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AlgU Controls Expression of Virulence Genes in *Pseudomonas syringae* pv. tomato DC3000

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ABSTRACT

Plant-pathogenic bacteria are able to integrate information about their environment and adjust gene expression to provide adaptive functions. AlgU, an extracytoplasmic function (ECF) sigma factor encoded by *Pseudomonas syringae*, controls expression of genes for alginate biosynthesis and genes involved with resisting osmotic and oxidative stress. AlgU is active while these bacteria are associated with plants, where its presence supports bacterial growth and disease symptoms. We found that AlgU is an important virulence factor for *P. syringae* pv. tomato DC3000 but that alginate production is dispensable for disease in host plants. This implies that AlgU regulates additional genes that facilitate bacterial pathogenesis. We used transcriptome sequencing (RNA-seq) to characterize the AlgU regulon and chromatin immunoprecipitation sequencing (ChIP-seq) to identify AlgU-regulated promoters associated with genes directly controlled by this sigma factor. We found that in addition to genes involved with alginate and osmotic and oxidative stress responses, AlgU regulates genes with known virulence functions, including components of the Hrp type III secretion system, virulence effectors, and the *hrpL* and *hrpRS* transcription regulators. These data suggest that *P. syringae* pv. tomato DC3000 has adapted to use signals that activate AlgU to induce expression of important virulence functions that facilitate survival and disease in plants.

IMPORTANCE

Plant immune systems produce antimicrobial and bacteriostatic conditions in response to bacterial infection. Plant-pathogenic bacteria are adapted to suppress and/or tolerate these conditions; however, the mechanisms controlling these bacterial systems are largely uncharacterized. The work presented here provides a mechanistic explanation for how *P. syringae* pv. tomato DC3000 coordinates expression of multiple genetic systems, including those dedicated to pathogenicity, in response to environmental conditions. This work demonstrates the scope of AlgU regulation in *P. syringae* pv. tomato DC3000 and characterizes the promoter sequence regulated by AlgU in these bacteria.

Pseudomonas syringae is a globally dispersed plant-pathogenic bacterium adapted to live in many diverse environments, which in some cases expose bacteria to stresses that can interfere with their growth and survival. Counteracting or tolerating these stresses requires coordinated expression of specific physiologic traits. These responses are controlled by environmental sensing and signal transduction systems that detect information about external conditions and induce changes in transcription. The extracytoplasmic function (ECF) sigma factors are one type of system providing this function in bacteria and are capable of inducing sets of genes (regulons) in response to environmental stimuli (1). ECF sigma factor activity is often controlled by a cytoplasmic membrane-bound anti-sigma factor that forms a complex with the ECF sigma factor (2). Under inducing conditions, the ECF sigma factor is liberated from the anti-sigma factor, enabling it to interact with RNA polymerase and activate gene expression.

P. syringae pv. tomato DC3000 encodes 10 ECF sigma factors (3). Five *P. syringae* pv. tomato DC3000 ECF sigma factors are dedicated to iron homeostasis and control expression of genes related to siderophore biosynthesis and/or uptake (3–6). The remaining five are classified as stress response ECF sigma factors based on sequence similarity to sigma factors encoded by other bacteria (3). The stress response group of *P. syringae* pv. tomato DC3000 ECF sigma factors includes AlgU, which was identified based on its role in controlling production of alginate, an exopolysaccharide composed of O-acetylated 1,4-linked β-D-mannuronic acid and its C-5 epimer, L-guluronic acid (7). Under certain conditions, alginate is secreted and contributes to the extracellular matrix, a component of bacterial aggregates and biofilms (8). In this context, alginate provides protection from a variety of external stresses, such as heat, desiccation, reactive oxygen species (ROS), and antimicrobials (9–11).

Regulation of alginate production has been intensively studied in *Pseudomonas aeruginosa*, where its aberrant constitutive expression is associated with a poor prognosis for cystic fibrosis patients infected with these bacteria (12, 13). AlgU regulation in *P. aeruginosa* also contributes to cell envelope homeostasis and is activated in response to perturbations within this compartment

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(14). *P. syringae* pv. tomato DC3000 contains all of the genes necessary for alginate production but lacks homologs to some of the regulatory genes found in *P. aeruginosa* (15, 16), and there are some differences with regard to the environmental stimuli that activate alginate production in these bacteria (17). In *P. syringae*, AlgU autoregulates its own expression and that of *algD* (18–20). Keith and Bender reported that *P. syringae* pv. syringae FF5 cells lacking *algU* were more sensitive to heat and oxidative stress and that these stresses, as well as osmotic stress and copper sulfate, stimulate transcription of *algU* (11). A global study of *algU*-dependent transcription in *P. syringae* pv. syringae B728a growing in plants and under a variety of *in vitro* conditions indicated that AlgU regulates genes for alginate biosynthesis as well as for type VI secretion and oxidative and osmotic stress responses (21). This study also found that AlgU-dependent transcription was induced in response to osmotic stress (21), and there is a great deal of overlapping regulation between the osmotic stress stimulon and the *algU* regulon in *P. syringae* pv. syringae B728a, suggesting that osmolarity is a key signal for AlgU activation in this pathovar (21, 22). But, the role of AlgU may be different in *P. syringae* pv. tomato DC3000 since key indicators of AlgU regulation (i.e., expression of alginate biosynthesis genes) were not induced by osmotic stress (22). These differences in AlgU-dependent regulation and sensing are likely indicative of the different strategies for environmental adaptation and plant association used by these pathovars.

P. syringae encodes systems dedicated to facilitating its pathogenic lifestyle, such as the hypersensitive response and pathogenicity (Hrp) system, which includes a type III secretion system (T3SS) and effectors (23). The Hrp system is a key component of pathogenesis because it helps to suppress plant immune responses (23, 24). We are interested in identifying additional systems that facilitate plant interactions and turn our focus to identifying genes regulated by AlgU in *P. syringae* pv. tomato DC3000.

AlgU is a leading regulator of gene expression for epiphytically and apoplastically growing *P. syringae* pv. syringae B728a (21). AlgU has an active role in regulating expression of genes in response to envelope stress on leaf surfaces (21), and AlgU-dependent expression correlates with ROS production *in planta* (25). *P. syringae* pathovars produce alginate *in planta* (26), and there is a positive correlation between the amount of alginate produced and the severity of disease symptoms (27). The proposed functions of alginate *in planta* include water absorption and nutrient acquisition, protection from antimicrobial compounds, protection from osmotic and oxidative stress, Ca^{2+} sequestration, and suppression of host defenses (27–29). However, the degree of the effect that alginate has on plant disease may depend on details of the specific interaction between individual *P. syringae* strains and plant hosts (19, 21, 30). For example, alginate production by *P. syringae* pv. syringae 3525 promotes symptom development, epiphytic growth, and pathogenic growth on bean (30). In contrast, *P. syringae* pv. glycinea PG4180, which causes bacterial blight on soybean, is not dependent on alginate production, but the presence of *algU* has a positive effect on virulence and bacterial growth in host plants (19, 21). Similarly, epiphytic and endophytic growths are reduced for *P. syringae* pv. syringae B728a *algU* mutants (21). Together, these results demonstrate that for some *P. syringae* strains, AlgU regulates genes besides those for alginate biosynthesis that support pathogenesis.

Recent work functionally linked the signal transduction cascade that activates AlgU with virulence-related functions in *P. syringae* pv. maculicola (31). Schreiber and Desveaux (31) found that the AlgW protease is a negative regulator of flagellar gene expression (*fliC*) and a positive regulator of the Hrp system expression, in addition to its role in promoting alginate expression. A mechanism explaining how AlgW controls gene regulation was not established, but the process likely involves AlgU. Additionally, based on the genetic relationship of *P. syringae* pv. tomato DC3000 and *P. syringae* pv. maculicola (32), this mode of regulation may function similarly in these two strains.

AlgU is homologous to *Escherichia coli* σ^E, which in addition to functions related to envelope stress also regulates many genes involved with pathogenesis and host-specific adaptation and survival (33). The same is likely true among the pseudomonads. For example, AlgU regulates around 300 genes in *P. aeruginosa* (14, 34) and more than 800 genes in *P. syringae* pv. syringae B728a (21). In the present study, we examined the AlgU regulon in *P. syringae* pv. tomato DC3000 and found that in addition to genes involved in alginate production, osmotic stress, and oxidative stress, AlgU coordinates expression of important virulence functions such as the Hrp system and others, such as flagella, that affect the outcome of the pathogenic processes. These data were used to construct a model of the AlgU-dependent promoter, which was tested by mutagenesis. These experiments demonstrate the relative importance of the conserved −10 and −35 elements with regard to AlgU-dependent transcriptional activity.

