THE APPLICATION OF POLYMERASE CHAIN REACTION FOR CHARACTERISING STRAINS OF *PSEUDOMONAS SYRINGAE* ISOLATED FROM NEW ZEALAND RIVERS

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ABSTRACT

Pseudomonas syringae is a complex group of bacteria, which comprises nine different genomospecies and over 50 pathovars. Strains of *P. syringae* have been isolated from some rivers and lakes in New Zealand. To determine whether these waterways act as reservoirs of plant pathogenic bacteria, 15 strains of *P. syringae* isolated from the Waikato River and Whakapapanui stream have been further characterised using several polymerase chain reaction (PCR) protocols. Five of those 15 strains belong to genomospecies 1, which comprises *P. syringae* pv. *syringae*, but none belongs to genomospecies 2. The protocol for detection of *P. syringae* pv. *papulans* was modified and is now specific for this pathovar. The identity of a strain isolated from the Waikato River as being *P. syringae* pv. *atrofaciens* has yet to be confirmed. None of the 15 strains studied belongs to the pathovars *papulans*, *actinidiae*, *tagetis*, *helianthii* or *theae*.

Keywords: bacterial ecology, genomospecies, *papulans*, *actinidiae*.

INTRODUCTION

Pseudomonas syringae is an important and complex taxonomic group that includes all fluorescent pseudomonad strains that are oxidase negative, arginine dihydrolase negative and induce a hypersensitive reaction when injected into tobacco plants (Palleroni 1984). *Pseudomonas syringae* includes many economically significant plant-pathogenic bacteria. A large number of plant species can be infected by strains of *P. syringae*, but every strain of *P. syringae* shows some host specificity and induces disease only in some plant species. To take into account the host specificity of the different strains of *P. syringae*, the word pathovar (pv.) has been coined. There are about 50 pathovars described for *P. syringae*. Based on DNA homology *P. syringae* is actually composed of at least nine different species, called "genomospecies" (Gardan et al. 1999; Marques et al. 2008). Since strains from different genomospecies cannot be distinguished phenotypically, eight genomospecies have not been named and the pathovar system of nomenclature is still commonly used. Unfortunately, strains of different pathovars cannot be distinguished by biochemical or physiological characteristics alone. Identification of a strain of *P. syringae* at the pathovar level usually requires a combination of physiological, biochemical and serological tests and pathogenicity assays. Because such a characterisation is difficult and laborious, many authors have tried to develop molecular tools such as polymerase chain reaction (PCR) protocols to identify strains of *P. syringae* that belong to one pathovar or one genomospecies (e.g. Musa et al. 2001; Kerkoud et al. 2002; Koh & Nou 2002; Kong et al. 2004).

Riffaud & Morris (2002) isolated a pathogen of cantaloupe, *P. syringae* pv. *aptata*, from retention basins used for irrigation of cantaloupe. The identification of such a source of inoculum is very important for the development of control methods. This is especially true of plant pathogenic bacteria for which there are only limited control

options. *Pseudomonas syringae* has been isolated from some rivers and lakes from the North Island of New Zealand (Vanneste et al. 2008). The aim of this research was to determine whether these waterways act as a reservoir of plant pathogenic bacteria. The approach taken was to develop a suite of methods to identify the pathovar or genomospecies of the strains of *P. syringae* isolated from the Waikato River and the Whakapapanui Stream. Since pathogenicity tests on several dozens of potential host plants is not practical, PCR protocols developed for specific identification of some genomospecies or pathovars were used.

MATERIALS AND METHODS

Bacterial strains

Ten strains isolated from the Whakapapanui Stream (Tongariro National Park) and five strains isolated from the Waikato River (Hamilton), described previously (Vanneste et al. 2008) as strains of *P. syringae* (based on their ability to produce a fluorescent pigment on a modified King's B medium (KBC; Mohan & Schaad 1987) and cause a hypersensitive reaction when infiltrated into a tobacco plant, the absence of a cytochrome c oxidase and their inability to utilise arginine) were used in this study.

