Phenotypically and Genotypically Heterogeneous Strains of *Pseudomonas syringae* Associated With Alfalfa Leaf Spot Disease in Iran

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Abstract

In this study, we provide a polyphasic characterization of 18 *Pseudomonas* spp. strains associated with alfalfa leaf spot symptoms in Iran. All of the strains were pathogenic on alfalfa, although the aggressiveness and symptomology varied among the strains. All strains but one were pathogenic on broad bean, cucumber, honeydew, and zucchini, whereas only a fraction of the strains were pathogenic on sugar beet, tomato, and wheat. Syringomycin biosynthesis genes (*syrB1* and *syrP*) were detected using the corresponding PCR primers in all of the strains isolated from alfalfa. Phylogenetic analyses using the sequences of four housekeeping genes (*gapA*, *gltA*, *gyrB*, and *rpoD*) revealed that all of the strains except one (Als34) belong to phylogroup 2b of *P. syringae sensu lato*, whereas strain Als34 placed within phylogroup 1 close to the type strain of

P. syringae pv. *apii*. Among the phylogroup 2b strains, nine strains were phylogenetically close to the *P. syringae* pv. *aptata* clade, whereas the remainder were scattered among *P. syringae* pv. *atrofaciens* and *P. syringae* pv. *syringae* strains. Pathogenicity and host range assays of the bacterial strains evaluated in this study on a set of taxonomically diverse plant species did not allow us to assign a “pathovar” status to the alfalfa strains. However, these results provide novel insight into the host range and phylogenetic position of the alfalfa-pathogenic members of *P. syringae sensu lato*, and they reveal that phenotypically and genotypically heterogeneous strains of the pathogen cause bacterial leaf spot of alfalfa.

Keyword: *Medicago sativa*

Since the beginning of the twenty-first century, forage plants have been increasingly grown on a large scale for animal feeding in Iran (Food and Agriculture Organization of the United Nations 2018). In particular, alfalfa (*Medicago sativa* L.) occupies >60% of the total areas dedicated to fodder plants with an annual production over 10 million tons. By 2014, the areas cultivated with alfalfa accounted for >600,000 ha in the country (Anonymous 2016). Commercial forage production is a new industry in Iran; hence, most of the biotic and abiotic constraints of these crops remain uninvestigated. In the spring of 2016, leaf spot symptoms were observed on alfalfa plants grown in different provinces in northwestern, central, and southern Iran. Symptomatic plants that were suspected to be infected with a bacterial agent were subjected to further analyses (Yaripour et al. 2018). A series of comprehensive field surveys was subsequently conducted across the alfalfa producing areas in Iran during the summer and early autumn of 2017 to address the causal agent(s) of the disease and to generate a precise distribution map for the respective pathogen(s). As a result, we have isolated and characterized *Xanthomonas alfalfae* subsp. *alfalfae* as the causal agent of alfalfa leaf spot in southern Iran (Yaripour et al. 2018). However, a number of Gram-negative non-xanthomonad bacterial strains were also recovered from the symptomatic plants in the northwestern, central, and southern areas of the country. Preliminary observations (e.g., colony characteristics on culture media; Schaad et al. 2001) revealed that the bacterial strains resembled members of *Pseudomonas* spp.

Two distinct *Pseudomonas* species infecting alfalfa plants have been described in the literature thus far (Gray and Hollingsworth 2015): alfalfa root discoloration and stunting caused by *P. marginalis*

pv. *alfalfae* (Shinde and Lukezic 1974) and alfalfa leaf spot and stem blight caused by taxonomically undetermined members of *P. syringae sensu lato* (Gray and Hollingsworth 2015; Harrison et al. 2016). Indeed, the alfalfa leaf spot pathogen has traditionally been described as *P. syringae* pv. *syringae* based on biochemical characteristics and host range assays (Gray and Hollingsworth 2015). Nevertheless, the phenotypic traits do not meet the complexities in the taxonomy of *P. syringae sensu lato* (Morris et al. 2019; Young 2010). By the beginning of the genomic era, Gardan et al. (1999) had described nine discrete genomospecies among the different pathovars of *P. syringae sensu lato* based on DNA-DNA hybridization and ribotyping. They noted that *P. syringae* (hereafter referred to as *P. syringae sensu stricto*) placed in genomospecies 1, which includes *P. syringae* pv. *syringae*, *P. syringae* pv. *aptata*, *P. syringae* pv. *lapsa*, *P. syringae* pv. *papulans*, *P. syringae* pv. *pisi*, *P. syringae* pv. *atrofaciens*, *P. syringae* pv. *aceris*, *P. syringae* pv. *panici*, *P. syringae* pv. *dysoxylis*, and *P. syringae* pv. *japonica*. Phylogenetic studies using multilocus sequence analysis (MLSA) and multilocus sequence typing (MLST) subsequently revealed that *P. syringae sensu lato* could be divided into five phylogroups; *P. syringae* is placed in phylogroup 2, which corresponds to genomospecies 1 of the Gardan et al. (1999) taxonomy (Bull et al. 2011; Hwang et al. 2005). As far as the alfalfa-pathogenic strains of *P. syringae* are concerned, complete genome sequence-based average nucleotide identity analyses showed that *P. syringae* strain ALF3 isolated from alfalfa is closely related to *P. syringae* pv. *aptata* DSM 50252 isolated from sugar beet, as well as *P. syringae* pv. *japonica* M301072 isolated from barley, and is less likely to belong to *P. syringae* pv. *syringae* (Harrison et al. 2016). The three latter pathovars (i.e., *P. syringae* pv. *aptata*, *P. syringae* pv. *japonica*, and *P. syringae* pv. *syringae*) as well as *P. syringae* pv. *atrofaciens*, *P. syringae* pv. *lapsa*, and *P. syringae* pv. *pisi* were considered as phylogroup 2b of *P. syringae* based on the MLSA scheme (Bull and Koike 2015; Newberry et al. 2019).

