Identification of *Pseudomonas syringae* pv. *actinidiae* strains causing bacterial canker of kiwifruit in the Anhui Province of China, and determination of their streptomycin sensitivities

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ABSTRACT. Bacterial canker, caused by *Pseudomonas syringae* pv. *actinidiae*, is one of the most severe diseases of kiwifruit. It has become an international pandemic and threatens the sustainable development of kiwifruit production in all main kiwi-growing regions worldwide. Streptomycin has been the major bactericide for the control of kiwifruit canker, especially in Anhui Province, one of the main kiwifruit

production regions in China. However, until now, no studies on the baseline sensitivity to streptomycin of field isolates of *P. syringae* pv. *actinidiae* from China have been available. During 2012-2013, a total of 102 single-colony *P. syringae* pv. *actinidiae* strains were isolated: 36, 12, 13, 26, and 15 strains from Yuexi, Jinzhai, Huoshan, Qianshan, and Taihu counties, respectively. All strains were confirmed by production of a 280-bp fragment using the specific primers PsaF1/R2 upon polymerase chain reaction amplification, followed by an assay for confirmation of pathogenicity to fulfill Koch's postulates. In this study, the streptomycin sensitivity of the 102 isolated strains was determined. The half-maximal effective concentration values for inhibition of growth by streptomycin were 0.03-0.42 µg/mL (average 0.12 ± 0.06 µg/mL). The baseline sensitivity curve was unimodal, representing range-of-variation factors of 14.0. No resistant subpopulation was identified among the strains used in the study. Thus, these sensitivity data could be used as a baseline for monitoring the shift in sensitivity of *P. syringae* pv. *actinidiae* populations to streptomycin in Anhui Province. Continuous resistance monitoring should be carried out, as streptomycin is an at-risk bactericide agent.

Key words: *Pseudomonas syringae* pv. *actinidiae*; Specific PCR; Streptomycin; Baseline sensitivity; Detection of kiwifruit bacterial canker

INTRODUCTION

Pseudomonas syringae pv. *actinidiae* is the causal agent of kiwifruit canker, which causes leaf spots, dieback, and canker symptoms on kiwifruit (Scortichini, 1994; Chapman et al., 2012), and has been shown to damage kiwifruit production worldwide (Chapman et al., 2012). According to previous reports, this disease was first reported in Asian countries such as Japan (Takikawa et al., 1989), the Hunan Province of China (Fang et al., 1990), and Korea (Koh et al., 1994), and was subsequently identified in European countries such as Italy (Ferrante and Scortichini, 2009), Portugal (Balestra et al., 2010), France (Vanneste et al., 2011a), Spain (Abelleira et al., 2011), Switzerland (EPPO, 2011), and Turkey (Bastas and Karakaya, 2012). Historically considered a minor disease, *P. syringae* pv. *actinidiae* rarely caused severe damage to kiwifruit until 2008, when a large outbreak occurred in Italy, and severe infections continue to spread to other countries (Mazzaglia et al., 2011; Chapman et al., 2012; Balestra et al., 2009, 2013; Vanneste et al., 2013). In Italy, the bacterium has been shown to cause severe disease symptoms, including leaf wilting; twig or cane dieback; cankers on canes, leaders, and trunks; plant wilting; and the production of an oozing red or white exudate (Ferrante and Scortichini, 2010; Bull et al., 2011). After just a few years, this disease has become an international pandemic and is threatening the sustainable development of kiwifruit production in all the main kiwi-growing regions worldwide. Under favorable conditions for an epidemic of this disease, *P. syringae* pv. *actinidiae* can destroy an entire kiwi tree within a few months.

