Hfq, a RNA Chaperone, Contributes to Virulence by Regulating Plant Cell Wall–Degrading Enzyme Production, Type VI Secretion System Expression, Bacterial Competition, and Suppressing Host Defense Response in *Pectobacterium carotovorum*

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Accepted 20 May 2018.

Hfq is a RNA chaperone and participates in a wide range of cellular processes and pathways. In this study, mutation of *hfq* gene from *Pectobacterium carotovorum* subsp. *carotovorum* PccS1 led to significantly reduced virulence and plant cell wall–degrading enzyme (PCWDE) activities. In addition, the mutant exhibited decreased biofilm formation and motility and greatly attenuated carbapenem production as well as secretion of hemolysin coregulated protein (Hcp) as compared with wild-type strain PccS1. Moreover, a higher level of callose deposition was induced in *Nicotiana benthamiana* leaves when infiltrated with the mutant. A total of 26 small (s)RNA deletion mutants were obtained among a predicted 27 sRNAs, and three mutants exhibited reduced virulence in the host plant. These results suggest that *hfq* plays a key role in *Pectobacterium* virulence by positively impacting PCWDE production, secretion of the type VI secretion system, bacterial competition, and suppression of host plant responses.

An essential host factor of the RNA bacteriophage Qbeta, known as Hfq, was first discovered in *Escherichia coli* a half century ago. In recent decades, Hfq was recognized as a member of a huge family of RNA-binding proteins found in almost every cellular organism from all three domains of life. Hfq belongs to the Sm and Lsm (Sm-like) protein families (Vogel and Luisi 2011) and it has a characteristic feature, i.e., a ring-like hexamer of quaternary multimeric architecture, including central components of messenger (m)RNA splicing machinery, scaffolds for RNA-decapping assembly, protective chaperones of small nucleolar RNAs, ribosomal RNAs, and transfer RNA precursors (Vogel and Luisi 2011). In bacteria, Hfq binds with RNAs and regulates gene expression in

posttranscriptional levels or RNA stability, using base-pairing interaction (Papenfert et al. 2013). The Hfq-binding sites for most mRNAs are unknown, but the structures of Hfq-RNA complexes revealed that the proximal and distal faces prefer U-rich (Robinson et al. 2014; Wang et al. 2011) and A-rich sequences (Link et al. 2009; Robinson et al. 2014), respectively. Moreover, Hfq could act alone to regulate gene expression (Chao and Vogel 2010), and, in some species, it has been reported that sRNAs could regulate with *trans*-encoded mRNA targets without an obvious Hfq homolog, which might provide leads for hidden proteins with Hfq-like function (Chao and Vogel 2010; Sharma et al. 2010). Importantly, Hfq regulates virulence in several animal pathogenic bacteria, such as *Bordetella pertussis*, *Listeria monocytogenes*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Yersinia pestis*, *E. coli*, and *Salmonella typhimurium* (Bibova et al. 2013; Chiang et al. 2011; Christiansen et al. 2004; Ding et al. 2004; Geng et al. 2009; Kendall et al. 2011; Sittka et al. 2007). Hfq also regulates the production of secondary metabolites to maintain a symbiotic relationship with the host nematodes in *Photorhabdus luminescens* (Tobias et al. 2017) and to promote symbiosis, competition, virulence, and biocontrol in rhizobacterium *Pseudomonas aeruginosa* M18 (Wang et al. 2012). However, in plant-pathogenic bacteria, the roles of Hfq remain only partially understood. The first Hfq-associated virulence phenotype was reported by Wilms in *Agrobacterium tumefaciens* in 2012 (Wilms et al. 2012). A deletion mutant of *hfq* in *Erwinia amylovora* Ea1189 significantly reduced virulence in both immature pear fruits and apple shoots (Zeng et al. 2013).

Pectobacterium carotovorum subsp. *carotovorum* is a bacterial phytopathogen that causes soft rot in several crops and ornamental plants worldwide, resulting in important economic losses (Ma et al. 2007; Mansfield et al. 2012). The secretion of plant cell wall–degrading enzymes (PCWDEs) and their regulatory systems associated with pathogenicity have been well studied (Charkowski et al. 2012; Hassan et al. 2013; Yasuhara-Bell et al. 2016). Although the roles of Hfq and sRNA interactions have been thoroughly examined in some enterobacteria strains (e.g., *E. coli* and *Salmonella typhimurium*), little was known about the role of Hfq in *P. carotovorum* subsp. *carotovorum*. In the present study, the *hfq* gene was found in *P. carotovorum* subsp. *carotovorum* PccS1 from a whole-genome sequencing analysis (*unpublished*). Phenotypic observations, quantitative reverse transcription-polymerase chain reaction (qRT-PCR), Western blotting, and RNA-seq analyses revealed that the *hfq* gene in *P. carotovorum* subsp. *carotovorum*

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Funding: This work was supported by Special Fund for Agro-Scientific Research in the Public Interest of China (grant number 201303015), China National Key Technology Support Program (2012BAD14B02) and Top-Notch Academic Programs Project of Jiangsu Higher Education Institutions (PPZY2015B157).

*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures and five supplementary tables are published online.

positively impacted expression of virulence-associated and some other related genes at the transcriptional level, and it also affected virulence, production of PCWDEs, and carbapenem, secretion of the type VI secretion system (T6SS), and responses to the host plant.

RESULTS

Hfq is crucial to the virulence

of *P. carotovorum* subsp. *carotovorum* PccS1.

