Applied and Environmental Microbiology, Apr. 2009, p. 2275–2283 Vol. 75, No. 8
0099-2240/09/$08.00+0 doi:10.1128/AEM.01964-08

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

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Received 23 August 2008/Accepted 3 February 2009

The xylem-limited, insect-transmitted bacterium *Xylella fastidiosa* causes Pierce's disease in grapes through cell aggregation and vascular clogging. GacA controls various physiological processes and pathogenicity factors in many gram-negative bacteria, including biofilm formation in *Pseudomonas syringae* pv. tomato DC3000. Cloned *gacA* of *X. fastidiosa* was found to restore the hypersensitive response and pathogenicity in *gacA* mutants of *P. syringae* pv. tomato DC3000 and *Erwinia amylovora*. A *gacA* mutant of *X. fastidiosa* (DAC1984) had significantly reduced abilities to adhere to a glass surface, form biofilm, and incite disease symptoms on grapevines, compared with the parent (A05). cDNA microarray analysis identified 7 genes that were positively regulated by GacA, including *xadA* and *hsf*, predicted to encode outer membrane adhesion proteins, and 20 negatively regulated genes, including *gumC* and an antibacterial polypeptide toxin gene, *cvaC*. These results suggest that GacA of *X. fastidiosa* regulates many factors, which contribute to attachment and biofilm formation, as well as some physiological processes that may enhance the adaptation and tolerance of *X. fastidiosa* to environmental stresses and the competition within the host xylem.

Xylella fastidiosa is a fastidious, xylem-limited, nonflagellated, insect-transmitted, gram-negative bacterium that causes many plant diseases, including Pierce's disease (PD) (7), a disease which is threatening the grape industry in California in particular. The disease process of PD is related to specific features of *X. fastidiosa*. It has the ability to adhere to the host (plant and insect) cell surfaces and to form biofilms that enable it to be specifically transmitted by insect vectors and to survive in and colonize the xylem tissue of plants (40). Cells of *X. fastidiosa* aggregate, form biofilms, and probably clog the host's vascular system, resulting in disease symptoms (32). To understand disease progression and to develop an effective disease control strategy, a better understanding of the complex interactions among the pathogen, plant, and insect vector is critical (21). However, very little is known about the basis of these complex interactions.

Pathogenic bacteria use gene regulatory mechanisms to rapidly respond to and survive in changing environments (47). Inside the xylem of plants, *X. fastidiosa* is exposed to a range of variable stress factors, such as changes in osmolarity, availability of nutrients, and agents generating reactive oxygen intermediates (1). To ensure survival, *X. fastidiosa* may respond to these stress situations via specific regulatory mechanisms. We are investigating regulatory pathways that contribute to the success of *X. fastidiosa* as a pathogen through mutagenesis of "global" regulatory genes that are known to coordinate expression of virulence-related factors in other pathogenic species. In a previous study, we constructed a mutant of *X. fastidiosa* defective in *algU*, encoding an alternate sigma factor that is highly conserved in gram-negative bacteria. The *algU* mutant had reduced cell-cell aggregation, attachment, and biofilm formation and lower virulence in grapevines (43). Microarray analysis showed that 42 genes had significantly lower expression in the *algU* mutant than in the wild type. This work identified several genes that could contribute to aggregation and biofilm formation as well as other physiological processes, such as virulence, competition, and survival.

An additional regulatory system identified in pathogenic and environmental bacteria is the two-component system of GacS and GacA, involved in sensing environmental signals (19). GacS is a putative sensor kinase that perceives environmental signals, and GacA is a response regulator, which functions as the transcriptional activator of one or more genes. Genes regulated by GacA include regulators of pathogenicity factors, and genes involved in quorum sensing, toxin production, motility, biofilm formation, and extracellular polysaccharide production in a wide range of pathogenic bacterial species, including *Pseudomonas syringae*, *Erwinia carotovora*, and *Pseudomonas aeruginosa* (4, 8, 38). The similarity between *gacA* of *X. fastidiosa* (designated $gacA_{Xf}$) and *gacA* of *P. syringae* (designated $gacA_{DC3000}$) suggests that, like $gacA_{DC3000}$, $gacA_{Xf}$ may regulate the pathogenicity of *X. fastidiosa* by acting as a global regulator during infection and the process of disease development. While a *gacA* homolog was identified in *X. fastidiosa*, a *gacS* homolog was not found, which suggests that there may be a specific regulatory role for *gacA* in *X. fastidiosa* (44). In this study, we cloned and characterized $gacA_{Xf}$ and

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 13 February 2009.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
Strains		
Escherichia coli DH5α	DH1 F$^-$Φ80Δ*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169*	N. T. Keen
X. fastidiosa		7
A05	Wild type	
DAC1984	Gentamicin cassette replacing most of *gacA* ORF (Δ*gacA*::Gm) of *X. fastidiosa* A05	This work
P. syringae pv. tomato		A. Chatterjee
DC3000	Wild type	
AC811	Km^r; $gacA_{DC3000}$ derivative of DC3000	A. Chatterjee
AC812	Tc^r Km^r; AC811 carrying pCPP$gacA_{Xf\text{-}Exp}$	This work
AC813	Tc^r Km^r; AC811 carrying plasmid pCPP47	This work
E. amylovora		A. Chatterjee
EC19	Wild type	
EC191	Gm^r; *gacA* derivative of EC19	A. Chatterjee
EC192	Tc^r Gm^r; EC191 carrying pCPP$gacA_{Xf\text{-}Exp}$	This work
EC193	Tc^r Gm^r; EC191 carrying plasmid pCPP47	This work
Plasmids		
pUC129	Ap^r; cloning vector	New England Biolabs
pUC$gacA_{Xf\text{-}Exp}$	Ap^r; a fragment including the *gacA* promoter and ORF of *X. fastidiosa* ($gacA_{Xf\text{-}Exp}$) cloned into pUC129	This work
pCPP47	Tc^r; broad-range plasmid	A. Chatterjee
pCPP$gacA_{Xf\text{-}Exp}$	Tc^r; $gacA_{Xf\text{-}Exp}$ cloned into pCPP47 constructed as $gacA_{Xf\text{-}Exp}$; expression plasmid	This work
pUC19841	Ap^r; mutagenized PCR fragment of the flanking regions of *gacA* ORF of *X. fastidiosa* cloned into pUC129	This work
pUC19842	Ap^r Gm^r; Gm cassette from pGEM-T-GM cloned into the AscI site of pUC19841	This work
pGEM-T Easy	Ap^r; cloning vector	Promega
pBBR1MCS-5	Gm^r; broad-range plasmid	S. Lindow
pGEM-T-GM	Ap^r Gm^r; Gm cassette from pBBR1MCS-5 cloned into pGEM-T	This work

