Identification, Virulence, and Distribution of Two Biovars of *Pseudomonas syringae* pv. *actinidiae* in New Zealand

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Abstract

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Pseudomonas syringae pv. *actinidiae*, the causal agent of bacterial canker of kiwifruit, was detected for the first time in New Zealand in November 2010. Only in Bay of Plenty, one of the four regions where this pathogen had been detected, did symptoms evolve beyond leaf spots, resulting in cane die-back, wilting of canes, and canker, sometimes leading to death of the vine. Molecular analysis (*cts* haplotype and BOX-polymerase chain reaction [PCR] electrophoretic pattern) of strains isolated from different regions of New Zealand revealed that two biovars could be distinguished. They have been called biovar 3 and biovar 4 to differentiate them from strains from Japan (biovar 1) or

Korea (biovar 2), which have a different *cts* haplotype or a different BOX-PCR pattern. Biovars 3 and 4 displayed different degrees of virulence, as measured by their ability to cause leaf spots on young, potted kiwifruit plants. Biovar 3, which has also been present in Italy since 2008 and in France, was found in the Bay of Plenty, where cane die-backs were observed. In contrast, no symptoms other than leaf spots have been observed in orchards where strains of biovar 4 have been isolated. We report the distribution and the disease progression of biovars 3 and 4 in New Zealand.

Actinidia deliciosa and *A. chinensis*, the two most economically important species of kiwifruit in the world, are both susceptible to *Pseudomonas syringae* pv. *actinidiae*, the causal agent of bacterial canker of kiwifruit. This disease was first recorded in Japan in 1984 (35) and was subsequently found in Korea (20) and Italy (33). Affected vines exhibit leaf spots, cane wilting, cane die-back, and cankers, which are sometimes associated with copious production of a red or milky-white exudate. Although production of a red exudate is a common symptom of bacterial canker, the pathogen is not easily isolated from it (41). A similar red exudate can also be produced from apparently healthy tissues or vines nonaffected by *P. syringae* pv. *actinidiae*. It is produced, for example, in reaction to environmental stress such as frost damage. In contrast, *P. syringae* pv. *actinidiae* is readily isolated almost as a pure culture from the milky-white exudate that is sometimes produced by infected tissues, suggesting that this exudate consists of bacteria being extruded from the plant tissues (42). When conditions are favorable for development of the disease, *P. syringae* pv. *actinidiae* can kill a kiwifruit vine within a few months. *P. syringae* pv. *actinidiae* affects all commercial cultivars of kiwifruit, including the green-fleshed kiwifruit *A. deliciosa* ‘Hayward’ and ‘Summer Kiwi’, and the yellow-fleshed kiwifruit *A. chinensis* ‘Jin Tao’, ‘Hort16A’, and

‘Soreli’. In Europe, orchards of *A. chinensis* are, in general, more affected than the orchards of *A. deliciosa* (2). Bacterial canker is currently the most destructive disease of kiwifruit worldwide.

In Asia, *P. syringae* pv. *actinidiae* has had a serious economic impact on kiwifruit production, in some cases limiting the cultivation of kiwifruit (21,34). Nevertheless, the effect of this disease in Asia has not been as destructive as its latest outbreak in Europe. Bacterial canker caused by *P. syringae* pv. *actinidiae* has been recently reported in several European countries: Italy (2,11), Portugal (3), France (40), Spain (1), Switzerland (8), and Turkey (4). This epidemic, which was first reported in Latina (Italy) in 2008, has been devastating for the local kiwifruit industry. In several cases, orchards were totally destroyed and plants had to be pulled out because of bacterial canker; this outbreak jeopardized the cultivation of kiwifruit and, in particular, of *A. chinensis*, in the most important growing regions of Italy. This is in contrast to the first outbreak of the disease in Italy (1992), which had a very limited economic impact.

Although the difference in economic impact between the Asian and recent Italian outbreaks could be the result of different climatic conditions, the differences between the Italian outbreaks of 1992 and 2008 are more difficult to explain, given that the environmental conditions were similar. Furthermore, the differences between those two Italian outbreaks could not be attributed to differences in host susceptibility, because the two major commercial species of kiwifruit present in Italy in 2008 (*A. deliciosa* Hayward and *A. chinensis* Hort16A) had been present in that country for several years. Because neither the host nor the environment can explain the difference in economic impact of the two Italian outbreaks, our attention has turned to the pathogen. Recent molecular analyses have shown that the populations of *P. syringae* pv. *actinidiae* detected during the 2008 outbreak in Italy were different from those in Japan, Korea, or Italy during the 1992 outbreak (12,28,41). Based on differences in the DNA sequence of the housekeeping

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gene coding for citrate synthase (*cts*) and BOX-polymerase chain reaction (PCR) electrophoretic patterns, the strains of *P. syringae* pv. *actinidiae* analyzed could be placed in two distinct groups (41). Strains isolated from Asia and those isolated in Italy before 2008 belong to one group, while all the strains isolated from Italy since 2008 belong to a second group. Because a major characteristic differentiating those two groups was differences in the DNA sequence of the *cts* gene, these groups were referred to as *cts* haplotype A and *cts* haplotype I (41).

In November 2010, *P. syringae* pv. *actinidiae* was isolated in New Zealand for the first time (10,42). It was isolated from leaves, canes, and flowers of kiwifruit showing typical signs of infection (10,42). Symptoms that led to the detection of *P. syringae* pv. *actinidiae* included angular leaf spots with or without a yellow halo and wilting of young shoots. In some cases, a white, milky exudate, from which the pathogen could be isolated, was also detected (42).

To establish the potential economic impact of this discovery, this project aimed to determine whether the strains of *P. syringae* pv. *actinidiae* isolated from New Zealand were similar to strains isolated overseas. Molecular analysis of strains isolated from different regions of New Zealand indicated that two groups of strains could be distinguished. These groups have been called biovar 3 and biovar 4 to separate them from strains with different molecular characteristics that were isolated from Japan (biovar 1) or Korea (biovar 2). Molecular characteristics (*cts* and *ompPI* sequences, BOX-PCR electrophoretic profiles, and presence of some effector genes such as *hopA1* and *avrD*), virulence (differences in virulence observed in the field could be replicated under laboratory conditions), and geographic distribution of the two biovars of *P. syringae* pv. *actinidiae* found in New Zealand have been determined and are presented.

Materials and Methods

Strains and media. The strains of *P. syringae* pv. *actinidiae*, including the 44 strains isolated from New Zealand and characterized during the course of the study, are presented in Table 1. The pathotype strain ICMP 9617 and strain ICMP 9855 were obtained from the International Collection of Microorganisms from Plants (ICMP) held by Landcare Research New Zealand. All the strains were maintained on King's B (KB) medium (19) at 28°C.