P. carotovorum subsp. *carotovorum* PccS1 was originally isolated from soft rotted leaves of colored calla (*Zantedeschia elliotiana*) in Nanjing, China in 2007 (Gu et al. 2009). The full-length sequence of the *hfq* gene was obtained from the whole-genome sequencing, and an alignment of the deduced Hfq amino acid sequence was obtained (Fig. 1A). The deduced amino acid sequence exhibited 94% identity with the Hfq sequence from *P. atrosepticum* SCRI1043. When compared with other species from Enterobacteriaceae, Vibrionaceae, Pseudomonadaceae, Burkholderiaceae, and Xanthomonadaceae families, the sequence exhibited 82 to 93% similarity (Fig. 1A). Similar to other bacterial Hfq proteins, Hfq from PccS1 contained conserved Sm1 and Sm2 motifs (Fig. 1A).

To demonstrate the roles of Hfq in PccS1 virulence, gene-deletion mutant Δhfq was constructed, and the pBBR vector (with or without the *hfq* fragment) was introduced into the *hfq* mutant. Growth of the mutant in Luria Bertani (LB) broth did not differ from the wild-type PccS1, but growth of the *hfq* mutant in minimal medium (MM) was significantly reduced in stationary phase as compared with the wild-type strain (Supplementary Fig. S1). Virulence assays in the leaves of Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), calla lily (*Z. aethiopica*), and colored calla (*Z. elliotiana*) indicated that the *hfq* mutant was almost non-pathogenic, whereas the wild-type strain caused dramatic rot symptoms in plant tissues 12 h after inoculation (hai). The reduced virulence of the *hfq* mutant could be fully restored (Fig. 1B). Statistical analyses of the lesion areas revealed significant differences in virulence between the *hfq* mutant and the wild-type strain PccS1 (Fig. 1C).

Hfq positively impacts PCWDEs and production of carbapenem, motility, and biofilm formation.

In previous studies, researchers found that PCWDEs, including proteases, pectate lyases, and cellulases, were the key virulence determinants of many phytopathogenic bacteria (Charkowski et al. 2012; Joshi et al. 2016; Mansfield et al.

Fig. 1. Deduced amino acid sequence of Hfq and virulence of the wild-type PccS1 and the mutants in *Pectobacterium carotovorum* subsp. *carotovorum*. **A**, Alignment of deduced amino acid sequences of *Pectobacterium carotovorum* subsp. *carotovorum* PccS1 Hfq with homologs from other bacterial strains in accordance with the National Center for Biotechnology Information. **B**, Tissue macerations of *P. carotovorum* subsp. *carotovorum* strains: PccS1 (wild-type), Δhfq (gene deletion mutant), $\Delta hfq(hfq)$ (Δhfq complemented with the *hfq* fragment), $\Delta hfq(pBBR)$ (Δhfq complemented with an empty vector carrying plasmid pBBR). Leaves of Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), calla lily (*Zantedeschia aethiopica*), and colored calla (*Z. elliotiana*) were inoculated with 10 μ l of diluted bacterial cells (optical density at 600 nm = 1.2) from cultures suspended in sterilized water. Leaves were inoculated with sterilized water as a negative control. Photographs were taken 16 h after inoculation (hai). Bar = 1.0 cm. **C**, Bars represent the maceration areas measured 16 hai. The data are shown as averages $\pm$ standard deviation (asterisks [**] indicate $P < 0.01$, versus the wild-type PccS1; Duncan's multiple range test).

2012). PCWDE activity assays showed that the *hfq* mutant exhibited significantly reduced pectate lyase (Pel) and cellulase (Cel) activities as compared with the wild-type PccS1 (Fig. 2A, B, and D) and showed almost no protease activity (Prt) (Fig. 2C and D). Enzyme activities were completely restored when the mutant was complemented (Fig. 2A to D). qRT-PCR analyses showed that expression of *priW*, *celV*, and *pelI* (encoding proteases, cellulases, and pectate lyases) decreased about 9, 4, and 1.5-fold, respectively, in the mutant as compared with the wild-type PccS1 (Fig. 2E). Previous studies indicated that at

least three known genes (*rsmA*, *kdgR*, and *hexA*) regulate the production of the PCWDEs in *P. carotovorum* subsp. *carotovorum* (Charkowski et al. 2012). Results of qRT-PCR analyses indicated that *hexA* expression was significantly increased in the absence of *hfq*, and expression of *rsmA* and *kdgR* was not altered significantly, whereas expression of both *flhC* and *flhD* decreased drastically (Fig. 2F). Western immunoblotting results further confirmed those of the qRT-PCR results (Fig. 2G).

It has been reported that *P. carotovorum* subsp. *carotovorum* produces carbapenem (Car), an antibiotic that inhibits the

growth of environmental bacteria, thus enhancing its competitive capacity during the infection process (Barnard et al. 2007; Coulthurst et al. 2005). A *carA-H* operon for Car synthesis and intrinsic resistance is present in the genome, and these genes are regulated by CarR, a LuxR-type regulator (Barnard et al. 2007; Coulthurst et al. 2005). In the present study, a cross-feeding assay to detect Car production was performed. Results indicated that Car production was significantly decreased in the *hfq* mutant and could be restored when the mutant was complemented (Fig. 2H and I). Expression levels of the Car biosynthetic genes *carA*, *carB*, *carC*, and *carD*, as well as the regulatory gene *carR*, in the mutant were reduced to about 1/10, 1/29, 1/13, 1/8, and 1/18, respectively, that of the wild-type strain (Fig. 2J).

