Syringomycin Production Among Strains of *Pseudomonas syringae* pv. *syringae:* Conservation of the *syrB* and *syrD* Genes and Activation of Phytotoxin Production by Plant Signal Molecules

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The *syrB* and *syrD* genes of *Pseudomonas syringae* pv. *syringae* are predicted to encode proteins that function in the synthesis and export of syringomycin, respectively. Using portions of the *syr* genes as DNA probes, both genes were shown to be conserved as single copies within a 15-kb or smaller DNA region among a broad spectrum of *P. s.* pv. *syringae* strains that produce syringomycin or one of its amino acid analogs, syringotoxin and syringostatin. Strains representative of *P. viridiflava* and six pathovars of *P. syringae* failed to hybridize with the gene probes, demonstrating that *syr* sequences are highly specific to *P. s.* pv. *syringae* and related nonpathogenic strains. Maximum parsimony analysis of restriction fragment length polymorphism profiles was used to evaluate relatedness among strains within the *syrB* and *syrD* gene region. A tree, conveying the smallest number of evolutionary changes among strains, revealed considerable diversity within the *syr* gene region; subclusters of strains were identified that appear to share specific qualities relevant to the plant-pathogen interaction. Because both the *syrB* gene and syringomycin production can be induced in response to plant signal molecules, 42 strains containing homologous *syr* sequences were tested for signal-mediated induction of toxin production. Over 90% of the toxigenic strains produced larger quantities of toxin when the plant signal molecules, arbutin and D-fructose, were added to syringomycin-minimal medium; 13 of the strains produced ≥10-fold higher toxin levels. Some strains, such as 5D428, produced toxin only in the presence of these signals. This demonstrates that nearly all strains of *P. s.* pv. *syringae* have a sensory mechanism for specific plant metabolites that modulate syringomycin, syringotoxin, or syringostatin production.

Additional keywords: antibiotic, phenolic signal, phytotoxin, RFLP analysis.

Toxin synthesis and secretion are prevalent among the pathovars of *Pseudomonas syringae*, suggesting that strong selective pressure for toxigenic strains exists in the plant environment (Gross 1991). Currently, six structural classes of toxins are recognized that induce either necrosis (i.e., syringomycin and syringopeptin) or chlorosis (i.e., coronatine, phaseolotoxin, tabtoxin, and tagetitoxin) of affected plant tissues. With the exception of *P. syringae* pv. *syringae*, which produces both syringomycin and syringopeptin (Ballio *et al.* 1991), only one class of toxin is produced by strains within a pathovar. It appears, therefore, that toxigenesis arose independently among the toxigenic groups of pseudomonads and reflects overall genetic differences (Hildebrand *et al.* 1982; Pecknold and Grogan 1973). Accordingly, genes required for phaseolotoxin production by *P. s.* pv. *phaseolicola* and coronatine production by *P. s.* pv. *tomato* and related pathovars are conserved among strains and serve as highly specific DNA probes in disease diagnosis (Cuppels *et al.* 1990; Schaad *et al.* 1989).

Syringomycin is a cyclic lipodepsinonapeptide that kills host cells by extensively disrupting ion transport across the plasmalemma due to activation of a cascade of physiological events (Takemoto 1992). Thus, it is not surprising that syringomycin production contributes to the virulence of *P. s.* pv. *syringae*, a pathovar possessing a broad host range among both monocot and dicot species (Bradbury 1986). For example, syringomycin-minus strains arising from single mutations within the *syrB* gene of strain B301D-R cause only small necrotic lesions in immature sweet cherry (*Prunus avium* L.) fruits (Mo and Gross 1991a; Xu and Gross 1988b). Based on quantitative evaluations of *syrB* mutants to the parental strain in pathogenicity tests, it was estimated that syringomycin production nearly doubled the virulence of *P. s.* pv. *syringae*. Syringomycin also exhibits antimicrobial activity, and the highly sensitive fungus *Geotrichum candidum* is used in routine bioassays for toxin production (Sinden *et al.* 1971).

Despite genetic evidence that syringomycin functions as an important virulence factor in *P. s.* pv. *syringae*, historical inconsistencies raise questions as to whether all wild-type strains that cause typical disease symptoms are capable of producing the toxin. The study by Otta and English (1971) is the most conspicuous; 25% of 351 strains that caused cankers in peach seedlings did not produce syringomycin, based on the failure to form characteristic zones of inhibition of *G. candidum* on potato-dextrose agar (PDA). In surveys for syringomycin production on PDA by strains isolated from pome and stone fruit trees that phenotypically resemble *P. s.* pv. *syringae*, roughly 80% were toxigenic (Gross *et al.* 1984;

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Latorre and Jones 1979; Roos and Hattingh 1983, 1987; Seemüller and Arnold 1978). Many of these strains were nonpathogenic, reflecting the heterogeneity of strains resembling *P. s.* pv. *syringae*. In fact, nonpathogenic strains of *P. syringae* that do not cause a hypersensitive reaction in tobacco are common epiphytes in nature (Baca *et al.* 1987), and yet some of these strains were reported to produce syringomycin (Gross and DeVay 1977b; Gross *et al.* 1984). Although syringomycin production may not be limited to pathogenic strains, one should not discount the premise that all highly virulent strains produce the phytotoxin *in situ* because laboratory-grown cells display phenotypic differences from cells grown on plants in nature (Wilson and Lindow 1993). Nevertheless, Young (1991) included a bioassay for syringomycin production on PDA as one of 11 tests for laboratory determination of the "syringae group." Such evidence suggests that genes involved in syringomycin synthesis and secretion are intrinsic to the genome of *P. s.* pv. *syringae* and related epiphytic strains that are nonpathogenic.

The recent discovery that specific plant metabolites activate expression of both the *syrB* gene and syringomycin biosynthesis in strain B3A-R demonstrates that virulence in *P. s.* pv. *syringae* is modulated by the perception of signals in the plant environment (Mo and Gross 1991b). Accordingly, an explanation for the failure of some wild-type strains to produce toxin in routine surveys on PDA may be that the crude potato extract either does not provide appropriate signal molecules or contains constituents that inhibit the signaling process. Two classes of plant metabolites display signal activity in *P. s.* pv. *syringae*, and both are commonly found in tissues of susceptible plants attacked by the bacterium. Certain phenolic β-glucosides serve as the primary signal, and a few sugars such as D-fructose enhance that activity in a manner resembling *vir* gene activation in *Agrobacterium tumefaciens* by acetosyringone and simple pyranose sugars (Ankenbauer and Nester 1990; Cangelosi *et al.* 1990; Shimoda *et al.* 1990). For example, when a transcriptional fusion between *syrB* and a promoterless *lac* operon was used as a reporter of gene activity in a derivative strain of B3A-R, the signal activity of arbutin at a concentration of 10 μM was enhanced fivefold by the addition (0.1%) of D-fructose; no *syrB*-inducing activity occurred in the absence of both signal molecules in the defined culture medium (Mo and Gross 1991b). The influence of plant signal molecules on syringomycin production by strain B3A-R was emphasized by the induction of 256 U/ml of toxin when both types of signals were present versus no production in their absence. The extent by which plant signal molecules govern expression of syringomycin production in representative wild-type strains of *P. s.* pv. *syringae* is not known.

Genetic analysis of syringomycin production in strain B301D led to the identification of three *syr* genes within a region spanning ~7 kb of the chromosome (Fig. 1) that appear to have an essential role in either synthesis or secretion of syringomycin (Quigley and Gross 1993; Quigley *et al.* 1993). The *syrB* and *syrC* genes appear to function in toxin synthesis, based upon the occurrence of signature enzymatic domains in the predicted SyrB and SyrC protein sequences (J.-H. Zhang, N. B. Quigley, and D. C. Gross, unpublished). In contrast, the *syrD* gene, which is transcribed in the orientation opposite to that of *syrB* and *syrC*, is predicted to encode a protein that belongs to the ATP-binding cassette (ABC) superfamily of transporter proteins (Quigley *et al.* 1993). Therefore, it is proposed that SyrD is embedded in the cytoplasmic membrane and functions as an ATP-driven efflux pump for the secretion of syringomycin. Based on such evidence, one can theorize that functional *syrB* and *syrD* genes would be conserved among toxigenic strains of *P. s.* pv. *syringae* and would be of use in studying diversity among strains isolated from disparate plant habitats.

