Production of Proteasome Inhibitor Syringolin A by the Endophyte Rhizobium sp. Strain AP16

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Syringolin A, the product of a mixed nonribosomal peptide synthetase/polyketide synthase encoded by the syl gene cluster, is a virulence factor secreted by certain Pseudomonas syringae strains. Together with the glidobactins produced by a number of betaproteobacterial human and animal pathogens, it belongs to the syrbactins, a structurally novel class of proteasome inhibitors. In plants, proteasome inhibition by syringolin A-producing P. syringae strains leads to the suppression of host defense pathways requiring proteasome activity, such as the ones mediated by salicylic acid and jasmonic acid. Here we report the discovery of a syl-like gene cluster with some unusual features in the alphaproteobacterial endophyte Rhizobium sp. strain AP16 that encodes a putative syringolin A-like synthetase whose components share 55% to 65% sequence identity (72% to 79% similarity) at the amino acid level. As revealed by average nucleotide identity (ANI) calculations, this strain likely belongs to the same species as biocontrol strain R. rhizogenes K84 (formally known as Agrobacterium radiobacter KB4), which, however, carries a nonfunctional deletion remnant of the syl-like gene cluster. Here we present a functional analysis of the syl-like gene cluster of Rhizobium sp. strain AP16 and demonstrate that this endophyte synthesizes syringolin A and some related minor variants, suggesting that proteasome inhibition by syrbactin production can be important not only for pathogens but also for endophytic bacteria in the interaction with their hosts.

Syringolin A was originally isolated from culture supernatants of the phytopathogenic gammaproteobacterium Pseudomonas syringae pv. syringae B301D-R based on its ability to elicit defense responses and pathogen resistance in rice plants (1, 2). It is a tripeptide derivative consisting an N-terminal valine and the two nonproteinogenic amino acids, 3,4-dehydrolysine and 5-methyl-4-amino-2-hexenoic acid; the first is N-acetylated with an unusual ureido-valine moiety, and the latter two form a 12-member macrolactam ring (Fig. 1A). Syringolin A is the major variant of a family of related compounds in which one or both valine residues can be replaced by isoleucine and/or 3,4-dehydrolysine can be replaced by lysine (3). Syringolin A was shown to be a virulence factor in the interaction of P. syringae strain B728a with its host plant Phaseolus vulgaris (bean) where loss of syringolin A production resulted in a significantly diminished lesion number (4). The elucidation of the mode of action of syringolin A revealed that it irreversibly inhibited all three proteolytic activities (i.e., the caspase-, trypsin-, and chymotrypsin-like activities) of the eukaryotic proteasome by covalent ether bond formation with the active-site N-terminal threonine residues of the catalytic β1, β2, and β5 subunits of the 20S core proteasome (4). Proteasome inhibition suppresses the action of many plant hormones, including defense reactions mediated by the important defense hormones jasmonic acid (JA) and salicylic acid (SA) (5–7).

Syringolin A is structurally similar to glidobactin A and related variants (syn. cepafungins) that were isolated more than 20 years ago from the betaproteobacterial strain K481-B101 (ATCC 53080; DSM 7029; formerly misidentified as Polyanium brachysporum) and an undefined species related to Burkholderia cepacia due to their antifungal and antitumor activities (8–11). Similar to syringolin A, glidobactin A consists of a 12-member ring structure and inhibits the eukaryotic proteasome by the same mechanism as syringolin A (4). In glidobactins, the ureido-valyl moiety is replaced by a fatty acid tail. Together, syringolin A and glidobactin A are the founding members of a novel class of proteasome inhibitors named syrbactins (12).

Syringolin A and its variants are synthesized by a mixed nonribosomal peptide synthetase/polyketide synthase (NRPS/PKS) encoded by a gene cluster comprising the five open reading frames (ORFs) sylA to sylE (Fig. 1B) (13). Whereas sylA encodes a LuxR-type transcriptional activator of the sylB gene and the sylCDE operon (14), sylE encodes a putative export facilitator involved in syringolin secretion. The sylC and sylD genes encode the NRPS/PKS responsible for syringolin biosynthesis, whereas sylB encodes a desaturase thought to mediate the conversion of lysine to 3,4-dehydrolysine in the ring structure. Based on the sequence and architecture of the syl gene cluster, an experimentally supported biosynthesis model of syringolin A was proposed which explains all structural features of the molecule (13, 15–18). The syringolin variants are the result of incomplete lysine desaturation by SylB and a relaxed specificity of the SylC NRPS module, which, in addition to valine, activates also isoleucine, although with reduced efficiency (3, 15). Cloning of the glidobactin A synthetase revealed a gene cluster (gllb genes) with high similarity in sequence and architecture to the syl gene cluster, allowing the postulation of a biosynthesis model analogous to the one for the syringolin variants (19).

