A simplified subtractive hybridization protocol used to isolate DNA sequences specific to *Xylella fastidiosa*

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A simplified protocol of subtractive hybridization based on the technique of L. M. Kunkel, A. P. Monaco, W. Middlesworth, H. D. Ochs & S. A. Latt (1985, *Proc Natl Acad Sci USA*, 82, 4778–4782) was used to obtain DNA sequences specific to *Xylella fastidiosa* isolated from diseased citrus plants. As a driver, DNA extracted from bacteria showing different degrees of relatedness was used: *Xy. fastidiosa* 788 isolated from another host (plum), *Xanthomonas campestris* pv. *campestris* and *Burkholderia gladioli* strains. A DNA fragment, f14, showing no hybridization to the driver DNA, was used as a probe specific to *Xy. fastidiosa* from citrus and oleander. This fragment was sequenced and the predicted protein showed 40% similarity to the central region of flagellin of *Escherichia coli* serotypes H1 and H12. A pair of internal primers (f14-1 and f14-2) was designed for amplification of *Xy. fastidiosa* DNA. These primers detected *Xy. fastidiosa* strains isolated from citrus and oleander and yielded an amplification product of about 600 bp. They were also able to detect the bacteria in extracts from citrus plants with or without symptoms of disease. No amplification reaction was obtained using DNA extracted from other species and pathovars of *Xanthomonas*, *Pseudomonas cichorii*, *Erwinia carotovora*, *Agrobacterium tumefaciens* and phytopathogens of citrus (*Xanthomonas axonopodis* pv. *citri*) and coffee (*Burkholderia andropogonis*, *P. cichorii*, *Pseudomonas syringae* pv. *garcae*). The isolation of a DNA fragment specific to *Xy. fastidiosa* from citrus showed that the simplified protocol of subtractive hybridization used in this work is potentially applicable to other micro-organisms.

Keywords: *Xylella fastidiosa*, subtractive hybridization, PCR detection

INTRODUCTION

One problem commonly found during the analysis and comparison of similar genomes is the isolation of differential DNA sequences. This situation arises in the design of specific probes and the analysis of related organisms or those differing by a mutant phenotype. Using phenol-accelerated competitive DNA hybridization, Kunkel *et al.* (1985) isolated specific fragments from the DNA of a male patient with an X

chromosome deletion. The method has been modified gradually by different authors, introducing more steps of competitive hybridization which permitted higher levels of enrichment of specific sequences (see, for example, Straus & Ausubel, 1990). In addition, adaptors ligated to the subtracted sequences were used for amplification by PCR. The method of subtractive hybridization has been widely used and has been employed in the phytopathology area mainly to isolate specific probes for the detection of certain pathogens, as described for *Pseudomonas* (Cook & Sequeira, 1991), *Erwinia* (Darrasse *et al.*, 1994), *Clavibacter* (Mills *et al.*, 1997) and *Xanthomonas* (Kufli & Cuppels, 1997).

In our work, a simplified procedure of subtractive

Abbreviation: CVC, citrus variegated chlorosis.

The GenBank accession number for the sequence reported in this paper is AF052587.

hybridization based on the method described by Kunkel *et al.* (1985) was used to isolate DNA sequences specific to *Xylella fastidiosa* isolated from diseased citrus plants. *Xy. fastidiosa* is a unique species in the genus and consists of several pathotypes causing diseases in economically important plants, including alfalfa, almond, grapevine, peach and plum (for a review see Hopkins, 1989; Purcell & Hopkins, 1996). New diseases associated with *Xy. fastidiosa* have recently been described in orange (Chang *et al.*, 1993; Hartung *et al.*, 1994) and coffee (Beretta *et al.*, 1996; Paradelo-Filho *et al.*, 1997; Lima *et al.*, 1998) trees in Brazil; these diseases were designated citrus variegated chlorosis (CVC) and coffee leaf scald, respectively. A rapid dissemination of CVC is occurring in the major citrus-growing areas in Brazil, with associated large economic losses. The extent of the damage in coffee plants is still undetermined.