Prior to the existence of genomic techniques, host range analysis was considered the most prominent approach for description of “pathovar” status within *P. syringae sensu lato* (Dye 1980; Young 2010; Young et al. 1978). However, the host range assay has a number of constraints, especially for identification of pathovars within *P. syringae* (Morris et al. 2019). For instance, *P. syringae* pv. *syringae* has the same host range as several other pathovars within the species, which complicates delineation of pathovar status to a given strain

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(Bradbury 1986; Newberry et al. 2016; Sedighian et al. 2014). As for the alfalfa leaf spot pathogen, severe incidences of the disease have rarely been reported around the world, and the causal agent represents only a very small fraction of the known *P. syringae* strains deposited in microbial culture collections (Harrison et al. 2016). Furthermore, according to the data available in NCBI GenBank and the Plant-Associated Microbes Database (PAMDB; Almeida et al. 2010), alfalfa-pathogenic strains of *P. syringae* have not been included in any of the comprehensive phylogenetic studies conducted for elucidation of the modern taxonomy of the species (Bull et al. 2011; Hwang et al. 2005; Sarkar and Guttman 2004). Hence, much of the molecular characteristics, phylogenetic position, and host range scheme of the alfalfa-pathogenic members of *P. syringae* remain to be deciphered.

The objectives of this study were to determine the phenotypic traits (e.g., biochemical feature and host range), molecular characteristics, and phylogenetic position of the *Pseudomonas* spp. strains associated with alfalfa leaf spot symptoms in Iran. With this aim, we performed a series of phenotypic tests and host range assays on a set of 11 agriculturally important plant species, whereas the sensitivity of the strains was evaluated against copper-based chemicals and antibiotics. MLSA and MLST were also performed using the sequences of four house-keeping genes (*gapA*, *gltA*, *gyrB*, and *rpoD*), and the genetic diversity and phylogenetic position of the strains was elucidated among *P. syringae sensu lato*. Our results revealed that *P. syringae* strains associated with alfalfa leaf spot disease belong to genetically diverse lineages among the species, and they are capable of infecting taxonomically diverse plant species, at least in greenhouse conditions.

Materials and Methods

Surveys, sampling, and bacterial isolation. Surveys were conducted during two consecutive years (May 2016 to September 2017) across alfalfa growing areas all over Iran (Table 1; Supplementary Fig. S1). This study follows a countrywide quarantine inspection program for the monitoring of bacterial diseases of annual crops (Mafakheri et al. 2017; Osdaghi 2014; Osdaghi et al. 2018a) and fruit

trees (Ansari et al. 2019; Zarei et al. 2019) in Iran. The surveying strategy and sampling procedure were described previously (Osdaghi et al. 2018b). Alfalfa plants with suspected bacterial disease symptoms were collected and brought to the laboratory for further analyses. Plant tissues were surface sterilized by dipping the tissue into 0.5% sodium hypochlorite for 20 seconds followed by two to three rinses in sterile distilled water (SDW). Small pieces of tissue were cut from the margin of the lesions with a sterile scalpel and were macerated in a few drops of SDW using a sterile mortar and pestle. A loopful of the resulting suspension was streaked onto nutrient agar (NA) and King B (KB; King et al. 1954) medium as described by Schaad et al. (2001). The plates were incubated at 25 to 27°C and examined for the growth of bacterial colonies 48 to 72 h postincubation. Pure cultures of the resulting bacterial strains were obtained by colony subculturing, and the strains were resuspended in SDW and stored at 4°C for further use. For long-term storage, the strains were maintained in 15% glycerol at -70°C.

Phenotypic characterization of the bacterial strains. Eighteen Gram-negative bacterial strains resembling members of *Pseudomonas* spp. were isolated from the symptomatic plants and subjected to standard phenotypic tests (Table 1). Catalase activity, aerobic/anaerobic growth, and colony characteristics on KB medium were determined. Furthermore, the LOPAT scheme (levan production, oxidase, potato soft rot, arginine dihydrolase, and hypersensitivity reaction in tobacco leaves) was determined as recommended previously (Lelliott et al. 1966; Schaad et al. 2001). Hypersensitivity reaction was evaluated on tobacco (*Nicotiana tabacum* 'Turkish') and geranium (*Pelargonium graveolens*) leaves using the bacterial suspension from a 48-h-old culture on KB medium at a concentration of 10⁸ CFU/ml. Utilization of mannitol, sorbitol, glucose, lactose, maltose, and sucrose; hydrolysis of aesculin and starch; liquefaction of gelatin; and tyrosinase activity of the strains were determined as described previously (Schaad et al. 2001). The reference strain *P. syringae* pv. *syringae* B728a isolated from snap bean (*Phaseolus vulgaris*) in the United States (Table 1; Feil et al. 2005) and the standard strain *P. syringae* pv. *aptata* M24 isolated from squash (*Cucurbita moschata*) in Iran (Sedighian et al. 2014) were

Table 1. Bacterial strains used in this study and their origin, host, date of isolation, and results of biochemical tests (i.e., LOPAT scheme) and sensitivity to copper compounds and antibiotics

Strain	Region	Host	Year	Fluorescence ^a	LOPAT ^b					Utilization of		Hydrolysis of aesculin	MIC (mg/liter) ^c			
					L	O	P	A	T	Mannitol	Sorbitol		Streptomycin	Rifampicin	Copper sulfate	Copper oxychloride
Als2	Marvdasht	Alfalfa	2016	Y to G	+	-	-	-	+	-	-	+	<20	<20	100	800
Als4	Marvdasht	Alfalfa	2016	C	+	-	-	-	+	-	-	-	<20	<20	100	500
Als28	Yasuj	Alfalfa	2016	YG	+	-	-	-	+	+	+	-	<20	<20	200	800
Als30	Yasuj	Alfalfa	2016	YG	+	-	-	-	+	+	+	+	<20	<20	100	1,500
Als33	Yasuj	Alfalfa	2016	YG	+	-	-	-	+	+	+	+	<20	<20	100	800
Als34	Boyer-Ahmad	Alfalfa	2016	YG	+	-	-	-	+	+	+	+	<20	<20	100	800
Als35	Boyer-Ahmad	Alfalfa	2016	YG	+	-	-	-	+	+	+	+	<20	<20	100	800
Als38	Boyer-Ahmad	Alfalfa	2016	G	+	-	-	-	+	+	+	-	<20	<20	200	1,500
Als51	Shiraz	Alfalfa	2016	YG	+	-	-	-	+	+	+	+	<20	<20	100	800
Als52	Shiraz	Alfalfa	2016	YG	+	-	-	-	+	+	+	+	<20	<20	100	800
Als53	Shiraz	Alfalfa	2016	YG	+	-	-	-	+	+	+	+	<20	<20	100	800
Als54	Shiraz	Alfalfa	2016	C	+	-	-	-	+	+	+	+	<20	<20	200	800
Als56	Shiraz	Alfalfa	2016	C	+	-	-	-	+	+	+	+	<20	<20	100	800
Als58	Shiraz	Alfalfa	2016	YG	+	-	-	-	+	-	+	+	<20	<20	100	800
Als59	Shiraz	Alfalfa	2016	DG	+	-	-	-	+	+	+	+	<20	<20	100	800
Als60	Urmia	Alfalfa	2017	DG	+ ^W	-	-	-	+	+	-	-	>100	>100	400	>3,000
Als61	Urmia	Alfalfa	2017	C	+ ^W	-	-	-	+	+	-	-	<20	<20	100	800
Als62	Urmia	Alfalfa	2017	YG	+	-	-	-	+	+	-	-	>100	>100	100	800
M29	Marand	Squash	2010	C	+	-	-	-	+	+	+	+	<20	<20	100	800
B728a	United States	Bean	1987	YG	+	-	-	-	+	+	+	+	<20	<20	100	800
CHA0	Switzerland	Tobacco root	1986	DG	+	+	-	+	-	+	+	+	<20	<20	200	>3,000

