GENETIC DIVERSITY OF *PSEUDOMONAS SYRINGAE* pv. *SYRINGAE* STRAINS, CAUSING BACTERIAL STEM BLIGHT DISEASE OF ALFALFA IN THE KURDISTAN PROVINCE OF IRAN

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SUMMARY

In 2008 and 2009, 66 isolates of *Pseudomonas syringae* pv. *syringae* were obtained from infected alfalfa leaf tissues collected from various locations of the Kurdistan province of Iran. These strains, together with the *Pseudomonas syringae* pv. *syringae* reference strain were tested for the presence of the *syrP* gene and were also phenotypically characterized. According to phenotypic properties, 87% and 13% of the strains belonged to LOPAT group 1a and 1b, respectively. Genetic diversity of all strains was assessed by repetitive sequence-based polymerase chain reaction (rep-PCR) using REP (repetitive extragenic palindromic), ERIC (enterobacterial repetitive intergenic consensus) and BOX primer sets. Cluster analysis of rep-PCR revealed three groups at a similarity level of 50%. Group 1 of rep-PCR including all strains belongs to LOPAT group 1a, whereas group 2 and 3 comprise strains of LOPAT group 1a and 1b. A specific band corresponding to *syrP* gene was produced by 84% of the strains belonging to LOPAT group 1a. Strains belonging to LOPAT group 1b produced only non-specific bands.

Key words: Alfalfa, phenotypic properties, *Pseudomonas syringae* pv. *syringae*, rep-PCR, *syrP* gene

INTRODUCTION

Bacterial stem blight disease of alfalfa (*Medicago sativa* L.) has been observed in the Kurdistan province of Iran since 2004. Preliminary isolation from infected tissues showed the presence of bacterial strains resembling *Pseudomonas syringae* pv. *syringae* (*Pss*) (Harighi, 2007). *Pss* is one of pathovars belonging to the heterogeneous species *Pseudomonas syringae* which was first isolated from lilac but causes disease in more than 44 plant species of unrelated plants (Bull *et al*., 2010, 2012; Young, 2010). Generally, biochemical and nutritional properties cannot distinguish at or below the pathovar level within *P. syringae* (Young and Triggs, 1994). Enterobacterial repetitive intergenic palindromic (ERIC) sequences, repetitive extragenic palindromic (REP) and BOX-like DNA sequences are dispersed throughout the genomes of diverse bacterial species (Versalovic *et al*., 1991). DNA-based techniques using primers corresponding to REP-, ERIC-, and BOX sequences (known as rep-PCR) generate strain-specific fingerprints that can differentiate *P. syringae* strains below species or subspecies level (Louws *et al*., 1994). The *syr* gene cluster of *Pss* encodes syringomycin synthetases. The *syrP* gene, which was predicted to encode a histidine kinase, was identified between *syrB1* and *syrD* (Zhang *et al*., 1997). Since *syr* genes are characteristic for *Pss*, PCR was done using *syrP*-based primers for strain identification. The objective of this study was to assess the genetic diversity of *P. syringae* strains isolated from alfalfa with stem blight symptoms sampled from crops growing in various locations of the Kurdistan province. All strains were classified according to physiological and biochemical properties, amplification of a DNA sequence corresponding to the *syrP* gene, then subjected to genomic fingerprinting using repetitive sequence polymerase chain reaction (rep-PCR).

MATERIALS AND METHODS

Bacterial isolation. Bacteria were isolated from infected tissues during April to July 2008 and 2009. At all locations, each plant was collected from a different field and each isolate was obtained from a different plant. Small tissues pieces were excised from the margins of leaf spots, surface-sterilized with 0.25% aqueous sodium hypochlorite for 30 sec, and rinsed in sterile-distilled water. Each tissue fragment was macerated in one ml of sterile distilled water for 10 min and the suspension was streaked onto King medium B (KB) (King *et al*., 1954) and incubated at 26-28°C. After 48-72 h, single fluorescent colonies were subcultured onto KB medium and stored. Several non-flourescent colonies were also recovered. Bacterial isolates were grown on KB for 24 h and stored at 4°C for a short period of time. For long-term storage, bacteria were grown in nutrient broth (NB, Difco) for 24 h and stored in 20% glycerol at −60°C.

