

# Characterization of the *argA* Gene Required for Arginine Biosynthesis and Syringomycin Production by *Pseudomonas syringae* pv. *syringae*

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Two types of necrosis-inducing lipodepsipeptide toxins, called syringomycin and syringopeptin, are major virulence factors of *Pseudomonas syringae* pv. *syringae* strain B301D. A previous study showed that a locus, called *syrA*, was required for both syringomycin production and plant pathogenicity, and the *syrA* locus was speculated to encode a regulator of toxin production. In this study, sequence analysis of the 8-kb genomic DNA fragment that complements the *syrA* phenotype revealed high conservation among a broad spectrum of fluorescent pseudomonads. The putative protein encoded by open reading frame 4 (ORF4) (1,299 bp) in the *syrA* locus region exhibited 85% identity to ArgA, which is involved in arginine biosynthesis in *Pseudomonas aeruginosa*. Growth of strain W4S2545, the *syrA* mutant, required supplementation of N minimal medium with arginine. Similarly, syringomycin production of *syrA* mutant W4S2545 was restored by the addition of arginine to culture media. Furthermore, the insertion of Tn5 in the genome of the *syrA* mutant W4S2545 was localized between nucleotides 146 and 147 in ORF4, and syringomycin production was complemented in *trans* with the wild-type DNA fragment containing intact ORF4. These results demonstrate that the *syrA* locus is the *argA* gene of *P. syringae* pv. *syringae* and that *argA* is directly involved in arginine biosynthesis and therefore indirectly affects syringomycin production because of arginine deficiency.

A notable feature of *Pseudomonas syringae* pv. *syringae*, an economically important pathogen causing necrotic infections of a broad range of plant species, is the production of two lipopeptide phytotoxins, syringomycin and syringopeptin (6). The toxins cause necrosis in host plants and are major virulence factors (17, 32). Syringomycin is a cyclic lipodepsinonapeptide composed of a 3-hydroxy fatty acid tail attached to a polar peptide head that contains nine amino acid residues, including arginine (6). Three forms of syringomycin that differ by the length of the 3-hydroxy fatty acid tail are produced (5). Syringomycin E (SRE), which contains a 3-hydroxy dodecanoic acid tail, is the major form produced by strain B301D of *P. syringae* pv. *syringae* (5). Syringomycin is synthesized via a nonribosomal peptide synthetase system (18, 43). The syringomycin (*syr*) gene cluster is located in a 55-kb DNA region in the strain B301D genome, and the *syr* gene cluster is composed of genes required for biosynthesis, regulation, and secretion of syringomycin (18, 23, 28, 43, 44).

Xu and Gross (40) described a gene called *syrA* that is an essential locus for syringomycin production and the pathogenicity of *P. syringae* pv. *syringae* B301D. The *syrA* mutant strain W4S2545 was generated by random Tn5 mutagenesis of the B301D genome and was selected due to the loss of syringomycin production and pathogenicity in pear seedlings and cherry fruits. Although the *syrA* mutant was unchanged with regard to its ability to elicit a hypersensitive reaction on tobacco leaves, the *syrA* mutant was significantly attenuated in its ability to grow in planta (41). Syringomycin production of strain

W4S2545 was restored both in *trans* and in *cis* by the introduction of cosmid pGX183 (41). The *syrA* gene was localized on an 8-kb *EcoRI-KpnI* DNA fragment in the B301D genome and was estimated to be 2.3 to 2.8 kb in size based on restriction enzyme mapping and Tn5 mutagenesis. Consequently, the *syrA* locus was predicted to be a regulatory gene that might control several pathogenicity genes (41). Recent sequence analysis found that the *syrA* locus was not located in the *syr* gene cluster (23, 33). The function of the *syrA* gene in syringomycin production was unclear prior to this work.

To define the function of the *syrA* locus in relation to syringomycin production and the pathogenicity of *P. syringae* pv. *syringae* strain B301D, the *syrA* gene region was cloned from pGX183 (41) and from the chromosome of *syrA* mutant W4S2545. Sequence analysis revealed significant nucleotide identity of the *syrA* gene locus to the *argA* gene of *Pseudomonas aeruginosa*, which is involved in arginine biosynthesis (9). The Tn5 transposon insertion in the strain W4S2545 genome was mapped and confirmed by sequencing. The results of this report demonstrate that strain W4S2545 is an arginine-auxotrophic mutant of strain B301D and that the *syrA* locus, renamed *argA*, encodes an *N*-acetylglutamate synthetase required for arginine biosynthesis. The conservation of the *argA* gene region among fluorescent pseudomonads is also discussed.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and growth conditions.** The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* strain DH10B (14) was used as a host for cloning and was cultured in Luria-Bertani medium (31) at 37°C. Strains of *P. syringae* pv. *syringae* were cultured at 25°C in nutrient broth-yeast extract (NBY) medium (38). Potato dextrose agar (PDA) supplemented with 0.4% Casamino Acids (16) was used in plate bioassays of *P. syringae* pv. *syringae* strains for syringomycin production. N minimal (NM)

