

## Identification of the *syr-syp* Box in the Promoter Regions of Genes Dedicated to Syringomycin and Syringopeptin Production by *Pseudomonas syringae* pv. *syringae* B301D

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The phytotoxins syringopeptin and syringomycin are synthesized by nonribosomal peptide synthetases which are encoded by the syringomycin (*syr*) and syringopeptin (*syp*) genomic island of *Pseudomonas syringae* pv. *syringae*. Previous studies demonstrated that expression of the *syr-syp* genes was controlled by the *salA-syrF* regulatory pathway, which in turn was induced by plant signal molecules. In this study, the 132-kb *syr-syp* genomic island was found to be organized into five polycistronic operons along with eight individual genes based on reverse transcriptional PCR and bioinformatic analysis. The transcriptional start sites of the *salA* gene and operons III and IV were located 63, 75, and 104 bp upstream of the start codons of *salA*, *syrP*, and *syrB1*, respectively, using primer extension analysis. The predicted  $-10/-35$  promoter region of operon IV was confirmed based on deletion and site-directed mutagenesis analyses of the *syrB1::uidA* reporter with  $\beta$ -glucuronidase assays. A 20-bp conserved sequence (TGtCccgN<sub>6</sub>cggGaCA, termed the *syr-syp* box) with dyad symmetry around the  $-35$  region was identified via computer analysis for the *syr-syp* genes/operons responsible for biosynthesis and secretion of syringomycin and syringopeptin. Expression of the *syrB1::uidA* fusion was decreased 59% when 6 bp was deleted from the 5' end of the *syr-syp* box in the promoter region of operon IV. These results demonstrate that the conserved promoter sequences of the *syr-syp* genes contribute to the coregulation of syringomycin and syringopeptin production.

Bacteria coordinate expression of functionally related genes to adapt to a rapidly changing environment (49). For example, enzymes that belong to the same metabolic pathway are always upregulated and downregulated together (49). Gene expression is most frequently modulated at the transcriptional level by the interaction of transcriptional factors and promoters in bacteria (47). Syringomycin (*syr*) and syringopeptin (*syp*) are lipodepsipeptide toxins produced by *P. syringae* pv. *syringae* through a nonribosomal peptide synthetase system (4). The *syr* and *syp* gene clusters dedicated to syringomycin and syringopeptin production are located adjacent to one another on the chromosome (Fig. 1) (44). The *syr-syp* genes are subjected to coordinated control by SalA and SyrF in response to environmental signals (29, 30). Similarly, coronatine biosynthesis genes are coregulated by transcriptional activator CorR, which binds to both *cmA* and *cfl/CFA* promoter regions, respectively, in *Pseudomonas syringae* (37, 53).

Generally, genes are transcriptionally coregulated because they share high similarity in their promoter regions so that the genes are controlled by the same sigma factors or regulators (10). For example, the *hrp* box has been identified for most *hrp* and type III effector genes controlled by the alternative sigma factor HrpL (16). A conserved sequence (TnrA box) has been

identified in the promoters of 17 TnrA-regulated genes in *Bacillus subtilis* (56). In *Escherichia coli*, LexA was reported to regulate 31 genes by binding to a consensus sequence of TAC TG(TA)<sub>5</sub>CAGTA in the promoter regions of the target genes (15). The PhoP/PhoQ two-component regulatory system controls the expression of more than 40 genes associated with virulence and fitness in *Salmonella enterica* (33). Apparently, coregulation is widely used in bacteria to maximize efficiency in utilizing resources and to enhance their competitiveness in the environment. This demonstrates that the conservation of the promoter regions is vital to the coordinated expression of the genes in one regulon.

Previous studies demonstrated that genes dedicated to biosynthesis, secretion, and regulation of syringomycin and syringopeptin production by *P. syringae* pv. *syringae* are coordinately regulated at the transcriptional level (30). Transcriptional analysis with 70-mer oligonucleotide microarrays, along with  $\beta$ -glucuronidase (GUS) assays and real-time PCR (RT-PCR) analysis, demonstrated that all of the *syr-syp* genes (Fig. 1) belong to the SalA regulon (30). Furthermore, all of the *syr-syp* genes belong to a stimulon activated by plant signal molecules (54). It was revealed that expression of the *syr-syp* genes was significantly higher in B301D than in the *salA* mutant (30) and was activated by the phenolic plant signal molecule arbutin (100  $\mu$ M). Genes activated included synthetase genes for syringomycin (i.e., *syrB1* and *syrE*) and syringopeptin (i.e., *sypA*, *sypB*, and *sypC*), four regulatory genes (i.e., *salA*, *syrF*, *syrG*, and *syrP*), and nine putative secretion genes (i.e., *syrD*, *sypD*, *pseA*, *pseB*, *pseC*, two *mtrC* homologs, ORF20 encoding an *oprM* homolog, and ORF21 encoding a putative threonine efflux protein) (Fig. 1), dedicated to production of the two toxins (30). Furthermore, all of the *syr-syp* genes responsible for bio-

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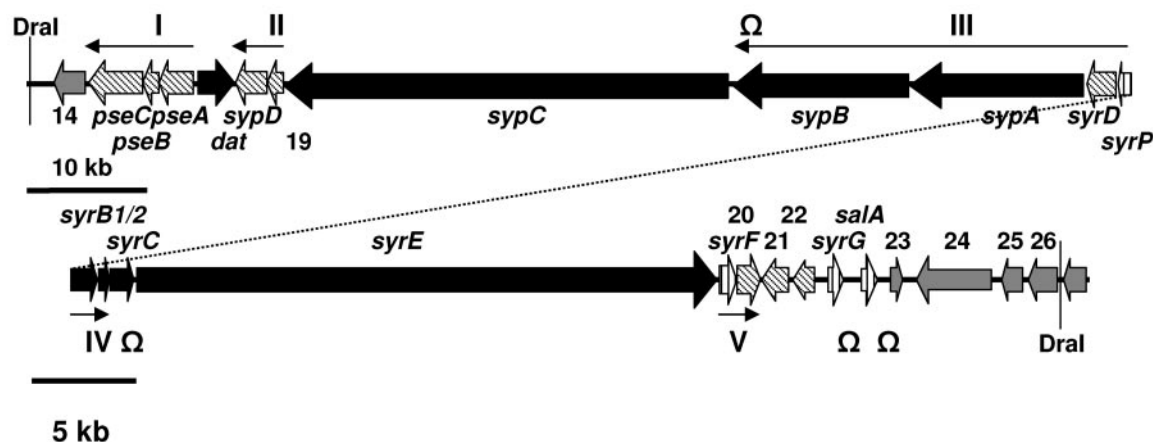


