Molecular characterisations of *Pseudomonas syringae* pv. *actinidiae* strains isolated from the recent outbreak of bacterial canker on kiwifruit in Italy

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Abstract *Pseudomonas syringae* pv. *actinidiae* (Psa) is the causal agent of bacterial canker on kiwifruit, a disease that affects both *Actinidia deliciosa* and *A. chinensis*. This disease has been reported in Japan, Korea and Italy. It has never been found in New Zealand. An outbreak of bacterial canker on kiwifruit has been recently reported in Latina, Italy. Strains isolated from this outbreak were compared with strains isolated from Japan, Korea and strains isolated from Italy in 1992. The specificity of the primers PsaF1/R2 and their usefulness in identifying strains of Psa were confirmed in this study. Fingerprinting by BOX-PCR allowed the distinction of strains of Psa tested from strains of the closely related pathovar *theae*. However, two different profiles were obtained for strains of Psa. Two haplotypes for the housekeeping gene *cts* (*gltA*) were also found among strains of Psa. Such variability might be used for further epidemiological studies.

Keywords *cts* gene, haplotype, BOX-PCR.

INTRODUCTION

Bacterial canker of kiwifruit, caused by the bacterium *Pseudomonas syringae* pv. *actinidiae* (Psa), is a disease that affects different species of *Actinidia* including *A. deliciosa* and *A. chinensis*. This pathogen was first described in Japan in 1984 (Serizawa et al. 1989). It was subsequently isolated in Korea (Koh & Lee 1992) and in Italy (Scortichini 1994). In spring, symptoms include production of drops of white exudate from infected tissues. More conspicuous are the cankers on the canes, leaders or trunks of infected vines, which can produce huge amounts of a red exudate. Later in the season, buds on infected canes will not develop or, if they do develop, the young fruits soon shrivel and the leaves wither before the cane dies. During the

growing season, small necrotic angular patches surrounded by a yellow halo develop on the leaves. The pathogen seems to overwinter in cankers and as an endophyte in infected tissues.

A recent outbreak of bacterial canker on kiwifruit occurred in the Latina region in Italy (Balestra et al. 2009; Ferrante & Scortichini 2009). This study sought to characterise strains of Psa isolated from Italy by BOX fingerprinting and by sequencing of a housekeeping gene. In the process, the specificity of the primers PsaF1/R2 (Rees-George et al. 2010) and their usefulness in identifying strains of Psa were confirmed. It was also confirmed that this pathogen has been present in Italy since 1992 and that it is not present in New Zealand.

MATERIALS AND METHODS

Strains and media

All the strains were maintained on King's B medium (King et al. 1954) at 28°C. The strains used in this study, including those isolated during the course of the study, are presented in Table 1 and Table 2. Isolations were made by streaking a drop of exudates collected from infected vines on plates of King's B medium, or by teasing out infected tissues in sterile water and streaking the resulting suspension on plates of King's B medium. The strains were purified twice at 28°C on King's B medium before being characterised. All the strains with an ICMP number were

obtained from the International Collection of Microorganisms from Plants (ICMP) held by Landcare Research, New Zealand.

Characterisation of strains of *Pseudomonas* isolated from kiwifruit

Strains of *Psa* share some characteristics that set them apart from strains of other *Pseudomonas* species and strains of other *P. syringae* pathovars. In particular, they are non fluorescent on King's B medium, they induce a hypersensitive reaction when infiltrated in tobacco plants, do not have a

Table 1 List of strains other than *Pseudomonas syringae* pv. *actinidiae* used in this study.

