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Identification and Relatedness of Coronatine-Producing *Pseudomonas syringae* Pathovars by PCR Analysis and Sequence Determination of the Amplification Products

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Production of the chlorosis-inducing phytotoxin coronatine in the *Pseudomonas syringae* pathovars atropurpurea, glycinea, maculicola, morsprunorum, and tomato has been previously reported. DNA hybridization studies previously indicated that the coronatine biosynthetic gene cluster is highly conserved among *P. syringae* strains which produce the toxin. In the present study, two 17-bp oligonucleotide primers derived from the coronatine biosynthetic gene cluster of *P. syringae* pv. glycinea PG4180 were investigated for their ability to detect coronatine-producing *P. syringae* strains by PCR analysis. The primer set amplified diagnostic 0.65-kb PCR products from genomic DNAs of five different coronatine-producing pathovars of *P. syringae*. The 0.65-kb products were not detected when PCR experiments utilized nucleic acids of nonproducers of coronatine or those of bacteria not previously investigated for coronatine production. When the 0.65-kb PCR products were digested with *Cla*I, *Pst*I, and *Sma*I, fragments of identical size were obtained for the five different pathovars of *P. syringae*. A restriction fragment length polymorphism was detected in the amplified region of *P. syringae* pv. atropurpurea, since this pathovar lacked a conserved *Pvu*I site which was detected in the PCR products of the other four pathovars. The 0.65-kb PCR products from six strains comprising five different pathovars of *P. syringae* were cloned and sequenced. The PCR products from two different *P. syringae* pv. glycinea strains contained identical DNA sequences, and these showed relatedness to the sequence obtained for the pathovar morsprunorum. The PCR products obtained from the pathovars maculicola and tomato were the most similar to each other, which supports the hypothesis that these two pathovars are closely related. In conclusion, the region amplified by PCR was highly effective for the detection of coronatine-producing *P. syringae* strains, and the sequence analysis of PCR products proved valuable in showing relatedness between strains and pathovars.

The chlorosis-inducing phytotoxin coronatine is produced by several pathovars of the phytopathogen *Pseudomonas syringae*, including atropurpurea, glycinea, maculicola, morsprunorum, and tomato, which infect ryegrass, soybean, crucifers, *Prunus* spp., and tomato plants (6, 20, 41). In addition to chlorosis, coronatine also induces stunting and hypertrophy of plant tissue and is important in the virulence of the pathovars that produce it (6, 15, 33). Although coronatine's mode of action remains obscure, it induces a number of responses in plants which can be reproduced by ethylene or indoleacetic acid, suggesting that the toxin alters host metabolism in a manner analogous to that of plant growth hormones (14, 17, 31).

Coronatine has an unusual structure and consists of two distinct chemical components, coronafacic acid and coronamic acid, both of which function as biosynthetic intermediates to coronatine (3, 24). Coronafacic acid, which is synthesized as a branched polyketide, is coupled via amide bond formation to coronamic acid, an ethylcyclopropyl amino acid derived from isoleucine (21, 27). Tn*5* mutagenesis, substrate feeding studies, and complementation analyses were recently used to characterize the coronatine biosynthetic gene cluster in *P. syringae* pv. glycinea PG4180. By this approach, regions of the cluster involved in the biosynthesis of coronafacic and coronamic acid and in the coupling of these two intermediates were identified (3).

Several investigators have utilized DNA hybridization experiments to demonstrate the conservation of the coronatine biosynthetic gene cluster in *P. syringae* pathovars which produce the phytotoxin (7, 10, 25, 39). Further relatedness among pathovars was obtained when Tn*5*-inactivated sequences from the coronatine gene cluster in *P. syringae* pv. tomato PT23.2 were used to mutate the plasmid-borne coronatine genes in pathovars atropurpurea, glycinea, and morsprunorum (7). The strong conservation of the coronatine biosynthetic gene cluster between pathovars suggested that the amplification of conserved regions of the cluster by PCR might be used for the specific detection of coronatine-producing bacteria.

