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Genetic and pathogenic diversity of *Pseudomonas syringae* strains isolated from cucurbits

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Abstract This work was done to determine the diversity of pathogenic *Pseudomonas syringae* pv. *lachrymans* and pv. *syringae* strains, isolated from cucurbit plants. Pathogenicity tests performed in this work on cucumber indicated that the 22 tested strains differed dramatically in virulence level. Genetic characterization using MLST loci, as well as ITS1, ERIC and REP fingerprinting, allowed the grouping of strains into four phylogenetic groups. After amplicon sequencing and BLAST alignment the collected strains comply with *P. syringae* pathovars: *lachrymans* and *syringae* and with saprophytic *P. fluorescens*. The strains of *Pseudomonas syringae* pv. *lachrymans* may be categorized into at least two types differing in both disease symptoms and molecular characteristics. We confirmed that MLST-loci-based grouping of strains corresponds well to grouping based on pathogenicity tests.

Keywords *Cucurbitaceae* · *Pseudomonas lachrymans* · MLST · Molecular differentiation

Abbreviations

ALS	Angular leaf spot
CFU	Colony forming unit
DSI	Disease severity index
ERIC	Enterobacterial repetitive intergenic consensus
HR	Hypersensitive reaction
ITS1	Inter-genic spacer 1
MLST	Multi-locus sequence typing
pv.	Pathovar
REP	Repetitive extragenic palindromic sequences
RH	Relative humidity

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Introduction

Pseudomonas syringae pv. *lachrymans,* a pathogen of cucumber (*Cucumis sativus* L.), is one of the pathovars belonging to the heterogeneous bacteria species *Pseudomonas syringae* (Young et al. 1996). It causes angular leaf spot (ALS), a very common cucumber disease. The symptoms of ALS consist of vein-limited water-soaked lesions, which later become necrotic, resulting in significant yield reduction (Bradbury 1986). Although this disease is ascribed to a single pathovar, there are numerous strains of this pathovar that vary from each other at biological, biochemical and genetic levels. Moreover, similar symptoms on cucumber may be caused by strains belonging to pathovar *syringae* (Olczak-Woltman *et al*. 2007).

Recent studies of *P. syringae* reveal that various strains differ dramatically in genome size, GC content, plasmid presence, and pathogenesis-related gene repertoire including effector-encoding genes (Baltrus *et al*. 2011; O'Brien *et al*. 2011). These variations illustrate *P. syringae* genome plasticity and indicate the dynamic nature of this species. Analysis of the 16S variable

region and of the Intergenic Spacer 1 (ITS1) region used to be commonly employed in bacterial strain differentiation and identification. In addition, intraspecific typing of bacteria can be done using the Multi-locus Sequence Typing (MLST) method. MLST is preferred for determination of genomic relatedness among bacterial strains because it allows comparison of sequences of housekeeping genes that encode proteins necessary for the microorganism's survival (Maiden *et al*. 1998; Urwin and Maiden 2003). MLST can be applied to *Pseudomonas syringae* (Sarkar and Guttman 2004). Another approach allowing bacterial isolate identification is based on PCR amplification of bacterial repetitive sequences (rep-PCR technique). In this approach Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences and Repetitive Extragenic Palindromic (REP) sequences are analyzed. These are short, repetitive DNA sequences with highly conserved central inverted repeats that are dispersed throughout the genomes of diverse bacterial species (Versalovic et al. 1991). Designing universal rep-PCR primers (ERIC and REP) allows generation of highly reproducible fingerprints that can differentiate strains below the level of species (Louws et al. 1994).

The aim of this study was to determine the genetic diversity among strains of *Pseudomonas* sp. collected from different cucurbit plants using the MLST method, ITS1 and rep-PCR primers. The molecular identification was verified by pathogenicity tests performed on cucumber and tobacco plants. Additionally, the aim of this study was to determine an efficient molecular technique for identification of various strains of *P. syringae* pv. *lachrymans*. We also looked for presence of plasmids in the collected bacterial strains and investigated whether there was a correlation between the plasmid's presence and the strain's increased pathogenicity and/or virulence.

Materials and methods

Bacterial strains The bacterial collection consisted of 22 *Pseudomonas* strains. Nine strains were isolated from cucumber leaves that were showing angular leaf spot symptoms, collected in Poland (Olczak-Woltman *et al*. 2007). The others were obtained from the European bacterial culture collections (Table 1). All strains were stored on nutrient agar slopes at 4 °C and only pure cultures were used in molecular analysis and assays.

Cucumber pathogenicity and virulence tests To verify the strain pathogenicity on cucumber and to define the virulence level, the seeds of susceptible to ALS *Cucumis sativus* inbred line B, selected from variety 'Borszczagowski', were individually sown into plastic pots filled with peat moss. The plants were maintained in a growth chamber at 25 °C during the day and 22 °C at night, with 16 h illumination under high pressure sodium lamps providing light intensity of 50 W m^{-2}. Inoculum was prepared by growing bacterial cells for 24 h on King B agar plates at 28 °C in the dark. The resulting colonies were suspended in sterile distilled water and adjusted to $OD_{600}=0.050$ previously established to be equal to concentration of 1×10^7 CFU ml^{-1}. Cucumber plants at the 2nd to 3rd leaf stage were inoculated by spraying the abaxial side of each leaf (Klement *et al*. 1990). Sterile water was used as a control. After inoculation, plants were kept in the dark at 100 % RH for 24 h, and then for 6 days in the growth chamber under light and with 90 % relative humidity. After 7 days, inoculated leaves were scored for disease severity and appearance of symptoms. The evaluation using a 1–9 disease severity index (DSI) where the most resistant plants were given the score of nine and the most susceptibility plants were given the score of one, was performed according to the protocol previously described (Olczak-Woltman *et al*. 2008). The pathogenicity tests of each strain were performed using a total of 24 plants (with four replications of six plants in each replication).

Pathogenicity verification The strain pathogenicity was verified using *Nicotiana benthamiana* as a host. The seeds of *N. benthamiana* were sown into plastic pots filled with peat moss. The growth chamber conditions and bacterial inoculum preparation were the same as for the cucumber pathogenicity and virulence tests. Nine-weeks-old tobacco plants were inoculated by infiltration of the abaxial side of each leaf (five separate points per leaf) with bacterial suspension. Infiltration using sterile water was used as a control. Each strain was tested on a separate tobacco plant. Additionally, some tobacco plants were inoculated with four different strains on the same leaf. Plants were kept in the dark at 100 % relative humidity for 24 h, and then for 48 h in the chamber with light (50 W m^{-2}) and 90 % relative

Table 1 Bacterial culture collection – name, place and plant of isolation, the HR results on tobacco and cucumber pathogenicity and virulence tests with DSI and symptoms description

No	Strain's name & pathovar	Source of strain		HR on tobbaco	Pathogenicity on cucumber			
		country	species		*P*	*V*	*DSI*	Symptoms description
1	Psl 814/98	Holland	cucumber	+	+	***	4,0	water-soaked, bacterial ooze
2	Psl CCM2857	Czech Rep	cucumber	+	+	***	4,0	water-soaked, bacterial ooze
3	Psl BG283	Holland	cucumber	–	+	*	8,1	few chlorotic lesions
4	Psl BG966	Holland	cucumber	+	+	**	5,5	necrotic and chlorotic lesions
5	Psl LMG5070	Belgium	cucumber	+	+	**	5,3	necrotic and chlorotic lesions
6	Pss 2905	Poland	stone fruits	+	+	**	6,5	tiny, chlorotic/necrotic lesions
7	Pss BG913	Holland	cucumber	+	+	**	5,9	pinpoint, chlorotic lesions,
8	Pss CCB37/09	Czech Rep	cucumber	+	+	**	6,6	dry, papery, necrotic lesions
9	Pss CCM2858	Czech Rep	watermelon	+	+	***	3,8	large necrotic lesions
10	Pss PD2021	Italy	pumpkin	+	+	**	6,8	dry, papery, necrotic lesions
11	Pss WK1/02	Poland	cucumber	+	+	**	5,9	dry, papery, necrotic lesions
12	Pss WW4/02	Poland	cucumber	+	+	*	8,3	few chlorotic lesions
13	Pss WW17/01	Poland	cucumber	+	+	**	6,5	pinpoint, chlorotic lesions
14	Pss WW27/01	Poland	cucumber	+	+	**	6,4	pinpoint, chlorotic lesions
15	Pss WW254/2	Poland	cucumber	+	+	**	5,3	pinpoint, chlorotic lesions
16	P.sp. PD3662	Holland	cucumber	+	+	*	7,1	limited necrotic lesions, halo
17	P.sp.WH6/01	Poland	cucumber	+	+	*	7,5	limited necrotic lesions, halo
18	P.sp.WM2/02	Poland	cucumber	+	+	*	7,6	limited necrotic lesions, halo
19	P.sp.YPG1293	Holland	cucumber	+	+	*	7,5	limited necrotic lesions, halo
20	Pf PD2760	Italy	*Cucumis* sp.	–	+	*	8,0	limited chlorotic lesions
21	Pf WH1/01	Poland	cucumber	–	+	*	8,4	limited chlorotic lesions
22	Pf WH2/01	Poland	cucumber	–	+	*	8,4	limited chlorotic lesions

