Global Small RNA Chaperone Hfq and Regulatory Small RNAs Are Important Virulence Regulators in Erwinia amylovora

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Hfq is a global small RNA (sRNA) chaperone that interacts with Hfq-regulated sRNAs and functions in the posttranscriptional regulation of gene expression. In this work, we identified Hfq to be a virulence regulator in the Gram-negative fire blight pathogen Erwinia amylovora. Deletion of hfq in E. amylovora Ea1189 significantly reduced bacterial virulence in both immature pear fruits and apple shoots. Analysis of virulence determinants in strain Ea1189Δhfq showed that Hfq exerts pleiotropic regulation of amylovoran exopolysaccharide production, biofilm formation, motility, and the type III secretion system (T3SS). Further characterization of biofilm regulation by Hfq demonstrated that Hfq limits bacterial attachment to solid surfaces while promoting biofilm maturation. Characterization of T3SS regulation by Hfq revealed that Hfq positively regulates the translocation and secretion of the major type III effector DspE and negatively controls the secretion of the putative translocator HrpK and the type III effector Eop1. Lastly, 10 Hfq-regulated sRNAs were identified using a computational method, and two of these sRNAs, RprA and RyhA, were found to be required for the full virulence of E. amylovora.

Erwinia amylovora is a Gram-negative bacterial plant pathogen and the causal agent of fire blight, a disease that occurs on rosaceous species, such as apples and pears. During infection, E. amylovora enters host plants through natural openings in flowers or shoot tips and is able to rapidly move within plant hosts in the vascular tissue and establish systemic infections. To date, many virulence factors of E. amylovora have been characterized, with the major determinants including the type III secretion system (T3SS), amylovoran exopolysaccharide production, biofilm formation, and motility (1). E. amylovora pathogenesis on apple trees is manifested through several distinct stages and interactions with living and nonliving host cells. The stigma surface of flowers is the primary site of multiplication of E. amylovora prior to infection of flowers through nectar ducts and internal invasion of the host (2). Infection of flowers by E. amylovora requires a functional T3SS, and motility is an important virulence factor affecting migration of cells downward from the stigma to the nectar ducts (3–5). The T3SS of E. amylovora is mainly required for the translocation of the effector protein DspE into plant cells; DspE is required for the pathogenicity of E. amylovora, multiplication in planta, and disease promotion by the alteration of host cell defenses (6–8). Genes associated with production of the T3SS and type III effector genes, including the alternate sigma factor hrpL, type III pilus hrpA, translocator hrpN, and effector dspE, are expressed at between 6 and 48 h of inoculation to flower stigmas (9), which correlates well with regulatory studies of the HrpL regulon performed in vitro (10). In addition to the T3SS and DspE, a third pathogenicity factor produced by E. amylovora is the exopolysaccharide amylovoran, which is an important component of biofilms (11, 12). One role of amylovoran is apparently to protect cells from exposure to antimicrobial compounds produced by the host in response to pathogen infection (11).

Infection of apple leaves and shoots by E. amylovora is a second common mode of infection that is initiated following cell entry through wounds. Leaf infection also requires the T3SS, as the initial interaction is with living parenchymal cells in leaf tissue. Systemic infection of apple and other hosts by E. amylovora is accomplished following invasion of xylem (the water-conducting tubes of plants), phloem, and the cortical parenchyma of stems. Xylem is composed of nonliving cells, and plant pathogens such as Xanthomonas albilineans that exclusively infect xylem do not possess a T3SS (13), suggesting that this determinant is of less importance for systemic infection by E. amylovora. In contrast, biofilm formation, which is not required for leaf infection, is critically important to the establishment of large cell populations in xylem tubes and to systemic movement of E. amylovora out of leaves and into apple stems (12, 14).

In order to successfully establish infections, E. amylovora utilizes a complicated regulatory network involving two-component signal transduction systems, alternate sigma factors, quorum sensing, and the second messenger cyclic di-GMP to collectively control the expression of virulence genes at different stages of infection (1, 4, 10, 15–18). In addition, the requirement at different times of infection for the T3SS and biofilm formation and the importance of cellular motility suggest that E. amylovora cells must be able to rapidly alter the production of distinct virulence factors in response to specific host cues.

One method utilized by bacteria to facilitate rapid responses to environmental changes is through the use of regulatory small RNAs (sRNAs). These noncoding RNAs range from 50 to 400 nucleotides (nt) and target specific mRNA transcripts in cells, effecting posttranscriptional regulation of the target mRNAs by either altering their translational efficiency or affecting mRNA stability, or both (19–21). The stability and functional activation of sRNAs are controlled by the RNA chaperone protein Hfq (22).

Hfq forms a hexameric ring structure and preferentially binds...
to U-rich sequences of sRNAs on the proximal side of its central core and to A-rich sequences on the distal face (23, 24). The sRNAs bound by Hfq target specific mRNAs in the bacterial cell and exert posttranscriptional regulatory effects. Two roles of Hfq in sRNA-mRNA interactions have been implicated: first, Hfq-sRNA binding enhances the stability of sRNAs; second, Hfq-sRNA binding also facilitates the imperfect base pairings of sRNAs to the 5’ untranslated regions (5’ UTRs) of their target mRNAs (20, 21, 25). The binding of sRNAs to the 5’ UTRs would either lead to translational repression of the target mRNAs, when the binding of sRNAs blocks the ribosomal binding site (RBS), or lead to translational activation, when the binding of sRNAs competes with inhibitory intramolecular base-pairing interactions (21). In addition, RsmB/CsrB-type sRNAs also play critical roles in the posttranscriptional regulation by binding to and sequestering the RsmA/CsrA (26, 27).

