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Assessment of subtractive hybridization to select species and subspecies specific DNA fragments for the identification of *Xylophilus ampelinus* by polymerase chain reaction (PCR)

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Abstract

Eighteen *Bsp143*I digested DNA fragments specific to *Xylophilus ampelinus* were cloned from a library enriched for *X. ampelinus* obtained after a subtractive hybridization step. It was also possible to clone specific DNA sequences directly after DNA digestion with *Bsp143*I probably because *X. ampelinus* is a unique bacterium. Nucleotidic sequences of four cloned specific fragments were determined. They did not share any homology with other DNA sequences in the EMBL/GeneBank database. Four primer sets were designed and tested for specificity to *X. ampelinus*. One primer set (Xamp 1.27) was a good candidate for a species-specific reagent in a procedure of identification of *X. ampelinus* using PCR. One primer set detected only Greek strains isolated from *Vitis vinifera* cv. Sultana. Genetic diversity within the *X. ampelinus* species can be used in further epidemiological studies on the bacterial necrosis of grapevine.

Introduction

Bacterial blight or bacterial necrosis of grapevine is caused by *Xylophilus ampelinus* (Panagopoulos, 1969) comb. nov. (Willems et al., 1987). This disease was first reported in Sicilia in 1879 and attributed to *Bacillus vitivorus*, and then in France where Ravaz (1895, 1896) first obtained typical symptoms after artificial inoculations. The identity of the causal agent was confused until 1966 when Panagopoulos showed that the causal agent was a very slow-growing bacterium characterized and described as *Xanthomonas ampelina* (Panagopoulos, 1969). Although this bacterium possessed features of the genus *Xanthomonas*, a taxonomic study including DNA–DNA and DNA–rRNA hybridizations showed that the bacterium which causes bacterial necrosis of grapevine belongs to the *Comamonadaceae* family in β-subdivision of *Proteobacteria* whereas *Xanthomonas* is located in the δ-subdivision of *Proteobacteria*. It was transferred to a new genus *Xylophilus* as *Xylophilus ampelinus* (Willems et al., 1987).

X. ampelinus has been detected in several Mediterranean countries (Greece, France, Spain, Italy, Turkey, Portugal) and in South Africa. It probably occurs also in Bulgaria, Yugoslavia, Austria, Switzerland, Tunisia, and Argentina where typical symptoms have been described (EPPO, 1984). *X. ampelinus* is a quarantine bacterium and phytosanitary measures are relevant for the exchange of grapevine materials. As direct inspection of planting material is likely to be unreliable, inspection is required in nurseries located in areas where the disease is known to occur (EPPO, 1984). Laboratory diagnosis of the disease is required because symptoms may vary considerably and can be confused with those of other diseases commonly found in vineyards in France, such as excoriosis caused by *Phomopsis viticola.*

No selective medium is available for the isolation of *X. ampelinus* and its slow growth on various agar media

hampers its recovery because of interference with saprophytic bacteria (Serfontein et al., 1997). Serological reagents were developed to be used in detection procedures. Polyclonal antisera were prepared and used in indirect immunofluorescence staining procedure (Ridé et al., 1977; Serfontein, pers. comm.). A monoclonal antibody raised by Gorris et al. (1989) was proposed as the basis of an ELISA test.

None of these methods has been widely accepted for routine testing, since they have low sensitivity and are incompatible with the detection of latent infections. Therefore, the need for a highly sensitive and specific assay to identify the bacterial necrosis of grapevine pathogen still exists. Amplification of specific DNA sequences by means of the polymerase chain reaction (PCR) has been successfully used for rapid identification of numerous plant pathogenic bacteria. The subtractive hybridization technique which consists of enrichment of specific DNA sequences of a target organism by trapping common DNA sequences with a closely related organism, was successfully used for several plant pathogenic bacteria (Cook and Sequeira, 1991; Seal et al., 1992; Darrasse et al., 1994).

The aims of this study were to clone specific DNA fragments, after a subtractive hybridization step, in order to design primers specific to *X. ampelinus* and to develop a highly specific diagnostic procedure for the rapid identification of *X. ampelinus*.

Materials and methods

Bacterial strains, culture media, and growth conditions

The characteristics and sources of the strains tested in this study are shown in Table 1. *X. ampelinus* was routinely cultured at 24 °C on YPGA medium (yeast extract, 7 g; bacto-peptone, 7 g; glucose, 7 g and agar, 15 g, H_2O 1000 ml, pH 7). *Escherichia coli* DH5α and derivative strains were stored at −80 °C and were grown at 37 °C in LB medium (Miller, 1972). All other bacterial species used were grown on YPGA medium.

