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Characterization of the Transcriptional Activators SalA and SyrF, Which Are Required for Syringomycin and Syringopeptin Production by *Pseudomonas syringae* pv. syringae

Nian Wang,† Shi-En Lu,‡ Angela R. Records, and Dennis C. Gross*

Department of Plant Pathology and Microbiology, Texas A&M University, College Station, Texas 77843

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Production of the phytotoxins syringomycin and syringopeptin by *Pseudomonas syringae* pv. syringae is controlled by the regulatory genes *salA* and *syrF*. Analysis with 70-mer oligonucleotide microarrays established that the *syr-syp* genes responsible for synthesis and secretion of syringomycin and syringopeptin belong to the SyrF regulon. Vector pMEKm12 was successfully used to express both SalA and SyrF proteins fused to a maltose-binding protein (MBP) in *Escherichia coli* and *P. syringae* pv. syringae. Both the MBP-SalA and MBP-SyrF fusion proteins were purified by maltose affinity chromatography. Gel shift analysis revealed that the purified MBP-SyrF, but not the MBP-SalA fusion protein, bound to a 262-bp fragment of the *syrB1* promoter region containing the *syr-syp* box. Purified MBP-SalA caused a shift of a 324-bp band containing the putative *syrF* promoter. Gel filtration analysis and cross-linking experiments indicated that both SalA and SyrF form homodimers in vitro. Overexpression of the N-terminal regions of SalA and SyrF resulted in decreased syringomycin production by strain B301D and reduced levels of β-glucuronidase activities of the *sypA*::*uidA* and *syrB1*::*uidA* reporters by 59% to 74%. The effect of SalA on the expression of the *syr-syp* genes is mediated by SyrF, which activates the *syr-syp* genes by directly binding to the promoter regions. Both SalA and SyrF resemble other LuxR family proteins in dimerization and interaction with promoter regions of target genes.

Syringomycin and syringopeptin production by *Pseudomonas syringae* pv. syringae is coordinately controlled by a common regulatory mechanism. Both toxins are lipodepsipeptides and are synthesized separately by modular nonribosomal peptide synthetases (24, 62, 72). Genes dedicated to the biosynthesis, secretion, and regulation of the two toxins are localized in the syringomycin (*syr*) and syringopeptin (*syp*) gene clusters, which are adjacent to one another on the chromosome (40, 62). Assembly of the two compounds is induced by plant signal molecules such as arbutin and D-fructose (48, 68). Previous studies demonstrated that the two-component GacS/GacA system is critical for the regulation of both toxins (29, 36). The *gacS* gene encodes a transmembrane protein, which functions as a histidine protein kinase that undergoes phosphorylation in response to environmental stimuli (30). GacA is a response regulator protein that is phosphorylated by GacS (27, 29). The regulation of syringomycin and syringopeptin by GacS/GacA is mediated by the downstream regulator SalA. Neither syringomycin nor syringopeptin was produced by a *salA* mutant (36, 42). Analysis with 70-mer oligonucleotide microarrays, along with β-glucuronidase (GUS) assays and quantitative real-time PCR (QRT-PCR) analysis, demonstrated that all of the *syr-syp* genes (Fig. 1) belong to the SalA regulon (42). The *syrF* gene, which is positively controlled by SalA, is also required for syringomycin and syringopeptin production (40). Consequently, both SalA and SyrF are critical for the coregulation of syringomycin and syringopeptin production.

Both SalA and SyrF belong to a family of transcriptional activators characterized by high sequence similarities to the C-terminal region of LuxR, which contains a helix-turn-helix (HTH) domain (33). The LuxR DNA-binding domain consists of four helix bundles in which the HTH motif comprises the second and third helices (17). The LuxR superfamily proteins are grouped into two major subfamilies on the basis of sequence similarity at the N terminus and by their functional regulatory mechanism. One subfamily consists of the autoinducer-binding regulators including LuxR (33), LasR (19), CarR (70), EsaR (67), CerR (55), and TraR (53), which are activated by homoserine lactones. The LuxR protein is one of the most studied autoinducer-binding regulators and is essential for quorum sensing in *Vibrio fischeri* (18). LuxR contains an autoinducer-binding domain at the N terminus, which interacts with an acyl-homoserine lactone (acyl-HSL), and an HTH DNA-binding motif at the C terminus (15). LuxR activates the *lux* operon, necessary for light generation, by binding to the 20-bp *lux* box centered at the −42.5 position relative to the *luxI* transcriptional start site (12). Accordingly, LuxR contacts both the α-subunit carboxy-terminal domain and the σ subunit of RNA polymerase as an "ambidextrous activator" (12). Evidence that LuxR functions by forming a multimer exists (7).

The other subfamily of LuxR-like proteins is composed of the response regulators of the two-component signal transduction systems, including NarL (38), FixJ (3), NarP (11), GacA (59), and UhpB (31). NarL, which activates the nitrate reduc-

* Corresponding author. Mailing address: Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843. Phone: (979) 845-8288. Fax: (979) 845-6483. E-mail: d-gross@tamu.edu.

† Present address: Plant and Microbial Biology Department, University of California, Berkeley, CA 94720.

‡ Present address: Department of Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS 39762.

