri*. Seven 18 bp oligonucleotide primers were designed and tested with DNA from *X. c.* pv *citri* and other strains of X. campestris associated with *Citrus* as templates in PCR reactions. Primer pair 2 / 3 directed specific amplification of target DNA of pathotype A of X. c. pv *citri*, but not of other pathotypes of *X. c.* pv *citri*. A buffer of pH 9.0 which contained 1 % Triton X-100 and 0.1 % gelatin was absolutely required for successful amplification of the GC rich target DNA.

KEYWORDS : Diagnostic Probe, Citrus Bacterial Canker, Pathogen Detection

Recent outbreaks of Citrus Bacterial Canker (CBC) in Florida, USA have stimulated a great deal of research into the biology of the pathogen *Xanthomonas campestris* pv *citri*. (reviewed by Stall and Civerolo, 1991). Because the pathogen is the target of international quarantine efforts (Anonymous, 1991) development of

rapid and reliable diagnostic procedures for this pathogen has been a priority. This task has been complicated by the presence of another distinct disease in Florida Citrus nurseries, citrus bacterial spot (CBS), caused by other strains of *Xanthomonas campestris* (Schoulteis it al., 1987). Although the taxonomic position of *Xanthomonas campestris* pv *citri* has been controversial (Gabriel et al., 1989; Young et al. 1991), there is widespread agreement that the strains of *X. campestris* which cause CBS are quite distinct from, and should not be confused with any of the several pathotypes (A-D) causing CBC.

We and others have shown that RFLP analysis of genomic DNA of these strains can separate the strains into groups which are entirely consistent with groups independently derived from other data (Gabriel et al. 1988; Hartung and Civerolo, 1989). This work was recently extended by a RFLP analysis of plasmid DNA from strains of *X.c.* pv *citri.* (Pruvost et al., 1992). During the course of this study, a 4.2 kb *Bam* HI fragment was found in 85.2% of the most pathogenic strains (Asiatic or CBC-A) of *X.c.* pv. *citri.* After molecular cloning from strain XC62 as pFL62.42, this fragment distinguished subtypes of *X. c.* pv. *citri* in Southern blots and importantly, did not cross react with strains of *X. campestris* which cause CBS.An internal 562 bp *Eco*RI fragment was cloned as pFL1 and the two fragments used to develop a rapid and sensitive dot blot assay for *X. c.* pv *citri* (Hartung, 1992).

The Polymerase Chain Reaction (PCR) (Saiki et al. 1988) allows rapid, specific and sensitive detection of DNA sequences, and thus is ideally suited for the detection of plant pathogens. We report the development of a PCR based assay for X. c. pv citri based on the DNA sequence of the *Eco*RI insert in pFL1.

MATERIAL AND METHODS

The *Eco*RI insert from pFL1 (Hartung 1992) was cloned into the sequencing vectors M13mp18 and M13mp19 by standard methods. The complete nucleotide sequence of both strands of the insert was determined by dideoxy sequencing using the Sequenase system (United States Biochemical, Cleveland, Ohio). Oligonucleotides 18 bp in length were designed using the computer program Nuc-it (Compu-Right, Gaithersburg, MD). Paired primers were selected which had low homology to other sequences in the target fragment and which had closely matched calculated thermal melting points. PCR assays were performed in a DNA Thermal Cycler (Perkin Elmer/Cetus) in 50 ul reactions which typically contained 50 ng genomic DNA, dNTP's at 200 uMolar and primers at 1 uMolar each. Three

reaction buffers were used: 10 mM Tris/Cl (pH8.3), 50 mM KCl, 3 mM Mg^{++} (Buffer I), Buffer I with 3% formamide and 7% glycerol (Buffer II) and 50 mM Tris/HCl (pH9.0), 20 mM NaCl, 1% Triton X-100 and 0.1 % gelatin and 3 mM Mg^{++} (Buffer III) (Barry et al., 1991). Denaturation was at 95 C for 70 seconds, annealing was at 45-65 C for 60 seconds and extension was at 72 C for 1 minute plus 2 seconds per cycle for 30 cycles. Aliquots containing 25% of the reaction were removed and subjected to agarose gel electrophoresis in 3% composite agarose gels (3 parts Nusieve : 1 part standard LE agarose (FMC, Rockland ME)) or 1.5 % LE agarose gels. The oligonucleotide primers were synthesized commercially (Genosys, The Woodlands, Texas). The bacterial strains used were described previously (Hartung, 1992).