Species	Strain number	Host plant	Country of origin
<i>Pseudomonas fluorescens</i>	ICMP 3953	Freesia	Netherlands
<i>P. fluorescens</i>	A506	Pear	USA
<i>P. s. pv. aceris</i>	ICMP 2802	Acer	USA
<i>P. s. pv. aptata</i>	ICMP 459	<i>Beta vulgaris</i>	USA
<i>P. s. pv. atrofaciens</i>	ICMP 4394	<i>Triticum aestivum</i>	New Zealand
<i>P. s. pv. berberidis</i>	ICMP 4116	<i>Berberis</i>	New Zealand
<i>P. s. pv. cannabina</i>	ICMP 2823	<i>Cannabis sativa</i>	Hungary
<i>P. s. pv. coronafaciens</i>	ICMP 3113	<i>Avena sativa</i>	UK
<i>P. s. pv. eriobotryae</i>	ICMP 4455	<i>Eriobotrya japonica</i>	USA
<i>P. s. pv. garcae</i>	ICMP 4323	<i>Coffea arabica</i>	Brazil
<i>P. s. pv. lachrymans</i>	ICMP 4464	<i>Cucumis sativus</i>	USA
<i>P. s. pv. lapsa</i>	ICMP 3947	<i>Zea L</i>	Unknown
<i>P. s. pv. morsprunorum</i>	ICMP 3016	<i>Prunus avium</i>	UK
<i>P. s. pv. papulans</i>	ICMP 4040	<i>Malus X domestica</i>	USA
<i>P. s. pv. persicae</i>	ICMP 5846	<i>Prunus persica</i>	France
<i>P. s. pv. persicae</i>	ICMP 7099	<i>Prunus persica</i>	New Zealand
<i>P. s. pv. pisi</i>	ICMP 2452	<i>Pisum sativum</i>	New Zealand
<i>P. s. pv. syringae</i>	ICMP 3523	<i>Lycopersicon esculentum</i>	Australia
<i>P. s. pv. sesami</i>	ICMP 763	Unknown	Yugoslavia
<i>P. s. pv. striafaciens</i>	ICMP 3961	<i>Avena sativa</i>	Unknown
<i>P. s. pv. tagetis</i>	ICMP 4091	<i>Tagetes erecta</i>	Zimbabwe
<i>P. s. pv. tabaci</i>	ICMP 2835	<i>Nicotiana tobacum</i>	Hungary
<i>P. s. pv. theae</i>	ICMP 3934	<i>Camellia sinensis</i>	Japan
<i>P. s. pv. theae</i>	ICMP 3923	<i>Camellia sinensis</i>	Japan
<i>P. s. pv. viburni</i>	ICMP 3963	<i>Viburnum</i>	USA

Table 2 List of strains of *Pseudomonas syringae* pv. *actinidiae* used in this study.

Strain number	Host plant	Country of origin	Year of isolation	Source
ISPAVE-B-019 ¹	<i>Actinidia deliciosa</i>	Italy	1994	M Scortichini
ISPAVE-B-020 ²	<i>A. deliciosa</i>	Italy	1994	M Scortichini
CRA-FRU 10.22	<i>A. chinensis</i>	Italy	2008	M Scortichini
CRA-FRU 2.2	<i>A. chinensis</i>	Italy	2009	M Scortichini
CRA-FRU 5.1	<i>A. deliciosa</i>	Italy	2009	M Scortichini
CRA-FRU 8.76	<i>A. deliciosa</i>	Italy	2009	M Scortichini
ICMP 9617 ³	<i>A. deliciosa</i>	Japan	1989	ICMP
ICMP 9853	<i>A. deliciosa</i>	Japan	1989	ICMP
K-Psa 2	<i>A. sp.</i>	Korea	unknown	GM Balestra
I -Psa H1	<i>A. deliciosa</i>	Italy	2008	GM Balestra
CFBP 7287	<i>A. deliciosa</i>	Italy	2008	GM Balestra
I- Psa H3	<i>A. deliciosa</i>	Italy	2008	GM Balestra
CFBP 7286	<i>A. chinensis</i>	Italy	2008	GM Balestra
I-Psa Z	<i>A. chinensis</i>	Italy	2008	GM Balestra
I-Psa 8	<i>A. chinensis</i>	Italy	2008	GM Balestra
I-Psa G	<i>A. chinensis</i>	Italy	2008	GM Balestra
CFBP 7285	<i>A. chinensis</i>	Italy	2008	GM Balestra
I-9.4.10-D1	<i>A. chinensis</i>	Italy	2010	This study
I-9.4.10-D4	<i>A. chinensis</i>	Italy	2010	This study
I-9.4.10-D3b	<i>A. chinensis</i>	Italy	2010	This study
I-9.4.10-E4	<i>A. chinensis</i>	Italy	2010	This study
I-9.4.10-E7	<i>A. chinensis</i>	Italy	2010	This study
I-9.4.10-E-Ab	<i>A. chinensis</i>	Italy	2010	This study

¹ Received as NCPPB 3871.

² Received as NCPPB 3873.

³ This is the pathotype of Psa.

cytochrome c oxidase or an arginine dehydrolase, do not hydrolyse esculin, do not rot potato and do not induce ice nucleation. The ability of a bacterial strain to induce a hypersensitive reaction when injected into tobacco plants was tested as described previously (Vanneste et al. 1990). Inoculum prepared in sterile water was infiltrated in the intercostal area of young and fully expanded leaves using an 18-gauge sterile needle.