^a Y = yellow, G = green, C = creamy, YG = yellowish green, and DG = deep green.

^b LOPAT = levan production, oxidase, potato soft rot, arginine dihydrolase, and hypersensitivity reaction in tobacco leaves. Plus and minus signs indicate positive and negative, respectively. The superscript "W" indicates "weak."

^c MIC = minimum inhibitory concentration.

used as controls in all of the biochemical and phenotypic assays. *P. fluorescens* strain CHA0 was also used as a negative control. All of the biochemical tests were repeated at least twice.

Sensitivity to antibiotics and copper compounds. The use of copper-based chemicals and commercial antibiotics is a primary approach to manage newly emerging bacterial pathogens around the world (Hwang et al. 2005), and this approach has been thought to work for emerging bacterial diseases in Iran (Osdaghi et al. 2017). On the other hand, copper-tolerant strains of *P. syringae* are increasingly being reported, advocating the need for novel approaches for the management of these pathogens (Lamichhane et al. 2018). Therefore, to find the minimum inhibitory concentration (MIC) of different antibacterial compounds, all of the bacterial strains obtained in this study were evaluated for sensitivity to different concentrations of streptomycin, rifampicin (20, 50, and 100 mg/liter), copper sulfate (20, 50, 100, 200, and 400 mg/liter), and the most commonly used copper-based bactericide in Iran, copper oxychloride (200, 500, 800, 1,500, and 3,000 mg/liter). The tests were performed using the procedure described by Kebede et al. (2014) with minor modifications as described previously (Osdaghi et al. 2016).

Pathogenicity tests and host range. All of the bacterial strains recovered in this study were evaluated for their pathogenicity on the host of isolation (alfalfa cultivar Bonab) as well as on cucumber (*Cucumis sativus* 'Beit Alpha'), broad bean (*Vicia faba* 'HBP-B'), honeydew (*Cucumis melo* var. *inodorus* 'Marygold'), mung bean (*Vigna radiata* 'Mashhad'), radish (*Raphanus raphanistrum* subsp. *sativus* 'Saxa'), sugar beet (*Beta vulgaris* 'Sanetta'), tomato (*Solanum lycopersicum* 'Sunseed 6189'), wheat (*Triticum aestivum* 'Zare'), and zucchini (*Cucurbita pepo* var. *cylindrica* 'Clarita-SL5-F1'). Plants were grown in 20-cm-diameter pots (three plants per pot) and they were maintained in the greenhouse at ambient conditions (25 to 28°C, 14-h natural light) as described previously (Osdaghi et al. 2015). A bacterial suspension consisting of 1×10^8 CFU/ml was prepared from 48-h-old culture grown on KB medium. Fifteen plants/strains were inoculated, and the inoculation was conducted on the plants when they had at least three expanded true leaves (20 to 35 days old, depending on the plant species). Bacterial suspensions were sprayed onto the fully expanded leaves, petioles, and stems by using an A7 mini rebuildable atomizer as described previously (Zarei et al. 2018). Inoculated plants were covered with transparent nylon bags for 24 h to favor bacterial entry through the stomata. The plants were maintained in greenhouse conditions with 90 to 95% RH for the next 24 h and were then transferred to the ambient conditions as described above. The inoculated plants were periodically monitored for symptom development until 4 weeks post-inoculation. The same number of plants were inoculated with the reference strain B728a, as well as *P. syringae* pv. *aptata* strain M24, using the method as described above. Negative control plants were treated in the same manner, although SDW was used instead of bacterial suspension. Koch's postulates were accomplished by reisolating the inoculated strains on KB medium from the inoculated plants showing symptoms. Confirmation of the reisolated bacteria was made using the colony characteristics on KB medium and *syfP*-specific PCR primers P1/P2 (Supplementary Table S1). Pathogenicity tests were repeated twice.

Detection of phytotoxin-producing genes. Different pathovars of *P. syringae* have evolved a set of phytotoxin genes to extend their host range or increase their survival on various unrelated plant species (Mo and Gross 1991; Scholz-Schroeder et al. 2001). To determine whether the phytotoxin-producing genes are present in the strains associated with alfalfa, PCR primers that specifically detect syringomycin biosynthesis enzyme 1 (*syfB1*) and syringomycin biosynthesis (*syfP*) genes of the syringomycin biosynthesis cluster were used as described previously (Mo and Gross 1991; Scholz-Schroeder et al. 2001). Strains B728a and CHA0 were used as positive and negative controls, respectively, in all of the PCR tests. Bacterial DNAs were extracted using the Expin Combo GP DNA extraction kit (GeneAll, Tic Tech Center, Singapore), as recommended by the manufacturer. The quality and quantity of the DNA was spectrophotometrically evaluated and adjusted to 50 ng/μl using a Nanodrop ND-100 (Nanodrop Technologies, Waltham, MA). For PCR reactions, the Universal

PCR Kit with Ampliqon Taq DNA Polymerase Master Mix Red (Ampliqon A/S, Odense, Denmark) was used according to the manufacturer's recommendations. For each strain, a 25-μl PCR including 50 ng of total DNA and 1 μl of each primer (10 pmol/μl) was used.

