

PCR Detection of Cyclic Lipodepsinonapeptide-Producing *Pseudomonas syringae* pv. *syringae* and Similarity of Strains†

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Many strains of *Pseudomonas syringae* pv. *syringae* produce one of four classes of small cyclic lipodepsinonapeptides: syringomycins, syringostatins, syringotoxins, or pseudomycins. These metabolites are phytotoxic and growth inhibitory against a broad spectrum of fungi. Their production is dependent upon the expression of conserved biosynthesis and export genes *syrB* and *syrD*, respectively. PCR and oligonucleotide primers specific for a 752-bp fragment of *syrB* were used to identify cyclic lipodepsinonapeptide-producing strains of *P. syringae* pv. *syringae*. In contrast, PCR amplification with primers based on *syrD* did not always correlate with possession of the *syrD* gene, as indicated by Southern blot analysis, or with cyclic lipodepsinonapeptide production. Sequence comparisons of 400 nucleotides from the *syrB* PCR-amplified fragments showed 94% plot similarity among 27 strains. In a sequence phenogram, syringostatin and syringotoxin producers were grouped apart from syringomycin-producing strain B301D, with sequences that differed by eight and nine conserved base substitutions, respectively. PCR amplification of the 752-bp *syrB* fragment offers rapid and accurate detection of cyclic lipodepsinonapeptide-producing strains, and its sequence provides some predictive capabilities for identifying syringotoxin and syringostatin producers.

Many strains of *Pseudomonas syringae* pv. *syringae* are known to produce cyclic lipodepsipeptides as secondary metabolites. These metabolites consist of small forms (approximately 1,200 Da) (34) and large forms (approximately 2,500 Da) (3), with the latter collectively known as syringopeptins. The small forms are cyclic lipodepsinonapeptides (CLPs) that possess hydroxylated acyl chains and the conserved tetrapeptide sequence dehydroaminobutanoic acid-hydroxyaspartic acid-chlorothreonine-serine. They include the syringomycins (14, 31), syringostatins (13, 23), syringotoxins (5), and pseudomycins (6). Conceivably, other CLP forms remain undiscovered. A strain of *Pseudomonas fuscovaginae* was recently reported to produce syringotoxin (12), showing that CLPs are not produced exclusively by *P. syringae* pv. *syringae*.

The CLPs are considered to be plant virulence factors and antifungal agents (16). They affect plant membrane activities (11, 34) and induce necroses at relatively high concentrations (22), but the relationship of these effects to plant diseases has not been clearly established. Certain *P. syringae* pv. *syringae* mutants that do not produce syringomycin in vitro still cause varying degrees of disease symptoms (21, 26, 30, 35). On the other hand, a few *P. syringae* pv. *syringae* isolates that are nonpathogenic on pear and cherry fruit produce antifungal agents presumed to be syringomycin (17, 27), and wheat isolate *P. syringae* M1 produces syringomycin E but does not cause disease symptoms on wheat (1). Two *P. syringae* strains that produce syringomycin E (10) are employed as biocontrol agents against fungal diseases on postharvest citrus (9, 10). Purified CLPs have potent antifungal activities against a broad spectrum of fungi including human pathogens (20, 33).

D. C. Gross and colleagues have shown that the CLP production genes, *syrB* and *syrD*, are conserved among *P. syringae*

pv. *syringae* strains (27, 28, 36). These genes are predicted to encode proteins that function in CLP synthesis and export, respectively. In view of the variabilities encountered in CLP production in vitro (27) and the difficulties in their chemical identification, the *syrB* and *syrD* genes offer a better means for identifying CLP producers. In addition, sequence differences in these genes offer the potential to identify strains that produce specific classes of CLPs, as suggested by restriction fragment length polymorphism (RFLP) analyses of Quigley and Gross (27).