In this study, we reexamine the toxigenicity of strains of *P. s.* pv. *syringae* isolated from a wide range of plants with the aim of clarifying the relationship of syringomycin production to phytopathogenesis. Included are several strains that cause typical disease symptoms despite the failure to produce syringomycin in standard assays on PDA. Using portions of the *syrB* and *syrD* genes as DNA probes, we demonstrate that both genes are conserved as single loci among a spectrum of *P. s.* pv. *syringae* strains and do not occur in the genomes of other pathovars of *P. syringae* that are representative of the known toxigenic classes of *P. syringae* pathovars. We also show that *syrB* and *syrD* sequences are conserved in strains of *P. s.* pv. *syringae* that produce either syringotoxin or syringostatin, which are amino acid analogs of syringomycin (Fig. 2) (Fukuchi *et al.* 1992b). In addition, we test the hypothesis that plant signal molecules modulate expression of syringomycin production in most strains of *P. s.* pv. *syringae*, and we demonstrate that some strains fail to produce toxin in the absence of plant signal molecules.

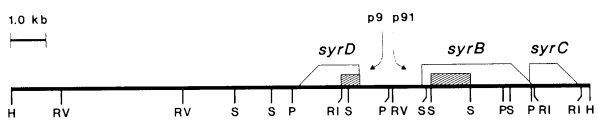

Fig. 1. Map positions of the *syrB* and *syrD* probe fragments relative to the restriction map of the 16-kb *Hin*dIII fragment carrying the *syrBCD* genes of *Pseudomonas syringae* pv. *syringae* strain B301D. The relative sizes, orientations, and positions of the *syrB*, *syrC*, and *syrD* genes are represented by lightly shaded boxes; the tapered end of each box indicates the 3′ end of the gene. Plasmids p9 and p91 contain the *syrD* (510 bp) and *syrB* (1,107 bp) probe fragments, respectively, that are defined by diagonally shaded boxes. Restriction sites are given for all enzymes used in restriction fragment length polymorphism analyses: *Eco*RI (RI), *Eco*RV (RV), *Hin*dIII (H), *Pst*I (P), and *Sal*I (S).

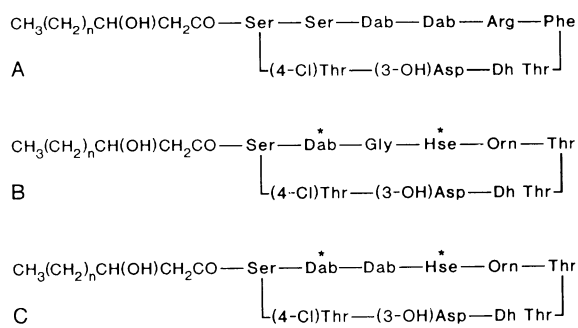

Fig. 2. Structures of syringomycin (**A**), syringotoxin (**B**), and syringostatin (**C**) produced by strains of *Pseudomonas syringae* pv. *syringae*. In the 3-hydroxy fatty acid moiety, n = 8 or 10. The stars identify the two amino acids in each toxin structure occurring as D-isomers. Abbreviations of amino acids: Arg, arginine; (3-OH)Asp, 3-hydroxyaspartic acid; (4-Cl)Thr, 4-chlorothreonine; Dab, 2,4-diaminobutyric acid; Dh Thr, dehydrothreonine; Gly, glycine, Hse, homoserine; Orn, ornithine; Phe, phenylalanine; Ser, serine; Thr, threonine.

RESULTS

Effect of plant signal molecules on induction of syringomycin, syringotoxin, and syringostatin production.

Of the 47 strains provisionally identified as *P. s.* pv. *syringae* (Table 1), 42 produced an antifungal metabolite characteristic of syringomycin and its amino acid analogs, syringotoxin and syringostatin (Fig. 2). Approximately 90% of these toxigenic strains (38 out of 42) produced larger quantities of the toxins in the presence of plant signal molecules. In general, there was at least a twofold increase in toxin yield with the addition of arbutin and fructose to syringomycin-minimal (SRM) medium (i.e., SRM_{AF}) (Gross 1985; Mo and Gross 1991b); 13 of the strains produced ≥10-fold higher toxin levels. The effect of plant signals was striking for strains B3A, SD19, 61, and W4N7, which essentially failed to produce toxin without addition of the plant signal compounds to SRM medium. Only two strains, W4N27 and B301D, produced equivalent amounts in the SRM and SRM_{AF} media; strain B301D is the source of the *syrB* and *syrD* gene probes. Although strains 5D4118 and B124 produced more toxin in SRM than in SRM_{AF}, very low yields of ≤36 U/ml were detected. Strains that produced syringotoxin (i.e., Ps268 and B457) and syringostatin (i.e., SY12) consistently produced higher levels of toxin in SRM_{AF} medium. The most conspicuous was strain B457, which produced 23-fold more syringotoxin.

Toxin production was more consistent in SRM_{AF} than on PDA or in potato-dextrose broth (PDB) and, consequently, was most indicative of toxigenicity (Table 1). Over 50% of

Table 1. Relevant characteristics of *Pseudomonas* strains used in this study

Strain identification	Plant source	Location	Bacteriocin group[a]	Virulence rating[b]	Presence of *syrB* and *syrD* sequences[c]	Toxin zone on PDA (mm)[d]	Syringomycin production (U/ml)[e] in:[f]		
							PDB	SRM	SRM_{AF}
Pseudomonas syringae pv. *syringae* and related *P. syringae* Strains isolated from lesions on plants									
B301D	Pear	England	8F	3	Yes	10.9	4,096	2,048	2,048
B3A	Peach	California	14	2	Yes	6.7	2,048	0	640
HS191	Millet	Australia	6C	3	Yes	2.5	256	80	480
PS17	Maize	Nebraska	6B	3	Yes	2.8	176	112	1,792
PS4A#1	Maize	Nebraska	6D	3	Yes	<1	40	66	576
SD202	Wheat	S. Dakota	ND[g]	3	Yes	11.4	1,280	1,152	3,072
PA105	Sugar beet	Unknown	ND	3	Yes	3.9	1,280	768	2,048
5D4171	Tomato	California	16	3	Yes	<1	224	13	1,792
5D4214	Rose	California	8A	3	Yes	9.1	1,024	56	1,280
5D428	Plum	California	8F	1	Yes	0	1	33	1,280
5D4113	Cherry	California	14	1	Yes	0	25	16	128
5D4118	Almond	California	14	1	Yes	0	0	36	9
5D4132	Apricot	California	14	1	Yes	0	16	288	896
Ps251	Plum	Unknown	8F	1	Yes	0	12	48	96
B728a	Bean	Wisconsin	ND	3	Yes	2.8	257	88	1,024
SC1	Sugarcane	Japan	ND	1	Yes	7.7	225	192	768
475	Maize	S. Dakota	6C	0	Yes	0	28	256	768
SD19	Sorghum	S. Dakota	ND	0	Yes	0	8	2	40
B124	Almond	California	None	0	Yes	0	8	6	0
61	Wheat	Delaware	ND	0	Yes	<1	0	1	99
761-5	Foxtail	S. Dakota	13	0 (HR^-)[h]	Yes	5.4	48	48	256
Ps268[i]	Lemon	California	6C	3	Yes	22.6	8,192	896	1,408
B457[i]	Orange	California	6C	3	Yes	18.6	2,048	45	1,024
SY12[j]	Lilac	Japan	ND	0	Yes	0	0	116	224

(*continued on next page*)

[a] Bacteriocin typing data from Gonzalez *et al.* (1981), Gross *et al.* (1984), Vidaver and Buckner (1978), and Vidaver *et al.* (1972).

[b] 0 = Nonpathogenic; 1 = weakly virulent (necrotic lesions of 1–3 mm in diameter with no other discoloration of fruits); 2 = moderately virulent (large sunken lesions ≥3 mm in diameter that were not coalesced but with some discoloration of surrounding tissue); 3 = highly virulent (very large lesions that usually were coalesced to encompass 50–100% of the fruit surface). Each value represents the average of two trials conducted on separate occasions; 10 fruits were inoculated per strain in each trial. Virulence was evaluated after 3 days of incubation at 20°C.

[c] Determined by homology to the *syrB* and *syrD* gene probes contained in plasmid clones p91 and p9, respectively. All strains were either positive (Yes) or negative (No) for both probes.

[d] Zones of inhibition of *Geotrichum candidum* as measured from the margin of the bacterial colony to the point of fungal growth. Each value represents the average of two trials conducted on separate occasions, with three plates per trial. PDA, potato-dextrose agar.