analyzed the phenotypic effects of a *gacA* deletion in *X. fastidiosa* (DAC1984). We also performed whole-genome microarray analysis of gene expression in the mutant in comparison with that in the parent strain and identified genes whose expression in vitro is controlled by GacA.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this work are listed in Table 1. For growth rate measurements, aggregation, adhesion, colony morphology determination, and biofilm formation, strains of *X. fastidiosa* were cultured on PD3 Gelrite medium (10, 43). After 7 days at 28°C, cells were harvested using 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. Cells used for pathogenicity tests were cultured for 5 days at 28°C on PW Gelrite medium (25, 43), then harvested, and adjusted to the same OD as mentioned above with sterile water. *Pseudomonas syringae* pv. tomato DC3000 and *P. syringae* strains AC811, AC812, and AC813 were maintained on Kings medium B (KmB) agar (27) at 28°C. *E. amylovora* strains EC19, EC191, EC192, and EC193 were maintained on LB agar at 28°C. When required, antibiotics were added as follows: ampicillin (Ap), 100 μg/ml; kanamycin (Km), 10 μg/ml; gentamicin (Gm), 10 μg/ml; spectinomycin, 50 μg/ml; and tetracycline (Tc), 10 μg/ml. All bacteria were stored in 15% glycerol at −80°C.

Cloning of *gacA* of *X. fastidiosa* ($gacA_{Xf\text{-}Exp}$). A 1,025-bp region of *X. fastidiosa* A05 genomic DNA containing the *gacA* promoter and an open reading frame (ORF) (PD1984) was amplified by PCR with Vent polymerase (New England Biolabs, MA) and primers $GacA_{Exp}$For and $GacA_{Exp}$Rev (see Table S1 in the supplemental material). The PCR-amplified fragment was cloned into the SmaI site of pUC129 to make pUC$gacA_{Xf\text{-}Exp}$. The PCR fragment insert was released from pUC$gacA_{Xf\text{-}Exp}$ and cloned into the NotI and SpeI sites of pCPP47 to construct pCPP$gacA_{Xf\text{-}Exp}$ (Table 1). The presence of the cloned PCR fragment (*gacA* promoter and ORF) in pUC$gacA_{Xf\text{-}Exp}$ and pCPP$gacA_{Xf\text{-}Exp}$ was confirmed by sequence comparison with the genomic DNA of *X. fastidiosa*.

Electroporation of bacteria. Electrocompetent cells of *P. syringae* pv. tomato AC811 and *E. amylovora* EC191 were prepared as described previously (4, 8). One microgram of the plasmid pCPP$gacA_{Xf\text{-}Exp}$ DNA in a volume of 5 μl was electroporated into 50 μl of *P. syringae* pv. tomato AC811 or *E. amylovora* EC191 electrocompetent 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, CA) with time constants of about 4 ms. *Pseudomonas* cells were plated on KmB agar (4, 27) supplemented with Km and Tc. One Km- and Tc-resistant clone was selected as *P. syringae* pv. tomato AC812. *Erwinia* cells were plated on LB agar (8) supplemented with Gm and Tc. One Gm- and Tc-resistant clone was selected as *E. amylovora* EC192. The pCPP47 DNA was electroporated into *P. syringae* pv. tomato AC811 or *E. amylovora* EC191 electrocompetent cells as negative controls to yield *P. syringae* pv. tomato AC813 or *E. amylovora* EC193 (Table 1). Cloned inserts in *P. syringae* pv. tomato AC812 and *E. amylovora* EC192 carrying pCPP$gacA_{Xf\text{-}Exp}$ were confirmed by PCR with primers $GacA_{Exp}$For/Rev (see Table S1 in the supplemental material).

HR and pathogenicity tests. Strains of *P. syringae* pv. tomato were grown on KmB agar overnight at 28°C, and *E. amylovora* was grown in LB broth overnight at 28°C. Bacterial cells at an approximate OD_{600} of 0.1 were pelleted and resuspended in water for hypersensitive reaction (HR) and pathogenicity tests. The procedure for HR in tobacco leaves was as previously described (4). Leaves of tobacco (*Nicotiana tabacum* L. cv. Samsun) were infiltrated with *P. syringae* cell suspensions at 5×10^6 to 1×10^7 CFU/ml. For pathogenicity tests, leaves of 5-week-old African violet plants were infiltrated with *E. amylovora* cell suspensions (1×10^6 CFU/ml) (49). Five plants with a total of 10 leaves were inoculated for each assay. Tobacco and African violet plants were kept on the benches in a greenhouse with 75% humidity and a photoperiod of 16 h at 28°C.

Construction of a *gacA* deletion mutant of *X. fastidiosa* (DAC1984). A crossover PCR strategy (49) was used to construct a Δ*gacA*::Gm mutant of *X. fastidiosa*. Two different asymmetric PCRs were performed to generate fragments to the left side (primers GacAA and GacAB) and right side (primers GacAC and GacAD) of the *gacA* ORF (PD1984) (see Table S1 in the supplemental material). The left and right PCR fragments were mixed, denatured at 95°C for 5 min, and annealed at overlapping barcode regions (indicated with italics in Table S1

in the supplemental material), including an AscI recognition site in primers GacAB and GacAC, at 25°C for 10 min. The mixture was further amplified by PCR with primers GacAA and GacAD to generate the final, mutagenized 1.1-kb fragment, which was cloned into pUC129 to make pUC19841 (Table 1). The DNA sequence of the PCR fragment in pUC19841 was confirmed by comparison with the genomic sequences of *X. fastidiosa.* A Gm cassette from pGEM-T-GM (Table 1) was excised and cloned into the AscI site of a 1.1-kb PCR fragment in pUC19841, resulting in the mutant construct pUC19842 (Table 1).

