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Characterization of Regulatory Pathways in *Xylella fastidiosa*: Genes and Phenotypes Controlled by *algU*▽†

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Many virulence genes in plant bacterial pathogens are coordinately regulated by "global" regulatory genes. Conducting DNA microarray analysis of bacterial mutants of such genes, compared with the wild type, can help to refine the list of genes that may contribute to virulence in bacterial pathogens. The regulatory gene *algU*, with roles in stress response and regulation of the biosynthesis of the exopolysaccharide alginate in *Pseudomonas aeruginosa* and many other bacteria, has been extensively studied. The role of *algU* in *Xylella fastidiosa*, the cause of Pierce's disease of grapevines, was analyzed by mutation and whole-genome microarray analysis to define its involvement in aggregation, biofilm formation, and virulence. In this study, an *algU*::*nptII* mutant had reduced cell-cell aggregation, attachment, and biofilm formation and lower virulence in grapevines. Microarray analysis showed that 42 genes had significantly lower expression in the *algU*::*nptII* mutant than in the wild type. Among these are several genes that could contribute to cell aggregation and biofilm formation, as well as other physiological processes such as virulence, competition, and survival.

Xylella fastidiosa is a xylem-limited, gram-negative, plant-pathogenic bacterium that is transmitted by insects such as the glassy-winged sharpshooter, *Homalodisca coagulata* (48). Strains of *X. fastidiosa* cause many diseases, including citrus variegated chlorosis, leaf scorch of live oak, pear leaf scorch, and Pierce's disease (PD) of grape (10, 46), which threatens the grapevine industry in the United States. The characteristic symptoms of PD are scorched leaves, matchstick (petioles attached to the cane after the scorched leaf blades have abscised), and green islands (patches of green tissue surrounded by brown tissue on infected canes). Eventually, the fruit desiccates, vine cordons die back, and diseased grapevines can die 2 to 3 years after infection (47). Genetic resistance to *X. fastidiosa* is not available in most commercial wine grape varieties, although several relatives of grape, such as *Vitis tiliifolia*, can harbor high populations of *X. fastidiosa* without typical symptoms of PD (16, 31). Currently, the control of insect vectors is the main management strategy for PD. Successful biocontrol of insect vectors depends on the insects' ecology and field environmental conditions. The identification of genetic factors that enable *X. fastidiosa* to express PD symptoms could lead to new disease management strategies, including new targets for disruption of the disease process.

Although the mechanisms of *X. fastidiosa* pathogenicity are not completely understood, the major symptoms of most of the diseases caused by this pathogen are similar to water stress, probably resulting from blockage of the xylem transport system (37). Previous studies have shown that *X. fastidiosa* is embedded in an extracellular translucent extracellular polysaccharide (EPS)-matrix biofilm within xylem vessels (44). These two observations suggest that *X. fastidiosa* cells can form bacterial aggregates (biofilm-like colonies) containing EPSs that occlude the xylem vessels, resulting in blockage of water transport and causing PD symptoms.

Sigma factors control virulence and pathogenicity factors in various bacterial pathogens in response to different environmental conditions (13, 52). *algU* encodes an alternate sigma factor, AlgU, that is highly conserved in gram-negative bacteria (17, 30) and confers tolerance to osmotic, oxidative, and heat stresses. Its role in the regulation of the biosynthesis of the EPS alginate has been extensively studied in the human pathogen *Pseudomonas aeruginosa* and the plant pathogen *P. syringae* (17, 30). Alginate may play a role in biofilm-related phenomena, including contribution to adhesion and antibiotic resistance in *P. aeruginosa* (17). Alginate is also involved in colonization by and dissemination of the plant pathogen *P. syringae* in planta (60). Although a homolog of *algU* is present in the genome of *X. fastidiosa* (53), its role in *X. fastidiosa* is unknown. In this study, we analyzed the effect of an insertional mutation in the *algU* gene of *X. fastidiosa*, performed whole-genome microarray analysis of gene expression in the mutant, and identified genes whose expression is controlled by *algU*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All of the bacterial strains and plasmids used in this work are listed in Table 1. For growth profile, aggregation, adhesion, colony morphology determination, and biofilm formation, bacterial strains were cultured on PD3 medium (12) supplemented with 0.8% Gelrite instead of agar. After 7 days at 28°C, cells were harvested with a scraper (Fisher Scientific, CA), washed and resuspended in 1 ml of PD3 broth, and adjusted to an optical density at 600 nm (OD_{600}) of 0.10. Bacterial cells used for pathoge-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Bacterial strains		
Escherichia coli DH5α	DH1 F^- φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169*	N. T. Keen
Xylella fastidiosa	Wild-type *X. fastidiosa* A05	10
X. fastidiosa algU::*nptII*	Tn*5* insertional mutation in *algU* homologue of *X. fastidiosa* A05	This work
Plasmids		
pGEM-T Easy	Ap^r; cloning vector	Promega
pUC129	Ap^r; cloning vector	New England BioLabs
pUC1284	Ap^r; 0.891-kb fragment including *algU* ORF cloned into pUC129	This work
pUC12841	Ap^r Km^r; Tn*5* insertion within *algU* ORF in pUC1284	This work

nicity tests were cultured for 5 days at 28°C on PW (27) Gelrite medium, harvested, and adjusted to the same OD_{600} as above with sterile water. When required, antibiotics were added as follows: ampicillin, 100 μg/ml; kanamycin, 10 μg/ml. All strains were stored in PD3 broth with 15% glycerol at −80°C.

