Regulation of Biosynthesis of Syringolin A, a *Pseudomonas syringae* Virulence Factor Targeting the Host Proteasome

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Many strains of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae* synthesize the virulence factor syringolin A, which irreversibly inactivates the eukaryotic proteasome. Syringolin A, a peptide derivative, is synthesized by a mixed nonribosomal peptide/polyketide synthetase encoded by five clustered genes, *sylA* to *sylE*. Biosynthesis of syringolin A, previously shown to be dependent on the GacS/GacA two-component system, occurs in planta and in vitro but only under still culture conditions in a defined medium. Here, we show that the *sylC*, *sylD*, and *sylE* genes of *P. syringae* pv. *syringae* B301D-R form an operon transcribed by promoter sequences located between the *sylCDE* operon and the *sylB* gene residing on opposite strands. Assays of overlapping *sylB* and *sylCDE* promoter deletions translationally fused to the *lacZ* gene defined promoter sequences required for gene activity both in vitro and in planta. Activation of both promoters depended on the *sylA* gene encoding a helix-turn-helix (HTH) LuxR-type transcription factor which was shown to directly bind to the promoters. Activity of the *sylA* gene, in turn, required a functional *salA* gene, which also encodes an HTH LuxR-type transcription factor. Furthermore, evidence is presented that acyl-homoserine lactone-mediated quorum-sensing regulation is not involved in syringolin A biosynthesis but that oxygen concentration appears to play a role.

Many strains of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae* secrete syringolins, a family of closely related cyclic peptide derivatives, in planta and under suitable in vitro culture conditions (Wäspi et al. 1998a, 1999). Spray application of syringolin A, the predominant variant, onto powdery mildew-infected wheat and *Arabidopsis* results in large transcriptome changes and programmed cell death at infection sites (Michel et al. 2006; Wäspi et al. 2001). Syringolin A was also shown to be an important virulence factor in the interaction of *P. syringae* pv. *syringae* B728a with bean (*Phaseolus vulgaris*) (Groll et al. 2008). Elucidation of syringolin A's mode of action revealed that it covalently binds to the active site residue of all three catalytic subunits of the eukaryotic proteasome by a novel mechanism, thereby irreversibly inhibiting proteasomal protein degradation (Groll et al. 2008).

Recently, experiments with bean and *Arabidopsis thaliana* revealed that proteasome inhibition by syringolin A suppresses the stomatal immune reaction and the salicylic acid-dependent host defense pathway (Schellenberg et al. 2010).

Syringolin A is a tripeptide derivative that consists of an N-terminal valine followed by the two nonproteinogenic amino acids 3,4-dehydrolysine and 5-methyl-4-amino-2-hexenoic acid, the latter two forming a 12-membered macrolactam ring structure. The N-terminal valine is linked by a urea moiety to the amino group of a second valine (Wäspi et al. 1998a). In minor variants, one or both valines can be substituted by isoleucine residues, and the 3,4-dehydrolysine by lysine (Wäspi et al. 1999). Syringolins are synthesized by a mixed nonribosomal peptide synthetase/polyketide synthase (NRPS/PKS) which is encoded by a gene cluster comprising five open reading frames (ORF) termed *sylA* to *sylE* (Amrein et al. 2004). These genes are sufficient to direct syringolin A biosynthesis when transferred to *Pseudomonas* strains lacking syringolin A biosynthesis genes (Ramel et al. 2009). The architecture of these genes and experimental evidence allowed researchers to postulate a biosynthesis model completely explaining syringolin A's structure (Amrein et al. 2004; Imker et al. 2009; Ramel et al. 2009). Whereas *sylC* and *sylD* encode NRPS and PKS modules of the syringolin A synthetase, the *sylB* gene is thought to be responsible for the desaturation of the 3,4-dehydrolysine residue (Amrein et al. 2004). The *sylA* gene encodes a helix-turn-helix (HTH) LuxR-type DNA-binding domain protein hypothesized to be a transcriptional regulator, whereas the *sylE* gene codes for a major facilitator superfamily protein postulated to represent the syringolin A exporter (Amrein et al. 2004).

The regulation of syringolin A biosynthesis appears to be complex. Syringolin A production was shown to be dependent on the *lemA* gene (Reimann et al. 1995; Wäspi et al. 1998a) encoding the sensor part of the GacS/GacA two-component system, which controls many aspects of pathogenicity such as phytotoxin and protease production and lesion formation (Hrabak and Willis 1992; Rich et al. 1992, 1994). Syringolin A is produced in planta, even if bacteria are infiltrated into non-host species, and in vitro under still culture conditions (Wäspi et al. 1998a) in a medium originally optimized for the production of the phytotoxin syringomycin (Gross 1985; Mo and Gross 1991a and b).

In order to elucidate the regulation of syringolin A biosynthesis, we demonstrate here that the *sylC*, *sylD*, and *sylE* genes are transcribed as an operon and that the intergenic region between the *sylB* and *sylCDE* genes, which reside on opposite DNA strands, contains the promoters responsible for transcription of these genes. We show that the product of the *sylA* gene directly activates the *sylB* and *sylCDE* genes, and that the *salA*

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gene, which also encodes an HTH LuxR-type DNA-binding domain protein (Kitten et al. 1998), is required for the activation of *sylA*. In addition, we present evidence that the acyl-homoserine lactone quorum-sensing (QS) system is not involved in syringolin A biosynthesis but that oxygen concentration appears to play a role.

RESULTS

The *sylC*, *sylD*, and *sylE* genes form an operon.

The syringolin A gene cluster consists of the five ORF *sylA* to *sylE* (Amrein et al. 2004). The arrangement of the *sylC*, *sylD*, and *sylE* genes suggests that they may be transcribed as an operon, likely regulated together by a promoter residing in the intergenic region between the *sylB* and *sylC* genes (Fig. 1A).

Amplification of the intergenic regions between *sylC*, *sylD*, and *sylE* from RNA by reverse-transcriptase polymerase chain reaction (RT-PCR) indicated that these genes are indeed transcribed as a single polycistronic mRNA (Fig. 1B). This finding was corroborated by RNA gel-blot analysis with RNA extracted from the wild-type strain carrying the *sylA* overexpression construct pOEAC (Ramel et al. 2009) using *sylC* and *sylE* fragments as hybridization probes. Both the *sylC* and *sylE* gene fragments labeled a band corresponding to a large RNA of a size consistent with the 18 kb expected for a transcript encompassing the *sylC*, *sylD*, and *sylE* ORF, which have a size of 3,984, 12,570, and 1,233 nucleotides, respectively (Fig. 1C). This result confirmed that these ORF are transcribed as an operon.

To identify promoter sequences expected to reside within the intergenic region between the *sylB* and *sylC* ORF, reporter genes were constructed in which the 221-bp intergenic sequence plus 18 and 5 bp of the coding regions of the *sylB* and *sylC* genes, respectively, was fused in frame with the *lacZ* ORF of

pME6014 in both orientations (Schnider-Keel et al. 2000). These constructs were named *psylBp-lacZ* and *psylCp-lacZ*, respectively. We note that, in *psylCp-lacZ*, not the first ATG codon but an in-frame ATG codon encoding the seventh amino acid of the *sylC* ORF, as originally annotated by Amrein and associates (2004), represents the translational initiation codon of the *sylC* gene (see below). Translational *lacZ* fusions after the first ATG were completely inactive.

The LuxR-type transcription factor SylA is a positive regulator of the *sylB* and *sylC* promoters.

