The RNA Chaperone Hfq Regulates Antibiotic Biosynthesis in the Rhizobacterium *Pseudomonas aeruginosa* M18

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The rhizosphere microbe *Pseudomonas aeruginosa* M18 shows strong antifungal activities, mainly due to the biosynthesis of antibiotics like pyoluteorin (Plt) and phenazine-1-carboxylic acid (PCA). The ubiquitous RNA chaperone Hfq regulates bacterial virulence and stress tolerance through global posttranscriptional regulation. Here, we explored the molecular mechanism by which Hfq controls antibiotic biosynthesis in *P. aeruginosa* M18. The robust downregulation of Plt biosynthesis by Hfq was mediated exclusively by the posttranscriptional downregulation of the *plt* transcriptional activator PltR. Hfq posttranscriptionally repressed *phzM* expression and consequently reduced the conversion of PCA to pyocyanin. However, Hfq positively controlled the *phz2* operon and PCA biosynthesis through both QscR-mediated transcriptional regulation at the promoter and an unknown regulation at the operator. Also, Hfq was shown to directly bind at the mRNA 5' untranslated leaders of *pltR*, *qscR*, and *phzM*. These three negatively regulated target genes of Hfq shared a similar secondary structure with a short single-stranded AU-rich spacer (a potential Hfq-binding motif) linking two stem-loops. Taken together, these results indicate that Hfq, potentially in collaboration with unknown small noncoding RNAs (sRNAs), tightly controls antibiotic biosynthesis through both direct post-transcriptional inhibition and indirect transcriptional regulation.

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, and polar-flagellum bacterium that inhabits diverse environments in soil, water, animals, and plants (38). This remarkable ecological diversity has greatly enriched the number of active *Pseudomonas* secondary metabolites, including antibiotics, virulence factors, and siderophores, which contribute to symbiosis, competition, virulence, or biocontrol (2, 14, 32). For any given *Pseudomonas* strain, the secondary metabolite profile depends both on the environmental fluctuations encountered during evolution and on its genome features and corresponding regulatory mechanisms. Like other bacteria, *Pseudomonas* spp., including *P. aeruginosa*, have also evolved multiple molecular mechanisms that regulate products of secondary metabolism in response to diverse environmental pressures and ecological competition (15, 24, 41, 49).

The *P. aeruginosa* strain M18 isolated from the rhizosphere was reported to simultaneously produce phenazine-1-carboxylic acid (PCA) and pyoluteorin (Plt), which can efficiently inhibit soil-borne phytopathogenic fungi (22, 53). Our previous comparative studies have shown that the predominant phenazine produced by M18 is PCA, while the clinically isolated *P. aeruginosa* strain PAO1 predominately accumulates pyocyanin (PYO), a derivative of PCA (19). PYO functions as an important virulence factor (25). There is also evidence that PYO is not required for the fungicidal action of *P. aeruginosa* (12). PCA has been shown to suppress root disease in *Pseudomonas* strains producing only PCA but not PYO, such as *Pseudomonas fluorescens* 2-79 (17) and *Pseudomonas chlororaphis* 30-84 (31). The differential production of these two phenazines by strains PAO1 and M18 is mainly due to temperature-dependent expression of *phzM*, a gene encoding an adenosylmethionine-dependent methyltransferase (19). The *phzM* gene, together with the flavin-dependent hydroxylase-encoding gene *phzS*, is necessary for conversion of PCA to PYO (32).

In *P. aeruginosa*, two copies of the *phz* gene cluster (*phzABCDEFGHI*) and a number of modifying genes (such as *phzM* and *phzS*) are responsible for the biosynthesis of PCA and its derivatives (29, 32). The other antibiotic produced by the strain M18 is Plt, which

is biosynthesized via a hybrid polyketide synthase-nonribosomal peptide synthetase pathway. The *plt* gene cluster is composed of the structural operon *pltLABCDEFG* and the *pltM* gene, the ATP-binding cassette (ABC) transport operon *pltHIJKNO*, and two regulatory genes, *pltR* and *pltZ*, divergently transcribed from these two operons in both *P. fluorescens* Pf-5 and *P. aeruginosa* M18 (5, 20–22, 35). Our previous studies have shown that Plt biosynthesis is strongly downregulated by three typical quorum-sensing (QS) systems (Las, Rhl, and PQS) in strain M18 (8, 30, 54). The activation of Plt biosynthesis and expression of the *plt* gene cluster are dependent on both the two-component signal transduction system GacS/GacA (11) and the Plt biosynthetic pathway-specific transcriptional activator PltR, which in turn must be translationally activated by GacA (21). The biosynthesis of PCA drastically increased in the Gac- or Las-inactivated mutant (8, 11), while it was almost entirely inhibited by inactivation of the PQS or Rhl system in strain M18 (30, 54). In addition, the PCA biosynthesis was negatively regulated by the LuxR-type regulatory protein QscR in *P. aeruginosa* M18 (50) and in other *P. aeruginosa* strains (9, 27). These data lead us to speculate that a unique and complex regulation network is involved in the differential control of secondary metabolites, including Plt and PCA biosynthesis, in *P. aeruginosa* M18, but this remains to be determined.

In addition to two-component signal transduction and cell density-dependent quorum sensing, the small noncoding RNA

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(sRNA) is another important class of regulator utilized by bacteria to coordinate expression of secondary metabolite genes in response to environmental and metabolic stresses (41, 51). The Hfq protein is a conserved RNA chaperone protein first characterized as a host factor (HF-1) for phage Q β RNA replication and subsequently shown to be widely distributed in the bacterial kingdom with multiple homologues in the annotated genomic database (3). As a bacterial homologue of the eukaryotic and archaeal Sm/Lsm proteins, Hfq is known largely for its global posttranscriptional regulation by binding AU-rich sequences of target mRNA and facilitating pairing between sRNAs and mRNAs (3). Most Hfq homologues are known to function as homohexamers with two independent RNA-binding motifs (3). The *hfq* mutants display pleiotropic phenotypes in *Escherichia coli*, *Salmonella enterica*, *Sinorhizobium meliloti*, and *Staphylococcus aureus*, including decreased growth rate, increased sensitivity to various environmental stressors, and attenuated virulence (7, 13).

The *P. aeruginosa* Hfq homologue (82 residues), which shares 92% identity with the *E. coli* Hfq (102 residues) in the N terminus, can functionally substitute for the *E. coli* Hfq to translationally activate *rpoS* mRNA expression in *E. coli* (43). The *P. aeruginosa* *hfq* mutant alters the production of elastase, catalase, and pyocyanin and even causes significant attenuation of virulence (42). Hfq also moderately stimulated translation of the QS response regulators RhlR and QscR during the bacterial stationary phase (44). In addition, Hfq has been confirmed to bind to and stabilize the sRNA RsmY, which in turn binds to the translational repressor RsmA (44). However, the molecular mechanisms of Hfq regulation of secondary metabolism, including antibiotic biosynthesis, remain to be determined.

This study was motivated by the observation that cell cultures from the *hfq* mutant exhibited a significantly deepened blue color in comparison to the parent strain M18. It has been reported that blue-green pigment produced by *P. aeruginosa* is mainly composed of PYO (blue) and pyoverdine (green) (33). The possibility of elucidating the unique gene regulatory network controlling secondary metabolism in *P. aeruginosa* M18 prompted a further investigation of the Hfq-mediated global regulation on the biosynthesis of PYO (blue pigment) and other antibiotics, including PCA and Plt.

