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Comparison of ethylene-producing *Pseudomonas syringae* strains isolated from kudzu (*Pueraria lobata*) with *Pseudomonas syringae* pv. *phaseolicola* and *Pseudomonas syringae* pv. *glycinea*

B. Völksch and H. Weingart
Friedrich-Schiller-Universität Jena, Institut für Mikrobiologie, Philosophenweg 12, D-07743 Jena, Germany (Fax: +49-3641-949302)

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Abstract

The relationships among strains of *Pseudomonas syringae* pv. *glycinea* (Psg) and *Pseudomonas syringae* pv. *phaseolicola* (Psp) isolated from kudzu (*Pueraria lobata*) and bean (*Phaseolus vulgaris*) were investigated. All strains tested showed a close phenotypic similarity, with the exception of the utilization of inositol and mannitol as well as the production of toxins. On this basis the strains could be divided into three groups. Group 1 consists of all strains of pathovar *glycinea*, group 2 includes all Psp strains isolated from kudzu, and all Psp strains isolated from bean belong to group 3. This grouping was also reflected in the genetic fingerprints using the polymerase chain reaction (PCR) with primers that anneal to dispersed repetitive bacterial sequences (rep-PCR). The rep-PCR generated fingerprints were unique for each of the three groups. The strains of group 2, Psp strains isolated from kudzu, possess certain characteristics of group 1 (ethylene production) and group 2 (phaseolotoxin production). The Psp strains from kudzu can be clearly differentiated from Psp strains isolated from bean. They utilize mannitol, produce ethylene, and are strongly pathogenic to kudzu, bean, and soybean. The results obtained show that the Psp strains from kudzu should be separated from the pathovar *phaseolicola* and should represent their own pathovar.

Introduction

Pseudomonas syringae (Ps) is a species subdivided to date into 57 pathovars on the basis of distinctive pathogenicity to one or more plant hosts (Young et al., 1991; Gardan et al., 1995). It is well known that many pathovars of Ps produce toxins with unique structures that appear to function in disease development. In some cases toxins are produced by only one Ps pathovar or by a cluster of closely related pathovars. Therefore, the distribution of the toxins across species and genera can help in the taxonomic classification of bacterial strains and could be an indication of their evolutionary significance.

Strains of *Pseudomonas syringae* pv. *glycinea* (Psg), the causal agent of bacterial blight of soybean (*Glycine max* (L.) Merr.), and strains isolated from the Japanese weed *Pueraria lobata* (Willd.) Ohwi (common name: kudzu) with halo blight symptoms generate ethylene at a very high rate (Weingart and Völksch, 1997). Goto and Hyodo (1987) reported that the kudzu strains (Psp-kudzu) are identical with *Pseudomonas syringae* pv. *phaseolicola* (Psp), the halo blight pathogen of bean (*Phaseolus vulgaris* L.), in their physiological and biochemical properties. The kudzu strains are able to produce ethylene like Psg strains and phaseolotoxin like Psp strains. Therefore, we have examined various properties of the bacteria isolated from these plants to study the degree of relatedness between these *Pseudomonas syringae* pathovars.

Materials and methods

Bacterial strains and growth conditions. The bacterial strains used in this study are described in Table 1. *Pseudomonas* strains were routinely grown on bouillon glycerol agar (Völksch et al., 1984) at 28 °C, and *Escherichia coli (E. coli)* on standard-1 agar (Merck) at 37 °C. The references cited in Table 1 indicate the specific studies or laboratories in which the strains were isolated and characterized.

Detection techniques for phytotoxins. The cultivation of strains was as described previously (Völksch et al., 1984). The strains were incubated at 18 °C in 2 ml of 5b-medium (Guthke et al., 1984). The culture filtrates were checked for phytotoxins. The coronatine production of the strains was screened by their ability to induce hypertrophy on potato tubers (Völksch et al., 1989). The phaseolotoxin production was determined with the agar plate diffusion test using *E. coli* N100 as indicator strain (Völksch et al., 1984). When L-amino acids were tested for their ability to antagonize the toxin, 25 μl culture filtrate and 25 μl 0.1% amino acid were together added into wells in the agar plates.