TABLE 1 Strains and plasmids used

Strain or plasmid	Relevant characteristic(s)	Reference
Strains		
PS1	*P. syringae* pv. tomato DC3000 wild type	15
PS554	*P. syringae* pv. tomato DC3000 Δ*algU*	This work
PS392	*P. syringae* pv. tomato DC3000 Δ*algD*	This work
PS519	*P. syringae* pv. tomato DC3000 Δ*algU mucAB* Δ*algD*	This work
PS368	*P. syringae* pv. tomato DC3000 Δ*algU mucAB*	77
Plasmids		
pJN105	P_{BAD}::empty Gm^r	78
pEM53	P_{BAD}::*algU* Gm^r	This work
pBB51	P_{BAD}::*algU*-FLAG Gm^r	This work
pZB48	pK18mobsacB::Δ*algU*	This work
pEM52	pK18mobsacB::Δ*algD*	This work
pK18mobsacB	Cloning vector for site-specific genomic deletions	38
pBS58	P_{nptII}::gateway expression vector	4
pBS59	P_{nptII}::gateway expression vector (gateway opposite orientation)	4

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. syringae* strains (Table 1) were grown at 28°C in LM (35) or Kings B (KB) medium (36) or on medium solidified with 1.5% (wt/vol) agar. Except where noted otherwise, rifampin (Rif), gentamicin (Gm), kanamycin (Km), and tetracycline (Tet) were used at 50 μg/ml, 10 μg/ml, 50 μg/ml, and 10 μg/ml, respectively. *E. coli* DH5α and TOP10 were used as the host for subcloning and other plasmid manipulations and grown at 37°C in LB medium or LB medium solidified with 1.5% (wt/vol) agar.

Molecular biology procedures. Molecular biology was performed according to standard methods, such as those described in the work of Sambrook and Russell (37). Vectors used for expression of AlgU or AlgU-

FLAG were constructed by Gateway *attL-attR* (LR) recombination between entry vectors containing these genes and the pBS1 destination vector as described in reference 4 and per the Gateway (Invitrogen) cloning product literature. The *algU* and *algD* genes were deleted from *P. syringae* pv. tomato DC3000 by marker exchange mutagenesis, using deletion constructs pZB48 and pEM52 for the *algU* and *algD* mutants, respectively. The deletion constructs were constructed by cloning ~1-kb regions flanking the coding sequences of these genes in pK18mobsacB (38). These mutations were designed to be nonpolar, and the deletions were in frame and eliminated all coding sequences except several codons at the 5′ and 3′ ends of the genes. The PSPTO_4381, PSPTO_1417, *algD*, PSPTO_1185, and PSPTO_1511 promoter regions were cloned by PCR and Topo cloning with the Gateway pENTR/D (Invitrogen). These plasmids were then used for LR cloning with pBS58 or pBS59 to introduce the cloned insert upstream of the *lux* operon as described in reference 4. The mutant derivatives of PSPTO_4381 were cloned using gBlocks (IDT, Coralville, IA), which were digested with AscI and NotI and ligated with a similarly digested P_{PSPTO_4381}::*lux* reporter construct. All plasmids and mutant clones were confirmed by DNA sequencing.

Plant virulence assays. *Solanum lycopersicum* 'Moneymaker' plants were germinated and grown in a greenhouse with approximately 16-h/8-h light/dark cycles. At 4 to 5 weeks after transplanting, leaves were inoculated with 3×10^4 CFU/ml bacterial suspension using blunt syringe infiltration. Bacteria were recovered from plants by taking samples of the leaf tissue at the site of infection using a no. 2 disk punch (3 disks, total area of 0.589 cm^2) at 5 days postinfection (dpi). Leaf disks were homogenized by mechanical disruption in 300 μl of 10 mM MgCl. Serial dilutions of the tissue homogenate were plated on the LM agar supplemented with Rif, and the number of CFU per square centimeter was calculated.

Alginate assay. The concentration of uronic acid-containing molecules produced by *P. syringae* pv. tomato DC3000 and derivative mutant strains was quantified using a modified carbazole assay at 55°C with borate to help enhance detection of mannuronic and guluronic acids (39, 40). Bacterial cells were grown in KB medium amended with Gm and 0.2% L-(+)-arabinose to optical densities at 600 nm (OD_{600}s) of between 0.3 and 0.7. Bacterial cell pellets were resuspended and diluted to a final OD_{600} of 1.2 in PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.8 ± 0.2). Thirty-five microliters of resuspended bacterial pellets was added to 300 μl sulfuric acid-borate on ice and mixed by vortexing at a low level. Ten microliters of a carbazole solution (1 mg/ml in 100% ethanol) was added, and mixtures were incubated for 50 min at 55°C and then cooled to room temperature. Two hundred microliters of each sample was transferred into 96-well clear-bottom plates, and the absorbance at 530 nm was measured using a BioTek Synergy 2 plate reader. A standard curve was prepared using brown alga alginic acid in 10 mM NaOH over the range of 10 to 90 μg/ml.

Reverse transcription-quantitative PCR (qRT-PCR). Overnight cultures of *P. syringae* pv. tomato DC3000 and mutant derivative strains were grown in KB with Gm, subinoculated at an OD_{600} of 0.1 in KB with 5 μg/ml Gm and 0.2% L-(+)-arabinose when appropriate, and grown to an OD_{600} of between 0.3 and 0.7. Cell pellets were prepared from 1 ml of culture and stored at −80°C. RNA was prepared from cell pellets using the RNeasy kit (Qiagen, Hilden, Germany) with the on-column DNase treatment option (Qiagen RNase-free DNase set). Eluted RNA was treated with Ambion DNase I RNase-free enzyme and purified with the Qiagen RNeasy minikit.

Real-time PCR was performed by using the My IQ5 sequence detection system (Bio-Rad) and iQ SYBR green Supermix (Bio-Rad) according to the manufacturer's protocols. One hundred nanograms of total RNA extracted from *P. syringae* pv. tomato DC3000 or mutant derivative strains was reverse transcribed in a thermocycler using qScript cDNA SuperMix (Quanta Biosciences) according to the manufacturer's instructions. The resulting total cDNA population was diluted 6-fold in nuclease-free water. Six microliters of the diluted total cDNA population was mixed with each primer (final concentration of 0.4 μM) and 10 μl of master mix in a 20-μl final volume. The PCR assay was carried out with one cycle at 95°C for 2 min and 30 s followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The amount of fluorescence that resulted from the incorporation of the SYBR green dye into double-stranded DNA was measured at the end of each cycle to determine the PCR kinetics for each sample. DNA contamination and the formation of primer dimers were assessed using a no-reverse-transcriptase (no-RT) control and no-template control, respectively. The production of nonspecific products was determined by the dissociation protocol included in the software provided with the My IQ5 real-time PCR machine. The resulting threshold cycle (C_T) values were calculated by the My IQ5 software and analyzed using the relative standard curve method (separate tubes) described in ABI user bulletin no. 2 (41). In each strain, the C_T values of each gene tested were normalized to the C_T values of housekeeping gene *gap-1* as described in reference 4.

RNA-seq. Strain PS368 containing pEM53 or pJN105 was streaked onto LM-Gm plates and incubated at room temperature for 2 days. These clones were used to start 25-ml KB cultures containing Gm and grown at 28°C overnight. The next day, cultures were subinoculated at an OD_{600} of 0.1 in 50 ml of KB with 5 μg/ml Gm and 0.2% L-(+)-arabinose. Cultures were grown to an OD_{600} of between 0.3 and 0.5. Cell pellets used to collect RNA were harvested by centrifugation at room temperature for 5 min at 5,000 × *g* and stored at −80°C. RNA was purified with Qiagen RNeasy minikit using the on-column DNase treatment option (Qiagen RNase-free DNase set) and treated with Ambion DNase I RNase-free enzyme. RNA was purified using Zymo Research RNA Clean & Concentrator-25, and then the purity and quality of the samples were analyzed on an Agilent Bioanalyzer. qRT-PCR was used to test for DNA contamination and for induction of *algD* and PSPTO_4381 genes. rRNA was removed with the Epicentre Ribo-Zero Magnetic kit (Gram-negative bacteria), and rRNA-depleted RNA was purified with the Zymo Research RNA Clean & Concentrator-25 kit. Transcriptome sequencing (RNA-seq) libraries were prepared using the Script Seq v2 RNA-seq library preparation kit per the manufacturer's instructions. The Agencourt AMPure XP system (Beckman Coulter) was used to purify both the cDNA and the RNA-seq library. RNA-seq libraries were sequenced by the Cornell Genomics Facility using the Illumina HiSeq sequencer operating in high-output mode.