Strain's sources: strains 814/98 and YPG1293 of Dutch origin were obtained from the Institute of Plant Protection, Poznan, Poland. Strain LMG5070 was obtained from the Microbiology Laboratory in Gent, Belgium. Strains BG283, BG913, and BG966 were obtained from the Nunhems Netherlands, Haelen, The Netherlands. Strains CCB37/09, CCM2857, and CCM2858 were obtained from AGRI, Olomouc, Czech Republic. Strains PD2021 and PD2760 of Italian origin and strain PD3662 of Dutch origin were obtained from the National Reference Centre, Plant Protection Service, Wageningen, The Netherlands. Strain 2905 of *P. syringae* pv. *syringae* was obtained from the Research Institute of Pomology and Floriculture in Skierniewice, Poland. Nine strains were isolated from cucumber leaves that were showing angular leaf spot symptoms, and were collected in central and southern (WW, WM and WK, WH, respectively) Poland (Olczak-Woltman *et al*. 2007)

The abbreviations of the species/pathovars names before the laboratory collections numbers means: *Psl* - Pseudomonas syringae pv. lachrymans; *Pss* - Pseudomonas syringae pv. syringe; *Pf* - Pseudomonas fluorescens; *P.sp.* - Pseudomonas sp.;

HR on tobacco=hypersensitivity reaction on *N.benthamiana*. (+)=HR present, and (−)=no HR. *P*=pathogenicity on susceptible cucumber accession line B. (+)=symptoms present, and (−)=no symptoms. *V*= the virulence level. ***=highly virulent strain, **=average level of virulence *=low virulent strain. *DSI*=disease severity index, where 9=no symptoms, and 1=the maximum of symptoms. Each strain was tested on 24 plants of susceptible cucumber accession line B, and the DSI is an average score of these 24 scores

humidity. The lesions were scored for the presence of hypersensitive reaction (HR) 3 d after inoculation.

Bacterial DNA isolation After growing the cultures for 24 h in LB liquid medium on a rotary shaker at 28 °C, total genomic DNA was extracted from 1 ml of culture using the DNA Genomic-tips 100/G kit (Qiagen, Germany), following the manufacturer's instructions. The DNA concentration was estimated by electrophoresis of 2 μl DNA, together with a DNA marker, on a 1 % agarose gel with 1× TEA buffer and stained with ethidium bromide.

Plasmid detection Plasmid bands were detected and visualised using Eckhardt gel electrophoresis technique

(Eckhardt 1978; modified by Maj *et al*. 2013). The bacterial cultures were grown overnight in LB liquid medium on a rotary shaker at 28 °C. The free-living cells contained in 1 ml of LB culture at OD_{620}=0.20 were harvested by centrifugation. The pellet was suspended in 0.5 ml of cold water and layered onto 1 ml of ice-cold 0.3 % sodium sarcosinate, centrifuged again, and the pellet was resuspended in the mixture (40 μl) of 10 mM Tris–HCl, 10 mM EDTA, and 20 % Ficoll (MT 400 000). The samples were held on ice for 10 min. A 0.75 % agarose gel with 0.5× TBE buffer was prepared by loading 25 μl of 10 % SDS containing xylene cyanole FF (1 mg ml^{-1}) into each origin slot and backtracking at 100 V for 30 min. Next, 10 μl of lysis solution (10 mM Tris–HCl, 10 mM EDTA, 0.4 mg ml^{-1} boiled RNase type A, 1 mg ml^{-1} lysosyme, and 1 mg ml^{-1} bromophenol blue) was mixed with each sample. Then 25 μl of such sample was loaded into the gel. The electrophoresis was run for 15 h and the gel was strained with ethidium bromide (0.5 μg ml^{-1}) for 20 min. Strain 1448A of *P.syringae* pv. *phaseolicola*, that contains two plasmids of known size (Joardar *et al*. 2005), was used as a positive control in plasmids visualization on the gel.

Amplification of the ITS1 region DNA amplification was performed using D21 and D22 primers (Manceu and Horvais 1997) according to the protocol of Sorensen et al. (1998) with modifications of Olczak-Woltman *et al*. (2007). The PCR amplifications were performed using a PTC-200 thermocycler (MJ Research, USA) with the following parameters: template denaturation at 94 °C for 1.5 min, primer annealing at 62 °C for 1 min, and DNA extension for 2 min at 72 °C. The PCR was repeated for 30 cycles, with a final extension step of 10 min at 72 °C. Amplicons were detected by electrophoresis on a 1 % agarose gels with 0.5× TBE buffer, stained with ethidium bromide and photographed using an UV trans-illuminator.

Amplification of the selected MLST loci Identification of *P. syringae* pv. *lachrymans* strains was done by PCR amplification using primers for the following housekeeping genes: aconitate hydratase B (*acnB*), citrate synthase (*cts*), glyceraldehyde-3-phosphate dehydrogenase (*gapA*), gyrase B (*gyrB*), phosphofructokinase (*pfk*), phosphoglucoisomerase (*pgi*) (Sarkar and Guttman 2004; Hwang *et al*. 2005). In addition, we performed an amplification of harpin elicitor (*hrpZ*) (Inoue and Takikawa 2006) using the same conditions as for the MLST loci. The characteristics of the used MLST loci and the harpin elicitor, i.e. the name, encoding protein, melting temperature, length, primer sequence, amplicon length and the author are listed in Table 2. Reactions (repeated twice) were carried out for all strains in the collection, in the same PCR and detection conditions as described for amplification of the ITS1 region.

Amplicons sequencing The successful PCR reactions, in which the resulting target PCR product was obtained, were collected and commercially sequenced (Genomed, Poland). Contigs were assembled using Sequencher 4.5 Software (Gene Codes, USA). Consensus sequences were submitted to the GenBank and accession numbers are provided in Table 3.

Strain differentiation using ERIC and REP fingerprinting Primer sequences corresponding to ERIC elements (ERIC1R: 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and ERIC2: 5′-AAGTAAGTGACTGGGGTGAGCG-3′) and corresponding to REP elements (REP1R-I: 5-′ IIIICGICGICATCIGGC-3′ and REP2-I: 5′-ICGICTTA TCIGGCCTAC-3′) were used (De Bruijn 1992). The reaction mixture (20 μl) consisted of 10× Dream Taq buffer (Thermo Scientific, Lithuania), 0.4 μM of dNTPs Mix, 0.75 μM of each primer, 1.0 U of DreamTaq DNA polymerase and 4 μl of bacterial suspension. The PCR amplifications were performed in a PTC-200 thermocycler (MJ Research, USA) with the following parameters: initial denaturation at 95 °C for 7 min, template denaturation at 94 °C for 1 min, primer annealing at 52 °C (ERIC) or at 41 °C (REP) for 1 min, and DNA extension for 8 min at 65 °C. The PCR was repeated for 30 cycles, with a final extension step of 15 min at 65 °C. Amplicons were detected by electrophoresis on a 1.5 % agarose gels with 0.5× TBE buffer, stained with ethidium bromide and photographed using a digital camera and a UV trans-illuminator.

