



## Identification of *strA-strB* Genes in Streptomycin-Resistant *Pseudomonas syringae* pv. *actinidiae* Biovar 2 Strains Isolated in Korea

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**Bacterial canker is a devastating disease of kiwifruit caused by the bacterium *Pseudomonas syringae* pv. *actinidiae*. Canker disease of kiwifruit in Korea has been controlled using streptomycin for more than two decades. Four streptomycin-resistant strains, belonging to biovar 2, which are found only in Korea, were collected between 2013 and 2014 from different orchards located in Jeju, Korea. The genetic background for streptomycin resistance among *P. syringae* pv. *actinidiae* strains were determined by examining the presence of *strA-strB* or *aadA*, which are genes frequently found in streptomycin-resistant bacteria, and a point mutation at codon 43 in the *rpsL* gene. All four streptomycin-resistant strains of *P. syringae* pv. *actinidiae* investigated in this study contained *strA-strB* as a resistant determinant. The presence of the *aadA* gene and a mutation in codon 43 of the *rpsL* gene was not identified.**

**Keywords :** bacterial canker, kiwifruit, *Pseudomonas syringae* pv. *actinidiae*, streptomycin resistance

Bacterial canker caused by *Pseudomonas syringae* pv. *actinidiae* (Psa) is a destructive disease of kiwifruit plants that has resulted in severe economic losses worldwide (Van-neste, 2017). Currently, the Psa population is divided into

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five subgroups called biovar 1, 2, 3, 5, and 6 (Sawada et al., 2016). However, evidence for the existence of a significant number of different populations within Psa has recently been reported (Mazzaglia et al., 2021). The strains belonging to biovar 1 were initially isolated from Japan and Italy in 1984 and 1992, respectively (Scortichini, 1994; Takikawa et al., 1989). Biovar 2 strains have been isolated only in Korea and cause severe economic losses on both green (*Actinidia deliciosa*) and yellow (*A. chinensis*) fleshed kiwifruit (Koh et al., 1994, 2010). The third population, biovar 3, which was first isolated in Italy in 2008 (Ferrante and Scortichini, 2010), has recently been responsible for a worldwide Psa pandemic (Balestra et al., 2013; Chapman et al., 2012; McCann et al., 2017). The biovar 5 and 6 strains were reported to occur in a limited region of Japan (Sawada et al., 2014, 2016). In Korea, biovar 2 or biovar 3 strains have been isolated from orchards infected with Psa (Lee et al., 2017).

Bacterial disease in plants has been controlled using chemical treatment methods and non-chemical strategies, such as cultural control practices, orchard management, activating the plant immune system, and breeding resistant varieties (Cameron and Sarojini, 2014). One of the chemical methods used to control bacterial pathogens in the field is spraying or a trunk injection of streptomycin (Koh et al., 2017). Streptomycin is an aminoglycoside antibiotic that inhibits protein synthesis in prokaryotes and has been used for agricultural purposes since the late 1950s (Sundin and Wang, 2018). The use of streptomycin for the control of bacterial pathogens on crops is legal in Asian countries, New Zealand, Israel, Canada, Mexico, and the United States, but its use in plant agriculture is prohibited in the European Union (Cameron and Sarojini, 2014). Streptomycin has been used since the 1990s to control bacterial canker of kiwifruit in Korea, the only country where the biovar 2 strain is isolated (Mazzaglia et al., 2012). Therefore, the

appearance of the streptomycin resistance trait in biovar 2 means that it is the result of adaptation that occurred in Korea.