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics <sup>a</sup>	Source or reference
<b>Strains</b>		
<i>E. coli</i> DH10B	F <sup>-</sup> <i>mcrA</i> $\Delta$ lacX74 ( $\phi$ 80dlacZ $\Delta$ M15) $\Delta$ ( <i>mrr-hsdRMS-mcrB</i> ) <i>deoR recA1 endA1 araD139 <math>\Delta</math>(<i>ara-leu</i>)7697 <i>galU galK</i> <math>\lambda^-</math> <i>rpsL nupG</i></i>	14
<i>P. syringae</i> pv. <i>syringae</i>		
B301D	Wild-type strain from pear	8
W4S2545	B301D-R::Tn5; Rif <sup>r</sup> Km <sup>r</sup>	40
B301DSL22	ORF5::nptII derivative of B301D; Km <sup>r</sup>	This study
B301DSL23	ORF6::nptII derivative of B301D; Km <sup>r</sup>	This study
BR132	<i>syrB1</i> ::Tn3HoHo1 derivative of B301DR; Pip <sup>r</sup>	43
<b>Plasmids</b>		
pBlueScript II SK	Cloning vector; Ap <sup>r</sup>	Stratagene
pUC18	Cloning vector; Ap <sup>r</sup>	42
pUCP26	Broad-host range vector; Tc <sup>r</sup>	39
pBR325	Cloning vector; Cm <sup>r</sup> Tc <sup>r</sup> Ap <sup>r</sup>	27
pGX15	pLAFR3 containing the 12-kb B301D genomic DNA fragment; Tc <sup>r</sup>	41
pJS100	pBlueScript II SK containing the 8-kb <i>EcoRI-KpnI</i> DNA fragment from pGX15; Ap <sup>r</sup>	This study
pSL101	pBlueScript II SK containing the 14-kb <i>EcoRI-KpnI</i> DNA fragment of the W4S2545 genome; Km <sup>r</sup> Ap <sup>r</sup>	This study
pSL102	pBlueScript II SK containing the 5-kb <i>BamHI-KpnI</i> DNA fragment from pGX15; Ap <sup>r</sup>	This study
pSL108	pUC18 containing the 3.6-kb <i>SalI-kpnI</i> fragment from pJS100; Ap <sup>r</sup>	This study
pSL113	pSL108 with the <i>nptII</i> insertion at the <i>MscI</i> site of ORF5; Km <sup>r</sup> Ap <sup>r</sup>	This study
pSL114	pSL108 with the <i>nptII</i> insertion at the <i>EcoRV</i> site of ORF6; Km <sup>r</sup> Ap <sup>r</sup>	This study
pSL115	pBR325 containing the disrupted <i>argE</i> gene with the <i>nptII</i> insertion from pSL113; Km <sup>r</sup> Tc <sup>r</sup>	This study
pSL116	pBR325 containing the disrupted ORF6 with the <i>nptII</i> insertion from pSL114; Km <sup>r</sup> Tc <sup>r</sup>	This study
pSL117	pUCP26 containing the 8-kb <i>EcoRI-KpnI</i> DNA fragment from pGX15; Tc <sup>r</sup>	This study

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

agar medium (38) was used for the investigation of arginine auxotrophy. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were added to media as required in the following concentrations: 100  $\mu$ g/ml for ampicillin, 100  $\mu$ g/ml for kanamycin, and 25  $\mu$ g/ml for tetracycline.

**DNA manipulations.** Routine procedures were used for preparing plasmid DNA and for subcloning DNA fragments into designated vectors (31). Genomic DNA was prepared with the cetyltrimethylammonium bromide protocol (10). A QIAEX II gel extraction kit (QIAGEN Inc., Valencia, Calif.) was used to recover DNA fragments for cloning and for use as hybridization probes. Electroporation of plasmids into cells of *E. coli* and *P. syringae* was completed with a Gene Pulser II (Bio-Rad Laboratories, Hercules, Calif.) as described by Scholz-Schroeder et al. (32).

**Sequence analysis.** To sequence the *syrA* gene region, plasmid pJS100 (Table 1) was generated from pGX15 (41) and mutagenized by using an EZ::TN <Kan-2> insertion kit according to the manufacturer's instructions (Epicentre Technologies Corp., Madison, Wis.). Based on mapping with restriction endonucleases, the plasmids with *nptII* insertions at different positions were selected for sequencing. Forward and reverse primers supplied with the EZ::TN <Kan-2> insertion kit were used for initial sequence reactions off each plasmid carrying an EZ::TN transposon. Sequencing was completed with primer walking when noncontiguous sequences existed. Primers were designed for primer walking with the PRIME program within the Wisconsin Sequence Analysis programs of Genetics Computer Group (GCG) package version 10.0 (12) and were synthesized by Operon Technologies, Inc. (Alameda, Calif.). Sequence reactions were completed by using fluorescence-based dideoxy terminators and Ampli-Taq polymerase and were run on an ABI PRISM 377 DNA sequencer (PerkinElmer Applied Biosystems, Inc., Norwalk, Conn.). At least triple coverage of sequencing reactions was achieved to generate consensus DNA sequence.