ChIP-seq. PS519 containing pBB51 or pJN105 (Table 1) was streaked on LM-Gm agar, incubated for 2 days at room temperature, used to inoculate 25 ml KB with Gm, and grown at 28°C with vigorous shaking overnight (O/N). The cultures were then diluted to an OD_{600} of 0.1 in 200 ml of KB with 5 μg/ml Gm and 0.2% L-(+)-arabinose and grown to an OD_{600} of between 0.4 and 0.5. Cultures were treated with 1% formaldehyde and quenched with 0.36 M glycine. Cells were washed twice with 50 ml cold Tris-buffered saline (TBS) and once with 1 ml cold TBS, and pellets were stored at −80°C. Pellets were lysed with 1 ml CelLytic B (Sigma-Aldrich), 10 μl Longlife lysozyme (G-Biosciences; catalog no. 786-037), and 10 μl of 100 mM phenylmethylsulfonyl fluoride (PMSF) in ethanol at 37°C for 15 min. Cell lysates were sonicated and fractionated by centrifugation to remove insoluble material. Cleared cell lysates were incubated for 2 h with anti-FLAG M2 affinity gel (Sigma-Aldrich; catalog no. A2220) and then washed three times with cold TBS in a Spin X column. DNA was eluted using 100 μl of 100 ng/μl FLAG peptide (Sigma-Aldrich; catalog no. F3290), and cross-links were reversed and protein was degraded by adding pronase and heating samples to 42°C for 2 h and then at 65°C for 6 h in a thermal cycler. DNA was purified using the Qiagen QIAquick PCR purification kit. Chromatin immunoprecipitation sequencing (ChIP-seq) libraries were prepared and sequenced by the Cornell Genomics Facility using the Illumina HiSeq operating in high-output mode.

Bioinformatic analysis. (i) Sequence alignment. Bowtie2 (42) 2.1.0 was used to align the Illumina reads against the *P. syringae* pv. tomato DC3000 (NC_004578.1, NC_004632.1, and NC_004633.1) and PhiX (NC_001422.1) genomes. The bowtie2-build command was used to index the genomes per documentation. Then, bowtie2 was invoked in a manner similar to the following: "bowtie2 -end-to-end -k5 -x GENOMES -U RAW_READS > ALIGNED_READS".

Custom scripts were used to remove ambiguous reads and make sinister and naive profiles for RNA-seq and ChIP-seq sequence data as described in the work of Filiatrault et al. (43).

(ii) RNA-seq analysis. The RefSeq genome annotation was used to define gene regions for differential analysis. A custom script was used to count the number of uniquely aligned reads whose 5′ end fell within each gene region for each of the samples. The R package DESeq2 (44) was used to analyze the magnitude and significance of the fold change in expression between the conditions. A false-discovery rate (FDR) cutoff of 0.01 was used to select the genes whose fold change was most supported. The output was further filtered to select those genes whose fold change was greater than 2 or less than 1/2.

(iii) Functional category enrichment analysis. To help discover biological functions or systems that might be regulated by AlgU, an enrichment analysis was performed to identify functional categories for which more genes were in AlgU's regulon than could be explained by chance. For this analysis, we used the R (45) package GOSEQ (46).

As input, we used the same set of genes as the RNA-seq analysis. Genes whose fold change was greater than 2 were assigned to the "up regulon," and those whose fold change was less than 1/2 were assigned to the "down regulon." These two sets were combined to form a single "regulon" set for the analysis. The results shown in Table S1 in the supplemental material break down the counts for each functional category into their "up" and "down" components. For functional categories, we used gene annotations downloaded from the Comprehensive Microbial Resource (CMR) (47) hosted by the J. Craig Venter Institute (JCVI), here referred to as the "JCVI functional categories." Of the 5,692 genes included in our analysis, 4,977 were assigned one or more JCVI functional categories; the remaining 715 were included in a "no function assigned" category. JCVI functional categories were downloaded from the CMR (47) hosted by JCVI on or about 8 July 2013. The CMR website (http://cmr.jcvi.org/) is offline and the data files are no longer available, but Table S2 in the supplemental material lists all of the functional annotations for *P. syringae* pv. tomato DC3000 downloaded from the CMR website.

With these inputs, GOSEQ was used to assign a *P* value to the enrichment of each functional category, relative to the entire set of genes. GOSEQ uses Fisher's exact test, modified to account for transcript length, on the number of genes in each functional category. As an approximation of its transcript length, we used the annotated length of each gene. The method of Benjamini and Hochberg (48) was used to compute a false-discovery rate (FDR) from the *P* values. Only functional categories with an FDR of <0.05 are reported.

(iv) ChIP-seq analysis. MACS2 (49, 50) (version 2.1.0.20140616) was used to identify regions of enrichment, or "peaks," in the sinister profile for the immunoprecipitation (IP)-treated AlgU-FLAG library (CHIP_FILE). In order to identify nonspecific enrichment, the sinister profile of the AlgU-FLAG lysate library (CONTROL_FILE) was used. First, the two profiles were converted to BED format, and then they were analyzed by MACS using the following command invocation: "macs2 callpeak -t CHIP_FILE -c CONTROL_FILE -f BED -seed 1 -g 6.1e6 -fix-bimodal -keep-dup all -q 0.05".

(v) AlgU promoter motif. Regions within 50 bp of the MACS-identified peaks were calculated, and overlapping regions were merged. Genomic sequences of each region were extracted and written to FASTA files. MEME (51) 4.10.0 with the parameters "-dna -revcomp -mod zoops -nmotifs NUM_MOTIFS -minsites 2 -maxsites NUM_SEQS -minw 6 -maxw 50 -nostatus" was used to analyze these sequences for conserved motifs.

AlgU binding motif functional analysis. Cultures of PS368 containing pEM53 or pJN105 transformed with the promoter::*lux* fusion constructs were grown in KB medium containing Km and Gm. These cultures were diluted 1:10 in KB medium with a final concentration of 0.2% L-(+)-arabinose, 25 ng/ml Km, and 5 ng/ml Gm. The luminescence and OD_{600} were measured on a BioTek Synergy 2 plate reader after shaking at room temperature for 6 h.

Accession number(s). Sequencing data for RNA-seq and ChIP-seq experiments are accessible at SRP076234 in the Sequence Read Archive (SRA).

RESULTS

***P. syringae* pv. tomato DC3000 AlgU promotes disease and *in planta* growth.** The function of alginate and AlgU in *P. syringae* plant disease has been tested previously (19, 21, 30). The results of these studies suggest that there is variability regarding the role of alginate and AlgU in promoting *P. syringae* virulence and growth in plants. To test whether alginate production or some other functions regulated by AlgU influence *P. syringae* pv. tomato DC3000 plant disease dynamics, we compared disease symptoms and bacterial growth of wild-type *P. syringae* pv. tomato DC3000 to those of *algD* and *algU mucAB* mutant derivatives on host tomato plants (Fig. 1). The *algD* gene encodes GDP mannose dehydrogenase, which catalyzes the committed step of alginate biosynthesis and is the first gene of a putative 12-gene operon that contains other alginate biosynthetic genes. The *algD* mutant is not capable of producing alginate (Fig. 2) but retains a functional copy of *algU*. The wild-type *P. syringae* pv. tomato DC3000 and its *algD* mutant caused similar symptoms and grew similarly in leaves. But, the *algU mucAB* mutant was impaired with regard to its ability to cause disease and grow in tomato relative to the wild type or the *algD* mutant. Leaves infected with the *algU mucAB* mutant showed reduced chlorosis and fewer necrotic spots, and this mutant grew to a lower density in leaves (Fig. 1). This result suggests that AlgU is important for plant interactions while alginate production is dispensable. If this is true, one prediction is that overexpression of AlgU would increase disease-associated phenotypes, even in cells not capable of producing alginate. We tested this idea by examining bacterial growth and symptoms of plants infected with the *algU mucAB algD* mutant transformed with the *algU* expression vector (pEM53) or empty vector control. We found that AlgU expression caused a dramatic increase in the number of necrotic spots and the amount of chlorosis (Fig. 1A). Additionally, the AlgU expression vector fully complemented the *in planta* growth of the *algU mucAB algD* strain (Student's *t* test, $P = 0.38$) but did not cause the strain to exceed the growth of the wild type (Fig. 1B). The finding that disease symptoms increased without a concomitant increase in the amount of bacterial growth suggests that the virulence of these cells was increased by AlgU expression.

Elimination of *mucAB* facilitates AlgU-dependent expression in the absence of inducer. Most *P. syringae* strains surveyed are nonmucoid, and AlgU-dependent phenotypes are not expressed under standard laboratory conditions (17). In the experiments described here, we chose to overexpress AlgU from a plasmid to study its function in *P. syringae* pv. tomato DC3000. To determine whether this plasmid system could be used to accomplish this goal, we compared alginate levels produced by *P. syringae* pv. tomato DC3000 strains transformed with a vector (pEM53) expressing *algU* to the levels produced by cells containing the empty vector control plasmid (pJN105). We found that alginate production increased only slightly in wild-type cells containing the AlgU expression plasmid (Student's *t* test, $P = 0.008$) (Fig. 2A). This result was further supported by comparing the difference in expression between the *algA* gene in wild-type cells containing the AlgU expression vector and that in empty vector controls (Fig. 2B). We observed a 4.1-fold difference between *algA*

