Characterization of the *salA*, *syrF*, and *syrG* Regulatory Genes Located at the Right Border of the Syringomycin Gene Cluster of *Pseudomonas syringae* pv. *syringae*

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Sequence analysis of the right border of the *syr* gene cluster of *Pseudomonas syringae* pv. *syringae* strain B301D revealed the presence of the *salA* gene 8,113 bp downstream of *syrE*. The predicted SalA protein of strain B301D differs by one amino acid from that of strain B728a. Two homologs of *salA*, designated *syrF* and *syrG*, were identified between *syrE* and *salA*. All three proteins contain helix-turn-helix DNA-binding motifs at their C termini and exhibit homology to regulatory proteins of the LuxR family. A *salA* mutant failed to produce syringomycin, whereas *syrF* and *syrG* mutants produced 12 and 50%, respectively, of syringomycin relative to the wild-type strain. The *salA*, *syrF*, and *syrG* mutants were significantly reduced in virulence, forming small, nonspreading lesions in immature cherry fruits. Translational fusions to the *uidA* gene were constructed to evaluate expression of *syrB1* in regulatory mutant backgrounds and to determine the relationship among the three regulatory loci. Expression of a *syrB1::uidA* fusion required functional *salA* and *syrF* genes and, in series, the expression of a *syrF::uidA* fusion required a functional *salA* gene. These results demonstrate that *salA* is located upstream of *syrF* in the regulatory hierarchy controlling syringomycin production and virulence in *P. syringae* pv. *syringae*.

A distinctive feature of *Pseudomonas syringae* pv. *syringae* is the production of two classes of lipopeptide phytotoxins, syringomycins and syringopeptins, that cause necrosis in host plants (Bender et al. 1999; Gross 1991). Both toxins exhibit a similar structure consisting of a polar peptide head attached to a hydrophobic fatty acid tail (Ballio et al. 1991; Fukuchi et al. 1992). Multimers of each toxin form pores in plasma membranes that result in transmembrane fluxes of cations and can cause lysis of cellular membranes. Consequently, the two toxins are not host specific but cause necrotic symptoms in plants parasitized by the bacterium (Bender et al. 1999; Hutchison

and Gross 1997). Biosynthesis mutants defective in both syringomycin and syringopeptin were reduced in virulence by 60 to 70% relative to the wild-type strain, demonstrating that the two toxins are major virulence determinants of *P. syringae* pv. *syringae* (Scholz-Schroeder et al. 2001a; Zhang et al. 1995).

Enzymatic analysis and DNA sequence analyses indicate that syringomycin and syringopeptin are synthesized separately by a nonribosomal thiotemplate mechanism (Grgurina and Mariotti 1999; Scholz-Schroeder et al. 2001b; Zhang et al. 1995). The syringomycin (*syr*) and syringopeptin (*syp*) gene clusters of *P. syringae* pv. *syringae* strain B301D are located adjacent to each other on an approximately 145-kb *DraI* fragment of genomic DNA (Scholz-Schroeder et al. 2001b). The *syr* gene cluster encompasses an approximately 55-kb DNA region (Fig. 1) (Scholz-Schroeder et al. 2001b). The most striking feature of the *syr* gene cluster is the presence of an enormous *syrE* gene predicted to encode a 1,039-kDa nonribosomal peptide synthetase containing eight amino acid activation modules (Guenzi et al. 1998). Other biosynthesis genes within the cluster include *syrB1*, which encodes the ninth activation module responsible for activation of L-threonine (Grgurina and Mariotti 1999; Zhang et al. 1995); *syrC*, which is predicted to function as a thioesterase; and *syrB2*, which is predicted to modify L-threonine bound to the *SyrB1* thiolation domain (Bender et al. 1999; Zhang et al. 1995). The *syrD* gene lies at the left border of the *syr* gene cluster and upstream of the *syp* gene cluster and encodes an ATP-binding protein associated with syringomycin and syringopeptin secretion (Grgurina et al. 1996; Quigley et al. 1993). The *syrP* gene, located between the *syrD* and *syrB1* genes, was proposed by Zhang and associates (1997) to encode an intermediate phosphate transmitter in a phosphorelay system of signal transduction. Recently, the regulatory gene *salA* (Kitten et al. 1998) was localized approximately 8.5 kb downstream of *syrE*, based on preliminary mapping analysis (Scholz-Schroeder et al. 2001b). However, the right border region of the *syr* gene cluster remains to be characterized. In contrast to the *syr* gene cluster, the *syp* gene cluster encompasses an approximately 80-kb DNA region with three very large peptide synthetase genes (*sypA*, *sypB*, and *sypC*) encoding 22 amino acid activation modules (B. K. Scholz-Schroeder, J. D. Soule, and D. C. Gross, unpublished data).

Regulation of syringomycin production is complex. In the bean strain *P. syringae* pv. *syringae* B728a, the *gacS/gacA* regulon plays a critical role in the expression of the *syrB1* synthetase gene (Hrabak and Willis 1992; Kitten et al. 1998; Rich

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et al. 1994). The *gacS* and *gacA* genes, respectively, encode a sensor and a response regulator of a two-component signal transduction system that controls syringomycin production and the ability to cause necrotic lesions in plants. Sequence analysis revealed that GacS is a transmembrane protein, which presumably functions as a histidine protein kinase that undergoes phosphorylation in response to environmental stimuli (Hrabak and Willis 1992). GacA is a cognate response regulator protein that contains a receiver domain (phosphorylation site) at the N terminus and a helix-turn-helix (HTH) DNA-binding motif at the C terminus (Rich et al. 1994). GacS/GacA homologs are widely conserved in fluorescent pseudomonads as a global regulation system that controls the expression of cellular functions such as production of protease, pectate lyase, N-acylhomoserine lactones, and various antimicrobial metabolites (Gaffney et al. 1994; Kitten et al. 1998; Pierson et al. 1998). The *salA* gene of strain B728a was recently characterized as a member of the *gacS/gacA* regulon (Kitten et al. 1998). Sequence analysis showed that the predicted SalA protein contains an HTH DNA-binding motif with similarity to response regulators of signal transduction systems such as DctR (Hamblin et al. 1993) and FixJ (Anthamatten and Hennecke 1991), but SalA lacks a receiver domain characteristic of response regulators. Expression analysis revealed that *gacS* and *gacA* positively regulate a *salA::lacZ* reporter gene fusion, and in turn, SalA activates the expression of a *syrB1::lacZ* reporter (Kitten et al. 1998).

Nutritional factors, such as inorganic phosphate and iron, and phenolic plant signal molecules control syringomycin production based on assays of antifungal activity to *Geotrichum candidum* and expression of a *syrB1::lacZ* reporter system in strain B301D (Gross 1985; Mo and Gross 1991a; Mo et al. 1995). Syringomycin production requires Fe^{3+} concentrations of 2 μM or greater and is suppressed by inorganic phosphate concentrations of 1 mM or greater based on toxin bioassays (Gross 1985). Furthermore, the *syrB1::lacZ* reporter system was used to demonstrate that Fe^{3+} concentrations greater than 2 μM are required for expression of the *syrB1* synthetase gene

(Mo and Gross 1991b). A significant discovery is that specific plant signal molecules are required for activation of the *syrB1-lacZ* transcriptional fusion and syringomycin production (Mo and Gross 1991a; Mo et al. 1995; Quigley and Gross 1994). Phenolic glycosides, such as arbutin, serve as the primary signals, and sugars, such as D-fructose, significantly enhance the phenolic signal activity (Mo and Gross 1991a; Mo et al. 1995). More importantly, the stimulatory effect of the plant signal molecules was demonstrated for most strains of *P. syringae* pv. *syringae* (Quigley and Gross 1994). Temperature and pH also influence syringomycin production (Gross 1985, 1991). The various environmental factors known to modulate syringomycin production are indicative of a complex regulatory cascade controlling lipodepsipeptide production in *P. syringae* pv. *syringae*.