The *sylA* gene encodes a putative protein with an HTH LuxR-type DNA-binding domain. The location of the *sylA* gene and the fact that syringolin A production was abolished if the *sylA* gene was disrupted by plasmid insertion (Amrein et al. 2004) led to the hypothesis that the *sylA* gene product may be a positive regulator of the *sylB* and *sylCDE* transcriptional units. To verify this hypothesis, a markerless *sylA* deletion mutant (Δ *sylA*) was constructed and transformed with the reporter constructs. In this mutant, the activities of the *sylB* and the *sylC* promoters were strongly decreased as compared with the wild type (Table 1).

Next, we placed the *sylA* gene under the control of the *lacZ* promoter onto the *sylB* and *sylC* reporter plasmids, resulting in the *sylA* overexpression constructs pOEAB and pOEAC, respectively. β -Galactosidase (β -gal) assays of the wild type and the Δ *sylA* mutant transformed with these constructs revealed a large increase of the *sylB* and *sylC* promoter activities compared with the reporter plasmids *psylBp-lacZ* and *psylCp-lacZ* in the wild-type background (Table 1). In addition, syringolin A concentrations of conditioned media from the former strains also increased with the same order of magnitude compared with the wild-type strain carrying the empty vector (data not shown). We conclude from these experiments that SylA is a positive regulator of syringolin A biosynthesis that directly or indirectly activates the *sylB* and *sylC* promoters.

Delimitation of essential *sylB* and *sylC* promoter regions.

To delimit sequences necessary for activity of the *sylB* and *sylC* promoters, overlapping deletions of the *sylB-sylC* intergenic DNA fragment were prepared by PCR amplification (Fig. 2A; Supplementary Table S1 shows the primer sequences) that were translationally fused to the *lacZ* reporter gene in pME6014. Results of β -gal assays with *Pseudomonas syringae* pv. *syringae* B301D-R strains transformed with these reporter plasmids and grown under still culture conditions are shown in Figure 3. For the *sylB* gene, fragments comprising 226 (full length), 153, 117, and 105 bp upstream of the translation initiation were active to approximately the same extent (Fig. 3A, black columns), whereas fragments of 90, 75, 67, and 57 bp only led to basal activities (open columns). For the *sylC* gene, promoter fragments encompassing 239 (full length), 175, and 124 bp of upstream sequence were active above basal levels

Fig. 1. *SylC*, *sylD*, and *sylE* are transcribed as an operon. **A**, Genomic region of *Pseudomonas syringae* pv. *syringae* B301D-R containing the *sylA* to *sylE* genes (accession number AJ548826). Boxes above and below the line denote open reading frames on the top and the bottom strand, respectively. **B**, Reverse-transcriptase polymerase chain reaction with primers amplifying a 359-bp *sylC* gene fragment or 368- and 378-bp fragments containing the *sylC/D* and *sylD/E* intergenic regions, respectively. Reactions did (+) or did not (-) contain reverse transcriptase. The arrows on the left denote the position of the 400-bp (upper) and 300-bp (lower) molecular weight markers. **C**, Top panels: autoradiogram of an RNA gel blot hybridized with a *sylC* and *sylE* probe, respectively. Positions of the 16S and 23S rRNAs are indicated. RNA was extracted from *P. syringae* pv. *syringae* B301D-R from which the complete *syl* gene cluster has been deleted (lane a) or from *P. syringae* pv. *syringae* B301D-R overexpressing the *sylA* gene (lane b). Bottom panels: ethidium bromide-stained gel.

Table 1. Effect of *sylA* on *sylB* and *sylC* reporter gene activity

Strain ^a	Reporter	β -Gal (MU) ^b	Comment
WT (psyBp and lacZ)	<i>sylB</i>	705 $\pm$ 37	...
Δ <i>sylA</i> (psyBp and lacZ)	<i>sylB</i>	96 $\pm$ 20	...
WT (psyCp and lacZ)	<i>sylC</i>	1,194 $\pm$ 108	...
Δ <i>sylA</i> (psyCp and lacZ)	<i>sylC</i>	106 $\pm$ 6	...
WT (pOEAB)	<i>sylB</i>	6,960 $\pm$ 149	<i>sylA</i> overexpression
Δ <i>sylA</i> (pOEAB)	<i>sylB</i>	7,128 $\pm$ 106	<i>sylA</i> overexpression
WT (pOEAC)	<i>sylC</i>	7,923 $\pm$ 249	<i>sylA</i> overexpression
Δ <i>sylA</i> (pOEAC)	<i>sylC</i>	9,600 $\pm$ 415	<i>sylA</i> overexpression

^a WT = wild type.

^b β -Galactosidase (β -Gal) activity. Means $\pm$ standard error ($n > 15$) in Miller units (MU).

(Fig. 3B, black columns), although activities seemed consistently to decrease with shorter fragment length. Fragments of 110, 96, 82, and 68 bp resulted in basal activities (open columns). The shortest active *sylB* and *sylC* promoter fragments (105 and 124 bp, respectively) overlap for 8 bp at their 5' ends (Fig. 2A).

To complement these results from in vitro cultures, β -gal activity was also monitored in planta after infiltration of bacteria harboring reporter plasmids into leaves of *Nicotiana benthamiana* using a colorigenic substrate (Hull et al. 1996; Teeri et al. 1989). The results were consistent with the ones obtained from in vitro cultures and identified the 105-bp *sylB* and 124-bp *sylC* promoter fragments as the minimal fragments necessary for promoter activity in planta (Fig. 4A).

Analysis of the 221-bp intergenic region with the Virtual Footprint program (Munch et al. 2005) predicted putative imperfect dyad binding sites on both strands with scores (9.6 and 9.79) for the *P. aeruginosa* HTH LuxR-type response regulator RhlR near the ends of the shortest active *sylB* and *sylC* promoter fragments (Fig. 2A, boxed; the binding sequence logo is depicted in Fig. 2B). This putative binding site is present in all active fragments but absent or truncated in all *sylB* promoter fragments exhibiting basal activities. This holds true also for the *sylC* promoter, except for the shortest active deletion (124 bp of upstream sequence), where it is severely truncated (Fig. 2A). Intriguingly, analysis with the Virtual Footprint program of this deletion clone, including the neighboring vector sequences, revealed that, by chance, a good RhlR binding site with a score of 10.68 was recreated around the EcoRI cloning site into which this deletion fragment was cloned (Fig. 2A), thus suggesting that this putative binding site may also be important for *sylC* transcriptional activation.

A maltose-binding protein-SylA fusion protein binds to *sylB/sylC* promoter fragments.

In order to determine whether the SylA positive regulator binds directly to the *sylB* and *sylC* promoter fragments, maltose-binding protein (MBP)-SylA fusion proteins were generated. The *sylA* gene was amplified by PCR, cloned in frame into the vectors pMal-c2X (for expression in *Escherichia coli*) and pMEKm12 (a derivative of pMal-c2X) for expression in *P. syringae* (Lu et al. 2002b) to yield pMalSylA and pMEKmSylA, respectively. The recombinant plasmids were transformed into *E. coli* DH10B and *P. syringae* pv. *syringae* B301D-R.

MBP-tagged proteins were purified from extracts of both organisms by maltose-affinity chromatography and subjected to gel-blot analysis using an anti-MBP antibody, which revealed bands of approximately 70 kDa, the expected size of the MBP-SylA fusion protein (data not shown). To test whether the MBP-SylA fusion protein was functional in vivo, pMEKmSylA was transformed into the *P. syringae* pv. *syringae* B301D-R Δ sylA deletion mutant and tested for complementation of the syringolin A-negative phenotype using a rice infiltration assay. We have previously established that infiltration of syringolin A or syringolin A-synthesizing bacteria into rice leaves results in the accumulation of *Pir7b* transcripts, whereas syringolin A-negative strains or mutants do not induce this gene. (Hassa et al. 2000; Ramel et al. 2009; Wäspi et al. 1998b, 1999). Gel-blot analysis of RNA from infiltrated rice leaves revealed that transformation of the Δ sylA mutant with pMEKmSylA complemented the mutant and led to strong *Pir7b* transcript accumulation, whereas no *Pir7b* transcripts were detected in leaves infiltrated with the Δ sylA mutant with or without the empty control vector (Fig. 5A). This result confirmed that the MBP-SylA fusion protein was functional in vivo.

