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Fast molecular detection of *Pseudomonas syringae* pv. *aesculi* in diseased horse chestnut trees

By O. Schmidt[1,3], U. Moreth[1], D. Dujesiefken[2], H. Stobbe[2] and O. Gaiser[2]

[1]Department of Wood Biology, University of Hamburg, Leuschnerstr. 91, 21031 Hamburg, Germany;
[2]Institute of Arboriculture, Brookkehre 60, 21029 Hamburg, Germany;
[3]E-mail: o.schmidt@holz.uni-hamburg.de (for correspondence)

Summary

A molecular technique was used to detect the bacterium *Pseudomonas syringae* pv. *aesculi* in horse chestnut trees (*Aesculus hippocastanum*), affected by the recently recognized European '*Pseudomonas* horse chestnut bark disease'. The technique helped identify the pathogen within 6 h of sample preparation including DNA extraction, polymerase chain reaction (PCR) and electrophoresis until gel documentation. PCR primer pairs derived from the gyrase B gene sequence were used. Because of the great similarity in the gyrase B gene sequences of the numerous closely related *P. syringae* pathovars, the primers were not only totally specific to the pathovar *aesculi*, but also detected a few other pathovars. The assumption that other bacteria should not occur at least near to a necrotic lesion of a horse chestnut tree was corroborated by sequence identity of the PCR products obtained with the gyrase B gene sequence of *P. syringae* pv. *aesculi*. Koch's postulates were fulfilled for an isolate of *P. syringae* pv. *aesculi* obtained from a diseased horse chestnut tree sampled in Hamburg in 2007.

1 Introduction

From 2002, stem bleeding on horse chestnut trees (*Aesculus hippocastanum* L.) caused by the bacterium *Pseudomonas syringae* pv. *aesculi* has become severe in several parts of Europe, especially in the Netherlands (Dujesiefken et al. 2008). Initial assumptions that a *Phytophthora* species was the primary causal agent were refuted when the bacterium *P. syringae* was proven to be associated with the disease (Dijkshoorn-Dekker 2005) and the pathovar *aesculi* described in the UK (Webber et al. 2006). The first report of the bacterium in Germany (Schmidt et al. 2008) was based on traditional microbiological isolation and subsequent molecular identification. DNA sequencing of the gyrase B gene to identify the pathovar, however, is time-consuming. Here, we report a rapid molecular technique, which identified the pathovar from a tree sample within 6 h of starting laboratory work.

2 Material and methods

After pilot tests, samples were taken from 12 *A. hippocastanum* growing at different geographical locations in Germany (Table 1). Tree no. 4 was planted in February 2008 in Reinbek near Hamburg and artificially infected in April 2008 to verify Koch's postulates. The other sampled hosts were trees growing in urban areas. All trees, including the artificially infected one, showed typical symptoms of horse chestnut bleeding cankers.

Samples were taken in November 2007 to October 2008 by cutting horizontally through the bark using a saw, into the outer xylem above and below a bleeding canker. Samples were carefully removed from the tree with a chisel on both vertical sides of the necrosis to

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Table 1. **Aesculus *hippocastanum* investigated for the presence of *Pseudomonas syringae* pv. *aesculi* using PCR primers.**

Tree no.	Origin	German state	Age (years)	Sampling	Primer G3/G4	Primer G5/G6
1	Mülheim	North-Rhine Westphalia	50–60	November 2007	+	+
2	Stuttgart	Baden-Württemberg	40	November 2007	+	+
3	Flensburg	North Schleswig-Holstein	30	January 2008	+	+
4	Reinbek	South Schleswig-Holstein	5	July 2008	+	nt*
5	Hamburg	Hamburg	15	July 2008	+	nt
6	Leer	West Lower Saxony	20	July 2008	+	nt
7	Flensburg	North Schleswig-Holstein	100	September 2008	+	nt
8	Flensburg	North Schleswig-Holstein	100	September 2008	+	nt
9	Flensburg	North Schleswig-Holstein	100	September 2008	+	nt
10	Flensburg	North Schleswig-Holstein	100	September 2008	+	+
11	Glückstadt	South Schleswig-Holstein	80–100	October 2008	+	+
12	Glückstadt	South Schleswig-Holstein	80–100	October 2008	+	+

*nt = not tested.

include the whole bark and approximately 1 cm depth of sapwood. Asepsis was unnecessary because of the subsequent use of specific polymerase chain reaction (PCR) primers. However, samples were either immediately processed or wrapped and frozen in aluminium foil at −18°C until use. Repeated freezing and thawing had an adverse effect on subsequent DNA extraction.

