The global regulator Hfq exhibits far more extensive and intensive regulation than Crc in *Pseudomonas protegens* H78

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**Abstract**
The biocontrol rhizobacterium *Pseudomonas protegens* H78 can produce a large array of antimicrobial secondary metabolites, including pyoluteorin (Plt), 2,4-diacetylphloroglucinol (DAPG), and pyrrolnitrin (Prn). Our preliminary study showed that the biosynthesis of antibiotics including Plt is activated by the RNA chaperone Hfq in *P. protegens* H78. This prompted us to explore the global regulatory mechanism of Hfq, as well as the catabolite repression control (Crc) protein in H78. The antimicrobial capacity of H78 was positively controlled by Hfq while slightly down-regulated by knockout of *crc*. Similarly, cell growth of H78 was significantly impaired by deletion of *hfq* and slightly inhibited by knockout of *crc*. Transcriptomic profiling revealed that *hfq* mutation resulted in significant down-regulation of 688 genes and up-regulation of 683 genes. However, only 113 genes were significantly down-regulated and 105 genes up-regulated by the *crc* mutation in H78. Hfq positively regulated the expression of gene clusters involved in secondary metabolism (*plt*, *prn*, *phl*, *hcn*, and *pvd*), the type VI secretion system, and aromatic compound degradation. However, Crc only positively regulated the biosynthesis of Plt but not other antibiotics. Hfq also regulated expression of genes involved in oxidative phosphorylation and flagellar biogenesis. In addition, Hfq and Crc activated transcription of *crcY/Z* sRNAs by feedback. In summary, Hfq processes far more extensive and intensive regulatory capacity than Crc and shows small cross-regulation with Crc in H78. This study lays the foundation for clarifying the Hfq and/or Crc-dependent global regulatory network and improving antibiotic production by genetic engineering in *P. protegens*.

**KEYWORDS**
antibiotic biosynthesis, catabolite repression control protein Crc, oxidative phosphorylation, PQQ-EDHs biosynthesis, RNA chaperone Hfq, type VI secretion system.
**INTRODUCTION**

*Pseudomonas protegens* H78 is a biocontrol bacterium from a screen of the oilseed rape rhizosphere in Shanghai. It can effectively suppress phytopathogenic fungi such as *Pythium ultimum* and *Fusarium oxysporum* by producing multiple antimicrobial secondary metabolites including pyoluteorin (Plt), 2,4-diacyltolophenol (DAPG), and pyrrolnitrin (Prn) (Takeuchi et al., 2014). The complete genome of *P. protegens* H78 has been sequenced and deposited in the GenBank database under the accession number CP013184 (Huang et al., 2017). Based on the characterization of the H78 complete genome, our previous research focused on exploring the pathway-specific and global regulatory mechanisms of secondary metabolism including antibiotic biosynthesis in *P. protegens* H78 (Liu et al., 2018; Wang et al., 2017; Wu et al., 2020; Zhang et al., 2019). The Gac/Rsm signal transduction regulatory system controls Plt biosynthesis via several different regulatory pathways, including the characterized GacS/A-RsmXYZ-RsmE-pltR/pltAB positive regulatory pathway and other uncharacterized regulatory pathways (Wang et al., 2017).

Most bacteria, including *Pseudomonas*, inhabit various environments and are exposed to rapid environmental and nutrient changes, which can be overcome by bacteria with their own broad metabolic diversity and rapid-response regulatory networks. An important regulatory mechanism, known as carbon catabolite repression (CCR), can facilitate the utilization of carbon sources in a hierarchical manner. When the growth medium contains more than one type of nutrient, the most suitable nutritional utilization pathway can be activated to be functional. CCR retains repression on less preferred carbon sources until the preferred one is consumed. In *Bacillus subtilis* the catabolite control protein A (CcpA) acts as a transcriptional repressor and mediates CCR, whereas in *Escherichia coli* the cyclic AMP receptor protein (CRP) in conjunction with cAMP mediates CCR (Deutscher, 2008; Gorke & Stulke, 2008; Rojo, 2010). The *Pseudomonas* CCR regulatory system is composed of the two-component regulatory system CbrA/CbrB, the small non-coding RNAs (sRNAs) CrcZ(Y), and the translational repressor Crc. The catabolite repression control (Crc) protein directly binds to the mRNAs of related target genes and generally represses the translation of these genes. In turn, the Crc protein can be sequestered by the CrcZ-type sRNAs and its repression action is thus relieved. At the top of the CCR system, the CbrA/B two-component system is responsible for the transcriptional activation of the CrcZ-type sRNAs. *Pseudomonas putida* and *P. protegens* possess two CrcZ-type sRNAs, CrcZ and CrcY (Moreno et al., 2012) while *Pseudomonas aeruginosa* only harbours CrcZ (Sonnleitner et al., 2009).

Hfq is an RNA chaperone protein and is widely present in eu- karyotes and prokaryotes. Hfq plays an important global regulatory role in bacterial physiology and metabolism. In *P. aeruginosa*, Hfq globally regulates metabolism (Sonnleitner & Blasi, 2014), quorum sensing (Sonnleitner et al., 2006), virulence (Fernandez et al., 2016; Sonnleitner et al., 2003), and stress responses (Vogt & Raio, 2014). Hfq, which can bind with both mRNA and sRNA, regulates gene expression through various mechanisms. It not only mediates base-pairing between sRNAs and target mRNAs for the antisense regulation but also directly binds to the 5‘ or 3’ untranslated region (UTR) of target mRNAs for independently regulating mRNA stability or translation (Huber et al., 2020; Updegrove et al., 2016).

The Crc protein also contributes in some of the regulatory functions performed by Hfq (Quiroz-Rocha et al., 2017; Rojo, 2010). Crc can be copurified with tagged Hfq in *P. aeruginosa* (Moreno et al., 2015; Sonnleitner et al., 2017). It can stabilize the binding of the A-rich motif of target mRNA with the distal side of Hfq to form a tripartite Hfq-RNA-Crc complex (Madhushani et al., 2015; Sonnleitner et al., 2017). In the presence of preferred carbon sources, Hfq and Crc bind the CA-motifs of mRNAs involved in catabolism of non-preferred substrates to inhibit ribosome binding and/or translation initiation. However, when preferred carbon sources are exhausted or absent, the carbon source-sensitive CbrA/CbrB two-component system activates transcription of sRNAs crcZ and/or crcY, containing multiple CA-motifs. The increased expression of crcZ(Y) sRNA leads to the capture of Hfq-Crc, which allows bacteria to grow on the non-preferred substrate (Liu et al., 2017; Moreno et al., 2012; Valentini et al., 2014).

Crc globally modulates, at the posttranscriptional level, the expression of various genes, specifically those involved in CCR. In *P. putida*, the crc mutation results in changes in the expression of about 134 genes, which are mainly involved in energy metabolism and substrate transfer across the membrane. Crc contributes to organize and enhance metabolism when the cell is cultivated on medium containing succinate and glucose as carbon sources (La Rosa et al., 2015; Moreno, Martinez-Gomariz, et al., 2009). In addition, the crc mutant is impaired in biofilm formation and motility (Linares et al., 2010; O’Toole et al., 2000).

