

# Molecular and phenotypic features of *Pseudomonas syringae* pv. *actinidiae* isolated during recent epidemics of bacterial canker on yellow kiwifruit (*Actinidia chinensis*) in central Italy

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*Pseudomonas syringae* pv. *actinidiae* (Psa) was identified as the causal agent of severe epidemics of bacterial canker on *Actinidia chinensis* (yellow kiwifruit) in central Italy occurring during 2008–9. A total of 101 strains were obtained from infected leaves, twigs, branches and trunks of cvs Hort16A, Jin Tao and CK3. Outbreaks were also found on *A. deliciosa* cv. Hayward. A representative set of 21 strains were compared with other Psa strains isolated from previous outbreaks in Japan and Italy as well as with *P. s.* pv. *syringae* strains obtained from *A. chinensis* and with strains of genomospecies 8. Repetitive-sequence PCR (rep-PCR) typing using BOX and ERIC primer sets revealed that all Psa strains obtained during 2008–9 showed the same fingerprinting profile. This profile, however, was different from those of strains previously isolated in Japan and Italy. Multilocus sequence typing (MLST) of *gapA*, *gltA*, *gyrB* and *rpoD* revealed a higher genetic variability among the strains than rep-PCR, with some of them showing the same sequence pattern although isolated from different areas, cultivars and years. None of the recently obtained strains possessed genes coding for phaseolotoxin or coronatine, and all had an effector protein, namely *hopA1*, differentiating them from the strains causing past outbreaks in Japan and Italy. All isolates were inhibited *in vitro* by copper-based compounds, antibiotics, geraniol, citronellol and by a chitin-based organic compound. The recent epidemics found in central Italy on yellow kiwifruit appear to have been caused by a different Psa population than those previously recorded in Japan, South Korea and Italy.

**Keywords:** *Actinidia chinensis*, *Actinidia deliciosa*, chitosan, genomospecies 8, multilocus sequence typing, repetitive-sequence PCR, terpenes

## Introduction

During spring and autumn 2008 and winter 2008–9, severe outbreaks of bacterial canker were observed on *Actinidia chinensis* (yellow kiwifruit) cvs Hort 16A and Jin Tao cultivated in central Italy (Latina province). The main typical symptoms were the oozing of reddish exudates along the main trunk and branches, reddening of the lenticels under the epidermis, leaf spots sometimes surrounded by a chlorotic halo, leaf wilting, twig dieback and plant wilting.

Many field surveys were performed to monitor the severity of the infection and *Pseudomonas syringae* pv. *actinidiae* (Psa) was consistently isolated from infected plant samples (Ferrante & Scortichini, 2009). This pathogen was isolated for the first time in the same area from *Actinidia deliciosa* cv. Hayward in 1992 (Scortichini, 1994), and from that time until 2008 it caused only sporadic damage (i.e. leaf spotting, twig dieback), always

towards *A. deliciosa*. Severe damage and/or epidemics were never observed. However, probably because of spread of bacterial inoculum, Psa was noticed more frequently during 2008–9 than in the past on *A. deliciosa*.

Information about the population structure of a pathogen during epidemics and preliminary assessment of a microorganism's sensitivity to different antimicrobials are fundamental prerequisites for planning effective field strategies to control the spread of a bacterium and to reduce the incidence of disease over a long-term period. With the above-mentioned aim in mind, strains of Psa were isolated from *A. chinensis* cvs Hort 16A and Jin Tao, from the pollinator cv. CK3, and from recent outbreaks of bacterial canker on *A. deliciosa* cv. Hayward in central Italy. To this set of strains were added two Psa strains obtained from a single case of bacterial canker on *A. chinensis* in northern Italy (Ravenna province) and two isolates of *P. s.* pv. *syringae* isolated from leaf spotting symptoms on *A. chinensis* cv. Hort16A in central Italy. A comparison with some strains of Psa inciting bacterial canker on *A. deliciosa* cv. Hayward in the past, isolated in Japan and Italy (Takikawa *et al.*, 1989;

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Scortichini, 1994), was also performed. Since it has been shown that Psa belongs to genomospecies 8, *sensu* Gardan *et al.* (1999), together with *Pseudomonas avellanae* and *P. s. pv. theae* (Scortichini *et al.*, 2002), representative strains of this genomospecies were also included for comparative purposes.

This paper reports on the molecular characterization and strain relationships of Psa cultures obtained using repetitive-sequence PCR performed with BOX and ERIC primer sets and multilocus sequence typing (MLST). The pathogen was also assessed for the occurrence of genes involved in toxin production (i.e. phaseolotoxin and coronatine) and synthesis of effector proteins. Finally, the *in vitro* sensitivity of the bacterium towards copper-based and organic compounds, as well as to antibiotics and terpenes, was tested.

## Materials and methods

### Isolation and bacterial strains

During 2008 and 2009, diseased leaf, twig, branch and trunk samples of *A. chinensis* cvs Hort16A, Jin Tao and CK3, and *A. deliciosa* cv. Hayward were processed to identify the causal agent of bacterial canker epidemics in central Italy. Bacteria were identified by following techniques already described and applied (Scortichini *et al.*, 2002, 2003; Ferrante & Scortichini, 2009). A total of 101 isolated strains of Psa and two of *P. s. pv. syringae* were obtained. Subsequently a subset of 21 representative Psa strains was chosen for assessing and comparing their molecular and phenotypic features. They were selected in order to test samples obtained from all cultivars, different orchards, different plant organs and from the 2 years of epidemics. Also included in the study were two Psa strains obtained from a single case of bacterial canker on *A. chinensis* in northern Italy (Ravenna province) in 2009, three Psa strains from Japan representing the first epidemic of bacterial canker that occurred in 1984 on *A. deliciosa* cv. Hayward (Takikawa *et al.*, 1989) and two Psa strains isolated in Italy in 1992 (Latina province) from *A. deliciosa* cv. Hayward (Scortichini, 1994). The list of strains used is reported in Table 1. All the strains were cultured on nutrient agar (Oxoid) supplemented with 5% sucrose (NSA), and incubated at 25–27°C.

