Genetic Diversity Among *Pseudomonas syringae* pv. *morsprunorum* Isolates from *Prunus mume* in Korea and Japan by Comparative Sequence Analysis of 16S rRNA Gene

Young Sun Lee¹, Hyun Seok Koh¹, San Ho Sohn¹, Young Jin Koh² and Jae Sung Jung¹*¹

¹Department of Biology; ²Department of Plant Medicine, Sunchon National University, Suncheon 540-742, Korea

(Received on March 4, 2012; Revised on May 7, 2012; Accepted on June 28, 2012)

Genetic diversity among *Pseudomonas syringae* pv. *morsprunorum* isolates from *Prunus mume* in Korea and Japan was investigated by comparative sequence analysis of the 16S rRNA gene. The strains included 24 field isolates recovered from *P. mume* in Korea along with seven Japanese strains. Two strains isolated from *P. salicina* in Japan, one strain from *P. avium* in the United Kingdom, and the pathotype strain were also used for comparison with their 16S rRNA gene sequences. Nearly complete 16S rRNA gene sequences were sequenced in all 35 strains, and three sequence types, designated types I, II and III, were identified. Eleven strains consisting of five Korean isolates, five Japanese strains, and one strain from the United Kingdom belonged to type I, whereas the pathotype strain and another 19 Korean isolates belonged to type III. Another four Japanese strains belonged to type II. Type I showed 98.9% sequence homology with type III. Type I and II had only two heterogeneous bases. The 16S rRNA sequence types were correlated with the races of *P. syringae* pv. *morsprunorum*. Type I and II strains belonged to race 1, whereas type III isolates were included in race 2. Sequence analyses of the 16S rRNA gene from *P. syringae* pv. *morsprunorum* were useful in identifying the races and can further be used for epidemiological surveillance of this pathogen.

**Keywords**: 16S rRNA, bacterial canker, *Prunus mume*, *Pseudomonas syringae* pv. *morsprunorum*

*Prunus mume* (Japanese apricot) is widely grown in the southern part of Korea for the purpose of large-scale fruit production in commercial orchards. Fruits of *P. mume*, called *maesil* in Korean, *ume* in Japanese, and *mei* in Chinese, have been used as a traditional medicine and health food throughout East Asia.

Several fungal and bacterial diseases have been recorded on *P. mume* trees in Korea (Korean Society of Plant Pathology, 2004). Of these diseases, bacterial canker, which debases fruit quality, is economically one of the most important diseases in the cultivation of Japanese apricot. In a previous work, we characterized the causal agent of bacterial canker of Japanese apricot in Korea using a polyphasic taxonomy approach and identified the pathogen as *Pseudomonas syringae* pv. *morsprunorum* (Kim et al., 2005). In addition to Japanese apricot, *P. syringae* pv. *morsprunorum* is also the main cause of bacterial canker in several *Prunus* species, such as sweet and sour cherry (Berkowicz and Rudolph, 1994; Jones, 1971; Latorre and Jones, 1979; Ménard et al., 2003; Paterson and Jones, 1991; Renick et al., 2008; Sundin et al., 1988) and Japanese plum (Roos and Hattingh, 1983).

Characterization of the genotypes of plant pathogens with respect to geographic origin is used not only to understand evolutionary properties but also to explain the worldwide migration of plant pathogenic bacteria (Boudon et al., 2005). To characterize bacterial populations, direct amplification and sequencing of the 16S rRNA gene has been established as the standard method in ecology studies on plant pathogens (Li et al., 1993).

*P. syringae* pv. *morsprunorum* comprises two highly homogeneous races, race 1 and race 2, on the basis of colony structure, gelatinase activity, and pathological characteristics (Freigoun and Crosse, 1975) and can be distinguished by biochemical, physiological, and serological methods (Vicente et al., 2004). In addition, as the rep-PCR fingerprints of the two *P. syringae* pv. *morsprunorum* races are distinctive, the genetic profiles generated by this method have been used to distinguish isolates of *P. syringae* pv. *morsprunorum* at the race level (Vicente and Roberts, 2007). Especially, BOX-PCR has been shown to be useful for identifying *P. syringae* pv. *morsprunorum* race 1 and race 2 (Gilbert et al., 2009).

The aim of the present study was to investigate the genetic diversity of representative *P. syringae* pv. *morsprunorum* isolates from Japanese apricot in Korea and Japan by comparative sequence analysis of the 16S rRNA genes as

*Corresponding author.
Phone) +82-61-750-3616, FAX) +82-61-750-5469
E-mail) jjung@sunchon.ac.kr
well as by race determination.