Collection of plant samples. Kiwifruit leaves analyzed for the presence of *P. syringae* pv. *actinidiae* usually showed symptoms of bacterial canker (i.e., small angular necrotic spots). However, leaves with other types of necrotic spots and symptomless leaves were also collected from orchards where the presence of *P. syringae* pv. *actinidiae* was either suspected or unknown (e.g., orchards located in proximity to orchards where *P. syringae* pv. *actinidiae* had been isolated). Leaves were sampled from every region of New Zealand where commercial kiwifruit orchards are located (Fig. 1). However, the majority of the samples came from the Bay of Plenty region of the North Island of New Zealand, which is the major growing area for kiwifruit and the area where the first outbreak of bacterial canker was reported. Because of the biosecurity status of *P. syringae* pv. *actinidiae* as an "unwanted organism" in New Zealand, the samples were collected, transported, and analyzed following protocols established by the Ministry for Primary Industries (MPI; formerly Ministry of Agriculture and Forestry [MAF]). The samples were double bagged immediately after collection and transported in a chilled polystyrene-insulated container to one of the following three Physical Containment Level 2 (PC2) laboratories: the Plant and Food Research Laboratory located in Hamilton (PFR), the Verified Laboratory Services laboratory (VLS) in Te Puke, or the MPI Plant Health and Environment Laboratory (PHEL) in Tamaki, Auckland. Between November 2010 and May 2011, samples from over 300 orchards nationwide were analyzed by PFR and over 900 were analyzed by PHEL; between 15 April 2011 and January 2012, more than 2,200 samples had been analyzed by VLS.

Bacterial isolation and biochemical characterization. Bacteria were isolated from leaves or canes using one of two methods.

Samples sent to PFR were isolated using the methods described by Vanneste et al. (42). Briefly, 100 µl of a washing solution obtained by vigorously rubbing each leaf for 2 min in 10 ml of sterile distilled water was plated on KB medium containing a 1% solution of cycloheximide at 5 ml/liter. When isolating from cane tissue, small sections were teased out in sterile water and the suspension was spread on plates of KB medium supplemented with cycloheximide. In both cases, after 28 h of incubation at 28°C, *P. syringae* pv. *actinidiae*-like colonies were purified and characterized as described below. Samples sent to PHEL were isolated using the methods described by Chapman et al. (5). Briefly, leaves were surface sterilized with 70% ethanol; then, small pieces of tissue from the margins of lesions were ground in bacteriological saline (0.85% [wt/vol] NaCl), then left at room temperature for 10 min. Aliquots of the macerated leaf tissue were streaked onto KB medium, incubated at 25°C overnight, and then subcultured onto KB medium to obtain single colonies.

Strains of *P. syringae* pv. *actinidiae* share some biochemical and molecular characteristics that set them apart from strains of other *Pseudomonas* spp. and strains of most other *P. syringae* pathovars. In particular, they are only slightly or nonfluorescent on KB medium, induce a hypersensitive reaction (HR) when infiltrated in tobacco plants, do not produce a cytochrome c oxidase or an arginine dihydrolase, produce levan, do not hydrolyze esculin, do not rot potato, do not produce syringomycin, and do not induce ice nucleation. The ability of a bacterial strain to induce an HR when injected into tobacco plants was tested as described previously (39). Absence of cytochrome c oxidase, a characteristic that differentiates *P. syringae* from other species of plant-pathogenic fluorescent *Pseudomonas*, was determined using Test Oxidase (Pro-Lab Diagnostic). For this assay, *P. fluorescens* (strain A506) (44) was used as a positive control and *P. syringae* pv. *syringae* (strain ICMP3523) was used as a negative control. Production of levan and of an arginine dihydrolase under anaerobic conditions and the ability to hydrolyze esculin were determined as described by Leliot et al. (23). *P. syringae* pv. *syringae* (ICMP 3523) was used as a positive control for production of levan and hydrolysis of esculin. For production of an arginine dihydrolase, *P. fluorescens* (A506) was used as a positive control. The ability to rot potato was determined as described previously (36). Water was used as a negative control and a strain of *Pectobacterium* sp. isolated from kiwifruit was used as a positive control. Ice nucleation activity was determined as described by Lindow et al. (25). *P. syringae* pv. *syringae* (ICMP 3523) was used as a positive control and *P. syringae* pv. *actinidiae* (ICMP 9855 and ICMP 9617) was used as a negative control. Production of syringomycin was determined according to Gross and DeVay (15), with the following modifications: bacterial strains were grown at 28°C for 48 h in the center of a plate of potato dextrose agar (PDA) (Difco; Becton, Dickinson and Company) before a plug of *Geotrichum candidum* (ICMP 5442) was placed 50 mm away from the edge of the bacterial growth. After 48 h of incubation at room temperature, inhibition of 1 mm or more around the bacterial colonies indicated syringomycin production. *P. syringae* pv. *atrofaciens* (ICMP 4394) was used as a positive control and *P. syringae* pv. *actinidiae* (ICMP 9855 and ICMP 9617) was used as a negative control. To assess resistance to streptomycin, KB medium was supplemented with streptomycin (100 ppm). To assess resistance or tolerance to copper, bacteria were resuspended in 1 mM solution of copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ at 250 mg/liter; BDH Laboratory Supplies) and incubated for 4 h at 28°C, before being spread on KB medium without copper.

Identification and characterization of *P. syringae* pv. *actinidiae* by PCR. Total DNA from isolated colonies was isolated at PFR with a cell disruptor FastPrep-24 MP using the ZR fungal/bacterial DNA kit (Zymo Research), and at PHEL using the DNeasy Blood and Tissue Kit (Qiagen). PCRs were performed at PFR on an Eppendorf Mastercycler Gradient, in a total volume of 20 µl, using Intron Maxime PCR premix (INTRON Biotechnology Inc.), to which 10 pmol of each primer and 1 µl containing 50–100 ng of genomic DNA were added. At PHEL, PCRs were performed