In this study, we show that the *hfq* mutant displayed significantly reduced PCA production and drastically increased PYO and Plt production. The Hfq protein strongly inhibited *phzM* expression at the posttranscriptional level, resulting in a significant decrease in the conversion of PCA to PYO. The strong downregulation of Plt biosynthesis by Hfq was exclusively mediated by the *plt* transcriptional activator PltR. However, *phz2* operon expression and PCA biosynthesis were strongly upregulated by Hfq, with two target sequences located at the promoter region (for receiving the QscR-mediated regulation) and the operator region. Moreover, Hfq was also shown to directly bind to the *phzM*, *qscR*, and *pltR* mRNA leaders. A new model is proposed to summarize Hfq-dependent pleiotropic regulation of antibiotic biosynthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and growth conditions. Bacterial strains, plasmids, and primers used in this study are listed in Tables S1 and S2 in the supplemental material. *E. coli* was routinely grown in LB medium at 37°C (37). *P. aeruginosa* and its derivatives were grown at 28°C in King's medium B (KMB) (23) or pigment-producing medium (PPM) (28).

When required, 20 $\mu\text{g ml}^{-1}$ X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) was used for blue/white colony screening, 5% sucrose was used for counterselecting the suicide plasmid pEX18Tc, 4 mg ml^{-1} ONPG (orthonitrophenyl- β -D-galactopyranoside) in 100 mM phosphate buffer (pH 7.0) was used for β -galactosidase assays, and 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside) was used for blue/white colony screening and promoter induction. For *P. aeruginosa* and its derivative strains, antibiotics were added at the indicated final concentrations ($\mu\text{g ml}^{-1}$): gentamicin (Gm), 45; kanamycin (Km), 50; spectinomycin (Sp), 100; tetracycline (Tc), 120; and chloramphenicol (Cm), 200. For *E. coli*, 50 $\mu\text{g ml}^{-1}$ Km, 10 $\mu\text{g ml}^{-1}$ Gm, 15 $\mu\text{g ml}^{-1}$ Tc, and 40 $\mu\text{g ml}^{-1}$ Cm were used.

DNA manipulations. All molecular biological methods not described in detail were performed using standard methods (37). Taq, LA Taq (TaKaRa), KOD Plus DNA polymerase (Toyobo), RNA reverse transcriptase, restriction endonucleases, DNA ligase, DNA molecular mass markers (MBI Fermentas), and other associated products were used as recommended by the manufacturers. Genomic DNA was extracted with the EZ-10 spin column genomic DNA isolation kit supplied by Bio Basic Inc. Plasmid DNA was purified using the TaKaRa miniBEST plasmid purification kit version 2.0 and the BioDev plasmid miniprep purification system B. DNA was recovered from the gel with the QIAquick gel extraction kit (Qiagen) and the AxyPrep DNA gel extraction kit (Axygen). DNA was synthesized and sequenced by Invitrogen Biotechnology Corporation and the Beijing HuaDa Genomics Institute.

Bioinformatics analysis of DNA and RNA sequence. A database search for similar nucleotide sequences was carried out with NCBI BLAST. Putative promoters were predicted with NNPP (Promoter Prediction by Neural Network) (36). MEME (<http://meme.nbcr.net/>) was utilized to search consensus sequence motifs (1). The maximum number of motifs to find is 3. The default distribution of motif occurrences is assumed to be zero or one occurrence per sequence. The optimum width of each motif ranged from 2 to 15 nucleotides (nt). RNA secondary structures were predicted at 28°C with Mfold 3.2. In cases where multiple structures were predicted, the most stable structure was chosen (57). IntaRNA (v1.2.5) (28°C, 10-continuous-base pairing, and other defaulting parameters) (39) was used to search for potential sRNAs interacting with the *phzM*, *qscR*, and *pltR* mRNAs.

Inactivation and complementation of the *hfq* gene in *P. aeruginosa* M18. The *P. aeruginosa* M18 *hfq* gene was deleted and replaced with a gentamicin resistance cassette by *in vitro* mutation (see Fig. 1A) and *in vivo* homologous recombination. Two fragments (479 bp and 468 bp) flanking the *hfq* open reading frame (ORF) were amplified with the KOD Plus DNA polymerase and two pairs of primers, Ph1 and Ph2 or Ph3 and Ph4 (see Table S2 in the supplemental material). Both the 3' end of the upstream fragment and the 5' end of the downstream fragment carried the same restriction site, PstI, for the insertion of the gentamicin resistance cassette. These two flanking PCR fragments were simultaneously cloned into the EcoRI-HindIII-digested pEX18Tc to generate the recombinant plasmid pEX-H1H2. The 825-bp gentamicin resistance cassette from pUCGm was then inserted into the PstI site of pEX-H1H2. The resulting plasmid, pEX-H1H2-Gm, was transformed from *E. coli* SM10 into *P. aeruginosa* M18 by biparental mating. Transconjugants were selected on LB plates containing Sp (to counterselect *E. coli* SM10), Gm, and 15% sucrose. After a second crossing-over, the Gm-resistant, Tc-sensitive, and sucrose-resistant recombinants with a deletion in *hfq* were obtained. The *hfq* mutant, designated M18hfq, was confirmed by PCR and sequencing with primers Ph1 and Ph4.

To complement the *hfq* mutant M18hfq, the recombinant expression plasmid pBBR-hfq was constructed as follows. The 636-bp fragment, which carries the entire 249-bp *hfq* coding region and its own promoter, was amplified from the M18 genomic DNA with the KOD Plus DNA polymerase and the primers PhfqB1 and PhfqB2 and then cloned into the *Pseudomonas-E. coli* shuttle vector pBBR1MCS at XhoI and HindIII sites.

Quantification of antibiotic production. Cells from fresh overnight cultures of *P. aeruginosa* M18 and its derivative strains were inoculated into 100 ml KMB in a 500-ml Erlenmeyer flask at a final concentration showing an optical density at 600 nm (OD_{600}) of 0.05 and then cultured at 28°C with shaking at 200 rpm. PCA and Plt were extracted with chloroform (pH 4.0) and ethyl acetate, respectively, and quantified with high-performance liquid chromatography (HPLC) as previously described (22). Quantification of PYO production was carried out at pH 7.0 in chloroform as previously described (10).

In this study, each experiment was independently repeated at least three times, using triplicate parallel samples within each experiment. Unless stated otherwise, each value represents the mean and standard deviation (SD) of three replicates. Student's *t* test in Microsoft Excel was used to evaluate the significance of difference.

Construction of *lacZ* reporter gene fusions. To investigate the regulatory functions of Hfq on relevant target operons or genes, including *phz1*, *phz2*, *phzM*, *plt*, *qscR*, and *pltR*, the *lacZ* reporter gene was fused to the promoters and/or operator regions of these genes, creating the *lacZ* translational (indicating the total regulatory level), transcriptional, and 5' untranslated region (UTR)-*lacZ* fusion vectors (see Table S1 in the supplemental material). Generally, the first several codons, operator, and promoter region of these genes were used for constructing the translational *lacZ* fusions in the vector pME6015 in which the promoter and first eight codons of the *E. coli lacZ* gene had been deleted. The transcriptional *lacZ* fusions were constructed by cloning the promoter region (upstream of the transcriptional start site [TSS]) into the plasmid pME6522 carrying the promoterless *lacZ* gene and its own ribosome binding site (RBS). Likewise, the 5' UTR-*lacZ* fusions were constructed by introducing the entire or partial operator region (+1 to ATG) into the plasmid pME9533, which harbors the *tac* promoter and a replaceable operator region upstream of *lacZ*.

