Oligonucleotide Microarray Analysis of the *SaIA* Regulon Controlling Phytotoxin Production by *Pseudomonas syringae* pv. *syringae*

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The *saiA* gene is a key regulatory element for syringomycin production by *Pseudomonas syringae* pv. *syringae* and encodes a member of the LuxR regulatory protein family. Previous studies revealed that *saiA*, a member of the GacS/GacA signal transduction system, was required for bacterial virulence, syringomycin production, and expression of the *syrB1* synthetase gene. To define the *SaIA* regulon, the spotted oligonucleotide microarray was constructed using gene-specific 70-mer oligonucleotides of all open reading frames (ORFs) predicted in the syringomycin (*syr*) and syringopeptin (*syp*) gene clusters along with representative genes important to bacterial virulence, growth, and survival. The microarray containing 95 oligos was used to analyze transcriptional changes in a *saiA* mutant (B301DSL07) and its wild-type strain, B301D. Expression of 16 genes was significantly higher (> twofold) in B301D than in the *saiA* mutant; the maximum change in expression was 15-fold for some toxin biosynthesis genes. Except for the *sylD* synthetase gene for syringolin production, all ORFs controlled by *SaIA* were located in the *syr-syp* genomic island and were associated with biosynthesis, secretion, and regulation of syringomycin and syringopeptin. The positive regulatory effect of *SaIA* on transcription of *sypA*, *syrB1*, *syrC*, and *sylD* was verified by reporter fusions or real-time polymerase chain reaction analysis. None of the genes or ORFs was significantly down-regulated by the *saiA* gene. These results demonstrated that a subgenomic oligonucleotide microarray is a powerful tool for defining the *SaIA* regulon and its relationship to other genes important to plant pathogenesis.

Pseudomonas syringae pv. *syringae* causes cankers, blossom blights, and leaf spots in several economically important plants (Young 1991). A distinctive feature of this bacterium is the production of two lipopeptide toxins, syringomycin and syringopeptin (Bender et al. 1999), which are synthesized separately by modular nonribosomal peptide synthetases (Guenzi et al.

1998; Scholz-Schroeder et al. 2003; Zhang et al. 1995). The two phytotoxins are major virulence factors in plant pathogenesis (Scholz-Schroeder et al. 2001a).

Genes dedicated to biosynthesis, secretion, and regulation of the two toxins are localized in an approximately 150-kb genomic island of the strain B301D chromosome. The syringomycin (*syr*) and syringopeptin (*syp*) gene clusters are located adjacent to one another on the chromosome and are approximately 55 and 90 kb in size, respectively (Lu et al. 2002; Scholz-Schroeder et al. 2001b, 2003). The predominant feature of the toxin clusters is the presence of genes encoding modular nonribosomal peptide synthetases for syringomycin (i.e., *syrB1* and *syrE*) and syringopeptin (*sypA*, *sypB*, and *sypC*) production (Guenzi et al. 1998; Scholz-Schroeder et al. 2003; Zhang et al. 1995). Each peptide synthetase is composed of one or more catalytic modules responsible for the addition of a specific amino acid to the growing peptide chain (Mootz et al. 2002). As predicted from the toxin structures, nine syringomycin and 22 syringopeptin modules were identified and each module was shown to possess adenylation, thiolation, and condensation domains (Guenzi et al. 1998; Scholz-Schroeder et al. 2003; Zhang et al. 1995). A few regulatory genes, including the *saiA*, *syrF*, and *syrG* genes, were identified within the *syr-syp* genomic island (Lu et al. 2002). Functional *saiA* and *syrF* genes are required for syringomycin production, and the *syrG* gene is not essential for toxin production but is associated with virulence of *P. syringae* pv. *syringae*. Three type I toxin efflux systems responsible for secretion of phytotoxins were predicted to occur within the *syr-syp* genomic island (Fig. 1) (Gross et al. 2003; Quigley et al. 1993). In addition, the flanking region of the right border of the *syr-syp* genomic island was sequenced and shown to encode a set of proteins sharing similarity to synthetases and a transporter of achromobactin (B728a genome, Joint Genome Institute) (Franza et al. 2002). The flanking region of the left border of the *syr-syp* genomic island also was sequenced and a few unknown open reading frames (ORFs) were predicted to be present (N. Wang and D. C. Gross, unpublished data). Altogether, a 180-kb genomic region containing the *syr* and *syp* genomic island was sequenced that encompassed approximately 3% of the genome of strain B301D.

Oligonucleotide microarray analysis is a powerful approach for studying transcriptional regulation (Rhodius and LaRossa 2003) and functional genomics (Giles and Kipling 2003; Kane et al. 2000; Lockhart et al. 1996; Tolstrup et al. 2003) and is applicable to studies of the regulatory network controlling expression of the *syr-syp* genomic island. DNA microarrays can be fabricated using genomic DNA (Gerhold et

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*The e-Xtra logo stands for “electronic extra” and indicates that Figures 1 and 2 are in color online and the HTML abstract available on-line contains a supplemental table of 70-mer oligonucleotide sequences not included in the print edition.

al. 1999; Okinaka et al. 2002), cDNA (Schena et al. 1995), polymerase chain reaction (PCR) products (de Souza et al. 2003; Richmond et al. 1999), and synthesized oligonucleotides (Giles and Kipling 2003; Kane et al. 2000; Lockhart et al. 1996; Tolstrup et al. 2003; Wagner et al. 2003). Transcriptional profiling by microarray analysis is increasingly being used in bacteria to define regulons (Britton et al. 2002; Cao et al. 2002; Laub et al. 2002; Schuster et al. 2003; Wagner et al. 2003) and stimulons (Price et al. 2001; Stewart et al. 2002; Zheng et al. 2001). For example, oligonucleotide microarray analysis was used by Wagner and associates (2003) to show that 626 genes were regulated by the quorum-sensing system in *Pseudomonas aeruginosa*. Currently, oligonucleotide microarrays have become popular, due to their high gene specificity, uniform characteristics, and lower expense (Hessner et al. 2004); consequently, a growing number of 60- to 70-mer oligonucleotide microarrays are available commercially.