Some of the features in the *P. putida* crc-null strain were also observed in *P. aeruginosa* PA01 hfq mutant, indicating that Crc and Hfq may work together. Moreover, recent findings proved that Hfq needs the assistance of Crc to carry out repressive regulation of gene expression during CCR (Moreno et al., 2015; Sonnleitner & Blasi, 2014; Sonnleitner et al., 2003). Recent chromatin immuno-precipitation sequencing (ChIP-Seq) studies also showed that the common regulatory scope of Crc and Hfq is much wider than initially expected in pseudomonads and Hfq was found to be cotranscriptionally attached with more than 600 emerging RNA transcripts most often with cooperation of Crc (Kambara et al., 2018). These findings demonstrate that Hfq and Crc display far more posttranscriptional regulatory functions than catabolite repression.

Our previous report showed that in *P. aeruginosa* M18 Hfq globally regulates the biosynthesis of antibiotics, including Plt, phenazine-1-carboxylic acid (PCA), and pyocyanin, through direct posttranscriptional inhibition of two transcriptional regulator genes (pltR and qscR) and one conversion enzyme gene (phzM). Most significantly, Plt biosynthesis was significantly up-regulated by the *hfq* mutation in M18, suggesting the negative regulation of Hfq on Plt biosynthesis. In contrast, in *P. protegens* H78 the biosynthesis of antibiotics including Plt, DAPG, and Prn was strongly inhibited by the *hfq*
mutation, implying that the Hfq chaperone plays an activator role in antibiotic biosynthesis of H78. This stimulated our research interest to explore the different regulatory mechanisms and networks of Hfq and Crc on antibiotic biosynthesis and other physiological and metabolic phenotypes in P. protegens.

In this study, the RNA-Seq-based transcriptomic analysis and related phenotype assay were chosen to explore the global regulatory profiles of Hfq and Crc on antibiotic biosynthesis and other physiological and metabolic phenotypes in P. protegens H78. The results demonstrate that the Hfq chaperone protein shows a far more extensive and intense regulatory profile than the transcriptional regulatory protein Crc in P. protegens H78. There is a small proportion of overlapping regulons under the similar or opposite control of Hfq and Crc and a larger number of regulons under the independent regulation of Hfq in P. protegens H78.

2 | RESULTS

2.1 | Different influences of Hfq and Crc on growth of P. protegens H78

The growth curves of P. protegens H78 and its crc or hfq mutant were measured in King’s medium B (KMB). As shown in Figure S1, the growth rate and cell density of the hfq mutant strain were significantly lower than those of its parental strain H78 in KMB. Similarly, the crc mutation caused a moderate inhibition of cell growth for H78 in KMB (Figure S1).

In addition, the influence of hfq or crc on the growth of P. protegens H78 was assessed in inorganic salt medium supplemented with glycerol, mannitol, maltose, xylose, glucose, inositol, or succinate as the sole carbon source. The results showed that in all tested media the hfq mutation could significantly inhibit cell growth of H78. Introduction of the E. coli-Pseudomonas shuttle empty vector pME6032 brought about an obvious inhibition on the H78 growth rate and cell density of the parental strain H78 in the media containing protocatechuate or succinate (Figure 1). Growth of the crc mutant was obviously inferior to that of the wild-type strain H78 in medium containing protocatechuate or succinate (Figure 1).

Complementation of the hfq or crc gene harboured by the vector pME6032 partially or completely restored the growth of the respective mutants. In addition, we observed that the hfq mutation completely inhibited the growth of H78 in medium supplemented with protocatechuate as the sole carbon source. In turn, the inhibited growth of H78hfq mutant was restored to the growth level of the wild-type strain harbouring the empty vector by the exogenous complementation of the hfq gene. Strong down-regulation of growth also occurred in the crc mutant and was reversed to the growth level of the wild-type strain harbouring the empty vector by the exogenous complementation of the crc gene in medium containing protocatechuate (Figure 1).

These results indicate that the growth of P. protegens H78 is significantly influenced by Hfq and moderately influenced by Crc. Hfq and Crc play a significant role in the ability of P. protegens H78 to utilize protocatechuate or succinate as the sole carbon source.

2.2 | Hfq contributes more than Crc to antimicrobial activities in P. protegens H78

Two pathogens, Bacillus subtilis and Gibberella saubinetii, were selected for assessing the influence of Hfq and Crc on the antimicrobial activity of P. protegens H78. The mutation of hfq gene (H78hfq) resulted in nearly complete loss of antimicrobial activity of H78 to B. subtilis. In contrast, the crc mutation had a minor effect on the antimicrobial activity of H78 against B. subtilis. Complementation of the hfq gene could restore the antibiotic activity of the hfq mutant to the wild-type level (Figure 2a, b). Similarly, the hfq mutation significantly reduced the antimicrobial activity of P. protegens H78 against G. saubinetii. Instead, the antimicrobial activity of H78crc mutant on G. saubinetii was slightly lower than that of the wild-type strain (Figure 2c, d). The results suggest the antifungal activity of H78 is significantly affected by Hfq and mildly affected by Crc.

2.3 | The range and number of genes regulated by Hfq are much larger than those by Crc in P. protegens H78

The RNA sequencing-based transcriptomic analysis was performed for the wild-type strain H78, the hfq mutant (H78hfq), and the crc mutant (H78crc) to explore the different regulatory profiles of Hfq and Crc in P. protegens H78. The Hfq-mediated transcriptomic profile suggested that 1,371 genes, which account for 21% of the 6,446 annotated genes in the H78 genome, were significantly differentially regulated by at least two-fold (p < .05) in the hfq mutant when compared with the wild-type strain H78. Among these Hfq-regulated genes, 688 genes were down-regulated and 683 genes were up-regulated by the hfq deletion (Tables S1 and S2). In contrast, the Crc-mediated transcriptomic profile showed that the crc mutation resulted in the significant up-regulation of 218 genes, which only accounts for 3.4% of total genes of the H78 genome. Of these Crc-regulated genes, 113 genes were down-regulated and 105 were up-regulated by the crc deletion in P. protegens H78 (Tables S3 and S4).

These results demonstrate that the transcriptomic consequence induced by Hfq was more extensive and intense than that induced by Crc. Comparison between the Hfq- and Crc-mediated transcriptomic profiles revealed that 30 genes were identically up-regulated, including those genes within the plt biosynthetic and transport gene cluster, and 45 genes were identically down-regulated by both Hfq and Crc. In addition, 19 genes that were up-regulated by the hfq mutation were conversely down-regulated at the transcript level in the H78crc mutant. In contrast, 12 genes, the transcript level of which was down-regulated by the hfq mutation, were up-regulated in H78crc (Figure 3a and Tables S5–S8).

Based on the PseudoCAP, the Hfq or Crc regulon was grouped into 21 functional categories. In each functional category, the number of down-regulated and up-regulated genes in the hfq or crc mutant was plotted in Figure 3b. The genes under the positive control
of Hfq were mainly involved in the biosynthesis and transport of secondary metabolites including antibiotics and siderophores, amino transport and metabolism, inorganic transport and metabolism cell wall/membrane/envelope biogenesis, transcriptional factors, regulators, and two-component systems. The genes that were negatively regulated by Hfq were mainly related to amino acid transport and metabolism, carbohydrate transport and metabolism, energy production, and conversion. The number of genes regulated by Crc was far less than that by Hfq in *P. protegens* H78. The Crc protein was mainly involved in the positive regulation of biosynthesis and transport gene clusters of secondary metabolites such as pyoluteorin and the negative regulation of amino acid metabolism and transport genes (Figure 3b).