For constructing the *phzM* and *phz2* fusions with *lacZ* (see Table S1 in the supplemental material), a series of gradually shortened fragments upstream of *phzM* (see Fig. 2A) and *phz2* (see Fig. 4A) were cloned into the EcoRI/PstI-digested plasmids pME6015 and pME6522 and the KpnI/PstI-digested plasmid pME9533 to generate two arrays of *phzM-lacZ* and *phz2-lacZ* translational, transcriptional, and 5' UTR-*lacZ* fusions. The *pltL-lacZ* transcriptional fusion plasmid (p6522-*pltLp*) containing the *pltL* promoter region (−85 to +1) was constructed based on the EcoRI/PstI-digested plasmid pME6522. Similarly, the PCR fragment covering the 46-bp *pltL* operator (+1 to ATG) and the first nine codons was cloned into the KpnI/PstI-digested pME9533, producing the *pltLo* (5' UTR)-*lacZ* fusion plasmid p9533-*pltL*. By cloning the PCR fragment containing the 63-bp operator region (+1 to ATG) and the first nine codons of *pltR* into the KpnI/PstI-digested plasmid pME9533, we constructed the *pltRo-lacZ* fusion plasmid p9533-*pltRo*. The *qscRo-lacZ* fusion (p9533-*qscRo*) was constructed by cloning the PCR fragment covering 545 bp upstream of the translational start site and the first 9 codons of *qscR* into pME9533. The *qscR'-lacZ* translational fusion plasmid (p6015-*qscR*) harboring the *qscR* operator and promoter region (−217 to +572 relative to the putative +1 position) was constructed based on the EcoRI/PstI-digested plasmid pME6015. The *qscRp-lacZ* transcriptional fusion plasmid (p6522-*qscRp*) containing the *qscR* promoter region (−126 to +1) was constructed based on the EcoRI/PstI-digested plasmid pME6522.

Site-directed mutagenesis of the AT-rich motif of the *phzM* and *pltR* leader regions. The *lacZ* reporter plasmids, in which the AT-rich motif (encoding a potential AU-rich Hfq-binding motif) of the *phzM* leader region was changed by site-directed mutagenesis, were constructed to assess the importance of this AT-rich motif for the Hfq regulation on *phzM* expression. The *phzM* leader mutant fragment (+130 to +297) containing a 4-nucleotide (nt) substitution (A142G, T144C, T146C, and T148C) in the AT-rich motif (see Fig. 2B) was PCR amplified with the mutant forward primer PphzM-M1-1 and the reverse primer PphzM-M1-2 (see Table S2 in the supplemental material) and subsequently cloned into the KpnI/PstI-digested pME9533 vector. The resulting plas-

mid pPMO-2-M1 was verified by sequencing, and the 4-nt mutant *phzM* leader fragment was shown to be located downstream of the *tac* promoter carried by the plasmid itself. In the same way, the *phzM* leader mutant fragment (+130 to +297) carrying a 2-nt substitution (T143C and T148C) (see Fig. 2B) was recombined into the KpnI/PstI-digested pME9533, yielding the plasmid pPMO-2-M2. The plasmid p9533-*pltRo*-M1 was constructed by cloning the 63-bp mutant operator region (+1 to ATG) of *pltR*, which carries a 5-nt replacement (A29G, U31C, U33C, U35C, and A37G) in the AT-rich spacer (see Fig. 8A), into the KpnI/PstI-digested plasmid pME9533.

β-Galactosidase assays. *P. aeruginosa* M18 and its derivative strains carrying the *lacZ* reporter plasmid (see Table S1 in the supplemental material) were inoculated from the overnight culture into 500-ml Erlenmeyer flasks containing 100 ml KMB to a final concentration with an OD_{600} of 0.05 and then cultured at 28°C with shaking at 200 rpm. Cells were harvested at different time points for assaying the β-galactosidase specific activities, according to the method of Miller (34) and as described by Huang et al. (22).

Expression and purification of the *P. aeruginosa* M18 Hfq protein. A 269-bp DNA fragment containing the 249-bp entire *hfq* ORF was PCR amplified with the proofreading enzyme KOD and then cloned into the NdeI-HindIII sites of plasmid pET28a. The resulting *hfq* recombinant expression plasmid pET28a-*hfq* (see Table S1 in the supplemental material) was confirmed by sequencing and transformed into *E. coli* BL21 (DE3) cells. Cell cultures were incubated at 37°C in LB medium to an OD_{600} of 0.8, induced with 0.5 mM IPTG, and subsequently grown at 16°C for 20 h. The cells were then harvested by centrifugation and resuspended in 30 ml nickel A buffer (20 mM imidazole, 300 mM NaCl, 25 mM Tris-HCl, pH 8.0), supplemented with 1 μg ml^{−1} leupeptin, 1 μg ml^{−1} aprotinin, and 50 μM phenylmethylsulfonyl fluoride (PMSF). Cell resuspension solution was slowly shaken at 4°C for 30 min, and cells were lysed using an ultrasonic cell disruptor. Subsequently, the cell lysate was loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) agarose column (GE Healthcare). The column was washed two times with 5 ml nickel buffer, and then Hfq protein was eluted with the elution buffer containing 500 mM imidazole. After the removal of imidazole by HPLC (Akta), the purified Hfq protein was stored in a buffer containing 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM dithiothreitol (DTT), and 1 mM EDTA.

In vitro synthesis of the unlabeled and biotin-labeled RNAs. The RNAs for electrophoretic mobility shift assays (EMSAs) were synthesized *in vitro* on Applied Biosystems 394 automated DNA/RNA synthesizers using cyanoethyl-diisopropylamino phosphoramidite chemistry and/or labeled at the 3' end with biotin by TaKaRa Biotechnology Co., Ltd. (Dalian, China). The RNA products were purified by anion-exchange HPLC. Seven RNA fragments are the *phzM* (nt +131 to +190 relative to +1), *phzM*-M1 (carrying a 4-nt substitution), *phz2*-1 (nt +50 to +89 relative to +1), *phz2*-2 (nt +92 to +131 relative to +1), *qscR* (−48 to −97 relative to ATG), *pltR* (nt +9 to +62 relative to +1), and *pltR*-M1 (carrying a 5-nt substitution) mRNA 5' UTRs.

EMSA of RNA-protein interaction. Electrophoretic mobility shift assay (EMSA) analysis was carried out with the Thermo Scientific LightShift Chemiluminescent RNA EMSA kit. The biotin-labeled RNA probes (1 nM) were incubated with increasing amounts of the purified Hfq protein in 20 μl binding buffer (100 mM HEPES at pH 7.3, 200 mM KCl, 10 mM MgCl₂, 10 mM DTT). For competition assays, a 1,000- or 1,500-fold molar excess of unlabeled RNAs was added into the binding reaction mixture as a competitor. After the addition of probe, EMSA reaction mixtures were incubated at room temperature for 30 min. After incubation, 10-μl samples were mixed with 2.5 μl of 5× loading buffer (50% glycerol, 0.1% bromophenol blue), and then loaded on a native 6% polyacrylamide gel. Electrophoresis was performed in 0.5× Tris-borate-EDTA (TBE) buffer at 100 V for 40 min. The separated protein-RNA conjugates were electrophoretically transferred from the gel to a positively charged nylon membrane (Ambion) by semidry transfer apparatus (Tanon). The transferred Hfq-RNA complexes and free RNAs were cross-linked to the membrane by a 320-nm UV-light cross-linking instrument. Finally, the biotin-la-

beled bands were detected by the Thermo Scientific chemiluminescent nucleic acid detection module. The stabilized streptavidin-horseradish peroxidase (HRP) conjugate was utilized to probe for the biotin-labeled RNAs transferred to nylon membrane. Then, an enhanced luminol-based substrate was catalyzed by HRP with optimized blocking and a wash step that together produce high sensitivity to light. Subsequently, the membrane with light was placed on a film cassette and exposed to X-ray film for 20 to 30 s.

