Nontoxigenic Strains of *Pseudomonas syringae* pv. *phaseolicola* Are a Main Cause of Halo Blight of Beans in Spain and Escape Current Detection Methods

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ABSTRACT

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From a collection of 152 pseudomonads isolated from diseased beans in Spain, 138 (91%) of the strains were identified as *Pseudomonas syringae* pv. *phaseolicola* and the rest as *P. syringae* pv. *syringae*. The *P. syringae* pv. *phaseolicola* strains produced typical water-soaked lesions on bean pods, although 95 of them did not produce phaseolotoxin in vitro. Ninety-four of these isolates did not produce the expected 0.5-kb product after polymerase chain reaction (PCR) amplification using primers specific for open reading frame (ORF) 6 of the phaseolotoxin (*tox*) gene cluster and did not contain DNA homologous to ORF 6 in

Southern hybridization experiments. To our knowledge, this is the first report of the widespread occurrence in the field of strains of *P. syringae* pv. *phaseolicola* lacking the *tox* cluster, which contrasts sharply with the general belief that *Tox*⁺ isolates are the only ones with epidemiological importance. Additionally, the *tox*⁻ isolates were not specifically detected by a commercial polyclonal antisera in an enzyme-linked immunosorbent assay. Accordingly, it is possible that the certification of seed lots as free of the pathogen cannot be reliably done in Spain, or in any other country where *tox*⁻ strains might occur frequently, using current PCR or serological protocols. The amplification of three avirulence genes by PCR allowed us to make predictions of the *P. syringae* pv. *phaseolicola* race structure, as confirmed by plant assays. Six races (races 1, 2, 5, 6, 7, and 9) were identified, with race 7 being the most prevalent (46.1%) followed by races 6 (21.3%) and 1 (9.0%). All the *tox*⁻ isolates contained gene *avrPphF*, typical of races 1, 5, 7, and 9.

The three major bacterial diseases of common bean (*Phaseolus vulgaris* L.) are caused by pathovars of *Pseudomonas syringae* and *Xanthomonas campestris* and result in economically important losses worldwide (31,41). In particular, *P. syringae* pv. *phaseolicola*, causing halo blight, is probably the most important bacterial pathogen of bean in Europe, the United States, and many other countries. Spain is not an exception. Some of these pathogens cause field epidemics (1,4,41). *P. syringae* pv. *phaseolicola* was first described in Spain in 1939 and it also appears to be the main cause of bacteriosis of bean, although *P. syringae* pv. *syringae* was reported to be of local importance in certain areas and bean cultivars (4,7). Although it is still considered a quarantine organism in Spain, *X. campestris* pv. *phaseoli* has been repeatedly isolated in the field (1,4; C. Asensio, unpublished data) and works are in progress to determine its importance for bean production.

Control of halo blight is difficult, and the only practical methods for its management are the use of pathogen-free seed and appropriate cultural practices and planting of resistant cultivars. The presence of extremely low levels of primary inoculum can initiate severe epidemics under favorable conditions, and in consequence, certification of seed as free of the pathogen requires the use of highly sensitive and specific methods. Detection and identification of *P. syringae* pv. *phaseolicola* has been done using different methods that include microbiological assays (15), nucleic

acid hybridization (36), and different serological methods (44,51), for which there are a number of commercially available antibodies. However, due to its effectiveness and low cost, several researchers have developed detection assays based on the amplification of specific DNA sequences by means of polymerase chain reaction (PCR) (2,28,37). Further, rapid-cycle, real-time PCR is currently being evaluated as a routine tool for the diagnosis of *P. syringae* pv. *phaseolicola* in bean seed (38). In all these cases, specific primers were designed from the available sequence of DNA coding for phaseolotoxin biosynthesis, which is organized as a large (>30 kb) gene cluster (*tox* cluster) (11,54). Phaseolotoxin is a non-host-specific toxin that induces chlorosis on leaves of several plant species by inhibition of ornithine carbamoyl transferase, a critical enzyme in the urea cycle. Toxin production is very specific to *P. syringae* pv. *phaseolicola*, although it also was described in a single bean isolate of *P. syringae* pv. *syringae* and in *P. syringae* pv. *actinidiae*, which causes a canker disease of kiwifruit (28,43,47). Nontoxigenic (*Tox*⁻) strains of *P. syringae* pv. *phaseolicola* have been described, and it is known that they are still pathogenic and occasionally occur in the field (17), although it is generally believed that *Tox*⁻ strains are of little or no epidemiological significance (30,31,37).