Sample preparation (10 min) for bacterial DNA extraction was as follows: sawdust was produced from the sample with an autoclaved metallic rasp (E. Magel, pers. comm.), which is normally used to remove horny skin. About 70 mg wet weight of sawdust was sampled and collected directly onto an aluminium foil. Extraction and purification of bacterial DNA from sawdust (cf. Table 1; 90 min) followed the instructions of the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Bacterial cells (cf. Table 2) were directly transferred to the PCR mix, as described by Schmidt et al. (2008).

Polymerase chain reaction (3 h) was performed with the Qiagen Tag Core Kit. PCR primers G3 and G4 were designed from the gyrase B gene sequence of *P. syringae* pv. *aesculi* (EMBL: AM886133; Schmidt et al. 2008). Prior to primer design, available gyrase B gene sequences were aligned with ClustalX to detect DNA regions of preference for the pathovar *aesculi*. The G3 forward primer is 5′-CGTCGGTGAAAGTGATACCAC-3′ and G4 reverse primer is 5′-CAGCGCAATCTCGACACCAAT-3′. Primers enclose a DNA region of 256 bp. The total length of the PCR product should be 298 bp. Because of critical remarks by a reviewer with regard to specificity, a second pair was designed which is located a little inside G3/G4, namely G5 (5′-ATACCACCGGTACTCAGATCC-3′) and G6 (5′-AATGCCGTCGTCGCGTTGAAT-3′). These primers enclose 224 bp. Several related *Pseudomonas* bacteria from the German Collection of Microorganisms and Cell Cultures (DSMZ) were tested with the primers, along with nine bacterial isolations 'epiphytic' on horse chestnut trees 7 to 12, and some unrelated bacteria from the laboratory collection (Table 2).

Polymerase chain reaction in the PTC-100 thermocycler (MJ Research, Watertown, MA, USA) comprised 35 cycles of 30 s at 94°C, 30 s at 59°C and 30 s at 72°C, with an initial denaturation of 4 min at 94°C (98°C for bacterial cells) and a final elongation of 7 min at 72°C. After agarose gel electrophoresis (30 min) in the submarine electrophoresis system Mupid-exU (Advance, Tokyo, Japan), PCR products were visualized with GelStar Nucleic Acid Gel Stain (Cambrex, Rockland, ME, USA) over UV (30 min.). PCR products were sequenced by Eurofins MWG Operon (Martinsried, Germany).

Table 2. **Bacteria tested for specificity with *Pseudomonas syringae* pv. *aesculi* primers (order according to gel in Fig. 2).**

Species/pathovar	Origin/isolate	Reaction with primers		Lane no. at the gel
		G3/G4	G5/G6	
Pseudomonas amygdali	DSM 6759	+	+[1]	1
P. syringae pv. *aptata*	DSM 50252	+	–	2
P. syringae pv. *morsprunorum*	DSM 50277	+	+[2]	3
P. syringae pv. *phaseolicola*	DSM 50287	–	–	4
P. syringae pv. *tomato*	DSM 50315	–	–	5
Bacillus mycoides	Laboratory collection/B4	–	–	6
Bacillus megaterium	Laboratory collection/B7	–	–	7
Erwinia carotovora	Laboratory collection/B14	–	–	8
Cellulomonas flavigena	Laboratory collection/DSM 20109	–	–	9
Pseudomonas fluorescens	Laboratory collection/DSM 50108	–	–	10
Pseudomonas fragi	Laboratory collection/Ilomba wood	–	–	11
Bacillus sp.	Laboratory collection/oak wood	–	–	12
Aureobacterium luteolum	Laboratory collection/ponded wood	–	–	13
Unknown	Epiphyte	–	–	14
Unknown	Epiphyte	–	–	15
Unknown	Epiphyte	–	–	16
Unknown	Epiphyte	–	–	17
Unknown	Epiphyte	–	–	18
Unknown	Epiphyte	–	–	19
Unknown	Epiphyte	–	–	20
Bacillus mycoides	Epiphyte	–	–	21
Unknown	Epiphyte	–	–	22
P. syringae pv. *aesculi*	Control isolate 3[3]	+	+	23
–	PCR control without DNA	–	–	24

Sequence deposition in EMBL: [1]FM 955596; [2]FM 955597.
[3]Isolate from a horse chestnut tree in Hamburg (Schmidt et al. 2008).