[e] Each value is the mean of two trials conducted on separate occasions; each trial consisted of duplicate cultures. Units (U) of toxin are as defined in Materials and Methods. Only one trial was performed for *P. viridiflava* and pathovars other than *P. s.* pv. *syringae*. In general, the SD for syringomycin yield was less than ±1 dilution point. Instead of syringomycin, syringotoxin (strains Ps268 and B457) and syringostatin (strain SY12) yields are shown.

[f] PDB, potato-dextrose broth; SRM, syringomycin-minimal medium; SRM_{AF}, SRM supplemented with arbutin and D-fructose to final concentrations of 100 μM and 0.1%, respectively.

[g] ND, not determined.

[h] HR^-, failed to induce a hypersensitive response in tobacco.

[i] Producer of syringotoxin.

[j] Producer of syringostatin.

the 42 toxigenic strains produced more toxin in SRM_{AF} than in PDB. Furthermore, 95% produced >10 U/ml in SRM_{AF} versus 86% in PDB. Eleven of the toxigenic strains failed to produce discernible zones of antifungal activity on PDA, and yet most of these strains produced large quantities of toxin in the SRM_{AF} medium. For example, strain 5D428 produced over 1,200 U/ml of toxin in SRM_{AF} medium and essentially no toxin in PDB or on PDA.

Induction of toxin production by plant signal molecules was not associated with the plant or geographic sources of strains (Table 1). When SRM was amended with arbutin and fructose, larger toxin yields were observed for strains isolated from dicot and monocot plants, for strains isolated as epiphytes and from disease lesions, and for strains isolated in Asia, Australia, and North America as compared to the yields in unamended SRM medium. Strains within 10 bacteriocin groups produced more toxin in SRM_{AF} than in SRM.

No antifungal activity was observed for strain W4N1 of *P. viridiflava* and strains representing six pathovars of *P. syringae* (Table 1). Except for strains of *P. s.* pv. *phaseolicola*, which failed to grow in the SRM and SRM_{AF} media, results were not compromised by the stringent growth conditions in SRM and SRM_{AF} media.

The *syrB* and *syrD* genes are conserved among *P. syringae* strains producing syringomycin, syringotoxin, or syringostatin.

Portions of the *syrB* and *syrD* genes that appear to function in syringomycin synthesis (J.-H. Zhang, N. B. Quigley, and D. C. Gross, unpublished) and export (Quigley *et al.* 1993),

Table 1. *Continued from preceding page.*

Strain identification	Plant source	Location	Bacteriocin group[a]	Virulence rating[b]	Presence of *syrB* and *syrD* sequences[c]	Toxin zone on PDA (mm)[d]	Syringomycin production (U/ml)[e] in:[f]		
							PDB	SRM	SRM_{AF}
Strains isolated as ice-nucleation-active epiphytes									
W4N9	Cherry	Washington	12	1	Yes	13.4	1,536	16	82
W4N15	Apple	Washington	8F	1	Yes	9.5	1,792	512	2,560
W4N27	Pear	Oregon	8B	2	Yes	12.8	3,072	1,792	1,792
W4N31	Pear	Oregon	8B	2	Yes	7.6	1,536	1,024	1,280
W4N44	Pear	Washington	8B	1	Yes	4.0	480	27	288
W4N45	Apple	Washington	6C	1	Yes	2.8	3,072	1,536	3,072
W4N53	Pear	Oregon	8B	1	Yes	0	21	768	2,048
W4N56	Pear	Oregon	6C	2	Yes	0.9	1,024	640	1,024
W4N65	Pear	Oregon	8B	2	Yes	4.9	3,072	768	2,048
W4N70	Pear	Oregon	14	3	Yes	4.4	3,584	640	1,024
W4N72	Pear	Oregon	8B	2	Yes	9.4	2,048	1,408	1,536
W4N79	Pear	Oregon	8B	3	Yes	12.3	1,536	96	1,408
W4N87	Apple	Washington	6C	1	Yes	0.6	1,536	128	1,280
W4N104	Cherry	Washington	13	1	Yes	2.3	4,096	1,536	3,584
W4N108	Cherry	Washington	8F	2	Yes	9.8	1,024	1,024	2,048
W4N5	Apple	Washington	6C	0	Yes	0	512	1,536	2,816
W4N7	Cherry	Washington	8B	0	Yes	12.8	80	0	48
W4N50	Pear	Oregon	8F	1	No	0	0	0	0
W4N30	Pear	Oregon	6C	0	No	0	0	0	0
W4N42	Pear	Washington	8F	0	No	0	0	0	0
W4N77	Pear	Oregon	8F	0	No	0	0	0	0
W4N47	Cherry	Washington	13	0 (HR^-)	No	0	0	0	0
W4N103	Cherry	Oregon	6C	0 (HR^-)	Yes	9.4	1,024	608	1,024
P. s. pv. *phaseolicola*									
4612-9	Bean	New Zealand	ND	0	No	0	0	0	0
G50-1	Bean	Unknown	ND	0	No	0	0	0	0
N4SP	Bean	Nebraska	ND	0	No	0	0	0	0
P. s. pv. *tabaci*									
PT113	Tobacco	Unknown	ND	0	No	0	0	0	0
ATCC 11528	Tobacco	Unknown	ND	0	No	0	0	0	0
P. s. pv. *tagetis*									
A1	Marigold	Wisconsin	ND	0	No	0	0	0	0
B4	Marigold	Netherlands	ND	0	No	0	0	0	0
P. s. pv. *tomato*									
CNBP1318	Tomato	France	ND	0	No	0	0	0	0
T6D1	Tomato	Ontario	ND	0	No	0	0	0	0
P. s. pv. *morsprunorum*									
C28 A2	Cherry	England	8F	1	No	0	0	0	0
C46 RF	Cherry	England	8F	1	No	0	0	0	0
P. s. pv. *pisi*									
PSP1	Pea	Idaho	ND	0	No	0	0	0	0
895A	Pea	England	ND	0	No	0	0	0	0
P. viridiflava									
W4N1	Peach	Washington	1	1	No	0	0	0	0

respectively, proved to be highly specific gene probes for toxigenic strains of *P. s.* pv. *syringae* and related nonpathogenic strains (Table 1). Plasmids p91, containing a 1,107-bp insert from *syrB*, and p9, containing a 510-bp insert from *syrD* (Fig. 1), hybridized only to the total DNA of the 42 strains identified as producers of syringomycin, syringotoxin, or syringostatin (Table 1). Five strains (i.e., strains W4N50, W4N30, W4N42, W4N47, and W4N77) isolated as ice-nucleation-active epiphytes of fruit trees and that produced no discernible antifungal toxin, showed no homology with either of the *syr* gene probes in Southern analysis. The probes were demonstrated to be highly specific for *P. s.* pv. *syringae* strains by the lack of homologous sequences in strains representative of *P. viridiflava* and in *P. syringae* pathovars *phaseolicola*, *tabaci*, *tagetis*, *tomato*, *morsprunorum*, and *pisi* (Table 1, Fig. 3A).

Relationship between virulence and toxigenicity in *P. s.* pv. *syringae*.

Pathogenicity tests in immature cherry fruits revealed that 32 of the 47 strains that phenotypically resembled *P. s.* pv. *syringae* caused necrotic lesions (Table 1). Syringomycin or syringotoxin was produced by 31 of the pathogenic strains, the exception being the weakly virulent strain W4N50. Moreover, W4N50 was the only strain causing necrotic lesions that did not harbor *syrB* and *syrD* sequences. The 13 strains found to be highly virulent produced ≥480 U/ml of toxin in SRM_{AF} liquid medium, whereas the 11 strains that produced <480 U/ml of toxin were either nonpathogenic or weakly virulent. Toxigenicity was determined not to be correlated with pathogenicity in cherry fruits because eight of the nonpathogenic strains produced toxin and contained sequences homologous to the *syrB* and *syrD* gene probes. In addition, strains 761-5

