COMPLETE GENOME SEQUENCING OF *Pseudomonas syringae* pv. *actinidiae* BIOVAR 3, P155, KIWIFRUIT PATHOGEN ORIGINATING FROM CHINA

SEQUENCIAMENTO COMPLETO DO GENOMA *Pseudomonas syringae* pv. *actinidiae* BIOVAR 3, P155, AGENTE PATOGÊNICO DO KIWI, ORIGINÁRIO DA CHINA

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ABSTRACT: *Pseudomonas syringae* pv. *actinidiae* is a bacterial pathogen of kiwifruit. Based on the results of the pathogenicity assay, we sequenced the strain *Pseudomonas syringae* (Psa3) P155 which possesses a series of virulence and resistance genes, CRISPR candidate elements, prophage related sequences, methylation modifications, genomic islands as well as one plasmid. Most importantly, the copper resistance genes *copA*, *copB*, *copC*, *copD,* and *copZ* as well as aminoglycoside resistance gene *ksgA* were identified in strain P155, which would pose a threat to kiwifruit production. The complete sequence we reported here will provide valuable information for a better understanding of the genetic structure and pathogenic characteristics of the genome of P155.

KEYWORDS: *Actinidia* sp. Bacterial canker. Complete sequence. Pathogenicity. Pac-Bio platform.

INTRODUCTION

Kiwifruit (*Actinidia* sp.), an economically important fruit, is cultivated worldwide for its taste and nutritional value (Fujikawa and Sawada 2016). Among the different cultivars of kiwifruit, the cv. 'Hongyang' (*A. chinensis var. chinensis*) is widely grown in southwestern China especially in Sichuan province (HUANG et al. 2013). However, the bacterial canker of kiwifruit, which is caused by *Pseudomonas syringae* pv. *actinidiae* (Psa) has recently caused severe economic losses, in some cases limiting the cultivation of kiwifruit (Vanneste 2017).

Strains of Psa are separated into biovars (VANNESTE 2017). Strains of biovar 1 producing phaseolotoxin, and were initially isolated from Japan and Italy before 2008; strains of biovar 2 producing coronatine but not phaseolotoxin; Strains of biovar 3 producing neither coronatine nor phaseolotoxin, are responsible for the most recent outbreaks (CUNTY et al. 2015b); Strains of biovar 4 producing neither coronatine nor phaseolotoxin, are less aggressive than strains of other biovars (Cunty et al. 2015a). However, the pathogenic mechanism of biovar 3 has not been clear yet. Besides, biovar 3 does not produce any known toxins (Vanneste 2017).

Whole genome sequencing has largely been used as a research tool (van EL et al. 2013). With the development of whole genome sequencing, virulence and antimicrobial resistance profile of bacteria can be predicted. Additionally, the sequence data also provide a level of strain discrimination and precision (LINDSEY et al. 2016). Whole genome sequencing will be useful for developing molecular tools for Psa detection (ANDERSEN et al. 2017, RUINELLI et al. 2017). Here, whole genome sequencing was also carried out to better clarify the detailed characteristics of Psa strain P155 employing the PacBio system.

MATERIAL AND METHODS

Detection of Psa

Four strains named P155 (from Erbian county), P185 (from Yingjing county), P203 (from Qionglai county), and P540 (from Cangxi county) were randomly isolated from different areas in Chengdu, China. Psa was confirmed by PCR with a series of primer sets including PsaF1/R2 (REESGEORGE et al. 2010), PsaJ-F/R, PsaK-F/R, *avrD*, Koh and Nou amplicon (GALLELLI et al. 2011) for these 4 strains. Then the strain was immediately frozen in glycerol at -70 ℃.

