An Emerging Disease Caused by *Pseudomonas syringae* pv. *phaseolicola* Threatens Mung Bean Production in China

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Abstract

An emerging bacterial disease with symptoms resembling those of halo blight is threatening mung bean production in China. This study was conducted to investigate the disease's geographic distribution in China using consecutive multiyear field surveys and to confirm the causative agents' identity. The surveys were conducted in 15 provinces covering seven geographic regions from 2009 to 2014. The survey results revealed that the emerging mung bean disease has rapidly spread and is prevalent in three of the main Chinese geographic regions, which contain more than 90% of the mung-bean-growing areas in China. To confirm the causal agent, diseased mung bean leaves were collected from the surveyed fields

and used to isolate the pathogen. A bacterium was consistently isolated from all of the collected leaves. Based on the phenotypic characteristics, the physiological and biochemical properties, pathogenicity tests, and fatty acid composition, in combination with specific polymerase chain reactions and 16S-23S ribosomal DNA sequence analyses, the bacterium was identified as *Pseudomonas syringae* pv. *phaseolicola*. To our knowledge, this is the first report of *P. syringae* pv. *phaseolicola* causing halo blight on mung bean in China. The results indicate that *P. syringae* pv. *phaseolicola* is likely of epidemiological significance on mung bean in China.

Mung bean (*Vigna radiata* L.) is an important warm-season legume crop widely grown in tropical and subtropical regions in Asia (Nair et al. 2013). It has been cultivated extensively in several geographic regions of China (Wang et al. 2009). Mung bean is considered a rich source of dietary protein, iron, carbohydrates, and micronutrients, as well as bioactive compounds with medicinal values (Dahiya et al. 2015). Additionally, its products have appeared in various processed foods which are popular in China, Korea, the Philippines, and Thailand (Dahiya et al. 2015; Silva et al. 2013; Tang et al. 2014; Zeng et al. 2011). Biotic stresses are major factors limiting the yield and quality of mung bean. To date, there are more than 20 diseases of mung bean documented in China (Zhu and Duan 2012). Numerous new mung bean diseases have been reported in China in recent years (Cui et al. 2014; Yue et al. 2015; Zhang et al. 2011).

Pseudomonas syringae is a widespread bacterial species comprising more than 60 pathovars with a broad host range (Young 2010). Most of the pathovars have highly restricted or specific host ranges, and are only pathogenic to a single plant genus or related host taxa (Lamichhane et al. 2015; Young 2010). However, the pathovar *P. syringae* pv. *phaseolicola* (Burkh.) has a relatively wide host range, infecting several crops, including *Phaseolus vulgaris*, *P. acutifolius*, *P. coccineus*, *P. lunatus*, *Cajanus cajan*, *Centrosema* sp., *Desmodium* spp., *Glycine max*, *Lablab purpureus*, *Lens culinaris*, *Macroptilium atropurpureum*, *Neonotonia wightii*, *Pachyrhizus erosus*, *Pisum sativum*, *Pueraria lobata*, *P. thunbergiana*, *Vigna angularis*, *V. radiata*, and *V. unguiculata* (Birch et al. 1981; Hunter and Taylor 2006; Patel and Walker 1965; Taylor et al. 1996). *Pseudomonas syringae* pv. *phaseolicola*, the causative agent of halo blight, is probably the most important bacterial pathogen of bean (*Phaseolus*

vulgaris) in Europe and the United States, as well as other countries (Rico et al. 2003). Bean halo blight disease was first observed in the United States in 1925. It reduced average yields by up to 45% and further losses occurred due to the poor quality of the infected pods (Arnold et al. 2011; Burkholder 1926; Webster et al. 1983). To date, the disease has been observed in most bean-growing regions worldwide and has become a highly destructive disease on bean. The bean halo blight pathogen favors cool (less than 25°C) and moist conditions. Severe epidemics of the disease can result in bean yield losses as high as 80 to 100% in regions with cool and humid climates (van Schoonhoven and Voysest 1989).