Based on their interaction with eight bean differential cultivars, nine races of *P. syringae* pv. *phaseolicola* have been differentiated involving five pairs of matching resistance-avirulence genes (Table 1) (45,49). Three of these avirulence (*avr*) genes (*avrPphB*, *avrPphE*, and *avrPphF*) were cloned and sequenced (16,42,48). Although *avrPphB* and *avrPphF* are present only in races expressing the corresponding phenotype, *avrPphE* is present in all the examined isolates but is only functional as an *avr* gene in races 2, 4, 5, and 7. The geographical distribution of races is not

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random, and races 3, 4, 5, 8, and 9 are not found, or only rarely, outside of Africa, whereas races 1, 2, 6, and 7 are distributed worldwide (45). Race 6, which is compatible with all the differential cultivars (Table 1), appears to be on average the predominant race worldwide (19,20,45), but race 8 is by far the most common in South Africa (5). Therefore, it is necessary to know beforehand the race structure of the pathogen in a given area, and to continuously monitor any possible deviations, in order to implement effective control measures based on the deployment of vertical resistance.

In the County of Castilla y León, the largest dry bean producing region in Spain, most of the bean crop is dedicated to high quality common bean landraces that are, in general, highly susceptible to bacterioses. Consequently, frequent disease outbreaks are a major constraint for bean production and cause the disappearance of valuable local bean cultivars (1). Ongoing breeding programs at Servicio de Investigación y Tecnología Agraria (directed by C. Asensio) aim to introduce durable resistance to these local bean genotypes. In this work, we focused on the identification and characterization of the local *Pseudomonas* populations in order to support the breeding programs and to develop appropriate molecular tools for the rapid identification of races. Our results demonstrate the prevalence in Spanish fields of nontoxigenic *P. syringae* pv. *phaseolicola*, which cannot be detected by PCR or enzyme-linked immunosorbent assay (ELISA) using the currently available methods. A preliminary report has been published (29).

MATERIALS AND METHODS

Strains and growth conditions. Reference strains of bacteria and fungi were obtained from international collections and are listed in Table 2. Unless otherwise indicated, *P. syringae* isolates were propagated on King's medium B (KMB) (18) at 28°C.

Isolation and identification of bacteria from diseased beans. Bacteria were isolated on KMB following standard methods (21) from bean leaves or pods with typical symptoms of bacterioses. From the original isolation plates, only fluorescent colonies were retained for this work. All isolates were purified by a series of single colony transfers on KMB and stored at -80°C in KMB plus 20% glycerol before their characterization.

Bacterial isolates were identified essentially as described (21, 39) following the LOPAT scheme (levan production, oxidase activity, potato soft rot, arginine dihydrolase activity, and tobacco hypersensitive response) (21), except that the degradation of potato tissue was not examined and the arginine dihydrolase test was carried out on petri dishes using anaerobic jars (3). We examined the utilization of diverse carbohydrates as the sole carbon source,

the degradation of aesculine, degradation of casein on milk-Tween (MT) medium (8), production of toxins, and pathogenic characteristics. Carbohydrates (D-mannitol, inositol, D-sorbitol, erythritol, L-tartrate, D-tartrate, and L-lactate) were added to Ayer's minimal medium (21) to a final concentration of 0.1% (wt/vol). Those isolates that did not show significant growth after 1 week of incubation at 28°C were considered negatives.

Symptomatology on pods and leaves of bean cv. Canadian Wonder, which is susceptible to all known races of *P. syringae* pv. *phaseolicola*, was examined as described (10,45). Excised pods were dipped in 70% ethanol, rinsed two times in distilled water, and inoculated with a toothpick. To confirm the pathogenicity of the isolates, the symptomatology on leaves of cv. Canadian Wonder was examined as described below for race identification. At least two replicate inoculations were made per isolate.

Toxin production. The production of antimetabolite toxins and phytotoxins was examined following previously published procedures (6,9) with slight modifications (3). For phaseolotoxin, the isolates were toothpick-inoculated on top of a lawn of *Escherichia coli* on solid Ayer's minimal medium (21) and incubated for 2 days at 22°C. To confirm the identity of the toxins, isolates also were tested on plates supplemented with 100 µl of a sterile 1% (wt/vol) solution of the amino acids L-ornithine, L-citrulline, and L-arginine. Isolates were considered to produce phaseolotoxin when the inhibition of *E. coli* growth was inverted on plates containing L-citrulline and L-arginine but not on plates with L-ornithine. The production of syringomycin was assessed by the capacity of the isolates to inhibit the growth of *Geotrichum candidum* F260 on potato dextrose agar plates (9).