In the present study, the earlier work of D. C. Gross and colleagues is extended by employing PCR protocols and oligonucleotide primers derived from the coding sequences of the *syrB* and *syrD* genes to quickly and reliably detect CLP-producing bacteria. In addition, PCR-amplified products from the *syrB* genes in 27 *P. syringae* strains were analyzed to determine relationships between base sequence and class of CLP produced.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. Strains B301D, 475, SD19, 761-5, Ps268, B458, W4N5, W4N7, W4N47, and W4N103 were obtained from D. Gross (Washington State University, Pullman). Strains Lilac and SY12 were obtained from S. Thompson (Utah State University) and A. Isogai (Nara Institute of Science and Technology), respectively. Strains ESC-10 and ESC-11 were obtained from C. Bull (USDA Agricultural Research Service, Fresno, Calif.). *P. syringae* pv. *tabaci* and *P. syringae* pv. *pisi* were obtained from A. Anderson (Utah State University). Other strains were isolated locally (Table 1).

DNA isolation. All strains were grown in potato dextrose broth (Difco Laboratories, Detroit, Mich.). Genomic DNA was isolated as described previously (2). After ethanol precipitation, nucleic acids were dissolved in sterile distilled water and stored at -20°C .

Southern blot analysis. Detection of the *syrB* and *syrD* genes was performed by Southern blot analysis unless the presence of the genes was previously confirmed. Purified genomic DNAs were digested with the *EcoRI* restriction enzyme, electrophoresed on 0.8% agarose gels, and transferred to Hybond-N+ membranes (Amersham, Arlington Heights, Ill.). Plasmids p91 (pUC18 containing a 1-kb *EcoRI-PstI* fragment of *syrB*) and p9 (pUC19 containing a 510-bp *SalI* fragment of *syrD*) were obtained from D. Gross (Washington State University). The *syrB* and *syrD* fragments were purified by gel electrophoresis and hybridized with the

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TABLE 1. Strains used and their properties related to *syrB* and *syrD* and to CLP produced

Strain	Plant source	<i>syrB</i> and <i>syrD</i> sequences ^a (reference)	PCR signal for:		CLP produced, if known (reference)
			<i>syrB</i>	<i>syrD</i>	
<i>P. syringae</i> pv. <i>syringae</i> and related <i>P. syringae</i> strains					
B301D	Pear	Yes (27)	+	+	Syringomycin (4, 31)
475	Maize	Yes (27)	+	+	
SD19	Sorghum	Yes (27)	+	+	
761-5	Foxtail	Yes (27)	+	+	
Ps268	Lemon	Yes (27)	+	+	Syringotoxin (18)
SY12	Lilac	Yes (27)	+	+	Syringostatin (24)
B458	Orange	Yes	+	+	Syringotoxin (5)
W4N5	Apple	Yes (27)	+	+	
W4N7	Cherry	Yes (27)	+	+	
W4N47	Cherry	No (27)	-	- ^c	
W4N103	Cherry	Yes (27)	+	+	
Lilac	Lilac	Yes	+	+	Syringostatin (25)
M1	Wheat	Yes (1)	+	+	Syringomycin (1)
M2	Wheat	Yes	+	+	Syringomycin (32)
M3	Wheat	Yes	+	+	
M4	Wheat	Yes	+	+	
M5	Wheat	Yes	+	+	
M6	Wheat	Yes	+	+	
M7	Wheat	Yes	+	+	
C1	Cherry	No	-	-	None
C2	Cherry	No	-	-	None
C3	Cherry	Yes	+	+	C3A (25)
FP13	Wheat	Yes	+	+	
FP14	Wheat	Yes	+	+	
FP17	Wheat	No	-	+	
FP18	Wheat	Yes	+	+	
FP20	Wheat	Yes	+	+	
FP21	Wheat	Yes	+	+	
FP22	Wheat	Yes	+	+	
ESC-10	Apple	Yes (10)	+	+	Syringomycin (10)
ESC-11	Pear	Yes (10)	+	+	Syringomycin (10)
pv. <i>lisi</i>	Unknown	No	-	- ^c	None
pv. <i>tabaci</i>	Unknown	No	-	- ^c	None
Other fluorescent pseudomonads from diseased plants					
SH-1	Cherry	ND ^b	-	+	None
95-39	Cherry	ND	-	-	None
95-54	Lilac	ND	-	+	None
KS1	Maize	No	-	-	None
KS2	Maize	No	-	-	None

^a As determined by Southern blotting.