Electrocompetent cells of *X. fastidiosa* strain A05 (7) were prepared according to published procedures (15). One to two micrograms of pUC19842 DNA in a volume of 5 μl was electroporated into the cells under the conditions described earlier. 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 Gm to select for replacement of the wild-type *gacA* ORF with Gm by homologous recombination. A Gm-resistant clone was selected as a potential Δ*gacA*::Gm mutant strain and named *X. fastidiosa* DAC1984.

X. fastidiosa A05 or DAC1984 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 Technologies, WI). The insertion of Gm in the genome of DAC1984 was confirmed by PCR using primers M13For/M13Rev and $GacA_{ORF}$ P1/P2, respectively (see Table S1 in the supplemental material). A fragment of 0.831 kb from A05 and a 2.22-kb fragment from DAC1984 were cut from gels, cloned into pGEM-T Easy (Promega, WI), and sequenced. The location of Gm in DAC1984 genomic DNA was determined by comparing the sequences of the cloned PCR fragment from DAC1984 with the sequences from A05, using Vector NTI (Invitrogen, CA).

Phenotypic analyses. The colony morphologies of *X. fastidiosa* A05 and DAC1984 were observed after 10 to 14 days of growth at 28°C on PD3 Gelrite plates. For cell attachment analysis, A05 and DAC1984 were grown in 50 ml of PD3 broth in 125-ml glass flasks on a shaker at 28°C for 6 to 10 days. In vitro growth curves in 3 ml of PD3 broth were determined after 3 to 21 days of growth at 28°C. Due to 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 turbidity at OD_{600}. Cell aggregation, biofilm formation, and lipopolysaccharide (LPS) gel analyses were done as described previously (3, 16, 29), with modifications as described previously for a comparison of the *X. fastidiosa* A05 wild-type strain and an *algU* mutant (43).

Tolerance of DAC1984 to desiccation stress in vitro. The sensitivities of *X. fastidiosa* A05 and DAC1984 to desiccation on filters were assessed using a modification of a previous procedure (37). Seven- to ten-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 Gelrite plates and incubated at 28°C for 3 weeks. The filters containing water only and 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 in vitro. Sensitivity to hydrogen peroxide (H_2O_2) or sodium hypochlorite (NaOCl) was examined as previously described (33). 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 plates on which 100 μl of 7-day-old cultures of *X. fastidiosa* A05 or DAC1984 was 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 used in each treatment, each treatment was repeated three times, and the results were averaged.

Pathogenicity assays with grapes. *X. fastidiosa* A05 and DAC1984 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 ten 20-μl drops of each suspension were used to inoculate five to ten canes on seedlings of *Vitis vinifera* L. cv. Pinot Noir by using a needle inoculation procedure as previously described (22). A water inoculation served as a negative control. The inoculated grapevines were kept on benches in a greenhouse and were observed for symptom development approximately every 2 weeks for 5 months after inoculation. The symptoms were rated on a visual scale from 0 to 5 as described previously (16), with 0 representing healthy grapevines without scorched leaves (water control) and 5 representing plants with heavy scorching or numerous matchstick symptoms, where the petiole remains attached to the cane after scorched leaf blades abscise and fall. The final disease index was an average for 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* A05 or DAC1984 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 Gm. After incubation for 21 days at 28°C, the identity of *X. fastidiosa* cells on PD3 Gelrite plates was confirmed by PCR using specific primers for *X. fastidiosa* A05, $GacA_{ORF}$P1/P2, and tapBPD1993P1/P2 (see Table S1 in the supplemental material).

To determine the bacterial populations, 16 weeks after inoculation, 2- to 3-cm petiole tissues of each vine inoculated with *X. fastidiosa* A05 and DAC1984 were harvested and treated as mentioned above. Tissues were tested using an enzyme-linked immunosorbent assay (ELISA) with a Patho-ScreenXF kit according to the manufacturer's instructions (Agdia, Inc., IN). 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* cells (Agdia, Inc., IN). The PD3-cultured *X. fastidiosa* A05 and DAC1984 cells were resuspended in phosphate-buffered saline (PBS) buffer (Agdia, Inc., IN) and used to confirm that ELISA worked equally well for quantifying the populations of the wild type and the mutant. Developed plates were measured at 650 nm using a SpectraMax microplate reader via SoftMaxPro (version 3.1.2; Molecular Devices Corp., CA). Bacterial populations were calculated by comparing the OD_{650} to that of the positive control (purified *X. fastidiosa* cells in PBS suspension).

RNA isolation, quantification, and RT-PCR. A modified hot-phenol RNA preparation procedure was used to extract total RNA from *X. fastidiosa* A05 and DAC1984 (28). 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 dissolved in RNase-free distilled H_2O and DNase treated using Turbo DNA-free DNase (2 U/μl) (Ambion, TX). To ensure that the RNA preparation was DNA free, a 1-μl aliquot of RNA (500 ng/μl) was used to amplify the *gacA* ORF by using $GacA_{ORF}$P1/P2 primers (see Table S1 in the supplemental material). The quality of isolated RNAs was determined by denaturing RNA formaldehyde gel electrophoresis (5). The expression of *gacA* was analyzed by reverse transcription-PCR (RT-PCR) with primers $GacA_{ORF}$mRNAFor/Rev (see Table S1 in the supplemental material), using the AccessQuick RT-PCR System per the manufacturer's instructions (Promega, WI).