A search in genome sequence databases revealed intact syl gene
clusters in the majority of sequenced strains belonging to phylogenetic group II of the *P. syringae* species complex (20–22), whereas, in addition to strain K481-B101, intact *glm*-like gene clusters were identified in the human pathogens *Burkholderia pseudomallei*, *B. ockhamensis* (betaproteobacteria), and *Photorhabdus asymbiotica* (gammaproteobacteria), as well as in the insect pathogen and nematode symbiont *Ph. luminescens* (18, 19). Thus, these strains seem capable of synthesizing syrbin-class proteasome inhibitors, and this was indeed shown for the genus *Photorhabdus*, which, in addition to glidobactin A (23, 24), also synthesizes the minor variant cepafungin I, the strongest proteasome inhibitor hitherto known (25).

Among recently deposited genome sequences we have discovered a *syl*-like gene cluster with unusual features in the genome of the alphaproteobacterial strain *Rhizobium* sp. strain AP16, which was isolated from the root endosphere of poplar trees (*Populus deltoides* [Eastern cottonwood]) (26). Here we present a functional analysis of this gene cluster and show that this endophyte synthesizes the proteasome inhibitor syringolin A under laboratory conditions in a medium which contains plant-borne phenolic compounds. We also demonstrate that a type II thioesterase encoded immediately downstream of the *sylB* homolog is involved in enhancing syringolin A biosynthesis yield. Furthermore, we demonstrate that the *SylC* homolog in this strain contains a tandem peptidyl-carrier protein (PCP) domain and that deletion of the second of these domains has no influence on syringolin production. Finally, we investigated the phylogenetic position of AP16 and show that this strain is closely related to the biocontrol agent *Rhizobium rhizogenes* K84 (formerly *Agrobacterium radiobacter* K84), which, interestingly, retains only remnants of the *syl*-like gene cluster.

### MATERIALS AND METHODS

#### Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *Pseudomonas syringae* were routinely grown in Luria-Bertani (LB) medium (tryptone at 10 g/liter, yeast extract at 5 g/liter, and NaCl at 5 g/liter). *Rhizobium* sp. strain AP16 was cultivated in rhizobium medium (mannitol at 10 g/liter, K2HPO4 at 0.5 g/liter, MgSO4 at 0.2 g/liter, NaCl at 0.1 g/liter, and yeast extract at 1 g/liter) unless stated otherwise. For syringolin production, SRM medium (27) supplemented with 0.5 g/liter yeast extract was used. For solid media, 2% of agar was added. Antibiotics were used in the following concentrations: chloramphenicol at 20 µg/ml, gentamicin at 50 µg/ml, rifampin at 50 µg/ml, and tetracycline at 15 µg/ml.

**RNA extraction and RT-PCR analysis.** Total RNA was extracted from bacteria grown as a lawn on SRM plates after 3 days in constant darkness using an RNeasy Protect Bacteria minikit (Qiagen, Hilden, Germany) with on-column DNase treatment as described in the manufacturer’s manual. Semiquantitative reverse transcriptase PCR (RT-PCR) was performed using a Qiagen OneStep RT-PCR kit (Qiagen, Hilden, Germany) as described in the manufacturer’s manual.

**Generation of markerless in-frame gene deletion mutants.** Fragments (700 bp long) of upstream and downstream sequences surrounding the target gene were amplified using the respective primer pairs (see Table S1 in the supplemental material) (primers P1 and P2 to amplify the upstream sequence and primers P3 and P4 amplify the downstream sequence). The primers were designed such that 18 to 30 bases of the original gene sequence, including the start and the stop codons, would be retained in order to generate an in-frame deletion. The two DNA fragments were then joined by overlap extension PCR using primers P1 and P4. The PCR product obtained was digested with the respective restriction enzymes then joined by overlap extension PCR using primers P1 and P4. The PCR product obtained was digested with the respective restriction enzymes.
enzymes, cloned into suicide vector pQ200KSΔPlac, and transformed into E. coli XL-1 Blue. After verification by sequencing, the resulting construct was transformed into E. coli S17-1 and further mobilized into Rhizobium sp. strain AP16 by biparental mating. Single recombinants were selected on plates containing gentamicin and, after verification by PCR, grown on plates with rhizobium medium supplemented with 10% sucrose in order to select double recombinants. Deletion of the target sequence was verified by PCR using the respective oriA and oriB primers and sequence determination.