Highly sensitive and reliable methods for detecting the bacterium are important in strategies to control the spread of the disease. Currently, the detection of CVC-*Xy. fastidiosa* is performed by visual inspection of plants, microscopy and serological techniques such as ELISA and the dot immunobinding assay (Garnier *et al.*, 1993; Lee *et al.*, 1992). The employment of these techniques is sometimes ambiguous and culturing the bacteria is a tedious and slow process. Due to the outbreak of CVC in Brazil, PCR-based techniques have been developed for *Xy. fastidiosa* (Minsavage *et al.*, 1994; Pooler & Hartung, 1995). More sensitive techniques permitting the detection of the bacterium before the development of symptoms are important to control the spread of CVC. Bacterial infection can occur during the grafting procedure from contaminated rootstocks, in the unprotected nursery, or in the field by sharp-shooter leafhopper insects, which are the natural vectors (for a review see Carlos *et al.*, 1997).

In this paper we describe a procedure of subtractive hybridization used to isolate DNA sequences specific to *Xy. fastidiosa*. Using this approach, a DNA probe hybridizing to CVC- and oleander-*Xy. fastidiosa* was identified. This fragment was sequenced and a pair of primers designed for the PCR detection of the bacterium isolated from citrus and oleander, and in extracts prepared from symptomatic or symptomless/inoculated citrus plants.

METHODS

Bacterial strains, growth conditions and plasmids. The strains used in this work are listed in Table 1. *Agrobacterium*, *Enterobacter*, *Erwinia* and *Escherichia coli* DH5 α strains were grown in LB medium (Sambrook *et al.*, 1989); *Pseudomonas*, *Burkholderia* and *Xanthomonas* strains were grown in NYG-agar medium (Turner *et al.*, 1984); and *Xylella* strains were grown in SPW-agar medium (Hartung *et al.*, 1994). The incubation temperature was 30 °C for all strains, except for *E. coli* (37 °C). The plasmid pBluescript KS+ (Stratagene) was used as a cloning vector.

DNA extraction. Ten milligrams of *Xy. fastidiosa* bacterial mass was collected from SPW-agar plates and placed into

1.5 ml microfuge tubes. The cellular mass was washed once in 500 μ l TAS buffer (50 mM Tris/HCl pH 8.0, 50 mM EDTA, 150 mM NaCl), centrifuged and the pellet was resuspended in 500 μ l lysis buffer (TAS plus 1% SDS and 150 μ g proteinase K ml⁻¹). The tubes were incubated at 50 °C for 1 h. The cell debris was extracted once with phenol and twice with chloroform and dialysed for 48 h against TE buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA). DNA concentration was determined by comparison with lambda DNA in ethidium-bromide-stained 0.8% agarose gel. Plasmid extraction was carried out using the alkaline lysis method (Sambrook *et al.*, 1989).

DNA manipulation and hybridization. Restriction enzyme digestion and ligation of DNA molecules were carried out following the manufacturers' specifications. General DNA manipulation procedures were carried out according to Sambrook *et al.* (1989). Small DNA fragments were visualized in 7% polyacrylamide/bis-acrylamide gels (49:1, w/w) in a Tris/borate/EDTA buffer system, using a silver staining method (Moreno *et al.*, 1985). In the slot-blot hybridization experiments, 1 μ g genomic DNA was placed onto nylon membranes using a hybri-slot filtration manifold. The DNA was denatured for 5 min (1.5 M NaCl, 0.5 NaOH), neutralized for 5 min (1.5 M NaCl, 0.5 M Tris/HCl pH 7.2, 1 mM EDTA), dried and baked for 2 h at 80 °C prior to the hybridization procedures. For Southern blot hybridization, 2 μ g digested genomic DNA was separated in a 0.8% agarose gel and blotted onto nylon membranes using the alkaline transfer method. Plasmid vectors harbouring DNA fragments (cloned into *Bam*HI sites) to be labelled were extracted, digested with *Pst*I and *Xba*I and separated in a 0.8% low-melting-point agarose gel. Purified fragments were labelled using the DIG DNA labelling kit and detection of bands was carried out using the chemiluminescent substrate CSPD [disodium 3-(4-methoxy-2,3-dioxetane-3,2'-bis(5'-chloro-1,3,5-triazine-2-yl)phenyl)phosphate] as recommended by the manufacturer (Boehringer Mannheim).

