Occurrence of White Top of Pea Caused by a New Strain of *Pseudomonas syringae* pv. *pisi*

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ABSTRACT

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A new bacterial disease has been observed on pea in Shizuoka prefecture, Japan, since 1981. The disease occurs in early autumn when pea plants grow vigorously. The disease is characterized by chlorosis and whitening of apical shoots, including leaflets, stipules, and young pods. Usually, these white top (WT) symptoms are associated with extensive water-soaked lesions on stems and on leaflets at the basal part of the diseased plants. Thirty-four bacterial isolates from WT plants were characterized and identified together with 16 strains of *Pseudomonas syringae* pv. *pisi* from common bacterial blight of pea. The bacteria were gram-negative rods, having one to six polar flagella. The results of LOPAT tests were + − − − +, showing that they belong to *P. syringe*. In stab inoculation on stems, the WT isolates produced WT symptoms with water-soaked spots 14 days after inoculation. The 16 *P. syringae* pv. *pisi* strains never induced WT symptoms and, on the contrary, caused the typical bacterial blight. WT isolates were not pathogenic on any other plants tested. Phenotypic properties differentiated WT isolates and *P. syringae* pv. *pisi* strains into two groups; one consists of WT isolates and *P. syringae* pv. *pisi* group A, the other is *P. syringae* pv. *pisi* group B. Two distinct fingerprint profiles were identified by repetitive sequence based-polymerase chain reaction. WT isolates and *P. syringae* pv. *pisi* group A belonged to the same fingerprint type in rep-PCR, whereas a distinct fingerprint was shown by strains of the *P. syringae* pv. *pisi* group B. We concluded that the WT isolates should be included in *P. syringae* pv. *pisi* as a distinct strain in symptom expression.

In 1981, a disease of unknown cause characterized by white top (WT) appearance was first observed on pea in the Izu district of Shizuoka Prefecture, Japan, one of the major pea-production areas. Symptoms developed as conspicuous apical shoot chlorosis and whitening, which did not resemble any diseases caused by known pathogens of pea. The symptoms of the WT disease usually are associated with water-soaked lesions typical of bacterial blight. The WT symptoms have some similarity with physiological disorder such as iron deficiency, though typical iron deficiency is interveinal chlorosis of new growth, which is somewhat different from WT symptoms. In addition, an iron supplement had no effect. The disease did not reappear until a new outbreak in 1996, when a bacterium was isolated consistently and the inoculation experiment demonstrated that the bacterium was pathogenic on pea, causing both WT symptoms and typical bacterial blight symptoms simultaneously.

Bacterial blight of pea caused by *Pseudomonas syringae* pv. *pisi* has been one of the most important problems of pea cultivation all over the world (4,21). The first record of bacterial blight of pea in Japan was made in 1935, and the disease has been sporadic in occurrence (23,25,31). No mention of WT symptom has been made previously (4, 21).

WT symptoms on pea resemble those of apical chlorosis on marigold caused by *P. syringae* pv. *tagetis.* Our preliminary study indicated that the bacterium isolated from pea belongs to the *P. syringae* group, though it had more similarity with *P. syringae* pv. *pisi* than with *P. syringae* pv. *tagetis* in phenotypic and pathogenic characteristics. Therefore, we initiated a study to further characterize the pathogen isolated from WT symptoms and to compare it with known *P. syringae* pathovars. A preliminary report has been made (30). This study provides a full description of this newly emerged bacterium causing WT of pea.

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MATERIALS AND METHODS

Symptoms. WT disease of pea occurs in early spring and early autumn when pea plants grow vigorously (Fig. 1A). In summer and winter, symptoms are hardly observed. The entire apical shoot, including leaflets, stipules, and young pods, is chlorotic (Fig. 1B). Symptoms initially appear as a slight yellowing at the margin of an expanding leaflet while veins and tips of the leaflet remain light green (Fig. 1C). Later, the whole apical shoot becomes light yellow, then white. There is a sharp, delineated boundary between apparently healthy green and symptom-expressing white tissues. As occurs with bacterial blight of pea, WT plants may display extensive water-soaked lesions on stems and on leaflets at the basal part of the infected plant where white discoloration is not observed (Fig. 1D). Water-soaked spots also are observed on chlorotic leaflets and pods (Fig. 1E). As an infected plant continues to grow, newly developed lateral shoots turn yellow to white. On the same affected plant, mature shoots never turn white. Earliest symptoms can be observed on seedlings 2 to 3 weeks after germination (Fig. 1F). The flowers formed on chlorotic shoots have whitish calyxes, but the petals are normal in color (e.g., a red flower). Formed pods are papery and unmarketable. Severely affected plants stop growing and are stunted. During our survey from 1996 to 2000, we could not observe the plant showing typical bacterial blight symptoms without WT symptoms.

Isolation of pathogen. Bacterial isolates were extracted from affected pea tissues during April to May and late August to October in 1996 to 2000. Small pieces of the plants were excised from chlorotic tissues, the boundary part of green and white tissues without water-soaking, and water-soaked lesions. The pieces were surface sterilized in 70% ethyl alcohol for 10 s, macerated in 100 µl distilled water, and streaked onto yeast-peptone agar

(YPA; yeast extract, 5 g; peptone, 10 g; agar, 15 g; distilled water, 1,000 ml; pH 6.8) plates. The plates were incubated for 2 to 3 days at 27°C. Single colonies were subcultured on slants of potato peptone-glucose agar (PPGA; infusion from 200 g of potato; peptone, 5 g; glucose, 5 g; $Na_2HPO_4 \cdot 12H_2O$, 3 g; KH_2PO_4, 0.5 g; NaCl, 3 g; agar, 15 g; distilled water, 1,000 ml) medium. The cultured bacterial cells were suspended in 10% skim milk with 1.5% sodium glutamate and stored at –20°C. The skim milk suspensions also were lyophilized for long-term storage.