Despite evidence that the *gacS*, *gacA*, *salA*, and *syrP* genes are involved in regulating syringomycin production, the regulatory cascade (or cascades) is largely unexplored. The *salA* gene of strain B301D has not been characterized, nor is its regulatory effect on syringopeptin production known. In this study, we hypothesize that other regulatory genes are present in the right border region of the *syr* gene cluster and that they control the expression of genes dedicated to biosynthesis of syringomycin, syringopeptin, or both toxins, thereby strongly influencing the virulence of *P. syringae* pv. *syringae*. To test our hypothesis, the right border of the *syr* gene cluster was identified from a genomic library of strain B301D, and approximately 20 kb of DNA downstream of *syrE* was sequenced and analyzed. The *salA* gene of strain B301D was characterized and compared with the *salA* locus of strain B728a. In addition, two *salA* homologs, *syrF* and *syrG*, were identified and characterized. Effects of mutations in the *salA*, *syrF*, and *syrG* genes on phytotoxin production and virulence are reported. Evidence is presented that the *salA* and *syrF* genes positively regulate expression of a *syrB1::uidA* reporter and that *salA* controls expression of the *syrF* gene fused to the *uidA* reporter. These results are discussed in regard to the regulatory cascade controlling syringomycin and syringopeptin production.

Fig. 1. Map of the 75-kb region on the chromosome of *Pseudomonas syringae* pv. *syringae* B301D containing *sypA*, all of the known *syr* biosynthesis genes, and the right border of the *syr* cluster. The positions and orientations of the known genes and potential open reading frames (ORFs) are shown as horizontal arrows. The solid red arrows indicate the regulatory genes, *salA*, *syrF*, *syrG*, and *syrP*. The solid pink, orange, and gray arrows indicate additional ORFs identified at the right border region of the *syr* gene cluster. Previously described *syr* and *syp* biosynthesis genes are represented by solid green arrows. The black vertical arrows indicate the positions of the *nptII* insertions. The yellow triangular flags indicate the positions of *uidA-aacCI* insertions. The locations of cosmids *p29* and *p634* are shown as horizontal lines. The restriction enzyme sites are indicated as follows: E = EcoRI, K = KpnI, D = DraI.

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Source
<i>Escherichia coli</i>		
DH10B	F ⁻ <i>mcrA</i> Δ <i>lacX74</i> (ϕ 80 <i>dlacZ</i> Δ M15) Δ (<i>mrr-hsdRMS-mcrB</i>) <i>deoR recA1 endA1 araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU galK</i> λ^- <i>rpsL nupG</i>	Grant et al. 1990
<i>Pseudomonas syringae</i> pv. <i>syringae</i>		
B301D	Wild type from pear	Cody et al. 1987
BR132	<i>syrB1::Tn3HoHo1</i> derivative of B301D-R; Pip ^r Rif ^r	Mo and Gross 1991
BR208	<i>sypA::Tn3HoHo1</i> derivative of B301D-R; Pip ^r Rif ^r	Scholz-Schroeder et al. 2001a
BR-DBL1	<i>sypA::nptII</i> derivative of BR132; Pip ^r Rif ^r Km ^r	Scholz-Schroeder et al. 2001a
B301DSL1	<i>syrF::nptII</i> derivative of B301D; Km ^r	This study
B301DSL6	<i>syrG::nptII</i> derivative of B301D; Km ^r	This study
B301DSL7	<i>sala::nptII</i> derivative of B301D; Km ^r	This study
B301DSL8	<i>syrB1::uidA-aacC1</i> in forward orientation, derivative of B301D; Gm ^r	This study
B301DSL9	<i>syrB1::uidA-aacC1</i> in reverse orientation, derivative of B301D; Gm ^r	This study
B301DSL10	<i>syrF::nptII</i> derivative of B301DSL8; Gm ^r Km ^r	This study
B301DSL11	<i>syrG::nptII</i> derivative of B301DSL8; Gm ^r Km ^r	This study
B301DSL12	<i>sala::nptII</i> derivative of B301DSL8; Gm ^r Km ^r	This study
B301DSL15	Marker-rescued derivative of strain B301DSL1	This study
B301DSL16	Marker-rescued derivative of strain B301DSL6	This study
B301DSL17	Marker-rescued derivative of strain B301DSL7	This study
Plasmid		
pBluescriptSK(+)	Cloning vector; Ap ^r	Stratagene, La Jolla, CA, U.S.A.
pBR325	Cloning vector; Cm ^r Tc ^r Ap ^r	Prentki et al. 1981
pLAFR3	Cosmid vector; Tc ^r	Staskawicz et al. 1987
pRK415	Broad host range vector; Tc ^r	Keen et al. 1988
pBI101	Binary vector containing <i>uidA</i> gene; Km ^r	Clontech, Palo Alto, CA, U.S.A.
pUCGM	Gentamycin resistance gene cassette; Gm ^r	Schweizer 1993
pBSL15	Kanamycin resistance gene cassette; Km ^r	Alexeyev 1995
pYM101	pUC19 carrying the 16-kb <i>HindIII</i> fragment containing <i>syrD</i> , <i>syrB1</i> , <i>syrB2</i> , and <i>syrC</i> ; Ap ^r	Quigley et al. 1994
p601D-1	pRK415 carrying the 8-kb <i>HindIII</i> fragment of pYM101 with deletion of two <i>EcoRV</i> fragments upstream of <i>syrB1</i> ; Tc ^r	J. H. Zhang and D. C. Gross, unpublished data
p601D-1-R	pRK415 carrying the 8.0-kb <i>HindIII</i> fragment of p601D-1 inserted in opposite orientation; Tc ^r	This study
p2395	pBR325 carrying the 13-kb <i>EcoRI</i> fragment containing Tn5 insertion at the end of <i>syrE</i> ; Km Tc ^r	Scholz-Schroeder et al. 2001b
pBS008	pUC18 carrying the 5.0-kb <i>KpnI-EcoRI</i> fragment of pYM101 containing partial <i>syrD</i> and the start of <i>sypA</i> ; Ap ^r	Scholz-Schroeder et al. 2001a
p29	pLAFR3 carrying a 16-kb fragment of <i>P. syringae</i> pv. <i>syringae</i> B301D genomic DNA; Tc ^r	This study
P634	pLAFR3 carrying a 24-kb fragment of <i>P. syringae</i> pv. <i>syringae</i> B301D genomic DNA; Tc ^r	This study
pSL2	pBI101 with the 0.85-kb <i>aacC1</i> gene of pUCGM inserted at the <i>EcoRI</i> site downstream of the <i>uidA</i> gene; Km ^r Gm ^r	This study
pSL4	pBluescriptSK(+) carrying the 5.8-kb <i>BamHI-KpnI</i> fragment of p29 containing <i>syrF</i> ; Ap ^r	This study
pSL5	pBluescriptSK(+) carrying the 6.2-kb <i>KpnI-HindIII</i> fragment of p29 containing <i>orf4</i> , <i>orf5</i> , and <i>syrG</i> ; Ap ^r	This study
pSL8	pBR325 carrying the 3.0-kb <i>EcoRI</i> fragment of p29 containing <i>sala</i> , Tc ^r Ap ^r	This study
pSL9	pBluescriptSK(+) carrying the 2.5-kb <i>HindIII-EcoRV</i> fragment of pSL5 containing <i>syrG</i> ; Ap ^r	This study
pSL19	pBR325 carrying the 5.8-kb <i>BamHI-KpnI</i> fragment of p29 with <i>nptII</i> insertion at the <i>BstEII</i> site of <i>syrF</i> ; Ap ^r Tc ^r Km ^r	This study
pSL20	pBR325 carrying the 2.5-kb fragment of pSL9 with <i>nptII</i> insertion at the <i>EcoRI</i> site of <i>syrG</i> ; Ap ^r Tc ^r Km ^r	This study
pSL21	pBR325 carrying the 3.0-kb fragment of p29 with <i>nptII</i> insertion at the <i>KpnI</i> site of <i>sala</i> ; Ap ^r Tc ^r Km ^r	This study
pSL26F	pBR325 carrying 5.0-kb fragment of p601D-1-R with the 3.2-kb <i>uidA-aacC1</i> fragment from pSL2 in-frame of <i>syrB1</i> at the <i>EagI</i> site in forward orientation; Gm ^r Ap ^r	This study
pSL26R	pBR325 carrying 5.0-kb fragment of p601D-1-R with the 3.2-kb <i>uidA-aacC1</i> fragment from pSL2 in-frame of <i>syrB1</i> at the <i>EagI</i> site in reverse orientation; Gm ^r Ap ^r	This study
pSL35	pRK415 carrying the 5.8-kb <i>BamHI-KpnI</i> fragment of p29 containing <i>syrF</i> ; Tc ^r	This study
pSL36	pRK415 carrying the 6.2-kb <i>KpnI-HindIII</i> fragment of p29 containing <i>orf4</i> , <i>orf5</i> and <i>syrG</i> ; Tc ^r	This study
pSL37	pRK415 carrying the 3.0-kb <i>EcoRI</i> fragment of p29 containing <i>sala</i> ; Tc ^r	This study
pSL38F	pLAFR3 carrying the 5.8-kb <i>BamHI-KpnI</i> fragment of p29 with the 3.2-kb <i>uidA-aacC1</i> fragment from pSL2 inserted at <i>BstEII</i> site in-frame of <i>syrF</i> in forward orientation; Tc ^r Gm ^r	This study
pSL38R	pLAFR3 carrying the 5.8-kb <i>BamHI-KpnI</i> fragment of p29 with the 3.2-kb <i>uidA-aacC1</i> fragment from pSL2 inserted at the <i>BstEII</i> site in-frame of <i>syrF</i> in reverse orientation; Tc ^r Gm ^r	This study
pSL39F	pLAFR3 carrying the 2.5-kb <i>HindIII-EcoRV</i> fragment of pSL9 with the 3.2-kb <i>uidA-aacC1</i> fragment from pSL2 inserted at the <i>EcoRI</i> site in-frame of <i>syrG</i> in forward orientation; Tc ^r Gm ^r	This study
pSL39R	pLAFR3 carrying the 2.5-kb <i>HindIII-EcoRV</i> fragment of pSL9 with the 3.2-kb <i>uidA-aacC1</i> fragment from pSL2 inserted at the <i>EcoRI</i> site in-frame of <i>syrG</i> in reverse orientation; Tc ^r Gm ^r	This study
pSL40F	pLAFR3 carrying the 3.0-kb <i>EcoRI</i> fragment of p29 with the 3.2-kb <i>uidA-aacC1</i> fragment from pSL2 inserted at the <i>KpnI</i> site in-frame of <i>sala</i> in forward orientation; Tc ^r Gm ^r	This study
pSL40R	pLAFR3 carrying the 3.0-kb <i>EcoRI</i> fragment of p29 with the 3.2-kb <i>uidA-aacC1</i> fragment from pSL2 inserted at the <i>KpnI</i> site in-frame of <i>sala</i> in reverse orientation; Tc ^r Gm ^r	This study
pSL51	pBR325 carrying the 5.8-kb <i>BamHI-KpnI</i> fragment of p29 at the <i>EcoRI</i> site; Tc ^r	This study
pSL52	pBR325 carrying the 6.2-kb <i>KpnI-HindIII</i> fragment of p29 at the <i>EcoRI</i> site; Tc ^r	This study