^b ND, the test was not done.

^c A 446-bp *syrD* fragment was amplified at an annealing temperature of 60°C.

enhanced chemiluminescence direct nucleic acid labeling and detection system (Amersham).

Oligonucleotide primers. The complete sequences of the *syrB* (36) and *syrD* (28) genes from *P. syringae* pv. *syringae* B301D have been derived. Two 21-mer oligonucleotides for each sequence were selected for amplification by PCR with Oligo primer analysis software (National Biosciences, Inc., Plymouth, Minn.). For *syrB*, the oligonucleotide sequences were 5'-CTTCCGTGGTCTTGATGAGG-3' (primer B1) and 5'-TCGATTTGCCGTGATGAGTC-3' (primer B2). These primers were located 787 and 1,539 bp, respectively, into the open reading frame of the *syrB* gene and yielded a 752-bp product (34). For *syrD*, the oligonucleotide sequences were 5'-AAACCAAGCAAGAGAAGAAGG-3' (primer D1) and 5'-GGCAATACCGAACAGGAACAC-3' (primer D2). These primers were located 466 and 912 bp, respectively, into the open reading frame of the *syrD* gene and yielded a 446-bp product (28).

PCR amplification. PCR amplification was performed in a total volume of 100 μ l. Each reaction mixture contained 1 \times PCR reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), a 0.5 μ M concentration of each primer, a 200 μ M concentration of each dNTP, 1.5 mM MgCl₂, 0.025 U of *Taq* DNA polymerase per μ l, 100 to 200 ng of genomic DNA, and 30 μ l of mineral oil to prevent evaporation. The PCR was run for 35 cycles. Each cycle consisted of template denaturation at 94°C for 1.5 min, primer annealing at 60°C for 1.5 min, and DNA extension for 3.0 min at 72°C. After the cycling period was finished, an additional extension of 10 min at 72°C was included. Small aliquots (5 μ l) of the PCR products were analyzed on 1% agarose gels. The remaining amplified DNAs were precipitated with three volumes of ethanol before digestion with the *SalI* restriction enzyme. The digested DNA fragments were also analyzed on agarose gels. Many samples were duplicated by using 10 μ l of whole-cell suspensions in place of purified DNAs and/or increasing the annealing temperature to 62°C.

Sequencing of PCR products and analysis. After amplification, the 752-bp *syrB* fragments from each strain were purified with a Wizard PCR Preps DNA purification system (Promega, Madison, Wis.). The sequence of each fragment was determined with primer B1 at the Utah State University Biotechnology Center (Logan). Sequences of 400 bp from 27 *P. syringae* strains were analyzed with the Wisconsin Sequence Analysis programs of the Genetics Computer Group (Madison, Wis.). The sequences were aligned with the Genetics Computer Group program PILEUP. Pairwise distances between the sequences were determined with the DISTANCES program by the Jukes-Cantor method. A phenogram was drawn based upon these distances with GROWTREE by the unweighted pair group method using arithmetic averages. Areas of similarity and divergence were determined by PLOTSIMILARITY. The sequence analysis started 810-bp into the open reading frame of *syrB*.

RESULTS

PCR analysis of bacterial strains. PCR amplification with the *syrB* primers yielded 752-bp fragments from all strains that were confirmed to have the *syrB* gene by Southern blot analyses (Table 1 and Fig. 1, upper panel). The 752-bp fragment was found to be absent when PCR was performed on DNAs isolated from strains that were confirmed *syrB* negative by