Bacteriological characterization. Thirty-four bacterial isolates were extracted from pea plat showing WT symptoms and were tested for bacteriological properties. For comparison, 16 strains of *P. syringae* pv. *pisi* and one each of strains of *P. syringae* pv. *syringae*, pv. *phaseolicola*, pv. *maculicola,* and pv. *coronafaciens* were included. Nine strains of an unidentified pathovar of *P. syringae* from water-soaked lesions on pea stems also were included for identification (Table 1). Phenotypic properties divided the sixteen strains of *P. syringae* pv. *pisi* into two groups, A (PP1, 8, 11, 13, 18, 28, 35, saya2, kuki1, and kuki5) and B (Pisum94-1, 94-2, 94-3, 94-4, MAFF311141, and 311144). Nine strains of an unidentified pathovar of *P. syringae* from pea plant formed another group, designated as *P. syringae* group C.

Colony morphology was observed on a YPA plate. Gram reaction was determined by a nonstaining method (28). Flagella were stained with 2% potassium phosphotungstate and observed with an electron microscope. Accumulation of poly-β-hydroxybutyrate (PHB) was determined by Pierce and Schroth's method (26). Fluorescent pigment production was determined on medium B of King et al. (19). An oxidation/fermentation (OF) test was carried out in the medium of Hugh and Leifson (18). In a test for indole production, bacteria were grown in peptone water and indole was detected with Kovac's reagent (13). Urease activity was determined on Christensen's urea agar plates (7). Phenylalanine deaminase activity was determined on the medium of Ewing et al. (11). Tests for hydrolysis of starch and casein digestion were performed as described in the Manual of Methods for General Bacteriology (13). For H_2S production, cultures were grown in peptone water and H_2S was tested using lead-acetate paper. Growth at 40°C was examined in peptone water. Reducing substances from sucrose and reaction in purple milk were tested by the methods described by Dye (10). Utilization of organic compounds as sole sources of carbon was determined using the modified medium of Ayers et al. (29), incorporating carbohydrates at 0.2% and the other compounds at 0.1%. A positive reaction was recorded when visible growth was observed. The