FIG. 1. Operon analysis of a 132-kb genomic DNA region of *P. syringae* pv. *syringae* B301D containing both syringomycin (*syr*) and syringopeptin (*syp*) gene clusters. The positions and orientations of the known and potential ORFs are shown as horizontal arrows. The black, diagonally striped, and vertically striped arrows represent genes that are predicted to be involved, respectively, in the synthesis, regulation, and secretion of the phytotoxins. The gray arrows represent the potential ORFs for which functions remain unknown. Operons I to V are indicated by thin black arrows. Putative rho-independent terminators are represented by  $\Omega$ .

synthesis and secretion of syringomycin and syringopeptin belong to the SyrF regulon (N. Wang and D. C. Gross, unpublished data). Both SalA and SyrF belong to the LuxR regulatory protein family (11, 29). The LuxR protein contains a helix-turn-helix DNA-binding motif at the C terminus and regulates target genes by binding to the *lux* box. The *lux* box is 20 bp in length with a dyad symmetry centered at the  $-42.5$  position relative to the transcriptional start site of *luxI* (12). It was predicted that similar sequences are present in the *syr-syp* promoter region and are responsible for the coregulation of syringomycin and syringopeptin production.

Despite progress made in understanding the regulation of syringomycin and syringopeptin production (4, 29, 30), the transcription pattern of the *syr-syp* genes remains largely unknown. In addition, the promoters of the *syr-syp* genes have not been studied in depth. The objectives of this study were to carry out a detailed transcriptional analysis of the *syr-syp* genes, including characterizing the operon structure, the transcriptional start sites, and the common characteristics of the promoter regions of the *syr-syp* genomic island. Efforts were also made to identify potential sigma factors that are associated with the promoter regions of the *syr-syp* genes.

#### 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 (17) was used for cloning and was cultured at 37°C in Terrific Broth (48) or on Luria broth agar (41). *P. syringae* pv. *syringae* strains were routinely cultured at 25°C in nutrient broth-yeast extract broth or on nutrient broth-yeast extract agar medium (51). For GUS assay experiments, *P. syringae* pv. *syringae* strains were cultured in potato dextrose broth medium (18). Antibiotics (Sigma Chemical Co., St. Louis, MO) were added to media in the following concentrations: 25  $\mu$ g of tetracycline per ml, 100  $\mu$ g of kanamycin per ml, 100  $\mu$ g of ampicillin per ml, and 5  $\mu$ g of gentamicin per ml.

**Operon analysis of the *syr-syp* genomic island using RT-PCR.** RT-PCR analysis was performed to define the transcripts for the *syr-syp* genes. For each pair of neighboring genes, specific primers (primer sequences are available upon request) were used to identify the putative transcript. For convenience, the RT-PCR products were named after the genes or open reading frames (ORFs) at the two ends of the transcript. Total RNA was prepared from strain B301D cells after 3 days of incubation on potato dextrose agar at 25°C using the RNeasy

Mini kit (QIAGEN Inc., Valencia, CA) according to the method described in the manufacturer's directions. RNA was treated further with RNase-Free DNase I (Promega Corporation, Madison, Wis.) after isolation. Total RNA (0.1  $\mu$ g) from strain B301D was used as a template, and the primers used for the RT-PCR analysis were designed based on the instructions of the manufacturer (primer sequence available upon request). RT-PCR was performed with a OneStep RT-PCR kit (QIAGEN Inc., Valencia, CA). After the reverse transcription reaction at 50°C for 30 min, PCR was carried out using the following conditions: 95°C for 15 min and 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 to 2 min (variable according to the length of the predicted products). PCR was performed to demonstrate that there was no DNA contamination in the RNA sample. For negative controls, template RNA was not added to the reaction mixtures. The RT-PCR products were subjected to electrophoresis with a 1.0% agarose gel. When no RT-PCR product was obtained with RNA as template, genomic DNA was used to test the fidelity of the primer pairs.

**Primer extension analysis.** Primer extension was performed with the Primer Extension System (Promega Corporation, Madison, WI), and the sequence marker was generated with the SequiTherm EXCEL II DNA sequencing kit (Epicentre, Madison, WI) according to the manufacturer's instructions. Oligonucleotides syrPPE, syrB1PE, and salAPE were 5' end labeled with [ $\gamma$ - $^{32}$ P]ATP (Perkin-Elmer Life Sciences, Inc., Boston, MA). The primer extension reaction was done with 1.0 pmol of labeled primer and 15  $\mu$ g of total RNA from strain B301D prepared as described above. The sequence ladders for the upstream regions of *syrP* and *syrB1* were obtained with pYM101 (38) as a template. Plasmid pSL8 (29) was used as a template for generating the *salA* sequence ladder.

**Computer analysis.** A program was designed to search for conserved sequences in the promoter regions of the *syr-syp* genes and operons controlled by *salA-syrF*. For an imperfect dyad symmetry, two end elements are separated by a short arbitrary sequence with variable lengths (length is zero for a perfect dyad symmetry). A combinatorial ( $l, d, w$ ) dyad symmetry model, where  $l$  is the length of an element,  $d$  is the maximal number of mismatches allowed between two corresponding elements, and  $w$  is the length of the arbitrary sequence, was used. We used  $l = 6$ ,  $d = 1$ , and  $w = 11$  in this study. Imperfect dyad symmetries were sought by identifying motifs that consist of two parts  $w_1$  and  $w_2$ , each of length  $l$ , so that  $w_1$  and  $w_2$  are almost perfect inverted repeats of each other and they are separated by a short region of variable lengths and content. The resultant sequences were aligned with T-Coffee (34).