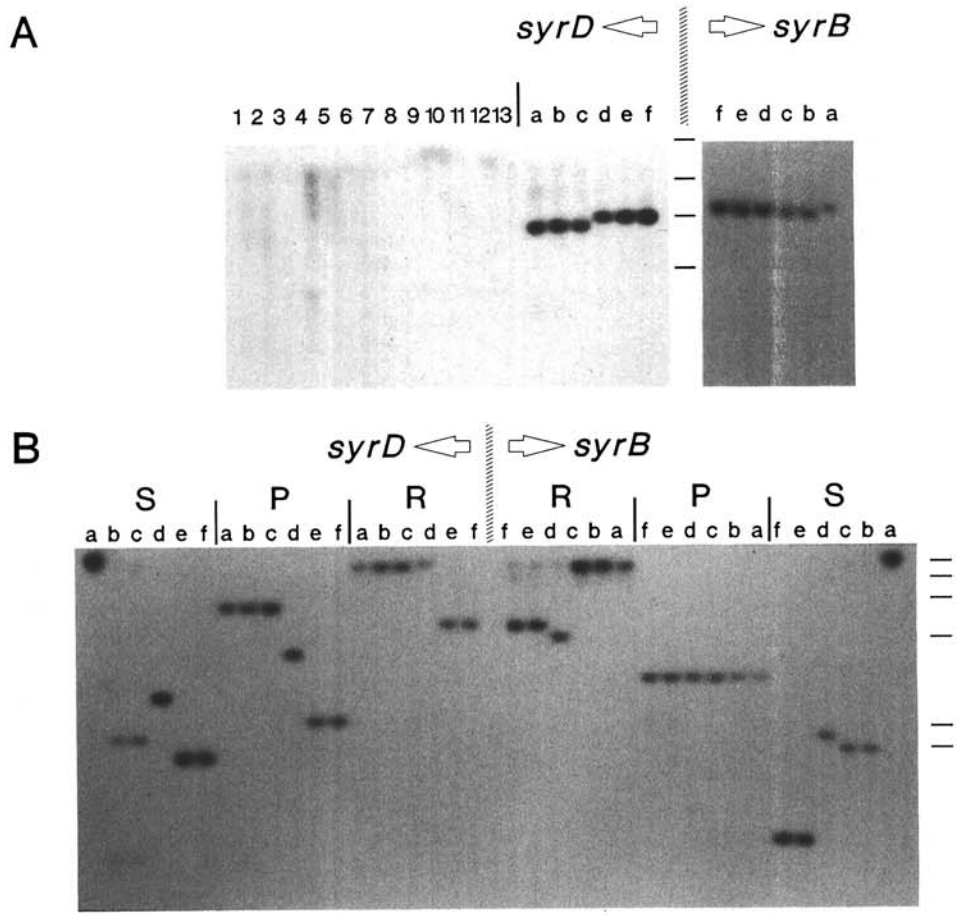

Fig. 3. Restriction fragment length polymorphism patterns of six toxigenic strains of *Pseudomonas syringae* pv. *syringae* in the *syrB* and *syrD* regions and lack of homologous sequences in toxigenic strains representative of other pathovars. Total DNAs were digested with restriction enzymes, and DNA fragments were separated by agarose gel electrophoresis and blotted to nitrocellulose membranes. The blots were hybridized with either the p9 *syrD* probe (left side of **A** and **B**) or the p91 *syrB* probe (right side of **A** and **B**). **A,** *Eco*RV digestions. Lanes 1–13 contain DNA from the following strains (groups of strains are followed in parentheses by the toxin they produce): *P. s.* pv. *pisi* 895A, PSP1 (no known toxin); *P. s.* pv. *morsprunorum* C46 RF, C28 A2, *P. s.* pv. *tomato* T6D1, CNBP1318 (coronatine); *P. s.* pv. *tagetis* A1, B4 (tagetitoxin); *P. s.* pv. *tabaci* ATCC 11528, PT113 (tabtoxin); *P. s.* pv. *phaseolicola* G50-1, 4612-9, N4SP (phaseolotoxin). Lanes containing DNA from *P. s.* pv. *syringae* strains are as follows: a, SY12 (syringostatin); b, Ps268; c, B457 (syringotoxin); d, SC1; e, B301D; f, B3A (syringomycin). **B,** DNAs were digested with *Sal*I (S), *Pst*I (P), or *Eco*RI (R) and loaded using the same labeling convention as in **A**. The *SalI/syrD* RFLP pattern of strains Ps268 (lane b) and B457 (lane c) consisted of a conspicuous 2.65-kb fragment plus a faint 1.25-kb fragment. The following products of a lambda-*Hin*dIII digest are indicated as mobility standards: **A,** 27.5 (annealing product of 23.1- and 4.4-kb fragments), 9.4, 6.6, and 4.4 kb; **B,** 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb.

and W4N103, which did not induce a hypersensitive response in tobacco, produced toxin and contained *syrB* and *syrD* sequences. Although strain W4N45 tested negative in tobacco hypersensitivity tests, presumably because of its unusual ability to agglutinate in water, it proved to be pathogenic to cherry fruits. In contrast to the nonpathogenic response of the syringostatin-producing strain SY12 in cherry fruits, the two syringotoxin-producing strains B457 and Ps268 were highly virulent, causing extensive necrotic lesions that encompassed 50–100% of the fruit surface by day 3.

Of the eight strains of *P. s.* pv. *syringae* originating from monocot plants, four were rated as highly virulent (Table 1). Strain SD202 isolated from wheat was the most impressive of the 13 highly virulent strains in cherry fruits, causing a total blackening of all 10 cherry fruits per trial by day 2. It was not unusual to observe a sudden discoloration of fruits within a span of 1–2 hr; this was attributed to phytotoxin production, since the blackened tissues were not yet invaded by the bacterium. Only strains rated as highly virulent caused similar blackening of fruits, although perhaps not as suddenly or not in all fruits within a replication, as observed for SD202. Furthermore, inoculations with strain SD202 resulted in more rapid and extensive systemic necrosis of the fruit stems than observed for fruits inoculated with other strains.

Strains of *P. viridiflava* and *P. s.* pv. *morsprunorum* caused small necrotic lesions in immature cherry fruits that resembled lesions formed by weakly virulent strains of *P. s.* pv. *syringae* (Table 1). However, lesions caused by *P. viridiflava* W4N1 developed more slowly than those caused by *P. s.* pv. *syringae*. Strains of pathovars *phaseolicola*, *tabaci*, *tagetis*, *tomato*, and *pisi* were negative in cherry fruit pathogenicity tests, as expected.

RFLP analysis of *P. s.* pv. *syringae* and related strains using the *syrB* and *syrD* gene-specific probes.

The specificity of the *syr* gene probes for *P. s.* pv. *syringae* strains that produce either syringomycin, syringotoxin, or syringostatin is illustrated in Figure 3A. In Southern blots of *Eco*RV-cleaved total DNA from six representative strains of *P. s.* pv. *syringae*, both the *syrB* and *syrD* probes hybridized to one *Eco*RV fragment of different sizes (Fig. 3A). In contrast, DNA from all 13 strains representing pathovars *pisi*, *morsprunorum*, *tomato*, *tagetis*, *tabaci*, and *phaseolicola* failed to hybridize with the *syrD* probe (Fig. 3A, lanes 1–13). Similarly, the *syrB* probe did not hybridize with DNA from any of the 13 strains representing the six pathovars (not shown).

The interval spanning approximately 8.5 kb from the *Sal*I site immediately downstream of the *syrD* gene to the *Hin*dIII site downstream of *syrC* in strain B301D (Fig. 1) has been sequenced (Quigley et al., 1993; J.-H. Zhang, N. B. Quigley, and D. C. Gross, unpublished). Restriction sites were identified from the sequence data and used as a guide in selecting enzymes for restriction fragment length polymorphism (RFLP) analysis in the *syr* region. RFLP analysis revealed that the *syrB* and *syrD* gene probes were homologous to a single *Hin*dIII fragment of between 11 and 35 kb in length in all 42 strains containing homologous *syr* gene sequences (Table 2). As observed in B301D (Fig. 1), both *syr* genes were clustered on a 16-kb *Hin*dIII fragment in over half of the strains (Table 2). Furthermore, a single *Eco*RI fragment (ranging in size from approximately 4.8 to 15 kb) in almost all strains was homologous to both probes. The only exceptions were strains SC1 and 61, which yielded identical *Eco*RI RFLP patterns but carried an *Eco*RI site between the *syrB* and *syrD* genes based on homology of the corresponding probes to 4.2- and 13.5-kb *Eco*RI fragments, respectively. Approximately half of the strains contained a 4.8-kb *Eco*RI fragment that hybridized to both probes, which was indicative of a close physical linkage between the *syrB* and *syrD* genes as observed in strain B301D (Fig. 1).