The region which encodes the coupling of coronafacic acid and coronamic acid was previously localized to a 2.3-kb region of the coronatine biosynthetic cluster (3). All mutations which inactivated coupling activity were localized within a single open reading frame which was designated *cfl* for coronafacate ligase (19a). In the present study, PCR primers derived from the coding sequence comprised by this open reading frame were evaluated for their ability to detect coronatine-producing bacteria. PCR products which were amplified from this region in five different *P. syringae* pathovars were further characterized by restriction endonuclease analysis and nucleotide se-

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TABLE 1. Bacterial strains used in the present study and PCR analysis

Organism and/or pathovar	Strain designation(s)	Group assignment[a]	PCR signal	Strain source and/or reference[b]
Agrobacterium tumefaciens	C58	III	−	D. A. Cooksey
Bacillus subtilis	BD170	III	−	H. Matzura
Erwinia amylovora	Ea1/79	III	−	13
Erwinia ananas		III	−	K. Naumann
Erwinia carotovora subsp. *atroseptica*	185	III	−	13
Erwinia carotovora subsp. *carotovora*	582	III	−	13
Erwinia chrysanthemi	533	III	−	13
Erwinia herbicola	NZ	III	−	K. Geider
Erwinia herbicola subsp. *synvitivora*	2035	III	−	13
Erwinia stewartii	DC283	III	−	D. Coplin
Escherichia coli	1100	III	−	K. Geider
Pseudomonas aeruginosa	B4	III	−	C. Bender
P. apii	1657	III	−	ICMP[c]
P. cepacia	945	III	−	C. Bender
P. cichorii	886-26	III	−	C. Bender
P. fluorescens	Pf5	III	−	C. Bender
P. syringae				
Pathovars				
Atropurpurea	1304	I	+	7
	4315, 4328, 4451, 4457	I	+	19a; ICMP
Glycinea	PG4180	I	+	7
	15a/90, 28a/90, 43a/90, 46a/90, 58a/90, S8/83, S30/85, S161/83	I	+	39
Glycinea	15a/89, 16a/90, 18a/90, 24a/90, 27a/90, 36a/90, 44a/90, 51a/90, 55a/90	II	−	39
Lachrymans	826	III	−	ICMP
Maculicola	438	I	+	26
	921, 2735	II	−	20; ICMP
Morsprunorum	567, 3712, 3714	I	+	20; ICMP
	133, 153, 634, 753	II	−	I. Roos
Papulans	Psp36	III	−	J. Norelli
Phaseolicola	0886-19	II	−	C. Bender
	6/0, 106/1, 181/4a, J80/2	II	−	B. Völksch
Pisi	1086-2	III	−	C. Bender
Syringae	PS51, PS61	II	−	3
	524, B1, C72	II	−	K. Geider
	J59, W50	II	−	B. Völksch
Syringae	FF5	III	−	C. Bender
Tomato	4325	I	+	23
	34	I	+	B. Völksch
Tomato	479	II	−	B. Völksch
	2843, 2846, 4933	II	−	23; ICMP
Tabaci	PTBR 2004	III	−	P. Shaw
P. viridiflava	5782-2	III	−	R. Stall
X. campestris				
Pathovars				
Campestris	1086-8	III	−	C. Bender
Dieffenbachiae	B400	III	−	N. Schaad
Nigromaculans	682-1	III	−	D. A. Cooksey
Phormiicola[d]	4293, 4294, 4297	II	−	22
Vesicatoria	XV10	III	−	C. Bender

[a] I, II, and III indicate coronatine producers, nonproducers of coronatine, and strains not previously examined for coronatine production, respectively.
[b] References for group I and II strains indicate studies or laboratories in which coronatine production was assessed.
[c] ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand.
[d] The three *X. campestris* pv. phormiicola strains were previously shown to synthesize coronafacoylvaline and coronafacoylisoleucine, but they lacked the ability to synthesize coronatine (22).

quencing. The implications of PCR analysis for detection of coronatine-producing bacteria are discussed.