Data analysis Polymorphisms generated by MLST and ITS1 in PCR reactions and visualised after sequencing were analysed using Geneious R7 software (Biomatters, USA). Genetic similarity (%) among the bacterial strains was calculated and the alignments were made using ClustalW method. Dendrograms were constructed using UPGMA clustering method. Polymorphisms generated by ERIC- and REP- PCR reactions were analysed

Table 2 The ITS1, *hrpZ* gene fragment and MLST primers used in this study

Name of the amplified region	Tm (°C)	Length (bp)	Sequence (5′→3~′)	Amplicon length (bp)	Reference
16S-23S ITS	54	20	AGCCGTAGGGGAACCTGCGG	550–574	Manceu and Horvais (1997)
	50	20	TGACTGCCAAGGCATCCACC		
acn	60	23	ACATCCCGCTGCACGCYCTGGCC	626	Sarkar and Guttman (2004)
	60	24	GTGGTGTCCTGGGAACCGACGGTG		
cts (*glt*)	64	24	GCCTCBTGCGAGTCGAAGATCACC	949–667	Hwang et al. (2005)
	62,8	24	CTTGTAVGGRCYGGAGAGCATTTC		
gapA	62	16	CGCCATYCGCAACCG	681	Sarkar and Guttman (2004)
	62	19	CCCAYTCGTTGTCGTACCA		
gyrB	62,9	23	TCBGCRGCVGARGTSATCATGAC	703	Hwang et al. (2005)
	60,7	23	TTGTCYTTGGTCTGSGAGCTGAA		
hrpZ	60	20	TTGGCTCAAGAGTTGACCCG	810	Inoue and Takikawa (2006)
	60	20	GCGCGTTGACCAGCAAGTTG		
pgi	60	25	TGCAGGACTTCAGCATGCGCGAAGC	559–589	Sarkar and Guttman (2004)
	60	25	CGAGCCGCCCTGSGCCAGGTACCAG		
pfk	63	20	ACCMTGAACCCKGCGCTGGA	785–815	Sarkar and Guttman (2004)
	63	20	ATRCCGAAVCCGAHCTGGGT		
rpoD	63	25	AAGGCGARATCGAAATCGCCAAGCG	532	Sarkar and Guttman (2004)
	63	25	GGAACWKGCGCAGGAAGTCGGCACG		

16S-23S ITS1=—the 16S-23S intergenic spacer region, *acn*=aconitate hydratase, *cts*=(*glt*) citrate synthase, *gapA*=glyceraldehyde-3-phosphate dehydrogenase, *gyrB*=gyrase B, *hrpZ*=harpin *hrpZ, pgi*=glucose-6-phosphate isomerase, *pfk*=1-phosphofructokinase, *rpoD*=sigma factor 70

using GelCompar II v.6.6 software (Applied Maths, USA). Genetic similarity (%) of bacterial strains patterns was calculated using the Dice coefficient and a dendrogram was constructed using the UPGMA clustering method.

Sequences were aligned to genome sequences of *P. syringae* strains collected in the JGI database (DOE Joint Genome Institute, USA) using BLAST algorithm.

Results

Hypersensitivity reaction on tobacco leaves All but four strains produced a HR on *N. benthamiana* leaves 3 d after infiltration, confirming their pathogenicity (Table 1, Fig. 1). Strains BG283, PD2760, WH1/01, and WH2/01 did not produce a HR on tobacco.

Pathogenicity and virulence tests on cucumber leaves The bacterial strains exhibited diverse pathogenicity and virulence levels when tested on susceptible cucumber accession line B (Fig. 2 a–f). *P. syringae* pv. *lachrymans* strains 814/98 and CCM2857 were the most virulent and caused typical angular leaf spot symptoms on cucumber leaves, i.e. water-soaked, later necrotic lesions with a large chlorotic halo, as well as the bacterial ooze on the abaxial side of the inoculated leaves (Fig. 2a). *P. s.* pv. *lachrymans* strains LMG5070 and BG966 were far less virulent on cucumber leaves, causing fast drying water-soaked lesions, and dry, light-coloured, papery lesions, with numerous pinpoint chlorotic lesions (Fig. 2b). Strain CCM2858 was a very virulent one, causing characteristic drying of the part of cucumber leaf, but although the lesions were very large in size, they differed dramatically from the lesions caused by other strains. Strain 2905 of pathovar *syringae* and strains BG913, WW17/01, WW27/01, and WW254/02 caused numerous pinpoint chlorotic or light-coloured necrotic lesions on cucumber leaves. In the case of strain BG913, these symptoms were the most severe, and in case of WW17/01 and WW27/01 less severe (Fig. 2d). Similar symptoms were caused by LMG5070 and BG966 strains. Strains CCB37/09, PD2021, and WK1/02 caused on cucumber large, dry, light-coloured necrotic lesions without chlorosis (Fig. 2e). Similar lesion, although smaller in size, were caused by strains PD3662, WH6/01, WM2/02, and YPG1293. Strains BG283 (Fig. 2c), PD2760, WH1/01, WH2/01 (Fig. 2f), and WW4/02 produced only very weekly chlorotic lesions, HR reactions, or did not produce any symptoms on cucumber leaves.

Table 3 Consensus sequences submitted to the NCBI GenBank with accession numbers

Strain name	16S-23S ITS	*acn* partial sequence	*cts* partial sequence	*gapA* partial sequence	*gyrB* partial sequence	*hrpZ* partial sequence	*pfk* partial sequence	*pgi* partial sequence	*rpoD* partial sequence
Psl 814/98	HQ171969	KJ158838	KJ158857	HQ171970	KJ158888	HQ171971	KJ158909	KJ158926	KJ158944
PslCCM2857		KJ158839	KJ158858	JN624869	KJ158889	JN624876	KJ158910		KJ158945
Psl BG283			KJ158859	KJ158878	KJ158890	JN624875		KJ158927	KJ158946
Psl BG966	JN624859	KJ158842	KJ158862	JN624867	KJ158893		KJ158913	KJ158930	KJ158949
PslLMG5070	JN624860	KJ158841	KJ158861	JN624868	KJ158892		KJ158912	KJ158929	KJ158948
Pss 2905	JN624865	KJ158855	KJ158876	JN624874	KJ158908		KJ158924	KJ158942	KJ158965
Pss BG913		KJ158844	KJ158865	JN624866	KJ158896		KJ158916	KJ158932	KJ158952
PssCCB37/09		KJ158846	KJ158867	KJ158879	KJ158898		KJ158917		KJ158954
PssCCM2858	JN624861	KJ158843	KJ158864	JN624870	KJ158895		KJ158915	KJ158933	KJ158951
Pss PD2021	JN624862		KJ158863	JN624871	KJ158894		KJ158914	KJ158931	KJ158950
Pss WK1/02		KJ158851	KJ158872	KJ158884	KJ158902		KJ158921	KJ158938	KJ158959
Pss WW4/02		KJ158850	KJ158871	KJ158883	KJ158901		KJ158920		KJ158958
PssWW17/01		KJ158854		KJ158886	KJ158905			KJ158940	KJ158962
PssWW27/01		KJ158849	KJ158870	KJ158882	KJ158900		KJ158919	KJ158937	KJ158957
PssWW254/02		KJ158853	KJ158874	KJ158885	KJ158904		KJ158923	KJ158939	KJ158961
P.sp.PD3662		KJ158840	KJ158860		KJ158891	JN624877	KJ158911	KJ158928	KJ158947
P.sp.WH6/01			KJ158869	KJ158881	KJ158899		KJ158918	KJ158936	KJ158956
P.sp.WM2/02		KJ158852			KJ158903		KJ158922		KJ158960
P.sp.YPG1293	JN624863	KJ158856	KJ158877	JN624872	KJ158906		KJ158925	KJ158943	KJ158964
Pf PD2760	JN624864	KJ158845	KJ158866	JN624873	KJ158897			KJ158934	KJ158953
Pf WH1/01		KJ158847	KJ158868	KJ158880				KJ158935	KJ158955
Pf WH2/01	HQ171972	KJ158848	KJ158873	HQ171973					

Amplification of the ITS1 region Amplification of the 16S-23S ITS1 region allowed us to obtain amplicons of 560–574 bp long in all strains. However, despite of several attempt sequencing of this fragment was possible only in case of 10 out of 22 strains.

Amplification of the hrpZ gene fragment Amplification of the *hrpZ* gene fragment produced 810 bp long amplicon. However, despite several attempts, this fragment was obtained and sequenced for only four strains: 814/98, BG283, CCM2857, and PD3662.

Amplification of the MLST loci Amplification of the 7 MLST loci resulted in informative amplicons that further facilitated grouping of strains. The obtained amplicons ranged from 530 to 970 bp, depending on the MLST locus (Table 2). Although the obtained amplicon quality was satisfactory, in the case of *acn*, *pgi,* and *rpoD* amplification failed in 1 strain, in case of *gyrB* and *pfk* - in 2 strains; in case of *gapA* – amplification of 3 strains failed. Amplicons for *cts* were obtained in all strains. The obtained amplicons were sequenced and the consensus sequences of all alleles were submitted to the NCBI GenBank (the consensus sequences symbols and numbers are provided in Table 3).

Strains differentiation by ERIC and REP fingerprinting ERIC and REP showed significant diversity in the banding patterns of all strains. Each pattern consisted of approximately 11–22 bands, ranging in size from 130 to over 3,000 bp. The strains' banding patterns differed from each other, although in case of ERIC (Figs. 4 and 5) and REP (data not shown) strains LMG 5070 and BG966 showed identical band patterns.