^a Cm^r, Tc^r, Ap^r, Km^r, Gm^r, Pip^r, and Rif^r = resistance to chloramphenicol, tetracycline, ampicillin, kanamycin, gentamycin, piperacillin, and rifampin, respectively.

RESULTS

Sequence and analyses of the right border region of the *syr* gene cluster.

To characterize the DNA region downstream of the *syrE* synthetase gene, a genomic library of *P. syringae* pv. *syringae* strain B301D was screened by colony hybridization with a probe localized to the 3' region of *syrE*. Plasmid p2395 is a 13-kb genomic subclone carrying a Tn5 insertion located 1.2 kb upstream of the *syrE* stop codon (Scholz-Schroeder et al. 2001b). A 1.1-kb *Cla*I fragment of p2395 was used as a probe for Southern hybridization to identify cosmid p29, which carries a 16-kb genomic DNA insert, from a *P. syringae* pv. *syringae* strain B301D library (Table 1). Cosmid p29 includes 4.5 kb at the 3' end of the *syrE* gene and approximately 11.5 kb of uncharacterized DNA downstream of *syrE*. A 3-kb *Eco*RI fragment of cosmid p29 was used in Southern hybridization to identify cosmid p634 (Fig. 1), which contains 24 kb of genomic DNA and overlaps p29 by approximately 4 kb. Restriction mapping analysis of p634 revealed a unique *Dra*I site that was used to delineate the right border region of the *syr-syp* gene clusters (Scholz-Schroeder et al. 2001b).

A 20.7-kb segment of DNA extending from the 3' end of *syrE* to the *Bam*HI site approximately 1.5 kb beyond the *Dra*I site in cosmid clone p634 was sequenced (Fig. 1). Sequence analyses revealed 10 potential open reading frames (ORFs) in this region. A notable feature of the 20.7-kb DNA region was the occurrence of an 852-bp ORF corresponding to the *sala* gene (Kitten et al. 1998), which was located 8,113 bp downstream of *syrE*. The B301D *sala* nucleotide sequence shared 99.3% identity to the *sala* sequence of strain B728a (Kitten et al. 1998). A conserved ribosomal-binding site was identified 6 to 9 bp (GGAA) upstream of the start codon of the *sala* ORF, and a potential rho-independent transcriptional terminator region was observed 213 to 244 bp downstream of the stop codon TGA.

Two additional homologs of *sala* were identified in the 8.1-kb region between *syrE* and *sala*. The first, designated *syrF*, was 828 bp in size and was mapped 97 bp downstream of *syrE*. A conserved ribosomal-binding site was identified 9 to 12 bp (AGGA) upstream of the start codon of *syrF*, but no rho-inde-

pendent or rho-dependent transcriptional terminator region was observed at the 3' end. The *syrF* gene exhibited 99.3% identity at the nucleotide level to *salC* (GenBank accession number AF297011), an uncharacterized ORF of *P. syringae* pv. *syringae* strain B728a. The second ORF with homology to *sala* identified in this region was designated *syrG*. The *syrG* gene was 786 bp in size, was mapped 6,192 bp downstream of *syrE*, and was preceded by a potential ribosomal-binding site 13 to 16 bp (GGAA) upstream of the start codon. A potential rho-independent terminator was located 100 to 150 bp downstream of the *syrG* stop codon. Similarly, the *syrG* gene shared 99.8% identity at the nucleotide level to *salB* (Gen Bank accession number AF299084), another uncharacterized ORF of *P. syringae* pv. *syringae* strain B728a. As observed for *syrC*, *syrP*, *syrE*, and the *syrB* operon, no typical σ^{70} -like promoter was present upstream of the *sala*, *syrF*, or *syrG* genes.

In addition to *sala*, *syrF*, and *syrG*, seven ORFs were identified in the 20.7-kb region downstream of *syrE* (Fig. 1). The start codon (ATG) of *orf1* overlapped the stop codon (TGA) of *syrF*, which indicates that *syrF* and *orf1* are organized in an operon. The predicted ORF1 protein containing 475 amino acids was most similar to outer membrane proteins associated with secretion systems in gram-negative bacteria. The OprM protein of *P. aeruginosa* (Nakajima et al. 2000) shared the highest degree of similarity to ORF1, with 67% identity. The protein deduced from *orf2* was only 123 amino acids in size, but it exhibited 31% identity to the N terminus of the CmaU protein associated with coronatine production by *P. syringae* pv. *glycinea* (Alarcón-Chaidez et al. 1999). Interestingly, a 603-bp DNA region between *orf1* and *orf2* showed 60% identity to part of an IS870-like element (Ullrich and Bender 1994). The predicted protein product of *orf3* contained 388 amino acids with 28% identity to the periplasmic fusion protein MexC of *P. aeruginosa* (Poole et al. 1996). Immediately downstream of *sala*, ORF4 was identified with 100% identity to ORF3 of strain B728a (Kitten et al. 1998). BLAST database searches with the predicted protein products of *orf5*, *orf6*, and *orf7* showed similarity to a DNA helicase-related protein (Smith et al. 1997), a protein from the GGDEF family containing response regulators (Hecht and Newton 1995; White et al. 1999), and a McpA chemotaxis protein (Kuroda et al. 1995), respectively. Composition analysis revealed that the G+C contents of *sala*, *syrF*, *syrG*, and other *orfs* in the approximately 20-kb DNA region downstream of *syrE* range from 53.1 to 60.4%. These values are not significantly different from those of *syrB1* (60.9%) (GenBank accession number U25130), *syrB2* (56.6%), *syrC* (59.8%), and *syrE* (61.8%) (GenBank accession number AF047828).