MATERIALS AND METHODS

Primers used for amplification and PCR analysis. The complete sequence of *cfl* was previously derived by the dideoxy method (32) and will be reported elsewhere (19a). Two 17-mer oligonucleotides with the sequences 5′-GGCGCTCCCTCG CACTT-3′ (primer 1) and 5′-GGTATTGGCGGGGGTGC-3′ (primer 2) were chosen for amplification by PCR; these were located 692 and 57 bp from the 3′ end of *cfl*, respectively (19a). The standard reaction mixture (50 μl) contained 25 pmol of each primer and 10 ng of DNA. Amplification included 37 cycles as described previously (8), with an annealing temperature of 67°C. Prior to digestion with restriction enzymes the PCR products were precipitated with three volumes of ethanol and analyzed on 0.8% agarose or 6% polyacrylamide gels.

Bacterial strains. The bacterial strains utilized for PCR analysis (Table 1) included coronatine producers (group I), nonproducers of coronatine (group II), and strains not previously examined for coronatine production (group III; unknowns). Coronatine production by *P. syringae* pv. atropurpurea (all strains), glycinea PG4180, maculicola 438, morsprunorum 567, 3712, and 3714, and tomato 4325 was verified by isolating organic acids from these strains and fractionating them by gas chromatography or high-performance liquid chromatography as described previously (5, 26). Coronatine production by *P. syringae* pv. glycinea 15a/90, 28a/90, 43a/90, 46a/90, 58a/90, S8/83, S30/85, and S161/83 and *P. syringae* pv. tomato 34 was determined by screening these strains for the ability to induce hypertrophy on potato tubers by using Völksch's modification (40) of Sakai's method (30). The lack of coronatine production in selected strains (group II) was confirmed by the absence of coronatine in organic acid extracts or the absence of hypertrophy in the potato bioassay. The references cited in Table 1 for group I and group II strains indicate the specific studies or laboratories in which coronatine production was assessed. Coronatine production in the remaining strains (group III) was not previously examined.

DNA isolation. All strains were grown in 5 to 10 ml of standard I nutrient broth (Merck) or King's medium B (18). Bacteria were lysed with lysozyme and sodium dodecyl sulfate, and DNA was purified as described previously (8). After ethanol precipitation, nucleic acids were dissolved in sterile distilled water and stored at −20°C.

Cloning of PCR products. Ethanol-precipitated nucleic acids from a standard assay were dissolved in 10 μl of distilled water and stored at −20°C. PCR products were cloned into vector pGEM-T, which was cut with *Eco*RV and thymidinylated at the 3′ ends as recommended by the manufacturer (Promega Corp., Madison, Wis.). Recombinant plasmids were then transformed into competent cells of *Escherichia coli* DH5α. Plasmid DNA was isolated from selected colonies, and inserts were examined by restriction endonuclease analysis.

Sequencing of PCR products. Recombinant plasmids containing PCR products were sequenced from selected clones by using the protocol specified by U.S. Biochemicals (Cleveland, Ohio) for Sequenase 2.0. Universal and reverse primers for *lacZ′* were used to sequence the cloned products. To confirm nucleotide substitutions found in the cloned fragments of various pathovars, the amplified DNA was purified immediately after PCR by the QIA quick-spin procedure (Qiagen, Chatsworth, Calif.). Primer 1 was used to derive approximately 300 bp of sequence from the PCR products obtained from each pathovar.