Pathogenesis assays

A cane inoculation assay was conducted as Hoyte's research (Hoyte et al. 2015). After incubation for 24 h (37℃), bacterial colonies were

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suspended in sterile water to a concentration of 1.0×10^9 CFU/mL. Distilled water was used as a negative control. Hongyang canes sampled P155 and kept at 4 ℃ until needed were used for the assay. Canes were cut into segments of 10 cm long and placed into separate onion bags and labeled (Each cane is cut 6 segments, 5 segments inoculated Psa, 1 segment as control). Transfer labeled onion bags of cane segments into 1% sodium hypochlorite for 5 min and washed with SDW. Transfer cane segments onto the dry paper towels in the plastic sushi trays while recording the position of each cane. Apply a small amount of paraffin jelly to each of the cut ends to reduce moisture loss. Using a triangular file, cut two notch wounds into each cane segment, each about 1.5 cm from the ends of the cane and on the upper facing surface. Transfer a 10μL droplet of Psa inoculums directly to each wound. Transfer SDW droplet (10μL) to the wound sites on the cane segments being used as a control. Add 15 mL SDW to paper towels in the base of the plastic sushi and put them in the laboratory where the temperature is controlled to 20-23℃ for 21 days. Use knife blades to expose the lesions at wounds, using the digital calipers to measure the full length of any clearly visible lesion that extends beyond the wound area. If no lesion is obvious, then measure the width of the wound itself. The pathogenicity data were verified by the ANOVA method with statistical tool Stata SE (https://www.stata.com/).

Whole genome sequencing

The strain of Psa named P155 was sequenced and assembled using third-generation sequencing based on the PacBio platform. Sequencing was performed at the Beijing Novogene Bioinformatics Technology Co., Ltd. The genes were annotated by RAST (BRETTIN et al. 2015).

Genome component prediction included the prediction of the coding gene, repetitive sequences, non-coding RNA, genomics islands, transposon, prophage, and clustered regularly interspaced short palindromic repeat sequences (CRISPR). We used the geneMarkS program to retrieve the related coding gene and the interspersed repetitive sequences were predicted using the RepeatMasker (http://www.repeatmasker.org/). The tandem Repeats were analyzed by the TRF (Tandem repeats finder). Transfer RNA (tRNA) genes were predicted by the tRNAscanSE Ribosome and RNA (rRNA) genes were analyzed by the rRNAmmer. The IslandPath-DIOMB program was used to predict the Genomics Islands and transposon PSI was used to predict the transposons based on the homologous blast method. The PHAST was used for the prophage prediction (http://phast.wishartlab.com/) and the CRISPRFinder was used for the CRISPR identification.

We used seven databases to predict gene functions. They were respective GO (Gene Ontology) (https://www.uniprot.org/help/gene_ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes) (https://www.kegg.jp/), COG (Clusters of Orthologous Groups) (http://www.ncbi.nlm.nih.gov/COG), NR (Non-Redundant Protein Database databases) (https://www.biostars.org), TCDB (Transporter Classification Database) (http://www.tcdb.org/browse.php) and Swiss-Prot (https://web.expasy.org/docs/swiss-prot). A whole genome Blast search (E-value less than 1e-5, minimal alignment length percentage larger than 40%) was performed against the above seven databases. For pathogenic bacteria, we added the pathogenicity and drug resistance analysis. We used VFDB (Virulence Factors of Pathogenic Bacteria) (http://www.mgc.ac.cn/VFs/) and ARDB (Antibiotic Resistance Genes Database) (http://ardb.cbcb.umd.edu/) to perform the above analysis. The genes of plasmid were annotated by RAST (BRETTIN et al. 2015).

Accession numbers

The sequences have been deposited in GenBank under the accession numbers CP032870 (chromosome) and CP032871 (plasmid).