Absence of a cytochrome c oxidase, a criterion that differentiates *P. syringae* from other species of plant pathogenic fluorescent *Pseudomonas*, was determined using Test Oxidase™ (Pro-

Lab Diagnostic, Richmond Hill, ON, Canada). For this assay, the strain of *P. fluorescens* A506 (Wilson & Lindow 1993) was used as a positive control and the strain of *P. syringae* pv. *syringae* ICMP3523 was used as a negative control.

Production of arginine dihydrolase under anaerobic conditions and the ability to hydrolyse esculin were determined as described by Lelliot et al. (1966). Inability to utilise arginine is a characteristic shared by all strains of *P. syringae* while hydrolysis of esculin is a characteristic of some pathovars only.

Ability to rot potato was carried out as follows: potato tubers were surface disinfected by immersion for 3 min in a 3% hypochlorite solution, rinsed with distilled water, sliced and placed in trays with moist paper towels. The strains to be tested were toothpicked into the flesh of the potato slice. Water was used as a negative control and a strain of *Pectobacterium* sp. isolated from kiwifruit was used as a positive control. The test was read after 48 h incubation at 28°C.

Ability to induce ice nucleation was tested after 30 sec and 1 min at -5°C as described by Lindow et al. (1978).

Polymerase chain reaction (PCR) experiments

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 50 to 100 ng of DNA of the strain of interest, 10 µM of each primer, 2.5 mM of each dNTP, 3 µl of 10x PCR buffer supplied with the DNA polymerase and 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 already identified as *Psa*, were both 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 containing 10 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 (ZR 1 kb DNA ladder from Zymo Research, USA) was used for size comparison.

To identify strains of *Psa*, the primers *Psa*F1 (5'-TTTTGCTTTGCACACCCGATTTT-3') and *Psa*R2 (5'-CACGCACCCTTCAATCAGGATG-3') were used as they yield an amplicon of 280 bp when tested on DNA from strains of *Psa* and *P.s. pv. theae* (*Pst*). The programme

published earlier (Rees-George et al. 2010) was employed, except that the number of cycles was increased from 30 to 35. For BOX-PCR fingerprinting, the BOX primer BOXA1R (5'-CTACGGCAAGGCGACCTGACG-3') was used with the BOX-PCR programme described by Louws et al. (1994).

Sequencing of the *cts* gene

The *cts* gene, also known as *gltA*, was sequenced using the primers *cts*-Fs (5'-CCCGTCGAGCTGCCAATWCTGA-3') and *cts*-Rs (5'-ATCTCGCACGSGTRTTGAACATC-3') after PCR amplification of the total DNA using the primers *cts*-Fp (5'-AGTTGATCATCGAGGGCGCWGCC-3') and *cts*-Rp (5'-TGATCGGTTTGATCTCGCACGG-3') following the protocol published by Sarkar & Guttman (2004). The sequences were compared with those of other strains of *Psa* deposited in GenBank® from the National Centre for Biotechnology Information (NCBI) (Table 3). Multiple alignments were performed with AlignX from VECTOR NTI 11 (Invitrogen).

RESULTS AND DISCUSSION

Isolation and identification of strains of *Psa* from infected material

Different bacterial species including some strains of *Pseudomonas* sp. and *Pantoea* sp. (data not shown) were isolated from *A. deliciosa* and *A. chinensis* kiwifruit vines producing a red exudate. Strains that exhibited all the characteristics of *Psa* (i.e. were non fluorescent on King's B medium, induced a hypersensitive reaction when infiltrated in tobacco plants, did not have a cytochrome c oxidase or an arginine dehydrolase, did not hydrolyse esculin, did not rot potato and did not induce ice nucleation) were also isolated. These strains were labelled *Psa* after confirmation that a 280 bp fragment could be obtained by PCR using the *Psa*F1/R2 primers. These strains could not be confused with strains of *Pst* that also yield a 280 bp amplicon with the *Psa*F1/R2 primers, because strains of *Pst* are, in contrast to strains of *Psa*, fluorescent on King's B medium (Takikawa et al. 1988). Isolation of *Psa* from red exudate was

Table 3 Accession numbers of the DNA sequences of the *cts* gene from different strains of *Psa* deposited in GenBank and used for comparison with the *cts* sequences obtained in this study.

