To successfully infect plant hosts, the collective regulation of virulence factors in a bacterial pathogen is crucial. Hfq is an RNA chaperone protein that facilitates the small RNA (sRNA) regulation of global gene expression at the post-transcriptional level. In this study, the functional role of Hfq in a broad host range phytopathogen
\textit{Pantoea ananatis} was determined. Inactivation of the \textit{hfq} gene in \textit{P. ananatis} LMG 2665\textsuperscript{T} resulted in the loss of pathogenicity and motility. In addition, there was a significant reduction of quorum sensing signal molecule acyl-homoserine lactone (AHL) production and biofilm formation. Differential sRNA expression analysis between the \textit{hfq} mutant and wild-type strains of \textit{P. ananatis} revealed 276 sRNAs affected in their abundance by the loss of \textit{hfq} at low (OD\textsubscript{600} = 0.2) and high cell (OD\textsubscript{600} = 0.6) densities. Further analysis identified 25 Hfq-dependent sRNAs, all showing a predicted Rho-independent terminator of transcription and mapping within intergenic regions of the \textit{P. ananatis} genome. These included known sRNAs such as ArcZ, FnrS, GlmZ, RprA, RyeB, RyhB, RyhB2, Spot42, and SsrA, and 16 novel \textit{P. ananatis} sRNAs. The current study demonstrated that Hfq is an important component of the collective regulation of virulence factors and sets a foundation for understanding Hfq-sRNA mediated regulation in the phytopathogen \textit{P. ananatis}.

\textbf{Keywords: Pantoea ananatis, plant pathogen, Hfq, sRNA, regulation, virulence}

\section*{INTRODUCTION}

\textit{Pantoea ananatis}, formerly described as the pineapple pathogen \textit{Erwinia ananas} (Serrano, 1928), is a Gram-negative bacterium belonging to the family \textit{Enterobacteriaceae}. To date, the occurrence of \textit{P. ananatis} has been reported from various ecological niches spanning both the aquatic and terrestrial environments, including fresh (Morohoshi et al., 2007) and marine water (Jatt et al., 2014) as well as the rhizosphere of crop plants (Oliveira et al., 2008; Marquez-Santacruz et al., 2010).
The bacterium exhibits ecologically diverse roles in association with its environment. For example, *P. ananatis* can be found as an epiphyte of crop and weed plants (Gitaitis et al., 2002) or as an endophyte in maize kernels (Rijavec et al., 2007) and rice seeds (Okuniishi et al., 2005). Moreover, the ability of *P. ananatis* to solubilize phosphate, and produce indoleacetic acid and siderophores, makes the bacterium an ideal plant growth-promoting agent in the production of pepper (Kang et al., 2007), soybean (Kuklinsky-Sobral et al., 2004), and sugarcane (da Silva et al., 2015).

*Pantoea ananatis* is better known as a phytopathogen affecting the yield of many economically important plant species that causes blight and dieback of *Eucalyptus* (Coutinho et al., 2002), maize leaf spot disease and brown stalk rot (Goszcynska et al., 2006; Pérez-y-Terrón et al., 2009; Alippi and López, 2010; Krawczyk et al., 2010), leaf blight and bulb rot of onion (Gitaitis and Gay, 1997; Schwartz and Otto, 2000; Goszcynska et al., 2007), palea browning and stem necrosis of rice (Azegami et al., 1983; Cother et al., 2004; Cortesi and Pizzatti, 2007), and fruit rot of netted melon (Kido et al., 2008). *P. ananatis* has also been considered an emerging plant pathogen due to increasing reports of disease outbreaks in the previously undescribed host and geographical regions (Coutinho and Venter, 2009). This emergence is likely to have resulted from the persistent nature of *P. ananatis* in diverse environments through its association with a wide range of non-host plant and even insect vectors (Gitaitis et al., 2003; Dutta et al., 2014).

The virulence factors that have been identified as necessary for pathogenesis of *P. ananatis* in onion are motility for attachment (Weller-Stuart et al., 2016) and quorum sensing (QS) for production of biofilm and exopolysaccharide (EPS) (Morohoshi et al., 2007). In addition, genomic regions named "HiVir" (Asselin et al., 2018) and "Onion Virulence Region" (Stice et al., 2018), encoding enzymes catalyzing phosphonate biosynthetic pathway and cell wall degradation, respectively, have been characterized in the onion pathogenic strains of *P. ananatis*. For successful infection by *P. ananatis*, a rapid and collective expression of these virulence genes in response to the surrounding environment is critical as it results in the modulation of cellular pathways that predispose the pathogen for infection, pathogenesis, and survival in the host.

Hfq is an RNA-binding protein that constitutes a key component of post-transcriptional gene regulation exhibited by small non-coding regulatory RNAs (sRNAs) (Vogel and Luisi, 2011). Hfq is a ring-like homohexameric protein that was initially identified as a host factor needed for the replication of RNA bacteriophage QB (Franze de Fernandez et al., 1968). It is now known that the chaperone Hfq is essential for the structural stabilization of the class of trans-acting sRNAs whose regulatory mechanisms are dependent on Hfq (Updegrove et al., 2016). The chaperone facilitates imperfect base-pairing between the sRNA and its cognate messenger RNA (mRNA), forming an Hfq–sRNA–mRNA complex that determines the fate of target mRNA translation (Gottesman and Storz, 2011; Storz et al., 2011). Suppression of the protein synthesis is achieved by the formation of a sRNA–mRNA duplex at the 5′-untranslated region (UTR) of the transcript by occlusion of ribosome binding and/or by recruiting ribonucleases for mRNA degradation (De Lay et al., 2013). Conversely, translation of the mRNA is enhanced by Hfq–sRNA complexes that alter the 5′-secondary inhibitory structure of an mRNA, making it more accessible for initiation of translation.

Hfq-dependent sRNAs are typically 50–300 nucleotides in length and are trans-encoded from their cognate mRNAs. They are mostly found in, but not limited to, the intergenic regions of bacterial chromosomes (Argaman et al., 2001; Chao et al., 2012; Guo et al., 2014), and are characterized by often possessing a Rho-independent terminator at the 3′-end, resulting in a poly-uridylic tail of sRNA that are recognized by Hfq (Otaka et al., 2011). The cellular functions modulated by Hfq–sRNAs are diverse, ranging from cell membrane integrity, acquisition, and metabolism of nutrients, motility, secretion systems, stress response, and virulence (Chao and Vogel, 2010). Their role in virulence regulation has been extensively studied in bacterial pathogens of animals. For example, in *Salmonella typhimurium*, motility and expression of the T3SS encoded within *Salmonella* pathogenicity island SPI-1 and SPI-2 are dependent on Hfq and contribute significantly to the adhesion and invasion of *Salmonella* into the host cells (Sittka et al., 2007, 2008) whereas in *Vibrio cholera*, Hfq and its trans acting sRNAs Qrr 1–4 regulate cholera toxin (CT) biosynthesis (Bardill and Hammer, 2012) and QS as an ultrasensitive switch to transition *V. cholerae* from low to high cell density mode for colonization and disease development (Lenz et al., 2004).