RESULTS

Detection and Pathogenicity

These 4 isolates were identified as Psa biovar 3, which possesses the target 280bp,492bp and 226bp DNA fragments with Primer sets Psa (ITS) F1/R2 (280bp), Koh and Nou (KN) amplicon (492bp) and *avrD*-F/R(226bp) to amplified respectively (Table 1; Figure 1). In other words, these 4 strains had all the characteristics of strains of Psa biovar 3. Among these 4 Psa 3 strains, it is obvious that P155, with a mean lesion diameter of 11.26 mm and variance of 1.16 mm, exhibited stronger and more stable pathogenicity when carrying out the kiwifruit cane canker in comparison with P185, P203, and P540 (Table 2; Figure 2).

Table 1. Psa Identification of P155, P185, P203, and P540

	Psa F1/R2	AvrDdpx-F/R		Psa J-F/R	Psa K-F/R
	ITS 280bp	KN amplicon 492bp	*avrD* 226bp	Psa 1 481bp	Psa 2 413bp
P155	+	+	+	-	-
P185	+	+	+	-	-
P203	+	+	+	-	-
P540	+	+	+	-	-
Psa1 (positive control)	+	+	+	+	-
Psa2 (positive control)	+	-	+	-	+
Psa3 (positive control)	+	+	+	-	-
Psa4 (positive control)	+	+	-	-	-
Negative control	-	-	-	-	-

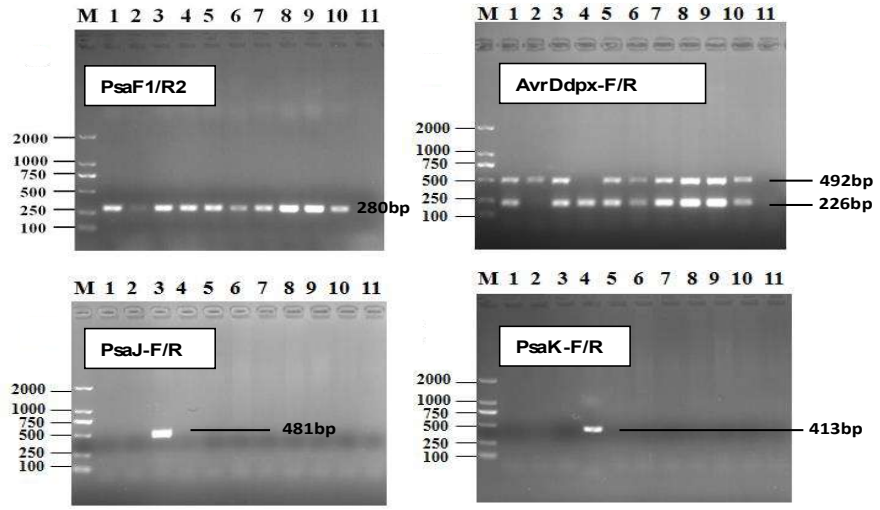

Figure 1 PCR identification of Psa with multiple primers.
M: Maker; **1:** Psa-3 (positive control strain); **2:** Psa-4 (positive control strain); **3:** Psa-1 (positive control strain); **4:** Psa-2 (positive control strain); **5:** P155; **6:** P185; **7:** P203; **8:** P540; **9:** P155; **10:** P185; **11:** negative control.

Table 2. Branch canker diameter caused by P155, P185, P203, and P540

Strain	**Diameter of branch canker (mm)**						**Mean ± SD (mm)**
P155	9.20	12.66	12.34	10.64	10.96	11.78	11.26 ± 1.16
P185	5.58	4.67	5.49	6.85	3.23	5.31	5.19 ± 1.09
P203	7.66	8.24	10.82	9.96	8.29	13.45	9.74 ± 1.99
P540	4.95	6.53	6.83	9.06	6.33	7.26	6.83 ± 1.23
Negative	2.90	1.99	2.76	2.25	2.25	2.00	2.36 ± 0.35