Serology. Double-antibody sandwich (DAS)-ELISAs were performed with a commercial kit (Loewe Biochemica GmbH, Germany) and following the manufacturer's instructions. Bacterial suspensions were prepared on buffered physiological saline (140 mM NaCl, 2 mM NaH₂PO₄, and 15 mM Na₂HPO₄) and adjusted to optical density (OD) at 600 nm of approximately 1 before applying them to antibody-coated plates. In every plate, *P. syringae* pv. *phaseolicola* strain 1449B was used as a positive control and *P. syringae* pv. *syringae* strains B728a and B86-17 as negative controls. The results were read on a plate reader (Multiskan EX; Thermo Electron, Finland) at 405 nm. In order to make comparisons among plates, we calculated the serological reaction (SR) for each isolate as described (40), using the formula SR = [(x - y)/(z - y)] × 100, where x is the average OD for the studied isolate, z is the average OD for the positive control, and y is the average OD for the negative controls. Results were considered negative when the SR was below 50. Strains were tested in at least two separate experiments with two replicates each.

TABLE 1. Partly validated model to explain observed interactions between races of *Pseudomonas syringae* pv. *phaseolicola* and cultivars of the host, bean

		Race/avr gene ^a								
		1	2	3	4	5	6	7	8	9
		avrPphF	•	•	•	avrPphF	•	avrPphF	•	avrPphF
		•	avrPphE	•	avrPphE	avrPphE	•	avrPphE	•	•
		•	•	avrPphB	avrPphB	•	•	•	•	•
		•	•	•	•	4	•	•	•	•
Cultivar	Resistance gene	•	5	•	•	•	•	•	5	5
Canadian Wonder	•	•	•	•	•	+	+	+	+	+
A52 (ZAA54)	•	•	•	4	•	+	+	+	+	+
Tendergreen	•	•	R3	•	•	+	+	+	+	+
Red Mexican UI3	R1	•	•	4	•	-	+	-	+	-
1072	•	R2	•	•	•	+	-	+	+	+
A53 (ZAA55)	•	•	R3	4	•	+	+	+	+	+
A43 (ZAA12)	•	R2	R3	4	5	+	-	-	-	-
Guatemala 196-B	R1	•	R3	4	•	-	+	-	+	-

^a (+), susceptible response; (-), resistant response; (•), gene absent; although homologues of *avrPphE* are present in the nine races (42), it is only functional as an avirulence gene in the races indicated. Avirulence/resistance matching genes: *avrPphF/R1*; *avrPphE/R2*; and *avrPphB/R3* (adapted from literature citation 49).

Molecular genetic techniques. PCR amplifications were performed in a total volume of 25 µl by mixing 20 pmol each primer, 0.15 mM each dNTP, 1× PCR reaction buffer, 1.5 mM MgCl₂, 1 unit of *Taq* DNA polymerase (Biotag; Bioline Ltd., London), and 5 µl of a bacterial cell suspension. Specific primers for the amplification of the ethylene forming enzyme gene (*efe*) (34), the coronafacate ligase gene from the coronatine cluster (22), *avrPphB* (42), *avrPphE* (42), *avrPphF* (48), open reading frame (ORF) 6 from locus *phtE* of the phaseolotoxin biosynthesis cluster (primers P 5.1 and P 3.1) (37,54), and *virPphA* (14) were as published previously. Amplifications were carried out using a RoboCycler Gradient Cycler (Stratagene, La Jolla, CA) and consisted of a denaturation step of 10 min at 94°C followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 10 min.

DNA was isolated from single colonies grown overnight in KMB with a DNA extraction kit (Puregene; Gentra Systems Inc., Minneapolis, MN). Southern transfer was done by a standard procedure (33), and hybridization of nylon filters was carried out with a chemiluminescence labeling and detection kit (Roche Diagnostics, Basel, Switzerland). Appropriate amplicons to be used as DNA probes were purified from gels and ligated to pGemT-Easy (Promega, Madison, WI); the identity of the cloned inserts was routinely confirmed by DNA sequencing. To label the probe, the fragment was amplified from the clone, purified from a 1% agarose gel, and labeled by random priming.