Despite the growing evidence of Hfq and Hfq-dependent sRNAs as a global post-transcriptional gene regulatory complex, the functionality of Hfq and its trans acting sRNAs in plant pathogenic bacteria has only been investigated in a few bacterial species to date, namely in *Agrobacterium tumefaciens* (Wilms et al., 2012a,b), *Burkholderia glumae* (Kim et al., 2018), *Dickeya dadantii* (Yuan et al., 2019), *Erwinia amylovora* (Zeng et al., 2013; Zeng and Sundin, 2014), *Pectobacterium carotovorum* (Wang et al., 2018), and *Xanthomonas* spp. (Schmidtke et al., 2013). Consequently, the functional role of Hfq and the diversity of Hfq-dependent sRNAs in phytopathogens remain largely elusive. We hypothesized that Hfq and Hfq-dependent sRNAs would play a critical role in *P. ananatis* pathogenesis, through direct regulation of specific virulence traits and through regulation of QS system. In this study, we functionally characterized the role of Hfq as a regulator in the production of acyl-homoserine lactones (AHLs), biofilm development, motility, and virulence, and identified the Hfq-dependent sRNAs that are potentially implicated in the regulation of the virulence traits of the ubiquitous plant pathogen *P. ananatis*.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

The bacterial strains and plasmids used in this study are listed in Table 1. *P. ananatis* LMG2665T and *Escherichia coli* DH5α strains were cultured in Luria-Bertani (LB) broth [1% (w/v) NaCl, 1% (w/v) tryptone, and 0.5% (w/v) yeast extract; pH 7.2] or on LB agar plates [LB broth amended with 1.5% (w/v) agar; pH 7.2] at 28...
and 37°C, respectively. The growth medium was supplemented with either ampicillin (100 µg/ml), chloramphenicol (50 µg/ml), gentamicin (20 µg/ml), or kanamycin (50 µg/ml) for plasmid DNA selection and maintenance.

**Generation of a *P. ananatis* hfq Mutant and Complemented Strains**

A mutant strain with chromosomal deletion of a single copy gene hfq (locus tag: PANa_RS17940) was constructed as previously described (Katashkina et al., 2009; Shyntum et al., 2015). The modification was made in the preparation of the knockout cassette which was amplified from the pKD13 plasmid using the Kan-F and Kan-R primers (Table 2) consisting of 50 bp homologous sequences of hfq flanking regions and 20 bp of kanamycin resistance gene priming sequences. Insertion of the kanamycin resistance gene was verified by Southern blotting, PCR amplification, and sequencing of the hfq region.

The promoter sequence of hfq determined in *E. coli* K12 MG1655 by Kim et al. (2012) was searched against the upstream sequence of hfq start codon in *P. ananatis* LMG2665T. An amplicon (1038 bp) containing the hfq gene (315 bp), its native promoter (58 bp), and flanking sequences (662 bp) was cloned into a pBRR1MCS-START vector (Obranić et al., 2013) restricted with *SmaI* and *BamHI* enzymes. Electromagnetic hfq deletion mutant *P. ananatis* was transformed with hfq complementing plasmid, pBRR1MCS::hfq and the resulting transformants were selected on the gentamicin amended LB agar.

The integrity of the hfq complementation was determined by plasmid extraction, PCR, and sequencing using Test-F and Test-R primers (Table 2).

**In vitro and in planta Growth Assay**

The growth of wild-type *P. ananatis* with an empty pBRR1MCS-5_START vector (WT), hfq deletion mutant with an empty pBRR1MCS-5_START vector (Δhfq), and hfq complementing pBRR1MCS-5_START::hfq (pBRR1MCS::hfq) strains of *P. ananatis* was monitored both in vitro and in planta conditions.

*Pantoea ananatis* strains grown overnight in LB broth were normalized to an OD<sub>600nm</sub> reading of 0.5. For *in vitro* growth assay, the normalized cultures were diluted 100-fold in fresh LB medium and incubated with shaking at 200 rpm. The absorbency of each culture was periodically measured. There were three replicates for each culture and the experiment was repeated twice.

The previously described red onion scale assay (Stice et al., 2018) was adapted for quantifying *in planta* growth of *P. ananatis* between the WT, Δhfq, and hfq complementing strains. In summary, sliced red onion (*Allium cepa* L) scales of approximately 9 cm² in area were surface sterilized in 3% bleach solution for 1 min and were rinsed twice in distilled water. Each scale was macerated in 1 ml of bacterial cells suspended in 1× PBS [0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.144% (w/v) NaHPO₄, 0.024% K₂HPO₄; pH 7.4] using a sterile pipette tip. Inoculated scales were placed on moistened paper towels in a surface sterilized container and were incubated at room temperature for 5 days. To quantify growth, three onion scales per strain were harvested at 24 h intervals. Each scale was macerated in 1 ml of 1× PBS and the extract was serially diluted and cells were enumerated on LB supplemented with gentamicin. Experiments were repeated in triplicate, and the results were presented as CFU/g of onion tissue. Sterile water was used as a negative control.

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**TABLE 1** A list of strains or plasmids used in this study.

| Strain or plasmid       | Characteristics<sup>a</sup>                                      | Source                               |
|-------------------------|------------------------------------------------------------------|--------------------------------------|
| **Strains**             |                                                                  |                                      |
| *Escherichia coli* DH5α | F<sup>−</sup> ϕ80lacZΔM15 ΔlacZYA-argFU169 recA1 endA1 hsdR17 (r<sup>e−</sup>, m<sup>k+</sup>) phoA, supE44 λ− thi−1 gyra96 relA1 | Invitrogen                           |
| *Chromobacterium violaceum* CV026 | ATCC 31532 derivative, cvil:Tn6xylE; Km<sup>r</sup>, Sm<sup>r</sup> | McClean et al., 1997                 |
| **Pantoea ananatis**    |                                                                  |                                      |
| LMG 2665<sup>T</sup>    | Wild-type                                                        | Serrano, 1928                        |
| LMG 2665<sup>T</sup> (pRSFredTER) | LMG 2665<sup>T</sup> transformed with pRSFredTER, Cm<sup>r</sup> | This study                           |
| LMG 2665<sup>T</sup> (pBRR1MCS-START-5) | LMG 2665<sup>T</sup> transformed with pBRR1MCS-START-5, Gm<sup>r</sup> | This study                           |
| LMG 2665<sup>T</sup> Δhfq | hfg deletion mutant, Km<sup>r</sup>                              | This study                           |
| LMG 2665<sup>T</sup> Δhfq-pBRR1MCS-5_START::hfq | LMG 2665<sup>T</sup> Δhfq transformed with pBRR1MCS-START-5::hfq, Km<sup>r</sup>, Gm<sup>r</sup> | This study                           |
| **Plasmids**            |                                                                  |                                      |
| pKD13                   | Broad-host range vector, mutagenesis cassette template, Km<sup>r</sup> | Datsenko and Wanner, 2000            |
| pRSFredTER              | Broad-host range vector, expresses bacteriophage λ red recombinase (bet, exo, gam) and sacB, Cm<sup>r</sup> | Katashkina et al., 2009              |
| pBRR1MCS-START-5        | Broad-host range vector, promoterless, Gm<sup>r</sup>            | Obranić et al., 2013                 |
| pBRR1MCS-START-5::hfq   | Broad-host range vector, promoterless, Gm<sup>r</sup>            | This study                           |