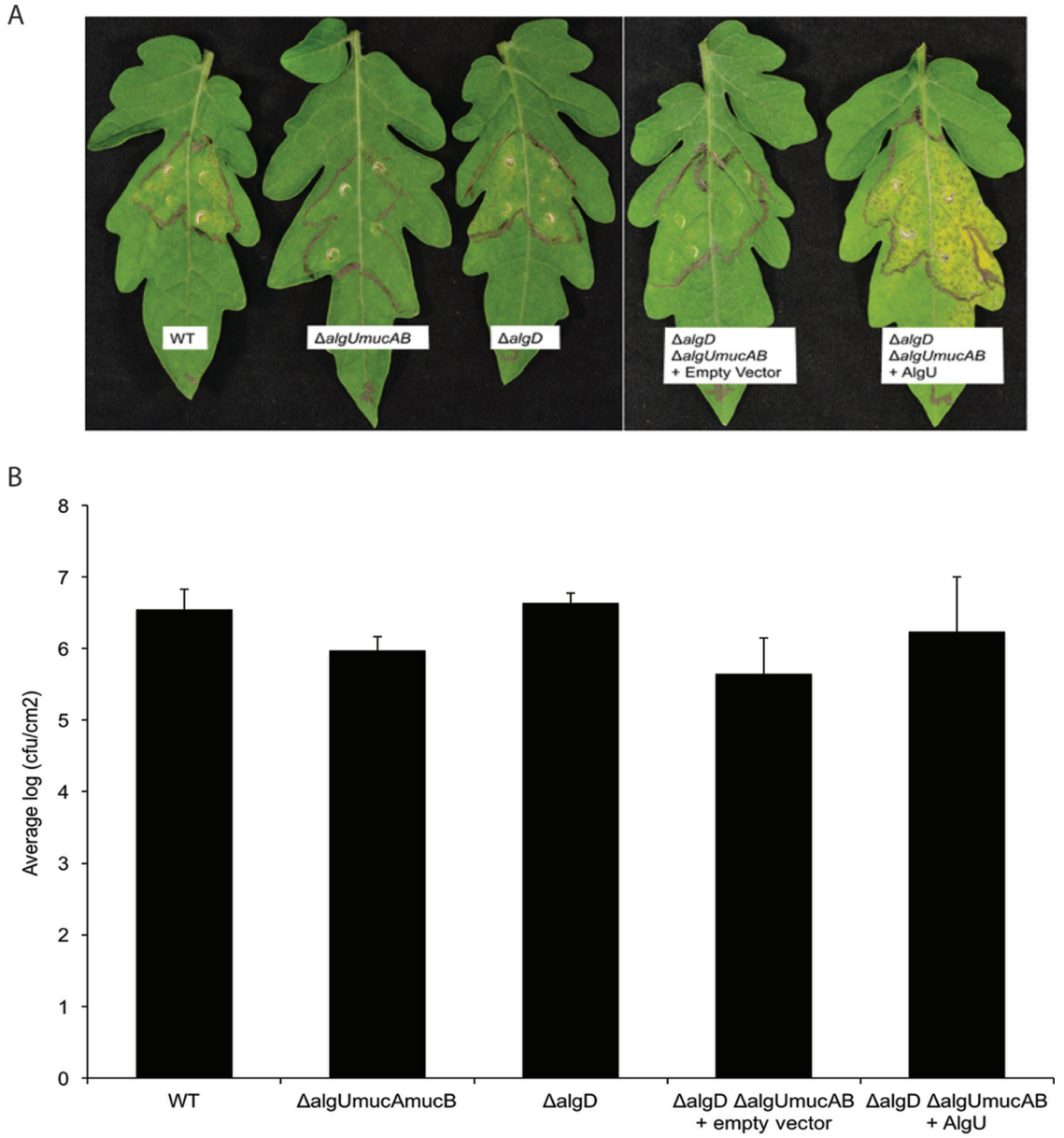

FIG 1 AlgU contributes to *in planta* growth and disease, but alginate has negligible effects. (A) Disease symptoms at 7 days on tomato (*Solanum lycopersicum* 'Moneymaker') leaves inoculated by syringe infiltration with 3×10^4 CFU/ml *P. syringae* pv. tomato DC3000 (WT), the isogenic deletion mutants, or mutants with the empty vector (pJN105) or the AlgU expression vector pEM53. Leaves shown are representative of symptoms observed on at least three plants infected on separate days. (B) Growth of *P. syringae* pv. tomato DC3000 and mutants in tomato at 5 days postinoculation. A 3×10^4-CFU/ml amount of *P. syringae* pv. tomato DC3000 or mutants was infiltrated into 4- to 5-week-old tomato plants. The number of bacterial cells per square centimeter of leaf tissue was determined at 5 days postinoculation. Error bars in Fig. 1, 2, and 6 indicate standard deviations.

transcript levels with and those without AlgU expression, which was less than expected with overexpression of the sigma factor.

The *algU* gene is in an operon that also contains *mucA* and *mucB*, the products of which function as an anti-sigma factor that suppresses AlgU activity under noninducing conditions (34, 52, 53). Hypermucoid *Pseudomonas* strains (e.g., clinical isolates of *P. aeruginosa*) often have mutations in *mucA* that dysregulate AlgU, resulting in excessive alginate production (54). Genes regulated by AlgU in *P. aeruginosa* are also highly expressed in *mucA* mutants (34, 55). Similarly, deletion of *mucB* can cause conversion to mucoidy (52). Because we are testing for AlgU-dependent effects under noninducing conditions, we hypothesized that the anti-sigma factor may be inhibiting AlgU activity and preventing robust induction of AlgU activity. To test this, we assessed *algU*-dependent phenotypes in a *P. syringae* pv. tomato DC3000 *algU mucAB* deletion strain containing either the AlgU expression vector or the empty control vector. In this case, we observed a large AlgU-dependent increase in alginate production (Fig. 2A) and *algA* gene expression in strains lacking the *mucAB* genes (Fig. 2B). As a control, we measured the carbazole reaction of an *algU mucAB algD* deletion strain unable to produce alginate. The results indicate 19 to 22 μg/ml of uronic acid being produced by this strain, which

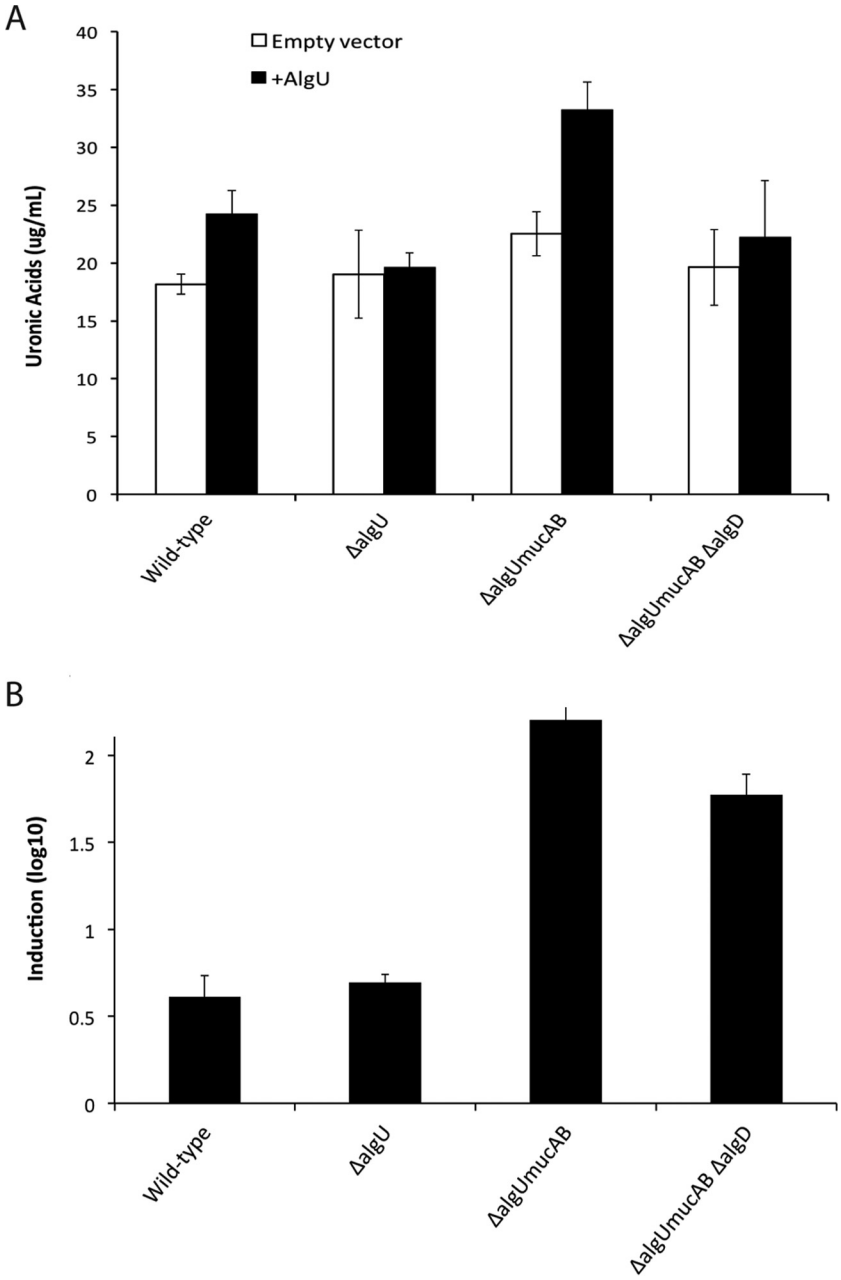

FIG 2 AlgU-dependent expression in *P. syringae* pv. tomato DC3000. AlgU function was determined by comparing alginate production (A) and *algA* gene expression (B) in wild-type *P. syringae* pv. tomato DC3000 and mutant strains containing the AlgU expression vector (pEM53) to those in strains containing the empty vector control (pJN105). (A) The alginate assay was used to determine the micrograms per milliliter of uronic acid-containing polysaccharides present in normalized cell suspensions. The *algU mucAB algD* deletion strain indicates background of other nonalginate cellular materials capable of reacting with the carbazole reagent. (B) qRT-PCR results showing the induction of the *algA* gene. Induction is the ratio of relative *algA* expression in cells containing the *algU* expression vector (pEM53) to that in cells containing the empty vector control plasmid (pJN105).

shows the background of other nonalginate cellular materials capable of reacting with the carbazole reagent.