Construction of an *algU*::*nptII* mutant. A 0.891-kb region of *X. fastidiosa* genomic DNA including the *algU* (PD1284) open reading frame (ORF) was amplified with Vent polymerase (New England BioLabs, Ipswich, MA) with primers algUP1 and algUP2 (see Table S1 in the supplemental material) and cloned into the SmaI site of pUC129 (Table 1) to make pUC1284. The PCR fragment in pUC1284 was sequenced to confirm the presence of an intact *algU* ORF. pUC1284 DNA was mutagenized with the Tn*5* transposon with the EZ::TN <KAN-2> insertion kit (Epicentre Biotechnologies, Madison, WI) as outlined by the manufacturer. A plasmid with a Tn*5* insertion within the *algU* ORF in pUC1284 was named pUC12841. Tn*5* insertions in pUC12841 were precisely mapped by sequencing with transposon primers Kan-2 FP-1 or Kan-2 RP-1 (see Table S1 in the supplemental material).

X. fastidiosa electrocompetent cells of strain A05 isolated from Temecula (10) were prepared according to previously published procedures (18). One to two micrograms of pUC12841 DNA in a volume of 5 μl was electroporated into the cells in a 0.1-cm-gap cuvette at 1.8 kV, 200 Ω, and a capacitance of 25 μF in a GenePulser (Bio-Rad, Hercules, CA) with time constants of about 4 ms. The electrocompetent cells alone and PD3 broth with no bacterial cells served as negative controls. Electroporated cells were grown for 24 h in PD3 broth with shaking and plated on PD3 Gelrite medium supplemented with 10 μg/ml kanamycin as previously described to select for replacement of wild-type *algU* with *algU*::*nptII* by homologous recombination (20).

Genomic DNA extraction and confirmation of the *algU*::*nptII* mutant. Wild-type *X. fastidiosa* or the *algU*::*nptII* mutant strain was cultured in 50 ml PD3 broth at 28°C for 7 to 10 days with or without antibiotics. The genomic DNAs were extracted with a MasterPure DNA purification kit (Epicentre Biotechnologies). The insertion of the construct into the genome of the *algU*::*nptII* mutant was confirmed by PCR with primers M13For/Rev and algUORF P1/P2, respectively (see Table S1 in the supplemental material). A. 0.891-kb fragment from the wild type and a 2.1-kb fragment from the mutant were cut from the gel, cloned into pGEM-T Easy (Promega, Madison, WI) (Table 1), sequenced, and compared with *X. fastidiosa* genomic sequences or Tn*5* transposon sequences with Vector NTI (Invitrogen, CA), respectively. The Tn*5* insertion within the *algU* ORF of *algU*::*nptII* genomic DNA was determined by sequencing with transposon primers Kan-2 FP-1 or Kan-2 RP-1 (see Table S1 in the supplemental material).

Colony morphology, growth curves, surface attachment, and cell aggregation. The colony morphologies of the *X. fastidiosa* wild-type and *algU*::*nptII* mutant strains were analyzed after 10 to 14 days of growth at 28°C by plating 100 μl of 0.10 OD_{600} cell suspensions on PD3 Gelrite plates. In vitro growth curves were determined in 3 ml of PD3 broth after 3 to 21 days of growth at 28°C. Because of the aggregation of the cells in broth, immediately after inoculation and 3, 6, 9, 12, 15, 18, and 21 days later, the cells were dispersed by repeated pipetting or vortexing. Cell concentration was determined by measuring the OD_{600}. For cell aggregation analysis, strains were grown in 25 ml of PD3 broth in petri dishes and incubated at 28°C without shaking for 4 days. After growth, the content of each petri dish was pipetted into a tube and the cells were dispersed by vortexing or pipetting and adjusted to an OD_{600} of 0.10. Two hundred microliters of each culture was then subcultured into eight tubes, each with 25 ml of fresh PD3 broth. Tubes were allowed to stand in the incubator without shaking. Three days after incubation, the tubes were vortexed and the OD_{540} (OD_t) was measured. The concentration of bacterial cells was also measured by determining the OD_{600}. These tubes were kept without shaking for 1 h to allow the bacterial cells to clump and settle. The OD_{540} of supernatants of the tubes (OD_s) was again measured. The relative percentage of cell aggregation was measured by using the following formula: % aggregation = $(OD_t - OD_s)/(OD_t \times 100)$ (5). This procedure was repeated for the remaining seven tubes of each culture at 6, 9, 12, 15, 18, 21, and 24 days after the initial incubation.

Biofilm formation. *X. fastidiosa* wild-type and *algU*::*nptII* mutant cells were cultured in PD3 broth and incubated at 28°C without shaking for 4 to 6 days. Bacterial cells were collected and adjusted to an OD_{600} of 0.10. One-hundred-fifty-microliter aliquots of each culture were added to wells of 96-well microtiter plates. The negative control consisted of PD3 broth without bacteria. Plates were incubated at 28°C without shaking. At 3, 6, 9, 12, and 15 days after incubation, biofilm formation on the wall of the wells was determined by a crystal violet staining method (33). Each treatment had three replications, and the data were averaged.

LPS gel analysis. Lipopolysaccharide (LPS) fractions were prepared by a mini-phenol-water extraction technique as described by Guihabert et al. (19). Twenty microliters of dissolved LPS in polyacrylamide gel electrophoresis (PAGE) sample buffer (0.3% Tris base, 0.2% glycerol, 0.05% bromophenol blue) was loaded and separated by deoxycholic acid-PAGE with 18% acrylamide in the bilayer stacking gel. Gels were silver stained and stored in water (28).

Tolerance of the *algU*::*nptII* mutant to desiccation stress in vitro. The sensitivity of wild-type *X. fastidiosa* and the *algU*::*nptII* mutant to desiccation on filters was assessed by a modification of the procedure described by Ophir and Gutnick (43). Seven- to 10-day-old cultures were collected and adjusted to an OD_{600} of 0.10 with sterile distilled water and serially diluted to 1×10^{4} CFU/ml. One milliliter of each dilution was vacuum filtered onto Millipore filters (no. HAWP04700; pore size, 0.25 μm; diameter, 3.5 cm). The filters were placed in petri dishes at 25°C for slow drying. At 0, 2, 4, 6, 8, 10, 12, and 14 days, filters were placed onto PD3 agar plates and incubated at 28°C for 3 weeks. Filters without dilutions, incubated for the same period of time, served as controls. The number of colonies on each filter was recorded. Each treatment consisted of five filters and was repeated three times.