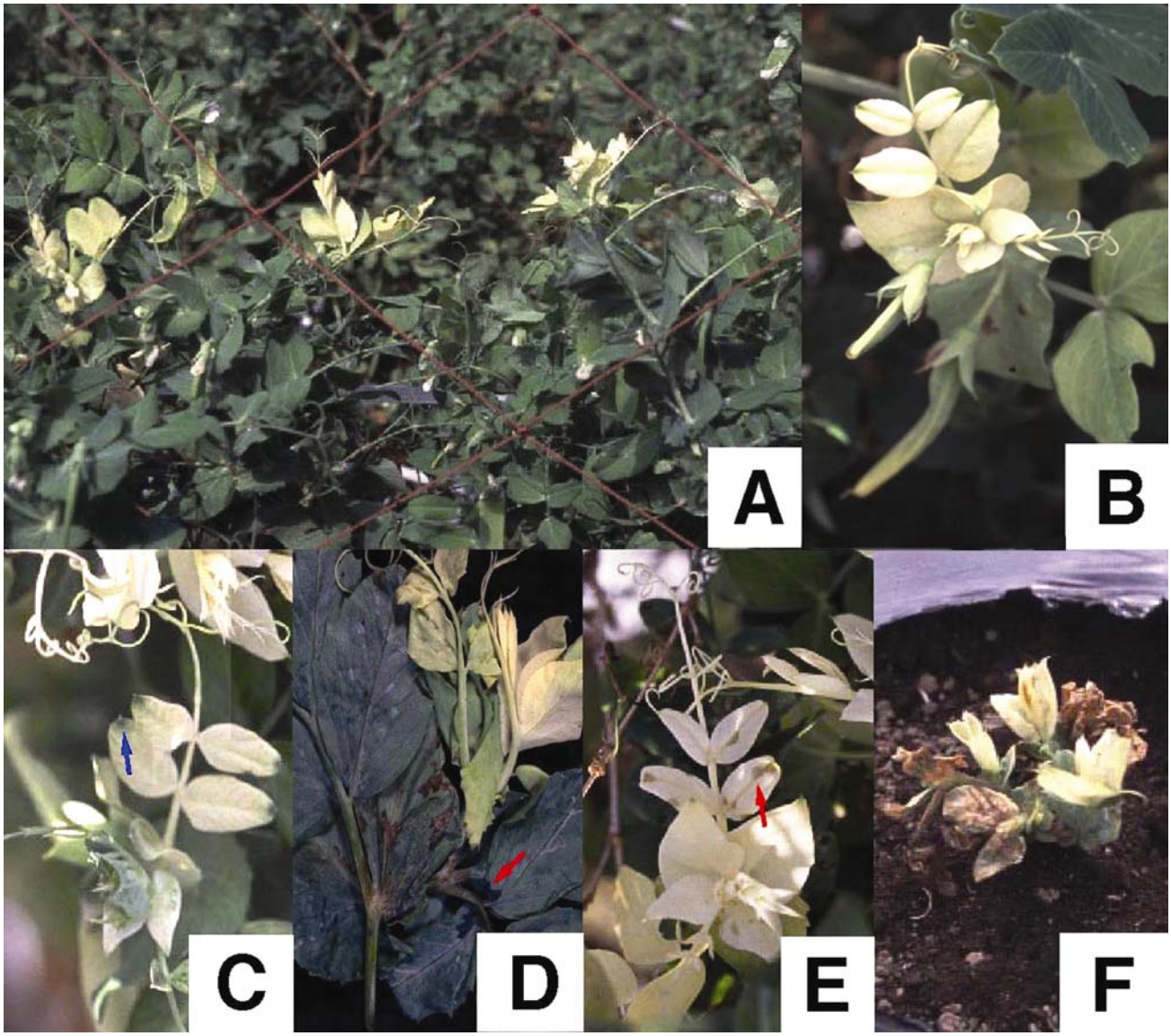

Fig. 1. Natural symptoms. **A,** Pea plants showing white top (WT) symptoms in the field. **B,** Typical WT symptoms on an apical shoot. **C,** Green veins on white leaflets (blue arrow). **D,** WT symptoms associated with water-soaked lesions (red arrow). **E,** Water-soaked spots on white leaflets (red arrow). **F,** WT symptoms on young pea seedlings.

followings tests were performed according to the methods described by Lelliott et al. (22): levan production, oxidase activity, potato soft rot, arginine dihydrolase, and tobacco hypersensitive reaction (LOPAT); nitrate reduction; tyrosinase activity; production of 2-keto-gluconate; hydrolysis of esculin, arbutin, and Tween 80; gelatin liquefaction; and lecithinase activity. Except when stated otherwise, bacterial strains were cultivated at 25°C in all tests.

Pathogenicity tests. Pathogenicity tests were performed on 2-week-old pea seedlings (cv. Izu-ichigou and Kelvedon Wonder) in the greenhouse at various seasons throughout the year. Seeds were germinated in beds and 1-week-old seedlings were transplanted into pots 8.0 cm in diameter. Culture (1 to 2 days old) on PPGA was scraped and suspended in sterile water at a concentration of approximately 1×10^8 CFU/ml and used as inoculum. For stab inoculation, stems, stipules, and leaflets were stabbed with a needle and a 10-µl droplet of bacterial suspension was placed over the wounded site. For spray inoculations, bacterial suspensions were sprayed using perfume misters with a 20-ml plastic bottle. In both inoculation experiments, sterile water was used as a control. Sprayed plants were kept humid overnight by holding them in a mist chamber and, subsequently, kept in a greenhouse.

Each strain also was inoculated to three detached pea pods placed in a 9-cm petri dish containing a filter paper sterilized by autoclaving. The pods were stabbed with a needle through bacterial suspension put on the pod surface. After inoculation, the filter paper was moistened with sterile distilled water and incubated at 27°C in a dark chamber for 7 days. The host range of the pathogen was tested by stab inoculation on the following plants: *Actinidia chinensis* (kiwi), *Arachis hypogaea* (peanut), *Brassica oleracea* var. *capitata* (cabbage), *Camellia sinensis* (tea), *Capsicum annuum* (green pepper), *Citrullus lanatus* (watermelon), *Citrus natsudaidai* (natsudaidai), *Cucumis sativus* (cucumber), *Eriobotrya japonica* (loquat), *Glycine max* (soybean), *Iris* × *germanica* (flag iris), *Lycopersicon esculentum* (tomato), *Morus bombycis* (mulberry), *Myrica rubra* (Chinese bayberry), *Nicotiana tabacum* (tobacco), *Oryza sativa* (rice), *Phaseolus vulgaris* (kidney bean), *Photinia glabra* (Japanese photinia), *Prunus mume* (Japanese apricot), *Prunus persica* (peach), *Syringa vulgaris* (lilac), *Tagetes erecta* (marigold), *Trifolium repens* (white clover), *Triticum aestivum* (wheat), *Vicia faba* (broad bean), and *Zinnia elegans* (zinnia). Annual seed plants were grown in pots in the greenhouse and were inoculated at the three- to five-true-leaf stage. Field-grown perennial trees and shrubs were inoculated on suckling shoots and leaves. Controls received the same treatment without bacterial cells. Plants were evaluated for any symptoms up to 1 month after inoculation.

16S ribosomal DNA sequence. Total bacterial genomic DNA was isolated by the miniscale cetyltrimethyl ammonium bromide (CTAB) purification method as described by Ausubel et al. (2). The nearly complete 16S ribosomal (r)DNA sequence of the WT strain PP105 was obtained by amplification of the genomic DNA with primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) (20). Sequencing reactions were performed using a SequiTherm EXCEL II-LC DNA Sequencing Kit (Epicentre Technologies, Madison, WI) as recommended by the manufacturer. Sequencing reactions were electrophoresed using a 4000L DNA sequencer (LI-COR, Lincoln, NE). A similarity search with the 16S rDNA sequence was performed against 16S rDNA sequences available in the DDBJ/EMBL/GenBank databases using the FASTA. The accession number for the nearly complete 16S rDNA sequence of PP105 is AB109218.