Fig. 2. A, Intergenic region between the *sylB* and *sylC* genes. Translation initiation codons of the *sylB* (TIB, bottom strand) and *sylC* (TIC, top strand) genes are shown in bold letters. Positions of promoter deletion breakpoints relative to the respective translational initiation codon are indicated with negative numbers above (*sylC*) or below (*sylB*) arrows. Fragments with background promoter activities are indicated with gray arrows, fragments with activities above background with black ones. A 64-bp-long subfragment used in MDAE experiments is marked with a bar between the DNA strands. The RhlR-like binding site residing within the 64-bp fragment as well as the one recreated by cloning the -124-bp fragment into the *EcoRI* cloning site (italics) are boxed. **B**, RhlR binding-sequence logo.

Binding of the MBP-SylA fusion protein to *sylB*/*sylC* promoter fragments was examined using a magnetic DNA affinity enrichment (MDAE) approach. A 244-bp fragment containing the intergenic region between the *sylB* and *sylC* genes as well as 18 and 5 bp of the *sylB* and *sylC* ORF was amplified by PCR using a biotinylated primer. As a negative control, a biotinylated internal 120-bp fragment of the *sylC* coding region was generated. The purified DNA fragments were immobilized on streptavidin-coated magnetic beads, which were then incubated with crude protein extracts from *P. syringae* pv. *syringae* B301D-R, *E. coli* expressing the MBP-SylA fusion protein, or MBP from the empty vector alone. Bound proteins were eluted and subjected to gel-blot analysis using MBP-specific antibodies. A band with the expected size of the fusion protein of approximately 70 kDa was observed with the *sylB*/*sylC* promoter probe but not with the negative control probe (Fig. 5B). Extracts from bacteria carrying the empty vector producing MBP alone (51 kDa) resulted sometimes in a faint background band in the expected size range. Next, a 64-bp-long subfragment (Fig. 2A, underlined) encompassing the sequence upstream of position -66 relative to the *sylB* gene and upstream of -91 relative to the *sylC* gene, and which contains the putative RhIR-like binding site, was tested in MDAE experiments. MBP-SylA bound to this fragment as well (Fig. 5B, right panel). Identical results were obtained with protein extracts from *P. syringae* pv. *syringae* B301D-R and *E. coli* harboring the respective MBP::SylA expression plasmids. These results strongly suggest that SylA is directly activating the *sylB* and *sylC* genes.

We also assayed fragments corresponding to the -105, -90, and -75 *sylB* promoter deletions. Although the -105 *sylB* promoter fragment bound the MBP::SylA fusion protein, the -90

fragment (Fig. 5C, left panel) and the -75 fragment did not. This result is in agreement with the results from the promoter activity assays (Fig. 3A) and further suggests that the putative RhIR-like binding element, which is present in the -105 deletion but severely truncated in the -90 deletion (Fig. 2A), is important for *sylB* activation by the *sylA* gene product. The corresponding results with *sylC* deletion fragments revealed that the -124 deletion fragment only weakly bound the MBP::SylA fusion protein, whereas the -110 fragment weakly bound the fusion protein equally as well as the MBP protein alone (i.e., unspecifically) (Fig. 5C, right panel). Results with the -95 and -82 fragments were similar to those obtained with the -110 deletion.

SalA is an upstream activator of syringolin A biosynthesis.

First, we examined the activity of the *sylA* promoter by constructing an *sylA*::*lacZ* translational fusion gene (*psylAp-lacZ*) that encompassed the 445 bp between the *sylA* translation initiation codon and the next upstream gene (which is highly conserved in *P. syringae* strains that lack a syringolin synthetase gene cluster, such as *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *phaseolicola*) and the first 86 bp of the *sylA* ORF. β -Gal assays of the wild type transformed with *psylAp-lacZ* and grown under still culture conditions showed an activity of approximately 300 Miller units (MU), whereas the same construct in the Δ *sylA* background led to an approximately 50% higher activity (Table 2). Therefore, we checked whether this might indicate negative feedback control of SylA on its own gene promoter by placing the *sylA* gene under the control of the *lacZ* promoter onto *psylAp-lacZ* to yield pOEAA. However, β -gal assays of the B301D-R wild-type strain carrying pOEAA exhibited activities similar to those carrying *psylAp-lacZ* (data not shown), thus not confirming the existence of a negative feedback loop.

Evidence from a microarray hybridization experiment reported in the literature (Lu et al. 2005) suggested that the *sylD* gene belonged to the regulon controlled by the HTH LuxR-type transcription factor SalA, which was reported to positively regulate syringomycin biosynthesis (Lu et al. 2002a; Wang et al. 2006). Therefore, we tested whether SalA was also involved in syringolin A biosynthesis. Thus, *psylAp-lacZ*, *psylBp-lacZ*, and *psylCp-lacZ* reporter plasmids were transformed into the *salA* transposon insertion mutant DSL7 (Lu et al. 2002a) and β -gal activities were determined. The activities of the *sylB* and *sylC* promoters were strongly decreased in the *salA* mutant compared with the wild type (Table 2), indicating that the *salA* gene was necessary for expression of *sylB* and *sylC*. Overexpression of *sylA* (using plasmid pOEAB and pOEAC) in the *salA* mutant background led to a dramatic increase of the *sylC* and *sylB* reporter, indicating that *salA* acts upstream of *sylA*. Consistent with this, syringolin A accumulation in conditioned media of the *salA* insertion mutant DSL7 was abolished but was restored by overexpression of *sylA* in this mutant (data not shown).

The *salA* gene under the control of the *lacZ* promoter was inserted into the *psylAp-lacZ*, *psylBp-lacZ*, and *psylCp-lacZ* reporter plasmids to yield pOESalAA, pOESalAB, and pOESalAC, respectively. When transformed into the Δ *sylA* mutant, pOESalAB and pOESalAC led to β -gal activities similarly low to those of *psylBp-lacZ* and *psylCp-lacZ* in the same genetic background, showing that overexpression of *salA* had no effect on the *sylB* and *sylC* promoters in the Δ *sylA* background (data not shown). In contrast, overexpression of *salA* in wild-type or *salA*-mutant bacteria transformed with pOESalAA consistently increased the activity of the *sylA* promoter approximately two- to threefold above the wild-type levels of approximately 300 MU, while the *sylA* reporter construct *psylAp-lacZ* led to β -gal activities of 172 MU in the *salA* mutant background (Table 2). The *sylA* reporter plasmid *psylAp-lacZ* in wild-type

Fig. 3. Promoter activities of **A**, *sylB* and **B**, *sylC* deletion fragments. Open bars indicate fragments resulting in background activity and black bars indicate fragments with activities above background. Means of data points measured at three representative culture time points (96, 120, and 144 h post inoculation [hpi]), each in 6 to 11 replicates, are given in Miller units (MU). Error bars indicate the standard error of the mean. Promoter fragments of the *sylB* and the *sylC* genes indicated for the 96-h time point apply analogously to the other time points.

bacteria led to blue staining of colonies in *N. benthamiana*, whereas no staining was detected in plants infiltrated with the *salA* mutant DSL7 carrying *psylAp-lacZ* (Fig. 4B). These findings suggest that SalA directly or indirectly activates the *sylA* gene, whose product then activates the *sylB* and *sylC* genes. Next, we tested whether SalA directly binds to the