Table 1. Strains of *Pseudomonas syringae* pv. *actinidiae* used in this study

Strain number	Host plant	Geographic origin	Year of isolation	Biovar	Source
ISPAVE-B-019 ^a	<i>Actinidia deliciosa</i>	Italy	1994	1	M. Scortichini
ISPAVE-B-020 ^b	<i>A. deliciosa</i>	Italy	1994	1	M. Scortichini
ICMP 9617 ^c	<i>A. deliciosa</i>	Japan	1989	1	ICMP ^d
ICMP 9855	<i>A. deliciosa</i>	Japan	1989	1	ICMP
NIAS 302143	<i>A. deliciosa</i>	Japan	1987	1	G. M. Balestra
K-Psa 2	<i>Actinidia</i> sp.	Korea	...	2	G. M. Balestra
CRA-FRU 10.22	<i>A. chinensis</i>	Italy	2008	3	M. Scortichini
CRA-FRU 2.2	<i>A. chinensis</i>	Italy	2009	3	M. Scortichini
CRA-FRU 5.1	<i>A. deliciosa</i>	Italy	2009	3	M. Scortichini
CRA-FRU 8.76	<i>A. deliciosa</i>	Italy	2009	3	M. Scortichini
I-Psa H1	<i>A. deliciosa</i>	Italy	2008	3	G. M. Balestra
CFBP 7287	<i>A. deliciosa</i>	Italy	2008	3	G. M. Balestra
I-Psa H3	<i>A. deliciosa</i>	Italy	2008	3	G. M. Balestra
CFBP 7286	<i>A. chinensis</i>	Italy	2008	3	G. M. Balestra
I-Psa Z	<i>A. chinensis</i>	Italy	2008	3	G. M. Balestra
I-Psa 8	<i>A. chinensis</i>	Italy	2008	3	G. M. Balestra
I-Psa G	<i>A. chinensis</i>	Italy	2008	3	G. M. Balestra
CFBP 7285	<i>A. chinensis</i>	Italy	2008	3	G. M. Balestra
219-8	<i>Actinidia</i> sp.	France	2011	3	41
I-9.4.10-D1	<i>A. chinensis</i>	Italy	2010	3	41
I-9.4.10-D4	<i>A. chinensis</i>	Italy	2010	3	41
I-9.4.10-D3b	<i>A. chinensis</i>	Italy	2010	3	41
I-9.4.10-E4	<i>A. chinensis</i>	Italy	2010	3	41
I-9.4.10-E7	<i>A. chinensis</i>	Italy	2010	3	41
I-9.4.10-E-Ab	<i>A. chinensis</i>	Italy	2010	3	41
I-6.5.10-4.4	<i>A. chinensis</i>	Italy	2010	3	41
I-25.10.6.6	<i>A. chinensis</i>	Italy	2010	3	41
I-6.5.10	<i>A. chinensis</i>	Italy	2010	3	41
I.23.6.10	<i>A. chinensis</i>	Italy	2010	3	41
10627	<i>A. chinensis</i>	Te Puke, NZ	2010	3	This study
10635	<i>A. chinensis</i>	Te Puke	2010	3	This study
10636	<i>A. deliciosa</i>	Te Puke	2010	3	This study
10638	<i>A. chinensis</i>	Te Puke	2010	3	This study
10639	<i>A. chinensis</i>	Te Puke	2010	3	This study
10641	<i>A. chinensis</i>	Te Puke	2010	3	This study
10642	<i>A. deliciosa</i>	Te Puke	2010	3	This study
10787	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11264	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11265	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11266	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11268	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11269	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11271	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11282	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11283	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11284	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11285	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11287	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11290	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11291	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11293	<i>A. deliciosa</i>	Te Puke	2010	3	This study
11297	<i>A. deliciosa</i>	Te Puke	2011	3	This study
11298	<i>A. deliciosa</i>	Te Puke	2011	3	This study
11301	<i>A. deliciosa</i>	Te Puke	2011	3	This study
11302	<i>A. deliciosa</i>	Te Puke	2011	3	This study
11303	<i>A. deliciosa</i>	Te Puke	2011	3	This study
11305	<i>A. deliciosa</i>	Te Puke	2011	3	This study
11311	<i>A. deliciosa</i>	Te Puke	2011	3	This study
11318	<i>A. chinensis</i>	Te Puke	2011	3	This study
11955	<i>A. deliciosa</i>	Pukehina, NZ	2011	3	This study
12030	<i>A. deliciosa</i>	Te Puke	2011	3	This study
12339	<i>A. deliciosa</i>	Te Puke	2011	3	This study
10850	<i>A. chinensis</i>	Motueka, NZ	2010	4	This study
10874	<i>A. chinensis</i>	Motueka	2010	4	This study
10880	<i>A. chinensis</i>	Motueka	2010	4	This study
10885	<i>A. chinensis</i>	Motueka	2010	4	This study
10984	<i>A. chinensis</i>	Hawke's Bay, NZ	2010	4	This study
10986	<i>A. chinensis</i>	Hawke's Bay	2010	4	This study
10988	<i>A. chinensis</i>	Hawke's Bay	2010	4	This study
11248	<i>A. chinensis</i>	Te Puke	2010	4	This study
11249	<i>A. chinensis</i>	Te Puke	2010	4	This study
11251	<i>A. chinensis</i>	Te Puke	2010	4	This study
11616	<i>A. deliciosa</i>	Te Puke	2011	4	This study

^a Also known as NCPPB 3871.^b Also known as NCPPB 3873.^c This is the pathotype of *P. syringae* pv. *actinidiae*.^d ICMP is the International Collection of Microorganisms from Plants held by Landcare Research New Zealand.

on GeneAmp 9700 thermal cyclers (Applied Biosystems) using GoTaq Green mastermix (Promega Corp.) and concentrations of primers and DNA comparable to those used at PFR. For identification of *P. syringae* pv. *actinidiae*, four sets of primers were used. The primers Psa F1 (5'-TTTTGCTTTGCACACCCGATTTT-3') and Psa R2 (5'-TTTTGCTTTGCACACCCGATTTT-3'), which yield a 280-bp amplicon with DNA from strains of *P. syringae* pv. *actinidiae*, were used following the protocol described by Rees George et al. (31) and modified by Vanneste et al. (41). This assay was further modified for high-throughput testing by adapting it to real-time PCR, utilizing SYBR Green technology and melting curve analysis. This is the assay VLS used. DNA extracted from leaf material was directly analyzed, bypassing the need to isolate and purify bacterial colonies before characterization. Leaf punches

were taken from suspect leaf spots and homogenized in buffer (VLS: Mini Beadbeater, BioSpec Products; PHEL: cetyltrimethylammonium bromide buffer followed by grinding on a Homex homogenizer). DNA was extracted from the resulting supernatant using manual (Plant Genomic DNA Mini Kit; Geneaid or Plant MiniKit; Qiagen) or automated (LabTurbo 48 or Kingfisher mL; Thermo Scientific) extraction platforms (VLS and PHEL, respectively, in both cases). The real-time PCR assay used 0.3 μ M Psa F1 and Psa R2 primers, 5 μ l of either Perfecta SYBR Green FastMix (Quanta BioSciences) (PFR) or SSO Fast EvaGreen supermix (Bio-Rad) (PHEL), and 2 μ l of extracted DNA in a 10- μ l final volume reaction. Real-time PCR was performed on either the Eco real-time PCR System (Illumina) (VLS) or the CFX96 real-time PCR system (Bio-Rad) (PHEL) using a thermal cycling pro-

Fig. 1. Location of commercial kiwifruit-growing regions in New Zealand. The areas labeled Katikati, Tauranga, Te Puke, Whakatane/Edgecumbe, Opotiki, and Waihi constitute the Bay of Plenty. In November 2010, *Pseudomonas syringae* pv. *actinidiae* was isolated from the areas which are underlined (Te Puke, Hawke's Bay, Waikato, and Nelson/Motueka).

gram of 95°C for 3 min followed by 43 cycles of 95°C for 5 s and 60°C for 20 s. Default melting curve analysis (80 to 95°C melt curve in 0.5°C increments, 2 s per increment) was then performed to verify the amplified product, because the 280-bp amplicon produced by *P. syringae* pv. *actinidiae* isolates generated a highly characteristic two-domain melting curve.