Phylogenetic analysis. To obtain precise and reliable data on the phylogenetic position of the bacterial strains isolated in this study, they were subjected to MLSA/MLST analysis. Four housekeeping genes (*gapA*, *gltA*, *gyrB*, and *rpoD*) were selected for MLSA as recommended in the literature (Bull et al. 2011; Hwang et al. 2005; Sarkar and Guttman 2004). These loci were shown to provide robust phylogeny and are sufficient to reliably resolve evolutionary relationships among *P. syringae sensu lato*. PCR parameters were the same as described above. The PCR products were sent to Bioneer Corporation (Daejeon, South Korea; <http://www.Bioneer.com>) to be sequenced via Sanger sequencing technology.

BLAST (<https://blast.ncbi.nlm.nih.gov/>) search on the NCBI GenBank database using the resulting DNA sequences revealed that the individual strains isolated in Iran had high sequence similarity (99 to 100%) to the pathotype strains of different *P. syringae* pathovars. Hence, the corresponding sequences of these four genes in a collection of 170 worldwide *P. syringae sensu lato* strains were retrieved from the GenBank and PAMDB databases and included in the phylogenetic analysis (Almeida et al. 2010; Bull et al. 2011; Hwang et al. 2005). Sequences of the four genes in a set of 12 Iranian *P. syringae* strains, recently isolated from stone fruits, were also included in the analyses (Ahmadi et al. 2018). Sequences of the corresponding genes were extracted from the draft genome sequence of *P. syringae* strain ALF3 isolated from alfalfa (Harrison et al. 2016). The sequences were concatenated following the alphabetical order of the genes, ending in a sequence of 1,819 bp: nucleotides 1 to 460 for *gapA*, 461 to 960 for *gltA* (500 bp), 961 to 1,439 for *gyrB* (479 bp), and 1,440 to 1,819 for *rpoD* (380 bp). Phylogenetic trees were constructed using the maximum likelihood method with MEGA7 software (Kumar et al. 2016). The model of evolution for maximum likelihood analysis was determined using the Modeltest tab in MEGA7. *P. fluorescens* was used to root the phylogenetic trees, and they were constructed with bootstrapping (1,000 replications).

Genetic diversity of the strains. DnaSP software version 6 was used to estimate the nucleotide diversity, the number of sequence types, and the minimum number of recombination events (Rozas et al. 2017). The class I neutrality tests (Tajima's *D*, Fu and Li's *D**, and Fu and Li's *F** statistics) were also calculated in order to detect departure from the mutation/drift equilibrium (Librado and Rozas 2009). Multiple methods were used to detect recombination events among the strains. A set of seven nonparametric detection methods (i.e., the Recombination Detection Program [RDP], Geneconv, MaxChi, Chimaera, BootScan, SiScan, and 3Seq) implemented in RDP version 4.80 (Martin et al. 2015) was used to carry out detection of potential recombinant sequences and identification of likely parental sequences. The analysis was performed with default settings, and the Bonferroni-corrected *P* value cutoff was set at 0.05. Recombination events were accepted when they were identified by at least four of seven detection methods (Martin et al. 2015). NeighborNet networks were constructed and the pairwise homoplasy index (PHI) was calculated using SplitsTree version 4.14.4 (Huson and Bryant 2006). These calculations used the entire data set for all of the individual genes as well as for the concatenated sequences (Huson and Bryant 2006). To visualize the relationships between DNA sequences of the strains, haplotype networks were inferred for individual housekeeping genes as well as the concatenated sequences using the TCS algorithm (Clement et al. 2002) implemented in PopART version 1.7 (Leigh and Bryant 2015). Geographic distribution of the strains collected in this study was employed into the haplotype network of each of the four housekeeping genes as described by Leigh and Bryant (2015).

GenBank accession numbers and strain availability. The sequenced nucleotides were deposited in GenBank under the following accession numbers: MK122663 to MK122680 for *gapA*, MK122681 to MK122698 for *gltA*, MK122699 to MK122716 for *gyrB*, and MH810252 to MH810269 for *rpoD*. Furthermore, pure cultures of

the representative strains recovered in this study were deposited in the French Collection for Plant-Associated Bacteria (CIRM-CFBP) culture collection and assigned accession numbers as follows: Als30 = CFBP 8612 and Als33 = CFBP 8613. All of the strains are also available in the bacterial collection of the Pennsylvania State University Department of Plant Pathology and Environmental Microbiology (supervised by Dr. Carolee T. Bull).

Results

Disease symptoms. Alfalfa growing areas located in northern (i.e., East Azerbaijan, West Azerbaijan, and North-Khorasan provinces), central (i.e., Isfahan, Zanjan, and Hamedan provinces), and southern (i.e., Fars, Kerman, Kohgiluyeh-Buyer-Ahmad, Khuzestan, and Sistan-Baluchestan provinces) areas of Iran were surveyed from May 2016 to September 2017 (Table 1). Dates of samplings within a given year varied across the sites as a result of different climatic conditions and agronomic practices. The range of the surveyed fields varied from 0.1 to 5 ha. Leaf spot symptoms resembling the bacterial infection were observed on the samples collected in Fars, Kerman, Kohgiluyeh-Buyer-Ahmad, and West Azerbaijan provinces (Table 1). Disease symptoms were observed on all of the aerial parts of the plants (e.g., leaves, stems, and sepals). On leaves, symptoms began as small (1 to 3 mm in diameter) water-soaked lesions that tended to be chlorotic and irregular in appearance until they became brownish green. Leaves bearing many lesions became dark-brown with an uneven torn edge. Leaf lesions were occasionally enlarged up to 5 mm in diameter and became necrotic and coalesced, which resulted in a blighted appearance on the plants. The latter was more frequently observed during the rainy season where dew formation was common in the early mornings, as well as in the fields where sprinkler or center pivot irrigation methods were used. Stem and petiole lesions were observed only on the severely infected alfalfa plants.