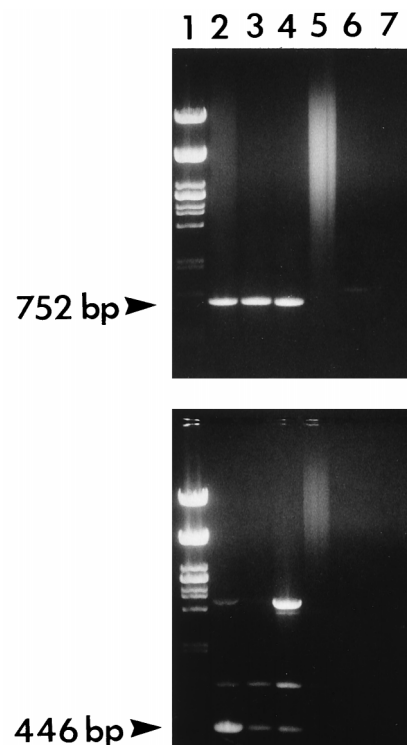


FIG. 1. PCR amplification of the 752-bp fragment of *syrB* (upper panel) and the 446-bp fragment of *syrD* (lower panel). The primer annealing temperature was 62°C. Lane 1, λ -*PstI*-digested DNA; lane 2, *P. syringae* pv. *syringae* B301D; lane 3, related *P. syringae* W4N7; lane 4, *P. syringae* pv. *syringae* SY12; lane 5, W4N47; lane 6, *P. syringae* pv. *lisi*; lane 7, *P. syringae* pv. *tabaci*. Products were separated on 1.0% agarose gels.

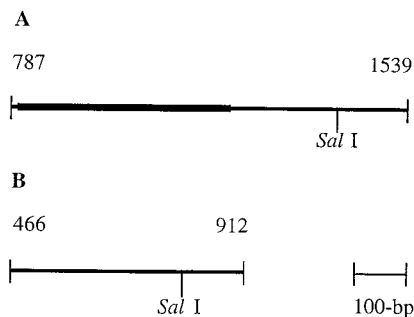


FIG. 2. Restriction maps of the *syrB* (A) and *syrD* (B) regions of *P. syringae* pv. *syringae* B301D amplified by PCR. The boldface line indicates the 400-bp region of *syrB* that was sequenced.

Southern blot analyses. Occasionally, nonspecific fragments of various sizes would be amplified. However, no consistent pattern was seen for the occurrence of these fragments, as they were found in strains both positive and negative for *syrB*, and most were eliminated by increasing the annealing temperature to 62°C.

As expected, the *syrD* primers amplified a 446-bp fragment (Fig. 1, lower panel). This amplification was seen with all strains that were confirmed to have *syrD* by Southern blot analyses, but it was not limited to these strains (Table 1). The *syrD*-negative strains for which the 446-bp fragment was amplified were *P. syringae* pv. *tabaci*, *P. syringae* pv. *pisii*, and *P. syringae* W4N47. This 446-bp fragment was not amplified in these strains when the annealing temperature was increased to 62°C (Fig. 1, lower panel). Another fragment of approximately 800 bp was often amplified.

The same results were obtained with whole-cell suspensions instead of purified genomic DNAs.

Restriction enzyme analysis of amplified products. The *SalI* restriction enzyme site found in the B301D product (Fig. 2A) was not always present in the *syrB*-amplified products from other strains. *SalI* digestion resulted in three distinguishable RFLPs: (i) one 752-bp fragment (no *SalI* site), (ii) two fragments of 621 and 131 bp whose sizes were equivalent to those predicted by the B301D restriction map (Fig. 2A), and (iii) two fragments whose sizes were estimated to be 410 and 342 bp.

The *SalI* restriction site in the *syrD*-amplified fragment of B301D (Fig. 2B) was conserved in all strains. The analysis yielded two fragments from all strains; the fragments were 321 and 125 bp in size.

