Rapid Diagnosis of *Pseudomonas syringae* pv. *papulans*, the Causal Agent of Blister Spot of Apple, by Polymerase Chain Reaction Using Specifically Designed *hrpL* Gene Primers

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ABSTRACT

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The identification and detection of *Pseudomonas syringae* pv. *papulans*, the causal agent of blister spot of apple, on apple leaves and fruit was achieved by polymerase chain reaction amplification of a specific DNA fragment of the *hrpL* sequence. The consensus primers *hrpL*₁ and *hrpL*₂ were designed based on the alignment of pseudomonad *hrpL* gene sequences available in nucleic acid data banks. This primer set produced a 631-bp amplicon from 37 of the 57 pseudomonads strains tested. These strains belonged to genomospecies 1 and 2, as described by Gardan et al. (8). The amplicon obtained from 30 of these strains was digested with eight restriction enzymes. Three different restriction patterns were pro-

duced from strains belonging to genomospecies 1, resulting in A1 and A2 patterns, while strains belonging to genomospecies 2 were characterized by a B pattern. Patterns A1 and A2 differed at only two sites, a *Bsp* 143I site located at nucleotide 360 and a *Mse*I site located at nucleotides 22–24. Group A2 consisted solely of *P. syringae* pv. *papulans* strains. The *hrpL* gene in *P. syringae* pv. *papulans* strain CFBP3323 was sequenced. Two primer sets, Pap1/Pap2 and Pap1/Pap3, were designed and tested for specificity to *P. syringae* pv. *papulans*. These primers amplified expected fragments of 242 and 303 bp, respectively. Pap1/Pap2 amplified a fragment only with *P. syringae* pv. *papulans* DNA, while Pap1/Pap3 amplified all tested strains belonging to genomospecies 1. A diagnostic procedure using the Pap1/Pap2 primer set was successful for the detection of *P. syringae* pv. *papulans* in diseased fruit and artificially inoculated leaves.

Pseudomonas syringae pv. *papulans* is the causal agent of blister spot of apple. It is the most important disease of the apple cv. Mutsu in the eastern United States (4), Canada (7), and Italy (2). Recently, blister spot was reported in Germany (21) and France (12). Epiphytic population studies of *P. syringae* in French pome orchards revealed the occurrence of *P. syringae* pv. *papulans* where natural symptoms had never been observed (12).

P. syringae is subdivided into pathovars differentiated primarily on the basis of their host plants and the symptoms that they cause. Each pathovar typically has strong biochemical, immunological, and DNA relatedness to several other pathovars, including *P. syringae* pv. *syringae*, which can be isolated from many plants (including apple), even though they are not pathogenic on these plants. Classical methods for the detection and identification of *P. syringae* pv. *papulans* rely on the isolation of the bacterium on nutrient agar medium and performance of specific biochemical, serological, and pathogenicity tests (3,8,12). These tests take time and are expensive. Therefore, molecular tools (18) for detecting and identifying this pathovar are a promising alternative.

The polymerase chain reaction (PCR) has been used widely in molecular biology for the past 15 years. It is a very powerful methodology which has great potential for use in the identification and detection of microorganisms because of its rapidity, sensitivity, and specificity. The specificity of PCR is determined mainly by the primers. Thus, sequence analyses of the genomes of pathogenic bacteria are critical for the design of specific primers. In this paper we describe the approach that we used to design primers

specific to *P. syringae* pv. *papulans*, using the *hrpL* gene as a target. This gene encodes an alternative sigma factor that belongs to the sigma-70-type sigma factor family (17) and is required for the expression of the *hrp* gene cluster in *P. syringae* (16), which encodes a type III secretion system essential for pathogenicity and host range (11). The sensitivity of a PCR assay is limited by the specificity of the primers used, the recovery of the target sequences, and the efficiency of the enzymatic reaction. *Taq* DNA polymerase is sensitive to inhibition by factors present in biological samples. Therefore it is evident that sample preparation should ensure both the removal or blocking of inhibitory substances and a high yield of amplifiable DNA. The objective of this work was to describe primers specific to *P. syringae* pv. *papulans* and to develop a reliable assay for the extraction of DNA and amplification by PCR of specific *P. syringae* pv. *papulans* target sequences from infected apple fruit and leaves.

MATERIALS AND METHODS

Bacterial strains. Eighteen strains of *P. syringae* pv. *papulans* isolated from different geographical areas (United States, Canada, Italy, and France) and 49 reference and type strains of other *P. syringae* pathovars and related species were used. In addition to the pseudomonads, isolates of four different species from rosaceous trees were tested (Table 1). All strains were stored as lyophilized stocks or frozen at –80°C in liquid King's medium B (KB) (13) with glycerol (40%, wt/vol) and were restreaked for colony uniformity on KB agar.

Sample preparation for direct PCR from cell cultures. Bacterial cells grown on KB agar for 24 h were resuspended in sterile distilled water. The cell suspensions (approximately 1×10^7 cells ml⁻¹) were boiled for 10 min and used for the PCR assay.