Identification of the *P. syringae* pv. tomato DC3000 AlgU regulon. AlgU has been reported as an essential and direct regulator of alginate biosynthesis, but as described above, the production of alginate had negligible effects on *P. syringae* pv. tomato DC3000 growth and virulence in plants (Fig. 1). This is consistent with the idea that AlgU regulates expression of additional genes in *P. syringae* pv. tomato DC3000 with roles in facilitating plant interactions. RNA-seq was used to determine the AlgU-dependent regulon and possibly identify *P. syringae* pv. tomato DC3000 functions that might affect virulence or other aspects facilitating plant interactions.

To test this, *P. syringae* pv. tomato DC3000 Δ*algU mucAB* cells containing the AlgU expression vector or the empty vector control were grown and the complete transcriptome was determined for each strain using RNA-seq. The AlgU regulon was determined using DESeq2 to calculate relative transcript abundance for each

TABLE 2 AlgU core regulon members[a]

Category	Locus tag	Gene	Function
Osmotic stress functions	PSPTO_0152	*osmC*	Osmoresponsive protein
	PSPTO_0462	*cbcV*	QAC metabolism and transport
	PSPTO_0463	*cbcW*	QAC metabolism and transport
	PSPTO_0464	*cbcX*	QAC metabolism and transport
	PSPTO_1631	*ggnC*	Compatible solute synthesis
	PSPTO_1632	*ggnB*	Compatible solute synthesis
	PSPTO_1633	*asnB*	Compatible solute synthesis
	PSPTO_3176	*lasB*	Protease (elastase)
	PSPTO_4575	*opuCA*	QAC metabolism and transport
	PSPTO_4576	*opuCB*	QAC metabolism and transport
	PSPTO_4577	*opuCC*	QAC metabolism and transport
	PSPTO_4578	*opuCD*	QAC metabolism and transport
Oxidative stress functions	PSPTO_0675	*cpoF*	Antioxidant enzyme
Alginate biosynthesis	PSPTO_1232	*algA*	Alginate synthesis
	PSPTO_1233	*algF*	Alginate synthesis
	PSPTO_1234	*algJ*	Alginate synthesis
	PSPTO_1235	*algI*	Alginate synthesis
	PSPTO_1236	*algL*	Alginate synthesis
	PSPTO_1237	*algX*	Alginate synthesis
	PSPTO_1238	*algG*	Alginate synthesis
	PSPTO_1239	*algE*	Alginate synthesis
	PSPTO_1240	*algK*	Alginate synthesis
	PSPTO_1241	*alg44*	Alginate synthesis
	PSPTO_1242	*alg8*	Alginate synthesis
	PSPTO_1243	*algD*	Alginate synthesis

[a] Orthologs of these *P. syringae* pv. tomato DC3000 genes were regulated by AlgU in *P. syringae* pv. syringae B728 (21) and *P. aeruginosa* PAO1 (34). QAC, quaternary ammonium compound.

gene and identify those that were differentially regulated between the two strains. This analysis identified a total of 965 AlgU-dependent genes for which expression changed 2-fold or more and had FDR values less than 1% (regulatory statistics of all differentially regulated genes are listed in Table S1 in the supplemental material). The majority of these genes (696 genes) were activated by AlgU, and 269 genes had lower expression in the AlgU expression strain.

Classification of AlgU regulon functions. Initial appraisal of the AlgU-dependent regulon indicated, as expected, that the alginate biosynthesis operon was under positive control by AlgU; expression of all genes of the 12-gene alginate biosynthetic operon was found to be elevated between 613- and 63-fold above the control. To gain a better understanding of the AlgU core regulon functions, we compared the *P. syringae* pv. tomato DC3000 AlgU-upregulated genes to those listed as similarly regulated in *P. syringae* pv. syringae B728a and *P. aeruginosa* PAO1 (the list of AlgU-regulated genes in these organism was compiled from Tables S1A and B in reference 34 and from Table S2 in reference 21). For this analysis, we compared the *P. syringae* pv. syringae B728a and *P. aeruginosa* PAO1 PseudolugeDB database orthologs (56) of the *P. syringae* pv. tomato DC3000 genes. This comparison indicated that all coregulated orthologs fell into only three functional categories: osmotic stress response, oxidative stress response, and alginate biosynthesis (Table 2). We also found that AlgU regulated a variety of membrane functions such as transport and environmental sensing in these bacteria, even though these categories were not found when requiring strict orthology (see Table S1 in the supplemental material). We also probed the set of *P. syringae* pv. tomato DC3000 AlgU-upregulated genes for statistically significant enrichment of specific functional categories. For this, we conducted an enrichment analysis based on Fisher's exact test (46) to identify JCVI functional role categories (47) present in the AlgU-regulated set significantly more frequently than the genome-wide statistics for those categories. The AlgU regulon members represent 115 JCVI categories, suggesting that AlgU controls diverse functions, and the enrichment analysis identified 4 categories that were significantly enriched (Fig. 3). The "Energy metabolism/Biosynthesis and degradation of polysaccharides" functional category is mainly composed of genes that contribute to osmotic stress responses and compatible solute synthesis, including *glgP*, *glgA*, *malQ*, and *glgX*. Notably, one of the most enriched categories contained genes coding for pathogenesis functions. Additionally, the majority of genes in the "Protein fate/Protein and peptide secretion and trafficking" category were also involved with encoding the Hrp T3SS.

Finding that pathogenesis functions were enriched in the AlgU regulon prompted us to compare the HrpL and AlgU regulons. HrpL is a sigma factor responsible for controlling expression of the Hrp system, which includes a T3SS and effectors necessary for these bacteria to cause disease in plants. We compared AlgU-upregulated genes to those upregulated by HrpL (57) and found that expression of 38% of HrpL-upregulated genes was also under AlgU control (Fig. 4 and Table 3). This set of genes upregulated by both AlgU and HrpL includes functions involved with regulation of transcription (*hrpL* and *sigX*), posttranslational regulation of Hrp system expression (*hrpV*), structural genes for the T3SS apparatus (*hrcC* and *hrcQb*), and T3SS substrates that include the

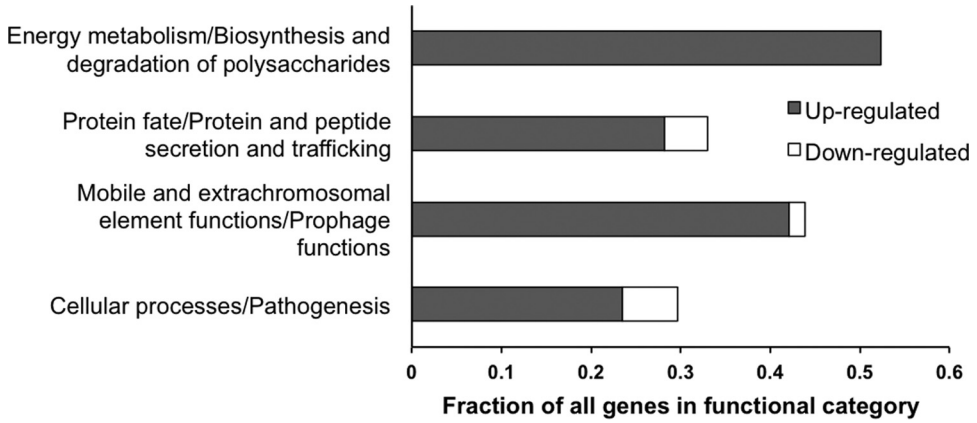

FIG 3 Functional categories enriched among AlgU-regulated genes. Enrichment analysis was used to identify JCVI functional categories (47) enriched among AlgU-regulated genes relative to the genome-wide statistics. The significant enrichment threshold was a *q* value of ≤0.05.

pilus protein gene (*hrpA1*) and effectors (*hopY1*, *avrPtoB*, *avrPto*, and *hopE1*).

There were 269 genes whose expression was reduced by 2-fold or more (FDR ≤ 0.01) in the AlgU expression strain (see Table S1 in the supplemental material). These genes are likely to be indirectly regulated because σ^{70} family members, like AlgU, are canonically thought of as positive regulators of gene expression. The functional categories assigned to the repressed portion of the regulon were generally similar to those of the induced genes. For example, genes encoding cell envelope and transport-related functions comprised a large fraction for both up- and downregulated genes (for the sets of genes with annotated functions). This can be interpreted as evidence that AlgU is responsible for coordinating changes in composition and function of the cell's envelope by increasing as well as decreasing the presence of certain proteins.

