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# The use of PCR melting profile for typing of *Pseudomonas syringae* isolates from stone fruit trees

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**Abstract** Of thirty fluorescent *Pseudomonas* isolates originating from symptomatic tissues of sweet (*Prunus avium*) and sour cherry (*Prunus cerasus*), plum (*Prunus domestica*), peach (*Prunus persica*) and apricot (*Prunus armeniaca*), 23 were identified as *P. syringae* using LOPAT tests. Further characterization of those isolates by GATTa and L-lactate utilization tests showed that 10 of them belonged to race 1, six to race 2 of *P. syringae* pv. *morsprunorum* (*Psm*) and six other isolates were identified as pathovar *syringae* (*Pss*). One isolate (791) was determined as atypical. Phenotypic determination and genetic analysis of studied isolates for toxin production revealed that isolates of *Pss* produced syringomycin, 3 *Psm* race 1 produced coronatine and 6 *Psm* race 2 produced yersiniabactin. Genetic diversity of all isolates was evaluated with the PCR melting profile (PCR MP) method. A dendrogram constructed with PCR MP patterns showed positive correlation with phenotypically distinguished pathovars. Isolates of *Psm* races 1 and 2 formed distinct, tight clusters, whereas *Pss* isolates were more heterogeneous. Isolate 791 was placed within *Pss* isolates. Bacteria identified as *Pss* caused more severe symptoms on immature cherry fruits compared to *Psm*, which corresponded to determined pathovars and races.

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*Pseudomonas syringae* is a polyphagous bacterium causing diseases of over 180 plant species, both annual and perennial, including fruit trees, ornamentals and vegetables (Agrios 2005). Depending on pathogenicity, the strains of *P. syringae* can be classified to over 50 pathovars (Young et al. 1996). On stone fruits, these are mainly pathovars *syringae* (causing bacterial canker particularly on sour cherry and belonging to genospecies 1) and *morsprunorum* (causing bacterial canker and gummosis mostly on sweet cherry and belonging to genospecies 2 and 3) (Young 1991; Gardan et al. 1999; Sobiczewski 1984; Burkowicz 1981; Vicente et al. 2004) were found. The pathogen attacks the branches and main trunk of the trees as well as buds, blossoms, leaves and fruits, causing reduction of yield and sometimes leading to death of the trees. The disease occurs in all major stone-fruit-growing areas of the world (Agrios 2005). Its diagnosis is commonly based on phenotypic characterization of causal agent, including pathogenicity (Bultreys and Gheysen 1999; Schaad et al. 2001, Vicente et al. 2004). For some pathovars of *P. syringae*, causing diseases on other plants (e.g. on

*Actinidia deliciosa* fruit), the diagnosis can be also confirmed by a PCR method with specific primers (Koh and Nou 2002). Recently, the PCR melting profile (PCR MP) method appeared to be very useful in genetic diversity studies of several bacterial genera, mainly Enterobacteriaceae (Masny and Płucienniczak 2003; Krawczyk et al. 2006, 2007). This method consists of three steps: digestion with restriction enzyme, ligation of oligonucleotide adapters corresponding to the restriction sites, and PCR amplification of the ligation products using low denaturation temperatures $T_d$ (80–88°C). Lowering of $T_d$ decreases the number of amplified fragments and leads to limited and specific amplification of less stable DNA fragments. This DNA fingerprinting method allows differentiation of bacteria, even at strain level, based on heterogeneity of their GC content and length of genomic DNA fragments obtained after digestion with endonucleases (Masny and Płucienniczak 2003).

The aim of our study was to characterize fluorescent Pseudomonas isolates originating from different symptomatic tissue of stone fruits in Poland using conventional and molecular methods and to determine their diversity by the PCR MP method. Thirty isolates were tested for the ability to induce hypersensitivity reaction (HR) on tobacco leaf cv. Samsun. $HR^+$ isolates were characterized using other LOPAT tests according to Lelliott and Stead (1987). To distinguish pathovars within identified *P.syringae* isolates, gelatin hydrolysis (G), aesculin hydrolysis (A), tyrosinase activity (T) and utilization of tartrate (Ta) tests and additionally L-lactate utilization test (Lattore and Jones 1979) were used. The pathogenicity of isolates was tested on immature sweet cherry fruits cv. Napoleon. Fruitlets, after disinfection by dipping in 50% ethanol, were inoculated (10 fruitlets per isolate) with a sterile needle immersed in a $10^8$ cfu/ml aqueous suspension of each isolate. After inoculation, fruitlets were placed on moist filter paper in sterile Petri dishes and incubated at 22°C for 4 days. The reference strains Pss 2905 (*Pss*), LMG 1247 (*Pss*), LMG 2222 (*Psm* race 1) and CFBP 3800 (*Psm* race 2) were included as positive controls and sterile distilled water was applied as a negative control. Symptoms on immature fruits were observed 96 h after inoculation.