Motility is an important virulence determinant in *P. carotovorum* subsp. *carotovorum* and many other pathogenic bacteria (Wang et al. 2009). In the present study, the *hfq* mutant exhibited almost

nonswimming motility on MM plates containing 0.3% agar (Fig. 3A) and cells of the mutant precipitated quickly when transferred to glass tubes after being cultured to an optical density at 600 nm (OD₆₀₀) of 2.0 (Fig. 3B). The reduced motility phenotype of the *hfq* mutant was partly restored when complemented (Fig. 3A and B).

Similarly, biofilm formation in the *hfq* mutant decreased to approximately 20% that of the wild-type PccS1 (Fig. 3C and D) and could be restored when complemented.

Hfq suppresses pathogen-associated molecular patterns (PAMP)-induced callose deposition in host plant cells.

PAMP can activate plant intrinsic defense responses, including callose-associated cell-wall modification (Hauck et al. 2003; Kim et al. 2011). To find out whether there was any difference between PccS1 and the *hfq* mutant in forming PAMP-induced callose deposition of plant cells, the wild-type PccS1 and the

Fig. 2. The activity of plant cell wall-degrading enzymes (PCWDEs) and production of carbapenem were assayed in *Pectobacterium carotovorum* subsp. *carotovorum* strains. PCWDE activities were determined visually as follows. **A**, Production of double haloes around colonies for pectate lyase (Pel), **B**, an off-white halo on a dark background around colonies for cellulose (Cel), and **C**, a clear halo on an opaque background around colonies for protease (Prt), 48 h after the bacterial cells were patched onto the assay plates. **D**, Bars indicate halo areas of Pel, Cel, Prt produced by the wild-type PccS1 and mutants. **E** and **F**, Bars represent relative messenger (m)RNA levels of genes encoding PCWDEs (*pel1*, *celV*, and *prtW*), regulatory proteins (*kdgR*, *rsmA*, *hexA*, *flhC*, and *flhD*) in wild-type PccS1 and *Δhfq*. The mRNA levels of these genes in the wild-type PccS1 were set to 1.0 to calculate the relative expression ratio. **G**, Protein expression levels of the regulatory proteins KdgR-FLAG, RsmA-FLAG, HexA-FLAG, and FlhD-FLAG in the wild-type PccS1 and the *hfq* mutant. RecA was used as the loading control. **H**, Activity of carbapenem (Car) produced by the wild-type PccS1 and *Δhfq*, *Δhfq(hfq)*, and *Δhfq(pBBR)* mutants that were tested visually, based on a transparent halo around the colonies caused by antibiotic cell killing 24 h after the cells were patched onto Luria Bertani agar plates mixed with a suspension of Car-sensitive *Escherichia coli* DH5α at a concentration of 500 μl per petri dish. **I**, Bars indicate halo areas of Car produced by the wild-type PccS1 and mutants. **J**, Bars represent relative mRNA levels of genes encoding proteins associated with the synthesis and regulation of Car (*carA-H* and *carR*) in the wild-type PccS1 and *Δhfq*. The mRNA levels of these genes in the wild-type PccS1 were set to 1.0 to calculate the relative expression ratio. The experiments were repeated three times with at least three internal replicates in each. The data are shown as averages ± standard deviation (one asterisk [*] indicates *P* < 0.05, two [**] indicate *P* < 0.01, versus the wild-type PccS1; Duncan's multiple range test).

Fig. 3. Hfq effects on motility, biofilm formation, and sedimentation in *Pectobacterium carotovorum* subsp. *carotovorum* PccS1. **A**, The swimming motility of cells visualized as a halo on a clear background at 24 h after patching on minimal medium plates containing 0.3% agar. **B**, Sedimentation of the cells in the glass tubes statically placed for 24 h at room temperature after being oscillated in Luria Bertani at 28°C to an optical density at 600 nm of 2.0. **C**, Biofilm development on an abiotic surface was visualized based on the cells attached on the inner wall of tubes and stained with crystal violet. **D**, Quantification of the biofilm was measured based on light absorbance at 575 nm in crystal violet eluted from the inner walls of the tubes. The experiments were repeated three times with at least five internal replicates in each. The data are shown as averages ± standard deviation (asterisks [**] indicate *P* < 0.01, versus the wild-type PccS1; Duncan's multiple range test).

mutant and complementation strains were infiltrated into *N. benthamiana* leaves, and localized callose deposition in the plant cell at 12 hai was observed by staining the bacteria-infiltrated leaves with aniline blue. Some PAMP-induced callose deposition was observed in *N. benthamiana* leaves infiltrated with the wild-type PccS1 or the complemented strain (Fig. 4A and C) and a much higher level of callose deposition occurred in leaves infiltrated with the mutant (Fig. 4B and D). In addition, PAMPs-induced callose deposition was almost abolished in *N. benthamiana* leaves infiltrated with *Pseudomonas syringae* pv. *tomato* DC3000 and sterilized water (Fig. 4E and F), as reported previously (Kim et al. 2011).

Hfq promotes the secretion of Hcp and significantly activates the expression of the core genes in the T6SS cluster.

It has been reported that the T6SS is a potential virulence factor in some gram-negative bacteria (Lin et al. 2014; Tian et al. 2015), and Hcp is the hallmark of a functional T6SS in many bacterial species. Hcp is a protein with dual functions, one as the T6SS structural component and the other as a direct effector of T6SS. It is delivered into the hosts as a toxin by some animal pathogenic bacteria (Hood et al. 2010; Kapitein

and Mogk 2013; Records 2011). To determine whether *P. carotovorum* subsp. *carotovorum* *hfq* affects the secretion of T6SS, Hcp-overexpressed strains of the wild-type PccS1, mutant, and complementation strains were constructed by introducing a plasmid carrying the *hcp* gene fused with an epitope (DYKDDDDK) at the C-terminus. Western blotting results showed that secretion of Hcp was almost abolished in the *hfq* mutant and was recovered in the complementation strains as compared with the wild type (Fig. 5A).