The other strains of *P. syringae* used in this study were obtained from the International Collection of Microorganisms from Plants (ICMP) held by Landcare Research New Zealand. They were used as positive or negative controls in the different PCR protocols and are presented in Table 1. They were maintained on King's B medium (King et al. 1954) at 28°C. Strains of other genera were also included in some of the experiments as negative controls: *Erwinia amylovora* ICMP 8865, *Pantoea agglomerans* P10c (Vanneste et al. 2002), *Pseudomonas fluorescens* A506 (Lindow et al. 1996) and *Xanthomonas campestris* pv. *pruni* 2.38 (J.L. Vanneste, laboratory collection).

TABLE 1: Strains of *Pseudomonas syringae* used as positive or negative controls in the polymerase chain reactions.

Species or pathovar	Strains	Host plant	Year of isolation	Geographical origin
<i>P. syringae</i> pv. <i>syringae</i>	ICMP 3523	<i>Lycopersicum esculentum</i>	1970	Australia
<i>P. s.</i> pv. <i>aptata</i>	ICMP 459	<i>Beta vulgaris</i>	1959	USA
<i>P. s.</i> pv. <i>lapsa</i>	ICMP 3947	<i>Zea sp.</i>	Not known	Not known
<i>P. s.</i> pv. <i>papulans</i>	ICMP 3881	Not known	Not known	USA
<i>P. s.</i> pv. <i>papulans</i>	ICMP 4044	<i>Malus domestica</i>	1968	Canada
<i>P. s.</i> pv. <i>pisi</i>	ICMP 2452	<i>Pisum sativum</i>	1969	New Zealand
<i>P. s.</i> pv. <i>atrofaciens</i>	ICMP 4394	<i>Triticum aestivum</i>	1968	New Zealand
<i>P. s.</i> pv. <i>panici</i>	ICMP 3955	Not known	Not known	Not known
<i>P. s.</i> pv. <i>japonica</i>	ICMP 6305	<i>Hordeum vulgare</i>	1951	Japan
<i>P. s.</i> pv. <i>helianthi</i>	ICMP 3549	<i>Helianthus annuus</i>	1969	New Zealand
<i>P. s.</i> pv. <i>tagetis</i>	ICMP 4091	<i>Tagetes erecta</i>	1972	Zimbabwe
<i>P. s.</i> pv. <i>theae</i>	ICMP 3923	<i>Camellia sinensis</i>	1970	Japan
<i>P. s.</i> pv. <i>theae</i>	ICMP 3934	<i>Camellia sinensis</i>	Not known	Japan
<i>P. s.</i> pv. <i>actinidiae</i>	ICMP 9617	<i>Actinidia deliciosa</i>	1984	Japan

Characterisation of the bacteria by Polymerase Chain Reaction (PCR)

All bacterial strains were characterised by PCR using about 20 ng of total DNA per reaction. Total DNA was isolated with a cell disruptor FastPrep®-24 from MP™ using the ZR fungal/bacterial DNA kit™ from Zymo Research, USA. PCRs were performed

on an Eppendorf Mastercycler® Gradient. The PCRs were carried out in 30 µl containing 1 µl of the DNA of the strain of interest, 2 µl of primers (1 µl of each primer, corresponding to 10 µM), 2 µl of dNTP mix (2.5 mM each) (supplied with the DNA polymerase), 3 µl of 10× PCR buffer supplied with the DNA polymerase, 21.8 µl of sterilised distilled water and 0.2 µl (corresponding to 1 unit) of *i-Taq*™ from INtRON Biotechnology, Inc. A negative control, in which the DNA solution was replaced by the same volume of water, and a positive control, in which the DNA was that of a strain that gave a positive reaction, were used for each experiment.

The PCR products were separated by horizontal gel electrophoresis using a Biokey Super Screener 120 Electrophoresis system (Innovation Sciences Limited, Dunedin, New Zealand) on 1% or 2% agarose (depending on the size of the PCR product) containing 100 ng/ml of ethidium bromide. From each reaction, 15 to 30 µl were loaded on the agarose gel and the DNA bands were visualised under UV light. On each gel, a DNA ladder (1 kb Plus DNA Ladder or 100 bp DNA Ladder from Invitrogen™) was used. The primers used in this study are presented in Table 2. Unless stated otherwise, the PCR programmes used to determine whether a strain belonged to genomospecies 1 or 2 or to pathovar *papulans* were those described by Kerkoud et al. (2002). The programmes used to determine whether a strain belong to the pathovar *aptata*, the pathovar *actinidiae* or the pathovars *helianthii* or *tagetis* were those described by Musa et al. (2001), Koh & Nou (2002) and Kong et al. (2004), respectively.