Sequence analysis was accomplished using the GCG package and Lasergene expert sequence analysis software version 5.0 (DNASTAR, Inc., Madison, Wis.). The following GCG programs were used: GELASSEMBLE for the assembly of nucleotide sequences, FINDPATTERNS for the identification of Shine-Dalgarno sequences, TERMINATOR for the prediction of rho-independent transcriptional terminators, and BESTFIT for the comparison of two sequences. Database searches for genes and proteins homologous to the predicted open reading frames (ORFs) were conducted by using the BLAST server at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>).

To evaluate the significance of the protein sequence similarities, the GAP program of GCG was run with the randomization option of 100. The Z scores were calculated by using the actual quality score minus the mean quality value of the randomized scores divided by the standard deviation of randomized score distribution. Protein homology was significant if the Z score value was larger than 6. The MEGALIGN program within the Lasergene software package was used for multiple sequence analysis.

**Complementation assays.** The growth of *P. syringae* pv. *syringae* *syrA* mutant strain W4S2545 (40) was evaluated on NM medium supplemented with a 1- $\mu$ g/ml concentration of L-glutamate, N-acetyl-L-glutamate, N-acetyl-L-ornithine, or L-arginine (Sigma Chemical Co.). Bacterial cells were grown with shaking in 4 ml of NBY medium for 6 h at 25°C, harvested by centrifugation, and diluted to a cell density of  $\sim 2 \times 10^8$  CFU/ml. NM agar medium was supplemented with 1  $\mu$ g of L-arginine/ml or selected intermediates in the arginine biosynthesis pathway. NM plates were inoculated with a 5- $\mu$ l droplet containing  $10^6$ ,  $10^4$ , or  $10^2$  cells per spot. Wild-type strain B301D of *P. syringae* pv. *syringae* was used as the reference strain for growth. The ability to produce visible colonies was recorded after the inoculated plates were incubated at 25°C for 96 h. Each experiment was repeated independently three times with three plates per replicate.

**Mutagenesis of the ORFs upstream of the *syrA* gene.** ORF5 and ORF6 were disrupted by the insertion of the *nptII* gene to generate nonpolar mutations. A 1.4-kb fragment containing the *nptII* gene from pBSL15 (1) was cloned into pSL108 at the *MscI* site to disrupt translation of ORF5, resulting in plasmid pSL113. Likewise, the *nptII* fragment was inserted in pSL108 at the *EcoRV* site to disrupt the translation of ORF6 and to generate plasmid pSL114. The *nptII* insertions in the two ORFs were confirmed by restriction endonuclease mapping and sequencing. The disrupted gene fragments from plasmids pSL113 and pSL114 were cloned into pBR325 at the *EcoRI* site that was previously blunted by T4 DNA polymerase to construct plasmids pSL115 and pSL116, respectively. Plasmids pSL115 and pSL116 were electroporated into *P. syringae* pv. *syringae* strain B301D for marker exchange mutagenesis as previously described by Scholz-Schroeder et al. (32). Homologous recombination of the disrupted gene fragments in the genome of *P. syringae* pv. *syringae* B301D was verified by Southern analysis (31).

**Assays for syringomycin production.** Syringomycin production by *P. syringae* pv. *syringae* strains was evaluated by using bioassays as previously described by Gross and DeVay (16). In brief, bacterial cells were grown overnight in NBY

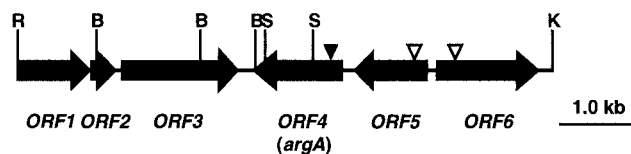


FIG. 1. Physical map of the 8,080-bp *syrA* gene region of *P. syringae* pv. *syringae* B301D. The positions and orientations of the potential ORFs are shown as horizontal arrows. The solid-black triangle indicates the position of the Tn5 insertion in strain W4S2545, and the open triangles mark the positions of the *nptII* insertions. Restriction enzyme sites are abbreviated as follows: R, *EcoRI*; B, *BglII*; S, *Sall*; and K, *KpnI*.

medium at 25°C. The cells were harvested by centrifugation, washed with sterile deionized water, and adjusted to a final cell density of  $\sim 2 \times 10^8$  CFU/ml. To test the effects of additions of L-arginine and selected intermediates of the arginine biosynthesis pathway on the restoration of syringomycin production of the *syrA* mutant, each PDA plate was supplemented with a 1- $\mu$ g/ml concentration of either L-glutamate, N-acetyl-glutamate, N-acetyl-L-ornithine, or L-arginine. The PDA plates were inoculated with 5  $\mu$ l of the bacterial suspension and incubated at 25°C for 4 days. The inoculated plates were oversprayed with *Geotrichum candidum* F-260 as an indicator organism and were further incubated for 24 h at 25°C. Zones inhibitory to the growth of *G. candidum* indicated syringomycin production. *P. syringae* pv. *syringae* wild-type strain B301D and *syrB1* mutant strain BR132 (43) were used as positive and negative controls, respectively, for syringomycin production. Each assay was repeated independently three times with three plates per replicate.

**Nucleotide sequence accession number.** The nucleotide sequence of the 8,080-bp DNA region (Fig. 1) is available in the GenBank database under accession number AY374326.