Phenotypic characteristics of the strains. Eighteen Gram-negative bacterial strains were isolated from the symptomatic alfalfa plants and subjected to phenotypic analysis. All of the strains were oxidase negative and catalase positive and had oxidative metabolism, whereas the colony characteristics of the strains varied from creamy (nonfluorescent) to deep green (highly fluorescent) on KB medium. Results of the LOPAT scheme are described in Table 1. All of the strains were positive for utilization of glucose, maltose, and sucrose,

as well as liquefaction of gelatin, whereas they were negative for the utilization of lactose, hydrolysis of starch, and tyrosinase activity. Furthermore, levan production, utilization of mannitol and sorbitol, as well as aesculin hydrolysis differed from one strain to another (Table 1). Hyper-sensitivity reaction was observed in all of the strains on tobacco and geranium leaves 24 to 36 h postinoculation. Based on the results of the phenotypic tests, the bacterial strains were identified as members of *P. syringae sensu lato*.

Sensitivity to antibiotics and copper compounds. All strains except Als60 and Als62 were sensitive to streptomycin and rifampicin at all the tested concentrations (MIC < 20 mg/liter). As for copper sulfate, although all of the strains grew at 20 and 50 mg/liter, only strains Als28, Als38, Als54, and Als60 were able to grow at 100 mg/liter (MIC = 200 mg/liter); all strains were sensitive to 400 mg/liter. Similarly, all strains but one grew at 200 and 500 mg/liter of copper oxychloride (MIC = 800 mg/liter). Strains Als30, Als38, and Als60 were resistant to 800 mg/liter of copper oxychloride (MIC = 1,500 mg/liter), whereas Als60 was resistant to 200 and 300 mg/liter of copper sulfate and copper oxychloride, respectively (Table 1).

Pathogenicity tests and host range. Symptomology and aggressiveness of the bacterial strains on the inoculated plant species varied between the strains, ranging from chlorotic areas to extended necrosis as described in Table 2. A given bacterial strain was considered pathogenic on a given plant species when two-thirds of the inoculated plants (at least 10 individual plants) showed the same symptoms. Although the strains isolated in Boyer-Ahmad (Als35 and Als38), Shiraz (Als51, Als52, Als53, Als54, Als56, and Als59), and Yasuj (Als28, Als30, and Als33) induced extended necrotic leaf spots on alfalfa plants (Table 2), those obtained from Marvdasht (Als2 and Als4) and Urmia (Als60, Als61, and Als62) incited only chlorotic areas on the leaves of the same plant species. The exceptions were strains Als34 and Als58, which were isolated in Boyer-Ahmad and Shiraz, respectively, and produced chlorotic areas (Table 2). Only strains Als34, Als51, and Als59 were pathogenic on tomato plants, whereas all strains except Als34 were pathogenic on broad bean, cucumber, honeydew, and zucchini. None of the strains was pathogenic on radish and wheat except for Als58, which induced necrotic leaf spots on wheat plants. Strains Als60, Als61, and Als62, which were isolated in Urmia County, were nonpathogenic on mung bean, whereas all of the remaining strains were

Table 2. Results of host range assays in *Pseudomonas syringae* strains used in this study

Strain	Evaluated plant species ^a										Sequence types ^b			
	Alfalfa	Sugar beet	Tomato	Zucchini	Cucumber	Honeydew	Mung bean	Broad bean	Wheat	Radish	<i>gapA</i>	<i>gltA</i>	<i>gyrB</i>	<i>rpoD</i>
Als2	1	NP	NP	3	3	3	2	3	NP	NP	1	1	1	1
Als4	1	NP	NP	3	3	3	2	3	NP	NP	2	2	2	2
Als28	3	3	NP	3	3	3	3	3	NP	NP	3	3	3	1
Als30	3	3	NP	3	3	3	3	3	NP	NP	4	4	4	1
Als33	3	3	NP	3	3	3	3	3	NP	NP	2	5	5	1
Als34	1	NP	3	NP	NP	NP	NP	NP	NP	NP	5	6	6	3
Als35	3	3	NP	3	3	3	3	3	NP	NP	6	7	7	4
Als38	3	3	NP	3	3	3	3	3	NP	NP	7	8	8	1
Als51	3	3	3	3	3	3	3	3	NP	NP	8	9	9	1
Als52	3	3	NP	3	3	3	3	3	NP	NP	4	9	10	5
Als53	3	3	NP	3	3	3	3	3	NP	NP	8	10	11	6
Als54	3	3	NP	3	3	3	3	3	NP	NP	9	11	12	7
Als56	3	3	NP	3	3	3	3	3	NP	NP	6	12	13	8
Als58	1	NP	NP	3	3	3	3	3	3	NP	10	13	14	9
Als59	3	3	3	3	3	3	3	3	NP	NP	6	13	14	6
Als60	1	NP	NP	3	3	3	NP	2	NP	NP	11	5	2	1
Als61	1	NP	NP	3	3	3	NP	2	NP	NP	12	14	15	10
Als62	1	NP	NP	3	3	3	NP	2	NP	NP	13	14	16	11
M29	1	3	NP	3	3	3	2	2	NP	NP	ND	ND	17	12
B728a	3	NP	3	3	3	3	3	2	NP	NP	14	15	18	13
CHA0	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	ND	ND	ND	ND

^a Bold numbers 1 through 3 indicate the following: 1 = chlorotic areas on the leaves, 2 = necrotic leaf spots, and 3 = extended necrotic areas. NP = nonpathogenic and ND = not determined.

^b Sequence types of four housekeeping genes (*gapA*, *gltA*, *gyrB*, and *rpoD*) in all of the strains indicated.

Alfalfa plants inoculated with the bacterial strains obtained in this study exhibited water-soaked lesions that gradually turned into chlorotic areas at 8 to 12 days postinoculation (dpi) (Supplementary Fig. S2A). Subsequently, dark-brown spots surrounded by chlorotic halos appeared on the leaves 20 to 25 dpi and extended to the leaf margins. Chlorotic areas were observed on the leaf surfaces of zucchini, honeydew, sugar beet, cucumber, and tomato plants 6 to 15 dpi (depending on the plant species) and turned to angular leaf spots 15 to 20 dpi. On wheat plants, tiny, irregular, and necrotic spots were observed

8 to 10 dpi, and the oval-shaped necrotic areas were observed while the spots were joining together. On broad bean plants, small, irregular, and tan spots were observed on the leaves 8 to 10 dpi. Furthermore, on mung bean plants, irregular small necrotic spots were observed on the leaves. Necrotic areas were surrounded by chlorotic haloes, whereas the center of the spots was grayish white to brown. The symptoms on cucurbit plants were similar to those induced by the standard strain M29 isolated from squash in Iran. Reference strain B728a was pathogenic on alfalfa, tomato, zucchini, cucumber, honeydew, mung bean, and broad bean and was nonpathogenic on sugar beet, wheat, and radish. All of the negative control plants remained healthy. Bacterial strains, as those originally inoculated, were consistently reisolated from symptomatic plants on KB medium and their identity was confirmed using *syrP*-specific PCR primers P1/P2 (*data not shown*).