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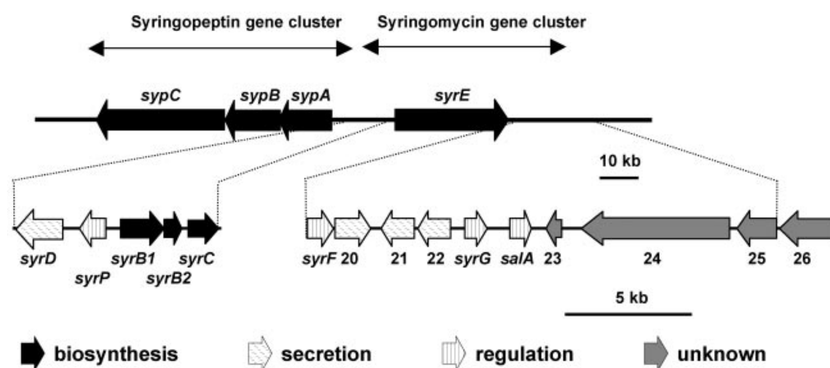

FIG. 1. Physical map of a 132-kb genomic island of *P. syringae* pv. syringae strain B301D containing both syringomycin (*syr*) and syringopeptin (*syp*) gene clusters. The positions and orientations of the known and potential ORFs are shown as horizontal arrows. The solid, diagonally striped, and vertically striped arrows represent genes that are predicted to be involved in the synthesis, secretion, and regulation of the phytotoxins, respectively. The gray arrows represent the putative ORFs for which functions remain unknown.

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tase operon in *Escherichia coli*, is one of the best-understood response regulators and is comprised of two domains, an amino-terminal receiver domain and a carboxyl-terminal effector domain (74). Unlike the proteins that respond to acyl-HSL, NarL is activated by phosphorylation signals (13). The NarL response regulator is phosphorylated at the N-terminal regulatory domain (74) and forms a dimer to recognize heptamer sequences, which are often present as pairs of inverted repeats in the promoter regions of target genes (45). Therefore, LuxR-type proteins from both subfamilies function similarly with regard to dimerization and interactions with promoter regions of target genes, despite sequence differences at the N terminus.

Sequence analyses of the SalA (284 amino acids) and SyrF (276 amino acids) proteins demonstrated that both proteins contain the HTH DNA-binding domain of the LuxR protein family at the C terminus (40). The C termini of SalA and SyrF exhibit 27 to 46% identity to LuxR (33), TraR (53), NarL (38), FixJ (3), and GerE (9, 40). Unlike LuxR, no autoinducer domain was identified at the N termini of the SalA and SyrF proteins (18), and unlike typical response regulators, SalA and SyrF lack the "acid pocket" composed of four highly conserved residues (Asp, Asp, Asp, and Lys) characteristic of response regulator receiver domains (52). Therefore, both SalA and SyrF belong to a novel LuxR subfamily (40).

Despite evidence that *salA* and *syrF* are required for syringomycin and syringopeptin production, the mechanisms behind SalA- and SyrF-activated expression of the *syr-syp* genes were largely unknown. In a recent study (69), a 20-bp conserved sequence (TGtCccgN6cggGaCA, termed the *syr-syp* box; the less conserved nucleotides in the consensus are in lowercase) with dyad symmetry around the −35 region was identified for the *syr-syp* genes/operons responsible for biosynthesis and secretion of syringomycin and syringopeptin. The −10/−35 regions of the *syr-syp* genes share high similarity with the σ^{70}-dependent promoter sequence. Apparently, 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 (69). It was hypothesized that SyrF controls the *syr-syp* genes by binding to their promoter regions. In this study, we demonstrate that the *syr-syp* genes are members of the SyrF regulon. In addition, we find that both SalA and SyrF resemble LuxR proteins with regard to dimerization and transcriptional activation of target genes by binding to their promoter regions. In particular, the control of the expression of the *syr-syp* genes by SalA is mediated by SyrF, which directly binds to the promoter regions of the *syr-syp* genes and activates their expression. This study provides an important foundation for understanding a novel LuxR subfamily of proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *P. syringae* pv. syringae strains and plasmids used in this study are listed in Table 1. Strains were routinely cultured in nutrient broth-yeast extract broth or on nutrient broth-yeast extract agar medium (66) at 25°C (*P. syringae* pv. syringae) or in Luria broth (LB) or on LB agar medium at 37°C (*E. coli* strain DH10B) (21). For microarray analysis, *P. syringae* pv. syringae strains were cultured on syringomycin minimal medium with exogenously added arbutin (100 μM) and D-fructose (0.1%) (SRM_{AF}) (23). For GUS assay experiments, *P. syringae* pv. syringae strains were cultured in potato-dextrose broth medium. Antibiotics (Sigma Chemical Co., St. Louis, MO) were added to media at the following concentrations: 25 μg of tetracycline per ml, 100 μg of kanamycin per ml, 100 μg of ampicillin per ml, and 5 μg of gentamicin per ml.

Microarray analysis. To test the effect of the mutation of *syrF* on the transcriptional expression of the *syr-syp* genes and representative genes associated with plant pathogenesis of *P. syringae* pv. syringae, microarray analysis was performed as described previously (42). Wild-type strain B301D and *syrF* mutant strain B301DSL1 of *P. syringae* pv. syringae were cultured with shaking at 25°C overnight in SRM_{AF} liquid medium (2 ml). Cells were harvested by centrifugation, washed twice with sterile deionized water, and then diluted with sterile deionized water to a concentration of approximately 2×10^8 CFU per ml. Cell suspensions (50 μl) were spread onto SRM_{AF} plates and were incubated at 25°C for 72 h prior to the recovery of cells. Total RNA was purified using a RiboPure-Bacteria kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. Total RNA (50 μg) was labeled with either Cy3-dUTP or Cy5-dUTP as described previously (42). Glass DNA microarrays containing a set of 70-mer oligonucleotides (42), designed and synthesized by QIAGEN (now available at Operon Biotechnologies, Inc., Huntsville, AL), were produced to represent genes contained in the *syr-syp* genomic island and other genes associated with virulence. The microarrays were used to quantify relative mRNA levels by parallel two-color hybridization according to protocols described in detail elsewhere previously (42). Briefly, hybridization was performed at 60°C overnight in a moist chamber. After washing, the slides were dried by centrifugation and scanned immediately using a GenePix 4000b scanner (Axon Instruments Inc., Foster City, CA) to visualize the hybridization images (42). Signal intensities and ratios were generated using GenePix Pro software, and the raw data were normalized using 16S rRNA genes as a standard. Microarray data with intensities reproducibly higher than that of the background level were selected for analysis. Hybridization