### Repetitive-sequence PCR

Strains of Psa obtained from *A. chinensis* and *A. deliciosa* during 2008–9 were compared with other Psa strains previously isolated in Japan and Italy from *A. deliciosa* by means of repetitive-sequence PCR (rep-PCR) typing using BOXA1R and ERIC primer sets (Table 2). In addition, they were also compared using rep-PCR with representative strains of genomospecies 8, namely *P. avellanae* and *P. s. pv. theae*, as well as with two *P. s. pv. syringae* strains isolated from *A. chinensis* in 2009 (Table 1). From each strain 50 ng genomic DNA were extracted by alkaline lysis for use as template. Briefly, a loopful of pure

colonies were suspended into Eppendorf tubes containing sterile saline (0.85% NaCl in distilled water) and mixed with a vortex. Subsequently, the tubes were centrifuged for 2 min at 10 000 g. Then, the pellet was suspended in 100 µL 0.05 M NaOH and heated at 95°C for 15 min. After a centrifugation of 2 min at 10 000 g, the supernatant was used as DNA template or stored at –20°C. Thermal cycling was carried out according to the protocol of Louws *et al.* (1994) in a Bio-Rad MJ Mini thermal cycler. PCR amplification was performed in triplicate. Products of PCR were separated by gel electrophoresis on 2.5% agarose (Invitrogen) and photographed using a Bio-Rad Gel Logic 100 transilluminator.

### Multilocus sequence typing

Multilocus sequence typing (MLST) was performed like rep-PCR in order to reveal the relationships among Psa strains. Gene fragments of *gapA*, *gltA*, *gyrB* and *rpoD* coding for glyceraldehyde-3-phosphate dehydrogenase, citrate synthase, DNA gyrase B and sigma factor 70, respectively, were amplified from genomic DNA of Psa and *P. s. pv. syringae* strains listed in Table 1. DNA was extracted using the alkaline lysis method described above. Gene fragments were amplified and sequenced with primers described by Sarkar & Guttman (2004) (Table 2). All PCR reactions were performed in a Bio-Rad MJ Mini thermal cycler. The annealing temperatures used for *gapA*, *gltA*, *gyrB* and *rpoD* were 54, 72, 62 and 76°C, respectively. All the PCR products were sent to Primm for sequencing. All ambiguous and terminal sequences were trimmed before data analysis. A neighbour-joining dendrogram was built with the concatenated data of the four housekeeping genes using SPLITSTREE4 software (Huson & Bryant, 2006) to infer the genetic relationships between the Psa strains. The two *P. s. pv. syringae* strains from *A. chinensis* were used as outgroups.

### Detection of effector genes

The presence of 12 effector genes in Psa and *P. s. pv. syringae* strains (Table 1) was evaluated by PCR using primers listed in Table 2. The effector primers were designed using the PRIMER3 program based on the sequences of *P. s. pv. tomato* DC3000 and *P. s. pv. phaseolicola* 1448A effector genes, and available at the *Pseudomonas-PlantInteraction* website (<http://www.pseudomonas-syringae.org>). For *brpK1*, *hopAF1* and *hopAN1* effectors, the primers described by Ferrante *et al.* (2009) were used. PCR was carried out in a total volume of 25 µL containing 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% TritonX-100 pH 9); 50 pmol each primer; 1.25 U GoTaq® DNA Polymerase (Promega); dNTPs at 0.2 mM each (Promega); 2 mM MgCl<sub>2</sub>; and 1 µL of 50 ng DNA extracted using the alkaline lysis method described above. All PCR reactions were performed in a Bio-Rad MJ Mini thermal cycler with the following cycling conditions: denaturation at 95°C for 5 min; 35 cycles of 30 s of annealing at 58°C and