The *salA* regulatory gene was shown to have a critical role in syringomycin production and virulence of *P. syringae* pv. *syringae*. Consequently, the *salA* gene is an obvious focal point of transcription/microarray analysis of the *syr-syp* genomic island. The *salA* gene originally was identified from *P. syringae* pv. *syringae* B728a, a bean strain causing brown spot disease; *salA* was determined to be required for syringomycin production in vitro and lesion formation in bean leaves (Kitten et al. 1998). The GacS/GacA global signal transduction system positively regulates expression of the *salA* gene, and a functional *salA* gene is required for expression of the *syrB1* synthetase gene of syringomycin (Kitten et al. 1998). A helix-turn-helix DNA-binding motif was identified from the C terminus of the putative SalA protein that exhibits significant homology to members of the LuxR regulatory proteins, such as GerE (Cutting and Mandelstam 1986) and DctR (Hamblin et al. 1993). Lu and associates (2002) demonstrated that the *salA* gene was localized 8.5 kb downstream of the *syrE* gene and that the putative SalA proteins from strains B301D and B728a differed by one amino acid. Furthermore, the *salA* gene was demonstrated to control expression of the *syrF* gene located between the *syrE* and *salA* genes (Fig. 1) and *salA* was associated with syringopeptin production (Lu et al. 2002).

Despite evidence that the *salA* gene is essential for syringomycin production and virulence of *P. syringae* pv. *syringae*, the members of the SalA regulon remain largely unknown. For example, it is unclear if the *salA* gene positively regulates ex-

pression of all the genes associated with the *syr-syp* genomic island. In addition, multiple pathogenesis-related factors of *P. syringae* have been demonstrated or predicted to be involved in plant pathogenesis, such as the Hrp type III secretion system (Collmer et al. 2002), phytohormone synthesis (Glickmann et al. 1998; Mazzola and White 1994), siderophore production (Buell et al. 2003), syringolin production (Wäspi et al. 1998), and pectate lyase production (Bauer and Collmer 1997). It remains to be determined if the *salA* gene is involved in regulation of these pathogenesis-related factors.

In this study, a microarray was constructed of 70-mer oligonucleotides that represent all ORFs identified in the *syr-syp* genomic island, as well as genes involved in production of factors associated with plant pathogenesis. Transcriptional profiles of a *salA* mutant, B301DSL7 (*salA::nptII*) (Lu et al. 2002), were compared with those of wild-type strain B301D. Evidence is presented that the *salA* gene controls expression of genes contributing to the synthesis, secretion, and regulation of both syringomycin and syringopeptin phytotoxins, as well as the *sylD* synthetase gene for syringolin. These results are discussed in regard to the definition of the SalA regulon and its role in plant pathogenesis by *P. syringae* pv. *syringae*.

RESULTS

Construction of an oligonucleotide microarray.

A subgenomic microarray of *P. syringae* pv. *syringae* was constructed using 70-mer oligonucleotides that represent genes of interest. Of 95 oligonucleotides, 19 were designed from genes and putative ORFs identified from the *syr* gene cluster, and 30 oligonucleotides were selected from genes and putative ORFs located in the *syp* gene cluster or its border region (Fig. 1). In addition, oligonucleotides were designed for genes involved in the Hrp type III secretion system (*avrP*, *virPphA*, *hrpL*, *hrpR*, *hrpS*, *hrpZ*, *hrpJ*, *hrpW*, and *hrpK*), phytohormone synthesis (*iaaM*, *iaaH*, and *efe*), alginate production (*algT* and *algD*), siderophore production (*pvdS*, *pvdD*, *fsc*, *acsD*, *chrB*, *chrD*, and *fur*), syringolin production (*sylD*), ice nucleation (*inaK*), pectate lyase (*ptl*), environmental stress (*rulA* and *sodB*), quorum sensing (*ahlI*), and global regulation (*gacS*, *gacA*, *rpoN*, *rpoS*, *gyrB*, and *sigX*). All the oligonucleotides representing genes and ORFs identified in the *syr-syp* genomic island were designed from strain B301D of *P. syringae* pv. *syringae*. For large synthetase genes, more than one oligonucleotide were designed and included in the subgenomic microarray. Of the 95 oligonucleotides, 26 were

Fig. 1. A physical map of a 155-kb genomic DNA region of *Pseudomonas syringae* pv. *syringae* B301D containing both the syringomycin and syringopeptin gene clusters. The red, green, and yellow arrows represent genes that are predicted to be involved in, respectively, biosynthesis, secretion, and regulation of the phytotoxins. The black arrows represent genes whose functions remain unknown. The numbers identify the potential opening reading frames (ORFs). The phenotypic results of disruption of genes or ORFs are designated with a flag pointed right for loss of syringomycin production, a flag pointed left for loss of syringopeptin production, and a double-sided flag for loss of both syringomycin and syringopeptin production. The restriction enzyme sites for *Hind*III (H), *Kpn*I (K), and *Dra*I (D) are identified.

selected from a genome sequence draft of *P. syringae* pv. *syringae* B728a, and 11 were from other strains of *P. syringae* pv. *syringae*. Oligonucleotides representing genes *ptl* (encoding pectate lyase; GenBank, AF219381) and *efe* (encoding ethylene biosynthetase; GenBank, AF101061) were designed from gene sequences identified from *P. syringae* pv. *glycinea* and *P. syringae* pv. *pisi*, respectively. In addition, oligonucleotides from various housekeeping genes (*gyrB*, *argA*, *argE*, *recA*, and 16S rDNA) were included as controls and for normalization. A random 70-mer oligonucleotide was synthesized and included as a negative control, which did not show homology to any region of the strain B728a genomic DNA sequence draft. The other negative control was designed from the *luc* gene encoding a firefly luciferase (GenBank, U47295). Melting temperature (T_m) values of these oligonucleotides were averaged at 78.97°C (74.74 to 81.77).