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PCR conditions for amplification of *hrpL* region of *P. syringae*. The nucleotide sequence of *P. syringae* pv. *syringae* Pss61 *hrpL* genes (27) (GenBank Accession No. U03854) was compared with that of *P. syringae* pv. *morsprunorum* PM7 *hrpL* genes (15) (GenBank Accession No. L3653). Primers were selected from conserved regions of these *hrpL* genes. Primers *hrpL*₁ (5'-GCGAGT-TGGTTCCAGACAG-3') and *hrpL*₂ (5'-GGTTATCGCTATAG-GGCTTGC-3') are located 44 bp upstream of the *hrpL* gene and 21 bp before the stop codon, respectively. Oligonucleotides were synthesized by Eurogentec, Seraing, Belgium. PCR reactions were carried out in a 100- μ l reaction volume. A volume of 10 μ l of boiled bacterial cells was added to 90 μ l of PCR reaction mixture, consisting of 10 mM Tris-HCl (pH 9); 50 mM KCl; 1% Triton X-100; 3 mM MgCl₂; dATP, dCTP, dGTP, and dTTP (0.25 mM each) (Eurogentec SA); 20 pmol of each primer; and 2 units of *Taq* DNA polymerase (Eurotaq, Eurogentec) at 25°C. PCR amplifications were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA) as follows: an initial denaturation step at 92°C for 2 min and then 35 cycles of 92°C for 1 min, 52°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 2 min. Amplified DNA fragments were examined by horizontal electrophoresis in 2% agarose gels (1% Seakem GTG plus 1% Nuseive GTG, FMC Bioproducts, Rockland, ME) in TBE buffer (19,26) with

10- μ l aliquots of PCR products. Gels were stained with ethidium bromide (1 μ g ml⁻¹) and photographed under UV light (312 nm).

DNA sequencing. The DNA fragment obtained after PCR of *P. syringae* pv. *papulans* strain CFBP3323 was isolated from the agarose gel and purified using a silica-based procedure (Ultra-Clean 15 DNA purification kit, Mo Bio Laboratories, Solana Beach, CA) as described by the manufacturer. The DNA fragments were cloned in the pGEM-T easy plasmid (Promega France) according to the manufacturer's instructions. Recombinant plasmids were introduced into *E. coli* strain DH5 α by transformation (10). Cloned DNA fragments were sequenced by using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and a model ABI377 automatic sequencer. The complete DNA sequence was determined by using T7 and SP6 primers on DNA fragments cloned in pGEM-T easy plasmid. Both strands of two clones were sequenced.

Restriction fragment analysis. The following enzymes were used: *Bsp*143I, *Hae*III, *Hha*I, *Msp*I, *Taq*I (Eurogentec); *Mse*I, *Tsp*509I (New England Biolabs, Ozyme, Montigny le Bretonneux, France); and *Sau*96I (GIBCO-BRL, Life Technologies, Eragny, France). PCR products (95 μ l) were ethanol-precipitated and resuspended in 100 μ l of sterile distilled water; 10 μ l was used for each digestion. Digestions were performed as described by the

TABLE 1. Bacterial strains used in this study and results of polymerase chain reaction (PCR) assays