Repetitive sequence based-polymerase chain reaction (rep-PCR). The rep-PCR was carried out as described by Rademaker et al. (27) with some modifications. Three sets of primers were tested; these were based on the enterobacterial repetitive intergenic consensus (ERIC) sequence (ERIC 1R [5′-ATGTAAGCTCCTGGGGATTCAC] and ERIC 2 [5′-AAGTAAGTGACTGGGGTGAGCG]), and on the repetitive extragenic palindromic (REP) element (REP 1R [5′-IIIICGICGICATCIGGC] and REP 2I [5′-ICGICTTATCIGGCCTAC]), and on the 154-bp BOX element (BOX A1R [5′-CTACGGCAAGGCGACGATGACG]). We carried out the PCR reactions in the following mixture (total, 10 µl): 1× Gitschier buffer (16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8, 6.7 mM $MgCl_2$, 6.7 mM EDTA, 30 mM β-mercaptoethanol), 4 µg of bovine serum albumin, 10% dimethyl sulfoxide (vol/vol), 50 pmoles of each primer, 0.2 mM each dNTP, 1 unit of *Taq* polymerase (Gene *Taq*, Nippon Gene, Japan), and 10 ng of bacterial genomic DNA. Each reaction set included a negative control containing water instead of the DNA template. The samples were amplified through 1 cycle at 95°C for 7 min; 30 cycles at 94°C for 1 min; 52°C (ERIC), 40°C (REP), or 53°C (BOX) for 1 min; and 65°C for 8 min, followed by 65°C for 16 min in a GeneAmp PCR System 2400 (Perkin-Elmer Cetus, Norwalk, CT). PCR products were separated by electrophoresis in 1.5% agarose gel at 70 V for 40 min in 1× Tris-acetate-EDTA buffer. Gels were stained with ethidium bromide and differences in DNA fingerprint patterns were assessed visually.

Table 1. Bacterial strains used in this study

Strains	SUPP no.[a]	Year	Group	Source
White top isolates				
PP101, 102, 103, 104, 105, 106, 107, 108, 109	1658 - 1666	1996	WT	This study
Pisum97-1, 97-2, 97-3, 97-4, 97-5, 97-6, 97-8, 97-91	1700 - 1707	1997	WT	This study
Pip1, 2, 3, 4, 5, 9		1998	WT	This study
Pip13, 14, 15, 16, 17		1999	WT	This study
Pip18, 19, 20, 21, 22, 23		2000	WT	This study
Pseudomonas syringae pv. *pisi*				
PP1, 8, 11, 13, 18, 28, 35	1667 - 1673	1978	A	Ohta (25)
saya2, kuki1, kuki5	1999 - 2001	1980	A	SUPP
Pisum94-1, 94-2, 94-3, 94-4	1696 - 1699	1994	B	SUPP
MAFF311141, 311144	1945 - 1946	1994	B	MAFF[b]
Pseudomonas syringae from pea				
Pisum-1	679	1984	C	SUPP
Pea8504, 8506, 8512	1736 - 1738	1985	C	SUPP
E3, 7, 13, 14, 17	1739 - 1743	1985	C	SUPP
P. syringae pv. *syringae* (Lilac)				
LOB2-1	458			SUPP
P. syringae pv. *phaseolicola* (Kidney been)				
BQH1	1139			SUPP
P. syringae pv. *maculicola* (Turnip)				
PMC8301	2206			SUPP
P. syringae pv. *coronafaciens* (Oat)				
AVPCO8101	196			SUPP

[a] All strains were preserved in the culture collection of Shizuoka University Plant Pathology (SUPP).
[b] From MAFF (Ministry of Agriculture, Forestry and Fishery, Japan) see reference 23.

RESULTS

Bacterial isolation and characterization. When we attempted to isolate bacteria from chlorotic tissues, they gave no bacterial growth. Large numbers of bacteria consistently were isolated from water-soaked lesions or the boundary part of green and white tissues without water soaking. Among the 34 isolates from plants with WT symptoms, 8 (Pip9, 14, 15, 17, 18, 19, 20, and 21) were isolated from the boundary of white and green tissues without water-soaked lesions and 26 were from classical water-soaked lesions. Later, they were revealed to be identical in almost all properties, including pathogenicity; there-

fore, they were collectively referred to as WT isolates. They were subjected to bacteriological tests together with 16 *P. syringae* pv. *pisi* strains (10 *P. syringae* pv. *pisi* group A and 6 *P. syringae* pv. *pisi* group B), 4 strains of *P. syringae* pathovars, and 9 unidentified strains from pea (*P. syringae* group C).