**Construction of GUS transcriptional fusions.** To identify the promoter region of operon IV, 5' *cis* promoter deletion constructs were made from pNW104, a transcriptional fusion construct containing the *syrB1* gene with a promoterless *uidA* gene insertion in the *EagI* site of *syrB1*. To construct pNW104, the EcoRI-HindIII fragment containing *syrB1::uidA-aacC1* was cloned into pUCP26 (55). Then, the *syrB1::uidA-aacC1* constructs were isolated as BglI, NarI, and DrdI fragments; polished; and cloned into the dephosphorylated SmaI site of pUCP26 to generate pNW104-1, pNW104-2, and pNW104-3, respectively (Table 1).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s) <sup>a</sup>	Source or reference
<i>Escherichia coli</i> DH10B	F <sup>-</sup> <i>mcrA</i> $\Delta$ lacX74 ( $\phi$ 80d $\Delta$ lacZ $\Delta$ M15) $\Delta$ ( <i>mrr-hsdRMS-mcrB</i> ) <i>deoR recA1 endA1 araD139</i> $\Delta$ ( <i>ara leu</i> )7697 <i>galKX</i> <sup>-</sup> <i>rpsL nupG</i>	17
<i>P. syringae</i> pv. <i>syringae</i> B301D B301DNW201	Wild type, from pear <i>rpoS::nptII</i> derivative of B301D; Km <sup>r</sup>	9 This study
Plasmids		
pBluescript SK(+)	Cloning vector; Ap <sup>r</sup>	Stratagene, La Jolla, CA
pUCP26	Cloning vector; Tc <sup>r</sup> Ap <sup>r</sup>	34a
pB101	Binary vector containing <i>uidA</i> gene; Km <sup>r</sup>	Clontech, Palo Alto, CA
pBSL15	Kanamycin resistance gene cassette; Km <sup>r</sup>	1
pUCGM	Vector containing gentamicin resistance gene cassette; Gm <sup>r</sup>	45
p601D-1	pRK415 carrying the 8-kb HindIII fragment of pYM101 with deletion of EcoRV fragment upstream of <i>sybI</i> ; Tc <sup>r</sup>	J. H. Zhang and D. C. Gross, unpublished data
pSL2	pB1101 with the 0.85-kb <i>aacC1</i> gene of pUCGM inserted at the EcoRI site downstream of the <i>uidA</i> gene; Gm <sup>r</sup> Km <sup>r</sup>	29
pSL8	pBR325 carrying the 3.0-kb EcoRI fragment of p29 containing <i>salA</i> ; Tc <sup>r</sup> Ap <sup>r</sup>	29
pSL103	pUC18 carrying an 8-kb EcoRI-KpnI fragment from pBS008 containing <i>sypA::uidA-aacC1</i> at BstZ171 partial <i>syd</i> and start of <i>sypA</i> ; Ap <sup>r</sup>	30
pBS008	pUC18 carrying an 8-kb EcoRI-HindIII fragment from BS008 with <i>sypA::uidA-aacC1</i> insertion at BstZ171 site of <i>sypA</i> ; Ap <sup>r</sup> Gm <sup>r</sup>	43
pYM101	pUC19 carrying a 16-kb HindIII DNA fragment from pYM1 containing 5' end of <i>syd</i> , all of <i>sydC</i> , <i>sybI</i> , <i>syb2</i> , <i>syd</i> , and <i>sypP</i> ; and 3' end of <i>sypA</i> ; Ap <sup>r</sup>	39
pSLB4	pUC18 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>sybI</i> at the EagI site in forward orientation; Ap <sup>r</sup> Gm <sup>r</sup>	S. E. Lu and D. C. Gross, unpublished data
pGEMT <sub>rpos</sub>	pGEM-Teasy vector carrying a 3.1-kb fragment containing amplified <i>rpoS</i> of B301D; Ap <sup>r</sup>	This study
pGEMT <sub>rpos</sub> Km	pGEM-Teasy vector carrying a 4.3-kb fragment containing amplified <i>rpoS</i> of B301D with <i>nptII</i> insertion at the ClaI site of <i>rpoS</i> ; Ap <sup>r</sup> Km <sup>r</sup>	This study
pBR325 <sub>rpos</sub> Km	pBR325 vector carrying a 4.3-kb fragment containing amplified <i>rpoS</i> of B301D with <i>nptII</i> insertion at the ClaI site of <i>rpoS</i> ; Tc <sup>r</sup> Km <sup>r</sup>	This study
pSL105	pUCP26 carrying a 8-kb EcoRI-HindIII fragment from BS008 with <i>sypA::uidA-aacC1</i> insertion at BstZ171 site of <i>sypA</i> ; Tc <sup>r</sup> Gm <sup>r</sup>	S. E. Lu and D. C. Gross, unpublished data
pNW105	pUCP26 carrying <i>sypA::uidA-aacC1</i> and the upstream DNA sequence containing <i>syd</i> , <i>sypP</i> , <i>sybI</i> , <i>syb2</i> , and 5' end of <i>sydC</i> ; Tc <sup>r</sup> Gm <sup>r</sup>	This study
pNW105-1	pUCP26 carrying <i>sypA::uidA-aacC1</i> , <i>syd</i> , <i>sypP</i> , and 335 bp upstream of the start Codon of <i>sypP</i> ; Tc <sup>r</sup> Gm <sup>r</sup>	This study
pNW105-2	pUCP26 carrying <i>sypA::uidA-aacC1</i> , <i>syd</i> , <i>sypP</i> , and 59 bp upstream of the start codon of <i>sypP</i> ; Tc <sup>r</sup> Gm <sup>r</sup>	This study
pNW104	pUCP26 carrying HindIII-EcoRI fragment from pSLB4 containing the 3.2-kb <i>uidA-aacC1</i> fragment from pSL2 in frame of <i>sybI</i> at the EagI site in forward orientation; Tc <sup>r</sup> Gm <sup>r</sup>	This study
pNW104-1	pUCP26 carrying <i>sybI::uidA-aacC1</i> and 391 bp upstream of the start codon of <i>sybI</i> ; Tc <sup>r</sup> Gm <sup>r</sup>	This study
pNW104-2	pUCP25 carrying <i>sybI::uidA-aacC1</i> and 276 bp upstream of the start codon of <i>sybI</i> ; Tc <sup>r</sup> Gm <sup>r</sup>	This study
pNW104-3	pUCP26 carrying <i>sybI::uidA-aacC1</i> and 218 bp upstream of the start codon of <i>sybI</i> ; Tc <sup>r</sup> Gm <sup>r</sup>	This study
pNW104-4	pUCP26 carrying <i>sybI::uidA-aacC1</i> and 180 bp upstream of the start codon of <i>sybI</i> ; Tc <sup>r</sup> Gm <sup>r</sup>	This study
pNW104-5	pUCP26 carrying <i>sybI::uidA-aacC1</i> and 111 bp upstream of the start codon of <i>sybI</i> ; Tc <sup>r</sup> Gm <sup>r</sup>	This study
pNW104-6	pUCP26 carrying <i>sybI::uidA-aacC1</i> and 218 bp upstream of the start codon of <i>sybI</i> with the potential -10 region was replaced with CTGCAG; Tc <sup>r</sup> Gm <sup>r</sup>	This study
pNW104-7	pUCP26 carrying <i>sybI::uidA-aacC1</i> and 218 bp upstream of the start codon of <i>sybI</i> with the potential -35 region was replaced with CTGCAG; Tc <sup>r</sup> Gm <sup>r</sup>	This study
pNW104-8	pUCP26 carrying <i>sybI::uidA-aacC1</i> and 146 bp upstream of the start codon of <i>sybI</i> ; Tc <sup>r</sup> Gm <sup>r</sup>	This study
pNW104-9	pUCP26 carrying <i>sybI::uidA-aacC1</i> and 218 bp upstream of the start codon of <i>sybI</i> with TGTCCTC of the potential <i>syd-syp</i> box was replaced with CTGCAG; Tc <sup>r</sup> Gm <sup>r</sup>	This study