Nucleotide sequence accession numbers. The complete genome of *P. aeruginosa* M18 was deposited in GenBank (GenBank accession no. CP002496). The locus tags of *hfq*, *phzM*, *qscR*, *phz2*, *phz1*, and *pltR* have the accession numbers PAM18_5055, PAM18_0728, PAM18_3144, PAM18_3143 to PAM18_3137, PAM18_0727 to PAM18_0721, and PAM18_2384, respectively.

RESULTS

Identification and inactivation of the *hfq* gene in *P. aeruginosa* M18. PCR amplification and sequencing analysis indicated that the *hfq* gene of *P. aeruginosa* M18 shares 99% sequence identity with *P. aeruginosa* PAO1 and 57% sequence identity with *E. coli*. The 249-bp M18 *hfq* gene encodes a 9.103-kDa protein. The *hfq* gene has two flanking genes, the *miaA* gene encoding a tRNA $\delta(2)$ -isopentenylpyrophosphate transferase and the PAM18_5054 ORF encoding a putative GTP-binding protein, both organized in the same direction in all *P. aeruginosa* strains.

To investigate the regulatory activities of Hfq on antibiotic biosynthesis in *P. aeruginosa* M18, we replaced the *hfq* gene with the gentamicin resistance cassette (Fig. 1A) through homologous recombination. The resultant *hfq* deletion mutant was validated by PCR and sequencing. Moreover, the *hfq* deletion was confirmed by semiquantitative reverse transcription-PCR (RT-PCR) to have no polar effect on the expression of flanking genes *miaA* and PAM18_5054 (Fig. 1B).

Growth of *P. aeruginosa* M18 impaired by the *hfq* deletion. The *hfq* deletion gave rise to a serious impairment in *P. aeruginosa* M18 growth in PPM as evidenced by a significant decline of OD₆₀₀ from 4.0 in wild-type M18 cultures to 1.0 in M18hfq (Fig. 1D). The PPM, in which glucose is utilized as a major carbon source, is generally used for PCA production (23). This impaired growth of the *hfq* mutant in PPM was restored to the wild-type growth level by ectopic expression of exogenous *hfq* via transformation of the plasmid pBBR-hfq (Fig. 1D), which was constructed by cloning the entire *hfq* ORF and its own promoter region into the *Pseudomonas-E. coli* shuttle vector pBBR1MCS. Due to the poor growth of the *hfq* mutant in PPM, *P. aeruginosa* M18 and its derivative strains were generally grown in KMB, in which the main carbon source is glycerol. When grown on KMB agar plates, the *hfq* mutant took twice as long to form colonies as did the wild-type strain M18. In KMB liquid medium, the *hfq* mutant exhibited an obvious decrease in cell growth compared to the parent strain M18 (Fig. 1E to G). Likewise, the decreased growth of the *hfq* mutant was restored to the wild-type level in KMB by transformation of the plasmid pBBR-hfq (see Fig. S1D in the supplemental material).

Strong and differential regulation of Hfq on antibiotic biosynthesis. *P. aeruginosa* M18, M18hfq, and their derivative strains were inoculated on KMB plates and incubated at 28°C for 60 h, and changes in pigment production were observed. As shown in Fig. 1C, the loss of *hfq* function gave rise to a deeper blue pigment, implying that the *hfq* mutant likely led to the overproduction of the blue pigment PYO. Furthermore, the introduction of an exogenous

hfq gene derived from the plasmid pBBR-hfq almost completely reversed this color change in the *hfq* mutant cultures relative to the wild-type strain (Fig. 1C). The same change in pigment production resulting from the *hfq* deletion was also observed in KMB liquid medium (data not shown).

To further determine whether the deeper blue color of the *hfq* mutant was caused by the overproduction of the blue pigment PYO, we quantitatively analyzed the production of PYO, PCA, and Plt in KMB. As expected, the *hfq* deletion mutant M18hfq displayed a significant enhancement of PYO production compared to the M18 parent strain. The PYO production by M18hfq strain reached a peak of 18.7 $\mu\text{g ml}^{-1}$ after 60 h of growth, while the wild-type M18 strain accumulated only 1.5 $\mu\text{g ml}^{-1}$ PYO within the same culture period (Fig. 1E). At the same time, we also observed that the accumulation of PCA was significantly reduced in the *hfq* deletion mutants (Fig. 1F). Based on these results, we speculated that the reduced PCA probably resulted from conversion of PCA to PYO in the *hfq* mutant. Of course, we cannot exclude the possibility that the biosynthesis of PCA itself was also reduced by the *hfq* deletion. The decreased amount of PCA (about 24 $\mu\text{g ml}^{-1}$ [Fig. 1F]) was still higher than the increased amount of PYO produced by strain M18hfq (about 17 $\mu\text{g ml}^{-1}$ [Fig. 1E]). In addition, the *hfq* deletion led to a nearly 7-fold increase in Plt production, and the peak of Plt production rose sharply from 33.8 $\mu\text{g ml}^{-1}$ in the wild-type M18 strain to 240.8 $\mu\text{g ml}^{-1}$ in the M18hfq strain (Fig. 1G). These data suggest that Hfq strongly downregulates the biosynthesis of PYO and Plt and significantly upregulates the biosynthesis of PCA in *P. aeruginosa* M18.

To further verify that the inhibition of PCA production and the overproduction of PYO and Plt are exclusively caused by the *hfq* deletion in the strain M18hfq, the *hfq* expression plasmid pBBR-hfq and the corresponding empty plasmid pBBR1MCS (as the control) were used to transform the M18 strain and the *hfq* deletion mutant M18hfq, and antibiotic production was quantified in KMB. Compared to the empty plasmid pBBR1MCS, *hfq* overexpression deriving from pBBR-hfq resulted in a significant increase in PCA production (see Fig. S1A in the supplemental material) but a significant reduction in Plt production (see Fig. S1B) in both the wild-type strain M18 and its *hfq* deletion mutant. PYO production of M18hfq was restored to the wild-type level by the exogenous *hfq* expression resulting from pBBR-hfq (see Fig. S1C). However, no obvious influence of *hfq* overexpression on PYO production was detected for M18/pBBR-hfq compared with M18/pBBR1MCS (see Fig. S1C), but this can be accounted for by the fact that the level of PYO produced by the M18 strain was originally very low. These results confirmed the regulation of antibiotic biosynthesis by the RNA chaperone Hfq in *P. aeruginosa* M18.

Hfq downregulated *phzM* expression at the posttranscriptional stage and consequently inhibited the conversion of PCA to PYO. Deletion of the global posttranscriptional regulator *hfq* substantially increased PYO production, suggesting that Hfq downregulates the PYO biosynthetic genes. We first examined the expression of *phzM*, a gene involved in the first step of conversion of PCA to PYO. To this end, a series of *phzM-lacZ* fusion plasmids (Fig. 2A; see also Table S1 in the supplemental material) were constructed and transformed into the wild-type M18 and *hfq* mutant M18hfq. The β -galactosidase (LacZ) activities of these transformed bacteria were then measured in KMB to compare *phzM* expression in the presence and absence of *hfq* (Fig. 2A). The LacZ activities expressed from the *phzM* (5' UTR)-*lacZ* fusion plas-