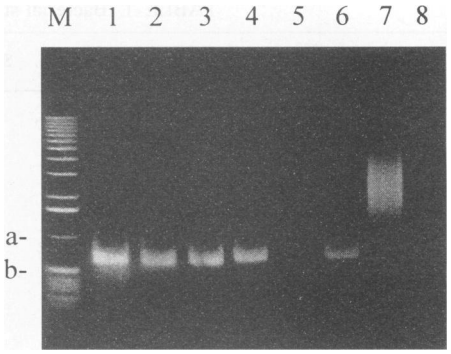

FIG. 1. PCR products amplified from various bacteria pathogenic to plants. Lane 1, *P. syringae* pv. atropurpurea 1304; lane 2, *P. syringae* pv. morsprunorum 3714; lane 3, *P. syringae* pv. maculicola 438; lane 4, *P. syringae* pv. glycinea PG4180; lane 5, *X. campestris* pv. phormiicola 4294; lane 6, *P. syringae* pv. tomato 4325; lane 7, *X. campestris* pv. phormiicola 4297; lane 8, *P. syringae* pv. syringae PS51. M, marker DNA (sizes: a, 1.0 kb; b, 0.52 kb). The samples were separated on a 0.8% agarose gel.

RESULTS AND DISCUSSION

PCR analysis of bacterial strains. PCR amplification with primers 1 and 2 resulted in the specific detection of a 0.65-kb fragment in all coronatine-producing strains (Fig. 1, lanes 1 through 4, 6; Fig. 2, lanes 1 through 5). The 0.65-kb fragment was absent when PCR was performed on DNAs isolated from nonproducers of coronatine and strains not previously investigated for coronatine production (groups II and III; Fig. 1, lanes 5, 7, and 8). Occasionally, a nonspecific zone of UV-reactive material larger than 1 kb was observed at the stringent annealing temperature and occurred regardless of the DNA source (Fig. 1, lane 7).

In the present study, all strains yielding the 0.65-kb PCR product were known to synthesize coronatine. The strong correlation between detection of nucleic acid sequences involved in coronatine production and active synthesis of the toxin has been previously demonstrated (7, 10, 39). Interestingly, strains which contain coronatine synthesis genes but do not produce the toxin have not been detected. These observations suggest that selection pressure supports the maintenance of the coronatine gene cluster and active biosynthesis of this toxin.

Ullrich et al. (39) reported the occurrence of coronatine-producing and coronatine-nonproducing strains of *P. syringae* pv. glycinea within a single soybean field in one vegetation period. Recently, Liang et al. (19) have shown that 98% of 319 *P. syringae* pv. morsprunorum strains isolated from cherry trees produced coronatine and contained DNA homologous to a coronatine-specific probe from the pathovar tomato. In syringomycin-producing *P. syringae* pv. syringae isolates, toxigenicity and pathogenicity could not be directly correlated, since some syringomycin-producing strains were found to be nonpathogenic to cherry trees (29). Coronatine was shown to significantly enhance virulence for the pathovar tomato (6), and it may be a pathogenicity factor for the pathovar atropurpurea (34). The coronatine biosynthetic cluster is generally encoded by large transmissible plasmids (90 to 110 kb) in *P. syringae* strains regardless of their geographic origin (5, 7, 34, 39). It is tempting to speculate that coronatine-nonproducing strains occur because they have not received the plasmid-encoded coronatine gene cluster. The primer set used in the