Pseudomonas syringae pv. *phaseolicola* is a serious seedborne pathogen, and even extremely low levels of seed infestation can result in severe epidemics under favorable weather conditions (Webster et al. 1983). Therefore, the only practical and effective control treatment for this disease is the use of pathogen-free seed (Taylor et al. 1979; Webster et al. 1983). Thus, the detection of *P. syringae* pv. *phaseolicola* in seed is essential for effective disease management. The certification of seed as pathogen-free requires highly sensitive and specific identification and detection methods for *P. syringae* pv. *phaseolicola*. To date, many countries, including China, have strict quarantine laws against *P. syringae* pv. *phaseolicola* due to its seedborne status (Li et al. 2009; Yang et al. 2015). The pathogen *P. syringae* pv. *phaseolicola* can produce a chlorosis-inducing toxin, phaseolotoxin, which causes the characteristic chlorotic halo surrounding water-soaked spots on infected leaves (Bender 1999; Mitchell 1976; Tamura et al. 2002). The phaseolotoxin synthetic gene clusters of *P. syringae* pv. *phaseolicola* have been isolated and identified (Mosqueda et al. 1990; Peet et al. 1986; Zhang and Patil 1997; Zhang et al. 1993). Subsequently, several simple and efficient methods for the specific detection of toxigenic strains of *P. syringae* pv. *phaseolicola* have been developed based on the phaseolotoxin gene cluster sequences (Audy et al. 1996; Mosqueda-Cano and Herrera-Estrella 1997; Prosen et al. 1993; Schaad et al. 1995). Nontoxigenic strains of *P. syringae* pv. *phaseolicola* were also found in the field (Rico et al. 2003). These strains were pathogenic and could produce typical water-soaked lesions but could not cause chlorotic halo symptoms or spread systemically in the plant. It was generally thought that they had little or no epidemiological significance to halo blight of bean (Schaad et al. 1995). In Spain, Rico et al. (2003) found that the nontoxigenic strains were a main causal agent of halo blight in bean. Nontoxigenic strains could not be detected using previous molecular

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and serological methods. Therefore, Rico et al. (2006) established a reliable method to distinguish toxigenic and nontoxigenic strains of *P. syringae* pv. *phaseolicola*. Primer pair P3004 L/P3004R, which amplified a 0.24-kb fragment only in nontoxigenic *P. syringae* pv. *phaseolicola* strains, has been developed (Oguiza et al. 2004; Rico et al. 2006). A duplex polymerase chain reaction (PCR) amplification with two primer pairs, P3.1/P5.1 and P3004 L/P3004R, specific for toxigenic and nontoxigenic strains of *P. syringae* pv. *phaseolicola*, respectively, could be simply and effectively used to identify and detect *P. syringae* pv. *phaseolicola* in seed (Rico et al. 2006).

Mung bean halo blight, caused by *P. syringae* pv. *phaseolicola*, was first recorded in the United States in 1971 (Schmitthenner et al. 1971). Subsequently, Patel and Jindal (1972) observed halo blight on mung bean in India. To date, mung bean halo blight disease has also been documented in Pakistan, Australia, and Tanzania (Akhtar 1988; Conde and Diatloff 1991; Taylor et al. 1996).

In China, halo blight caused by *P. syringae* pv. *phaseolicola* on bean was first reported in Heilongjiang Province in 1987, and it resulted in more than 50% yield losses (Li and Zhang 1990). Recently, halo blight disease of bean was observed in Yunnan Province, although it is still considered a quarantine organism in China (Chen and Zhou 2013). In 2009, an emerging bacterial disease resembling halo blight was observed on mung bean plants grown in the northeastern region of China during our field disease survey of food legumes (Zhu and Duan 2012). The diseased mung bean plants showed typical symptoms on infected leaves that were similar to those of halo blight (Harveson and Schwartz 2007). Subsequently, the disease rapidly spread and emerged in many mung-bean-planting areas, which caused serious mung bean yield losses, making the disease a major threat to mung bean production in China. Thus, it is necessary to investigate the geographic distribution of this disease in order to know its prevalence and response to the disease control.

The objective of this study was to investigate the geographic distribution of the emerging disease resembling halo blight on mung

bean in China through consecutive multiyear field surveys and characterize the causal agent of the emerging disease using morphological observations, physiological and biochemical tests, and molecular characteristics, including specific PCR amplification reactions and 16S-23S ribosomal DNA (rDNA) sequence analyses, as well as pathogenicity tests.

Materials and Methods

Field surveys of disease distribution. Our previous field observation indicates that the emerging disease resembling halo blight is becoming a major threat to mung bean in China. In this study, the distribution of mung bean halo blight in China was conducted by consecutive multiyear field surveys in 15 provinces, including all mung-bean-growing areas and covering seven geographic regions of China: northeastern, northern, northwestern, eastern, central, southwestern, and southern China (Fig. 1). The surveys commenced in 2009 and continued until 2014, spanning 6 years. Consecutive multiyear field surveys will demonstrate the disease occurrence and prevalence as well as symptom development process on mung bean.