Table 1 List of *Pseudomonas* strains used in this study

Species/pathovar	Strain name <sup>a</sup>	Country	Year of isolation	Host plant-cultivar
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 10.22	Italy-Latina	2008	<i>Actinidia chinensis</i> -Hort16A
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 10.26	Italy-Latina	2008	<i>A. chinensis</i> -Hort16A
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 8.52	Italy-Latina	2008	<i>A. chinensis</i> -CK3
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 8.43	Italy-Latina	2008	<i>A. chinensis</i> -Hort16A
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 8.51	Italy-Latina	2008	<i>A. chinensis</i> -Hort16A
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 8.57	Italy-Latina	2009	<i>A. chinensis</i> -Hort16A
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 10.6	Italy-Latina	2009	<i>A. chinensis</i> -Hort16A
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 10.10	Italy-Latina	2009	<i>A. chinensis</i> -Hort16A
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 10.28	Italy-Latina	2009	<i>A. chinensis</i> -Hort16A
<i>P. syringae</i> pv. <i>actinidiae</i>	4252 A.1	Italy-Ravenna	2009	<i>A. chinensis</i>
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 10.25	Italy-Latina	2009	<i>A. chinensis</i> -Hort16A
<i>P. syringae</i> pv. <i>actinidiae</i>	4649.1	Italy-Ravenna	2009	<i>A. chinensis</i>
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 1.2	Italy-Latina	2009	<i>A. chinensis</i> -CK3
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 3.1	Italy-Latina	2009	<i>A. chinensis</i> -Hort16A
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 10.5	Italy-Latina	2009	<i>A. chinensis</i> -Hort16A
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 10.14	Italy-Latina	2009	<i>A. chinensis</i> -Jin Tao
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 10.15	Italy-Latina	2009	<i>A. chinensis</i> -Jin Tao
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 10.16	Italy-Latina	2009	<i>A. chinensis</i> -Jin Tao
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 10.24	Italy-Latina	2009	<i>A. deliciosa</i> -Hayward
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 5.1	Italy-Latina	2009	<i>A. deliciosa</i> -Hayward
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 8.69	Italy-Latina	2009	<i>A. deliciosa</i> -Hayward
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 8.75	Italy-Latina	2009	<i>A. deliciosa</i> -Hayward
<i>P. syringae</i> pv. <i>actinidiae</i>	CRA-FRU 8.80	Italy-Latina	2009	<i>A. deliciosa</i> -Hayward
<i>P. syringae</i> pv. <i>actinidiae</i>	NCPPB 3871	Italy	1994	<i>A. deliciosa</i> -Hayward
<i>P. syringae</i> pv. <i>actinidiae</i>	NCPPB 3873	Italy	1994	<i>A. deliciosa</i> -Hayward
<i>P. syringae</i> pv. <i>actinidiae</i>	KW11 <sup>T</sup> = NCPPB 3739	Japan	1984	<i>A. deliciosa</i> -Hayward
<i>P. syringae</i> pv. <i>actinidiae</i>	KW31	Japan	1984	<i>A. deliciosa</i> -Hayward
<i>P. syringae</i> pv. <i>actinidiae</i>	KW30 = NCPPB 3740	Japan	1984	<i>A. deliciosa</i> -Hayward
<i>P. avellanae</i>	BPIC 631 <sup>T</sup>	Greece	1986	<i>Corylus avellanae</i>
<i>P. avellanae</i>	CRA-FRU PA111	Italy	2006	<i>C. avellanae</i>
<i>P. syringae</i> pv. <i>theae</i>	SUPP 68	Japan	1983	<i>Thea sinensis</i>
<i>P. syringae</i> pv. <i>theae</i>	NCPPB 2598 <sup>T</sup>	Japan	1970	<i>T. sinensis</i>
<i>P. syringae</i> pv. <i>syringae</i>	CRA-FRU 10.31	Italy	2009	<i>A. chinensis</i>
<i>P. syringae</i> pv. <i>syringae</i>	CRA-FRU 10.32	Italy	2009	<i>A. chinensis</i>

<sup>T</sup>: type-strain.

<sup>a</sup>CRA-FRU: Culture Collection of C.R.A.-Centro di Ricerca per la Frutticoltura, Roma, Italy. BPIC: Benaki Phytopathological Institute Collection, Kiphissia-Athens, Greece. KW and SUPP: Culture Collection of the Shizuoka University, Japan. NCPPB: National Collection of Plant Pathogenic Bacteria, Fera, York, UK.

extension at 72°C for 1 min; and 5 min of final extension at 72°C. Annealing temperatures of 64°C for *hrpK1* and *hopAF1* and 68°C for *hopAN1* amplification were used. Amplification products were separated in 1% agarose gels in Tris-acetate-EDTA (TAE) buffer 0.5×, and visualized by a Bio-Rad Gel Logic 100 UV transilluminator. Presence/absence of a band of the expected size was taken as indication of the presence or absence of the gene in the genome of the tested strain. *Pseudomonas syringae* pv. *tomato* DC3000 and *P. s. pv. phaseolicola* 1448A were used as positive controls.

### Detection of phytotoxins

The presence of the *cfl* gene coding for coronatine and of fragments of the *tox-argK* gene cluster coding for phasolotoxin was checked by PCR amplification in all the *Psa* cultures obtained in this study. The PCR reactions were performed as described in the referential papers (Prosen

*et al.*, 1993; Bereswill *et al.*, 1994; dos Marques *et al.*, 2000; Sawada *et al.*, 2002). All the primers used are listed in Table 2. The separation of PCR products and gel visualization were performed as described above. *Pseudomonas syringae* pv. *tomato* DC3000 and *P. s. pv. phaseolicola* 1448A, as well as *Psa* KW11 isolated in Japan in 1984 and NCPPB 3871 isolated in Italy in 1992, were used as positive controls. Presence/absence of a band of the expected size was taken as indication of the presence or absence of the gene in the genome of the tested strain.