Susceptibility to oxidative stress. Sensitivity to hydrogen peroxide (H_2O_2) or sodium hypochlorite (NaOCl) was examined as described by Martin et al. (38). Millipore filter disks (diameter, 6 mm) were soaked with 10 μl of H_2O_2 (3 or 12%, vol/vol) or NaOCl (3 or 6%, vol/vol) and placed on PD3 Gelrite plates on which 100 μl of 7-day-old cultures of wild-type *X. fastidiosa* or the *algU*::*nptII* mutant were spread with a glass rod. The diameters of the inhibition zones surrounding the impregnated disks were measured after 14 to 21 days of incubation at 28°C. Three disks were placed in each treatment, each treatment was repeated three times, and the results were averaged.

Pathogenicity assays on grapes. Wild-type *X. fastidiosa* and the *algU*::*nptII* mutant were grown on PW Gelrite medium for 5 days at 28°C, suspended in sterile deionized water, and adjusted to an OD_{600} of 0.10. Five to 10 20-μl drops of each suspension were used to inoculate 5 to 10 canes on plants of *Vitis vinifera* var. Pinot Noir by a needle inoculation procedure as previously described (24). Water inoculation served as a negative control. The inoculated vines were kept on the benches in a greenhouse with 75% humidity. The vines were observed for symptom development approximately every 2 weeks for 5 months after inoculation. The symptoms were rated on a visual scale of 0 to 5 as described before (19). Briefly, 0 represented healthy grape vines without scorched leaves (water control) and 5 represented plants with all leaves with heavy scorching or numerous matchsticks. The final disease index was an average of 10 independent replications for each *X. fastidiosa* strain.

Recovery and determination of populations of *X. fastidiosa* from inoculated grapes. To recover and confirm the bacteria in inoculated grapes, 12 weeks after inoculation, petiole tissues (2 to 3 cm) from each vine inoculated with either *X. fastidiosa* wild-type or *algU*::*nptII* mutant cells were harvested at the inoculated points, as well as 25 cm and 50 cm above the inoculation points. Tissues were washed once with deionized water containing Tween 20; surface sterilized for 1 min in 20% commercial bleach, 1 min in 2% sodium hypochlorite, and 1 min in 70% ethanol; and rinsed three times in sterile deionized water. The samples were ground in 100 μl of sterile deionized water and cultured on PD3 and PW Gelrite media with or without kanamycin. After incubation for 21 days at 28°C, the identity of *X. fastidiosa* cells on PD3 Gelrite plates was confirmed by PCR with primers specific for wild-type *X. fastidiosa*, i.e., algUORFP1/P2 and tapBPD1993P1/P2 (see Table S1 in the supplemental material) (data not shown).

To determine the bacterial populations 16 weeks after inoculation, 2- to 3-cm petiole tissues of each vine inoculated with the *X. fastidiosa* wild-type and mutant strains were harvested and treated as described above. Tissues were tested by enzyme-linked immunosorbent assay (ELISA) with a PathoScreenXF kit according to the manufacturer's (Agdia Inc., IN) instructions. The antibodies used in the Agdia ELISA system are a mixture of polyclonal antibodies raised to whole cells of three serologically distinct isolates of *X. fastidiosa* (Agdia Inc.). The PD3-cultured *X. fastidiosa* wild-type and *algU*::*nptII* mutant cells were resuspended in phosphate-buffered saline (PBS; Agdia Inc.) and used to confirm that the ELISA worked equally well for quantifying the wild-type and mutant populations. Developed plates were measured at 650 nm with a SpectraMax microplate reader via SoftMaxPro (version 3.1.2; Molecular Devices Corp., CA). Bacterial populations were calculated via OD_{650} determination in comparison to the positive control (purified *X. fastidiosa* cell PBS suspension).

SEM. *X. fastidiosa* wild-type and mutant cells in grapevine xylem were examined by scanning electron microscopy (SEM) (57). Petiole samples were collected above the inoculation points from symptomatic grapevines 12 weeks after inoculation. Preparation and observation of the samples by SEM were carried out in the University of California Riverside Central Facility for Advanced Microscopy and Microanalysis. Petioles of five leaves from symptomatic grapevines were cross-sectioned with a fine razor blade and immersed for 24 h in a modified Karnovsky solution. Samples were washed twice with ultrapure water for 30 min, and sections were transferred to a 1% aqueous solution of osmium tetroxide and incubated for 24 h at 4°C. Samples were subsequently dehydrated for 20 min each in an alcohol solution series (20, 30, 40, 50, 60, 70, 80, 90, and 100%), and the solvents were removed by vacuum (critical point dried) (57). The samples were mounted according to the manufacturer's instructions and observed with a Philips XL30 ESEM-FEG electron microscope. All images were recorded at a working distance of 9 mm according to the standard procedure with an accelerating voltage of 20 kV.

RNA isolation, quantification, and RT-PCR. A modified hot-phenol RNA preparation procedure was used to extract total RNA from *X. fastidiosa* wild-type and mutant strains (7, 32). Bacterial cultures were incubated in 50 ml of PD3 broth at 28°C for 5 days under constant agitation. After the hot-phenol extraction, RNA was suspended in RNase-free distilled H_2O and DNase treated with the Turbo DNA-free DNase (2 U/μl) (Ambion, TX). To ensure that the RNA preparation was DNA free, an aliquot of 1 μl of RNA (500 ng/μl) was then used to amplify the ORF of *algU* with algUORFP1/P2 primers (see Table S1 in the supplemental material). The quality of isolated RNAs was determined by denaturing RNA formaldehyde gel electrophoresis (7). The expression of *algU* was analyzed by reverse transcription (RT)-PCR with the AccessQuick RT-PCR system by following the manufacturer's (Promega) instructions.