Plant DNA preparation. DNA from plant extracts was obtained by grinding approximately 300 mg citrus or coffee petioles under liquid nitrogen in a chilled mortar and pestle. The following steps were the same as described by Doyle & Doyle (1990). After precipitation, the DNA pellet was suspended in 40 μ l TE buffer and 4 μ l was used in the PCR reactions. The extracts were prepared from healthy and CVC-affected *Citrus sinensis* adult plants growing in the field, as well as from healthy seedlings. Symptomless citrus seedlings artificially inoculated with *Xy. fastidiosa* were also used. These seedlings were inoculated 6 months in advance, using different procedures (J. Rodrigues Neto, unpublished data). Leaves from symptomatic and healthy adult coffee (*Coffea arabica*) plants or seedlings of cv. Mundo Novo and cv. Catuai were provided by the Instituto Biológico, Campinas, SP, Brazil by O. Guerreiro Filho (IAC, Campinas, SP, Brazil).

Subtractive hybridization and cloning. Tester genomic DNA of *Xy. fastidiosa* isolated from citrus showing CVC symptoms was totally digested with *Sau*3AI and fragments between 0.5 and 2.0 kb were purified from a 0.8% low-melting-point agarose gel. Approximately 1 mg driver DNA was sheared by sonication using an Ultrasonic Homogenizer (Cole-Parmer), until fragments with an average size of 500 bp were obtained as the major component. As a driver, DNA from *Xy. fastidiosa* 788, *Xanthomonas campestris* pv. *campestris* and *Burkholderia gladioli*, was used. Ten micrograms of driver DNAs were mixed with 100 ng tester DNA (100:1, w/w) and denatured in a water bath at 100 °C for 10 min. The mixture

Table 1. Bacterial strains used

Strain	Hosts	Origin and other references*
<i>Agrobacterium tumefaciens</i>	Wide range of plants	IBSBF 304 = ICMP 5793; ATCC 4720
<i>Burkholderia andropogonis</i>	<i>Coffea arabica</i> cv. Catuí amarelo	IBSBF 166 = ICMP 6779; NCPPB 6943
<i>Burkholderia gladioli</i> pv. <i>gladioli</i>	<i>Gladiolus</i> sp.	IBSBF 604 = ICMP 9382
<i>Enterobacter dissolvens</i>	<i>Zea mays</i>	IBSBF 1289 = ICMP 1570
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>	<i>Solanum tuberosum</i>	IBSBF 863 = ICMP 5702; ATCC 15713; LMG 2404
<i>Pseudomonas</i>		
<i>P. cichorii</i>	<i>Coffea arabica</i>	IBSBF 587 = ICMP 9276; NCPPB 3109
<i>P. syringae</i> pv. <i>garcae</i>	<i>Coffea arabica</i>	IBSBF 1297
<i>P. syringae</i> pv. <i>syringae</i>	<i>Syringa vulgaris</i>	IBSBF 451 = ICMP 3023; LMG 1247; ATCC 19310
<i>Xanthomonas</i>		
<i>Xa. axonopodis</i> pv. <i>citri</i>	<i>Citrus sinensis</i>	IBSBF 223
<i>Xa. axonopodis</i> pv. <i>phaseoli</i>	<i>Phaseolus vulgaris</i>	LMG 7455
<i>Xa. campestris</i> pv. <i>campestris</i>	Crucifers	Ca110 (Destefano & Rosato, 1991)
<i>Xa. translucens</i> pv. <i>translucens</i>	<i>Hordeum vulgare</i>	LMG 876
<i>Xa. translucens</i> pv. <i>undulosa</i>	<i>Triticum aestivum</i>	IBSBF 1026
<i>Xylella fastidiosa</i>		
A1, A4, E4, E6, F5, H9, B1, B7, B9, JB1, C6, C10	<i>Citrus sinensis</i>	Fundecitrus
CF01, CF02, CF03, 11752, 12288	From <i>Coffea arabica</i>	Fundecitrus; IAPAR
GP01	From <i>Vitis vinifera</i>	IBSBF 789 = ATCC 33107
OL01	From <i>Nerium oleander</i>	Fundecitrus
PL9746	From <i>Prunus salicina</i>	IAPAR 9746
PL788	From <i>Prunus salicina</i>	IBSBF 788 = ICMP 8735

