

PHENOTYPIC AND MOLECULAR PROPERTIES OF *PSEUDOMONAS SYRINGAE* pv. *SYRINGAE* THE CAUSAL AGENT OF BACTERIAL CANCKER OF STONE FRUIT TREES IN KURDISTAN PROVINCE

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SUMMARY

Since 2005, a disease similar to bacterial canker of stone fruit trees was observed in some areas of Kurdistan province, Iran. A total of 12 bacterial isolates were obtained from infected tissues of apricot and peach trees. According to biochemical, physiological and whole cell protein patterns, isolates were identified as *Pseudomonas syringae* pv. *syringae*. Some molecular properties of the isolates were studied. Electrophoretic plasmid analysis revealed that isolates contained 1-3 plasmids with estimated mol. wt of 371.4, 93.1 and 16.5 kb. Mutant strains were produced using plasmid curing methods. These mutant strains were not mucoid on nutrient agar containing 5% sucrose. Compared with wild type strains, mutants were sensitive to ampicillin, and mutant strains were more actively motile than wild type strains. Polymerase chain reaction (PCR) was used to detect *virB1* and *virD4* genes. The predicted 1453 and 513 bp PCR products were obtained for *virD4* and *virB1* genes, respectively, when a 93.1 kb plasmid was used as template DNA.

Key words: *P. syringae* pv. *syringae*, PCR detection, plasmid curing, *virD4*, *virB1*.

INTRODUCTION

Since 2005, severe dieback and canker disease were observed on apricot (*Prunus armeniaca* L.) and peach (*Prunus persicae* L.) trees in some areas of the Kurdistan province of Iran. Losses resulted from death of buds and flowers or from tree decline due to the development of cankers on branches. The symptoms of this disease were similar to those of bacterial canker of stone fruit trees produced by *Pseudomonas syringae* pv. *syringae* van Hall.

P. syringae pv. *syringae* can cause disease in over 180 species of plants in several unrelated genera (Bradbury,

1986). Strains are identified on the basis of phenotypic and molecular tests. Most bacterial strains harbour one or several plasmids which show unique properties. Examples are pPSR12 (Cu^r, Co^r, As^r, conferring stable mucoid colony form, Kidambi *et al.*, 1995), pOSU900 (cryptic plasmid, loss does not affect pathogenicity toward bean, Mukhopadhyay *et al.*, 1990) and pPSR14 (Cu^r, streptomycin^r, Sundin *et al.*, 1994). Pss strain A2 contains a 68-kb conjugative plasmid from the pPT23A family that transfers resistance to copper and streptomycin (Sundin and Bender, 1993).

The two main objectives of this study were to identify and characterize bacteria isolated from various *Prunus* species in Kurdistan province, and investigate the presence of *vir* genes in plasmids found in these strains.

MATERIALS AND METHODS

Source and isolation of bacteria. Bacteria were isolated from cankers and stem lesions of peach and apricot trees from April to May in 2005 and 2006 from Sanandaj, Kamyaran and Marivan in the western Kurdistan province. Individual samples were randomly selected from different trees. Symptomatic tissues were surface-sterilized with 0.25% aqueous sodium hypochlorite for 30 sec and rinsed in sterile distilled water. Small pieces of tissue were excised from canker margins, macerated in 3 ml of sterile distilled water for 30 min and the resulting suspensions were streaked onto nutrient agar (NA). Plates were incubated at 25 to 27°C and after 3-4 days single colonies were subcultured on NA or King's medium B (KB) (King *et al.*, 1954).

Identification and characterization of bacteria. Morphology was determined by examining strains grown on NA plates. Gram reaction was determined by potassium hydroxide solubility test (KOH test). Strains were grown on KB and fluorescent pigment production was evaluated using a UV transilluminator. Levan formation, oxidase reaction, potato soft rot, arginine dihydrolase and induction of the hypersensitive reaction in tobacco leaves (LOPAT tests), were done as described by Lelliott *et al.* (1966). For other biochemical and physiologi-

cal tests (Table 1) methods described by Schaad *et al.* (2001) were used. Acid production from carbon sources or utilization of amino acids used methods described by Dye (1968). Isolates which were fluorescent on KB and +---+ for LOPAT tests were further characterized.