The disease is very difficult to control because of its extensive distribution, severe effects, broad host base, and high pathogenicity, resulting in serious yield and economic losses. As the major kiwifruit cultivars *Actinidia deliciosa* and *Actinidia chinensis* are both very sus-

ceptible to this disease, *P. syringae* pv. *actinidiae* can affect all of the commercial cultivars of kiwifruit, such as the green-fleshed kiwifruits *A. deliciosa* 'Hayward' and 'Summer Kiwi', and the yellow-fleshed kiwifruits *A. chinensis* 'Jin Tao', 'Hort16A', and 'Soreli'. This also leads to limited opportunities to screen for new resources conferring resistance to this disease. Furthermore, because little is known about natural hosts, the infection process, and colonization sites of the pathogen, it is still not clear what the pathogenic mechanism is, resulting in high costs and poor efficiency of control. Therefore, the application of bactericides becomes an indispensable complementary tool, especially for regions planting susceptible cultivars (Xu et al., 2010). During a survey, we found that most kiwifruit growers sprayed bactericides to control this disease. Streptomycin is the most commonly used bactericide used to control kiwifruit bacterial canker in China (Xu et al., 2010). Streptomycin is an aminoglycoside antibiotic and has been widely used in the treatment of human and animal bacterial diseases (Sundin and Bender, 1993). Since it was introduced for crop protection in 1955, streptomycin has been the major antibiotic for controlling plant diseases caused by Gram-negative bacteria such as those causing fire blight (*Erwinia amylovora*), soft rot diseases of cut flowers and potato seed pieces (*Pectobacterium* spp formerly *Erwinia* spp), fruit-spotting or blossom-blast symptoms on apple, pear, and related landscape trees (*P. syringae*), and bacterial spot of pepper and tomato (*Xanthomonas campestris* pv. *vesicatoria*) (McManus et al., 2002). China is one of the main kiwifruit-production regions worldwide and canker has been the most effective disease limiting production of kiwifruit in this country. However, until now, no studies on the baseline sensitivity to streptomycin of field isolates of *P. syringae* pv. *actinidiae* from China have been available. This provides an opportunity to study the baseline sensitivity of *P. syringae* pv. *actinidiae* strains to streptomycin, although it has already been used for control of this disease in China.

Establishment of the baseline sensitivity is a useful tool for monitoring sensitivity shifts and effectiveness of resistance management strategies, and is one of the major tasks undertaken by scientists working in the chemical control of pests, forming a significant part of the registration process for pesticides (Russell, 2004). The objectives of this study were to: i) identify the pathogen by polymerase chain reaction (PCR) amplification of the 16S rDNA region, and ii) establish the baseline sensitivity to streptomycin of *P. syringae* pv. *actinidiae* strains that were never exposed to this bactericide.

MATERIAL AND METHODS

Collection of plant samples and isolation of *P. syringae* pv. *actinidiae*

The leaves, twigs, and canes of kiwifruit with symptoms of bacterial canker were collected from the major growing regions for kiwifruit of Anhui Province, China, including Yuexi, Jinzhai, Huoshan, Qianshan, and Taihu counties during 2012-2013.

Bacterial strains were isolated from leaves, twigs, or canes using the following methods: leaf samples were isolated as described by Vanneste et al. (2011b) with slight modification. Briefly, 100 µL washing solution was obtained by vigorously rubbing each leaf for 2 min in 10 mL sterile distilled water and plated on nutrient agar (NA) medium containing 1% cycloheximide at 5 mL/L. NA medium consisted of 5 g polypeptone, 1 g yeast powder, 3 g beef extract, 10 g sucrose, and 17 g/L agar, with the pH adjusted to 7.0 with 1 M NaOH. Small sections of twig or cane tissue were teased out in sterile water and the suspension was spread

onto NA plates supplemented with cycloheximide. After 28 h incubation at 28°C, *P. syringae* pv. *actinidiae*-like colonies were purified by picking up single colonies. One single-colony isolate of *P. syringae* pv. *actinidiae* was obtained from each leaf, twig, or cane, yielding a total of 102 single-colony strains (Table 1). A standard *P. syringae* pv. *actinidiae* strain was kindly provided by Prof. Gen-Jia Tan (Anhui Agricultural University), which was used as a positive control. Bacterium identification was based on colony morphology and was confirmed by sequencing the 16S rDNA and by a pathogenicity assay.