The *Sala*, *SyrF*, and *SyrG* regulatory proteins are members of the *LuxR* family.

Sequence analyses of the *Sala*, *SyrF*, and *SyrG* proteins of strain B301D, which are, respectively, 284, 276, and 262 amino acids in length, revealed homology to prokaryotic regulatory proteins. The *Sala* sequences of strains B301D and B728a differed by one amino acid (i.e., Ser 75 in B301D *Sala* and Asp 75 in B728a *Sala*). The B301D *Sala* protein showed 27% (Z score = 40.9) and 26% (Z score = 43.9) identity to the entire protein sequences of *SyrF* and *SyrG*, respectively, with the greatest sequence conservation occurring in the C-terminal region. In contrast, *SyrF* and *SyrG* exhibited 49% (Z score = 101.2) identity to one another. The HTH DNA-binding domains, frequently observed in prokaryotic regulatory proteins, were identified at the C termini of *Sala*, *SyrF*, and *SyrG*. In contrast, a potential PAS domain, named from founding eukaryotic members PER, ARNT, and SIM (Gu et al. 2000), was

Fig. 2. Relative inhibition due to production of syringomycin by different *Pseudomonas syringae* pv. *syringae* strains. The strains were incubated on potato dextrose agar for 4 days and then oversprayed with *Geotrichum candidum*. Zones of inhibition were measured from the margins of colonies. Inhibition due to syringomycin production by the mutants is shown relative to inhibition by the parental strain B301D. Differences between treatments were determined by Tukey's analysis of variance. Different letters indicate statistically significant differences ($\alpha = 0.01$). Vertical bars indicate standard error of the mean. Column 1, B301D (parental strain); 2, B301DSL1 (*syrF* mutant); 3, B301DSL6 (*syrG* mutant); 4, B301DSL7 (*sala* mutant); and 5, BR132 (*syrB1* mutant).

identified at the N terminus of the predicted SalA protein (residues at 20 to 81), but not in SyrF or SyrG. The PAS domain of SalA shared 38% similarity (Z score = 5.0) to the B repeat region of the PAS domain (Gu et al. 2000) of the oxygen sensor protein FixL (Gong et al. 1998). Database searches with BLAST demonstrated that approximately 60 amino acids in the C-terminal regions of SalA, SyrF, and SyrG shared significant similarity to that of known regulatory proteins. As observed for the B728a SalA protein (Kitten et al. 1998), the C terminus of SalA from B301D exhibited significant identity to GerE (43%; Cutting and Mandelstam 1986), DctR (37%; Hamblin et al. 1993), and RcsB (36%; Jayaratne et al. 1993). In comparison, the C-terminal sequences of both SyrF and SyrG exhibited 27 to 38% identity to RcsB, GerE, DctR, and FixJ (Anthamatten and Hennecke 1991), which are members of the LuxR family (Bairoch 1993; Prosite Database). In addition, a pattern search showed that the SalA, SyrF, and SyrG proteins contain the consensus pattern of the LuxR family (Bioinformatics Group). Finally, the conserved C-terminal regions of SalA, SyrF, and SyrG shared 20 to 27% identity to three possible transcriptional regulators of *P. aeruginosa* PAO1 (GenBank accession numbers PA2899, PA3599, and PA1760 respectively) that were predicted to be members of the LuxR family of regulatory proteins (Pseudomonas Genome Project).

Further analyses of the C-terminal regions of SyrF, SyrG, and SalA identified a three-element fingerprint that provides a signature for the HTH DNA-binding motif of LuxR bacterial regulatory proteins (Bairoch 1993; Prosite Database) in SalA (211 to 254 residues), SyrF (218 to 261 residues), and SyrG (201 to 244 residues). Phylogenetic analysis indicated that SyrF, SyrG, and SalA are most closely related to the response regulator subfamily exemplified by FixJ and DctR (data not shown). However, four highly invariant residues in the amino-terminal regions of members of the response regulator subfamily corresponding to Asp11, Asp12, Asp55, and Lys105 in FixJ (Parkinson and Kofoid 1992) were not observed in SyrF, SyrG, or SalA. Similarly, five highly conserved amino acids in the amino-terminal regions of members of an autoinducer-binding subfamily (Fuqua et al. 1996) corresponding to the highly conserved Trp59, Tyr69, Asp79, Pro80, and Gly121 of LuxR were not found in SyrF, SyrG, or SalA.

Site-directed insertional mutagenesis of the *salA*, *syrF*, and *syrG* genes and effects on syringomycin production.

Nonpolar mutations were constructed in the *salA*, *syrF*, and *syrG* genes by insertion of the *nptII* cassette, which encodes kanamycin resistance, at the unique *KpnI*, *BstEII*, and *EcoRI* sites of the three respective ORFs. Marker exchange mutagenesis of the *nptII*-disrupted ORFs individually into the genome of strain B301D generated *salA* mutant B301DSL7, *syrF* mutant B301DSL2, and *syrG* mutant B301DSL6. Southern analysis verified recombination of the *nptII* disruption of each gene in the genome of *P. syringae* pv. *syringae* B301D (data not shown).

Strains B301DSL7, B301DSL2, and B301DSL6 carrying nonpolar insertions of the *nptII* cassette were affected in syringomycin production based on reductions in inhibitory activity to *Geotrichum candidum* (Fig. 2). The *salA* mutant B301DSL7 exhibited no detectable antifungal activity toward *G. candidum*. In contrast, the parental strain B301D produced a large zone of inhibition approximately 14 mm in radius. The *syrF* mutant B301DSL1 produced a small zone of inhibition approximately 2 mm in radius, which corresponds to 12% of the toxin produced by parental strain B301D. The *syrG* mutant B301DSL6 produced a zone of inhibition approximately 8 mm

in radius, which is about one half the size of the zone produced by parental strain B301D. Control strains BR132, a *syrB1* mutant, and BR-DBL1, a *syrB1-sypA* double mutant, failed to produce zones of antifungal activity as reported previously (Scholz-Schroeder et al. 2001a; Zhang et al. 1995). Marker rescue of *salA*, *syrF*, and *syrG* mutants with the corresponding wild-type genes resulted in strains B301DSL15, B301DSL13, and B301DSL14, which were as inhibitory to *G. candidum* as the wild-type strain B301D. Similarly, wild-type levels of inhibition were observed for B301DSL1, B301DSL6, and B301DSL7 complemented *in trans* with pRK415 carrying wild-type *salA*, *syrF*, and *syrG* genes, respectively (data not shown).

The *salA* and *syrF* are essential for expression of the *syrB1* synthetase gene.

A fusion of the *syrB1* synthetase gene to the *uidA* gene encoding β -glucuronidase (GUS) activity was used as a reporter to evaluate possible regulatory effects of *salA*, *syrF*, and *syrG* on expression of a gene dedicated to syringomycin biosynthesis. The *syrB1::uidA* translational fusion was constructed by insertion of the 3.2-kb *uidA-aacC1* fragment (from pSL2) at the *EagI* site of the *syrB1* ORF (Fig. 1) and selection with Gm^r. Sequence analysis verified that the *uidA* reporter was inserted in-frame 456 bp downstream of the start codon of *syrB1*. Marker exchange of the *syrB1::uidA* reporter into the genome of strain B301D resulted in strain B301DSL8, which expressed high levels of GUS activity when cultured on potato dextrose agar (PDA) and assayed using 5-bromo-4-chloro-3-indolyl- β -D-glucuronide as substrate. In contrast, strain B301DSL9, generated by marker exchange of the *uidA-aacC1* construct inserted in the reverse orientation to *syrB1* transcription, failed to express GUS activity. To determine the effects of *nptII*-disrupted *syrF*, *syrG*, and *salA* mutations on *syrB1* expression, constructs pSL19, pSL20, and pSL21 were introduced into the B301DSL8 genome by marker exchange, resulting in respective strains B301DSL10, B301DSL11, and B301DSL12 (Table 1). Southern analyses with *uidA* and *nptII* probes verified the presence of both the *syrB1::uidA* reporter and the *nptII*-disrupted genes in the genomes of the double mutant strains B301DSL10, B301DSL11, and B301DSL12 (data not shown). Based on failure to inhibit *G. candidum* in standard bioassays, all marker exchange mutants failed to produce syringomycin.