### Sensitivity to copper-based and organic compounds

The *in vitro* sensitivity of *Psa* towards copper-based and organic compounds was checked towards all 101 strains. For each strain, a loopful of a 24-h-old single colony grown onto NSA was suspended in sterile saline (0.85% NaCl in distilled water) to a concentration,

Table 2 Tests and list of primers used in this study

Test	Primer name	Primer sequence	Reference
Effector genes detection	avrPto-F	5'-GAACTCCCCAGACCGAGTTA-3'	This study
	avrPto-R	5'-CCAGTGTCTTTGCGTATGTG-3'	
	avrD1-F	5'-CGATGGTTTCAAACATGTGG-3'	
	avrD1-R	5'-TGTGCGTGACTGACAAGTGA-3'	
	avrAE1-F	5'-GGAAGCATCATTGACCCAAC-3'	
	avrAE1-R	5'-GACTGTCAGGTGACCGGAAT-3'	
	hopA1-F	5'-CGGCAAGAGGTACGAGATTC-3'	
	hopA1-R	5'-TTCAATGCCTTTAGCGTGTG-3'	
	hopB1-F	5'-AGGCTATTATCCGCCAACCT-3'	
	hopB1-R	5'-TCTTGCAACAGGATGCTCAC-3'	
	hopC1-F	5'-TCTGGACACATCGGAAAACA-3'	
	hopC1-R	5'-GGAAGGGTAGGCCTGAGTTC-3'	
	hopD1-F	5'-GTTACTGAGCTCGCCAGACC-3'	
	hopD1-R	5'-TGGTGGCTACATGCAACAAT-3'	
	hopF2-F	5'-GGTAATATTTGCGGCACCTC-3'	
	hopF2-R	5'-AATCTCCCAGTCGCATTTTG-3'	
	hopG1-F	5'-TAATGCCTCGCCTAAGATGG-3'	
	hopG1-R	5'-AGCTCACCTTTCAGGCACAT-3'	
Effector genes detection	hopAN1-F	5'-GCGCACCGTGAGAGCAT-3'	Ferrante <i>et al.</i> , (2009)
	hopAN1-R	5'-GTCAGCGGGTGGTTCTGC-3'	
	hopAF1-F	5'-CAAGCAGAAAGACGGCATC-3'	
	hopAF1-R	5'-GCACACGCGACAGCAATG-3'	
	hrpK1-F	5'-GACARTGCCGACAAGGACK-3'	
	hrpK1-R	5'-ATCKGCGGTTTGCAGAGACT-3'	
rep-PCR	BOXA1R	5'-CTACGCGCAAGGCGACGCTGACG-3'	Louws <i>et al.</i> , (1994)
	ERIC1R	5'-ATGTAAGCTCCTGGGGATTAC-3'	
	ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3'	
MLST	GapA-F	5'-CGCCATYCGCAACCCG-3'	Sarkar & Guttman, (2004)
	GapA-R	5'-CCCAYTCGTTGTGCTACCA-3'	
	GyrB-F	5'-MGGCGGYAAGTTCGATGACAAYTC-3'	
	GyrB-R	5'-TRATBKCAGTCARACCTTCRCGSGC-3'	
	GltA-F	5'-AGTTGATCATCGAGGGCGCWGCC-3'	
	GltA-R	5'-TGATCGGTTTGATCTCGCACGG-3'	
	RpoD-F	5'-AAGGCGARATCGAAATCGCCAAGCG-3'	
	RpoD-R	5'-GGAACWKGCAGGAGTCGGCACG-3'	
Phaseolotoxin detection	PHA19	5'-CGTCTGTAAACAGTTGATCC-3'	dos Marques <i>et al.</i> , (2000)
	PHA95	5'-GAATCCTTGAATGCGAAGGC-3'	Prosen <i>et al.</i> , (1993)
	HM6	5'-CGTGTCCGTGGATAAAAGC-3'	Sawada <i>et al.</i> , (2002)
	HM13	5'-GTTGAATTTCACTACCCG-3'	
	OCTF-03	5'-GACCGTCAAGGAAGAATTCGGGCGC-3'	
	OCTR	5'-CGACCTTGTTGACCTCCCG-3'	
Coronatine detection	CFLF	5'-GGCGCTCCCTCGCACTT-3'	Bereswill <i>et al.</i> , (1994)
	CFLR	5'-GGTATTGGCGGGGTGC-3'	

photometrically adjusted, of  $1-2 \times 10^6$  CFU mL<sup>-1</sup>. Subsequently, 10 µL of the bacterial suspension were spotted onto NSA medium amended with the following compounds tested at the dose recommended by the manufacturer and proven as effective for the control of diseases in open field conditions: copper oxychloride (25 or 32% Cu content) at 300 g hL<sup>-1</sup>, copper oxychloride (35% Cu) at 200 g hL<sup>-1</sup>, copper hydroxide (7% Cu) at 80 mL hL<sup>-1</sup>, copper hydroxide (33% Cu) at 150 mg hL<sup>-1</sup>, copper sulphate (5.5 or 20% Cu) at 200 mL hL<sup>-1</sup>, Cu (7.6 or 15%) plus EDTA at 150 g hL<sup>-1</sup>, chitin extract plus zinc (0.05%) and boron (0.05%), grapefruit seed extract at

150 mL hL<sup>-1</sup> and garlic extract at 150 mg hL<sup>-1</sup>. The plates were incubated at 25–27°C. The growth of the bacterial colonies was checked up to 5 days after spotting onto NSA medium. After this period, the complete absence of growth was taken as indicative of the bactericidal activity of the compound tested. The experiments were replicated twice.