Microarray hybridizations and microarray data analysis. The gene expression profiles of wild-type *X. fastidiosa* and the *algU*::*nptII* mutant were analyzed with a NimbleGen prokaryotic gene expression array (NimbleGen System Inc., WI). DNA microarray chips were designed with 24-mer oligonucleotides according to the available *X. fastidiosa* genomic sequences. The expression levels of RNAs were averaged from three technical replications in a single hybridization experiment. The raw data were analyzed with the ArrayStar FirstLight. The expression levels of 2,188 genes under treatment (*algU*::*nptII*) and control (wild type) were analyzed (21). The hybridization signal intensity obtained from the wild-type or mutant RNA was normalized according to the total signal strength. The normalized hybridization signals were log plot analyzed for reliability (21) and were statistically analyzed by Student's *t* test ($P < 0.001$) for differential expression. The normalized signal intensity of the mutant was divided by that of the wild type to calculate the mutant/wild-type (M/W) ratio. M/W ratios obtained from individual hybridization experiments were averaged to give the final M/W ratio. Genes having ≥1.5 or ≤0.66 final M/W ratios were selected as mutated gene up-regulated or mutated gene down-regulated, respectively.

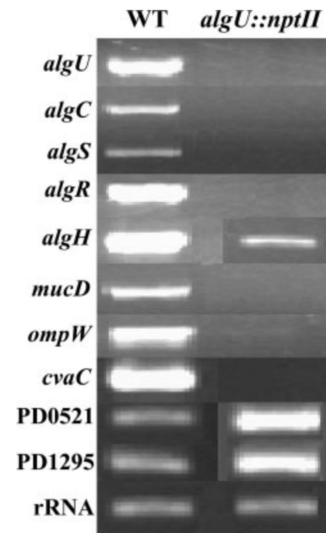

FIG. 1. RT-PCR of genes differentially expressed between wild-type (WT) *X. fastidiosa* and the *algU*::*nptII* mutant. rRNAs were detected in the *algU*::*nptII* mutant and the wild type in this RT-PCR condition. The *algU*, *algC*, *algS*, *algR*, *algH*, *mucD*, *ompW*, and *cvaC* (PD0216) RNAs were decreased in the mutant compared to the wild type, and the PD0521 and PD1295 RNAs were slightly increased in the *algU*::*nptII* mutant.

Validation of microarray data. To validate the differential expression data obtained in microarray analysis, RT-PCR and PCR experiments were performed with specific primers designed to amplify an internal region of the ORF of each target gene (see Table S1 in the supplemental material). Several up-regulated and potential virulence-related genes were chosen, and primers were designed for their ORFs according to the *X. fastidiosa* Temecula1 genome sequences. cDNA was amplified from stored DNase-cleaned RNAs with the AccessQuick RT-PCR system by following the instructions of the manufacturer (Promega). The amplification conditions used were 45 min at 45°C for RT; 35 cycles of 2 min at 55°C for initial denaturation, 1 min at 55°C for annealing, and 2 min at 72°C for extension; and a final extension of 10 min at 72°C. Five microliters of the reaction mixture was run in agarose gels, and products were stained with ethidium bromide.

RESULTS

Confirmation of the mutational insertion and physiological properties of an *algU*::*nptII* mutant. The *algU*::*nptII* mutant had the Tn*5* transposon inserted in the *algU* ORF 79 bp downstream from the ATG start codon. After streaking five to eight times on PD3 Gelrite medium with 10 μg/ml kanamycin, the mutant still grew well, indicating that the mutant had stable genetic characteristics. cDNAs amplified by RT-PCR with algUORFP1/P2 (see Table S1 in the supplemental material) showed that there was no expression of *algU* within the *algU*::*nptII* mutant cells, while strong expression was detected in wild-type cells (Fig. 1). The in vitro growth curves of the wild-type and *algU*::*nptII* strains over 21 days were similar (data not shown). In PD3 broth, the wild-type strain formed large aggregates whereas the *algU*::*nptII* mutant grew in less aggregated clumps (Fig. 2A). An OD assay was used to quantify the effect of the *algU*::*nptII* mutation on cell-to-cell aggregation and showed that the percentage of aggregated cells of *algU*::*nptII* was significantly lower than that of the wild type (Fig. 2B).

Cells of the wild type attached to the surface of the flasks

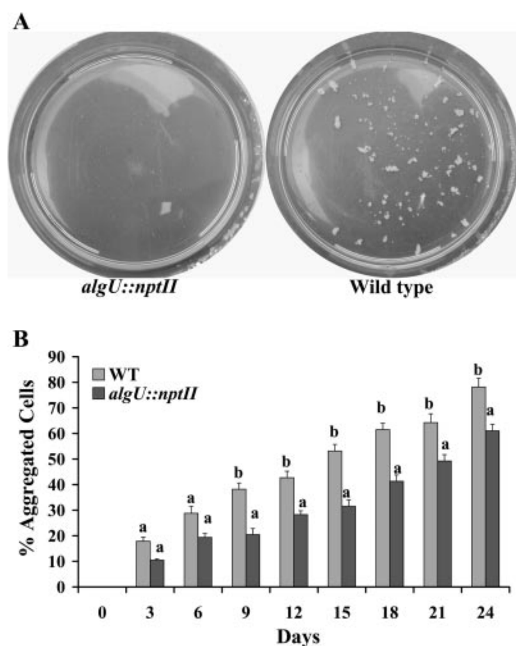

FIG. 2. Cell-to-cell aggregation of wild-type *X. fastidiosa* and the *algU*::*nptII* mutant. (A) Cell-to-cell aggregations of the *algU*::*nptII* mutant (left) and wild-type *X. fastidiosa* (right) in PD3 broth in petri dishes. (B) Quantitative assessment of cell-to-cell aggregation of wild-type (WT) *X. fastidiosa* or the *algU*::*nptII* mutant by an OD assay as previously described (5). Three replicates were used in each experiment. For each assay time, different letters indicate significant differences (Student's *t* test, $P < 0.05$) between the wild type and the mutant.

and formed wide rings, while the *algU*::*nptII* mutant cells attached to the surface formed lighter rings (data not shown). This indicated that the mutant had a reduced surface attachment ability, resulting in reduced biofilm formation. The ability of the *algU*::*nptII* mutant to form biofilm was investigated further by a crystal violet staining method. The wild type formed more biofilm in PD3 medium than did the mutant (Fig. 3). Deoxycholate-PAGE analysis showed that there was no significant alteration in the purified LPS profile of the *algU*::*nptII* mutant grown in vitro compared with the LPS profile of the wild type (data not shown).