Sequencing and analysis of *gyrB* and *rpoD*. Primer pairs *gyrB*-L (5'-AAGTATCCGGCGGCTTG-3') and *gyrB*-R (5'-GTG-GTCGCGACCTTGTG-3') and *rpoD*-L (5'-CGCCAAACGTATCGAAGAA-3') and *rpoD*-R (5'-GCTATTTTCAGGCCGGTTT-3') were designed from published sequences of *gyrB* (EMBL Accession No. AB016375 [35]) and *rpoD* (EMBL Accession No. AB039500 [53]), respectively, from strains of *P. syringae* pv. *phaseolicola*. Genes *gyrB* and *rpoD* were amplified as described previously from the nontoxigenic *P. syringae* pv. *phaseolicola* strains CYL233, CYL275, CYL309, CYL314, CYL325, and CYL352. Partial sequences of 534 and 572 nucleotides were determined at MWG-Biotech AG (Ebersberg, Germany) directly from the purified *gyrB* and *rpoD* PCR fragments, respectively, using the corresponding amplification primers. Sequences were combined for the same strain and treated as a single 1106 nucleotide sequence, assuming that analysis using longer sequences results in better resolution and reliability (53). Sequences were aligned with the corresponding combined sequence fragments of a range of *P. syringae* pathovars and other pseudomonads (53) using ClustalW (46). Phylogenetic trees were constructed with ClustalW using the neighbor-joining method (32). The nucleotide sequences were deposited in the nucleotide sequence database as EMBL Accession Nos. AJ564779 to AJ564784 for *gyrB* and AJ564785 to AJ564790 for *rpoD*.

Race identification of *P. syringae* pv. *phaseolicola* strains. Isolates were inoculated on primary leaves of line 1072 of *P. acutifolius* of bean cvs. Canadian Wonder, A52, Tendergreen, Red Mexican UI3, A53, A43, and Guatemala 196-B as described previously (45). Infection was scored according to a previously defined five-point scale (12). At least six replicate plants were inoculated for each combination of isolate and cultivar.

RESULTS

Isolation and identification of *P. syringae* from diseased beans. Sampling was carried out from 1993 to 2001 in commercial fields over the entire bean production zone (4,162 ha in 1998) from Castilla y León County (94,147 km²), Spain, mainly from common bean landraces. From our own experience, these cultivars are highly susceptible to bacteriosis in the field, although it is not known if they display resistance to any particular race of *P. syringae*. Bacteria were isolated from leaves and pods

showing necrotic or water-soaked lesions typical of bacteriosis, either surrounded or not by chlorotic haloes, and only fluorescent isolates were kept for further characterization. The 152 isolates examined in this work were fluorescent on KMB, levan positive, oxidase and arginine dihydrolase negative, and elicited a hypersensitive reaction on tobacco (LOPAT group Ia), and were therefore considered *P. syringae* (21).

Fourteen of the isolates (9.2%) displayed a biochemical pattern typical of *P. syringae* pv. *syringae*, produced syringomycins but not phaseolotoxin, and induced sunken brown lesions upon inoculation onto pods of bean cv. Canadian Wonder (Table 3). These isolates were in consequence identified as *P. syringae* pv. *syringae* and were not further characterized.

The remaining 138 isolates (90.8%) were considered *P. syringae* pv. *phaseolicola* because they showed typical metabolic characteristics of this pathovar and their inoculation onto pods of universally susceptible cv. Canadian Wonder resulted in typical water-soaked lesions 2 to 3 days after inoculation (Table 3). Additionally, all of them produced specific amplification bands by PCR using primers specific for *virPphA*, shown to be essential for pathogenicity on beans (13), and for *avrPphE*. These isolates did not produce syringomycins and did not produce amplification bands with primers specific for coronatine genes and for the ethylene forming enzyme (Table 3), which has been described in *P. syringae* pv. *glycinea* and in kudzu isolates of *P. syringae* pv. *phaseolicola* (52). Assimilation of mannitol is usually negative for *P. syringae* pv. *phaseolicola*, although all the isolates from kudzu tested positive, as did some isolates from other hosts, including bean (23,50). However, 72% of the Spanish isolates were able to utilize mannitol as the sole carbon source (Table 3). Noticeably, the 94 isolates that lacked the phaseolotoxin gene ORF 6 (*tox*; described below) tested positive, whereas 38 of the 44 isolates containing ORF 6 tested negative as expected.

Phaseolotoxin detection. Unexpectedly, only 43 of the *P. syringae* pv. *phaseolicola* isolates produced phaseolotoxin (Table 3), as determined by the *E. coli* inhibition bioassay. The remaining 95 isolates (68.8%) did not cause the inhibition of *E. coli* or, if they did, it was not specifically reverted by citrulline or arginine.