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference(s)
Strains		
Escherichia coli DH10B	F$^-$ *mcrA* Δ*lacX74* (ϕ80d*lac*ZΔ*M15*) Δ(*mrr-hsdRMS-mcrB*) *deoR recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL nupG*	21
Pseudomonas syringae pv. syringae		
B301D	Wild type, from pear	22
B301DSL1	*syrF*::*nptII* derivative of B301D; Km^r	40
B301DSL7	*salA*::*nptII* derivative of B301D; Km^r	40
B301DSL8	*syrB1*::*uidA-aaaC1*, derivative of B301D; Gm^r	40
B301DSL29	*sypA*::*uidA-aaaC1*, derivative of B301D; Gm^r	42
Plasmids		
pGEM-T Easy	Cloning vector; Ap^r	Stratagene, La Jolla, CA
pUCP26	Cloning vector; Tc^r Ap^r	71
pBR325	Cloning vector; Cm^r Tc^r Ap^r	54
pRK415	Broad-host-range cloning vector; Tc^r	20
pMEKm12	*E. coli* and *P. syringae* pv. syringae overexpression vector; Km^r	41
pSL2	pBI101 with the 0.85-kb *aacC1* gene of pUCCGM inserted at the EcoRI site downstream of the *uidA* gene; Km^r Gm^r	40
pSL8	pBR325 carrying the 3.0-kb EcoRI fragment of p29 containing *salA*; Tc^r Ap^r	40
pSL9	pBluescript SK(+) carrying a 2.5-kb HindIII-EcoRV fragment of pSL5 containing *syrG*; Ap^r	40
pSL21	pBR325 carrying the 3.0-kb fragment of p29 with *nptII* insertion at the KpnI site of *salA*; Ap^r Tc^r Km^r	40
pSL82	pMEKm12 carrying the *syrF* gene in-frame fused to *malE*; Km^r Pip^r	This study
pSL83	pMEKm12 carrying the *salA* gene in-frame fused to *malE*; Km^r Pip^r	This study
pNWB1probe	pGEM-T Easy vector carrying a 262-bp DNA fragment containing the confirmed *syrB1* promoter region; Ap^r	This study
pNWFprobe	pGEM-T Easy vector carrying a 324-bp DNA fragment containing the putative *syrF* promoter region; Ap^r	This study
pNWSalANE	pUCP26 carrying an EcoRI-KpnI fragment from pSL21, containing the 1.273 kb upstream of and 0.681 kb downstream of the start codon of *salA*; Tc^r	This study
pNWSyrF	pUC18 carrying a 0.86-kb PCR fragment of *syrF* with BamHI and PstI added at the 5′ and 3′ ends, respectively; Ap^r	This study
pNWSyrFSDM	pUC18 carrying a 0.86-kb PCR fragment of *syrF* with a second BamHI site added by site-directed mutagenesis; Ap^r	This study
pNWSyrFNE	pUCP26 carrying 0.571 kb of the 5′ end of *syrF*; Tc^r	This study

experiments were conducted four times, and each slide contained duplicate arrays.

QRT-PCR analysis. The effects of the mutation of *syrF* on the expression of *sypA*, *sypB*, *syrB1*, *syrC*, *sypD*, *syrD*, *sylD*, *hrpR*, *hrpZ*, and *recA* observed in the microarray analysis were verified by QRT-PCR using the QuantiTect SYBR Green RT-PCR kit (QIAGEN Inc., Valencia, CA). Total RNA from wild-type strain B301D and *syrF* mutant strain B301DSL1 was purified as described above. Primers used for QRT-PCR were designed using the Lasergene Expert sequence analysis package (DNAstar, Madison, WI) and are available upon request. Primers specific for the 16S rRNA gene were used for normalization. QRT-PCR was performed three times as described previously (42).

Construction of plasmids pSL82 and pSL83 for expression of the maltose-binding protein (MBP)-SyrF and MBP-SalA fusion proteins. For overexpression of the SyrF and SalA proteins, the *syrF* and *salA* genes were amplified with PCR and cloned into vector pMEKm12 (41). The primer pairs used for amplification of *syrF* and *salA* were FF-EcoRI and FR-HindIII and AF-EcoRI and AR-HindIII, respectively. The amplified fragments contained EcoRI and HindIII restriction sites and were digested with EcoRI and HindIII for insertion into pMEKm12 to generate pSL82 and pSL83 (Table 1) for overexpression of SyrF and SalA, respectively.

Expression and purification of SyrF and SalA proteins. Proteins were expressed in *E. coli* strain DH10B by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (0.3 mM, 25°C, 6 h) and purified via maltose affinity chromatography according to the manufacturer's instructions (New England Biolabs, Beverly, MA). Protein concentrations were measured using the Bradford assay (4). Fusion proteins were overexpressed from B301D by the same methods described above for *E. coli*, except that B301D cells were induced with 5 mM IPTG at 25°C for 6 h.