Phytotoxin-producing genes. The *syrB1*-specific primer pair B1/B2 directed the amplification of a 757-bp DNA fragment in all of the tested bacterial strains. Similarly, the expected 601-bp DNA fragment was amplified using the *syrP*-specific primer pair P1/P2 in all of the bacterial strains. Detection of these two DNA fragments indicated syringomycin biosynthesis in the bacterial strains tested in this study.

Phylogenetic analyses. BLAST search on the NCBI GenBank database using the partial sequences of four individual housekeeping genes revealed that the strains isolated in Iran had a sequence identity of 99 to 100% with the pathotype strains of phylogroup 2b of *P. syringae sensu lato* (i.e., *P. syringae* pv. *aptata* CFBP 1617^{PT}, *P. syringae* pv. *syringae* LMG 1247^{PT}, *P. syringae* pv. *atrofaciens* LMG 5095^{PT}, and *P. syringae* pv. *pisi* NCPPB 2585^{PT}). The exception was Als34, which showed 99 to 100% sequence similarity in all of the individual genes to the pathotype strains of *P. syringae* pv. *apii* NCPPB 1626^{PT} and *P. syringae* pv. *tomato* CFBP 2212^{PT}, both of which belong to phylogroup 1 of *P. syringae sensu lato*. The phylogenetic tree constructed using the concatenated sequences of four housekeeping genes confirmed the results obtained from the BLAST analyses. All of the strains isolated in Iran (except for Als34) clustered within phylogroup 2b of *P. syringae sensu lato*, although they were scattered through different clades of the species (Fig. 1). Strain Als34 clustered separately and placed among phylogroup I, close to the pathotype strain of *P. syringae* pv. *apii* NCPPB 1626^{PT}. Similar results were obtained when the sequences of individual housekeeping genes were subjected to phylogenetic analyses (*data not shown*).

Most of the strains isolated in this study were placed in phylogroup 2b along with the pathotype strains of *P. syringae* pv. *aptata* CFBP

Fig. 2. TCS multilocus haplotype network generated using the PopART program from the concatenated sequences of four housekeeping genes (*gapA*, *gltA*, *gyrB*, and *rpoD*) in 18 *Pseudomonas syringae* strains obtained in this study. Hatch marks along the branches indicate the number of mutations. Each shade indicates a different geographic area.

1617^{PT} and *P. syringae* pv. *atrofaciens* LMG 5095^{PT}. For instance, four strains isolated in Shiraz (Als51, Als52, Als53, and Als54) were placed in a common cluster close to alfalfa strain Alf3 isolated in the United States, whereas strains Als30 and Als33 isolated in Yasuj were clustered among *P. syringae* pv. *aptata* strains isolated from sugar beet (CFBP 1617^{PT} and CFBP 1906), ornamental pear (FF5), and wheat (DSM 5022). Strain Als28 isolated in Yasuj was clustered with zucchini strain 1188-1 isolated in the United

States, but strains Als58 and Als59 isolated in Shiraz were clustered in a separate subgroup. Strain Als38 was adjacent to the branch containing the pathotype strain of *P. syringae* pv. *atrofaciens* (LMG 5095^{PT}) and the stone fruit strains Pss72, Pss92, and Pss102 isolated in western Iran. Furthermore, five strains (Als2 and Als4, isolated in Marvdasht; and Als60, Als61, and Als62, isolated in Urmia) were clustered in a common branch, which could not be delineated to any of the pathovars within phylogroup

Fig. 3. NeighborNet network generated from the concatenated sequences of *gapA*, *gltA*, *gyrB*, and *rpoD* genes of *Pseudomonas syringae* strains obtained in this study as well as the strains previously isolated in Iran by Ahmadi et al. (2018). Parallel lines indicate conflicting phylogenetic relationships and recombination among the strains. All strains isolated in this study but Als34 were clustered in a monophyletic group showing no detectable recombination signal in between the strains. Three of 12 Iranian stone fruit strains were clustered close to the strains isolated in this study, whereas the remaining nine stone fruit strains were divided into two distinct groups.

2b. Two strains (Als35 and Als56) isolated in Boyer-Ahmad and Shiraz, respectively, were placed in the cluster containing the pathotype strain of *P. syringae* pv. *syringae* (LMG 1247^{PT}) and three strains isolated from lilac (*Syringa vulgaris*). Finally, strain Als34 was placed in phylogroup I, close to the pathotype strains of *P. syringae* pv. *apii* (NCPBP 1626^{PT}) and *P. syringae* pv. *tomato* strain T03Dps (Fig. 1).

Genetic diversity and MLST. The strains isolated from alfalfa carried multiple sequence types in the sequences of four housekeeping genes (Table 2), and none of the strains shared the same multilocus haplotype with the strains retrieved from GenBank. Haplotype networks were generated using PopART version 1.7 for the four individual genes (Supplementary Fig. S3) as well as the concatenated data set of sequences (Fig. 2). In the *gapA* gene sequences, the strain pairs Als4/Als33, Als30/Als52, and Als51/Als53 shared the same sequence type. Furthermore, strains Als35, Als56, and Als59 shared the same sequence type with the strains *P. syringae* pv. *aptata* 601 (isolated from sugar beet), *P. syringae* pv. *syringae* FTRS-W7 (isolated from *Prunus mume*), as well as the lilac strains of *P. syringae* pv. *syringae* (i.e., L177, LOB2-1, and LMG 1247^{PT}). As for the *gltA* gene sequences, strain groups Als4/Als61, Als51/Als52, Als33/Als60/*P. syringae* pv. *syringae* FF5 (isolated from *Pyrus calleryana* in the United States), Als58/Als59/*P. syringae* pv. *syringae* Alf3, Als34/*P. syringae* pv. *tomato* T03Dps (isolated from tomato in Germany), and Als30/*P. syringae* pv. *lachrymans* 1188-1 (isolated from zucchini in the United States) shared the same sequence types. In the *gyrB* gene sequences, only the strain pairs Als4/Als60 and Als58/Als59 shared the same sequence type. Strain Als34 also shared the same sequence type with the pathotype strain of *P. syringae* pv. *apii* CFBP2103^{PT} isolated from celery in the United States. In the *rpoD* gene sequences, Als2, Als28, Als30, Als33, Als38, Als51, and Als60 shared the same sequence type, whereas strains Als53 and Als59 shared another sequence type. None of the strains retrieved from GenBank/PAMDB shared the same sequence types with the strains isolated in this study in the *rpoD* gene sequences. Furthermore, none of the strains isolated in Iran shared the same multilocus haplotype with one another (Figs. 1 and 2).