	Strain number (CFBP) ^a	Host	Origin	PCR results ^b with:		
				<i>hrpL</i> primers	Pap1/Pap2 primers	Pap1/Pap3 primers
<i>Pseudomonas syringae</i> genomospecies 1 ^c						
<i>P. syringae</i> pv. <i>syringae</i>	1392 ^T	<i>Syringa vulgaris</i>	U.K.	+ A1	—	+
	1391	<i>Pyrus communis</i>	U.K.	+ A1	—	+
	3077	<i>Pyrus communis</i>	France	+ A1	—	+
	602	<i>Malus sylvestris</i>	France	+ A1	—	+
	2559	<i>Malus sylvestris</i>	France	+ A1	—	+
<i>P. syringae</i> pv. <i>aptata</i>	1617	<i>Beta vulgaris</i>	U.S.	+ A1	—	+
<i>P. syringae</i> pv. <i>lapse</i>	1731	<i>Triticum aestivum</i>	Unknown	NT	—	+
<i>P. syringae</i> pv. <i>papulans</i>	1754	<i>Malus sylvestris</i>	Canada	+ A2	+	+
	3324	<i>M. sylvestris</i> cv. Mutsu	Canada	+ A2	+	+
	5040	<i>M. sylvestris</i> cv. Mutsu	Canada	+ A2	+	+
	5041	<i>M. sylvestris</i> cv. Mutsu	Canada	+ A2	+	+
	5731	<i>M. sylvestris</i> cv. Mutsu	U.S.	+ A2	+	+
	3323	<i>M. sylvestris</i> cv. Mutsu	U.S.	+ A2	+	+
	5043	<i>M. sylvestris</i> cv. Mutsu	U.S.	+ A2	+	+
	5044	<i>M. sylvestris</i> cv. Mutsu	U.S.	+ A2	+	+
	5045	<i>M. sylvestris</i> cv. Mutsu	U.S.	+ A2	+	+
	5046	<i>M. sylvestris</i> cv. Mutsu	U.S.	+ A2	+	+
	5047	<i>M. sylvestris</i> cv. Mutsu	U.S.	+ A2	+	+
	5048	<i>M. sylvestris</i> cv. Mutsu	U.S.	+ A2	+	+
	5049	<i>M. sylvestris</i> cv. Mutsu	U.S.	+ A2	+	+
	5134	<i>M. sylvestris</i> cv. Mutsu	Italy	+ A2	+	+
	5135	<i>M. sylvestris</i> cv. Golden Delicious	Italy	+ A2	+	+
	5728	<i>M. sylvestris</i> cv. Golden Delicious	Italy	+ A2	+	+
	5132	<i>M. sylvestris</i> cv. Golden Delicious	France	+ A2	+	+
	5133	<i>M. sylvestris</i> cv. Golden Delicious	France	+ A2	+	+
<i>P. syringae</i> pv. <i>pisi</i>	2105	<i>Pisum sativum</i>	New Zealand	+ A1	—	+
<i>P. syringae</i> pv. <i>atrofaciens</i>	2213	<i>Triticum aestivum</i>	New Zealand	+ A1	—	+
<i>P. syringae</i> pv. <i>aceris</i>	2339	<i>Acer</i> sp.	U.S.	+ A1	—	+
<i>P. syringae</i> pv. <i>panici</i>	2345	<i>Panicum</i> sp.	U.S.	+ A1	—	+
<i>P. syringae</i> pv. <i>dysoxyl</i>	2356	<i>Dysoxylum spectabile</i>	New Zealand	+ A1	—	+
<i>P. syringae</i> pv. <i>japonica</i>	2896	<i>Hordeum vulgare</i>	Japan	+ A1	—	+
<i>P. syringae</i> genomospecies 2						
<i>P. savastanoi</i>	1670 ^T	<i>Olea europea</i>	Yugoslavia	+ B	—	—
<i>P. ficusectae</i>	3224 ^T	<i>Ficus erecta</i>	Japan	NT	—	—
<i>P. meliae</i>	3225 ^T	<i>Melia azedarach</i>	Japan	+ B	—	—
<i>P. amygdali</i>	3340 ^T	<i>Prunus amygdalus</i>	Greece	NT	—	—
<i>P. syringae</i> pv. <i>phaseolicola</i>	1390	<i>Phaseolus vulgaris</i>	Canada	+ B	—	—
<i>P. syringae</i> pv. <i>ulmi</i>	1407	<i>Ulmus</i> sp.	Yugoslavia	NT	—	—

(continued on next page)

^a CFBP, Collection Française de Bactéries Phytopathogènes. T, type strain.

^b PCR restriction fragment length polymorphism (RFLP) groups. PCR results obtained with *hrpL*₁/*hrpL*₂, Pap1/Pap2, and Pap1/Pap3 primers: +, positive; —, negative; NT, not tested.

^c Genomospecies according Gardan et al. (8).

supplier but with an excess of enzyme (5 units per reaction). Restricted DNA was analyzed by horizontal electrophoresis in 3% agarose gels (1% SeaKem GTG plus 2% Nuseive GTG, FMC Bioproducts) as described above. The technique permitted the clear visualization and estimation of the length of DNA fragments longer than 70 bp and permitted the visualization of poorly resolved fragments between 40 and 70 bp long.

Data analysis. The restriction maps were determined from restriction fragment length polymorphisms (RFLPs) by comparison with those inferred from the *hrpL* sequence of *P. syringae* pv. *papulans* strain CFBP3323. In this study, restriction and nucleotide sites were numbered starting from the first nucleotide identified in the sequence of strain CFBP3323 (Fig. 1).

PCR detection of *P. syringae* pv. *papulans* in infected pome tissues. Artificially inoculated apple fruit was used for direct detection of *P. syringae* pv. *papulans* in plant tissues. Detached immature fruit of the apple cv. Golden Delicious was inoculated in the laboratory by vacuum infiltration (12) with a suspension of bacteria (10^7 CFU ml⁻¹) in sterile distilled water. Controls were inoculated with sterile distilled water. Inoculated fruit was enclosed in clear plastic bags and incubated in a growth chamber (25°C, 8 h darkness a day) for 4 weeks. From each infected fruit, transverse sections with blister spots were individually transferred to 1.5-ml sterile Eppendorf tubes and crushed in 200 µl of sterile distilled water. The bacterial cells were allowed to diffuse from

plant tissues for 15 min. Undiluted and 10-fold-diluted preparations were used directly for PCR detection. DNA was also extracted by the following procedure: 20 µl of soaking liquid was added to 80 µl of lysis buffer, containing 0.2M Tris HCl (pH 7.5), 0.25 M NaCl, 0.25 M EDTA, sodium dodecyl sulfate (0.5%, wt/vol), and polyvinylpyrrolidone 360 (2%, wt/vol), after Edwards et al. (8), and left at room temperature for 10 min; DNA was extracted from the lysis solution by a silica-based procedure (Ultraclean 15 DNA purification kit, Mo Bio Laboratories), as described by the manufacturer. A Genereleaser treatment was applied to DNA samples before PCR, as described by the manufacturer (Bioventures, Murfreesboro, Tennessee).