Gel mobility shift assays. A 262-bp DNA fragment containing the confirmed *syrB1* promoter region was amplified by PCR with primers syrB1RP and syrPFP using B301D genomic DNA as a template (69). The fragment was cloned into the pGEM-T Easy vector (Promega, Madison, Wisconsin) to generate plasmid pNWB1probe, which allowed for sequencing confirmation with a T7 primer. The DNA fragment was end labeled with [γ-^{32}P]ATP (Perkin-Elmer Life Sciences, Inc., Boston, MA) using T4 polynucleotide kinase (Promega, Madison, WI) at 37°C for 60 min. The labeled fragment was used as a probe (about 5 nM) by incubation with increasing amounts of SyrF-MBP or SalA-MBP for 10 min at room temperature in 10 μl of TGED binding buffer (50 mM Tris-HCl [pH 8.0], 5% [vol/vol] glycerol, 0.1 mM EDTA, 1 mM dithiothreitol) containing 20 μg of poly(dI-dC)/ml and 200 μg of bovine serum albumin/min (58). Next, 1% formaldehyde was added to the reaction mixture and kept for 10 min at room temperature to stabilize the protein-DNA interaction. Reaction mixtures were resolved on a 6% (wt/vol) nondenaturing polyacrylamide gel in Tris-borate-EDTA buffer at room temperature at 200 V. Competition experiments using 500 times more unlabeled probe were performed as described previously (58).

Similarly, a 324-bp DNA fragment containing the intergenic region of *syrE* and *syrF* was synthesized by PCR with primers syrERTF and syrFRP, labeled with [γ-^{32}P]ATP, and used to study the interaction with purified SalA protein.

Sephacryl S-200 gel filtration. Purified MBP-SalA (1 ml) was loaded and fractionated on a column (2.5 cm in diameter by 80 cm in length) packed with Sephacryl S-200 High Resolution (Amersham Biosciences, Piscataway, NJ) at a flow rate of 1.3 ml/min. The column was preequilibrated with elution buffer (50 mM Tris-HCl, pH 7.4) and calibrated with gel filtration molecular mass standards (12 to 200 kDa) (Sigma, St. Louis, MO). Eluted fractions (3 ml) were analyzed for the presence of MBP-SalA using Western blotting with polyclonal antibody to MBP. Polyclonal antiserum with antibodies for MBP was

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TABLE 2. Microarray analysis of the SyrF regulon and relevant genes

Gene[b]	Ratio (±SEM)[a]	Gene product	Reference or source
syrD	8.22 (±1.07)	ATP-binding secretion protein	57
syrP	17.76 (±1.94)	Homolog of histidine kinase	73
syrB1	23.06 (±2.37)	Syringomycin synthetase	72
syrB2	23.04 (±2.92)	Syringomycin biosynthesis enzyme	72
syrC	8.56 (±1.76)	Syringomycin biosynthesis enzyme	72
syrE-1	2.79 (±0.46)	Syringomycin synthetase	24
syrE-2	2.76 (±0.47)	Syringomycin synthetase	24
syrE-3	2.51 (±0.28)	Syringomycin synthetase	24
syrF	1.31 (±0.16)	LuxR family bacterial regulator	40
ORF20	2.23 (±0.10)	Putative outer membrane protein	40
ORF21	2.45 (±0.20)	Hypothetical protein	40
ORF22	3.27 (±0.37)	Membrane protein	40
syrG	1.75 (±0.46)	LuxR family regulatory protein	40
salA	1.89 (±0.14)	LuxR family bacterial regulator	40
sypA	7.56 (±2.61)	Syringopeptin synthetase	62
sypB-1	8.49 (±0.46)	Syringopeptin synthetase	62
sypB-2	2.78 (±0.80)	Syringopeptin synthetase	62
sypC1	2.07 (±0.22)	Syringopeptin synthetase	62
sypC2	2.58 (±0.22)	Syringopeptin synthetase	62
ORF19	2.21 (±0.09)	Putative membrane protein	Kang and Gross, unpublished
sypD	2.39 (±0.32)	Putative ABC transporter	Kang and Gross, unpublished
dat	2.69 (±0.45)	Aminotransferase	Kang and Gross, unpublished
pseA	2.20 (±0.58)	Putative outer membrane protein	32
pseB	1.68 (±0.33)	Efflux membrane fusion protein	32
pseC	1.42 (±0.12)	RND-type efflux protein	32
sylD	2.44 (±0.39)	Putative syringolin synthetase	2
16S rRNA gene	1 (±0.0)	16S ribosomal RNA	50

[a] Ratios were determined by comparing gene transcription (measured by signal intensity) in *P. syringae* pv. syringae B301D to that of B301DSL1 cultured on SRM_{AF} medium. Data shown in the table are the means of four independent experiments with the standard errors of means (SEM).

[b] Additional genes tested showed no significant differences in expression levels between *P. syringae* pv. syringae strains B301D and B301DSL1. These included housekeeping genes *sigX*, *algT*, *algD*, *sodB*, and *inaK* (ratios ranging from 1.05 to 1.78); siderophore genes *pvdS*, *pvdE*, *fsc*, *acsD*, *cbrB*, *cbrD*, and *fur* (0.99 to 1.48); global regulatory genes *gacS*, *gacA*, *rpoN*, *rpoS*, and *rpoD* (1.25 to 1.96); phytohormone synthesis genes *iaaM* and *iaaH* (1.40 and 1.35, respectively), environmental stress gene *rulA* (1.19); quorum-sensing gene *ahlI* (1.53); as well as 39 other genes analyzed previously by Lu et al. (42).

purchased from New England Biolabs (Beverly, MA). Western blot analyses were performed according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ). The protein content present in fractions was estimated by the Bio-Rad (Hercules, CA) protein assay, a modification of the Bradford procedure (4).