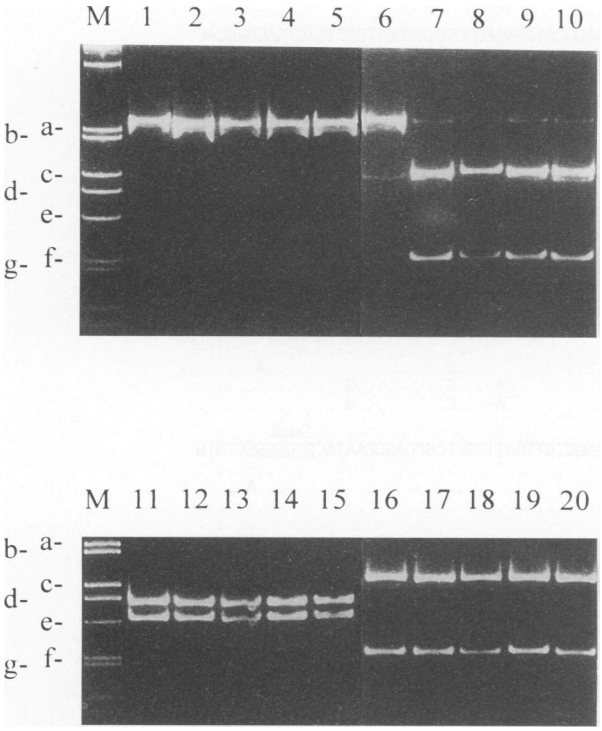

FIG. 2. Restriction enzyme analysis of PCR products. Lanes 1 to 5, uncut PCR products; lanes 6 to 10, *Pvu*I-digested PCR products; lanes 11 to 15, *Sma*I-digested PCR products; lanes 16 to 20, *Cla*I-digested PCR products. M, marker DNA (fragment sizes: a, 517 bp; b, 506 bp; c, 396 bp; d, 344 bp; e, 298 bp; f, 220 bp; g, 201 bp). Lanes 1, 6, 11, and 16, amplification product from DNA of *P. syringae* pv. atropurpurea 4457; lanes 2, 7, 12, and 17, that from DNA of *P. syringae* pv. glycinea 43a/90; lanes 3, 8, 13, and 18, that from DNA of *P. syringae* pv. tomato 4325; lanes 4, 9, 14, and 19, that from DNA of *P. syringae* pv. morsprunorum 3714; lanes 5, 10, 15, and 20, that from DNA of *P. syringae* pv. maculicola 438. The samples were separated on a 6% polyacrylamide gel.

present study could be used to monitor coronatine producers and to follow plasmid transfer and gene exchange for coronatine synthesis in populations of *P. syringae* pathovars.

Primers 1 and 2 specifically amplified a portion of *cfl*, which encodes the enzyme responsible for amide bond formation between coronafacic acid and coronamic acid. It is interesting to note that *cfl* lacks rigid specificity. Mutants blocked in coronamic acid biosynthesis were shown to excrete elevated levels of coronafacoylvaline and coronafacoylisoleucine, which contain valine and isoleucine substituted for coronamic acid (3, 45). Recently, several strains of *Xanthomonas campestris* pv. phormiicola were shown to produce coronafacoylvaline and coronafacoylisoleucine, but these strains lacked the capacity to produce coronatine (22). Substrate feeding studies indicated that *X. campestris* pv. phormiicola produces coronafacic acid and contains a functional *cfl* gene (22). However, the absence of amplified products (Fig. 1, lanes 5 and 7) indicates that the *cfl* gene in *X. campestris* pv. phormiicola has diverged considerably from the *cfl* homolog in *P. syringae*. The divergence of the biosynthetic clusters in *X. campestris* pv. phormiicola and *P. syringae* pv. glycinea has been further demonstrated in Southern hybridizations (4).

Restriction enzyme analysis of selected amplification products. To further clarify whether the 0.65-kb amplification

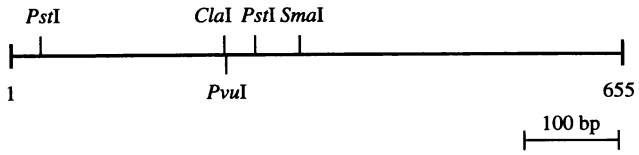

FIG. 3. Restriction map of the region amplified by PCR.