Whole-cell protein analysis. Soluble whole-cell protein extracts were obtained for each strain as described by Laemmli (1970). SDS-PAGE was performed using a Cleaver midi apparatus (Cleaver Scientific, Rugby, UK) and 12% (w/v) polyacrylamide gels electrophoresed vertically at 20 mA. The gels were stained for 1 h with 50% methanol, 10% acetic acid and 0.1% Coomassie blue in distilled water. The gels were destained using the same solution without dye for 15-20 min followed by 7% acetic acid. Gels were then photographed. A known strain of *P. syringae* pv. *syringae*, 3023^T (International Collection of Microorganisms from Plants (ICMP) was used as reference strain.

Pathogenicity tests. bacteria isolated from infected tissues were grown in nutrient broth (NB) at 28°C for 24 h. Cells were centrifuged (5 min at 7000 rpm) and the pellet resuspended in sterile distilled water to a concentration of approximately 1×10^8 CFU/ml as determined by OD reading ($A_{600} = 0.05$) with a spectrophotometer (Jenway, Essex, UK). Bacteria were injected into branches of one-year-old apricot and peach seedlings using a sterile syringe. Sterile distilled water was used as control. Plants were maintained in a greenhouse at 20-30°C with 90-98% relative humidity until symptoms were assessed 21-28 days after inoculation. Bacteria were isolated from the lesions produced and characterized as above.

Plasmid extraction. Plasmids were extracted using the alkaline lysis method of Birnboim and Doly (1979) with some modifications. A single colony from a 24-h-old bacterial culture was grown in 5 ml sterile LB medium. Tubes were placed on a shaker for 24 h at 28°C. Bacterial cells were recovered by centrifugation at 14000 rpm for 1.5 min and the pellet was resuspended in 200 µl of ice-cold solution I (50 mM glucose, 25 mM Tris.HCl pH 8.0, 10 mM EDTA pH 8.0). Subsequently 200 µl of solution II (1 ml of 0.4 M NaOH, 1 ml of 2% SDS) was added and the tubes were mixed by gentle inversion. Then 200 µl of ice-cold solution III (1.15 ml glacial acetic acid, 2.85 ml distilled water, 6 ml 5M potassium acetate pH 4.8) was added and shaken gently to mix. The tubes were then centrifuged at 14000 rpm for 5 min. The supernatant was transferred into a clean 1.5 ml Eppendorff tube. Plasmids were precipitated by adding 2 vol of ice-cold absolute ethanol, then placed on ice for at least 15 min. The tubes were centrifuged for 15 min (14000 rpm at 4°C) and the plasmid pellet was washed with 1 ml of 70% ethanol, dried, and resus-

ended in 50 µl TE buffer (10 mM Tris.Cl, pH 7.5, 1 mM EDTA, pH 8.0) containing RNase to a final concentration of 20 µg/ml. Plasmids were examined by electrophoresis in 0.8% agarose gels at 10 v/cm in 1x Tris-acetate-EDTA buffer. Gels were stained with ethidium bromide and DNA was visualized with a gel imaging system. All DNA fragments were isolated from the gel using silica fine powder or a DNA extraction kit (Fermentas, Vilnius, Lithuania) according to manufacturer's instructions and used as template DNA in PCR reactions.