<sup>a</sup>Cm<sup>r</sup>, Gm<sup>r</sup>, Km<sup>r</sup>, and Sm<sup>r</sup> represent chloramphenicol, gentamicin, kanamycin, and streptomycin resistance, respectively.
| Primer name          | Sequence (5′–3′)                                                                 | Length (nt) |
|---------------------|--------------------------------------------------------------------------------|-------------|
| **Mutagenesis**     |                                                                                   |             |
| Kan-F               | ACGTCGCTTATATAAAAAGACCGAGATGGAAGACCT                                              | 70          |
|                     | GACGCTTTCCGATGCGATTGTAGCCTGGAAGCT                                               |             |
| Kan-R               | TTACGCAATTTTTTTTCGAGAACACTGTGTTTCTCAAA                                           | 70          |
|                     | GCAACAAACAAACAATACCGCCGGATTCGCCTGGAACC                                             |             |
| Test-F              | TATGGCCGAAACATGGGTG                                                              | 18          |
| Test-R              | TGCTACCCGCTATACCAAGGG                                                            | 21          |
| Southernblot-F      | GCGATTTGTTAGGGCTGAGCT                                                           | 21          |
| Southernblot-R      | TCGGATGGAAGCGGGCTTTTGTG                                                          | 23          |
| **Complementation** |                                                                                   |             |
| Comp-F              | AAAAGGATTCGAGGGCTGAGCGGTGTTATACATGG                                             | 31          |
| Comp-R              | CGGTCAAAACAGCAGCTAAACCTCG                                                       | 23          |
| **qRT-PCR**         |                                                                                   |             |
| ffh-F               | CATTGAGATCAAACACCGTGG                                                             | 20          |
| ffh-R               | TGCGCGAGTGGCTGCTGCT                                                             | 19          |
| Arcz-F              | GCAAGTTGTTACCAATACCC                                                            | 20          |
| Arcz-R              | GGTGCGCTATACGGC                                                               | 17          |
| FnR5-F              | GTGATGCTGCTAAGCTCCA                                                             | 17          |
| FnR5-R              | GTTAGCCGGGTATTTTJC                                                              | 17          |
| GlmZ-F              | CATAAACCTGGGAAGCTG                                                           | 19          |
| GlmZ-R              | AGCAGGTGTAAGCTAGG                                                              | 18          |
| RprA-F              | TACCATGTTCCTATGTGG                                                              | 20          |
| RprA-R              | GATGGGCAAAGACTCAC                                                              | 18          |
| RynB2-F             | TCCTGGCTATCGGCTACG                                                             | 19          |
| RynB2-R             | GGCCTGCTAATAATACCTGGAAGCC                                                        | 24          |
| RyeB-F              | CGAAAGCCTCTTTTATTGCA                                                            | 20          |
| RyeB-R              | AGGATAGAACACGTTCC                                                               | 17          |
| pPAR237-F           | GTGCGAAGAGCAGGGTGA                                                              | 17          |
| pPAR237-R           | CTTTTGGGCGCAGCTC                                                               | 17          |
| pPAR238-F           | CTGAGAACACGACCCC                                                              | 17          |
| pPAR238-R           | GATGTTACTGTTGAGTGTTCC                                                          | 20          |
| pPAR395-F           | TGCGGCAAATCGATGG                                                              | 18          |
| pPAR395-R           | CGGACACCTCTGTTAAAGG                                                             | 17          |
| **5′-RACE**         |                                                                                   |             |
| Linker nested-F     | GAAGAGACTGCTAGCTAGGAAGG                                                         | 19          |
| FnRS-R              | GTTAGCCGGGCTATTTTCC                                                             | 17          |
| FnRS-nR             | AGACAAATATGGGAGCGCAACGG                                                         | 20          |
| GlmZ-F              | AGCAGGTGTAAGCTAGG                                                              | 18          |
| GlmZ-nR             | CGAAGGTGATGCCGACTCAACG                                                                 | 23          |
| pPAR237-R           | ACTTTGGGCGCAGCTGTTGCA                                                          | 21          |
| pPAR237-nR          | CGGACACCTCTGCTAGCTAGG                                                           | 21          |
| pPAR238-R           | TCAAGTTGCTCGGCGCAGCTAGCT                                                        | 23          |
| pPAR238-nR          | TCTCTGTGTTGCTGCTGTTG                                                           | 20          |
| pPAR395-R           | CGGACACCTCTGTTAAAGG                                                             | 17          |
| pPAR395-nR          | CCTAAATGACTCCTCCAAACAGG                                                        | 22          |

**Virulence Assay**

Virulence assay was performed as previously described for *in planta* growth assay. The vertical diameter of the water-soaked lesion on onion scales inoculated with WT, Δhfq, and Δhfq complementing *P. ananatis* strains was measured at 3 days post inoculation (dpi). The virulence assay was repeated twice, and there were three technical replicates for each *P. ananatis* strains.

**Motility Assay**

Overnight cultures of *P. ananatis* strains (WT, Δhfq, and pBBR1MCS::hfq) were normalized to OD<sub>600nm</sub> = 0.5 and 1 µl of each culture was inoculated in the center of the soft agar [0.5% (w/v) NaCl, 1% (w/v) tryptone, and 0.3% (w/v) agar; pH 7.2]. The inoculated plates were incubated at 28°C, and swimming motility was determined after 24 h. Negative control plates were
inoculated with sterile water. The swimming motility experiment was repeated three times with three biological replicates in each experiment.

**Bioassay Detection of Acyl-Homoserine Lactones**

Formation of AHL by WT, Δhfq, and hfq complementing strains of *P. ananatis* was determined using experimental procedures adapted from McLean et al. (1997). An aliquot (0.5 ml) of AHL reporter strain *Chromobacterium violaceum* (*C. violaceum* 026) grown in LB overnight was spread plated on LB agar plates and air-dried. Thereafter, three wells were (three replicates) created on each plate by puncturing the agar with a sterile cork-borer and inoculated with 100 µl of cell-free filtrate of *P. ananatis* WT, Δhfq, and hfq complementing strains overnight cultures. The inoculated plates were incubated at 28°C for 48 h. The formation of violacein (purple halo) by CV026, around the inoculated wells, served as a negative control. Thereafter, the inoculated 96-well plates were inverted to remove the excess LB broth, air-dried, and incubated at 60°C for 40 min to heat-fix the biofilms. The biofilms were stained with 1% crystal violet (220 µl) for 15 min before being rinsed with distilled water. After rinsing and air-drying the microplate, 220 µl of ethanol:acetone in 8:2 ratio was added to the wells to solubilize the crystal violet dye for 20 min at room temperature. The solubilized biofilm was measured at OD$_{600}$ using Safire Microplate Reader (Tecan, Research Triangle Park, NC, United States), and this assay was repeated twice and carried out in three technical replicates.