Fig. 4. Qualitative β -galactosidase activity of bacteria carrying promoter-*lacZ* fusions in leaves of *Nicotiana benthamiana*. Leaves were infiltrated with *Pseudomonas syringae* pv. *syringae* B301D-R at 10^5 CFU/ml carrying the indicated promoter-reporter constructs. Leaves were stained with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside after 72 to 96 h. **A**, Leaves with bacteria carrying *sylB* (Bp) and *sylC* (Cp) promoter deletion reporter constructs were stained for equal amounts of time. **B**, *N. benthamiana* leaves infiltrated with wild-type (left panel) and *salA*-mutant bacteria (right panel) harboring the *psylAp-lacZ* reporter plasmid. **C**, *N. benthamiana* leaves infiltrated with wild-type (WT) and *Psyl3425*-ortholog (*anr* homolog)-negative bacteria carrying a full-length *sylC::lacZ* reporter construct stained for the same amount of time.

sylA promoter region using MDAE. The *salA* gene was cloned into pMEKm12 as a translational fusion to MBP, resulting in pMEKmSalA. This plasmid complemented the *salA* mutant DSL7 with regard to syringolin A production as monitored by *Pir7b* transcript accumulation upon infiltration of transformed bacteria into rice leaves, thus confirming that the MBP-SalA fusion protein was functional in vivo (Fig. 5A). However, no binding of MBP-SalA to the putative *sylA* promoter fragment

Fig. 5. Promoter binding assays with maltose-binding protein (MBP)-SylA and MBP-SalA fusion proteins. **A**, RNA gel-blot analysis of *Pir7b* transcript accumulation in rice leaves 16 h after infiltration of the wild-type *Pseudomonas syringae* pv. *syringae* B301D-R strain (lane 1), the Δ *sylA* mutant (lane 2), the Δ *sylA* mutant containing the empty vector pMEKm12 (lane 3), the Δ *sylA* mutant complemented with pMEKmSylA (lane 4), the *salA* insertion mutant DSL7 complemented with pMEKmSalA (lane 5), DSL7 containing the empty vector pMEKm12 (lane 6), and DSL7 (lane 7). Top panel: autoradiogram; bottom panel: ethidium-bromide-stained gel before blotting. The gel blot was probed with a radiolabeled *Pir7b* cDNA probe. **B**, Gel-blot analysis of protein eluates after magnetic DNA affinity enrichment (MDAE) using an MBP-specific antibody for immunodetection. Left panel: a 244-bp *sylB/sylC* promoter fragment or a 120-bp control fragment derived from the *sylC* coding region was bound to magnetic beads and incubated with extracts of *P. syringae* pv. *syringae* B301D-R carrying the empty vector (lane 1), MBP-SylA encoding pMEKmSylA (lane 2), and a 1:1 mixture of extracts from bacteria carrying pMEKmSylA or pMEKmSalA, respectively (lane 3). Right panel: MDAE with extracts from *Escherichia coli* carrying the empty vector (lane 1) or pMalSylA (lane 2) using a 64-bp subfragment. **C**, MDAE with *E. coli* extracts (lane 1, empty vector; lane 2, pMalSylA) using the indicated *sylB* (left panel) and *sylC* (right panel) promoter deletion fragments. The position and size in kDa of marker proteins is indicated on the left of panels in B and C.

Table 2. Effect of *salA* on *sylA*, *sylB*, and *sylC* reporter gene activity

Strain ^a	Reporter	β -Galactosidase activity ^b	Comment
WT (<i>psylAp-lacZ</i>)	<i>sylA</i>	299 $\pm$ 28	...
Δ <i>sylA</i> (<i>psylAp-lacZ</i>)	<i>sylA</i>	445 $\pm$ 25	...
WT (<i>psylBp-lacZ</i>)	<i>sylB</i>	763 $\pm$ 59	<i>n</i> = 9
WT (<i>psylCp-lacZ</i>)	<i>sylC</i>	1,237 $\pm$ 33	<i>n</i> = 9
DSL7 (<i>psylBp-lacZ</i>)	<i>sylB</i>	76 $\pm$ 9	<i>salA</i> mutant, <i>n</i> = 9
DSL7 (<i>psylCp-lacZ</i>)	<i>sylC</i>	209 $\pm$ 14	<i>salA</i> mutant, <i>n</i> = 9
DSL7 (pOEAB)	<i>sylB</i>	7,778 $\pm$ 218	<i>salA</i> mutant, <i>sylA</i> overexpression
DSL7 (pOEAC)	<i>sylC</i>	6,717 $\pm$ 340	<i>salA</i> mutant, <i>sylA</i> overexpression
WT (pOESalAA)	<i>sylA</i>	761 $\pm$ 41	<i>salA</i> overexpression
DSL7 (pOESalAA)	<i>sylA</i>	592 $\pm$ 35	<i>salA</i> mutant, <i>salA</i> overexpression
DSL7 (<i>psylAp-lacZ</i>)	<i>sylA</i>	172 $\pm$ 16	<i>salA</i> mutant

^a WT = wild type.

^b Means $\pm$ standard error (*n* > 15, except stated otherwise) in Miller units (MU).

encompassing 445 bp of sequence upstream of the *sylA* translation initiation codon and the first 86 bp of the coding region was observed (data not shown). Binding to this fragment was also not observed after incubation with a 1:1 mixture of extracts containing MBP-SalA and MBP-SylA, respectively.

Because these results may indicate that the action of SalA on the *sylA* gene is indirect, we considered two other genes encoding HTH LuxR-type regulatory proteins, *syrF* and *syrG*, as candidates possibly involved in *syl* gene regulation. *SyrF* was reported to be directly activated by the SalA regulator and to activate, in turn, genes controlling syringomycin biosynthesis (Wang et al. 2006). *SyrG* is physically located adjacent to the *salA* gene and was reported to exhibit a twofold reduced expression level in an *salA* mutant. In addition, an *syrG*-negative mutant exhibited reduced virulence on cherry fruits (Lu et al. 2002a).

Thus, we generated *syrF* and *syrG* insertion mutants (*SyrF_KO* and *SyrG_KO*) in which the HTH LuxR-type DNA-binding domains of the putative gene products were interrupted. Upon transformation of the *sylA* and *sylC* reporter plasmids *psylAp-lacZ* and *psylCp-lacZ* into these mutants, β -gal activities similar to those in the wild type carrying the same plasmids were observed. Furthermore, rice infiltration assays with the *syrF* and *syrG* mutants exhibited wild-type *Pir7b* transcript levels as determined by RNA gel-blot analysis (data not shown). Thus, there is no evidence that *syrF* and *syrG* play a role in the regulation of syringolin A biosynthesis.

Why does syringolin A accumulate in still but not in shaken cultures?

Syringolin A can be isolated from conditioned media of *P. syringae* pv. *syringae* B301D-R grown under still culture conditions in SRM_{AF} minimal medium (Wäspi et al. 1998a). Typically, 20 to 40 mg/liter can be obtained after 7 to 10 days at 28°C. In conditioned rich media (shaken and unshaken) or in shaken SRM_{AF} cultures, syringolin A accumulates only to concentrations barely detectable by direct high-performance liquid chromatography (HPLC) analysis (<2 mg/liter). Overexpression of the plasmid-borne *sylA* gene under the control of the *lacZ* promoter results in greatly increased syringolin A production in both still and shaken SRM_{AF} cultures (typically 200 to 400 mg/liter). Correspondingly, *sylA*-overexpressing *sylB::lacZ* and *sylC::lacZ* reporter plasmids (pOEAB and pOEAC) resulted in high β -gal activities in shaken cultures, in contrast to *psylBp-lacZ* and *psylCp-lacZ* not overexpressing *sylA* (data not shown). One obvious difference between still and shaken culture conditions is the cell density reached. Typically, in still cultures, a plateau at an optical density at 600 nm (OD₆₀₀) of 0.4 to 0.5 is reached after 3 to 4 days of growth at 28°C which is maintained until day 6 or 7 but declines thereafter. Because shaken cultures in SRM_{AF} reach much higher cell densities (up to an OD₆₀₀ of 4 after 4 days, and declining thereafter) and are much better aerated, we considered the possibility that syringolin A production might be either under negative QS control or inhibited by high oxygen concentration.