### Isolation of syringolin from bacterial cultures and high-performance liquid chromatography (HPLC) analysis

For *P. syringae*, liquid cultures (5 ml each) were grown overnight in LB medium supplemented with tetracycline at 28°C on a shaker (220 rpm/min). The density was adjusted to an OD₆₀₀ of 0.2, and 4-ml aliquots were used to inoculate 40 ml distilled water per plate. Cells were harvested by centrifugation and resuspended in 40 ml of 70% isopropanol overnight at 4°C. Isopropanol was then removed, and the beads were washed with another 900 µl of 70% isopropanol overnight at +4°C. Isopropanol was then removed, and the beads were washed with another 900 µl of 70% isopropanol for 20 min at room temperature with shaking (200 rpm). The two eluates were combined and filtered through a Millex GP filter unit (Millipore, Molsheim, France) (0.22 µm pore size), and 1 ml of the reaction mixture was evaporated to dryness using a Savant SpeedVac (Fisher Scientific Switzerland, Reinach, Switzerland). Dry material was resuspended in 300 µl of MeCN, filtered again through a Millex GP filter unit, and stored at +4°C until further use.

HPLC analysis was performed as follows. A 20-µl volume of filtrate was injected into a 250-mm-by-4.6-mm Nucleosil 100 C18, 5-µm-pore-size column (Dr. Maisch GmbH, Ammerbuch, Germany) which was connected to an UltiMate 3000 HPLC system (Thermo Fisher, Olten, Switzerland). Isocratic separation of components was performed with 20% acetonitrile–0.06% trifluoroacetic acid (TFA) in water at a flow rate of 1 ml/min.

### MS and nuclear magnetic resonance (NMR).

Low-resolution electrospray ionization–mass spectrometry (ESI) mass spectra were measured with a quadrupole ion trap instrument (Esquire LC; Bruker Daltonik GmbH, Bremen, Germany). The solutions (about 0.1 to 1 µmol/ml in MeCN) were continuously introduced through the electrospray interface at a flow rate of 5 µl min⁻¹. Mass spectrometry (MS) acquisitions were performed at normal resolution (0.6 U at half peak height) under ion charge control (ICC) conditions (10,000) in the mass range of m/z 100 to 2,000. To get representative mass spectra, 8 scans were averaged.

High-resolution (6,000) electrospray ionization–mass spectrometry (HR-ESI-MS) was performed on a Finnigan MAT 900 (Thermo Finnigan, San Jose, CA) double-focusing magnetic sector mass spectrometer. The samples were dissolved in CH₃OH (50 µl). PPG425 and PPG10000 (Aldrich, Steinheim, Germany) served for calibration.

All 1H and 13C NMR spectra were recorded at room temperature using a Bruker Avance 600 MHz instrument at 600 MHz (1H) and 151 MHz (13C). Samples (ca. 2 mg) were dissolved in d₄-methyl sulfoxide (d₄-MSO). Spectra were calibrated with regard to the proton chemical shift (δ = 0 ppm) and carbon chemical shift (δ = 0 ppm) of tetramethylsilane.

### Phylogenetic tree generation.

Nucleotide sequences of the following four housekeeping genes were extracted from NCBI GenBank (http://www.ncbi.nlm.nih.gov/GenBank): DNA gyrase subunit B (*gyrB*), glyceraldehyde triphosphate dehydrogenase (*gap*), citrate synthase (*ctsY*), and RNA polymerase sigma factor 70 (*rpoD*). Accession numbers of the included genomes are listed in Table S2 in the supplementary material. Nucleotide sequences were aligned using the MUSCLE algorithm (28) and concatenated. Phylogenetic tree construction was done using MEGA 5.2.2 software (29) with the neighbor-joining method (Saitou–Nei algorithm) and 1,000 bootstrap cycles.