Because the maximum likelihood phylogenies showed conflicting topologies, phylogenetic networks were generated using the NeighborNet algorithm for all of the individual genes (Supplementary Fig. S4) as well as the concatenated data set of sequences (Fig. 3). When all *P. syringae* strains isolated in this study were analyzed along with the strains isolated from stone fruits in Iran, the NeighborNet network confirmed recombination in the data set of concatenated sequences (Fig. 3). The topology of the NeighborNet network was in congruence with that of the phylogenetic tree constructed using the concatenated sequences of the four genes (Fig. 1). Recombination was observed between the strains isolated in this study and the strains isolated from stone fruits in Iran (parallel lines in the NeighborNet network; Fig. 3). Similar results were observed when the individual housekeeping gene sequences were analyzed using the same method.

Sequence variation statistics and diversity parameters are shown in Table 3. Eighteen *P. syringae* strains in this study consisted of 13, 14, 16, and 11 sequence types (haplotypes) in the sequences of *gapA*,

gltA, *gyrB*, and *rpoD*, respectively (Table 3). The highest nucleotide diversity was observed in the *gyrB* gene sequences ($P_i = 0.03325$). Tajima's *D* and Li's *D** and *F** statistics showed that there was a significant departure from the mutation drift equilibrium in the sequences of *gapA* and *rpoD* genes (Table 3). The negative values of neutrality indices in the strains suggest a recent selective sweep and/or population expansion after a recent bottleneck. In addition, recombination events were detected using DnaSP in all four housekeeping genes within the strains isolated in Iran, while the number of recombination events was greater in *gyrB* than in the remaining three genes (Table 3). The PHI test found statistically significant evidence for recombination ($P < 0.00001$) among the concatenated sequences of the four genes (Table 3). However, the same test on the individual gene sequences did not detect any evidence for recombination in the strains isolated in Iran (Table 3). The test of recombination using the seven nonparametric detection programs in RDP version 4 revealed significant evidence of recombination only in the *gyrB* gene sequences.

Discussion

In this study, through a series of comprehensive field surveys and multiphasic molecular phylogenetic analyses, we characterized the bacterial strains associated with alfalfa leaf spot symptoms in Iran. Host range assays accomplished by MLSA-based phylogenetic analyses revealed that different lineages of *P. syringae sensu lato* are responsible for alfalfa leaf spot disease. To our knowledge, this is the first comprehensive host range assay and phylogenetic analysis on the alfalfa-pathogenic members of *P. syringae* around the globe, which provides novel insight into the genetic diversity of *P. syringae sensu lato*. Different pathovars of *P. syringae* were reported as the causal agents of leaf spot diseases on annual crops and fruit trees in Iran (Ahmadi et al. 2018; Rahimian 1995; Sedighian et al. 2014). However, until recently, no precise information was available from the host range and taxonomic position of the strains. A leaf spot disease was reported on cucurbits plants in northwestern Iran in 2014, and the causal agent was identified as *P. syringae* pv. *aptata* based on the phylogeny of *gyrB* and *rpoD* gene sequences (Sedighian et al. 2014). We included a representative squash strain (M29) in our study and noted that the host range and phylogenetic position of the squash strain were in congruence with those of strains Als28, Als30, Als33, Als51, Als52, Als53, and Als54 isolated in this study. All of these strains were phylogenetically closely related to *P. syringae* pv. *aptata*, the pathogen of sugar beet plants around the globe (Nikolić et al. 2018). Similar results were obtained by Newberry et al. (2016) who noted that the watermelon strains of *P. syringae* isolated in Florida were phylogenetically closely related to the pathotype strain of *P. syringae* pv. *aptata*. On the other hand, strain Als34 was clustered in phylogroup 1 of *P. syringae sensu lato* (closely related to the type strains of *P. syringae* pv. *apii* NCPBP 1626^{PT} and *P. syringae* pv. *tomato* CFBP 2212^{PT}) and had a distinct host range, being pathogenic only on alfalfa and tomato. *P. syringae* pv. *tomato* was reported in central Iran (Varamin, Tehran Province), causing bacterial speck of tomato (Ghobakhloo and Rahimian 2000); however, the causal agent was identified only using the phenotypic assays, and no further information is currently available regarding the host range and distribution of the pathogen in the region. Alfalfa

Table 3. Sequence variation statistics and diversity parameters of *Pseudomonas syringae* strains used in this study, calculated using DnaSP 5.10 software^a

Sequence set	N	H	S (%)	P_i (π)	NM (η)	HD	Neutrality tests			R	Mean ϕ_w (phi test) ^b	R ^b	P^b
							Tajima's D	Fu and Li's D*	Fu and Li's F*				
Concatenated	1,819	18	226 (12.4)	0.02217	236	1.00	-1.75757 (NS)	-2.36068 (NS)	-2.53802 (NS)	18	0.155	Yes	0.000**
<i>gapA</i>	460	13	56 (12.1)	0.01944	56	0.96	1.86445 ($P < 0.05$)*	-2.45590 ($P < 0.05$)*	-2.65104 ($P < 0.05$)*	3	0.115	No	0.195 (NS)
<i>gltA</i>	500	14	54 (10.8)	0.01936	55	0.974	-1.63111 (NS)	-2.22663 (NS)	-2.38338 (NS)	5	0.123	No	0.210 (NS)
<i>gyrB</i>	479	16	75 (15.6)	0.03325	82	0.987	-1.38788 (NS)	-1.63570 (NS)	-1.81596 (NS)	7	0.124	No	0.910 (NS)
<i>rpoD</i>	380	11	42 (11.0)	0.01542	44	0.856	-2.24031 ($P < 0.01$)**	-3.29433 ($P < 0.02$)**	-3.46709 ($P < 0.02$)**	1	0.0714	No	0.087 (NS)

^a Calculated according to Librado and Rozas (2009). N = number of nucleotides, H = number of haplotypes, S = total number of segregating sites, P_i = nucleotide diversity, NM = number of mutations, HD = haplotype (gene) diversity, R = minimum number of recombination events, and NS = not significant. The asterisks by the *P* values indicate statistical significance of the data in 0.05 (*) and 0.01 (**).