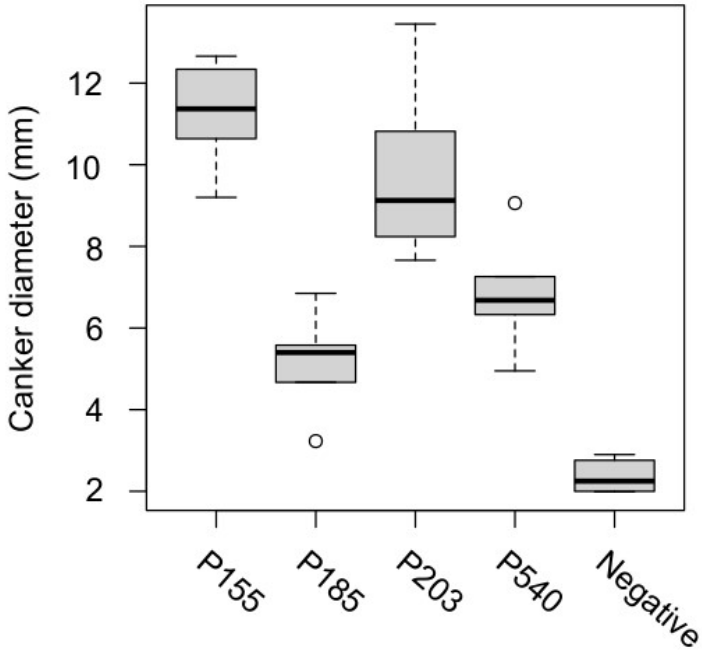

Figure 2. Canker diameter (mm) caused by each strain (including P155, P185, P203, P540, and negative) in the pathogenicity test.

Whole genome sequencing

Genome information and gene component of *P. syringae* strain P155

Because of its strong pathogenicity on *Actinidia* sp., P155 was selected and sequenced. 272,181 single reads of an average length of 7,768bp were obtained. After filtering of raw data, 2 contigs with an N50 of 6,538,506bp were obtained. BLASTn search revealed that *P. syringae* strain P155 is closely related to these Psa biovars 3 strains such as CRAFRU 14.08 (*P. syringae*; accession CP019732.1; 96% coverage; 99% identity), which was isolated from kiwifruit, NZ-47 (*P. syringae*; CP017009.1; 95% coverage; 99% identity), which was isolated from kiwifruit, NZ-45 (*P. syringae*; CP017007.1; 96% coverage; 99% identity), which was isolated from kiwifruit, ICMP 18884(*P. syringae*; CP011972.2; 96% coverage; 99% identity), which was isolated from kiwifruit, ICMP 18708 (*P. syringae*; CP012179.1; 96% coverage; 99% identity), which was isolated from kiwifruit and CRAFRU 12.29 (*P.syringae*; accession CP019730.1; 96% coverage; 99% identity), which was also isolated from kiwifruit. The *P. syringae* strain P155 has a single circular chromosome that was 6,529,859bp in length, corresponding to 150-fold genome coverage, with GC content of 58.4% and encodes 65 tRNA, 16 rRNA, and 17 sRNA. 6,138 protein-coding genes were predicted in P155. The general genomic features of P155 were summarized in Figure 3. The GIs were abundant in strain P155 which had 32 GIs with an average length of 14,730bp. In addition, 35 CRISPR candidate elements and 7 prophage related sequences, ranging from 19 kb to 74 kb, were also predicted in this strain.

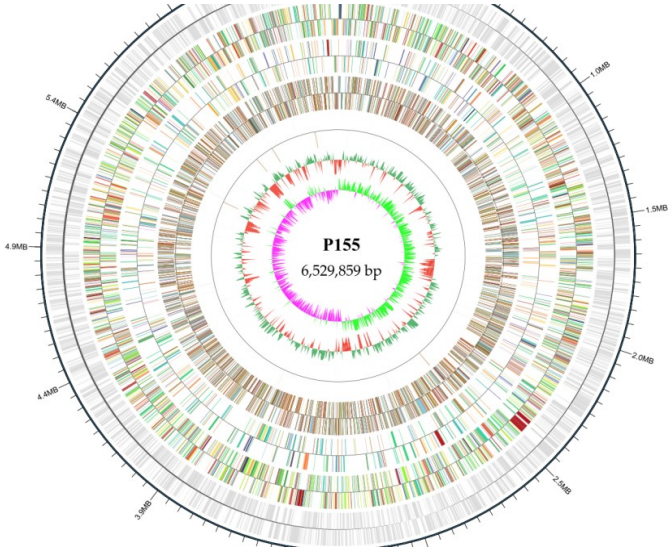

Figure 3. Genome map of Psa biovar 3 (P155).