<sup>a</sup> Km<sup>r</sup>, Gm<sup>r</sup>, Pip<sup>r</sup>, Rif<sup>r</sup>, Tc<sup>r</sup>, Ap<sup>r</sup>, and Cm<sup>r</sup>, resistance to kanamycin, gentamicin, piperacillin, rifampin, tetracycline, ampicillin, and chloramphenicol, respectively.

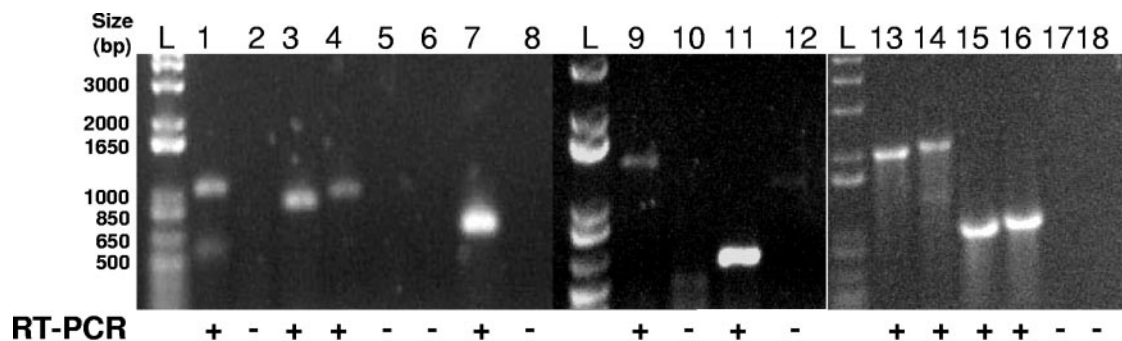


FIG. 2. Results of RT-PCR analysis to define the operons in the *syr-syp* genomic island. RT-PCR analysis was performed with total RNA isolated from *Pseudomonas syringae* pv. *syringae* B301D incubated on PDA medium for 3 days at 25°C and subjected to agarose gel electrophoresis. The RT-PCR products were named after the ORFs that they cover and are numbered as follows: 1, *syrP-syrD*; 2, *syrC-sypD*; 3, ORF19-*sypD*; 4, *syrB2-syrC*; 5, *sypC-ORF19*; 6, *syrC-syrE*; 7, *syrF-ORF20*; 8, *syrE-ORF20*; 9, *sypA-sypB*; 10, *sypB-sypC*; 11, *syrB1-syrB2*; 12, *pseB-ORF14*; 13, *sypA-syrP* (primer 1); 14, *sypA-syrP* (primer 2); 15, *pseA-pseB*; 16, *pseB-pseC*; 17, *dat-sypD*; 18, ORF21-ORF22. A “+” indicates that two genes are in one operon, whereas a “-” indicates that two genes are not in one operon. The “L” represents lanes with a DNA ladder.

To further define the promoter region of *syrB1*, a HindIII site was introduced at a different location in the upstream region of *syrB1* using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's directions. Primers B8SDM1F and B8SDM1R were used for construct pNW104-4, while B8SDM2F and B8SDM2R were used for construct pNW104-5. Constructs pNW104-4 and pNW104-5, with different promoter regions, were obtained by subcloning the HindIII-EcoRI fragments into pUCP26. Similarly, to test the effect of deletion of the upstream sequence from the -42 region on expression of the *syrB1::uidA* reporter, primers B8SDM6F and B8SDM6R were used to generate plasmid pNW104-8. By deletion of the upstream sequence from the -42 region, 13 bp of the total 20 bp of the *syr-syp* box was deleted in the promoter region of operon IV. Plasmid pNW104-4 was used as a template, in conjunction with primers B8SDM3F and B8SDM3R, to generate pNW104-6, in which the -10 region (TGAAAT) was replaced with CTG CAG. Similarly, plasmid pNW104-7 was generated with primers B8SDM4F and B8SDM4R, in which the -35 region (GACAGACGC) was changed to CTG CAG. To further test the effect of mutation of the *syr-syp* box, primers B8SDM5F and B8SDM5R were used to generate plasmid pNW104-9, in which TGTCCTC at the 5' end of the *syr-syp* box was replaced with CTGCAG. All site-directed mutations were verified by DNA sequencing using the primer syrB1PE. The plasmids used for GUS assays are listed in Table 1.