Subtractive hybridization

A scheme displaying steps of subtractive hybridization is shown in Figure 1. Sequences present in the target DNA not present in the driver DNA were enriched by the removal of common DNA. The technique employed was based on increasing the rate of reassociation of DNA molecules by the presence of a high concentration of inorganic phosphate. The DNAs of two strains of *X. ampelinus* were used as target DNA separately, strain CFBP 1192 and CFBP 2292 respectively. A mixture of DNAs of *Acidovorax avenae* CFBP 2446, *Acidovorax delafieldii* CFBP 2442, *Acidovorax testosteroni* CFBP 2436, *Acidovorax vallerianelleae* 3052-1 and *Commamonas acidovorus* CFBP 2444 strains were used as driver DNA. Fifty micrograms of target DNA were digested with 50 U of the *Bsp143*I endonuclease (Eurogentec SA, Seraing, Belgium) in a final volume equal to 100 µl at 37 °C for 3 h. The digested DNAs were ethanol precipitated and redissolved in 100 µl of phosphate buffer 1.2 M ($NaHPO_4 \cdot 12H_2O$, 42.97 g; $NaH_2HO_4 \cdot 2H_2O$, 18.4 g; distilled water 100 µl; pH 6.2). The average size of digested fragments was equal to 250 bp. Three hundred micrograms of driver DNA mixture in 1 ml of TE8 (Tris–OH, 10 mM; EDTA, 1 mM; pH 8) were sheared by ultrasonication (15 W for 40 s, Model Sonifier 450 Branson Ultrasonics, Danbury, CT, USA) to a size range of 600–2000 bp. The sheared DNA was ethanol precipited and dissolved in 1 ml of phosphate buffer 1.2 M. One hundred micrograms of driver DNA were mixed with 1.8 g of target DNA. The volume was adjusted to 1 ml with phosphate buffer 1.2 M. The DNA mixtures were denatured at 100 °C for 10 min and they were allowed to reassociate for 16 h at 86 °C and 3 h at 76 °C successively. The reassociated DNA mixtures were dialysed extensively against 5 mM Tris–HCl buffer pH 8 at 4 °C, precipitated with ethanol and redissolved in 50 µl sterile distilled water. Ligations of reassociated DNA in pUC18 were carried out overnight at 15 °C. Each reaction mixture contained 7 µg of substracted mixture and 0.15 µg of phosphatase treated *Bam*H1-digested pUC18 DNA and 6 U of T4 DNA ligase (Eurogentec SA). Aliquots of ligation mixtures were transformed into competent *E. coli* DH5α cells (Hanahan, 1983). Transformants were selected on LB plates supplemented with ampicillin (40 µg/ml), X-gal (40 µg/ml) and IPTG (100 µg/ml). Transformants containing recombinant plasmids formed white colonies. White colonies were picked out with sterile tooth picks and streaked onto LB agar supplemented with ampicillin, X-gal and IPTG in order to check the purity.

DNA probes

DNA probes were made from recombinant plasmids by PCR amplifications using oligonucleotide primers

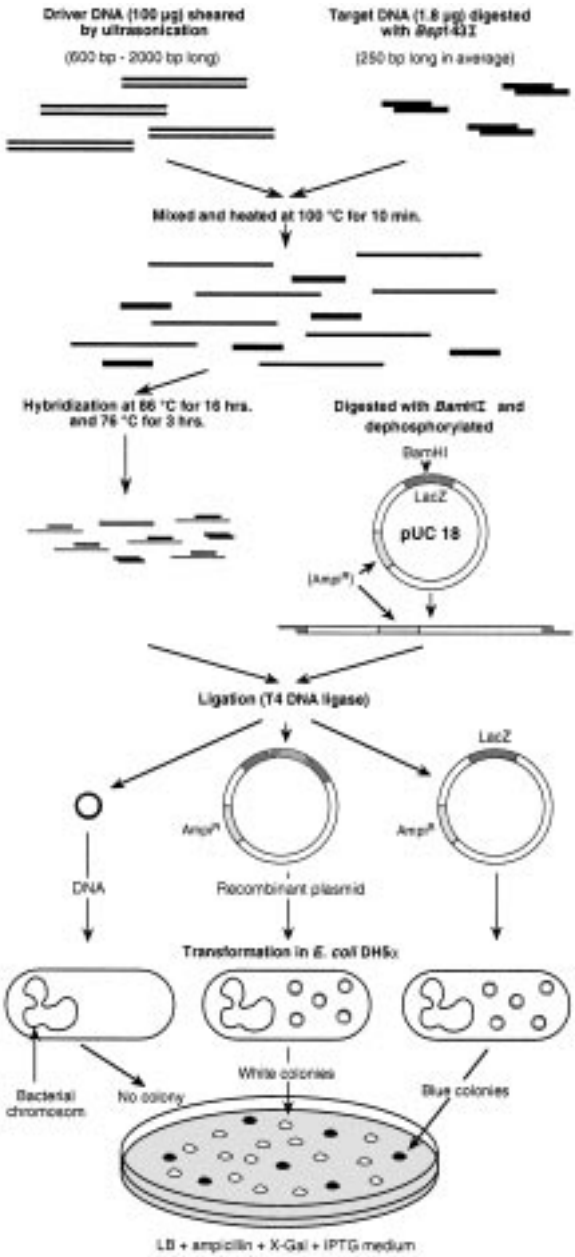

Figure 1. A diagram showing the serial steps of subtractive hybridization. Driver DNA is sheared by ultrasonication while target DNA is digested with *Bsp143*I. Both DNA are mixed and heated to be denatured. Hybridization of homologous fragments is carried out at 86 and 76 °C for 16 and 3 h successively. *Bsp143*I ended DNA fragments are cloned into *BamH*I digested pUC18 and competent *E. coli* DH5*α* cells are transformed with recombinant plasmids.