## RESULTS

**Sequence analysis of the *syrA* gene region.** To characterize the genomic DNA region that restored the phenotype of the *syrA* mutation, an 8,080-bp DNA *EcoRI*-*KpnI* fragment of pGX15 was sequenced from pJS100 (Table 1). Sequence analysis revealed six potential ORFs in the region (Fig. 1). A striking feature of the 8,080-bp region was the presence of ORF4 and ORF5, which exhibited significant homology, respectively, to the *argA* and *argE* genes involved in arginine biosynthesis in prokaryotes (9). The other four ORFs in this region were also conserved among pseudomonads (25, 36).

ORF4 shared the highest identity to the *argA* gene, encoding N-acetylglutamate synthetase in bacteria (9). This ORF, 1,299 bp in length, was preceded by a potential Shine-Dalgarno sequence (ACAGG) 11 bp upstream of the start codon (ATG) of the ORF, and a rho-independent transcriptional terminator was identified 58 bp downstream of the stop codon (TAA). Nucleotide sequence analysis revealed that ORF4 exhibited 84 and 80% identity to the *argA* genes of *Pseudomonas putida* KT2440 (25) and *P. aeruginosa* PAO1 (36), respectively. ORF4 was predicted to code for a protein 432 amino acids in size. Database searches demonstrated that the putative protein from ORF4 shared the highest identity (88%; Z score, 364) to the ArgA protein of *P. putida* KT2440 (25). In addition, this protein encoded by ORF4 showed identities of 85, 52, and 49% to the putative ArgA proteins of *P. aeruginosa* PAO1 (36), *Yersinia pestis* KIM (11), and *E. coli* K-12 (29), respectively. The N-acetylglutamate synthetase encoded by *argA* is the first enzyme of the arginine biosynthesis pathway in bacteria and

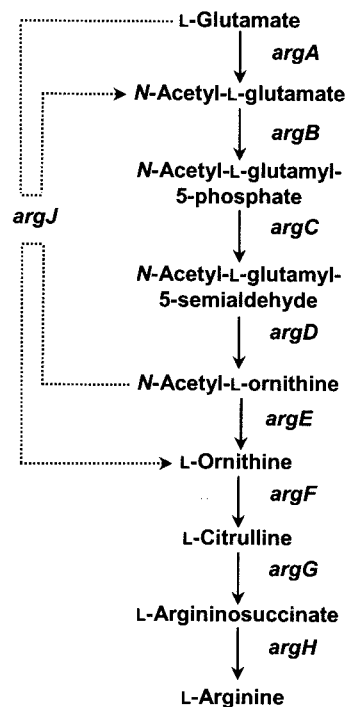


FIG. 2. Pathway of arginine biosynthesis in bacteria modified from the work of Cunin et al. (9). Gene designations in the arginine biosynthesis pathway are *argA* (N-acetylglutamate synthetase), *argB* (N-acetylglutamate 5-phosphotransferase), *argC* (N-acetylglutamate 5-semialdehyde dehydrogenase), *argD* (N-acetylornithine aminotransferase), *argE* (acetylornithine deacetylase), *argF* (anabolic ornithine carbamoyltransferase), *argG* (argininosuccinate synthetase), *argH* (argininosuccinase), and *argJ* (ornithine acetyltransferase). The dashed line indicates that the acetyl group of N-acetyl-L-ornithine is recycled to form N-acetylglutamate by ArgJ in the cyclic pathway.

catalyzes the acetylation of L-glutamate with acetyl coenzyme A (Fig. 2).

ORF5 exhibited significant homology to the *argE* gene, predicted to code for acetylornithine deacetylase in bacteria (7, 9). This ORF was 1,149 bp in length and had a conserved Shine-Dalgarno sequence (AGGAAG) 5 bp upstream of the start codon (ATG) of the ORF. A potential rho-independent transcriptional terminator was predicted 79 bp downstream of the stop codon (TGA). Likewise, ORF5 exhibited 78 and 77% identity to genes designated *argE* in the genomes of *P. aeruginosa* PAO1 (36) and *P. putida* KT2440 (25), respectively. ORF5 was predicted to encode a protein 382 amino acids in size which shares 81 and 78% identity to the putative ArgE proteins of *P. putida* KT2440 (25) and *P. aeruginosa* PAO1 (36), respectively. In addition, the putative protein encoded by ORF5 exhibited 49% identity to the well-characterized acetylornithine deacetylase (ArgE) of *E. coli* (7, 21). The acetylornithine deacetylase encoded by *argE* is known to catalyze deacetylation of N-acetylornithine into ornithine in the arginine biosynthesis pathway of *E. coli* (Fig. 2) (9).