**RESULTS**

The *syl*-like gene cluster of *Rhizobium* sp. strain AP16. BLAST searches in microbial genome databases at NCBI revealed the draft genome of *Rhizobium* sp. strain AP16 (AP16) to contain a region (GenBank accession no. AJVM01000074) encompassing a gene cluster with high similarity in sequence and architecture to the syringolin A synthetase-encoding genes of *P. syringae* B301D-R and a number of other *P. syringae* strains (Fig. 1B) (13). The proteins encoded by PMI03_05099 to PMI03_5102 exhibit 55% to 65% sequence identity (72% to 79% similarity) to the products of

**TABLE 1 Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype, relevant characteristic(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhizobium</em> sp. strain AP16</td>
<td>Wild-type strain, Chf⁺</td>
<td>This study</td>
</tr>
<tr>
<td><em>Rhizobium</em> sp. strain AP16, ΔsylC</td>
<td>Deletion mutant of PMI03_05100</td>
<td>This study</td>
</tr>
<tr>
<td><em>Rhizobium</em> sp. strain AP16, ΔsylC</td>
<td>Deletion mutant of PMI03_05097</td>
<td>This study</td>
</tr>
<tr>
<td><em>Rhizobium</em> sp. strain AP16, ΔsylC</td>
<td>Double-deletion mutant of PMI03_05096 and PMI03_05097</td>
<td>This study</td>
</tr>
</tbody>
</table>

Plasmid

| pQ200KSΔPlac | Suicide vector; p15A ori; Gent⁰, SacB⁻, Δlac promoter | This study |

[^1]: Ap, ampicillin; Chl, chloramphenicol; Gent, gentamicin; Km, kanamycin; Nal, nalidixic acid; Tc, tetracycline.
the sylB to sylE genes that are responsible for biosynthesis and export of syringolin A in P. syringae strains. The AP16 gene cluster lacks a homolog of the sylA gene encoding a transcriptional activator which in P. syringae B301D-R controls expression of the sylB gene and the sylCD operon. This suggests that transcriptional regulation is not conserved between the two species. In addition to these genes, the AP16 gene cluster contains three open reading frames (ORFs) (PMI03_05098, PMI03_05097, and PMI03_05096) on the bottom strand downstream of the sylB homolog PMI03_05099 which are not present in P. syringae syl gene clusters (Fig. 1B). The relatively close spacing of these ORFs (78 bp between PMI03_05099 and PMI03_05097; 69 bp between PMI03_05097 and PMI03_05096) may suggest that they are transcribed as an operon together with PMI03_05099, which would point to a function of these additional genes in the biosynthesis of the putative product controlled by the gene cluster. The annotation of PMI03_05098 appears doubtful as it is predicted to encode a short peptide of 35 amino acids with no similarity to known proteins and overlaps 25 nucleotides of PMI03_05097, which encodes a type II (stand-alone) thioesterase. Type II thioesterases are known to release stalled NRPS modules that have been loaded with a type II (stand-alone) thioesterase. Type II thioesterases are known to release stalled NRPS modules that have been loaded with a nonextendable moiety (30, 31). The third ORF, PMI03_05096, encodes a hypothetical protein of unknown function belonging to the 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) superfamily of proteins (cl01268). Certain members of this superfamily catalyze the isomerization of unsaturated ketones (32).

Because of the highly conserved domain structure and amino acid specificity as revealed by the “10-amino-acid codes” (33) of the corresponding adenylation domains in the AP16 SyIC (PMI03_05100) and SyID (PMI03_05101) homologs, the AP16 gene cluster is predicted to control the biosynthesis of a syringolin A-like proteasome inhibitor. The closely spaced additional genes downstream of the sylB homolog PMI03_05099 and the unusual tandem thiolation domains in the SyIC homolog PMI03_05100 might be involved in decoration and/or biosynthesis efficiency of the putative product.