TABLE 2. Tolerance of wild-type *X. fastidiosa* and the *algU*::*nptII* mutant to desiccation stress in vitro

Genotype	Survival rate (%) of *X. fastidiosa* cells after drying on filters[a] for:				
	0 days	2 days	4 days	6 days	8 days
Wild type	100	51.4§	7.9§	0.7¶	0¶
algU::*nptII*	100	17.6¶	0.7¶	0¶	0¶

[a] Data are averages of three independent replications for each treatment. Five filters per dilution were used in each treatment. For each assay date, different symbols indicate a significant difference (Student's *t* test, $P < 0.05$) between the wild-type and mutant survival rates. The data for days 10 to 14 were all 0 and are not shown.

Tolerance to oxidative and desiccation stresses. When wild-type *X. fastidiosa* and the *algU*::*nptII* mutant were exposed to desiccation stress in vitro in petri dishes, the survival rate of the mutant differed significantly from that of the wild type (Table 2), indicating that the mutant died off faster than the wild type. No significant differences in tolerance to oxidative stress (sensitivity to hydrogen peroxide or sodium hypochlorite) were observed between the mutant and wild-type strains (data not shown).

Pathogenicity tests and recovery from infected plants. Grapevines inoculated with the *algU*::*nptII* mutant showed significantly less severe disease symptoms 12 to 20 weeks after inoculation than those inoculated with the wild type (Fig. 4). Water-inoculated control grapevines did not show any PD symptoms. All diseased grapevines were positive, and the asymptomatic water control grapevines were negative for the presence of *X. fastidiosa* when the plants were examined by ELISA. *X. fastidiosa* wild-type and *algU*::*nptII* mutant cells were also examined by SEM in grapevine xylem vessels 12 weeks after inoculation (data not shown), confirming the successful survival of cells of wild-type *X. fastidiosa* and the *algU*::*nptII* mutant in grapevine xylem vessels.

Bacteria were reisolated from macerated inoculated grapevine petioles on PD3 and PW Gelrite media. The bacterial genotypes were confirmed as wild-type *X. fastidiosa* or the *algU*::*nptII* mutant by PCR amplification with primers algUORFP1/P2 and tapBPD1993P1/P2 (see Table S1 in the supplemental material) (data not shown).

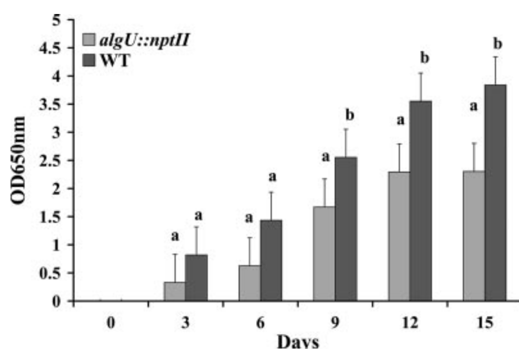

FIG. 3. Analysis of biofilm formation of wild-type (WT) *X. fastidiosa* and the *algU*::*nptII* mutant. Three replicates were used in each experiment. For each assay time, different letters indicate significant differences (Student's *t* test, $P < 0.05$) between the wild type and the mutant.

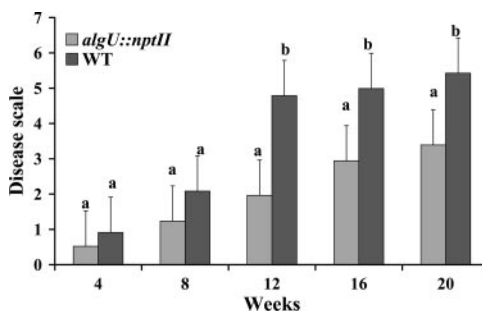

FIG. 4. PD progression in grapevines inoculated with wild-type *X. fastidiosa* and the *algU*::*nptII* mutant. Disease severity was based on a visual disease scale of 0 to 5 and was assessed 4, 8, 12, 16, and 20 weeks after inoculation (19). The data are an average of 10 independent replications. For each time point, different letters indicate significant differences (Student's *t* test, $P < 0.05$) between the wild type and the mutant.

TABLE 3. Bacterial populations in grapevines 16 weeks after inoculation

Strain A05 genotype	Population (10^6 CFU/g of tissue)[a]		
	At inoculated point	Above inoculated points	
		25 cm	50 cm
Wild type	9.087 ± 2.6§	0.609 ± 0.18§	0.646 ± 0.28§
algU::*nptII*	1.267 ± 2.1¶	0.067 ± 0.013¶	0.063 ± 0.031¶

[a] Data are averages of 10 independent replications for each treatment. Each treatment had five samples. For each assay point, different symbols indicate a significant difference (Student's t test, $P < 0.05$) between the wild-type and mutant populations. The data for asymptomatic water control were all 0 and are not shown.