Most features printed on the oligonucleotide microarray showed uniform and strong intensities of fluorescence when hybridized with sheared genomic DNA of strain B301D (data not shown). As expected, negative controls of the nucleotide representing the luciferase gene and the random oligonucleotide only produced the background noise as observed for the 3× SSC buffer control (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). In addition, four spots representing *virPphA* (Jackson et al. 1999), *ptl* (Bauer and Collmer 1997), *avrP*, and *efe* (Fukuda et al. 1992) showed signals that were comparable to the background level, suggesting the four genes may not be present in the B301D genome. Accordingly, the four gene fragments could not be detected using PCR amplification from the genomic DNA of strain B301D. Neither the *ptl* nor *efe* genes were identified from the genomic sequence draft of strain B728a.

Overall transcriptional profiles of genes included in the subgenomic microarray.

All the genes dedicated to toxin production were expressed in the wild-type strain B301D at a high level when cultured on potato-dextrose agar (PDA) medium (Fig. 2; Table 1). Previous studies revealed that expression of a *syrB1::lacZ* fusion in *P. syringae* pv. *syringae* BR132 reached a peak after a 3-day incubation on media conducive to toxin production (Mo and Gross 1991a and b). Consequently, total RNA was extracted from bacterial cells grown on PDA for 72 h and was used for transcriptome analysis. To estimate expression level of genes of interest, the background signal was calculated from the intensities of two negative controls, as well as from probes representing the *virPphA*, *ptl*, *avrP*, and *efe* genes that were not present in the B301D genome. Hybridization intensity analysis showed that signals of the *syrB1* and *syrE* synthetase genes for syringomycin production by strain B301D were observed to be 127 and 59 times higher than the background. Likewise, intensities of the other *syr* genes, including the *syrB2*, *syrD*, *syrP*, and *syrC* genes, averaged 52 times higher (ranging from 20 to 127) than the background. Signals from transcripts of the *sypA*, *sypB*, and *sypC* synthetase genes were 40, 44, and 17 times higher than the background, respectively. A few genes located outside the *syr-syp* genomic island expressed high levels (5 to 17 times higher than the *recA* gene) on PDA medium, such as *sigX*, *algD*, *algT*, *sodB*, and *inaK* (Fig. 2).

The most significant result of the oligonucleotide microarray analysis was the identification of genes that were positively regulated by the *sala* gene. To define the *sala* regulon, the transcriptional profile of a *sala* mutant (B301DSL7) (Lu et al. 2002) was compared with the wild-type strain B301D. As observed in the scatter diagram shown in Figure 2, 20 spots rep-

Fig. 2. Log plot of microarray signals obtained using B301D and B301DSL7 (*sala*) probes (cells grown on potato-dextrose agar for 72 h at 25°C). Each spot on the array is represented by a dot in the scatter plot. The means of signal intensity of the B301D probe (Cy5) were plotted against those of the B301DSL7 probe (Cy3). The top line represents a threshold Cy5/Cy3 ratio of twofold and the bottom line shows a threshold Cy5/Cy3 ratio of one-half. These data were derived from six replicates. The red and brown spots indicate expression profiles of genes associated with the *syr* and *syp* clusters, respectively. The syringol biosynthesis gene *syID* is identified by a green spot. Blue spots indicate genes associated with a variety of functions, including Hrp type III effectors and secretion, siderophore biosynthesis, alginate, phytohormone synthesis, and cellular housekeeping processes.

representing 16 arrayed genes were expressed significantly greater than twofold in strain B301D compared with that in strain B301DSL7; all, except for the *sylD* gene, were located within the *syr-syp* island. In this study, none of the genes or ORFs included in this array was observed to be negatively controlled by the *salA* gene. These results were consistently observed in dye-swap experiments.

Effect of the *salA* mutation on transcription of genes located in the *syr-syp* genomic island.

Genes dedicated to biosynthesis, secretion, and regulation of syringomycin and syringopeptin were up-regulated by the *salA* gene. First, mutation in the *salA* gene resulted in significant reductions of transcripts for biosynthesis genes of syringomycin and syringopeptin, including the *syrB1*, *syrB2*, *syrC*, *syrE*, *sypA*, *sypB*, and *sypC* genes (Guenzi et al. 1998; Scholz-Schroeder et al. 2003; Zhang et al. 1995) (Fig. 1; Table 1). All oligonucleotides designed from different locations in the same gene (e.g., *syrE*, *sypB*, or *sypC*) showed significant reductions in the *salA* mutant as compared with strain B301D, although the fold changes from the oligonucleotides of each gene were slightly different. Second, the transcriptional levels of genes involved in secretion of syringomycin and syringopeptin were significantly reduced in the *salA* mutant as compared with strain B301D, which include *syrD* (Quigley et al. 1993) and *sypD* (Gross et al. 2003). A similar effect of the *salA* mutation was observed on ORFs 20 and 22, which were predicted to encode an outer membrane protein and a cytoplasmic membrane protein, respectively (Lu et al. 2002). A resistance-nodulation-division (RND) transporter encoded by *pseA*, *pseB*, and *pseC* was identified and was demonstrated to be associated with secretion of syringopeptin (Kang and Gross 2003). However, changes in transcriptional levels of *pseA*, *pseB*, and *pseC* were not significant between strains B301DSL7 and B301D under the conditions of this study. Third, a positive regulatory effect of *salA* was observed on *syrF* but not on *syrG*, which corresponds to the results obtained using translational fusions to a *uidA* reporter gene (Lu et al. 2002). The *syrP* gene (Zhang et al. 1997) was expressed at a high level on the PDA medium and was reduced in transcription by about 20 times in the *salA* mutant. In addition, one ORF, named *dat*, located downstream of *sypD* (Fig. 1) was predicted to encode a protein with significant homology to aminotransferases (Ikai and Yamamoto 1997) involved in the formation of the modified amino acid, 2,4-diaminobutyric acid (D. C. Gross, unpublished data). Mutation in the *salA* gene resulted in a reduction of the transcriptional level of the *dat* gene by more than sixfold (Table 1). Similarly, transcription of ORFs 21 and 26 was decreased in the *salA* mutant, which shared similarity to *cmaU* (Alarcón-Chaidez et al. 1999) and *mcpA* (Kuroda et al. 1995), respectively. In contrast, transcription of some ORFs identified in the genomic island, such as ORFs 5, 6, 7, 23, 24, and 25, was not affected by a mutation in the *salA* gene (Table 1).

Effect of the *salA* mutation on transcription of genes located outside the *syr-syp* genomic island.