#### Sensitivity to antibiotics and terpenes

In order to verify the sensitivity to antibiotic and terpenes, the 101 *Psa* strains were also tested against kanamycin,

gentamycin sulphate, ampicillin, tetracycline, streptomycin sulphate, geraniol and citronellol. To determine the minimum bactericidal concentration (MBC), the antibiotics were added to NSA medium at a concentration of 100, 50, 25 or 10 mg L<sup>-1</sup>. Bacterial suspensions (10 µL at 10<sup>6</sup> CFU mL<sup>-1</sup>) were spotted onto the medium. The plates were incubated for 72 h at 25–27°C. The complete inhibition of bacterial growth onto NSA medium was considered as evidence of antibiotic sensitivity. To check the inhibitory activity of terpenes and to determine the MBC, the procedures described by Scortichini & Rossi (1991) were followed. Briefly, geraniol and citronellol were added to 523 liquid medium at a concentration of 25, 50, 100, 150 or 200 mg L<sup>-1</sup>. Dimethylsulphoxide was added to completely dissolve the terpenes in the liquid medium. Then, the Eppendorf tubes were inoculated with 200 µL of 1–2 × 10<sup>7</sup> CFU mL<sup>-1</sup> bacterial suspension and shaken with a vortex. The tubes were incubated at 25–27°C for 72 h. Each day, a loopful of the broth was streaked onto NSA medium to check the bacterial growth. In the absence of growth after 72 h a further sample from the MBC was determined.

## Results

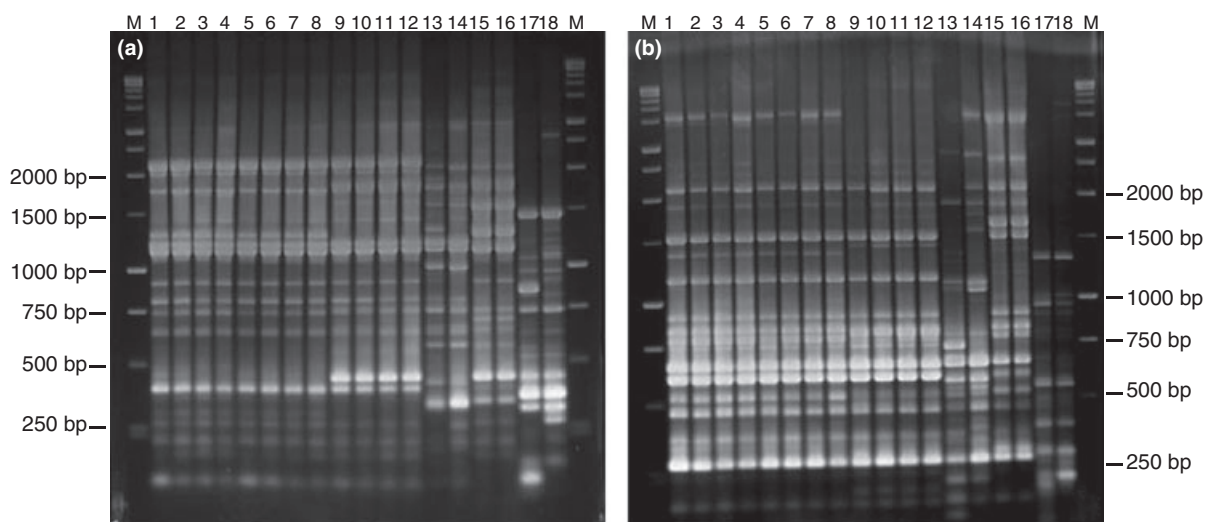
### Isolation and pathogenicity test

Isolations from diseased specimens of *A. chinensis* and *A. deliciosa* yielded 101 Psa isolates, from all plant organs affected by the disease (i.e. leaves, twigs, branches and

trunks). All isolated strains were levan-positive, oxidase-, potato soft rot- and arginine dehydrolase-negative, and induced the hypersensitive response on tobacco leaves after 24 h of infiltration (group Ia of LOPAT test). In addition, they did not produce fluorescent pigment on medium B of King *et al.* (1954) (KB) and were arbutin- and tyrosine-negative. The isolated strains caused the complete wilting of *A. chinensis* Hort16A plants 10–12 days after inoculation. They did not induce any symptoms on lemon fruits. The two *P. s. pv. syringae* strains obtained from leaf spotting on *A. chinensis* cv. Hort16A in central Italy in 2009 showed the same LOPAT response as Psa isolates, but produced fluorescent pigments on KB and were arbutin- and tyrosine-positive. In addition, they induced necrotic lesions on lemon fruits 7–8 days after inoculation. When inoculated onto *A. chinensis* twigs, they only caused a necrotic lesion to form surrounding the site of inoculation and never caused the wilting of the plant. During the study, potato soft rot-positive bacteria were never isolated.

### Rep-PCR and multilocus sequence typing

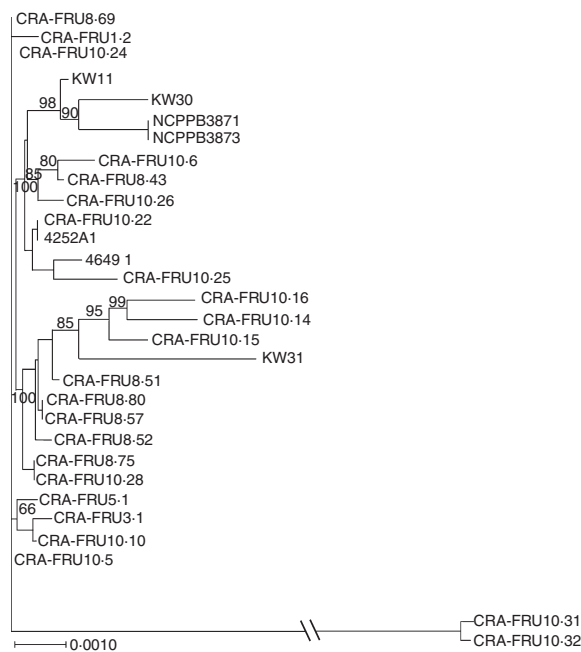
Confirmation of strain identity was also obtained by means of rep-PCR. All 101 Psa isolates obtained from *A. chinensis* and *A. deliciosa* isolated in central Italy during 2008–9 showed the same pattern profile upon rep-PCR performed using both BOX and ERIC primer sets. By contrast, these strains were slightly different from those isolated from past cases of bacterial canker