The Koh and Nou primers (forward: 5'-CACGATACATGGGCTTATGC-3' and reverse: 5'-CTTTTCATCCACACTCCG-

3'), which yield a 492-bp amplicon, were used following the protocol published by Koh and Nou (22). The duplex protocol of Gallelli et al. (13) which, in addition to the Koh and Nou primers, uses the primers AvrDpx-F (5'-TTTCGGTGGTAACGTTGGCA-3') and AvrDpx-R (5'-TTCCGCTAGGTGAAAAATGGG-3') to amplify a 226-bp portion of the *avrD1* gene, was carried out as described by the authors (13). In addition, amplification of a 896-bp fragment of the 16S rDNA was carried out using the PCR pri-

Fig. 2. Scoring system used for assessment of the virulence of *Pseudomonas syringae* pv. *actinidiae*, shown on leaves of *Actinidia deliciosa* 'Bruno'. Leaves were scored from 0 to 5 according to the percentage of leaf surface showing necrosis: (A) 0% necrosis was scored 0, (B) 1–10% of the leaf area necrosed was scored 1, (C) 11–25% necrosis was scored 2, (D) 26–50% necrosis was scored 3, (E) 51–75% necrosis was scored 4, and (F) 76–100% necrosis was scored 5.

mers 11F (5'-GTTTGATCMTGGCTCAG-3') (14) and 907R (5'-CCGTCAATTCMTTGGAGTTT-3') (30) as described earlier (14).

The *cts* gene, also known as *gltA*, was amplified using the primers *cts*-Fp (5'-AGTTGATCATCGAGGGCGCWCC-3') and *cts*-Rp (5'-TGATCGGTTTGATCTCGCACGG-3') and amplicons were then sequenced using the primers *cts*-Fs (5'-CCCGTCGAGCTGCCAATWCTGA-3') and *cts*-Rs (5'-ATCTCGCACGGS GTRTTGAACATC-3'), as described earlier (32). BOX-PCR was carried out using the primer BOXAIR (5'-CTACGGCAAGGC GACCTGACG-3'), as described by Louws et al. (26). The presence of the *hopA1* effector gene was detected by PCR using primers *hopA1*-F (5'-CGGCAAGAGGTACGAGATTC-3') and *hopA1*-R (5'-TTCAATGCCTTTAGCGTGTG-3') (12).

When needed, PCR products were separated by horizontal gel electrophoresis on 1 or 2% agarose containing ethidium bromide or SYBR Safe DNA gel stain (Molecular Probes) at 10 ng/ml. Between 5 and 20 µl of each PCR reaction was loaded, along with

two lanes containing a DNA ladder (100-bp DNA ladder from Invitrogen or the ZR 100-bp DNA ladder from Zymo Research).

Sequencing of amplicons. DNA suspensions containing an amplicon to be sequenced were purified using a DNA Clean and Concentrator kit (Zymo Research; PFR) or QIAquick PCR Purification Kit (Qiagen; PHEL). DNA sequencing for PFR was conducted at the Waikato DNA Sequence Facility, Waikato University, New Zealand, and for PHEL at EcoGene, Auckland, New Zealand. When required, the sequences obtained were compared with sequences deposited in GenBank from the National Center for Biotechnology Information using the program BLAST 2.2.25 (45). Multiple alignments were performed with AlignX from VECTOR NTI 11 (Invitrogen). All DNA sequences were deposited in GenBank (JQ934440 to JQ934486).

Pathogenicity assay. A suspension containing the bacterial strain to be tested at approximately 10^9 CFU/ml was made in water from freshly grown colonies on KB plates. These suspensions were sprayed on the abaxial side of unwounded leaves. At least six 3- to 4-month-old seedlings of *A. deliciosa* 'Bruno' or Hayward or of *A. chinensis* Hort16A were used per assay. All fully developed leaves were inoculated. When the assays were carried on 1-year-old plants of *A. chinensis* 'Meteor', five plants per treatment were used. The plants were kept at an average temperature of approximately 20°C in plastic chambers to maintain the relative humidity. After 14 or 21 days, each leaf was scored using a 0-to-5 scale reflecting the percentage of the surface area covered with necrotic leaf spots (Fig. 2). All the leaves in a given experiment were scored by the same person. The average score of all the inoculated leaves on a single plant was calculated. The score for a strain was determined as the average score of all the plants of the same cultivar inoculated with that strain. Average leaf severity scores were compared by analysis of variance in Genstat v14 (VSN International). The means were separated by Fisher's least significant differences (5% level).

Results

Isolation of *P. syringae* pv. *actinidiae* from different regions of New Zealand. Since November 2010, bacteria that have the biochemical characteristics of *P. syringae* pv. *actinidiae* (non- or only slightly fluorescent on KB medium, induce HR when infiltrated in tobacco plants, do not have a cytochrome c oxidase or an arginine dehydrolase, produce levan, do not rot potato, do not produce syringomycin, and do not induce ice nucleation) have been isolated from kiwifruit leaves showing angular necrotic leaf spots on vines located in several regions of New Zealand (Fig. 1). In some cases, such bacteria were also isolated from leaves that did

Fig. 3. Agarose gel electrophoresis of total DNA from strains of *Pseudomonas syringae* pv. *actinidiae* after BOX-polymerase chain reaction. Lane 1, 100-bp Zymo Research DNA ladder; lane 2, strain 10638; lane 3, 10636; lane 4, 10642; lane 5, 10635; lane 6, 10641; lane 7, 10640; lane 8, 10787; lane 9, 10885; lane 10, 10850; lane 11, 10874; lane 12, 10880; lane 13, 219-1; lane 14, ICMP 9855; and lane 15, I-Psa8. Strains in lanes 1-8 and lanes 13 and 15 are *P. syringae* pv. *actinidiae* biovar 3. Strains in lanes 9-12 are *P. syringae* pv. *actinidiae* biovar 4. The strain in lane 14 is *P. syringae* pv. *actinidiae* biovar 1. Arrows indicate DNA bands unique to strains belonging to the same biovar.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

Fig. 4. Agarose gel electrophoresis of *Pseudomonas syringae* pv. *actinidiae* after duplex polymerase chain reaction following the protocol described by Gallelli (13). Lanes 1 and 26, 100-bp ladder; lane 2, water control; lane 3, strain 11293; lane 4, 11302; lane 5, 11303; lane 6, 10986; lane 7, 11311; lane 8, 11301; lane 9, 11955; lane 10, 11616; lane 11, 12339; lane 12, 10885; lane 13, 11268; lane 14, 11285; lane 15, 11282; lane 16, 11264; lane 17, 10639; lane 18, 11271; lane 19, 12030; lane 20, 11297; lane 21, 10984; lane 22, 11290; lane 23, 11248; lane 24, 11318; and lane 25, empty. Strains in lanes 4, 6, 11, 12, 16, 19, and 21 are *P. syringae* pv. *actinidiae* biovar 4; the others are *P. syringae* pv. *actinidiae* biovar 3.

not show any symptoms but where other vines on the orchard were showing leaf spots.