No significant effect of the *salA* mutation was observed on genes located outside the *syr-syp* genomic island, except for the *sylD* gene (Table 1). The *sylD* gene encodes a nonribosomal peptide synthetase dedicated to biosynthesis of the syringolin peptide elicitor that induces resistance in rice plants (Amrein et al. 2004; Wäspi et al. 1998). Transcription of the *sylD* gene in a *salA* mutant (B301DSL7) was decreased by about eight times as compared to that in the wild-type strain B301D (Table 1). No significant effect of the *salA* mutation was observed on genes involved in the Hrp type III secretion system, phytohormone synthesis, alginate production, sidero-

phore production, ice nucleation, environmental stress, or quorum sensing. It was observed that mutation in the *salA* gene did not affect transcription of any global regulators or sigma factors included in this study. A few genes not controlled by the *salA* regulon were highly expressed when cells were grown on PDA medium, which included genes associated with ice nucleation activity (*inaK*), alginate production (*algD*, *algT*), and environmental stress (*sodB*) (Fig. 2).

Validation of microarray data.

Expression analysis of a *sypA::uidA* reporter strain of *P. syringae* pv. *syringae* confirmed positive regulation of the *salA* gene on transcription of the *sypA* synthetase gene of syringopeptin (Fig. 3). To evaluate expression of the *sypA* gene, reporter strain B301DSL29 was generated by forward insertion of a *uidA-aaCC1* fragment (Lu et al. 2002) into the *sypA* gene, generating a transcriptional fusion of the *uidA* gene to the 5' end of the *sypA* gene. Recombination of the *uidA-aaCC1* fragment into the chromosome of strain B301DSL29 was verified by Southern analysis. Strain B301DSL29 exhibited a high level of activity of β -glucuronidase (GUS) on PDA supplemented with 5-bromo-4-chloro-3-indolyl- β -D-glucuronide. To investigate the effect of the *salA* mutation on expression of the *sypA::uidA* reporter, the *salA* gene of strain B301DSL29 was disrupted by marker exchange of a *salA::nptII* construct (Lu et al. 2002) to generate strain B301DSL30 (*sypA::uidA*, *salA::nptII*). Southern analysis confirmed disruption of the *salA* gene in strain B301DSL30. As shown in Figure 3, strain B301DSL29 (*sypA::uidA*) exhibited high levels of GUS activity, averaging 410 units per 10^8 CFU after three days of incubation at 25°C. In contrast, strains B301DSL30 (*sypA::uidA*, *salA::nptII*) and B301D did not produce GUS activity. This result verified the positive regulation of the *salA* gene on the *sypA* synthetase gene of syringopeptin observed in microarray analysis.

The effect of the *salA* mutation on expression of *syrC*, *sylD*, and *recA* was validated by using real-time (RT)-PCR. Microarray analysis showed that transcriptional levels of *syrC*, *sylD*, and *recA* are 12.27-, 10.92-, and 0.88-fold higher, respectively, in the wild-type strain B301D than in the *salA* mutant (Table 1). RT-PCR analysis demonstrated that transcriptional levels of *syrC* and *sylD* were 11.6- and 9.0-fold higher, respectively, in strain B301D as compared with that in the *salA* mutant. As expected, no significant changes of the *recA* and *inaK* transcriptional levels were observed between the mutant and wild-type strain from the RT-PCR analysis. These RT-PCR results agreed with the magnitudes of the changes determined from microarray analysis.

Fig. 3. The effect of mutation in the *salA* regulatory gene on expression of the *sypA::uidA* fusion. Strains B301D, B301D29 (*sypA::uidA*), and B301DSL30 (*sypA::uidA*; *salA::nptII*) were incubated for 72 h at 25°C in potato-dextrose broth medium. Vertical bars indicate standard error of the means for triplicate cultures.

DISCUSSION

The results of this study demonstrated that the oligonucleotide-based subgenomic microarray is a powerful approach for gene expression profiling of virulence-associated genes in *P. syringae*. One of the earliest microarray studies of gene expression in a plant pathogenic bacterium was by Okinaka and associates (2002). They constructed a genomic DNA microarray containing random 3-kb clones of the *Erwinia chrysanthemi* 3937 genome and identified plant up- and down-regulated clones relative to growth on Luria-Bertani (LB) culture medium. However, limitations of the strategy were observed, including missing pathogenesis-related genes and over-represented clones (Okinaka et al. 2002). In contrast, the oligonucleotide-based microarray was demonstrated to have several advantages, such as gene specificity, enhanced flexibility in design, cost-effectiveness, and uniformity (Denef et al. 2003; Hessner et al. 2004) as compared with a random DNA array. In this study, a subgenomic microarray system was successfully used to demonstrate that the SalA regulon controls expression of the syringomycin and syringopeptin gene clusters and that expression of other important virulence genes, such as those associated

with Hrp type III secretion, are independent of SalA activity. As complete genome sequences of *P. syringae* strains become available, the 70-mer oligonucleotide microarray approach will be especially valuable to defining the complete set of genes composing a regulon such as that controlled by SalA, as influenced by changing environmental conditions.

Genes involved in biosynthesis, secretion, and regulation of syringomycin and syringopeptin are members of the SalA regulon. These genes are expressed at high levels on petri plates of PDA, a medium conducive to toxin production (Gross and DeVay 1977). With the exception of the *sylD* gene for syringolin, it appears that the genes positively regulated by the SalA protein are clustered in the *syr-syp* genomic island. The expression results obtained from microarray analysis indicate that the *syr-syp* genomic island consists of the DNA region extending from ORF14 to ORF26 (Fig. 1). Although it is possible that horizontal transfer of the *syr-syp* genomic island may occur in *P. syringae* strains, it is important to recognize that the *syr-syp* genomic island is not found in the genome of *P. syringae* pv. *tomato* DC3000 (Gross et al. 2003) and appears to not be present in several other pathovars of *P. syringae* (Quigley and Gross 1994).