Sequences alignment The obtained ITS1, *hrpZ*, and MLST sequences were aligned to sequences deposited in the JGI base. The alignment of 10 obtained ITS1 amplicons allowed us to divide strains into four groups.

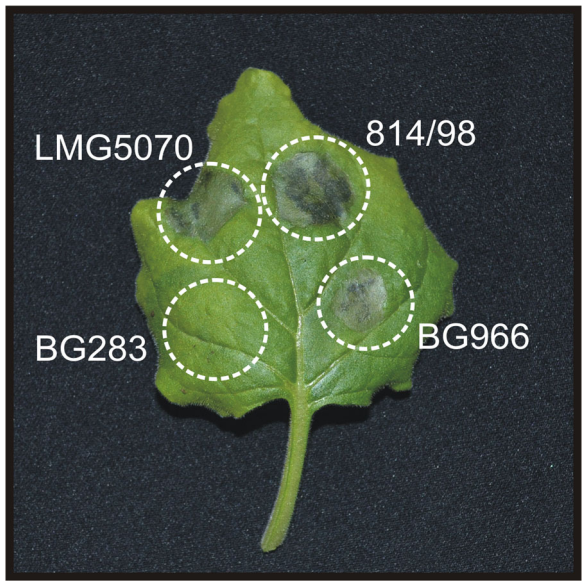

Fig. 1 The results of the HR test performed on *Nicotiana benthamiana* with visualisation of differences in pathogenicity. A tobacco leaf three days after infiltration. This leaf was infiltrated with four different strains, all classified as *P.s.* pv. *lachrymans*: 814/98 of high virulence level, LMG5070 and BG966 of medium virulence level, and BG283 of low virulence level

Based on the ITS1 loci analysis, strain 814/98 exhibited 99 % of genetic similarity to *lachrymans* strain Pla107 deposited in the JGI base. Strains LMG5070 and BG966 showed the highest genetic similarity to *lachrymans* strain Pla106. Strains CCM2858, PD2021, and WW4/02 exhibited the highest genetic similarity to strains of pathovar *syringae*, and the referenced strain 2905 confirmed its classification in this pathovar. Strains PD2760, WH1/01, and WH2/01 turned to be similar to *Pseudomonas fluorescens*.

The *hrpZ* amplicons were obtained only for strains 814/98, BG283, CCM2857, and PD3662. The alignment to the sequences deposited in the JGI database suggested that all these strains belong to pathovar *lachrymans*. In the case of 814/98 and CCM2857 the alignment indicated the highest similarity to *lachrymans* strain Pla107.

The MLST alignment allowed a very accurate strain grouping. All obtained MLST amplicons classified strains into the same pathovar, either *lachrymans* or *syringae*, or into *P.fluorescens*. Strain 814/98 according to loci *gapA*, *gyrB*, *rpoD*, *cts*, *acn*, *pgi*, and *pfk* was classified as pathovar *lachrymans*, with the highest similarity to strain Pla107. The same results were obtained for CCM2857, except that for locus *pgi* an amplicon was not generated. Strain BG283 exhibited the highest genetic similarity to strain Pla107 when using loci *gyrB*, *rpoD*, *cts*, and *pgi*. There were no amplicons for loci *acn* and *pfk*; and for locus *gapA* exhibited the highest similarity to pathovar *syringae*. This suggests that this strain may be classified as such but does not have to belong to pathovar *lachrymans*. Strains LMG5070 and BG966 in all tested MLST loci showed the highest similarity to pathovar *lachrymans* strain Pla106 and to pathovar *tomato*. Strain 2905 confirmed the highest similarity to pathovar *syringae* in all tested MLST loci. The same result was obtained for strain WW4/02 (although this does not correlate with the pathogenicity tests) and for strain WK1/02 (except for *gyrB* locus, where it was

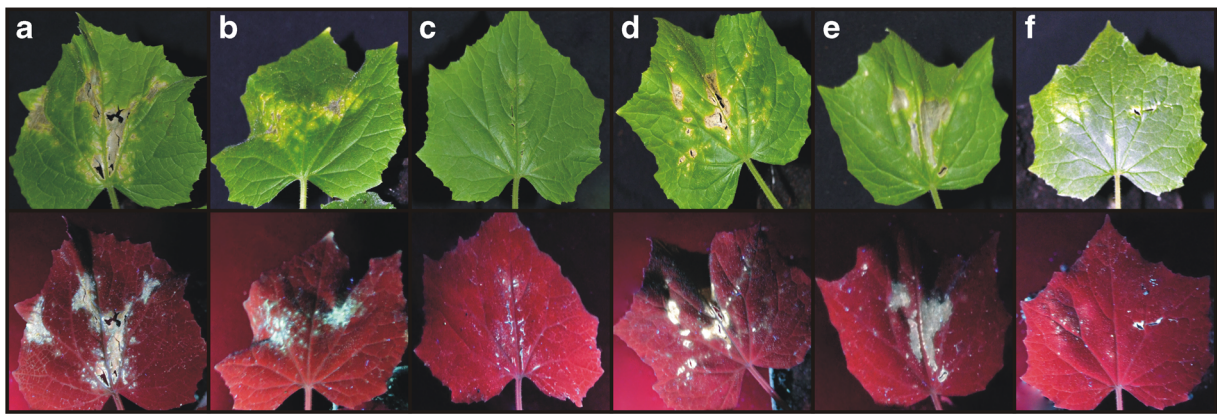

Fig. 2 The results of the pathogenicity and virulence tests performed on cucumber leaves of susceptible line B. Pictures were taken under day light (upper line) and under UV light (lower line—red leaves). The following strains are presented: **a** – Psl 814/98, **b** – Psl BG966, **c** – Psl BG283, **d** – Pss WW27/01, **e** – Pss WK1/02, **f** – Pf WH2/01. All strains were tested on line B - susceptible to angular leaf spot. Leaves were sprayed with inoculum containing 1×10^{7} CFU ml^{-1} and results were recorded 7 days after inoculation

similar also to pathovar *pisi*). Most of the strains that exhibited high similarity to pathovar *syringae* in 5 or 6 loci (i.e. BG913, CCB37/09, CCM2858, WW17/01, WW27/01, WW245/02) showed similarity to pathovar *aptata* at the *pfk* locus, and to pathovar *pisi* at the *acn* locus (strains WW17/01, WW27/01, and WW254/02). Strain PD2021 showed high similarity to pathovar *syringae* at 5 loci, failed to produce amplicons at the *acn* locus and showed high similarity to pathovar *pisi* at the *pfk* locus. Four strains: PD3662, WH6/01, WM2/02, and YPG 1293, exhibited similarity to various pathovars at various MLST loci, that is to *lachrymans* (Pla106 and/or Pla107), *syringae*, *tomato*, *maculicola*, *aesculi*, *actinidiae*, or *P.fluorescens*. Therefore classification of these strains into one certain pathovar was not possible. Three strains in our collection (PD2760, WH1/01, and WH2/02) turned out to be *P.fluorescens* strains or at least exhibited the highest genetic similarity to this species at 6 MLST loci for PD2760, 5 loci for WH1/01 and 3 MLST loci for WH2/01.

Analysis of genetic similarity Sequences of all MLST loci were used to construct phylogenetic trees (dendrograms) of genetic similarity. Based on the constructed dendrograms and alignment results collected strains were divided into four main groups (Figs. 5, 6 and 7). The first main group consisted of strains that belonged to pathovar *lachrymans* (strains 814/98, BG283, BG966, CCM2857, and LMG5070). These strains may be further divided into two separate clusters. The first cluster consisted of strains 814/98, BG283 and CCM2857, genetically very similar to each other, as well as to reference *lachrymans* strain Pla107 (JGI database). However, they differed in pathogenicity on cucumber, as strain BG283 exhibited only very week virulence. The second cluster consisted of two strains of *P.s.* pv. *lachrymans:* BG966 and LMG5070. These strains were very similar to each other and to reference *lachrymans* strain Pla106 (Figs. 6 and 7). The second main group is composed of pathovar *syringae* strains. This group consists of BG913, CCB37/09, CCM2858, PD2021, WK1/02, WW17/01, WW27/01, WW254/02, and WW4/02 and the reference *syringae* strain 2905 (Figs. 6 and 7). In this group, strain WW4//02 is an outlier, and strains WW17/01 and WW27/01, as well as PD2021 and CCB37/09 form two sub-groups (Fig. 7); this classification correlates with pathogenicity test results. The third main group consisted of three strains of *P.fluorescens*. The strains PD2760, WH1/01, and WH2/01 were classified as *P.fluorescens* based on the high genetic similarity to sequences of this species deposited at the JGI database. These strains exhibit high genetic similarity to each other, and form a separate branch on dendrograms (Fig. 5, 6 and 7). The last group (PD3662, WH6/01, WM2/02, and YPG 1293) contains strains that do not exhibit similarity to any given pathovar. However, in pathogenicity tests these strains show similarities, and MLST assays form a separate dendrogram branch for them (Fig. 7).