To identify the promoter region of operon III, 5' *cis* promoter deletion constructs were made from pSL105, a transcriptional fusion construct of the *sypA* gene with a promoterless *uidA* gene insertion in the BstZ171 site. To construct pSL105, the 3.2-kb HindIII-BglII fragment containing the *uidA-aacC1* genes from pSL2 was polished with T4 DNA polymerase and inserted at the BstZ171 site of pBS008 to generate pSL103. Then, the EcoRI-HindIII fragment-containing portion of the *syrD-sypA::uidA-aacC1* fusion was subcloned into pUCP26. The resulting construct, pSL105, lacks the promoter region for expression of the *sypA* gene and was used to define the promoter by inserting different upstream fragments. Variable regions of the operon III promoter, from the EcoRI site of *syrD* to the regions upstream or downstream of *syrP*, were amplified by PCR. The forward primer syrPR6 was used for all amplifications. Reverse primers containing an EcoRI restriction site were paired with syrPR6 for amplification of promoter variants of operon III. The resulting amplicons were digested with EcoRI and cloned into the EcoRI site of pSL105. All constructs were verified by sequencing using primer syrDF7 to confirm that the orientations were correct. The reverse primers used to generate the constructs were syrCRTR1 (pNW105), syrBR11 (pNW105-1), and syrBR24 (pNW105-2) (primer sequences are available upon request).

**GUS assays.** The effects of *cis* elements on expression of the *syrB1::uidA* or *sypA::uidA* gene fusions were evaluated by fluorometric analysis of GUS activity. The GUS assays were performed as described previously (29). All assays for GUS activity were performed three times on separate days with duplicate cultures for each treatment.

## RESULTS

**Operon analysis of the *syr-syp* genomic island.** Of the 21 genes associated with syringomycin and syringopeptin produc-

tion, 13 were organized into polycistronic operons including *pseA-pseB-pseC* (operon I, 5.7 kb), *sypD-ORF19* (operon II, 3.2 kb), *syrP-syrD-sypA-sypB* (operon III, 35.6 kb), *syrB1-syrB2* (operon IV, 3.0 kb), and *syrF-ORF20* (operon V, 2.3 kb). Operon III appeared to be one of the biggest operons identified in bacteria (Fig. 1). The *syrP*, *syrD*, *sypA*, and *sypB* genes are transcriptionally joined as demonstrated by RT-PCR analysis (Fig. 2, lanes 1, 4, 9, 13, and 14). No *sypB-sypC* product was obtained with primers based on the 3' and 5' sequences of these genes, respectively (Fig. 2, lane 10). The two weak bands observed in lanes 10 and 12 resulted from trace amounts of DNA present in the RNA samples based on their disappearance upon treatment with DNase I. All other RT-PCR products were not affected by DNase I treatment (data not shown). To ensure that negative RT-PCR data were not the result of internal problems with primers, genomic DNA was used as a template to check the fidelity of the primers. For all of the primer pairs, PCR products were obtained as predicted. These results demonstrate that *syrP*, *syrD*, *sypA*, and *sypB* are in one operon. Besides the five operons, *dat*, *sypC*, *syrE*, ORF21, and ORF22 are transcribed individually based on RT-PCR analysis (Fig. 2, lanes 2, 5, 6, 8, 10, 17, and 18). The *syrC* gene is transcribed individually even though a *syrB2-syrC* RT-PCR product was observed (Fig. 2, lane 4) (57). Apparently, this is because the promoter of *syrC* overlaps with the 3' end of *syrB2*, which makes the readthrough of *syrB2-syrC* possible. Two typical rho-independent terminators, located after the *syrP-syrD-sypA-sypB* operon and the *syrC* gene, were identified by the Find-Term program (Softberry) (Fig. 1). The *salA* and *syrG* genes were previously shown by Lu et al. (29) to be independent genes, bringing the total number of transcriptional units for the *syr-syp* genomic island to 13 (Fig. 1).

**Determination of the transcriptional start sites and promoters of the *salA* gene and operons III and IV.** Once the transcriptional units of the *syr-syp* genomic island were identified, the transcription start sites of the *salA* gene and operons III and IV were defined by primer extension analysis. A transcript was obtained for operon IV with primer syrB1PE, which is complementary to a region comprising 3 and 20 nucleotides upstream and downstream of the start codon of operon IV, indicating a transcriptional start site 104 nucleotides upstream of the *syrB1* translational start codon (Fig. 3). For operon III,

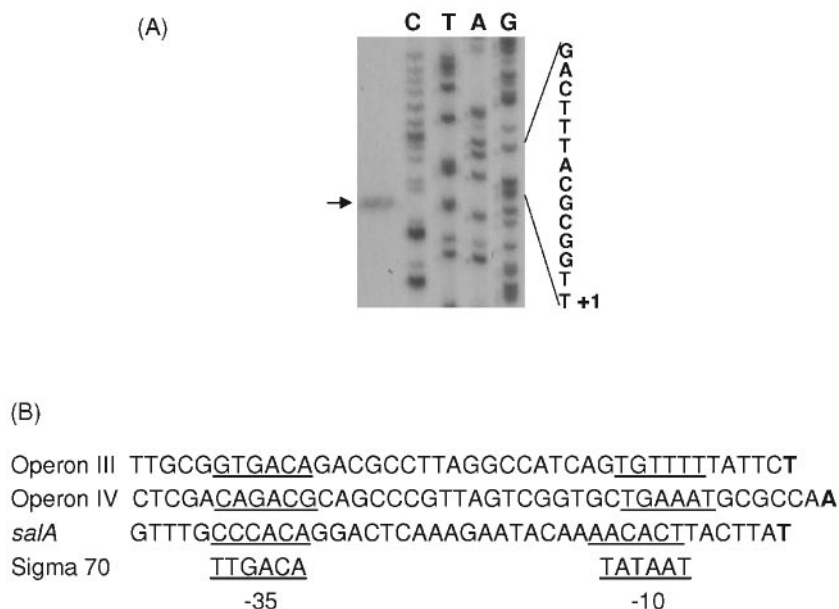


FIG. 3. Promoter analysis of operons III and IV and the *salA* gene. (A) Primer extension analysis of total RNA from *P. syringae* pv. *syringae* B301D to define the promoter region of operon IV (*syrB1-syrB2*). The 104-bp cDNA products (indicated by a black arrow) of operon IV mapped the transcriptional start site to a thymine residue shown with +1. (B) Comparison of putative promoter sequences of the *syr-syp* genes. The predicted promoter sequences of operon III, operon IV, and the *salA* gene are based on the defined transcriptional start sites. Conserved sequence motifs corresponding to the -35 and -10 sites are underlined. The typical  $\sigma^{70}$ -dependent promoter sequence is listed at the bottom.