RESULTS AND DISCUSSION

Because the DNA sequence of the 562 bp target fragment was 60.6 % G+C, and the target sequence was part of a plasmid, difficulty in achieving amplification was anticipated. Because buffer composition can effect PCR results, the standard PCR buffer recommended by Perkin Elmer/Cetus was compared to the same buffer supplemented with the cosolvents glycerol and formamide (Sarkar et al. 1990; Smith et al., 1990) and to a third buffer (Barry et al. 1991). Primer pairs 2/3, 4/5, 6/7 and 1/5 were expected to prime amplification of products of 222 bp, 462 bp, 478 bp and 261 bp respectively when homologous (XC62) DNA was used as the target template (Figure 1). No amplification of homologous target DNA was achieved using Buffers I or II at any annealing temperature from 45 C - 65 C except for primer pair 1/5 which successfully primed amplification of its target sequence when annealed at 65 C. In contrast, specific amplification products were produced in Buffer III at all annealing temperatures from 45 C to 65 C with all four primer pairs. The results from the 55 C annealing reaction are typical (Figure 2). Specificity was improved when the annealing temperature was 60 C.

Primer pair 2/3 was used to amplify target sequences in genomic DNA from 12 CBC-A strains originally isolated in 12 countries. This primer pair did not find target sequences in DNA from 5 strains of pathotype B,C and D of *X.c.* pv. *citri* (Figure 3) nor in DNA from 4 strains of *X. campestris* associated with CBS. A product was found when DNA from a single strains of *X. c.* pv *vignicola* and *X.c.* pv *bilvae* were tested. These results are consistent with hybridization analysis of these same strains (Hartung, 1992), in which pathotype B, C and D strains gave

consistently weaker results than pathotype A strains in dot blot assays, and produced homologous bands of different size in Southern blots.

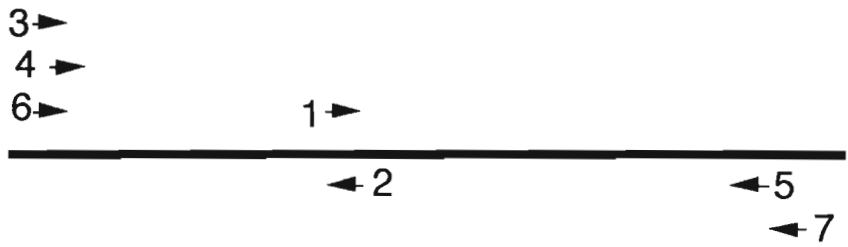

Figure 1. Diagram of the 562 bp target region for PCR amplification of *X. c.* pv. *citri* DNA showing the relative positions and orientations of the 18 bp oligonucleotide primers 1-7.