Identification of *P. syringae* pv. *actinidiae* by PCR

Total DNA from each of the collected strains was isolated according to Zhang et al. (2011) with slight modifications. Briefly, after the bacteria were grown in NB (NA minus agar) at 28°C for 24 h, a bacterial suspension aliquot (1.5 mL) was centrifuged at 12,000 g for 10 min. The pellet was resuspended in 567 μL TE buffer [10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0]. Proteinase K and sodium dodecyl sulfate were added to final concentrations of 100 μg/mL and 5 g/L, respectively. After incubation for 1 h at 37°C, sodium chloride and hexadecyltrimethylammonium bromide were added to each preparation to final concentrations of 0.7 M and 10 g/L, respectively. The preparations were incubated at 65°C for 10 min, and DNA was extracted with chloroform/isoamyl alcohol (24:1 by volume). Samples were shaken for 10 min and centrifuged at 12,000 g for 20 min. DNA was re-extracted with phenol/chloroform/isoamyl alcohol (25:24:1 by volume) and centrifuged as described above. DNA was precipitated by adding 0.6 volumes isopropanol and incubating at -20°C for 30 min. Samples were centrifuged at 12,000 g for 20 min, and the pellets were washed with 1 mL 70% ethanol and centrifuged again. The DNA was dried at room temperature, and the pellet was then re-dissolved in 50 μL sterilized ultrapure water and stored at -20°C.

Based on the conserved 16S-23S rDNA intertranscribed spacer (ITS) region of *P. syringae* pv. *actinidiae*, the PCR primers *Psa*F1 5' - TTT TGC TTT GCA CAC CCG ATT TT - 3' and *Psa*R2 5' - CAC GCA CCC TTC AAT CAG GAT G - 3' were used for specific amplification of the target fragment from *P. syringae* pv. *actinidiae* by PCR, according to previous report (Rees-George et al., 2010). PCR mixtures contained total DNA, 1.5 mM MgCl$_2$, 200 μM of each dNTP, and 2.5 units *Taq* DNA polymerase. PCRs were performed in a DNA Engine System PT-200 (Bio-Rad, Watertown, MA, USA) programmed for 2.5 min at 94°C, followed by 30 cycles of 0.5 min at 94°C, 1 min at 52°C, 1.5 min at 72°C, a final extension for 8.5 min at 72°C, and a 4°C hold. PCR products were separated by electrophoresis on 1.5% agarose gels in 40 mM Tris-acetate, pH 8.0, and 1 mM EDTA (TAE) buffer. The PCR products were purified using a DNA Gel Extraction Kit (AxyPrep™, Corning, Hangzhou, China) according to manufacturer instructions, and sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

Pathogenicity assay

A suspension containing the bacterial strain to be tested at approximately 10⁹ CFU/mL was made in water from colonies freshly grown on NA plates. These suspensions were sprayed on the abaxial side of unwounded leaves. At least six 3 to 4-month-old seedlings were

used per assay. All fully developed leaves were inoculated. When the assays were carried out on 1-year-old *A. chinensis* 'Jintao' plants, 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, the necrotic spots on each leaf were observed and the *P. syringae* pv. *actinidiae* strain was re-isolated as described above.

Baseline streptomycin sensitivity of *P. syringae* pv. *actinidiae* strains from Anhui Province

The sensitivities of *P. syringae* pv. *actinidiae* strains to streptomycin were determined based on the *in vitro* inhibition of bacterial growth in NB medium (Li et al., 2006; Xu et al., 2010). Briefly, each of the bacterial strains was grown in NB at 28°C until the late logarithmic growth phase, and then the suspension was diluted to a density of approximately 10^7 CFU/mL. Aliquots (100 μL) of the suspension were added to 25 mL NB medium in 50-mL Erlenmeyer flasks containing various concentrations of streptomycin (0, 0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, or 1.28 μg/mL). The inoculated flasks were placed on an orbital shaker (28°C, 170 rpm) and the density of the flasks containing 0 and 0.08 μg/mL streptomycin was checked at each 12-h interval. When the concentration of bacterial suspension in the control flask reached approximately 10^8 CFU/mL, the values of optical density at 600 nm ($OD_{600\,nm}$) of the bacterial suspensions in all flasks were measured with a nephelometer (WCY-WOG, Baoli, Beijing, China). Each treatment concentration was prepared in triplicate and the experiment was performed twice. A regression equation was derived by correlating the log of inhibitor concentration of streptomycin and the probit of inhibition percentage of the average OD value of *P. syringae* pv. *actinidiae* strains. The effective concentration for 50% inhibition (EC_{50}) of *P. syringae* pv. *actinidiae* strains was calculated from the regression equation for each of the 102 strains. A histogram of EC_{50} values was plotted for all isolates, and the shape of the frequency distribution was analyzed by examining the EC_{50} histogram regarding curve shape, range, and mean values, as well as the ratio of the highest to lowest values.