Production of syringomycin (SR) and other toxic lipodepsipeptides (TLP) was evaluated on peptone-glucose-NaCl agar medium with the yeast *Rhodotorula pilimanae* MUCL30397 as an indicator strain (Bultreys and Gheysen 1999). To detect the genes responsible for toxin production, three primer sets were used: cfl1 and cfl2 for amplification of a gene for coronatine with a 650 bp product (Bereswill et al. 1994), PSYE2 and PSYE2R for detection of yersiniabactin with a 943 bp product (Bultreys et al. 2006) and a primer pair for amplification of *syrB* for syringomycin (SR) with a 752-bp product (Sorensen et al. 1998). Isolation of bacterial DNA was done according to the method of Aljanabi and Martinez (1997). Amplifications were conducted according to methods presented in the above papers with slight modification of annealing temperatures: 70°C, 45 s for cfl primers, 66°C, 45 s, for PSYE primers and 71°C, 45 s for *syr*B primers.

To determine genetic diversity of all *P. syringae* strains, a slightly modified method of PCR MP described by Masny and Płucienniczak (2003) was used. The digestion of 200 ng of DNA with *Pst*I endonuclease (10 U/μl, Promega) was performed in conditions according to manufacturer instructions. Digested DNA was ligated with two oligonucleotides forming an adaptor PstI and PstII according to Waugh et al. (1997). PCR amplifications were performed with primer Pst1 (Waugh et al. 1997) and GoTaq DNA polymerase (Promega Corporation, Madison, USA) with denaturation temperature ranged from 86°C to 88°C in a Biometra T3000 thermocycler (Biometra, Germany). PCR products were separated on a 1.5% agarose gel in TBE buffer. Each amplification band was scored as 1 (present) or 0 (absent), to determine a distance matrix for a Pearson correlation coefficient. A dendrogram using the UPGMA algorithm was constructed. Each reference strain was included for comparison with the stone fruit isolates.

Of 30 fluorescent bacterial isolates, 23 induced HR on tobacco leaves 24 h after infiltration. Using other LOPAT tests, all $HR^+$ isolates were identified as *Pseudomonas syringae*—group Ia. Based on the results of GATTa and L-lactate utilization tests, 10 isolates were classified as race 1 of *P. syringae* pv. *morsprunorum* (*Psm*), 6 as race 2 of this pathovar and 6 other isolates as pathovar *syringae* (*Pss*). Isolate 791 showed atypical characteristic as it utilized L-lactate and did not hydrolyze aesculine (Table 1). All *Pss* isolates, produced SR and /or other TLP, virulence factors used by some researchers as a marker for identification (Grgurina et al. 1996; Fukuchi et al. 1990). None of isolates belonging to *Psm* produced SR, which is in

agreement with descriptions by Schaad et al. (2001) and Janse (2005). The presence of *syrB* for production of SR by *Pss* isolates was confirmed by PCR. A specific 650 bp fragment for the coronatine gene (*cfl*) was found in three isolates characterized as *Psm* race 1 and in the reference strain of *Psm* LMG 2222 (Table 1). Although 4 other *Psm* race 1 isolates do not posses this gene, they appeared to be pathogenic to cherry fruitlets. Some COR-defective ($COR^-$) mutants were still pathogenic, however, the severity of symptoms was significantly less than those for COR producers (Xu and Gross 1988; Ullrich et al. 1993; Bender et al. 1999). The yersiniabactin gene was detected only in *Pseudomonas syringae* pv. *morsprunorum* race 2