Rings from the outside as follows: (1) scale marks (unit, Mb), (2) genomic island, (3) protein-coding genes on the forward strand colored by COG category, (4) protein-coding genes on the reverse strand (same color scheme as the second circle), (5) rRNA genes, (6) GC content (deviation from average), and (7) GC skew in blue (below average) and yellow (above average).

Virulence factors and resistance genes located in P155

In this study, there are 15 virulence effectors genes were predicted in P155 including *hopA1*, *hopI1*, *hopN1*, *hopM1*, *hopAE1*, *hopZ3*, *hopAA1*, *hopAU1* and *avrP*, *rpoD*, *acnB*, *qyrB*, *hrpL*, *hrpW,* and *avrD1*.

A variety of antimicrobial resistance genes were identified on the chromosome of P155. Our strain P155 possesses 27 multiple resistance genes, including *emrD* (aminoglycoside), *mexA* (aminoglycoside/tigecycline/fluoroquinolone), *mexB* (aminoglycoside/ fluoroquinolone/tetracycline), *mexE* (chloramphenicol), *mexF* (chloramphenicol/fluoroquinolone), *mexD* (glycylcycline/roxithromycin/erythromycin), *macB* (macrolide), *smeC* (fluoroquinolone), *smeD* (fluoroquinolone), *vanA* (vancomycin/teicoplanin), *acrB* (aminoglycoside/glycylcycline/macrolide/acriflavine) and *ksgA* (kasugamycin).

Amounts of heavy metal resistance genes were also identified in P155, including *arsB* (arsenic), *arsH* (arsenic), *copA* (copper), *copB* (copper), *copC* (copper), *copD* (copper), *copZ* (copper), *corA* (magnesium/cobalt), *rcnA* (nickel), *corC* (magnesium/cobalt), *terA* (tellurium), *terB* (tellurium), *terC* (tellurium), *terD* (tellurium/zinc) and *terZ* (tellurium), which resistant to As (arsenic), Cu (copper), Mg (magnesium), Ni (nickel), Co (cobalt), Te (tellurium) and Zn (zinc) respectively. The key factors in P155 were exhibited in Figure 4.