To demonstrate how Hfq influences the secretion of T6SS and virulence in *P. carotovorum* subsp. *carotovorum*, mRNA levels of the 17 genes in the T6SS cluster were determined by qRT-PCR. Results showed that transcriptional levels of these genes were all significantly reduced by more than 85% in the mutant as compared with the wild-type (Fig. 5B).

Hfq globally affects gene expression in *P. carotovorum* subsp. *carotovorum* PccS1.

All the aforementioned results indicated that Hfq plays an important role in regulating *P. carotovorum* virulence. Next, global gene expression of the wild-type PccS1 and the *hfq* mutant was compared using RNA-seq analyses at the

Fig. 4. Callose deposition in the *Nicotiana benthamiana* leaves infiltrated with bacterial strains. Portions of the *N. benthamiana* leaves were stained with aniline blue for callose deposition at 12 h after infiltration of the bacterial cells (10^8 CFU·ml⁻¹). **A**, *Pectobacterium carotovorum* subsp. *carotovorum* wild-type strain PccS1, **B**, *Δhfq*, **C**, *Δhfq(hfq)*, **D**, *Δhfq(pBBR)*, **E**, *Pseudomonas syringae* pv. *tomato* DC3000, and **F**, sterilized water as a negative control. The experiments were repeated three times with at least five internal replicates in each.

midexponential growth phase. A total of 452 of 4,419 genes (approximately 10%) exhibited significantly differential expression (≥ 2 fold) at the transcript level in the *hfq* mutant as compared with PccS1. Among the 452 Hfq-regulated genes, 134 genes were upregulated and 318 genes were down-regulated. These results suggest that Hfq more often acts as an activator than as a repressor in *P. carotovorum*. Further analysis revealed that the 452 Hfq-dependent genes belonged to at least 16 functional categories (Fig. 6), including lipid transport and metabolism (16.0%), carbohydrate transport and metabolism (15.2%), energy production and conversion (12.9%), inorganic ion transport and metabolism (9.2%), and general function prediction (9.0%) (Fig. 6). In addition, results of qRT-PCR analyses of genes associated with Hfq-mediated regulation, including PCWDEs (*pel1*, *PccS1_0814*; *celV*, *PccS1_3690*; *prtW*, *PccS1_2900*), regulatory proteins (*hexA*, *PccS1_4093*), Car synthesis and regulation (*carA*, *PccS1_0882*; *carB*, *PccS1_0881*; *carC*, *PccS1_0880*; *carD*, *PccS1_0879*; *carR*, *PccS1_0883*), were consistent with the RNA-seq data (Supplementary Table S1).

Three sRNAs (*ArcZ*, *CsrB*, and *SraG*) regulate virulence in PccS1.

Based on the complete genome sequence of *P. carotovorum* subsp. *carotovorum* PccS1, 27 sRNAs were predicted, using Rfam. The sequences of the 27 sRNAs and differential expression of sRNAs in the *hfq* deletion mutant versus that of wild-type PccS1 by qRT-PCR are listed in Supplementary Table S2. To analyze the role of sRNAs in the virulence of PccS1, 26 deletion mutants of the predicted sRNAs were constructed and were tested in the host plant. Results indicated that three mutants ($\Delta arcZ$, $\Delta csrB$, and $\Delta sraG$) were significantly attenuated in virulence, whereas other mutants did not alter in virulence (Fig. 7A and B).

DISCUSSION

Hfq is required for *P. carotovorum* subsp. *carotovorum* to grow in MM and for virulence.

It has been demonstrated that Hfq participates in a wide range of cellular processes and pathways, including regulation of translation by sRNA, resistance to various environmental

Fig. 5. Effects of Hfq on functional type VI secretion system (T6SS) in *Pectobacterium carotovorum* subsp. *carotovorum*. **A**, Western blot analyses of the supernatant and cell pellet of *hfp::flag* fusion strains of the wild-type PccS1, the *hfp* mutant, and the complementation strain. RecA was used as the loading control. **B**, Bars indicate relative messenger (m)RNA levels of genes in the cluster of the T6SS in the wild-type PccS1 and the *hfp* mutant. The mRNA levels of these genes in the wild-type were set to 1.0 to calculate the relative expression ratio. The experiments were repeated three times with at least five internal replicates in each. The data are shown as averages $\pm$ standard deviation (asterisks [**] indicate $P < 0.01$, versus the wild-type PccS1; Duncan's multiple range test).

stresses, cellular metabolism, and virulence (Cui et al. 2013; Feliciano et al. 2016; Sonnleitner and Bläsi 2014; Wroblewska and Olejniczak 2016). In the present study, Hfq is involved in *P. carotovorum* for growth in amino acid limiting conditions, as reported in many animal and plant pathogenic bacteria that showed a growth defect in the absence of Hfq, such as *Listeria monocytogenes*, *Brucella melitensis*, *Agrobacterium tumefaciens* (Christiansen et al. 2004; Cui et al. 2013; Wilms et al. 2012). This growth defect of both the wild-type and the *hfq* mutant in MM might result from the limitation of carbon resource. Meanwhile, it might also result from improved concentration of metal ions in MM, same as the lower growth rate of *Proteus mirabilis* cultured in LB supplemented with NaCl at 5% (Wang et al. 2014). The decreased growth rate of the *hfq* mutant did not influence the cell density to reach the threshold level of infection, but the virulence of the *hfq* mutant was almost nonpathogenic (Fig. 1B). It indicated that Hfq was fully required for virulence in *P. carotovorum* subsp. *carotovorum*.