Because Hfq is a global sRNA chaperone and binds to sRNAs with diverse functions, its regulation in bacteria is often pleiotropic. Genes controlled by Hfq encode diverse traits, including cell membrane protein composition, cell surface structures, stress tolerance, motility, and sugar, nitrogen, and fatty acid metabolism (28). Recent reports have also shown that Hfq and sRNAs play important roles in virulence regulation in animal pathogens (28, 29). For example, in Vibrio cholerae, Hfq is required for intestinal colonization of suckling mice (30), and in enterohemorrhagic Escherichia coli (EHEC), Hfq negatively controls T3SS-encoding genes in the locus of enterocyte effacement (LEE) (31). A reduced-virulence phenotype in hfq mutants was also commonly observed in other Gram-negative pathogens, including Brucella abortus, Francisella tularensis, Neisseria meningitidis, Pseudomonas aeruginosa, and Yersinia pestis, and in the Gram-positive pathogen Listeria monocytogenes (28). Although Hfq has been implicated in controlling virulence in many bacterial pathogens, the regulatory targets of Hfq vary among species, from regulation of the T3SS (31, 32) and stress tolerance (33, 34) to biofilm formation (35). In some cases, the regulatory function of Hfq is still not clear (28).

Although the contribution of Hfq and Hfq-regulated sRNAs to virulence has been described in detail in bacterial pathogens of animals, the role of Hfq and Hfq-regulated sRNAs in virulence and host colonization has been reported in only one bacterial plant pathogen, Agrobacterium tumefaciens (36). We hypothesized that Hfq and Hfq-regulated sRNAs would regulate the critical components of pathogenesis in E. amylovora, including the T3SS and amylovoran exopolysaccharide biosynthesis. In this study, we constructed an hfq deletion mutant in E. amylovora Ea1189 and demonstrated that Hfq is an important virulence regulator. By combining virulence assays, electron microscopy analyses, and effector translocation and secretion assays, we showed that the virulence regulation of Hfq is exerted through its control of amylovoran biosynthesis, biofilm formation, motility, and the T3SS. Finally, 10 potential Hfq-regulated sRNAs were identified in the E. amylovora genome, and 2 of them were shown to be important for virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and culture conditions. The bacterial strains and plasmids used in this study and their relevant characteristics are listed in Table 1. The sequences of the oligonucleotide primers used for cloning and mutations are listed in Table S1 in the supplemental material. All strains were stored at −80°C in 15% glycerol and cultured in LB media at the following concentrations: gentamicin, 15 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 15 μg/ml; ampicillin, 100 μg/ml.

Luria-Bertani (LB) medium at 28°C. For biofilm assays, strains were cultured in 0.5 × LB broth. For Northern blot assays and protein secretion assays, strains were cultured in LB broth at 28°C overnight and then cultured in Hrp-inducing minimal medium (41), which induces the expression of the T3SS regulon. When required, antibiotics were added to the media at the following concentrations: gentamicin, 15 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 50 μg/ml; and ampicillin, 100 μg/ml.

Deletion mutagenesis of hfq and sRNA-encoding genes. E. amylovora chromosomal deletion mutants were constructed using the red recombinase method (38). Briefly, recombination fragments consisting of 50-nucleotide homology arms of flanking regions of hfq or sRNA-encoding genes flanking a chloramphenicol resistance cassette were amplified from the plasmid pKD4. PCR products were purified by gel purification and electroporated into E. amylovora Ea1189 expressing recombinase genes from the helper plasmid pKD46. Mutants were selected on LB medium amended with kanamycin. Mutations of target genes were confirmed by PCR and sequencing. Deletion of sRNA-encoding genes in E. amylovora was based on their sequence homologies to corresponding sRNA-encoding genes in E. coli.

Virulence assays. The virulence of wild-type strain Ea1189 and mutant strains was tested using an immature pear fruit assay and an apple shoot assay as previously described (17, 42). Briefly, for the immature pear fruit assay, bacteria were inoculated on wounded immature pears at a concentration of 1 × 10^6 CFU ml⁻¹, and the pears were incubated at 25°C under high-relative-humidity conditions. Lesion diameters were measured at 3, 5, and 7 days postinoculation. Bacterial populations within immature pear fruits were quantified at 3, 27, and 51 h postinoculation.

TABLE 1 Bacterial strains and plasmids used in this study and their relevant characteristics

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
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<tr>
<td>Escherichia coli DH5a</td>
<td>F⁻λφ80lacZΔM15 K12ΔlacZYA-argF/ lacF169 endA1 recA1 bsdR17 (r6K-m5') deoR thi-1 supE44 gyrA96 relA1 k</td>
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<td>Erwinia amylovora</td>
<td></td>
<td></td>
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*Cmr, Km⁺, Cmr⁺, Ap⁺, chloramphenicol, kanamycin, gentamicin, and ampicillin resistance, respectively.\n
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For the apple shoot assay, the youngest apple leaves were inoculated by cutting with scissors dipped in a bacterial suspension of 2 $\times$ 10^8 CFU ml$^{-1}$. The progression of symptoms was observed at 3, 8, and 14 days postinoculation. All assays were repeated three times, with five biological replicates in each experiment. Statistical analyses of treatment means was done by one-way analysis of variance, and mean separation (P < 0.05) was accomplished using Fisher’s protected-least-significant-difference test.

**Amylovoran production assay and motility assays.** The amylovoran concentration in supernatants of bacterial cultures was quantified using a turbidity assay with cetylpyridinium chloride (CPC) as previously described (42). Briefly, cells from overnight LB medium cultures were harvested by centrifugation, washed with phosphate-buffered saline (PBS), and inoculated into MBMA medium (43) with 1% sorbitol. The supernatant of the MBMA culture was tested for the amylovoran concentration by adding 50 $\mu$l of CPC (50 mg ml$^{-1}$) per ml of supernatant sample, followed by measuring the optical density at 600 nm (OD$_{600}$). The experiments were repeated three times with four biological replicates in each experiment.