Most known streptomycin resistance determinants in bacteria encode enzymes that modify streptomycin by phosphorylation or adenylation. The *strA*, *strB*, and *aadA* genes are the three most widely distributed streptomycin resistance determinants (Sundin and Wang, 2018). Two phosphotransferase genes, *strA* and *strB*, which encode aminoglycoside-3"-phosphotransferase and aminoglycoside-6-phosphotransferase, respectively, were often linked to form a tandem gene pair, *strA-strB*. This gene pair is distributed worldwide among diverse gram-negative bacteria isolated from plants, animals, humans, and diverse environment samples (Petrova et al., 2008; Sundin and Bender, 1996; Tancos et al., 2016; van Overbeek et al., 2002). Additionally, the gene encoding an aminoglycoside adenylyltransferase, *aadA*, has been associated with streptomycin resistance in many clinical and phytopathogenic bacteria (Clark et al., 1999; Sundin and Wang, 2018; Xu et al., 2013).

Another resistance mechanism involves a point mutation at the target site of streptomycin. Streptomycin binds to the S12 protein, which is encoded by the *rpsL* gene, of the 30S ribosomal subunit, thereby inhibiting protein synthesis. A change in nucleotides at codon 43 of the *rpsL* gene from AAA, which encodes lysine in streptomycin-sensitive *E. amylovora* strains, to AGA (arginine), AAT/C (asparagine), or ACA (threonine) conferred a resistance phenotype to the antibiotic (Cameron and Sarojini, 2014; Chiou and Jones, 1995; Sundin and Wang, 2018).

The objective of this study was to identify the mechanism of streptomycin resistance in Psa biovar 2 strains isolated in Korea. Two possibilities for the resistance mechanism, enzymatic inactivation of the antibiotic and target site modification, were investigated.

The Psa strains used in this work were isolated between

2013 and 2014 from symptomatic kiwifruit plants in orchards located in Jeju, Korea (Table 1). The samples were surface sterilized using sodium hypochlorite, rinsed with sterile water, cut into small sections, and incubated in sterile water for 10 min with agitation. The suspensions were spread over peptone sucrose medium (PS; 20 g peptone, 20 g sucrose, and 15 g agar per liter), and the plates were incubated at 28°C for 24-48 h. The bacterial colonies were picked and transferred to PS agar to verify the pathogen. Identification of Psa and determination of biovars were conducted, as described previously (Lee et al., 2017). The strains identified as Psa were assessed for streptomycin resistance by streaking onto PS agar supplemented with 100 µg/ml of streptomycin. Streptomycin resistance was evaluated by observing the colonies on streptomycin-amended plates. Four streptomycin-resistant Psa strains were finally selected from different orchards, all of which belonged to biovar 2. Streptomycin-susceptible Psa strain JJ18 was included as a negative control.

The presence of *strA*, *strB*, and *aadA* genes in the four streptomycin-resistant strains was investigated by PCR using the primers developed by van Overbeek et al. (2002). Two primer pairs, aph(3")-F/R and aph(6)-F/R, targeting two aminoglycoside phosphotransferase genes, were used for PCR amplification. A primer set, strA-F and strB-R, designed from *strA* and *strB*, respectively, were used to determine the existence of a linked *strA-strB* structure (Palmer et al., 1997). The *aadA* gene was searched using the primer set, ant(3")-F/R. A pair of primers, rpsL212-F/R (Russo et al., 2008), was used to amplify the internal fragment of *rpsL* from the Psa strains. Table 2 lists the primer sequences and annealing temperatures for PCR. A PCR reaction mixture, 50 µl in total volume, consisted of 10 mM Tris (pH 8.0), 2.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 2.0 U of *Taq* polymerase (Bioneer, Daejeon, Korea), 10 mM of forward and reverse primers, and 20 ng of template DNA. The PCR reactions were performed in a Thermal Cycler Dice (Takara

**Table 1.** Bacterial strains used in this study and summary of PCR results

Bacteria	Strain	Year of isolation	Sm <sup>a</sup>	<i>strA</i> <sup>b</sup>	<i>strB</i> <sup>b</sup>	<i>strA - strB</i> <sup>b</sup>	<i>aadA</i> <sup>b</sup>
<i>Pseudomonas syringae</i> pv. <i>actinidiae</i>	KSH1351	2013	R	+	+	+	-
	OYJ1351	2013	R	+	+	+	-
	KTS1431	2014	R	+	+	+	-
	KBD14311	2014	R	+	+	+	-
	JJ18	2008	S	-	-	-	-
<i>Escherichia coli</i> <sup>c</sup>	DNRP545	2015	R	+	+	+	-
	DMRP5410	2015	R	-	-	-	+

<sup>a</sup>Sm, streptomycin; R, resistant; S, sensitive.