Microarray hybridizations and microarray data analysis. The gene expression profiles of *X. fastidiosa* A05 and DAC1984 were analyzed by NimbleGen prokaryotic gene expression arrays (NimbleGen System, Inc., WI). DNA microarray chips were designed with a 24-mer oligonucleotide according to the available *X. fastidiosa* genomic sequences. The expression levels of RNA were averaged from three technical replications in a single hybridization experiment. The raw data were analyzed using the ArrayStar FirstLight software program. The expression levels of 2,188 genes under treatment (DAC1984) and control (A05) were analyzed (17). The hybridization signal intensity obtained from A05 or DAC1984 RNA was normalized according to the total signal strength. The normalized hybridization signals were log plot analyzed for signal reliability (17) and were statistically analyzed by Student's *t* test ($P < 0.001$) for differential expression. The normalized signal intensity of DAC1984 was divided by that of A05 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 number. Genes having final M/W ratios of ≥ 1.5 or ≤ 0.66 were selected as positively or negatively regulated mutated genes, respectively.

Validation of microarray data. To validate the differential expression data obtained in microarray analysis, RT-PCR experiments were performed with specific primers designed to amplify internal regions of the ORFs of the target genes (see Table S1 in the supplemental material). Several positively regulated or negatively regulated genes were chosen, and primers were designed for their ORFs according to the *X. fastidiosa* Temecula1 genome sequences. cDNA was amplified from stored RNA by using the AccessQuick RT-PCR system (Promega, WI) according to the manufacturer's instructions. The amplification conditions used were as follows: 45 min at 45°C for reverse transcription; 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.

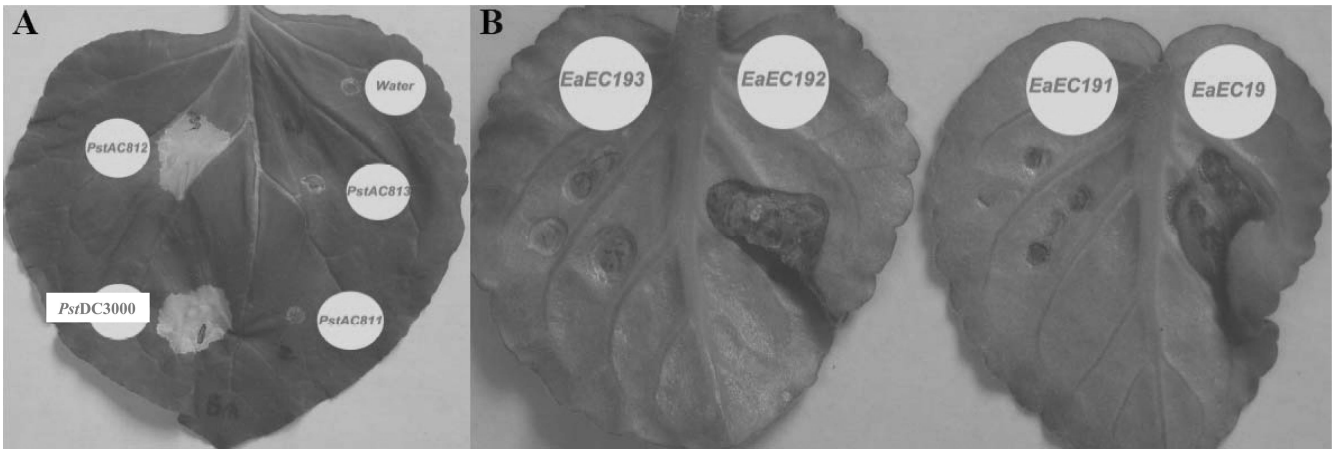

FIG. 1. Complementation of GacA function by $gacA_{Xf}$ in *gacA* mutants of *P. syringae* and *E. amylovora.* (A) Effect of $gacA_{Xf}$ in *gacA* mutant *P. syringae* pv. tomato AC811 (PstAC811) on the elicitation of the HR in tobacco leaf (*Nicotiana tabacum* L. cv. Samsun). Leaf panels were infiltrated with bacterial cell suspensions at 5×10^6 to 1×10^7 CFU/ml. Site 1, *P. syringae* pv. tomato DC3000 (wild type); site 2, *P. syringae* pv. tomato AC811 (*gacA* mutant); site 3, *P. syringae* pv. tomato AC812 (*gacA* mutant complemented with cloned $gacA_{Xf}$); site 4, *P. syringae* pv. tomato AC813 (*gacA* mutant with cloning vector alone); and site 5, water. (B) Effect of $gacA_{Xf}$ in *gacA* mutant *E. amylovora* EC191 (EaEC191) in causing of disease symptoms in Africa violet. Leaves were infiltrated with bacterial cell suspensions at 1×10^6 CFU/ml. Site 1, *E. amylovora* EC19 (wild type); site 2, *E. amylovora* EC191 (*gacA* mutant); site 3, *E. amylovora* EC192 (*gacA* mutant complemented with cloned $gacA_{Xf}$); site 4, *E. amylovora* EC193 (*gacA* mutant with the cloning vector alone).

RESULTS

Complementation of the HR and pathogenicity in heterologous pathogens with $gacA_{Xf}$. An alignment of the predicted amino acid sequences of GacA from *X. fastidios*a and *P. syringae* pv. tomato DC3000 showed that the sequences are 211 amino acids in length and are 43% identical and 69% similar overall (data not shown). In the HR and pathogenicity test experiments, *P. syringae* pv. tomato DC3000 (*P. syringae* wild type) and *P. syringae* pv. tomato AC812 (*P. syringae gacA* mutant complemented with cloned *gacA* from *X. fastidiosa*) elicited typical HR in tobacco (*Nicotiana tabacum* L. cv. Samsun), whereas the water control, *P. syringae* pv. tomato AC811 (*P. syringae gacA* mutant) and *P. syringae* pv. tomato AC813 (*P. syringae gacA* mutant carrying only the plasmid vector used to clone *X. fastidiosa gacA*) did not (Fig. 1), confirming that $gacA_{Xf}$ restored the elicitation of HR in tobacco. *E. amylovora* EC19 (the *E. amylovora* wild type) and *E. amylovora* EC192 (an *E. amylovora gacA* mutant complemented with cloned *gacA* from *X. fastidiosa*) produced disease symptoms in African violet leaves. In contrast, *E. amylovora* EC191 (an *E. amylovora gacA* mutant) and *E. amylovora* EC193 (an *E. amylovora gacA* mutant carrying only the plasmid vector used to clone *X. fastidiosa gacA*) failed to produce disease symptoms (Fig. 1), suggesting that $gacA_{Xf}$ restored the ability of *E. amylovora* EC192 to cause disease in African violet leaves. This demonstrated that $gacA_{Xf}$ can complement *gacA* deficiencies of *P. syringae* and *E. amylovora* in regulating the HR and pathogenicity.