PCR detection of *P. syringae* pv. *papulans* in mixed leaf extracts. Leaves of the apple cv. Golden Delicious (3 g) were crushed in 18 ml of extraction buffer (8.5 mM NH₄H₂PO₄, 13.5 mM KCl, and 8.3 mM MgSO₄·7H₂O, pH 7.2). Tenfold serial dilutions of a suspension of *P. syringae* pv. *papulans* (approximately 6×10^6 CFU ml⁻¹) were made in leaf extract. Samples (900 µl) of the bacterial suspensions were filtered through a paper disk (ref. 321861, Schleicher and Schuell, Dassel, Germany), and the bacteria were collected by centrifugation at $13,000 \times g$ for 10 min. The pellet was resuspended in 100 µl of Edwards's buffer and treated like infected pome tissue extracts.

PCR conditions for specific identification and detection of *P. syringae* pv. *papulans*. The designed primers were tested on a

TABLE 1. (continued from preceding page)

	Strain number (CFBP) ^a	Host	Origin	PCR results ^b with:		
				hrpL primers	Pap1/Pap2 primers	Pap1/Pap3 primers
<i>P. syringae</i> pv. <i>mori</i>	1642	<i>Morus alba</i>	Hungary	+ B	—	—
<i>P. syringae</i> pv. <i>sesami</i>	1671	<i>Sesamum indicum</i>	Yugoslavia	NT	—	—
<i>P. syringae</i> pv. <i>tabaci</i>	2106	<i>Nicotiana tabacum</i>	Hungary	+ B	—	—
<i>P. syringae</i> pv. <i>morsprunorum</i>	1647	<i>Prunus avium</i>	Italy	+ B	—	—
<i>P. syringae</i> pv. <i>glycinea</i>	2214 ^T	<i>Glycine max</i>	New Zealand	+ B	—	—
<i>P. syringae</i> pv. <i>ciccaronei</i>	2342	<i>Ceratonia siliqua</i>	Italy	NT	—	—
<i>P. syringae</i> genomospecies 3						
<i>P. syringae</i> pv. <i>tomato</i>	2212 ^T	<i>Lycopersicon esculentum</i>	U.K.	—	—	—
<i>P. syringae</i> pv. <i>persicae</i>	1573 ^T	<i>Prunus persica</i>	France	—	—	—
<i>P. syringae</i> pv. <i>antirrhini</i>	1620 ^T	<i>Antirrhinum</i> sp.	U.K.	—	—	—
<i>P. syringae</i> pv. <i>maculicola</i>	1657 ^T	<i>Brassica oleracea</i>	New Zealand	—	—	—
<i>P. syringae</i> pv. <i>viburni</i>	1702	<i>Viburnum</i> sp.	U.S.	—	—	—
<i>P. syringae</i> pv. <i>berberidis</i>	1727	<i>Berberis</i> sp.	New Zealand	—	—	—
<i>P. syringae</i> pv. <i>apii</i>	2103	<i>Apium graveolens</i>	U.S.	—	—	—
<i>P. syringae</i> pv. <i>delphinii</i>	2215	<i>Delphinium</i> sp.	New Zealand	—	—	—
<i>P. syringae</i> pv. <i>passiflorae</i>	2346	<i>Passiflora edulis</i>	New Zealand	—	—	—
<i>P. syringae</i> pv. <i>lachrymans</i>	2440	<i>Cucumis sativum</i>	U.S.	—	—	—
<i>P. syringae</i> pv. <i>philadelphii</i>	2898	<i>Philadelphus coronarius</i>	U.K.	—	—	—
<i>P. syringae</i> genomospecies 4						
<i>P. syringae</i> pv. <i>porri</i>	1908	<i>Allium porrum</i>	France	—	—	—
<i>P. syringae</i> pv. <i>garcae</i>	1634	<i>Coffea arabica</i>	Brazil	—	—	—
<i>P. syringae</i> pv. <i>striaefaciens</i>	1674	<i>Avena sativa</i>	Unknown	—	—	—
<i>P. syringae</i> pv. <i>coronafaciens</i>	2216 ^T	<i>Avena sativa</i>	U.K.	—	—	—
<i>P. syringae</i> pv. <i>atropurpurea</i>	2340 ^T	<i>Lolium multiflorum</i>	Japan	—	—	—
<i>P. syringae</i> pv. <i>oryzae</i>	3228	<i>Oryza sativa</i>	Japan	NT	—	—
<i>P. syringae</i> genomospecies 5						
<i>P. syringae</i> pv. <i>tremae</i>	3229	<i>Trema orientalis</i>	Japan	NT	—	—
<i>P. syringae</i> genomospecies 6						
<i>P. viridiflava</i>	2107 ^T	<i>Phaseolus</i> sp.	Switzerland	—	—	—
<i>P. syringae</i> pv. <i>primulae</i>	1660	<i>Primula</i> sp.	U.S.	NT	—	—
<i>P. syringae</i> genomospecies 7						
<i>P. syringae</i> pv. <i>tagetis</i>	1694 ^T	<i>Tagetes erecta</i>	Zimbabwe	—	—	—
<i>P. syringae</i> pv. <i>helianthi</i>	2067	<i>Helianthus annuus</i>	Mexico	NT	—	—
<i>P. syringae</i> genomospecies 8						
<i>P. syringae</i> pv. <i>theae</i>	2353	<i>Thea sinensis</i>	Japan	—	—	—
<i>P. syringae</i> genomospecies 9						
<i>P. syringae</i> pv. <i>cannabina</i>	2341	<i>Cannabis sativa</i>	Hungary	—	—	—
Other species						
<i>Erwinia amylovora</i>	1430	<i>Pyrus communis</i>	France	—	—	—
<i>Erwinia herbicola</i>	1189	<i>Malus sylvestris</i>	U.S.	—	—	—
<i>Pantoea agglomerans</i>	3615	<i>Wisteria floribunda</i>	Japan	—	—	—
<i>Xanthomonas arboricola</i> pv. <i>pruni</i>	3566	<i>Prunus armeniaca</i>	France	—	—	—