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Table 1. Bacterial strains isolated from alfalfa leaves with disease symptoms

Isolate number and place of isolation[a]	Detection of *syrP*[b]	Fluorescence on KB	Ice nucleation activity	LOPAT results[c]					GATTa results[d]			
				L	O	P	A	T	G	A	T	Ta
Del, De2, De3, De4, De5, De6, De7, De8, De9, De10, De15, De21, De26, De27, De28, De29, De31, De33, De34, De36, De40, Ka111, Sr86, Sa89, *Pss* reference strain	+	+	+	+	–	–	–	+	+	+	–	–
De17, De19, De20, Sr76, Sa94	–	–	–	+	–	–	–	+	+	+	–	–
De22, De23, De24, De25, De35,Sa93, Ka99, Ka103, Ka105, Ka106, Ka107, Ka109, Ka112, Ka113, Ka117, Ka118	+	+	–	+	–	–	–	+	+	+	–	–
De37, De38, De39, Ka110	+	+	+	+	–	–	–	+	+	–	–	–
Ma 43, Ma44, Ma45	–	+	–	–	–	–	–	+	+	+	–	–
Ma42, Ma46, Ma50, Ma55, Ma57	–	+	+	–	–	–	–	+	+	+	–	–
Sr77,Sr78, Sr84, Sr85	–	–	+	+	–	–	–	+	+	+	–	–
Sa95, Or119, Or120, Or121	+	+	–	+	–	–	–	+	+	–	–	–
Ka102	+	–	–	+	–	–	–	+	+	–	–	–

[a]De= Dehgolan; Ma= Marivan; Sr= Sarvabad; Sa=Sanandaj; Ka= Kamyaran; Or= Oraman. [b]Strains for which the *syrP* gene amplification product (576 bp) was detected. [c]Results of LOPAT tests (L, levan production; O, oxidase reaction; P, potato soft rot; A, arginine dihydrolase; T, tobacco hypersensitivity). [d]Results of GATTa tests (G, gelatin liquefaction; A, aesculin hydrolysis; T, tyrosinase activity; Ta, tartrate utilisation).

Characterization and identification of bacterial isolates. Biochemical and physiological tests were performed according to previously described methods (Schaad *et al*., 2001). All isolates were tested for levan production, oxidase reaction, the ability to rot potato slices, arginine dihydrolase and tobacco hypersensitivity (LOPAT tests) (Lelliott *et al*., 1966). All isolates were further characterized for gelatine and aesculin hydrolysis, tyrosinase activity, L-tartrate utilization (GATTa tests) and ice nucleation activity as described by Schaad *et al.* (2001). Acid production from carbon sources or utilization of amino acids was tested according to Dye (1968). *P. syringae* pv. *syringae* ICMP 3475^T, obtained from the International Collection of Microorganism from Plants (ICMP, Auckland, New Zealand) was used as reference strain.

Pathogenicity tests. According to phenotypic groups identified, five bacterial strains (De31, De35, Ma44, Ma55 and Sr78) and *Pss* reference strain were selected for pathogenicity tests. Strains were grown in nutrient broth at 27°C for 48 h. Cultures were centrifuged (5 min, 7000 rpm), the pellet was suspended in sterile-distilled water to a concentration of approximately 1×10^7 CFU ml^{-1}. Four-week-old alfalfa plants (cv. Hamedani) were spray-inoculated with a hand-held mister. Sterile water was used as control. After inoculation, plants were kept humid for 48 h by holding them in a mist chamber and subsequently, maintained in a greenhouse at 24-26°C, 16 h light and 95% RH. Symptoms were assessed up to 6 weeks after inoculation. Bacteria were re-isolated from the inoculated tissues and characterized as previously described.