To measure bacterial swimming motility, cells from overnight cultures were collected by centrifugation, resuspended in PBS, and diluted in sterile water. The diluted bacterial suspension was plated onto the center of swirling agar plates (10 g tryptone, 5 g NaCl, 3 g agar per liter). Swarming diameters were measured at 18 h postincubation. The experiments were repeated three times with four biological replicates in each experiment.

**Biofilm quantification by crystal violet staining and analysis using SEM.** To quantify the amount of biofilm by crystal violet staining, bacterial strains were cultured in 0.5 $\times$ LB broth in a 24-well plate with a glass coverslip placed in each well at a 30$^\circ$ angle. After 48 h of incubation at 28$^\circ$C, the bacterial culture was removed from the wells, 10% crystal violet was added to the wells, and the plate was incubated at room temperature for 1 h. Glass coverslips were rinsed with water, air dried for 2 h, and eluted with 200 $\mu$l of elution solution (40% ethanol, 10% glacial acetic acid). The solubilized crystal violet in the elution solution was quantified by measuring the light absorbance at OD$_{595}$ using a Safire microplate reader (Tecan, Research Triangle Park, NC). The experiment was repeated three times with 12 replicates in each experiment.

For the observation of biofilm formation using scanning electron microscopy (SEM), strains to be tested were cultured in 100 $\mu$l of 0.5% LB broth in a 96-well plate with a 300 mesh–test electron microscopy copoy (TEM) gold grid in each well (G300-Au; Electron Microscopy Sciences). The plates were incubated at 28$^\circ$C for 48 h, and 100 $\mu$l of paraformaldehyde-glutaraldehyde (2.5% of each compound in 0.1 M sodium cacodylate buffer; Electron Microscopy Sciences) was added to each well. The mixture was incubated at room temperature for 1 h, and grids were successively dehydrated in 25, 50, 75, and 90% ethanol for 30 min each and in 100% ethanol three times for 15 min each. Grids were then dried to the critical point using a critical point drier (Balzers CPD, Lichtenstein) and mounted on aluminum mounting stubs (Electron Microscopy Sciences). Samples were then coated with osmium using a pure osmium coater (Neoc-an; Meiwa Shoji Co. Ltd., Japan). Images were taken on a JEOL 6400V scanning electron microscope (Japan Electron Optics Laboratories) equipped with an LaB6 emitter (Noran EDS) using analySIS software (Soft Imaging System, GmbH).

**Protein purification and analyses.** Strains were cultured in 50 ml LB broth overnight at 28$^\circ$C. Cells were harvested by centrifugation, washed with 20 ml of PBS, and resuspended in 100 ml Hrp-inducing minimal medium. After 48 h of induction in Hrp-inducing minimal medium, culture supernatant was collected by centrifugation, phenylmethylsulfonyl fluoride (PMSF) was added to a concentration of 0.5 mM, and the resulting solution was filtered through a 0.22-$\mu$m-pore-size filter (Stericup; Millipore, Billerica, MA) to obtain a cell-free supernatant. The cell-free supernatant was concentrated to 1 ml using an Amicon 15-ml centrifugal filter unit (10-kDa-molecular-mass cutoff). Proteins was extracted from the cell-free supernatant using a previously described method (44) with modifications. Proteins from the concentrated supernatant were extracted twice by mixing with 0.5 volume of water-saturated phenol at 4°C with agitation for 30 min. Phases of the mixture were separated by centrifugation; the lower phases of the phenol fractions from each extraction were combined, and the proteins in the phenol fraction were precipitated by adding 5 volumes of 100 mM ammonium acetate in methanol. Samples were incubated overnight at −20°C, followed by centrifugation at 13,000 × g at 4°C for 30 min. The protein pellets were resuspended in 50 $\mu$l of water and reproprecipitated by adding 500 $\mu$l of cold acetone. Samples were incubated overnight at −20°C. Protein pellets were collected by centrifugation at 13,000 × g at 4°C for 30 min and resuspended in 50 $\mu$l of water with 0.5 mM PMSF. Protein concentrations were measured with a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL) and were adjusted to 1 $\mu$g $\mu$l$^{-1}$. For the SDS-PAGE analysis of proteins from the E. amylovora secretome, proteins were separated using a Mini-PROTEAN 3 system (Bio-Rad, Hercules, CA), and gels were stained with Coomassie blue. Protein bands of interest were excised from the Coomasie blue-stained polyacrylamide gels and identified by mass spectrometry (MS) using a Thermo Scientific LTQ linear ion trap mass spectrometer. For Western blot analysis, 8 $\mu$g of proteins of each sample was analyzed using anti-CyaA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-DnaA antibody was used as a lysis control. The band intensities of proteins of interest following Western blot analysis were quantified using ImageJ software (http://rsweb.nih.gov/).

**Hypersensitive response and DspE-CyaA translocation assay.** For hypersensitive response (HR) assays, strains were cultured in LB broth overnight, harvested by centrifugation, and washed with 0.5× PBS twice. Cells were resuspended in 0.5× PBS and adjusted to a concentration of 1 $\times$ 10^7 CFU ml$^{-1}$. Approximately 100 $\mu$l of cell suspension was infiltrated into 9-week-old Nicotiana benthamiana leaves using a needleless syringe, and the HR was observed at 16 h after infiltration.

The DspE-CyaA translocation assay was performed as previously described (40). Briefly, bacterial strains carrying DspE-CyaA fusion plasmids pRLT201 and pRLT8 were cultured in LB medium overnight, washed with 0.5× PBS, and resuspended in 0.5× PBS. Cells were adjusted to a concentration of 6 $\times$ 10^7 CFU ml$^{-1}$ and were infiltrated into the youngest three fully expanded leaves of an 8-week-old Nicotiana tabacum plant. After 20 h postinoculation, leaf disks were collected using a 1-cm hole puncher and were immediately frozen in liquid nitrogen. Cyclic AMP (cAMP) was extracted from the leaf disks by grinding leaf disks in liquid nitrogen and resuspending them in 325 $\mu$l 1.1 M HCl. cAMP levels in the supernatants were then quantified using a cyclic AMP enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI). Protein levels in leaf pellets were measured using the Bradford method. The final cAMP concentration of each sample was the measured cAMP level adjusted by the amount of proteins (pg cAMP/µg protein).