'universal' (GTT TTC CCA GTC ACG AC) and 'reverse' (AAC AGC TAT GAC CAT GA) flanking the *BamH*I site of pUC18. Reaction volumes (50 µl) contained 1× PCR buffer [75 mM Tris–HCl, pH 9; 20 mM $(NH_4)_2SO_4$; 0.1% (w/v) Tween 20], 0.6 µM of each primer, 3 mM $MgCl_2$, 0.14 mM of dATP, dCTP, dGTP; 0.133 mM dTTP, 0.007 mM digoxigenin-11-dUTP; Goldstar DNA polymerase (Eurogentec SA)

Table 1. Bacterial strains used and analysis of primers specificity

Bacterial strains	Host plant	Isolation (country and date)	PCR amplification with primers				
			1.27A/ 1.27B	1.27A/ 1.27C	1.3A/ 1.3B	2.0A/ 2.0B	1.19A/ 1.19B
Xylophilus ampelinus							
CFBP 1192 T[a]	*Vitis vinifera* cv. Sultana	Greece, 1966	+	+	+	+	+
CFBP 1193	*Vitis vinifera* cv. Sultana	Greece, 1966	+	+	+	+	+
CFBP 1194	*Vitis vinifera* cv. Sultana	Greece, 1969	+	+	+	+	+
CFBP 1313	*Vitis vinifera* cv. Ugni Blanc	France, 1971	+	+	+	+	−
CFBP 1393	*Vitis vinifera* cv. Ugni Blanc	France, 1969	+	+	+	+	−
CFBP 1394	*Vitis vinifera* cv. Ugni Blanc	France, 1969	+	+	+	+	−
CFBP 1796	*Vitis vinifera* cv. Ugni Blanc	France, 1975	+	+	+	+	−
CFBP 1797	*Vitis vinifera* cv. Grenache	France, 1975	+	+	+	+	−
CFBP 1798	*Vitis vinifera* cv. Grenache	France, 1975	+	+	+	+	−
CFBP 1799	*Vitis vinifera* cv. Maccabeu	France, 1975	+	+	+	+	−
CFBP 1800	*Vitis vinifera* cv. Alicante	France, 1976	+	+	+	+	−
CFBP 1802	*Vitis vinifera* cv. Alicante	France, 1968	+	+	+	+	−
CFBP 1803	*Vitis vinifera* cv. Carignan	France, 1976	+	+	+	+	−
CFBP 1833	*Vitis vinifera* cv. Grenache	France, 1976	+	+	+	+	−
CFBP 1834	*Vitis vinifera* cv. Grenache	France, 1975	+	+	+	+	−
CFBP 1835	*Vitis vinifera* cv. Maccabeu	France, 1975	+	+	+	+	−
CFBP 1836	*Vitis vinifera* cv. Ugni Blanc/ Rupestris du Lot	France, 1975	+	+	+	+	−
CFBP 1837	*Vitis vinifera*	France, 1975	+	+	+	+	−
CFBP 1841	*Vitis vinifera* cv. Ugni Blanc	France, 1977	+	+	+	+	−
CFBP 1842	*Vitis vinifera* cv. Valensi	France, 1977	+	+	+	+	−
CFBP 1926	*Vitis vinifera* cv. Grenache	Spain, 1978	+	+	+	+	−
CFBP 1927	*Vitis vinifera* cv. Maccabeu	Spain, 1978	+	+	+	+	−
CFBP 1928	*Vitis vinifera* cv. Quiebratinaja	Spain, 1978	+	+	+	+	−
CFBP 1938	*Vitis vinifera* cv. Maccabeu	Spain, 1978	+	+	+	+	−
CFBP 1939	*Vitis vinifera* cv. Grenache	France, 1978	+	+	+	+	−
CFBP 1942	*Vitis vinifera* cv. Maccabeu	Spain, 1978	+	+	+	+	−
CFBP 2059	*Vitis vinifera* cv. Italia/ Rupestris du Lot	France, 1981	+	+	+	+	−
CFBP 2060	*Vitis vinifera* cv. Seybel	France, 1981	+	+	+	+	−
CFBP 2061	*Vitis vinifera* cv. Grenache	France, 1978	+	+	+	+	−
CFBP 2098	*Vitis vinifera* cv. Grenache	France, 1979	+	+	+	+	−
CFBP 2266	*Vitis vinifera* cv. Clairette	France, 1983	+	+	+	+	−
CFBP 2289	*Vitis vinifera* cv. Ugni Blanc	France, 1983	+	+	+	+	−
CFBP 2290	*Vitis vinifera* cv. Ugni Blanc	France, 1983	+	+	+	+	−
CFBP 2291	*Vitis vinifera* cv. Ugni Blanc	France, 1983	+	+	+	+	−
CFBP 2292	*Vitis vinifera* cv. Ugni Blanc	France, 1983	+	+	+	+	−
CFBP 2293	*Vitis vinifera* cv. Ugni Blanc	France, 1983	+	+	+	+	−
CFBP 2294	*Vitis vinifera* cv. Ugni Blanc	France, 1983	+	+	+	+	−
CFBP 2295	*Vitis vinifera* cv. Ugni Blanc	France, 1983	+	+	+	+	−
CFBP 2358	*Vitis vinifera* cv. Ugni Blanc	France, 1983	+	+	+	+	−
CFBP 2359	*Vitis vinifera* cv. Ugni Blanc	France, 1984	+	+	+	+	−
CFBP 2393	*Vitis vinifera* cv. Ugni Blanc	France, 1975	+	+	+	+	−
CFBP 2394	*Vitis vinifera* cv. Grenache	France, 1975	+	+	+	+	−
CFBP 2398	*Vitis vinifera* cv. Italia	France, 1984	+	+	+	+	−
CFBP 2399	*Vitis vinifera* cv. Baco	France, 1984	+	+	+	+	−
CFBP 2400	*Vitis vinifera* cv. Ugni Blanc	France, 1984	+	+	+	+	−
CFBP 2730	*Vitis vinifera*	South Africa, 1972	+	+	+	+	−
CFBP 2731	*Vitis vinifera*	South Africa, 1972	+	+	+	+	−
CFBP 2748	*Vitis vinifera* cv. Grenache	France, 1985	+	+	+	+	−