TABLE 2. Reference bacterial strains and fungi used in this study

Strain	Characteristics	Source or reference
<i>Escherichia coli</i> CECT831	Sensitive to phaseolotoxin	Colección Española de Cultivos Tipo, Spain
<i>Geotrichum candidum</i> F260	Sensitive to syringomycin	A. de Vicente
<i>Pseudomonas syringae</i> pv. <i>glycinea</i> 49a/90	Race 4; coronatine producer; 1990, Germany	M. Ullrich
4180	Race 4; coronatine producer; 1975, New Zealand	(24)
pv. <i>phaseolicola</i> 1281A	Race 1; 1984, UK	(45)
882	Race 2; 1975, USA	(45)
1310A	Race 3; 1984, Tanzania	(45)
1302A	Race 4; 1984, Rwanda	(45)
1375A	Race 5; 1985, Kenya	(45)
1299A	Race 6; 1984, Tanzania	(45)
1449B	Race 7; 1985, Ethiopia	(45)
2656A	Race 8; 1990, Lesotho	(45)
2709A	Race 9; 1990, Malawi	(45)
pv. <i>syringae</i> B728a	Wild type; Rif ^r ; isolated from <i>Phaseolus vulgaris</i> , USA	G. W. Sundin
B86-17	Wild type; isolated from <i>Phaseolus vulgaris</i> , USA	G. W. Sundin
pv. <i>tabaci</i> CFBP1621	Wild type	C. Manceau

Other researchers have described the natural existence of *Tox*⁻ strains of *P. syringae* pv. *phaseolicola* (24,37,50) as resulting either from point mutations or, apparently less frequently, from the absence of part or all of the *tox* cluster. However, although they are still pathogenic and occasionally occur in the field (17), it is generally believed that *Tox*⁻ strains are of little or no epidemiological significance (30,31,37). This is the basis for the widespread use of DNA sequences from the *tox* cluster as a target for the detection, by PCR or DNA hybridization, of *P. syringae* pv. *phaseolicola* in seed lots (2,28,36,37). Accordingly, we examined if the *Tox*⁻ isolates identified here could be detected by PCR using primers P 5.1 and P 3.1, which are specific for phaseolotoxin genes and were designed for a highly sensitive enrichment PCR assay, BIO-PCR, for seed sample processing (37). All the *Tox*⁺ and one of the *Tox*⁻ isolates produced the expected 0.5-kb band after PCR amplification (Table 3; Fig. 1). The remaining 94 *Tox*⁻ *P. syringae* pv. *phaseolicola* isolates, as well as isolates from pathovars *glycinea*, *syringae*, and *tabaci*, did not produce any amplification band or occasionally produced some nonspecific weak bands of higher size than expected. Southern hybridization experiments of genomic DNA using the P 5.1-P 3.1 amplicon from strain 1449B as a probe showed that these latter isolates did not contain sequences homologous to the probe (Fig. 1). This suggests that the majority of the *Tox*⁻ *P. syringae* pv. *phaseolicola* isolates native to Spain examined here might lack part of or the entire phaseolotoxin biosynthesis cluster and, in consequence, might remain undetected after PCR examination of contaminated seed lots.

Because *Tox*⁻ isolates of *P. syringae* pv. *phaseolicola* are only rarely reported (30,37,50), it is arguable that the Spanish non-toxicogenic isolates lacking *tox* DNA (*tox*⁻) might belong to a different pathovar. We therefore conducted a phylogenetic analysis by using partial sequences of the genes for DNA gyrase B subunit (*gyrB*) and σ^{70} factor (*rpoD*) from six representative *tox*⁻ isolates. Both proteins are ubiquitous in bacteria and essential for cell growth and were used previously for phylogenetic analysis of the genus *Pseudomonas* (53). A total of 534 nucleotides for *gyrB* and 572 nucleotides for *rpoD* were identical to the corresponding sequences of four strains each of *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *glycinea* (53). The neighbor-joining tree (data not shown) resulting from the analysis of the combined partial *gyrB* and *rpoD* sequences was essentially identical to the previously constructed tree (53) and clustered the six *tox*⁻ isolates together

with *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *glycinea* and well apart from other *P. syringae* pathovars.