Transcriptome data showed that Hfq and Crc exhibited the same positive regulation on the Plt biosynthetic and transport operons. However, the expression of *phi* genes for DAPG biosynthesis was shown to be oppositely regulated by Hfq and Crc. The *phi* gene expression was positively regulated by Hfq and negatively regulated by Crc. In addition, Hfq and Crc also showed opposite regulatory effects on the expression of both *pedD-ppqI* and *cyoA–E* gene clusters, which was negatively regulated by Hfq and positively regulated by Crc (Figure 3c).

### Table

| Condition          | H78 | H78hfq | H78crc | H78-pME6032 | H78hfq-p6032hfq | H78crc-p6032crc |
|--------------------|-----|--------|--------|-------------|-----------------|----------------|
| LB (24 h)          | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) |
| Glycerol (36 h)    | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) |
| Mannitol (36 h)    | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) |
| Maltose (36 h)     | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) |
| Xylose (36 h)      | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) |
| Glucose (36 h)     | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) |
| Inositol (36 h)    | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) |
| Protocatechuate (72 h) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) |
| Succinate (72 h)   | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) | ![Image](MemePlate.png) |

**Figure 1** Effect of Hfq and Crc on the ability of *Pseudomonas protegens* H78 to utilize different carbon sources as the sole carbon source. The cell growth of six strains, *P. protegens* H78, H78hfq (hfq mutant), H78crc (crc mutant), H78-pME6032 (H78 harbouring the empty vector pME6032), H78hfq-p6032hfq (H78hfq harbouring the hfq expression vector p6032hfq), and H78crc-p6032crc (H78crc harbouring the crc expression vector p6032crc), was assayed in the minimal medium plate supplemented with the sole carbon sources glycerol, mannitol, maltose, xylose, glucose, inositol, protocatechuate, or succinate at 28 °C. The collected and washed cells from overnight culture were first diluted to the same starting density (OD$_{600}$ = 0.05) and then successively diluted 10 times. Two microlitres of sample was inoculated on the minimal medium and cultivated at 28 °C. LB, Luria-Bertani medium.
Regulatory mechanism of secondary metabolism, including antibiotic biosynthesis, was the focus of our research. Transcriptomic profiles revealed that the gene clusters involved in secondary metabolism, including antibiotic and siderophore biosynthesis, were one of the most important regulatory functional categories of Hfq and Crc.

According to the transcriptomic results, the deletion of hfq gene resulted in a 6.3–96.7-fold reduction in the transcriptional level of all genes within the Plt biosynthetic operon pltLABCDEFG. Compared with the parental strain H78, the hfq mutant was significantly down-regulated by 9.4- and 3.0-fold in the expression of pltR and pltM genes (Table S1). Similarly, transcription of the Plt ABC transport operon pltIJKNOP was reduced by 2.5–14.1-fold due to the hfq mutation. Knockout of crc resulted in a 3.1–4.1-fold decrease in the transcriptional level of all genes in the Plt biosynthetic operon pltLABCDEFG. However, the transcription level of pltR and pltM only decreased 1.7 and 1.6 times, respectively, due to the crc mutation. The crc mutation moderately lowered the transcriptional level of pltIJKNOP by 1.9–3.0 times. Expression analysis of the pltL::lacZ in-frame fusion reporter further confirmed that Hfq exhibited strong positive regulation on the expression of the pltLABCDEFG biosynthetic operon (Figure 4b). As shown in Figure 4b, the crc mutation resulted in a significant decrease in pltL::lacZ fusion expression. The results suggest that the Crc protein also plays a positive regulatory role in Plt biosynthesis. Correspondingly, we quantitatively analysed Plt production of H78 and its hfq or crc mutant grown in KMB. As expected, the hfq-deleted strain was completely inhibited in Plt biosynthesis, whereas the H78crc mutant strain showed a significant decrease in Plt production compared with the parental strain H78 (Figure 4a). We speculate that Hfq and Crc coregulate the expression of pltR and thus affect the pltLABCDEFG transcription and Plt biosynthesis. In this regulation, Hfq may play a major role while Crc has an auxiliary role.

Like Plt, DAPG biosynthesis was also completely inhibited by the hfq deletion in H78. However, the crc mutation only caused a slight down-regulation of DAPG biosynthesis (Figure 4c). The DAPG biosynthetic, transport, and regulatory gene cluster consists of five operons, phlACBD, phlE, phlF, phlG, and phlH. The phlACBD operon is responsible for DAPG biosynthesis. Unexpectedly, only three genes within the phl gene cluster were significantly down-regulated by the hfq deletion. These three genes, phlA, phlF, and phlG, were down-regulated by 2.2-, 3.6-, and 8.2-fold, respectively, in the hfq mutant (Tables S1 and S2). Similarly, only these three genes were significantly regulated at the transcript level by the crc mutation. However, what is different is that phlA and phlF were significantly up-regulated by 2.5-fold while phlG was down-regulated by 2.3-fold in the crc mutant when compared with the wild-type H78 (Tables S3 and S4). The phlA: lacZ fusion expression analysis further confirmed that Hfq positively regulates the expression of the phlACBD biosynthetic operon while Crc has a slight negative regulatory effect on the expression of the phlACBD operon (Figure 4d). Crc might be able to independently perform this regulatory function in the absence of Hfq.
Another two operons, prnABCD and hcnABC, are responsible for the biosynthesis of Prn and HCN, respectively. The transcriptome data showed that the deletion of the hfq gene resulted in a 1.5- to 4.1-fold decrease in transcription of the prnABCD operon and a 3.3- to 3.7-fold decrease in the hcn operon in H78. However, the knock-out of the crc gene had no significant effect on the transcription of the prn and hcn operons. The expression analysis of prnA-lacZ and hcnA-lacZ in-frame translational fusions further confirmed that the expressions of prn and hcn operons are positively regulated by Hfq, but are not influenced by Crc in H78.

Pyochelin, a main fluorescent iron carrier produced by Pseudomonas, is biosynthesized by the pchDHIEFKCBA gene cluster. Transcriptome data showed that all genes of the pchDHIEFKCBA gene cluster were strongly down-regulated by 7.7- to 25.6-fold at the transcript level in the hfq mutant when compared with the wild-type H78. In contrast, the transcript level of pchDHIEFKCBA genes relative to the wild-type strain H78. The highly regulated and well-characterized gene clusters are indicated.

**FIGURE 3**

Transcriptional profiles of *Pseudomonas protegens* H78 and its hfq or crc mutant grown to the late exponential phase (OD<sub>600</sub> = 5.0–6.0) in King's medium B for 18 hr at 28 °C with shaking at 200 rpm. (a) The number of genes that were significantly up-regulated or down-regulated (fold change ≥ 2, *p* < .05) due to the hfq or crc deletion. (b) Functional classification of significantly up-regulated (red bars) or down-regulated (blue bars) genes in the H78hfq and H78crc mutants when compared with the wild-type H78 strain. (c) Scatter plots representing a comparison of transcriptomes of *P. protegens* H78 and its hfq or crc mutant. The x axis shows the gene number in the H78 genome; the y axis represents the log<sub>2</sub> fold change of transcript abundance of each gene in the H78hfq mutant or H78crc mutant relative to the wild-type strain H78. The highly regulated and well-characterized gene clusters are indicated.
was less affected and decreased by 1.2- to 2.2-fold in the crc mutant when compared with the H78 strain (Tables S1 and S3). The results suggest that Hfq is a global positive regulator in secondary metabolism including antibiotic and siderophore biosynthesis while the Crc-mediated regulatory action is much smaller than that the Hfq-mediated regulatory action.