Detection technique for ethylene. The strains were grown overnight in 5a-medium (Völksch et al., 1984) at 28 °C. 1 ml from the culture was transferred in a sterile 5 ml syringe sealed with a rubber cap and incubated on a rotary shaker at 90 rpm and room temperature for 2 h. After incubation gas samples (1 ml) were withdrawn with a gas-tight syringe, and ethylene was determined with a gas chromatograph (Shimadzu GC-14A) equipped with an activated aluminia column and a flame ionization detector. Bacterial number was turbidometrically estimated at 578 nm. Ethylene production was expressed in nl h^{-1} $cell^{-1}$.

Biochemical and physiological tests. The presence of oxidase, arginine dihydrolase, catalase and pectolytic activity, fluorescent pigment production, levan formation, acid production from glucose, reduction of nitrate, and the hypersensitivity reaction on tobacco leaves were tested as described by Lelliott et al. (1966) and Bradbury (1988). The utilization of carbon compounds was checked on the medium C of Dye (Dye, 1968) with bromocresol purple for the substrates inositol, mannitol, and D-sorbitol, and with phenol red for the substrates L-arginine, sarcosine, and betaine. The compounds were added as concentrated filter-sterilized solutions to give final concentrations of 0.5%.

Pathogenicity tests. The plants grew in individual pots in a greenhouse at 20–30 °C with supplemental light for a 14 h photoperiod. Soybean (cv. Maple Arrow), bean (cv. Red Kidney), and kudzu plants were inoculated by spraying the abaxial surfaces of leaves using a glass atomizer until water-soaking appeared. Bacterial suspension (about 10^6 cfu ml^{-1}) was applied to the first fully expanded trifoliate leaves of soybeans and beans (about 20-day-old) and to young trifoliates of kudzu. Plants were observed daily for development of symptoms. Bacterial populations in leaves were monitored by punching 5 discs (7 mm diameter) from the inoculated area. These discs were homogenized in 5 ml isotonic NaCl and serially diluted for plating onto KING's medium B (King et al., 1954).

Polymerase chain reaction (PCR)-technique. Genomic fingerprints of the Psg- and Psp- strains were determined by means of repetitive element (rep)-PCR (Louws et al., 1994). Primer sequences corresponding to Enterobacterial Repetitive Intergenic Consensus (ERIC) elements (ERIC1R: 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and ERIC2: 5′-AAGTAAGTGACTGGGGTGAGCG-3′) were used (Versalovic et al., 1991). The standard reaction mixture (50 μl) contained 67 mM Tris/HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 8 μg bovine serum albumin, 5% dimethylsulfoxide, 400 μM each of dNTPs, 1.5 units Taq DNA polymerase (Pharmacia), 100 pmol of each primer. About 10^6 bacterial cells were directly applied to the reaction mixture. Amplification included initial denaturation (95 °C, 5 min) followed by 30 cycles of denaturation (94 °C, 1 min), annealing (52 °C, 1 min), and extension (65 °C, 5 min) with a single final extension (65 °C, 15 min). PCRs were carried out with the Gene ATAQ Controller from Pharmacia. The amplification products were separated on 1.2% agarose gels, stained with ethidium bromide, and photographed under UV illumination.

Results and discussion

Phytotoxin production. Culture filtrates of the strains were tested for their ability to inhibit the growth of *E. coli* to detect phaseolotoxin production as well as to cause a visible outgrowth of potato tuber tissue to detect coronatine production. Ethylene production was determined in the late logarithmic growth phase of

Table 1. Bacterial strains used in this study

Species and strain designation	Host isolated from / location	Source or reference
Pseudomonas syringae pv. *glycinea*:		
8/83, 16/83	Soybean / Germany 1983	B. Völksch
19/84, 26/G/84	Soybean / Germany 1984	B. Völksch
27/G/B/85, 21/O/85	Soybean / Germany 1985	B. Völksch
33a/90, 43a/90	Soybean / Germany 1990	B. Völksch
1a/94, 12a/94	Soybean / Germany 1994	B. Völksch
PG4180	Soybean / New Zealand	C. Bender
R2	Soybean / USA	K. Naumann[1]
Pseudomonas syringae pv. *phaseolicola*:		
1321, Hb-1b	Bean / unknown	K. Naumann
106/1	Bean / Germany 1977	Arndt et al., 1989
6/0, 26/0, M2/1, F2	Bean / Germany 1980	Arndt et al., 1989
268/16	Bean / Germany 1981	Arndt et al., 1989
664, 666, 667, 668, 669	Kudzu / USA	K. Rudolph[2]
KZ2w, PK2	Kudzu / Japan 1981	Takikawa et al., 1988
KZ8401	Kudzu / Japan 1984	Y. Takikawa
KZ1H, KZ1S	Kudzu / Japan 1995	Y. Takikawa
Escherichia coli:		
N100		K. Rudolph