FIG 1 Regulation of cell growth and antibiotic biosynthesis by Hfq in *P. aeruginosa* M18. (A) The construction map of the *P. aeruginosa* M18 *hfq* mutant (M18hfq). The *hfq* gene in *P. aeruginosa* M18 was replaced with the gentamicin resistance cassette by homologous recombination. (B) No polar effect of the *hfq* deletion on the expression of its flanking genes, *miaA* and PAM18-5054, was detected by semiquantitative RT-PCR in *P. aeruginosa* M18. (C) The *hfq* deletion stimulated blue pigment production in *P. aeruginosa* M18. The tested strains were grown on KMB plates for 60 h. The six tested strains were *P. aeruginosa* M18, M18hfq, and their four derivative strains carrying the *hfq* expression plasmid pBBR-hfq or the empty plasmid pBBR1MC5 (abbreviated as pBBR) as the control. (D) Cell growth assay of *P. aeruginosa* M18, M18hfq, and their derivative strains carrying pBBR-hfq or the empty plasmid pBBR in PPM. The *hfq* deletion caused a significant impairment of M18 growth, which in turn was recovered to the wild-type level by the exogenously expressed Hfq from the plasmid pBBR-hfq. (E) PYO production (solid symbols) and cell growth (open symbols) of strain M18 and its *hfq* mutant M18hfq in KMB. PYO production was significantly enhanced and cell growth was significantly decreased by the *hfq* deletion in KMB. (F) PCA production (solid symbols) and cell growth (open symbols) of the strains M18 and M18hfq in KMB. PCA production by M18hfq was almost entirely reduced in KMB. (G) Plt production (solid symbols) and cell growth (open symbols) of the strains M18 and M18hfq in KMB. The *hfq* deletion resulted in a marked increase of Plt production in KMB.

mids pPMO-1 and pPMO-2 were markedly elevated in the *hfq* mutant M18hfq compared to the parental strain M18. Although LacZ expression was relatively low, the *hfq* deletion resulted in an obvious increase in *phzM'*-*lacZ* translational fusion expression (pPML). In contrast, the wild-type strain M18 and the *hfq* mutant

M18hfq did not show a significant difference in LacZ expressed from the *phzMp-lacZ* transcriptional fusion plasmid pPMC or from the *phzM0-lacZ* fusion plasmids, pPMO-3 and pPMO-4. Based on these results, the target regulatory element of Hfq was localized to the *phzM5'* UTR from bp +130 to +190 downstream

this AU-rich motif is required for Hfq binding and Hfq-mediated posttranscriptional regulation, two mutants of this AT-rich motif in the plasmid pPMO-2 were constructed by PCR. The mutants contained either 4-nt substitutions (A142G, T144C, T146C, and T148C) or 2-nt substitutions (T143C and T148C). The strains M18 and M18hfq transformed with pPMO-2 or its AT-rich motif mutants (pPMO-2-M1 and -M2) were assayed for LacZ activity in KMB (Fig. 2C). Indeed, negative regulation of Hfq on the *phzM*-2-*lacZ* fusion (pPMO-2) expression was reduced or even entirely eliminated by the two AU-rich motif mutants. This result further suggested that the AU-rich motif within the *phzM* mRNA 5' UTR plays a key role in posttranscriptional repression by Hfq. It is also noteworthy that the 4-nt substitutions (M1) in the AT-rich motif brought about a significant increase in LacZ expression (pPMO-2-M1) in the wild-type strain M18 (Fig. 2C). This further demonstrates that the AU-rich motif in the *phzM* 5' UTR is critical for the Hfq-mediated inhibition of *phzM* expression. In addition, a relatively minor decline in the *lacZ* fusion expression from pPMO-2-M2 was observed owing to the 2-bp substitution in the AT-rich

motif in the wild-type strain M18 by an unknown mechanism (Fig. 2C).

Hfq directly binds to the *phzM* mRNA 5' leader between nt +130 and +190. To explore whether Hfq directly binds to the *phzM* mRNA 5' UTR, we expressed and purified the Hfq protein (Fig. 3A) and carried out an EMSA to assess the binding activity of Hfq for the 60-nt biotin-labeled 5' UTR covering nt +130 to +190 downstream of the *phzM* TSS that also carries a potential Hfq-binding AU-rich motif. It needs to be mentioned that the presence of a His₆ tag in the recombinant 9.1-kDa Hfq protein caused a larger delay in gel mobility on SDS-PAGE (Fig. 3A). This phenomenon frequently occurs in other recombinant proteins with His₆ tags. As shown in Fig. 3B, when 1 nM biotin-labeled *phzM* mRNA leader was incubated with increasing amounts of Hfq (0 to 24 μ M) in a 20- μ l reaction system, four bands (C1 to C4) were clearly observed, corresponding to four Hfq-*phzM* complexes. As presented in lanes 2 to 9 of Fig. 3B, with increasing Hfq protein, the Hfq-*phzM* complex bands with lower mobility gradually increased in intensity, while the complex bands with higher mobility gradually decreased in intensity and eventually disappeared above 20 μ M Hfq. Concomitant with the increased Hfq binding, the biotin-labeled free *phzM* mRNA gradually decreased and finally was almost completely bound by 24 μ M Hfq protein. In addition, the *phzM* mRNA itself was also observed to form 2 to 3 oligomers, which were sequentially reduced in number and intensity as the Hfq concentration was increased, eventually disappearing completely above 20 μ M (Fig. 3B, lanes 1 to 7). A 1,500-fold molar excess of unlabeled *phzM* mRNA almost entirely displaced the biotin-labeled *phzM* mRNA from Hfq (Fig. 3B, lane 10), thus demonstrating the specificity of the Hfq-*phzM* mRNA interaction. Also, we tested whether a 4-nt replacement in the AU-rich spacer (Fig. 2B) affects the binding affinity between the *phzM* mRNA leader and the Hfq protein. As expected, the mutated *phzM* mRNA leader displayed significantly weakened binding affinity with Hfq compared with the wild-type *phzM* (Fig. 3C). A higher proportion of 1 nM mutated *phzM* remained unbound by even 24 μ M Hfq (Fig. 3C). In contrast, 1 nM wild-type *phzM* began to be strongly bound by only 0.8 μ M Hfq and finally was almost entirely bound by 24 μ M Hfq (Fig. 3B). In summary, the results depicted in Fig. 3B and 3C strongly suggest that the *P. aeruginosa* M18 Hfq protein can directly bind to the *phzM* mRNA 5' UTR between +130 and +190, a region that contains a typical Hfq-binding AU-rich motif.

Hfq contributed to the transcriptional and posttranscriptional upregulation of *phz2* expression. In *P. aeruginosa* M18, two sets of phenazine biosynthetic gene clusters, *phzA1* to *phzG1* and *phzA2* to *phzG2*, share over 98% identity in amino acid coding sequences but possess distinct upstream 5' noncoding sequences, including promoter and regulatory elements, implying distinct regulatory mechanisms. The preceding results (Fig. 1E and F) imply that the total decrease in PCA production of the *hfq* deletion mutant may be due mainly to the enhanced conversion of PCA to PYO and may also be owing partly to inhibition of the biosynthesis of PCA itself. The following experiments were designed to determine which of the two *phz* operons is under the control of Hfq and to further investigate the underlying regulatory mechanism of Hfq-mediated upregulation of PCA biosynthetic genes. The *phz1'*-*lacZ* and *phz2'*-*lacZ* translational fusion plasmids (pMP1L and pMP2L), which carry the *phz1* fragment from bp -340 to +361 and the *phz2* fragment from bp -344 to +227,