All these strains, regardless of their geographic origin, produced a 280-bp amplicon after PCR using the primers PsaF1/R2 and a 492-bp amplicon when using the Koh and Nou primers. Although both sets of primers were designed to specifically amplify DNA from *P. syringae* pv. *actinidiae*; neither of them is entirely specific. For example, they can give an amplicon of the right size and similar sequence with strains of other pathovars, such as *P. syringae* pv. *theae* (31,37). The DNA sequences of the Psa F1/R2 amplicon, which corresponds to part of the 16S-23S ribosomal RNA intergenic spacer, of five of the strains isolated in 2010 (10627, 10880, 10988, 11251, and 11955) were 100% similar to one another and to the corresponding DNA fragment of the pathotype strain ICMP 9617 (GenBank AY342165) and to that of the strains isolated from Japan (ICMP 9855), Korea (NIAS 302143 and K-Psa2), France (219-8), and Italy (ISPaVe 019, ISPaVe020, E7, I.25.10.6.6, I.6.5.10, and I.23.6.10).

The DNA sequences of a 800-bp fragment of the 16SrDNA of the nine strains 10627, 10880, 10850, 10988, 11251, 11266, 10638, 10787, and 11298 were 100% similar to one another and to the 16SrDNA sequences of ICMP 9617, the pathotype strain of *P. syringae* pv. *actinidiae* (GenBank AB001439.1).

To confirm that those strains were *P. syringae* pv. *actinidiae*, five strains (10627, 10880, 10988, 11251, and 11955) were inoculated onto young Bruno or Hort16A seedlings. They all induced small angular necrotic leaf spots, similar to those seen in infected orchards, and from which *P. syringae* pv. *actinidiae* could readily be reisolated. These results confirmed the isolates to be *P. syringae* pv. *actinidiae*.

On that basis, *P. syringae* pv. *actinidiae* was determined to be present in several regions of New Zealand, including Te Puke in the Bay of Plenty, Hawke's Bay, Waikato, and Motueka in the South Island (Fig. 1). However, during spring (November) 2010, shoot die-back and wilting of canes, sometimes accompanied by production of a red exudate, was detected in only a few orchards of *A. chinensis* Hort16A in the Bay of Plenty. *P. syringae* pv. *actinidiae* could be isolated from such infected canes but not from the red exudate. Similar symptoms on *A. deliciosa* Hayward vines caused by *P. syringae* pv. *actinidiae* were not detected until autumn (May) 2011. It was striking that systemic cane infections developed on only a few orchards in the Bay of Plenty, in relatively close proximity to where the first affected orchards were found, but not on Hort16A in the other regions, where presence of *P. syringae* pv. *actinidiae* had been confirmed (Waikato, Hawke's Bay, and Motueka) but where symptoms had not progressed beyond leaf spots.

To determine whether this difference in symptom development was due to the pathogen or the environment, strains isolated from the Bay of Plenty were compared with strains isolated from other

regions of New Zealand or from other parts of the Bay of Plenty where systemic cane infection had not been observed.

Identification of two haplotypes of *P. syringae* pv. *actinidiae* present in New Zealand. Analysis of the DNA sequence of the *cts* gene of 44 strains isolated from different regions of New Zealand revealed that there were two different sequences, or *cts* haplotypes, differing by 6 bp (Supplementary Figure S1). One of the *cts* haplotypes was identical to that found earlier in strains isolated from Italy (2008 outbreak) (41) and from France (2010 outbreak) (36). Only strains isolated from Hort16A orchards in Te Puke that showed cane die-back had this *cts* haplotype, whereas all other New Zealand strains shared a second, previously undescribed, haplotype. These *cts* haplotypes have been numbered from 1 to 3 to reflect the chronology of their identification. The *cts* haplotype of the strains of *P. syringae* pv. *actinidiae* isolated from Japan and from Italy before 2008 is referred to as *cts* haplotype 1 (previously called haplotype A) (41). Although at least one strain isolated from Korea shares that haplotype, other strains isolated from Korea have a different haplotype, which is called haplotype 2. These two hap-

Fig. 5. Morphological characteristics of different biovars of *Pseudomonas syringae* pv. *actinidiae* and *P. syringae* pv. *theae* on King's B medium (A) under natural light or (B) under UV light. Strains 10880 (*P. syringae* pv. *actinidiae* biovar 4), ICMP 9617 (*P. syringae* pv. *actinidiae* biovar 1) and pathotype of *P. syringae* pv. *actinidiae*, ICMP3923 (*P. syringae* pv. *theae*), and 10627 (*P. syringae* pv. *actinidiae* biovar 3) are in quadrants denoted I, II, III, and IV, respectively.

Fig. 6. Pathogenicity of strains of *Pseudomonas syringae* pv. *actinidiae* of biovar 3 and biovar 4 on seedlings of A, 'Bruno'; B, 'Hort16A'; or C, 'Hayward'. Results were read 14 days after inoculation for Bruno seedlings and 21 days after inoculation for the two other cultivars. The y axis is the score severity (as defined in Figure 2). Strains 11955, 11930, 10643, and 10627 are *P. syringae* pv. *actinidiae* biovar 3 and strains 11249, 10988, 10850, 11251, and 10880 are *P. syringae* pv. *actinidiae* biovar 4. Vertical bars represent standard errors of the means. Columns with the same letter are not significantly different at 5%. A, $P < 0.001$, least significant difference (LSD) (5%) = 0.299; B, $P < 0.001$, LSD (5%) = 0.553; C, $P < 0.001$, LSD (5%) = 0.595.

lotypes differ from each other by 2 bp. The *cts* haplotype 2 is also shared by strains isolated from Italy since 2008, from France, and from the New Zealand orchards with shoot die-back. This haplotype was previously called haplotype I (41). The *cts* haplotype of strains from orchards distributed throughout New Zealand and that has never been described before is referred to as *cts* haplotype 3. In New Zealand, there is a clear correlation between *cts* haplotypes and symptom development in orchards of *A. chinensis* Hort16A. In every orchard where shoot die-back was observed, strains with *cts* haplotype 2 were isolated, whereas strains with *cts* haplotype 3 were only ever isolated from *A. chinensis* orchards where the disease had not progressed beyond leaf spots. On *A. deliciosa*, the correlation between symptom development and *cts* haplotype was less clear because the development of shoot die-back was less common and took longer to develop. However, strains with *cts* haplotype 3 were never isolated from *A. deliciosa* vines showing cane wilting or die-back; only strains of *cts* haplotype 2 have ever been isolated from vines showing such symptoms.