Table 1. Continued

Bacterial strains	Host plant	Isolation (country and date)	PCR amplification with primers				
			1.27A/ 1.27B	1.27A/ 1.27C	1.3A/ 1.3B	2.0A/ 2.0B	1.19A/ 1.19B
CFBP 2875	*Vitis vinifera* cv. Ugni Blanc	France, 1988	+	+	+	+	−
CFBP 2876	*Vitis vinifera* cv. Ugni Blanc	France, 1988	+	+	+	+	−
CFBP 2877	*Vitis vinifera* cv. Clairette	France, 1984	+	+	+	+	−
CFBP 3674	*Vitis vinifera* cv. Sultana	Greece, 1966	+	+	+	+	+
CFBP 3675	*Vitis vinifera*	Greece, 1966	+	+	+	+	−
CFBP 3677	*Vitis vinifera*	Greece, 1966	+	+	+	+	−
CFBP 3678	*Vitis vinifera* cv. Sultana	Greece, 1966	+	+	+	+	+
CFBP 3681	*Vitis vinifera* cv. Sultana	Greece, 1966	+	+	+	+	+
CFBP 3682	*Vitis vinifera* cv. Sultana	Greece, 1966	+	+	+	+	+
CFBP 3683	*Vitis vinifera* cv. Sultana	Greece, 1966	+	+	+	+	+
CFBP 3684	*Vitis vinifera* cv. Sultana	Greece, 1966	+	+	+	+	+
CFBP 3685	*Vitis vinifera* cv. Sultana	Greece, 1966	+	+	+	+	+
CFBP 3686	*Vitis vinifera* cv. Sultana	Greece, 1966	+	+	+	+	+
CFBP 3688	*Vitis vinifera* cv. Sultana	Greece, 1974	+	+	+	+	+
CFBP 3689	*Vitis vinifera* cv. Mavrodafni	Greece, 1977	+	+	+	+	−
CFBP 3690	*Vitis vinifera* cv. Sultana	Greece, 1977	+	+	+	+	−
CFBP 3691	*Vitis vinifera* cv. Sideritis	Greece, 1977	+	+	+	+	−
CFBP 3692	*Vitis vinifera* cv. Sideritis	Greece, 1977	+	+	+	+	−
Acidovorax avenae							
CFBP 2446	*Oryzae sativa*	Nepal, 1909	−	−	−	−	−
Acidovorax delafreldii							
CFBP 2442T		1970	−	−	−	−	−
Acidovorax testosteroni							
CFBP 2436T		1956	−	−	−	−	−
Acidovorax vallelanelleae							
SO52-1		France, 1989	−	−	−	−	−
Commomonas acidovorarans							
CFBP 2444T			−	−	−	−	−
Escherichia coli							
DH5α			−	−	−	−	−
DH5α (pUC18)			−	−	−	−	−
Agrobacterium tumefaciens							
CFBP 2179	*Vitis vinifera*	France, 1982	−	−	−	−	−
Erwinia amylovora							
CFBP 1430	*Crataegus* sp.	France, 1972	−	−	−	−	−
Erwinia carotovora							
CFBP 2136	*Solanum tuberosum*	France, 1976	−	−	−	−	−
Pseudomonas syringae							
CFBP 1392T	*Syringa vulgaris*	UK, 1950	−	−	−	−	−
Xanthomonas campestris							
CFBP 2350T	*Brassica oleracea*	UK, 1957	−	−	−	−	−
Xanthomonas fragariae							
CFBP 2157T	*Fragaria* sp.	USA, 1960	−	−	−	−	−
Ralstonia solanacearum							
CFBP 2972	*Solanum tuberosum*	Martinique, 1986	−	−	−	−	−

[a]CFBP, collection Française de Bactéries Phytopathogènes, INRA, Angers, France; T, type strain.

1.25 U and 5 µl of previously boiling bacterial suspension as template DNA.

The mixture was subjected to 35 cycles of the following incubations: 30 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C in a MJ research PT150 Thermocycler. Unincorporated nucleotides were removed using a Quiaquick PCR purification kit (Quiagen S.A.) according to the recommendation of the manufacturer.

Dot blots

Specificity of cloned DNA was checked by dot blot on GeneScreen Plus® hybridization transfer membranes (NEN Research Products). Nylon membranes were placed in a 2× SSC solution for 5 min. Five microlitres of heat denaturated DNA (500 µg/ml) of each strain to be tested were plotted onto the membranes. The membranes were air dried and DNA was fixed under UV light 120 mJ/cm^2 for 25 s.

Membrane hybridizations

Membranes were prehybridized for 4 h at 42 °C in a solution containing 5× SSC, blocking reagent 2% (w/v) (Boehringer-Mannheim). N-lauroyl sarcosine 0.1%, SDS 0.1%, formamide 50% in water. Hybridizations were performed in the same solution supplemented with 10 µl (100 ng) of digoxygenin-labelled DNA overnight at 42 °C. Membranes were then washed twice in 2× SSC, 0.1% SDS for 5 min at room temperature and twice in 0.1× SSC, SDS 0.1% for 15 min at 68 °C. After hybridization with homologous DNA the modified probes was detected by using a direct immunoenzymatic reaction. The anti-digoxygenin alkaline phosphatase conjugate was bound to the digoxygenin residues of the probe. The alkaline phosphatase activity was visualized by a chromogenic substrate: 5-bromo-4-chlor-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT) according to the procedure recommended by Boehringer-Mannheim.