Expression of the *syrB1::uidA* reporter was significantly reduced in both the *salA* (B301DSL12) and *syrF* (B301DSL10) mutants based on quantitative GUS assays with 4-methylumbelliferyl- β -glucuronide (MUG) as substrate (Table 2). Strain B301DSL8 carrying only the *syrB1::uidA* reporter produced high levels of GUS activity averaging 1,225 units per 10⁸ CFU after 3 days of incubation at 25°C. The *salA* mutant B301DSL12 exhibited no detectable GUS activity, as was observed for control strains B301D and B301DSL9. The *syrF* mutant B301DSL10 produced 40 units of GUS activity per 10⁸

Table 2. Effects of *syrF*, *syrG*, and *salA* mutations on *syrB1::uidA* expression

Strain	Genotype ^a	β -Glucuronidase activity (units per 10 ⁸ CFU $\pm$ standard error)
B301D	Wild type	4.1 $\pm$ 2.2
B301DSL8	<i>syrB1::uidA</i> (+)	1225.3 $\pm$ 51.3
B301DSL9	<i>syrB1::uidA</i> (-)	10.8 $\pm$ 2.6
B301DSL10	<i>syrB1::uidA</i> (+); <i>syrF::nptII</i>	40.0 $\pm$ 5.0
B301DSL11	<i>syrB1::uidA</i> (+); <i>syrG::nptII</i>	1185.3 $\pm$ 127.8
B301DSL12	<i>syrB1::uidA</i> (+); <i>salA::nptII</i>	8.1 $\pm$ 3.1

^a The symbols "+" and "-" indicate insertion of the *uidA* reporter in forward or reverse orientation, respectively, relative to transcription.

CFU, which is only 3% of the activity measured for the parental strain B301DSL8. In contrast, the *syrG* mutant B301DSL11 expressed high levels of GUS activity (1,185 units per 10⁸ CFU), compared with parental strain B301DSL8 (Table 2).

The *salA* gene activates the expression of the *syrF* gene.

Translational fusions of the *salA*, *syrF*, and *syrG* genes with the *uidA* gene were employed as reporters to evaluate the possible regulatory relationships between *salA*, *syrF*, and *syrG*. Sequence analysis confirmed that the *uidA* gene was inserted in-frame into pSL40F (*salA::uidA*), pSL38F (*syrF::uidA*), and pSL39F (*syrG::uidA*) (Table 1), indicating that translation of these genes would produce fusion proteins with GUS. Strain B301D, containing the plasmids pSL40F, pSL38F, and pSL39F, expressed high levels of GUS activity when cultured on PDA plates and assayed with 5-bromo-4-chloro-3-indolyl- β -D-glucuronide as the substrate. In contrast, strain B301D containing the plasmid pSL40R, pSL38R, or pSL39R, which carry insertion of the *uidA* reporter in reverse orientation relative to transcription, exhibited no detectable GUS activity in the same assays. The transcriptional orientation of the GUS fusions in pSL40F, pSL38F, and pSL39F were opposite to that of the LacZ α fragment encoded by pLAFR3. Consequently, the *plac* promoter in the pLAFR3 vector does not regulate expression of the GUS fusion in these plasmids. Recovery of pSL40F, pSL38F, and pSL39F from 72-h still cultures of bacteria confirmed maintenance of the plasmids.

Based on quantitative GUS assays, expression of the *syrF::uidA* reporter carried by pLAFR3 was significantly reduced in the *salA* mutant (B301DSL7) as compared with expression in wild-type B301D (Table 3). Strain B301D carrying the *syrF::uidA* reporter on pSL38F produced 3,552 units of GUS activity per 10⁸ CFU after 72 h of incubation at 25°C. Similarly, strain B301DSL1 (*syrF* mutant) carrying pSL38F also produced high levels of GUS activity (2,791 units per 10⁸ CFU). In contrast, strain B301DSL7 (*salA* mutant), carrying the plasmid pSL38F produced only 195 units of GUS activity per 10⁸ CFU, which is 5% of the activity exhibited in strain B301D containing pSL38F. These results demonstrate that the presence of a functional *salA* gene is necessary for *syrF* expression. Strain B301DSL6 (*syrG* mutant) containing pSL38F produced GUS activity similar to that of B301D carrying pSL38F, which suggests that *syrG* exhibits no effect on *syrF* expression.

Effects of *syrF* and *salA* mutations on *syrG* expression and of *syrF* and *syrG* mutations on *salA* expression were also evaluated by GUS activity assays. Strains B301DSL1 (*syrF* mutant) and B301DSL7 (*salA* mutant) carrying pSL39F (*syrG::uidA*) produced GUS activities of 2,269 units per 10⁸ CFU and 1,969 units per 10⁸ CFU, respectively, which are not significantly different from that of B301D carrying pSL39F (2,636 units per 10⁸ CFU). These results indicate that the *syrF* and *salA* genes do not regulate *syrG* expression. Similarly, strains B301DSL1 (*syrF* mutant) and B301DSL6 (*syrG* mutant) carrying pSL40F (*salA::uidA*) expressed GUS activity of 4,223 units per 10⁸ CFU and 3,346 units per 10⁸ CFU, respectively, which is not significantly different from that of B301D carrying pSL40F (4,411 units per 10⁸ CFU). Thus, neither *syrF* nor *syrG* affects expression of the *salA* gene.

Effects of *salA*, *syrF*, and *syrG* mutations on syringopeptin production.

Double-mutant strains B301DSL10 (*syrB1-syrF*), B301DSL12 (*syrB1-salA*), and B301DSL11 (*syrB1-syrG*) were used to evaluate the effects of mutations in regulatory genes on syringopeptin production. Syringomycin production was eliminated in the three strains by disruption of the *syrB1* gene. Consequently, residual antimicrobial activity against *Bacillus megaterium* strain Km resulted from syringopeptin production (Scholz-Schroeder et al. 2001a).

Strains B301DSL10 and B301DSL12, carrying nonpolar insertions of the *nptII* cassette in *syrF* and *salA*, respectively, were reduced in syringopeptin production (Fig. 3). Strain B301DSL10 produced an inhibitory zone 10.7 mm in radius against *B. megaterium*, or 65.8% of the size of the inhibitory zone produced by strain B301DSL8 (*syrB1* mutant). Similarly, strain B301DSL12 produced an inhibitory zone 11.7 mm in radius against *B. megaterium*, which is 66.4% of the size of the zone produced by parental strain B301DSL8. In contrast, no significant difference was observed between the sizes of the inhibitory zones produced against *B. megaterium* by the *syrG* mutant B301DSL11 (18.3 mm) and the parental strain B301DSL8 (18.3 mm). Strain BR-DBL (*syrB1-sypA* double mutant) did not form an inhibitory zone, as reported previously (Scholz-Schroeder et al. 2001a).

Effects of mutations in the *salA*, *syrF*, and *syrG* genes on virulence.

Strains B301DSL7, B301DSL1, and B301DSL6, carrying nonpolar insertions of the *nptII* cassette, were significantly reduced in virulence relative to wild-type strain B301D, as indicated by lesion diameter in immature sweet cherry fruits (Fig. 4). Lesions formed by *salA* mutant strain B301DSL7 averaged 1.0 mm in diameter by day 4, only 17% of the size of lesions formed by strain B301D. The *salA* mutant was comparable in virulence to strain BR-DBL (*syrB1-sypA* double mutant) and significantly less virulent than strain BR132 (*syrB1* mutant) ($\alpha = 0.05$). In contrast, lesions produced by strain B301DSL1 (*syrF* mutant) averaged 3.0 mm in diameter, which is 53% of the size of lesions formed by wild-type strain B301D. The *syrF* mutant formed significantly larger lesions than strains B301DSL7 and BR-DBL and was comparable in virulence to the *syrB1* mutant strain BR132 ($\alpha = 0.05$). Similarly, lesions produced by strain B301DSL6 (*syrG* mutant) averaged 2.3 mm in diameter, or 40% of the size of lesions formed by wild-type strain B301D. Virulence of the *syrG* mutant was not significantly different from that of strains B301DSL1 and BR132 ($\alpha = 0.05$). In addition, the virulence of strains B301DSL1 and B301DSL6 was comparable to that of BR208, a *sypA* mutant ($\alpha = 0.05$). Lesions produced by strains B301DSL15 (rescued *salA* mutant), B301DSL16 (rescued *syrF* mutant), and B301DSL17 (rescued *syrG* mutant) were not significantly different in size from those of wild-type strain B301D (data not shown).