2.5 | The Hfq regulon involved in multiple ABC transport systems

In the Hfq-mediated transcriptome profile, many gene clusters encoding ABC transport systems, which are involved in the transport of mineral and organic ions (spb, cysATW, tauABC, potABCD, and...
opuCA/CB/CC), oligosaccharide and polyol (mtlEFGK), monosaccharide (gtsABC/malK and rbsABCD), amino acid (gltUKL, aapJMPQ, liv-FGHJM and metI2N2Q2), and C4-succinate and fumarate (dctAMPQ), varied considerably at the transcriptional level due to hfq mutation (Figure S2; Tables S1 and S2). The gene clusters related to sulphate/thiosulphate (spb, cysATW), taunine (tauABC), and polyamine transport (potABCD) were significantly up-regulated in the hfq mutant strain, while the gene clusters related to glycine betaine/proline and osmoprotectant transport were slightly down-regulated, as shown in Figure S2a and Tables S1 and S2. The mtlEFGKDYZ gene cluster involved in mannnol uptake and metabolism was significantly up-regulated by 10.2- to 24.0-fold in the H78hfq mutant compared with the wild-type H78 (Figure S2b and Table S2). The gts and rbs operons, which are required for the transport of glucose/mannose and ribose/autoinducer/β-xylene, respectively, showed a significant up-regulation at the transcript level due to the hfq deletion (Figure S2c and Table S2). The gltUKL operon encoding a glutamate/aspartate transporter was up-regulated by 3.8- to 6.3-fold because of the hfq deletion. In addition, the hfq mutation resulted in a substantial up-regulation in both the γ-amino acid transporter operon aapJQMP (9.5–18.5-fold) and the branched-chain amino acid transporter operon livLHMGF (4.6–5.9-fold) (Figure S2d and Table S2). In addition, the transcript level of the metQ2I2N2 operon, which is involved in the uptake of methionine, was slightly up-regulated in the hfq mutant (Figure S2d and Table S2). The dctPQM and dctA transport operons, which are involved in the uptake of succinate and fumarate were up-regulated by 9.3–24.2 times and 2.4 times, respectively, at the transcript level in the hfq mutant when compared with the H78 strain (Figure S2e and Table S2).

2.6 Negative regulation of Hfq on expression of the pca operon involved in degradation of aromatic compounds

As shown by the transcriptomic result, the pca operon (pcaR-pcaK-catU-pcaFHG-H78_01383-pcaBDC), which is involved in the degradation pathway of aromatic and hydroaromatic compounds, was significantly up-regulated by 2.5- to 17.3-fold due to the hfq mutation in H78 (Figure S3a). Most notably, the 4-hydroxybenzoate transporter gene pcaK was up-regulated by 17.3-fold in the hfq mutant when compared with the H78 strain. In contrast, the transcript levels involved in the degradation pathways of aromatic and hydroaromatic compounds were not influenced by the crc mutation. The pcaK-lacZ in-frame translational fusion reporter plasmid was constructed to further assess the influence of Hfq and Crc on the pca expression. The result showed that the hfq mutation resulted in a significant increase in the pcaK expression, which is consistent with the transcriptome data (Figure S3a). This confirmed that Hfq could negatively regulate the expression of pcaK at the transcriptional level. In addition, the pcaK-lacZ fusion expression was significantly up-regulated by the crc mutation (Figure S3b). However, the transcriptomic result showed that the crc mutation had little effect on the transcript level of pcaK. We speculate that Crc, as a posttranscriptional regulator, may directly inhibit the pcaK expression at the posttranscriptional level.

2.7 Hfq positively regulates the gene expression of type VI secretion systems

In the H78 genome, the gene cluster covering from H78_06284 to H78_06304 encodes a type VI secretion system (T6SS) (Figure 5b), which corresponds to the H1-T6SS of three T6SSs in P. aeruginosa (Gottesman, 1996). The RNA-Seq analysis results showed that all genes within the H1-T6SS gene cluster were significantly down-regulated by 2.1- to 10.6-fold in the hfq mutant relative to the wild-type strain H78 (Figure 5a). However, the crc deletion had no obvious influence on the expression of the H1-T6SS gene cluster. To further confirm the positive regulation of Hfq on the H1-T6SS gene cluster, three lacZ reporter plasmids, which carry the tssA-lacZ, tssE-lacZ, and fha-lacZ translational fusions, respectively, were constructed. As shown in Figure 5c, the expression of the tssA, tssE, and fha genes was significantly down-regulated in the hfq mutant compared with its parental strain H78. However, the expression of these three genes in the crc mutant showed little change when compared with the wild-type H78 (Figure 5c). These results are consistent with the transcriptome data. It is demonstrated that Hfq could positively regulate the expression of T6SS, while Crc had no significant effect on the transcription of T6SS.

2.8 Hfq negatively regulates the expression of genes involved in oxidative phosphorylation

In Pseudomonas, the oxidative phosphorylation system consists of five redox enzyme complexes that transfer electrons from NADH and succinate to oxygen (Figure S4a). Four gene clusters involved in oxidative phosphorylation, nuoA-N, ctaE-C, ccoH-N2, and atpC-B, were significantly up-regulated by 3.9–5.2, 9.5–15.8, 1.5–4.0, and 2.0–2.4 times, respectively (Table 1). However, the crc mutation had no significant impact on the oxidative phosphorylation-related gene clusters except cyoABCDE. The cyoABCDE gene cluster was down-regulated by 3.1–3.6 times due to the crc deletion. The consumption of NADH increases with the increase in oxidative phosphorylation. Correspondingly, we measured the level of NADH produced by three strains, H78, H78hfq, and H78crc. As shown in Figure S4b, the hfq mutation resulted in a significant decrease in NADH content, which was in turn restored by the exogenous complementation of the hfq gene. In contrast, no obvious influence was caused by the crc mutation (Figure S4b).

2.9 The opposite regulatory effect of Hfq and Crc on the PQP-EDH gene expression

As a rhizosphere resident and biological control strain, Pseudomonas spp. are exposed to a variety of volatile organic compounds (VOCs)
from different sources. To capture and metabolize these VOCs effectively, *Pseudomonas* has evolved to express two pyrroloquinoline quinone-dependent ethanol dehydrogenases (PQQ-EDHs) (Muckschel et al., 2012; Wehrmann et al., 2017). Transcriptomic data showed that the mutation of *hfq* resulted in a strong up-regulation of the *ped* gene cluster by 5.0- to 208.9-fold. In contrast, the *crc* deletion gave rise to a 1.1- to 23.7-fold decrease in the transcript abundance of the *ped* gene cluster (Figure 6a). In addition, in the *pqq* gene cluster, the *pqqBCDEM* operon was significantly up-regulated by 3.0–6.6-fold at the transcript level due to the *hfq* mutation. More significantly, the *yiiM*-pqqI-H78_05873 operon in the *hfq* mutant was up-regulated 135.8- to 369.0-fold when compared with the parental strain H78 (Figure 6b). However, the expression of the *pqq* gene cluster was not influenced by the *crc* mutation.