Hfq influences PCWDE production and virulence through activation of FlhDC, which represses HexA in *P. carotovorum* subsp. *carotovorum*.

Researchers have shown that PCWDEs are key virulence determinants in *P. carotovorum* subsp. *carotovorum* and other soft-rot Enterobacteriaceae, including *P. atrosepticum* and *Dickeya dadantii* (Charkowski et al. 2012; Toth et al. 2003). In this study, we found that Hfq significantly promoted the production of PCWDEs (Fig. 2A to C and E), and this is the first evidence of the role of Hfq in the PCWDE production. We speculate that Hfq regulates the production of PCWDEs, thus acting as a virulence determinant in *P. carotovorum* subsp. *carotovorum*.

Researches have also shown that expression of the PCWDE genes is negatively regulated by *rsmA*, *kdgR*, and *hexA* in *P. carotovorum* subsp. *carotovorum* (Charkowski et al. 2012; Cui et al. 2008; Liu et al. 1999; Mukherjee et al. 1998). Researchers further indicated that the hexameric complex (FlhD₄C₂), comprised of the products of the master regulatory flagellar genes *flhC* and *flhD*, negatively regulates the expression of *hexA* (Charkowski et al. 2012; Chatterjee et al. 2009;

Cui et al. 2008), and the complex activates the production of PCWDEs in *P. carotovorum* subsp. *carotovorum* Ecc71 (Cui et al. 2008) and prodigiosin in *Serratia* sp. strain ATCC 39006 (Hampton et al. 2016). Our data (Fig. 2F) suggest the following molecular regulatory pathway of Hfq required for virulence in *P. carotovorum* subsp. *carotovorum* PccS1 (Fig. 8): i) Hfq negatively regulates the expression of *hexA* and positively regulates *flhCD* and ii) the altering of these two regulatory proteins contributes to the production of PCWDEs.

Moreover, in previous studies, flagellar motility and biofilm formation were characterized as accessory virulence determinants in many animal and plant bacterial pathogens, including *P. carotovorum* subsp. *carotovorum* (Cui et al. 2008; Esquivel and Pohlschroder 2014; Gauger et al. 2007; Horne et al. 2016; Schiano et al. 2010; Siryaporn et al. 2014). In this study, our results are consistent with those reported previously in *Serratia* sp. strain ATCC 39006 (Hampton et al. 2016). Though the hypermotility of *Yersinia pseudotuberculosis* was observed on semisolid agar plates in the absence of *hfq*, it was confirmed that the hypermotility might not be mediated through changes in flagellar expression (Schiano et al. 2010). In addition, evidence supporting the role of twitching motility in biofilm formation was found in *Pseudomonas aeruginosa* (O'Toole and Kolter 1998a), and surface attachments induce *Pseudomonas aeruginosa* virulence (Siryaporn et al. 2014). Here, the extremely reduced biofilm formation and swimming motility of the *hfq* mutant further demonstrated that Hfq is important for virulence in *P. carotovorum* subsp. *carotovorum*.

Hfq regulates Car biosynthesis in *P. carotovorum* subsp. *carotovorum*.

Car, a β -lactam antibiotic, is isolated from gram-negative bacteria, including *P. carotovorum* subsp. *carotovorum*, *Serratia* sp. strain ATCC39006, and *Photobacterium luminescens* TT01 (Coulthurst et al. 2005). The *carA-H* operon, which is responsible for Car production and intrinsic resistance, has been found in some *P. carotovorum* subsp. *carotovorum* strains and in *Serratia* sp. strain ATCC39006 (Coulthurst et al. 2005). This operon is controlled by a LuxR-type transcriptional

Fig. 6. Functional classification of Hfq-dependent genes in *Pectobacterium carotovorum* subsp. *carotovorum* analyzed using RNA-seq. A total of 452 Hfq-dependent genes were grouped into different functional classes. Bars represent the percentages of genes from different functional categories from the total number of differentially expressed genes in Δhfq compared with those of the wild-type PccS1.

activator that is encoded by *carR* upstream of the operon (Barnard et al. 2007). Genome sequencing of *P. carotovorum* subsp. *carotovorum* PccS1 indicated that it possesses entire genes associated with the operon and *carR*. Our results suggest that Hfq is essential for the production of Car in *P. carotovorum* subsp. *carotovorum* PccS1, as reported in *Serratia* sp. strain ATCC39006 (Wilf et al. 2013). It has been demonstrated that Car aids the promotion of environmental competitive activities during the infection process (Barnard et al. 2007; Coulthurst et al. 2005). A consequence of Car production suggests that Hfq is also essential for accessory processes associated with virulence in *P. carotovorum* subsp. *carotovorum*.

Hfq activates T6SS to deliver Hcp in *P. carotovorum* subsp. *carotovorum*.