Genomic DNA extraction. A single colony per strain was grown in 5 ml LB medium. Cells were collected in 1.5 ml Eppendorf tubes and the pellets were left at 20°C for 30 min, resuspended in 200 µl TE buffer and incubated with 8 µl lysozyme (10 µg/ml) at 37°C for 30 min. The cells were then lysed by addition of 40 µl of 4M sodium perchlorate, 24 µl of 10% SDS and 8 µl of proteinaseK (20 µg/ml stock) and after mixing by inverting, incubated at 45°C for 2 h. The DNA was precipitated by adding 2 vol absolute ethanol, incubated at -20°C for 30 min and centrifuged for 5 min (13000 rpm at 4°C). The pellet was washed with 1 ml 70% ethanol, dried and resuspended in 500 µl TE buffer.

DNA was mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), inverted at room temperature to mix completely, then the solution was centrifuged for 3 min. The aqueous layer was then transferred to a clean tube and the procedure repeated three times. The aqueous layer was extracted once with chloroform:isoamyl alcohol (24:1). DNA was precipitated by adding 2 vol of absolute ethanol and 0.1 vol 3M sodium acetate (pH 4.8), then incubated at -20°C overnight. It was subsequently centrifuged for 5 min, washed with 1 ml 70% ethanol, dried and resuspended in 50 µl TE buffer with RNase (10 mg/ml stock) to a final concentration of 20 µg/ml.

Polymerase chain reaction. PCR was carried out as described by Sambrook *et al.* (1989) with some modifications. The sets of primers used were DR (5'-CACTTCAGACGCTTTGTC-3'), DF (5'-CAGC-TACGGGCAACAG-3') and BR (5'-GGGAGTTGAG-GCTTCTTC-3'), BF (5'-AAAAAGCTGGTGGCAGAG-3') designed on the *virD4* and *virB1* sequences of pPSR1 (Sundin *et al.*, 2004), respectively. PCR was carried out in a 25 µl reaction vol containing 12.5 µl of master mix (Fermentas, Vilnius, Lithuania), 1 µl of each primer (10 pmol/µl), 1 µl of template DNA (5 ng/µl for plasmid DNA and 10 ng/µl for genomic DNA) and 10.5µl of sterile distilled water. A thermal cycler (Bio-Rad MJ mini, Hercules, CA, USA) and the following protocol were used: initial denaturation step of 94°C for 4 min followed by 25 cycles of 94°C for 45 sec, 55°C (*virB1*) or 56°C (*virD4*) for 30 sec, 72°C for 45 sec and a

final extension step of 72°C for 10 min. Amplified fragments were examined by electrophoresis in 1% agarose gels at 10 v/cm for 45 min in 1x Tris-acetate-EDTA buffer. Gels were stained with ethidium bromide and DNA was visualized with a gel imaging system.

Plasmid curing. Freezing-thawing was carried out as described by Calcott (1982) with some modifications. Bacterial strains were grown in LB medium for 24 h. The samples were maintained at -20°C for 20 min, then thawed by warming in water bath at 55°C. After freezing-thawing 10 times, serial dilutions of samples were prepared and 10^{-3} - 10^{-5} dilutions were plated on LB agar medium. Plates were incubated at 27°C for 24-48 h.

Motility test. Motility behavior of wild type and mutant strains was observed in nutrient broth containing 0.2- 0.3% agar. Stab-inoculated plates were incubated at 28°C for up to two days. Motility behavior was measured after 10, 24 and 48 h.

RESULTS

Bacterial isolation and characterization. A total of 12 fluorescent pseudomonad strains were recovered from infected tissues of peach and apricot trees. Colonies on NA were round, slightly convex, white and 2-3 mm in diameter. All strains were gram-negative, aerobic and showed fluorescence on KB. The result of LOPAT tests (levan production, oxidase reaction, potato soft rot, arginine dihydrolase, and tobacco hypersensitivity) were +---+ respectively, showing that the bacterial isolates belonged to *P. syringae* (Lelliott *et al.*, 1966). Based on other biochemical and physiological properties the bacterium was ultimately identified as *Pseudomonas syringae* pv. *syringae* (Table 1) (Brenner *et al.*, 2005).