**Table 1** The origin and characteristics of studied *Pseudomonas syringae* isolates[a]

| No. of isolate | Host | Year of isolation | Results of | | Group | Syringomycin | | Coronatine[b] | Yersinia-bactin[c] |
|---|---|---|---|---|---|---|---|---|---|
| | | | GATTa tests | L-lactate utilization | | inhibition zone of *R.pilimanae* | *syr*B presence | | |
| 701A | sweet cherry | 2005 | − − + + | − | *Psm* race 1 | − | − | + | − |
| 702 | plum | 1994 | − − + + | − | *Psm* race 1 | − | − | − | − |
| 704 | sweet cherry | 1994 | − − + + | − | *Psm* race 1 | − | − | + | − |
| 710 | sweet cherry | 1996 | − − + + | − | *Psm* race 1 | − | − | + | − |
| 755 | plum | 1999 | − − + + | − | *Psm* race 1 | − | − | − | − |
| 771 | plum | 1999 | − − + + | − | *Psm* race 1 | − | − | − | − |
| 782 | sweet cherry | 2001 | − − + + | − | *Psm* race 1 | − | − | − | − |
| 787 | plum | 2001 | − − + + | − | *Psm* race 1 | − | − | − | − |
| 788 | plum | 2001 | − − + + | − | *Psm* race 1 | − | − | − | − |
| 793 | plum | 2001 | − − + + | − | *Psm* race 1 | − | − | − | − |
| 701 | sour cherry | 1994 | + − − − | − | *Psm* race 2 | − | − | − | + |
| 719 | sour cherry | 1997 | + − − − | − | *Psm* race 2 | − | − | − | + |
| 732 | sour cherry | 1997 | + − − − | − | *Psm* race 2 | − | − | − | + |
| 733 | sour cherry | 1997 | + − − − | − | *Psm* race 2 | − | − | − | + |
| 745 | sour cherry | 1999 | + − − − | − | *Psm* race 2 | − | − | − | + |
| 764 | sour cherry | 1999 | + − − − | − | *Psm* race 2 | − | − | − | + |
| 791 | sour cherry | 2001 | + − − − | + | Atypical[d] | − | − | − | − |
| 702A | plum | 2005 | + + − − | + | *Pss* | + | + | − | − |
| 753 | apricot | 1999 | + + − − | + | *Pss* | + | + | − | − |
| 757 | plum | 1999 | + + − − | + | *Pss* | + | + | − | − |
| 760 | sour cherry | 1999 | + + − − | + | *Pss* | + | + | − | − |
| 762 | apricot | 1999 | + + − − | + | *Pss* | + | + | − | − |
| 763 | sour cherry | 1999 | + + − − | + | *Pss* | + | + | − | − |
| 2905 | sour cherry | 1978 | + + − − | + | Reference strain of *Pss* | + | + | − | − |
| LMG 1247 | *Syringa vulgaris* | | + + − − | + | Reference strain of *Pss* | + | + | − | − |
| LMG 2222 | *Prunus avium* cv. Napoleon | | − − + + | − | Reference strain of *Psm* race 1 | − | − | + | − |
| CFBP 3800 | *Prunus cerasus* | | + − − − | − | Reference strain of *Psm* race 2 | − | − | − | + |

[a] Based on LOPAT tests all isolates were identified as *Pseudomonas syringae* group 1a

[b] PCR detection of the *cfl* gene involved in coronatine production

[c] PCR detection of the *irp1* gene involved in yersiniabactin production

[d] based on PCR MP determined as *Pss*

+ positive, − negative

**Fig. 1** Symptoms on immature cherry fruitlets caused by *Pseudomonas syringae* isolates: (left) water-soaked superficial lesions—pathovar *morsprunorum*; (right) deep black/brown necrosis—pathovar *syringae*

isolates and in reference strain CFBP 3800 (Table 1). The production of this toxin by race 2 is propably due to better adaptation to its hosts (Haag et al. 1993; Bultreys et al. 2006).

Generally, two types of symptoms were observed on inoculated cherry fruitlets. All isolates identified as *Pss* produced deep brown-black necroses, whereas isolates from *Psm* race 1 and *Psm* race 2 caused water-soaked, superficial lesions (Figs. 1 and 3). Some of the *Pss* isolates caused larger necroses than reference strain Pss 2905. Isolate 791 did not produce water soaked lesions but necrosis typical for *Pss*. However, necrotic size was the smallest one out of all *Pss* isolates tested. Of all the bacteria studied, isolates 760, 762 and 702A, identified as *Pss* showed the highest virulence (produced the largest necroses), whereas isolates: 793, 771 and 701A identified as *Psm* race 1 gave the smallest superficial lesions (data not shown). No symptoms developed on fruitlets treated with sterile water. Greater virulence of *Pss* than *Psm* isolated from *Prunus* also was demonstrated in a cherry plantlet assay by Liang et al. (1994).