TABLE 2: Name, size of DNA product in base pairs (bp), specificity and reference of the Polymerase Chain Reaction primers used in this study.

Name	Size of product (bp)	Specificity	Reference
hrpL1 and hrpL2	631	genomospecies 1 and 2	Kerkoud et al. 2002
Pap1 and Pap2	242	<i>P. syringae</i> pv. <i>papulans</i>	Kerkoud et al. 2002
hrpZf1 and hrpZr2	900	<i>P. syringae</i> pv. <i>aptata</i>	Musa et al. 2001
TAGTOX-9FP1 and TAGTOX-9FR1	507	<i>P. syringae</i> pv. <i>tagetis</i> and pv. <i>helianthii</i>	Kong et al. 2004
TAGTOX-10FP1 and TAGTOX-10FR1	733	<i>P. syringae</i> pv. <i>tagetis</i> and pv. <i>helianthii</i>	Kong et al. 2004
Forward primer and Reverse primer	492	<i>P. syringae</i> pv. <i>actinidiae</i>	Koh & Nou 2002
JLVLF3 and JLVLR8	280	Genomospecies 2	J.L. Vanneste, unpublished data

RESULTS AND DISCUSSION

None of the non *P. syringae* strains gave a positive reaction with any of the protocols (set of primers and corresponding PCR programme) used or developed in this study. Of the 15 strains analysed, four strains isolated from the Waikato River and one strain isolated from Whakapapanui stream gave a positive reaction with the primers hrpL1 and hrpL2, which are specific for strains of genomospecies 1 and 2. No PCR product was detected when the total DNA of those five strains was used in a PCR experiment using the primers JLVLF3 and JLVLR8, which lead to a 280 bp DNA band from all and only the strains belonging to genomospecies 2. Therefore, those five strains most probably belong to genomospecies 1, which is represented by *P. syringae* pv. *syringae*. However, they could belong to any of the pathovars that constitute genomospecies 1, which include the pathovars *syringae*, *aptata*, *lapsa*, *papulans*, *pisi*, *atrofaciens*, *aceris*, *dysoxyli*, *panici* or *japonica*. This also indicates that no strains of genomospecies 2, which includes the pathovars *phaseolicola*, *morsprunorum*, *erobotryae*, *tabaci*, *lachrymans*, *sesami*, *ulmi*, *mori*, *lachrymans*, *cerasicola* and *glycinea* were present in this sample. However, this

was a limited sample of strains, and those strains were isolated using the medium KBC, which might not allow strains of all pathovars to grow equally well (Morris et al. 2008; Vanneste et al. 2008).

When the protocol described by Musa et al. (2001) to identify *P. syringae* pv. *aptata* was used, in addition to *P. syringae* pv. *aptata* ICMP 459, strains of *P. syringae* pv. *pisi* ICMP 2452, *atrofaciens* ICMP 4394, *panici* ICMP 3955 and *japonica* ICMP 6305 also gave a PCR product of ca 900 bp. One of the strains isolated from the Waikato River gave a positive reaction with this PCR protocol. However, when leaves of silver beet, which is one of the host plant of *P. syringae* pv. *aptata* (Koike et al. 2003), were inoculated with this strain, no symptoms were observed after 10 days incubation at room temperature (data not shown). This suggests that this strain is not a strain of *P. syringae* pv. *aptata*. This will be confirmed with further pathogenicity tests on other potential host plants and by multilocus sequencing typing (MLST). Sarkar & Guttman (2004) have shown that analysis of the DNA sequence of seven housekeeping genes using MLST represents another powerful tool for the characterisation of strains of *P. syringae*.