collection of *P. syringae* pv. *papulans* strains, related pseudomonads, and nonpseudomonad bacteria associated with rosaceous trees (Table 1). PCR components were 0.3 µl of *Taq* DNA polymerase (Appligène, 5 units/µl), 5 µl of 10× buffer, and 3.5 µl of dNTP (10 µM each); 5 µl of MgCl₂ (25 mM); and 1 µl of primer Pap1/Pap2 or Pap1/Pap3 (20 µM) in a final volume of 50 µl. Amplification was carried out as follows: one step at 92°C for 2 min, followed by 35 cycles at 92°C for 1 min, 64°C for 30 s, and 72°C for 1 min, and then one step at 72°C for 1 min. Amplified products were detected by agarose gel electrophoresis, as described above.

	<i>Tsp509 I</i> <i>MseI</i>			
1	ATAGGGCTTG	CACTCCATCA	ATTAAGGCAA	GTCCAGAGGC
41	AAGTCCTGCT	CCCGAATCTT	<i>Bsp143I</i> GTGATCCTTG	ATGCAACCGA
	Pap1		<i>MspI</i>	<i>MspI Tsp509I</i>
81	ACCACGCCAA	CCATCGTCAT	CCGCCGGTAT	CCGGCAATTG
	<i>HaeIII Bsp143I Sau96</i>			
121	ACGGCCGATC	AGGTCCAGAT	GCTCAGAGCG	TTCATCCAGA
161	AGCGGGTGAA	GAACGCTGAT	GATGTGGATG	ACATCCTGCA
	<i>TaqI</i>			
201	ATGCGTGTTC	CTCGAGGCGT	TACGCAATGA	GCACAAGTTT
241	CAGCACGCCA	GCAAACCGCA	AACCTGGTTG	TGTGGCATTG
	<i>HhaI Bsp143I</i>		Pap2	
281	CGCTGAATCT	GATCCGCAAC	CACTTTCGCA	AAATGTATCG
321	CCAGCCCTAT	CAGGAAAGCT	GGGAAGACGA	GGTCCATACG
	<i>Sau96</i>	<i>TaqI Pap3</i>		
361	GACCTGGAGT	CGAACGGCGA	TATACCCAT	CAGGTAGACG
	<i>HaeIII</i>		<i>HaeIII TaqI</i>	
401	GCCACCGACA	GCTGCGACGC	GTCATAGAGG	CCATCGACTG
441	CCTGCCGACG	AATATGCAGA	AGGTTCTGGA	AGTTTCTCTG
	<i>Tsp509 I</i>			
481	GAAATGGATG	GCAATTATCA	GGAAACCGCC	AACATGCTCG
	<i>Bsp143I</i>		<i>MspI</i>	
521	GTGTTCCGAT	CGGCACTGTC	CGTCCCGAC	TGTCCCGGGC
561	ACGGGTGCAG	CTCAAGCAAC	AGATAGACCC	GTTCCCT

Fig. 1. Nucleotide sequence of an internal fragment of the *hrpL* gene of *Pseudomonas syringae* pv. *papulans* strain CFBP3323. Sequence of primer Pap1 and complementary sequences of primers Pap2 and Pap3, designed to detect *P. syringae* pv. *papulans*, are in bold letters. Also shown are the restriction sites of *Tsp509I*, *MseI*, *Bsp143I*, *MspI*, *HaeIII*, *Sau96*, *TaqI*, and *HhaI*.