* ATCC, American Type Culture Collection, Manassas, VA, USA; Fundecitrus, Fundo Paulista de Defesa da Citricultura, Araraquara, SP, Brazil; IAPAR, Instituto Agronômico do Paraná, Londrina, PR, Brazil; IBSBF, Instituto Biológico, Seção de Bacteriologia Fitopatológica, Campinas, SP, Brazil; ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand; LMG, Culture Collection of the Laboratorium voor Microbiologie, Rijksuniversiteit, Gent, Belgium; NCPPB, National Collection of Plant-pathogenic bacteria, CSL, York, England.

was transferred to a 50 ml centrifuge tube, 50 µl phenol was added and the final volume adjusted to 500 µl with TE buffer. The phenol aqueous emulsion permits increase in the rate of DNA-DNA reassociation many thousand times faster than under standard conditions (Kohne *et al.*, 1977). The optimum rate is obtained by vigorous shaking of the emulsion. In the present case, a vortex mixer at medium speed was used for 48 h. The whole mixture was cleaned using phenol and chloroform and the products were ligated to *Bam*HI-digested pBluescript KS+ cloning vector and electrotransformed into *E. coli* DH5α strain. White colonies were selected by plating the cells after transformation onto LB-agar medium supplemented with ampicillin (60 µg ml⁻¹) and X-Gal (40 µg ml⁻¹).

DNA sequencing and analysis. Fragments cloned into the pBluescript KS+ vector were sequenced using the Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham) and the primers M13 and Reverse (Gibco-BRL). The samples were read in an automatic sequencer ABI Prism 377 (Perkin Elmer). For DNA sequence and primer analysis the Gene Runner, version 3.0 (Hastings Software), DNAsIS version 6.0 (Hitachi Software Engineering) and Oligo structure, version 3.3 (National BioScience) were used.

PCR conditions for amplification using the designed primers (f14). Bacterial DNA or cells were amplified using the Thermal

Cycler (Perkin Elmer) in the following conditions: 10–20 ng genomic DNA, 1.0–1.5 mM MgCl₂, 100 µM dNTPs, 1–5 pmol of each primer, 0.5 units *Taq* DNA polymerase and buffer as recommended (Gibco-BRL) and water to 25 µl. The PCR cycling condition was: 1 × (94 °C/2 min); 40 × (94 °C/30 s, 62–64 °C/30 s and 72 °C/45 s).

RESULTS

Cloning CVC-*Xy. fastidiosa*-specific DNA by subtractive hybridization

This technique was used to selectively clone DNA sequences of CVC-*Xy. fastidiosa* which are absent from *Xy. fastidiosa* from another host (plum) and related bacteria. The method used is shown in Fig. 1. The driver DNAs, from different bacterial species, were chosen based on the different levels of relatedness to *Xy. fastidiosa*. One was a strain of *Xy. fastidiosa* isolated from another host (plum); *Xa. campestris* is a phyto-pathogenic bacterium close related to *Xy. fastidiosa*; and *B. gladioli*, formerly *Pseudomonas gladioli*, is a more distantly related pathogen. The rationale of using DNA from different pathogens is to exhaust the house-

Fig. 1. Diagram of the simplified subtractive hybridization used to isolate DNA sequences specific to *CVC-Xy. fastidiosa*.

keeping and pathogenicity-related genes that they might have in common. In the first screening, 47 white colonies were isolated and subjected to plasmid extraction and restriction analysis using *HindIII* and *SstI*, which cut at the flanking edges of the cloned fragment. Most of the clones possessed inserts of 200–300 bp, which were discarded. Four recombinant plasmids had insert sizes between 600 and 700 bp and these inserts (f9, f14, f40 and f45), showing differences in the restriction sites for *BglI* and *HaeII*, were tested as probes specific for *CVC-Xy. fastidiosa*.