To test the first possibility, a markerless QS-negative mutant was constructed in *P. syringae* pv. *syringae* B301D-R, in which the orthologs of the adjacent *ahlI* autoinducer and *ahlR* regulator genes of the closely related *P. syringae* pv. *syringae* B728a strain (Quinones et al. 2004) were deleted. Transformation of the *sylB* and *sylC* reporter plasmids *psylBp-lacZ* and *psylCp-lacZ* into this mutant revealed reporter gene activities not significantly different from those observed in the wild-type background under both still and shaken SRM_{AF} cultures (data not shown). In addition, HPLC analysis of conditioned media from mutant and wild-type cultures contained similar syringolin A concentrations under all conditions (not shown). Thus,

we conclude that syringolin A biosynthesis is not dependent on the *ahlI/ahlR* QS system.

To examine the dependency of syringolin A production in vitro on oxygen concentration, *sylC* reporter activities (*psylCp-lacZ* in the *P. syringae* pv. *syringae* B301D-R wild type) were compared in shaken SRM_{AF} cultures under normal and low oxygen conditions. Cultures were grown for 64 h under normal shaken conditions (i.e., culture flasks were capped with a lid allowing gas exchange). Thereafter, half of the culture flasks were flushed with N₂ and sealed with parafilm to reduce gas exchange. All cultures were then shaken for another 46 h. *sylC::lacZ* reporter activity increased significantly upon oxygen limitation (Fig. 6), indicating that oxygen concentration does play a role in the regulation of syringolin A biosynthesis.

A well-known regulator of responses to limiting oxygen concentrations is the *E. coli* Fnr protein and its *P. aeruginosa* homologue, Anr (Blumer and Haas 2000; Green et al. 2009; Sawers 1999). Under anaerobic conditions, Fnr forms a dimer that binds to DNA and can either activate or repress genes (Bauer et al. 1999; Melville and Gunsalus 1996). The only close homologue of Anr in *P. syringae* pv. *syringae* B728a is YP_236495 (86% identity and 94% similarity) encoded by *Psyr_3425* (GeneID 3368952). In order to examine the role of the *Psyr_3425* ortholog in the regulation of syringolin A biosynthesis, a markerless deletion mutant was generated in the *P. syringae* pv. *syringae* B301D-R background, which then was transformed with the *psylCp-lacZ* and *psylAp-lacZ* reporter genes. Reporter activities of the respective plasmids in the mutant background were not significantly different from those measured in the wild-type background under both still and shaken culture conditions (data not shown). Furthermore, infiltration of wild-type and mutant strains into *N. benthamiana* leaves led to indistinguishable β -gal activity staining (Fig. 4C). In addition, both the wild type and the *Psyr_3425*-negative strains led to essentially the same *Pir7b* transcript levels upon infiltration into rice leaves. In summary, these results indicate that the Anr ortholog of *P. syringae* pv. *syringae* B301D-R does not play a role in the regulation of syringolin A biosynthesis.

DISCUSSION

Syringolin A biosynthesis can be conferred to the saprophytic *P. putida* by transformation with a cosmid harboring the

Fig. 6. Effect of oxygen concentration on *sylC::lacZ* reporter activity. Promoter activities were measured in *Pseudomonas syringae* pv. *syringae* B301D-R bacteria transformed with the *psylCp-lacZ* reporter construct. Cultures were first shaken for 64 h under aerated conditions. Then, oxygen was excluded from half of the cultures for another 46 h of shaking incubation (open bar), while the other half was shaken under aerated conditions (black bar). Average β -galactosidase activities in Miller units (MU) are given ($n = 5$). Error bars indicate the standard error of the mean.

sylA-E gene cluster, indicating that no other specific genes are necessary (Ramel et al. 2009). Here, we presented evidence that *sylC*, *sylD*, and *sylE* form an operon. The activity of the *sylCDE* operon and the *sylB* gene on the opposite strand depends on the *sylA* gene and is greatly increased upon overexpression of the latter.

Overlapping deletions of the *sylB/sylC* intergenic region allowed us to delimit minimal promoter fragments necessary to drive *sylB::lacZ* and *sylC::lacZ* reporter genes. The 226-bp full-length *sylB* promoter (comprising a 221-bp intergenic DNA and a 5-bp *sylC* coding region) translationally fused to the *lacZ* gene resulted in an activity of approximately 700 MU. Essentially the same activities were also observed with the shorter fragments of 153, 117, and 105 bp. Shorter fragments of 90, 75, 67, and 57 bp resulted in the same basal activity of approximately 200 to 300 MU, suggesting that the sequence between 105 and 90 bp contains elements important for promoter activity. These results were corroborated in planta upon infiltration of bacteria harboring these reporter constructs. Blue colonies inside the leaves appeared only with promoter deletions shown to be active in in vitro cultures, whereas no blue staining was detected with the reporter genes resulting in basal activities in vitro. This 15-bp region overlaps with a putative binding site for *P. aeruginosa* RhlR, the HTH-LuxR-type response regulator of a QS system in *P. aeruginosa* from position -100 to -85 relative to the *sylB* translational start site (5'-CTGTAAAAATAGCAG on the *sylB* strand). Because SylA also exhibits a putative HTH LuxR-type DNA binding domain, it seems likely that this site is important for *sylB* gene activation. Indeed, this element (5'-CTGCTATT TTTTACAG on the *sylC* coding strand) appears also to be essential for *sylC* promoter activity. Although the shortest active *sylC* promoter deletion (124 bp of upstream sequence) contained only a severely truncated version, a new high-scoring putative binding site was fortuitously created together with a flanking vector sequence, which likely explains the activity of this fragment in vitro and in planta. These data are essentially in agreement with the MDAE experiments using promoter fragments and protein extracts from *E. coli* and *P. syringae* pv. *syringae* B301D-R which produce an MBP::SylA fusion protein. It is worthwhile to mention that all our efforts to perform MDAE experiments using affinity-purified MBP::SylA proteins, or to perform electrophoretic mobility shift assays with them, remained unsuccessful, which may indicate that either the MBP::SylA fusion protein is instable or binding only occurs in the presence of one or more additional factors which, however, would have to be present in extracts of both *E. coli* and *P. syringae* pv. *syringae* B301D-R.

Whereas the results of the deletion analysis suggest that, apart from the small region containing the RhlR-like binding motif, no other upstream sequence element is important for *sylB* transcription, the consistently higher activity of the 239-bp (full-length) *sylC* promoter as compared with the -175 and -124 deletions (Fig. 2A) suggests that other sequence elements enhancing *sylC* transcriptional activity reside between positions -239 and -124. Virtual Footprint analysis revealed putative *P. aeruginosa* integration host factor (IHF) binding sites (5'-CAATGCGT between -195 and -188 on the *sylC* coding strand, and 5'-CAACGCAT between -186 and -193 on the bottom strand relative to the *sylC* translation initiation codon) as the only notable feature we observed in this region. IHF can induce DNA bends (U-turns) at its binding site and is known to influence transcription (Dillon and Dorman 2010; Huo et al. 2009; Macchi et al. 2003). It remains presently unknown if these sites play a role in *sylC* transcriptional regulation.