DNA sequencing

The cloned DNA fragments were sequenced by using a *Taq* Dye Deoxy terminator cycle sequencing kit [Applied Biosystems, Inc., Foster City (USA)] and a model ABI 377 automatic sequencer. DNA sequences were determined by using 'universal' and 'reverse' primers on DNA fragment cloned into pUC18 plasmid.

PCR amplification

Five microlitres of bacterial suspension in sterile distilled water were boiled for 5 min and added to 45 µl of reaction mixture (75 mM Tris–HCl pH 9, 20 mM $(NH_4)_2SO_4$, Tween 20 0.1% (w/v), 0.6 µM of each primer, 5 mM $MgCl_2$, 0.14 mM of dATP, dCTP, dTTP and dGTP, 1.25 U red GoldStar DNA polymerase (Eurogenec SA). PCR was carried out with 35 cycles of 94 °C for 30 s, 60 °C for 45 s and 72 °C for 45 s. Following agarose gel electrophoresis and ethidium bromide staining, PCR products were visualized under UV light (300 nm).

Results

Cloning of DNA fragments obtained by subtractive hybridization

Two subtractive hybridization experiments were done with two different strains of *X. ampelinus*. The virulent type strain CFBP 1192 isolated in Greece in 1966 from *Vitis vignifera* cv. Sultana and another virulent strain CFBP 2292 isolated in France in 1983 from *V. vignifera* cv. Ugni Blanc. A direct cloning experiment of *Bsp143*I digested DNA of strain CFBP 2292 into dephosphorylated *Bam*H1-digested pUC18 without subtractive hybridization step was also performed.

Eighty-five and 48 clones were analysed for the occurrence of insert DNA after the subtractive hybridization experiment performed with *X. ampelinus* strains CFBP 1192 and CFBP 2292, respectively. Fifty clones obtained after direct cloning were analysed concurrently. Forty, 14 and 18 of which had DNA inserts and were amplified when using universal and reverse primers flanking the cloning site of pUC18, respectively. These DNA inserts had a size range of 100–350 bp (data not shown).

Fifty-eight of these clones were used as DNA templates to make digoxygenin-labelled probes (31 and 12 from subtractive hybridization performed with CFBP 1192 and with CFBP 2292 respectively and 15 from direct cloning of CFBP 2292 Sau3A digested DNA). The specificity of these probes was tested against five strains of *X. ampelinus*, the five non-target strains used to make the driver DNA set and DNA samples extracted from a mixture of saprophytic bacteria isolated from healthy leaves and stems of grapevine. Specific probes were obtained in the three experiments with the two

strains and with and without subtractive hybridization step. Fifteen out of 31 probes (48.4%) made with subtracted DNA of CFBP 1192 strain hybridized specifically to DNA of *X. ampelinus* strains when 3 probes on 12 (25%) and 5 on 15 (33.3%) made with subtracted and non-subtracted DNAs of CFBP 2292 strains respectively hybridized also specifically to DNA of all *X. ampelinus* strains tested (Table 2). Non-specific probes were also isolated in all protocols: 35.5–66.6% of cloned probe after subtractive hybridization and 40% of probes obtained by direct cloning hybridized with 1–4 non-target strains in addition to *X. ampelinus* strains. Eight to 20% of probes did not hybridize to any DNA template. Finally, probes which hybridized to only a part of target strains were obtained in the two experiments. Two probes obtained after subtractive hybridization step with DNA cloned from CFBP 1192 strain hybridized only with CFBP 1192 DNA and one probe obtained after direct cloning CFBP 2292 DNA hybridized with DNA of CFBP 2292, CFBP 2877 and CFBP 1802 (Table 1, Figure 2).

DNA sequencing

Three probes which hybridized specifically to all five *X. ampelinus* tested were sequenced: Xamp 1.27 and Xamp 1.3 cloned from *X. ampelinus* strain CFBP 1192, and Xamp 2.0 cloned from *X. ampelinus* strain CFBP 2292. The Xamp 1.19 probe which hybridized only with the DNA of the origin-strain CFBP 1192 was also sequenced (Figure 3). Sequenced probes were ranged from 136 to 314 bp. For each probe, the GeneBank and the EMBL database were screened with BLAST 2.0 software (Altschul et al., 1997) for homologies. No significant homology was observed for any of the sequences.

PCR amplification

Synthetic oligonucleotide primers were designed for each sequenced DNA fragment (Figure 3). PCR amplifications were performed under high-stringency conditions: 60 °C annealing temperature, which was compatible with the *Tm* of the primers and the high GC content of *X. ampelinus* (68–69%). The sets of primers 1.27A/1.27B, 1.27A/1.27C, 1.3A/1.3B and 2.0A/2.0B amplified expected fragments of 310, 265, 131 and 153 bp respectively with all *X. ampelinus* strains tested (Figure 4, Table 1).