Presence of plasmids Either plasmids or mega-plasmids were detected in 10 strains (Fig. 3). However, each strain contained a different set of plasmids. The presence or lack of plasmids, and their number and/or length, did not correlate with pathogenicity tests or with molecular

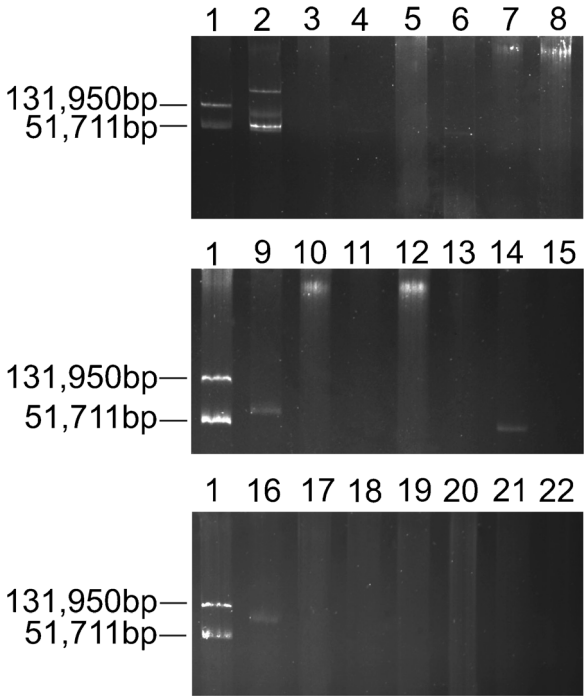

Fig. 3 The plasmid bands isolated from the collected *Pseudomonas* strains together with a positive control. Eckhardt gels for the 23 *Pseudomonas* sp. strains. Lane 1 – a positive control *Pseudomonas syringae* pv. *phaseolicola* 1448A strain (contains two plasmids of know size – 51,711 kb and 131,950 kb), than the collected strains: lane 2 – Psl 814/98, lane 3 – Psl CCM2857, lane 4 – Psl BG283, lane 5 – Psl LMG5070, lane 6 – Psl BG966, lane 7 – Pss WK1/02, lane 8 – Pss WW254/02, lane 9 – Pss BG913, lane 10 – Pss CCB37/09, lane 11 – Pss CCM2858, lane 12 – Pss PD2021, lane 13 – Pss 2905, lane 14 – Pss WW17/01, lane 15 – Pss WW27/01, lane 16 – Pss WW4/02, lane 17 – Pf PD2760, lane 18 – Pf WH1/01, lane 19 – Pf WH2/01, lane 20 – P.sp. PD3662, lane 21 – P.sp. WM2/02, lane 22 – P.sp. WH6/01

characterizations. Strain 814/98 possessed four plasmids estimated to be about 40 kb, 50 kb, 100 kb, and 200 kb in size. The other strains possessed one plasmid each: strains BG283, BG966, and WW17/01 had a plasmid about 40–50 kb in size, strain BG913 about 80–90 kb, and strain WW4/02 about 100 kb in length. Strains WK1/02, WW254/02, CCB37/09, and PD2021 likely possessed a mega-plasmid which showed as a smeared high molecular weight band on the gel. Plasmid detection and visualisation was performed in a comparison to referenced strain *P.s*. pv. *phaseolicola* 1448A (Fig. 3). There was no obvious association between plasmid presence and strain pathogenicity or virulence.

Discussion

Genetic characterization and clustering, as well as fingerprinting of plant pathogenic bacteria by various molecular methods has been the subject of numerous papers (Versalovic et al. 1994; Weingart and Völksch 1997; Sarkar and Guttman 2004; Inoue and Takikawa 2006; Olczak-Woltman *et al.* 2007; Lee *et al*. 2012; Martín-Sanz et al. 2012). These studies showed that the MLST technique and rep-PCR are useful as methods for identification and classification of strains belonging to *Pseudomonas syringae* (Hwang *et al*. 2005; Louws *et al*. 1994). Therefore we decided to use these methods to group *Pseudomonas* strains isolated primarily from cucumber and kept in our Department's collection.

The genetic diversity of *Pseudomonas* strains isolated from cucumber and other cucurbit plants was assessed using the MLST technique, rep-PCR method, and amplification of the ITS1 region. The last, however, is not very useful in *P. syringae* pv. *lachrymans* diagnostics due to the multiple copies of the ITS1 region and the intergenomic DNA variation in this region.

The MLSTs of seven housekeeping genes chosen for use in this study have been known to reveal higher genetic diversity within collection of *Pseudomonas* strains than rep-PCR fingerprinting (Ferrante and Scortichini 2010). In this study we confirmed, that the MLST method is indeed a powerful tool for accurate strain clustering that correlates well with both pathogenicity and virulence assessments.

One of the objectives of this study was identification of an efficient technique for differentiation of pathovar *lachrymans* strains from among other strains isolated from cucumber plants. Moreover, we investigated the presence of plasmids in pathovar *lachrymans* and looked for a correlation between plasmid presence and virulence. We grouped and characterized the pathovar *lachrymans* strains with regard to pathogenicity and molecular tests results. We want to stress that it was not easy to collect strains of pathovar *lachrymans* because many strains isolated from *Cucumis* species turned to belong to pathovar *syringae*.

According to the results, the sequencing, clustering and alignment analysis of our collected cucurbit strain collection allows their division into four main groups. The first main group consists of strains that belong to pathovar *lachrymans*. These strains differ in the level of virulence and ERIC- and REP-PCR fingerprinting patterns (Figs. 2 and 4). Strains 814/98 and CCM2857 are the most virulent. The analysis of the MLST amplicons showed their high genetic similarity. The product of amplification of the *hrpZ* gene fragment was present in both strains. Although they differ in the presence or absence of plasmids (four in 814/98 and none in CCM2857), they can be treated as a basic cluster for pathovar *lachrymans* based on the pathogenicity tests. The alignment with the JGI database sequence indicated high similarity to the *lachrymans* strain Pla107 that was characterized as highly virulent (Baltrus *et al*. 2011). The high virulence of both strains and difference in plasmids content suggest no correlation between plasmids presence and virulence for pathovar *lachrymans*.

Two strains: LMG5070 and BG966 that showed high molecular similarity to each other and caused similar, but week symptoms on cucumber, did not produce the *hrpZ* gene fragment. Sequence alignment to strains deposited in the JGI database indicated significant similarity to pathovar *lachrymans* strain Pla106 (both for the MLST and the ITS1) described as less virulent and genetically separate from strain Pla107 (Baltrus *et al*. 2011). As before, despite of several attempts, it was not possible to isolate the same plasmids in both of these strains. In contrast to Pla107 and strains 814/98 and CCM2857, both strain Pla106 and strains LMG5070 and BG966 showed very high genetic similarity to pathovar *tomato*. We suggest therefore, that there are two different types of *P.s.* pv. *lachrymans* strains, that correspond to the Pla107 or Pla106 type respectively (Baltrus *et al*. 2011). Strains Pla107-like cause more developed symptoms on cucumber leaves, and exhibit some genetic similarity to pathovar *syringae*. Strains Pla106-like, are molecularly similar to pathovar *tomato*,