Table 1. Microarray analysis of the SalA regulon

Genes ^a	Ratio (SEM) ^b	Gene product	References
<i>syrD</i>	7.47 (±1.78)	ATP-binding secretion protein	Quigley et al. 1993
<i>syrP</i>	16.94 (±3.77)	Homologue of histidine kinase	Zhang et al. 1997
<i>syrB1</i>	3.99 (±1.46)	Syringomycin synthetase	Zhang et al. 1995
<i>syrB2</i>	17.39 (±4.43)	Syringomycin biosynthesis enzyme	Zhang et al. 1995
<i>syrC</i>	12.27 (±2.53)	Syringomycin biosynthesis enzyme	Zhang et al. 1995
<i>syrE-1</i>	9.90 (±2.97)	Syringomycin synthetase	Guenzi et al. 1998
<i>syrE-2</i>	5.17 (±0.72)	Syringomycin synthetase	Guenzi et al. 1998
<i>syrE-3</i>	7.85 (±0.97)	Syringomycin synthetase	Guenzi et al. 1998
<i>syrF</i>	2.88 (±0.57)	LuxR family bacterial regulator	Lu et al. 2002
<i>ORF20</i>	4.32 (±0.84)	Putative outer membrane protein	Lu et al. 2002
<i>ORF21</i>	2.67 (±0.70)	Hypothetical protein	Lu et al. 2002
<i>ORF22</i>	5.34 (±1.77)	Membrane protein	Lu et al. 2002
<i>syrG</i>	2.03 (±0.46)	LuxR family regulatory protein	Lu et al. 2002
<i>salA</i>	1.35 (±0.16)	LuxR family regulatory protein	Lu et al. 2002
<i>ORF23</i>	0.55 (±0.17)	Hypothetical protein	Lu et al. 2002
<i>ORF24-1</i>	0.91 (±0.23)	Hypothetical protein	Lu et al. 2002
<i>ORF24-2</i>	1.29 (±0.09)	Hypothetical protein	Lu et al. 2002
<i>ORF25</i>	1.18 (±0.12)	Hypothetical protein	Lu et al. 2002
<i>ORF26</i>	2.94 (±0.47)	Unknown (chemotaxis protein)	Lu et al. 2002
<i>sypA</i>	9.40 (±3.08)	Syringopeptin synthetase	Scholz-Schroeder et al. 2003
<i>sypB-1</i>	17.66 (±3.30)	Syringopeptin synthetase	Scholz-Schroeder et al. 2003
<i>sypB-2</i>	7.09 (±1.24)	Syringopeptin synthetase	Scholz-Schroeder et al. 2003
<i>sypC1</i>	2.58 (±0.40)	Syringopeptin synthetase	Scholz-Schroeder et al. 2003
<i>sypC2</i>	3.28 (±0.64)	Syringopeptin synthetase	Scholz-Schroeder et al. 2003
<i>ORF19</i>	1.74 (±0.27)	Putative membrane protein	Gross et al. 2003
<i>sypD</i>	4.31 (±1.12)	Putative ABC transporter	Gross et al. 2003
<i>dat</i>	6.22 (±1.14)	Aminotransferase	Gross, unpublished data
<i>pseA</i>	2.06 (±0.32)	Putative outer membrane protein	Kang and Gross 2003
<i>pseB</i>	1.59 (±0.19)	Membrane fusion efflux protein	Kang and Gross 2003
<i>pseC</i>	1.27 (±0.23)	Membrane fusion efflux protein	Kang and Gross 2003
<i>ORF14</i>	2.31 (±0.30)	Putative chemotaxis protein	N. Wang and D. C. Gross, unpublished data
<i>ORF13</i>	2.14 (±0.34)	Unknown	N. Wang and D. C. Gross, unpublished data
<i>ORF12</i>	0.81 (±0.14)	Sensor protein	N. Wang and D. C. Gross, unpublished data
<i>ORF11</i>	0.94 (±0.15)	Amino acid deaminase	N. Wang and D. C. Gross, unpublished data
<i>ORF10</i>	0.75 (±0.22)	Hypothetical protein	N. Wang and D. C. Gross, unpublished data
<i>ORF9</i>	1.02 (±0.19)	Hypothetical protein	N. Wang and D. C. Gross, unpublished data
<i>ORF8</i>	0.50 (±0.18)	Hypothetical protein	N. Wang and D. C. Gross, unpublished data
<i>ORF7</i>	0.75 (±0.21)	Hypothetical protein	N. Wang and D. C. Gross, unpublished data
<i>ORF6</i>	1.08 (±0.19)	Hypothetical protein	N. Wang and D. C. Gross, unpublished data
<i>ORF5</i>	0.73 (±0.16)	Hypothetical protein	N. Wang and D. C. Gross, unpublished data
<i>ORF4</i>	0.86 (±0.31)	Hypothetical protein	N. Wang and D. C. Gross, unpublished data
<i>ORF3</i>	0.82 (±0.23)	Hypothetical protein	N. Wang and D. C. Gross, unpublished data
<i>ORF2</i>	0.92 (±0.38)	Hypothetical protein	N. Wang and D. C. Gross, unpublished data
<i>ORF1</i>	2.66 (±1.32)	Hypothetical protein	N. Wang and D. C. Gross, unpublished data

Continued on next page

^a The *psyr* numbers stand for ORFs predicted by www.jgi.doe.gov/JGI_microbial.

^b Data shown in the table are the means of six replicates of the three independent experiments with the standard errors of means (SEM).

The *salA* gene encodes a key regulator for syringomycin production. Previous studies revealed that syringomycin production was eliminated in a *salA* mutant and that the *salA* gene positively controlled expression of the synthetase gene *syrB1* and the regulatory gene *syrF* (Lu et al. 2002). In this study, transcriptional levels of both the *syrB1* and *syrF* genes in the *salA* mutant were significantly lower than those of the wild-type strain B301D, which is consistent with previous studies (Lu et al. 2002). In addition to *syr* genes, transcription of *syp* genes is positively regulated by *salA*. It was previously observed that mutation in the *salA* gene affected syringopeptin production based on phytotoxin bioassays (Lu et al. 2002). The results of this study suggest that the effect of the *salA* gene on syringopeptin production is via the positive regulation of *salA* on transcription of both synthetase genes (e.g., *sypA*, *sypB*, and *sypC*) and secretion (e.g., *sypD*) genes. Expression of the *pseA* gene in strain B301D was twofold higher than that in the *salA* mutant; however, statistical analysis showed that the difference in expression level was not significant (Table 1). Further analyses are needed to investigate regulation of the *salA* gene on gene expression of the RND transporter system. It is not surprising that both syringomycin and syringopeptin are coregulated

by *SalA*, because the two toxins contribute significantly to virulence of *P. syringae* pv. *syringae* (Scholz-Schroeder et al. 2001a).