Specificity of the selected clones from the subtractive library

To assess the specificity of each of the four fragments obtained and the homology among these potential probes, slot-blot hybridization experiments were carried out. Four membranes were prepared using DNA from *Xy. fastidiosa* strains isolated from citrus (A1, C6 and E4), from the strains used as drivers in the subtractive

hybridization (*Xy. fastidiosa* PL788, *Xa. campestris* pv. *campestris* and *B. gladioli* pv. *gladioli*) and the isolated fragments f9, f14, f40 and f45. Each membrane was hybridized individually using one of the four labelled fragments (f9, f14, f40 and f45) as probes. No homology was detected among the four fragments. All probes showed no hybridization to the DNA from *Xa. campestris* and *B. gladioli* used as driver DNA. However, three of them (f9, f40 and f45) hybridized to DNA from *Xy. fastidiosa* PL788. The fragment f14 showed no hybridization to any driver DNA and therefore it was chosen for further analysis. Slot-blot experiments were carried out to confirm its specificity to *CVC-Xy. fastidiosa*. DNA of five strains of *Xy. fastidiosa* from different hosts (citrus A1, coffee CF01, oleander OL01, plum PL788 and grapevine GP01) and strains of different species and genera (*Xanthomonas axonopodis* pv. *citri*, *Xa. axonopodis* pv. *phaseoli*, *Xa. campestris* pv. *campestris*, *Xanthomonas translucens* pv. *translucens*, *Xa. translucens* pv. *undulosa*, *Pseudomonas cichorii*, *Pseudomonas syringae* pv. *garcae*, *P. syringae* pv.

Fig. 2. Hybridization experiments using f14 as a probe. (a) Slot-blot using DNA from different isolates of *Xy. fastidiosa* and control strains of different genera: A1, citrus A1; A2, coffee CF01; A3, oleander OL01; A4, plum PL788; A5, grapevine GP01; A6, *Xa. axonopodis* pv. citri; A7, *Xa. axonopodis* pv. phaseoli; A8, *Xa. campestris* pv. campestris; A9, *Xa. translucens* pv. translucens; A10, *Xa. translucens* pv. undulosa; A11, *P. cichorii*; A12, *P. syringae* pv. garcae; B1, *P. syringae* pv. syringae; B2, *A. tumefaciens*; B3, *B. gladioli* pv. gladioli; B4, *B. andropogonis*; B5, *En. dissolvens*; B6, *Er. carotovora* subsp. *carotovora*; B7, *E. coli* DH5 α ; B8, fragment f14. (b) Southern blot of *Sau*3AI-digested DNA. Lanes: 1–12, CVC-*Xy. fastidiosa* A1, A4, E4, E6, F5, H9, B1, B7, B9, JB1, C6, C10, respectively; 13–15, OL01, DH5 α and f14 fragment, respectively; M, DNA molecular mass markers (1.0 kb ladder, Gibco-BRL). (c) Slot-blot using citrus plant extracts. Lanes: 1, symptomatic adult plant; 2, healthy adult plant; 3, symptomless/inoculated seedling; 4, healthy seedling.

syringae, *Agrobacterium tumefaciens*, *B. gladioli* pv. *gladioli*, *Burkholderia andropogonis*, *Enterobacter dissolvens*, *Erwinia carotovora* subsp. *carotovora* and *E. coli* DH5 α were tested with probe f14. There was hybridization with DNA from strains A1 (from CVC), OL01 (from oleander) and *E. coli* DH5 α (Fig. 2a). No hybridization was detected with *Xy. fastidiosa* DNA from coffee (CF01), plum (PL788), grape (GP01), or with DNA from the other bacteria tested. Confirmation of the slot-blot experiments was carried out by Southern blot hybridization using 12 other strains of *Xy. fastidiosa* from CVC. The DNA was digested with *Sau*3AI and the results showed the expected hybridization band of about

670 bp (Fig. 2b). Strains OL01 and DH5 α , which hybridized with f14 in the previous slot-blot hybridization tests, were included in these Southern experiments. OL01 strain showed a hybridization band of 0.5 bp whereas *E. coli* DH5 α showed a higher molecular mass and less intense band of about 1.6 kb (arrowhead in Fig. 2b).