All strains tested for pathogenicity produced water-soaked lesions on branches 3-4 weeks after inoculation. Bacterial strains were re-isolated from infected tissues and the biochemical properties were those of *P. syringae* pv. *syringae*. Analysis of whole cell protein patterns of the bacterial isolates showed that all had the same pattern, which was very similar to that of the *P. syringae* pv. *syringae* reference strain 3023^T (Fig. 1).

Genetic characterization. After plasmid isolation and electrophoresis, gels were analyzed using One Dscan software (Scanalytics, version 1.3). The strains analysed had one to three plasmids with an estimated molecular weight of 371.4, 93.1 and 16.5 Kb (Fig. 2). Primers corresponding to *virD4* and *virB1* genes annealed to genomic DNA and to each plasmid (template DNA). After PCR, the expected DNA fragments for *virB1* and *virD4*, of 513 and 1453 bp, respectively, were obtained when the plasmid with estimated molecular weight of

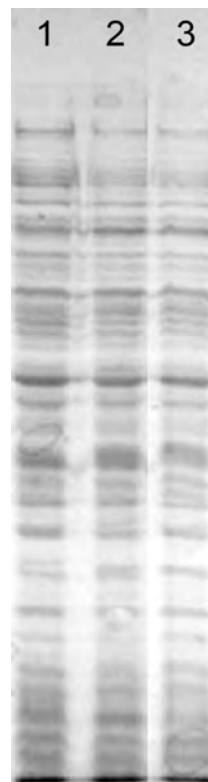


Fig. 1. Electrophoretic protein pattern of *P. syringae* pv. *syringae*. Lane 1, *P. syringae* pv. *syringae* 3023; lanes 2 and 3, *P. syringae* pv. *syringae* isolates from peach and apricot, respectively.

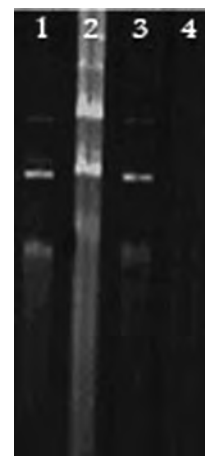


Fig. 2. Plasmid profiles of *P. syringae* pv. *syringae* recovered from stone fruit trees. Lanes: 1, 2 and 3, bacterial strains isolated from stone fruit trees; lane 4, bacterial strain cured of plasmids.

93.1 Kb was used as template DNA (Fig. 3A, B). The nucleotide composition of the PCR product corresponding to *virD4* was checked by sequencing.

Plasmid curing. After freezing and thawing, plasmids were cured from all strains tested. All strains produced white, flat colonies, 1-2 mm in diameter on NA medi-

Table 1. Physiological and biochemical properties of *P. syringae* pv. *syringae* strains isolated from apricot and peach in Kurdistan province, Iran.

Test	Bacterial isolates	<i>P. s.pv. syringae</i> *	Test	Bacterial isolates	<i>P. s.pv. syringae</i> *
Gram reaction	-	-	Glucose	+	+
Levan production	+	+	Sucrose	+	+
Oxidase	-	-	Glycerin	+	+
Potato soft rot	-	-	Sorbitol	+	+
Arginine dihydrolase	-	-	Inositol	+	+
Hypersensitivity reaction	+	+	Raffinose	-	-
Catalase	+	+	Maltose	-	-
Hydrolysis of:			Sorbose	-	NT
Starch	-	-	Lactose	-	-
Gelatin	-	-	Galactose	+	+
Tween 80	+	+	Fructose	d	+
Casein	-	NT	Arabinose	-	-
Aescolin	+	+	Trehalose	-	-
Lecithine	-	-	Xylose	-	+
Pink production colony on YDC	-	-	Mellibiose	-	-
3% NaCl tolerance	+	+	Mannose	d	+
4% NaCl tolerance	-	NT	Rhamnose	-	-
Urease	+	+	Salicin	-	-
Acetoeine production	-	NT	Cellobiose	-	-
H ₂ S production from peptone	-	NT	Utilization of:		
H ₂ S production from cystein	-	NT	Adonitol	-	-
Nitrate reduction	-	-	Dulcitol	-	-
Methyl red	-	NT	Mannitol	-	+
Growth at 4°C	+	+	Malonate	-	+
Growth at 41°C	-	-	Benzoate	-	-
Ketolactose	-	NT	Propionate	-	-
Oxidative/fermentative (O/F)	O	O	Tartrate	-	-
Indole production	-	NT	Ethanol	-	-
Acid production from:					