Nucleotide sequence analysis. For the 27 strains with positive PCR signals for *syrB*, the sequence plot similarity between the 400-nucleotide fragments was 94%. Most of the differences were base substitutions. Percent differences in the sequences ranged from 12.5% between B301D and W4N5 to 0% among three different groups. Strains that were grouped together with identical sequences were Lilac and SY12 (syringostatin producers); M1, M2, M4, and M7; and FP13, FP14, FP18, and FP20 (Fig. 3). Strains within the latter two groups were isolated from the same plant hosts in similar locations, but strains Lilac and SY12 were isolated separately from lilac hosts in Kaysville, Utah, and Japan, respectively. The sequences of syringotoxin-producing strains Ps268 and B458 differed by 1.5%. Nucleotide analysis grouped the 27 strains into three major branches (Fig. 3). One branch contained the known syringostatin (Lilac and SY12)- and syringotoxin (Ps268 and B458)-producing strains. Compared to the sequence of syringomycin-producing strain B301D, the sequences of the syringostatin producers differed by 9.9% and those of the syringotoxin producers, Ps268 and

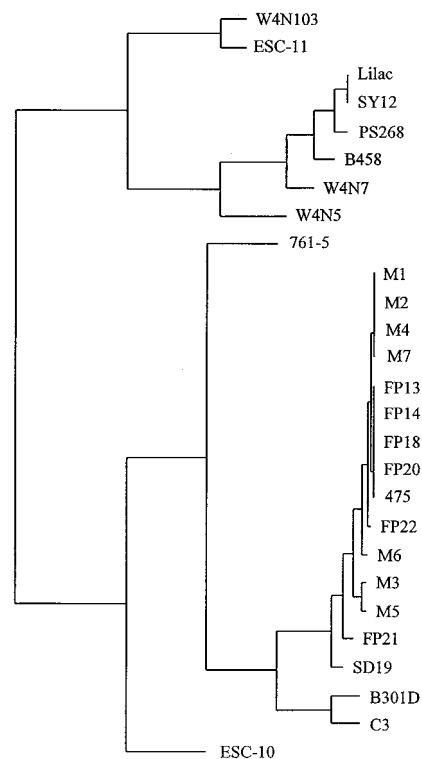


FIG. 3. Phenogram of sequences of the 400-bp fragments of *syrB* from 27 different strains. The lengths of horizontal lines represent relative numbers of nucleotide substitutions assumed under the Jukes-Cantor model.

B458, differed by 9 and 10.2%, respectively. Nine and eight base substitutions were unique to the syringostatin- and syringotoxin-producing strains, respectively, compared to B301D (Table 2). Other strains that were grouped with the syringostatin- and syringotoxin-producing strains were W4N7 and W4N5.

Strains that have been identified as producing syringomycin were found throughout the other two branches. Strain C3, which produces a new CLP, C3A, was placed near syringomycin-producing B301D. The percent sequence difference between these strains was 2.8%.

TABLE 2. Comparison of *syrB* fragment base substitutions unique to syringostatin and syringotoxin producers with corresponding bases of syringomycin-producing strain B301D

Open reading frame nucleotide position	Base of:				
	B301D	Syringostatin producer		Syringotoxin producer	
		Lilac	SY12	PS268	B458
887	C	G	G	G	G
936	C	T	T	T	T
987	G	A	A	A	A
1002	A	G	G	G	G
1011	G	T	T	T	T
1065	G	C	C	C	C
1083	T	C	C	C	C
1123	G	A	A	G	G
1129	C	T	T	T	T

DISCUSSION

A PCR method was developed and evaluated for its ability to identify *P. syringae* pv. *syringae* strains that produce CLPs. It is based on the known conservation of the peptide biosynthesis gene, *syrB*, in this group of organisms (27) and was shown to specifically detect strains that produce syringomycin, syringostatin, and syringotoxin. A similar PCR-based approach was developed and used to identify analogous coronatine-producing strains of *P. syringae* (8). In addition to their specificity and reliability, these methods offer the advantages of speed, sensitivity, and simplicity compared to approaches based on DNA hybridization. Thus, PCR amplification of the 752-bp *syrB* fragment documented in this report could be exploited for applications such as the detection and quantitation of CLP-producing bacteria in host plants. Examples of such applications are the use of PCR-based assays to detect *Erwinia amylovora* in fruit tree tissues for diagnosis of fire blight disease (7) and the detection and localization of phenazine and 2,4-diacetylphloroglucinol antibiotic-producing fluorescent pseudomonads in soils (29).