3 Results and discussion

Brenner et al. (2005) listed 37 pathovars of *P. syringae* (pv. *aesculi* was not listed), stating that 'many of the pathovars may not be distinguishable from *P. syringae* except for their host range' and that 'few phenotypic characters are taxonomically useful'. Gardan et al. (1999) studied 48 pathovars of *P. syringae* and quoted Bradbury (1986) that 'it is impossible to identify correctly each of the pathovars by means of routine biochemical tests'. Thus, Schmidt et al. (2008) reported the detection of the pathovar *aesculi* by sequencing the gyrase B gene and subsequent identification by BLAST comparison with deposited sequences. However, DNA sequencing takes several days. For quicker routine diagnosis of the bacterium, 'species-specific PCR' was adopted, which works well with several species of wood-decay fungi (Moreth and Schmidt 2000).

All *A. hippocastanum*, which were macroscopically assumed to be infected by *P. syringae* pv. *aesculi* (cf. Table 1), showed the presence of the bacterium using specific PCR primers. Figure 1 shows an electrophoresis gel with the PCR products obtained with primers G3 and G4 from trees 4 to 6. In most tests, two elutions of the DNA extraction procedure were used to obtain a suitable template concentration for PCR. Both elutions provided

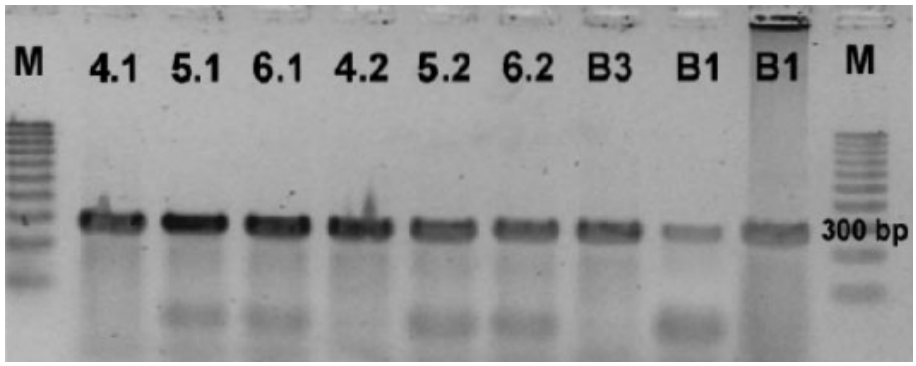

Fig. 1. Gel with DNA bands of the partial gyrase B gene from *Pseudomonas syringae* pv. *aesculi* obtained with primers G3 and G4. Lanes 4 to 6 = tree number (cf. Table 1). Lanes 1 and 2 = first and second DNA elution. B1 = unpurified bacteria outgrown from sawdust of tree 4 using different template amounts. B3 = pure culture of *P. syringae* pv. *aesculi* from the isolation in 2007. M = Marker.

sufficient template DNA in the gel shown in Fig. 1. Lane B3 was derived from isolate 3 of *P. syringae* pv. *aesculi*, which was obtained earlier from a diseased tree in Hamburg (Schmidt et al. 2008). Lanes labelled 'B1' show the PCR products from an unpurified bacterial colony, cultured from the sawdust of tree 4. The size of all DNAs was approximately 300 bp, as expected by primer design. It may be assumed, therefore, that the DNA bands on the gel represent the partial gyrase B gene sequence of *P. syringae* pv. *aesculi*, which was confirmed by sequencing the PCR products.

As a result of great similarities of the gyrase B gene sequences between pathovars of *P. syringae*, however, the G3 and G4 primers were not specific to the pathovar *aesculi*. BLAST of the combined sequence of these primers suggested that pv. *morsprunorum* was also detected and, with few nucleotide divergences, further pathovars (*tomato*, *myricae*, *aptata*, *eriobotryae*) and species (*P. amygdali*, *P. tremae*).

To increase primer specificity, a further primer pair (G5 and G6) was used for some of the infected trees (Table 1). BLAST of the combined G5/G6 sequence showed that these primers detected only pathovars *aesculi* and *morsprunorum*, as well as to two depositions of *P. syringae*, sequences of which were identical to the *morsprunorum* sequence. Among the nine discrete genomospecies of *P. syringae* delineated by Gardan et al. (1999), pathovars *aesculi*, *morsprunorum*, *eriobotryae*, *myricae* and *P. amygdali* belong to genomospecies 2, pathovar *aptata* to genomospecies 1, pv. *tomato* to genomospecies 3, and pv. *tremae* to genomospecies 5.

Both primer pairs were also tested against some related and unrelated bacteria. Not all pathovars related to *P. syringae* pv. *aesculi* (Gardan et al. 1999) were available for this test. Likewise, the DNA databases do not contain the gyrase B gene sequence of all described *P. syringae* pathovars, which would be suitable for a theoretical specificity test by BLAST.