**Rhizobium sp. strain AP16 produces syringolin A and related variants.** We sought to detect transcripts corresponding to the syl-like gene cluster of the AP16 strain under various growth conditions. The strain grew reasonably well on plates with rhizobium medium both at 28°C and 18°C but hardly grew on LB plates. Best growth was obtained on SRMAF medium supplemented with 0.5 g/liter yeast extract (SRMAF). RNA was extracted from bacteria collected from rhizobium medium or SRMAF plates after incubation at 18°C or 28°C for 3 days in the dark and used as a template for RT-PCR using primers specific for the individual ORFs (PMI03_05096 through PMI03_05102, except PMI03_05098, which was not considered further). None of these genes was detectably expressed in bacteria grown on plates with either medium at 28°C. In contrast, growth on SRMAF plates at 18°C revealed transcripts corresponding to PMI03_05097 to PMI03_05102, whereas PMI03_05096 transcripts were not detected (Fig. 1C). On rhizobium medium plates, transcripts were hardly detectable. Therefore, SRMAF plates were used for all further experiments. Amplification by RT-PCR of the intergenic region between PMI03_05100 and PMI03_05101 was not successful (Fig. 1C), indicating that these genes, unlike their homologs in syringolin A-producing P. syringae strains, were not transcribed as an operon (14). Similarly, the intergenic region between PMI03_05097 and PMI03_05099 was also not detected in the extracted RNA.

To detect the putative product of the AP16 syl-like gene cluster, bacteria were grown as a lawn on SRMAFY plates at 18°C in the dark for 5 days, scraped off the plates, and subjected to our standard syringolin extraction and detection procedure (see Materials and Methods). Samples were subjected to HPLC analysis. For comparison of HPLC profiles, a PMI03_05100 (sylC homolog) in-frame deletion mutant was constructed (AP16ΔsylC) and analyzed in parallel. As shown in Fig. 2, several peaks were detected in wild-type extracts that were absent in the AP16ΔsylC mutant. Comparison with the HPLC profile of extracts from P. syringae B301D-R suggested that major peaks A and D in Rhizobium sp. strain AP16 extracts corresponded to syringolin A and syringolin D of B301D-R (3). Peak A from strain AP16 was collected and subjected to LC-MS analysis, which revealed a quasimolecular ion with an m/z of 494.2974, corresponding to the formula C₁₉₂H₁₆₂N₄O₆ (hydrogen adduct, m/z 494.2973; standard deviation [SD], 0.32 ppm), which is identical to syringolin A. The 1H and 13C NMR data of peak A were also essentially identical to those of syringolin A isolated from P. syringae B301D-R (Table S3 in the supplemental material). We conclude from these results that Rhizobium sp. strain AP16 synthesizes the proteasome inhibitor syringolin A under the culture conditions used. Interestingly, when AP16 was grown on plates in the light (16 h light/8 h dark), no syringolin A was detected (Fig. S1 in the supplemental material). Syringolin A was also not produced when AP16 was grown in liquid culture, irrespective of the light conditions.

ESI-MS of the second-largest AP16 peak, peak D, revealed a quasimolecular ion with an m/z of 508.2, which is identical to the hydrogen adduct of syringolin D, thus confirming the tentative identification given above. In syringolin D, ureido-valine is replaced by ureido-isoleucine (3). The comparison of the HPLC profiles showed that AP16 produced significantly less syringolin B, C, and E relative to syringolin A than was observed in P. syringae B301D-R (Table 2). In syringolin B, the 3,4-dehydroxyisoeucine moiety in the ring structure is replaced by lysine, which, according to the biosynthesis model, results from incomplete lysine desaturation by the sylB gene product, most likely before incorporation (13, 17). Thus, lysine desaturation seems considerably more efficient in strain AP16 than in P. syringae B301D-R. This explains also the reduced abundance of syringolin E in AP16, as this variant com-
Another unusual feature of the thiolation domains enhances the biosynthesis efficiency of syringolin. In particular, in the apparently more efficient desaturation of lysine to 3,4-dehydrolysine in strain AP16 compared to *P. syringae* B301D-R, in-frame mutants carrying in-frame deletions of PMI03_05097 (Δ05097) or both of PMI03_05096 and PMI03_05097 (Δ05096-05097) were constructed. Comparison of the HPLC profiles of these two mutants and that of the wild-type AP16 strain did not exhibit significant differences with regard to relative variant abundances (Fig. 3A and Table 2), thus suggesting that the presence of PMI03_05096 alone or in combination with PMI03_05097 did not cause the different variant abundances observed in *P. syringae* B301D-R and AP16. However, both mutants reproducibly produced reduced amounts of syringolin A: in mutants Δ05097 and Δ05096-05097, syringolin A levels reached 59% ± 0.4% (*P* = 0.002, Student’s *t* test, *n* = 3) and 49% ± 11% (*P* = 0.004, Student’s *t* test, *n* = 3) of the values observed in the wild-type AP16 strain, respectively, suggesting that the PMI03_05097 gene enhances the biosynthesis efficiency of syringolin.