**Biofilm Quantification**

The biofilm of WT, Δhfq, and hfq complementing strains of *P. ananatis* was quantified as previously described by Santander and Biosca (2017) with slight modifications. An aliquot of 160 µl broth culture diluted to an OD$_{600nm}$ of 0.5 in half-strength LB [0.5% (w/v) NaCl, 0.5% (w/v) tryptone, and 0.25% (w/v) yeast extract; pH 7.2] was made into each well of a polystyrene 96-well microplate (NuncTM MicroWellTM, Thermo Scientific, Waltham, MA, United States) and incubated for 24 h under static conditions. Eight replicates per *P. ananatis* strain were included in each experiment with sterile half-strength LB broth serving as a negative control. Thereafter, the inoculated 96-well plates were inverted to remove the excess LB broth, air-dried, and incubated at 60°C for 40 min to heat-fix the biofilms. The biofilms were stained with 1% crystal violet (220 µl) for 15 min before being rinsed with distilled water. After rinsing and air-drying the microplate, 220 µl of ethanol:acetone in 8:2 ratio was added to the wells to solubilize the crystal violet dye for 20 min at room temperature. The solubilized biofilm was measured at OD$_{600}$ using Safire Microplate Reader (Tecan, Research Triangle Park, NC, United States), and this assay was repeated three times.

**RNA Extraction and Transcriptomic Analysis**

Total RNA of *P. ananatis* WT and Δhfq strains grown in LB broth was extracted at OD$_{600nm}$ readings of 0.2 (T1 = low cell density) and 0.6 (T2 = high cell density) using the mirNeasy Mini kit (Qiagen, Hilden, Germany). Genomic DNA was removed by including an on-column DNase digestion step during the RNA extraction. The purity (A$_{260}/A_{280}$) of extracted RNA was measured by Nanodrop2000 (Thermo Scientific, Sugarland, TX, United States) and RNA integrity was determined by Agilent2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States). Illumina Truseq Small RNA Library (Illumina, San Diego, CA, United States) preparation was performed on the RNA samples, and deep sequencing of the library was conducted on Illumina HiSeq2500 platform (single-end, 1 × 50 bp) by Macrogen (South Korea).

**Bioinformatic Analysis and sRNA Identification**

Raw sequencing reads (BioProject accession number: PRJNA550544) were stringently trimmed and filtered using Trimmmomatic (Bolger et al., 2014) to remove adapter sequences and low quality reads. Following adapter trimming and filtering, quality was verified using FastQC (Andrews, 2010) and reads were mapped to the *P. ananatis* LMG20103 genome (De Maayer et al., 2010) using Bowtie2 (Langmead and Salzberg, 2012), as the genome of LMG 20103 was the only *P. ananatis* genome with a complete annotation at the time of analysis. For sRNA identification, a custom python script (Supplementary Data Sheet S1, see the section “genic_filter.py” in the Supplementary Material) was compiled to remove reads that mapped to coding sequences, ribosomal RNA, and transfer RNA, or within 120 bases upstream or downstream of these features from the resulting sequence alignment map (SAM) files. The purpose of the 120 base pair buffer was to reduce the number of sRNAs identified that originated from extended 5′- or 3′-UTR regions. All wild-type sequencing replicates from the same sampling time point were merged into a single gene-filtered SAM file for sRNA identification.

To identify putative sRNAs from gene-filtered SAM files, a custom python script (Supplementary Data Sheet S1, see the section "peak_ID.py" in the Supplementary Material) was used to calculate per base depth relative to the genome-wide per-base sequencing depth by replicate, which was also normalized to library size. A threshold of 10-fold increased abundance above background with a minimum length of 10 nucleotides was chosen for sRNA identification. Using the script, putative sRNAs at the low cell density and high cell density sampling time points were identified and the lists of sRNAs were merged using a custom python script (Supplementary Data Sheet S1, see the section “mergeList.py” in the Supplementary Material), combining any overlapping identified sRNAs into a single sRNA to generate a single list of putative *P. ananatis* sRNAs (pPARs sRNA).

**Computational Prediction of Rho-Independent Terminators**

Following established criteria (Zeng and Sundin, 2014), Rho-independent terminators were searched in the *P. ananatis* LMG20103 genome using a custom python script (Supplementary Data Sheet S1, see the section “RI_term.py” in the Supplementary Material). Briefly, the search was conducted in an effort to detect poly-T regions with at least six continuous Ts and for those that had at least four GC base pairs in the last six bases before the poly-T stretch. Of these, those that had at least 50% GC content in the last 25 bases before the poly-T were considered to be putative Rho-independent terminators.

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*Shin et al.*
Differential sRNA Expression in *P. ananatis* LMG 2665 WT vs. Δ*hfq*

Using the genomic coordinates from the BLAST + search of the pPAR sRNAs against the *P. ananatis* LMG2665 genome (Adam et al., 2014), a gene format file (gff) for all the pPAR sRNAs was generated. The sRNA sequencing reads that had been trimmed and filtered were mapped to the LMG2665 genome using Bowtie2 (Langmead and Salzberg, 2012). The mapped reads were sorted using SAMTools (Li et al., 2009) and the number of reads mapping to pPAR sRNAs in the LMG2665 genome was counted using HTSeq (Anders et al., 2015). Read counts tables were analyzed for statistically significant differential expression of pPAR sRNAs between WT- and Δ*hfq*-mutant samples at corresponding time points using the DESeq R package which utilizes a negative binomial distribution model (Anders and Huber, 2010; R Core Team, 2013). Resulting genes with a false-discovery rate of 0.05 were considered differentially expressed.

**sRNA Conservation Analysis**

The bacterial genomes were downloaded from NCBI and searched using BLAST+ (Camacho et al., 2009) with all pPAR sRNA sequences as queries. Because BLAST uses local alignment, the global percent identity to pPAR sRNAs was calculated by multiplying the percent identity by the length of the BLAST alignment and dividing by the length of the pPAR sRNA. Heatmaps showing percent identity of sRNA by genome were generated using ClustVis (Metsalu and Vilo, 2015).

**qRT-PCR Validation of sRNA Expression**

To validate the expression of putative sRNAs identified, a quantitative RT-PCR was conducted on a subset of sRNAs. The 2 μg of total RNA extracted from the two time points (low and high cell density) was converted to cDNA using random primers using the High-Capacity cDNA Synthesis Kit (Applied Biosystems, Carlsbad, CA, United States). Subsequently, PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Carlsbad, CA, United States) was used to quantify expression levels of the selected sRNAs real time in QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Carlsbad, CA, United States). The list of primers used for qRT-PCR is found in Table 2. The relative expression of sRNA was calculated using 2−ΔΔCT method (Livak and Schmittgen, 2001) with the gene *ffh* encoding a signal recognition particle protein, serving as an endogenous mRNA control (Takle et al., 2007; Sibanda et al., 2018).