### 2.10 Hfq negatively regulates the flagellar biogenesis gene expression

Referring to the KEGG flagellar assembly model, in *P. protegens* H78 the flagellum-related proteins can be divided into five parts: flagellar basal-body rod proteins (H78_01720 to 01726 and H78_04786 to 04789), flagellar hook-associated proteins (H78_01737 to 01740), flagellar motor switch proteins (H78_01754 to 01762), flagellar basal body rod proteins (H78_01776 to 01785) (Figure S5f). Transcriptomic profiling showed that the expression of almost all flagellar biosynthetic, assembly, and regulatory genes were significantly up-regulated in the *hfq* deficient strain when compared with its parental strain H78. At the same time, the transcription level of chemotaxis-related genes (H78_01777 to 01783) (Figure S5f) was significantly increased due to *hfq* mutation. In contrast, all flagella-related genes were not significantly changed by the *crc* mutation.
| GeneID     | Gene name | Product                                           | Hfq-fold change | Crc-fold change |
|------------|-----------|---------------------------------------------------|-----------------|-----------------|
| **NADH dehydrogenase**                        |           |                                                   |                 |                 |
| H78_04188  | nuoA      | NADH-quinone oxidoreductase subunit A             | 4.39            | 0.99            |
| H78_04189  | nuoB      | NADH dehydrogenase subunit B                      | 4.35            | 0.95            |
| H78_04190  | nuoC      | Protein NuoC                                      | 4.73            | 1.22            |
| H78_04191  | nuoE      | NADH dehydrogenase subunit E                      | 4.67            | 1.24            |
| H78_04192  | nuoF      | NADH dehydrogenase subunit F                      | 4.35            | 1.19            |
| H78_04193  | nuoG      | NADH dehydrogenase subunit G                      | 4.84            | 1.12            |
| H78_04194  | nuoH      | NADH-quinone oxidoreductase subunit H             | 4.83            | 1.05            |
| H78_04195  | nuoI      | NADH dehydrogenase subunit I                      | 5.12            | 1.23            |
| H78_04196  | nuoJ      | NADH dehydrogenase subunit J                      | 4.94            | 0.94            |
| H78_04197  | nuoK      | NADH:ubiquinone oxidoreductase subunit K          | 4.72            | 0.84            |
| H78_04198  | nuoL      | NADH-quinone oxidoreductase subunit L             | 4.71            | 1.00            |
| H78_04199  | nuoM      | NADH-quinone oxidoreductase subunit M             | 3.91            | 1.05            |
| H78_04200  | nuoN      | NADH-quinone oxidoreductase subunit N             | 5.17            | 1.07            |
| **Succinate dehydrogenase**                  |           |                                                   |                 |                 |
| H78_01822  | sdhC      | Succinate dehydrogenase subunit C                 | 1.96            | 1.06            |
| H78_01823  | sdhD      | Succinate dehydrogenase                           | 1.57            | 0.97            |
| H78_01824  | sdhA      | Succinate dehydrogenase                           | 2.27            | 1.13            |
| H78_01825  | sdhB      | Succinate dehydrogenase iron-sulphur subunit     | 2.58            | 1.15            |
| **Cytochrome c reductase**                  |           |                                                   |                 |                 |
| H78_05294  | petB      | Ubiquinol–cytochrome C reductase, cytochrome C1 subunit | 3.19           | 0.84            |
| H78_05295  | petA      | Putative cytochrome b                             | 3.59            | 0.71            |
| H78_05296  | petA      | Ubiquinol–cytochrome C reductase, iron-sulphur subunit | 4.25           | 0.50            |
| **Cytochrome c oxidase**                     |           |                                                   |                 |                 |
| H78_00063  | ctaC      | Cytochrome c oxidase subunit II                   | 12.59           | 1.35            |
| H78_00064  | coxA      | Cytochrome c oxidase subunit I                    | 15.84           | 1.36            |
| H78_00065  | ctaG      | Cytochrome c oxidase assembly protein             | 13.46           | 1.72            |
| H78_00066  | ctaE      | Cytochrome c oxidase subunit III                  | 9.52            | 0.98            |
| H78_00070  | ctaE      | Cytochrome c oxidase assembly protein             | 11.27           | 1.34            |
| H78_00071  | ctaE      | Protoheme IX farnesytransferase                   | 9.07            | 1.31            |
| H78_05248  | ctaE      | Protoheme IX farnesytransferase                   | 9.85            | 0.28            |
| H78_05249  | ctaE      | Cytochrome o ubiquinol oxidase subunit IV         | 8.88            | 0.29            |
| H78_05250  | ctaE      | Cytochrome o subunit III                          | 9.88            | 0.28            |
| H78_05251  | ctaE      | Cytochrome o subunit I                            | 8.99            | 0.32            |
| H78_05252  | ctaE      | Cytochrome o ubiquinol oxidase subunit II         | 10.09           | 0.28            |
| H78_02053  | ctaE      | Protein CcoH                                      | 2.53            | 1.19            |
| H78_02054  | ctaE      | Putative ferredoxin                               | 3.96            | 1.12            |
| H78_02055  | ctaE      | Putative cytochrome c                             | 2.22            | 0.76            |
| H78_02056  | ctaE      | Cytochrome c oxidase subunit IV                   | 2.88            | 0.82            |
| H78_02057  | ctaE      | Cbb3-type cytochrome c oxidase subunit II         | 2.15            | 0.81            |
| H78_02058  | ctaE      | Cytochrome c oxidase subunit I                    | 2.38            | 0.76            |
| H78_02059  | ctaE      | Cytochrome c oxidase subunit III                  | 1.57            | 1.25            |
| H78_02060  | ctaE      | Cytochrome c oxidase, Cbb3-type, CcoQ subunit     | 1.72            | 1.40            |
| H78_02061  | ctaE      | Cbb3-type cytochrome c oxidase subunit II         | 1.45            | 1.44            |

(Continues)
due to the
transcription of flagella- related genes. Crc, is an important global regulatory system of carbon metabolism system, the CrcZY (X) sRNAs, and the translational regulatory protein
Crc, could positively regulate the transcription of flagella-related genes.

2.11 | Positive regulation of Hfq and Crc on expression of the crcZ/Y sRNAs

Bacterial sRNAs are important transcriptional and posttranscriptional regulators. In Pseudomonas spp., the Cbr/Crc signal transduction cascade, which in turn consists of the CbrA/B two-component system, the CrcZY (X) sRNAs, and the translational regulatory protein Crc, is an important global regulatory system of carbon metabolism and other cell activities. The transcriptome data showed that the hfq deletion almost completely inhibited the expression of crcY and crcZ. The hfq deletion resulted in 4,780-fold down-regulation of the crcZ gene and 280-fold down-regulation of the crcY gene (Table S1). The results of quantitative reverse transcription PCR, which were consistent with the transcriptome data, showed that the expression of crcZ and crcY significantly decreased by 3,333 times and 201 times due to the hfq mutation, respectively (Figure 7a). To further verify the effects of hfq and crc genes on the transcription of crcZ and crcY, the crcZp-lacZ and crcYp-lacZ transcriptional fusion plasmids were constructed and measured for β-galactosidase activity in H78 and its hfq or crc mutant. The mutation of hfq resulted in a 10-fold decrease in the transcription of both crcY and crcZ. Similarly, the transcription levels of crcY and crcZ genes were also significantly down-regulated in the crc mutant compared with the wild type (Figure 7b,c).