The colonies of all isolates and reference strains studied were grayish white and butyrous, 1 to 2 mm in diameter on YPA after 3 days at 27°C, with an entire margin, circular and convex. They were gram-negative, aerobic, non-sporing, straight rods, and motile by means of 1 to 6 polar flagella. PHB granules were not accumulated. In LOPAT tests, all isolates and reference strains were positive for levan production and tobacco hypersensitive reaction, but negative for oxidase, arginine dihydrolase, and potato soft rot. WT isolates and the *P. syringae* pv. *pisi* group A strains did not produce fluorescent pigment on King's B medium, but *P. syringae* pv. *pisi* group B, *P. syringae* group C, and four other pathovars did. All WT isolates and *P. syringae* pv. *pisi* strains gave positive reactions in the following tests: oxidative metabolism of glucose, reducing substances from sucrose, and catalase and Tween 80 hydrolysis. In purple milk reaction, WT isolates, *P. syringae* pv. *pisi* group A, and *P. syringae* group C showed digestion and alkali production. All WT isolates and reference strains gave negative reactions in the following tests: lecithinase and phenylalanine deaminase, H_2S production, indole production, growth at 40°C, nitrate reduction, and starch hydrolysis. WT isolates and *P. syringae* pv. *pisi* groups A and B utilized glucose, galactose, fructose, mannose, sucrose, xylose, ribose, glycerol, mannitol, sorbitol, inositol, acetate, citrate, gluconate, glycerate, *meso*-tartrare, glutarate, L-malate, succinate, quinate, saccharate, caprate, peralgonate, *p*-hydroxybenzonate, γ-aminobutyrate, L-arginine, L-serine, L-leucine, triacetin, sarcosine, and trigonelline. They did not utilize D-arabinose, lactose, maltose, melibiose, melezitose, L-rhamnose, cellobiose, trehalose, adonitol, dulcitol, starch, salicin, α-methyl-D-glucoside, D-tartrate, L-tartrate, formate, n-butyrate, propionate, mesaconate, sebacate, anthranilate, linolenate, β-alanine, L-valine, L-isoleucine, and tyrosine. WT isolates and *P. syringae* pv. *pisi* groups A and B utilized homoserine. *P. syringae* pv. *pisi* group B showed negative reactions on esculin and arbutin hydrolysis, casein digestion, gelatin liquefaction, and alkali production and digestion in purple milk, and did not utilize erythritol, DL-lactate, and betain, whereas WT isolates and *P. syringae* pv. *pisi* group A showed positive reactions in these tests. *P. syringae* group C did not utilize homoserine but utilized D-tartrate. The WT isolates were homologous in these tests except for brown pigment production. In all, 21 WT isolates, PP105, 106, 107, 109, Pip1 to 5, 9, and 13 to 23, produced diffusible brown pigment on many media, including YPA and King's B medium, whereas 13 WT isolates, PP101 to 104, PP108, and Pisum97-1 through 97-91, did not. *P. syringae* group C was indistinguishable from the pv. *syringae* strain except for positive utilization of D-tartrate. WT isolates were clearly distinguished from pvs. *phaseolicola*, *maculicola,* and *coronafaciens* in the properties shown in Table 2.

Pathogenicity tests. Despite the difference of the position of isolation, all of the WT isolates behaved equally in pathogenicity tests on pea. Through stab inoculation on stems, WT isolates initially produced symptoms only around the wounds in the form of dark, water-soaked elliptical areas about 4 days after inoculation. WT isolates produced distinctive fan-like, water-soaked spots along the veins on stipules and leaflets which were similar to classical bacterial blight symptoms (Fig. 2A). As the plant inoculated with WT isolates grew, newly expanding leaves became yellow 1 week after inoculation (Fig. 2B). Apical shoots became chlorotic to whitish in the following week (Fig. 2C). Later, lateral shoots also became white (Fig. 2D). The WT plant showed remarkable stunting (Fig. 2E). *P. syringae* pv. *pisi* group A and B strains also caused typical bacterial blight symptoms but the inoculated pea plants never produced WT symptoms. When *P. syringae* group C strains were inoculated, water-soaked lesions were formed at the inoculation sites on stem and leaflet about 4 days after inoculation, and they developed into brown necrosis.

When inoculations were made in midsummer and midwinter, WT isolates and *P. syringae* pv. *pisi* groups A and B produced leaf spot and blight, and did not cause chlorosis. When pea plants inoculated with WT isolates were kept under shade, WT symptoms hardly appeared, indicating the requirement of light for the symptom development.

By spray inoculations, all WT isolates and *P. syringae* pv. *pisi* group A and B strains produced small, shiny, water-soaked spots on leaflets and stems. Within a few days, these spots developed into irregularly shaped lesions on leaflets or dark-green elliptical lesions on stems, invading stipules. Spray-inoculated plants did not develop WT symptoms if stems or stipules were not wounded.

When inoculated on mature pods, WT isolates and *P. syringae* pv. *pisi* group A and B strains produced a dark, water-soaked spot that later turned brown, sunken, and surrounded by a narrow, wa-

Table 2. Major bacteriological properties of *Pseudomonas syringae* strains isolated from pea in comparison with related pathovars

	Strains[a]							
Tests	**WT**	**Ppi (A)**	**Ppi (B)**	**Ps (C)**	**Pss**	**Psp**	**Psm**	**Psc**
Gram reaction	–[b]	–	–	–	–	–	–	–
OF test	O	O	O	O	O	O	O	O
PHB accumulation	–	–	–	–	–	–	–	–
Growth at 40°C	–	–	–	–	–	–	–	–
Fluorescent pigment	–	–	+	+	+	+	+	+
Gelatin liquefaction	+	+	–	+	+	–	–	+
Esculin hydrolysis	+	+	–	+	+	–	–	+
Purple milk reaction	KD	KD	–	KD	KD	–	–	KD
Levan	+	+	+	+	+	+	+	+
Oxidase	–	–	–	–	–	–	–	–
Potato soft rot	–	–	–	–	–	–	–	–
Arginine dihydrolase	–	–	–	–	–	–	–	–
Tobacco HR	+	+	+	+	+	+	+	+
Reducing substances from sucrose	+	+	+	+	+	+	+	+
Nitrate reduction	–	–	–	–	–	–	–	–
Utilization of								
Sucrose	+	+	+	+	+	+	+	+
Trehalose	–	–	–	–	–	–	–	–
Erythritol	+	+	–	+	+	–	–	+
Inositol	+	+	+	+	+	–	+	+
Sorbitol	+	+	+	+	+	–	+	+
D-Tartrate	–	–	–	+	–	–	+	–
L-Tartrate	–	–	–	–	–	–	–	–
DL-Lactate	+	+	–	+	+	–	+	–
β-Alanine	–	–	–	–	–	–	–	–
Valine	–	–	–	–	–	–	–	–
Betaine	+	+	–	+	+	+	+	+
Homoserine	+	+	+	–	–	–	–	–

[a] Abbreviations for strains: WT = White top isolates; Ppi (A) and (B) = *P. syringae* pv. *pisi* groups A and B; Ps (C) = *P. syringae* from pea; Pss = *P. syringae* pv. *syringae*; Psp = *P. syringae* pv. *phaseolicola*; Psm = *P. syringae* pv. *maculicola*; and Psc = *P. syringae* pv. *coronafaciens*.