^b These parameters were calculated using SplitsTree version 4.14.4 (Huson and Bryant 2006).

leaf spot was reported in a restricted area of western Iran in 2007, and the causal agent was described as *P. syringae* pv. *syringae* only based on the phenotypic assays (Harighi 2007). Our results elucidated the exact taxonomic position of the alfalfa leaf spot pathogen among *P. syringae sensu lato*.

Among *P. syringae sensu stricto*, complexities in the population structure and host range make it difficult to describe the exact span of traditionally defined pathovars (Bull and Koike 2015; Morris et al. 2019; Young 2010). Furthermore, given the possibility of horizontal transfer of genetic factors implicated in pathogenicity of the strains, the assumption that a pathovar is a physiologically and genetically homogenous taxonomic unit is proposed to be no longer universally valid in *P. syringae sensu stricto* (Morris et al. 2000, 2019). According to the results of comprehensive host range assays of 134 *P. syringae* strains on 15 to 22 plant species (conducted in four separate tests from 1998 to 2017), Morris et al. (2019) noted that the “artificial” host range of phylogroup 2 strains is broader than previously assumed. This led them to propose that the pathovar-based designations of the strains within *P. syringae* do not correspond to the underlying biology of the species. Practically, it is impossible to determine a complete host range of a given bacterial strain; hence, the host range of a candidate strain is normally determined against a pre-selected panel of plant species that are taxonomically related to the host of isolation (Bull and Koike 2015; Dye 1980). This approach would lead to a situation in which many potential hosts of a given pathogen remain undetermined. Host range assays in greenhouse conditions showed that the alfalfa strains isolated in this study were capable of inducing symptoms on several annual crops within a taxonomically diverse set of plant families (e.g., Amaranthaceae, Cucurbitaceae, Fabaceae, and Solanaceae). Strain Als58 was also pathogenic on wheat (Poaceae), although none of the strains were pathogenic on radish (Brassicaceae). Although MLSA has shed light on the phylogenetic position of the strains, we were unable to delineate the exact “pathovar” status to the alfalfa leaf spot pathogen because they were capable of infecting all of the predefined hosts of *P. syringae* pv. *aptata*, *P. syringae* pv. *atrofaciens*, *P. syringae* pv. *syringae*, and *P. syringae* pv. *tomato* (Bull and Koike 2015; Dye 1980; Young 2010).

Results of the host range assays obtained in this study were in congruence with those of Morris et al. (2019), especially in the case of reference strain B728a, which was shared in both studies. Our assays showed that strain B728a was pathogenic on alfalfa, broad bean, cucumber, mung bean, honeydew, zucchini, and tomato in greenhouse conditions. Likewise, Morris et al. (2019) showed that the same strain was pathogenic on broad bean, buttercup, clover, cowpea, eggplant, geranium, melon, mustard, onion, pea, pepper, sorghum, soybean, and tomato. Strain B728a has traditionally been considered to have a restricted host range and is capable of inducing nonhost resistance on other species such as tomato (Chien et al. 2013). It could be hypothesized that this presumption is attributable mostly to the restricted plant species/cultivars on which the strain has been evaluated. On the other hand, regarding the different inoculation methods used in this study and that used by Morris et al. (2019) (spray inoculation versus infiltration/injection), the broad host range of strain B728a in both experiments shows that the inoculation method does not significantly affect the artificial host range span of the strain, which could be the case in all of the strains isolated from alfalfa in this study. All of these findings illustrate the issues that must be fixed before host range testing can effectively contribute to the taxonomy and identification of *P. syringae* strains on a routine basis. There are major obstacles to host range testing that may explain why it has rarely been used for identification of pathovars of *P. syringae*. For instance, no standard set of host plants (i.e., species and cultivars) for differentiating pathovars of *P. syringae* has been proposed. Furthermore, choice of inoculation method and environmental conditions (e.g., relative humidity) should be standardized to each of the plant species to facilitate the globalization of the results obtained by different scientists (Gullino et al. 2009).

P. syringae pv. *syringae* is capable of infecting up to 40 plant species (Bradbury 1986; Young 2010). Although one can contemplate that the earlier claims of wide host range were probably attributable

to misidentification of the causal agent, current assays supported by complete genome sequence-based analyses also confirmed the overlapping continuum of the host range between traditionally defined pathovars within *P. syringae sensu stricto* (Bradbury 1986; Hesse et al. 2018; Morris et al. 2019; Newberry et al. 2019; Vinatzer et al. 2017). Furthermore, the definition of *P. syringae* pv. *syringae* needs to be reconsidered, given that it has been described only upon the host range-based “pathovar” system. Indeed, strains that are proven to be pathogenic to lilac (the original host of *P. syringae* pv. *syringae*) also infect several other plant species, although there are strains with the same determinative characteristics of the pathovar that do not attack lilac (Young 1991, 2010). We have seen the same situation for the strains isolated in this study, in which a number of strains that were fairly pathogenic on the host of isolation (alfalfa) were severely pathogenic on a set of Cucurbitaceae and Fabaceae plant species (Table 2).

In conclusion, this study provides novel insight into the diversity of *P. syringae sensu lato* in terms of host range and phylogenetic position of alfalfa-pathogenic strains, which were absent in the previously reported comprehensive and polyphasic studies within the species (Bull et al. 2011; Hwang et al. 2005; Sarkar and Guttman 2004). With increasing reliance on complete genome sequencing, several strains among *P. syringae sensu lato* were reported to be distinct from any pathotype strain within the species, suggesting that the existing pathotype strains represent only a fraction of the genomic diversity of the species (Hesse et al. 2018). Results of this study, in congruence with previous reports (Morris et al. 2019), advocate a need to revisit the taxonomy of plant pathogenic members of *P. syringae*, which will result in improved detection and identification methods of plant diseases in twenty-first century agriculture.

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