**5′-Rapid Amplification cDNA Ends Analysis**

The 5′-Rapid Amplification cDNA Ends (RACE) analysis was conducted on the selected putative sRNAs to capture their transcription start sites (TSS). Total RNA (up to 15 μg) of *P. ananatis* strains grown to high density (OD₆₀₀ = 0.6) was extracted as above mentioned (see the section “RNA Extraction and Transcriptomic Analysis”). The resulting RNA was ligated to 300 pmol of RNA linker: GACGAGCACGAGGACACUGACAUUGGAGGGAGAGUAG AAA in the presence of RNA 5′-pyrophosphohydrolase (RppH) (New England BioLabs, Ipswich, MA, United States) and T4 RNA ligase (New England BioLabs, Ipswich, MA, United States) at 37°C for 4 h. The linker-ligated RNA was purified using Trizol-chloroform (2:1) extraction method, as described by Rio et al. (2010). The resulting RNA was ethanol precipitated and suspended in 10 μl of RNase-free water. The cDNA of linker-ligated RNA was synthesized as previously described (see the section “5′-RACE”).

**Secondary Structure and mRNA Target Prediction**

The secondary structures of sRNAs, of which their TSS have been determined by 5′-RACE analysis, were predicted *in silico* using RNAfold web server (Hofacker, 2003). The putative target mRNAs of novel sRNAs pPAR237, pPAR238, and pPAR395 and their putative interacting domains were computationally predicted using CopraRNA and IntaRNA (Wright et al., 2014). The above information is presented in Supplementary Figure S6 and Supplementary Table S4, respectively.

**Image and Statistical Analysis**

Images resulting from motility, AHL detection, and virulence assays were analyzed in ImageJ (Schneider et al., 2012) for measurement of halos and lesion diameter. Statistical analyses are performed with R 3.2.6 (R Core Team, 2013) and significance of the data (*P* < 0.05) were determined by analysis of variance (ANOVA) and Tukey’s honestly significantly difference (HSD) tests. Except where otherwise mentioned, all data shown in this study represent mean values and error bars represent standard error (SE) of the samples.

**RESULTS**

**hfq Mutation Negatively Affects Growth**

To investigate the functional role of Hfq in the pathogenesis of *P. ananatis*, an *hfq* deletion mutant (Δ*hfq*) was constructed by replacing the *hfq* gene with a kanamycin resistance marker (the section “Materials and Methods”). Southern blotting (Supplementary Figure S1) and PCR amplification of the *hfq* region (Supplementary Figure S2) verified a single insertion of the antibiotic marker in the *hfq* mutant strain. For the construction of the *hfq*-complementing plasmid, *hfq* promoter sequence of *E. coli* K12 was used to search for *hfq* promoter in *P. ananatis* and a highly conserved *hfq* promoter sequence (93% nucleotide identity) of *P. ananatis* compared to that of *E. coli* K12 was found overlapping in the coding region of the adjacent gene *miaA*.

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1. [http://rna.tbi.univie.ac.at](http://rna.tbi.univie.ac.at)
2. [http://rna.informatik.uni-freiburg.de](http://rna.informatik.uni-freiburg.de)
In vitro growth analyses of *P. ananatis* WT, Δhfq, and hfq complementing strains cultured in LB medium showed that the hfq mutation affected the growth of *P. ananatis*. The hfq mutant exhibited a slower growth rate relative to the WT and hfq-complementing strains, but similar cell density was reached at stationary phase as the both strains (Supplementary Figure S3). Similarly, in planta growth curves at 12 h showed that WT, Δhfq, and hfq complementing strains of *P. ananatis* exhibited comparable cell densities to one another (Supplementary Figure S4B) which were at sufficient levels for the onset of symptoms by 3 dpi (Supplementary Figure S4A).

**Loss of Hfq Attenuates Virulence in *P. ananatis***

Virulence assay on red onion scales demonstrated clearing of the red pigment and formation of a water-soaked lesion in the onion scales inoculated with WT *P. ananatis* while no disease symptoms were observed on the scales infected with the *P. ananatis* Δhfq mutant (Figure 1). The impaired virulence of the *P. ananatis* Δhfq mutant was restored to the wild-type levels by trans expression of hfq gene on the plasmid pBBR1MCS-5:start::hfq. The finding that the *P. ananatis* Δhfq-mutant strain was able to attain in planta population densities equivalent to WT (Supplementary Figure S4) suggests that the lack of disease symptoms is not associated with a growth defect, and that hfq is required for virulence of this strain when inoculated into red onion.

**Hfq Regulates Motility, AHL Production, and Biofilm Formation**

To determine whether the *P. ananatis* Hfq regulates virulence traits, swimming motility, production of AHL molecules, and biofilm were quantified in WT, Δhfq, and hfq-complementing strains of *P. ananatis*. The results show that *P. ananatis* hfq mutant was impaired in swimming motility relative to the wild-type strain, as determined by the size of the halo that formed on the soft agar (Figure 2). In addition, AHL production, as determined by the production of the purple pigment violacein by the *C. violaceum* 026 biosensor demonstrated a statistically significant reduction in the size of the purple halo formed by the hfq-mutant strain relative to the wild type, indicating a significant reduction in AHL production by the mutant strain (Figure 3). Furthermore, a threefold reduction (*P* < 0.05) in the biofilms formed by the hfq-mutant strain relative to the WT.
P. ananatis was also observed (Figure 4). These findings are consistent with previous studies that showed that AHL molecules are needed as a signal for QS to regulate biofilm formation in P. ananatis (Morohoshi et al., 2007; Sibanda et al., 2016). The phenotypic defects resulting from loss of hfq, which were restored to wild-type levels by trans-complementation of hfq, suggest that Hfq regulates the production of multiple virulence traits in P. ananatis.

Identification of Putative sRNAs
Due to impaired motility, AHL production, biofilm formation, and virulence caused by the loss of Hfq in P. ananatis LMG 2665, a sRNA sequencing analysis was conducted to identify the regulatory sRNAs that are dependent on Hfq for stability and function. Deep sequencing of the sRNA transcriptomes of WT and Δhfq-mutant P. ananatis strains at low (OD600 = 0.2) and high cell density (OD600 = 0.6) time points resulted in a total of 172.03 million reads. Following trimming of adapters and filtering for high-quality reads (Phred score =30), 66.74 million reads were retained, of which 83.2% mapped uniquely to the P. ananatis LMG20103 genome. Following removal of the reads that mapped to protein coding genes, rRNAs, or tRNAs, 9.72 million reads remained for the sRNA identification and analysis. The distribution of reads across the WT and Δhfq-mutant P. ananatis strains, each with three technical replicates at low and high cell density time points, are included in Supplementary Table S1.

For identification of sRNAs in the transcriptome dataset, the WT sequencing data were utilized and calculated for the per-base depth across the genome relative to the genome-wide average per-base depth. To select a threshold that would allow for sensitive detection of sRNAs while also filtering out noise in the sequencing data, the number of putative sRNAs identified across a broad range of signal-to-noise thresholds was calculated. A strong linear relationship ($R^2 = 0.9981$) between Log10(Threshold) and Log10(# sRNAs identified) was found (Supplementary Figure S5), and a signal-to-noise threshold ratio of 10 was selected for calling of putative sRNAs from the sequencing data. Using this threshold, a total of 615 pPARs sRNAs was identified. Of these, 425 pPARs were identified in both time points, 90 were identified only in the low cell density time point, and 100 only in the high cell density time point in P. ananatis LMG2665.
Characterization of pPAR sRNAs

The 615 identified pPARs were further classified as intergenic, antisense, or overlapping. The classification resulted in 249 intergenic pPAR sRNAs, 302 antisense pPAR sRNAs, and 64 overlapping pPAR sRNAs (Figure 5A). The mean length of pPAR sRNAs was 66.4 bases with a median of 42 bases (Figure 5B) and mean GC content of pPAR sRNAs was 52.3% with a median of 52.2% (Figure 5C). Both of these are quite close to the genome average of 53.7% GC bases (De Maayer et al., 2010). Of note, seven pPAR sRNAs had GC content below 30%, and 14 pPAR sRNAs had GC content above 70%, suggesting the potential horizontal acquisition of the genomic regions containing these sRNAs.