Sample collection and pathogen isolation. To determine the identity of the pathogen causing the emerging disease, infected mung bean leaves with severe symptoms of halo blight, necrotic spots surrounded by a yellow halo, were collected from the surveyed fields located in the northeastern, northern, northwestern, eastern, central, southwestern, and southern regions of China (Fig. 1).

To isolate the putative causal agent, the boundary areas of the lesion tissues on mung bean leaves were cut into small pieces, which were then surface disinfested in 2% sodium hypochlorite for 2 min, followed by rinsing three times in sterile distilled water. The disinfested pieces were crushed in sterile 1.5-ml tubes containing 600 µl of sterile distilled water. The resulting suspensions or dilutions were streaked onto King's B (KB) medium plates (King et al. 1954). The plates were incubated at 28°C. After 3 to 5 days of incubation, white colonies that fluoresced under UV light were

Fig. 1. Geographic distribution of mung bean halo blight as determined by consecutive multiyear field surveys in China. Gray, shaded regions represent provinces in which the surveys were conducted. Black dots indicate cities or counties where mung bean halo blight has occurred.

purified using single-colony subcultures. The cultured bacterial cells were suspended in KB plus 15% glycerol and stored at -80°C until further use.

Pathogenicity tests. Nine representative isolates from nine provinces were selected for pathogenicity testing. The pathogenicity tests were performed on 2-week-old seedlings of Chinese elite mung bean ‘Jilv 7’, which appears highly susceptible to the pathogen in the affected fields. Pathogen-free seed of Jilv 7 were kindly provided by Prof. Tian Jing at Hebei Academy of Agricultural and Forestry Sciences, Hebei Province, China. Seed were sown in paper cups (10 cm in diameter) filled with a 3:1 mixture of vermiculite and peat moss. Five seeds were sown per pot. The planted pots were placed in a greenhouse with temperatures of 23 to 27°C and natural daylight. For preparing inoculum, the nine representative isolates were cultured on nutrient broth agar (NA) medium for 48 h. The inoculum was made by suspending the bacterial cells in sterile tap water to an approximate cell concentration of 1×10^8 CFU/ml measured by a ultraviolet spectrophotometer. The plants having the first fully expanded trifoliate leaves were inoculated using a leaf water-soaking method (Schuster 1955). Bacterial suspensions were sprayed onto the underside of leaf surfaces using a sprayer connected to a high-pressure air compressor (Haiou, Jiangyan City, China). Plants inoculated with sterile tap water were used as controls. Three hours after inoculation, the plants were placed in a mist room at 18 to 20°C for 48 h. Misting occurred for 30 min every 2 h by an automatically controlled centrifugal humidifier, which maintained leaf wetness without excessive runoff. Then, the plants were removed to a greenhouse maintained at 18 to 22°C until the plants were rated for disease symptoms. After disease symptom development (i.e., 10 days after inoculation), bacteria were reisolated from infected tissues to complete Koch’s postulates.

Phenotypic characteristics. Nine representative isolates from nine provinces were selected for morphological observations. The colony morphology of the nine isolates was observed on KB and NA media growing at 28°C for 72 h. Bacterial cell morphology from the colony growth on NA at 28°C for 24 h was characterized using a transmission electron microscope (HITACHI H-7500; Tokyo).

Physiological and biochemical properties. Nine representative isolates were physiologically and biochemically analyzed. For the physiological analysis, the carbon source utilization of the isolates were determined using mannitol, inositol, sorbitol, erythritol, betaine, anthranilate, homoserine, trigonelline, D-tartrate, L-tartrate, and L-lactate (Schaad et al. 2001). For the biochemical analysis, gram staining reactions and the levan formation, oxidase reaction, potato soft rot, arginine dihydrolase, tobacco hypersensitive (LOPAT) tests were conducted following the methodology described by Lelliott et al. (1966). Each test was repeated at least twice.

Fatty acid composition. The nine bacterial isolates were inoculated onto NA medium and grown at 25°C for 24 h, then transferred onto trypticase soy broth agar (Sigma-Aldrich, St. Louis) and grown at 28°C for 48 h to determine the fatty acid composition. The cellular fatty acids of the nine isolates were extracted and their fatty acid

methyl esters (FAME) were separated according to the standard Microbial Identification System (MIDI) protocol (Microbial ID, Inc., Newark, DE). The FAME were then dissolved in hexane and analyzed by gas chromatography using an Agilent 6890 N GLC (Agilent Technologies, Palo Alto, CA) equipped with a 7683 injector, a G2614A autosampler, a flame ionization detector, and hydrogen as carrier gas. Conditions (temperature and time) for the gas chromatography analyses are set by MIDI (Sherlock version 4.5; Microbial ID, Inc.). FAME peaks were analyzed using MIDI, Sherlock MIS4.5 (microbial identification system) and LGS4.5 (library generation software).