Fig. 2. Gel electrophoresis of an *hrpL* DNA fragment of *Pseudomonas syringae* digested with *Bsp143I* and amplified by polymerase chain reaction. Lanes 1 to 4: *P. syringae* pv. *papulans* strains CFBP3323, CFBP1754, CFBP5132, and CFBP3324. Lane 5: *P. syringae* pv. *syringae* strain CFBP1392. Lane 6: *P. syringae* pv. *aptata* strain CFBP1617. Lane 7: *P. syringae* pv. *lapsa* strain CFBP1731. Lane L: DNA size markers (100-bp ladder, Life Technologies).

RESULTS

Nucleotide sequence of a segment of the *hrpL* gene of *P. syringae* pv. *papulans* strain CFBP3323. The 597-bp segment encoding part of the *hrpL* gene of *P. syringae* pv. *papulans* strain CFBP3323 was sequenced in both directions. Four restriction sites for *Bsp143I*, three restriction sites apiece for *HaeIII*, *TaqI*, and *Tsp509I*, two restriction sites apiece for *MspI* and *Sau96*, and one restriction site apiece for *HhaI* and *MseI* were located in the sequence (Fig. 1). Their locations were verified by analysis of the electrophoretic patterns of digested amplified DNA fragments. *hrpL* sequences of *P. syringae* pv. *syringae* strain Pss61 (27) (GenBank Accession No. U03854) and *P. syringae* pv. *phaseolicola* strain 1302A (20) (GenBank Accession No. U16817) were used to precisely locate restriction sites which were not present in strain CFBP3323.

Restriction enzyme analysis of the amplified *hrpL* gene fragments of *P. syringae*. Stringent PCR conditions with primers *hrpL*₁ and *hrpL*₂ allowed the amplification of a single DNA fragment of the expected size (631 bp) for only 37 of the 57 pseudomonad strains tested (Table 1). All tested strains that produced a DNA amplicon belonged to either genomospecies 1 or genomospecies 2, as defined by Gardan et al. (9). Digestion of the PCR products with eight restriction enzymes (*Bsp143I*, *HaeIII*, *HhaI*, *MseI*, *MspI*, *Sau96*, *TaqI*, and *Tsp509I*) revealed polymorphisms in restriction patterns (as an example, results of digestion with *Bsp143I* are shown in Figure 2). Analysis of the diversity within this DNA fragment was performed using 30 restriction sites. Three different patterns were identified. Patterns A1 and A2 dif-

Fig. 3. Amplification of *Pseudomonas syringae* strains by polymerase chain reaction with primer set Pap1/Pap2. Lanes 1–8: *P. syringae* pv. *papulans* strains CFBP1754, CFBP3324, CFBP3323, CFBP5043, CFBP5134, CFBP5135, CFBP5132, and CFBP5133. Lane 9: negative control. Lane 10: *P. syringae* pv. *syringae* strain CFBP1392. Lane 11: *P. savastanoi* strain CFBP1670. Lane 12: *P. syringae* pv. *tomato* strain CFBP2212. Lane 13: *P. syringae* pv. *garcae* strain CFBP1634. Lane 14: *P. viridiflava* strain CFBP2107. Lane 15: *P. syringae* pv. *tagetis* strain CFBP1694. Lane L: DNA size markers (100-bp ladder, Life Technologies).

TABLE 2. Detection level of *Pseudomonas syringae* pv. *papulans* with PCR and plating assays

Dilution of bacterial suspension ^a	PCR		Plating ^b	
	Water	Leaf extract	Water	Leaf extract
0	+	+	+	— ^c
10 ⁻¹	+	+	+	—
10 ⁻²	+	+	+	—
10 ⁻³	+	+	+	—
10 ⁻⁴	+	+	30 ^d	—
10 ⁻⁵	—	—	—	—
10 ⁻⁶	—	—	—	—

^a The initial concentration of the bacterial suspension was 6 × 10⁶ CFU ml⁻¹.
^b Plating assays were performed on King's medium B.
^c Colonies of *P. syringae* pv. *papulans* were undetectable among colonies of epiphytic bacteria, which numbered 1 × 10⁶ CFU ml⁻¹ in the leaf extract suspension.
^d Average number of colonies detected on King's medium B (in 50 µl), corresponding to 6 × 10² CFU ml⁻¹.

ferred at only two sites (*Bsp*143I at nucleotide 360 was present in A1 but absent in A2, and *Mse*I at nucleotides 22–25 was absent in A1 but present in A2). Pattern A2 was formed only by strains of *P. syringae* pv. *papulans*. Patterns A1 and A2 differed from pattern B at 22 and 21 of 30 sites, respectively. Patterns A1 and A2 were observed for all tested pathovars belonging to genomospecies 1 of *P. syringae* (including pathovars *syringae*, *aptata*, *lapsa*, *pisi*, *atrofaciens*, and *papulans*), whereas pattern B was observed for the seven tested members of genomospecies 2 (*P. savastanoi*, *P. meliae*, and *P. syringae* pvs. *phaseolicola*, *mori*, *tabaci*, *morsprunorum*, and *glycinea*). These results suggested that it is possible to identify nucleotide sequences specific to *P. syringae* pv. *papulans* in the *hrpL* gene.