[1] Strain collection of the Bundesanstalt für Züchtungsforschung an Kulturpflanzen, Aschersleben, Germany.
[2] Strain collection of the Institut für Pflanzenpathologie und Pflanzenschutz, Göttingen (GSPB), Germany.

the strains since the production is growth associated (Weingart and Völksch, 1997).

Out of 18 tested culture filtrates of Psp strains, 6 strains isolated from bean, and 6 strains isolated from kudzu showed an inhibitory effect on the growth of *E. coli*. The inhibition zones were clear and could be reversed by L-arginine and L-citrulline, but not by L-ornithine. Strains from both host plants produced the same phytotoxin, phaseolotoxin, which inhibits ornithine carbamoyltransferase an enzyme of the ornithine cycle. It catalyzes the conversion of ornithine to citrulline (Patil et al., 1970). Culture filtrates of some Psg strains (27/G/B/85, 1a/94) also produced inhibition zones on *E. coli* lawns. However, these inhibition zones were diffuse and smaller in contrast to zones caused by phaseolotoxin. The inhibition could be compensated by L-isoleucine and L-leucine but not by L-arginine and L-citrulline. These strains presumably excreted small amounts of L-valine. L-valine individually inhibited the growth of the indicator *E. coli* by blocking the enzyme acetohydroxy acid synthase (Umbarger, 1978). Since this enzyme catalyzes a reaction not only in the L-valine biosynthesis but also in the L-isoleucine/L-leucine biosynthesis this growth inhibition could be reversed by L-isoleucine and L-leucine.

All strains of Psg examined were unable to produce phaseolotoxin. However, 8 out of the 12 Psg strains produced coronatine, and all tested Psp strains failed to produce coronatine (Table 2).

In contrast to the formation of phaseolotoxin and coronatine, all tested Psg strains and all Psp strains isolated from kudzu produced ethylene (Table 2). Fifty Psg strains from various sources were tested for ethylene production (data not shown). The Psg and the kudzu strains of Psp showed a similarly high production rate for ethylene of about 5×10^{-7} nl h^{-1} $cell^{-1}$.

Pathogenicity. The Psg strains as well as the Psp strains isolated from kudzu caused similar disease symptoms on soybean plants in contrast to the Psp strains isolated from bean which only induced very weak chlorosis similar to lesions formed by water infiltration (Figure 1). However, in bean plants Psp strains independent of their host origin produced typical disease symptoms but the Psg strains induced dif-

Table 2. Characteristics of *Pseudomonas syringae* pv. *glycinea* and *phaseolicola*

Pseudomonas syringae pathovars and strains	Toxins			Utilization of:	
	Coronatine	Phaseolotoxin	Ethylene	Inositol	Mannitol
glycinea					
8/83	+	–	+	+	+
19/84	+	–	+	+	+
26/G/84	+	–	+	+	+
27/G/B/85	+	–	+	+	+
33a/90	+	–	+	+	+
43a/90	+	–	+	+	+
12a/94	+	–	+	+	+
PG4180	+	–	+	+	+
16/83	–	–	+	+	+
21/O/85	–	–	+	+	+
1a/94	–	–	+	+	+
R2	–	–	+	+	+
phaseolicola (kudzu)					
664	–	+	+	–	+
666	–	+	+	–	+
668	–	+	+	–	+
KZ2w	–	+	+	–	+
KZ1H	–	+	+	–	+
KZ1S	–	+	+	–	+
PK2	–	–	+	–	+
KZ8401	–	–	+	–	+
667	–	–	+	–	+
669	–	–	+	–	+
phaseolicola (bean)					
1321	–	+	–	–	–
6/0	–	+	–	–	–
26/0	–	+	–	–	–
106/1	–	+	–	–	–
268/16	–	+	–	–	–
F2	–	+	–	–	–
Hb–1b	–	–	–	–	–
M2/1	–	–	–	–	–

ferent reactions (Figure 2). The coronatine producers caused water soaked lesions which turned into intensive chlorosis (typical symptoms) (Figure 2). However, after inoculation of strains unable to produce coronatine, no typical symptoms appeared. On kudzu leaves all three strains caused disease symptoms (chlorosis which enlarged and became necrotic) similar to disease lesions on their host plants (Figure 3). After inoculation of kudzu with Psp from bean or Psg, however, the symptoms developed 2–3 days later.