Twenty-four isolates from a collection of 92 isolates obtained from Japanese apricot trees affected by bacterial canker were selected randomly as representative of the different geographical areas of Korea. Physiological and biochemical tests including LOPAT and GATTa were performed according to the procedures described by Vicente et al. (2004). All strains tested showed characteristics of *P. syringae pv. morsprunorum*. Seven Japanese strains of *P. syringae pv. morsprunorum* isolated from Japanese apricot along with two strains from Japanese plum (*P. salicina*) were obtained from the National Institute of Agrobiological Sciences (NIAS, Japan). Pathotype strain of *P. syringae pv. morsprunorum* was obtained from the National Institute of Agrobiological Sciences (NIAS, Japan). The reaction mixture contained 5 µl of 10X buffer (100 mM Tris-HCl, pH 8.0, 500 mM KCl, 25 mM MgCl₂), 200 µM each of deoxynucleoside triphosphates, 20 pmol each of forward and reverse primers, 2.0 U of Taq DNA polymerase (Bioneer, Korea), and 50 ng of purified genomic DNA as a template. The reactions for 16S rDNA were routinely grown at 28°C on peptone-sucrose medium (20 g of peptone, 20 g of sucrose per 1 liter) in the broth or solid state. Total DNA from bacteria was isolated using a genomic DNA extraction kit (Bioneer, Korea). The 16S rDNA gene was amplified by using the universal bacterial primer pair 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525r (5'-AAAGGAGGTGATCCAGCC-3') (Lane, 1991). The primer BOXA1R (5'-CTACGGCAAGGCGACCTACCA-3') and BOX1525R (5'-AAAGGAGGTGATCCAGCC-3') described previously (Versalovic et al., 1994) was used for BOX-PCR. DNA was amplified in a total volume of 50 µl using a PCR Thermal Cycler (Takara Shozo, Japan). The reaction mixture contained 5 µl of 10X buffer (100 mM Tris-HCl, pH 8.0, 500 mM KCl, 25 mM MgCl₂), 200 µM each of deoxynucleoside triphosphates, 20 pmol each of forward and reverse primers, 2.0 U of Taq DNA polymerase (Bioneer, Korea), and 50 ng of purified genomic DNA as a template. The reactions for 16S rDNA were performed using the following program: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and an extension at 72°C for 30 s. Reactions were terminated after a final 5 min elongation at 72°C. PCR product was purified using a PCR purification kit (Bioneer, Korea), and the nucleotide sequence of the amplified DNA was determined by Solgent Co. (Korea). The BOX-PCR program comprised: one cycle at 95°C for 7 min; 30 cycles at 94°C for 1 min, 53°C for 1 min, and 65°C for 8 min; finally one cycle at 65°C for 15 min (Louws et al., 1994).

BOX-PCR allowed for distinction of *P. syringae pv. morsprunorum* race 1 and race 2 strains since they showed constant and specific BOX patterns. BOX primer gave reproducible PCR profiles consisting of bands ranging in size from approximately 900 bp to 6 kb. The Box-PCR pattern of the *P. syringae pv. morsprunorum* race 1 strains were grouped in only one pattern with 6 bands. The patterns of *P. syringae pv. morsprunorum* race 2 isolates were also uniform and were clearly distinctive from those of race 1 (Fig. 1). In the present work, *P. syringae pv. morsprunorum* LMG 2222 and LMG 5075, which were identified previously as *P. syringae pv. morsprunorum* race 1 and race 2, respectively, by BOX-PCR (Gilbert et al., 2009), were used as reference strains. Among the unclassified strains, 15 strains from Korea and Japan belonged to *P. syringae pv. morsprunorum* race 1 as the reference strain LMG 2222, whereas 18 isolates from Korea were included in *P. syringae pv. morsprunorum* race 2 as the pathotype strain of *P. syringae pv. morsprunorum* LMG 5075 (Fig. 1, Table 2). Both *P. syringae pv. morsprunorum* races were clearly different from the patterns of *P. syringae pv. syringae* and *P. syringae pv. actinidiae* (Fig. 1, lane 36, 37).

The sequence data of the thirty five strains of *P. syringae pv. morsprunorum*, including the thirty one Japanese apricot isolates and four strains from other *Prunus* plants, were

| Strains | Geographic origin | Host plant |
|---------|------------------|------------|
| LMG5075 | pathotype strain | Prunus domestica |
| MKS1    | Gangjin, Korea   | *P. mume*  |
| MKO1    | Gokseong, Korea  | *P. mume*  |
| MGD3    | MGD4, MGD6      | *P. mume*  |
| MBA3    | Boseong, Korea   | *P. mume*  |
| MSW3    | Sunchon, Korea   | *P. mume*  |
| MJA5    | Jangheung, Korea | *P. mume*  |
| MHS2    | Haenam, Korea    | *P. mume*  |
| MSM1    | Sacheon, Korea   | *P. mume*  |
| MJA1    | Jinju, Korea     | *P. mume*  |
| MHA1    | Hadong, Korea    | *P. mume*  |
| MWA1    | Hwasun, Korea    | *P. mume*  |
| MAF301433 | Aomori, Japan | *P. salicina*
| MAF301443 |  | *P. mume*  |
| MAF301444 |  | *P. mume*  |
| MAF301448 |  | *P. mume*  |
| MAF301610 |  | *P. mume*  |
| MAF301447 |  | *P. mume*  |
| MAF301436 |  | *P. mume*  |
| MAF301437 |  | *P. mume*  |
| MAF301445 |  | *P. mume*  |
| MAF301446 |  | *P. mume*  |
| LMG2222 | United Kingdom | Prunus avium |
| LMG 1247 | pathotype strain | Syringa vulgaris |
| KW11    | pathotype strain | Actinidia delicosa |