In addition, we performed a genome-wide computational search for putative Rho-independent terminators that are associated with the transcription termination of Hfq-dependent sRNAs (Otaka et al., 2011). The results revealed that there were 5,002 poly-T stretches with at least 6 continuous Ts and 2,437 of these had four or more GC base pairs in the last 6 bases before the poly-T. A total of 1,842 of poly-T stretches had approximately 50% GC content in the final 25 bases before the poly-T, meeting the established criteria of Rho-independent terminators (Zeng and Sundin, 2014). Based on these criteria, only 569 were associated with protein-coding genes and 69 were associated with the identified pPAR sRNAs. The key features of select pPAR sRNAs are presented in Table 3 and for full data, including genomic coordinates and sRNA sequences, refer to Supplementary Table S2.

Identification of Hfq-Dependent sRNAs

Because trans-encoded sRNAs have been shown to depend on RNA chaperone proteins such as Hfq for stability and activity (Vogel and Luisi, 2011), an hfq mutant was included in the sRNA sequencing experiment in order to determine pPAR sRNAs that are dependent on or influenced by the loss of Hfq. The analysis of hfq to WT samples from both low cell density and high cell density samples identified a total of 276 pPAR sRNAs affected in abundance by Hfq. Sixty-four pPAR sRNAs were affected in abundance by loss of hfq in both cell density samples, 58 pPAR sRNAs in low cell density samples, and 154 pPAR sRNAs only in high cell density samples (Figure 6). Of all the Hfq-dependent pPAR sRNAs, 145 had decreased abundance and 131 had increased abundance in the hfq mutant relative to wild type. Overall, results indicate that Hfq affects the abundances of numerous pPAR sRNAs either positively and/or negatively. Supplementary Table S3 lists all pPAR sRNAs affected...
FIGURE 4 | Effect of deletion of hfq on the biofilm-forming ability of *P. ananatis*. (A) Biofilms formed by the wild-type (WT), *hfq* mutant (*Δhfq*), and *hfq* complementing (*Δhfq* (pBBR1MCS::*hfq*)) strains of *P. ananatis* LMG 2665T in 96-well microtiter plate after 24 h incubation under static conditions. (B) Quantification of the biofilms formed by the (WT), *Δhfq*, and *Δhfq* (pBBR1MCS::*hfq*) strains after 24 h using crystal violet (1%) staining method. The absorbance of solubilized biofilms stained with crystal violet was measured at an optical density wavelength of 600 nm. An asterisk denotes significance differences (*P* < 0.05) in the amount of biofilm formed by *Δhfq* relative to WT *P. ananatis*.

FIGURE 5 | Characterization of *Pantoea ananatis* sRNAs (pPAR sRNA). (A) A total of 615 putative *P. ananatis* LMG 2665T sRNAs were classified into 302 antisense, 64 overlappings, and 249 intergenic pPAR sRNAs. (B) The mean length of pPAR sRNAs was 66.4 bases with a median of 42 bases. (C) The mean GC content of pPAR sRNAs was 52.3% with a median of 52.2%.

in abundance by loss of *hfq* as well as corresponding fold changes in both low and high cell densities.

Of the pPAR sRNAs affected by the loss of *hfq*, 41 have predicted Rho-independent terminators. Of these, 25 are intergenic and 16 are antisense, consistent with the classical model that Hfq-dependent sRNAs are frequently intergenic (Vogel and Luisi, 2011). Among the sRNAs detected in intergenic regions and Hfq-dependent with Rho-independent terminator, 9 known sRNAs and 16 novel sRNAs were identified. The known sRNAs included ArcZ, FnrS, GlmZ, RprA, RyeB/SdsR, RyhB, RyhB2, Spot42, and SsrA. The depth plots for a number of selected known and novel pPAR sRNAs of interest were generated, showing per-base sequencing depth across the length of the sRNA (Figure 7). Several pPAR sRNAs have certain regions...
| sRNA locus ID | sRNA name | Strand | Start | End | Length (nt) | Classification | RI-terminator | Hfq-dependent |
|---------------|-----------|---------|-------|-----|-------------|----------------|---------------|---------------|
| pPAR009       |           | –       | 97939 | 98052 | 113         | Intergenic     | Yes           | Yes           |
| pPAR026       | glmZ      | +       | 183885 | 184074 | 189         | Intergenic     | Yes           | Yes           |
| pPAR035       |           | –       | 241229 | 241262 | 33          | Antisense      | Yes           | No            |
| pPAR052       |           | –       | 318391 | 318427 | 36          | Intergenic     | Yes           | Yes           |
| pPAR063       | arcZ      | –       | 497228 | 497409 | 181         | Intergenic     | Yes           | Yes           |
| pPAR089       |           | –       | 518137 | 518185 | 48          | Overlapping    | Yes           | Yes           |
| pPAR091       |           | –       | 758889 | 758998 | 109         | Intergenic     | Yes           | No            |
| pPAR143       |           | –       | 1192217| 1192317| 100         | Overlapping    | Yes           | Yes           |
| pPAR155       |           | –       | 1306802| 1306998| 109         | Antisense      | Yes           | Yes           |
| pPAR165       |           | –       | 1480479| 1480511| 32          | Intergenic     | Yes           | No            |
| pPAR184       |           | –       | 1706868| 1706902| 34          | Antisense      | Yes           | Yes           |
| pPAR204       |           | –       | 1900966| 1901000| 34          | Antisense      | Yes           | Yes           |
| pPAR205       | rprA      | –       | 1918007| 1918133| 126         | Intergenic     | Yes           | Yes           |
| pPAR237       |           | –       | 2187335| 2187526| 191         | Intergenic     | Yes           | Yes           |
| pPAR241       |           | –       | 2226977| 2227034| 57          | Intergenic     | Yes           | Yes           |
| pPAR245       | fnrS      | –       | 2254671| 2254972| 121         | Intergenic     | Yes           | Yes           |
| pPAR246       | ryhB      | –       | 2257249| 2257331| 82          | Intergenic     | Yes           | Yes           |
| pPAR287       |           | –       | 2451765| 2451968| 203         | Antisense      | Yes           | No            |
| pPAR307       |           | –       | 2750610| 2750660| 50          | Intergenic     | Yes           | Yes           |
| pPAR320       |           | –       | 3095161| 3095223| 62          | Intergenic     | Yes           | No            |
| pPAR322       |           | –       | 3146708| 3146885| 177         | Intergenic     | Yes           | Yes           |
| pPAR332       |           | –       | 3235861| 3235937| 76          | Antisense      | Yes           | Yes           |
| pPAR343       | ssrA      | +       | 3274707| 3274936| 229         | Intergenic     | Yes           | Yes           |
| pPAR345       |           | –       | 335859 | 335962 | 103         | Overlapping    | Yes           | Yes           |
| pPAR364       |           | –       | 3475041| 3475225| 184         | Intergenic     | Yes           | No            |
| pPAR388       |           | –       | 3747994| 3748044| 50          | Antisense      | Yes           | No            |
| pPAR394       |           | –       | 3822243| 3822884| 41          | Intergenic     | Yes           | Yes           |
| pPAR395       |           | –       | 3822866| 3822977| 111         | Intergenic     | Yes           | Yes           |
| pPAR404       |           | –       | 3929664| 3929700| 36          | Antisense      | Yes           | No            |
| pPAR418       |           | –       | 4001816| 4001864| 48          | Overlapping    | Yes           | No            |
| pPAR433       | ryhB      | –       | 4105322| 4105419| 97          | Intergenic     | Yes           | Yes           |
| pPAR442       |           | –       | 4239633| 4239719| 86          | Intergenic     | Yes           | No            |
| pPAR447       |           | –       | 4256795| 4256882| 87          | Intergenic     | Yes           | No            |
| pPAR457       |           | –       | 4330842| 4330892| 50          | Overlapping    | Yes           | Yes           |
| pPAR463       |           | –       | 4372873| 4372966| 93          | Intergenic     | Yes           | No            |
| pPAR464       | spf       | –       | 4373881| 4373992| 111         | Intergenic     | Yes           | Yes           |
| pPAR470       |           | –       | 4388229| 4388352| 123         | Overlapping    | Yes           | Yes           |
| pPAR479       |           | –       | 4457282| 4457307| 25          | Intergenic     | Yes           | Yes           |
| pPAR509       |           | –       | 122956 | 123006 | 50          | Intergenic     | Yes           | No            |
| pPAR511       |           | –       | 132823 | 132844 | 21          | Antisense      | Yes           | Yes           |
| pPAR521       |           | –       | 392904 | 392917 | 71          | Antisense      | Yes           | No            |
| pPAR525       |           | –       | 477597 | 477633 | 36          | Intergenic     | Yes           | No            |
| pPAR535       |           | –       | 809520 | 809548 | 28          | Antisense      | Yes           | Yes           |
| pPAR544       |           | –       | 900462 | 900550 | 88          | Antisense      | Yes           | No            |
| pPAR582       |           | –       | 2552244| 2552278| 34          | Intergenic     | Yes           | No            |
| pPAR608       |           | –       | 3962774| 3962823| 49          | Antisense      | Yes           | Yes           |
| pPAR626       |           | –       | 4293304| 4293392| 88          | Intergenic     | Yes           | No            |
| pPAR632       |           | –       | 4459587| 4459632| 45          | Antisense      | Yes           | No            |