The RFLP patterns were compared with the B301D restriction map, on which there are single *Eco*RV and *Pst*I sites between the *syrB* and *syrD* genes, an internal *Sal*I site in *syrD*, and *Sal*I sites flanking the *syrB* region used as a probe (Fig. 1). With the exception of strains PA105 and W4N27, which appeared to carry both *syr* probe homologs on a single *Eco*RV fragment of approximately 17 kb (data not shown), all strains tested carried their *syrB* and *syrD* probe homologs on different *Eco*RV fragments. Similarly, different *Pst*I fragments hybridized to the two *syr* probes in all strains except PA105, W4N9, and W4N27, which appeared to carry both probe homologs on single *Pst*I fragments of approximately 8.1, 10.3, and 5.6 kb, respectively. The *Eco*RI, *Pst*I, and *Sal*I RFLP patterns for six representative *P. s.* pv. *syringae* strains are shown in Figure 3B.

For most strains, digestion with *Sal*I gave the smallest fragments with homology to the *syr* probes (Fig. 3B). The *syrB* probe was homologous to a single *Sal*I fragment of between 1.25 and 5.35 kb in all strains tested, except for SY12 and W4N65. DNA of strains SY12 and W4N65 could not be digested with *Sal*I despite numerous attempts that employed a variety of enzyme concentrations and digestion conditions. These strains appeared to have a *Sal*I site protection mechanism that most likely involves a base-specific methylase ac-

Table 2. Relationship between *Hin*dIII and *Eco*RI fragment size (kb) and homology to the *syrB* and *syrD* gene probes among 42 strains of *Pseudomonas syringae*[a]

	Restriction enzyme and *syr* gene probe			
	*Hin*dIII		*Eco*RI	
Strain[b]	*syrB*	*syrD*	*syrB*	*syrD*
PA105	11	11	4.8	4.8
PS17, 475	11	11	13.5	13.5
SC1,* 61	14.5	14.5	4.2	13.5
SY12*	14.8	14.8	11.5	11.5
W4N7, W4N44	15	15	7.4	7.4
Ps268,* B457,* W4N45, W4N108	15	15	11.5	11.5
HS191, PS4A#1, SD19, W4N9	15	15	13.5	13.5
B301D,* B3A,* 5D4171, 5D4214, 5D4113, 5D4118, 5D4132, Ps251, B728a, B124, W4N31, W4N53, W4N56, W4N65, W4N70, W4N72, W4N79	16	16	4.8	4.8
761-5, W4N5	16	16	13.5	13.5
W4N87, W4N103, W4N104	16	16	15	15
W4N27	23	23	4.8	4.8
W4N15	25	25	13.5	13.5
5D428	35	35	4.8	4.8
SD202	35	35	13.5	13.5

[a] Plasmids p91 and p9 were used as *syrB*- and *syrD*-specific probes, respectively.

[b] The restriction fragment length polymorphism patterns of strains marked with a star are shown in Figure 3.

tivity (Nelson and McClelland 1991). Among the other strains, all but two had an internal *SalI* site within the *syrD* region (Fig. 1), based on the detection of two *SalI* fragments that hybridized to the p9 probe. The *SalI*/*syrD* RFLP pattern of most strains consisted of fragment pairs of 2.35 and 1.17 kb, or 2.65 and 1.25 kb (e.g., Fig. 3B *syrD* lanes b and c under S), or a doublet of 2.25 kb (e.g., Fig. 3B *syrD* lanes e and f under S). Strains PA105 and W4N27 both carried *syrD*-homologous fragment pairs of 2.25 and 1.17 kb; the fragment pairs in strains W4N15 and W4N103 were 2.65 and 1.2 kb, and 6.5 and 1.13 kb, respectively. Strains SC1 and 61 were exceptional in that each carried only one 3.4-kb *SalI* fragment with homology to the *syrD* probe (e.g., Fig. 3B *syrD* lane d under S).

The *syr* gene probes were used to distinguish *P. s.* pv. *syringae* strains that produce different analogs of syringomycin. Six strains for which the toxin structures have been resolved (Ballio *et al.* 1990, 1991; Fukuchi *et al.* 1992a,b) as syringomycin (B3A, B301D, SC1), syringotoxin (B457, Ps268), or syringostatin (SY12) were subjected to RFLP analysis (Fig. 3). RFLP patterns resulting from most combinations of enzyme and probe distinguished readily the syringomycin-producer strains from those that produced syringotoxin or syringostatin (Fig. 3B). However, only the *Eco*RV/*syrB* combination produced patterns that clearly distinguished between syringotoxin- and syringostatin-producer strains (observed in the six lanes at the right of Fig. 3A). The RFLP patterns observed for the two syringotoxin-producer strains were indistinguishable in all restriction digests.

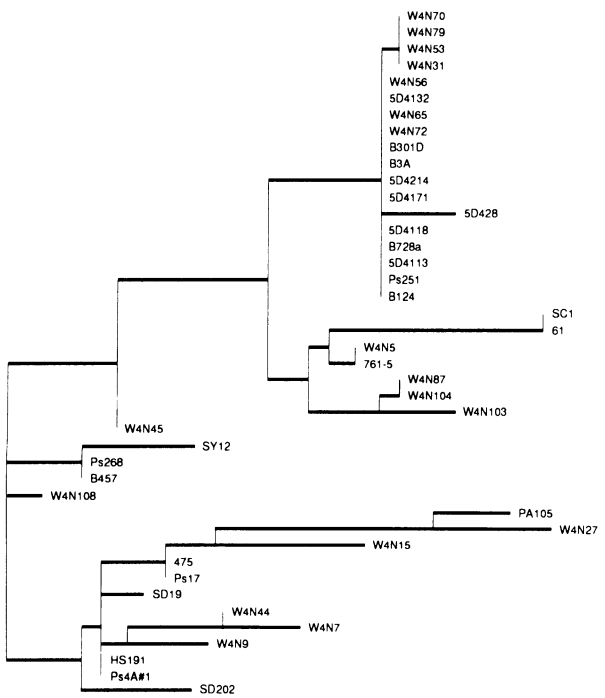

Fig. 4. Maximum parsimony tree based on restriction fragment length polymorphism (RFLP) analysis of 42 strains of *Pseudomonas syringae* using plasmids p91 and p9 as *syrB*- and *syrD*-specific gene probes, respectively. The tree summarizes the combined RFLP data for both *syr* probes obtained by Southern analysis of genomic DNA cleaved by *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, and *Sal*I. Scaled branches are shown as thick lines; lengths of these branches are proportional to the numbers of molecular changes. The tree is unrooted using the midpoint method, and character-state optimization was by the minimum *f*-value method. Goodness-of-fit statistical analysis of the tree showed a consistency index of 0.833 and a retention index of 0.921.d

Maximum parsimony and distance matrix analyses of RFLP data.

Maximum parsimony analysis of the RFLP profiles of 42 strains of *P. syringae* was used to evaluate relatedness among strains within the *syrB* and *syrD* gene region. The maximum parsimony tree shown in Figure 4 is based on the RFLP data generated using both *syr* probes and all five restriction enzymes; the tree infers the minimum amount of evolutionary change within the *syr* gene region. Because the horizontal lengths of branches in Figure 4 are proportional to the numbers of molecular changes, considerable diversity within the *syr* gene region was evident among the 42 *P. syringae* strains analyzed. When the *syrB* and *syrD* RFLP data were analyzed separately, the maximum parsimony trees closely resembled the tree in Figure 4. In addition, distance matrix analysis of the combined *syrB* and *syrD* RFLP data generated consensus trees that did not differ appreciably in the number and composition of strain clusters.

Approximately 40% of the strains were clustered together with strain B301D (Fig. 4), indicating that the restriction site locations observed in B301D (Fig. 1) are highly conserved in the vicinity of *syrB* and *syrD*. Because there were some missing RFLP data for strains W4N70, W4N79, W4N53, and W4N31, it would not be surprising that analysis of further RFLP data would place these strains in the B301D cluster. Strains PA105 and W4N27 were located at the greatest distance from the B301D cluster (Fig. 4). Strain PA105 is representative of *P. s.* pv. *syringae* isolates from sugar beet, which are sometimes designated as *P. s.* pv. *aptata* (Otta and English 1971; Pecknold and Grogan 1973); such strains are clustered in serotype VI of Otta and English (1971), which included <4% of *P. s.* pv. *syringae* strains tested. None of the strains originally isolated from monocot plants was placed in the B301D cluster. In particular, strains SD202, HS191, Ps4A#1, SD19, 475, and Ps17 (originally isolated from holcus spot lesions of foxtail, maize, millet, and wheat) were clustered in relatively close proximity to one another. Nevertheless, three strains from monocots (i.e., SC1, 61, and 761-5) displayed very different RFLP profiles. Strains that are producers of syringotoxin (i.e., Ps268 and B457) or syringostatin (i.e., SY12) formed a distinct cluster in the maximum parsimony tree.