<sup>b</sup>+ and - indicate positive and negative PCR amplification.

<sup>c</sup>A reference for *E. coli* strains is Lim et al. (2018).

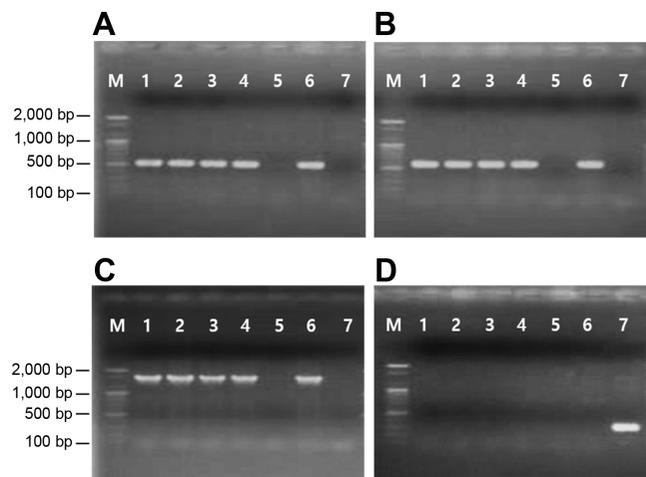
**Table 2.** Primers used for amplifying the streptomycin resistance genes

Primer name	Nucleotide sequence (5'→3')	Expected size (bp)	Annealing temp. (°C)	Target gene	Reference
aph(3'')-F	GCT CAA AGG TCG AGG TGT GG	515	55	<i>strA</i>	van Overbeek et al. (2002)
aph(3'')-R	CCA GTT CTC TTC GGC GTT AG				
aph(6)-F	GAC TCC TGC AAT CGT CAA GG	560	55	<i>strB</i>	van Overbeek et al. (2002)
aph(6)-R	GCA ATG CGT CTA GGA TCG AG				
StrA-F	TGA ATC GCA TTC TGA CTG GTT	1,571	58	<i>strA-strB</i>	Palmer et al. (1997)
StrB-R	GCT AGA TCG CGT TGC TCC TCT				
ant(3'')-F	CAG CGC AAT GAC ATT CTT GC	295	55	<i>aadA</i>	van Overbeek et al. (2002)
ant(3'')-R	GTC GGC AGC GAC A(C/T)CC TTC G				
rpsL212-F	CGT ACG CAA AGT TGC AAA AA	212	55	<i>rpsL</i>	Russo et al. (2008)
rpsL212-R	GGA TCA GGA TCA CGG AGT GT				

Bio Inc., Shiga, Japan) under the following temperature conditions: 95°C for 5 min, 30 cycles of 95°C for 30 s, 30 s at each annealing temperature, and 72°C for 30 s, followed by a final extension at 72°C for 7 min. The PCR products were electrophoresed and visualized on a 1.2% agarose gel stained with ethidium bromide.

Two primer pairs, aph(3'')-F/R and aph(6)-F/R, amplified 515 bp and 560 bp of the internal fragments of the *strA* and *strB* genes, respectively, from streptomycin-resistant Psa strains and *Escherichia coli* DNRP545. On the other hand, no amplification occurred in the streptomycin-sensitive strain (Fig. 1A and B). In addition, the 1,571 bp amplicon was produced by PCR in streptomycin-resistant Psa using the forward primer designed from *strA* and the reverse primer from *strB*, indicating that the two genes are linked in tandem to form a gene complex (Fig. 1C). No amplification products were obtained from streptomycin-resistant Psa strains when the primers specific for *aadA* were used for PCR. Only *E. coli* DMRP5410 used as a positive control produced the expected 295 bp amplicon (Fig. 1D). Hence, the mechanism of resistance in Psa is due to the presence of the *strA-strB* gene cluster (Table 1).