Design of oligonucleotides specific to *P. syringae* pv. *papulans*. The DNA sequence of the *hrpL* gene fragment of *P. syringae* pv. *papulans* strain CFBP3323 was used to perform a BLAST search of nucleotide data banks. The most similar sequence found was the *hrpL* gene of *P. syringae* pv. *pisi* (GenBank Accession No. U95530). Three primers—Pap1 (5'-AACCACGCCAACCATCGTCA-3'), Pap2 (5'-GCGATACATTTTGGCGAAAGTGGTTG-3'), and Pap3 (5'-GCCGTTTCGACACCAGTCC-3')—were selected from regions where the number of mismatches was highest (Fig. 1). Pap3 was from the region where the restriction site *Bsp*143I₍₃₆₀₎ was located in the *hrpL* sequence of *P. syringae* pv. *pisi*. The primer sets Pap1/Pap2 and Pap1/Pap3 amplified fragments of the expected sizes, 242 and 303 bp, respectively, from *P. syringae* pv. *papulans* strains.

Primer specificity. The designed primers were tested on a collection of pseudomonads and bacteria common to rosaceous trees (Table 1). PCR was performed under high-stringency conditions (annealing at 64°C for 30 s), which were compatible with the temperature melting of the primers and the high G+C content of *hrpL* clusters. The primer set Pap1/Pap2 was specific for *P. syringae* pv. *papulans*; only *P. syringae* pv. *papulans* strains amplified the 242-bp fragment (Fig. 3 and Table 1). However, the primer set Pap1/Pap3, which initiated amplification of a 303-bp DNA fragment from *P. syringae* pv. *papulans* strains, produced a fragment of the same size from all the other *P. syringae* genomospecies 1 pathovars that were tested (Table 1). Five repetitions of the experiments confirmed the reproducibility of these results.

Sensitivity of PCR detection using apple leaf tissue. Amplification products were detected using a 1×10^{-4} dilution of bacterial suspension in sterile distilled water or crushed leaf liquid (Table 2). The level of detection corresponded to 600 CFU ml⁻¹. The same detection level was obtained in leaf extracts, indicating that the DNA extraction procedure removed PCR inhibitors contained in the leaf extracts (Fig. 4 and Table 2). *P. syringae* pv. *papulans* was not detected by plating bacterial suspensions made in leaf extracts on KB because epiphytic bacteria outgrew the pathogen.

PCR detection in blister spots on fruit. No amplification product was obtained when PCR was performed directly on crude

extracts of blister spots. However, a characteristic DNA fragment was observed when the extract was diluted 10-fold in sterile distilled water or when the DNA in the extract was purified as described in the Methods section (Fig. 5). DNA amplicons were digested with *Taq*I, and restriction products were separated by electrophoresis in agarose gel. Two fragments (108 and 134 bp) were observed in all cases (data not shown), which indicates that the amplicons correspond to the targeted DNA template of the *hrpL* gene.

DISCUSSION

The pathogenicity-related gene *hrpL* displayed a nucleotide sequence diversity similar to that observed in genes encoding house-keeping functions, such as *gyrB* and *rpoD* (25), suggesting that the *hrpL* gene is a good candidate for analysis of the evolutionary course of *P. syringae sensu lato*. Our results agree with those reported by Sawada et al. (25). Primers *hrpL*₁ and *hrpL*₂, used to amplify the *hrpL* gene, allowed the amplification of a DNA fragment of *P. syringae* pathovars belonging to Sawada's *P. syringae* groups 2 and 3 only, but not those of group 1, based on *gyrB*, *rpoD*, *hrpL*, and *hrpS* DNA sequences (25). The RFLP data on the *hrpL* gene shows a correlation between the observed RFLP groups A and B and genomospecies 1 and 2, described by Gardan et al. (9). These observations confirm that the evolution of the *hrpL* gene parallels speciation within the fluorescent oxidase-negative *Pseudomonas* group.