No significant differences were observed for bacterial population sizes recovered from cherry fruits inoculated with parental strain B301D and mutant strains B301DSL1, B301DSL6, and B301DSL7. For each strain, approximately 10⁸ CFU per fruit were recovered at day 3 from inoculated cherry fruits.

Table 3. Effects of *salA* and *syrG* mutations on *syrF::uidA* expression

Strain	Genotype	Plasmid	β -Glucuronidase activity (units per 10 ⁸ .CFU $\pm$ standard error)
B301D	Wild type	pLAFR3	12.6 $\pm$ 7.3
B301DSL1	<i>syrF::nptII</i>	pSL38F (<i>syrF::uidA</i>)	2,791.0 $\pm$ 166.0
B301DSL6	<i>syrG::nptII</i>	pSL38F (<i>syrF::uidA</i>)	2,608.8 $\pm$ 427.3
B301DSL7	<i>salA::nptII</i>	pSL38F (<i>syrF::uidA</i>)	195.2 $\pm$ 18.7
B301D	Wild type	pSL38F (<i>syrF::uidA</i>)	3,551.9 $\pm$ 314.5

DISCUSSION

Three regulatory genes, *salA*, *syrF*, and *syrG*, located at the right border region of the *syr* gene cluster, were identified as members of the LuxR family of regulatory proteins. Sequence analysis revealed the presence of HTH DNA-binding motifs in the C termini of SalA, SyrF, and SyrG. The HTH motif has been observed in many regulatory proteins (Pabo and Sauer 1992), which are divided into more than 10 groups, including the LuxR, AraC, and MarR families based on sequence similarity (European Bioinformatics Institute). The SalA, SyrF, and SyrG proteins most closely resembled members of the LuxR regulatory family, such as DctR (Hamblin et al. 1993) and FixJ (Anthamatten and Hennecke 1991), especially within the last 60 residues of the C terminus. NarL, a member of the LuxR family, is a transcription factor that both activates and represses the genes involved in nitrate and nitrite metabolism in *Escherichia coli* (Stewart and Parales 1988). Analysis of the crystal structure of NarL defined a four-helix ($\alpha 7$, $\alpha 8$, $\alpha 9$, and $\alpha 10$) fold for the 62-residue DNA-binding domain at the C terminus (Baikalov et al. 1996) that was designated an effector domain (Stock et al. 2000). The approximately 60-residue region of the C terminus containing the four helices and their turns, called a three-element fingerprint, provides the signature for the HTH motif of the LuxR family of bacterial regulatory proteins (European Bioinformatics Institute). In contrast, the signature of regulatory proteins of the AraC family is a two-element fingerprint. The evidence that the SalA, SyrF, and SyrG proteins exhibit the highest similarity to DctR and FixJ and contain the three-element fingerprint suggests that they are members of the LuxR family.

Despite evidence that the SalA, SyrF, and SyrG proteins contain the fingerprint of the LuxR family, they lack conserved residues at the N termini characteristic of the two major LuxR subfamilies. The regulatory proteins of the LuxR family are grouped into two major subfamilies on the basis of sequence similarity and functional mechanism. One subfamily is composed of the response regulators of two-component signal transduction systems, such as FixJ (Anthamatten and Hennecke 1991) and DctR (Hamblin et al. 1993). Four highly invariant residues (Asp, Asp, Asp, and Lys) (Parkinson and Kofoid 1992), referred to as the acid pocket characteristic of a receiver domain, were not observed in the N-terminal regions of SalA, SyrF, and SyrG. Thus, they do not appear to belong to the response regulator subfamily. The other subgroup is composed of autoinducer-binding regulators, including LuxR (Devine et al. 1989) and PhzR (Pierson et al. 1995), which are activated by homoserine lactones (Fuqua et al. 1996; Pierson et al. 1998). Also, five of seven highly conserved residues (Fuqua et al. 1996) in the second subfamily, corresponding to Trp59, Tyr69, Asp79, Pro80, and Gly121 in LuxR, were not observed in the *P. syringae* proteins. Although SalA positively regulates expression of SyrF, SalA does not affect synthesis of homoserine lactones (Kitten et al. 1998). Thus, they also do not appear to belong to the autoinducer-binding regulator subfamily. These results suggest that SalA, SyrF, and SyrG may represent a new subfamily of the LuxR family.

The importance of the *salA* gene in the development of disease symptoms in immature cherry fruits was demonstrated by an approximate 80% reduction in virulence of the *salA* mutant relative to the wild-type strain. The significant reduction of virulence resulting from mutation of the *salA* gene is, at least in part, attributed to the following factors. First, based on activity of the reporter GUS fusion, *salA* positively regulated expression of the syringomycin synthetase gene *syrB1*. Consequently, mutation in the *salA* gene eliminated syringomycin production, which is a major virulence determinant of *P. syrin-*

gae pv. *syringae* (Scholz-Schroeder et al. 2001a; Zhang et al. 1995). The effects of the *salA* mutation in B301D on expression of the *syrB1* gene and syringomycin production are consistent with those caused by a *salA* mutation in strain B728a (Kitten et al. 1998). Second, the *salA* mutant also was reduced in production of syringopeptin, another major virulence determinant of the pathogen (Scholz-Schroeder et al. 2001a). Third, based on GUS activity, *salA* positively regulated expression of the *syrF* gene, and *syrF* was also associated with virulence (discussed below). Collectively, these results indicate that SalA functions as a transcriptional activator of more than one gene or operon with corresponding effects on virulence of *P. syringae* pv. *syringae*.

The *syrF* gene was critical for syringomycin production and exhibited a less profound effect on syringopeptin production. The *syrF* mutant strain was approximately 88% less inhibitory to *G. candidum* in vitro than the wild-type strain, and this loss of inhibitory activity was correlated with a significant reduction in expression of the *syrB1* gene. Interestingly, sequence analysis revealed that the stop codon of *syrF* overlaps the start codon of *orf1*, which indicates that translation of the *orf1* is coupled to that of *syrF*. In translationally coupled genes, the 30S ribosome reinitiates translation of the second gene without dissociating from the mRNA transcribed from the first gene. As a result, reinitiation of translation is more efficient (Oppenheim and Yanofsky 1980). ORF1 is a homologue of OprM, a component of a prokaryotic type I secretion system in *P. aeruginosa* (Guan et al. 1999). Mutation of ORF1 in strain B301D resulted in smaller zones of inhibition to *G. candidum* (data not shown), suggesting that biosynthesis and secretion genes for syringomycin may be coordinately regulated. Although bioassay results showed that the *syrF* mutant was reduced in syringopeptin production, the regulatory mechanism controlling syringopeptin synthesis remains unknown. However, mutations in *salA* and *syrF* genes exhibited reduced effects on syringopeptin production compared with syringomycin production, suggesting it might be distinct from the regulation of syringomycin synthesis.