T6SS was found to be required for the delivery of pathogenicity effector proteins into the host cell (Basler et al. 2013; Gallique et al. 2017; Hood et al. 2010; Kapitein and Mogk 2013; Pukatzki et al. 2006). Additional studies of T6SS function revealed that T6SS is a versatile tool with roles in virulence (de Pace et al. 2011; Mulder et al. 2012; Records 2011). Our data showed that the PccS1 mutant of the T6SS-cluster deletion significantly attenuated virulence in the host plant (*unpublished*), thus indicating that T6SS plays an important role in *P. carotovorum* subsp. *carotovorum* virulence. In the present

study, our results suggested that inactivated *hfq* significantly repressed the expression of all core genes in the T6SS cluster (Fig. 5A) and led to complete loss of delivering Hcp (Fig. 5B). Therefore, our results confirmed the important role of Hfq in the virulence of PccS1 by affecting expression of T6SS. However, the regulatory mechanisms underlying the relationship between Hfq and T6SS require further study.

Hfq depresses host cell wall-based defense to promote host leaf maceration in *P. carotovorum* subsp. *carotovorum*.

PAMPs can trigger plant defense responses referred to as PAMP-triggered immunity (PTI) and accumulation of callose is a molecular marker for PTI (Boudsocq et al. 2010). Researchers indicated that *Pseudomonas syringae* DC3000 suppressed callose deposition in plant leaves, whereas the effects of *P. carotovorum* WPP14 and the T3SS mutants of WPP14 on PAMP-induced callose deposition were similar, as they were not able to completely inhibit PAMP-induced callose deposition in the host plant of *N. benthamiana* (Kim et al. 2011). Similarly, we found that inoculation of PccS1 slightly induced callose deposition in *N. benthamiana* leaves (Fig. 4), but a higher level of callose deposition was observed in *N. benthamiana* leaves inoculated with the *hfq* mutant. These results suggested that Hfq is involved in the suppression of tobacco cell-wall reinforcement. Thus, decreased virulence in

Fig. 7. A, Images indicate the virulence of the wild-type PccS1 and the small RNA individual deletion mutants, as determined on the leaves of Chinese cabbage (*Brassica rapa* subsp. *pekinensis*). Photographs were taken 16 h after inoculation. Bar = 1.0 cm. **B**, Maceration areas caused by the wild-type PccS1 and the mutants. The virulence assays were conducted three times using five to ten leaf pieces in each assay. The data are shown as averages $\pm$ standard deviation (asterisks [**] indicate $P < 0.01$, maceration caused by the mutants versus that by PccS1 on the same host plant; Duncan's multiple range test).

Three sRNAs (ArcZ, CsrB, and SraG) regulate virulence in a Hfq-dependent or -independent manner in *P. carotovorum* subsp. *carotovorum*.

According to previous studies, ArcZ is a conserved, Hfq-binding sRNA and is highly conserved in the 3' region but more divergent in the 5' region among some Enterobacteriaceae genera (Supplementary Fig. S2) (Mandin and Gottesman 2010; Papenfort et al. 2009). As many other sRNAs, intracellular accumulation of ArcZ is critically dependent on Hfq at the level of transcript stability (Papenfort et al. 2009). In the present study, Hfq might regulate the virulence of *P. carotovorum* by controlling the stability of ArcZ, though the mechanism of ArcZ on virulence regulation requires further study.

regulation of secondary metabolism in *Photorhabdus luminescens* via HexA (Tobias et al. 2017). Here, we confirm that Hfq negatively regulates HexA to activate CrsB in *P. carotovorum* infection on host plants, suggesting that Hfq indirectly facilitates CrsB expression.

The other sRNAs with no influence on virulence participate in many other regulatory processes, linking with Hfq directly or indirectly, and further studies in *P. carotovorum* subsp. *carotovorum* are required.

The present study has highlighted the roles of Hfq in the global regulation of virulence factors in the plant pathogen, *P. carotovorum*. Hfq acts as a global regulator to not only regulate key virulence determinants (PCWDEs), but it also regulates Car production, biofilm formation, motility, T6SS, and callose deposition in plant cells. These findings will provide new evidence for our understanding of pleiotropic roles of Hfq in *P. carotovorum* virulence (Fig. 8).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this study are summarized in Supplementary Table S3. PccS1 and the derivative strains were cultured in LB medium (Wang et al. 2018), LB agar plate (LA), or MM (Kersey et al. 2012) at 28°C, and *E. coli* in LB at 37°C. When required, the antibiotics were added to the media at the following final concentrations: rifampicin (Rif) at 100 µg·ml⁻¹, kanamycin (Km) at 50 µg·ml⁻¹, and gentamicin (Gm) at 50 µg·ml⁻¹. The OD of the culture was measured by a BioPhotometer (Eppendorf, Hamburg, Germany) at 600 nm.

Mutant construction and complementation.

Gene knockout mutants were constructed and complemented as previously described (Wang et al. 2018). Briefly, plasmids pEX18Gm carried the 300- to 600-bp fragment cloned from upstream and downstream of the target genes, using the relevant primers (Supplementary Table S4), and were ligated by a Km cassette amplified from pET30, and then, the recombinant vectors were introduced into *E. coli* S17-1λpir to generate a donor strain. With the wild-type strain PccS1 used as the recipient, transconjugants were constructed by homologous recombination. To select candidate gene-deletion mutants, transconjugants were plated on LB plate containing 10% sucrose (Solarbio, Beijing, China), 100 µg·ml⁻¹ Rif, and 50 µg·ml⁻¹ Km. To construct complementation strains, the target gene and its promoter region were amplified and were cloned into pBBR1-MCS5. The generated vector carrying the target gene was introduced into each respective mutant by conjugation, as described above. The complemented strains were selected based on Gm resistance. All constructs were verified by PCR and sequencing. The complemented strains carrying the target gene fusion with an epitope tagging fragment (DYKDDDDK) at the C terminus were additionally confirmed by Western immunoblotting for the expected FLAG-tagged protein (de Bruin et al. 2011; Jiang et al. 2017).