Primer sets 1.27A/1.27B, 1.27A/1.27C and 1.3A/1.3B were specific for *X. ampelinus*. All *X. ampelinus* strains but no other strains amplified these fragments (Table 1). However, primer set 2.0A/2.0B, which allowed the amplification of a 153 bp DNA fragment with all *X. ampelinus* strains, amplified a larger non-specific fragment with a few saprophytic bacteria isolated from healthy grapevine (data not shown). Furthermore, only a few strains were amplified with primer set 1.19A/1.19B. This result was in agreement with the data obtained by dot blot hybridization test performed with the corresponding Xamp 1.19 probe. Only 12 *X. ampelinus* strains (18%) were amplified. They were all isolated from cv. Sultana in Greece. Furthermore, all *X. ampelinus* strains isolated from cv. Sultana except one (strain CFBP 3690) showed the specific amplicon with primer set 1.19A/1.19B

Table 2. Specificity patterns of probes obtained after subtractive hybridization with strains CFBP 1192 and CFBP 2292 and after direct cloning of *Bsp143*I-digested DNA of the strain CFBP 2292

Specificity patterns			Number of probes		
X. ampelinus	*Acidovorax* and *Commamonas*[a]	Grapevine microflora[b]	Subtractive hybridization		Direct cloning
			CFBP 1192	CFBP 2292	CFBP 2292
5/5	0/5	−	15 (48.4%)	3 (25%)	5 (33.3%)
5/5	1–4/5[c]	+/−	11 (35.5%)	8 (66.6%)	6 (40%)
0/5	0/5	−	3 (9.7%)	1 (8.3%)	3 (20%)
1–3/5[d]	0/5	−	2 (6.5%)	0	1 (6.6%)
Total			31	12	15

[a] *A. avenae, A. delafreldii, A. testosteroni, A. vallerianelleae* and *C. acidovorans*;
[b] Bacterial isolates from healthy grapevine;
[c] Each probe listed on the line did not hybridize to all *Acidovorax* and *Commamonas* strains tested (only 1–4 according to the probe);
[d] Each probe listed on the line did not hybridize to all four *X. ampelinus* strains tested (only 1–3 according to the probe).

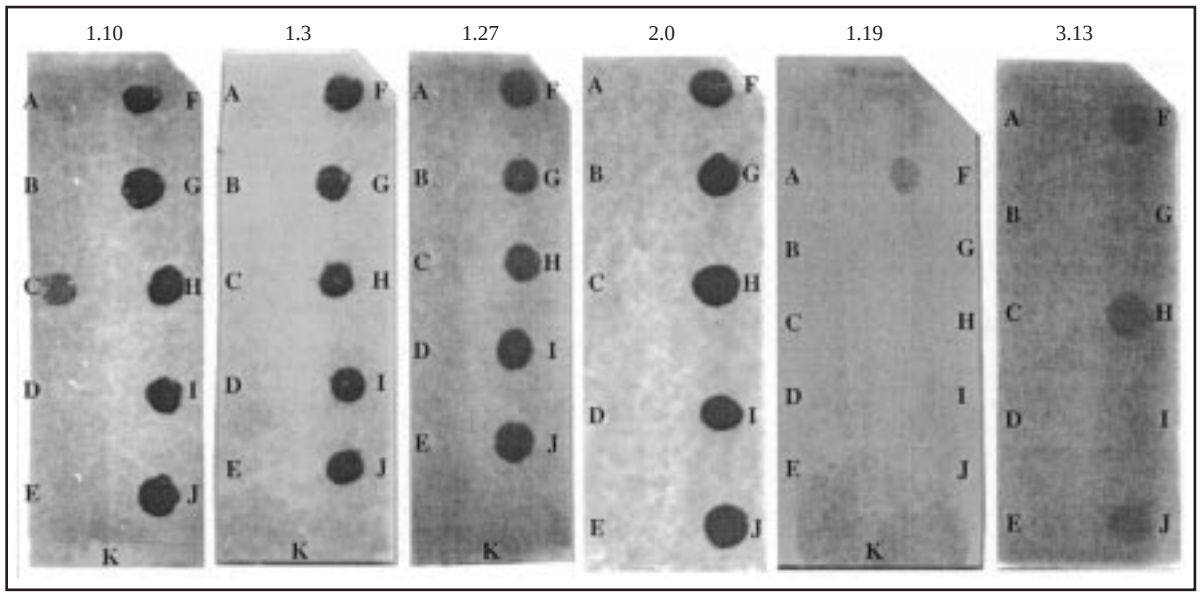

Figure 2. Dot blots of DNA of five *X. ampelinus* strains (F: CFBP 2292; G: CFBP 1192; H: CFBP 2877; I: CFBP 2398; J: CFBP 1802), of *A. vallerianelleae* SO52-1 (A), *A. testosteroni* CFBP 2436 (B), *A. avenae* CFBP 2446 (C), *A. delafieldii* CFBP 2442 (D), *C. acidovorans* CFBP 2444 (E) and DNA extracted from a mixture of bacteria isolated from healthy grapevine (K). Approximately 2.5 μg of DNA were used for each dot. Each membrane was probed with digoxigenin-dUTP labelled DNA from cloned DNA inserts: probes 1.10, 1.3, 1.27 and 1.19 were obtained after subtractive hybridization with *X. ampelinus* CFBP 1192; probe 2.0 after subtractive hybridization with *X. ampelinus* CFBP 2292, probe 3.13 after direct cloning of DNA fragment of *X. ampelinus* CFBP 2292.

(Table 1). The known non-target bacteria used to test primer specificity belonged to the main Gram-negative plant pathogenic groups (*Pseudomonas*, *Xanthomonas*, *Agrobacterium* and *Erwinia*) and to the saprophytic microflora of grapevine. Forty-six bacterial colonies were isolated on YBGA from healthy grapevine collected in August and October 1996. These bacteria were partially characterized. Half of them were Gram positive. Ninety per cent of the Gram-negative strains had a catalase activity and 10% fermented the glucose. None of these bacterial strains amplified any fragment with any of the primers tested.