+, 85% or more positive; -, 15% or less positive; NT, not tested. d, 15-85% are positive

*. Data selected from Brenner *et al.* (2005), Sands *et al.* (1970)

um. All mutant strains were non-mucoid on 5% sucrose agar. Compared with wild type strains, mutants were sensitive to ampicillin. Motility test in NB medium plus 0.3% agar showed that mutant strains were more actively motile than wild type strains (data not shown).

DISCUSSION

This is the first report of a canker disease of stone fruit trees in the Kurdistan province of Iran caused by *P.*

syringae pv. *syringae*, as shown by the consistent association of a fluorescent pseudomonad with the disease and its identification based on phenotypic properties and whole-cell protein patterns compared with the reference strain of *P. syringae* pv. *syringae*. Biochemical and physiological characteristics as well as whole-cell protein patterns indicated that the bacterial strains isolated from apricot and peach trees formed a homogenous group. There were some differences in the physiological characteristics of these isolates compared with isolates previously reported (Brenner, 2005), particularly in the uti-

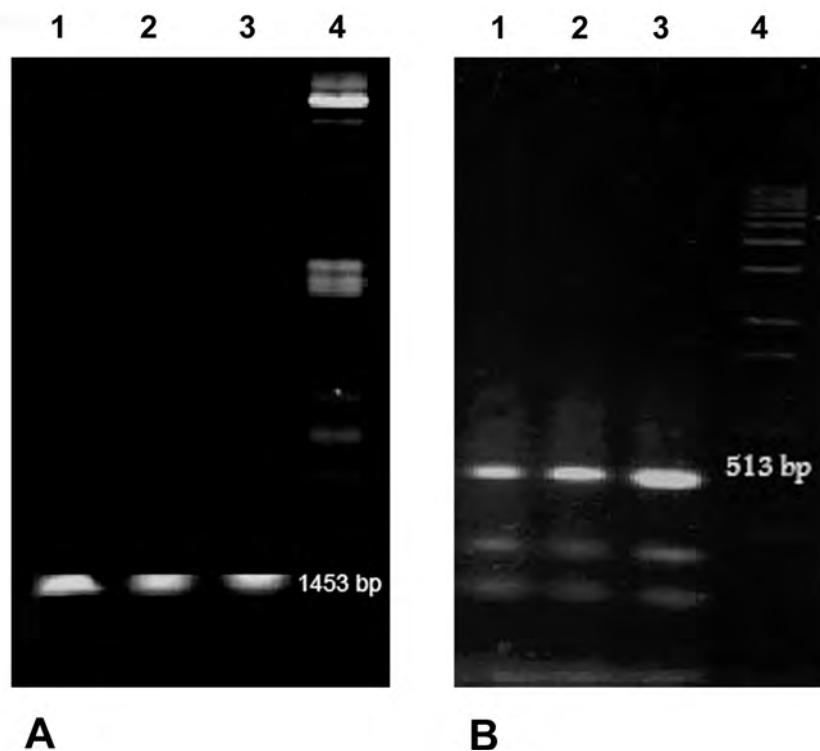


Fig. 3. Electrophoretic analysis of PCR products corresponding to gene *virD4* (A) and gene *virB1* (B). A. lanes *P. syringae* pv. *syringae* recovered from peach (lanes 1 and 2) and from apricot (lane 3). Lane 4, size marker. B. *P. syringae* pv. *syringae* isolated from peach (lanes 1 and 2) and from apricot (lane 3). Lane 4, size marker.

lization of mannitol and xylose. Whole-cell protein pattern analysis, however, revealed that all strains had high similarity level and were grouped in one cluster.