Virulence assay.

Virulence assays were performed as previously described (Andrade et al. 2008; Jiang et al. 2017; Li et al. 2010) on 45-day-old leaves detached from Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), calla lily (*Zantedeschia aethiopica*), and colored calla (*Z. elliotiana*) plants grown in a greenhouse (temperature varying from 22 to 28°C, 16-h day length, and approximately 80% relative humidity). The assays were repeated three times using at least five leaf pieces in each.

PCWDE activity and carbapenem assay.

PCWDE activity assays were essentially performed as previously described (Barnard et al. 2010; Jiang et al. 2017). Briefly, appropriated cultures were harvested and were diluted in LB broth to an OD₆₀₀ of 1.0. Two microliters of the cell suspension was spotted onto protease assay plates (LA containing 10% sterilized skimmed milk) as well as pectate lyase and cellulase assay plates, as previously described (Barnard et al. 2010). PCWDE production was visualized based on haloes that occurred around the bacterial colonies when cells were spotted onto assay plates and incubated for 48 h at 28°C. Finally, plates were photographed and areas (in square centimeters) of the haloes were measured. The experiments were repeated three times with at least five replicates in each.

Car assays were performed as described previously (Thomson et al. 1997). Briefly, a 500-µl suspension of Car-sensitive *E. coli* DH5α (OD₆₀₀ = 1.0) was mixed with LA in each petri dish before solidification, then, a bacterial lawn was formed. Cells of wild-type PccS1 and the mutants were appropriated, cultured,

suspended, and adjusted in sterilized water to OD₆₀₀ = 1.0. Two microliters of diluted cells was spotted onto the plates with a bacterial lawn. Plates were incubated at 28°C for 24 h. When cross-feeding occurred, a clear halo appeared around patched cells, areas (in square centimeters) of the haloes were measured and photographs were taken. The assays were repeated three times with at least five internal replicates in each.

Motility assay.

Motility of the strains was performed as previously described with some modifications (Fan et al. 2011; Wang et al. 2009). Appropriated cultures of PccS1 and mutants were harvested and were suspended in sterilized water to an OD₆₀₀ of approximately 1.0. Cell motility was visualized based on swimming haloes on a MM plate (containing agar at 3 g-per liter) 24 h after 2.5 µl of the cell suspension patched onto. Motility was also monitored by observing precipitation rate of the cells. Cells were cultured in LB at 28°C to an OD₆₀₀ of 2.0, 5 ml of the each culture was transferred to glass tubes, and static-placed for 24 h at room temperature. Finally, sedimentation of the cells was observed and the tubes were photographed. Each motility assay was repeated three times with at least five replicates.

Biofilm formation assay.

Qualitative observation and quantitative determination of biofilm formation on an abiotic surface were performed using crystal violet, as previously described (Jiang et al. 2017; O'Toole and Kolter 1998b; Zeng et al. 2013). Each assay was repeated three times with at least five replicates.

qRT-PCR.

Transcript levels of target genes were measured by qRT-PCR as previously described (Jiang et al. 2017; Kersey et al. 2012). Cells of the wild-type PccS1 or mutants were cultured in LB from a 1% dilution of overnight cultures to an OD₆₀₀ of 1.0. Three milliliters of the cultures were centrifuge-pelleted at 4°C. After washing three times in RNA-free water, total RNA of each strain was extracted using the E.Z.N.A. bacterial RNA kit (OMEGA, Norcross, GA, U.S.A.), and 1 µg of RNA was then used to synthesize cDNA according to the manufacturer's instructions for the PrimeScript RT reagent (perfect real time) kit (TaKaRa, Dalian, China). The quality and concentration of RNA and cDNA was verified using microspectrophotometer NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, U.S.A.). Then qRT-PCR was performed in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA, U.S.A.), using AceQ qPCR SYBR green master mix (Vazyme, Nanjing, China). Gene-specific primers are listed in Supplementary Table S5. The housekeeping gene *recA* was used as an endogenous control for assessing expression (Kersey et al. 2012; Wang et al. 2018). Data were analyzed by the ABI 7500 software (v.2.0.6).

RNA-seq.

RNA samples of the wild-type PccS1 and the *hfq* mutant that passed the verification of quality and concentration, as the above described in qRT-PCR, were used to generate cDNA libraries using the NEBNext Ultra Directional RNA library prep kit for Illumina (Illumina, San Diego, CA, U.S.A.). The cDNA libraries were purified using AMPure XP beads (Illumina). Quality of cDNA libraries was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.A.), and quantitation of cDNA libraries was determined with ABI 7500 real time PCR system (Applied Biosystems) using KAPA SYBR green fast universal 2× qPCR master mix kit (Vazyme). Then, pair-end (PE) index libraries were constructed according to the manufacturer's protocol of NEB Next Ultra Directional

RNA Library Prep Kit for Illumina (Illumina). Libraries with different indexes were multiplexed and loaded on an Illumina HiSeq 2500, according to manufacturer's instructions (Illumina). Sequencing was carried out using a 2 × 125 PE configuration.