Genomic DNA preparation. For genomic DNA preparation, a single colony of each strains was grown in LB medium at 28°C for 24 h. A 5 ml suspension was exposed to SDS/lysozyme and DNA extracted treating twice with phenol:chloroform:isoamyl alchohol (25:24:1) and once with chloroform: isoamyl alchohol (24:1). Finally DNA was precipitated with 0.1 vol. of 3M sodium acetate (pH 4.8) and two vol. of absolute ethanol overnight at –20°C. DNA was centrifuged at 14,000 rpm for 5 min, washed with 70% ethanol and resuspended in 50 µl TE buffer [with RNAse (10 mg ml^{-1} stock)] to a final DNA concentration of 20 µg ml^{-1}.

Repetitive PCR genomic fingerprinting (rep-PCR). Genomic fingerprints were determined for each strain as described (Louws *et al*., 1994), using primers corresponding to REP, ERIC and BOX sequences. Amplification was carried out in a 25 µl reaction volume containing 12.5 µl of master mix (Fermentas, Lithuania), 1 µl of each primer (10 pmol/µl), 1 µl (approximately 40 ng) of genomic DNA and 10.5 µl of sterile distilled water. PCR was performed in a Bio Rad MJ mini thermocycler (BioRad, USA) with the following program: one cycle at 95°C for 2 min, 35 cycles at 92°C for 30 sec, annealing at 40°C (REP), 50°C (ERIC) and 53°C (BOX) for 1 min, extension at 65°C for 8 min, followed by a final extension at 65°C for 8 min. PCR products were separated by gel electrophoresis on 1.5% agarose in 1X TAE buffer, stained with ethidium bromide and visualized on a gel imaging system (UVIdoc, UK). PCR reactions were repeated at least three times.

DATA analysis. Differences in amplified fragments of each strain were assessed visually. Cluster analysis was performed on a similarity matrix produced, using the Dice's coefficient (Dice, 1945) and subjected to the unweighted pair group method with arithmetic average clustering algorithm (UPGMA), using NTSYSpc software, version 2.02e (Exeter Software, USA).

Detection of *syrP* gene. The complete sequence of the *syrP* gene of *Pss* B301D strain is known (Zhang *et al*., 1997). Primers PF (5'-GCGCGGTCTTGTTTCGGG-GA-3') and PR (5'- TCTCGCAGCTCCTCGCCCAT-3') were designed for PCR amplification using GeneFisher software (Giegerich *et al*., 1996). These primers were located 552 and 1128 bp, respectively, into the *syrP* gene ORF and yielded a 576 bp product. PCR amplification was performed in a total volume of 25 µl under the following conditions: 1 cycle at 93°C for 3 min, 35 cycles at 93°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min; and a final extension at 72°C for 6 min.

RESULTS

Bacterial isolation, identification and characterization. Bacterial streaming from infected tissues was consistently observed. A total of 66 isolates were obtained from infected tissues. All isolates were Gram-negative, rod-shaped and aerobic. Most strains (58 out of 66, i.e. 87%) performed as expected for *P. syringae* LOPAT group 1a (+ --- +), and eight strains (13%) displayed traits typical of *P. syringae* group 1b (– --- +). Ten of 58 strains belonging to group 1a were non-flourescent on King B medium, whereas all strains of group 1b were flourescent on the same medium. All strains belonging to LOPAT groups 1a and 1b were positive for tobacco hypersensitivity reaction.

Pathogenicity test. The pathogenicity of five selected strains belonging to LOPAT group 1a and 1b was tested. These strains exhibited diverse pathogenicity on susceptible alfalfa plants. Three to four weeks post inoculation, symptoms similar to bacterial blight were observed on the inoculated tissues. Bacterial strains belonging to LOPAT group 1a and positive for detection of the *syrP* gene, produced water-soaked spots on the leaves, which turned necrotic with a chlorotic halo. Bacterial strains identified as group 1b and 1a in which the *syrP* gene was not detected, produced chlorotic lesions on the leaves. *P. syringae* pv. *syringae* reference strain produced symptoms similar to the LOPAT group 1b strains. No symptoms developed on control plants. Isolations were made from the lesions and the bacterial colonies were successfully identified.