Characterization of the two *cts* haplotypes present in New Zealand. When the total DNA of 59 strains, including the 44 strains analyzed previously, were analyzed by BOX-PCR, three electrophoretic patterns were detected (Fig. 3). Strains from Japan and Korea, independently of their haplotype, displayed a similar pattern that was different from the pattern displayed by non-Korean strains of *cts* haplotype 2. As noted previously, there were

very few differences between the BOX-PCR electrophoretic pattern of strains with *cts* haplotypes 1 and 2 (41). Most of the DNA banding differences occurred between 1.5 and 3 kb. Strains of *cts* haplotype 2 had five unique bands, compared with strains of *cts* haplotype 1, which had only two unique bands. The differences between the BOX-PCR electrophoretic patterns of strains of *cts* haplotype 3 and strains of *cts* haplotypes 1 and 2 were greater than the differences between the patterns from *cts* haplotype 1 and 2. Two major bands between 1.2 and 1 kb were not present in strains of *cts* haplotype 3; however, these strains had an additional band of 1.5 kb (Fig. 3). The other differences between BOX-PCR profiles were less noticeable and were generally DNA bands between 1.5 and 3 kb.

When using the duplex PCR protocol developed by Gallelli et al. (13), the 15 strains of *cts* haplotype 1 and the 32 New Zealand strains of *cts* haplotype 2 analyzed produced two amplicons of the expected size: 492 bp for the Koh and Nou amplicon and 226 bp for the *avrD1* amplicon (Fig. 4). In contrast, the 13 strains of *cts* haplotype 3 produced only the 492-bp amplicon, suggesting that the *avrD1* gene is not present in strains of *cts* haplotype 3. Furthermore, the DNA sequence of the 492-bp Koh and Nou amplicon was not identical for all strains (Supplementary Figure S2). The sequences of five strains of *cts* haplotype 2 (including two strains isolated from Italy) were 100% identical to one another and differed from the sequences of the other strains of *P. syringae* pv. *actinidiae* by 5 to 7 bp. Base pairs at position 191, 200, and 214

Fig. 7. Progression of bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* biovar 3 in the Bay of Plenty (North Island, New Zealand) from November 2010 until November 2011.

were always A, T, and C, respectively, for strains of *cts* haplotype 2 and G, C, and T, respectively, for strains of haplotypes 1 and 3. DNA sequence analysis revealed that all sequences were similar (98 to 100 %) to the sequence of the ompP1 found in *P. syringae* pv. *tomato* (GenBank FR863686.1), which codes for the outer membrane protein P1.

The gene *hopA1* was detected by PCR in all 44 strains isolated from New Zealand but was not detected in strains of *cts* haplotype

1, including those strains from Italy isolated in 1992 and in strains isolated from Korea. Previous reports that this gene was present in strains isolated in Italy in 1992 (36) were based on strains NCPPB 3871 and NCPPB 3873, which were shown not to be *P. syringae* pv. *actinidiae* (31).

The morphology of strains of *cts* haplotype 3 was slightly different from that of the three other haplotypes (Fig. 5). Strains of *cts* haplotype 1 seemed completely nonfluorescent when grown on KB

Fig. 8. Map of New Zealand showing the distribution of *Pseudomonas syringae* pv. *actinidiae* biovar 3 and biovar 4 as of March 2012.

medium and exposed to ultraviolet light, whereas those of the *cts* haplotype 2 were variable, sometimes showing some light-blue fluorescence. Strains of the *cts* haplotype 3 consistently produced a green fluorescent pigment. None of the strains of *cts* haplotype 1 or 2 hydrolyzed esculine but all the strains of haplotype 3 did hydrolyze this compound. None of the 20 strains isolated from New Zealand, randomly selected and representing both haplotypes, was found to be resistant to streptomycin or copper.

Difference in virulence of the two *cts* haplotypes found in New Zealand. When the New Zealand strains of *cts* haplotype 2 (11930, 10643, and 10627) were inoculated onto kiwifruit seedlings of *A. deliciosa* Bruno, they caused significantly more disease than when similar plants were inoculated with the strains of *cts* haplotype 3 (11249, 10850, 11251, and 10880; Fig. 6A). There were no significant differences between strains of the same haplotype (Fig. 6A). To determine whether this was a specific interaction between bacterial strains and Bruno seedlings, two strains of each *cts* haplotype (11955 and 10627 for *cts* haplotype 2 and 10988 and 11251 for *cts* haplotype 3) were inoculated onto *A. deliciosa* Hayward and *A. chinensis* Hort16A seedlings. In both cases, strains of *cts* haplotype 2 produced significantly more leaf spots than those of *cts* haplotype 3 (Fig. 6B and C). Similar results were obtained when the strains were inoculated onto potted *A. chinensis* Meteor plants (*data not shown*).

Distribution of *cts* haplotype 2 and 3 of *P. syringae* pv. *actinidiae* in New Zealand. Molecular characterization of *P. syringae* pv. *actinidiae* strains (in particular, BOX-PCR electrophoretic pattern and high-resolution melting analysis) isolated from different regions of New Zealand allowed mapping and inference of the progression of *cts* haplotype 2 and *cts* haplotype 3 in New Zealand (Fig. 7). From November 2010 until August 2011, *cts* haplotype 2 seemed to be moving relatively slowly and over short distances. Up to early August 2011, its distribution was limited to the Te Puke region: a 10-by-20 km area. However, from September 2011 to November 2011, this pathogen appeared to move more rapidly. It was detected for the first time in Tauranga on 9 August, Waihi on 12 September, Katikati on 27 September, Whakatane on 29 September, and Opotiki on 20 October. By November 2011, 1 year after *P. syringae* pv. *actinidiae* was first detected in New Zealand, the entire Bay of Plenty was affected by strains of *cts* haplotype 2, this haplotype having been isolated from orchards spreading from Waihi to Opotiki, which in a straight line are 145 km away from each other. Until mid-November 2011, the *cts* haplotype 2 form of the pathogen was restricted to the Bay of Plenty; however, by the end of November 2011, it had been detected on three orchards in the south of Auckland. In contrast, the distribution of *cts* haplotype 3 does not appear to have changed between November 2010 and November 2011, although there is some evidence that orchards in the Te Puke region previously infected with *cts* haplotype 3 have been overcome with *cts* haplotype 2. Strains of *cts* haplotype 3 could not be detected in orchards where *cts* haplotype 2 was isolated, suggesting that strains of *P. syringae* pv. *actinidiae* haplotype 2 displaced the strains of haplotype 3. The distribution of *P. syringae* pv. *actinidiae* *cts* haplotype 2 and 3 as of March 2012 is presented in Figure 8.