The *syrG* gene was associated with syringomycin but not syringopeptin production and, thus, also contributes to the virulence of *P. syringae* pv. *syringae*. The *syrG* mutant produced an antifungal zone against *G. candidum* that was 50% smaller

Fig. 3. Relative inhibition due to production of syringopeptin by different *Pseudomonas syringae* pv. *syringae* strains. The strains were incubated on peptone-glucose-NaCl agar for 4 days and then oversprayed with *Bacillus megaterium*. Zones of inhibition were measured from colony margins. Inhibition due to production of syringopeptin by the mutants is presented as a percentage of that of the parental strain B301D. Differences between treatments were determined by Tukey's analysis of variance. Different letters for each treatment indicate statistically significant differences ($\alpha = 0.01$). Vertical bars indicate standard error of the mean. Column 1, B301DSL8 (*syrB1* mutant); 2, B301DSL10 (*syrF-syrB1* double mutant); 3, B301DSL11 (*syrG-syrB1* double mutant); 4, B301DSL12 (*salA-syrB1* double mutant); and 5, BR-DBL1 (*syrB1-sypA* double mutant).

than the wild-type strain, which indicates that *syrG* is associated with syringomycin production. However, there was no effect of the *syrG* mutation on expression of *syrB1::uidA*, which suggests that the *syrG* gene does not regulate transcription of the *syrB* operon. It remains unknown whether *syrG* is involved in posttranscriptional regulation of *syrB1* or in regulation of other genes that are critical for synthesis and secretion of syringomycin. No effect of the *syrG* mutation was detected on syringopeptin production in this study. In immature sweet cherry fruit assays, a syringomycin mutant was reduced 26% in virulence (Scholz-Schroeder et al. 2001a), however, the *syrG* mutant was 40% as virulent as the wild-type strain. These results suggest *syrG* may also be involved in regulation of other genes associated with virulence of *P. syringae* pv. *syringae*.

The *salA*, *syrF*, and *syrG* genes are highly conserved in different strains of *P. syringae* pv. *syringae*. Strain B728a is the causal agent of brown spot of bean (Hirano et al. 1995), but it also can cause necrotic lesions on artificially inoculated immature cherry fruits (Quigley and Gross 1994). In contrast, strain B301D, a canker pathogen of stone fruit trees, did not cause disease on bean (B. K. Scholz-Schroeder and D. C. Gross, unpublished data). The *salA* gene of B301D exhibits 96.7% identity to that of B728a, and only one amino acid, at residue 75, differs between the two predicted SalA proteins. This residue, which is not located in the conserved HTH region, is a serine in B301D SalA and an asparagine in B728a SalA. Furthermore, both serine and asparagine are uncharged amino acids. Consequently, this difference in both of the putative SalA proteins is not predicted to change the function or specificity of either of the two proteins. Recently, the sequences of *salB* (accession number AF299084) and *salC* (accession number AF297011), which reside within the *syrE* and *salA* region of strain B728a, were deposited in the GenBank database. The deduced SalC is seven amino acids longer at the N terminus than the predicted SyrF, but the remainders of the two proteins are identical. Similarly, the SalB is six amino acids longer at the N terminus than the predicted SyrG, and the remainder of SalB

differs from SyrG only at residue 110, which is not located in the conserved HTH regions.

Characterization of *salA*, *syrF*, and *syrG* provides a valuable foundation for defining the organization of the regulatory complex for syringomycin production by *P. syringae* pv. *syringae*. A global, two-component regulatory system, *gacS/gacA*, positively regulates syringomycin production and is mediated by the *salA* gene in strain B728a (Kitten et al. 1998). This study demonstrated that the B301D SalA plays a critical role in the transcription of *syrB1*, syringomycin production, and virulence of strain B301D. More interestingly, we revealed that SalA is required for transcription of *syrF*, and a functional SyrF is essential for transcription of *syrB1* and syringomycin production. Furthermore, there was no effect of mutations in either *syrF* or *syrG* on expression of *salA*, and a *salA* mutant was reduced significantly in virulence compared with a *syrF* mutant ($\alpha = 0.01$). These findings indicate that *salA* is located downstream of *gacS/gacA* and upstream of *syrF* in the regulatory hierarchy of syringomycin production and virulence of *P. syringae* pv. *syringae*. The *syrG* gene product is clearly associated with syringomycin production but may also control other genes that are involved in virulence of *P. syringae* pv. *syringae*. The *salA* and *syrF* mutants exhibited less profound effects on syringopeptin than on syringomycin in strain B301D, suggesting that regulation of the two toxins may be distinct. Future work will determine whether SalA controls *syrB1* transcription directly or is mediated by *syrF* and whether SyrF directly binds the promoter region of the *syrB* operon.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strain DH10B (Grant et al. 1990) was used for cloning and was cultured at 37°C in Luria-Bertani (LB) broth or on LB agar. *P. syringae* pv. *syringae* strains were cultured at 25°C on nutrient broth–yeast extract (NBY) agar medium (Vidaver 1967). PDA containing 0.4% casamino acids and 1.5% glucose (Gross and DeVay 1977) and peptone-glucose-NaCl agar (Bultreys and Gheysen 1999) were used in bioassays of *P. syringae* pv. *syringae* strains for production of syringomycin and syringopeptin, respectively. Potato dextrose broth supplemented with FeCl₃ at a final concentration of 5 μ M was used to culture cells for an assay of GUS activities. Antibiotics (Sigma Chemical Co., St. Louis, MO, U.S.A.) were added to media in the following concentrations: 25 μ g of tetracycline per ml, 100 μ g of kanamycin per ml, 100 μ g of ampicillin per ml, and 5 μ g of gentamycin per ml.

DNA manipulations.

Screening of the genomic library of *P. syringae* pv. *syringae* strain B301D was completed by using standard colony-blotting procedures (Sambrook et al. 1989). Routine procedures were used for preparing plasmid DNA and for subcloning DNA fragments into designated vectors (Sambrook et al. 1989). Cells of *E. coli* strain DH10B were made competent by a standard protocol (Sambrook et al. 1989). A gel extraction kit (Qiagen, Inc., Valencia, CA, U.S.A.) was used to isolate DNA fragments for subcloning and for use as hybridization probes. Primers for sequencing were synthesized by Operon Technologies, Inc. (Alameda, CA, U.S.A.) and sequencing reactions were analyzed using fluorescence-based dideoxy terminators and Ampli-Tag polymerase (Perkin-Elmer Applied Biosystems, Inc., Norwalk, CT, U.S.A.). Sequencing reactions were run on an ABIPRISM 377 DNA Sequencer (Perkin-Elmer Applied Biosystems).

Fig. 4. Relative virulence of mutant strains of *Pseudomonas syringae* pv. *syringae* as compared with parental strain B301D. Fruits were wounded at three sites and inoculated at each site with a 2.5- μ l droplet containing approximately 5×10^3 cells. Lesion diameter was measured after incubation for 4 days at 20°C. Relative virulence of the mutants is presented as a percentage of that of the wild-type strain B301D. Differences among strains were analyzed by Fisher's least significant difference (LSD) test ($\alpha = 0.05$, LSD = 0.2). Different letters for each strain indicate statistically significant differences ($\alpha = 0.05$). Vertical bars indicate the standard error of the mean. Column 1, B301D (parental strain); 2, B301DSL7 (*salA* mutant); 3, B301DSL1 (*syrF* mutant); 4, B301DSL6 (*syrG* mutant); 5, BR132 (*syrB1* mutant); 6, B301DR::208 (*syaA* mutant); and 7, BRDBL1 (*syrB1-syaA* double mutant).

Sequence analysis.

The nucleotide and deduced protein sequences were analyzed using the Wisconsin Package, version 10.0 (Genetics Computer Group, Madison, WI, U.S.A.). GELASSEBLE was used to assemble nucleotide sequences, FINDPATTERNS to identify Shine-Dalgarno sequences, and TERMINATOR to identify transcription terminators. BESTFIT and GAP were used to compare two nucleotide or protein sequences and calculate Z scores. NETBLAST was used to identify nucleic acid sequences and proteins homologous to query sequences. ORF analyses were conducted using ORF Finder on the Web site of the National Center for Biotechnology Information or Clone-Map, version 2.11 (CGC Scientific, Inc., Ballwin, MO, U.S.A.).

Mutagenesis of *syrF*, *syrG*, and *salA*.