The sensitivity of PCR amplifications with primers sets 1.27A/1.27B, 1.27A/1.27C and 1.3A/1.3B were tested with 10-fold serial dilutions of exponentially growing *X. ampelinus* cells in distilled water. Fifty microlitres of each dilution (10^{-1}–10^{-7}) were plated on YPGA plates and colony forming units (cfu) were monitored after incubation for 5 days at 24 °C. Five replicates were done at each dilution. Bacterial suspensions were boiled for 10 min, and 10 μl samples were used as templates for PCR amplification with each set of primers. Five replicates were done at each dilution. The bacterial concentration in the starting suspension was assessed as $4.4 \pm 1.22 \times 10^7$ cfu/ml. The detection thresholds were assessed equal to $4.4 \pm 1.22 \times 10^4$ cfu/ml in 5 replicates out of 5 for the set 1.27A/1.27B and $4.4 \pm 1.22 \times 10^3$ cfu/ml for the set 1.27A/1.27C but in only 3 replicates out of 5, when the threshold was higher ($4.4 \pm 1.22 \times 10^5$ cfu/ml) with the set of primer 1.3A/1.3B (Table 3).

Discussion

Almost 50% (18/42) of DNA fragments cloned after subtractive hybridization were specific to *X. ampelinus* strains. We could conclude that the subtractive step was very effective for selecting specific fragments in the bacterial genome. However, one-third (5/15) of DNA fragments cloned into pUC18 plasmid directly after restriction were also specific to *X. ampelinus* strains. These two percentages were not significantly different from each other and it cannot be concluded that subtractive hybridization was necessary for selecting species-specific DNA fragments of this unique bacterium which is of *X. ampelinus*. We assume that the selected specific DNA fragments were all specific to

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Xamp1.19
            1.19A
GATCTACCTG GTGCCGTGTC CGCACTGCGG CCACCACCAC CCGCTGGAGC  50
TGGACAACTT CCGCTACCGC CGCGACCCTG AGACCGGTTT TATGGATGGC 100
GCCTGGTTTG TCTGCCCCGA TTGCGGCAGC GAGATC
                        1.19B

Xamp1.27
      1.27A
GATCGCAAGA AATCCCGATG ATAAATACCG AAAACTCATG CGTCAGGCGC  50
TTGAAGTGAT TATCGGAAGA GAAGTGGAAT TAAAACCAAA ACAACCAGAA 100
GACATAGTGG TAGACACATC AAGTGAAAAA AAAGATATGA TTGACGGCGT 150
TTTAAAAATA CTTCCCAGAT ATGCAAAAGG AGAAAGTATG ACTTCTTTGA 200
GAAAGGATTT TCCTAATATC ACTACCTATT TGTTAAATAG TAGGCGTAGC 250
      1.27C
GACGCCAACG GAGCGAAATG CTTGTTGTCT CAATTTACAA CAGAACAACG 300
AAGGGAATTT GATC
1.27B


Xamp1.3
            1.3A
GATCCGATGT AGCCGGACGT ACCGTATCTA ATGCTTCAAC CGCGATGCGC  50
GCAGGTAGAG GTTTTCTTCA AGACTTCAGC CGTCGCGGAG TGGCTTTGGA 100
AAGCATCGAT GCCACGGAAA ACGAAACTTT CGTCAATGTG ATC
            1.3B
Xamp2.0
            2.0A
GATCGTGTGG CGATAATCGT GACGGCCCCC CTGACGCGCA ACGAAGAGTG  50
GATGGCTTTC GGACAAGGCG AACTGAAAGT GTTCGTAGAC GGCGCACTGC 100
ATCTGCGTGA CGCGCAATGG CGGTAGTGCG TATAAGCACT GCCGCCGCGC 150
TCTTTCAAAC GAGATC                          2.0B
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Figure 3. Nucleotide sequences of *X. ampelinus* specific fragments, Xamp 1.19, Xamp 1.27, Xamp 1.3 and Xamp 2.0. Nucleotides shown in bold print identify the internal forward (A suffix) and reverse (B and C suffix) primers used to amplify each fragment.