ELISA. Because most of the Spanish isolates of *P. syringae* pv. *phaseolicola* cannot be detected by the currently available PCR protocols, we tested the specificity of other available detection techniques. Isolates were tested by DAS-ELISA using one of the several commercial antibodies available for the detection of *P. syringae* pv. *phaseolicola* and using two strains of *P. syringae* pv. *syringae* as negative controls. The polyclonal antibody used reacted as expected with all the isolates that contained the phaseolotoxin gene cluster, producing clear positive reactions, with SR mean values ranging between 82 and 113. Conversely, clear negative reactions were observed for all the *tox*⁻ isolates (mean SR values between 0.2 and 13) and for two *P. syringae* pv. *glycinea* (mean SR values of 0 and 3). Our results, therefore, indicate that the Spanish isolates lacking *tox* DNA cannot be detected using a commercial ELISA test and suggest that other commercial antibodies also might fail to detect this type of *P. syringae* pv. *phaseolicola* isolate.

Race identification. We wanted to set up a method to allow the rapid identification of races as well as to assess the diversity of races in the sampled area. The previous molecular characterization of *P. syringae* pv. *phaseolicola* isolates (7,23) appears to indicate that races are polyphyletic, which could make the identification of race-specific molecular markers problematical. We therefore decided to examine the amplification by PCR of *avr* gene bands characteristic of races (Table 1). Selected genes were *avrPphB*, which is present only in races 3 and 4 (16), *avrPphE*, producing an amplification product 104 bp larger in race 8 (42), and *avrPphF*, which is carried only by races 1, 5, 7, and 9 (48). From the 138 isolates examined, 108 isolates (78.3%) contained gene *avrPphF* and were tentatively assigned to the group of races 1, 5, 7, and 9. The remaining 30 isolates (21.7%) were tentatively included in races 2 or 6, because no isolates were found that could be assigned to races 3, 4, or 8.

The reactions of 89 randomly selected isolates on the bean differentials resulted in a race assignment (Table 4) that widely agreed with the PCR analyses: 51 isolates (57.3%) were assigned to races 1, 5, 7, and 9, while 21 isolates (23.6%) were included in races 2 and 6. In addition, we found no isolates that corresponded to races 3, 4, or 8, as was predicted from the PCR results described previously. The *avr* gene content of all these isolates, as examined by PCR, was as expected for the race in which they

TABLE 3. Characteristics and identification of 152 *Pseudomonas syringae* isolates from infected beans in Spain

Characteristics	Reference <i>P. syringae</i> pvs.				Number of field <i>P. syringae</i> isolates		
	<i>phaseolicola</i> 1449B	<i>syringae</i> B728a	<i>glycinea</i> 49a/90	<i>tabaci</i> CFBP1621	<i>pv. phaseolicola</i>	<i>pv. syringae</i>	
					44	94	14
Utilization of ^a							
D-Mannitol	—	+	+	+	+6/-38	+	+
Inositol	—	+	+	+	—	—	+
D-Sorbitol	—	+	+	+	—	—	+
Erythritol	—	+	—	—	—	—	+
L+Tartrate	—	—	—	+	—	—	—
D+Tartrate	—	—	—	+	—	—	—
L-Lactate	—	+	—	—	—	—	+
Esculin hydrolysis	—	+	+	+	—	—	+
Casein hydrolysis	—	+	—	—	—	—	+
Symptoms	ws ^b	sb ^b	HR ^b	HR	ws	ws	sb
Production of							
Phaseolotoxin	+	—	—	—	+43/-1	—	—
Syringomycins	—	+	—	—	—	—	+
Phaseolotoxin genes ^c	+	—	—	—	+	—	—
Coronatine genes ^c	—	—	+	—	—	—	—
<i>efe</i> gene ^c	—	—	+	—	—	—	—

^a Utilization of compounds as the sole carbon source.

^b Symptoms scored 4 days after stab inoculation of pods of the universally susceptible bean cv. Canadian Wonder; ws, water-soaked lesions with occasional bacterial ooze; sb, sunken brown lesions; and HR, hypersensitive reaction.

^c Examined by polymerase chain reaction with specific primers.

were classified by the plant assays (Table 1). However, 17 additional isolates, including 16 *tox*⁻ isolates that contained *avrPphF*, produced sets of reactions that were not compatible with any of the known races. The patterns of these reactions appeared to warrant their classification in at least five new putative races (data not shown).

In general, race 7 was the most abundant (46.1%) followed by races 6 (21.3%) and 1 (9.0%), while only one or two isolates each were found for races 2, 5, and 9. Noticeably, all the *tox*⁻ isolates contained gene *avrPphF* and, in consequence, the majority of the race 1 and 7 isolates belonged to this group. We did not find any obvious correlation between the race isolated and the place, date, or cultivar of isolation.