The *syrF*, *syrG*, and *salA* genes were each disrupted by insertion of the *nptII* gene to construct nonpolar mutations. A 1.4-kb fragment containing the *nptII* gene from pBSL15 (Alexeyev 1995) was inserted into pSL4 at the *BstEII* site to disrupt translation of *syrF* and into pSL9 at the *EcoRI* site to disrupt translation of *syrG*. The 1.4-kb *nptII* cassette was inserted into pSL8 at the *KpnI* site to disrupt translation of *salA*, generating plasmid pSL21. The *nptII* insertions in the three constructs were confirmed by sequencing. The *syrF::nptII* and *syrG::nptII* fragments were subcloned into pBR325, generating plasmids pSL19 and pSL20, respectively. Plasmids pSL19, pSL20, and pSL21 were electroporated into *P. syringae* pv. *syringae* for marker exchange mutagenesis as previously described by Scholz-Schroeder (2001) using a Gene Pulser II (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Colonies acquiring resistance to kanamycin and not tetracycline were confirmed by Southern analysis for marker exchange mutagenesis. For marker rescue, the wild-type fragments of the *syrF* and *syrG* genes were isolated from pSL4 and pSL5, respectively, and cloned into the *EcoRI* site of pBR325 by blunt-end ligation to generate pSL51 and pSL52. Plasmids pSL51, pSL52, and pSL8 were electroporated into *syrF*, *syrG*, and *salA* mutants, respectively, for marker rescue of genotypes. Colonies sensitive to kanamycin and tetracycline were selected and confirmed as rescued strains by Southern analysis.

Construction of GUS translational fusions.

The *uidA* gene encoding GUS was inserted into the *syrB1*, *sypA*, *syrF*, *syrG*, and *salA* genes in-frame to monitor gene expression in wild-type or mutant strains of *P. syringae* pv. *syringae*. Plasmid pSL2 was constructed by insertion of a 0.85-kb *SmaI* fragment containing the *aacC1* gene, a gentamycin resistance gene from pUCGM (Schweizer 1993), into the polished *EcoRI* site downstream of the promoterless *uidA* gene from pBI101 (Clontech Laboratories, Inc., Palo Alto, CA, U.S.A.). The 3.2-kb *XbaI-BglII* fragment containing the *uidA-aacC1* genes from pSL2 was polished with T4 DNA polymerase (Life Technologies, Inc., Gaithersburg, MD, U.S.A.) and inserted at the *EagI* site of pSL25 in both forward and reverse orientations in-frame of *syrB1*. The *syrB1::uidA-aacC1* constructs were isolated as an *EcoRI-HindIII* fragment, polished, and cloned into the polished *EcoRI* site of pBR325 (Prentki et al. 1981) to generate pSL28F and pSL28R. The 3.2-kb *SmaI-BglII* fragment from pSL2 was polished with T4 DNA polymerase and inserted at the *BstEII* site of pSL4 in both forward and reverse orientations in-frame of *syrF*. The *BamHI-StuI* fragment was isolated and inserted into the *BamHI-SmaI* site of pBlueScriptSK. The *syrF::uidA-aacC1* constructs in forward and reverse retentions were recovered as *BamHI-EcoRI* fragments and inserted into pLAFR3 to generate pSL38F and pSL38R. The 3.2-kb *SmaI-BglII* fragment from pSL2 was polished and then inserted into the polished *EcoRI* site of pSL9 in

both forward and reverse orientations in-frame of *syrG*. The forward and reverse *syrG::uidA-aacC1* constructs were recovered as a polished *HindIII-EcoRV* fragment and inserted into pLAFR3 to generate pSL39F and pSL39R. The *SmaI-BglII* fragment from pSL2 was inserted into the *SmaI* site of pSL8 in both forward and reverse orientations in-frame of *salA*. The forward and reverse *salA::uidA-aacC1* constructs were recovered as an *EcoRI* fragment and inserted into pLAFR3 to generate pSL40F and pSL40R (Table 1). The orientations of *uidA-aacC1* insertion in all target genes were confirmed by sequencing. The *syrB1::uidA-aacC1* constructs carried by pSL26F and pSL26R were introduced into the genome of strain B301D by marker exchange to generate strains B301D8 and B301D9. The plasmids pSL19, pSL20, and pSL21 carrying the respective mutated *syrF*, *syrG*, and *salA* genes disrupted with a kanamycin resistance cassette were introduced into strain B301DSL8 by electroporation. The mutated *syrF*, *syrG*, and *salA* genes were recombined into the genome of strain B301DSL8 by marker exchange mutagenesis to generate strains B301DSL10, B301DSL11, and B301DSL12, respectively. The introductions of all disrupted genes into the B301D genome were confirmed by Southern blot analyses.

The strains of *Pseudomonas* carrying the *uidA* gene *in trans* and *in cis* were screened by a modified color development protocol (Stomp 1992). Strains tested were cultured on PDA plates for 2 days at 25°C. Cells were transferred into tubes containing 10 µl of reagent mix (0.1 M NaPO₄; 10 mM EDTA, pH 7.0; 0.5 mM potassium ferricyanide; 0.5 mM potassium ferrocyanide; 1.0 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide; 0.1% Triton X-100) and incubated for 30 min at 37°C. Development of a blue color in the mixture indicated the expression of GUS.

Analysis of *P. syringae* pv. *syringae* strains for phytotoxin production.

The *syrF*, *syrG*, and *salA* mutants were evaluated for production of syringomycin and syringopeptin by using standard bioassays for the respective phytotoxins as previously described by Scholz-Schroeder and associates (2001a). *P. syringae* pv. *syringae* strain B301D and strain BR132 (*syrB1* mutant) were used as positive and negative controls, respectively, for syringomycin production. Syringopeptin production was determined for the *syrF*, *syrG*, and *salA* mutants (Table 1) of strain B301DSL8, which carries the *syrB1::uidA-aacC1* reporter fusion and does not produce syringomycin. Strains B301DSL8 and BR-DBL (*syrB1-sypA* double mutant) were used as positive and negative controls, respectively, for syringopeptin production. The plasmids pSL4, pSL5, and pSL8, carrying the respective wild-type *syrF*, *syrG*, and *salA* genes, were used for *in trans* complementation experiments. All bioassays were repeated three times with five plates per replicate.

Assays for GUS activity.

The effects of *syrF*, *syrG*, and *salA* mutations on expression of *syrB1::uidA* and *sypA::uidA* gene fusions were quantified by fluorometric analysis of GUS activity. An optimized protocol was developed based on comparisons of different media, time courses, and incubation conditions. Bacterial cells were cultured with shaking in potato dextrose broth (PDB; 4 ml) for 14 h at 25°C, harvested by centrifugation, and then diluted to an optical density at 600 nm of 0.6 with PDB containing 20% glycerol. The cell suspension was dispensed in a volume of 500 µl and stored at -80°C as stock cells. A stock cell suspension of 50 µl was used to inoculate PDB (4 ml) and incubated for 72 h at 25°C without shaking. Cells from 150 µl of the culture were collected by centrifugation, resuspended in 500 µl of GUS extract buffer (GEB; Xiao et al. 1992), and lysed by soni-

cation (The Virtis Company, Inc., Gardiner, NY, U.S.A.) for 10 s. Cell lysate (6 µl) was added to GEB (54 µl) supplemented with 1 mM MUG (Sigma Chemical Co., St. Louis, MO, U.S.A.). The reaction mixture was incubated for 30 min at 37°C, and a 25-µl aliquot of the reaction mixture was combined with 975 µl of stop buffer to terminate the reaction. GUS activity was measured by using a Fluorolite-1000 microplate reader (Dynatech, Denkendorf, Germany). One unit of activity is defined as 1 pmol of methylumbelliferone formed per minute (Xiao et al. 1992). Bacterial cell densities were measured by absorption at A_{600} . All assays for GUS activity were repeated on four independent occasions.

Virulence assays in immature cherry fruits.

The virulence of *syrF*, *syrG*, and *salA* mutants of *P. syringae* pv. *syringae* was evaluated in immature cherry fruits as previously described by Scholz-Schroeder and associates (2001a). Inoculated cherry fruits were incubated for 4 days at 20°C. Virulence was evaluated by measurement of the diameters of necrotic lesions formed at each inoculated site. Parental strain B301D, *syrB1* mutant strain BR132, and *syrB1-sypA* double mutant strain BR-DBL1 were included as controls in the analyses. Bacterial populations in the cherry fruits were determined as described by Mo and Gross (1991b). For each experiment, 10 cherry fruits were inoculated per treatment, and the experiment was repeated three times.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Bioinformatics Group Web site: <http://www.isrec.isb-sib.ch>
 European Bioinformatics Institute: <http://srs.ebi.ac.uk/>
 National Center for Biotechnology Information:
<http://www.ncbi.nlm.nih.gov/>
 Prosite database: <http://expasy.cbr.nrc.ca/prosite>
 Pseudomonas Genome Project: <http://www.pseudomonas.com>