(Continued)
TABLE 3 | Continued

| sRNA locus_ID | sRNA name | Strand | Start | End | Length (nt) | Classification | RI-terminator | Hfq-dependent |
|--------------|-----------|--------|-------|-----|-------------|----------------|---------------|--------------|
| pPAR638      | –         | +      | 58679 | 58728 | 49          | Intergenic     | Yes           | Yes          |
| pPAR642      | –         | –      | 111611| 111679| 63          | Overlapping    | No            | Yes          |
| pPAR667      | –         | +      | 812084| 812142| 58          | Overlapping    | Yes           | Yes          |
| pPAR679      | –         | +      | 1035446| 1035471| 25         | Intergenic     | Yes           | Yes          |
| pPAR699      | –         | –      | 1501874| 1501915| 41         | Antisense      | Yes           | Yes          |
| pPAR714      | –         | –      | 2049786| 2049816| 30         | Antisense      | Yes           | Yes          |
| pPAR719      | –         | –      | 2171734| 2171781| 47         | Intergenic     | Yes           | Yes          |
| pPAR724      | –         | +      | 2341919| 2341964| 45         | Antisense      | Yes           | Yes          |
| pPAR726      | –         | –      | 2353498| 2353521| 23         | Intergenic     | Yes           | Yes          |
| pPAR732      | sdsR/ryeA | –      | 2435078| 2435148| 70         | Intergenic     | Yes           | Yes          |
| pPAR765      | –         | –      | 3763787| 3763821| 34         | Antisense      | Yes           | Yes          |
| pPAR793      | –         | –      | 4420191| 4420213| 22         | Intergenic     | Yes           | Yes          |
| pPAR796      | –         | –      | 4502685| 4502709| 24         | Intergenic     | Yes           | Yes          |

aBased on location and position in genome of strain LMG20103 (2665 co-ordinates are found in Supplementary Table S1). RI terminator indicates the presence of a Rho-independent terminator sequence downstream of the sRNA sequence.

Experimental Validation and Characterization of Individual sRNAs

Expression of the arcZ, fnrS, glmZ, rprA, ryeB, ryhB2, pPAR237, pPAR238, and pPAR395 sRNA genes was quantified in the P. ananatis hfq-mutant strain relative to WT using qRT-PCR (Figure 9). The resulting expression profile of aforementioned sRNA transcript levels (except glmZ and ryhB2) was decreased in the absence of hfq, which was in agreement with the depth plots analysis (Figure 7). In WT P. ananatis, glmZ expression is likely repressed at low cell density (OD<sub>600nm </sub>= 0.2) and increased at high cell density (OD<sub>600nm </sub>= 0.6) in a Hfq-dependent manner. Similarly, Hfq may negatively affect ryhB2 expression, as the abundances of ryhB2 transcript in WT at low and high cell density conditions were both low relative to the hfq-mutant ryhB2 levels. The TSS of FnrS, GlmZ, pPAR237, pPAR238, and pPAR395 was determined by 5′-RACE analysis. Their predicted structures, sequence, and targets are reported in Supplementary Figure S6 and Supplementary Table S4.

DISCUSSION

In the present study, we investigated the functional role of Hfq in the pathogenesis of the Gram-negative phytopathogen P. ananatis, and demonstrated that Hfq is important for motility, AHL and biofilm formation, and virulence of the pathogen. We also identified several putative sRNAs, which include known and novel sRNAs that are Hfq-dependent for their abundances in P. ananatis. The pleiotropic phenotypes caused by hfq mutation is due to global post-transcriptional gene regulation operated by Hfq and Hfq-dependent sRNAs that modulate stress response and virulence of numerous bacterial pathogens (Chao and Vogel, 2010). The ability of P. ananatis to survive in diverse ecological niches and to successfully infect susceptible plant hosts requires a
FIGURE 7 | Sequencing read depth plots for selected Pantoea ananatis sRNAs (pPAR sRNA). Per-base sequencing read depth across the length of sRNAs, normalized to the genome-wide average per-base read depth was plotted for selected P. ananatis LMG 2665T sRNAs. Solid black lines represent sRNA sequencing depth in wild-type (WT) P. ananatis at low cell density (OD$_{600nm}$ = 0.2) and dashed black lines represent sRNA sequencing depth in WT at high cell density (OD$_{600nm}$ = 0.6). Solid gray lines represent sRNA sequencing depth in hfq mutant P. ananatis (Δhfq) at low cell density (OD$_{600nm}$ = 0.2) and dashed gray lines represent sRNA sequencing depth in Δhfq at high cell density (OD$_{600nm}$ = 0.6).

timely and collective regulation of cellular functions in response to environmental conditions.