To gain further understanding of the mechanisms that could explain why the *algU*::*nptII* mutant had reduced virulence, bacterial populations and bacterial movement in infected grapevines were estimated from ELISAs. The ELISA showed no cross-reaction with any healthy tissue tested. Preliminary experiments showed that the ELISA used to quantify the *X. fastidiosa* populations worked equally well for wild-type and mutant cultures. Bacterial populations at inoculation points and at 25 cm and 50 cm above inoculation points were estimated from ELISAs by comparing the OD_{650} with that of the positive control *X. fastidiosa* with known concentrations (Table 3). An OD_{650} of the *X. fastidiosa* positive control (purified *X. fastidiosa* cells in a PBS suspension) of 1 represented approximately 1×10^4 CFU/ml. The average bacterial populations were calculated by comparing their OD_{650} values to that of the positive control and dividing by the average weight of 2- to 3-cm sampled petioles. There were no *X. fastidiosa* cells detected in the asymptomatic water-inoculated control grapevines. The cell population of the *algU*::*nptII* mutant was less than that of the wild type at 25 cm and 50 cm above inoculation points (Table 3). The actual populations could have been larger than we reported, since those were calculated on the basis of *X. fastidiosa* cultures in PBS rather than plant sap. Plant sap could lower ELISA detection. However, it is the relative difference between the wild type and the mutant that is significant (Table 3). These data suggest that the mutated *algU* gene may affect the growth and possibly the movement of *X. fastidiosa* inside the xylem, resulting in reduced pathogenicity.

DNA microarray analysis of gene expression in vitro. The expression levels of 2,188 genes were monitored in the wild-type and *algU*::*nptII* mutant strains. The expression levels of RNAs were averaged from three technical replications in a single hybridization experiment. The normalized hybridization signals formed a linear pattern after the log plot analysis, indicating that the hybridization signals were stable, repeatable, and reliable (data not shown). rRNA of *X. fastidiosa* was detected in wild-type *X. fastidiosa* and the *algU*::*nptII* mutant (Fig. 1), indicating that this RT-PCR condition was reliable. Differential expression of *algU*, *algC*, *algS*, *algR*, *algH*, *mucD*, *ompW*, *cvaC* (PD0216), PD0521, and PD1295 was validated by RT-PCR (Fig. 1). Forty-three genes were differentially expressed in the *algU*::*nptII* strain compared to the wild type (Table 4). One gene (PD1926), predicated to encode a fimbrial protein, was shown to be negatively regulated by *algU* in the *X. fastidiosa* wild type. The other 42 differentially expressed genes appear to be positively regulated by *algU* in the *X. fastidiosa* wild type, including the following: genes predicted to function in macromolecular metabolism, intermediary metabolism, cell structure, and cellular processes; mobile gene elements; genes that encode conserved and hypothetical proteins; ORFs with undefined functions; and pathogenicity, virulence, and adaptation genes, according to the functional groups (Table 4).

DISCUSSION

An *X. fastidiosa algU*::*nptII* mutant did not differ in growth rate in vitro compared to the wild type, but it had reduced aggregation and attachment to surfaces, suggesting that *algU* may play roles in the synthesis of proteins and other molecules which are related to attachment. The *algU*::*nptII* mutant had reduced biofilm formation and reduced virulence on grapevine, suggesting that *algU* may play a role in biofilm and virulence. The mechanism of resistance of grapevines to infection includes the restriction of *X. fastidiosa* to fewer xylem vessels (15, 41). Decreased vessel-to-vessel movement of cells inside the xylem results in a delay of systemic infection and disease development (19, 25). The populations of the *algU*::*nptII* mutant at 25 cm and 50 cm above the inoculation points were reduced compared to those of the wild type (Table 3) but were also significantly smaller at the inoculation points. Thus, we cannot determine from these data specifically whether the *algU*::*nptII* mutant had decreased vessel-to-vessel movement inside the xylem. In vitro desiccation stress tolerance in plant pathogens has been correlated with resistance to dehydration in vivo and an increased ability to form microbial biofilms (22, 51). Wild-type *X. fastidiosa* tolerated desiccation stress for about 2 to 3 days, while the *algU*::*nptII* mutant did not tolerate desiccation as well as the wild type. The mechanism of toleration to desiccation related to *algU* in *X. fastidiosa* remains to be determined.

AlgU is a member of a family of alternative sigma factors, σ^E (*rpoE*), which are only distantly related to σ^{70} (49, 50). Environmental stresses induce the transcription of *algU* in *P. aeruginosa* (52), and virulence or persistence factors are controlled by σ^E (AlgU) (13). *X. fastidiosa* is exposed to a range of variable stress factors inside the xylem of plants (1), such as changes in osmolarity, availability of nutrients, and agents generating reactive oxygen intermediates. Gene expression profiles of the *algU*::*nptII* mutant of *X. fastidiosa* compared to those of the wild type via microarray analysis revealed that *algU* regulates various factors that could contribute to survival under the environmental conditions present in the xylem.

Although several of the *P. aeruginosa* alginate genes (*algA*, *algD*, *algG*, *algF*, *algI*, and *algJ*) were not found in the *X. fastidiosa* genome (53), other genes involved in alginate biogenesis in *P. aeruginosa*, such as *mucD* (PD1286), *algR* (PD1153), *algH*(PD1276), *algC*(PD0120), and *algS* (PD0347), are present and had decreased expression in the *algU*::*nptII* mutant of *X. fastidiosa*. In *P. aeruginosa*, the *algC* gene encodes a bifunctional enzyme that is involved in alginate production (phosphomannomutase activity) and LPS production (phosphoglucomutase activity) (11). Thus, the function of alginate homolog genes in *X. fastidiosa* may be involved not in alginate biosynthesis but rather in the synthesis of LPS or another form of EPS, either of which could play a role in biofilm formation and cell attachment. However, the purified LPS profile of the

TABLE 4. Genes differentially expressed in the *X. fastidiosa algU*::*nptII* mutant in vitro, organized by functional groups