**RNA isolation, qRT-PCR, and Northern blot analysis.** Total bacterial RNA from cultures grown in Hrp-inducing medium was isolated by using the RNaseasy minikit method (Qiagen, Valencia, CA) and treated with Turbo DNA-free DNase (Ambion, Austin, TX). cDNA was synthesized from 1 $\mu$g of DNase-treated total RNA using TaqMan reverse transcription (RT) reagents (Applied Biosystems, Foster City, CA). SYBR green PCR master mix (Applied Biosystems) was used for real-time PCRs to quantify the cDNA levels of target genes. The oligonucleotide primer sequences used in quantitative RT-PCR (qRT-PCRs) are listed in Table S1 in the supplemental material. recA was used as an endogenous control for data analysis (45). Data were collected using a StepOne Plus real-time PCR system (Applied Biosystems) and analyzed using the Relative Expression software tool as described previously (36).

**5’ rapid amplification of cDNA ends (RACE) assay.** Twelve micrograms of total bacterial RNA from E. amylovora Ea1189 was treated with tobacco acid pyrophosphatase (Epipcentre, Madison, WI) at 37°C for 0.5 h, following which 300 pmol of RNA oligonucleotide linker was added. An extraction with a 25:24:1 (vol/vol) solution of water-saturated phenol-chloroform-isoamyl alcohol (P-C-I) was added to the tobacco acid pyro-
phosphatase-treated sample in a 2:1 (vol/vol) ratio, followed by vigorous shaking for 30 s and centrifugation at 13,000 × g for 15 min. RNA was pelleted from the aqueous phase by adding 3 volumes of ethanol containing 0.3 M sodium acetate, followed by incubation on ice for 1 h and centrifugation at 13,000 × g at 4°C for 40 min. The RNA pellet was then dissolved in 14 μl of RNase-free H₂O. Purified RNA-linker mix was de-natured at 90°C for 2 min and was ligated by T4 RNA ligase (New England Biolabs, Ipswich, MA). The ligated RNA-linker mix was purified by the P-C-I extraction again and was dissolved in 10 μl of RNase-free H₂O. cDNA was synthesized by SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) using random hexamers following the instructions of the kit. The cDNA as the template and RNA linker primer and primers specific for the ryhA and rprA genes. cDNA of ryhA and rprA was gel purified and sequenced to map the 5’ end of the transcript.

Nucleotide sequence accession numbers. The ryhA and rprA sequences from E. amylovora Ea1189 were deposited in GenBank with accession numbers KC357251 and KC357252, respectively.

RESULTS

Hfq is an important virulence regulator in Erwinia amylovora.

To determine whether Hfq plays a role in virulence regulation of E. amylovora, an hfq deletion mutant (EAM0436) was constructed and the levels of virulence of wild-type strain E. amylovora Ea1189 and E. amylovora mutant strain Ea1189Δhfq were compared in immature pears and in apple shoots (Fig. 1). In the immature pear fruit assay, Ea1189 caused dramatic necrosis symptoms with associated ooze production at 5 days postinoculation (dpi), while Ea1189Δhfq caused significantly reduced necrosis symptoms that were restricted to the inoculation site (Fig. 1A). In apple shoots at 8 dpi, Ea1189 caused complete necrosis of the inoculated leaf and surrounding leaves and exhibited systemic spreading within the shoot, resulting in an overall wilt symptom (Fig. 1B). In contrast, Ea1189Δhfq caused only slight necrosis at the inoculation site (denoted by the arrow) and showed no systemic movement to other parts of the plant (Fig. 1B). Enumeration of bacterial populations at the early stage of infection indicated that Ea1189Δhfq failed to rapidly multiply following inoculation and exhibited significantly reduced populations compared to wild-type Ea1189 at 3, 27, and 51 h postinoculation (Fig. 1C). The reduced-virulence phenotype and reduced growth following inoculation into pears of Ea1189Δhfq were partially complemented by plasmid pMLhfq, which encoded the hfq gene from Ea1189 (Fig. 1A and C).

Hfq regulates amylovoran production, motility, and biofilm formation. We next conducted experiments to assess the involvement of Hfq in regulating critical virulence factors in E. amylovora. We first compared the amylovoran production, motility, and biofilm formation in strains Ea1189, Ea1189Δhfq, and Ea1189Δhfq/pMLhfq. Production of amylovoran was detected in Ea1189 but not in Ea1189Δams, a deletion mutant of the amylovoran biosynthesis operon (Fig. 2A). Compared to Ea1189, Ea1189Δhfq exhibited a significant reduction in amylovoran production that was complemented by pMLhfq (Fig. 2A). Similar to the observation of amylovoran production, bacterial swarming motility was also significantly reduced in Ea1189Δhfq to about 0.5 that in Ea1189 (Fig. 2B). The reduced-motility phenotype in Ea1189Δhfq was also restored by pMLhfq (Fig. 2B).

To confirm the regulation of amylovoran biosynthesis and mo-
tility by Hfq, the expression of two genes in the amylovoran biosynthesis operon, \textit{amsG} and \textit{amsK}, along with one gene encoding flagellin, \textit{fliC}, was compared in strains \textit{Ea1189}, \textit{Ea1189/H9004/hfq}, and \textit{Ea1189/ams}. Consistent with the phenotypic observations, the mRNA levels of \textit{amsG}, \textit{amsK}, and \textit{fliC} were all reduced in \textit{Ea1189/H9004/hfq} and restored in \textit{Ea1189/H9004/hfq/pMLhfq} compared to \textit{Ea1189}, measured by qRT-PCR. (D) Biofilm formation of \textit{Ea1189}, \textit{Ea1189/H9004/hfq}, \textit{Ea1189/H9004/hfq/pMLhfq}, and \textit{Ea1189/ams}. Bacterial strains were incubated with glass coverslips in static cultures of 0.5× LB broth. The biofilm formed on the coverslips was stained with crystal violet and quantified by measuring light absorbance at OD$_{600}$. Asterisks indicate significant differences ($P<0.05$) compared to \textit{Ea1189}.