Sequence analysis of the *rpsL* gene in Psa was conducted to compare the nucleotide sequences of the gene of the streptomycin-resistant strains with those of the sensitive strain. After amplification, the PCR products of the *rpsL* gene were purified using an AccuPrep PCR Purification Kit (Bioneer). Nucleotide sequencing was performed in both the forward and reverse directions at SolGent Co. (Daejeon, Korea). A comparison of the nucleotide sequences of the PCR products amplified with rpsL212-F/R primers showed that all resistant strains had identical sequences to that of the sensitive one (data not shown). The four streptomycin-resistant strains had identical sequences to the susceptible strain JJ18 at a codon 43 of the *rpsL* gene coding for the



**Fig. 1.** PCR detection of *strA* (A), *strB* (B), *strA-strB* (C), and *aadA* (D) genes. Lane M, size markers (100 bp ladder); lanes 1-5, strains of *Pseudomonas syringae* pv. *actinidia*: KSH1351 (lane 1), OYJ1351 (lane 2), KTS1431 (lane 3), KBD14311 (lane 4), and JJ18 (lane 5); lane 6-7, strains of *Escherichia coli*: DNRP545 (lane 6) and DMRP5410 (lane 7).

amino acid, lysine (AAA). This indicates that streptomycin resistance in Psa is not due to a mutation in *rpsL*.

Of the 120 Psa strains isolated from Korea in 1998, there was no streptomycin-resistant strain. On the other hand, four *P. marginalis* strains isolated at the same place showed resistance to streptomycin, and three of them had the *strA-strB* gene pair as the resistant determinant. The resistance of one strain was attributed to a mutation in the target gene, *rpsL*, encoding a ribosomal protein S12 (Han et al., 2003). Hence, the *strA-strB* gene pair in the epiphytic bacterium, such as *P. marginalis*, may have migrated to Psa via horizontal gene transfer. No streptomycin-resistant strain was observed among the 21 Psa strains isolated in Korea in 1999 (Lee et al., 2005). In contrast, the streptomycin-

cin-resistant Psa strains isolated in Japan had the *strA-strB* genes or were caused by a mutation in the *rpsL* gene (Han et al., 2003).

The use of streptomycin in plant agriculture has resulted in the development of streptomycin resistance in pathogens worldwide (Förster et al., 2015; Sundin and Wang, 2018). Many studies of streptomycin resistance in phytopathogenic bacteria had been conducted with *E. amylovora*. The mechanism of streptomycin resistance found mainly in *E. amylovora* differed according to the regions where the bacteria had been isolated. Streptomycin resistance through a mutation in the *rpsL* gene was described in the isolates from New Zealand, Mexico, and several states in the United States, including California, Oregon, Washington, Idaho, Michigan, and Utah (Chiou and Jones, 1995; Nischwitz and Dhiman, 2013; Ponce de León-Door et al., 2013). Meanwhile, the *strA-strB* gene pair was the most common determinant of resistance in Michigan and New York (McGhee et al., 2011; Russo et al., 2008; Tancos et al., 2016). On the other hand, the genes associated with streptomycin resistance in some *E. amylovora* and *Clavibacter michiganensis* have not been identified, suggesting the existence of a new resistance mechanism (Lyu et al., 2019; Ponce de León-Door et al., 2013).

Our results indicate that streptomycin resistance of Psa biovar 2 strains isolated in Korea is due to the *strA-strB* genes. To understand the origin and acquisition of the streptomycin resistance determinant in Psa biovar 2, it is necessary to investigate resistance in the soil, rhizosphere, and phyllosphere microbiome. The comparative sequencing analyses of resistance genes may provide clues to understanding possible relationships such as horizontal gene transfer between Psa and coinhabiting streptomycin-resistant microorganisms.

## Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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