Four additional ORFs (ORF1, ORF2, ORF3, and ORF6) in the *syrA* gene region were predicted to code for conserved proteins in pseudomonads (Fig. 1; Table 2). These four potential ORFs had the same transcriptional orientation, which was opposite to that of ORF4 and ORF5. ORF1 was 1,032 bp in

TABLE 2. Genes and ORFs identified in the *argA* gene region of strain B301D

ORF	Homology <sup>a</sup>			Predicted function
	Homolog	Identity (%)	Z score	
ORF1	Tex (part)	91	262	S1 RNA binding domain protein
ORF2	PP0244	76	86	Unknown
ORF3	GshA	74	392	Glutamate-cysteine ligase
ORF4	ArgA	88	364	<i>N</i> -Acetylglutamate synthetase
ORF5	ArgE	81	328	Acetylornithine deacetylase
ORF6	PP5187	75	255	Unknown

<sup>a</sup> Homology to genes or ORFs of *P. putida* KT2440 (26).

length, and the putative protein product exhibited 90% identity to the C terminus of the S1 RNA binding domain protein (PP0245) of *P. putida* KT2440 (25) and 85% identity to the C terminus of a hypothetical protein (PA5201) of *P. aeruginosa* (36). ORF2 was 384 bp in size, and the predicted protein from this ORF showed 76 and 72% identity to proteins PP0244 of *P. putida* and PA5202 of *P. aeruginosa*, respectively, whose functions are unknown. ORF3, 1,608 bp in size, was predicted to code for a protein that shared the highest identity (78%) to a putative glutamate-cysteine ligase (PP0243) of *P. putida* KT2440 and 76% identity to the glutamate-cysteine ligase (PA5203) of *P. aeruginosa*. ORF6, which was 1,455 bp in length, was localized upstream of ORF5, and the putative protein from ORF6 shared 75 and 67% identity to proteins of *P. putida* KT2440 (PP5187) and *P. aeruginosa* PAO1 (PA5209), respectively, whose functions are unknown.

**ORF4 was disrupted by the insertion of a Tn5 transposon in the *syra* mutant genome.** An *EcoRI*-*KpnI* DNA fragment 14 kb in length and containing a Tn5 transposon was cloned from the genome of the *syra* mutant W4S2545 to localize the transposon in the genome. Sequence analysis of the 14-kb DNA fragment carrying a Tn5 transposon (5.8 kb) demonstrated that the sequence from this fragment was identical to the 8.1-kb *EcoRI*-*KpnI* DNA fragment of pGX183 (41). Mapping and further sequence analysis revealed that the Tn5 transposon was inserted within ORF4 between nucleotides 146 and 147 relative to the start codon of ORF4 (Fig. 1).

Mutation of ORF4 resulted in a lack of syringomycin production of *P. syringae* pv. *syringae* B301D. The *syra* mutant W4S2545 failed to produce syringomycin (40, 41) on PDA medium. As expected, cells of strain W4S2545 containing plasmid pSL117, which is pUCP26 (39) carrying the entire ORF4 DNA fragment, produced a zone of inhibition to *G. candidum* that was comparable to that of the wild-type strain B301D (data not shown). These results show that a functional ORF4 is critical for syringomycin production by *P. syringae* pv. *syringae*.

To evaluate the effect of mutations in ORF5 and ORF6 on syringomycin production, insertions of the *nptII* gene fragment at the unique *EcoRV* and *SmaI* sites of the two respective ORFs constructed mutations in both ORF5 and ORF6. The mutations in the two ORFs are nonpolar due to the lack of a transcriptional terminator in the *nptII* gene (1). As a result, the

risk of polar effects of mutation on downstream ORFs was eliminated. The *nptII*-disrupted ORFs were individually recombined into the genome of *P. syringae* pv. *syringae* B301D by marker exchange mutagenesis, generating ORF5 mutant B301DSL22 and ORF6 mutant B301DSL23 (Table 1). Southern analysis verified the recombination of the *nptII* disruption of each ORF in the genomes of resultant mutants. Plate bioassays of syringomycin production on PDA showed that both strains B301DSL22 (ORF5::*nptII*) and B301DSL23 (ORF6::*nptII*) produced zones of inhibition approximately 10 mm in radius, which was not significantly different from that produced by the wild-type strain B301D (data not shown). These data show that neither ORF5 nor ORF6 is required for syringomycin production by *P. syringae* pv. *syringae*.

**Growth restoration of strain W4S2545 with supplementation of L-arginine and its biosynthetic intermediates.** Growth of *syra* mutant W4S2545 was tested on NM agar medium after 96 h of inoculation. No visible colony growth was observed on plates inoculated with a droplet containing 10<sup>6</sup>, 10<sup>4</sup>, or 10<sup>2</sup> cells of strain W4S2545, unlike the normal growth observed for wild-type strain B301D. The results show that strain W4S2545 is an auxotrophic mutant of *P. syringae* pv. *syringae*. In contrast, both strains B301DSL22 (ORF5::*nptII*) and B301DSL23 (ORF6::*nptII*) grew on NM plates as well as did wild-type strain B301D (data not shown), which demonstrated that disruption of either ORF5 or ORF6 did not result in an auxotrophic phenotype.

Supplementation of the NM agar medium with L-arginine restored the growth of strain W4S2545. Based on sequence analysis of the *syra* gene region and the location of the Tn5 insert in the W4S2545 genome, this strain was predicted to be an arginine-auxotrophic mutant. Growth measurements were performed on NM agar plates supplemented with L-arginine or one of the intermediates in the arginine biosynthesis pathway (Fig. 2). The results demonstrated that addition of L-arginine restored the growth of the *syra* mutant. Similarly, colonies of the *syra* mutant were observed after incubation for 96 h on the NM agar medium supplemented with *N*-acetyl-L-ornithine. As expected, supplementation of L-glutamate could not restore the growth of strain W4S2545. However, the growth of strain W4S2545 could not be stimulated by supplementation of *N*-acetyl-glutamate, which was also reported for *argA* mutants of *P. aeruginosa* (19) and *Salmonella enterica* serovar Typhimurium (13).