\textbf{FIG 2} Effect of Hfq on virulence-related functions in \textit{E. amylovora}. (A) Amylovoran production of \textit{E. amylovora} \textit{Ea1189}, \textit{Ea1189/H9004/hfq}, and \textit{Ea1189/ams}. Bacterial strains were cultured in MBMA medium for 2 days, and the amount of amylovoran produced was quantified using the cetylpyrimidinium chloride (CPC) assay. (B) Swarming motility of \textit{Ea1189}, \textit{Ea1189/H9004/hfq}, and \textit{Ea1189/H9004/hfq/pMLhfq}. Bacterial strains were inoculated at the center of swarming agar plates (0.3% agar), and the swarming diameters were measured at 18 h postinoculation. Asterisks indicate significant differences ($P<0.05$) compared to \textit{Ea1189}. (C) Relative amount of \textit{amsG}, \textit{amsK}, and \textit{fliC} mRNA in \textit{Ea1189}, \textit{Ea1189/H9004/hfq}, and \textit{Ea1189/H9004/hfq/pMLhfq} compared to \textit{Ea1189}, measured by qRT-PCR. (D) Biofilm formation of \textit{Ea1189}, \textit{Ea1189/H9004/hfq}, \textit{Ea1189/H9004/hfq/pMLhfq}, and \textit{Ea1189/ams}. Bacterial strains were incubated with glass coverslips in static cultures of 0.5× LB broth. The biofilm formed on the coverslips was stained with crystal violet and quantified by measuring light absorbance at OD$_{600}$. Asterisks indicate significant differences ($P<0.05$) compared to \textit{Ea1189}.

Hfq limits cell attachment to solid surfaces while promoting cell aggregation and biofilm maturation. The crystal violet staining method used as described above measures the amount of cells attaching to and presumably forming biofilms on glass coverslips but does not provide additional information regarding the biofilm structure and complexity. To test whether the increased biofilm formed by strain \textit{Ea1189/H9004/hfq} was similar in structure to that formed by the wild type, we examined the biofilm structures of \textit{Ea1189}, \textit{Ea1189/H9004/hfq}, and \textit{Ea1189/H9004/hfq/pMLhfq} produced \textit{in vitro} on 300-mesh gold grids using SEM. After 52 h, the majority of \textit{Ea1189} cells formed highly structured cell aggregates that attached to the grid rim and expanded into the center space of the mesh (Fig. 3A). In addition to the aggregated cells, single, nonaggregated cells individually attached to the grid surfaces were observed. Compared to \textit{Ea1189}, the majority of cells of \textit{Ea1189/H9004/hfq} observed were nonaggregated individual cells that formed a lawn evenly covering the grid surfaces. Fewer cell aggregates with less complex structures were observed for \textit{Ea1189/H9004/hfq} than for \textit{Ea1189} (Fig. 3B). The complemented strain \textit{Ea1189/H9004/hfq/pMLhfq} showed fewer cells attached to the grid surfaces and formed fewer cell aggregates than \textit{Ea1189} (Fig. 3C). Greatly reduced cell attachment and aggregation on the TEM grids were observed in the \textit{Δams} strain (unpublished data). Detailed structures of cell aggregates of \textit{Ea1189}, \textit{Ea1189/H9004/hfq}, and \textit{Ea1189/H9004/hfq/pMLhfq} were further characterized by SEM under higher magnification ($×14,000$).
In the biofilm formed by Ea1189, large amounts of extracellular fibrillar material were observed among cell aggregates (Fig. 3D, indicated by the arrow). The amount of this material was greatly reduced in Ea1189/H9004 hfq (Fig. 3E) and was restored in Ea1189/H9004 hfq/pMLhfq (Fig. 3F, denoted by the arrow).

In order to quantify the density of individual, nonaggregated cells of strains Ea1189 and Ea1189/H9004 hfq attaching to the grid surfaces, the cell density of nonaggregated cells attached to the grid surfaces was determined. Compared to Ea1189, a significant increase in density of individually attached cells was observed in Ea1189/H9004 hfq (Fig. 4A). This phenotype could be complemented by pMLhfq. We also quantified the percentage of the mesh space covered by the aggregated cells expanding from grid rims. Aggregated cells of Ea1189/H9004 hfq covered only 15% of the mesh spaces, whereas cell aggregation in Ea1189 covered 26% of the mesh space, on average (Fig. 4B). Although the average percent mesh space covered by Ea1189/H9004 hfq/pMLhfq was not restored (5%), the majority of cells attached to the grid surfaces were observed to be in the form of aggregated cells, similar to the wild type. These results indicate that Hfq negatively controls bacterial attachment while positively controlling cell aggregation in *E. amylovora*.

Taken together, our results suggest that the regulation of the biofilm by Hfq occurs on two different levels: negative control over initial attachment and positive regulation of cell aggregation and of biofilm maturation.

Hfq controls T3SS by upregulating the secretion and translocation of DspE and downregulating the secretion of HrpK and Eop1. To determine if Hfq regulated the normal functioning of T3SS, strains Ea1189, Ea1189Δhfq, Ea1189Δhfq/pMLhfq, and Ea1189ΔT3SS were infiltrated into leaves of the non-host plant *Nicotiana benthamiana* and compared for their abilities to elicit the HR. The HR is a phenotype of programmed cell death exhibited by non-host plants in response to bacterial pathogens (46). At 16 h postinfiltration, a typical HR was observed at the site of infiltration of Ea1189 but not Ea1189ΔT3SS (Fig. 5A). A reduction in the intensity of the HR was observed in the leaf area infiltrated with Ea1189Δhfq; this reduction was restored to wild-type levels in the complemented strain Ea1189Δhfq/pMLhfq (Fig. 5A). These results suggest that Hfq is important for the normal function of the T3SS in *E. amylovora*.