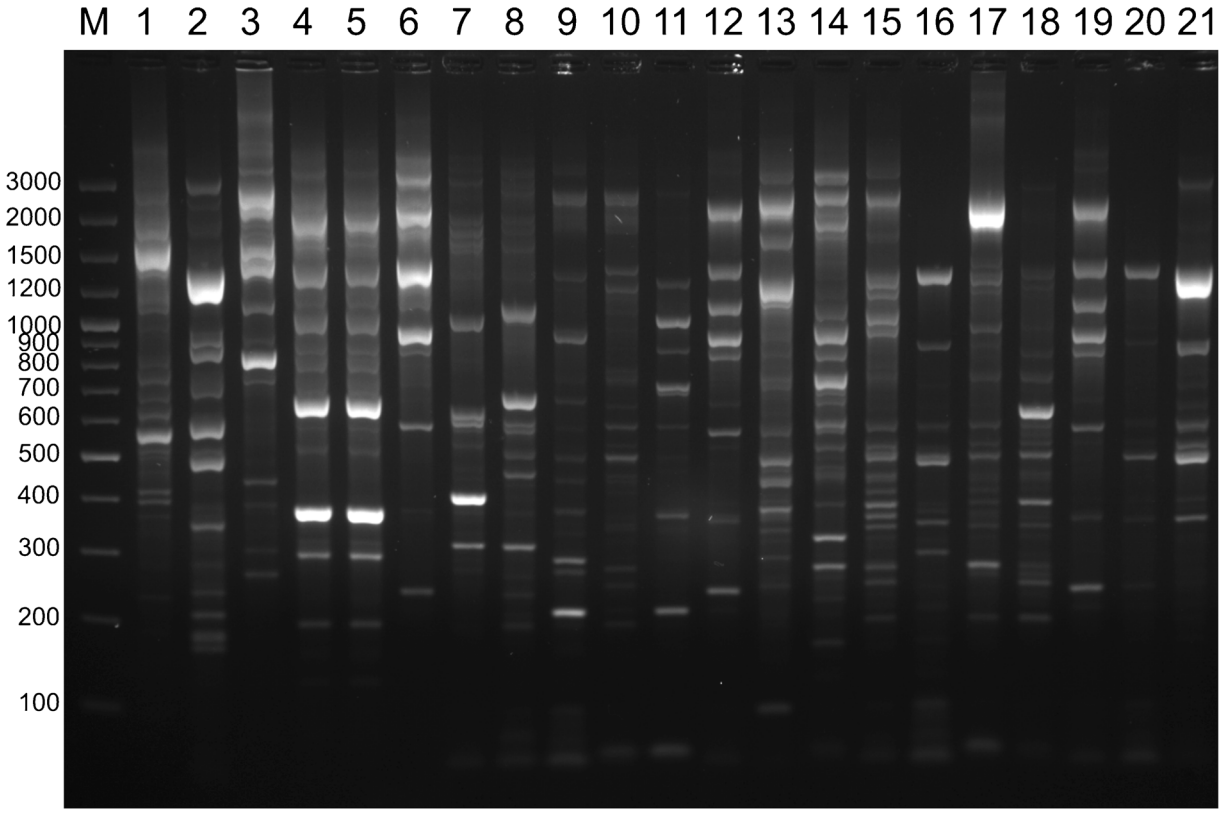

Fig. 4 The ERIC PCR fingerprinting pattern of the collected *Pseudomonas* strains. Lane M – 100 bp DNA molecular weight standard. Lines 1–21 – the following bacterial strains: lane 1 – Psl 814/98, lane 2 – Psl CCM2857, lane 3 – Psl BG283, lane 4 – Psl LMG5070, lane 5 – Psl BG966, lane 6 – P.sp. PD3662, lane 7 – P.sp. WM2/02, lane 8 – P.sp. WH6/01, lane 9 – Pss 2905, lane 10 – Pss CCM2858, lane 11 – Pss BG913, lane 12 – Pss PD2021, lane 13 – Pss CCB37/09, lane 14 – Pss WW4/02, lane 15 – Pss WW17/01, lane 16 – Pss WW27/01, lane 17 – Pss WK1/02, lane 18 – Pss WW254/02, lane 19 – Pf PD2760, lane 20 – Pf WH1/01, lane 21 – Pf WH2/01

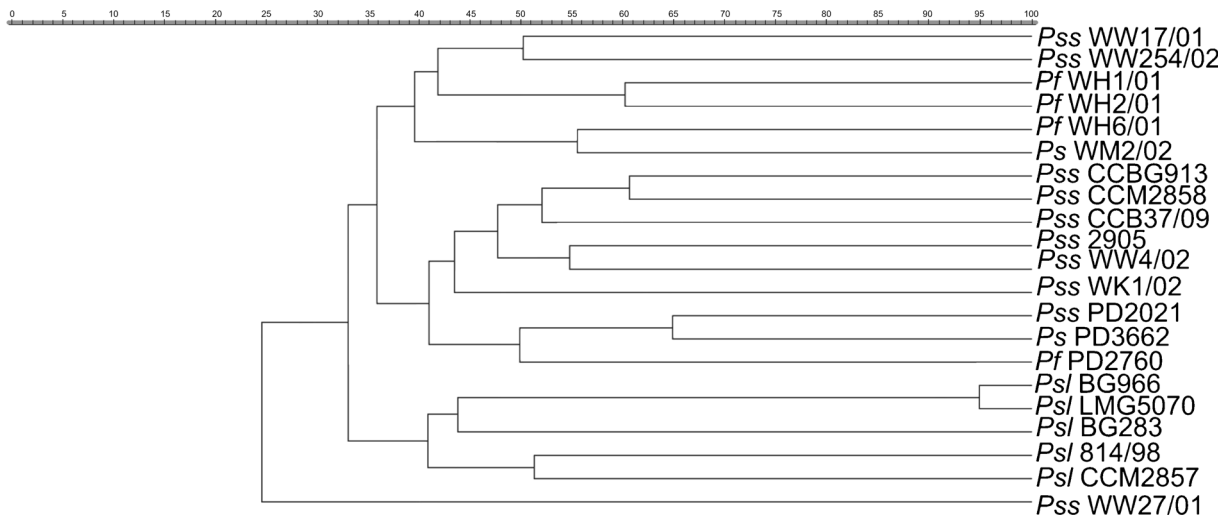

Fig. 5 The dendrogram of the genetic similarity (%) of the collected strains based on the ERIC PCR fingerprinting. The dendrogram was constructed using GelCompare II software, using the Dice coefficient and the UPGMA clustering method

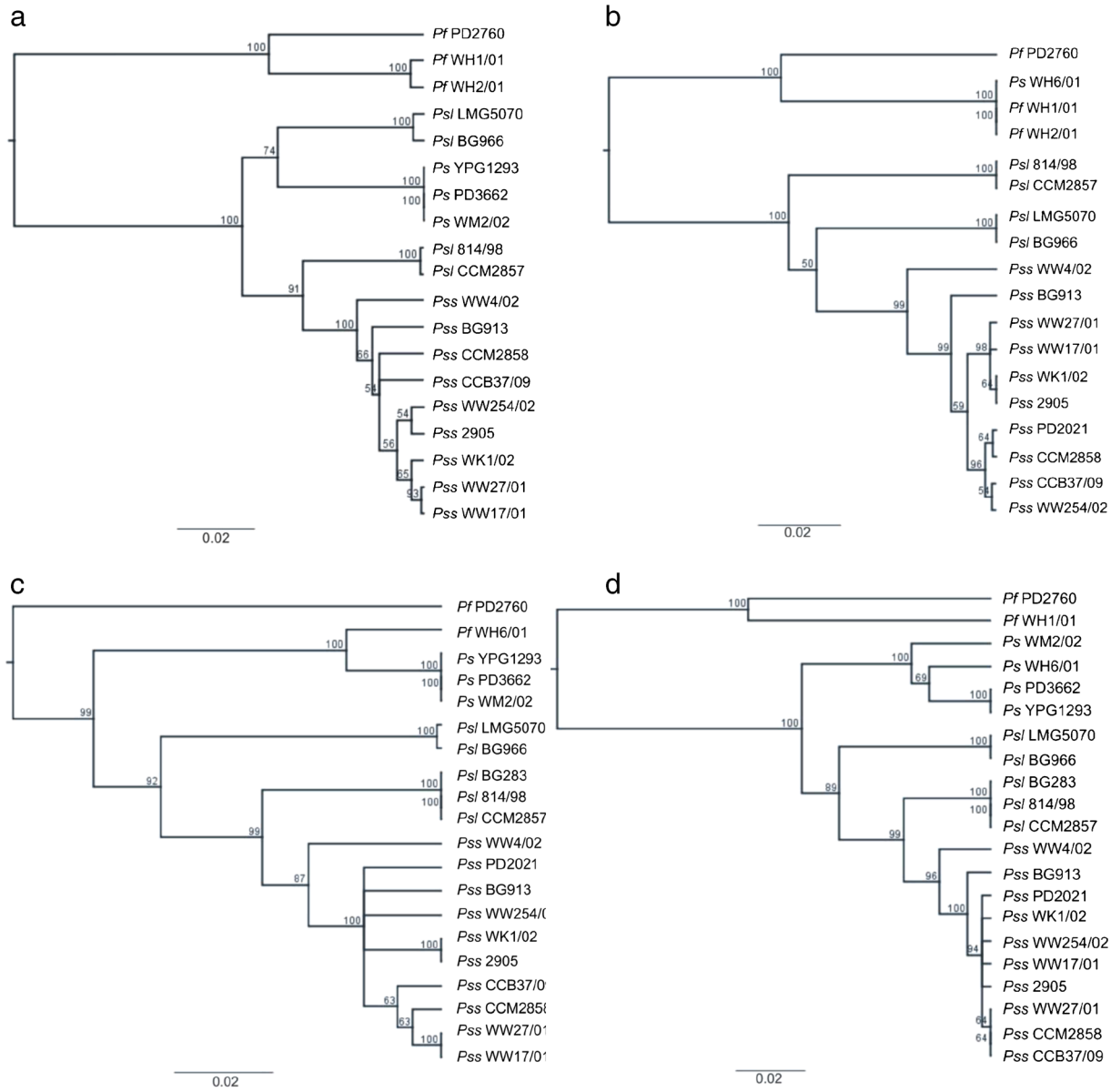

Fig. 6 The dendrograms of the genetic similarity (%) of the collected bacterial strains based on the selected MLST *loci*. a – *acn*, b – *gapA*, c – *gyrB*, d – *rpoD*. Each dendrogram was constructed using Geneious R7 software, with the Tamura-Nei evolutionary model and the UPGMA clustering method

are much less virulent on cucumber, and cause only weak symptoms.