The tested strains of genomospecies 1, which contains numerous pathovars, including the heterogeneous *P. syringae* pv. *syringae*, appeared to be quite uniform in the RFLP pattern of the *hrpL* gene. All pathovars displayed the same pattern except for *P. syringae* pv. *papulans* strains. However, *P. syringae* pv. *papulans* is not very different from the other pathovars of genomospecies 1, as it differed in only two restriction sites out of 30, which is very low in comparison with the 20 polymorphic sites noted between the groups A1-A2 and B. Additional polymorphic markers that would identify other pathovars or groups of pathovars probably exist in the *hrpL* nucleotide sequence, but RFLP analysis performed on 30 sites, which represent 120 nucleotides, did not reveal this polymorphism. DNA polymorphism analysis of the whole *hrpL* sequence of several pathovars of *P. syringae*, performed by Cournoyer et al. (6), indicated a division between *P. syringae* pv. *syringae* strain Pss61 and *P. syringae* pv. *pisi* strains. Furthermore, two lineages were described within *P. syringae* pv. *pisi*, based on *hrpL* sequence data analysis, indicating that intrapathovar polymorphism can be observed at this locus. However, a BLAST search in nucleotide data banks showed that there were few differences between pathovars; the sequence of *P. syringae* pv. *papulans* strain CFBP3323 differed from that of *P. syringae* pv. *pisi* by only 14 or 15 nucleotides out of 584 (97% identity).

Furthermore, our data indicate that *P. syringae* pv. *papulans* forms a uniform group, as all tested strains displayed exactly the

Fig. 4. Sensitivity of a polymerase chain reaction assay for detection of *Pseudomonas syringae* pv. *papulans* in apple leaf homogenate using primer Pap1/Pap2. Bacteria were added to healthy apple leaf extract and diluted in water to 1×10^{-6} . Lanes 1 to 6: dilutions of *P. syringae* cells ranging from 6×10^5 to 6 CFU ml⁻¹. Lane T-: healthy leaf sample. Lane L: DNA size markers (100-bp ladder, Life Technologies).

Fig. 5. Three representative results (R1, R2, and R3) of a polymerase chain reaction assay for direct detection of *Pseudomonas syringae* pv. *papulans* from blister spot lesions on apple fruit. Lane 0: undiluted crude sample. Lane 1: 10-fold diluted crude sample. Lanes 2 and 3: DNA purified from the crude and diluted samples, respectively. Lane T-: healthy fruit samples. Lane L: DNA size markers (100-bp ladder, Life Technologies).

same RFLP pattern. This result led us to design oligonucleotides specific for the *hrpL* gene as a diagnostic tool for *P. syringae* pv. *papulans*. The *hrp* region has already been used to design primers specific to plant-pathogenic bacteria, especially *Xanthomonas* (14). Three primers (Pap1, Pap2, and Pap3) were designed for *P. syringae* pv. *papulans*. Pap1 was designed as a forward primer and tested with Pap2 and Pap3 as reverse primers. Although Pap3 was designed with the discriminating *BspI43I*₍₃₆₀₎ site located at the 3' end, the primer set Pap1/Pap3 amplified all tested strains belonging to genomospecies 1 (Table 1). However, the primer set Pap1/Pap2 specifically amplified all strains of *P. syringae* pv. *papulans*. Pap1/Pap2 was designed after sequence alignment analysis. The Pap1/Pap2 primer set is a good tool for the identification of *P. syringae* pv. *papulans*, as it detected all strains of this pathovar, whatever the phenotypic polymorphism detected in these strains by biochemical and serological tests (12).

Hence, we developed two primer sets that allowed us to identify either *P. syringae* pv. *papulans* (Pap1/Pap2) or all *P. syringae* pathovars belonging to genomospecies 1 (Pap1/Pap3). Genomospecies 1 contains *P. syringae* pv. *syringae*, which groups pathogenic and nonpathogenic strains common on numerous plants, including apple. These two sets of primers represent good tools for monitoring the occurrence of these two *P. syringae* pathovars associated with apple. The specific Pap1/Pap2 primer set could be used for further epidemiological studies.

In Europe, apple blister canker has been observed in Italy and, recently, in Germany (21). Apple blister damage has been reported mainly on apple cv. Mutsu in North America and in Italy. However, other cultivars, such as Fuji, Golden Delicious, and Gala, are susceptible when young leaves or fruit is inoculated with *P. syringae* pv. *papulans* (12). In Germany, severe damage to cv. Delbarestivale was reported in several orchards in the Stuttgart area (21). The identity of the pathogen as strains of *P. syringae* pv. *papulans* was confirmed by PCR using the Pap1/Pap2 primer set (data not shown). The host range of *P. syringae* pv. *papulans* appears to be larger than the single cv. Mutsu, even under field conditions.

Enrichment procedures (26) have been developed to purify bacterial cells prior to PCR. In this study, pre-enrichment steps were not examined because one of our objectives was rapid detection, and pre-enrichment can be time consuming and labor-intensive. The PCR limit of detection of *P. syringae* pv. *papulans* was approximately 6×10^2 CFU ml⁻¹. Similar detection limits have been described for the identification of *P. syringae* pv. *pisi* (1), *P. corrugata* (5), and *Xanthomonas fragariae* (22,23).

In conclusion, a reliable and sensitive PCR assay has been developed for the detection of *P. syringae* pv. *papulans* on diseased fruit and leaves. It is a potentially useful tool for epidemiological studies of blister spot of apple.

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