Pathovar-specific PCR detection of the isolates. Total bacterial genomic DNA was extracted from the nine representative isolates grown on NA at 25°C for 72 h using the TIANamp Bacteria DNA Kit (Tiangen Biotech Co., Ltd., Beijing) according to the manufacturer’s instructions. DNA of the nine isolates was amplified using four pathovar-specific primer pairs: HB14F/HB14R (Audy et al. 1996), 62a/63a (Mosqueda-Cano and Herrera-Estrella 1997), P3.1/P5.1 (Schaad et al. 1995), and P3004 L/P3004R (Rico et al. 2006). Detailed information on the primers used in this study is listed in Table 1.

PCR amplification assays were conducted in a total volume of $20\ \mu\text{l}$, containing 50 ng of genomic DNA, $2.5\ \mu\text{l}$ of $10\times$ PCR buffer ($20\ \text{mM}\ \text{MgCl}_2$), $0.2\ \text{mM}$ each dNTP, $1.5\ \text{U}$ of *Taq* DNA polymerase (Tiangen Biotech Co., Ltd.), and $0.2\ \mu\text{M}$ primer mixture. PCR assays were carried out at 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 50 to 68°C (depending on the primer-specific annealing temperature) for 30 to 90 s, and 72°C for 1 to 2 min; with a final extension at 72°C for 10 min, using a thermal cycler (Biometa, Goettingen, Germany). PCR products were separated by electrophoresis on a 1% agarose gel with $1\times$ Tris-borate-EDTA buffer.

16S-23S rDNA sequence and phylogenetic analysis. The 16S-23S rDNA internal transcribed spacer (ITS) regions of three representative isolates (LDI7, LDI8, and LDI9) were amplified with the universal primer pair 1493F/23R (AGTCGTACCAAGGTAGCCGT/GTGCCAAGGCATCCACC) (Li and de Boer 1995). PCR assays and amplification were carried out as described above. The resulting purified amplicons produced by 1493F/23R were sequenced. Sequencing reactions were performed at the Beijing Genomics Institute. The resulting sequences were analyzed as query using the BLAST algorithm against the National Center for Biotechnology Information (NCBI) GenBank database. A phylogenetic analysis was performed using the neighbor-joining method, and a phylogenetic tree was constructed by bootstrapping using Mega 6 software (Tamura et al. 2013).

Results

Field surveys of disease distribution. In 2009, a field survey of diseases affecting food legume crops was initially conducted in the northeastern region of China during their growing season. An emerging bacterial disease resembling halo blight was observed to be severely affecting mung bean in experimental and commercial fields located in Wulanhaote City and Tuquan County in Inner Mongolia Province; Baicheng City and Songyuan County in Jilin Province;

Table 1. Four primer pairs used for pathovar-specific polymerase chain reaction detection of *Pseudomonas syringae* pv. *phaseolicola* in this study

Primer pair	Primer sequence 5’-3’	Size (bp)	Gene	References
HB14F/HB14R	CCGGTCTGCTCGACATCGACATCGTGCCAC	1,400	Phaseolotoxin gene cluster	Audy et al. 1996
62a/63a	CAACTCCGACACCAGCGACCGACCGAGC	...	...	...
	CAATGAAGATTACAAGCCTG	1,000	<i>argK</i>	Mosqueda-Cano and Herrera-Estrella 1997
P3.1/ P5.1	GCTAGCTATCAGGGGACGAC	...	...	...
	TGTTTCGGCCAGAGGCACTCATG	500	<i>phtE</i>	Schaad et al. 1995
P3004 L/P3004R	AGCTTCTCCTCAAAACACCTGC	...	...	...
	CTGTCTGGCAGCCACTACAAAG	240	AJ568001	Rico et al. 2006
	CTGTCTGGCAGCCACTACAAAG	...	...	...

Harbin City, Qiqihaer City, and Duerbote County in Heilongjiang Province; and Shenyang City and Fuxin County in Liaoning Province. The disease symptoms were extremely similar to those of halo blight of bean caused by the quarantined plant pathogen *P. syringae* pv. *phaseolicola*, which had been reported on bean crops in a few locations in China (Chen and Zhou 2013; Li and Zhang 1990).