Data analysis

Data from repeated experiments were combined for analysis because variances between experiments were homogeneous. All data were processed with the SIGMASTAT Statistical Software Package (SPSS Science, version 11, Chicago, IL, USA). EC_{50} values were calculated from the sensitivity tests described above from the fitted regression line of the log-transformed percent inhibition values plotted against the log-transformed bactericide concentrations (Chen et al., 2012).

RESULTS

Isolation and identification of *P. syringae* pv. *actinidiae* from different regions of Anhui Province

A total of 102 single-colony strains were isolated for this study; 36, 12, 13, 26, and 15 strains were from Yuexi, Jinzhai, Huoshan, Qianshan, and Taihu counties, respectively (Table

1). The overall isolation frequencies were 81.8, 42.86, 46.43, 72.22, and 12.20%, respectively, suggesting that kiwifruit canker outbreaks were more severe in Yuexi and Qianshan than in the other sampled counties during 2012-2013. This was also consistent with our previous survey in these counties (unpublished data), as the weather conditions in these mountain areas (Yuexi and Qianshan) are favorable for kiwifruit canker epidemics.

All the generated *P. syringae* pv. *actinidiae*-like strains showed 100% similarity to the standard *P. syringae* pv. *actinidiae* strain and the previous published strains as determined by National Center for Biotechnology Information (NCBI) blasting against 16S rDNA sequences (Figure 1), and all strains yielded a 280-bp fragment after PCR amplification using the primers PsaF1/R2, which distinguish *P. syringae* pv. *actinidiae* from other bacteria found on kiwifruit (Figure 2). To confirm that the strains were *P. syringae* pv. *actinidiae*, strains were inoculated onto young *A. chinensis* 'Jintao' seedlings. All strains induced small angular necrotic leaf spots, similar to those seen in seedlings infected by the standard *P. syringae* pv. *actinidiae* strain, and from which *P. syringae* pv. *actinidiae* could readily be re-isolated. Together, these results confirmed the 102 isolates to be *P. syringae* pv. *actinidiae*.

Table 1. Collection and isolation of *Pseudomonas syringae* pv. *actinidiae* strains in five counties of Anhui Province from 2012 to 2013.

Lacation	Sample year		Overall (by location)
	2012[a]	2013	
Yuexi	17 (21)	19 (23)	36 (44)
Jinzhai	6 (15)	6 (13)	12 (28)
Huoshan	7 (16)	6 (12)	13 (28)
Qianshan	12 (22)	14 (14)	26 (36)
Taihu	9 (22)	6 (19)	15 (41)

[a]The number of *Pseudomonas syringae* pv. *actinidiae* strains isolated is listed and the total number of samples collected is given in parentheses.
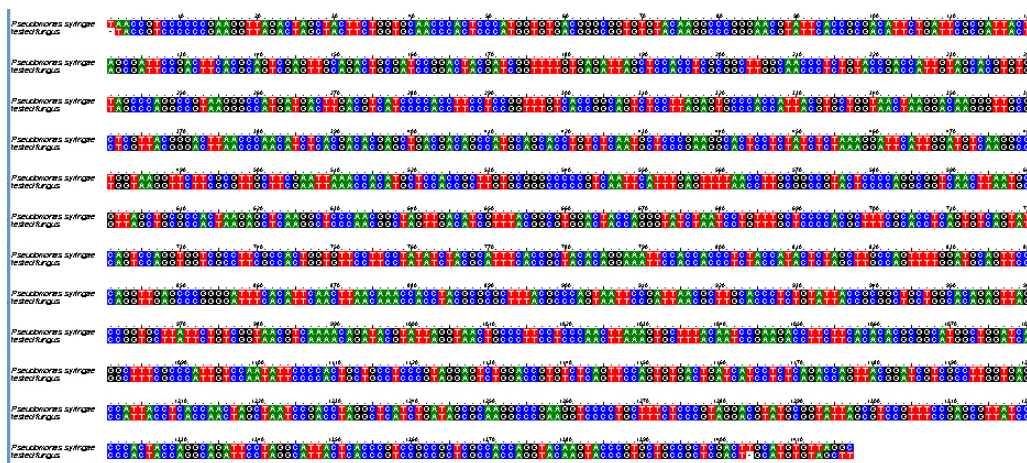