There was no apparent segregation of strains according to bacteriocin group in the tree derived from maximum parsimony analysis of RFLP data (Table 1, Fig. 4). For example, strains belonging to bacteriocin groups 6C, 8A, 8B, 8F, 14, and 16 were placed in the same cluster as B301D. In addition, there was no segregation of strains that corresponded to virulence ratings in cherry pathogenicity tests. The 13 strains rated as highly virulent were distributed throughout the maximum parsimony tree, as were the nonpathogenic strains.

DISCUSSION

The concept that syringomycin production is intrinsic to *P. s.* pv. *syringae* was substantiated by our evidence that

genes implicated in toxin synthesis and export are conserved and that plant signal molecules modulate toxigenesis in a diverse spectrum of strains. The presence of both *syrB* and *syrD* gene sequences was correlated strictly with a capacity to produce syringomycin or an amino acid analog of the toxin. This includes phytopathogenic strains of historical interest (Otta and English 1971, Gross *et al.* 1984), such as 5D428, which failed to produce toxin in standard assays on PDA or in PDB but produced high quantities in SRM_{AF}. Nevertheless, syringomycin production was not restricted to *P. s.* pv. *syringae*; several nonpathogenic strains produced toxin and harbored DNA sequences homologous to the *syr* gene probes. Although one can argue that some of these strains may be pathogenic to a plant other than cherry, they are representative of the phenotypic and genetic diversity existing among epiphytic strains of *P. syringae* that most closely resemble *P. s.* pv. *syringae* (Hirano and Upper 1990). The genetic similarity of the nonpathogenic strains to *P. s.* pv. *syringae* is emphasized by the fact that pathovars of *P. syringae* representative of the other major toxigenic classes do not contain homologous *syr* sequences. Consequently, the *syr* gene probes should prove to be valuable tools in disease diagnosis and in analyzing the genetic relationships among *P. s.* pv. *syringae* strains and affiliated nonpathogenic strains.

It appears that nearly all strains of *P. s.* pv. *syringae* have a sensory mechanism for specific plant metabolites that modulate syringomycin production. With some strains, the regulatory effect on toxigenesis was extraordinary, as documented by more than 10-fold higher toxin yields in SRM amended with arbutin and D-fructose as signal molecules. Control of toxigenesis by plant signals is not surprising because it ensures that the bacterium is attuned to a dynamic plant environment. There is now extensive documentation that plant phenolic compounds serve as regulators of gene expression in the Rhizobiaceae and control processes critical to the plant-microbe interaction (Peters and Verma 1990). Although few details are known about the sensory mechanism in *P. s.* pv. *syringae*, the perception of specific phenolic glycoside signals and enhanced sensitivity in the presence of specific sugars common to plants resembles the process of *vir* gene activation in *A. tumefaciens* (Ankenbauer and Nester 1990; Mo and Gross 1991b). However, it was surprising to observe that about 90% of the *P. s.* pv. *syringae* strains responded to arbutin and D-fructose as plant signal molecules. Not only syringomycin producers, but also producers of syringotoxin and syringostatin were stimulated to produce more toxin in SRM_{AF} than in SRM. Although arbutin occurs in the foliar tissues of many plant species such as pear (*Pyrus communis* L.) (Miller 1973), most of the strains activated in our study originated from plants lacking arbutin. This suggests that monocot and dicot plant hosts harbor phenolic glycoside signals that share certain chemical features recognized by a broad spectrum of *P. s.* pv. *syringae* strains, thereby activating toxin production with little or no impact on host range. Accordingly, the bacterium is favored in the plant environment by having a sensory mechanism that detects specific phenolic glycosides that signal the bacterium to rapidly activate virulence genes, and yet it can aggressively attack a wide range of plants since they all contain phenolics with the fundamental chemical structures responsible for signal activity.

Although PDA and PDB have been long used in standard assays for syringomycin production, more consistent production of toxin by a broad range of strains occurred in SRM_{AF} medium. All toxigenic strains as identified by homology to the *syr* gene probes were positive in SRM_{AF}, the only exception being strain B124, which produced barely detectable toxin in the other *in vitro* assays. In contrast, 10 and 26% of the toxigenic strains essentially failed to produce toxin in PDB and on PDA, respectively. Several factors may account for the performance of strains in a medium based on a crude potato tuber extract, but the composition and balance of plant signal molecules from potato may be a critical factor in why strains such as 5D428 fail to produce toxin in such assays. For example, hydroquinone and saligenin formed by cleavage of the β-glucosidic linkage of arbutin and salicin signals, respectively, display no signal activity and inhibit expression of *syrB* (Mo and Gross 1991b). In addition, esculin, which contains a coumarin ring, displayed only moderate signal activity of about 33% as compared to arbutin, illustrating the relationship of chemical structure to signal activity. Phenolic compounds also have been identified that antagonize signal transduction in *Bradyrhizobium* and *Rhizobium* spp. (Kosslak *et al.* 1990; Peters and Verma 1990), and it is possible that certain strains of *P. s.* pv. *syringae* are especially sensitive to constituents occurring in extracts from potato tubers that inhibit a step critical to signal transduction.

It was not surprising to observe among *P. s.* pv. *syringae* strains no direct correlation between virulence in cherry pathogenicity tests and amount of toxin produced in SRM_{AF}. However, all 13 highly virulent strains produced large quantities of toxin in SRM_{AF}. We were especially impressed by strains such as SD202, which caused a sudden blackening of cherry fruit resembling necrosis caused by release of massive amounts of phytotoxin. Because virulence represents a complex mixture of traits in addition to phytotoxin production, toxigenic strains that are avirulent could arise by a single mutation in a global regulatory gene. Although most of the strains we analyzed were stored as lyophilized cultures to avoid generating cultural variants, some of the strains of historical interest (e.g., B124, Ps251, and SD19) were maintained for several years in other laboratories as actively growing cultures and may now show little resemblance to the wild-type progenitor strain in toxin production, virulence, or both. Since mutations in *hrp* genes are unlikely to have a direct effect on syringomycin production, nonpathogenic strains 761-5 and W4N103, which also failed to cause a hypersensitive response, could simply arise in nature due to a mutation in one of several *hrp* genes (Huang *et al.* 1991).

The *syrB* and *syrD* genes that appear to be dedicated to toxin synthesis and export, respectively, lie in close proximity to one another in the genomes of a broad spectrum of strains. Indeed, all 42 toxigenic strains in our study carried single copies of the two genes within a 15-kb region or less. There is growing evidence that a tight clustering of antibiotic biosynthesis and export genes is common among microorganisms and that such a physical organization permits coordinated regulation and expression of the full antibiotic pathway (Gross 1991). The SyrD protein shows the greatest similarity with bacterial proteins that compose the secretion subfamily of ABC transporters. The 510-bp *syrD* fragment used as a probe is derived from a gene segment that encodes the N-terminus of SyrD (Quigley *et al.* 1993). In secretion-directed

ABC transporter proteins such as HlyB, the N-terminal domains define target specificity (Higgins *et al.* 1990); consequently, one can speculate that the portion of *syrD* used as a probe participates in the recognition and binding of syringomycin to SyrD and that the periplasmic loops within the N-terminus of SyrD participate in the translocation process. The 1,107-bp *syrB* fragment used as a probe encodes a portion of the SyrB N-terminus that contains conserved core sequences (J.-H. Zhang, N. B. Quigley, and D. C. Gross, unpublished) characteristic of all other peptide synthetase domains (Marahiel 1992). This region of SyrB appears to specifically bind and activate one of the structural amino acids contained in syringomycin. The identity of the amino acid activated by SyrB remains to be determined. Although the *syrB* probe fragment can distinguish *P. s.* pv. *syringae* strains that produce different analogs of syringomycin, SyrB may be involved in a biosynthetic step that is common to all strains.