Psp strains from kudzu were highly virulent on kudzu as well as on soybean and bean. These strains produced chlorosis on these plants within the first three days after inoculation. Chlorosis intensified to the 5–7th day. Later the chlorotic spots changed to brown and necrotic lesions and were surrounded by intensive yellow halos.

All used strains grew very well in these plants. The bacterial population of the Psp isolated from kudzu reached the stationary phase with about $10^8 - 5 \times 10^8$ cfu cm^{-2} of leaf area in all three plants. The Psg strains independent of their coronatine production reached a bacterial population of about 10^8 cfu cm^{-2} of leaf area in their original host plant. In non-host plants the coro-

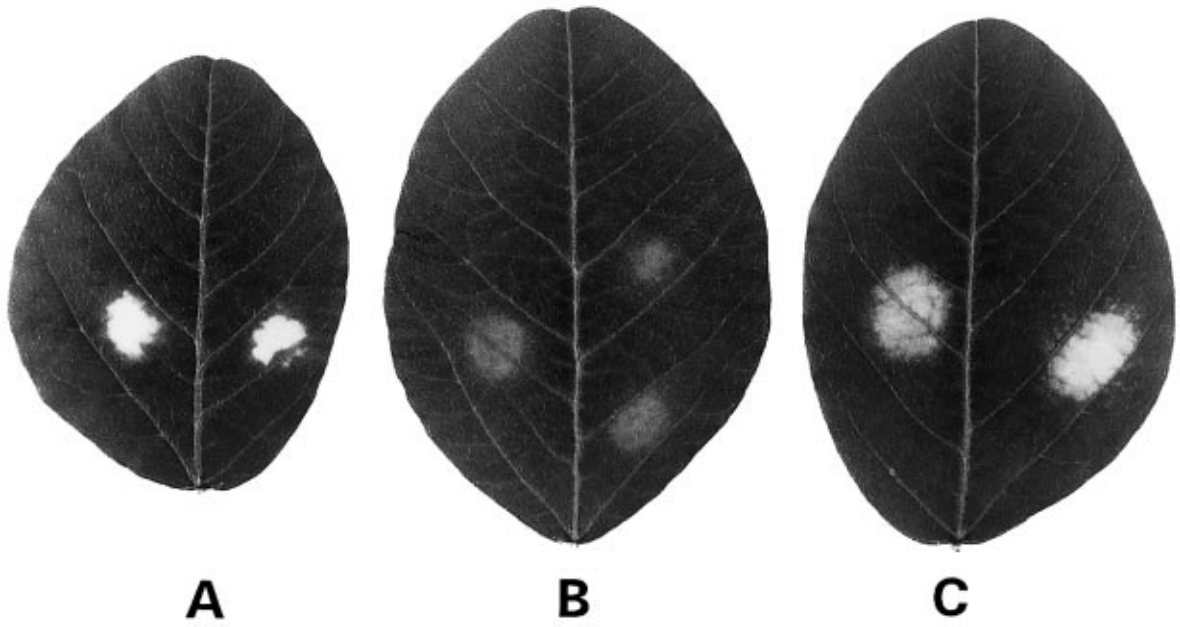

Figure 1. Comparison of symptom development in soybean leaves inoculated with *Pseudomonas syringae* pv. *phaseolicola* KZ2w (from kudzu) (A), 6/0 (from bean) (B) and *glycinea* 8/83 (C) after 7 days.

natine producing Psg population developed to about $10^7 - 5 \times 10^7$ cfu cm^{-2} of leaf area. The Psg population which did not produce coronatine was about one order of magnitude lower than the coronatine producers in bean plants (kudzu was not investigated). Psp strains from bean reached approximately the same final populations in its original host and in kudzu (about 10^8 cfu cm^{-2} of leaf area). Their development in soybeans was similar to that of Psg in non-host plants.