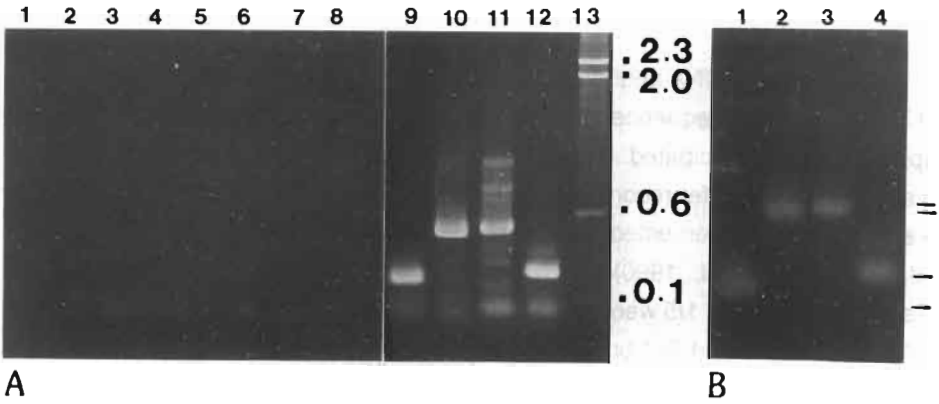

Figure 2 (A) Successful amplification of target DNA is dependent on the reaction buffer. Lanes 1-4: Buffer I; Lanes 5-8; Buffer II; Lanes 9-12: Buffer III. Primer pair 2/3 was used for samples 1,5 and 9; pair 4/5 for samples 2,6 and 10; pair 6/7 for samples 3, 7 and11 and pair 1/5 for samples 4,8 and 12. Annealing temperature was 55 C. **(B)** Same as Fig. 2 (A), lanes 9-12 except the annealing temperature was 60 C. The size of Lambda/*Hin* dIII standards is given in the margin. The template DNA was 50 ng of *Eco*RI digested pathotype A (XC62) DNA for all reactions.

Although amplification was successful with genomic DNA of pathotype A of *X. c.* pv *citri* , yield was improved if the DNA was digested with *Bam* HI or *Eco* RI prior to amplification *(not shown)*. This is probably due to the supercoiled (plasmid) state of the target DNA. However amplification of target DNA was successful when starting with intact bacteria *(not shown)* as described previously for other species (Barry et al., 1991). The composition of the reaction buffer was critical for successful amplification of *X. c.* pv *citri* DNA. Buffer III differs from the standard

PCR buffer by having a higher pH and by incorporating 1% Triton X-100 and 0.1% gelatin. We do not know which of these ingredients is most responsible for the success of the amplification.

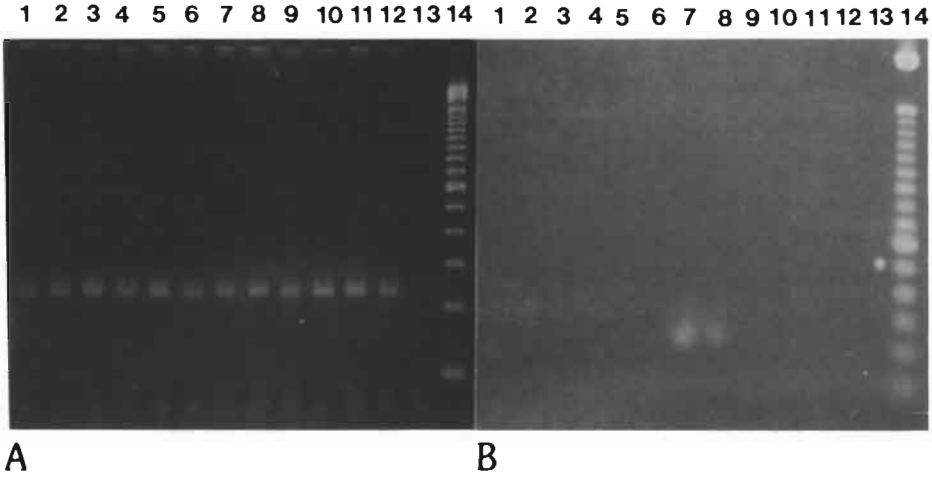

Figure 3 Specific amplification of target in DNA from pathotype A of *X.c.* pv. *citri* by the the polymerase chain reaction. **(A)** Template DNA was from pathotype A strains of *X. c.* pv. *citri* from 12 countries. **(B)** Template DNA was from pathotype B,C and D strains of *X.c.* pv. *citri* (lanes 1-5), pvs *alfalfae, bilvae* and *vignicola* (lanes 6-8), and Florida CBS strains (lanes 9-12). The no DNA control reaction was in lanes 13. Primer pair 2/3 was used, and annealing was at 58 C. The BRL 100 bp ladder was run in lanes 14, with the lowest band of 100 bp. The position of the predicted 222 bp product is marked in the margin.