**Syringomycin production of strain W4S2545 was restored by arginine supplementation.** The effects of supplementation of L-glutamate, *N*-acetyl-glutamate, *N*-acetyl-L-ornithine, and L-arginine on syringomycin production were measured by using the standard procedure of plate assays (Fig. 3). The cells of strain W4S2545 growing on the PDA plates supplemented with either L-arginine or *N*-acetyl-L-ornithine produced zones (8 to 10 mm in radius) inhibitory to *G. candidum* that were comparable to those of the wild type (12 mm in radius) (Fig. 3). In contrast, no inhibition zone was observed when the cells were cultured on PDA plates supplemented with either L-glutamate or *N*-acetyl-L-glutamate (Fig. 3). These results showed that the addition of L-arginine or its intermediates, except *N*-acetyl-L-glutamate, restored syringomycin production by strain W4S2545 on PDA medium. In addition, it was found that the radius of the inhibition zone produced by strain B301D on PDA plates

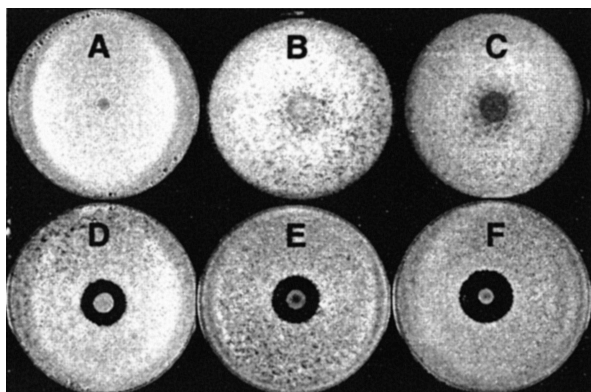


FIG. 3. Plate bioassays for syringomycin production by *P. syringae* pv. *syringae* strain W4S2545. The PDA plates were supplemented to a final concentration of 1 µg/ml with L-glutamate (B), N-acetyl-L-glutamate (C), N-acetyl-L-ornithine (D), or L-arginine (E). As controls, plates A and F were supplemented with sterile water. Plates A to E were inoculated with strain W4S2545, and plate F was inoculated with strain B301D as a control. The inoculated plates were incubated for 4 days at 25°C and oversprayed with *G. candidum*. Syringomycin production is observed by zones of inhibition of *G. candidum*. It was observed that the indicator fungus grew on colonies of strain W4S2545 on the PDA plates supplemented with water, L-glutamate, and N-acetyl-L-glutamate but not with the other compounds tested due to syringomycin production.

supplemented with 1 µg of L-arginine/ml was increased approximately 20% compared to that without the addition of L-arginine (data not shown).

**Conservation of the *argA* gene region in pseudomonads.** Comparative analyses revealed that the *argA* gene regions of related strains of *P. syringae* are conserved. The sequence of the *argA* gene region of a bean strain of *P. syringae* pv. *syringae*, B728a, was nearly identical to that of strain B301D, with an overall nucleotide substitution rate of ~0.7%, and all of the

putative proteins from this region of strain B301D were identical to that of strain B728a (<http://www.jgi.doe.gov>), which reveals conservation of the *argA* regions in closely related strains that differ in host specificity (16). More variation in the *argA* gene region was observed between *P. syringae* pv. *syringae* B301D and *P. syringae* pv. *tomato* DC3000. The *argA* gene region was identified in the genome sequence of strain DC3000 approximately 350 kb downstream of *dnaA*, the chromosomal replication initiator gene (<http://www.tigr.org>; GenBank accession number, AE016853). The gene organization of the *argA* region in strain DC3000 was the same as that in strain B301D; however, putative proteins of strain DC3000 exhibited ~93% identity to those of strain B301D on average. In addition, an overall nucleotide substitution rate of ~13.3% was observed for strains DC3000 and B301D in the corresponding *argA* (*syrA*) gene regions. Deletions also were identified in the *argA* (*syrA*) gene region of strain DC3000, compared to that of strain B301D. Most of the deletions occurred within noncoding regions, and the biggest deletion, 96 bp in length, was found between *gshA* and *argA* in DC3000. All deletions within an ORF did not result in a translational frameshift.