[b] + = positive reaction; – = negative reaction; O = oxidative metabolism of glucose; D = digestion; and K = alkali production.

ter-soaked zone 0.4 to 0.7 cm in diameter about 1 week after inoculation. *P. syringae* group C developed dry and brown spots at the inoculation site. None of the strains developed chlorosis with pod inoculation. One WT isolate, PP105, was selected for determination of its host range. The isolate was not pathogenic on any other plants tested, except zinnia. When PP105 was inoculated at the hypocotyls of a zinnia seedling, the youngest leaf showed mild chlorotic after 7 days but recovered 4 to 5 days later without induction of any water-soaked symptoms. Necrotic lesions were induced when *P. syringae* group C strains were inoculated onto young shoots of peach.

16S rDNA sequence. The 16S rDNA sequence (1,477 bp) of WT isolate PP105 was determined. The most closely related sequence in databases by FASTA analysis was *P. syringe* pv. *syringae* (Z76669), with 99.8% identity over 1,400 bases. 16S rDNA sequences of other known *P. syringae* showed similarities of 99.1 to 99.7%.

rep-PCR. Genomic fingerprints were generated for the tested bacteria. Data from three primer sets were scored visually, taking differences of the presence or absence of major bands into account. More than 20 DNA fragments, ranging from 0.2 to 6.0 kb in size, were amplified with ERIC-, BOX-, and REP-PCR primers. Based on genomic fingerprints using ERIC primers, two distinct groups were identified for WT isolates and *P. syringae* pv. *pisi* strains. They were arbitrarily designated as PCR type I and II. Type I contained WT isolates and *P. syringae* pv. *pisi* group A strains (Table 1 and Fig. 3, lane 1 to 11). Type II consisted of *P. syringae* pv. *pisi* group B strains (Table 1 and Fig. 3, lanes 12 and 13). Type I exhibited some unique bands compared with type II, such as bands of approximately 500 and 200 bp bands in ERIC-PCR (Fig. 3), whereas type II produced bands of approximately 600 and 100 bp. WT isolates and *P. syringae* pv. *pisi* group A strains produced the same fingerprints in BOX- and REP-PCR as well as in ERIC-PCR (*data not shown*). The reference strains and *P. syringae* group C strains differed from either type I or II fingerprints in all primer sets.

DISCUSSION

Although the disease which has occurred in Japan since 1996 has similarities with