Strain BG283 posed some problems with classification. This strain produced the *hrpZ* gene fragment, indicating high similarity to Pla107 strain type and in the MLST-based dendrogram was grouped into pathovar *lachrymans* type Pla107, but possessed one plasmid of the same length as BG966 (type Pla106), and in the rep-PCR based dendrogram was grouped into type Pla106 (Figs. 5, 6 and 7). However, since it exhibited extremely low level of virulence on cucumber and no HR on tobacco leaves (Fig. 1) this classification is not clear. Perhaps strain BG283 is *lachrymans*, but lost its virulence, or maybe it even does not belong to pathovar *lachrymans*.

The second main group consists of strains that are unequivocally pathovar *syringae*. The occurrence of this pathovar on cucumber, and its characteristics consisting of atypical numerous pinpoint chlorotic lesions, or dry

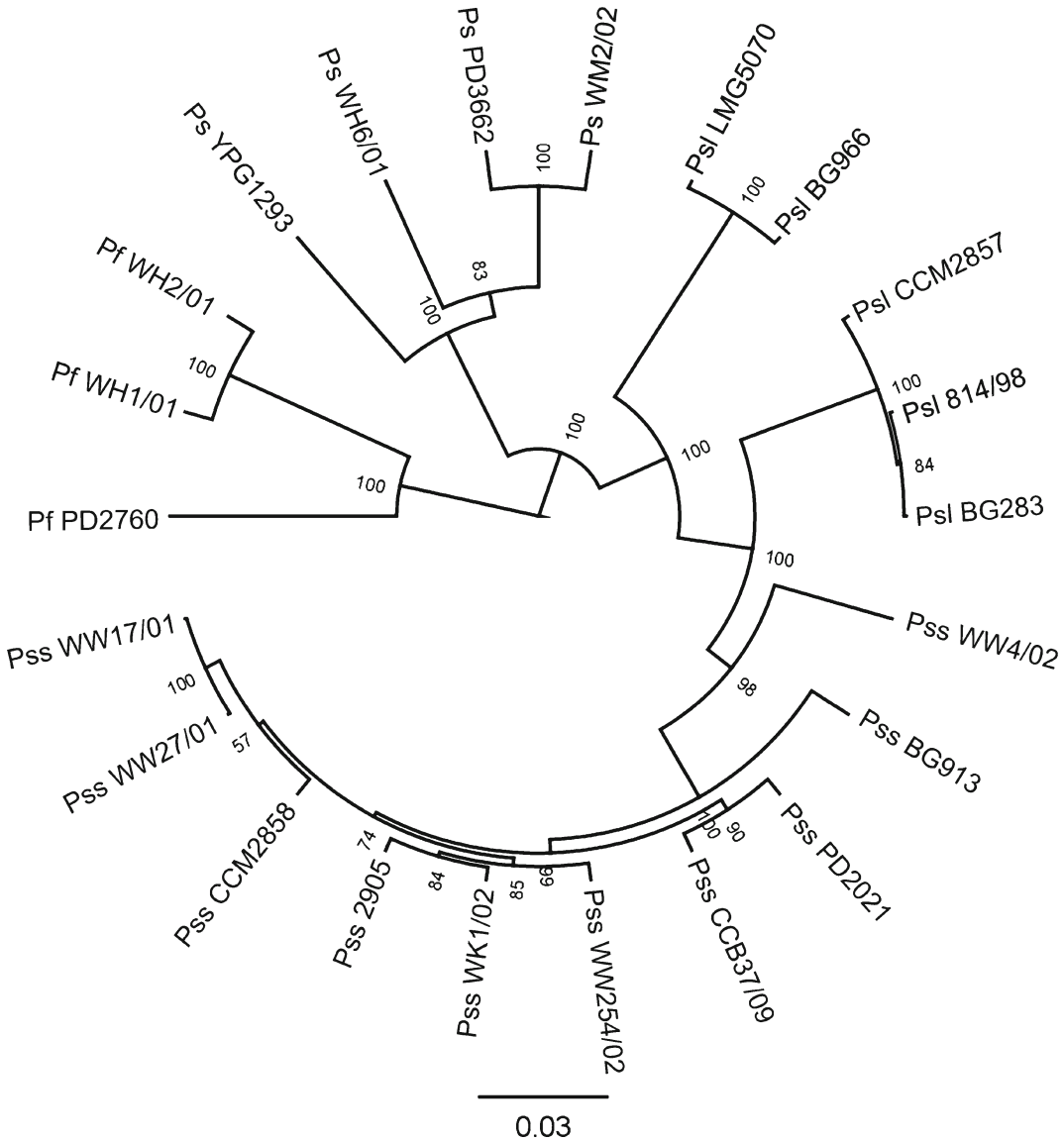

Fig. 7 The dendrogram of the genetic similarity (%) of the collected bacterial strains based on the results of all seven analyzed in this study MLST loci together. The dendrogram was constructed using Geneious R7 software, with the Tamura-Nei evolutionary model and the UPGMA clustering method

necrotic lesions without water-soaking has been described previously (Olczak-Woltman *et al.* 2007), and the classification into pathovar *syringae* has been verified by amplification of the *syrB* gene fragment (Olczak-Woltman *et al.* 2007). In this study ten strains were classified as pathovar *syringae* (Fig. 7). These strains may further be divided into sub-groups. The reference strain *P.s.* pv. *syringae* 2905, together with cucumber strains BG913, WW17/01, WW27/01, and WW254/02 cause numerous pinpoint, chlorotic or lightly-coloured necrotic lesions on cucumber leaves. These symptoms were most intensive for BG913 and less intensive in the case of WW17/01 and WW27/01 (Fig. 2d). Interestingly, partly similar symptoms were caused by pathovar *lachrymans* strains LMG5070 and BG966.

Strains CCB37/09, PD2021, and WK1/02 caused large, dry, lightly-coloured necrotic lesions without chlorosis on cucumber leaves (Fig. 2e). Strain WK1/02 has been previously classified as more distant from the 2905 strain than the other pathovar *syringae* strains

(Olczak-Woltman *et al.* 2007). The other two strains (CCM2858 and WW4/02) genetically similar 100 % to strains of pathovar *syringae* deposited at the JGI database, differ dramatically from the others in terms of the symptom type on cucumber. At the one end of the spectrum, strain CCM2858 was one of the most virulent in the collection and, at the other end, strain WW4/02 displayed nearly no virulence. Finally, there are three strains in our collection that caused either very week or no specific symptoms on cucumber, caused no HR on tobacco, and were classified into *P.fluorescens,* as they showed high genetic similarity to sequences of this species deposited in the JGI database.

The MLST loci used in this study were used previously as phylogenetic markers. They appear to provide a sound method for classification of *Pseudomonas* strains isolated from cucumber. The *gyrB*-based phylogenetic tree for the local isolates was topologically almost identical to the tree based on the rep-PCR fingerprinting, while the phylogenetic tree based on the *rpoB* and *rpoD* gene sequences revealed clearly different patterns of variation (Sarris *et al.* 2012). Our results indicate that although the *gyrB* is indeed one of the best markers for *P.syringae* pv. *lachrymans*, each method produces a somewhat different phylogenetic tree (Figs. 5 and 6) and perhaps the most reliable tree is obtained when several phylogenetic trees are combined into one (Fig. 7). Based on the ERIC and BOX PCR amplifications, strains belonging to *P.s.* pv. *syringae* appeared as the most heterogeneous and intermingled in relation to host plant, year and place of isolation (Weingart and Völksch 1997; Scortichini et al. 2003; Kałużna et al. 2010). We conclude that the best reflection of the strains biological properties, i.e. pathogenicity and virulence, are displayed when applying the MLST method.