rep-PCR analysis. All primer sets yielded reproducible genomic PCR profiles with bands ranging in size from *ca.* 0.25 to 3 kb. Representative genomic patterns are shown in Figs. 1A and B. For UPGMA analysis, 19, 19 and 18 bands were scored for the BOX, ERIC and REP primer sets, respectively. UPGMA cluster analysis of the combined data obtained in the rep-PCR experiments using Dice's coefficient revealed that the strains separated into three clusters related at a similarity level of approximately 50% (Fig. 2). Cluster I included all strains belonging to LOPAT group 1a as well as *Pss* reference strain. Clusters II and III corresponded to strains belonging to LOPAT group 1b and 1a. These groupings did not relate with the geographic origin of the strains.

Detection of *syrP* gene. PCR amplification of the *syrP* gene was attempted from all the tested strains. Most of the strains of LOPAT group 1a (84%) produced the expected fragment of 576 bp in size which indicated the presence of *syrP* gene. Strains belonging to LOPAT group 1b, cluster II and III produced one or four unexpected fragments, respectively (Fig. 3).

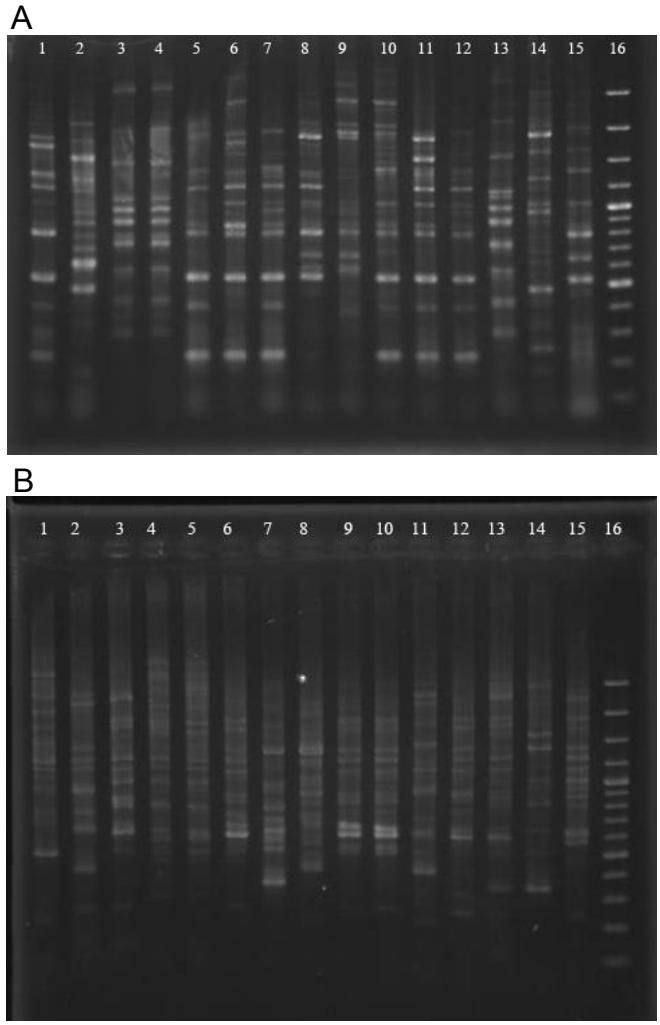

Fig. 1. A. PCR fingerprint patterns of genomic DNA from selected bacterial strains using ERIC-(a) and REP-(b) primers. Lane 1, De5; lane 2, De20; lane 3, Ma42; lane 4, Ma44; lane 5, De34: lane 6, De7; lane 7, De8: lane 8, De25; lane 9, Sa93; lane 10, Ka117; lane 11, Ka107; lane 12, Ka103; lane 13, Sa95; lane 14, De19; lane 15, *P. s.* pv. *syringae* reference strains; lane 16: 1 Kb DNA ladder. B. PCR fingerprint patterns of genomic DNA from selected strains of infected tissue using REP primers. Lane 1: Ma50; lane 2, De39; lane 3, Ka112: lane 4, De36: lane 5, Sa95; lane 6, De29: lane 7, Sa93; lane 8, De19; lane 9, Ka107; lane 10, Ka103; lane 11, Sr84; lane 12, De4; lane 13, De31; lane 14, Sr78; lane 15, *P. s.* pv. *syringae* reference strain; lane 16, 1 Kb DNA ladder.