Using the PCR MP technique it was possible to show the diversity of the *P. syringae* isolates. The denaturation temperature 86.5°C gave 8 to 15 of fragments allowing for grouping of all isolates (Fig. 2). Strain LMG 1247 yielded the lowest number of fragments and LMG 2222 and 710 the highest number of fragments. A denaturation temperature of 88.0°C was too high because too many bands were obtained, but at 86.0°C only 4–11 fragments were produced (data not shown). A dendrogram based on PCR MP patterns of isolates and reference strains

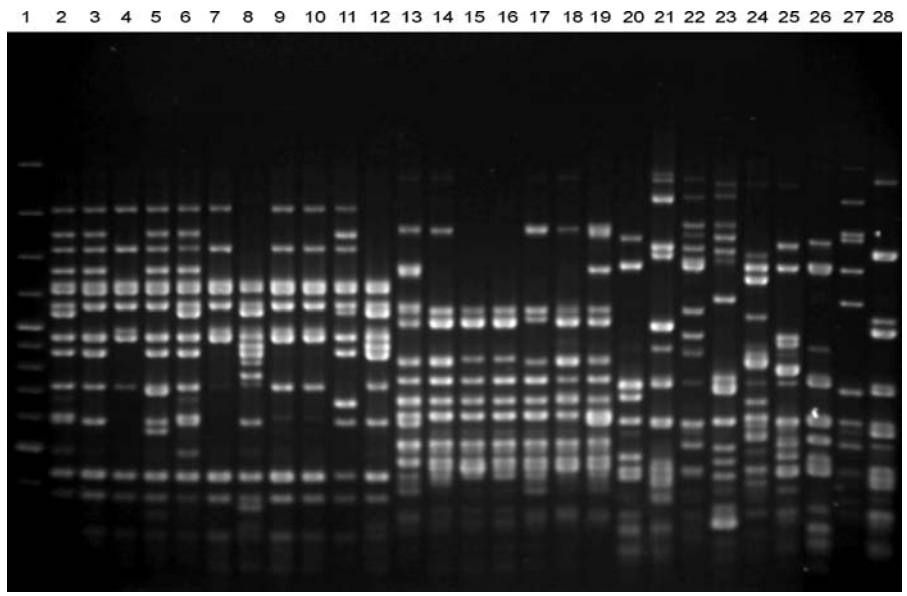


**Fig. 2** Electrophoretic patterns obtained after PCR MP of fluorescent psuedomonads with primer Pst1 Lane 1 – M – marker 100 bp Ladder (Fermentas,#SM0321); pv. *morsprunorum* race 1 isolates: 2 – LMG 2222, 3 – 701A , 4 – 702 , 5 – 704 , 6 – 710, 7 – 755,8 – 771, 9 – 787, 10 – 788, 11 – 782, 12 – 793; pv. *morsprunorum* race 2 isolates: 13 – CFBP 3800, 14 – 719, 15 – 732, 16 – 733, 17 – 745, 18 – 701, 19– 764; pv. *syringae* isolates: 20 – LMG 1247, 21 – 2905, 22 – 760, 23 – 791, 24 – 702A, 25 – 763, 26 – 753, 27 – 757, 28 – 762