Functional group and gene[d]	ORF	Description	M/W ratio[a,b]	Expression in mutant[c]
Macromolecule metabolism				
Protein metabolism/degradation				
clpS	PD0664	ATP-dependent Clp protease adaptor; posttranslational modification	0.448	Lower
clpA	PD0665	ATP-dependent Clp protease subunit; posttranslational modification	0.485	Lower
clpB	PD1685	ATP-dependent Clp protease subunit; posttranslational modification	0.421	Lower
mucD	PD1286	Periplasmic protease	0.616	Lower
Protein metabolism/chaperones				
grpE	PD1371	Heat shock protein GrpE	0.40	Lower
dnaK	PD1370	Heat shock protein Hsp70; cochaperones are DnaJ and GrpE	0.378	Lower
RNA metabolism/ribosomal proteins				
rpmH	PD2123	50S ribosomal protein L34; unknown function	0.449	Lower
rplW	PD0439	50S ribosomal protein L23; unknown function	0.499	Lower
rplP	PD0444	50S ribosomal protein L16; unknown function	0.426	Lower
rpmC	PD0445	50S ribosomal protein L29; unknown function	0.479	Lower
rplN	PD0447	50S ribosomal protein L14; unknown function	0.477	Lower
rpsN	PD0450	30S ribosomal protein S14; unknown function	0.244	Lower
rpsH	PD0451	30S ribosomal protein S8; unknown function	0.256	Lower
rplF	PD0452	50S ribosomal protein L6; unknown function	0.323	Lower
rpsE	PD0454	30S ribosomal protein S5; unknown function	0.437	Lower
rpmD	PD0455	50S ribosomal protein L30; unknown function	0.348	Lower
rpsM	PD0458	30S ribosomal protein S13; unknown function	0.394	Lower
rpmB	PD0488	50S ribosomal protein L28; unknown function	0.436	Lower
rpmG	PD0489	50S ribosomal protein L33; unknown function	0.353	Lower
rpmE	PD0749	50S ribosomal protein L31; unknown function	0.350	Lower
Intermediary metabolism				
Energy metabolism, carbon/tricarboxylic acid cycle				
gltA	PD0750	Citrate synthase; energy production and conversion	0.496	Lower
Regulatory functions/sigma factors and other regulatory components				
csrA	PD0095	RsmA homologue; regulates the production of virulence determinants	0.403	Lower
Regulatory functions/two-component systems				
algR	PD1153	Two-component system; regulatory protein	0.617	Lower
Regulatory functions/activators/repressors				
algH	PD1276	Transcriptional regulator	0.652	Lower
Cell structures				
Surface structures				
	PD1926	Fimbrial protein, pilus assembly protein	2.478	Higher
Membrane components/inner membrane				
algC	PD0120	Phosphomannomutase	0.3	Lower
Membrane components/outer membrane				
mopB	PD1709	Outer membrane protein	0.479	Lower
ompW	PD1807	Outer membrane protein	0.391	Lower
Cellular processes				
Transport/protein, peptide secretion				
secB	PD1065	Type II secretion system, preprotein translocase	0.409	Lower
Transport/carbohydrates, organic acids, alcohols				
algS	PD0347	Sugar ABC transporter ATP-binding protein	0.41	Lower
Transport/cations				
bfr	PD1672	Bacterioferritin; ferritin-like proteins	0.178	Lower
Mobile genetic elements				
Phage-related functions and prophages				
hfq	PD0066	Host factor-I protein; ubiquitous RNA-binding protein Hfq	0.32	Lower

Continued on following page

TABLE 4—*Continued*

Functional group and gene[d]	ORF	Description	M/W ratio[a,b]	Expression in mutant[c]
Pathogenicity, virulence, and adaptation				
Toxin production and detoxification				
cvaC[e]	PD0216	Colicin V precursor; antibacterial polypeptide toxin	0.40	Lower
hspA	PD1280	Heat shock protein (Hsp)	0.469	Lower
Hypothetical/conserved hypothetical proteins				
ND[f]	PD0159	Unknown	0.479	Lower
ND	PD0521	Unknown	0.439	Lower
ND	PD1354	Unknown	0.392	Lower
ND	PD0968	Unknown	0.495	Lower
ND	PD1028	Unknown	0.425	Lower
ND	PD1058	Putative transcriptional regulatory protein	0.484	Lower
ND	PD1295	Putative integral membrane protein; involved in cell shape determination	0.469	Lower
ND	PD1668	Putative integral membrane protein; involved in cell shape determination	0.413	Lower
ORFs with undefined category				
ND	PD1667	HesB-like protein; unknown function	0.462	Lower

[a] The hybridization signal intensity (mean of three technical replicates) obtained with the mutant was divided by that obtained with the wild type to obtain the M/W ratio.
[b] The normalized hybridization signals for those genes between the wild type and mutant are all statistically significantly different as analyzed by Student's *t* test ($P < 0.001$).
[c] Genes having >1.5 or <0.66 final M/W ratios were designated as having higher or lower expression in the mutant, respectively.
[d] Genes were detected on the basis of *X. fastidiosa* Temecula1 genomic sequences at the NCBI website.
[e] Currently annotated as colicin V precursor.
[f] ND, no designation.

algU::*nptII* mutant grown in vitro were not significantly altered compared with that of the LPS from the wild type by the assay used. The mechanism by which *algU* regulates the synthesis of other EPSs or LPS in *X. fastidiosa* remains to be determined.

Previous studies showed that unique structural components of bacterial cells, such as the cell wall, outer membrane proteins, or actively secreted proteins, may be associated with bacterial pathogenicity or suppressing host defenses (4, 42, 45). In *X. fastidiosa*, expression of the outer membrane proteins MopB and OmpW appears to be positively regulated by AlgU. Since SecB is also positively regulated by AlgU, the secretion of other proteins by the type II, *sec*-dependent secretion system may be affected by AlgU.