product was specifically involved in coronatine biosynthesis, we used the nucleotide sequence of *cfl* (19a) to select restriction enzymes which should digest the amplified product. On the basis of the nucleotide sequence, sites for *Cla*I, *Pst*I, *Pvu*I, and *Sma*I should be located within the amplified region (Fig. 3). These enzymes digested the amplified products from coronatine-producing strains of the pathovars glycinea, maculicola, morsprunorum, and tomato into restriction fragments of the expected sizes, i.e., 0.43 and 0.23 kb for *Pvu*I and *Cla*I (Fig. 2, lanes 7 through 10 and 17 through 20), 0.35 and 0.31 kb for *Sma*I (lanes 12 through 15), and 0.40 and 0.22 kb for *Pst*I (results not shown). A 0.03-kb *Pst*I fragment was predicted from the nucleotide sequence but was too small to be detected. Interestingly, the PCR products obtained from all coronatine-producing strains of *P. syringae* pv. atropurpurea lacked the *Pvu*I site (Fig. 2, lane 6) but contained the adjacent *Cla*I site (Fig. 2, lane 16). The strong conservation of this polymorphism among the atropurpurea strains is quite remarkable, since these strains were isolated from several different grass species in Japan, Great Britain, and Canada (34, 43). No other polymorphisms were detected in the amplified DNAs of the five coronatine-producing pathovars.

Nucleotide sequence analysis of PCR products. The complete nucleotide sequences for selected PCR products were obtained after these products were cloned into pGEM-T. The amplified products from six coronatine-producing strains, comprising five different pathovars, each had a length of 655 bp (Fig. 4). The nucleotide exchanges observed in the first 300 bp of the amplified products were confirmed by direct sequencing of the purified PCR products. The nucleotide sequence of the PCR-amplified product obtained from *P. syringae* pv. glycinea PG4180 is shown as a reference in Fig. 4. The sequence of the PCR product obtained from PG4180 was essentially identical to the nucleotide sequence of *cfl* in this region (19a).

The nucleotide sequence of the cloned PCR product derived from *P. syringae* pv. glycinea 43a/90 was identical to that obtained for PG4180 (Fig. 4). The identity of the sequences from PG4180 and 43a/90, which were isolated from diseased soybeans in New Zealand and Germany, respectively, is remarkable considering the geographical isolation of these two strains. However, cloned PCR products from pathovar morsprunorum strain 3714, pathovar atropurpurea strain 4457, pathovar tomato strain 4325, and pathovar maculicola strain 438 contained 3, 8, 17, and 21 substitutions, respectively, at various positions throughout the PG4180 sequence (Fig. 4). The *Pvu*I polymorphism of pathovar atropurpurea strain 4457 is due to a single nucleotide exchange at position 234, in the enzyme recognition site at positions 230 to 235. The adjacent *Cla*I site from position 228 to 233 was not affected (Fig. 4). Sixteen of the substituted nucleotides in the PCR product derived from pathovar tomato strain 4325 were identical to substituted nucleotides in pv. maculicola 438 (Fig. 4). Recently there has been considerable debate regarding the classification of the pathovars tomato and maculicola. Since these two pathovars have similar biochemical characteristics and partially overlapping host ranges, some researchers have argued for