**Figure 1** Representative repetitive-sequence PCR fingerprint patterns for genomic DNAs of *Pseudomonas syringae* pv. *actinidiae* strains isolated from *Actinidia chinensis* and *A. deliciosa* during 2008–9 in various epidemics in central Italy (lanes 1–8) obtained by using BOX (a) and ERIC (b) primer sets. The strains were compared with *P. s. pv. actinidiae* strains previously isolated in Japan (lanes 9 and 10) and Italy (lanes 11 and 12) and with strains belonging to genomospecies 8, namely *P. avellanae* (lanes 13 and 14) and *P. s. pv. theae* (lanes 15 and 16), as well as with two *P. s. pv. syringae* strains obtained from *A. chinensis* (lanes 17 and 18). M: molecular size marker 1-kb DNA ladder (Promega). Lane 1: CRA-FRU 8-43; lane 2: CRA-FRU 3-1; lane 3: CRA-FRU 10-14; lane 4: CRA-FRU 10-15; lane 5: CRA-FRU 1-2; lane 6: CRA-FRU 8-52; lane 7: CRA-FRU 8-69; lane 8: CRA-FRU 8-75; lane 9: KW 11; lane 10: KW 30; lane 11: NCPPB 3871; lane 12: NCPPB 3873; lane 13: BPIC 631; lane 14: CRA-FRU PA 111; lane 15: SUPP 68; lane 16: NCPPB 2598; lane 17: CRA-FRU 10-31; lane 18: CRA-FRU 10-32 (see also Table 1).

occurring on *A. deliciosa* in Japan and Italy (Fig. 1). In particular, some differential PCR products occurred between 1000 and 1500 bp and between 250 and 500 bp with the BOX primers (Fig. 1a), and between 1000 and 1500 bp with the ERIC primers (Fig. 1b). Rep-PCR also revealed the genetic diversity of the two *P. s. pv. syringae* obtained from *A. chinensis* and the overall similarity of the other strains belonging to genomospecies 8, namely *P. avellanae* and *P. s. pv. theae*.

MLST of the four housekeeping genes revealed more variability among the *P. s. pv. actinidiae* strains than rep-PCR. The neighbour-joining dendrogram obtained from the concatenated data is shown in Fig. 2. No clear strain clustering was observed, even though the three strains isolated from *A. chinensis* cv. Jin Tao were very closely related among them. Remarkably, 22 Psa strains showed a distinct MLST sequence profile. However, it is worth noting that some other strains had matching sequences. In fact, two strains, one isolated in Latina province in 2008 (CRA-FRU 10-22) and one in Ravenna province in 2009 (4252 A.1), were a matching pair, as were some strains obtained in 2009 from *A. chinensis* cv. Hort16A and *A. deliciosa* cv. Hayward (i.e. CRA-FRU 8-57 matched CRA-FRU 8-80 and CRA-FRU 10-28 matched CRA-FRU 8-75). The two Psa strains isolated in Italy in 1992 were the same as each other, but different from the three strains isolated in Japan in 1984, as well as from those of the recent epidemics. The two *P. s. pv. syringae*



**Figure 2** Neighbour-joining dendrogram obtained with the concatenated fragments of the four housekeeping genes of *Pseudomonas syringae* pv. *actinidiae* strains used in multilocus sequence typing analysis, namely *gapA*, *gltA*, *gyrB* and *rpoD* (see also Table 1). Two *P. s. pv. syringae* strains (CRA-FRU 10-31 and 10-32) were used as outgroups. Bootstrap confidence values higher than 60 are presented at each node.

strains isolated from *A. chinensis* were very different from all the Psa strains. All 120 MLST sequences were deposited in the NCBI database, with the following accession numbers: *Psa-gapA*: FN651845–FN651852 and FN652922–FN651941; *Psa-gltA*: FN651799–FN651806 and FN652843–FN652862; *Psa-gyrB*: FN651827–FN651834 and FN652883–FN652902; *Psa-rpoD*: FN651791–FN651798 and FN652863–FN652882; *P. syringae* pv. *syringae*: FN652914–FN652921.

### Detection of effectors and phytotoxins

The detection of 12 effector proteins revealed one remarkable difference between Psa strains obtained in Italy during 2008–9 and the other Psa strains isolated in Japan and Italy in 1984 and 1992, respectively (Table 3). The effector *hopA1* was detected in the Psa strains isolated during 2008–9, but not in strains isolated from past epidemics of bacterial canker of kiwifruit in Japan and Italy (Fig. 3). The *P. s. pv. syringae* strains isolated from *A. chinensis* in 2009 did not show the presence of three effector genes, namely *avrD1*, *hopA1* and *hopD1* (Fig. 3).