Discussion

A. deliciosa Hayward and *A. chinensis* Hort16A are the two main commercial cultivars of kiwifruit that are grown in New Zealand and elsewhere in the world. Strains of *P. syringae* pv. *actinidiae* isolated in New Zealand from Hayward and Hort16A were characterized and grouped into two different biovars based on their molecular and pathogenic characteristics. The pathogen was most often isolated from leaves showing necrotic spots. Necrotic spots on kiwifruit leaves can be elliptical, angular, or irregular; large or pinpoint; and with or without a yellow halo surrounding the lesion. The most characteristic leaf spots from which *P. syringae* pv. *actinidiae* could be isolated were small, angular, and confined by leaf veins. However, no absolute correlation between spot morphology and presence or absence of *P. syringae* pv. *actinidiae* could be

determined (42). *P. syringae* pv. *actinidiae* was also isolated from leaves without leaf spots or other signs of infection. This suggests that *P. syringae* pv. *actinidiae* can live as an epiphyte on kiwifruit. This ability to live epiphytically, a characteristic shared by other pathovars of *P. syringae* (17), might help to explain the rapid spread of the disease in Italy (38) and New Zealand.

P. syringae pv. *actinidiae* has also been isolated from flowers, including asymptomatic flowers, and from canes (42). The first symptoms on canes of *A. deliciosa* were found several months after the same symptoms had been easily detectable on *A. chinensis*. The same situation was found in Italy and in France, where the most affected vines in the field were always *A. chinensis* cultivars. In New Zealand up until May 2011, only Hort16A orchards in one small, well-defined region of Te Puke exhibited cane die-back. Other Hort16A orchards, including those in regions where *P. syringae* pv. *actinidiae* had been found, did not show these symptoms. The first orchards of *A. deliciosa* displaying similar symptoms of cankers and die-back were located in that same area of Te Puke. The fact that regions with similar host plants and similar climate did not seem to be affected by the disease to the same extent suggested that pathogens with different degrees of virulence could be present in New Zealand.

Strains of *P. syringae* pv. *actinidiae* isolated in New Zealand were originally characterized by their *cts* haplotype. The *cts* gene is one of seven housekeeping genes selected for multi-locus sequence typing (MLST) analysis to study the evolutionary relationship among strains of different pathovars of *P. syringae* (32). Housekeeping genes are necessary for bacterial survival; they evolve mostly by neutral mutations, and do not usually transfer horizontally. Therefore, they are very well suited to establish phylogenetic relationships between bacterial strains (18,32). This set of seven genes was later reduced to the four genes *cts*, *rpoD*, *gyrB*, and *gapA* without any significant reduction in phylogenetic resolution (18). Recently, the phylogenetic relationship of 120 strains of *P. syringae* established by MLST analysis using the concatenated sequences of the four genes *cts*, *rpoD*, *gyrB*, and *gapA* was compared with that obtained using the DNA sequence of the *cts* gene alone (29). The strains clustered identically although, on three occasions, clades represented by one strain each were merged into neighboring clades. Therefore, the relationship among strains of *P. syringae* can be determined, to a certain extent, using only the DNA sequence of the *cts* gene. Using that approach, strains of *P. syringae* pv. *actinidiae* isolated from Asia (Japan and one strain from Korea) and the latest Italian outbreak of bacterial canker were organized in two different groups (38,41). These groups were called *cts* haplotype A and *cts* haplotype I, a description which is now outdated. In this article, *cts* haplotypes A and I are called *cts* haplotypes 1 and 2, respectively. Strains of *cts* haplotype I were responsible for the Italian outbreak of 2008, while the previous Italian outbreaks and the one documented in Asia (Japan and Korea) were linked to strains of *cts* haplotype A (41). The separation of the strains of *P. syringae* pv. *actinidiae* isolated in Italy after 2008 into their own distinct group, separate from the strains isolated in Asia or Italy before 2008, was supported by MLST analysis of housekeeping genes (12), MLST analysis of housekeeping type III effector and phytotoxin genes (5), rep-PCR and IS50-PCR (28) and by analysis of whole-genome sequence of three strains of *P. syringae* pv. *actinidiae*: one isolated from Japan, one from Italy in 1992, and one from Italy in 2009 (27). Since the 2010 study (41), most strains isolated from Korea were found to carry *cts* haplotype 2 (the haplotype of strains isolated from Italy after 2008) but to have a BOX-PCR electrophoretic pattern similar to those of strains isolated from Japan (*data not shown*). This argues for the strains isolated in Japan and those isolated in Korea to be in two different groups. The separation of the strains of *P. syringae* pv. *actinidiae* isolated in Korea from strains isolated in Japan or Italy before 2008 is supported by analysis of the draft genome sequence of one strain of *P. syringae* pv. *actinidiae* isolated from Korea with the genome of three strains isolated from Japan, two from China, three from Italy in 2008, and one from Portugal (27). It is also

supported by MLST analysis of housekeeping, type III effector and phytotoxin genes (5).

When strains from the New Zealand outbreak were analyzed, a new *cts* haplotype associated with a unique BOX-PCR electrophoretic pattern was found. Other characteristics linked to this haplotype include absence of the effector *avrD1*, production of a green fluorescent pigment on KB medium, ability to hydrolyze esculin, and a lower virulence on several cultivars of *A. deliciosa* (Hayward and Bruno seedlings) or *A. chinensis* (Hort16A seedlings and 1-year-old Meteor potted plants). However, the most important characteristic of this haplotype is that, in the field, strains of this haplotype have never been linked to any symptom other than leaf spotting.