Inactivation of hfq in bacteria generally results in pleiotropic effects, of which growth retardation is common. Decreased growth rate has been reported in hfq-attenuated bacteria such as Acinetobacter baumannii (Kuo et al., 2017), Haemophilus influenzae (Hempe et al., 2013), Yersinia enterocolitica (Kakoschke et al., 2016), and the plant pathogens A. tumefaciens (Wilms et al., 2012a) and P. carotovorum (Wang et al., 2018). This phenotype was consistent with the P. ananatis hfq deletion mutant growing in vitro; however, this alteration did not prevent P. ananatis from entering logarithmic growth phase and eventually reaching the wild-type cell density at a stationary phase which was also observed in planta (Supplementary Figure S4). Unlike the E. amylovora hfq mutant (Zeng et al., 2013), which exhibited reduced growth in an immature pear fruit infection model, the P. ananatis hfq mutant strain was able to reach a population density comparable to that of the wild-type strain when inoculated into onion, indicating that the abolishment of virulence in the hfq-mutant P. ananatis was not due to a growth defect.
The loss of *hfq* gives rise to impairment of important virulence determinants such as motility in bacterial pathogens. Impaired motility affects the overall fitness of a bacterium as a pathogen as it disables attachment and dispersal of the pathogen in the host. This, in turn, results in the diminished invasion, colonization, and hence virulence (Sittka et al., 2007; Kulesus et al., 2008). In enterobacterial pathogens, Hfq and Hfq-dependent sRNAs control flagellar-based motility. For example, in *E. coli*, multiple sRNAs including ArcZ, OmrAB, OxyS, and RyeB/SdsR have been shown to modulate the expression and/or translation of *flhDC*, the master regulator of flagellar biosynthesis (De Lay and Gottesman, 2012). In the phytopathogen, *E. amylovora*, the sRNAs ArcZ, OmrAB, and RmaA have been found to regulate *flhDC* at both transcriptional and post-transcriptional levels (Schachterle and Sundin, 2019; Schachterle et al., 2019). In this way, the integration of different environmental cues is achieved through several sRNAs, allowing fine-tuning of flagellar expression and production.
The lack of swimming motility displayed by the *P. ananatis* hfq mutant clearly demonstrates the role of Hfq in regulating flagellar motility. In a previous study by Weller-Stuart et al. (2016), a *P. ananatis* flgK mutant deficient in flagellar assembly enzyme, FlgK, was abolished in swimming motility and pathogenicity in onion seedlings. Together with the current study, these findings suggest that flagellar motility is required for the virulence of *P. ananatis*, and this trait is regulated by functional Hfq. Similarly, in *P. carotovorum* (Wang et al., 2018) and *Serratia* sp. ATCC 39006 (Wilf et al., 2013; Hampton et al., 2016), attenuation of flagellar motility was observed in hfq-deletion mutant strains, as an expression of the flhDC genes was dependent on Hfq. Given that sRNAs namely, ArcZ, OmrAB, and RyeB/SdsR, were also identified in our sRNA sequencing data (Supplementary Table S2), and that their wild-type transcript levels are dependent on the functional copy of hfq (Figure 9), we hypothesize that Hfq, in conjunction with the identified sRNAs, may regulate the flagellar motility of *P. ananatis* in a similar manner as the other enterobacterial species.

In addition to impaired motility, disruption of hfq in Gram-negative pathogens often results in reduced biofilm formation (Kulesus et al., 2008; Monteiro et al., 2012; Zeng et al., 2013; Wang et al., 2018). One possible explanation for this phenotype is an effect on QS-mediated regulation of motility and biofilm formation. As biofilm formation is a developmental and co-operative process, the process necessitates cell to cell communication that enables perception of the signals generated from the community. The signal or information is packaged in the form of autoinducer molecules, acylated homoserine lactones (AHLs), or may be communicated to the QS circuit by secondary signaling molecule such as cyclic dimeric guanosine monophosphate (c-di-GMP) (Castiblanco and Sundin, 2016). Through protein phospho-relay, signals resulting from high cell density reach Hfq-dependent sRNAs which initiate the modulation of AHL synthesis in *P. ananatis* (2016). A total of 276 sRNAs were identified in the vicinity of the hfq gene (Figure 9). In contrast to the wild-type expression levels, expression of pPAR237 and pPAR238 was almost non-existent in the two cell density conditions in the *P. ananatis* hfq-mutant strain (Figure 9). The decreased transcript levels of pPAR237 and pPAR238 in hfq-mutant relative to WT *P. ananatis* were validated experimentally by qRT-PCR, reinforcing the idea that the expression of these sRNAs is dependent on cell density and Hfq. Potential pairing sites of pPAR237 and pPAR238 to earn1 and earnR were predicted in silico using IntaRNA (Supplementary Figures S7B–D). Further experimental confirmation of their interaction will indicate the role of pPAR237 and pPAR238 in QS through modulation of AHL synthesis in *P. ananatis*. Moreover, it will be also interesting to determine whether there are other upstream and downstream transcriptional or translational regulators of putative sRNAs pPAR237 and pPAR238. This is the case in *V. cholera* and *Pseudomonas aeruginosa* whose Hfq-dependent sRNA Qrr 1,2,3, and 4 and RsmY are transcriptionally activated by LuxO and GacA, respectively, and are used to repress transcription of hapR or sequester translational regulator RsmA (Lenz et al., 2004; Kay et al., 2006; Tu and Bassler, 2007; Brencic et al., 2009).

To date, factors that contribute to the pathogenicity of *P. ananatis* have been characterized, resulting in an expansion in our understanding of virulence mechanisms of this pathogen. A collective regulation of all virulence traits seems likely for the success and persistence of *P. ananatis* in hostile environments, and this can be achieved through Hfq and its global regulatory networks constituted by Hfq-dependent sRNAs. Overall, this study provided valuable insights into the essential role of Hfq in regulating different virulence traits of *P. ananatis*. A total of 276 sRNAs were identified that are affected in abundance by Hfq at low and high cell density conditions. These sRNAs include those that are well characterized as well as novel putative sRNAs that may possess novel function involved in the QS of *P. ananatis*.

**DATA AVAILABILITY**

The datasets generated for this study can be found in the NCBI BioProject PRJNA550544.

**AUTHOR CONTRIBUTIONS**

GS, JS, DS, LM, TC, and GWS conceived and designed the present study. GS conducted the mutagenesis, phenotypic assays, and sRNA validation experiments. JS compiled the custom python script and performed the bioinformatic analyses of sRNA sequencing data. GS and JS wrote the manuscript in consultation with DS, LM, TC, and GWS. All authors contributed to and approved the final version of the manuscript.
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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02075/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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