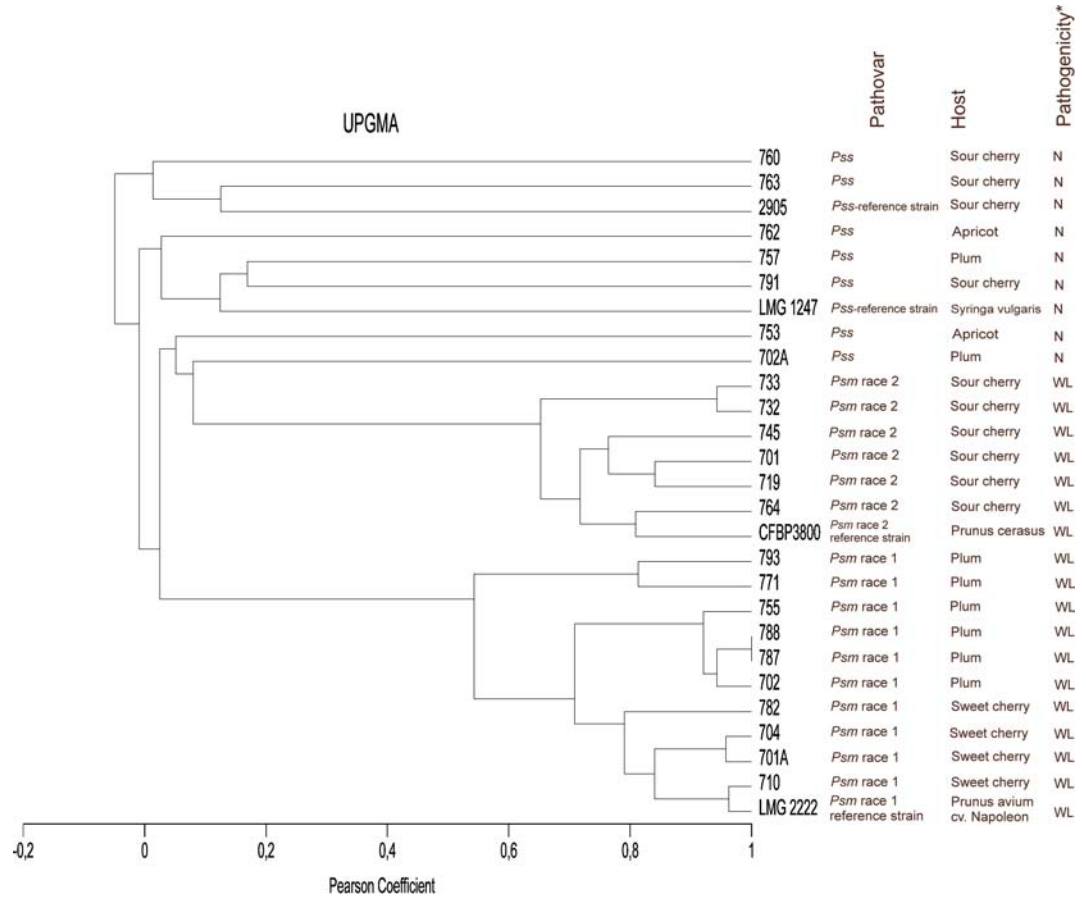


**Fig. 3** Dendrogram reflecting the relationship of studied bacteria based on PCR MP analysis. *N—necrosis and WL—water-soaked lesions, symptoms produced by strains in pathogenicity test on immature cherry fruits

show clearly that isolates and respective strains of *Psm* races 1 and 2 formed separate clusters, but those of *Pss* were more heterogeneous (Fig. 3). *Psm* races 1 and 2 were located in separate groups having higher similarity in their PCR MP patterns than those of the *Pss* group (Pearson coefficient >0.749). Isolate 791 was placed within *Pss* isolates. Various molecular methods have been applied for identification and differentiation of *P.syringae* pathovars (Weingart and Völksch 1997; Clerc et al. 1998; Ménard et al. 2003; Hwang et al. 2005). The PCR MP fingerprinting method is a new tool recently used to determine the genetic diversity of human- and plant-associated bacteria (Masny and Płucienniczak 2003; Olczak-Woltman et al. 2007, Krawczyk et al. 2006, 2007). This method was untested on isolates of the causal agent of bacterial canker on stone fruit trees. Using PCR MP, the *P. syringae* isolates we studied were grouped into clusters that strongly correspond with phenotypically differentiated pathovars and races. Within *Pss* isolates, heterogeneity was found. An important point is that the atypical isolate 791, that did not produce syringomycin but induced necroses on cherry fruitlets, was grouped with other *Pss* isolates. Phenotypic heterogeneity of *Pss* also was reported by others who found this pathovar to have characteristics overlapping with those of other pathovars (Sarkar and Guttman 2004; Sawada et al. 1999). Genetic heterogeneity of pathovar *syringae* was shown by Weingart and Völksch (1997), Gilbert et al. (2009); Ménard et al. (2003); Vicente et al. (2004) and Renick et al. (2008) using mainly rep-PCR methods. PCR MP was used to differentiate pathogenic pseudomonads isolated from cucumber and gave better discrimination than other techniques such as PCR-RFLP of the ITS region and analysis of the

*syr*B gene fragment (Olczak-Woltman et al. 2007). In studies with the clinical bacteria *Escherichia coli* and *Staphylococcus aureus*, the results obtained by using PCR MP were in agreement with simultaneously used restriction analysis of genomic DNA followed by pulsed-field gel electrophoresis (REA-PFGE) (Krawczyk et al. 2006, 2007). PCR MP appeared to be as good or better for purposes of epidemiological and genetic relations as those currently considered being a "gold standard", such as REA-PFGE which is recommended as a reference method for molecular typing (Van Belkum et al. 1998).

The results of our studies showed that PCR MP can be used not only for determination of genetic variability of *Pseudomonas syringae* isolates causing bacterial canker on stone fruit trees, but also as a quick, simple, and cost-effective method for identification and classification of isolates to pathovars and races. Thus, PCR MP could be used in tandem with rep-PCR methods or by itself to classify and examine the genetic variability of fluorescent pseudomonads from stone fruits with bacterial canker.

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