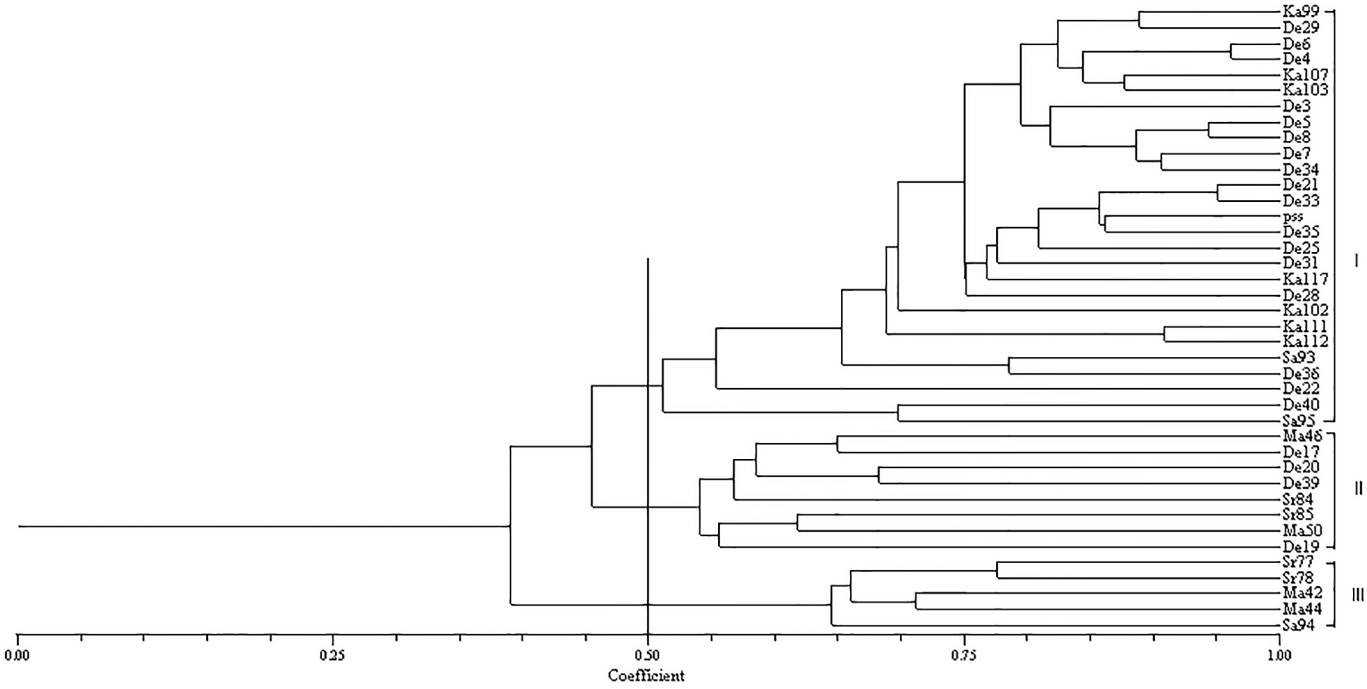

Fig. 2. Dendrogram of genetic relatedness of the rep-PCR fingerprint patterns generated by 40 bacterial strains isolated from alfalfa plants. Cluster analysis was performed using Dice's coefficients.