A single gene that encodes a predicted fimbrial protein (PD1926) was shown to be negatively regulated by *algU*. PD1926 is located in the gene cluster PD1922 to PD1928 (58), which includes homologs of PilD (PD1922), PilC (PD1923), PilA (PD1924), PilB (PD1927), PilR (PD1928), and PilS (PD1929), which are thought to function in the biogenesis and twitching motility of type IV pili in *P. aeruginosa* (26, 39). Mutations in *pilA*, *pilB*, and *pilR* of *X. fastidiosa* resulted in a twitching-minus phenotype (34, 40). It is predicted that PD1926 is a gene involved in the formation or function of type IV pili of *X. fastidiosa*, but its specific contribution is not known. Since *algU* appears to negatively regulate PD1926, the mutation in *algU* might be predicted to enhance its role in the twitching phenotype. We did not measure twitching motility directly, but the *algU* mutant had reduced populations at distances away from the inoculation points of grapevines, which is not consistent with enhanced motility (40). However, the *algU* mutant achieved significantly smaller populations in grapevine overall, even at the inoculation points, so the effects on motility were not necessarily apparent in our assays. The *algU* mutant also had reduced cell-cell aggregation, attachment, and biofilm formation. If PD1926 is involved in the formation of type IV pili and has enhanced expression in the *algU* mutant, then the observed adherence phenotype of the mutant is consistent with the recent finding that mutants of *X. fastidiosa* lacking type IV pili had enhanced cell-cell aggregation and biofilm formation, which appears to be primarily the role of the shorter type I pili (34). The long type IV pili may partially mask the adhesion functions of shorter type I pili.

Several genes that encode ribosomal protein subunits were shown to be positively regulated by *algU* in this study, indicating that *algU* may also be involved in regulating the normal physiological metabolism of *X. fastidiosa*. Genes involved in physiological metabolism under stress, such as heat shock protein genes *cplS*, *clpA*, *clpB*, *dnaK*, *grpE*, and *hspA*; iron storage and detoxification gene *bfr*; and the energy-producing citrate synthase gene *gltA*, had decreased expression in the *algU*::*nptII* mutant. Plant pathogenic bacteria probably regulate heat shock proteins and iron acquisition mechanisms to help them adapt to the harsh environmental conditions present within hosts (59). Cellular homeostasis of iron is essential for preventing iron toxicity in eukaryotes and most prokaryotes. Bacterioferritin is one of three types of ferritin-like proteins in bacteria (55). Bacterioferritin might be involved indirectly in the resistance to redox stress in *P. aeruginosa* (35). *bfr* (PD0066) of *X. fastidiosa* was positively regulated by *algU* and is predicted to encode a bacterioferritin that may play a role in the acquisition of iron or protection against oxidative stress. In this study, there were no significant differences in sensitivity to oxidative stress between wild-type *X. fastidiosa* and the *algU*::*nptII* mutant, so *bfr* may be more likely to function in iron acquisition than in oxidative stress in this pathogen.

cvaC (PD0216), which encodes a colicin V precursor pro-

tein, was identified as positively regulated by AlgU in *X. fastidiosa* in this study. The colicin V precursor is an antibacterial polypeptide toxin that acts against closely related sensitive bacteria (23, 54). It was reported that the expression of *cvaC* was detected in the pathogenic condition but not in the nonpathogenic condition of *X. fastidiosa* (14). Previous studies showed that there are diverse endophytic bacterial populations inside the xylem of plants (3, 9); thus, it is predicted that successful colonization of xylem of grapevine by *X. fastidiosa* may depend on the ability of *X. fastidiosa* to compete with other indigenous microbes for essential nutrients (2). AlgU may play a role such competition in *X. fastidiosa* by regulation of *cvaC*.

RsmA is a homolog of CsrA, which is an RNA-binding protein of *Escherichia coli*. Mutation of *csrA* in *E. coli* resulted in enhanced biofilm formation (29). In a previous study, an *rsmA* mutant of *X. fastidiosa* also formed more biofilm compared with the wild type (8), suggesting that RsmA represses biofilm formation. RsmB is an untranslated RNA molecule that antagonizes RsmA activity in *E. coli* (36). *rsmB* (PD1761) is present in *X. fastidiosa* Temecula1 (53); thus, there may be the RsmA/RsmB posttranscriptional regulation system in *X. fastidiosa*. In this study, DNA microarray analysis showed that the expression of *rsmA* and another gene, *hfq*, that encodes an RNA-binding protein was lower in the *algU*::*nptII* mutant than in the wild type, indicating that *rsmA* and *hfq* are positively regulated by the alternative sigma factor AlgU in *X. fastidiosa*. A decline in RsmA expression is expected to exert a positive effect on the production of biofilm, but the *algU*::*nptII* mutant had a reduced ability to form biofilm.

Hfq, also called host factor I, is an abundant RNA-binding protein and can be involved in the translational regulation of target mRNAs by regulating the stability of RNAs (36). In *P. aeruginosa* PAO1, Hfq may indirectly affect quorum sensing (QS) and biofilm formation by regulating the RsmA/RsmY system (6, 56). Hfq binds to and stabilizes RsmY RNA. The stabilized RsmY RNA then binds to and inactivates RsmA, which would release the negative effect of RsmA on the expression of QS and biofilm formation (56). The absence of Hfq affects the expression of QS and biofilm formation in the reverse way (56).

RsmA (PD1208), RsmB (PD1761), and Hfq (PD0066) are present in *X. fastidiosa* Temecula1 (53). In this study, the expression of *rsmA* and *hfq* was lower in the *algU*::*nptII* mutant, while the expression of *rsmB* was not significantly different. Hfq may be involved in regulating the RsmA/RsmB system of *X. fastidiosa*, as in the RsmA/RsmY system of *P. aeruginosa*. Mutation of *algU* caused lower expression of *hfq*, which would result in reduced stabilization of *rsmB* RNA and a lack of inactivation of RsmA by Hfq-stabilized *rsmB* RNA. There would be a lower level of RsmA in the *algU*::*nptII* mutant, but it could be more active than if Hfq were expressed at normal levels. This could help to explain the decreased biofilm formation in the *algU*::*nptII* mutant. Thus, it is predicted that the biofilm formation in *X. fastidiosa* is regulated by *algU* through a complex Hfq/*rsmB*/*rsmA*-mediated system.

The *X. fastidiosa algU*::*nptII* mutant was affected in the expression of many physiological metabolism genes, acid resistance genes, and membrane-permeating protein genes, which may contribute to maintaining normal physiological metabolism and adaptation to the poor nutrient conditions of the xylem. The specific roles of these genes in xylem colonization should be investigated through mutagenesis and functional assays.

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