Most of the *P. syringae* strains harboured one or several plasmids the majority of which belonged to the pPT23A plasmid family (Zhao *et al.*, 2005). Some behavioural and phenotypic properties of *P. syringae* strains are encoded by these plasmids. Examples are resistance to Cu and antibiotics (Sundin and Bender, 1996), resistance to ultraviolet radiation (Kim and Sundin, 2000), phytotoxin production (Bender *et al.*, 1987; Alarcon-Chaidez *et al.*, 1999), growth factor production (Glickmann *et al.*, 1998), and chemotaxis (Gonzalez *et al.*, 1984; Vivian *et al.*, 2001).

The complete nucleotide sequence was determined of pPSR1 which comprises *virD4* and *virB1* genes (Sundin *et al.*, 2004). Our bacterial isolates had one to three plasmids but PCR showed that *virD4* and *virB1* genes are located on a plasmid with estimated molecular weight of 93.1 Kb. These genes encode proteins involved in the type IV secretion system (T4SS) and play a role in conjugation. T4SS is one of the five major secretion systems in gram-negative bacteria and is thought to be a translocation system ancestrally related to conjugation system of gram-negative and -positive bacteria (Cao and Saier, 2001). T4SSs has a role in conjugation, DNA uptake and release, and effector translocation systems (Ding *et al.*, 2003). The type IVA secretion system of

Agrobacterium tumefaciens consists of 11 proteins encoded by the *virB* operon and the VirD4 protein; the genetic organizations of these structural genes in plasmids pPSR1 are similar.

Based on our results, we anticipate that the gene organization on the 93.1 Kb plasmid identified in *P. syringae* pv. *syringae* would be similar to that of pPSR1. Compared with wild type, mutant strains produced dry colonies on nutrient agar plus 5% sucrose and there was a significant decrease in levan formation. Because of deletion of all three plasmids by freeze-thawing, it was not ascertained which genes encoding changes in colony morphology, exopolysaccharide production and motility behavior of mutant strains are located on which plasmids. However, it can be hypothesized (although experimental evidence is lacking) that genes encoding these phenotypic properties are located on plasmids.

REFERENCES

- Alarcon-Chaidez F.J., Penaloza-Vazquez A., Ullrich M., Bender C.L., 1999. Characterization of plasmids encoding the phytotoxin coronatine in *Pseudomonas syringae*. *Plasmid* **42**: 210-220.
- Bender C.L., Stone H.E., Sims J.J., Cooksey D.A., 1987. Reduced pathogen fitness of *Pseudomonas syringae* pathovar tomato Tn5 mutants defective in coronatine production. *Physiological and Molecular Plant Pathology* **30**: 273-284.