Strain name	Year of isolation	Geographic origin	Genbank reference number
ICMP 9617	1989	Japan	FN651801.1, GU58933
NCPBP 3871 ¹	1992	Italy	FN651799.1
NCPBP 3873 ¹	1992	Italy	FN651800.1
CRA-FRU 10.22	2009	Italy	FN651804.1
CRA-FRU 5.1	2009	Italy	FN652857.1
CRA-FRU 8.75	2009	Italy	FN652859.1

¹These sequences were deposited by M. Scortichini and the strains were most probably the same as those he provided for the present study. They are not the same as those obtained from the National Collection of Plant Pathogenic Bacteria in York (UK); they should be renamed ISPAVE-B-019 and ISPAVE-B-020.

not always possible, indicating that the pathogen was either below the level of detection or not consistently associated with those symptoms. Several vines from New Zealand producing a red exudate were also analysed; *Psa* was never isolated from those vines. This is not surprising since *Psa* has never been isolated from New Zealand. These results indicate that production of a red exudate is not a specific symptom of bacterial canker in kiwifruit and support the conclusion that New Zealand is free of *Psa*.

Molecular characterisation of *Psa* strains isolated from Italy

Except for the strains of *Psa* and *Pst*, none of the 21 strains of *P. syringae*, representing 19 different previously untested pathovars, gave a 280 bp DNA fragment after PCR with the *PsaF1/R2* primers. This, added to the 29 other strains of *P. syringae* tested earlier (Rees-George et al. 2010), confirms the specificity of the primers *PsaF1/R2*. Furthermore, all the strains of *Psa* tested gave a positive reaction with these primers. This includes strains isolated from Italy in 1992, 2008, 2009 and 2010. Previously the only strains labelled as *Psa* strains isolated from Italy tested were the strains NCPBP 3871 and NCPBP 3873 received from the National Collection of Plant Pathogenic Bacteria in York (UK). These were determined by multi-locus sequence typing analysis and biochemical characterisation not to be *Psa* (Rees-George et al. 2010). Strains with the

same number (NCPBP 3871 and NCPBP 3873) were also received from M. Scortichini (CRA-FRU Rome). In contrast to the strains received from the National Collection of Plant Pathogenic Bacteria, these strains did exhibit all the characteristics of *Psa*: non fluorescent on King's B medium, induced a hypersensitive reaction when infiltrated in tobacco plants, did not have a cytochrome c oxidase or an arginine dehydrolase, did not hydrolyse esculin, did not rot potato and did not induce ice nucleation. They also gave a 280 bp DNA fragment after PCR with the *PsaF1/R2* primers. To avoid confusion with the strains received from the National Collection of Plant Pathogenic Bacteria, these two strains of *Psa* were given back, for the purpose of this study, their original name ISPAVE-B-019 and ISPAVE-B-020. Since these strains were isolated in Italy in 1992, their identification as strains of *Psa* confirms that the outbreak on *A. deliciosa* between 1992 and 1994 in Italy was caused by *Psa*.

Differences between *Psa* and *Pst* and variability between strains of *Psa*

Amplification by PCR using as primers consensus sequences of short intergenic repeated sequences found in plant pathogenic bacteria (rep-PCR) results in fingerprints that can be specific of a species or a pathovar (e.g. Marques et al. 2008). BOX-PCR is one type of rep-PCR that has been used to identify different pathovars of *P. syringae* and, in the case of *P. s. pv. morsprunorum*, to

identify strains from each of the two races of this pathovar (Gilbert et al. 2009). BOX PCR was used to try to differentiate strains of Psa from strains of Pst, something that cannot be done by PCR using the primers PsaF1/R2. The BOX PCR fingerprints from strains of Psa were similar to those of Pst (Figure 1), which is not surprising since these two pathovars are closely related (Vanneste et al. 2009). However, there are

Figure 1 Agarose gel electrophoresis of total DNA from strains of *Pseudomonas syringae* pv. *actinidiae* and pv. *theae* after polymerase chain reaction (PCR) using the primer BOX A1R. Lane 1: *P. s.* pv. *theae* ICMP 3923, Lane 2: *P. s.* pv. *theae* ICMP 3934, Lane 3: *P. s.* pv. *actinidiae* CFBP 7287, Lane 4: *P. s.* pv. *actinidiae* I-Psa H1, Lane 5: *P. s.* pv. *actinidiae* K-Psa 2, Lane 6: *P. s.* pv. *actinidiae* ICMP 9853, Lane 7: *P. s.* pv. *actinidiae* ICMP 9617 and Lane L: DNA ladder. The arrows indicate differences in the fingerprint of *P. s.* pv. *actinidiae* and *P. s.* pv. *theae* strains.