In vitro cross-linking. In vitro cross-linking experiments were performed with 20 ng/μl of purified SyrF-MBP or SalA-MBP protein in a 20-μl volume of cross-linking buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) (63). Purified MBP from *E. coli* was used as a negative control. The proteins were cross-linked with 1% formaldehyde for 20 min at room temperature, and the reactions were stopped by the addition of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) buffer (0.045 M Tris-Cl, pH 6.8, 10% glycerol, 1% SDS, 0.01% bromophenol blue, 0.05 M dithiothreitol) (60) and incubation for 10 min. The samples were heated for either 30 min at 37°C to maintain the formaldehyde cross-links or 20 min at 95°C to destroy them before samples were loaded onto a 10% SDS-PAGE gel. The samples were transferred onto a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ) after electrophoresis and immunoblotted using polyclonal antibody to MBP and MBP-SyrF, respectively. Polyclonal antiserum with antibodies recognizing SyrF-MBP was commercially produced by immunization of rabbits with purified SyrF protein (Pacific Immunology Corp., CA). Western blots were performed as described above.

Overexpression of the N-terminal domains of SalA and SyrF in B301D. To test the effect of overexpression of the N-terminal region of SalA, a 1.954-kb EcoRI-KpnI fragment from pSL21 was cloned into the EcoRI-KpnI sites of pUCP26 in a forward orientation to generate pNWSalANE. The fragment contains the 1.273 kb upstream of and the 0.681 kb downstream of the *salA* start codon. To test the effect of overexpression of the N-terminal region of SyrF, plasmid pNWSyrFNE, carrying the 0.57-kb 5′ end of *syrF*, was constructed. In brief, primers syrFPF4, which contains a BamHI site, and syrFPR5, which contains a PstI site, were used to amplify *syrF* using B301D genomic DNA as a template. The resulting DNA fragment was cloned into pUC18 (72), and the construct was named pNWSyrF. A BamHI site was introduced into pNWsyrF 571 bp downstream of the *syrF* start codon via the QuikChange site-directed mutagenesis kit using primers syrF571BamHIF and syrF571BamHIR, generating pNWSyrFSDM. A BamHI fragment from pNWsyrFSDM containing 571 bp of 5′ *syrF* was then subcloned in the forward orientation into pUCP26 to generate pNWSyrFNE. All of the constructs were verified by DNA sequencing with appropriate primers. B301D cells were transformed with pNWSalANE and pNWSyrFNE in order to test the effects of SalA or SyrF overexpression on syringomycin production with a standard bioassay, as described previously (61). Additionally, constructs pNWSalANE and pNWSyrFNE were transformed separately into B301DSL8 and B301DSL29 to test the effect of overexpression of the N-terminal regions of SalA and SyrF on GUS activities of the *syrB1*::*uidA* and *sypA*::*uidA* reporters. GUS assays were performed as described previously (40).

RESULTS

Identification of the SyrF regulon. Analysis of a 70-mer oligonucleotide microarray revealed that 16 *syr-syp* genes responsible for biosynthesis and secretion of syringomycin and syringopeptin were down-regulated greater than twofold in strain B301DSL1, a *syrF* mutant, compared with wild-type strain B301D (Table 2). Changes in expression levels of the biosynthesis genes for syringomycin (i.e., *syrB1* and *syrE*) (72) and syringopeptin (i.e., *sypA*, *sypB*, and *sypC*) (62) ranged from 2.1- to 23.1-fold. Seven putative secretion genes (i.e., *syrD*, ORF19, ORF20, ORF21, ORF22, *sypD*, and *pseA*) were repressed by as much as 8.2-fold in B301DSL1. In addition to the genes in the *syr-syp* genomic island, the expression of *sylD*, which is responsible for biosynthesis of syringolin (2), changed 2.4-fold. However, the changes of expression levels (*n*-fold) for the two regulatory genes (i.e., *salA* and *syrG*) located at the

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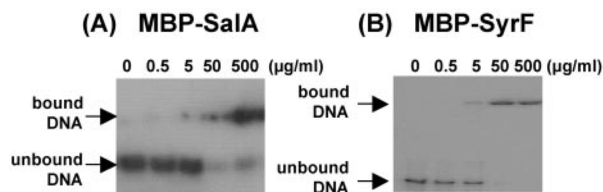

FIG. 2. Gel electrophoretic mobility shift analysis of the binding of SalA and SyrF with the regulatory regions of various genes. MBP-SyrF and MBP-SalA were expressed in *E. coli* and purified with maltose affinity chromatography. The regulatory regions of target genes were amplified by PCR and end labeled with [γ-^{32}P]ATP. The end-labeled probes were incubated with the purified MBP-SalA and MBP-SyrF proteins at the indicated concentrations and subjected to nondenaturing polyacrylamide gel electrophoresis. (A) The 324-bp fragment (5 nM) containing the putative promoter region of *syrF* was incubated without or with increasing concentrations of MBP-SalA. (B) The 262-bp fragment (5 nM) containing the promoter regions of *syrB1* and *syrP* was incubated without or with increasing concentrations of MBP-SyrF.