Raw reads were first filtered using statistical software Trimmomatic (v. 0.30) to remove low quality sequences and were re-evaluated using software FastQC (v. 0.10.1), and clean reads were then assembled. Reads were mapped back to the transcriptome of PccS1 using the alignment software bowtie2 (2.1.0). The number of mapped clean reads for each unigene was then counted and normalized into a FPKM value (fragment per kilobases per million reads) (Mortazavi et al. 2008). Differentially expressed genes were identified using statistical analysis among the libraries as described previously (Shen et al. 2012). *P*-value threshold in multiple tests was determined by the false discovery rate (FDR) (Benjamini et al. 2001; Liu et al. 2015). In the present study, differential expressed unigenes between the samples of the wild-type PccS1 and the *hfq* mutant were screened with a threshold of FDR ≤ 0.05 and an absolute value of log₂-fold ≥ 1 (Hu et al. 2016; Liu et al. 2015; Wang et al. 2015).

Callose deposition assay.

Callose deposition in the leaves of *Nicotiana benthamiana* was essentially performed as previously described (Hann and Rathjen 2007; Kim et al. 2011). The appropriated cultures were pelleted, were washed three times, and were suspended in sterilized water to an OD₆₀₀ of 1.2 (10⁸ CFU·ml⁻¹). The suspensions were infiltrated into the leaves of 6-week-old *N. benthamiana* potted plants growing in the greenhouse. The leaves were detached 12 h after infiltration, were dipped in 95% ethanol to be cleared of pigment at 37°C, and were subsequently washed twice in 70% ethanol and twice in sterilized water. Pigment-cleared leaves were stained in a solution of aniline blue 0.01% (wt/vol) in 150 mM K₂HPO₄ (pH 9.5) for 5 h in the dark. Finally, stained leaves were examined with epifluorescence (Olympus America Inc., Melville, NY, U.S.A.) and were photographed. Sterilized water and *Pseudomonas syringae* pv. *tomato* DC3000 were used as controls. Each assay was repeated at least three times with at least three replicates.

Western blot.

To visualize outside translocation of Hcp interrupted or not in the absence of *hfq*, the plasmid (pBBR1-MCS5) carrying the fragment of *hcp* fusion with an epitope tagging fragment (DYKDDDDK) at the C terminus was introduced into the wild-type PccS1 and the *hfq* mutant, for Western blotting as previously described (Basler et al. 2012; de Bruin et al. 2011; Jiang et al. 2017; Sonnleitner and Bläsi 2014). Overnight cultures were diluted to 1:100 in 25 ml of fresh LB. Cells were harvested by centrifugation at 6,000 × *g* for 20 min for intracellular protein assay when OD₆₀₀ reached 0.8, while supernatants were collected for extracellular secreted protein assay. Pellets were washed three times in phosphate buffered saline (PBS) and were resuspended in radioimmunoprecipitation assay (RIPA) lysates (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), added at 10 µl per milligram of cells). Phenylmethanesulfonyl fluoride, protease inhibitors, and phosphatase inhibitors were added to the samples at a ratio of 1:100 (vol/vol) of RIPA. The mixture was incubated on ice for 3 h, and then, was centrifuged at 13,000 × *g* for 5 min. The supernatants were obtained and heated at 98°C for 5 min, then dispensed for future analyses. For extraction of secreted proteins, the supernatants of the cultures were passed through a 0.2-µm filter to remove bacterial cells. Proteins from the supernatants were precipitated by adding 100% trichloroacetic

acid to a final concentration of 10%, and were then pelleted by centrifugation (13,000 × *g*) at 4°C for 20 min. The pellets were washed three times in ice-cold acetone and were resuspended in 1× PBS. Samples were heated at 98°C for 5 min, were dispensed, and were stored at -80°C for further analyses.

Samples were loaded onto a 12% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, U.S.A.) for polyacrylamide gel electrophoresis, and were then transferred to a polyvinylidene difluoride membrane (Millipore, Red Bank, NJ, U.S.A.). The membranes were blocked with 5% milk in Tris-buffered saline containing Tween 0.05% (TBST, pH = 7.5) for 1 h at room temperature, and were hybridized with a 1:5,000 dilution of primary protein antibody, DYKDDDDK-Tag (3B9) Mouse mAb (Abmart, Berkeley Heights, NJ, U.S.A.) for 2 h at room temperature with 3% milk in TBST. The membrane was then washed with TBST for 5 min three times and was incubated with a 1:10,000 dilution of goat anti-mouse immunoglobulin G-horseradish peroxidase (IgG-HRP) (Abmart) at room temperature for 1 h and were then washed three times with TBST. Peroxidase was detected by ECL Western blotting substrate (Bio-Rad). The membrane was incubated with a 1:5,000 dilution of goat anti-rabbit IgG-HRP (Abmart), after it had been hybridized with a 1:3,000 dilution of anti-RecA antibody (ab63797) (Abcam, Cambridge, U.K.), which was used as loading control and followed the same steps as above.

sRNA identification and sequence.

Based on the genome sequence, we used Rfam (Griffiths-Jones et al. 2005) to identify sRNAs in *P. carotovorum* subsp. *carotovorum* PccS1. Rfam is a database of noncoding RNAs (ncRNAs) represented by multiple sequence alignments. It aims to facilitate the identification and classification of new members of known sequence families and distributes annotation of ncRNAs. To confirm the sRNAs predicted by Rfam, we also blasted the sequences in the National Center for Biotechnology Information database.

Data were statistically analyzed using SPSS 14.0 (SPSS Inc., Chicago). The hypothesis test of percentages (Duncan's multiple range test, *P* = 0.05 or 0.01) was used to determine significant difference in the assays described above.

ACKNOWLEDGMENTS

We would like to thank F. Zhao (University of Illinois) for his critical review of the final manuscript and Z. Cui and G. Qian (Nanjing Agricultural University) for their tools and technical assistance. We also acknowledge the reviewers whose comments and suggestions helped us improve the manuscript.

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