Therefore, consecutive multiyear surveys were subsequently conducted from 2010 to 2014 across all of the mung-bean-production regions in China (Fig. 1). The comprehensive survey results showed that the disease occurred in 37 cities or counties in nine provinces: Heilongjiang, Jilin, Liaoning, Inner Mongolia, Beijing, Hebei, Shanxi, Shaanxi, and Xinjiang (Fig. 1; Supplementary Table S1). However, the emerging disease on mung bean was not observed in the remaining six provinces (Shandong, Henan, Jiangsu, Anhui, Hubei, and Chongqing; Fig. 1). The emerging mung bean disease occurred in an area between the northern latitudes of 37.48° and 48.01° and eastern longitudes of 87.56° and 128.05°, which includes three

main mung-bean-growing regions: northeastern, northern, and northwestern China (Fig. 1). Generally, the average yields of the fields infected by the emerging disease were reduced 30 to 50% according to our survey, with yield loss of more than 80% in the most severely infected fields. The highest incidence of the disease was 100%, causing complete yield losses in Yulin City in Shaanxi Province and Xuanhua County in Hebei Province.

Development of disease symptoms. Mung bean is commonly sown from 15 May to 10 June in three main mung-bean-growing regions: northeastern, northern, and northwestern China. The emerging disease, resembling halo blight, on mung bean was initially observed in early to mid-June. At first, disease symptoms were observed on the lower surfaces of the primary leaves, appearing as small, dark-brown, water-soaked spots (Fig. 2A). Then, the spots rapidly became visible on the upper surfaces of the infected primary leaves. With the lesion expansion, the center of the spot became necrotic and turned gray or white (Fig. 2B). The lesions were also observed on the central vein of

Fig 2. Natural symptoms of halo blight caused by *Pseudomonas syringae* pv. *phaseolicola* on mung bean grown in fields. **A**, Small dark-brown, water-soaked spots and vein necrosis on the lower surface of a primary leaf. **B**, Lesions with a gray or white necrotic central area on the upper surface of a primary leaf. **C**, Large circular or irregular dark-brown patches on a primary leaf. **D**, Primary leaf blight. **E**, Small water-soaked spots surrounded by chlorotic yellow-green halos on the upper surface of a leaflet. **F**, Water-soaked spots on the lower surface of a leaflet. **G**, Small necrotic spots surrounded by large yellow-green halos on the upper surface of a leaflet. **H**, Angular dark-brown necrotic spots on the upper surface of an old leaf in the late growth stage. **I**, Large necrotic areas on the lower surface of an old leaf. **J**, Water-soaked spots or dark-brown to black streaked lesions along pod sutures.

the primary leaves, leading to vein necrosis. Sometimes, large circular or irregular dark-brown patches appeared on the primary leaves (Fig. 2C), and the lesions expanded and merged together with time, finally resulting in leaf blight (Fig. 2D). With the appearance growth of trifoliolate leaves on plants, the typical halo blight symptoms were observed on trifoliolate leaves (Fig. 2E to G). Small water-soaked spots first appeared on leaflets. Then, the center of the spots soon became necrotic, turned brown to black, and were surrounded by chlorotic zones of yellow-green halos (Fig. 2E to G; Supplementary Fig. S1). Generally, the necrotic centers remained small in size (1 to 2 mm in diameter). However, the surrounding halos could enlarge to 2 cm in diameter and were visible on both sides of the leaflets. Young leaflets were the most susceptible, and infected young leaflets became distorted, crinkled, and chlorotic. In severe cases, young seedlings developed systemic chlorosis, and were stunted or even died. Spots on older leaves were usually angular and lacked the halo. The spots could enlarge and coalesce into large necrotic areas with the onset of cool and rainy weather (Fig. 2H to I). The disease was also observed on mung bean petioles and stems. Symptoms began as water-soaked spots, and later extended longitudinally, turning into brown streaks. Mung bean pods were infected after production; the initial symptoms were small water-soaked spots or streaks along the pod sutures, and these lesions turned dark brown to black with age (Fig. 2J).

Pathogen isolation and pathogenicity tests. Bacterial isolates were consistently obtained from collected leaves having halo-blight-like symptoms. Nine representative isolates from nine provinces were selected for pathogenicity tests on Jilv 7 mung bean. The results indicated that all of the isolates were pathogenic to Jilv 7. Typical small, water-soaked spots first appeared on the leaves 5 days after inoculation. Then, the lesions enlarged, turning irregular and brown, with a narrow yellow border or large halo within 10 days. These symptoms were similar to those observed in the field. By contrast, no symptoms were observed on control plants inoculated with sterile tap water. The bacteria were reisolated from the inoculated plants and their characteristics were identical to those of the inoculated isolates. This verified Koch's postulates.