Strains of *P. syringae* pathovars that exhibit some host specialization can be grouped into races. However, none of the different *cts* haplotypes of *P. syringae* pv. *actinidiae* can be differentiated by their ability to cause disease on some species, pathovar, or germplasm of *Actinidia*. They all cause disease to all cultivars of *A. deliciosa* and *A. chinensis* tested today and no plant other than kiwifruit plant has been found susceptible to these pathogens. To use the geographic origin of the first isolate of a *cts* haplotype to label all the strains of that haplotype, such as Italian strains or Asian strains (as has been done in some media), would be confusing and inadequate. Different haplotypes are present in the same country. For example, strains of *cts* haplotypes 1 and 2 have been isolated from Italy, and strains of *cts* haplotypes 2 and 3 have been isolated from New Zealand. Furthermore, such a labeling could also suggest a geographic origin for new outbreaks of the disease. This would be misleading because the geographic origin of the first isolate of a *cts* haplotype might not be the origin of later outbreaks caused by that *cts* haplotype. The appellation of PsA V and LV (which have also been used in the media) are not adequate either because they do not distinguish strains of *cts* haplotypes 1 and 2. Therefore, we propose to use the term biovar to distinguish the different groups of strains of *P. syringae* pv. *actinidiae*. Four biovars can be identified, each being defined by a combination of molecular characteristics, including *cts* haplotype and BOX-PCR electrophoretic pattern, and, sometimes, biochemical characteristics such as fluorescence or ability to hydrolyze esculin. Strains from biovar 1 were initially isolated in Japan and Italy prior to 2008, while the strains from biovar 2 were isolated from Korea. These strains share a similar BOX-PCR pattern but have different *cts* sequences. Biovars 1 and 2 can also be separated by DNA sequence differences at other housekeeping (*acn*, *pfk*, and *gapA*) and effector (*avrD1* and *hrpK1*) genes (5). Furthermore, strains from biovar 1 produce phaseolotoxin but not coronatine, whereas strains of biovar 2 produce coronatine but not phaseolotoxin (5,16). No strains of *P. syringae* pv. *actinidiae* biovars 1 or 2 have been isolated in New Zealand to date. Biovar 3 corresponds to *cts* haplotype 2; it comprises the strains of *P. syringae* pv. *actinidiae* isolated from Italy after 2008, France, and New Zealand. It also probably comprises the strains isolated from Portugal, Spain, Switzerland, and Turkey, based on virulence observations in the field and the geographic proximity of these countries to Italy. Outbreaks caused by biovar 3 have had the greatest economic impacts, in both Italy and New Zealand. Strains from biovar 3 do not produce coronatine or phaseolotoxin, nor do strains from biovar 4. Biovar 4 comprises strains isolated in New Zealand that never lead to symptoms other than leaf spots. Strains of biovar 4 have also been present in Western Australia since 1992, where it has been mistaken for *P. viridiflava*, a pathogen of limited economic impact, for almost 20 years (7). The limited symptoms caused by strains of biovar 4 render this pathogen inconspicuous. Similarly, strains of *P. syringae* pv. *actinidiae* biovar 4 may have been in New Zealand for many years, because the presence of biovar 4 in commercial kiwifruit orchards has never been associated with any symptoms other than leaf spots. This hypothesis is supported by the relatively wide distribution of this biovar in New Zealand by December 2010. Moreover, there is some diversity in the sequence of the *ompP1* amplicon of biovar 4 strains, although no genetic diversity within biovar 4 has been de-

tected among five strains characterized at a further 15 housekeeping or effector genes (5).

In contrast, the rapid spread and conspicuous symptoms associated with biovar 3 in New Zealand strongly suggests that this pathogen is a much more recent introduction. In a single year, it has spread from a few orchards centered in Te Puke to the entire Bay of Plenty kiwifruit-growing region, and has recently moved to at least one other region in New Zealand. If biovar 3 had been introduced into New Zealand much earlier than November 2010, it is difficult to imagine that it would not have spread more widely than the few orchards in which it was first detected in November 2010, or that the obvious symptoms it causes on *A. chinensis* (cane die-back, wilting, canker, and production of a red exudate) would have been overlooked by orchardists for a long period of time.

There are fewer molecular differences between strains of biovars 1, 2, and 3 than between those strains and strains of biovar 4. This is true for the DNA sequence of the *cts* gene, the BOX-PCR electrophoretic pattern, and the DNA sequence of the *ompP1* gene. This has been confirmed by analyzing the sequences of 15 housekeeping and effector genes in the four biovars of *P. syringae* pv. *actinidiae* (5).

Strains of biovar 4 are associated with a very low degree of virulence in the field and in the laboratory; they are biochemically different from strains of the other biovars of *P. syringae* pv. *actinidiae* (e.g., production of a strong green fluorescent pigment on KB medium and hydrolysis of esculin). They also have a lower homology at the *cts* gene to biovars 1, 2, and 3 than those biovars have with *P. syringae* pv. *theae* (GenBank accession AY610752.1). Therefore, it is reasonable to question whether biovar 4 is a pathovar of *P. syringae* closely related to but separate from *P. syringae* pv. *actinidiae*. However, biochemical characteristics are not indicative of a specific pathovar. Pathovars are defined by their host range; to date, there are no indications that strains of biovar 4 have a host range different from that of the other strains of *P. syringae* pv. *actinidiae*.

The duplex PCR developed by Gallelli et al. (13) can differentiate between strains of the two biovars of *P. syringae* pv. *actinidiae* present in New Zealand. No amplicons were obtained with the *avrD1* primers for strains of biovar 4, indicating that this gene is not present in those strains. This has been confirmed by PCR (5) using the set of primers for *avrD1* designed by Ferrante and Scorticini (12). The protocol developed by Gallelli et al. (13) may allow rapid identification by PCR of the two biovars of *P. syringae* pv. *actinidiae* present in New Zealand without the need to isolate and sequence DNA fragments from a pure bacterial culture. However, it also detects the same false positives as those obtained when using the Koh and Nou primers. When using those primers, an amplicon of the same size as that obtained for strains of *P. syringae* pv. *actinidiae* (492 bp) was obtained with some strains of *P. syringae* pv. *syringae*, *P. syringae* pv. *tomato* (31), and *P. syringae* pv. *theae* (37). There are now several PCR protocols that have been developed specifically to distinguish strains of biovar 3 from those of biovar 4 (J. F. Mackay, S. Dowlut, J. R. Chapman, and J. L. Vanneste, unpublished data).

Strains from biovar 4 do not seem to possess the effector *avrD1*. Whether the presence or absence of a single effector (*avrD1*) can explain the differences in virulence observed in the field and in the laboratory assays has yet to be determined, because this is just one of many molecular differences between the two biovars of *P. syringae* pv. *actinidiae* present in New Zealand (5).

This study clearly shows that two biovars of *P. syringae* pv. *actinidiae* are present in New Zealand. These two biovars are different from the two biovars identified in Asia (Japan and Korea). Biovars 1 to 4 correspond to the recently defined multi-locus sequence analysis (MLSA) groups PsA1, PsA2, PsA3, and PsA4, respectively (5). In addition to Japan, Korea, and Europe, *P. syringae* pv. *actinidiae* has been identified in Chile (9) and China (6,24,43). The virulent biovar 3 found in New Zealand has also been present in Italy since 2008 (41), France (41), Chile (5), and China (5). However, it is possible that some as-yet-uncharacterized biovars of

P. syringae pv. *actinidiae* are also present in these countries, especially China, which is the center of origin of most kiwifruit species, and for which only a single isolate of *Psa* has currently been characterized.

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