DISCUSSION

We characterized a collection of 152 fluorescent pseudomonads isolated between 1993 and 2001 in north-central Spain from lesions on field-grown common bean landraces. Similar to many other bean-growing regions of the world (31,41), *P. syringae* pv. *phaseolicola* was the most abundant bacterial pathogen found, representing 91% of the total isolates, while the remaining were *P. syringae* pv. *syringae* (Table 3). The majority (68.8%) of the *P. syringae* pv. *phaseolicola* isolates did not produce phaseolo-

toxin (Table 3), which contrasts sharply with the general belief that *Tox*⁺ isolates are the only ones with epidemiological importance (30,31,37). Additionally, all but one of the nontoxigenic isolates lacked DNA homologous to ORF 6 (Table 2), a putative fatty acid desaturase gene that is essential for the biosynthesis of phaseolotoxin (54). Identification of the *tox*⁻ as *P. syringae* pv. *phaseolicola* was unambiguously confirmed by comparison of *gyrB* and *rpoD* sequences from six representative *tox*⁻ isolates to those of other *P. syringae* pathovars and *Pseudomonas* spp. (53).

The absence of DNA homologous to ORF 6 in the *tox*⁻ strains is noteworthy, because the primers used in current PCR protocols for the detection and identification of this pathogen are based on this DNA sequence (37). In a separate study (J. A. Oguiza, A. Rico, L. Rivas, L. Sutra, A. Vivian, and J. Murillo, unpublished data), we showed that strains lacking ORF 6 also lacked DNA homologous to the genes in the known borders of the *tox* cluster, *argK* and *amtA*. This suggests that the *tox* cluster was probably acquired only by some *P. syringae* pv. *phaseolicola* strains and not by others and indicates the impracticality of using primer sets directed to other regions of the *tox* cluster for the detection or the identification of this pathogen (23,25,28). Furthermore, a commercial kit for the specific detection of *P. syringae* pv. *phaseolicola* by DAS-ELISA also failed to detect the Spanish isolates. Because the kit contains a polyclonal antibody, our results suggest the existence of significant differences between the isolates containing the *tox* cluster and those putatively lacking it and could imply that other commercial antibodies also might fail to detect the *tox*⁻ strains. In support of this, a selection of the *tox*⁻ isolates characterized here did not react with another polyclonal antisera produced against whole-cell preparations of a *Tox*⁺ *P. syringae* pv. *phaseolicola* strain (F. J. Legorburu and I. Ruiz de Galarreta, personal communication). In consequence, it is possible that the certification of seed lots as free of the pathogen cannot be reliably done in Spain, or in any other country where these kinds of strains might occur frequently, using current PCR or serological protocols.

To our knowledge, this is the first report of the widespread occurrence in the field of nontoxigenic *P. syringae* pv. *phaseolicola* strains. Although very rarely, nontoxigenic isolates are reported in the literature (37,50) and were responsible for the occurrence of some outbreaks of the so-called “halo-less” halo blight in Australia (17). However, even though one of these isolates produced no detectable toxin in the culture medium, the presence in the medium of a trace of phaseolotoxin was established unequivocally (24), indicating that these isolates putatively contained the phaseolotoxin biosynthesis cluster. It is possible that the isolates lacking *tox* DNA represent the original population of *P. syringae* pv. *phaseolicola* in Spain. In support of this, since 1987 (1) we have seen a noticeable increase in the number of isolates belonging to the primitive race 2, which was later divided into races 2, 6, and 8 (45). This increase was particularly noticeable in areas where commercial cultivars were planted, suggesting that this could have contributed to the introduction of these new races. The increase in the frequency of race 6 isolates could be due to their capacity to infect a wider range of bean cultivars or to its capacity to produce phaseolotoxin. However, the predominance of *tox*⁻ isolates is evidence against a role of phaseolotoxin in virulence.

Fig. 1. Detection of a sequence specific for the phaseolotoxin biosynthesis cluster in *Tox*⁺ and *Tox*⁻ Spanish field isolates of *Pseudomonas syringae* pv. *phaseolicola* (Pph). **A**, Electrophoretic analysis of polymerase chain reaction amplification products obtained with primer pair P 5.1-P 3.1, directed to open reading frame 6 from the *phtE* locus. C+, *P. syringae* pv. *phaseolicola* 1449B used as a *Tox*⁺ control; Pgy, *P. syringae* pv. *glycinea* 49a/90 used as a *Tox*⁻ control; and M, 1-kb marker. **B**, Southern blot hybridization of genomic DNA digested with *Eco*RI. The amplicon generated from strain 1449B with primers P 5.1-P 3.1 was cloned, purified, labeled with digoxigenin, and used as a probe. Sizes are indicated to the left in kilobases.