FIG 3 Hfq directly binds to the *phzM* mRNA 5' UTR. (A) Expression and purification of the Hfq protein. SDS-PAGE analysis of the overall protein profile (left) and the purified Hfq protein (right) from the cell culture of *E. coli* BL21 carrying the *hfq* overexpression plasmid pET28a-*hfq*. M, protein molecular mass marker; Control, without IPTG induction; Hfq (left), with IPTG induction. (B) Direct binding of Hfq to the *phzM* mRNA 5' UTR (+131 to +190) assessed by EMSA. Biotin-labeled *phzM* mRNA 5' UTR (1 nM) was incubated with increasing amounts (0 to 24 μ M) of Hfq protein as described in Materials and Methods. Hfq-*phzM* complexes are shown as C1 to C4. For competition reactions, 1.5 μ M unlabeled *phzM* was included in the binding reaction mixture in molar excess as indicated in lane 10. (C) Influence of a 4-nt replacement (Fig. 2B) within the AU-rich spacer on the binding affinity between the *phzM* mRNA leader (+131 to +190) and the Hfq protein. Biotin-labeled *phzM-M1* (1 nM) was incubated with increasing amounts (0 to 24 μ M) of Hfq protein as described in Materials and Methods. Hfq-*phzM-M1* complexes are designated C1 to C3.

respectively, were utilized to assess the regulation of these two gene clusters. These two *lacZ* gene reporter plasmids were introduced into strains M18 and M18_{hfq}, and the LacZ activities of the four strains were measured in KMB (Fig. 4A and B). The

FIG 4 Inhibition of *phz2* expression by the *hfq* deletion and identification of two target regulatory sequences in the *phz2* operon leader. (A) Construction map for a series of *phz2-lacZ* translational, transcriptional, and 5' UTR-*lacZ* fusion plasmids and β -galactosidase activity assay for the M18 or M18hfq strain harboring these *lacZ* fusion plasmids after 15 h of growth. The *hfq* deletion caused a significant decrease (indicated with asterisks) in the expression of the *phz2-lacZ* fusions, including pMP2L, p6522-*phz2po*-2, p6522-*phz2p*-5, and p9533-*phz2o*-6. Two target regulatory regions of Hfq on the *phz2* operon were located at bp -78 to $+1$ upstream and bp $+1$ to $+113$ downstream of the *phz2* TSS. (B) Assay for β -galactosidase activities expressed from the *phz1'-lacZ* translational fusion (pMP1L) in the strains M18 and M18hfq. The *hfq* mutant did not alter the *phz1'-lacZ* translational fusion expression originating from pMP1L.

phz2'-lacZ translational fusion expression from pMP2L was reduced by 96% in the *hfq* deletion mutant (29.7 Miller units of LacZ activity) compared to that in the M18 strain (691 Miller units) (Fig. 4A), while no significant difference in the total translational *phz1'-lacZ* fusion expression (pMP1L) was detected between the wild-type strain and M18hfq (Fig. 4B). From these results, it appears that Hfq may preferentially upregulate the expression of the *phz2* operon and the fraction of PCA produced through *phz2* operon activation, while much of the other fraction of PCA biosynthesized by the *phz1* operon is likely converted into PYO through the promoted *phzM* expression following Hfq deletion. In addition, quantitative RT-PCR (qRT-PCR) analysis was performed to quantify the accumulation of *phz2* mRNA in the early stationary phase of growth in M18 and M18hfq. The results, depicted in Fig. S2 in the supplemental material, suggest that the *hfq* deletion resulted in a remarkable decrease in *phz2* mRNA level in M18hfq compared to M18.

To further define the target regulatory elements of Hfq within the *phz2* operon 5' UTR, we constructed a set of *phz2-lacZ* fusion plasmids and compared LacZ expression between M18 and M18hfq in KMB (Fig. 4A). The *hfq* deletion caused a significant decrease in LacZ activity expressed from the *phz2po*-2-*lacZ* fusion plasmid p6522-*phz2po*-2, the transcriptional fusion plasmid p6522-*phz2p*-5, and the 5' UTR-*lacZ* fusion plasmid p9533-*phz2o*-6. In contrast, there was no obvious difference in LacZ activity between M18 and M18hfq strains transformed with the

transcriptional fusion plasmids p6522-*phz2p*-3 and p6522-*phz2p*-4 or the 5' UTR-*lacZ* fusion plasmid p9533-*phz2o*-7 (Fig. 4A). Based on these data, it can be concluded that there are two key regulatory fragments mediating positive regulation by Hfq located from bp -78 to $+1$ upstream and from bp $+1$ to $+113$ downstream of the *phz2* transcript TSS, suggesting that Hfq upregulates *phz2* expression at both the transcriptional and posttranscriptional levels.

The 5' UTR (nt $+1$ to $+113$) of *phz2* mRNA was not found to contain an AU-rich potential Hfq-binding motif in a single-stranded spacer region between two stem-loops (see Fig. S4A in the supplemental material). Two high-AU fragments (nt $+50$ to $+90$ and nt $+90$ to $+130$) did not show measurable binding with the Hfq protein (see Fig. S4B and S4C), suggesting that the *phz2* mRNA is probably not a direct target of Hfq regulation and that other transcriptional or posttranscriptional regulators are more likely to mediate the positive regulation by Hfq on the nt $+1$ -to $+113$ operator region of the *phz2* operon.

QscR mediated the transcriptional upregulation of Hfq on *phz2* expression. It has been widely reported that Hfq, an sRNA chaperone protein, regulates expression of target genes typically at the posttranscriptional level (3). The finding that the *phz2* expression was upregulated by Hfq at the transcriptional level suggests that transcriptional regulation by Hfq may be mediated indirectly through a transcriptional regulator controlling *phz2* expression. The LuxR family transcriptional regulator QscR has been reported to negatively control *phz* expression (9, 50) and *phz2* transcription

FIG 5 QscR mediated the upregulation of *phz2* transcription. (A to C) β -Galactosidase activities, which were expressed from the *qscR'*-*'lacZ* translational fusion (A) (p6015-qscR), *qscRp-lacZ* transcriptional fusion (B) (p6522-qscRp), and *qscRo-lacZ* fusion (C) (p9533-qscRo), were assayed in the strains M18 and M18hfq in KMB. The *hfq* mutation caused a significant increase in the expression of *qscR'*-*'lacZ* translational (A) and *qscRo-lacZ* (C) fusions. (D to F) β -Galactosidase activities, which were expressed from the *phz2'*-*'lacZ* translational fusion (D) (pMP2L), *phz2p-5-lacZ* transcriptional fusion (E) (p6522-phz2p-5), and *phz2o-6-lacZ* fusion (F) (p9533-phz2o-6), were assayed in the M18 strain and the *qscR* mutant M18qscR in KMB. Expression levels of both the *phz2'*-*'lacZ* translational fusion (D) and the *phz2p-5-lacZ* transcriptional fusion (E) were significantly increased in the *qscR* mutant M18qscR compared to the wild-type M18 strain. (G) β -Galactosidase activities expressed from the *phz1p-lacZ* transcriptional fusion (pMP1C) were assayed in the strains M18 and M18qscR in KMB. QscR did not exert a significant influence on the *phz1p-lacZ* transcriptional fusion expression.

(27) and so is one candidate mediating the positive regulation of *phz2* transcription by Hfq. To test this hypothesis, the *qscR-lacZ* translational, transcriptional, and 5' UTR-*lacZ* fusion expression deriving from the plasmids p6015-qscR, p6522-qscRp, and p9533-qscRo, respectively, was assessed in M18 and M18hfq strains growing in KMB (Fig. 5A to C). The *hfq* deletion brought about a remarkable enhancement in the expression of *qscR'*-*'lacZ* translational fusion (Fig. 5A) and *qscRo-lacZ* fusion (Fig. 5C) throughout the experimental period compared to the M18 strain. A slight increase was also observed in the *qscRp-lacZ* transcriptional fusion expression in the *hfq* mutant strain compared to the wild-type strain (Fig. 5B), implying that Hfq may indirectly exert a low level of transcriptional downregulation on the *qscR* gene expression. These results suggest that *qscR* expression was downregulated by Hfq mainly at the posttranscriptional level. The qRT-PCR results also indicated a substantial increase in the accumulation of *qscR* mRNA at the early stationary phase in the *hfq* mutant compared to the parent strain (see Fig. S2 in the supplemental material).