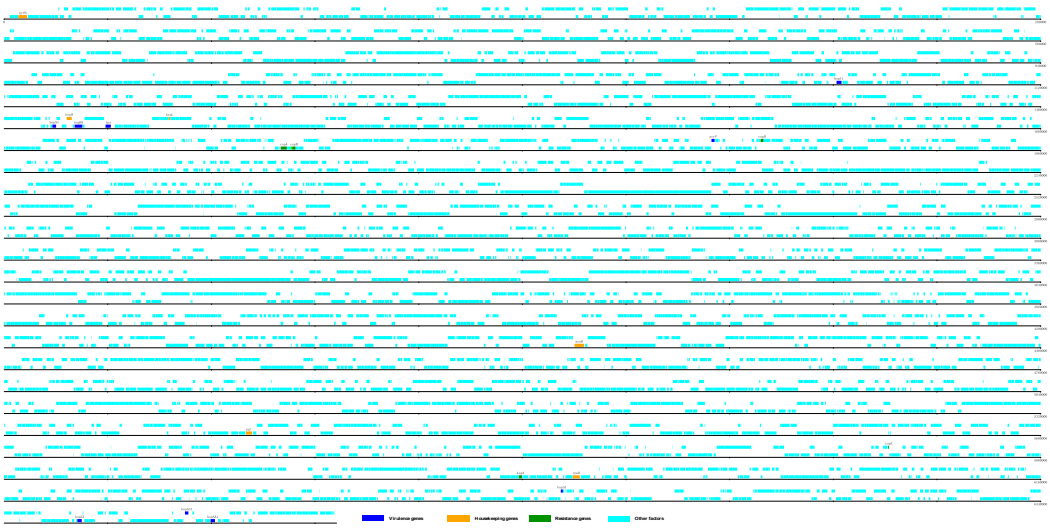

Figure 4. Overview of the key factors that exist in P155.

Apart from 1,411 uncharacterized proteins, 6,183 genes were annotated by COG databases. These genes encoding energy production and conversion, amino acid transport and metabolism, carbohydrate transport and metabolism, and transcription respectively that accounted for a large proportion (each more than 20%). In addition, high read coverage of P155 enabled methylation modifications to be annotated, and 1,001 m4C (4-methyl-cytosine) and 1,421 m6A (6-methyl-adenosine) residues were identified.

Overview of pLKQG722

The plasmid pLKQG722 was 77,771bp in length with GC content of 56.0%. The plasmid pLKQG722 comprised 97 open reading frames (orf). In plasmid pLKQG722, 6 IS3/IS911 were identified in pLKQG722 by the NCBI-IS database. However, no resistance gene or virulence gene was found in pLKQG722. The overview of plasmid pLKQG722 was exhibited in Figure S2. BLASTn search revealed that plasmid pLKQG722 is most closely related to plasmid pPsa20586 (accession CP017008.1; 100% coverage; 99% identity), which was carried by a *P. syringae* strain isolated from *A. deliciosa* in New Zealand, plasmid ICMP 18708 (accession CP017008.1; 100% coverage; 99% identity), which was carried by a *P. syringae* strain isolated from *A. deliciosa* in New Zealand, plasmid ICMP 18884 (accession CP017008.1; 100% coverage; 99% identity), which was carried by a *P. syringae* strain isolated from *A. deliciosa* in New Zealand, plasmid pPsa22180a (accession CP017008.1; 100% coverage; 99% identity), which was carried by a *P. syringae* strain isolated from *A. deliciosa* in New Zealand.

DISCUSSIONS

The identification of Psa resistant cultivars in a breeding program is considered an important strategy for disease control (KIM et al. 2016). In this study, a series of specific primer sets were employed for Psa confirmation simply based on PCR detection, which is more than 16srRNA sequencing for Psa confirmation. On the other hand, it is dramatic that all the 4 Psa belong to Psa 3, which indicated that Psa 3 might be a prevalent species that cause canker to local kiwifruits.

After the whole genome sequencing, 15 plant virulence factors were predicted in P155. With these virulence genes, including *hopA1*, *hopI1*, *hopN1*, *hopM1*, *hopAE1*, *hopZ3*, *hopAA1*, *hopAU1,* and *avrP*, *rpoD*, *acnB*, *qyrB*, *hrpL*, *hrpW* and *avrD1*, no wondering its high pathogenicity on *A. actinidiae* by causing branch canker. Meanwhile, these virulence genes have conserved avirulence and virulence activities (LIN et al. 2006). The housekeeping genes, *rpoD*, *acnB*, *qyrB*, *hrpL*, *hrpW,* and *avrD1*, we found in P155 was reported to make its sequence more conserved than that of the other genes considered (GALLELLI et al. 2011). For example, the *hrpL* gene, which codes for a putative sigma factor, is considered a good candidate for inferring the phylogenetic relationship between phytopathogenic bacteria and has been used to investigate the genetic relationships of *P. syringae* spp. (LORETI et al. 2008). On the other hand, the

type III secreted effectors (T3SE) were predicted in P155. Since T3SEs are some of the most important genes for understanding how Psa causes disease (TEMPLETON et al. 2015). Therefore, the pathogenic significances in this strain P155 warrant further investigation.