Pathotypes B,C and D are considered less virulent than pathotype A of *X.c.* pv *citri*, are much less widely distributed (Stall and Civerolo, 1991), and so pose less of a threat to the citrus industry. Thus the clear detection of pathotype A and non-detection of Pathotypes B,C and D represents a useful complement to the previous dot blot assays (Hartung , 1992). None of the primer pairs found targets in CBS strains tested. Experiments are in progress to determine the level of sensitivity which can be achieved with this assay system. Pathotype A has also been detected directly in washes from citrus canker lesions with this PCR technique.

LITERATURE CITED

Anonymous. 1990. Quarantine Procedure: Xanthomonas campestris pv. citri. **Bulletin of the European and Mediterranean Plant Protection Organization** 20:263-272.

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** 1:51-56.

Civerolo, E.L. 1984. Bacterial Canker Disease of Citrus. **J. of the Rio Grande Valley Horticultural Society** 37:127-146.

Gabriel, D.W., Hunter, J.E., Kingsley, M.T., Miller, J.W. and Lazo, G.L. 1988. Clonal Population Structure of *Xanthomonas campestris* and Genetic Diversity among Citus Canker Strains. **Molecular Plant - Microbe Interactions** 1:59-65.

Gabriel, D.W., Kingsley, M.T., Hunter, J.E. and Gottwald, T.R. 1989. Reinstatement of *Xanthomonas citri* (ex Hasse) and *X. Phaseoli* (ex Smith) to Species and Reclassification of all *X. campestris* pv *citri* strains. **International J. of Systematic Bacteriol.** 39:14-22.

Hartung, J. S. and Civerolo, E.L. 1989. Restriction Fragment Length Polymorphisms Distinguish *Xanthomonas campestris* strains isolated from Florida citrus nurseries from *X.c.* pv *citri.* **Phytopathology** 79:793-799.

Hartung, J.S. 1992. Plasmid-Based Hybridization Probes for Detection and Identification of *Xanthomonas campestris* pv. *citri.* **Plant Disease** 76:*in press.*

Pruvost, O.P., Hartung, J.S., Civerolo, E.L., Dubois, C. and Perrier, X. 1992. Plasmid DNA Fingerprints Distinguish Pathotypes of *Xanthomonas campestris* pv. *citri*, the Causal Agent of Citrus Bacterial Canker Disease. **Phytopathology** 82:485-490.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. 1988. Primer -Directed Enzymatic Amplification of DNA with thermostable DNA Polymerase. **Science** 239:487-491.

Sarkar, G., Kapelner, S. and Sommer, S. 1990. Formamide can dramatically improve the specificity of PCR. **Nucleic Acids Research** 18:7465.

Schoulteis, C.L., Civerolo, E.L., Miller, J.W., Stall, R.E., Krass, C.J., Poe, S.R. and DuCharme, E.P. 1987. Citrus Canker in Florida. **Plant Disease** 71:388-395.

Smith, K. T., Long, C. M., Bowman, B. and Manos, M. 1990. **Amplifications** 5: 16-17.

Stall, R.E. and Civerolo, E.L. 1991. Research Relating to the Recent Outbreak of Citrus Canker in Florida. **Annual Review of Phytopathology** 29:399-420.

Young, J.M., Bradbury, J.F., Gardan, L. Gvodzyak, R.I., Stead, D.E., Takikawa, Y. and Vidaver, A.K. 1991. Comment on the reinstatement of *Xanthomonas citri* (ex Hasse 1915) Gabriel et al. 1989 and *X. phaseoli* (ex Smith 1897) Gabriel et al. 1989: Indication of the Need for Minimal Standards for the Genus *Xanthomonas.* **International J. of Systematic Bacteriology** 41:172-177.