The *phz2p-5-lacZ* transcriptional fusion expression from the plasmid p6522-phz2p-5, which contains the promoter region

(-78 to +1) of the *phz2* operon (Fig. 4A), was assayed in strain M18 and the *qscR* mutant M18qscR to confirm the negative regulation of *phz2* transcription by QscR (Fig. 5E). The *phz2p-5-lacZ* transcriptional fusion expression was significantly enhanced, although not greatly increased, in the *qscR* mutant compared to the wild-type strain (Fig. 5E). Similarly, the *phz2'*-*'lacZ* translational fusion (pMP2L) expression, which may reflect the total regulatory level of the *phz2* operon, was also significantly enhanced by the *qscR* mutation (Fig. 5D). Not unexpectedly, no significant difference in the *phz2o-6-lacZ* fusion expression from the plasmid p9533-phz2o-6 was observed between the M18 strain and the *qscR* mutant (Fig. 5F). In addition, the *qscR* mutation did not bring about obvious influence on the expression of *phz1p-lacZ* transcriptional fusion (pMP1C) (Fig. 5G). These data strongly suggest that QscR downregulated the transcription of its neighboring *phz2* operon in M18. Furthermore, an alignment analysis revealed a *lux* box, a characteristic motif of the LuxR regulon, located from bp -56 to -37 upstream of the TSS of the *phz2* operon (Fig. 6A). Taking these data together, we conclude that the transcriptional upregulation of *phz2* expression by Hfq was mediated mainly by the transcriptional regulator QscR.

FIG 6 The direct binding of Hfq to the *qscR* mRNA 5' UTR. (A) DNA sequence analysis suggested that a conserved *lux* box (indicated by boxed nucleotides), a potential target motif of LuxR-type regulators, including QscR, is located immediately upstream of the –35 element of the *phz2* promoter. The +1 indicates the transcriptional start site for the *phz2* operon. (B) The mRNA leader covering 97 nt upstream of the translational start site of *qscR* was predicted by Mfold to display an RNA secondary structure containing an AU-rich single-stranded spacer (shown with gray-shaded nucleotides) linking two stem-loops. (C) Direct binding of Hfq to the *qscR* mRNA 5' UTR (–48 to –97 relative to the *qscR* AUG) containing the AU-rich region assessed by EMSA. Biotin-labeled *qscR* RNA (1 nM) was incubated with increasing amounts (0 to 32 μM) of Hfq protein as described in Materials and Methods. The retarded bands represent the Hfq-*qscR* complexes. For competition reactions, 1 μM unlabeled *qscR* was included in the binding reaction mixture in molar excess as indicated in lane 12.

The secondary structure prediction of the *qscR* mRNA leader showed an AU-rich single-stranded sequence linking two stem-loops (Fig. 6B), which is similar to the two RNA secondary structures in the mRNA leaders of *phzM* (Fig. 2B) and *pltR* (see Fig. 8A), two other negatively regulated targets of Hfq. An EMSA was carried out to assess the binding affinity of Hfq for the *qscR* mRNA 5' UTR, which ranges from nt –48 to –97 upstream of the translation start site AUG of *qscR* and contains the AU-rich region (Fig. 6B and C). The Hfq protein showed strong binding activity with the *qscR* 5' UTR (Fig. 6C).

Hfq negatively regulated the expression of Plt biosynthetic genes exclusively through the *plt* transcriptional activator PltR. The above results (Fig. 1G) clearly demonstrated that the RNA chaperone protein Hfq strongly inhibited pyoluteorin biosynthesis in *P. aeruginosa* M18. The actual molecular mechanism by

which Hfq negatively controlled Plt biosynthesis remains unknown, however. To identify the target regulatory region of Hfq within the intergenic region between divergently transcribed *pltLABCDEFG* and *pltR*, the *pltLA'*-*'lacZ* translational (pMEAZ), *pltLp-lacZ* transcriptional (p6522-*pltLp*), and *pltLo-lacZ* (p9533-*pltLo*) fusion plasmids were introduced separately into the strains M18 and M18hfq, and the LacZ activities were measured in KMB. As shown in Fig. 7C, no significant difference was detected in the *pltLo-lacZ* fusion expression between the strains M18 and M18hfq. However, the *hfq* mutation brought about a notable increase in the total translational *pltLA'*-*'lacZ* fusion expression (Fig. 7A). More significantly, a 2- to 3-fold increase in the *pltLp-lacZ* transcriptional fusion expression was observed in the M18hfq strain compared to the wild-type strain (Fig. 7B). Consistent with the pattern of LacZ expression, the result from qRT-PCR analysis showed that the *hfq* deletion caused a drastic increase of *pltA* mRNA accumulation in early stationary growth phase of the strain M18hfq compared to the wild-type strain (see Fig. S2 in the supplemental material). Based on these results, it is reasonable to deduce that Hfq is likely to exert an indirect negative regulation on *pltLABCDEFG* transcription in *P. aeruginosa* M18.

Like QscR-mediated transcriptional regulation of *phz2* expression, it could also be logically assumed that the transcriptional repression on *pltLABCDEFG* expression by Hfq is mediated by an intermediary transcriptional regulator. The LysR-type pathway-specific transcriptional activator PltR of the divergently transcribed *plt* operon (22, 35) is just such a candidate. Indeed, PltR has been reported to play an important role in Gac/Rsm signal transduction and in the Las/Rhl QS global regulatory network, especially the GacS/GacA two-component regulatory system, involved in Plt biosynthesis in *P. aeruginosa* M18 (21). To confirm the hypothesis that Hfq indirectly downregulated *plt* operon transcription through PltR, the translational (*pltR'*-*'lacZ*), transcriptional (*pltRp-lacZ*), and *pltRo-lacZ* fusion plasmids were separately transformed into the wild-type strain M18 and the *hfq* mutant strain M18hfq, and β-galactosidase assays were performed (Fig. 7D to F). The *pltRp-lacZ* transcriptional fusion expressions (pRZCF) were not significantly different between the strains M18 and M18hfq (Fig. 7E). However, the LacZ activity, which was expressed from both the *pltR'*-*'lacZ* translational fusion (pRZLF) and the *pltRo-lacZ* fusion (p9533-*pltRo*) was significantly enhanced in the *hfq* mutant (Fig. 7D and F). Consistent with these results, the accumulation of *pltR* mRNA in M18hfq was significantly elevated by about 2-fold in strain M18 (see Fig. S2 in the supplemental material). In summary, the RNA chaperone, Hfq, strongly downregulated *pltR* expression at the posttranscriptional level and consequently controlled the transcription of the *pltLABCDEFG* operon.