There was some confusion and unexpected results when we analyzed the plasmid content. There are publications that suggest pathogenicity islands are plasmid-borne (Jackson *et al.* 1999; Gibbon *et al.* 1999; Ma *et al.* 2007). However, in this study some highly virulent strains had no plasmid, and some non-virulent strains had a plasmid (Fig. 3). This leads to a conclusion that in the case of *Pseudomonas syringae* pv. *lachrymans*, additional analysis should be performed to better describe the plasmid/virulence relationship.

Overall, the results of this study indicate that pathogenicity tests combined with molecular analysis are accurate in differentiation and classification of plant pathogenic bacteria. We demonstrated that *P. syringae* pv. *lachrymans* is genetically diverse, which was exhibited by both pathogenicity and molecular tests. In case of some strains isolated from cucumber it is difficult to say which pathovar they belong to, because the sequences of various loci exhibit similarity to genomes of different strains. In general, the obtained results confirm the genome architecture diversity and dynamics of *P.syringae* shown by Baltrus *et al.* (2011). There is a great need to sequence more genomes of different pathovars of the plant pathogen *Pseudomonas syringae* to further study its complex composition.

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References

Baltrus, D. A., Nishimura, M. T., Romanchuk, A., Chang, J. H., Mukhtar, M. S., Cherkis, K., Roach, J., Grant, S. R., Jones, C. D., & Dangl, J. L. (2011). Dynamic evolution of pathogenicity revealed by sequencing and comparative genomics of 19 *Pseudomonas syringae* isolates. *PLoS ONE, 7*, 1–22.

Bradbury, J. F. (1986). Guide to plant pathogenic bacteria. (Ferry Lane, Kew, Surrey, CAB International Mycological Institute), pp.154–177.

De Bruijn, F. J. (1992). Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Applied and Environmental Microbiology, 58*, 2180–2187.

Eckhardt, T. (1978). A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid, 1*, 584–588.

Ferrante, P., & Scortichini, M. (2010). Molecular and phenotypic features of *Pseudomonas syringae* pv. *actinidiae* isolated during recent epidemics of bacterial canker on yellow

kiwifruit (*Actinidia chinensis*) in central Italy. *Plant Pathology, 59*, 954–962.

Gibbon, M. J., Sesma, A., Canal, A., Wood, J. R., Hidalgo, E., Brown, J., Vivian, A., & Murillo, J. (1999). Replication regions from plant-pathogenic *Pseudomonas syringae* plasmids are similar to ColE2-related replicons. *Microbiology, 145*, 325–334.

Hwang, M. S., Morgan, R. L., Sarkar, S. F., Wang, P. W., & Guttman, D. S. (2005). Phylogenetic characterization of virulence and resistance phenotypes of Pseudomonas syringae. *Applied and Environmental Microbiology, 71*, 5182–5191.

Inoue, Y., & Takikawa, Y. (2006). The *hrpZ* and *hrpA* genes are variable, and useful for grouping *Pseudomonas syringae* bacteria. *Journal of General Plant Pathology, 72*, 26–33.

Jackson, R. W., Athanassopoulos, E., Tsiamis, G., Mansfield, J. W., Sesma, A., Arnold, D. L., Gibbon, M. J., Murillo, J., Taylor, J. D., & Vivian, A. (1999). Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native pathovar phaseolicola. *Proceedings of the National Academy of Sciences, 96*, 10875–10880.

Joardar, V., Lindeberg, M., Jackson, R. W., & Buell, C. R. (2005). Whole-genome sequence analysis of *Pseudomonas syringae* pv. *phaseolicola* 1448A reveals divergence among pathovars in genes involved in virulence and transposition. *Journal of Bacteriology, 187*, 6488–6498.

Kałużna, M., Ferrante, P., Sobiczewski, P., & Scortichini, M. (2010). Characterization and genetic diversity of *Pseudomonas syringae* from stone fruits and hazelnut using repetitive-PCR and MLST. *Journal of Plant Pathology, 92*, 781–787.

Klement, Z., Mavridis, A., Rudolph, K., & Vidaver, A. (1990). Inoculation of plant tissues. In Z. Klement, K. Rudolph, & D. S. Sands (Eds.), *Methods in phytobacteriology* (pp. 94–124). Budapest: Akadémiai Kiadó.

Lee, Y. S., Koh, H. S., Sohn, S. H., Koh, Y. J., & Jung, J. S. (2012). Genetic diversity among *Pseudomonas syringae* pv. *morsprunorum* isolates from *Prunus mume* in Korea and Japan by comparative sequence analysis of 16S rRNA gene. *Plant Pathololgy Journal, 28*, 295–298.

Louws, F. J., Fulbright, D. W., Stephens, C. T., & de Bruijn, F. J. (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied Environmental Microbiology, 60*, 2286–2295.

Ma, Z., Smith, J. J., Zhao, Y., Jackson, R. W., Arnold, D. L., Murillo, J., & Sundin, G. W. (2007). Phylogenetic analysis of the pPT23A plasmid family of *Pseudomonas syringae. Applied and Environmental Microbiology, 73*, 1287–1295.

Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M., & Spratt, B. G. (1998). Multilocus sequence typing a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences USA, 95*, 3140–3145.

Maj, A., Dziewit, L., Czarnecki, J., Wlodarczyk, M., Baj, J., Skrzypczyk, G., Giersz, D., & Bartosik, D. (2013). Plasmids of carotenoid-producing *paracoccus* spp. (*Alphaproteobacteria*) - structure, diversity and evolution. *PLoS ONE, 8*, e80258.

Manceu, C., & Horvais, A. (1997). Assessment of genetic diversity among strains of *Pseudomonas syringae* by PCR-restriction fragment length polymorphism analysis of rRNA operons with special emphasis on *P. syringae* pv. *tomato. Applied and Environmental Microbiology, 63*, 498–505.

Martín-Sanz, A., Pérez de la Vega, M., Murillo, J., & Caminero, C. (2012). Genetic, biochemical and pathogenic diversity of *Pseudomonas syringae* pv. *pisi* strains. *Plant Pathology, 61*, 1063–1072.

O'Brien, H. E., Desveaux, D., & Guttman, D. S. (2011). Next-generation genomics of *Pseudomonas syringae. Current Opinion in Microbiology, 14*, 1–7.

Olczak-Woltman, H., Masny, A., Bartoszewski, G., Plucienniczak, A., & Niemirowicz-Szczytt, K. (2007). Genetic diversity of *Pseudomonas syringae* pv. *lachrymans* strains isolated from cucumber leaves collected in Poland. *Plant Pathology, 56*, 373–382.

Olczak-Woltman, H., Schollenberger, M., Mądry, W., & Niemirowicz-Szczytt, K. (2008). Evaluation of cucumber (*Cucumis sativus* L.) cultivars grown in Eastern Europe and progress in breeding for resistance to angular leaf spot (*Pseudomonas syringae* pv. *lachrymans*). *European Journal of Plant Pathology, 122*, 385–393.

Sarkar, S. A., & Guttman, D. S. (2004). Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Applied and Environmental Microbiology, 70*, 1999–2012.

Sarris, P. S., Trantas, E. A., Mpalantinaki, E., Ververidis, F., & Goumas, D. E. (2012). *Pseudomonas viridiflava*, a multi host plant pathogen with significant genetic variation at the molecular level. *PLoS ONE, 4*, 1–12.

Scortichini, M., Marchesi, U., Dettori, M. T., & Rossi, M. P. (2003). Genetic diversity, presence of the *syrB* gene, host preference and virulence of *Pseudomonas syringa* pv. *syringae* strains from woody and herbaceous host plants. *Plant Pathology, 52*, 277–286.

Sorensen, K. N., Kim, K.-H., & Takemoto, J. Y. (1998). PCR detection of cyclic lipodepsinonapeptide-producing *Pseudomonas syringae* pv. *syringae* and similarity of strains. *Applied and Environmental Microbiology, 64*, 226–230.

Urwin, R., & Maiden, M. C. (2003). Multi-locus sequence typing: a tool for global epidemiology. *Tends in Microbiology, 11*, 479–487.

Versalovic, J., Koeuth, T., & Lupski, J. R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research, 19*, 6823–6831.

Versalovic, J., Schneider, M., de Bruijn, F. J., & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology, 5*, 25–40.

Weingart, H., & Völksch, B. (1997). Genetic fingerprinting of *Pseudomonas syringae* pathovars using ERIC-, REP-, and IS50-PCR. *Journal of Phytopathology, 145*, 339–334.

Young, J. M., Saddler, G. S., Takikawa, Y., De Boer, S. H., Vauterin, L., Gardan, L., Gwozdyak, R. I., & Stead, D. E. (1996). Names of plant pathogenic bacteria 1864–1995. *Review of Plant Pathology, 75*, 721–763.