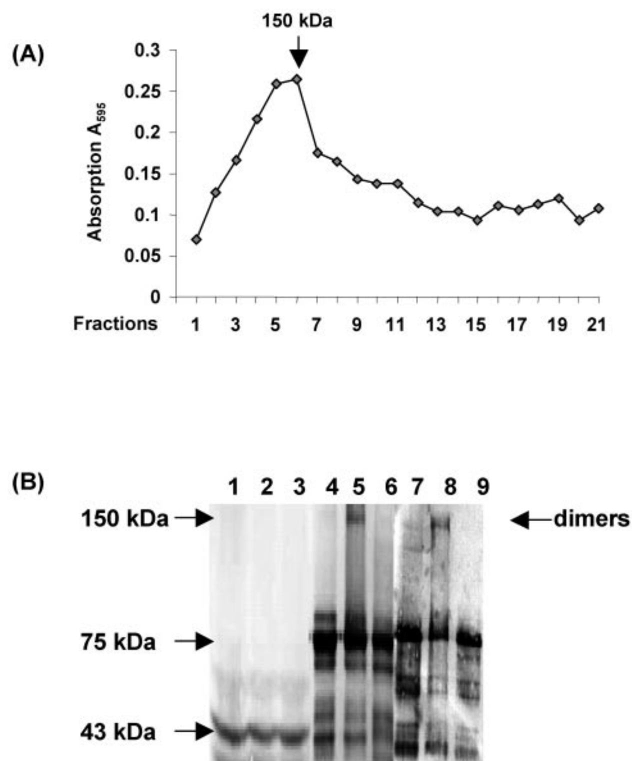

FIG. 3. Gel filtration or in vitro cross-linking of the MBP, SalA-MBP, and SyrF-MBP proteins showing dimerization of the SalA and SyrF proteins. Fractions of MBP-SalA purified with maltose affinity chromatography were separated by gel filtration on a Sephacryl S-200 column and analyzed by SDS-PAGE. (A) Absorption of MBP-SalA at 595 nm with the Bradford assay. (B) In vitro cross-linking of MBP, SalA-MBP, and SyrF-MBP proteins. Lanes: 1, MBP without cross-linking; 2, MBP with cross-linking; 3, MBP treated at 95°C for 20 min after cross-linking; 4, MBP-SalA without cross-linking; 5, MBP-SalA with cross-linking; 6, MBP-SalA treated at 95°C for 20 min after cross-linking; 7, MBP-SyrF without cross-linking; 8, MBP-SyrF with cross-linking; 9, MBP-SyrF treated at 95°C for 20 min after cross-linking. The resulting proteins were resolved by 10% SDS-PAGE, transferred onto a Hybond-P polyvinylidene difluoride membrane, and immunoblotted using a polyclonal antibody to MBP or MBP-SyrF.

right border of the *syr-syp* genomic island (Fig. 1) were below the twofold threshold.

In this study, none of the genes or open reading frames (ORFs) included in the array other than those identified above displayed changes in expression levels of more than twofold (Table 2). Housekeeping genes such as *sigX* (5), *algT* (34), *algD* (14), *sodB* (26), and *inaK* (39), located outside of the *syr-syp* genomic island, were expressed at high levels in SRM_{AF} medium with no significant differences in expression levels between B301D and B301DSL1. Genes involved in siderophore production (i.e., *pvdS* [49], *pvdE* [47], *fsc* [50], *acsD* [16], *cbrB* [44], *cbrD* [44], and *fur* [25]), environmental stress (*rulA*) (75), quorum sensing (*ahlI*) (35), global regulation (i.e., *gacS*, *gacA*, *rpoN* [1, 8], *rpoS* [28], and *rpoD* [64]), phytohormone synthesis (*iaaM* and *iaaH*) (46), and alginate production (*algD*) (37) were not affected by the mutation of *syrF*.

The regulation patterns of *syrF* defined by QRT-PCR were similar to those determined by microarray analysis. QRT-PCR analyses indicated that transcriptional expression levels for *sypA*, *sypB*, *syrB1*, *syrC*, *sypD*, *syrD*, *sylD*, and *recA* changed 5.5-, 11.3-, 9.7-, 3.4-, 2.8-, 2.9-, 2.1-, and 1.2-fold, respectively, for B301DSL1 compared to B301D grown in SRM_{AF}. Microarray analysis revealed that the changes for these genes at the transcriptional level were 7.6-, 5.6-, 23.1-, 8.6-, 2.4-, 8.2-, 2.4-, and 1.5-fold, respectively (Table 2). QRT-PCR was repeated three times, with consistent results.

Apparently, SyrF acts as a transcriptional activator controlling all of the *syr-syp* genes responsible for synthesis and secretion of toxins, as demonstrated by analysis with 70-mer oligonucleotide subgenomic microarrays (Table 2). This is consistent with the fact that most LuxR-type regulators act as transcription activators (17).

Interactions of the SalA and SyrF proteins with the *syr-syp* promoter regions. MBP-SyrF and MBP-SalA fusion proteins were overproduced in *E. coli* strain DH10B. MBP-tagged proteins were purified by maltose affinity chromatography, and analysis of the purified proteins on a 10% SDS-PAGE gel revealed the overexpression of products that were approximately 75 kDa in size (data not shown). Preliminary data showed that MBP does not interact with the 262-bp DNA fragment containing the confirmed *syrB1* promoter region. Therefore, the purified MBP-SalA and MBP-SyrF fusion proteins were used to study the interactions between the SalA or SyrF protein and the *syr-syp* promoter regions. Purified MBP-SyrF caused a single band shift when the 262-bp DNA fragment (Fig. 1) was incubated in the presence of increasing concentrations of MBP-SyrF (Fig. 2). The retarded band caused by MBP-SyrF was lost in competition assays in which 500-fold more unlabeled probe was used. Purified MBP-SalA did not cause a band shift of the same 262-bp DNA fragment (data not shown), but it caused the retardation of a 324-bp DNA fragment containing the intergenic region of *syrE* and *syrF* (Fig. 2).