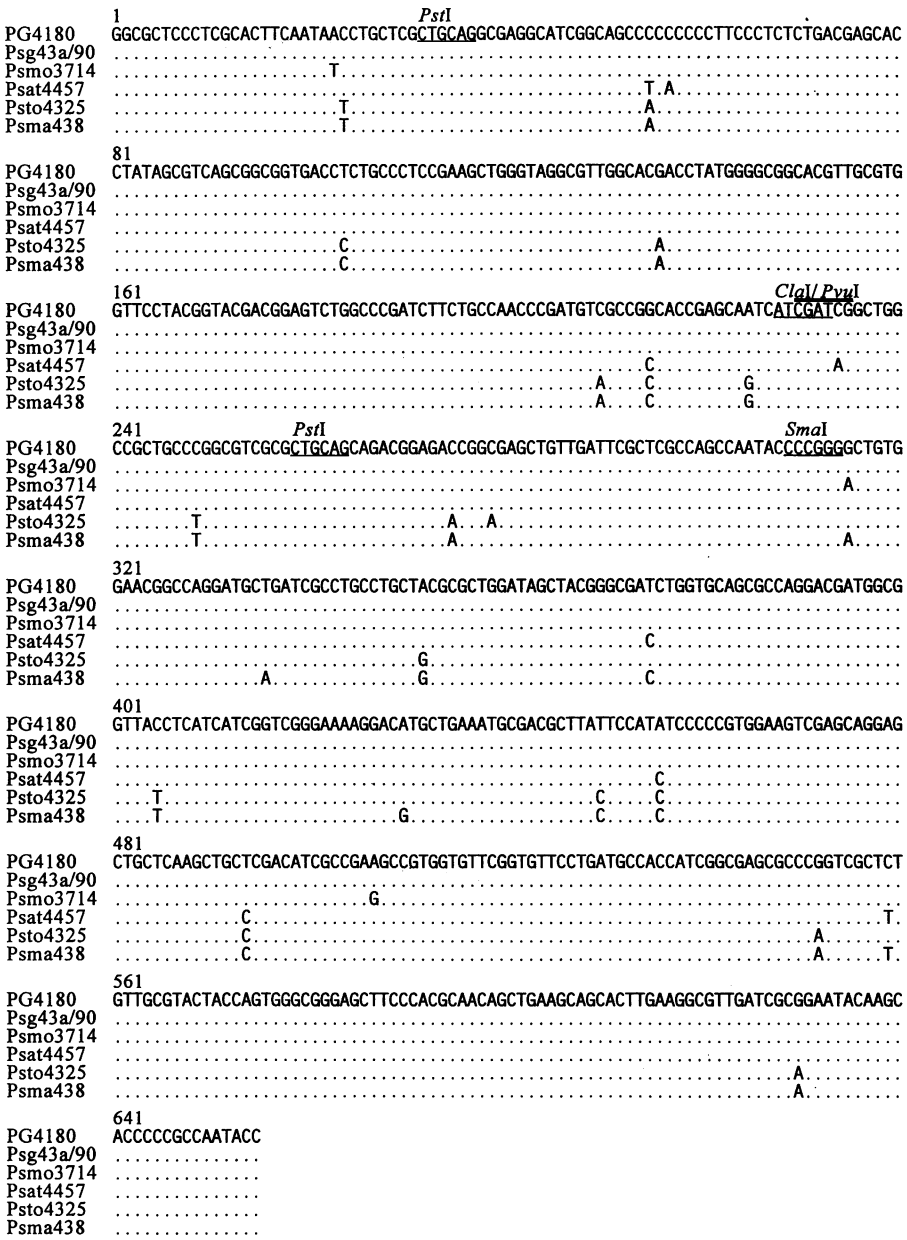

FIG. 4. Nucleotide sequences of PCR products cloned from *P. syringae* pv. glycinea (Psg) PG4180 and 43a/90, *P. syringae* pv. morsprunorum (Psmo) 3714, *P. syringae* pv. atropurpurea (Psat) 4457, *P. syringae* pv. tomato (Psto) 4325, and *P. syringae* pv. maculicola (Psma) 438. The recognition sites of enzymes *Cla*I, *Pst*I, and *Sma*I are underlined, and the *Pvu*I site is marked by a heavy line.

their classification as one pathovar (38). However, the pathovar tomato is not pathogenic on crucifers, and this has resulted in the continued classification of maculicola and tomato as distinct pathovars (41). Hendson et al. have provided some interesting insights into the evolutionary relationships of these two pathovars. Their data indicate that some strains of the pathovars tomato and maculicola are very closely related and may be diverging from common ancestral groups (16). The similarities in the sequence data for pathovar tomato strain 4325 and pathovar maculicola 438 suggest a high degree of relatedness between the two pathovars.