The transcription of crcZ and crcY genes from their respective promoters has been confirmed to rely on the transcriptional activation of the two-component system response regulator CbrB. To further investigate whether Hfq and Crc regulate the transcription of crcY and crcZ through CbrA/B, we constructed the cbrA-lacZ translational fusion plasmid. However, no noticeable changes were observed in the cbrA-lacZ expression between the wild-type H78 and the crc mutant. The hfq mutation slightly down-regulated the expression of cbrA-lacZ (Figure 7c,d). These results suggested that Hfq and Crc regulate the expression of crcZ and crcY independently from CbrB. In addition, the hfq-lacZ and crc-lacZ fusion plasmids were constructed to study the mutual influence between Hfq and Crc. As shown in Figure 7e,f, the mutation of crc had no significant effect on the expression of hfq, while hfq mutation caused a slight reduction in the expression of the crc gene.

3 | DISCUSSION

The earlier studies showed that Hfq plays an important regulatory role in many aspects of cell physiology and metabolism, including cell motility (Lai et al., 2018; Schachterle et al., 2019), morphology, quorum sensing (Sonleitner et al., 2006; Yang et al., 2015), virulence (Ding et al., 2004; Sittka et al., 2007; Sonleitner et al., 2003), and stress responses (Arce-Rodriguez et al., 2016). Crc has been identified only in the Pseudomonas genus and various related genera (Morales et al., 2004; Rojo, 2010). Crc globally modulates the expression of many genes involved in carbon metabolism and other cell activities. Initially, Crc was thought to bind to mRNA alone (Moreno, Marzi, et al., 2009; Sonleitner et al., 2009). However, recent results suggest that Hfq first recognizes the A-rich motif of target mRNAs (−AANAANAA−) in the target mRNAs to prevent their
translation. When the preferred carbon source is depleted, an unidentified signal can trigger the autophosphorylation of CbrA sensor kinase, thereby phosphorylating the CbrB response regulator. These events eventually lead to the expression of sRNAs CrcZ/Y, which in turn sequester Crc and Hfq, thus achieving the translation of target mRNA (Garcia-Maurino et al., 2013; Nishijyo et al., 2001). In this regulatory process, cells undergo a series of physiological and metabolic changes, including the activities of specific regulatory factors, enzymes, and transporters associated with the use of secondary carbon sources (Sanchez-Hevia et al., 2018; Schachterle et al., 2019).

Previous studies have shown that CrcY/Z may sequester Hfq–Crc and prevent their binding to the target RNA. In this study, we found that Hfq could also activate the transcription of crcZ/Y genes in P. protegens. It is implied that Hfq may depend on CbrB to regulate the expression of crcZ/Y genes. However, based on the results of this study we deduce that Hfq could regulate the expression of crcZ/Y independently from CbrB in P. protegens H78. To uncover the regulatory mechanism of Hfq activating crcZ/Y transcription needs further study. At the same time, we found that Hfq can slightly positively regulate the expression of crc gene, but the regulatory effect of crc on hfq gene was not significant. A proposed model for the regulatory network of Hfq, Crc, and CrcZ/Y in P. protegens H78 is shown in Figure 8. Hfq plays a far more extensive and intensive regulatory role than the translational regulatory protein Crc in P. protegens H78. A small portion of overlapping regulons were under the similar or opposite regulation by Hfq and Crc while a larger number of regulons were independently regulated by Hfq in H78. Both cell growth and antimicrobial activity were under the significant positive control by Hfq but only slightly up-regulated by Crc. In addition, the transcription of crcY/Z sRNAs can be activated by feedback from Hfq and Crc, independently of the CbrA/B two-component system.

The biocontrol activity by Pseudomonas and other bacteria is mainly related to the biosynthesis of secondary metabolites, including antibiotics and siderophores. In P. protegens H78, Hfq globally activates the biosynthesis of most secondary metabolites while Crc only positively regulates the biosynthesis of Plt. In contrast, in the
rhizobacterium *P. aeruginosa* M18, Hfq was shown to directly bind to the 5′ untranslated leaders of *pltR* and *phzM* mRNAs to negatively regulate the biosynthesis of Plt and repress the conversion of PCA to pyocyanin, respectively. The positive regulation of Hfq on PCA biosynthesis is mediated by the transcriptional repressor QscR in M18 (Wang et al., 2012). In *P. protegens* H78, the unknown regulatory pathway and molecular mechanism involved in the activation of antibiotic biosynthesis by Hfq aroused our interest in further research. In other *Pseudomonas* spp. and biocontrol bacteria, Hfq also plays an important role in the global regulation of antibiotic biosynthesis.

**FIGURE 7** Regulation of Hfq and Crc on the expression of CrcZ/Y small RNAs and the CbrA/B two-component system and mutual regulation between Hfq and Crc. (a) The influence of Hfq on the transcriptional levels of *crcZ* and *crcY* was determined by quantitative reverse transcription PCR. (b, c) β-Galactosidase expression from the *crcZp-lacZ* (b) and *crcYp-lacZ* (c) transcriptional fusion plasmids was measured in H78, H78hfq, and H78crc. (d) Effects of Hfq and Crc on the crcA/B expression were assessed by measuring the β-galactosidase expression from the *cbrA′-lacZ* fusion plasmid in H78, H78hfq, and H78crc. (e) β-Galactosidase activity expressed from the *hfq′-lacZ* fusion was assayed between H78 and H78crc. (f) β-Galactosidase activity expressed from the *crc′-lacZ* was measured between H78 and H78hfpq.

**FIGURE 8** Proposed global regulatory model of Hfq and Crc in *Pseudomonas protegens* H78. Bold arrows, positive control; bold lines with flatted end, negative control; dashed arrow, refer to other *Pseudomonas* spp.
and other secondary metabolisms. In *P. fluorescens* 2P24, the *hfq* mutation reduced both DAPG biosynthesis and rhizosphere colonization ability (Wu et al., 2010). In *Lysobacter enzymogenes* OH11, *Hfq* significantly down-regulated the production of antibacterial antibiotic WAP-8294A2 (Xu et al., 2015). In *Serratia plymuthica* A153 and 4Rx5, the *hfq* mutant resulted in the complete loss of both oocyclin A biosynthesis and antifungal activity (Matilla et al., 2015). The biosynthesis of pyochelin, as a main fluorescent siderophore produced by *Pseudomonas*, was strongly inhibited by the *hfq* mutation and slightly down-regulated by the *crc* mutation in H78. Similarly, the *hfq* inactivation obviously reduced siderophore production in *Burkholderia glumae* (Kim et al., 2018). In *P. putida*, *Hfq* and *Crc* are involved in the control of iron homeostasis (Sanchez-Hevia et al., 2018).

*Hfq* and *Crc* are closely related to carbon metabolism. The carbon source, as an essential nutrient for microbial growth, also affects the production of antibiotics. In *P. fluorescens* F113, sucrose, mannitol, and fructose were shown to promote DAPG biosynthesis, whereas glucose and sorbose can reduce DAPG production (Shanahan et al., 1992). Compared with other carbon sources, PCA yield was highest in the medium with glucose as carbon source in *P. aeruginosa* M18G (He et al., 2008). Some antibiotics, such as DAPG, are produced at the onset of the stationary phase and other antibiotics (Pkt, phenazine-1-carboxamide) during the exponential phase (Jin et al., 2016; Shanahan et al., 1992; Wang et al., 2017). RpoS, as the stationary phase sigma factor, regulates cell survival in response to the cessation of growth (stationary phase) and provide cross protection against environmental stress (Venturi, 2003). In *P. protegens* Pf-5, the rpoS mutation resulted in the overproduction of Pkt and DAPG and the inhibition of Pkn biosynthesis (Sarnigue et al., 1995). A recent study has shown that *Hfq* activates the expression of rpoS by enhancing the stability of dsrA sRNA in *E. coli* (Kim et al., 2019). Further research is needed to ascertain whether RpoS is involved in the regulation of antibiotic biosynthesis by *Hfq* in *P. protegens* H78.