The discovery that the syringolin biosynthesis gene *sylD* was controlled by the *salA* gene expanded the role of the *SalA* regulon in controlling expression of peptide metabolites important to the host-pathogen interaction. Syringolin was described by Dudler and associates (Hassa et al. 2000; Wäspi et al. 1998) as a peptide produced by *P. syringae* pv. *syringae* B301D that induced the hypersensitive response on nonhost plants including rice and wheat and was speculated to constitute a virulence factor. Recently, a gene cluster responsible for biosynthesis and secretion of the peptide was characterized and shown to be composed of four genes (*sylA*, *sylB*, *sylC*, and *sylD*) (Amrein et al. 2004). It is speculated that the *salA* gene controls all genes essential for syringolin production. It has been frequently observed that one key regulator can control multiple genetic loci that might not be clustered physically (Hamon et al. 2004; Joyce et al. 2004; Martínez-Antonio and Collado-Vides 2003). For example, the *hrpL* gene was predicted to encode an alternative sigma factor (Xiao et al. 1994) and was localized to the *hrpL* gene cluster (Alfano et al. 2000). Interestingly, multiple

Table 1. Continued from preceding page

Genes ^a	Ratio (SEM) ^b	Gene product	References
<i>hrpL</i>	1.18 (±0.14)	Alternative sigma factor	Alfano et al. 2000
<i>hrpR</i>	1.03 (±0.10)	Regulatory factor	Xiao et al. 1994
<i>hrpS</i>	0.84 (±0.38)	Positive regulatory factor	Xiao et al. 1994
<i>hrpK</i>	1.16 (±0.09)	Unknown	N. Wang and D. C. Gross, <i>unpublished data</i>
<i>hrpJ</i>	0.56 (±0.15)	Flagellar biogenesis	Lidell and Hutcheson 1994
<i>hrpZ</i>	1.09 (±0.12)	Harpin	He et al. 1993
<i>hrpW</i>	0.77 (±0.08)	Type III effector	Charkowski et al. 1998
<i>queA</i>	0.78 (±0.05)	Exchangeable effector locus	Alfano et al. 2000
<i>EEL-chp</i>	1.04 (±0.22)	Type III chaperone protein	N. Wang and D. C. Gross, <i>unpublished data</i>
<i>hrmA</i>	0.96 (±0.17)	Avr (effector) proteins	Alfano et al. 2000
<i>gacS</i>	1.06 (±0.11)	Sensor protein	Willis et al. 1990
<i>gacA</i>	0.76 (±0.19)	Regulator protein	Rich et al. 1994
<i>rpoS</i>	1.01 (±0.17)	RNA polymerase sigma factor	Miller et al. 2001
<i>rpoD</i>	0.91 (±0.14)	Principle sigma factor	Sawada et al. 1997
<i>rpoN</i>	0.75 (±0.13)	Putative sigma-54 protein	Alarcón-Chaidez and Bender 2001
<i>sigX</i>	1.04 (±0.05)	Sigma factor	Brinkman et al. 1999
<i>psyr020094</i>	1.70 (±0.28)	Putative peptide synthetase	JGI-DOE microbial index
<i>psyr020651</i>	0.84 (±0.12)	Putative peptide synthetase	JGI-DOE microbial index
<i>sylD</i>	10.92 (±2.69)	Putative syringolin synthetase	Amrein et al. 2004
<i>algT</i>	0.78 (±0.07)	Alternative sigma factor	Keith and Bender 1999)
<i>algD</i>	1.00 (±0.26)	GDP-mannose dehydrogenase	Fakhr et al. 1999
<i>iaaM</i>	1.33 (±0.17)	Tryptophan monooxygenase	Mazzola and White 1994
<i>iaaH</i>	1.60 (±0.18)	Indoleacetamide hydrolase	Mazzola and White 1994
<i>ahlI</i>	1.90 (±0.50)	Acyl homoserine lactone synthetase	Dumenyo et al. 1998
<i>inaK</i>	0.90 (±0.16)	Ice nucleation protein	Jung et al. 1998
<i>pvdS</i>	1.32 (±0.14)	Putative acetylase	Mossialos et al. 2002
<i>pvdE</i>	1.01 (±0.04)	Pyoverdine synthetase	McMorran et al. 1996
<i>fur</i>	0.89 (±0.10)	Ferric uptake regulator	Hassett et al. 1996
<i>lccC</i>	0.75 (±0.10)	Levansucrase	Li and Ullrich 2001
<i>cbrB</i>	0.96 (±0.07)	Ferrisiderophore permease	Mahe et al. 1995
<i>cbrD</i>	1.07 (±0.22)	ATP-binding unit in ABC transport	Mahe et al. 1995
<i>acsD</i>	0.70 (±0.23)	Achromobactin biosynthetase	Franza et al. 2002
<i>fsc</i>	0.94 (±0.12)	Ferric siderophore receptor	Nelson et al. 2002
<i>SR-dat</i>	0.54 (±0.20)	Diaminobutyrate transaminase	Ikai and Yamamoto 1997
<i>psyr020143</i>	1.90 (±0.43)	Putative peptide synthetase	JGI-DOE microbial index
<i>tex</i>	1.01 (±0.09)	S1 RNA binding domain protein	Nelson et al. 2002
<i>gshA</i>	0.95 (±0.08)	Glutamate-cysteine ligase	Nelson et al. 2002
<i>argA</i>	0.76 (±0.10)	N-acetylglutamate synthetase	Lu et al. 2003
<i>argE</i>	0.92 (±0.08)	Acetylornithine deacetylase	Lu et al. 2003
<i>SA-ORF6</i>	1.06 (±0.07)	Unknown	Lu et al. 2003
<i>ruIA</i>	1.02 (±0.14)	Radiation tolerance	Sundin et al. 2000
<i>sodB</i>	0.48 (±0.21)	Iron-superoxide dismutase	Hassett et al. 1993
<i>gyrB</i>	1.00 (±0.07)	DNA gyrase subunit B	Sawada et al. 1997
<i>recA</i>	0.88 (±0.11)	Principal sigma factor	Kokjohn and Miller 1985
<i>luc</i>	1.02 (±0.36)	Luciferase	Coronado et al. 1994
<i>negc10</i>	0.96 (±0.17)	Random primer	Qiagen, Valencia, CA, U.S.A.
16S rDNA	1.00 (±0.07)	16S ribosomal RNA	Nelson et al. 2002