We have previously established that syringolin A biosynthesis is controlled by the GacS/GacA two-component system and is abolished in a *gacS* (formerly designated *lemA*) mutant

(Reimann et al. 1995; Wäspi et al. 1998a). The GacS/GacA global regulatory system has been found in numerous bacteria, including plant pathogens, where it was shown to control a plethora of phenotypes, among them pathogenicity, virulence, toxin production, and ecological fitness (Chatterjee et al. 2003; Heeb and Haas 2001; Hirano and Upp 2000; Hirano et al. 1997; Hrabak and Willis 1992, 1993; Willis et al. 1990). In the bean pathogen *P. syringae* pv. *syringae* B728a, some of the phenotypes observed in a *gacS* mutant, including loss of syringomycin production and lesion formation, could be restored by overexpressing the *salA* gene, whose expression was shown to depend on the GacS/GacA system (Kitten et al. 1998).

Here, we showed that syringolin A biosynthesis also is mediated by the *salA* gene product. Inactivation of *salA* resulted in negligible syringolin A production in in vitro cultures and to only basal activity of the *sylB::lacZ* and *sylC::lacZ* reporter genes. *SalA* acts upstream of *sylA* in the regulatory cascade, because overexpression of *sylA* in the *salA* mutant resulted in very high activities of the *sylB::lacZ* and *sylC::lacZ* reporter genes. The moderate *sylA::lacZ* reporter activity observed in the wild type was reduced to approximately 50% in in vitro cultures of the *salA* mutant whereas, in planta, no blue staining was observed. In contrast, overexpression of *salA* led to a two- to threefold increase of the *sylA::lacZ* reporter activity above wild-type levels, indicating that *SalA* directly or indirectly activates the *sylA* gene. We were not able to demonstrate direct binding of a MBP-*SalA* fusion protein, which was functional in vivo, to the *sylA* promoter. This may be due to experimental circumstances, or, alternatively, it may indicate that the activation is indirect via the activation of one or more additional transcription factors. The most likely candidates we considered were the *syrF* and *syrG* genes. Both encode HTH LuxR-type regulators, reside at the border of the large syringomycin/syringopeptin gene cluster, and were shown by microarray analysis to belong to the *SalA* regulon (Lu et al. 2005). In addition, *SyrF* was shown to activate *syr-syp* genes and bind to an *syrB1* promoter fragment, while *SalA* was necessary for *syrF* activity and bound to its promoter region (Wang et al. 2006). However, both the *syrF* and *syrG* mutants we generated showed wild-type behavior with regard to syringolin A production in planta and *sylA* and *sylC* reporter gene activity in vitro and, therefore, were ruled out as regulators of syringolin A biosynthesis. Because the microarray used by Lu and associates (2005) contained only 95 gene probes, the *SalA* regulon may well comprise additional unknown genes.

In wild-type bacteria, syringolin A accumulated to considerable amounts in conditioned SRM_{AF} media only under still culture conditions, where lower cell densities are reached and oxygen availability is limited; therefore, we considered QS and oxygen concentration as possible negative regulators of syringolin A biosynthesis. However, an *ahlI/ahlR* deletion mutant exhibited a wild-type phenotype in both still and shaken cultures, indicating that these genes are not involved in syringolin A biosynthesis. Similarly, QS was reported not to be involved in the regulation of syringomycin biosynthesis (Dumenyo et al. 1998; Kinscherf and Willis 1999).

In contrast to the QS system, oxygen concentration seems to play a role in syringolin A biosynthesis, as evidenced by the fact that the *sylC::lacZ* reporter gene resulted in a two- to threefold higher β -gal activity in shaken cultures under oxygen-limiting conditions compared with well-aerated cultures. In *P. aeruginosa*, the Fnr-type oxygen sensor *Anr* is known to mediate the regulation of many pathways activated or repressed by low oxygen tension (Green et al. 2009; Trunk et al. 2010); therefore, we deleted the *anr* ortholog in *P. syringae* pv. *syringae* B301D-R. However, no significant effect of this deletion was detected, either on *sylA::lacZ* and *sylC::lacZ* reporter gene readouts under all culture conditions and upon infiltration

into *N. benthamiana*, or on *Pir7b* transcript levels upon infiltration into rice leaves.

The potential of oxygen limitation as an inducer for the production of secondary metabolites by microbes in culture has long been recognized (Clark et al. 1995; Rollins et al. 1988). In the case of *P. syringae* pv. *syringae*, which is a leaf pathogen, the biological significance of the apparent oxygen sensitivity of syringolin A biosynthesis is not obvious. During the day, when stomates are open and photosynthesis takes place, oxygen is hardly limiting, even though the bacteria seem to form dense localized colonies (Fig. 4), which may develop biofilm-like properties. During the night, when stomata are closed and no photosynthesis occurs, this may be different. However, it is also possible that oxygen limitation in vitro activates a pathway leading to syringolin A biosynthesis which in planta is activated by other stimuli. Clearly, the picture obtained of the regulation of syringolin A biosynthesis remains incomplete. In order to identify additional genes involved, a more complete exploration of the *salA* regulon using *P. syringae* pv. *syringae* B728a whole-genome microarrays and the development of an efficient screening method for the identification of syringolin A-negative mutants would be helpful.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

Bacterial strains and plasmids used in this study are listed in Table 3. Unless otherwise stated, bacteria were grown in Luria-Bertani (LB) medium (tryptone at 10 g/liter, yeast extract at 5 g/liter, and NaCl at 10 g/liter) containing the appropriate antibiotics (ampicillin at 100 µg/mg, gentamicin at 50 µg/ml, kanamycin at 50 µg/ml, or tetracycline at 10 µg/ml) on a shaker at 28°C (*P. syringae* pv. *syringae*) or 37°C (*E. coli*).

Syringolin A-inducing culture conditions.

Cultures of *P. syringae* pv. *syringae* (5 ml each) were grown overnight in SRM_{AF} medium (1% D-glucose, 0.1% D-fructose, 100 µM arbutin, 0.4% L-histidine, 0.8 mM MgSO₄, 10 µM FeCl₃, and 0.8 mM potassium phosphate, pH 7) (Gross and DeVay 1977; Mo and Gross 1991b) and tetracycline at 10 µg/ml, if appropriate, at 28°C on a shaker (220 rpm/min) overnight. The density was adjusted to 0.2 OD₆₀₀ and 1-ml aliquots were used to inoculate 100-ml SRM_{AF} cultures in 250-ml Erlenmeyer flasks, which were incubated at 28°C for 5 to 7 days without shaking.

For low-oxygen condition experiments, 100-ml cultures were grown in 250-ml Erlenmeyer flasks on a shaker (220 rpm) for 64 h. Then, culture flasks were flushed with N₂, the cap was removed, and the flask was sealed with parafilm to impede gas exchange. Cultures were then shaken for another 46 h.

Detection of syringolin A production.

Syringolin A in conditioned media was detected by HPLC analysis, as described (Ramel et al. 2009). Syringolin A production by bacteria in planta was detected by infiltrating bacteria into leaves of 2- to 3-week-old rice (*Oryza sativa* 'Loto') plants and analyzing accumulation of *Pir7b* transcripts by RNA gel blots 16 h after infiltration, as described (Ramel et al. 2009). *Pir7b* encodes an esterase (Wäspi et al. 1998b) whose corresponding transcript accumulates only upon infiltration of *P. syringae* pv. *syringae* producing syringolin A. Syringolin A was originally isolated based on *Pir7b* induction (Wäspi et al. 1998a).

Extraction and analysis of RNA from bacteria.