The results of this study showed that the regulatory effect of *Hfq* protein is much greater than that of *Crc* protein. *Hfq* plays a pivotal role in the transcriptional regulation of diverse metabolic and physiological functions in *P. protegens* H78, including secondary metabolism involved in the biosynthesis of antibiotics and siderophores, type VI secretion systems, ABC transport systems, oxidative phosphorylation, flagellar biogenesis, and degradation of aromatic compounds and volatile organic compounds. The *hfq* mutant displayed significant differential expression of the 21% genes of the H78 genome. In contrast, the *crc* mutation only resulted in significant changes in the transcription of 228 genes involved in secondary metabolism, including in antibiotic and siderophore biosynthesis, and aromatic compound and volatile organic compound degradation (Figure 8). Recently published proteomic and transcriptomic studies showed that *Hfq* and *Crc* control the iron homeostasis in *P. putida* (Sanchez-Hevia et al., 2018). Similarly, Sonnleitner et al. demonstrated that 332 and 149 genes were differentially expressed in the *hfq* mutant and *crc* mutant strains, respectively, compared with the parental strain *P. aeruginosa* PAO1. In addition, four transcripts of 104 overlapping transcripts showed the opposite regulation between the *hfq* and *crc* mutants when compared with PAO1 (Sonnleitner et al., 2018).

It has been reported that *Hfq* can facilitate formation of more stable Hfq–RNA complexes (Moreno et al., 2015). However, interestingly, in the present study *Hfq* and *Crc* had opposite regulatory effects on some of the same target genes in *P. protegens* H78. Nineteen genes were up-regulated in the *hfq* mutant, but down-regulated in the *crc* mutant. In contrast, 12 genes were down-regulated in the *hfq* mutant, but up-regulated in the *crc* mutant. DAPG biosynthesis was positively regulated by *Hfq*, but negatively regulated by *Crc*. In addition, *Hfq* and *Crc* also exhibited opposite regulatory effects on both the pedD-ppq1 and cyoA- E gene clusters. The transcription of the pedD-ppq1 and cyoA- E genes clusters was negatively regulated by *Hfq* and positively regulated by *Crc*. It has been reported that the purified *Crc* protein can specifically bind to the alkS mRNA fragment spanning from +1 to +43 nucleotides (relative to the translation start site) in *P. putida* (Moreno et al., 2007). Similarly, the *Crc* protein could specifically bind to a benR mRNA fragment and inhibit its translation in *P. putida* (Moreno & Rojo, 2008). We therefore speculate that the expression of some target genes regulated by *Crc* may be independent of *Hfq* in *P. protegens* H78.

The β-ketoadipate pathway, as the most common degradative pathway of aromatic and hydroaromatic compounds, exists widely in bacteria and fungi (Morales et al., 2004). This study showed that the transcription of the pcaR-pcaC gene cluster involved in the β-ketoadipate pathway was positively regulated by *Hfq* but not influenced by *Crc*. PcaK not only mediates the uptake of 4-HBA and PCA, but also plays a role in the chemotaxis of these two compounds. Transcriptomic results showed that *Crc* had no effect on the transcription level of *pcaK*. Unexpectedly, lacZ reporter analysis showed that the expression of pcaK was negatively regulated by *Crc*, which implies *Crc* may exert indirect regulation on pcaK at the posttranscriptional level. Previous studies have shown that *Crc* can inhibit the translation of the transcriptional activator gene *benR*, which is responsible for the activation of the benABCD genes cluster in *P. putida* (Morales et al., 2004; Moreno & Rojo, 2008). Similarly, *Crc* also plays an important role in the inhibition of the degradation of aromatic compounds in *Acinetobacter baylyi* (Zimmermann et al., 2009). Thus, we speculate that *Crc* is likely to bind to the pcaK mRNA and inhibit its translation at the posttranscriptional level in *P. protegens* H78.

Recent studies have shown that *Hfq* acts as a translational repressor during CCR in *P. aeruginosa* and several catabolic genes were translationally silenced by *Hfq* due to the availability of succinate as preferred carbon source (Sonnleitner & Blasi, 2014). When succinic acid was depleted, the expression of the Hfq-binding sRNA CrcZ increased (Hernandez-Arranz et al., 2016; Sonnleitner et al., 2009), thus sequestering *Hfq* and relieving the CCR. Unexpectedly, the results of this study showed that the growth of *hfq* mutant was significantly impaired in an inorganic salt medium using glycerol, mannitol, maltose, xylose, glucose, inositol, or succinate as the sole carbon source. Furthermore, the *hfq* mutant showed no growth on the medium with protocatechuate added as the sole carbon source. These results demonstrate that the carbon source may not be the
main factor affecting the growth of *P. protegens* H78. Recent studies provide proof that the RNA-binding protein Hfq plays an important role in ribosome biogenesis and affects the accuracy of translation (Andrade et al., 2018; Sharma et al., 2018). We speculate that the hfq mutation results in a higher level of immature 30S ribosomes and error-prone translation, which in turn affects growth.

In this study, we demonstrated that Hfq plays a far more extensive and intensive regulatory role than Crc in *P. protegens* H78. A small proportion of overlapping regulons were similarly or oppositely regulated by Hfq and Crc. In contrast, a larger number of regulons were independently regulated by Hfq in H78. Future work will focus on clarifying the molecular regulatory mechanisms and pathways of Hfq and Crc on antibiotic biosynthetic gene expression and improving antibiotic production through genetic and metabolic engineering.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Bacterial strains, plasmids, primers, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table S9. The oligonucleotide primers are described in Table S10. *P. protegens* and its derivative strains were incubated at 28 °C in KMB. *E. coli* growth was grown at 37 °C in Luria-Bertani (LB) medium. When required, antibiotics were used at the following concentrations: kanamycin (Km) 50 μg/ml, ampicillin (Amp) 100 μg/ml, and tetracycline (Tc) 40 μg/ml for *P. protegens* and 15 μg/ml for *E. coli*.

### 4.2 | DNA manipulations

All molecular biological experiments were performed using standard methods. Plasmid DNA was purified using the TaKaRa miniBEST plasmid purification kit (Ver. 2.0). DNA was recovered from the gel using the TaKaRa MiniBEST Agarose Gel DNA Extraction Kit (Ver. 4.0). Easy Taq Mix DNA polymerase (TransGen) and Primer STAR Max DNA Polymerase (TaKaRa) were used in PCR. Similarly, restriction endonucleases (NEB), Solution I (TaKaRa), and In-Fusion HD Cloning Kit (TaKaRa) were used according to the suppliers’ instructions. DNA was sequenced and synthesized by the Shanghai Sunny Biotech Co.

### 4.3 | Deletion and complementation of *hfq* and *crc* in *P. protegens* H78

The encoding region spanning +29 to +216 bp (relative to ATG) of the hfq gene was deleted in the H78hfq mutant by homologous recombination. Both sides of the hfq open reading frame (ORF) were amplified with primers hfq-F1/hfq-R1 and hfq-F2/hfq-R2, respectively (Table S10). The 454 bp upstream fragment and 485 bp downstream fragment were further ligated through an overlap PCR with two primers (hfq-R1 and hfq-F2) with a 5’ end homologous region. Then, the 939 bp fused PCR fragment was cloned into XbaI-HindIII-digested pK18mobsacB using an In-Fusion HD Cloning Kit. The recombinant plasmid, named pK18-hfq, was transformed into *P. protegens* H78 from *E. coli* S17-1 (λpir) by conjugation. The single crossover clones were selected on LB plates containing 100 μg/ml Amp and 50 μg/ml Km, and the double crossover clones were selected on LB plates containing 100 μg/ml Amp and 15% sucrose. The *hfq* mutant H78hfq was confirmed by PCR analysis and sequencing. The *crc* mutant H78crc was also constructed using the same method by deleting the +69 bp to +752 bp (relative to ATG) region in the *crc* ORF.