The ability of f14 to detect *Xy. fastidiosa* was also tested in plant extracts. Samples of 4 μ l plant extracts were slot-blotted and used in hybridization tests. Probe f14 detected the bacteria in extracts prepared from symptomatic and symptomless/inoculated citrus plants (Fig. 2c).

Primer design and their use in PCR tests

The entire fragment f14 was sequenced and a total of 669 bp was found. Homology in the nucleotide sequence was found with a small stretch (21 nucleotides) of an unidentified region of the *Bacillus subtilis* genome and to several stretches of the *E. coli* flagellin gene (*fliC*). A search for homology in the deduced amino acid sequence of f14 revealed similarity to the FliC protein (flagellin) from morphotype E flagellar filaments of *E. coli* serotypes H1 and H12 (Schoenhals & Whitfield, 1993). The amino acid sequences of H1 and H12 flagellin are very similar, differing only by 10 amino acid residues. The central region of the flagellin molecule of *E. coli* is variable, giving rise to serotype-specific epitopes and the terminal regions are highly conserved in *E. coli* and other bacterial species (Harshey *et al.*, 1989; Smith & Selander, 1990; Wei & Joys, 1986). The identity of F14 to H1 was 28% (59/209) and similarity was 40% (85/209). The intriguing result was that this similarity was detected within the variable region of the flagellin of *E. coli*. The alignment of F14 and FliC sequences (Fig. 3a) showed several gaps and the similarity in the amino acid sequence seems to occur randomly. There are no major domains and in the best cases there is a sequence of five identical contiguous amino acids.

Based on the flanking sequences, a pair of oligonucleotides was designed for PCR experiments. The selected sequences were: F14-1, 5'-ACC GTT GGC GTC CGT ATA GG-3'; and F14-2, 5'-GAC ATG GCT GCT CAC CTG G-3' (Fig. 3b). An amplification product of about 600 bp was expected by using these primers. These primers were tested with five strains of *Xy. fastidiosa* isolated from different hosts (A1, OL01, CF01, PL788 and GP01). Only strains A1, from citrus, and OL01, from oleander, showed the diagnostic band of 600 bp (Fig. 4a). The amplification products were blotted and hybridized with the probe f14 and strong bands were observed with A1 and OL01 strains (Fig. 4a). A further distinction between the PCR products of *Xy. fastidiosa* from citrus and oleander, made by a subsequent digestion with *Sau*3AI, showed that there was an internal site for this enzyme only in the fragment from the OL01 strain. All CVC-*Xy. fastidiosa* strains presented in Table 1 were subsequently tested with these

Fig. 3. (a) Alignment of the predicted amino acid sequence of the f14 DNA sequence to the flagellin gene (*flaC*) of *E. coli* serotypes H1 and H12. (b) Partial nucleotide sequence of f14 showing the annealing positions of primers f14-1 and f14-2 (underlined) and the *Sau*3AI sites (in bold).

primers and the same 600 bp product was observed for all strains. An unexpected result was obtained with *Xy. fastidiosa* isolated from coffee. Three strains (CF01, CF02 and 11752) isolated in the São Paulo State gave no amplification products; however, two strains (12288 and 11782) from another state (Paraná) amplified the 600 bp fragment. The specificity of these primers was also tested using DNA from different bacterial species (*E. coli* DH5 α , *Xa. axonopodis* pv. *citri*, *Xa. axonopodis* pv. *phaseoli*, *Xa. campestris* pv. *campestris*, *Xa. translucens* pv. *translucens*, *Xa. translucens* pv. *undulosa*, *P. cichorii*, *P. syringae* pv. *garcae*, *P. syringae* pv. *syringae*, *A. tumefaciens*, *B. gladioli* pv. *gladioli*, *B. andropogonis*, *En. dissolvens* and *Er. carotovora* subsp. *carotovora*) and no amplification products were detected (Fig. 5).