Physiological properties of DAC1984 in vitro. The replacement of the *gacA* ORF with a Gm cassette in the genome of DAC1984 was confirmed by electrophoresis after PCR amplification of the modified *gacA* gene (data not shown). Sequence analysis indicated that Gm physically replaced most of the *gacA* ORF, from only 25 bp downstream from the ATG start codon to 35 bp upstream from the TGA terminal codon of the *gacA* ORF. RT-PCR analysis using $GacA_{ORF}$mRNAFor/Rev (see Table S1 in the supplemental material) showed that there was no *gacA*-sized RT-PCR fragment within DAC1984 cells (insertion of the Gm cassette made the fragment too large to be detectable in this assay), but strong expression was detected in A05 cells (Fig. 2). After being streaked five to eight times on PD3 Gelrite medium with 10 μg/ml Gm, DAC1984 still grew

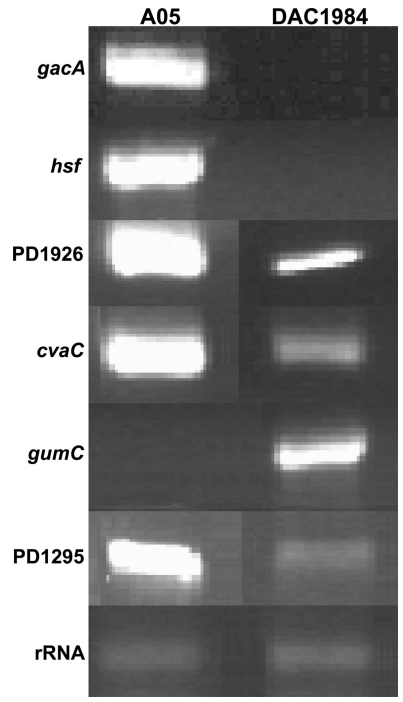

FIG. 2. RT-PCR of genes differentially expressed between wild-type *X. fastidiosa* A05 and *gacA* mutant DAC1984.

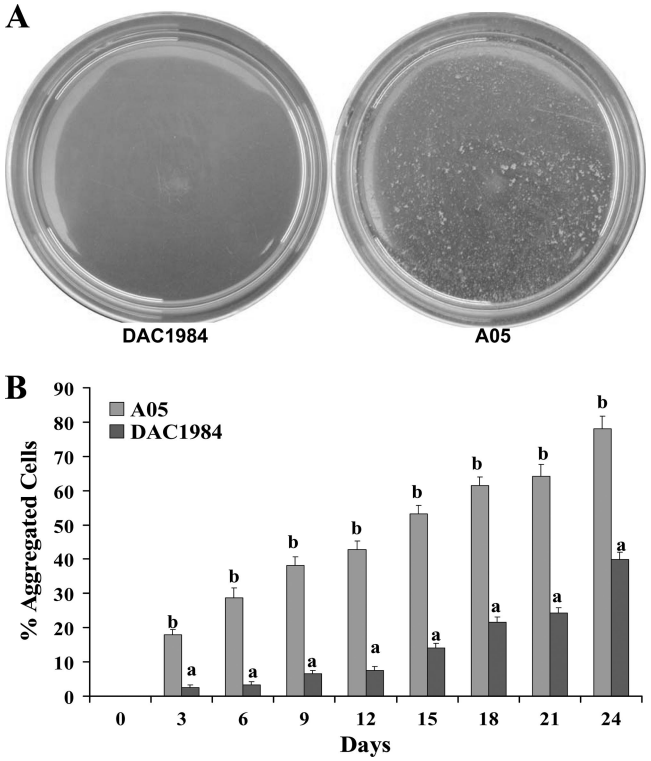

FIG. 3. Cell-to-cell aggregation of wild-type *X. fastidiosa* A05 and *gacA* mutant DAC1984. (A) Cell-to-cell aggregation of *X. fastidiosa* DAC1984 (left) and A05 (right) in PD3 broth in petri dishes. (B) Quantitative assessment of cell-to-cell aggregation of *X. fastidiosa* A05 or DAC1984 by an OD assay as described previously (3, 29). 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.

well and was indistinguishable from the parent, indicating that the mutant was stable.

In vitro growth curves of *X. fastidiosa* A05 and DAC1984 over 21 days were similar, with cell concentrations of both the wild type and the *gacA* mutant increasing from an OD_{600} of approximately 0.15 at 3 days to an OD_{600} of 0.55 at 21 days (data not shown). There was no obvious difference in colony morphology between A05 and DAC1984, but DAC1984 had less sticky colonies on PD3 Gelrite medium when touched with a bacteriological loop (data not shown). In PD3 broth, visual observation showed that colonies of A05 formed large aggregates, whereas DAC1984 grew in less aggregated clumps (Fig. 3). An OD assay was used to quantify the effect of the *gacA* deletion on cell-to-cell aggregation and showed that the percentage of aggregated cells of DAC1984 was significantly lower than that of A05 (Fig. 3).