Figure 2 shows the gel with the PCR products from the 23 bacteria listed in Table 2. Primer pair G3/G4 (Fig. 2, top) responded to the control *P. syringae* pv. *aesculi* (lane 23) and to the pathovars *aptata* (lane 2) and *morsprunorum* (lane 3), as expected by BLAST of the primers. Primer pair G5/G6 (Fig. 2, bottom) detected *P. syringae* pv. *aesculi* (lane 23) and pv. *morsprunorum* (lane 3). With regard to the other tested species, only *P. amygdali* (lanes 1) responded to both primer pairs (cf. Table 2). BLAST of the sequence of strain DSM 6759, however, showed 99% homology to depositions of *P. syringae* pv. *morsprunorum* and only 96% to *P. amygdali*. There was no reaction to the other tested pathovars of *P. syringae* (lanes 4 and 5), as not to the bacteria from the laboratory collection

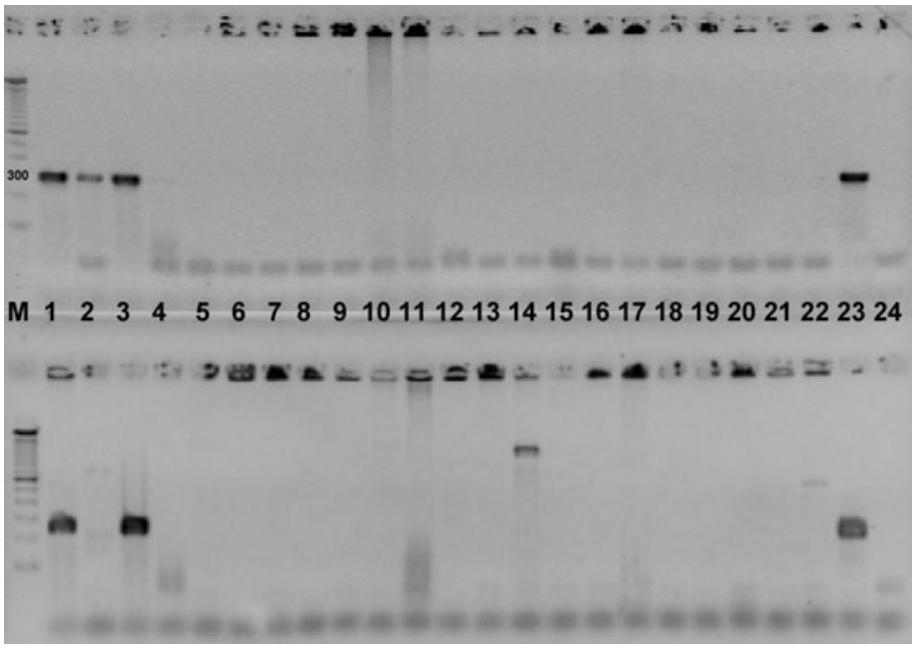

Fig. 2. Gels with DNA bands of the partial gyrase B gene from several bacteria. Lane numbers according to Table 2. PCR products were obtained with primers G3 and G4 (top) and primers G5 and G6 (bottom). M = Marker (bp).

(lanes 6 to 13), nor to bacteria isolated from samples located around necroses ('epiphytes', lanes 14 to 22) of six horse chestnut trees. The band at about 1350 bp in lane 14 is an obvious error caused by a laboratory contamination.

The reported specific host range of *P. syringae* pathovars (Brenner et al. 2005) led to the hypothesis that other pathovars of *P. syringae* and other *Pseudomonas* species with a similar gyrase B sequence should not occur close to a necrotic lesion on *A. hippocastanum*. This hypothesis was verified by sequencing the PCR products. All sequences, including that from the unpurified bacterial colony in Fig. 1, showed 99% to 100% homology to the deposited sequence of *P. syringae* pv. *aesculi*.

Figure 1 (lanes 4.1, 4.2 and B1) also shows that Koch's postulates were fulfilled for the isolate of *P. syringae* pv. *aesculi* from the diseased horse chestnut tree sampled in Hamburg in 2007 (Schmidt et al. 2008): the pure culture isolated from the diseased tree caused disease when introduced into a healthy tree, was re-isolated from the inoculated diseased experimental tree and identified as identical to the original specific causative agent. The same was proven for the British isolate of *P. syringae* pv. *aesculi* (Webber et al. 2006).

In summary, the specific primers can be used in routine investigations of diseased *A. hippocastanum* in tests for *P. syringae* pv. *aesculi*. The results also showed that the method may be used in investigations to detect *P. syringae* pathovars *aptata* and *morsprunorum* in their specific host plants. In principle, each pathovar of *P. syringae* should be detectable using suitable primers. A totally specific primer pair for the pathovar *aesculi* could not be designed from the partial sequence of the gyrase B gene of this bacterium. Ongoing experiments may focus on other parts of the genome.

Acknowledgements

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