primer extension indicated a transcriptional start site 75 nucleotides upstream of the start codon of *syrP* (data not shown). This analysis was performed using a primer complementary to a region comprising 14 and 37 nucleotides upstream of the initiation codon of operon III. For the *salA* gene, the transcriptional start site was located 63 nucleotides upstream of the *salA* translational start, using primer *salAPE*, which is complementary to a region comprising 11 and 34 nucleotides upstream of *salA* (data not shown).

The transcript of operon III was initiated at a thymine residue 75 bp upstream of the translational start site of *syrP*. The transcriptional start site of operon III suggested a putative promoter region, GTGACAN<sub>18</sub>TGTTTT (Fig. 3). Similarly, the transcriptional start site of operon IV was localized to an adenine residue 104 bp upstream of *syrB1* and suggested a putative promoter region, CAGACGN<sub>18</sub>TGAAAT (Fig. 3). Both promoters share high similarity to the  $\sigma^{70}$  consensus promoter sequences of gram-negative bacteria (5). The transcript of *salA* was initiated at a thymine residue 63 bp upstream of the translational start site of *salA*. The transcriptional start site of *salA* suggested a promoter region, CCCACAN<sub>17</sub>AACACT, which also shares some similarity with the typical  $\sigma^{70}$  consensus promoter sequences (5).

**Identification of the *syr-syp* box.** One imperfect dyad symmetric sequence (20 bp in length) was observed to overlap with the -35 regions of operon III based on computer analysis (Fig. 3). Similar sequences were found in the potential promoter regions of the *syr-syp* genes and operons responsible for synthesis and secretion of syringomycin and syringopeptin (Fig. 4). The consensus sequence TGtCccgN6cggGaCA is named the "*syr-syp* box."

**Deletion and site-directed mutagenesis analysis of the promoter region of operon IV.** The effects of the mutation of the

*cis* elements on expression of the *syrB1::uidA* fusion are shown in Fig. 5. Deletion constructs were generated from 817 bp (pNW104), 391 bp (pNW104-1), 276 bp (pNW104-2), 218 bp (pNW104-3), 180 bp (pNW104-4), and 111 bp (pNW104-5) upstream of the translational start site of *syrB1*. The resulting GUS activities of these constructs in B301D were 2,434, 1,974, 1,890, 1,800, 1,813, and 71 U/10<sup>8</sup> CFU, respectively. This indicates that the 69-bp region from 111 to 180 bp upstream of the start codon of *syrB1* is critical for expression of the *syrB1::uidA* fusion. When the putative -10 region TGAAAT was replaced with CTGCAG (pNW104-6), expression of the *syrB1::uidA* reporter in B301D was decreased about 85%. When the putative -35 region GACAGACGC was replaced with CTGCAG (pNW104-7), expression of the *syrB1::uidA* fusion in B301D

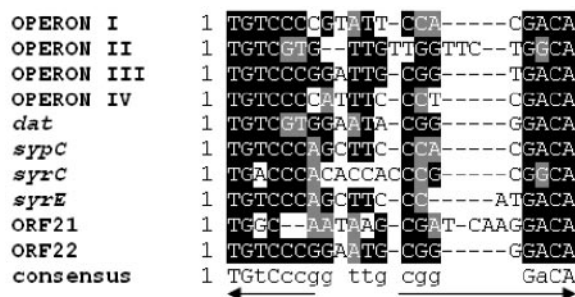


FIG. 4. Alignment of the *syr-syp* box elements identified in strain B301D of *P. syringae*. The dyad-symmetric DNA regions around the -35 region of the *syr-syp* genes were aligned using T-Coffee, and conserved sites are highlighted. The inverted repeats are indicated by arrows. The less conserved nucleotides in the consensus are represented in lowercase. The box shade figure was generated with BOX-SHADE 3.21 ([www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)).

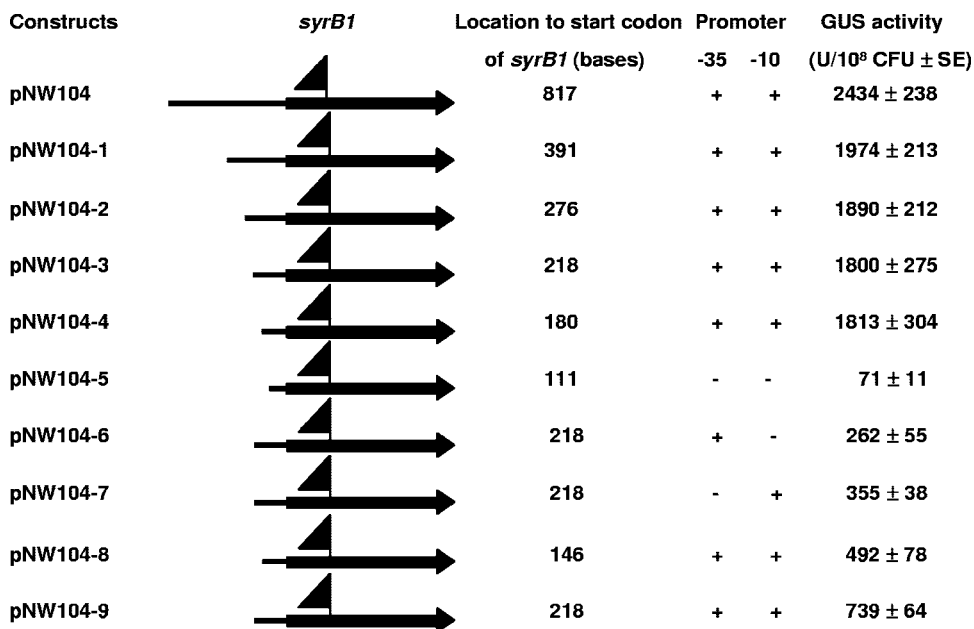


FIG. 5. Mutagenesis of the promoter region of operon IV evaluating the effect of *cis* elements on expression of a *syrB1::uidA* transcriptional fusion. The *syrB1* gene is represented by a black arrow. The *uidA* construct was inserted into the *EagI* site of *syrB1*. The *uidA* construct is represented by a flag. Plasmids containing the *syrB1::uidA* fusion with specific deletions or mutations were electroporated into cells of strain B301D for GUS assays. The transcriptional start site for operon IV was mapped at a position 104 nucleotides upstream of the *syrB1* start codon. All assays were repeated on three occasions with duplicate cultures.