Both SalA and SyrF form dimers in vitro. Gel filtration analysis of MBP-SalA obtained through maltose affinity chromatography revealed a peak that corresponded to a molecular mass of about 150 kDa (Fig. 3A). This peak represented the majority of the MBP-SalA protein in a dimerized state. Cross-linking assays with purified MBP-SalA indicated that MBP-

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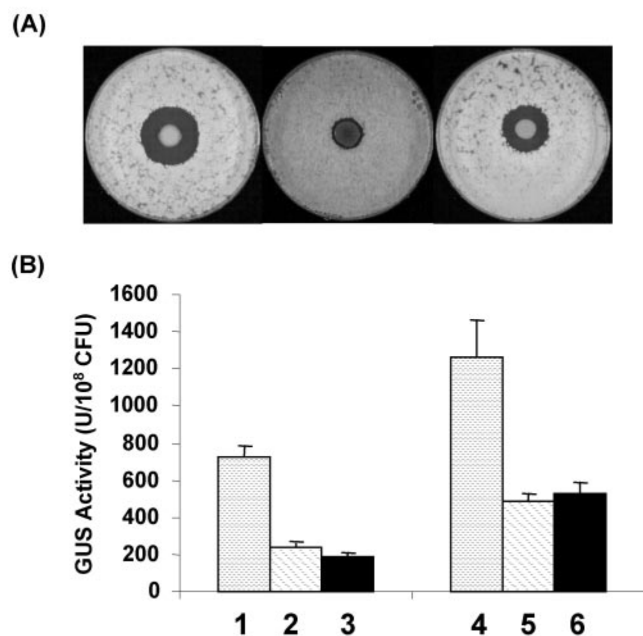

FIG. 4. Effects of overexpression of the N-terminal domains of SalA and SyrF on syringomycin production by B301D and expression of the *syrB1*::*uidA* and *sypA*::*uidA* reporters. (A) Bacterial strains were cultured on potato-dextrose agar medium supplemented with 25 μg/ml of tetracycline for 4 days. The inoculated plates were oversprayed with *Geotrichum candidum* and then incubated overnight. Left, B301D (pUCP26); middle, B301D (pNWSalANE); right, B301D (pNWSyrFNE). (B) Strains were incubated for 72 h at 25°C in potato-dextrose broth medium and tested for GUS activity. 1, B301DSL8 (pUCP26); 2, B301DSL8 (pNWSalANE); 3, B301DSL8 (pNWSyrFNE); 4, B301DSL29 (pUCP26); 5, B301DSL29 (pNWSalANE); 6, B301DSL29 (pNWSyr FNE). Vertical bars indicate standard errors of the means for triplicate cultures.

SalA forms a dimer, which migrated as an approximately 150-kDa fragment and was distinguishable from the 75-kDa fragment of the MBP-SalA monomer (Fig. 3B). No dimers were observed when only MBP was subjected to cross-linking assays (Fig. 3B). Similarly, a dimer of about 150 kDa was observed for MBP-SyrF after cross-linking. The disappearance of the dimers upon boiling (Fig. 3B, lanes 6 and 9) demonstrated that the formation of the dimers was indeed the result of cross-linking by 1% formaldehyde.

Overexpression of the N-terminal regions of SalA and SyrF. Overexpression of the N-terminal regions of SalA and SyrF in B301D decreased the sizes of syringomycin zones of inhibition of *Geotrichum candidum* from 8 mm to 1 mm and 3 mm, respectively (Fig. 4A). Expression of the *syrB1*::*uidA* reporter in strain B301DSL8 was reduced from approximately 1,200 U/10^8 CFU to about 500 U/10^8 CFU, a 62% decrease, by overexpression of the N-terminal region of SalA (Fig. 4B). Similarly, expression of the *sypA*::*uidA* reporter decreased from 381 U/10^8 CFU to 131 U/10^8 CFU, a 67% reduction (Fig. 4B). Furthermore, overexpression of the N-terminal two-thirds of SyrF lowered the GUS activities of *sypA*::*uidA* and *syrB1*::*uidA* by about 74% and 59%, respectively. These results are probably due to the fact that the nonfunctional heterodimers formed by the natural proteins and the truncated proteins interfere with the binding of the promoter regions. Both toxin bioassays and GUS assays were performed in triplicate, with consistent results.