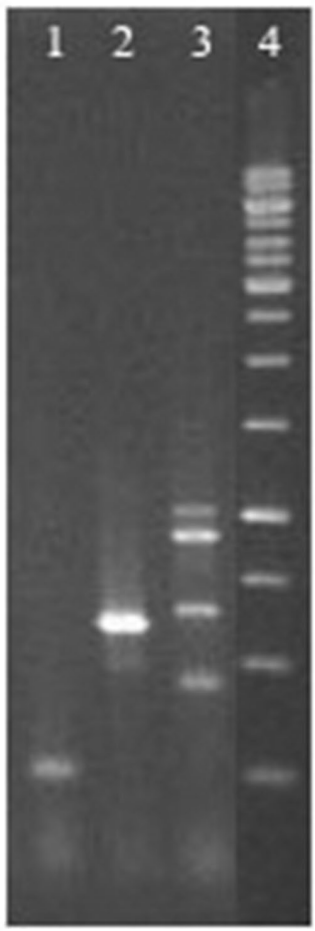

Fig. 3. PCR amplification product using primers corresponding to *syrP* gene. Lane 1, cluster II; lane 2, cluster I; lane 3, cluster III; lane 4, l Kb DNA ladder.

DISCUSSION

Bacterial stem blight of alfalfa has been observed in Kurdistan province since 2004. A preliminary study showed that this disease is caused by *Pseudomonas syringae* pv. *syringae* (Harighi, 2007). In this study we have ascertain that two groups of *P. syringae* classified as group 1a and 1b according to physiological and biochemical properties cause alfalfa stem blight. The biochemical and physiological properties of bacterial strains from alfalfa showed that all belong to *P. syringae*, the majority of which being identified as *Pss*. There were some differences in the biochemical properties of the presently investigated alfalfa isolates and those previously reported for *Pss* (Brenner *et al*., 2005). In this study, the cophenetic correlation coefficient for REP, ERIC and BOX was 0.89, 0.83 and 0.92, respectively. When these data were combined as recommended (Rademaker *et al*., 2000), the combined genetic similarity dendrogram of REP, ERIC and BOX primers had higher cophenetic correlation coefficient than the independent dendrograms (r = 0.93), showing that clustering is more accurate and that there is genetic diversity among strains isolated from various locations. Our results show that rep-PCR can differentiate between *P. syringae* group 1a and 1b strains, thus it could be used as a reliable method for identifying and differentiating strains from closely related bacterial species. A previous study had demonstrated that rep-PCR can differentiate *P. syringae* strains at the sub-species level (Louws *et al*., 1994; Weingart and Völksch, 1997). Toxin-specific primers were shown to be useful for the identification and differentiation of certain pathovars (Sorensen *et al*., 1998). Although syringomycin

is one of the major virulence factors of *Pss* not all bacterial strains produce this compound (Zeller *et al*., 1997). The production of syringomycin and the presence of *syr* genes have been used as a determinative characteristic to identify *Pss* (Little *et al*., 1998; Sorensen *et al*., 1998). Therefore, primers corresponding to *syrP* gene were used for the first time to confirm the identity of representative strains under investigation.

In this study, 84% of the strains belonging to LOPAT group 1a produced the expected fragment when primers for the amplification of the *syrP* gene were used. Pathogenicity of selected strains belonging to this group induced typical symptoms of alfalfa bacterial stem blight disease. By contrast, selected strains belonging to LOPAT group 1b failed to produce the expected *syrP* fragment and showed significant differences in disease symptoms. After incubation of inoculated plants with group 1b strains for the same period of time, only tiny chlorotic lesions developed. A previous study had shown that *syrP* gene regulates syringomycin production by *Pss* and that a *syrP* mutant is less virulent than the wild-type strain (Zhang *et al*., 1997). Thus, differences between disease symptoms severity among group 1a and 1b strains may reflect the failure of toxin production by 1b strains. Nucleotide sequences of the *syrP* gene is unique among *Pss* strains. Therefore based on the present results the primers designed in this study can be used to differentiate *P. syringae* pv. *syringae* from related bacteria.

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