was reduced about 80%. Once the -10 and -35 regions were confirmed by deletion analysis, the effect of the *syr-syp* box on expression of the *syrB1::uidA* fusion was tested by deletion from the -42 region and mutation of the 5' end of the *syr-syp* box. When the upstream sequence of the -42 region was deleted (pNW104-8), expression of the *syrB1::uidA* fusion in B301D was reduced about 73%. Expression of the *syrB1::uidA* reporter was lowered 59% when TGTCCC of the *syr-syp* box was replaced with CTGCAG (pNW104-9).

**Deletion analysis of the promoter region of operon III.** Effects of the deletions of *cis* elements on expression of the *sypA::uidA* fusion are shown in Fig. 6. When the upstream sequence was intact as shown in pNW105 and pNW105-1, the GUS activities were 796 and 719 U/10<sup>8</sup> CFU, respectively. Expression of the *sypA::uidA* reporter was decreased 94% when the upstream sequence was deleted from 59 bp upstream of the translational start

site of operon III (pNW105-2). Based on the deletion analysis, the promoter region was defined to a region between 59 and 335 bp upstream of the translational start site of operon III. Further deletion analysis of the downstream sequence from the translational start site of operon III showed results similar to pNW105-2. Plasmid pSL105 carries the intergenic region of *sypA* and the 3' end of *syrD*. The GUS activity for pSL105 was 54 U/10<sup>8</sup> CFU. This indicates that the *sypA* promoter is not at the intergenic region of *sypA* and *syrD* but rather that *sypA* shares the same promoter as *syrP* and *sypD*.

DISCUSSION

Conservation of the promoter regions in bacteria is vital for coordinated expression of genes of complementary pathways in response to the environment (5, 26). Expression of the *syr-syp* genes is coordinately controlled by plant signal

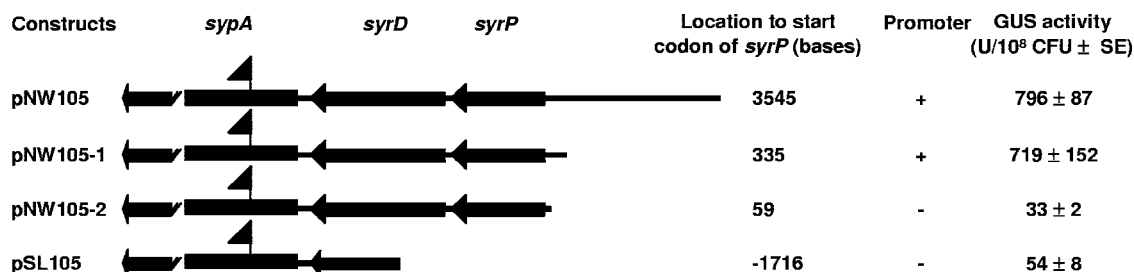


FIG. 6. Deletion analysis of the promoter region of operon III by evaluation of the effect of *cis* elements on expression of a *sypA::uidA* transcriptional fusion. The *syrP*, *syrD*, and *sypA* genes are represented by black arrows. The *uidA* construct was inserted into the *BstZ17I* site of *sypA*. The *uidA* construct is represented by a flag. Plasmids containing the *sypA::uidA* fusion with specific deletions were electroporated into cells of strain B301D for GUS assays. The transcription start site for operon III was mapped at a position 75 nucleotides upstream of the *syrP* start codon. All assays were repeated on three occasions with duplicate cultures.

molecules through the SalA-SyrF pathway in *P. syringae* pv. *syringae* B301D (54). It was established in this study that the conserved sequences, including the  $-10/-35$  sequence and the *syr-syp* box, in the promoter regions of the *syr-syp* genes contribute to the coregulation of syringomycin and syringopeptin production. The  $-10/-35$  sequence and the *syr-syp* box were required for expression of the *syrB1::uidA* reporter, and similar sequences have been identified in the upstream region of other *syr-syp* genes and operons, which are responsible for the biosynthesis and secretion of syringomycin and syringopeptin.

The first step to understand the regulatory mechanism of coregulation of the *syr-syp* genes was to determine the operon structure of the *syr-syp* genomic island. The 132-kb *syr-syp* genomic island was divided into five operons and eight individual genes (Fig. 1). For the five operons in the *syr-syp* genomic island, the intergenic lengths range from  $-4$  to 241 bp. This is consistent with the fact that adjacent open reading frames within an operon are usually separated by no more than 300 bp (13). In fact, the start codon of ORF20 overlaps with the stop codon of *syrF* (Fig. 1). This overlap provides strong evidence that *syrF*-ORF20 belong to one operon (Fig. 1) (25). A BLAST search indicated that ORF20 encodes an outer membrane protein, which is predicted to be involved in syringomycin or syringopeptin secretion. In addition, operons usually contain genes that are functionally related to each other (13, 31). Operon II (i.e., *sypD* and ORF19) is predicted to encode components of an efflux system including an ATP-binding cassette (ABC) transporter (*sypD*) and a periplasmic membrane protein (ORF19) (23). Operon IV (i.e., *syrB1* and *syrB2*) is involved in the synthesis of syringomycin, with *syrB1* encoding a synthetase protein (32) and *syrB2* encoding a non-heme Fe<sup>II</sup>  $\alpha$ -ketoglutarate- and O<sub>2</sub>-dependent halogenase (50, 57). Previous studies demonstrate that operons are often conserved among bacterial lineages (22, 31). This is true for operon I (i.e., *pseA*, *pseB*, and *pseC*) because BLAST search results (2) indicate that the *pseA-pseB-pseC* operon is conserved among many bacterial genomes, including *Dechloromonas aromatica* and *Ralstonia solanacearum* (40). Similarly, *sypD*-ORF19 operon II appears to be conserved in a number of prokaryotes, such as *Pseudomonas fluorescens* Pf-5 (36), *E. coli* (24), and *Yersinia pseudotuberculosis* (7). A rho-independent terminator is widely used to predict the end of one transcription unit (14, 27). Rho-independent terminators were found downstream of the stop codons of *sypB*, *syrC*, *salA*, and *syrG* (29) (Fig. 1). Besides the five operons mentioned above, eight genes (i.e., *dat*, *sypC*, *syrC*, *syrE*, ORF21, ORF22, *syrG*, and *salA*) are transcribed as monocistronic units, as suggested by the RT-PCR experiments, bioinformatic analysis, and translational analysis. These data demonstrate that the *syr-syp* genomic island contains at least 13 different promoter regions.