P. syringae pv. *syringae* B301D-R transformed with pOEAC was grown on a shaker in 50 ml of SRM_{AF} medium overnight

at 28°C. Bacteria were pelleted and resuspended in 1.6 ml of cold trizol solution (38% phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, and 5% glycerol [vol/vol]). After centrifugation at 20,000 × g for 10 min at 4°C, the supernatant was recovered and vigorously shaken with 300 µl of chloroform for 15 min at room temperature. After centrifugation for 8 min at 20,000 × g at 4°C, 800 µl of the supernatant was mixed with 400 µl each of isopropanol and 0.8 M sodium citrate + 1.2 M NaCl solution. The mixture was incubated at -20°C overnight and RNA was pelleted by centrifugation for 8 min at 12,000 × g, washed with 75 % ethanol, air dried, and dissolved in 60 µl of diethylpyrocarbonate-treated water. RNA gel-blot analysis, ³²P-labeling of probes, and hybridization were performed according to standard procedures (Ausubel et al. 1987). The probe fragments were amplified from *sylC* and *sylE* genes with primers *sylC*_N_f, *sylC*_N_r and *sylE*_N_f, *sylE*_N_r, respectively.

RT-PCR reactions with 1.5 to 2 µg of total RNA were performed using the OneStep RT-PCR Kit (Qiagen, AG, Hombrechtikon, Switzerland) according to the manufacturer's protocol using primers RT_*sylCD*_f, RT_*sylCD*_r (for the *sylC/sylD* intergenic region) and RT_*sylDE*_f, RT_*sylDE*_r (for the *sylD/sylE* intergenic region).

Construction of mutants.

Gene fragments of *syrF* (accession number AF372703; ORF position 59 to 889; fragment 127 to 802) and *syrG* (accession number AF372703; ORF position 6,136 to 6,924; fragment 6,249 to 6,827) were amplified with primer pairs *syrF*_KO_f/r and *syrG*_KO_f/r from genomic DNA of *P. syringae* pv. *syringae* B301D-R and cloned into the suicide vector pJQ200KS using the restriction sites *Bam*HI and *Xho*I. Recombinant plasmids were transformed into *E. coli* S17-1 and mobilized into *P. syringae* pv. *syringae* B301D-R. Colonies carrying chromosomally integrated plasmids were selected for kanamycin resistance and plasmid insertions at the correct site were verified by PCR, as described (Amrein et al. 2004).

The construction of the markerless Δ*sylA* mutant has been described (Ramel et al. 2009). Markerless Δ*ahlI*/*ahlR* and Δ*anr* mutants in the *P. syringae* pv. *syringae* B301D-R background were constructed in an analogous way, whereby primers were designed according to the genome sequence of *P. syringae* pv. *syringae* B728a (accession number NC_007005.1, *ahlI* GeneID 3367123, *ahlR* GeneID 3367124, and *anr* GeneID 3368952 [*Psy*r_3425 ortholog]). In each case, flanking fragments were amplified using primer pairs P1/P2 and P3/P4, which were joined by overlap extension PCR using primer pair P1/P4. Mutants were verified by PCR using primers flanking the deleted sequences.

Construction of promoter-reporter fusion genes.

Syl promoter fragments were amplified by PCR from B301D-R genomic DNA. Primer sequences were designed according to the sequence of the *syl* gene cluster (accession number AJ548826). All promoter fragments were flanked by *Eco*RI and *Pst*I sites and cloned into pME6014, resulting in translational fusions to the *lacZ* gene named *psylAp-lacZ*, *psylBp-lacZ*, and *psylCp-lacZ*. The *sylA*-overexpressing *sylA* and *sylB* reporter plasmids pOEAA and pOEAB were constructed analogously to pOEAC, as described (Ramel et al. 2009). In short, the *sylA* ORF was put under the control of the *lacZ* promoter and placed onto the reporter plasmids *psylAp-lacZ*, *psylBp-lacZ*, and *psylCp-lacZ*, respectively, in opposite orientation. Similarly, the *salA*-overexpressing *sylA* (pOEsa-lAA), *sylB* (pOEsa-lAB), and *sylC* (pOEsa-lAC) reporters were constructed by amplifying the *salA* ORF (accession number

AF372703, position 7,960 to 8,829) from B301D-R genomic DNA using primers *salA_OE_f* and *salA_OE_r*. The PCR product was cloned into pME6001^k behind the *lacZ* promoter to yield pOESalA^k, from which the *lacZp-salA* fusion gene was amplified using primers *SalA_OE_r* and *lacZp_OL_sylAp* (or *lacZp_OL_sylBp* or *lacZp_OL_sylCp*, respectively). Then, the *sylA*, *sylB*, and *sylC* promoter fragments were amplified from *psylAp_lacZ*, *psylBp_lacZ*, and *psylCp_lacZ*, respectively, with the respective primer pairs *syl[A/B/C]p_OL_lacZp/syl [A/B/C]p_r*. These fragments were joined to the corresponding *lacZp-salA* fusion fragments by overlap extension PCR with primer pairs *salA_OE_r/syl[A/B/C]p_r*, digested with *EcoRI* and *PstI*, and cloned into the polylinker of pME6014. All constructs were verified by sequencing.

Quantitative β-gal assays.

Quantitative β-gal assays were performed as described (Miller 1972) with some modifications. Samples of bacterial cultures were harvested from 72 to 168 h post inoculation (hpi) in intervals of approximately 24 h. Z-buffer (1 ml) (60 mM Na₂HPO₄ × 2H₂O, 40 mM NaH₂PO₄ × H₂O, 10 mM KCl, 1 mM MgSO₄ × 7 H₂O, and 50 mM β-mercaptoethanol), 20 μl of 0.1% sodium dodecyl sulfate, 40 μl of chloroform, and 100 to 300 μl of bacterial culture were mixed by inversion. The suspension was centrifuged at 1,000 × g for 5 min. To 1 ml of supernatant, 60 μl of reagent solution (60 mM Na₂HPO₄ × 2H₂O, 40 mM NaH₂PO₄ × H₂O, and 1.4% [wt/vol] ortho-nitrophenyl-β-D-galactoside) was added. The reaction was stopped by adding 150 μl of 1 M Na₂CO₃. Absorption was measured at OD₄₂₀ and MU were calculated according to the formula MU = 1,000 × OD₄₂₀ × 1.21/t × V × OD₆₀₀ × 1.7, where t = reaction

time in min and V = volume of bacterial suspension added; the formula was adapted for the reagent volumes used here. Experiments were repeated independently two or three times, each with three to five replicate cultures. Data are usually shown as the average from the measurements at 96, 120, and 144 hpi, when values normally have reached a plateau.

β-gal assay in planta.

Three-week-old *N. benthamiana* plants grown at 25°C in a greenhouse under a regime of 16 h of light and 8 h of darkness were infiltrated with a suspension of bacteria transformed with reporter constructs at an OD₆₀₀ of 0.0002 (approximately 10⁵ bacteria/ml) using a syringe without a needle. Leaves were harvested after 72 to 96 hpi. Leaf disks (1 cm) were punched out from infiltrated areas and vacuum infiltrated with 1% glutaraldehyde in potassium phosphate buffer (0.2 M NaPO₄, pH 7) to inactivate the endogenous β-gal of the plants (Teeri et al. 1989). Leaf disks were washed three times in potassium phosphate buffer and incubated overnight with 1 mM 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside. Stained disks were washed once in potassium phosphate buffer and incubated in acetone/methanol (1:3) for 3 h at 37°C to remove chlorophyll. After a final wash in 100% ethanol, leaf disks were arranged on glass slides in 50% glycerol for observation under the light microscope.

MBP-SylA and MBP-SalA fusion proteins.