*Strain names with MAAF were obtained from the gene bank of the Japanese Ministry of Agriculture, Forestries, and Fisheries. LMG, BCCM/ICM/LMG Bacterial Collection.*
16S rDNA of Pseudomonas syringae pv. morsprunorum

aligned using the jPHYDIT program (available at http://plaza.snu.ac.kr/~jchun/jphydit/). As demonstrated in Table 2, sequence differences among the 16S rRNA genes due to substitutions occurred at 16 nucleotide positions. These differences allowed division of the 35 P. syringae pv. morsprunorum strains into three types, namely types I, II, and III. Type I showed 98.9% homology with type III. Type I and II showed only two base differences. Since direct sequencing was used in this work, the intraspecific heterogeneity of the 16S rRNA gene sequences was not due to multicopy rRNA operons which have different nucleotide compositions. Interestingly, as shown in Fig. 1, type I and II strains belonged to P. syringae pv. morsprunorum race 1, whereas the P. syringae pv. morsprunorum race 2 isolates were all type III in the 16S rRNA gene sequence typing. These results suggest that the 16S rRNA gene sequence can be used in identifying P. syringae pv. morsprunorum race 1 and race 2, based on the clear relationship between 16S rRNA gene sequence and race for all members tested in this work. Both races of P. syringae pv. morsprunorum were isolated from Japanese apricot in Korea, but race 2 was not found in Japanese strains isolated from P. mume and P. salicinia (Japanese plum). In the UK both races were isolated on sweet cherry and wild cherry (Freigoun and Crosse, 1975; Vicente et al., 2004). The 16S rRNA gene sequences of P. syringae pv. morsprunorum: MWW1 (type I), MAFF301436 (type II) and LMG 5075 (type III) were deposited in the GenBank database under the accession numbers of GU997635, GU997636 and GU997634, respectively.

Table 2. Nucleotide signatures of three 16S rDNA types identified in P. syringae pv. morsprunorum strains

| 16S rDNA position | P. syringae pv. morsprunorum |
|-------------------|----------------------------|
| 472 473 474 601 1005 1006 1007 1009 1010 1017 1020 1021 1022 1035 1136 1216 | LMG2222, MWW1, MWW2, MGD3, MGD4, MHA2, MHH1, MAFF301433, MAFF301444, MAFF301447, MAFF301448, MAFF301610 |
| type I (race 1) | G A T G C T C C G T T G A A C |
| type II (race 1) | T G A A A T |
| type III (race 2) | A T C A T C C T T A G G A G C |

*Numbers refer to positions based on the E. coli system. Dots indicate identical nucleotides with type I strains of pv. morsprunorum.
UK strain (LMG2222) from *P. avium*. Among the 24 Korean strains examined, 18 belonged to type III, which included the pathotype strain (LMG5075) isolated from *P. domestica*. This result demonstrates that the differences in 16S rRNA gene sequence were not correlated with differences in host plants. Four strains (MAFF301436, MAFF301437, MAFF301445 and MAFF301446) isolated from *P. mume* in Japan were belonged to Type II. A guanine at position 601 and cytosine at position 1216 in type I were replaced by an adenine and thymine in type II, respectively. Type I and II strains, which belonged to race 1, were considered to be closely related to each other and might have diverged recently. Bacterial 16S rRNA genes generally contain nine hypervariable regions, termed V1-V9, that demonstrate sequence diversity among different bacterial species (Van de Peer et al., 1996). As shown in Table 2, sequence variations among the *P. syringae* pv. *morsprunorum* strains mainly occurred at V3 and V6 regions spanning nucleotides 433–497 and 986–1043 (numbering based on the *E. coli* system), respectively (Chakravorty et al., 2007). Although the number of *P. syringae* pv. *morsprunorum* strains used in this study to represent Japanese strains isolated in *P. mume* was relatively small, it is interesting that type III was not found in Japan while type II was not found in Korea. These results indicate that variations in the 16S rRNA gene sequence could be used for molecular typing of this species in epidemiological aspects.

### Acknowledgements

This study was carried out with the support of “Cooperative Research Program for Agriculture and Technology Development (PJ006686072012)”, RDA, Republic of Korea.

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