The promoters of the *syr-syp* genes and operons share high similarity with each other. Both promoters of operons III and IV share high similarity with the typical  $\sigma^{70}$ -like promoters (35).  $\sigma^{70}$ -like promoter sequences were observed for all of the *syr-syp* genes and operons (data not shown). The predicted *syr-syp* box sequence appears to be the key regulatory sequence for the coregulation of the *syr-syp* biosynthesis and secretion genes, because this conserved sequence was found in the promoter regions of 10 *syr-syp* genes/operons (Fig. 4). The conserved inverted repeat sequence TGtCccgN<sub>6</sub>cggGaCA, which

overlaps with the  $-35$  region, is predicted to be the binding site of the SyrF protein. SyrF belongs to the LuxR-FixJ family and shares significant homology with the 2.4 region of RNA polymerase sigma factor 70, which interacts with the DNA at the  $-35$  region (29, 35). Additionally, the feature of dyad symmetry of the *syr-syp* box is consistent with the fact that LuxR family proteins form dimers and bind to the inverted repeat sequence overlapping with the  $-35$  region (8, 12). The fact that SyrF forms dimers in vitro (Wang and Gross, unpublished) further supports the idea that the *syr-syp* box with its dyad symmetry is the binding site for SyrF. It is not surprising that the promoter regions of *salA*, *syrF*, and *syrG* do not contain the consensus sequence as observed for operons III and IV, because *salA*, *syrF*, and *syrG* do not belong to the SyrF regulon (Wang and Gross, unpublished). For the *syr-syp* promoters analyzed, there was great disparity in the extent to which each promoter resembles the consensus sequence, except for the eight nucleotides at the end of the *syr-syp* box (Fig. 3). A similar sequence with imperfect dyad symmetry has been reported for the TnrA box (TGTNANAWWTMTNACA) in *B. subtilis* (56). In addition, *las-rhl* box-like sequences (a 20-bp imperfect inverted repeat sequence) were identified in the promoter regions of 73 quorum-sensing-controlled genes in *Pseudomonas aeruginosa* using computer analysis (52). The *las-rhl* box sequence is different from the *syr-syp* box sequence and is not found in the promoter regions of genes/operons dedicated to secretion and synthesis of syringomycin and syringopeptin. This is consistent with the fact that syringomycin and syringopeptin production is not controlled by quorum sensing (28). A similar approach was used to search the whole genome of *P. syringae* pv. *syringae* B728a (20) for the conserved repeat sequence TGtCccgN<sub>6</sub>cggGaCA. Because B728a and B301D are closely related strains and share high similarity in their sequence (30), all of the *syr-syp* box sequences identified in B301D are conserved in B728a. In addition, the *syr-syp* box sequence was identified in the upstream regions of only three genes, namely, two hypothetical genes and an *nhaA* homolog encoding an Na<sup>+</sup>/H<sup>+</sup> antiporter protein. Although the roles of these three genes are unknown in regards to syringomycin and syringopeptin production, analysis of the B728a genome suggests that the *syr-syp* box sequence is not commonly found outside of the *syr-syp* gene cluster. Furthermore, it was observed that no *syr-syp* box sequence was found in the promoter regions of the syringolin gene cluster (3) despite evidence that *sylD* is controlled by *salA* (30).

The sigma factors involved in the regulation of the *syr-syp* genes and operons are yet to be determined. Originally, it was thought that  $\sigma^S$  might be the sigma factor involved in controlling production of syringomycin and syringopeptin since it controls the transcription of a considerable number of secondary metabolites (6, 21). Nevertheless, the fact that disruption of *rpoS* (data not shown) did not affect the production of syringomycin and syringopeptin indicates that *rpoS* is not the sigma factor responsible for transcriptional initiation of the *syr-syp* genes.  $\sigma^{70}$  might be the sigma factor responsible for the transcription of the *syr-syp* genes (35). The primary sigma factor  $\sigma^{70}$  is involved in the production of antibiotics and 2,4-diacetylphloroglucinol by *Pseudomonas fluorescens* CHA0 (42). In addition, LuxR interacts with  $\sigma^{70}$  to recruit RNA polymerase to the *luxI* promoter (46). However, it is still possible that

some alternative sigma factors belonging to the  $\sigma^{70}$  family recognize the *sy*-*sy* promoters (19).

In conclusion, a 20-bp *sy*-*sy* box sequence with imperfect dyad symmetry is required for expression of operon IV containing the *sy**B1* gene. We observed a similar sequence in the promoter regions of all of the coregulated *sy*-*sy* genes/operons responsible for synthesis and secretion of syringomycin and syringopeptin. Current research is analyzing the interaction of the *sy*-*sy* box with two key activators, SalA and SyrF, to understand the regulatory mechanism controlling *sy*-*sy* gene expression. A full appreciation of the regulation of syringomycin and syringopeptin production will shed light on at least one of the mechanisms that *P. syringae* uses to coordinate expression of distinct virulence factors.

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