DISCUSSION

Previous studies demonstrated that production of both syringomycin and syringopeptin is coordinately controlled by a complex regulatory cascade including GacS/GacA (29), SalA (36, 40), and SyrF (40). This study established that the *syr-syp* genes involved in synthesis and secretion of both toxins belong to the SyrF regulon and that both SalA and SyrF function in a manner similar to that of LuxR proteins. Both SalA and SyrF form dimers and interact with the promoter regions of their target genes, the *syr-syp* genes, based on the following evidence: (i) analysis with a subgenomic 70-mer oligonucleotide microarray, along with QRT-PCR, indicated that the *syr-syp* genes responsible for biosynthesis and secretion of syringomycin and syringopeptin belong to the SyrF regulon; (ii) gel mobility shift analysis showed that purified MBP-SyrF, but not the MBP-SalA fusion protein, bound to a 262-bp fragment containing the *syr-syp* box; (iii) purified MBP-SalA caused a shift in mobility of a 324-bp band containing the putative *syrF* promoter; (iv) gel filtration analysis and cross-linking experiments revealed that both SalA and SyrF formed dimers in vitro; and (v) syringomycin production by B301D was decreased and GUS activities of the *sypA*::*uidA* and *syrB1*::*uidA* reporters were reduced by 59% to 74% by overexpression of the N-terminal regions of SalA and SyrF. This study provides a valuable foundation for an understanding of the regulatory mechanism of a unique subfamily of LuxR proteins.

Dimerization is critical for transcriptional factors such as TraR (43) and NarL (45) to bind to promoter regions of target genes. Both SalA and SyrF resemble LuxR proteins with regard to dimerization, as evidenced by gel filtration (Fig. 3A) and cross-linking analyses (Fig. 3B). Syringomycin production by B301D and expression of the *syrB1*::*uidA* and *sypA*::*uidA* reporters were decreased by overexpression of the N-terminal region of SalA and SyrF (Fig. 4). This result can be explained by the fact that the N-terminal regions of SalA and SyrF are responsible for dimerization. Consequently, overexpression of the N-terminal region results in the formation of a nonfunctional heterodimer that cannot bind to the promoter region. Overexpression of the N-terminal domain of LuxR interferes with luminescence in *Vibrio fischeri* (7). Residues 116 to 161 in the N-terminal domain of LuxR are critical for its oligomerization (7). In addition, the N-terminal domain of TraR (residues 119 to 156) is required for dimerization, which is a requisite for the binding of TraR to the *tra* box (43). TrlR, a truncated TraR homolog lacking the C-terminal HTH DNA-binding domain, inhibits the function of TraR by forming an inactive heterodimer with the TraR protein (76). The dimerization of SyrF, LuxR (7), and TraR (56) is consistent with the existence of inverted repeat sequences in the promoter regions of the *syr-syp* genes (69), *luxI* (12), and the *tra* operon (77).

SalA and SyrF resemble other LuxR proteins with regard to regulation by binding to the promoter regions of target genes (17, 40). Purified MBP-SyrF binds to a 262-bp fragment containing a 20-bp sequence with dyad symmetry (TGTCccgN6cg

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gGACA) overlapping with the −35 region of *syrB1* (Fig. 2) (69). The *syr-syp* box is required for expression of the *syrB1*::*uidA* fusion and is identified in the promoter regions of the *syr-syp* genes/operons responsible for biosynthesis and secretion of syringomycin and syringopeptin (69). It is possible that SyrF binds to the *syr-syp* box, although no direct evidence of binding of the inverted repeats is available. LuxR is known to bind to the *lux* box, which is 20 bp in length with dyad symmetry centered at the −42.5 position relative to the transcriptional start site of *luxI* (12). In addition, the HTH domains of SyrF and LuxR share significant homology with the 2.4 region of the σ subunit of RNA polymerase, which interacts with the −35 region (40, 51).

Unlike LuxR, an autoinducer is not required for the binding of a target DNA sequence or for dimerization by purified MBP-SalA and MBP-SyrF. Most LuxR autoinducer-binding proteins are involved in quorum sensing to respond to cell population density by binding to acyl-homoserine lactone signal molecules (18). Interaction with acyl-homoserine lactone triggers conformational changes that stimulate dimerization and DNA binding (78). Purified LuxR binds specifically to DNA containing a *lux* box in the presence of *N*-(3-oxohexanoyl)-homoserine lactone (65). Binding of 3-oxo-octanolyl-homoserine lactone is required for the dimerization of TraR and its interaction with the *tra* box, an 18-bp palindromic element, in *Agrobacterium tumefaciens* (78). In vitro expression of TraR without an autoinducer does not form a dimer and does not bind to the *tra* box (56). The fact that an autoinducer is not required for dimerization of SalA or SyrF or for their binding to target DNA sequences corresponds with the fact that neither SalA nor SyrF contains an acyl-HSL autoinducer-binding domain and acyl-HSL is not required for the function of either SalA or SyrF. This is consistent with the observation that mutation of *ahlI* (6), which is responsible for the production of acyl-HSL, did not affect production of syringomycin (N. Wang and D. C. Gross, unpublished data). Response regulators of the LuxR family of proteins, such as NarL and FixJ, form dimers and bind to promoter regions in the presence of phosphorylation signals but not in the presence of an autoinducer (10, 45). The phosphorylation status of functional SalA and SyrF proteins remains unknown, and it is not clear whether they are activated by phosphorylation for the response regulator NarL as described previously (74) or by some unknown signal molecule. The data presented above clearly indicate that SalA and SyrF resemble LuxR proteins, even though they are not activated by autoinducers, and their phosphorylation status remains unknown (40).

In conclusion, both SalA and SyrF are similar to LuxR proteins with regard to dimerization and interactions with promoter regions of target genes. SalA regulates the *syr-syp* genes by forming a dimer and interacting with the *syrF* promoter. SyrF then activates the *syr-syp* genes directly by binding to their promoter regions. Results from this study provide evidence that SyrF is the key transcriptional factor in the activation of the *syr-syp* genes for *P. syringae* pv. syringae, allowing the bacterium to adapt to a rapidly changing environment. This study is the first report to delimit the regulatory mechanism of a unique LuxR subfamily of proteins.

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