None of the Psa strains isolated in central Italy from *A. chinensis* or *A. deliciosa* showed the presence of genes coding for phaseolotoxin or coronatine. The positive controls *P. s. pv. phaseolicola* 1448A, Psa KW11 (i.e. phaseolotoxin) and *P. s. pv. tomato* DC3000 (i.e. coronatine) showed the presence of PCR products corresponding to such genes (Fig. 4). Similarly to KW11, Psa NCPPB 3871 and NCPPB 3873, isolated in Italy in 1992 showed the presence of genes for phaseolotoxin but not for coronatine.

### Sensitivity to antimicrobial compounds

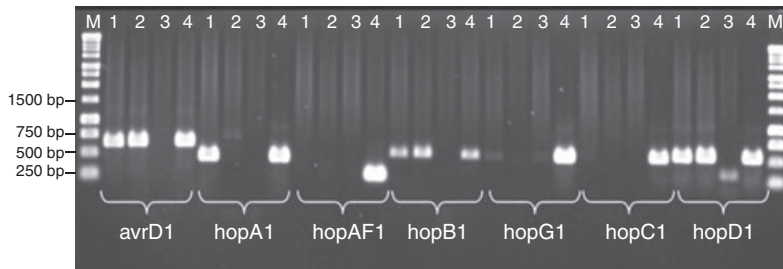
All copper-based compounds tested at the doses recommended by the manufacturers showed *in vitro* bactericidal activity by completely inhibiting the growth of all Psa strains. Among the organic compounds, only the chitin-based compounds (i.e. chitosan) inhibited the *in vitro* growth of all strains at 150 g hL<sup>-1</sup>. The garlic and grapefruit seed extracts did not show any bactericidal effect. All the antibiotics tested inhibited the *in vitro* growth of Psa strains. The MBC was 10 mg L<sup>-1</sup> for kanamycin, gentamycin sulphate, tetracycline and streptomycin sulphate, and 25 mg L<sup>-1</sup> for ampicillin. The two terpenes tested also showed an *in vitro* inhibitory effect towards Psa, with an MBC of 50 mg L<sup>-1</sup> for geraniol and 200 mg L<sup>-1</sup> for citronellol.

### Discussion

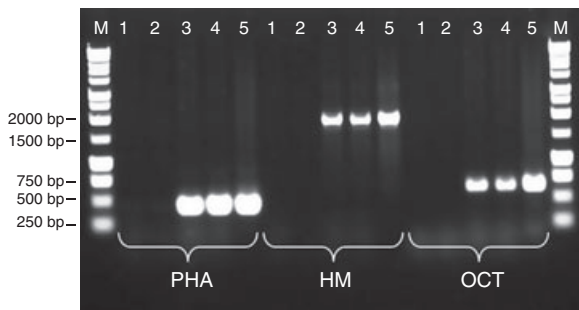
The genetic variability of Psa strains isolated during epidemics of bacterial canker on *A. chinensis* and *A. deliciosa* occurring in central Italy in 2008–9 was assessed by means of rep-PCR using BOX and ERIC primer sets, and MLST of four housekeeping genes, *gapA*, *gltA*, *gyrB* and *rpoD*. Rep-PCR did not reveal any differences among

**Table 3** Effector genes detection in *Pseudomonas syringae* pv. *actinidiae* strains isolated from *Actinidia deliciosa* and *A. chinensis*, and *Pseudomonas syringae* pv. *syringae* strains obtained during different epidemics in central Italy

Effector gene	<i>P. s.pv. actinidiae</i>		<i>P. s. pv. syringae</i>
	Italy 2008–2009 (this study)	Italy 1994 (Scortichini, 1994) Japan 1984 (Takikawa <i>et al.</i> , 1989)	( <i>A. chinensis</i> , 2009)
<i>avrPto1</i>	–	–	–
<i>avrD1</i>	+	+	–
<i>avrAE1</i>	+	+	+
<i>hopA1</i>	+	–	–
<i>hopB1</i>	+	+	–
<i>hopC1</i>	–	–	–
<i>hopD1</i>	+	+	–
<i>hopF2</i>	–	–	–
<i>hopG1</i>	–	–	–
<i>hrpK1</i>	+	+	+
<i>hopAF1</i>	–	–	–
<i>hopAN1</i>	+	+	+



**Figure 3** Representative gel for the detection of effector genes in *Pseudomonas syringae* pv. *actinidiae* strains isolated from *Actinidia chinensis* and *A. deliciosa* during 2008–9 (lane 1: CRA-FRU 8-43) and from *A. deliciosa* in Japan and Italy in 1984 and 1994 (lane 2: KW11). In the analysis were also included *P. s. pv. syringae* strains from *A. chinensis* isolated in 2009 (lane 3: CRA-FRU 10-31) and positive control strains, namely *P. s. pv. tomato* DC3000 (lane 4, effectors *hopA1*, *hopAF1*, *hopB1*, *hopG1*, *hopC1* and *hopD1*) and *P. s. pv. phaseolicola* 1448A (lane 4, effector *avrD1*). M: molecular size marker 1-kb DNA ladder (Promega).