Phenotypic characteristics. The colonies of the isolates grown on KB and NA plates for 72 h were creamy white, flat, and circular, with 1- to 3-mm diameters and entire margins (Fig. 3A). The bacterium was gram negative and produced fluorescent pigment on the KB medium under UV light (Fig. 3B). The bacteria were long and rod-shaped, 2.2 to 5.5 by 0.8 to 0.9 μm in size (Fig. 3C). One to several polar flagella existed at both ends of the bacterial cell (Fig. 3C).

Physiological and biochemical properties. For carbon source utilization, all of the isolates assimilated trigonelline and betaine but were negative for mannitol, inositol, sorbitol, erythritol, anthranilate, homoserine, D-tartrate, L-tartrate, and L-lactate. These results were consistent with the nutritional requirements of the pathogen *P. syringae* pv. *phaseolicola*. In LOPAT tests, all isolates were positive for levan production and the tobacco hypersensitive reaction but

negative for oxidase, potato soft rot, and arginine dihydrolase. These results corresponded to the LOPAT group containing *P. syringae* pathovars.

Fatty acid composition. Twenty-seven different fatty acids were detected in the nine representative isolates from mung bean. All of the isolates contained 10:0 3OH, 12:00, 12:0 2OH, 12:0 3OH, SF3 (16:1 w7c/16:1 w6c), 16:00, 18:1 w7c, and 18:00, and these eight major fatty acids accounted for more than 90% of the total fatty acids. The fatty acid composition analysis of the nine isolates using the MIDI microbial identification system (version 4.5) and library generation software (version 4.5) identified all of the isolates as *P. syringae* with similarity indexes of 0.503 to 0.954, which were good matches at the species level (Table 2).

Pathovar-specific PCR detection. Nine representative isolates were identified using four pathovar-specific primers for *P. syringae* pv. *phaseolicola* (Table 1). The primers HB14R/HB14F and 62a/63a amplified target fragments of 1,400 and 1,000 bp, respectively, from all of the isolates (Fig. 4). A duplexed PCR amplification using the primer pairs P5.1/P3.1 and P3004 L/P3004R produced 500- and 240-bp fragments in all of the isolates, respectively.

16S-23S rDNA sequence and phylogenetic analysis. Three isolates (LDI7, LDI8, and LDI9) were selected for 16S-23S rDNA sequence analyses. The resulting 16S-23S rDNA sequences of the three isolates were deposited into GenBank (NCBI accession numbers KF888742, KF888743, and KF888744). The 16S-23S rDNA sequences of the three isolates were identical and showed 99% identity with those of reported *P. syringae* pv. *phaseolicola* strains (NCBI accession numbers CP000058, D86378, and AY342163) and some *P. syringae* pathovars present in the NCBI database (Fig. 5).

Based on all data for bacterial morphology, physiological and biochemical properties pathogenicity tests, fatty acid composition, and molecular characteristics on pathovar-specific PCR amplification results and the 16S-23S rDNA sequence analysis, the causal agent of the emerging disease on mung bean was identified as *P. syringae* pv. *phaseolicola*.

Table 2. Fatty acid methyl ester identification of nine bacterial isolates from mung bean grown in nine provinces in China

Isolates	Geographic origin	Similarity index	Identity
LDI1	Shenmu, Shaanxi	0.752	<i>Pseudomonas syringae</i>
LDI2	Shenyang, Liaoning	0.767	<i>P. syringae</i>
LDI3	Baicheng, Jilin	0.503	<i>P. syringae</i>
LDI4	Qiqihaer, Heilongjiang	0.642	<i>P. syringae</i>
LDI5	Fangshan, Beijing	0.556	<i>P. syringae</i>
LDI6	Wulumuqi, Xinjiang	0.754	<i>P. syringae</i>
LDI7	Datong, Shanxi	0.592	<i>P. syringae</i>
LDI8	Tuqan, Inner Magnolia	0.803	<i>P. syringae</i>
LDI9	Xuanhua, Hebei	0.954	<i>P. syringae</i>

Fig. 3. Colony formation and a micrograph of a *Pseudomonas syringae* pv. *phaseolicola* isolate from mung bean. **A**, Two-day-old colonies on nutrient broth agar medium. **B**, Bacterium produces fluorescent pigment on King's B medium observed under UV light. **C**, Morphology of a bacterial cell observed using a transmission electron microscope (scale bar: 2 μm).