Genomic fingerprinting and nutritional characteristics. Primers corresponding to conserved DNA sequences of ERIC elements were used to generate genomic fingerprints of Psg, Psp isolated from bean, and Psp isolated from kudzu (Figure 4). These PCRs yielded multiple distinct DNA products, ranging in sizes from approximately 400 to 5000 bp, and three unique fingerprint patterns, one for Psg and the other two for Psp isolated from bean and Psp isolated from kudzu, respectively. These profiles were highly reproducible. All tested Psg strains as well as all tested Psp strains from bean and from kudzu, respectively, showed nearly identical PCR-generated patterns within their groups (Figure 4, lanes 1 to 5 versus lanes 6 to 12 versus lanes 13 to 17). The strains used were obtained from geographically distinct locations or isolated from the same geographic area at different times (Table 1). However, the fingerprint patterns of the two pathovars were found to be highly similar but clearly different by contrast with other pathovars (data not shown), suggesting that these pathovars are closely related.

The Psg strains, Psp strains isolated from kudzu, and Psp strains isolated from bean could be distinguished on the basis of the utilization of mannitol and inositol as carbon sources (Table 2). The utilization of sorbitol, L-arginine, sarcosine, and betaine were without diagnostic value because they showed considerable between-strain variation (betaine) or because they gave all positive (L-arginine, sarcosine) or all negative (sorbitol) results. Psg strains used inositol and mannitol. Psp strains isolated from kudzu utilized mannitol but not inositol. The Psp strains from bean could not utilize either mannitol or inositol.

Conclusions

The relationships among the two ethylene-producing pathovars were examined by their biochemical properties, toxin production, and genomic fingerprints by using rep-PCR.

Three groups of strains, not corresponding to pathovar grouping, could be defined on the basis of this characterization. All strains tested had identical phe-