The AP16 *syl*C homolog PMI03_05100 exhibits tandem thiolation domains. Another unusual feature of the *syl*-like gene cluster of strain AP16 is that the NRPS module encoded by PMI03_05100, the AP16 *syl*C homolog, contains two tandemly arranged thiolation domains (also known as peptide carrier proteins or PCPs). Tandem thiolation domains have been described as acyl carrier proteins (ACPs) in a number of PKS modules and polyunsaturated fatty acid (PUFA) synthetases, where they have been implicated in the enhancement of product formation (34–37).

To determine whether the tandem thiolation domains had an influence on syringolin production, in-frame deletion mutants of PMI03_05100 were generated that lacked either the first (ΔPCP1) or the second (ΔPCP2) thiolation domain. HPLC analyses of the mutants and the wild type revealed that the ΔPCP2 mutant produced 107% ± 12% of the wild-type AP16 level (*P* = 0.43, Student’s *t* test, *n* = 3), which does not represent a significant difference. Relative variant abundances in the ΔPCP2 mutant were also the same as in the wild type (Fig. 3B and Table 2). In contrast, syringolin biosynthesis was completely abolished in the ΔPCP1 mutant (Fig. 3B). Thus, a single PCP (PCP1) appears to have been sufficient for full syringolin A biosynthesis under the culture conditions used. The syringolin-negative phenotype of the ΔPCP1 mutant could be due to improper folding of this internal deletion protein, or it may indicate that PCP2 is unable to substitute for PCP1.

**Rhizobium sp. strain AP16 is closely related to *Rhizobium rhizogenes* K84.** *Rhizobium rhizogenes* K84 (formerly *Agrobacterium radiobacter* K84 [38]) has been commercially used as a biocontrol agent against crown gall disease for decades (39). As visualized in the phylogenetic tree shown in Fig. 4, *Rhizobium* sp. strain AP16 is closely related to *R. rhizogenes* K84. In fact, direct comparison of the complete draft genome of *R. rhizogenes* sp. strain AP16 (GenBank accession no. AVM000000000; 96 contigs) with the complete genome sequence of *R. rhizogenes* K84 (GenBank accession no. CP000628 and CP000629 for chromosomes 1 and 2, respectively) using the average nucleotide identity (ANI) calculator (http://enve-omics.ce.gatech.edu/ani/) yielded an identity value of 98.5%, strongly suggesting that the two strains belong to the same species (40). This conclusion was corroborated by a comparison using the genome-to-genome distance calculator GGDC 2.0 program GBDP2_BLASTPLUS (http://ggdc.dsmz.de/distcalc2.php [40, 41]), which assigned the two genomes to the same species with a probability of 98.97%.

### TABLE 2 Production of syringolin minor variants in relation to syringolin A

<table>
<thead>
<tr>
<th>Strain</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. syringae</em> pv. syringae B301D-R</td>
<td>10.0 ± 1.0</td>
<td>30.2 ± 2.0</td>
<td>37.9 ± 2.4</td>
<td>9.2 ± 1.3</td>
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<tr>
<td><em>Rhizobium</em> sp. strain AP16</td>
<td>2.6 ± 0.3</td>
<td>5.3 ± 0.4</td>
<td>27.6 ± 2.0</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td><em>Rhizobium</em> sp. strain AP16 (Δ05097)</td>
<td>3.0 ± 1.0</td>
<td>6.3 ± 2.2</td>
<td>24.8 ± 1.8</td>
<td>1.1 ± 0.9</td>
<td></td>
</tr>
<tr>
<td><em>Rhizobium</em> sp. strain AP16 (Δ05096-05097)</td>
<td>2.7 ± 1.0</td>
<td>6.6 ± 1.3</td>
<td>27.4 ± 0.6</td>
<td>0.6 ± 0.3</td>
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<tr>
<td><em>Rhizobium</em> sp. strain AP16 (Δ05097)</td>
<td>1.5 ± 0.6</td>
<td>5.7 ± 0.5</td>
<td>25.6 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td></td>
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</tbody>
</table>

Data represent average relative peak area ± standard deviation derived from HPLC profiles (*n* = 3).