several differences in those fingerprints, which allow the differentiation of strains from these two pathovars (Figure 1). Although BOX-PCR is not practical for diagnostic purposes, it allows differentiation between Psa and Pst. The BOX PCR fingerprint is not identical for all strains of Psa (data not shown). The level of variability and its significance for further epidemiological studies are still being studied. Differences in fingerprinting by BOX PCR between strains of Psa and Pst and within the pathovar *actinidiae* have been reported before (Scortichini et al. 2002; G.M. Balestra, Università degli Studi della Tuscia, personal communication 2010).

The *cts* gene is a housekeeping gene that encodes a citrate synthase. It is part of the core genome of *P. syringae* and it has been used in multi-locus sequence typing analysis of strains of *Pseudomonas* (Sarkar & Guttman 2004). In previous studies, it has proven to be a good indicator of the genetic diversity of populations of *P. syringae* (Morris et al. 2010). When the sequence of the *cts* gene from the strains I-Psa 8, CFBP 7285, CFBP 7286, CFBP 7287, I-Psa H1, I-Psa H3, I-Psa G, ICMP 9617 and K-Psa K2 were compared with those of the *cts* gene from other strains of Psa deposited in GenBank, two haplotypes were found that differed by only two base pairs. In the first haplotype, called haplotype I, a cytosine is in positions 239 and 420, while in the other haplotype, called A, the cytosine at position 239 is replaced by a thymine, and the cytosine at position 420 is replaced by an adenine (Figure 2). The Japanese, the Korean and the two Italian strains isolated in 1992 belonged to haplotype A, while all the other Italian strains belonged to haplotype I. Although the number of strains studied was relatively small, the existence of at least two haplotypes for the *cts* gene in strains of Psa might help in determining the origin of the different Italian outbreaks. This approach will be pursued with more strains of Psa and using other housekeeping genes, such as *gyrB*, *rpoD* and *gapA*.

CONCLUSIONS

Psa cannot always be isolated from kiwifruit vines

(1) TAGCGGTCTGACCGCCACCGGCCGCGTTTCACATTTGACCCTGGTTTCATGTCC
 (53) ACGGCCTCTTGGCGAGTCGAAGATCACCTACATCGATGGTGACAACGGAATTCT
 (106) GCTGCACCGCGGCTACCCGATCGAACAACCTGGCCGAGCAGTCCGATTATCTC
 (159) GAGACCTGCTACCTGTTGCTCAACGGCGAGCTGCCAACCGCCGAACAGAAAG
 (212) CCCAGTTCGTGGCCGTGGTCAAGAACCAC*ACGATGGTTCACGAACAACCTCAA
 (265) GACCTTCTTCAACGGCTTTCGCCGTGACGCCACCCGATGGCCGTCATGTGCG
 (317) GTGTAGTCGGCGCCCTGTCGGCGTTCTACCACGATTCGCTGGACATCAATAAC
 (370) CCGCAGCACCGCGAAATTTTCGGCTGTACGCCTGGTCGCCAAGATGCCGACC**
 (421) CTGGCAGCGATGGTCTACAAGTACTCCATGGGCCAACCCATGATGTACCCGCG
 (474) CAACGACCTCAGCTACGCCGAAAACCTTCCTGCACATGATGTTCAACACGCCGT
 (527) GCGAGATCA

Figure 2. DNA sequence of the *cts* gene from strains of *Pseudomonas syringae* pv. *actinidiae* (Haplotype I), based on the sequences deposited in Genbank and sequences obtained in this study. In Japanese, Korean and Italian strains isolated in 1992, the C noted with * is replaced by a T and the C noted with ** is replaced by an A (Haplotype A).

showing red exudate. Production of a red exudate by kiwifruit vines is not a symptom that is specific to bacterial canker. *Psa* has not been found in New Zealand but has been present in Italy since 1992. The primers *PsaF1/R2* are specific for *Psa* and *Pst*. Strains from these two pathovars can be differentiated by BOX-PCR. Some variability at the molecular level, as illustrated by the presence of several BOX-PCR fingerprints and at least two *cts* haplotypes within the pathovar *actinidiae*, might be used for further epidemiological studies.

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