Discussion

Mung bean is an important food legume and is grown widely in China. In 2009, a new bacterial disease on mung bean, with symptoms resembling those of halo blight, was first found in a major mung bean production area in northeastern China. In a previous study, halo blight also was reported on bean in the northeastern region of China

Fig. 4. Polymerase chain reaction amplification patterns of nine representative mung bean isolates of *Pseudomonas syringae* pv. *phaseolicola* using four pathovar-specific primers. **A**, Primers HB14R/HB14F amplified a 1,400-bp fragment in every isolate. **B**, Primers 62a/63a amplified a 1,000-bp fragment. **C**, Primers P5.1/P3.1 (above) and P3004 L/P3004R (below) amplified 500- and 240-bp fragments, respectively, in a duplexed reaction. Lane M indicates the DNA marker ladder D2000 (A and B) and 100-bp marker ladder (C). Lanes 1 to 9 represent nine mung bean isolates of *P. syringae* pv. *phaseolicola*: LDI1, LDI2, LDI3, LDI4, LDI5, LDI6, LDI7, LDI8, and LDI9, respectively.

(Li and Zhang 1990). Because of the discovery of an emerging disease, field surveys were conducted to discern the distribution of the new mung bean disease in China. The results indicated that the halo-blight-like disease had become one of the most important disease of mung bean in China.

Generally, halo blight disease of bean prefers low temperatures at night. The temperature range of 16 to 20°C was optimal for halo formation and systemic chlorosis on leaves (Patel and Walker 1963). According to our surveys, the geographic distribution of the mung bean halo-blight-like disease covers the northeastern, northern, and northwestern regions of China. From June to August, these three geographic regions have low daily mean temperatures of 13 to 22°C and high daily mean temperatures of 26 to 31°C. These weather conditions were favorable for disease development and epidemics.

In the field, we observed symptoms of the emerging disease on mung bean that were similar to those of halo blight described on bean and mung bean (Harveson and Schwartz 2007; Patel and Jindal 1972). In addition, we observed lesion development on the primary leaves, old leaves, petioles, leaf veins, and pods through our multi-year surveys (Fig. 1). Based on our observations, the typical halo surrounding the necrotic spot did not form on the primary leaves. The formation of the chlorotic halo surrounding spots on infected leaves is caused by the phaseolotoxin of *P. syringae* pv. *phaseolicola* (Bender 1999; Mitchell 1976; Tamura et al. 2002). Phaseolotoxin is a nonspecific phytotoxin, which is a potent inhibitor of ornithine carbamoyltransferase and ornithine decarboxylase. Their inhibition could cause chlorophyll synthesis suppression and chlorophyll destruction, respectively, which leads to the chlorotic symptoms (Bachmann et al. 1998; Bender 1999; Ferguson and Johnston 1980). The production of phaseolotoxin is regulated by temperature, and the optimal temperature for phaseolotoxin production is 18°C. For bean, Turner and Mitchell (1985) found that clear halo symptoms only develop on the primary leaves of the youngest seedlings 6 to 7 days after germination. However, the halo lesions did not appear on primary leaves of more than 14-day-old seedlings, in which the chlorophyll concentration had reached its maximum. Therefore, halos could only

Fig. 5. Phylogenetic analysis of 16S-23S ribosomal DNA sequences of mung bean bacterial isolates (LDI7, LDI8, and LDI9) and reported *Pseudomonas* species and *P. syringae* pathovars. The tree was rooted using the bacterium *Xanthomonas campestris*. Neighbor-joining bootstrapping values are shown above of branching nodes (1,000 bootstraps).

be induced in those leaves that were expanding and synthesizing chlorophyll in cool weather conditions (Patel and Walker 1963).

The infection of primary leaves could be induced from infected seed. Therefore, early discrimination and diagnosis of mung bean halo-blight-like symptoms on primary leaves is conducive to the early eradication or control of disease spread in the field. Epidemics of mung bean halo blight are closely associated with planting seed already infected by *P. syringae* pv. *phaseolicola* in the field (Schmitthenner et al. 1971).