Figure 1. Similarity analysis of the isolated *Pseudomonas syringae* pv. *actinidiae* strain with previous *Pseudomonas syringae* pv. *actinidiae* strain by blasting in NCBI with the partial 16s rDNA (GenBank accession No.: JQ957916.1).

Figure 2. Specific detection of *Pseudomonas syringae* pv. *actinidiae* strain with a 280-bp fragment. Marker: DL2000; *lane 1 = Pseudomonas syringae* pv. *actinidae*; *lane 2 = Pseudomonas syringae* pv. *glycinea*; *lane 3 = Pseudomonas syringae* pv. *tomato*; *lane 4 = Pseudomonas aeruginosa*; *lane 5 = Pseudomonas fluorescens*; *lane 6 = Escherichia coli*; *lane 7 = Bacillus subtilis*; *lane 8 = Agrobacterium tumefaciens*; *lane 9 = Xanthomonas oryzae pv. oryzicola*; *lane 10 = Xanthomonas oryzae* pv. *oryzae*; *lane 11 = Acidovorax avenae* subsp *Citrulli*; *lane 12 = Corynebacterium michiganensis*; *lane 13 = Pectobacterium stewartii*; *lane 14 = Pectobacterium amylovora*; *lane 15 = Pectobacterium carotovora*; lane 16 = *Claribacter michiganense* subsp *Sepedonicum*; *lane 17 = Liberobacter asianticum*; *lane 18 = Ralstonia solanacearum*; *lane 19 = Curtobacterium flaccumfaciens*; *lane 20 = Agrobacterium rhizogenes*; *lane 21 = Pantoca stewartii*; *lane 22 = Pesudomonas syringae* pv. *lachrymans*; *lane 23 = H$_2$O*.

Baseline streptomycin sensitivity of *P. syringae* pv. *actinidiae* strains from Anhui Province

The sensitivity to streptomycin of the 102 *P. syringae* pv. *actinidiae* strains isolated from Anhui Province was tested. The EC$_{50}$ values for streptomycin inhibition of bacterial growth were 0.03-0.42 µg/mL (average EC$_{50}$ value 0.12 ± 0.06 µg/mL) (Figure 3). No evidence was found for geographical variation in the sensitivity of *P. syringae* pv. *actinidiae* to streptomycin, and the sensitivity of the tested isolates remained unchanged from 2012 to 2013 (data not shown). The baseline sensitivity curve was unimodal (Figure 1), representing range-of-variation factors of 14.0. No resistant subpopulations were identified among the strains used in the study. Thus, these sensitivity data are suitable for use as a baseline for monitoring the shift of sensitivity of *P. syringae* pv. *actinidiae* populations to streptomycin in Anhui Province.
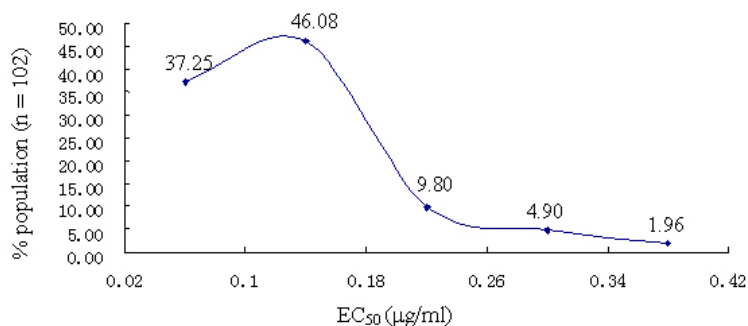

Figure 3. Frequency distribution of sensitivity (baseline sensitivity) of 102 *Pseudomonas syringae* pv. *actinidiae* strains to streptomycin.