SylA and *salA* genes were amplified by PCR using primer pairs *sylAFP_f/r* and *salAFP_f/r*, respectively, and cloned into the vectors pMal-c2X and pMEKm12 to yield pMalSylA and pMEKmSylA for expression in *E. coli* and *P. syringae*

Table 3. Bacterial strains and plasmids

Strains	Genotype, relevant characteristics	Reference
<i>Escherichia coli</i>		
DH10B	<i>F⁻ endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ⁻</i>	Grant et al. 1990
XL1-Blue	<i>endA1 gyrA96(nal^r) thi-1 recA1 relA1 lac glnV44 F⁺ [::Tn10 proAB⁺ lacI^q Δ(lacZ)M15]</i>	Bullock et al. 1987
S17-1	<i>(thi pro hsdR recA; chromosomal RP4 [Tra⁺ Tc^s Km^s Ap^s]);</i> used as donor strain in biparental matings	Simon et al. 1983
<i>Pseudomonas syringae</i> pv. <i>syringae</i>		
B301D-R	Wild type, isolated from pear, Rif ^r	Xu and Gross 1988
DSL7	<i>salA::nptIII</i> insertion mutant in B301D-R, Kan ^r	Lu et al. 2002a
ΔsylA	<i>sylA</i> deletion mutant in B301D-R	Ramel et al. 2009
SyrF_KO	<i>syrF</i> plasmid insertion mutant in B301D-R, Tet ^r	This study
SyrG_KO	<i>syrG</i> plasmid insertion mutant in B301D-R, Gm ^r	This study
Δahl/ahlR	ahlI/ahlR (quorum sensing system) double deletion mutant in B301D-R	This study
Δanr	Anr homologue deletion mutant in B301D-R (ortholog of Psyr_3425 of <i>P. syringae</i> pv. <i>syringae</i> B728a)	This study
Plasmids		
pMal-c2x	Commercial <i>E. coli</i> overexpression vector, designed to produce maltose-binding protein fusions, Amp ^r	New England Biolabs
pMEKm12	<i>P. syringae</i> pv. <i>syringae</i> overexpression vector, derivative of pMal-c2x. Amp ^r , Kan ^r	Lu et al. 2002b
pME3087	Suicide vector, ColE1 replicon, Tet ^r	Voisard et al. 1994
pJQ200KS	Suicide vector, p15A ori, Gm ^r	Quandt and Hynes, 1993
pME6014	<i>LacZ</i> reporter plasmid, used for quantitative promoter activity measurements, Tet ^r	Schnider-Keel et al. 2000
psylAp_lacZ	<i>sylA</i> promoter translationally fused to the <i>lacZ</i> gene of pME6014, Tet ^r	This study
psylBp_lacZ	<i>sylB</i> promoter translationally fused to the <i>lacZ</i> gene of pME6014, Tet ^r	This study
psylCp_lacZ	<i>sylC</i> promoter translationally fused to the <i>lacZ</i> gene of pME6014, Tet ^r	This study
pME6001	<i>P. syringae</i> pv. <i>syringae</i> overexpression vector, Gm ^r	Blumer et al. 1999
pOEAK	pME6001 derivative, overexpressing <i>sylA</i> , Gm ^r , Kan ^r	Ramel et al. 2009
OESalA ^k	pME6001 derivative, overexpressing <i>salA</i> , Gm ^r , Kan ^r	This study
pOEAC	pME6014 derivative containing a <i>lacZp::sylA</i> gene and the <i>sylCp::lacZ</i> reporter gene, Tet ^r	Ramel et al. 2009
pOEAA	pME6014 derivative containing a <i>lacZp::sylA</i> gene and the <i>sylAp::lacZ</i> reporter gene, Tet ^r	This study
pOEAB	pME6014 derivative containing a <i>lacZp::sylA</i> gene and the <i>sylBp::lacZ</i> reporter gene, Tet ^r	This study
pOESalAApp	pME6014 derivative containing a <i>lacZp::salA</i> gene and the <i>sylAp::lacZ</i> reporter gene, Tet ^r	This study
pOESalABp	pME6014 derivative containing a <i>lacZp::salA</i> gene and the <i>sylBp::lacZ</i> reporter gene, Tet ^r	This study
pOESalACp	pME6014 derivative containing a <i>lacZp::salA</i> gene and the <i>sylCp::lacZ</i> reporter gene, Tet ^r	This study

^a Rif^r, Kan^r, Tet^r, Gm^r, and Amp^r indicate resistance to rifampicin, kanamycin, tetracycline, gentamicin, and ampicillin, respectively.

pv. *syringae* B301D-R, respectively. After verification by sequencing, they were transformed into *E. coli* DH10B and *P. syringae* pv. *syringae* B301D-R. Bacterial cultures (3 × 200 ml) in LB with the appropriate antibiotic were grown to an OD₆₀₀ of 0.5 to 1 before expression of the fusion gene was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.3 mM for *E. coli* and 5 mM for *P. syringae* pv. *syringae*. Cultures were further incubated for 5 and 7 h (*E. coli* and *P. syringae* pv. *syringae*, respectively) before bacteria were pelleted by centrifugation (4,000 × g, 20 min, 4°C) and resuspended in a total of 22.5 ml of binding buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.01% Triton X-100). The suspension was supplemented with protease inhibitors (one tablet per 10 ml of Complete Mini Protease Inhibitor Cocktail; Roche Applied Science, Rotkreuz, Switzerland). Cells were broken with a French press and cell debris was removed by centrifugation (10,000 × g, 30 min, 4°C). The protein concentration in the supernatant was estimated by measuring the absorption at 280 nm. On average, extracts contained protein at approximately 35 mg/ml (*E. coli*) and 25 mg/ml (*P. syringae* pv. *syringae*). To verify the correct size of fusion proteins (MBP-SylA, 69 kDa; MBP-SalA, 74 kDa; and MBP, 51 kDa), protein extracts were analyzed on protein gel blots using an anti-MBP antibody (New England Biolabs, Ipswich, MA, U.S.A.) according to standard procedures (Ausubel et al. 1987).

MDAE.

MDAE was carried out with minor modifications as described (Koebnik et al. 2006). To amplify biotinylated promoter probes, biotinylated primers were used. Purified biotinylated DNA (10 pmol) in 200 µl of H₂O was added to an equal volume of magnetic beads (Dynabeads; Invitrogen, San Diego, CA, U.S.A.) suspension prepared as described (Koebnik et al. 2006). The suspension was incubated with slight agitation for 2 h at room temperature, washed three times for 5 min with wash buffer (10 mM Tris-HCl [pH 7.5] and 2 M NaCl), and resuspended in 1.1 ml of binding buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.01% Triton X-100) containing 18 mM maltose and a 10-fold (wt/wt) excess of salmon sperm DNA (Sigma-Aldrich, Buchs, Switzerland) over fragment DNA. Finally, the suspension was mixed with 900 µl of whole protein extract from *P. syringae* pv. *syringae* B301D-R or *E. coli* overexpressing MBP, MBP-SylA, or MBP-SalA protein. The mixture was gently agitated for 45 min at room temperature. Beads were then washed with binding buffer (three times, 5 min; in the second wash step, competitor DNA was added) and proteins were eluted twice with 100 µl of elution buffer (20 mM Tris-HCl [pH 7.5], 2 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.01% Triton X-100). The two eluates were combined and concentrated to approximately 40 µl using Vivaspin 500 (MWOC 10,000; Sartorius AG, Göttingen, Germany). Proteins were subjected to gel-blot analysis using anti-MBP antibody according to standard procedures (Ausubel et al. 1987).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Virtual Footprint software suite: prodoric.tu-bs.de/vfp
 Prodoric database: www.prodoric.de/matrix.php?matrix_acc=MX000011