As is typical of *X. fastidiosa*, cells of A05 attached to the surfaces of flasks and formed wide rings, but DAC1984 cells attached to the surfaces formed rather light rings (data not shown). This suggested that DAC1984 has a reduced surface attachment ability, resulting in reduced biofilm formation. Biofilm formation by DAC1984 was investigated further with a crystal violet staining method. *X. fastidiosa* A05 formed more biofilm in PD3 broth than did DAC1984 (Fig. 4). Deoxycholate-polyacrylamide gel electrophoresis analysis showed that there was no significant difference between the purified-

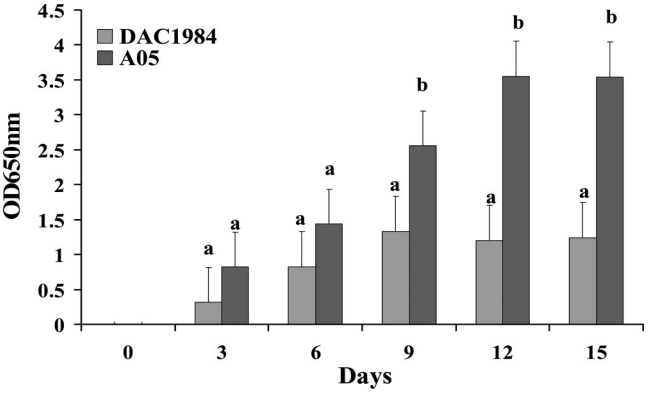

FIG. 4. Analysis of biofilm formation of wild-type *X. fastidiosa* A05 and *gacA* mutant DAC1984 by a crystal violet staining method (29). Biofilm cells were stained with crystal violet, the amount of stain was dissolved with ethanol, and the resulting absorbance was measured at OD_{650}. Three replicates 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.

LPS profile in DAC1984 grown in vitro and LPS from A05 (data not shown).

Tolerance to oxidative and desiccation stresses. No significant differences were observed in tolerance to oxidative stress (sensitivity to hydrogen peroxide or sodium hypochlorite) between mutant and wild-type strains (data not shown). When *X. fastidiosa* A05 and DAC1984 were exposed to desiccation stresses in vitro in petri dishes, the survival rate of DAC1984 was significantly lower than that of A05 (Table 2).

Pathogenicity tests and recovery from infected plants. Compared with grapevines inoculated with wild-type *X. fastidiosa* A05, grapevines inoculated with DAC1984 developed significantly less severe disease symptoms 12 to 20 weeks after inoculation (Fig. 5). Water-inoculated control grapevines did not show any PD symptoms. All diseased grapevines were positive, and the asymptomatic water control vines were negative, for the presence of *X. fastidiosa* when the plants were tested by ELISA. Bacteria were reisolated from macerated, inoculated grapevine petioles on PD3 and PW Gelrite media. The bacterial genotypes were confirmed as *X. fastidiosa* A05 or DAC1984 by PCR amplification with primers for $GacA_{ORF}$P1/P2 and primers tapBPD1993P1/P2 (see Table S1 in the supplemental material) (data not shown).

To gain further understanding of the reduced virulence of the *gacA* mutant, bacterial populations and bacterial move-

TABLE 2. Tolerance of wild-type *X. fastidiosa* and DAC1984 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[¶]
DAC1984	100	20.6[¶]	0.7[¶]	0[¶]	0[¶]

[a] Data are averages for three independent replications for each treatment. Five filters per dilution were used in each treatment. For each assay date, the presence of different symbols indicates 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.

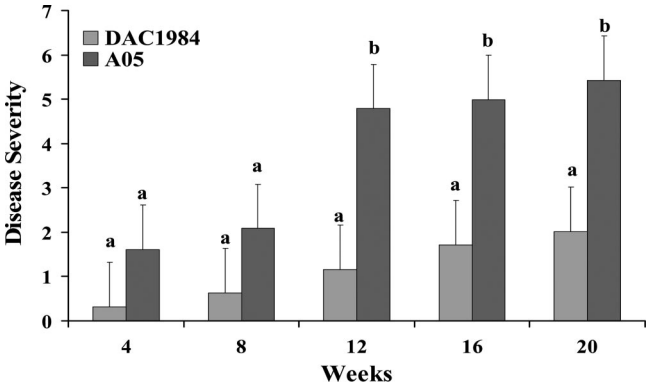

FIG. 5. PD progression in grapevines inoculated with wild-type *X. fastidiosa* A05 and *gacA* mutant DAC1984. Disease severity was based on a visual disease scale of 0 to 5 and was assessed 4, 8, 12, 16, and 20 weeks after inoculation (16). The data are averages for 10 independent replications. For each time point, different letters indicate significant differences (Student's *t* test; $P < 0.05$) between the wild type and the mutant.

ment 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* population worked equally well for A05 and DAC1984 cultures. Bacterial populations at inoculation points as well as at 25 cm and 50 cm above inoculation points were estimated from ELISAs by comparing the OD_{650} with that of the *X. fastidiosa* positive control with known concentrations (Table 3). One OD_{650} of the *X. fastidiosa* positive control (purified *X. fastidiosa* cells in PBS suspension) represented approximately 1×10^4 CFU/ml. The average bacterial populations were calculated by comparing the OD_{650} to that of the positive control and divided 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 populations of DAC1984 were less than those of A05 at 25 cm and 50 cm above inoculation points (Table 3). The actual populations could have been higher than we reported, since those were calculated based on *X. fastidiosa* cultures in PBS buffer 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). Those data suggest that the mutated *gacA* gene may affect the growth and possibly the movement of *X. fastidiosa* inside the xylem, resulting in reduced pathogenicity.

In vitro gene expression profiling of DAC1984. The expression levels of 2,188 genes in *X. fastidiosa* A05 and DAC1984 were monitored using a cDNA microarray. The expression levels of RNA were averaged from three 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). Twenty-seven genes were differentially expressed in DAC1984, compared with what was found for A05 (Table 4). Several putative pathogenicity-related genes, such as *xadA* (PD0731), encoding an outer membrane protein, *hsf* (PD0744), encoding a surface protein, and *gumC* (PD1395), involved in the biosynthesis of fastidian gum, were regulated by *gacA* in *X. fastidiosa*. *oprO* (PD0264), *actP* (PD1294), *cvaC* (PD0216), fimbrial protein gene PD1926, and hypothetical protein gene PD1295 were positively regulated by *gacA* in *X. fastidiosa*. Several genes were negatively regulated by *gacA* in *X. fastidiosa*, including genes encoding phage-related proteins, hypothetical conserved proteins, biotin synthesis (*bioI*), lysophospholipase-secretory lipase (PD1702 and PD1703), membrane fusion proteins, acriflavin resistance proteins, membrane-bound glucose dehydrogenase, and polyvinylacohol dehydrogenase, according to the functional groups (Table 4). RT-PCR was used to validate that there were lower expression levels of *hsf*, *cvaC* (PD0216), fimbrial protein gene PD1926, and PD1295 in the *gacA* mutant and higher expression levels of *gumC* (Fig. 2). Detection of rRNA at similar levels in *X. fastidiosa* A05 and DAC1984 (Fig. 2) indicated that the RT-PCR condition was reliable.