- Birnboim H., Doly J., 1979. A rapid extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**: 1513-1525.
- Bradbury J.F., 1986. *Pseudomonas syringae* pv. *syringae*. Guide to Plant Pathogenic Bacteria, pp. 175-177. CAB International Mycological Institute, Kew, Surrey, UK.
- Brenner D.J., Krieg N.R., Staley J.T., 2005. *Bergey's Manual of Systematic Bacteriology*, 2nd Ed. Springer, New York, NY, USA.
- Calcott P.H., 1982. Transient loss of plasmid mediated mercuric ion resistance after freezing and thawing of *Pseudomonas aeruginosa*. *Ohio Journal of Science* **82**: 59-66.
- Cao T.B., Saier M.H., 2001. Conjugal type IV macromolecular transfer systems of gram-negative bacteria: organismal distribution, structural constraints and evolutionary conclusions. *Microbiology* **147**: 3201-3214.
- Ding Z., Atmakuri K., Christie P.J., 2003. The outs and ins of bacterial type IV secretion substrates. *Trends in Microbiology* **11**: 527-535.
- Dye D.W., 1968. A taxonomic study of the genus *Erwinia* 1. The Amylovora group. *New Zealand Journal of Science* **11**: 590-607.
- Glickmann E., Gardan L., Jacquet S., Hussain S., Elasmri M., Petit A., Dessaux Y., 1998. Auxin production is a common feature of most pathovars of *Pseudomonas syringae*. *Molecular Plant-Microbe Interaction* **11**: 156-162.
- Gonzalez C.F., Layher S.K., Vidaver A.K., Olsen R.H., 1984. Transfer, mapping, and cloning of *Pseudomonas syringae* pv. *syringae* plasmid pCG131 and assessment of its role in virulence. *Phytopathology* **74**: 1245-1250.
- Kidambi S.P., Sundin G.W., Palmer D.A., Chakrabarty A.M., Bender C.L., 1995. Copper as signal for alginate synthesis in *Pseudomonas syringae* pv. *syringae*. *Applied and Environmental Microbiology* **61**: 2172-2179.
- Kim J.J., Sundin G.W., 2000. Regulation of the *rulAB* mutagenic DNA repair operon of *Pseudomonas syringae* by UV-B (290 to 320 nanometers) radiation and analysis of *rulAB*-mediated mutability in vitro and in planta. *Journal of Bacteriology* **182**: 6137-6144.
- King E.O., Ward W.K., Raney D.E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine* **44**: 301-307.
- Laemmli U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.
- Lelliott R.A., Billing E., Hayward A.C., 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. *Journal of Applied Bacteriology* **29**: 470-489.
- Mukhopadhyay P., Mukhopadhyay M., Mills D., 1990. Construction of a stable shuttle vector for high-frequency transformation in *Pseudomonas syringae* pv. *syringae*. *Journal of Bacteriology* **172**: 477-480.
- Sambrook J., Fritsch E.F., Maniatis T., 1989. *Molecular cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor, New York, NY, USA.
- Sands D.C., Schroth M.N., Hildebrand D.C., 1970. Taxonomy of phytopathogenic Pseudomonads. *Journal of Bacteriology* **101**: 9-23.
- Schaad N.W., Jones J.B., Chun W., 2001. *Laboratory Guide for Identification of Plant Pathogenic Bacteria*. 3rd ed. APS Press, St. Paul, MN, USA.
- Sundin G.W., Bender C.L., 1993. Ecological and genetic analysis of copper and streptomycin resistance in *Pseudomonas syringae* pv. *syringae*. *Applied and Environmental Microbiology* **59**: 1018-1024.
- Sundin G.W., Demezas D.H., Bender C.L., 1994. Genetic and plasmid diversity within natural populations of *Pseudomonas syringae* with various exposures to copper and streptomycin bactericides. *Applied and Environmental Microbiology* **60**: 4421-4431.
- Sundin G.W., Bender C.L., 1996. Molecular analysis of closely related copper- and streptomycin-resistance plasmids in *Pseudomonas syringae* pv. *syringae*. *Plasmid* **35**: 98-107.
- Sundin G.W., Mayfield C.T., Zhao Y., Gunasekera T.S., Foster G.L., Ullrich M.S., 2004. Complete nucleotide sequence and analysis of pPSR1 (72,601bp), a pPT23A-family plasmid from *Pseudomonas syringae* pv. *syringae* A2. *Molecular Genetics and Genomics* **270**: 462-475.
- Vivian A., Murillo J., Jackson R.W., 2001. The roles of plasmids in phytopathogenic bacteria: mobile arsenals? *Microbiology* **147**: 763-780.
- Zhao Y., Ma Z., Sundin G.W. 2005. Comparative genomic analysis of the pPT23A plasmid family of *Pseudomonas syringae*. *Journal of Bacteriology* **187**: 2113-2126.

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