DspE is the most important type III effector protein and is required for the pathogenicity of *E. amylovora*. To examine whether the translocation of DspE is affected by the deletion of hfq, the N-terminal portion of DspE from positions 1 to 737 (DspE1-737), which is required for translocation by the T3SS (40),
was fused with the reporter domain CyaA. The translocation of DspE1-737–CyaA into N. tabacum was compared in strains Ea1189 and Ea1189/H9004 hfq by measuring the cAMP levels in plants (Fig. 5B). The translocation of a previously reported nontranslocatable construct (DspE1-15–CyaA) (40) by Ea1189 was measured as a negative control. At 20 h postinoculation, cAMP was detected in plant tissues inoculated with Ea1189 (88.0 ± 5.7 pg cAMP/g protein) but was not detected following inoculation with Ea1189 producing DspE1-15–CyaA (2.3 ± 0.4 pg cAMP/g protein) (Fig. 5B). Consistent with the HR assay result, the cAMP level in plant tissues inoculated with Ea1189Δhfq containing DspE1-737–CyaA was reduced to about one-third of the level in plant tissues inoculated with Ea1189 (32.0 ± 3.7 pg cAMP/g protein) (Fig. 5B). The results from the HR assay and translocation assay both indicate that Hfq positively controls the translocation of the T3SS effector DspE into plant cells and is important for the normal function of the T3SS.

To test whether the reduced translocation of DspE1-737–CyaA in Ea1189/H9004 hfq was because of a reduced secretion of DspE, Ea1189 and Ea1189/H9004 hfq producing DspE1-737–CyaA were cultured in Hrp-inducing minimal medium, and the culture supernatant was examined for the presence of DspE1-737–CyaA by Western blotting using anti-CyaA antibody. A reduction in secretion of DspE1-737–CyaA (band intensity, 0.77, the level for Ea1189) was observed in Ea1189/H9004 hfq (Fig. 5C).

To test whether the secretion of other type III-secreted proteins was affected by Hfq, we conducted a secretome analysis of strains Ea1189, Ea1189/H9004 hfq, Ea1189/H9004 hfq/pMLhfq, and Ea1189/hrpL. Strains were cultured in Hrp-inducing minimal medium, and the profile of proteins from the culture supernatant was examined by SDS-PAGE. DspE (band 1, at 198 kDa; protein identity was confirmed by MS) was detected in both Ea1189 and Ea1189Δhfq but not in Ea1189/hrpL (Fig. 6A). Two proteins, identified in Fig. 6A as bands 2 and 3, showed enhanced secretion in Ea1189Δhfq but were not secreted by Ea1189ΔhrpL; these proteins were selected as potential T3SS-secreted proteins regulated by Hfq. The
were further characterized using MS. Protein 2, an 80-kDa protein, and protein 3, a 44-kDa protein, were identified as HrpK, a putative type III translocator protein, and Eop1, a type III secreted effector protein, respectively. Using a qRT-PCR assay, significantly enhanced levels of mRNA of hrpK and the Eop1 gene compared to the levels for the wild-type strain were also detected (Fig. 6B). The enhanced mRNA levels could be restored in the hfq-complemented strain (Fig. 6B). These results indicate that Hfq controls the production and secretion of T3SS-secreted proteins, including HrpK and Eop1.

Identification of 10 Hfq-regulated sRNAs in *E. amylovora* by computational analysis. To identify Hfq-regulated sRNAs in *E. amylovora*, sequences of 27 previously characterized Hfq-regulated sRNAs in *Escherichia coli*, another member of the Enterobacteriaceae family, were used in a BLAST search for homologs in the genome of *E. amylovora* ATCC 49946 (GenBank accession number FN666575). Candidate sRNA-encoding genes were selected on the basis of the following criteria: 50% or greater sequence homology, the presence of an sRNA sequence immediately upstream of a Rho-independent terminator, and location in the intergenic regions of the chromosome. Homologs of 10 of the total of 27 sRNA genes from *E. coli* were found in *E. amylovora* (Table 2).

<table>
<thead>
<tr>
<th>sRNA (or alternate name)</th>
<th>% sequence identity to homolog in <em>E. coli</em></th>
<th>Predicted size (nt)</th>
<th>Location in <em>Erwinia</em> genome&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Presence of Rho-independent terminator</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>spf (spot42)</td>
<td>99.1</td>
<td>108</td>
<td>52709–52816</td>
<td>Yes</td>
<td>Regulator of DNA polymerase I activity, gal operon</td>
</tr>
<tr>
<td>rprA</td>
<td>73.0</td>
<td>111</td>
<td>1771945–1771835 (c)</td>
<td>Yes</td>
<td>Translational activator of RpoS, biofilm formation</td>
</tr>
<tr>
<td>omrA-omrB</td>
<td>57.5</td>
<td>79</td>
<td>3009425–3009347 (c)</td>
<td>Yes</td>
<td>Outer membrane protein regulator</td>
</tr>
<tr>
<td>micA</td>
<td>79.8</td>
<td>84</td>
<td>2872079–2872162</td>
<td>Yes</td>
<td>Outer membrane protein regulator</td>
</tr>
<tr>
<td>gcvB</td>
<td>73.2</td>
<td>209</td>
<td>2962739–2962947</td>
<td>Yes</td>
<td>Regulator of periplasmic ABC transporter</td>
</tr>
<tr>
<td>glmZ</td>
<td>70.4</td>
<td>198</td>
<td>218841–219038</td>
<td>Yes</td>
<td>Translational activator of GlmS (aminotransferase)</td>
</tr>
<tr>
<td>ryeA (sraC)</td>
<td>58.6</td>
<td>251</td>
<td>2132789–2133039</td>
<td>No&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Unknown function</td>
</tr>
<tr>
<td>ryhB</td>
<td>73.3</td>
<td>93</td>
<td>1981747–1981655 (c)</td>
<td>Yes</td>
<td>Regulator of iron uptake</td>
</tr>
<tr>
<td>ryhA (arcZ, sraH)</td>
<td>62.3</td>
<td>121</td>
<td>3399430–3399550</td>
<td>Yes</td>
<td>Translational activator of RpoS</td>
</tr>
<tr>
<td>micM (sroB)</td>
<td>62.2</td>
<td>85</td>
<td>1149212–1149296</td>
<td>Yes</td>
<td>Negative regulator of DpiA/DpiB (TCST5)</td>
</tr>
</tbody>
</table>