For detection of *Xy. fastidiosa* in citrus plant extracts, 4 μ l of each extract was used in the amplification reaction. The primers successfully detected the presence of the bacterium in extracts prepared from symptomatic

or symptomless/inoculated citrus plants (Fig. 4b). There was no amplification when extracts from healthy citrus plants were used.

Sensitivity of detection of the primers f14

To determine the detection level of f14 primers by PCR, tenfold serial dilutions were prepared from an initial cell suspension of *Xy. fastidiosa* A1 (OD₆₀₀ 0.33) and 4 μ l of each suspension was used in the PCR reactions. Bacteria were detected up to a 10⁴-fold dilution. Detection was based on the presence of the diagnostic band in agarose gel, for which the total volume of the amplification reaction was loaded. Given that an OD₆₀₀ of 0.25 corresponds to 10⁷–10⁸ cells ml⁻¹ (Minsavage *et al.*, 1994), the PCR reaction using f14 primers was able to detect 5–50 cells, which is quite similar to the sensitivity described by Minsavage *et al.* (1994).

Fig. 4. (a) Agarose gel electrophoresis of PCR products from *Xy. fastidiosa* strains using primers f14 and the Southern blot hybridization with the probe f14. Lanes: M, DNA molecular mass markers (1.0 kb ladder, Gibco-BRL); 1–7, f14 fragment cloned into pBluescript KS+ vector, A1, OL01, CF01, PL788, GP01 and control reaction lacking DNA template, respectively. (b) Agarose gel electrophoresis of PCR products from citrus plant extracts using primers f14. Lanes 1–4, symptomatic adult plant, symptomless/inoculated seedling, healthy adult plant and control reaction lacking DNA template, respectively.

Fig. 5. Agarose gel electrophoresis of the PCR products from different bacterial species using the f14 primers. Lanes: M, DNA molecular mass markers (1.0 kb ladder, Gibco-BRL); 1, CVC-*Xy. fastidiosa*; 2–15, *E. coli* DH5 α , *Xa. axonopodis* pv. *citri*, *Xa. axonopodis* pv. *phaseoli*, *Xa. campestris* pv. *campestris*, *Xa. translucens* pv. *translucens*, *Xa. translucens* pv. *undulosa*, *P. cichorii*, *P. syringae* pv. *garcae*, *P. syringae* pv. *syringae*, *A. tumefaciens*, *B. gladioli* pv. *gladioli*, *B. andropogonis*, *Er. dissolvens*, *Er. carotovora* subsp. *carotovora*, respectively.

DISCUSSION

The use of diagnostic methods to identify the presence of *Xy. fastidiosa* in plants is an important step in the implementation of measures to restrict the dissemination of the disease in the field or to a non-infected area. The spread of CVC in the major citrus-growing areas in Brazil, mainly in the São Paulo State, can be considered alarming since approximately 35 % of citrus plants are presently affected (www.fundecitrus.com.br). The use of subtractive hybridization to obtain probes specific for phytopathogenic bacteria has been described and in general the protocols used require construction of a genomic library, various steps of hybridization and labelling. The primers f14 described herein were obtained using a protocol for fast and easy genomic subtraction. The technique, as used previously by