The phylogenetic relatedness of the five coronatine-producing pathovars was judged by their nucleotide sequence homologies in the amplified 0.65-kb fragments (Table 2). Accordingly, the pathovars glycinea and morsprunorum seem to be closely related, as are the pathovars tomato and maculicola. Both pairs of pathovars are distant in relatedness to each other, whereas the pathovar atropurpurea was located in an intermediate position to the other four pathovars. Many of the nucleotide substitutions observed in these sequences were silent on the amino acid level (Fig. 5). Other changes converted nonpolar amino acids into polar amino acids. Apparently, the divergences observed in the amino acid sequence do not interfere with the *cfl* function.

PCR analysis is widely used for identification of bacteria, viruses, and DNA fragments in eukaryotes (1, 2, 9, 11, 12, 37,

TABLE 2. Percent differences in the nucleotide sequences of the DNA fragments amplified from various pathovars[a]

Pathovar	% Difference from nucleotide sequence of DNA from pathovar:			
	Morsprunorum	Atropurpurea	Tomato	Maculicola
Glycinea	0.5	1.2	2.6	3.2
Morsprunorum		1.7	3.1	3.4
Atropurpurea			2.7	2.7
Tomato				0.9

[a] The strains used for calculation are the same as those listed in the legend to Fig. 4.

42). We previously used PCR assays to specifically detect the fire blight pathogen *Erwinia amylovora* in extracts of tissue obtained from infected plants (8). PCR amplification of DNA has many potential applications in phytopathology and was recently used to assist in the classification of mycoplasma-like organisms which cause plant disease (36). Since the pathovar classification is based on the ability of phytopathogenic strains to cause distinct disease symptoms in plants (44), PCR analyses and other nucleic acid detection methods have special merit when susceptible host plants are unavailable or difficult to infect.

DNA hybridizations and PCR analyses which utilize sequences involved in phytotoxin biosynthesis have been used to successfully detect *P. syringae* pv. phaseolicola and syringae. A DNA probe and PCR primers derived from a region involved in phaseolotoxin production were used to detect *P. syringae* pv. phaseolicola in bean seed (28, 35). Furthermore, the *syrB* and *syrD* genes, which function in syringomycin biosynthesis and export, respectively, were shown to specifically hybridize to *P. syringae* pv. syringae strains which produced syringomycin and related metabolites. Neither probe showed homology to other pathovars of *P. syringae*, suggesting that *syrB* or *syrD* might be used to specifically detect *P. syringae* pv. syringae (29).

A DNA hybridization probe derived from the coronatine gene cluster was previously used to detect *P. syringae* pv. tomato in tomato leaf and fruit lesions. All *P. syringae* pv. tomato strains isolated hybridized with the probe (10). However, strains of *P. syringae* pv. glycinea, maculicola, morsprunorum, and tomato which do not synthesize coronatine have been identified (19a, 23, 39). Consequently, DNA probes and primer sets derived from the coronatine biosynthetic gene cluster will not effectively detect coronatine-nonproducing strains of these pathovars. Although the primer set of the present study can be specifically utilized for the detection of coronatine-producing *P. syringae* strains, some caution is needed in the interpretation of results in which no amplification is obtained.

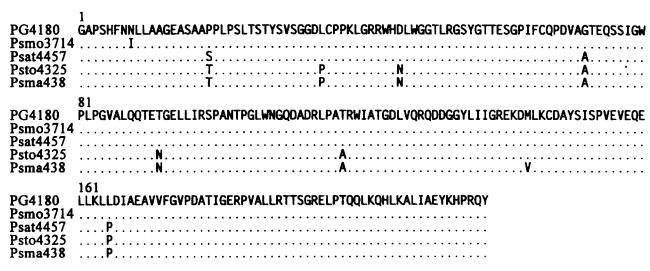

FIG. 5. Amino acid alignment deduced from the nucleotide sequences (Fig. 4 and legend) of the amplified fragments.

ACKNOWLEDGMENTS

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