Twenty of the downregulated genes were annotated with functions related to chemotaxis and motility. Notably, the *fliC* gene, which encodes the flagellin protein, was strongly repressed (18-fold) in the AlgU expression strain. Flagellin has important virulence-related functions because epitopes within this protein are recognized by plant cells as a pathogen-associated molecular pattern (PAMP), and it is a potent elicitor of plant basal immunity (58–61). Global analysis comparing gene expression profiles of *P. syringae* pv. syringae B728a cells indicated that genes involved in motility, chemosensing, and chemotaxis were repressed in cells growing in plant apoplasts (62). These data suggest that AlgU may serve an important function in repressing flagellar expression *in*

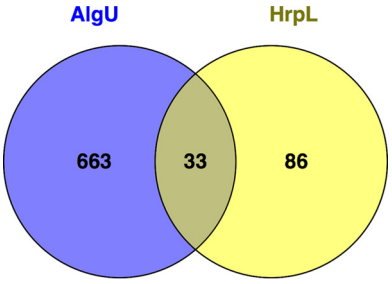

FIG 4 Intersection of AlgU- and HrpL-upregulated genes.

TABLE 3 Genes upregulated by HrpL and AlgU

Locus tag	Gene	Functional role
PSPTO_0061	*hopY1*	Type III effector
PSPTO_1206		TonB-dependent siderophore receptor
PSPTO_1233	*algF*	Alginate biosynthesis
PSPTO_1236	*algL*	Alginate biosynthesis
PSPTO_1378	*hrpH*	Lytic transglycosylase
PSPTO_1381	*hrpA1*	Hrp pilus
PSPTO_1382	*hrpZ*	Harpin/translocator
PSPTO_1383	*hrpB*	Type III secretion protein
PSPTO_1386	*hrpE*	Type III secretion protein
PSPTO_1389	*hrcC*	Outer membrane type III secretion protein
PSPTO_1391	*hrpV*	Negative regulator of Hrp expression
PSPTO_1396	*hrcQb*	Type III secretion protein
PSPTO_1404	*hrpL*	Sigma factor
PSPTO_1405	*hrpK1*	Type III helper protein/translocator
PSPTO_1507		Hypothetical protein
PSPTO_2298	*sigX*	Sigma factor
PSPTO_2678	*hopP1*	Type III helper protein/translocator
PSPTO_2697		Oxidoreductase zinc-binding protein
PSPTO_2734		Acetyltransferase family protein
PSPTO_2769		Lipoprotein
PSPTO_3087	*avrPtoB*	Type III effector
PSPTO_3157		Hypothetical protein
PSPTO_3296		Transporter
PSPTO_3490		Sugar ABC transporter periplasmic sugar binding protein
PSPTO_3500	*iolC*	Myoinositol metabolism
PSPTO_3527		Hypothetical protein
PSPTO_3692		TonB-dependent siderophore receptor
PSPTO_3700		Oxidoreductase
PSPTO_4001	*avrPto*	Type III effector
PSPTO_4331	*hopE1*	Type III effector
PSPTO_5015		Lyase
PSPTO_5255	*cynT*	Carbonic anhydrase
PSPTO_5353	*shcA*	Type III chaperone protein

TABLE 4 Expression of AlgU-regulated genes[a]

Gene	Fold change in expression (relative to empty vector) with:		Fold change in expression for WT vs FLAG-tagged AlgU
	AlgU	AlgU-FLAG	
algD	4,524.3	406.4	11.1
PSPTO_2207	655.7	160.1	4.1
algA	108.3	16.4	6.6
spot42	12.1	6.6	1.8
algR	4.6	2.9	1.6

[a] Fold changes were calculated by comparing expression of indicated genes in strains containing AlgU expression vectors or the empty vector control by qRT-PCR. WT, wild type.

planta, helping to avoid detection and immune response by limiting the presentation of this PAMP.

AlgU ChIP-seq shows evidence of direct regulation. The RNA-seq experiment revealed the set of genes regulated by AlgU, which is composed of genes that are directly regulated by AlgU and a secondary set of genes that are likely controlled by other regulators upregulated by AlgU. Direct regulation involves sigma factor binding DNA at promoter sequences upstream of genes. These sites were identified using ChIP-seq and provide a basis for classifying AlgU regulon members as directly controlled by AlgU or potentially regulated by some other mechanism.

A vector (pBB51) expressing a C-terminally FLAG-tagged AlgU derivative (AlgU-FLAG) was constructed for this ChIP-seq experiment. The function of the AlgU-FLAG was confirmed by qRT-PCR, which showed that the fusion protein was capable of gene regulation, but the magnitude of AlgU-FLAG-dependent gene induction was reduced between 1.5- and 11-fold relative to the wild-type AlgU (Table 4).

In this AlgU ChIP-seq experiment, *P. syringae* pv. tomato DC3000 Δ*algU mucAB* Δ*algD* cells containing the AlgU-FLAG expression vector (pBB51) or the empty vector equivalent (pJN105) were grown under the same conditions used for the differential transcriptome experiment described above. DNA from these cells collected before and after immunoprecipitation was sequenced and aligned with the *P. syringae* pv. tomato DC3000 genome sequence. The locations of DNA sequences enriched by the FLAG-mediated immunoprecipitation (ChIP-seq peaks) were identified by comparing the sequences of the AlgU-FLAG samples before and after FLAG immunoprecipitation using MACS2 (49, 50). A total of 204 AlgU ChIP-seq peaks were found with an FDR cutoff of ≤0.05. Ninety-seven of these peaks were located upstream (within 500 bases upstream or 20 bases downstream of the 5′ end) of AlgU-regulated genes (see Table S1 in the supplemental material). Except in seven cases, the AlgU ChIP-seq peaks were associated with positive regulation by AlgU, as would be expected from a sigma factor.

A conserved AlgU DNA binding motif is present in locations bound by AlgU. The AlgU ChIP-seq peaks are indicative of protein binding at discrete genomic sites. These types of interactions are often a function of specific nucleotide-amino acid contacts (63). In these cases, there is conservation of nucleotide sequences present in each location bound by the protein. We used DNA sequence alignments to identify conservation among AlgU binding sites. Sequences bound by AlgU were aligned, and a sequence logo derived from this information is shown in Fig. 5A. This motif was generated from alignment that included sequences from 82 of the 204 AlgU ChIP-seq peaks. This *P. syringae* pv. tomato DC3000 AlgU binding motif is 28 bp in length and shows evidence of conserved −10 and −35 boxes separated by a 16-bp spacer region with minimal sequence conservation. Overall, the pattern of conservation is typical for promoters used by this type of sigma factor, and the motif is similar to the AlgU- and RpoE-regulated promoters of *P. aeruginosa* (Fig. 5B) and *E. coli* (Fig. 5C), respectively (33, 64). Together, these data suggest that the DNA binding motif identified here could represent the AlgU-regulated promoter for *P. syringae* pv. tomato DC3000. Based on this, we refer to this motif as the AlgU-dependent promoter motif throughout the remainder of the paper.

AlgU-dependent promoter motif confers AlgU-dependent transcription. The AlgU-dependent promoter motif was tested to determine whether it has a functional role in AlgU-dependent transcription. In this experiment, five *P. syringae* pv. tomato DC3000 genomic loci containing the motif were cloned upstream of a promoterless *lux* operon (65) as described in reference 4. This method allows for DNA elements to be isolated from other genomic features and to test whether they might be responsible for transcriptional activity observed by the other methods, such as those described above. The five regions chosen for this test were each associated with AlgU binding in the ChIP-seq, and the downstream genes were differentially regulated by AlgU in the RNA-seq experiment.

Reporter expression from these fusion constructs was compared in *P. syringae* pv. tomato DC3000 Δ*algU mucAB* cells transformed with either the AlgU expression vector (pEM53) or the empty vector control (pJN105). The results show that each of these DNA sequences elicited robust (between 10- and 19-fold) AlgU-dependent expression (Fig. 6A). We were surprised to find that the *algD* promoter had intermediate expression relative to the other promoters tested. The PSPTO_4381 promoter region had the highest expression ratio when comparing the *lux* expression in cells expressing AlgU with that in the empty expression control.

These data show that AlgU-dependent promoter activity can be detected in DNA sequences isolated from the genomic context and provide a basis for choosing an AlgU-dependent promoter to serve as a model for testing the contribution of individual nucleotides to promoter function. The PSPTO_4381 promoter fusion was chosen for this fine-scale examination because it showed strong AlgU-dependent promoter activity and had very low background expression in the absence of AlgU. Low background expression suggests that this sequence lacks other promoters that could complicate analysis. Additionally, the region contained a good representative of the AlgU-dependent promoter motif and could serve as a model for testing the function of this class of promoter in *P. syringae* and other pseudomonads.