DISCUSSION

Kiwifruit canker is an internationally important disease, as it occurs in most of the kiwifruit-growing regions worldwide and causes severe damage to kiwifruit production. Identification of its pathogen, *P. syringae* pv. *actinidiae*, is very useful for the understanding of this disease. Specific PCR primers for one-step identification of *P. syringae* pv. *actinidiae* had not been provided in early studies (Sawada et al., 1997; Koh and Nou, 2002; Scortichini et al., 2002) and were not available until recently (Rees-George et al., 2010). PsaF1/R2 and PsaF3/R4 were designed to be complementary to a portion of the 16S-23S rDNA ITS region, and were shown to amplify a DNA fragment from strains of *P. syringae* pv. *actinidiae*, but not from 56 strains of bacteria from six genera and 17 species (Rees-George et al., 2010), permitting quick identification of the pathogen isolated from kiwifruit tissues. Although PsaF1/R2 and PsaF3/R4 were able to distinguish *P. syringae* pv. *actinidiae* from other pseudomonads isolated from kiwifruit orchards, and from a number of other bacteria, these primers were not able to distinguish *P. syringae* pv. *actinidiae* from *P. syringae* pv. *theae*, a strain of the tea pathogen. However, because *P. syringae* pv. *theae* is unlikely to be found on kiwifruit, primers PsaF1/R2 and PsaF3/R4 are recommended for screening bacteria isolated from kiwifruit tissue.

In this study, we used the primers PsaF1/R2 for specific amplification of the 280-bp fragment from the isolated *P. syringae* pv. *actinidiae*-like strains from kiwifruit. As expected, each of the *P. syringae* pv. *actinidiae*-like strains yielded the 280-bp fragment. To further confirm their identity as *P. syringae* pv. *actinidiae* strains, we performed a pathogenicity assay to fulfill Koch's postulates. All strains caused small angular necrotic leaf spots, similar to those seen in infected orchards, and from which *P. syringae* pv. *actinidiae* could readily be re-isolated, suggesting that the isolated bacteria were *P. syringae* pv. *actinidiae*.

Chemical control remains an important tool for the control of kiwifruit canker, because most cultivars planted in Anhui Province are susceptible to this disease. Streptomycin has been the most commonly used bactericide for the control of kiwifruit canker in China for decades. To sustainably control this disease, it is necessary to establish the baseline sensitivity of *P. syringae* pv. *actinidiae* strains to streptomycin, which would give us an important database to understand its activity and monitor its possible sensitivity shifts after years of application. In this study, the baseline sensitivity of 102 *P. syringae* pv. *actinidiae* strains to streptomycin was established by a growth-inhibition assay. The results showed that the EC_{50} values for streptomycin ranged from 0.03 to 0.42 µg/mL and exhibited a unimodal frequency distribution. As none of these strains had ever been exposed to streptomycin, these sensitivity data could be used as baseline sensitivity measure for monitoring any future changes in sensitivity to streptomycin in *P. syringae* pv. *actinidiae* populations in the Anhui Province of China.

Taken together, these results suggest that streptomycin is an appropriate tool for the management of kiwifruit canker. Continuous resistance monitoring of *P. syringae* pv. *actinidiae* populations to streptomycin should be carried out since it is an at-risk bactericide (Xu et al., 2010). In addition, a mixture of streptomycin with other groups of bactericides such as copper-based bactericides (bismerthiazol, thiram, etc.) should be applied to avoid or delay the development of resistance.

In summary, this is the first report of the baseline sensitivity of *P. syringae* pv. *actinidiae* strains to streptomycin, providing us with an important database to understand its activity and monitor its possible sensitivity shift after additional years of application. In addition, the PCR results revealed that the primers PsaF1/R2 are suitable for specific detection of *P. syringae* pv. *actinidiae*.

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