Differentiation of pathovars of *P. syringae* is an important part of disease diagnosis, but it can be difficult to do, especially when critical information about host range and pathogenicity are unavailable. The use of pathovar-specific DNA probes promises to revolutionize the detection and identification of isolates from natural sources. Thus far, the only pathovar-specific DNA probes available are based on genes involved in toxigenesis. For example, DNA probes have been developed that specifically identify coronatine- or phaseolotoxin-producing strains of *P. syringae* (Cuppels *et al.* 1990; Schaad *et al.* 1989). The 8.3-kb phaseolotoxin gene probe was specific for *P. s.* pv. *phaseolicola* (Peet *et al.* 1986), and the 5.3-kb coronatine gene probe was restricted to the closely related *P. syringae* pathovars *atropurpurea*, *glycinea*, *maculicola*, and *tomato* (Cuppels *et al.* 1990). The specificity of the *syr* probes for *P. s.* pv. *syringae* and related epiphytic strains further demonstrates that genes dedicated to phytotoxin synthesis or export are highly pathovar-specific and could serve as powerful tools in disease diagnosis. Genomic DNA from pathovars that commonly coexist with *P. s.* pv. *syringae*, such as *P. s.* pv. *morsprunorum* on stone fruit trees (Lattore and Jones 1979) and *P. s.* pv. *pisi* on peas (Mazarei and Kerr 1990) failed to hybridize with the *syr* probes. The lack of *syr* gene homologs in *P. s.* pv. *pisi* is particularly significant because this pathovar shares the highest overall DNA homology to *P. s.* pv. *syringae* (Hildebrand *et al.* 1982; Pecknold and Grogan 1973). Rich *et al.* (1992) used a large DNA fragment of 6.1 kb containing the *lemA* gene of *P. s.* pv. *syringae* strain B728a as a probe to identify seven RFLP groups among 80 strains of *P. s.* pv. *syringae* isolated from bean leaves. Unfortunately, the *lemA* probe was not specific, hybridizing to all fluorescent pseudomonads tested including *P. aeruginosa*. Consequently, the regulatory protein predicted to be encoded by *lemA* (Hrabak and Willis 1992, 1993) and to control syringomycin production appears to be high in the regulatory hierarchy of pseudomonads and not dedicated to toxigenesis. Nevertheless, flanking DNA may have been responsible for hybridization in some strain/probe combinations. The obvious advantage to using our *syr* probes in RFLP studies is that they contain no extraneous flanking DNA sequences and are associated with a pathovar-specific virulence trait.

The maximum parsimony tree, constructed from the RFLP profiles of 42 strains, conveys the smallest number of evolutionary changes among strains within the *syrBD* region (Fig. 4). The tree revealed subclusters of strains that appear to share specific qualities relevant to the plant-pathogen interaction. For example, a large proportion of the strains exhibit the same RFLP profile as strain B301D; all of these strains originated from dicot plants. Several of these strains, including B301D and B3A, have been tested for pathogenicity to monocots; the strains failed to grow in maize leaves and cause holcus spot disease (Gross and DeVay 1977a). Consequently, it would not be surprising to find that strains within this subcluster are restricted to dicot hosts such as cherry. Furthermore, strains originally isolated from holcus spot lesions were clustered and may reflect a general capacity, such as with strains Ps17 and HS191, to cause typical disease symptoms in both monocot and dicot plants (Gross and DeVay 1977a). Denny *et al.* (1988) assessed the genetic diversity among a sampling of monocot and dicot strains of *P. s.* pv. *syringae* by RFLP analysis, using random segments of the genome of a strain of *P. s.* pv. *tomato*. Two strain clusters were observed that were restricted to either monocot or dicot strains. In our study, three strains from monocots (i.e., SC1, 61, and 761-5) displayed very different RFLP profiles from other strains of monocot origin. Although strains 61 and 761-5 appear to be nonpathogenic (Table 1) (Gross and DeVay 1977a), SC1 was originally isolated from Japanese sugarcane (Fukuchi *et al.* 1992b) and is weakly virulent in tests of immature cherry fruits. Thus, the monocot strains may form two genetically distinct strain clusters. The placement of strain 61 at a relatively long distance from most strains of *P. s.* pv. *syringae* is especially noteworthy because it is the source of the *hrp* gene cluster most extensively studied in a fluorescent pseudomonad (Huang *et al.* 1991).

Another prominent observation is that the syringotoxin- or syringostatin-producing strains (B457, Ps268, and SY12) were placed on a distinct branch of the maximum parsimony tree (Fig. 4). Because syringotoxin and syringostatin structurally differ by only one amino acid (Fig. 2), it is interesting that they display similar RFLP profiles in the *syrBD* region. Furthermore, one can conjecture that RFLP analysis can be used to distinguish strains that produce either syringotoxin or syringostatin. Unfortunately, SY12 is the only strain known to produce syringostatin; consequently, more extensive surveys of syringotoxin-producing strains are needed to test this hypothesis. Evidence suggests that syringomycin is produced by a large majority of *P. s.* pv. *syringae* strains (Gross 1991); known syringomycin-producing strains, such as B301D, HS191, SC1, PA105, and 761-5 (Ballio *et al.* 1990, 1991; Gross and DeVay 1977b; Fukuchi *et al.* 1992b), were distributed throughout the branches of the tree (Fig. 4).

In conclusion, the *syrB* and *syrD* genes are conserved among a diverse spectrum of *P. s.* pv. *syringae* strains, and their presence is strictly correlated with production of syringomycin or one of its amino acid analogs. We also substantiate the importance of plant signal molecules in controlling toxin production. Therefore, it is not surprising that surveys of wild-type strains typically identify strains that do not produce toxin *in vitro*, since the media used are, or can be, devoid of plant signal molecules that activate the syringomycin biosynthetic apparatus. We intentionally included several strains that historically failed to produce toxin *in vitro*, despite the capability to infect and cause typical disease symptoms, and demonstrated that they were toxigenic. Although

the weakly virulent strain W4N50 did not produce syringomycin and lacked sequences homologous to either of the *syr* probes, it perhaps belongs to a different pathovar such as *P. s.* pv. *morsprunorum* that causes similar infections of cherry fruits (Latorre and Jones 1979). RFLP analysis using the *syr* probes documented the extensive genetic diversity among *P. s.* pv. *syringae* and related nonpathogenic strains, but it also revealed strain clusters that possibly reflect specialized plant-pathogen interactions. These results are a valuable foundation for exploring the genetic diversity among populations of *P. s.* pv. *syringae*.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions.

The *Pseudomonas* strains used in this study are listed in Table 1 along with their original plant source and location. Approximately half of the *P. s.* pv. *syringae* and related *P. syringae* strains were isolated from plant disease lesions; the remainder (identified by the W4N prefix) were isolated between 1980 and 1982 as ice-nucleation-active epiphytes from pear, apple, and sweet cherry trees in the Yakima Valley of Washington and the Hood River Valley of Oregon (Gross *et al.* 1984). Strains SC1 and SY12 produce syringomycin and syringostatin, respectively, and were received from K. Suyama (Tokyo University of Agriculture, Tokyo). The syringotoxin-producing strain B457 was obtained from A. K. Chatterjee (University of Missouri, Columbia). Strains B728a and 61 were provided by D. K. Willis (University of Wisconsin, Madison) and A. Collmer (Cornell University, Ithaca, NY), respectively. Strain PA105 was included to represent sugar beet strains that have been called *P. s.* pv. *aptata* and are documented as producers of syringomycin (Gross and DeVay 1977a,b; Otta and English 1971). Phaseolotoxin-producing strains 4612-9 and G50-1 of *P. s.* pv. *phaseolicola* were obtained from S. Patil (University of Hawaii, Honolulu), and strain N4SP was obtained from A. K. Vidaver (University of Nebraska, Lincoln); coronatine-producing strains of *P. s.* pv. *tomato* were obtained from D. A. Cuppels (Agriculture Canada, London, ON); and tabtoxin-producing strains of *P. s.* pv. *tabaci* and tagetitoxin-producing strains of *P. s.* pv. *tagetis* were obtained from D. K. Willis. Strains of *P. s.* pv. *pisi* were obtained from L. A. Hadwiger (Washington State University, Pullman). All other *Pseudomonas* strains were previously described (Gross *et al.* 1984; Gross and DeVay 1977a,b; Vidaver *et al.* 1972) and were maintained as lyophilized stocks in the laboratory strain collection.

Escherichia coli strain DH5α (Hanahan 1985) was used as the host for recombinant plasmids containing *syr* gene sequences. Plasmids pUC18 and pUC19 (Yanisch-Perron *et al.* 1985) were used as high-copy-number cloning vectors. The source of the *syrB* and *syrD* gene sequences was pYM101 (Quigley *et al.* 1993), which consists of pUC19 carrying a 16-kb *Hin*dIII fragment of genomic DNA from strain B301D (Mo and Gross 1991a).