hrpL-regulated genes encoding effectors were found scattered in the genome of *P. syringae* pv. *tomato* DC3000 (Buell et al. 2003; Fouts et al. 2002). Other virulence-associated genes may be present in the genome of *P. syringae* pv. *syringae* that belong to members of the SalA regulon. Recently, a draft sequence of the 6.08-megabase genome of *P. syringae* pv. *syringae* B728a was assembled, and genome annotation is underway. The B728a genome project will allow the construction of a high-density oligonucleotide microarray that could be used for genomewide analysis of the SalA regulon of *P. syringae* pv. *syringae*.

Differences in ratios of expression for oligonucleotides representing different locations of the same gene were observed, although they were consistent in showing the same overall response due to *salA* mutation. For example, three oligonucleotides designed from different regions of the *syrE* gene showed 9.90-, 5.17-, and 7.85-fold changes in expression (Table 1). Similar results were observed for the *sypB* and *sypC* genes. Such variation was reported in a study by Wagner and associates (2003) and was attributed to RNA degradation. Alternatively, differences of expression levels observed from the oligonucleotides of one gene may result from the secondary structures of the mRNA flanking the oligonucleotides. For example, the effects of the secondary structures of regions flanking the oligonucleotides were detected in studies of single nucleotide polymorphisms using oligonucleotide microarrays (Anthony et al. 2003). It was suggested that the sequences used for identifying oligonucleotides were filtered with a 100-nt window across the target part of a gene to reduce effects of the flanking regions that have the potential to form stable secondary structures of cDNA (Tolstrup et al. 2003).

In conclusion, the SalA regulon plays an essential role in plant pathogenesis of *P. syringae* pv. *syringae*. Results of this study demonstrated that the SalA regulon is composed of genes associated with production of syringomycin, syringopeptin, and syringolin. An important observation is that all genes identified as part of the SalA regulon are absent from the genome of strain DC3000. Although the *salA* gene was reported to be present in strain DC3000 (Chatterjee et al. 2003), this is unlikely because the overall homology of the ORF (identified as PSPT02833) to the original SalA protein is low (less than 32% identity). Previous studies revealed that the *salA* gene was controlled by the GacS/GacA global regulatory system that controls multiple cellular functions associated with plant interactions (Kitten et al. 1998). Thus, results of this study provide evidence that the *salA* gene represents a vital junction in the GacS/GacA regulatory system as it controls expression of genes essential for plant pathogenesis.

MATERIALS AND METHODS

Bacterial growth conditions and RNA extraction.

Strains of *P. syringae* pv. *syringae* were cultured with shaking at 25°C overnight in 5 ml of potato-dextrose broth medium (Gross and DeVay 1977). To extract total RNA for analysis of gene transcription, cells were harvested by centrifugation and then diluted with sterile distilled water to a concentration of 2×10^8 CFU/ml. Cells from 50 μ l of the suspension, containing approximately 10^6 cells, were plated on PDA containing 4% casamino acids (Gross and DeVay 1977), and the inoculated plates were incubated at 25°C for 72 h. Bacterial total RNA was extracted, using a RiboPure bacteria kit (Ambion, Inc., Austin, TX, U.S.A.) as described by the manufacturer. Briefly, a pellet with 1×10^{10} cells was suspended with 350 μ l of RNawiz and then was lysed by vortex with zirconia beads. The lysate was then mixed with chloroform and the resulting mixture was centrifuged at $10,000 \times g$. RNA in the upper aqueous phase was recovered by 100% ethanol and bound to a silica

filter. The RNA bound to the filter was washed to remove contaminants, and eluted with RNase-free water.

Oligonucleotide design and synthesis.

A set of 70-mer oligonucleotides representing the *syr-syp* genes of *P. syringae* pv. *syringae* and other genes of interest were designed and synthesized by Qiagen (Valencia, CA, U.S.A.). The oligonucleotides were selected based on the genome sequence draft of *P. syringae* pv. *syringae* B728a or on the genomic sequences of genes of *P. syringae* pv. *syringae* B301D or closely related strains of *P. syringae*. All oligonucleotides were selected using the following criteria: i) T_m values of all oligonucleotides are within $\pm 5^\circ\text{C}$; ii) each oligonucleotide is within 750 to 1,000 bases from the 3' end of the available gene sequence; iii) each oligonucleotide is scanned for contiguous single nucleotide base repeats (less than seven bases) and hairpin stems (less than nine bases); iv) BLAST search to establish less than 70% identity to all other genes; and v) less than 20 contiguous repeats to any other gene in the genome sequence draft of strain B728a. The 70-mer oligonucleotides were synthesized at the 0.2 μ mol scale and were dispensed at 96-well plates.

Microarray slide printing and blocking.

Microarray slides were printed on poly-L-lysine-coated glass slides (CEL Associates, Inc. Pearland, TX, U.S.A.). The oligonucleotides were solubilized to a final concentration of 40 μ M with $3\times$ SSC and were transferred into 96-well plates. All oligonucleotides were spotted in duplicate on one glass slide, using an OmniGrid 100 microarrayer (Genomic Solutions, Ann Arbor, MI, U.S.A.). Printing quality was checked using a microscope and scanner. Printed microarrays were fixed with a UV cross linker at 250 mJ and then were baked at 80°C for 3 h. Blocking of microarray slides was performed using succinic anhydride as recommended by Qiagen. To further check the quality of the printed microarrays, two aliquots (0.5 μ g per aliquot) of the sheared genomic DNA (approximately 2 kb) of strain B301D were labeled with Cy3 and Cy5, respectively, and were hybridized to the microarray to measure signal intensities as described below.