Further analyses revealed the presence of gene reorganizations among the related species of pseudomonads in the *syrA* (*argA*) gene region (Fig. 4). The *argA* region was identified in the genome sequence of *P. aeruginosa* strain PAO1 approximately 400 kb upstream of the *dnaA* gene (<http://www.pseudomonas.com>; GenBank accession number, AE004091). Compared with the *syrA* gene region of strain B301D, strain PAO1 carried an extra region, ~1.2 kb in size (between ORF4 and ORF5 of strain B301D) (Fig. 4), encoding PA5202. The other extra region, ~2.0 kb in size (between ORF5 and ORF6 of strain B301D), encoded hypothetical proteins PA5207 and PA5208. Interestingly, gene rearrangement in the *argA* gene region was observed when *P. syringae* pv. *syringae* B301D and *P. putida* KT2440 were compared (<http://www.tigr.org>) (Fig. 4). The

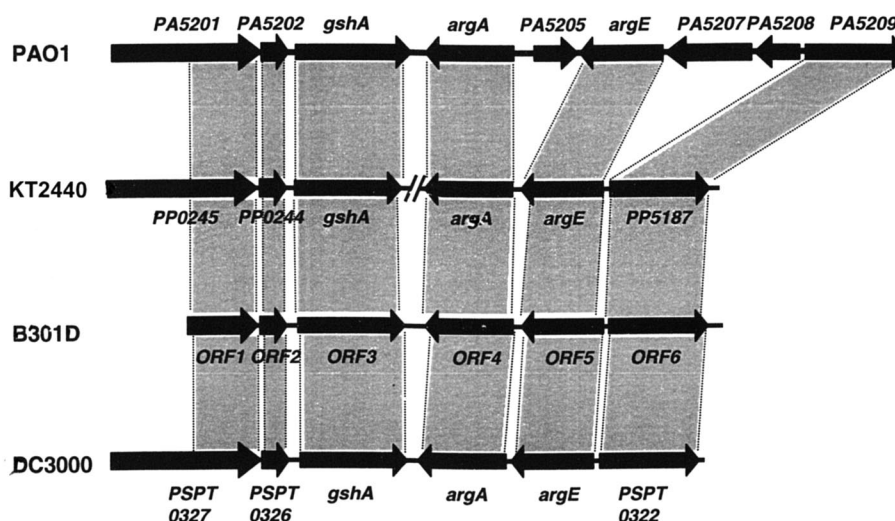


FIG. 4. Comparison of the gene organization in the *argA* (*syrA*) gene region of strain B301D with the corresponding *argA* region of related pseudomonads. The following strains were included in the comparative analysis: *P. syringae* pv. *syringae* B301D, *P. syringae* pv. *tomato* DC3000, *P. putida* KT2440, and *P. aeruginosa* PAO1. Gray boxes connect homologous ORFs. For strain KT2440, the “//” symbolizes an ~560-kb DNA fragment that separates the locus into two parts in the genome of strain KT2440. Gene regions and designations were obtained from the following websites: <http://www.tigr.org> for strain DC3000, <http://www.pseudomonas.com> for strain PAO1, and <http://www.tigr.org> for strain KT2440.

*argA* gene region of strain KT2440 was divided into two parts. The location of three ORFs, which corresponds to the strain B301D chromosome region containing ORF1, ORF2, and ORF3, was identified in the genome sequence of strain KT2440 approximately 300 kb downstream of the *dnaA* gene (<http://www.tigr.org>; GenBank accession number, AE015451). The other region, carrying *argA*, *argE*, and a homolog of ORF6 of B301D, was observed approximately 270 kb upstream of the *dnaA* gene. As a result, the two parts of the *argA* gene regions observed in strain B301D flank both sides of the origin of replication of the strain KT2440 chromosome.

## DISCUSSION

Phenotypic and genetic analyses demonstrated that the *syrA* locus is equivalent to the *argA* gene, which encodes *N*-acetylglutamate synthetase in bacteria. The putative protein from the *syrA* gene shared a high similarity to the known ArgA proteins of *P. putida* KT2440 (25), *P. aeruginosa* PAO1 (36), *Y. pestis* Kim (11), and *E. coli* K-12 (29). The ArgA protein, an *N*-acetylglutamate synthetase, catalyzes the first step of the arginine biosynthesis pathway and converts L-glutamate to *N*-acetyl-L-glutamate (9, 19). Mutation in the *syrA* locus resulted in the failure of *P. syringae* pv. *syringae* to grow on minimal medium and to produce syringomycin. The phenotype of W4S2545 was recovered after supplementation of the media with arginine and its intermediates and by complementation in *trans* with the wild-type DNA fragment containing the entire *syrA* gene. *N*-Acetyl-L-glutamate, one of the intermediates of arginine biosynthesis, was not able to restore the phenotype of the *syrA* mutant (W4S2545) in this study. The same results were observed for an *argA* mutant of *P. aeruginosa*, and it was predicted that *N*-acetyl-L-glutamate is deacetylated outside the cytoplasm to facilitate uptake into the cell (19). Finally, the *syrA* gene was the best-fit hit in the genomic DNA sequence draft of strain B728a (<http://www.jgi.doe.gov>) when the known *argA* sequences were used to search a genomic sequence database. These results demonstrated that strain W4S2545 is an arginine auxotrophic mutant and that the *syrA* phenotype resulted from a mutation of the *argA* gene. Consequently, the *syrA* gene is renamed the *argA* gene.