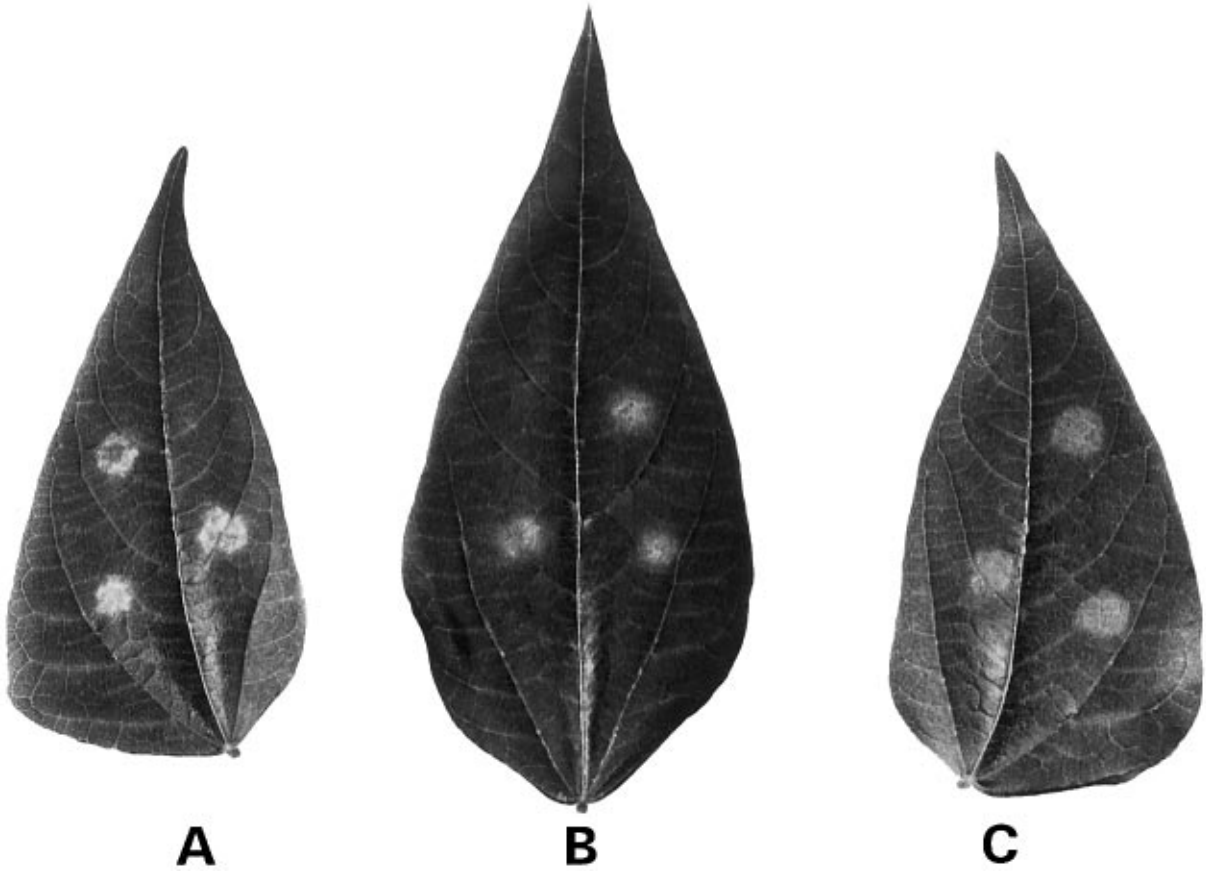

Figure 2. Comparison of symptom development in bean leaves inoculated with *Pseudomonas syringae* pv. *phaseolicola* KZ2w (from kudzu) (A), 6/0 (from bean) (B) and *glycinea* 8/83 (C) after 7 days.

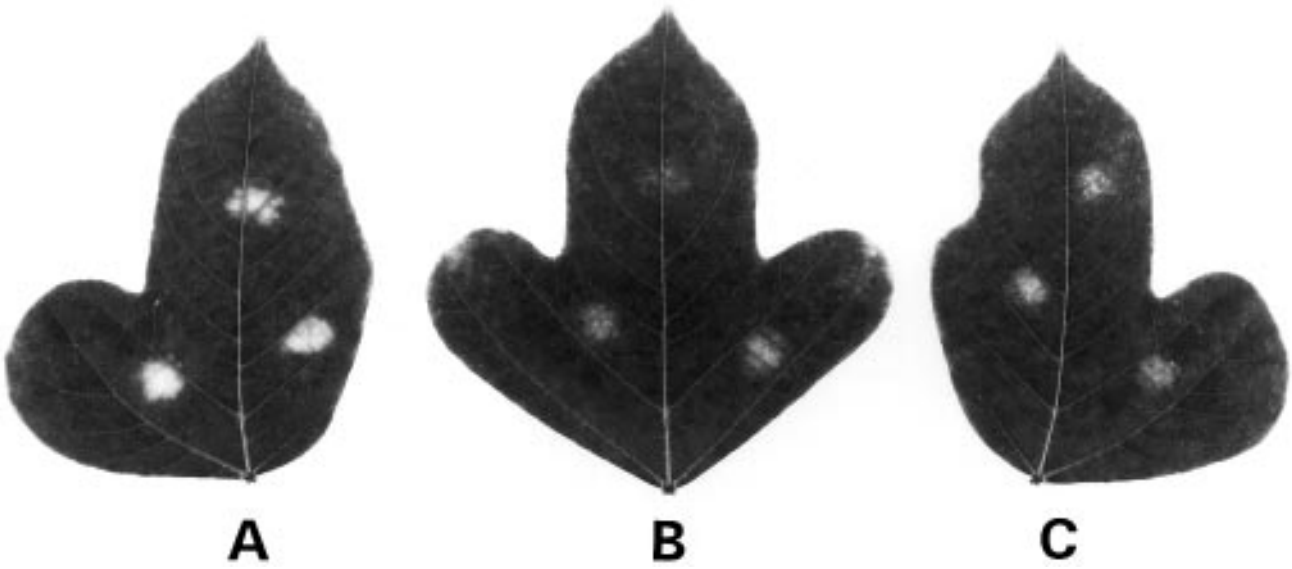

Figure 3. Comparison of symptom development in kudzu leaves inoculated with *Pseudomonas syringae* pv. *phaseolicola* KZ2w (from kudzu) (A), 6/0 (from bean) (B) and *glycinea* 8/83 (C) after 7 days.