To complement the hfq and crc mutants (H78hfq and H78crc), two fragments, which carry the entire ORFs and their own promoters of hfq and crc genes, respectively, were cloned into the pME6032 plasmid. These two recombinant expression plasmids were named p6032hfq and p6032crc, respectively.

### 4.4 | RNA isolation, RNA-Seq, and data analysis

For RNA-Seq, overnight cultures of *P. protegens* H78, H78hfq, and H78crc were inoculated in 100 ml of King’s B liquid medium at 28 °C with shaking at 200 rpm. For every strain, three replicates were set. After 18 hr of incubation, H78 and its mutants were collected at the late exponential phase (OD$_{600}$ = 5.0–6.0) by centrifuging at 4 °C. RNA extraction and sequencing were performed by the Shenzhen HuDa Genomics Institute (Shenzhen, China). After removing rRNAs using the RiboZer or RNA depletion kit, the RNAs were fragmented into 130–170 nucleotides. The first and second cDNA strands were amplified, purified, a poly(A) tail was added, and further used in constructing the sequencing library. Cluster generation and de novo sequencing were performed using the Hiseq 2500 System. The mRNA expression abundances of H78, H78hfq, and H78crc were calculated using the fragments per kilobase of mRNA per million reads (FPKM) method. Differentially expressed genes between the H78 group and the H78 mutant groups were screened by NOISeq with the standard deviation probability $\geq 0.8$ (or $p < .05$), fold change ≥2.

### 4.5 | Quantification of Plt and DAPG production

Overnight cultures of *P. protegens* H78 and its derivative strains were incubated in KMB at 28 °C and 200 rpm. The cultures were sampled at different time points to quantify the Plt and DAPG. When measuring the yield of DAPG, the culture was acidified with trifluoroacetic acid (9:1 vol/vol). Plt and DAPG were extracted using ethyl acetate (1:1 vol/vol). Production of Plt and DAPG was quantified by high-performance liquid chromatography according to a previous method (Huang et al., 2004; Zhang et al., 2014), respectively.
4.6 | Construction of lacZ reporter vectors and quantification of β-galactosidase expression

To investigate the influence of Hfq and Crc on the relevant target operons, the in-frame translational fusion reporter plasmids of 15 genes ( pltL, prmA, hcnA, phiA, flgF, flgB, tssA, tssE, fha, pedE, pedF, hqf, crc, yilM, and pcaK) and the lacZ gene were constructed by cloning the promoter/operator and first several codons of these genes upstream of the lacZ gene of the EcoRI/PstI-digested plasmid pME6015, respectively (Table S9). In the pME6015 plasmid, the lacZ gene lacks its own promoter, operator, and first seven codons. Two transcriptional fusion reporter plasmids carrying crcYp-lacZ and crcZp-lacZ were constructed by cloning the promoter regions of crcY and crcZ upstream of the promoterless lacZ gene of the EcoRI/PstI-digested plasmid pME6522, respectively.

P. protegens H78 and its derivative strains carrying different fusion plasmids were inoculated from the pre-overnight culture into 100 ml KMB in 500-ml Erlenmeyer flasks with a final OD 600 of 0.05 and then cultured at 28 °C with shaking at 200 rpm. Samples were harvested at different time points, and the β-galactosidase activity was assayed according to the method of Miller (Sambrook & Russell, 2001).

4.7 | Assessment of carbon source utilization of P. protegens H78

The ability of H78 and its derivative strains to utilize different carbon sources, including glycerol, mannitol, maltose, xylose, glucose, inositol, proteacetate, and succinate, as the sole carbon source was assessed in minimal media. The LB agar plate was set as the control. The minimal medium consisted of the following chemicals: NaCl (0.58 g/L), (NH₄)₂SO₄ (5.95 g/L), KH₂PO₄ (2.99 g/L), Na₄HPO₄·12H₂O (17.17 g/L), MgSO₄·7H₂O (0.246 g/L), vitamin B₁ (0.01 mg/L), FeCl₂·6H₂O (16.7 mg/L), ZnCl₂ (1.7 mg/L), CuCl₂·2H₂O (0.43 mg/L), CaCl₂ (4.3 mg/L), CoCl₂·6H₂O (0.6 mg/L), and H₂MoNa₂O₆·6H₂O (0.6 mg/L). The different carbon sources (4 g/L) were used as the sole carbon source in minimal media. The media were solidified with 1% (wt/vol) agarose.

Overnight culture was washed twice with minimal medium. The collected cells were first diluted to the same optical density (OD 600 = 0.05) and then successively diluted 10 times. A 2 μl diluted sample was inoculated on the minimal medium plate and incubated at 28 °C.

4.8 | Quantitative PCR

Bacterial samples for quantitative reverse transcription PCR (RT-qPCR) were cultured according to the bacterial cultivation procedure for RNA-Seq analysis. The total RNAs were extracted from the cells of H78 and its hqf or crc mutant using the phenol-chloroform extraction method and reverse transcribed to cDNA using a PrimeScript RT Master Mix Reagent Kit with gDNA Eraser. The resulting cDNA was amplified and quantified according to the previous method (Gottesman, 1996). The 16S rRNA gene was used as a reference. The calculated threshold cycle (Ct) of each gene was normalized to the Ct value of the 16S rRNA gene. The differential expression of genes between H78 and H78hfq or H78crc was compared by the 2⁻ΔΔCt method (Livak & Schmittgen, 2001).

4.9 | Detection of antimicrobial activity

Overnight cultures of P. protegens H78 and its derivative strains were adjusted to an OD 600 of approximately 5.0 for assaying their antimicrobial activity against B. subtilis, and then 8 μl samples were spotted onto an LB plate that had been already covered with B. subtilis. Similarly, for the antimicrobial activity against G. saubinetii, overnight cultures of P. protegens H78 and its derivatives were diluted to an OD 600 of 5.0 for the assay with G. saubinetii. First G. saubinetii was transferred to the centre of the YPG (containing 5 g/L yeast extract, 10 g/L peptone, and 40 g/L glucose) plate and 10 μl of P. protegens culture was streaked on the edge of plate. The plate was incubated at 28 °C until pathogenic fungi reached the edge of the plate.

4.10 | Accession number of nucleic acid sequence and RNA-Seq data

DNA sequences involved in this study were extracted from the P. protegens H78 complete genome (GenBank accession no. CP013184). The RNA-Seq data of H78 versus H78hfq and H78 versus H78crc have been deposited in the Gene Expression Omnibus database (accession nos. GSE141556 and GSE141557, respectively), in NCBI.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The RNA-Seq data of H78 versus H78hfq and H78 versus H78crc have been deposited in the Gene Expression Omnibus database at https://www.ncbi.nlm.nih.gov/geo/ with accession numbers GSE141556 and GSE141557, respectively. Other data that support the findings of this study are available from the corresponding author upon reasonable request.

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