To test the function of the motif, individual nucleotides were replaced with G↔T or C↔A, creating transversion mutations at each position of the AlgU-dependent promoter motif and the flanking positions. The mutated promoters were cloned as *lux* fusions, and expression of the reporter was compared to the wild-type PSPTO_4381 promoter fusion in the presence of the AlgU expression vector (Fig. 6B). The results show that for the sequence matching the AlgU-dependent promoter motif upstream of PSPTO_4381, the most conserved nucleotides generally have the largest influence on the transcriptional activity. This correlation is expected if the conservation represented in the motif is related to

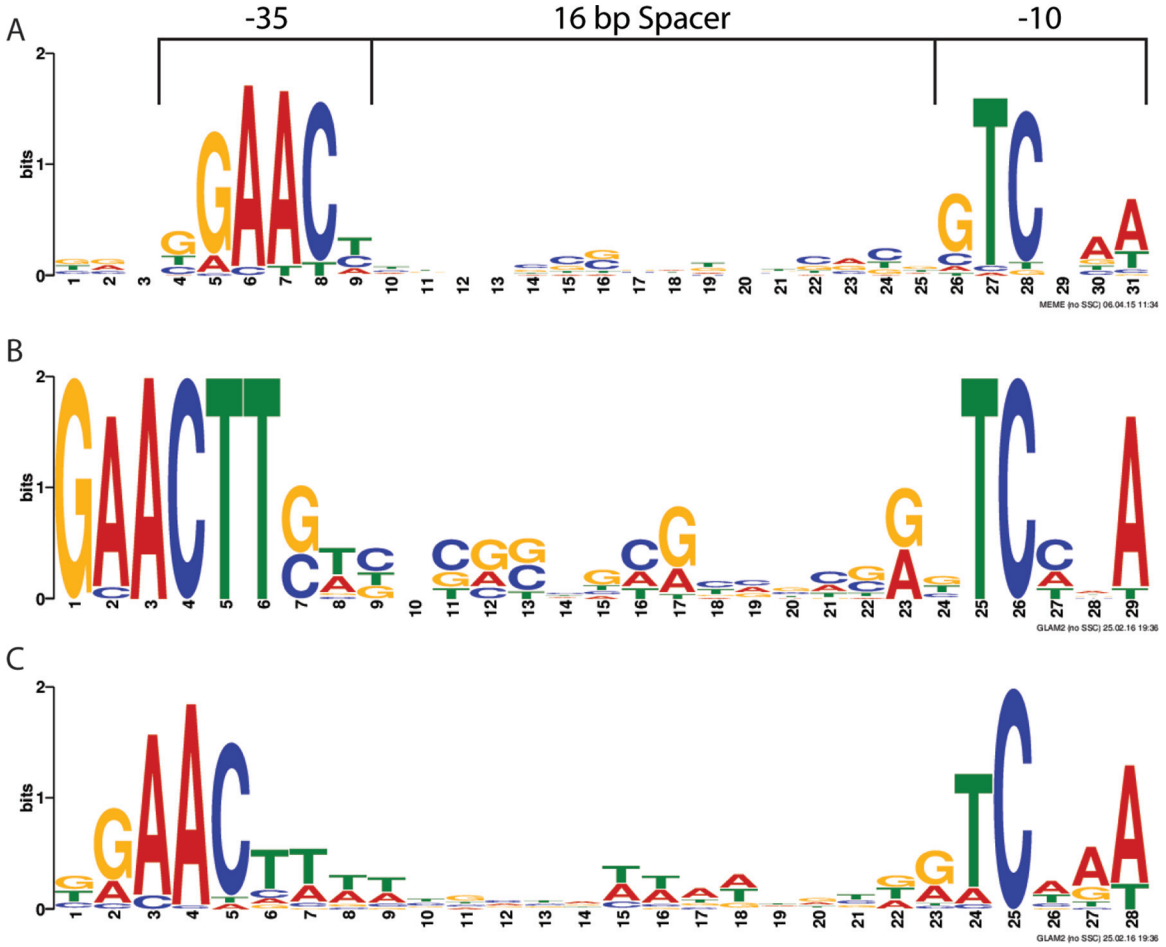

FIG 5 AlgU DNA binding motif. (A) *P. syringae* pv. tomato DC3000 AlgU binding site motif. This motif was identified by using MEME to align 50 bp on each side of the 204 ChIP-seq peaks that passed the FDR cutoff of 0.05. (B and C) *P. aeruginosa* AlgU (B) and *E. coli* RpoE (C) DNA binding site motifs derived from published data sets in references 76 and 33, respectively.

promoter function. Additionally, portions of the motif that have lower conservation (such as the 5′ portion of the −10 box) and positions flanking the motif generally have less influence on the promoter function. Together, these data demonstrate the functionality of an AlgU-regulated promoter and show the relative contribution of each position of the motif and of positions flanking the −10 and −35 boxes.

DISCUSSION

Plant-pathogenic bacteria like *P. syringae* pv. tomato DC3000 are exposed to a wide range of stresses throughout their life cycle. These bacteria survive as free-living organisms in the environment and transition to a pathogenic lifestyle upon encountering an appropriate host plant. These environments potentially present bacterial cells with various stresses and/or opportunities from which cells capable of responding stand to benefit. For example, plant tissues provide a nutrient-rich, moist, and sheltered environment. But, bacteria must also be able to tolerate or suppress antimicrobial plant immune functions. For *P. syringae*, surviving and proliferating in this environment require activation of the bacterial virulence program, a multifactorial process mediated by transcriptional regulators (66) and expression of virulence factors (phytotoxins, T3SS, and effectors). Here, we present several lines of evidence suggesting that AlgU is an important mediator of this process.

P. syringae pv. tomato DC3000 disease progression was influenced by AlgU, but eliminating alginate production did not compromise bacterial growth or symptoms in plants (Fig. 1). Additionally, supplementary expression of AlgU from a plasmid dramatically increased disease symptoms and fully complemented *in planta* growth but did not cause the strain's growth to exceed that of the wild type, suggesting that the virulence of each cell was increased. Taken together, we interpret these data as evidence supporting the idea that, in addition to alginate-related genes, AlgU also controls expression of other genes important for pathogenesis in response to plant-derived or -associated signals. It is not clear what signals activate AlgU *in planta*, but *P. syringae* pv. tomato DC3000 AlgU-dependent expression temporally correlates with plant cell-generated oxidative bursts associated with immune defense response (25). Presumably, AlgU activation is a response to stress and perturbations in the bacterial cell envelope (67).

The role of alginate in plant disease differs among pathovar-host interactions (19, 30), but in most of the pathosystems where it has been tested, alginate can be eliminated without altering disease progression or bacterial survival *in planta* (19, 21). This dis-