Kunkel *et al.* (1985), allowed isolation of four human specific fragments among the 81 clones analysed. No specific buffers were required, nor time-consuming purification steps to obtain clean material for cloning. Although various fragments were cloned in the present work, most of them were of too small a size for further detection using amplification in a PCR-based method and therefore they were not analysed. The small size of these fragments indicates that the isolation of fragments between 0.5 and 2.0 kb was not efficient and more steps of purification of the planned fragment size would improve the differential library cloning. In addition, the transformation procedure in general is more efficient for small inserts (Ferreira *et al.*, 1995), which helps to explain the high frequency of the small inserts obtained. The four clones examined showed no hybridization to the DNA from *Xa. campestris* pv. *campestris* and *B. gladioli* pv. *gladioli* used as drivers; however, three of the four clones hybridized to the DNA of the third driver strain (*Xy. fastidiosa* PL788). It is possible that using a higher ratio of driver DNA or performing a second round of hybridization would improve the recovery of the differential clones. In fact a ratio of 1:200 was used in the original protocol (Kunkel *et al.*, 1985). The primers f14 obtained were specific to CVC-*Xy. fastidiosa* and the low threshold of detection will improve the early diagnosis of symptomless and infected plants.

Specific primers for the detection of *Xy. fastidiosa* from citrus, obtained by differential RAPD products, have already been described (Pooler & Hartung, 1995) and they also amplify *Xy. fastidiosa* from coffee (data not shown). Indeed, *Xy. fastidiosa* strains isolated from these two hosts are considered closely related (Beretta *et al.*, 1996; Lima *et al.*, 1998; Paradelo-Filho *et al.*, 1997), but in our work, the f14 primers showed a differential screening among coffee strains. Three coffee-*Xy. fastidiosa* strains isolated in the São Paulo State were not detected by hybridization or PCR experiments. However, two other strains isolated in the Paraná State were detected by PCR. The isolation of other strains from coffee is under way to verify if populations from these major coffee-producing states are distinguishable. The lack of homology of f14 and/or the lack of amplification of *Xy. fastidiosa* from grape and plum and different bacterial species indicated that the f14 sequence might be only rarely found within the Gram-negative phyto-bacteria such as *Xanthomonas* and *Pseudomonas*. Common bacterial phytopathogens of citrus (*Xa. axonopodis* pv. *citri*) and coffee (*B. andropogonis*, *P. cichorii* and *P. syringae* pv. *garcae*) were also tested and showed no amplification product. Amplification using f14 primers was also obtained to the *Xy. fastidiosa* from another host (oleander). The amplification product of 600 bp was similar to that obtained with CVC-*Xy. fastidiosa*; however, the Southern hybridization experiments showed a smaller hybridizing band in oleander-*Xy. fastidiosa*, indicating differences in the *Sau3AI* restriction sites between both fragments. No other strains from oleander were tested, since detection of oleander

leaf scorch is recent (Purcell & Hopkins, 1996) and only one strain was available. A faint band of hybridization was detected with DNA from *E. coli* DH5 α , possibly due to various stretches of homology detected between f14 and the *fliC* gene of *E. coli*.

The sequencing of f14 showed an unexpected similarity in the deduced amino acid sequence to the flagellin of *E. coli* serotypes H1 and H12 (Schoenhals & Whitfield, 1993). Since *Xy. fastidiosa* does not have a flagellum (Wells *et al.*, 1987), it is plausible to assign a different function to f14. The flagellin molecule of *E. coli* presents the antigenic determinant for the H antigen which is routinely identified by the H antigen-specific antibodies in the slide agglutination test (Lawn, 1977). Although the similarity between the F14 and FliC predicted protein sequence appears to be fortuitous, a slide agglutination test was performed using two strains of CVC-*Xy. fastidiosa* and the results were negative (data not shown).

The simplified subtractive hybridization technique presented here was effective for the isolation of the DNA sequence specific for CVC-*Xy. fastidiosa*. This sequence was highly specific to the *Xy. fastidiosa* isolated from citrus and oleander and appears not to be essential since it is absent in the bacteria isolated from other hosts. The approach used was easy to perform and is potentially applicable for the isolation of specific genes in this little-studied species and also other micro-organisms.

ACKNOWLEDGEMENTS

We would like to thank Fundecitrus and IAPAR (R. P. Leite) for providing the strains of *Xy. fastidiosa*; to CAPES for the fellowship granted to H. Ferreira, to Dr O. Guerreiro Filho (IAC) for supplying the coffee plants and to Dr A. Dias for the slide agglutination tests.

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Received 8 February 1999; revised 31 March 1999; accepted
12 April 1999.