The sequences of the PCR-amplified *syrB* fragments from 27 *syrB*-positive strains were compared. As observed previously by Quigley and Gross (27) using genomic DNA hybridization analyses, *syrB* is polymorphic. This sequence polymorphism is likely related to variations in the CLPs produced by the different *P. syringae* strains. *SyrB* is postulated to be a peptide synthetase based on its shared homology with adenylate-forming enzymes that catalyze peptide antibiotic and siderophore biosyntheses (36). Specifically, six signature core sequences together resemble those of amino acid-activating domains of thiotemplate-employing peptide synthetases. One specific region (residues 310 to 333) falls within the PCR-amplified sequences analyzed in the present study and was suggested to be responsible for binding and activating serine for syringomycin biosynthesis (36). However, recent studies with enzymatically active preparations show that *SyrB* activates threonine (which is subsequently chlorinated) and not serine (15, 19). Analogous regions occur in peptide synthetases encoded by *grsB* and *HTS-1* of *Bacillus brevis* and *Cochliobolus carbonum*, respectively, which activate proline, and *EntF* of *Escherichia coli*, which activates serine (36). Within this region of *syrB*, we observed two different base substitutions when comparing syringomycin-producing strain B301D with syringostatin- and syringotoxin-producing strains (Table 2). Syringostatin and syringotoxin differ from each other by a single amino acid (at corresponding positions, syringostatin has diaminobutanoic acid and syringotoxin has glycine) and differ significantly from syringomycin in hydroxyamino acid content (34). Syringostatin and syringotoxin, but not syringomycin, possess homoserine and threonine, and they contain one serine residue instead of syringomycin's two. All possess chlorothreonine flanked by hydroxyaspartic acid (on the N-terminal side) and serine. If the *syrB* sequence variations observed in the present study do not contribute to these hydroxyamino acid differences, they may instead reflect evolutionary divergence of the conserved activation domain for chlorothreonine. A close genetic relatedness between syringostatin and syringotoxin producers was also noted by Quigley and Gross (27) based on genomic RFLP analyses.

Partial sequence analysis of the 752-bp *syrB* fragments offers some limited capability for predicting the kind of CLP produced. As mentioned above, four separately isolated syringostatin and syringotoxin producers showed high levels of sequence identity that led to close groupings in a sequence phenogram (Fig. 3). Two other *P. syringae* pv. *syringae* strains,

W4N5 and W4N7, were also grouped closely with the syringostatin and syringotoxin producers. Although the CLPs from these two strains have not been chemically identified, the probability is high that they each synthesize either syringostatin or syringotoxin. In contrast, syringomycin-producing strains occur throughout the phenogram, thus precluding the use of this fragment sequence to predict producers of this CLP. A similar lack of sequence relatedness in syringomycin producers was observed by Quigley and Gross (27), who found that syringomycin-producing strains B301D and SC-1 occupied different branches of maximum-parsimony trees based on RFLP analyses with *syrB* and *syrD* probes.

Recently isolated *P. syringae* C3 produces a novel CLP with a unique amino acid composition (25) and, accordingly, is not grouped closely with producers of CLPs of known structure. In regard to plant source, *P. syringae* strains isolated from cereals formed a tight cluster in the sequence phenogram (Fig. 3). These included strains with the M and FP prefixes isolated from wheat and strains 475 and SD19 from maize and sorghum, respectively. However, the foxtail isolate, strain 761-5, differed significantly from the other monocot strains. A similar relationship among monocots was observed by Quigley and Gross (27) using RFLP maximum-parsimony analyses.

The *syrD* oligonucleotide primers were effective in detecting the presence of the *syrD* gene, but they were not always specific to known CLP-producing strains at an annealing temperature of 60°C. At the higher annealing temperature, 62°C, the *syrD* primers were more specific and may be useful for detecting new CLP-producing strains. The *syrD* gene is expected to code for an ATP-binding transporter (28), and the lack of specificity of the observed 446-bp fragment may reflect the ubiquity of this or a similar transporter. Also, the observation of additional amplified products derived from the *syrD* primers (Fig. 1) suggests the occurrence of other related transporter genes in CLP producers.

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