DISCUSSION

A *gacA* mutant of *X. fastidiosa* (DAC1984) did not differ in growth rate in vitro from A05, but it had reduced abilities to aggregate, attach to surfaces, form biofilms, and incite disease symptoms in grapevine. DAC1984 had decreased movement inside plants, associated with decreased disease development (23). The populations of DAC1984 at 25 cm and 50 cm above the inoculation points were reduced compared with those of A05 but were also significantly lower at the inoculation points. Thus, we cannot determine from these data specifically whether DAC1984 was defective for vessel-to-vessel movement versus growth rate inside the xylem.

It was reported that cell surface structures such as LPSs and extropolysaccharides play an important role in the attachment process and biofilm formation (14). However, the purified LPS profile of DAC1984 was not significantly altered compared with LPS from A05 by the assay used.

The extracellular polysaccharide, fastidian gum, of *X. fastidiosa* is thought to be synthesized by nine enzymes encoded by the *gumBCDEFHJKM* operon (44). In *Xanthomonas campestris* pv. campestris, a related operon is responsible for production of the extracellular polysaccharide xanthan gum, and xanthan-deficient mutants have reduced virulence (26). The finding of a related operon in *X. fastidiosa* suggests that fastidian gum may also be an important virulence factor, as in *X. campestris* pv. campestris (9). Fastidian gum in *X. fastidiosa* may be involved in biofilm formation that is thought to benefit

TABLE 3. Bacterial populations in grapevines 16 weeks after inoculation

Genotype	Population (10^6 CFU/g of tissue)[a]		
	At inoculation point	Above inoculation point	
		25 cm	50 cm
Wild type	$9.087 \pm 2.6^{\S}$	$0.609 \pm 0.18^{\S}$	$0.646 \pm 0.28^{\S}$
DAC1984	$1.004 \pm 3.1^{\P}$	$0.028 \pm 0.012^{\P}$	$0.002 \pm 0.0023^{\P}$

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

TABLE 4. Genes differentially expressed in *X. fastidiosa* DAC1984 in vitro, organized by functional groups

Functional group and gene[d]	ORF	Description	M/W ratio[a,b]	Expression level in mutant[c]
Cell structure				
Membrane components/outer membrane; *oprO*	PD0264	Porin O precursor	0.24	Lower
Surface structures	PD1926	Fimbrial protein-pilus assembly protein	0.12	Lower
Cellular processes; transport/other; *actP*	PD1294	Acetate permease	0.337	Lower
Intermediary metabolism; energy metabolism and carbon/electron support	PD2039	Oxidoreductase	2.75	Higher
Biosynthesis of small molecules; cofactors, prosthetic groups, and carrier biosynthesis/biotin; *bioI*	PD1688	Cytochrome P450-like enzyme	2.45	Higher
	PD1703	Hypothetical protein-conserved domains: lysophospholipase	6.31	Higher
	PD1702	Hypothetical protein-conserved domains: secretory lipase	5.29	Higher
Pathogenicity, virulence, and adaptation				
Toxin production and detoxification				
cvaC	PD0215	Colicin V precursor: antibacterial polypeptide toxin	3.63	Higher
cvaC[e]	PD0216	Colicin V precursor: antibacterial polypeptide toxin	0.51	Lower
Outer membrane protein				
xadA	PD0731	Outer membrane protein: autotransporter adhesion	0.11	Lower
hsf	PD0744	Surface protein: autotransporter adhesion	0.31	Lower
Exopolysaccharides; *gumC*	PD1395	Exopolysaccharide biosynthesis	3.17	Higher
Hypothetical conserved/hypothetical proteins	PD0243	Conserved domains: membrane fusion protein	3.01	Higher
	PD0244	Conserved domains: acriflavin resistance protein	2.7	Higher
	PD0956	V8-like Glu-specific endopeptidase	4.77	Higher
	PD1299	Conserved domains: polyvinyl alcohol dehydrogenase	6.18	Higher
	PD1295	Putative membrane protein: unknown function	0.20	Lower
	PD0521	Unknown	3.24	Higher
	PD0657	Unknown	3.28	Higher
	PD0743	Unknown	6.62	Higher
	PD0955	Unknown	2.67	Higher
Mobile genetic elements/phage-related functions and prophages	PD0911	Phage-related proteins: unknown function	6.04	Higher
	PD0912	Phage-related proteins: unknown function	8.41	Higher
	PD0917	Phage-related proteins: unknown function	3.94	Higher
	PD0924	Phage-related proteins: unknown function	6.39	Higher
	PD0925	Phage-related proteins: unknown function	9.12	Higher
	PD0930	Phage-related proteins: unknown function	3.96	Higher

[a] The hybridization signal intensity (mean for 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 the mutant are all statistically significantly different, as analyzed by Student's *t* test ($P < 0.001$).

[c] Genes having final M/W ratios of >1.5 or <0.66 were designated as having higher or lower expression levels 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 a colicin V precursor.

X. fastidiosa survival inside xylem of plant and insect vectors (9). GumC may be responsible for regulating gum polymerization or secretion of fastidian gum (12). There was an increased expression level of *gumC* in DAC1984, indicating that *gumC* is repressed by GacA in vitro. The expression of *gumC* was significantly suppressed under high cell densities of *X. fastidiosa*, while other *gum* genes were induced (42), suggesting that *gumC* may be involved in the early stage of regulating or secreting fastidian gum, which would benefit the early steps of biofilm formation in planta.