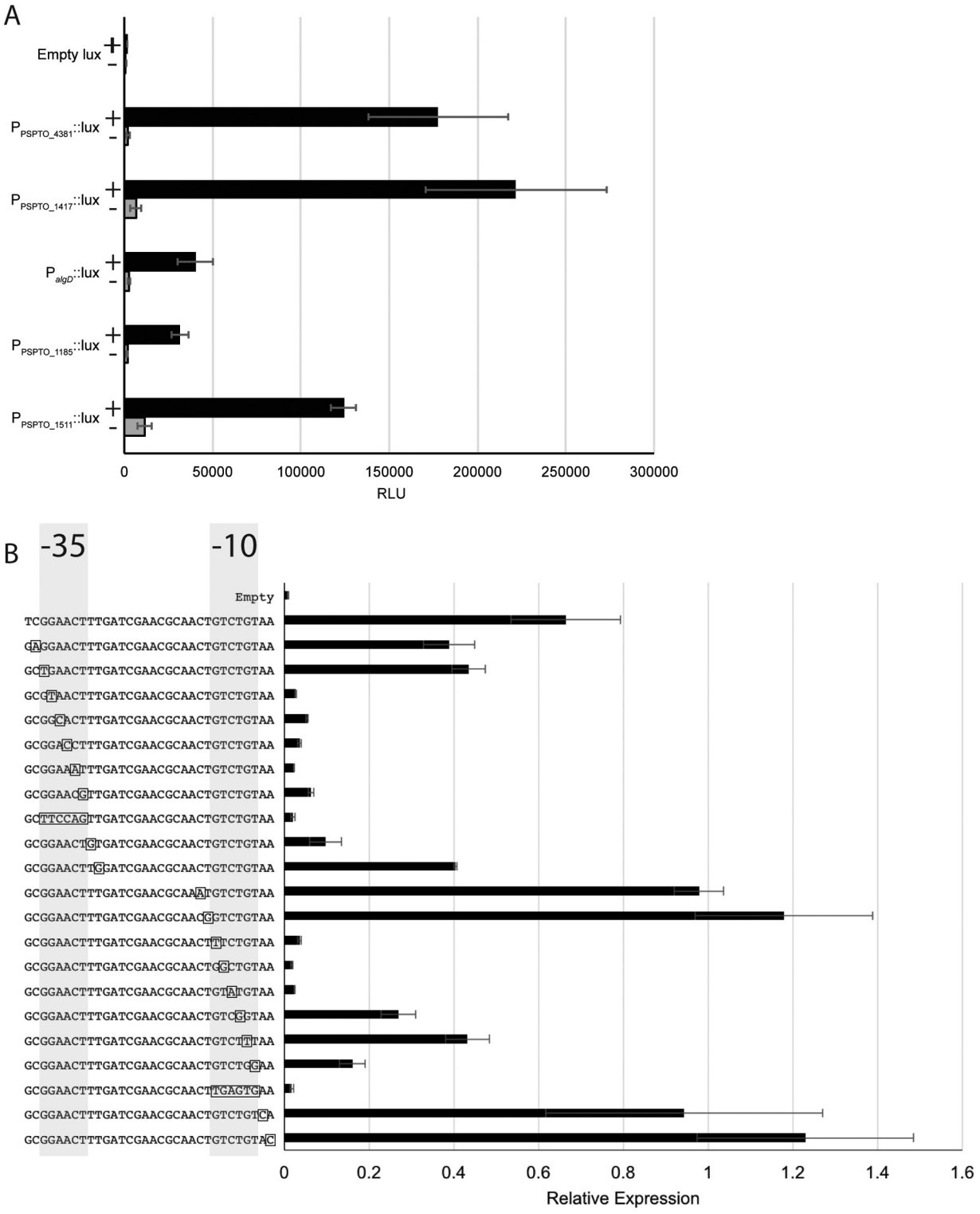

FIG 6 AlgU-dependent promoter motif transcriptional activity. (A) Transcriptional activity of *lux* promoter fusions in strains containing *algU* (+) or empty vector (−). Relative light units (RLU) are luminescence/OD_{600}. (B) Transcriptional activity of the wild-type P_{PSPTO_4381}::*lux* fusion and mutated derivatives. The boxed nucleotides indicate the mutated positions. Relative expression was calculated by comparing the RLU observed for each promoter with the RLU of the wild-type promoter (RLU_x/RLU_{WT}).

pensability likely results from the multifactorial and redundant functions that mediate disease. Alginate is thought to modulate plant immune responses by sequestration of apoplastic Ca^{2+} (28), but its contribution to disease may not have been apparent in this experiment, possibly because the Hrp effectors efficiently suppress immune function in host tomato plants (60, 68, 69), minimizing the need for other functions that suppress immune response propagation. The implication of this idea is that alginate's function in suppressing plant immune responses may be more apparent when the infection involves plants that are partially resistant to the pathogen.

AlgU is activated in response to periplasmic or cell envelope stress through an intricate regulatory cascade that involves proteins functioning in the periplasm and cytosolic compartments (14, 34, 70). MucA and MucB form a complex that transduces envelope stress information across the cytoplasmic membrane to modulate AlgU activity. In this study, *algU* was expressed from a plasmid using the arabinose-inducible P_{Bad} promoter. We were surprised to find that only marginal AlgU-dependent activity was detected using this expression strategy in wild-type *P. syringae* pv. tomato DC3000. However, much more robust AlgU activity was obtained by deleting the *mucAB* genes. This suggests that the anti-sigma factor activity of *mucAB* is capable of inactivating AlgU even when the sigma factor is expressed from a multicopy plasmid. This is consistent with the model that MucA and MucB work together to permanently inactivate AlgU molecules by targeting the sigma factor for proteolytic degradation (71). A similar result was observed in *P. syringae* pv. syringae FF5, where AlgU function could not be complemented in *trans* using a plasmid containing *algU*, *mucA*, and *mucB* but it could be complemented with a plasmid containing *algU* alone (11).

AlgU is similar to σ^E from *E. coli*, which regulates expression of genes involved with membrane homeostasis as well as other functions (33). Comparison of σ^E-regulated functions in *E. coli* and other enteric bacteria revealed that this sigma factor has the core and accessory functions in each organism (33). This idea posits that the sigma factor has adapted to regulate expression of a core set of functions that are conserved in all related species encoding an ortholog of the sigma factor and to regulate an accessory set of functions that differ between species and provide for the cell's needs when growing in a specific niche to which it has adapted (33). Here, we find evidence that the AlgU core function is dedicated to regulation of genes associated with alginate biosynthesis, oxidative stress, and osmotic stress resistance.

There is good evidence that AlgU is active in *P. syringae* pv. syringae B728a cells exposed to osmotic stress (21). However, based on data presented here and published data (22), osmotic stress exposure affects *P. syringae* pv. syringae B728a and *P. syringae* pv. tomato DC3000 cells differently. For example, only one *P. syringae* pv. tomato DC3000 gene (1/12 genes) that functions in alginate production was induced by salt stress (22); in contrast, all of these were strongly induced by AlgU in the present study (see Table S1 in the supplemental material). In this case, the one salt-induced alginate biosynthesis gene (*algC*) is influenced by AlgR, a response regulator, which is activated by salt stress in other pseudomonads (72). Additionally, the patterns of regulation for T3SS genes and related functions were also very different between NaCl- and AlgU-dependent conditions. Nearly all of these important pathogenesis genes (23/24) were regulated differently in comparison of AlgU- and salt-dependent gene expression in *P. syringae* pv. tomato DC3000, including 9 genes that were induced by AlgU but repressed in osmotic stress (22). But, as with many biological systems, the genes controlled by these processes are not entirely mutually exclusive. For example, we found that 44/102 NaCl-regulated genes (22) were also regulated by AlgU, and this set included genes that function in osmoadaptation, such as genes responsible for quaternary ammonium compound transport and metabolism and compatible solute synthesis. This suggests that AlgU has a role in osmoadaptation, but full activation of the *P. syringae* pv. tomato DC3000 AlgU regulon may be influenced by additional regulatory inputs.

The accessory regulon contains genes that enable bacterial survival under conditions specific to a particular pathogen-host interaction or other environments to which the bacterium has adapted (33). We found that 9% of the AlgU-regulated genes with annotated functions were explicitly dedicated to pathogenesis. The ability of *P. syringae* to infect and cause disease in plants is dependent on a type III secretion apparatus and a set of proteinaceous effector molecules that are translocated into plant cells at the site of infection (23). These factors are collectively known as the hypersensitive response and pathogenicity (Hrp) system because of the system's role in overcoming plant immunity in host plants to support pathogenesis and for conferring the hypersensitive response in noncompatible interactions. The expression of the Hrp system is controlled by a regulatory cascade that uses the σ^{54}-dependent activator and ECF sigma factor HrpRS and HrpL, respectively (66). These regulators function to activate *hrp* gene expression in response to plant-derived signals, possibly small organic acids (73). The data presented here indicate that in *P. syringae* pv. tomato DC3000, AlgU can be involved with Hrp system induction/expression. We found that a substantial portion of the HrpL-dependent regulon was upregulated by AlgU, including the *hrpRS* and *hrpL* genes. MACS2 (49, 50) analysis of AlgU ChIP-seq detected enrichment upstream of *hrpRS* (see Table S1 in the supplemental material), suggesting that *hrpRS* may be directly regulated by AlgU.

The requirement of AlgU for full disease symptom development and bacterial growth *in planta* is consistent with the hypothesis that AlgU is active in plants during infection and that genes regulated by AlgU contribute to disease processes. The finding that Hrp system expression is upregulated by AlgU in *P. syringae* pv. tomato DC3000 suggests that this portion of the AlgU regulon is responsible for the virulence deficit associated with the Δ*algU* mutant. However, it is unclear whether pathogenesis gene regulation is entirely responsible. For example, there was no evidence of AlgU-dependent regulation of *hrp* genes in other *P. syringae* pathovars (e.g., *P. syringae* pv. *syringae* strains), and yet they display a similar virulence defect (21).

We found 269 genes downregulated more than 2-fold in cells expressing AlgU. This type of regulation is most likely the result of AlgU-activated genes that encode negative regulators. The *fliC* gene had one of the largest differences in expression in comparing the $AlgU^+$ and $AlgU^-$ cells (18-fold downregulated by AlgU). Current models of flagellar regulation propose that AlgU downregulates *fliC* expression by inducing *amrZ*, which represses *fleQ* expression; in the absence of FleQ, the *fliC* gene is not expressed (74, 75). AlgU binding was detected upstream of *amrZ* along with a 3.4-fold AlgU-dependent upregulation. However, based on data presented here, the model is unlikely to apply to *P. syringae* pv. tomato DC3000. We did not observe a change in *fleQ* expression,

and *amrZ* overexpression did not reduce motility (data not shown). The mechanism for this regulation has yet to be determined, but these data are consistent with a model in which AlgU reduces flagellar gene expression in response to plant signals, thereby reducing PAMP exposure to the benefit of the microbe during infection (31).

The data presented here may be useful for understanding how *P. syringae* pv. tomato DC3000 coordinates the expression of multiple systems to assist microbial survival under changing environmental conditions. We found that AlgU coordinates expression of multiple systems that collectively aid bacterial survival and growth in plant tissue and the development of disease symptoms. This included activation and repression of functions that reprogram the bacterial cells, enabling them to exploit plants for nutrients and shelter from other environmental stressors.

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Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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