Based on the prediction of resistance genes, the *P. syringae* strain was shown to be resistant to fluoroquinolone, aminoglycoside, tigecycline, tetracycline, chloramphenicol, roxithromycin, erythromycin, macrolide, vancomycin, teicoplanin, glycylcycline, acriflavine and kasugamycin. It is noteworthy that the gene *ksgA* in P155 could invalidate the kasugamycin, an aminoglycoside antibiotic that prevents the *Actinidia* from getting canker disease (GHODS et al. 2015). Therefore, the kasugamycin, an aminoglycoside antibiotic, would not be chosen as the pesticides to disinfect this strain P155. In addition, P155 was also resistant to several heavy metals, which mostly caused by the intensive use of heavy metals and biocides recently (SUTTERLIN et al. 2018). Of note, P155 carries a copper resistance operon encoding *CopA*, *CopB*, *CopC*, *CopD,* and the regulatory *CopRS* pair. According to the report, the only effective method of control is to use antibacterial copper compounds (POULTER et al. 2017). Hence the copper resistance would make such strains pose a major threat to kiwifruit production.

In this study, we found the methylation modifications in P155. On the other hand, methylated residues have been reported for a genome from *P. syringae* (TEMPLETON et al. 2015). At present, a high level resistance to a broad spectrum of aminoglycoside antibiotics can arise through methylation modifications at the drug binding site in the bacterial 30S ribosomal subunit decoding center (ZELINSKAYA et al. 2011). These resistance determinants, combined with methylation modifications, thus pose a new threat to the usefulness of aminoglycosides as antibiotics, which has the same effect as gene *ksgA*.

The prophages related sequences in P155 should also be paid attention for further investigation since the horizontal transfer of DNA is a common method for acquiring new DNA in certain strain. In this study, the P155 possessed more than one phage tail protein, which indicated their ever target activities by phages. It was well known that the mobile elements that exist in bacteria such as plasmids make the dissemination of resistance genes serious (BLAIR et al. 2015). Fortunately, no resistance gene was found in plasmid pLKQG722 while 6 insert sequences were possessed by this plasmid, which seems would not pose a threat to the dissemination of resistance genes in our plasmid. Besides, it is noteworthy that our plasmid is more closely related to plasmids that all come from New Zealand.

In conclusion, we characterized the strain P155 which possesses a series of virulence and resistance genes, CRISPR candidate elements, prophage related sequences, methylation modifications, genomic islands as well as the plasmid. Most importantly, the copper resistance genes *copA*, *copB*, *copC*, *copD,* and *copZ* as well as an aminoglycoside resistance gene *ksgA* were identified in strain P155, which would pose threats to kiwifruit production. The complete sequence we reported here will provide valuable information for a better understanding of the genetic structure and pathogenic characteristics of the genome of P155.

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RESUMO: *Pseudomonas syringae* pv. *actinidiae* agente causal do cancro bacteriano do kiwi. Com base nos resultados do teste de patogenicidade, foi sequenciado um isolado de *Pseudomonas syringae* (Psa3) P155, que abriga a uma série de genes de virulência e resistência, elementos candidatos CRISPR, sequências relacionadas a profagos, modificações na metilação, ilhas genômicas, e também um plasmídeo. O mais importante foram os genes de resistência ao cobre, *copA*, *copB*, *copC*, *copD* e *copZ*, bem como, o gene de resistência aminoglicosídea *ksgA* identificados na estirpe P155, os quais representariam uma ameaça à produção de kiwi. A sequência completa relatada fornecerá informações valiosas para uma melhor compreensão da estrutura genética e as características patogênicas do genoma de P155.

PALAVRAS-CHAVE: *Actinidia* sp. Casca bacteriana. Patogenicidade. Plataforma Pac-Bio. Sequência completa.

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