TABLE 4. Race identification of 89 *Pseudomonas syringae* pv. *phaseolicola* isolates by pathogenicity tests and distribution of races among isolates containing or lacking open reading frame (ORF) 6 from the phaseolotoxin gene cluster

ORF 6	Number of isolates per race										Total
	1	2	3	4	5	6	7	8	9	?	
Present	1	2	0	0	0	19	6	0	0	1	29
Absent	7	0	0	0	1	0	35	0	1	16	60
Total	8	2	0	0	1	19	41	0	1	17	89

Indeed, nontoxigenic mutants of *P. syringae* pv. *phaseolicola* are still pathogenic and their virulence is comparable to that of the wild type (26,27), except that there is no formation of chlorotic haloes; however, there is limited evidence that phaseolotoxin might contribute to systemic movement of bacteria in planta (26). Likewise, phaseolotoxin appears to contribute to the formation of chlorotic halo lesions in kiwifruit canker produced by *P. syringae* pv. *actinidiae*, but not for production of other symptoms or for the multiplication of the pathogen in planta (43). It is possible however, that the toxin provides an ecological advantage that explains its acquisition by two different pathovars. For instance, because phaseolotoxin inhibits a key metabolic pathway, it is feasible that it might act by inhibiting the growth of potential competitors. Nevertheless, it would be necessary to obtain appropriate experimental evidence to support the role, if any, of phaseolotoxin in virulence.

Race 6 appears to be predominant worldwide (19,20,45); however, more than 78% of the Spanish isolates contained *avrPphF* and only 21% belonged to race 6. An important asymmetry is that all the *tox*⁺ isolates contained *avrPphF*, whereas the majority of the *Tox*⁺ isolates lacked it. The prevalence of *avrPphF* in the Spanish population is striking and currently difficult to explain. This gene is embedded in a plasmid-borne pathogenicity island (13) and has been shown to increase the virulence of the race 7 isolate 1449B to bean cv. Tendergreen and to different cultivars of soybean (48). Additionally, *avrPphF* also restricts the host range of the bacterium by inciting a resistance reaction in bean cultivars containing the resistance gene *RI*, such as Red Mexican and Guatemala 196-B. It is then possible that *avrPphF* was acquired only by certain groups of isolates because it confers a selective advantage, such as increased virulence to the local bean genotypes or to other alternative hosts. Alternatively, *avrPphF* might have been originally inherited by the ancestor of *P. syringae* pv. *phaseolicola* and has been selectively lost only by certain isolates. This last possibility is likely taking into account the close phylogenetic relationships of strains containing or lacking *tox* DNA.

The *avr* gene content of the individual isolates, as determined by PCR using primer pairs specific for three *avr* genes, allowed us to make predictions about the race structure of the native bacterial population that agreed in general with the results obtained by plant assays. This PCR method is valuable in that it determines the existence in the bacterial population of particular *avr* genes, which are responsible for specific plant resistance phenotypes that might be of interest to plant breeders. Of the five genes postulated to explain the existence of nine races in *P. syringae* pv. *phaseolicola*, only three *avr* genes were cloned and sequenced (Table 1). Because we used primers to detect only these last three avirulence genes, the method only gives a broad idea of the groups of races present, although it might be used accurately to detect the presence of races 3, 4, and 8, whose distribution is limited. As more *avr* genes are characterized, the method may be refined to precisely identify each race. In our case, both PCR and plant assays showed a preponderance of isolates containing *avrPphF*, which is characteristic of races 1, 5, 7, and 9, and evidenced the absence of isolates belonging to races 3, 4, and 8. A source of discrepancy that lowered the predictive value of the *avr*-PCR method was the existence of 19% of isolates, most of which contained *avrPphF*, that could not be classified in any of the known races (Table 4). Other researchers have reported the existence of isolates whose race could not be established (7,20,23,45). As these results and ours indicate, it is possible that there are several new, as of yet, uncharacterized races of *P. syringae* pv. *phaseolicola*. This is further supported (5) by the inconsistencies between reactions observed after leaf and pod inoculation for certain isolates, but not for others, suggesting the existence of new *avr* genes or alleles in the *P. syringae* pv. *phaseolicola* population that could determine new races.

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