**Figure 4** Representative gel for the detection gene fragments of phaseolotoxin in *Pseudomonas syringae* pv. *actinidiae* strains isolated from *Actinidia chinensis* and *A. deliciosa* in Italy during 2008–9 (lane 1: CRA-FRU 8-43; lane 2: 8-69), from *A. deliciosa* in Japan in 1984 (lane 3: KW 11) and Italy in 1994 (lane 4: NCPPB 3871) as well as from the positive control *P. s. pv. phaseolicola* 1448A (lane 5). Note that the *P. s. pv. actinidiae* strains isolated in central Italy during 2008–9 did not show the presence of such gene fragments. M: molecular size marker 1-kb DNA ladder (Promega).

these strains even though they could be clearly discriminated from Psa strains previously isolated from *A. deliciosa* in Japan and Italy, as well as from two *P. s. pv.*

*syringae* strains isolated from leaf spotting occurring on *A. deliciosa* in 2009. This confirmed once more the overall similarity of the phytopathogens belonging to the genomospecies 8 *sensu* Gardan *et al.* (1999), namely Psa, *P. avellanae* and *P. s. pv. theae*.

MLST revealed a higher genetic variability within the Psa strains than rep-PCR, even though the overall similarity among the strains was still high. In MLST analysis, the Psa strains previously isolated from *A. deliciosa* in Japan and Italy gave different results from those obtained recently in central Italy. Interestingly, some Psa strains obtained in different years from different areas of *A. chinensis* cultivation (i.e. central and northern Italy) or isolated from different *Actinidia* species, were identical in the total length of the four gene fragments analysed.

When all results obtained by means of rep-PCR and MLST are assessed it is clear that (i) the epidemics occurring in central Italy on *A. chinensis* and *A. deliciosa* during 2008–9 were caused by a different population of Psa than those previously found in Japan (Takikawa *et al.*, 1989) and Italy (Scortichini, 1994), (ii) strains of this new population can cross-infect either the *A. chinensis* cvs Hort16A, Jin Tao and CK3 or *A. deliciosa* cv. Hayward, (iii) such a population is currently present in central Italy

(Latina province) and in at least one site of northern Italy (Ravenna province), and (iv) the two *P. s.* pv. *syringae* strains isolated from *A. chinensis* gave quite different results from Psa in the tests performed herein.

The diversity of the Psa population currently causing severe damage to *A. chinensis* and *A. deliciosa* was confirmed by the detection of genes coding for phaseolotoxin, coronatine and effector proteins. In fact, none of the strains isolated in Italy during 2008–9 possessed gene fragments of the *argK-tox* cluster coding for the phaseolotoxin. By contrast, such gene fragments were present in the strains previously isolated in Japan and Italy (Takikawa *et al.*, 1989; Scortichini, 1994). In addition, the strains recently isolated in Italy did not show the presence of the gene coding for coronatine, as was found for the strains isolated in South Korea from *A. deliciosa* (Han *et al.*, 2003a). These findings would apparently indicate that the epidemics recently observed in Italy on *A. chinensis* and on *A. deliciosa* were caused by a population of the pathogen apparently also different from that previously found in South Korea (Koh *et al.*, 1994).

This study also ascertained that one effector gene, namely *hopA1*, is present in all Psa strains recently causing epidemics in central Italy to both *A. chinensis* and *A. deliciosa*, but absent from the strains previously isolated in Japan and in Italy (Takikawa *et al.*, 1989; Scortichini, 1994). However, the effector pattern for the other 11 proteins was identical in all these strains. The two strains of *P. s.* pv. *syringae* obtained from *A. chinensis* matched only three of the effector genes present in *P. s.* pv. *actinidiae*.

It cannot be established with certainty from which inoculum source the recent epidemics of bacterial canker on *A. chinensis* originated. The possibility that the inoculum infecting the *A. deliciosa* plants in 1992 (Scortichini, 1994) remained in the area and infected *A. chinensis* is not supported by the molecular typing of the recently isolated strains. Alternatively, this pathogen could have been introduced via apparently healthy propagative plant material, but clear evidence supporting this hypothesis is currently lacking. From the surveys performed, it seems that the epidemics started after frosts occurring in many areas of *A. chinensis* cultivation in November 2007 and February 2008. Subsequently, the exudates oozing out of the branches and trunks could have been displaced by the rain and wind frequently occurring during spring. Moreover, hail storm events would also have contributed to induce wounds along the branches and the trunk. These factors could explain the spread of the disease over a large area.

This study also revealed that, so far, all the Psa strains collected in Italy during the epidemics of 2008–9 are not resistant to copper-based or antibiotic compounds. In fact, *in vitro* tests clearly showed that all the strains were completely inhibited by such substances. It is, however, worth remembering that this pathogen can acquire resistance to both copper and streptomycin, as previously demonstrated in Japan and South Korea (Goto *et al.*, 1994; Han *et al.*, 2003b). Furthermore, the present study

found that Psa is also sensitive to two terpenes, geraniol and citronellol, with MBCs of 50 and 200 mg L<sup>-1</sup>, respectively. These natural compounds were previously found to inhibit the *in vitro* growth of *Erwinia amylovora*, another destructive bacterial plant pathogen (Scortichini & Rossi, 1991).

Interestingly, it was also found that an organic compound, chitosan, obtained from the shell of shrimps and other sea crustaceans, supplemented with 0.05% boron and 0.05% zinc, behaved like the copper-based compounds *in vitro* by inhibiting the growth of the bacterium. Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, and is commonly used as a plant growth enhancer. It is known that chitosan can control fungal infections (Linden *et al.*, 2000). This is apparently the first report of the *in vitro* effectiveness of chitosan towards a plant pathogenic bacterium. Field experiments using chitosan are under way to evaluate the possibility of effectively limiting the spread of bacterial canker on *Actinidia* spp. in central Italy.

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