Another of the strains isolated from the Waikato River, and previously identified as belonging to genomospecies 1, was positive when using the protocol described as specific for *P. syringae* pv. *papulans* (Kerkoud et al. 2002; Vanneste & Yu 2006). However, using a range of strains of different pathovars, *P. syringae* pv. *atrofaciens* ICMP 4394 also gave a positive reaction with this protocol. By increasing the annealing temperature from 64°C to 75°C, only the strains of *P. syringae* pv. *papulans* gave a DNA product of the expected size (242 bp). To make sure that this modified protocol was specific for all strains of *P. syringae* pv. *papulans*, it was tested with the 17 strains of this pathovar available in New Zealand. All of them gave a positive result (data not shown). None of the other strains tested including the strain from the Waikato river and *P. syringae* pv. *atrofaciens* ICMP 4394 gave a positive reaction with this modified protocol. Therefore, this Waikato river strain is not a strain of *P. syringae* pv. *papulans*. To establish whether this strain belongs to the pathovar *atrofaciens*, it will be necessary to carry out a pathogenicity assay on wheat plants, the host plant of *P. syringae* pv. *atrofaciens*.

None of the strains isolated reacted positively with the protocol specific for *P. syringae* pv. *actinidiae* (Koh & Nou 2002). Using this same protocol, a positive reaction, i.e. a 220 bp band of DNA, was detected when the total DNA of the two strains of *P. syringae* pv. *theae* (ICMP 3923, ICMP 3934) was used. The pathovars *theae* and *actinidiae* have initially been put into genomospecies 8 and 3, respectively. However, they might belong to the same genomospecies, which would be consistent with strains of those two pathovars giving a positive result with the protocol developed by Koh & Nou (2002) and with those strains showing a very high level of similarity at the DNA level for the genes *hrpA*, *hrpB*, *hrpS* and *hrpZ* (Inoue & Takikawa 2006) and for the genes *hrpL*, 23S rDNA and for the ITS sequences (data not shown). If the protocol for detection of *P. syringae* pv. *actinidiae* also detects strains of *P. syringae* pv. *theae*, then none of the strains isolated from either the Waikato River or the Whakapapanui Stream were *P. syringae* pv. *actinidiae* or *P. syringae* pv. *theae*. None of the strains tested gave a positive response when PCR protocols developed by Kong et al. (2004) specific for *P. syringae* pv. *tagetis*, and *P. syringae* pv. *helianthi* were used. While strains of *P. syringae* pv. *helianthi* have been isolated in New Zealand, *P. syringae* pv. *tagetis* is listed by MAF Biosecurity New Zealand as an unwanted organism. It is therefore encouraging that no such strain was detected in the present sample.

This study confirmed that strains of plant pathogenic bacteria similar to those found on cultivated plants (strains of genomospecies 1) are present in New Zealand waterways. The research has also demonstrated that none of the strains tested were *P. syringae* pv. *papulans*, *P. syringae* pv. *actinidiae* or *P. syringae* pv. *tagetis*, which are listed as unwanted organisms by MAF Biosecurity New Zealand. A close relationship at the DNA level between strains of *P. syringae* pv. *actinidiae* and *P. syringae* pv. *theae* is supported by the results obtained in the present study. It was also shown that some of the PCR protocols published as pathovar specific were actually not specific. An important outcome of the

present study is that one of these protocols has been slightly modified such that it is now specific for the detection of *P. syringae* pv. *papulans*, an organism labelled as unwanted by MAF Biosecurity New Zealand.

In conclusion, of the 15 strains isolated from the Waikato River and Whakapapanui stream, none were pv. *papulans*, *actinidiae*, *tagetis*, *helianthii* or *theae*, but five strains were identified as being from genomospecies 1. Further characterisation of one of these strains suggests it may be pv. *atrofaciens*, but additional investigations are needed to confirm this. Some strains could not be characterised due to the limited number of protocols available and by the specificity of these protocols. A further analysis of these strains using different tools such as MLST might provide more information about their identity. However, strains of *P. syringae* that do not belong to any of the pathovars already described are found regularly (Gilbert et al. 2009), and it would not be surprising if such strains were present in the Waikato River and Whakapapanui stream samples. The identification at the pathovar level of strains present in waterways in New Zealand will help to better understand the link between *P. syringae* and the water cycle, as initially proposed by Morris et al. (2007), and might help to develop control strategies for some of these pathogens.

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