Probe labeling.

Procedures for probe labeling and oligonucleotide microarray hybridization were optimized as follows. Purified total RNA (50 μ g) was mixed with 3 μ g of random hexamers (Qiagen) and was incubated at 65°C for 10 min. Reactions were held at room temperature for 10 min to allow the primers and the RNA template to anneal. To each reaction, the following were added: 2 μ l of Cy5/Cy3-dCTP (1 mM) (Amersham Biosciences, Piscataway, NJ, U.S.A.), 3 μ l of 0.1 M dithiothreitol, 6 μ l of $5\times$ reverse transcriptase buffer, 2 μ l of dNTP mixture (15 mM dATP, dTTP, and dGTP; 5 mM dCTP), and 200 U of Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA, U.S.A.). The reactions were incubated at 42°C for 2 h in the dark. The RNA was hydrolyzed with 1.5 μ l of 1 M NaOH at 65°C for 10 min and then was neutralized with 1 M HCl. A pair of Cy5/Cy3-labeled probes was purified together with a PCR purification kit (Qiagen), and probes were eluted from filters with 30 μ l of sterile distilled water. The labeled probes (approximately 27 μ l) were added to hybridization buffer containing $3\times$ SSC, 1% bovine serum albumin, and 0.25% sodium dodecyl sulfate (SDS). Probes were denatured at 95°C for 2 min and then were applied to slides. Hybridization was conducted in a moist chamber at 60°C for 14 h. After hybridization, the slides were washed for 4 min in $2\times$ SSC and 0.1% SDS, for 4 min in $1\times$ SSC and 0.1% SDS, and for 4 min in $0.2\times$ SSC. The slides were dried with centrifugation (10

min, 1,000 × g). The washed slides were scanned by using a GenePix 4000b (Axon Instruments Inc., Foster City, CA, U.S.A.) to visualize the hybridization images. The scanner PMT settings of two channels (532 nm for Cy3 and 635 nm for Cy5 acquisition, respectively) were adjusted based on the ratio of 16S rDNA expression (the 16S RNA is expected to be equal between different strains). The 16S rRNA that was expressed to similar levels in both strains B301D and B301DSL7 based on RNA gel analysis was used to normalize the microarray expression data.

Microarray data analysis.

Signal intensity and ratios were obtained using GenePix Pro software provided with the scanner. Oligonucleotide spots with signals lower than 100 after subtracting background were flagged and eliminated for further analysis. The raw data were normalized with probe intensities of 16S rRNA, which was transcribed to similar levels in both strains as a constitutively expressed gene. Hybridization experiments were performed in triplicate. Each slide contained duplicate arrays.

Microarray validation

by RT-PCR analysis and GUS assays.

Regulation of the *salA* gene on expression of *syrC* and *sylD* observed in microarray analysis was examined by qualitative real-time PCR using QuantiTect SYBR green RT-PCR kit (Qiagen). Primers used for RT-PCR were designed using the Lasergene expert sequence analysis package (DNASTar, Madison, WI, U.S.A.) according to primer criteria as described by Qiagen. The primers specific for 16S rRNA were generated for normalization controls. All primers used for RT-PCR are listed in Table 2. For each primer pair, the linearity of detection was confirmed to have a correlation coefficient of at least 0.98 ($r^2 > 0.98$) over the detection area by measuring a fivefold dilution curve with RNA isolated from bacterial cells. An aliquot containing 100 ng of total RNA as template was used for reverse transcription at 50°C for 30 min, followed by initial activation of HotStar DNA polymerase (95°C, 15 min). RT-PCR was performed in 40 cycles (94°C for 15 s, 55°C for 30 s, and 72°C for 30 s), followed by melting curve analysis as recommended for the RT-PCR kit of Qiagen to validate primer gene specificity.

Regulation of the *salA* gene on expression of the *sypA* gene was verified by GUS assays. A 3.2-kb *uidA-aacC* fragment of pSL02 (Lu et al. 2002) was cloned into pBS08 (Scholz-Schroeder et al. 2001) at the *Bst*Z17I site, to generate a transcriptional fusion of *uidA* to *sypA*. The resulting construct was inserted into pBR325 at the *Eco*RI site previously blunted by T4 DNA polymerase to generate plasmid pSL200. A *sypA::uidA* reporter was constructed by introduction of pSL200 into *P. syringae* pv. *syringae* B301D to generate strain B301DSL29 by marker exchange mutagenesis. Similarly, pSL08 (Lu et al. 2002), which carries a disrupted *salA* gene by insertion of *nptII*, was used to generate strain B301DSL30 (*sypA::uidA*, *salA::nptII*) to evaluate the effect of mutation of

salA on *sypA* expression. Homologous recombination in the resultant strains was verified by Southern analysis. GUS assays were performed as described by Lu and associates (2002).

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Table 2. Primers used for quantitative real-time PCR analysis

Name	Sequence	Resource
syrCF	ACCTGCAAGCGATGTTCTC	This study
SyrCR	TGCCAGCTCGGTCTTGTTC	This study
sylDF	ACTATCGCGCTCGTGTCCAA	This study
syrDR	CAGCCCGATACCGTCAGAAA	This study
inaKF	CGGCGATAGAAGCAAACTC	This study
inaKR	GCCGGCCGTCAAGATACT	This study
recAF	CTTCGGTACGCTGGACA	This study
recAR	AACTCGGCCTGACGGAAC	This study
16SF	ACACCGCCCGTCACACCA	Kang and Gross 2003
16SR	GTTCCCTACGGCTACCTT	Kang and Gross 2003

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AUTHOR RECOMMENDED INTERNET RESOURCES

Joint Genome Institute (JGI) and United States Department of Energy (DOE) homepage: www.jgi.doe.gov
 JGI-DOE *Pseudomonas syringae* pv. *syringae* B728a genome sequence project: genome.jgi-psf.org/draft_microbes/psesy/psesy.home.html
 JGI-DOE microbial index site: www.jgi.doe.gov/JGI_microbial
 Qiagen website: www.qiagen.com
 Agilent Technologies website: www.chem.agilent.com