<sup>a</sup>(c), the sequence is present on the complementary strand of the chromosomal sequence.

<sup>b</sup>This observation is consistent with the observation of ryeA in *E. coli*.

![FIG 7](https://jb.asm.org/) Examination of expression of sRNAs in *E. amylovora*. Detection of the sRNAs rprA, ryhA, omrAB, ryhB, and gcvB in Ea1189 and Ea1189Δhfq at 6 and 12 h postinoculation. Bacterial strains were cultured in Hrp-inducing minimal medium for 6 and 12 h prior to total RNA isolation. Each specific sRNA (and 5S rRNA control) in the total RNA sample was detected by Northern hybridization.
was complemented by plasmids carrying ryhA T3SS-encoding genes, was nonpathogenic (Fig. 8B). The reduced-mutant, with deletion of the alternate sigma factor that regulates virulence phenotype observed in Ea1189 rprA/H9004 hfq of Ea1189 but was still significantly higher than the lesion diameters of Ea1189 rprA/H9004 reduced to a level similar to that of Ea1189 hfq. The mean lesion diameter of Ea1189/H9004 rprA/H9004 was also reduced virulence compared to Ea1189 (Fig. 8A and B). However, two sRNA mutants, those with the ΔryhA and ΔrprA mutations, showed significantly reduced virulence compared to Ea1189 (Fig. 8A and B).

The transcriptional start sites of ryhA and rprA were determined using a 5′RACE assay (see Fig. S1 in the supplemental material). Based on our identification of the ryhA and rprA transcriptional start sites, we determined that the deletions constructed in strains Ea1189ΔrprA and Ea1189ΔryhA encompassed 80% and 100% of the sequences of these sRNAs (see Fig. S2 in the supplemental material). In addition, an examination of the expression of flanking genes mtgA and arcB for ryhA and ppsA, respectively, and EAM1647 for rprA indicated that the deletions of ryhA and rprA did not affect the expression levels of these genes (see Fig. S3 in the supplemental material).

At 5 dpi, the mean lesion diameter of Ea1189ΔryhA was reduced to a level similar to that of Ea1189Δhfq. The mean lesion diameter of Ea1189ΔrprA was also reduced to about half of that of Ea1189 but was still significantly higher than the lesion diameters of Ea1189Δhfq and Ea1189ΔryhA (Fig. 8B). The Ea1189ΔhrpL mutant, with deletion of the alternate sigma factor that regulates T3SS-encoding genes, was nonpathogenic (Fig. 8B). The reduced-virulence phenotype observed in Ea1189ΔrprA and in Ea1189ΔryhA was complemented by plasmids carrying rprA and ryhA, respectively (see Fig. S4 in the supplemental material). These results indicate that the Hfq-regulated sRNAs RyhA and RprA may work together with the sRNA chaperone Hfq and collaboratively control virulence in E. amylovora.

MATERIALS AND METHODS

Discussion

In this study, we showed that Hfq regulated all of the known essential pathogenicity determinants in E. amylovora, including type III secretion, translocation of the major effector DspE, and production of amyllovoran EPS. Hfq is a known regulator of virulence in many bacterial pathogens and is also important in the response to various environmental stress factors in pathogens and other bacteria (28). However, the role of Hfq as a virulence regulator is not universal among pathogens. For example, in Staphylococcus aureus, an hfq mutant, virulence for the worm Caenorhabditis elegans was not affected (48), and in Neisseria gonorrhoeae, the virulence of an hfq mutant was not altered in cell culture models (49). Biofilm formation is critical for E. amylovora to develop large populations in apple xylem and to move systemically through the host (12, 14, 42). The Ea1189Δhfq mutant appears to be unique among E. amylovora biofilm mutants in that although the mutant produced a decreased amount of amyllovoran, it exhibited an altered enhanced biofilm phenotype. However, further examination of biofilm formation by this mutant indicated that Ea1189Δhfq exhibited a hyperattachment phenotype that presumably led to an artifact result suggesting an increase in biofilm formation on a polystyrene surface. In addition, although Ea1189Δhfq cells were capable of aggregation, the fibrillar matrix material characteristic of wild-type E. amylovora Ea1189 biofilms was not present. Thus, it is likely that Ea1189Δhfq cells are not

FIG 8 Analysis and quantification of virulence of E. amylovora deletion mutants of hfq and sRNAs. (A) Virulence of E. amylovora Ea1189, Ea1189Δhfq, and Ea1189ΔhrpL. and sRNA mutants Ea1189ΔryhA, Ea1189ΔrprA, Ea1189Δspf, Ea1189ΔmicA, Ea1189ΔomrA/B, Ea1189ΔryhB, Ea1189ΔsroB, Ea1189ΔryeA, and Ea1189ΔglmZ in immature pears at 5 dpi. (B) Average lesion diameters of immature pears (± standard error) inoculated with each E. amylovora deletion mutant. Sample means were compared by an analysis of variance and separated using the Student t test. The presence of different letters indicates that the means were significantly different (P < 0.05).