![Fig 3](http://aem.asm.org/Downloaded from [aem.asm.org](http://aem.asm.org/) on October 16, 2017 by guest)
Interestingly, *R. rhizogenes* K84 carries a deletion remnant of the *syl* gene cluster on chromosome 2 consisting of the sequence encoding the last 102 amino acids of the PMI03_05101 (*sylD*) homolog and the complete PMI03_05102 (*syle*) homolog (annotated as *YP_002541055* in *R. rhizogenes* K84) (7). The deletion encompasses the rest of the *syl* gene cluster and the part of the PMI03_05095 homolog encoding the N-terminal 43 amino acids.

**DISCUSSION**

We have demonstrated that the AP16 *syl*-like gene cluster indeed governs the biosynthesis of the proteasome inhibitor syringolin A and some related variants. In comparison to *P. syringae* B301D-R, AP16 produces approximately 7-fold less syringolin B and only vanishing amounts of syringolin E in relation to syringolin A. SytB, which is homologous to dihydrorhizobitoxin desaturase (*sylD*), is thought to be responsible for the desaturation of lysine to 3,4-dehydrolysine (13). This reaction probably occurs before incorporation into the peptide structure by the first NRPS module of SytD, because its activation domain adenylates 3,4-dehydrolysine more quickly and more efficiently than lysine (17). The low abundance of syringolin B and E in AP16 shows that lysine is incorporated into the peptide at a much lower rate than in *P. syringae* B301D-R. This difference seems to be due to the intrinsic properties of the corresponding NRPS modules of the two species. The alternative hypothesis we initially entertained, namely, that the stand-alone thioesterase encoded by PMI03_05097 might lower the rate of incorporation of the nonpreferred lysine residue, had to be discarded because the deletion of PMI03_05097 had no influence on the variety ratios (Table 2).

Syringolin C and D are isomers differing in the substitution of the ring-proximal or the distal valine residue by isoleucine (Fig. 1A) (3). Whereas the abundance ratio of these two variants in *P. syringae* B301D-R is 0.8, it is 0.2 in AP16 (Table 2); i.e., syringolin C has 4-fold-lower abundance in the latter strain. *P. syringae* SytC was experimentally shown to be the NRPS starter module to activate valine and, to a lesser extent, isoleucine and to form an ureido-dipeptide, whereby the ureido carbonyl group is derived from hydrogen carbonate/carbon dioxide (15, 16). In contrast to *P. syringae*, in AP16, valine is much less likely to be substituted by isoleucine at the ring-proximal position than at the distal position. This appears to be an intrinsic property of the SytC homolog encoded by PMI03_05100 and is not due to the unusual tandem PCP domains, because the ΔPCP2 mutant and the wild type have very similar absolute variant abundances (Fig. 3B and Table 2). Thus, the second PCP in PMI03_05100 seems to be redundant for any aspect of syringolin biosynthesis under the experimental conditions used. Apart from the protein encoded by PMI03_05100, only one other NRPS module exhibiting tandem PCP domains was identified by a search through the NCBI RefSeq protein sequence database (GenBank accession no. WP_007460164 from *Streptomyces* sp. strain W007); that module, however, remains uncharacterized. As mentioned above, tandem thiolation domains (ACPs) have been described in some type I PKS modules as well as in polyunsaturated fatty acid (PUFA) synthetases. In some cases, they have been demonstrated to improve product yield by working either serially or in parallel (34–37).

AP16 has been isolated from the endosphere of poplar roots. Many bacteria have been found to maintain endophytic relationships with their host plants (43–45). Plants have been shown to respond with local defense reactions to colonization by endophytes (43, 46–48). Such colonization also results in elevated levels of SA. Compatible rhizobia produce specific nodulation (Nod) factors, which in turn suppress SA production, thus downregulating defense responses (49). Based on our analysis, the genome of AP16 lacks any known *nod* genes. In addition, despite the presence of a seemingly complete type III secretion system (NCBI accession AJVM1000009), we did not detect any known rhizobial or *Pseudomonas* type III effector proteins, which are normally involved in suppression of host immune responses (50). Thus, it is tempting to speculate that in AP16, syringolin A could potentially substitute for type III effectors or Nod factors with regard to host defense suppression during the colonization of poplar roots to at least some extent. This statement is, however, purely speculative, and further experiments are needed to clarify this point. This would involve establishing an infection model in Eastern cottonwood seedlings, the plant species from which the bacterium was originally isolated.

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