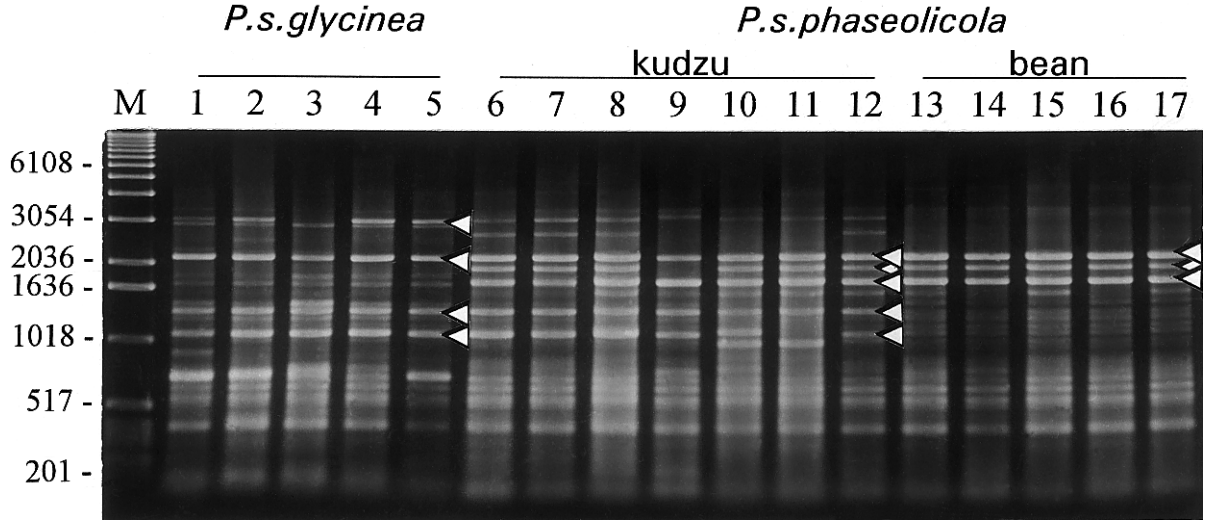

Figure 4. rep-PCR fingerprint patterns of *Pseudomonas syringae* pv. *phaseolicola* and *glycinea* strains (Lanes: 1, Psg 8/83; 2, Psg R2; 3, Psg 33a/90; 4, Psg 16/83; 5, Psg PG4180; 6, Psp 664; 7, Psp 667; 8, Psp 666; 9, Psp PK2; 10, Psp KZ1S; 11, Psp KZ1H; 12, Psp KZ2w; 13, Psp 1321; 14, Psp 6/0; 15, Psp Hb-1b; 16, Psp 106/1; 17, Psp M2/1; M, 1 kb DNA ladder).

notypic characteristics, except for their ability to utilize mannitol and inositol as carbon sources and their toxin production. Group 1, consisting of all Psg-strains, used inositol and mannitol; they produced ethylene and most of these strains produced coronatine, too. Group 2, consisting of all Psp-strains isolated from kudzu, utilized mannitol but not inositol; they could also produce ethylene like the strains of group 1, none of them could produce coronatine, but most of them were able to form phaseolotoxin. Group 3 was similar to group 2 in phaseolotoxin production, but did not form ethylene and could use neither inositol nor mannitol; this group includes all Psp-strains isolated from beans (Table 2). This grouping was also reflected in the ERIC-PCR generated patterns of representative strains of the groups. Very similar profiles were found for the strains of these groups suggesting that the strains were highly related. However, the groups could be distinguished by some typical bands accentuated by the arrowheads (Figure 4).

The phenotypic differences observed appear to correlate well with the formation of typical bands in the rep-PCR generated profiles. The strains from kudzu possess certain characteristics of the Psg-group as well as of the Psp bean-group. In addition, the kudzu-strains are highly virulent on its host-plant, kudzu, as well as on beans and soybeans with clear disease symptoms and high population levels at stationary phase. Our analysis revealed that the Psg- and Psp-strains are closely related. However, the Psp strains from kudzu differed significantly from strains isolated from bean plants in some characteristics. Therefore, it was proposed that the Psp-strains isolated from kudzu should be classified as a new pathovar of *Pseudomonas syringae*.

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