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capable of formation of mature biofilms. Since biofilm formation is important for the establishment of large populations of E. amylovora in apple xylem (12, 14, 42), we hypothesize that the lack of biofilm formation by Ea1189Δhfq resulted in an inability of these cells to establish in apple xylem and move systemically through the host. Furthermore, our results suggest that multiple in vitro and in vivo experiments are necessary for the characterization of mutants affecting biofilm formation in bacterial pathogens.

The regulatory effect of Hfq on biofilm formation had been studied in a few bacterial species prior to this study. In Moraxella catarrhalis, when the Δhfq and wild-type M. catarrhalis strains were mixed and inoculated in a continuous-flow biofilm system, the number of Δhfq cells clearly predominated over the number of wild-type cells in the population recovered from the biofilm after an overnight incubation (50). This observation is consistent with our finding that Ea1189Δhfq exhibited a hyperattaching phenotype on polystyrene solid surfaces compared to the wild-type strain Ea1189. Similarly, increased biofilm formation was observed in the absence of Hfq in Yersinia pestis (35). However, in uropathogenic Escherichia coli (UPEC), a mutation of hfq caused reduced biofilm formation when examined by a crystal violet staining method, which suggests a general activating effect of Hfq on biofilm formation (51). These observations indicate that biofilm regulation by Hfq may vary in different pathogens.

Biofilm formation is a complex developmental process that typically involves four phases: planktonic phase, attachment phase, maturation phase, and detachment phase (52). The transition between different phases is tightly regulated by multiple mechanisms, including the second messenger molecule cyclic di-GMP, and by quorum sensing (53, 54). Our observation that Hfq negatively controls bacterial attachment while positively controlling the production of amylovoran and biofilm maturation may provide insight into the transition of biofilm development processes. The attachment or adhesion to solid surfaces is the initial and prerequisite step of biofilm formation. This could be seen in our observation that much reduced attachment and biofilm formation were observed in Ea1189Δhfq/pMLhfq compared to Ea1189 when a multicopy plasmid, pMLhfq, was used for complementation. However, although attachment is an important step of biofilm formation, it has been proposed in animal pathogens that bacterial adhesion to host epithelial cells may also come at a cost due to the possibility of inducing host immunity (55). To minimize host recognition and possible host immune responses while establishing colonization, bacterial animal pathogens likely regulate the transition from initial attachment to biofilm maturation. Our work indicates that Hfq and potentially Hfq-regulated sRNAs may play important roles in this transition.

The delivery of effector proteins from bacteria into plant cells requires the processes of secretion and translocation. The fact that the translocation of DspE-CyaA in Ea1189Δhfq was reduced to ~0.3-fold of that of wild-type Ea1189 while its secretion was reduced to only ~0.77-fold of that of Ea1189 suggests that Hfq may also affect the translocation of DspE, in addition to regulating its secretion. Increased secretion of HrpK, a type III translocator protein (56), was observed in Ea1189Δhfq. The disruption of the production of HrpK, which is important for effector translocation, may be the reason for the reduced DspE translocation observed in Ea1189Δhfq. Similar to the downregulation of the expression of hrpK and the Eop1 gene observed in this work, Hfq was also reported to repress the production of type III effectors encoded on the LEE pathogenicity island in EHEC strains (31). Further study is needed to characterize the detailed mechanism of control of the T3SS by Hfq and small RNAs.

We observed pleiotropic regulation of amylovoran production, biofilm formation, motility, and the T3SS in E. amylovora by Hfq. These observations imply that Hfq-regulated sRNAs may likewise be important virulence regulators in E. amylovora. To determine the role of Hfq-regulated sRNAs in virulence regulation, we first identified 10 sRNAs using computational analysis and found 2 of them (RprA and RyhA) that contribute to the virulence of E. amylovora. Both RprA and RyhA were previously demonstrated to positively regulate translation of the stationary-phase sigma factor RpoS in E. coli (57). To test whether the reduced virulence in strains Ea1189ΔrprA and Ea1189ΔryhA was due to a potential downregulation of rpoS, a ΔrpoS mutant was constructed and its virulence phenotype was compared with that of Ea1189 in an immature pear fruit assay (data not shown). A similar level of virulence was observed in Ea1189ΔrpoS and Ea1189, which is consistent with a previous observation that RpoS is not involved in the induction of fire blight disease symptoms by E. amylovora (58). This suggests that the regulation of virulence by RprA and RyhA is likely mediated via targets different from those in E. coli. We are currently examining the regulatory mechanisms of these sRNAs. Also of note, prior to our study, Schmidtko et al. identified one virulence-related sRNA, sX12, in the plant-pathogenic bacterium Xanthomonas campestris (59). Neither RprA nor RyhA in E. amylovora shares any sequence homology with sX12.

In conclusion, we provide evidence that, similar to its role in animal pathogens, the RNA chaperone Hfq also plays important roles in controlling virulence in the plant-pathogenic bacterium E. amylovora. Compared to previously characterized regulators such as Hrpl, the master regulator for T3SS (10), and RcsBCD, the key regulator of amylovoran production (42), Hfq appears to more broadly regulate many virulence determinants, including amylovoran production, biofilm formation, T3SS translocation and secretion, and bacterial motility. Thus, Hfq and the sRNAs that it regulates likely play a central role in the fine-tuning of virulence gene expression in E. amylovora. During fire blight pathogenesis, E. amylovora cells colonize and grow on the relatively nutrient-rich stigma surface, on the high-osmotic flower nectharode, within the leaf apoplast, and in the potentially low-nutrient vascular system (1). Transitions between these various host environments may occur over relatively short time scales, thus necessitating the ability to rapidly alter the production of virulence factors via posttranscriptional regulation. Our identification of pleiotropic virulence effects in Ea1189Δhfq and of individual sRNAs with strong effects on virulence provides a promising start for characterization of the detailed regulatory mechanisms of Hfq and Hfq-regulated sRNAs in the virulence of E. amylovora.

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