Genes involved in surface structures and attachment components, such as *hsf*, *xadA*, and fimbrial protein gene PD1926, were positively regulated by GacA in *X. fastidiosa*. Several Hsf-like surface proteins are predicted to occur in the *X. fastidiosa* genome (44). *hsf* (PD0744) has a high level of similarity to the *hsf* adhesin gene of the human pathogen *Haemophilus influenzae* (46). *hsf* of *H. influenzae* encodes surface fibrils with an adhesion mechanism different from that of type I fimbriae and is responsible for the attachment to human epithelial cells (46), thus allowing a tight contact between the pathogen and the host cell. Previous studies showed that fimbriae, pili, and Hsf surface fibrils were coexpressed in *X. fastidiosa* (45). Higher expression levels of *hsf* were detected under the pathogenic condition but not under the nonpathogenic condition (13), suggesting that Hsf may play a role in the virulence of *X. fastidiosa* through initial adhesion to xylem cell walls and may be involved in the initial process of biofilm formation.

xadA homologs are present in the genomes of the plant pathogens *X. fastidiosa*, *Xanthomonas oryzae* pv. oryzae, and *Xanthomonas campestris* pv. vesicatoria (36, 41, 44), encoding a predicted fimbrial outer membrane protein. XadA is predicted to be an autotransporter, secreted by the type V secretion system (20). A *xadA* mutant of the rice pathogen *X. oryzae* pv. oryzae was deficient for virulence and had changes in colony morphology (41). A *xadA* mutant of *X. fastidiosa* had a 100-fold reduction in ability to attach to surfaces, compared with the wild type (31). The expression of *xadA* was decreased in DAC1984, possibly resulting in a reduced ability to adhere to xylem cell walls.

A gene encoding a predicted fimbrial protein (PD1926) was shown to be positively regulated by GacA. PD1926 is located in the gene cluster comprising PD1922 to PD1928 (48), which includes homologs of PilD (PD1922), PilC (PD1923), PilA (PD1924), PilB (PD1927), PilR (PD1928), and PilS (PD1929), thought to function in the biogenesis of type IV pili and twitching motility in *P. aeruginosa* (24, 34). Mutations in *pilA*, *pilB*, and *pilR* of *X. fastidiosa*, which still possessed type I pili only, resulted in a twitching-negative phenotype and could not colonize upstream vascular regions in planta (11, 30, 35). However, the mutants of *pilB* had enhanced biofilm formation (35). 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. If PD1926 contributes to the formation of type IV pili, then our finding that the *gacA* mutant had reduced cell-cell aggregation and biofilm formation is not consistent with the recent finding by Li et al. (30), indicating that mutants of *X. fastidiosa* lacking type IV pili had enhanced cell-cell aggregation and biofilm formation. The long type IV pili are thought to partially mask the adhesion functions of shorter type I pili (30). Our previous work with AlgU, which negatively regulates PD1926, was more consistent with the findings of Li et al., since an *algU* mutant had reduced cell-cell aggregation and biofilm formation (43). However, in addition to PD1926, expression levels of *hsf* and *xadA* were reduced in the *gacA* mutant, which would be expected to reduce the initial surface attachment and the process of biofilm formation. Therefore, the reduced-adherence phenotype of the *gacA* mutant is consistent with the reduced expression of several genes encoding surface structures and attachment.

One gene predicted to encode a colicin V precursor, *cvaC* (PD0215), was negatively regulated by GacA, but another *cvaC* gene (PD0216) was positively regulated by GacA in *X. fastidiosa* in this study. Colicin V is an antibacterial polypeptide toxin that acts against closely related sensitive bacteria (18). *X. fastidiosa* has three colicin-like precursor protein genes, PD0215, PD0216, and PD0217, and genes that should encode a complete colicin V secretory machinery (44, 48). There is also a *cvi* homolog, PD0214, in *X. fastidiosa*, predicted to encode a colicin V immunity protein (44, 50). In other studies of *cvaC* gene expression in *X. fastidiosa*, *cvaC* (PD0215) and *cvaC* (PD0217) were induced by glucose (39), and expression levels of *cvaC* (PD0216) were higher in later stages of colonization of citrus (13). In addition, *cvaC* (PD0216) was positively regulated by AlgU in our previous study (43). Since there are diverse endophytic bacterial populations inside the xylems of grape and citrus plants that may influence colonization by *X. fastidiosa* (2, 6), *cvaC* may play a role in competing with indigenous microbes in colonization of the xylem.

GacA in *X. fastidiosa* was shown to regulate genes contributing to attachment and biofilm formation as well as various physiological processes. Our previous work showed that AlgU also regulates genes involved in similar phenotypes, but there was little overlap in the genes regulated by these two proteins (43). GacA may contribute to the attachment process by positively regulating production of the nonfimbrial adhesion proteins Hsf and XadA and by regulating *gumC*, which may be involved in early stages of regulating or secreting fastidian gum. AlgU positively regulates different surface proteins, such as MopB and OmpW, and negatively regulates the predicted fimbrial protein PD1926, which is positively regulated by GacA.

GacA and AlgU also regulated genes predicted to contribute to various metabolic processes and antimicrobial competition. Most of the specific genes predicted to encode these functions were different for the GacA-versus-AlgU regulatory pathways, except for the colicin V precursor gene *cvaC* (PD0216), a gene encoding a putative membrane protein of unknown function (PD1295), and an additional ORF of unknown function (PD0521). Specific roles of genes regulated by GacA and AlgU are being investigated through mutagenesis and functional assays.

ACKNOWLEDGMENTS

Thanks to Steven Lindow (Department of Plant and Microbial Biology, University of California, Berkeley, CA) for providing plasmid pBBR1MCS-5 and Arun K. Chatterjee (Department of Plant Microbiology & Pathology, University of Missouri, Columbia, MO) for providing *gacA* mutants of *P. syringae* and *E. amylovora* and related plasmids. Thanks to Zhenyu Jia (Department of Pathology & Laboratory Medicine, University of California, Irvine, CA) for helping with analysis of the microarray data.

This project was supported by grants from the California Department of Food and Agriculture and the University of California Agricultural Experiment Station.

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