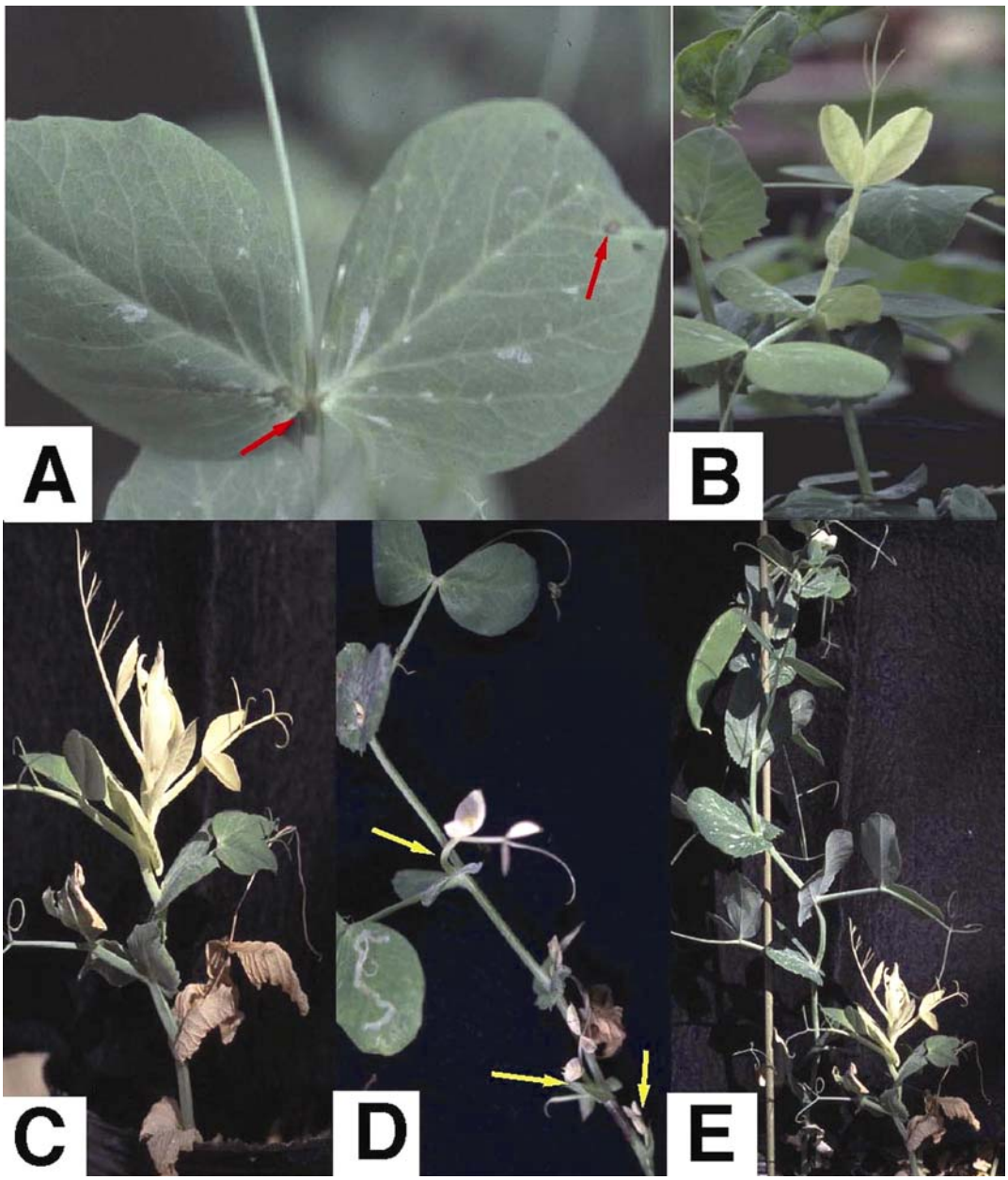

Fig. 2. Symptom expression by artificial inoculation. **A,** Water-soaked spots on leaflets (red arrows) 4 days after inoculation. **B,** Chlorotic symptoms on apical shoot 1 week after inoculation. **C,** White top (WT) symptoms on apical shoot 2 weeks after inoculation. **D,** WT symptoms on lateral shoot (yellow arrows) 2 weeks after inoculation. **E,** Stunting of inoculated plant compared with healthy one.

the symptoms of classical bacterial blight of pea, it is characterized by chlorotic and whitening of the apical shoot, including leaf, stipule, and pod. We propose that the common name of this disease be called bacterial white top (WT) of pea.

The causal agent of WT of pea is a gram-negative rod, aerobic, non-sporing, and motile, with one to six polar flagella. The pathogen does not accumulate PHB granules, and does not grow at 40°C. These results indicate the pathogen should be included in the genus *Pseudomonas*. The pathogen is characterized as levan production positive, oxidase negative, potato soft rot negative, arginine dihydrolase negative, and tobacco hypersensitive reaction positive. Therefore, the pathogen belongs to Group Ia of *P. syringae* (22). The pathogen does not utilize trehalose, β-alanine, and valine, does not reduce nitrate, utilizes sucrose, and produces reducing substances from sucrose. These characters further support the inclusion of the pathogen in *P. syringae.* Sequence analysis of the 16S rDNA of a WT isolate clearly indicates that it is a member of *P. syringae*.

Several studies have shown that *P. syringae* pv. *pisi* is heterogeneous in terms of phenotypic and genetic properties (1,14). Grondeau et al. reported that most strains produced fluorescence, did not hydrolyze esculin, and did not utilize DL-lactate, whereas aberrant strains showed opposite results in these tests. The characteristics of WT and *P. syringae* pv. *pisi* group A strains coincide with the latter, and *P. syringae* pv. *pisi* group B strains coincide with the former. All of the WT isolates and *P. syringae* pv. *pisi* groups A and B can utilize homoserine, utilization of which is one of the characteristics of pv. *pisi* (16).

By inoculation, the WT isolates first produced water-soaked lesions indistinguishable from the typical symptom of bacterial blight; and, later, they caused the typical WT symptoms after 14 days. *P. syringae* pv. *pisi* group A and B strains produced dark, water-soaked spots, and *P. syringae* group C strains developed dry and brown spots on inoculation sites. *P. syringae* pv. *pisi* groups A and B and *P. syringae* group C never developed WT symptoms. On pod inoculation, all of the strains caused dark-brown, sunken spots, but not WT symptoms. In spray inoculation, uninjured plants did not develop WT symptoms. We speculate that the WT pathogen can infect from wounded areas and can affect the growing point, but cannot cause WT in mature tissues. WT and *P. syringae* pv. *pisi* group A and B strains were not pathogenic on other plants. *P. syringae* group C strains produced necrosis on young peach twig. Our preliminary examination detected a part of syringomycin synthetase genes in the genome of *P. syringae* group C strains by Southern hybridization. Therefore, *P. syringae* group C strains were identified as *P. syringae* pv. *syringae*, the cause of brown spot disease of pea (21).

Specific genomic fingerprints generated with rep-PCR are an effective taxonomic tool that is able to identify pathovars and strains of phytopathogenic bacteria (17,27,36). The use of three rep-PCR methods (ERIC-, BOX-, and REP-PCR) enabled us to distinguish genomic variation among WT isolates and *P. syringae* pv. *pisi* strains. WT isolates and *P. syringae* pv. *pisi* group A strains gave the same fingerprints in all primer sets. *P. syringae* pv. *pisi* group B strains gave significant differences. rep-PCR also could be used to distinguish pv. *pisi* from other *P. syringae* pathovars.

All of the above-mentioned results indicated that the pathogen of WT was identical with *P. syringae* pv. *pisi* group A strains expect for symptom expression. Therefore, we concluded that the WT isolates should be included in pv. *pisi* as a distinct strain. Our results confirmed the diversity of *P. syringae* pv. *pisi*. WT strains and *P. syringae* pv. *pisi* group strains never were isolated in a single plant or a field.