A functional *argA* gene is critical for syringomycin production of *P. syringae* pv. *syringae*. Xu and Gross (40, 41) demonstrated that a mutation in the *syrA* (*argA*) gene eliminated syringomycin production by *P. syringae* pv. *syringae*. Subsequently, Grgurina et al. (14) confirmed that reverse-phase high-performance liquid chromatography analysis of the *syrA* mutant W3S2545 failed to produce syringomycin (15). This phenotype was recovered in *trans* and in *cis* from strain W4S2545 (*argA*::Tn5) by the introduction of the wild-type *syrA* gene, which was further confirmed in *trans* in this study. Although strain W4S2545 was able to grow as well as its wild-type strain on PDA medium, no measurable syringomycin was produced. The apparent explanation is that PDA medium contained a limited amount of arginine that supports the growth of the *argA* mutant but not syringomycin production. Because arginine is one of nine amino acids in the syringomycin molecule produced by strain B301D (5), mutation of the *argA* gene resulted in a corresponding failure to produce syringomycin. This explanation was supported by the observation that syrin-

gomyacin production by wild-type strain B301D was increased approximately 20% with the addition of arginine to PDA medium.

Arginine biosynthesis as a primary metabolic system is notable for its complexity and variability at the genetic level among prokaryotes (9) (Fig. 2). Two patterns for arginine biosynthesis were proposed for prokaryotes: a linear pathway and a cyclic pathway based on whether *argJ* is involved in the biosynthesis pathways (4, 9). The *argJ* gene is absent and the *argE* gene is required for arginine biosynthesis in the linear pathway, which was found in *Enterobacteriaceae* (9) and *Sulfolobus solfataricus* (37). In contrast, all other prokaryotes, including *P. aeruginosa*, were shown or predicted to employ the cyclic pathway, in which a functional *argJ* gene is able to complement the *argE* mutant (4, 9). In addition, the ArgJ proteins are classified into two categories: bifunctional and monofunctional (4, 9). A bifunctional ArgJ protein is capable of complementing both *argA* and *argE* auxotrophs as observed for *Bacillus stearothermophilus* (30), *Bacillus subtilis* (26), and *Neisseria gonorrhoeae* (24). In contrast, the monofunctional *argJ* gene product is unable to restore arginine production to an *argA* mutant as observed for *Streptomyces coelicolor* (20). An intact *argJ* gene was found in the draft genomic sequence of *P. syringae* pv. *syringae* strain B728a (<http://www.jgi.doe.gov>). Therefore, as in *P. aeruginosa* (19), it is predicted that *argJ* is monofunctional in *P. syringae* pv. *syringae* based on the fact that *argA* is required for arginine biosynthesis. However, more extensive mutagenesis and biochemical analyses are needed to characterize the function of the predicted *argJ* gene in *P. syringae*.

The *argA* regions in pseudomonads are conserved (Fig. 4). All six ORFs identified in the strain B301D genome were highly conserved, with identities ranging from 67 to 91% among a broad spectrum of fluorescent pseudomonads. The 8-kb region of strains B301D and B728a of *P. syringae* pv. *syringae* shared more than 99% identity at the nucleotide level, which was similar to the nucleotide conservation among different strains of *P. aeruginosa* (35). In comparison, *P. syringae* pv. tomato strain DC3000 showed higher variability in the *argA* region, with a 13.5% nucleotide substitution rate, than strains B301D and B728a of *P. syringae* pv. *syringae*. Another interesting observation about the *argA* region was that gene rearrangements occurred in the genome of *P. putida* KT2440. Consequently, the 8-kb region was split into two parts that flanked both sides of the putative replication origin (i.e., *dnaA*) of the strain KT2440 chromosome.

The ability to synthesize arginine is critical for pathogenicity of *P. syringae* pv. *syringae* B301D. Xu and Gross (41) demonstrated that mutant strain W4S2545 of *P. syringae* pv. *syringae* developed significantly smaller populations, peaking at approximately 10<sup>4</sup> CFU per fruit 3 days after inoculation (10<sup>8</sup> CFU per fruit for wild-type strain B301D) and failed to cause disease on sweet cherry fruits even though the cells of this strain still possessed the ability to cause a hypersensitive reaction on tobacco leaves. In contrast, BR132 (a *syrB1* mutant), defective in syringomycin production, grew in planta to levels comparable to those of the wild-type strain yet was reduced in virulence by approximately 40% (43). Results of this study reveal that strain W4S2545 was an arginine auxotroph of *P. syringae* pv. *syringae*. Considering all of the above observations together,

the loss of pathogenicity of strain W4S2545 results primarily from an inability for growth in planta due to arginine deficiency. In fact, it has long been known that auxotrophic mutants of pathogenic bacteria are oftentimes reduced in virulence or lose plant pathogenicity altogether. In the early 1960s, for example, Garber (as reported in reference 22) observed that mutants of *P. syringae* pv. tabaci that were unable to synthesize certain amino acids lost virulence. Similarly, effects of virulence and pathogenicity were observed for certain auxotrophic mutants of *P. syringae* pv. syringae strains, namely, PS9020 (2) and B728a (3) and *P. syringae* pv. phaseolicola strains PP7010 (2) and S2 (34). However, the effects of various auxotrophic mutants requiring different amino acids on pathogenesis varied greatly. They ranged from being indistinguishable from those of the wild-type strain to a complete loss of pathogenicity. Results of this study show that the *syrA* (*argA*) mutant W4S2545 was nonpathogenic due to arginine auxotrophy.

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