Until now, halo blight on mung bean had been reported in only a few countries. In previous studies, the causal agent of halo blight on mung bean was identified as *P. syringae* pv. *phaseolicola* (syn. *P. phaseolicola*) based on morphological, physiological, and biochemical properties (Akhtar 1988; Patel and Jindal 1972; Schmitthenner et al. 1971). In this study, the identity of the pathogen causing halo-blight-like symptoms on mung bean in China was also determined to be *P. syringae* pv. *phaseolicola* based on comprehensive phenotypic, physiological, and biochemical characteristics; pathogenicity tests; fatty acid composition; and molecular analyses. The phenotypic, physiological, and biochemical characteristics; pathogenicity test results; fatty acid composition; and molecular sequence analyses of the mung bean isolates corresponded to those of *P. syringae* pv. *phaseolicola* isolates reported in previous studies (Akhtar 1988; Güven et al. 2004; Jeon et al. 2006; Patel and Jindal 1972; Sawada et al. 1997).

Morphologically, we found that the bacterial cell size was somewhat different than that of the previously reported *P. syringae* pv. *phaseolicola* isolates from mung bean and kudzu vine. Akhtar (1988) measured the size of mung bean isolates as 1.2 to 6.0 by 1.4 to 3 μm . Jeon et al. (2006) reported that the kudzu vine isolates were 1.5 by 0.7 to 0.8 μm . These differences may be caused by different origins, hosts, or cultural conditions. Some factors such as temperature, nutrients, and the osmolarity of the growth medium have been reported to affect the size of bacteria (Monier and Lindow 2003).

In the analysis of carbon source utilization, our isolates did not assimilate mannitol. The isolates' carbon source utilization corresponded to that of bean isolates of *P. syringae* pv. *phaseolicola* (Birch et al. 1981; Güven et al. 2004; Völksch and Weingart 1997) but contrasted with those of Indian mung bean (Patel and Jindal 1972), yam bean (Birch et al. 1981), and kudzu vine (Jeon et al. 2006; Völksch and Weingart 1997) isolates.

In this study, three pathovar-specific primer pairs (HB14R/HB14F, 62a/63a, and P5.1/P3.1) targeting toxigenic isolates of *P. syringae* pv. *phaseolicola* that contained phaseolotoxin gene clusters, amplified expected target fragments in our mung bean isolates (Audy et al. 1996; Mosqueda-Cano and Herrera-Estrella 1997; Schaad et al. 1995) (Fig. 4). The results indicated that the nine representative isolates belonged to toxigenic strains of *P. syringae* pv. *phaseolicola* containing a phaseolotoxin gene. Whereas the primer pair P3004 L/P3004R did not yield the expected results (Rico et al. 2006), the primers P3004 L/P3004R amplified a 240-bp target fragment in our isolates (Fig. 4) which had previously been observed only in nontoxigenic strains (Oguiza et al. 2004). The primer pair P3004 L/P3004R may lack the specificity to discriminate between toxigenic and nontoxigenic isolates of *P. syringae* pv. *phaseolicola* from different origins or genetic backgrounds.

Previous studies revealed that *P. syringae* pv. *phaseolicola* isolates from mung bean and bean have different pathogenic capabilities, nutritional requirements, and genetic characteristics (Marques et al. 2000; Patel and Jindal 1972; Schmitthenner et al. 1971). This might be because mung bean originated from the old world (India) and bean from the new world (America). Patel and Jindal (1972) hypothesized that mung bean and bean isolates of *P. syringae* pv. *phaseolicola* evolved independently from some common saprophytic ancestors and that the host played an important role in the nature of the parasitic, nutritional, and other changes that accompanied evolution. In this study, our mung bean isolates also showed a few minor differences compared with various host isolates of *P. syringae* pv. *phaseolicola* in morphology, carbon source utilization, and

molecular characteristics, although our mung bean isolates also caused *P. syringae* pv. *phaseolicola*-associated symptoms, including water-soaked lesions on bean cotyledons and pods as determined by pathogenicity tests (data not shown). A further comparative analysis of mung bean and bean isolates of *P. syringae* pv. *phaseolicola* should be conducted to reveal all of the differences.

A comprehensive field survey of the geographic distribution of the emerging disease, halo blight on mung bean, was conducted in China. The survey results revealed that mung bean halo blight has been rapidly spreading and is prevalent in the three major mung-bean-producing regions (northeastern, northern, and northwestern China). By our multiyear field survey, the development of halo blight symptoms on mung bean was clearly observed and demonstrated on leaves, stems, petioles, and pods. The causal agent of the disease was confirmed as *P. syringae* pv. *phaseolicola* using morphological, physiological, and biochemical characteristics; pathogenicity tests; as well as specific PCR amplification assays and 16S-23S rDNA sequence analyses. To our knowledge, this is the first report of mung bean halo blight caused by *P. syringae* pv. *phaseolicola* in China.

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