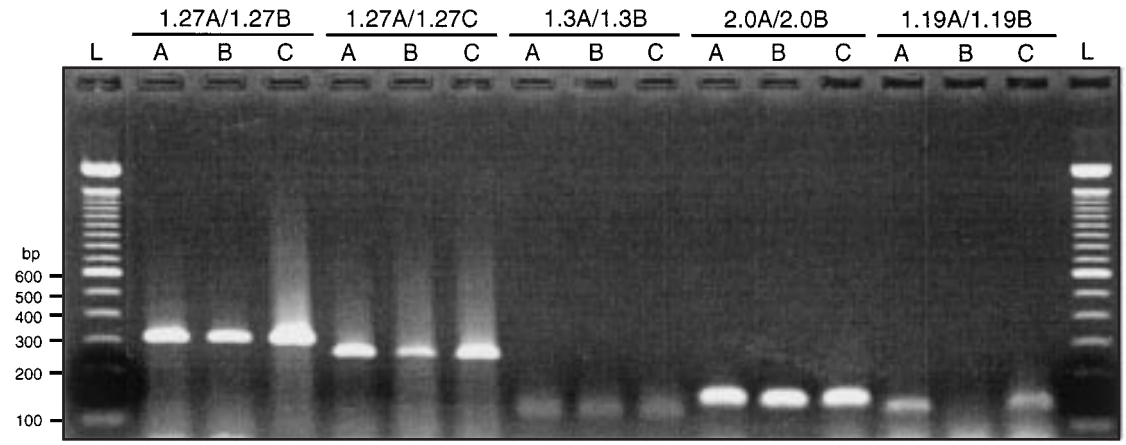

Figure 4. PCR amplifications of *X. ampelinus* strain CFBP 1192 (A), *X. ampelinus* strain CFBP 2292 (B), *X. ampelinus* strain CFBP 3681 (C) with primer sets 1.27A/1.27B, 1.27A/1.27C, 1.3A/1.3B, 2.0A/2.0B, 1.19A/1.19B which give expected DNA fragments of 310, 265, 118 and 153 bp, respectively, for all strains. Primer set 1.19A/1.19B did not allow the amplification of the specific 131 bp amplicon with strain CFBP 2292. L: 100 bp DNA ladder (Eurogentec S.A.).

Table 3. Sensitivity of PCR reactions on bacterial cell suspensions in water of strains CFBP 1192 with three sets of primers

Bacterial concentration ($4.4 \pm 1.22 \times 10^n$ cfu/ml)	Primer sets		
	1.27A/ 1.27B	1.27A/ 1.27C	1.3A/ 1.3B
$n = 7$	5/5[a]	5/5	5/5
$n = 6$	5/5	5/5	5/5
$n = 5$	5/5	5/5	3/5
$n = 4$	5/5	5/5	0/5
$n = 3$	0/5	2/5	0/5
$n = 2$	0/5	0/5	0/5

[a]Proportion of replicates (out 5) showing a specific signal on agarose gel after ethydium bromide staining. Five microlitre of bacterial suspensions were used in each PCR amplification reaction. The detection threshold is equal to 4.4×10^4 cfu/ml for the primer sets designed from Xamp 1.27 DNA fragment and superior to 4.4×10^5 cfu/ml for the primer set 1.3A/1.3B.

X. ampelinus, although the specificity of selected DNA fragments was checked with a limited library e.g. five target strains, five non-target strains and DNA isolated from a mixture of saprophytic bacteria. These saprophytic bacteria were unrelated to *X. ampelinus* and 50% of isolates, which were Gram positive, represented more than 90% of bacterial colonies recovered on agar medium.

The apparent inefficiency of the subtractive hybridization step for selecting a specific DNA fragment is probably due to the origin of the *X. ampelinus* species. *X. ampelinus* is the only species in the genus *Xylophilus*. Furthermore, this genus is characterized by a very high degree of binding (D) in DNA pairing experiments (Willems et al., 1987). DNA/DNA hybridization of *X. ampelinus* strains yielded 96–100% D. In contrast, the degrees of binding were very low with strains belonging to the closest phylogenetically related bacteria. *Acidovorax avenae* gave less than 20% D with *X. ampelinus* (Willems et al., 1987). These data indicate that a large part of the *X. ampelinus* genome does not share homology with other known bacteria; the failure to find any homology in the EMBL/GeneBank database supports this observation. The subtractive hybridization technique described in this paper was very similar to this described by Seal et al. (1992) for selecting specific DNA probes for the detection of *Ralstonia solanacearum*. It is not a very selective technique in comparison with techniques described for other plant-associated bacteria (Bjourson et al., 1992; Cook and Sequeira, 1991; Strauss and Ausubel, 1990; Darrasse et al., 1994). However, it was efficient to allow the enrichment in specific DNA fragments making their selection easier than by the direct cloning approach in the selection of *P. syringae* pathovar-specific DNA probes (unpublished data). *P. syringae* pathovars are more closely related to each other than *X. ampelinus* and other *Comamonadaceae* members, so the efficiency of the selective hybridization step could be significant.

Several primer sets designed in this work were highly specific for *X. ampelinus*. They detected all *X. ampelinus* strains regardless of the cultivar and the geographic origin. This indicates that *X. ampelinus* forms an homogenous genomic group. However, the pattern of primer set 1.19 showed the occurrence of a genomic diversity within the species. This primer set detected all strains isolated from cv. Sultana except one. All bacterial strains isolated from cv. Sultana were isolated in Greece. The bacterial population identified by the primer set 1.19 might be either a Greek population or a cultivar-specific population of *X. ampelinus*. Cultivar Sultana is very susceptible to the bacterial necrosis and has been very common in Greece where the disease has occurred. The occurrence of Greek strains isolated from other cultivars but not identified by the primer set 1.19 implicates occurrence of a cultivar-specific population. However, cv. Sultana is sensitive to all strains of *X. ampelinus* and Sultana strains are pathogenic on other cultivars tested (Panogopoulos, 1987). More information is required to demonstrate a specific interaction between the strains identified with the primer set 1.19 and the cv. Sultana.

Although all the species-specific primer sets described could be used as molecular reagents for identification of *X. ampelinus* using PCR, the nucleotide sequence Xamp 1.27 seems to be the best candidate for selection of primers for PCR because the two sets of primers designed into this sequence showed the lower limits of detection.

In conclusion, several molecular reagents have been designed which can be used for the identification of *X. ampelinus* species and for setting up a direct detection procedure in grapevine samples based on PCR. The genetic diversity pointed out with the primer set 1.19 indicates that ecological studies can be undertaken on populations of *X. ampelinus*.

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