WT symptoms were observed in early spring and early autumn. Increased WT incidence sometimes was observed after passage of a typhoon. Damage caused by typhoons may be involved with increased WT development, confirming the need for wounding in experiments. In addition, the pea plants were damaged by heavy frost and had warm rain 10 days before the appearance of WT symptoms in February. Development of bacterial blight is known to depend largely on weather. Infection and spread are favored by wet and cold conditions, and disease spreads during the growing season, being dispersed from plant to plant mainly by wind-driven rain (34,35). It is inferred that development of WT could occur in a similar way. Bacterial blight of pea caused by *P. syringae* pv. *pisi* is seedborne. Field observations indicate that seeds from affected plants tend to show the WT symptom at small seedling stage. It is suggested that the WT pathogen also is capable of seed transmission. Infected plant debris also may carry the pathogen. The disease may be controlled by the same methods used for bacterial blight (21).

The effect of environmental conditions such as light and temperature also was suggested. In inoculation experiments under shade conditions, faint chlorotic symptoms appeared after about 3 weeks, much later than under normal lighting conditions. In midsummer and midwinter, inoculated plants developed typical bacterial blight symptoms, but rarely become yellow or white. Some *P. syringae* pathovars produce phytotoxins which are involved in the development of symptoms such as halo and chlorosis (24). Toxin production and symptom development have been reported to depend on temperature and sunlight (6,8,9).

As mentioned above, the pathogenic bacterium could not be isolated from the chlorotic tissues but could be isolated from the lesions, which were indistinguishable from the classical blight symptoms. Therefore, we speculated that WT strains may produce toxin-like substances or some other chlorosis-inducing factors which may translocate from the water-soaked lesion to the apical shoot. *P. syringae* pv. *pisi* group A and B strains may not have such activities. A similar occurrence has been reported for *P. syringae* pv. *tabaci* and "*P. angulata*". At first, the two tobacco pathogens were given independent names based on their symptom expressions. Later, the

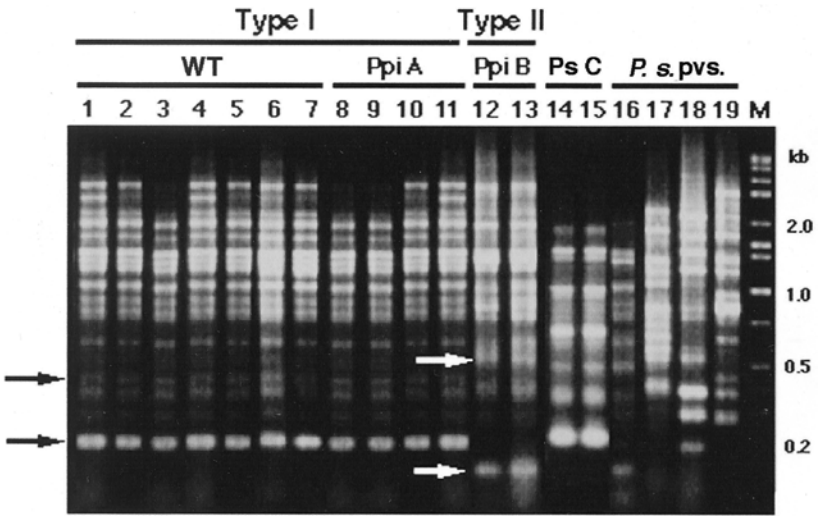

Fig. 3. Repetitive sequence based-polymerase chain reaction (rep-PCR) fingerprints obtained from *Pseudomonas syringae* pv. *pisi* (Ppi) strains and closely related pathovars with the enterobacterial repetitive intergenic consensus (ERIC) primers. Arrows indicate specific bands in each polymorphic types. M, DNA size marker; lanes 1 to 7, white top (WT) strains; lanes 8 to 11, Ppi group A strains; lanes 12 to 13, Ppi group B strains; lanes 14 to 15, *P. syringae* (Ps) group C strains; lanes 16 to 19, *P. syringae* pv. *syringae*, pv. *phaseolicola*, pv. *maculicola,* and pv. *coronafaciens*, respectively.

two pathogens were proved to be identical in bacteriological properties and host ranges. The differences in symptom expression was attributed to tabtoxin production, which is lost easily during cultivation of *P. syringae* pv. *tabaci* (33). Therefore, "*P. angulata"* was considered to be a nontoxigenic variety of *P. syringae* pv. *tabaci* (3,5). During our experiments, a spontaneous mutant of a WT isolate has been obtained by subculturing and causes watersoaked lesions without WT symptoms expression (*data not shown*). It is impossible to distinguish this mutant from *P. syringae* pv. *pisi* group A strains in any of its bacteriological properties, PCR fingerprints, or symptom expression on pea plant. We consider that it would be better not to place WT strain in a new pathovar.

Apical chlorosis of marigold incited by *P. syringae* pv. *tagetis* resembles WT in symptom expression, although pv. *tagetis* (TAG) differs from WT strains in bacteriological properties such as levan production (WT+, TAG–); gelatin liquefaction (WT+, TAG–); utilization of sucrose, DL-lactate, and erythritol (WT+, TAG–); and utilization of D-tartarate and ethanol (WT–, TAG+) and in host ranges (15,32); and belonging to a genomic group different from *P. syringae* pv. *pisi* (12). *P. syringae* pv. *tagetis* is not reported in Japan. Thus, we conclude that the WT strain is distinct from *P. syringae* pv. *tagetis*. There still remains some possibility that the WT strains and *P. syringae* pv. *tagetis* share a similar mechanism of symptom expression. Analysis of toxin production and its genetic background is underway.

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