Pseudomonas strains were cultured routinely in nutrient broth-yeast extract (NBY) liquid and agar media (Vidaver 1967); long-term preservation of strains was as described by Gross *et al.* (1984). Late exponential phase cells grown in NBY broth at 25° C on a rotary shaker (250 rpm) were used for preparation of whole-cell (total) DNA. Cells grown in NBY were used also as inocula in assays for virulence in cherry fruits and for syringomycin production *in vitro*. Cells were washed twice in sterile deionized water and then suspended to approximately 10^8 cfu/ml in sterile deionized water (Xu and Gross 1988a). PDA and PDB media, used in tests for syringomycin production, contained 1.5% glucose and 0.4% vitamin-free casamino acids (Difco Laboratories, Detroit, MI) (Gross 1985). In tests for induction of syringomycin production by plant signal molecules, SRM and SRM_{AF} media were prepared as described by Mo and Gross (1991b). SRM_{AF} medium is complete SRM medium supplemented with 100 μM arbutin (Sigma Chemical Co., St. Louis, MO) and 0.1% D-fructose (J. T. Baker, Inc., Phillipsburg, NJ). *E. coli* strains were cultured and maintained as described by Quigley *et al.* (1993). The pUC18/19 plasmids and derivatives were maintained in *E. coli* by growth at 37° C on Luria-Bertani (LB) agar or in LB broth (Sambrook *et al.* 1989) supplemented with ampicillin (25 μg/ml, Sigma).

Assays for syringomycin production.

All *P. syringae* strains and *P. viridiflava* W4N1 were assayed for syringomycin production in PDB, SRM, and SRM_{AF} as described by Gross (1985) and Gross and DeVay (1977b). *G. candidum* F-260 was used in bioassays to determine yield of syringomycin, syringotoxin, and syringostatin (in U/ml) from 50-ml liquid cultures. The standing cultures, incubated for 5 days at 25° C, were killed with an equal volume of acetone and acidified with concentrated HCl (100 μl). After removal of cellular debris by centrifugation (11,700 *g*, 15 min), the supernatant solutions were concentrated to 2.0 ml by flash evaporation and assayed for syringomycin activity. A unit of syringomycin activity is defined as the minimum amount of toxin contained within a 10-μl droplet that completely inhibits the growth of *G. candidum* within the area of application on PDA (Sinden *et al.* 1971). This also was used to define units of syringotoxin and syringostatin activity. Duplicate cultures of each strain were prepared in all three liquid media, and the experiments were repeated on separate occasions. Because strains of *P. viridiflava* and pathovars other than *P. s.* pv. *syringae* do not produce syringomycin, they were tested on one occasion only. The method of Gross and DeVay (1977a) was used to assay for syringomycin production on PDA. Individual strains were tested on three PDA plates, and each plate was spotted at two sites with 5 μl of the same inoculum prepared for liquid media. All cultures were incubated for 5 days at 25° C before being oversprayed with a spore suspension of *G. candidum*. Zones of inhibition of *G. candidum* were measured from the margins of bacterial colonies. PDA assays for syringomycin production were repeated on a separate occasion.

Virulence assays and tobacco hypersensitivity tests.

All *Pseudomonas* strains were tested for pathogenicity in immature sweet cherry (*P. avium* L. cv. Bing) fruits by the method described by Xu and Gross (1988a) as modified by Mo and Gross (1991a). Briefly, cherry fruits were inoculated at three sites with 5×10^4 cells (within a 2.5-μl droplet) per inoculation site. After 3 days of incubation at 20° C, fruits were evaluated for disease symptoms. Virulence of strains was rated according to the following scale: nonpathogenic

strains = 0; weakly virulent = 1; moderately virulent = 2; and highly virulent = 3. Fruits inoculated with nonpathogenic strains resembled fruits injected with sterile water. Weakly virulent strains caused necrotic lesions of 1–3 mm in diameter with no other discoloration of fruits. Moderately virulent strains formed large sunken lesions ≥3 mm in diameter that were not coalesced but were found with some discoloration of surrounding tissue. Highly virulent strains formed very large lesions that usually coalesced to encompass 50–100% of the fruit surface. Ten fruits per strain were inoculated in each trial; the pathogenicity tests of all strains were repeated once on a different day. Fruits were stored no longer than 1 wk before use in pathogenicity tests.

Leaves of *Nicotiana tabacum* cv. White Burley were used to test strains for the ability to induce a hypersensitive reaction as described by Gross *et al.* (1984).

Preparation and manipulation of DNA.

Plasmid DNA was prepared according to Ish-Horowicz and Burke (1981). Total DNA was isolated from 1 ml of late exponential phase NBY culture as described by Nakamura *et al.* (1979). Plasmid and total DNA were digested with restriction enzymes diluted in general-purpose potassium glutamate buffer at the appropriate dilution for the specific enzyme (Hanish and McClelland 1988).

Selection of *syrB* and *syrD* gene-specific hybridization probes.

Plasmids p91 and p9 were chosen as probes for *syrB*- and *syrD*-specific DNA, respectively, and maintained in *E. coli* DH5α. Both plasmids contain DNA sequences from strain B301D that were obtained from the pYM101 clone described by Quigley *et al.* (1993). The p91 construct is pUC18 carrying a 1,107-bp *Sal*I internal fragment of the *syrB* gene, and p9 is pUC19 carrying a 510-bp fragment containing 500 bp of the 5′-end of *syrD* (to the *Eco*RI site) and 10 bp of upstream DNA (Fig. 1). The insert fragment in p9 was the product of *Bal*31 deletion of a larger fragment initiated at the *Eco*RV site upstream of *syrD*.

Southern hybridization.

Plasmid DNA (p91 or p9) was labeled to approximately 10^8 cpm/μg by nick translation with the Klenow fragment of DNA polymerase I (Boehringer-Mannheim, Indianapolis, IN) and [α-^{32}P]dCTP (>3,000 Ci/mM; New England Nuclear, Boston, MA) as described by Sambrook *et al.* (1989). Restriction fragments of total DNA were resolved by electrophoresis in 0.7–0.8% Tris-borate agarose gels (Sambrook *et al.* 1989); *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, and *Sal*I were purchased from Gibco BRL (Gaithersburg, MD). Molecular size standards (in kb) consisted of linear bacteriophage lambda DNA digested with *Hin*dIII. DNA fragments were transferred to BA85 nitrocellulose membranes (Schleicher & Schuell, Keene, NH) by Southern blotting, baked, and hybridized with radioactively labeled intact plasmid DNA, as described by Sambrook *et al.* (1989). Hybridizations were conducted overnight at 42° C in 0.4 M NaCl containing 20% redistilled and deionized formamide (U.S. Biochemical Corp., Cleveland, OH). The membranes were washed at 25° C in 2× SSC (i.e., 0.3 M NaCl, 30 mM trisodium citrate) containing 0.1% sodium dodecyl sulfate, and blotted before baking. These moderately stringent conditions were calculated to permit an optimal rate of hybridization for DNA fragments that contained an average sequence mismatch of up to 25% (Meinkoth and Wahl 1984); the average G + C content of *P. syringae* genomic DNA is ~60% (Quigley *et al.* 1993). Preliminary experiments using a higher hybridization stringency (permitting less that 10% mismatch) gave very weak signals between the *syr* probes and DNA from *P. s.* pv. *syringae* strains. Lower stringencies (permitting ~40% mismatch) gave unacceptably high nonspecific background hybridization. Kodak X-Omat RP film (Eastman Kodak Co., Rochester, NY) was exposed to the radioactive membranes at –80° C.

Analysis of RFLP profiles.

The RFLP profiles of 42 strains of *P. syringae* generated by hybridization to the *syrB*- and *syrD*-specific gene probes were analyzed by both maximum parsimony and distance matrix methods to evaluate relatedness among strains. For each strain, RFLP profiles were recorded for genomic DNA cleaved by *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, and *Sal*I by measuring the sizes of DNA bands that hybridized to the *syr* gene probes. The sizes of homologous DNA fragments were used to construct multistate data matrices (42 strains by five restriction enzyme digestions) for both the *syrB* and *syrD* gene probes. Hybridizing DNA fragments for the 42 *P. syringae* strains were assigned a number code, with 1 corresponding to the largest fragment within each enzyme-probe combination. Maximum parsimony analysis of the RFLP data was performed by using the PAUP 3.1.1 computer program (Swofford 1993). The output tree was rooted at the midpoint of the longest pathway between two strains. Distance matrix analysis of RFLP data was performed using the NTSYS-pc version 1.70 computer software package (Applied Biostatistics Inc., Setauket, NY). Similarity matrices were generated using the SIMQUAL program and used as input for cluster analyses by the SAHN program. The UPGMA, single-link, and complete-link clustering methods were used, and outputs were displayed as consensus trees for comparisons of relatedness among strains in the *syr* gene region.

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