Hfq directly bound to the 5' UTR of *pltR* mRNA. Similar to the negative regulation of PYO production and *phzM* expression by the Hfq protein, Plt production by the *plt* operon was subject to strong negative regulation by Hfq. The only difference is that the latter regulation was mediated exclusively by the *plt* transcriptional activator PltR. On the basis of this similar negative regulation by Hfq on *phzM* and *pltR*, the *pltR* mRNA 5' UTR was assumed to share a similar RNA secondary structure with the *phzM* mRNA leader. As shown in Fig. 8A, the 63-nt *pltR* mRNA leader (+1 to ATG) indeed forms an almost identical RNA secondary structure where a 9-bp single-stranded AU-rich linker (a potential Hfq-binding motif) is flanked by two stem-loop structures. This

FIG 7 Hfq indirectly downregulated *plt* transcription through posttranscriptional repression on *pltR*. (A to C) β -Galactosidase activities, which derive from the *pltA'*-*lacZ* translational fusion (A) (pMEAZ), *pltLp*-*lacZ* transcriptional fusion (B) (p6522-*pltLp*), and *pltLo*-*lacZ* fusion (C) (p9533-*pltLo*), were assayed in the wild-type strain M18 (●) and the *hfq* mutant M18hfq (▲) in KMB. The *hfq* deletion significantly enhanced the expression of both the *pltA'*-*lacZ* translational fusion (A) and the *pltLp*-*lacZ* transcriptional fusion (B) in the *hfq* mutant compared to the wild-type strain M18. However, there was no significant difference in the *pltLo*-*lacZ* fusion expression (C) between M18 and M18hfq. (D to F) β -Galactosidase activities, which were expressed from the *pltR'*-*lacZ* translational fusion (D) (pRZLF), *pltRp*-*lacZ* transcriptional fusion (E) (pRZCF), and *pltRo*-*lacZ* fusion (F) (p9533-*pltRo*), were assayed in the wild-type strain M18 (●) and the *hfq* mutant M18hfq (▲) in KMB. The *hfq* mutation resulted in a significant increase in expression of both the *pltR'*-*lacZ* translational fusion (D) and the *pltRo*-*lacZ* fusion (F). However, the *hfq* mutation had no obvious impact on the *pltRp*-*lacZ* transcriptional fusion expression (E).

type of mRNA secondary structure is likely to be a typical characteristic of Hfq regulons. We thus questioned whether a 5-nt replacement in the AU-rich spacer of the *pltR* mRNA leader affects the regulatory effect of Hfq on *pltR*. As shown in Fig. 8B, the *LacZ* expression from the plasmid p9533-*pltRo*-M1 did not show significant difference between the wild-type strain M18 and its *hfq* mutant strain M18hfq, demonstrating that the regulatory effect of Hfq on *pltR* was diminished by the mutated AU-rich spacer.

An EMSA was performed to assess the direct interaction of Hfq with the *pltR* mRNA leader (Fig. 8C). The retarded bands representing the Hfq-*pltR* complexes were clearly observed in the EMSA gel, revealing the strong binding activity between the Hfq protein and the *pltR* mRNA. The number and density of Hfq-*pltR* complexes gradually increased as the Hfq concentration was raised from 0 to 5.6 μ M. When Hfq was above 8.0 μ M, stronger binding between Hfq and *pltR* was observed, and gels showed lower Hfq-*pltR* complex mobility and fewer complexes (Fig. 8C). A 1,500-fold excess of unlabeled *pltR* (Fig. 8C, last lane) almost entirely displaced the biotin-labeled *pltR* from the Hfq-RNA complex. Also, the data in Fig. 8D show that the mutated *pltR* mRNA leader with a 5-nt substitution (Fig. 8A) was still able to be bound by Hfq, but with significantly reduced binding affinity. A higher proportion of 1 nM mutated *pltR* mRNA leader remained unbound by Hfq at any concentration up to 28 μ M (Fig. 8D). In contrast, 1 nM wild-type *pltR* mRNA leader could be entirely bound by only 12 μ M Hfq (Fig. 8C, lane 7). These results strongly suggested that Hfq directly bound to the *pltR* mRNA leader and consequently inhibited *pltR* mRNA expression.

DISCUSSION

There is growing evidence that the RNA chaperone Hfq functions as an important global posttranscriptional regulator in many as-

pects of cell physiology and metabolism, including virulence and stress responses (3, 7). Here, we investigated the molecular mechanisms by which Hfq regulated antibiotic biosynthesis in the rhizosphere-isolated *P. aeruginosa* strain M18. The *hfq* deletion mutant M18hfq exhibited several significant phenotypes, including reduced PCA production, elevated PYO and Plt production, and impaired growth (almost entirely inhibited growth in PPM). As summarized in a proposed model in Fig. 9, the differential and strong regulation of Hfq on antibiotic biosynthesis was shown to occur at both the direct posttranscriptional level and the indirect transcriptional level mediated by other regulators. Hfq posttranscriptionally downregulated *phzM* expression and consequently inhibited the conversion of PCA to PYO. Hfq was also shown to posttranscriptionally inhibit the mRNA expression of *pltR* and *qscR*, thereby indirectly exerting strong negative regulation on the *plt* transcription and positive regulation on the *phz2* transcription. In addition, our previous studies have suggested that Plt can autoinduce the expression of its own biosynthetic operon *pltLABCDEFG* and is probably exported from the cell by the ABC (ATP-binding cassette) transport system PltHIJKNO (20). All three important QS systems, Las, Rhl, and PQS, are involved in strong and differential regulation of antibiotic biosynthesis in *P. aeruginosa* M18 (8, 30, 54). Furthermore, *phzM* and its regulatory genes *lasI* and *ptsP* are expressed in a temperature-dependent manner (19).

Hfq has predominantly emerged as an RNA chaperone and posttranscriptional regulator (3). Although a few studies have shown that Hfq can independently regulate gene expression by impacting polyadenylation or translation of mRNAs (7, 16, 26) or directly regulating target gene expression at levels other than mRNA (45, 46), we tend to assume that Hfq, potentially in coop-

FIG 8 Hfq directly bound to the *pltR* mRNA 5' UTR. (A) The *pltR* mRNA 5' UTR (+1 to ATG) was predicted by Mfold to fold into a characteristic secondary structure with a short AU-rich single-stranded spacer linking two stem-loops. A mutant of the *pltR* mRNA 5' UTR, M1, which carried a 5-nt replacement in the AU-rich spacer, was used for EMSA, and its corresponding DNA was utilized to construct the reporter plasmid p9533-*pltRo*-M1. (B) Influence of a 5-nt replacement within the AU-rich spacer on the Hfq regulation of *pltR*. β -Galactosidase activities, resulting from the *pltR-lacZ* fusion reporter plasmid p9533-*pltRo* or p9533-*pltRo*-M1, were determined in the strains M18 and M18hfq in KMB. The regulatory effect of Hfq on *pltR* was diminished by a 5-nt replacement within the AU-rich spacer. (C) Direct binding of Hfq to the *pltR* mRNA 5' UTR. Biotin-labeled *pltR* (1 nM) was incubated with increasing amounts (0 to 32 μ M) of Hfq protein as described in Materials and Methods. Hfq-*pltR* complexes are designated C1 to C5. For competition reactions, 1.5 μ M unlabeled *pltR* was included in the binding reaction mixture in molar excess as indicated in lane 13. (D) A 5-nt replacement (M1) in the AU-rich spacer significantly reduced the binding affinity between the *pltR* mRNA 5' UTR and Hfq. Biotin-labeled *pltR*-M1 (1 nM) was incubated with increasing amounts (0 to 28 μ M) of Hfq protein as described in Materials and Methods. Hfq-*pltR*-M1 complexes are designated C1 to C3.

eration with other unknown *cis*- or *trans*-encoded sRNAs, post-transcriptionally downregulated the *phzM*, *qscR*, and *pltR* expression. Therefore, we have attempted to screen the sRNA candidates controlling antibiotic biosynthesis of *P. aeruginosa* M18 from

those reported or predicted homologous *Pseudomonas* sRNAs. Forty-two homologous *Pseudomonas* sRNAs (20 detected by RT-PCR or Northern blotting and 22 predicted) (41) were respectively searched by intaRNA (v1.2.5) (39) for short stretches of 10 nucleotides complementary to the *phzM*, *qscR*, and *pltR* mRNA leaders. Twelve (4 and 8), 9 (3 and 6), and 4 (1 and 3) sRNAs, including CrcZ, PhrY, PhrW, RgsA, and SsrS et al., were respectively predicted to probably target the *phzM*, *qscR*, and *pltR* mRNA leaders (see Table S3 in the supplemental material). It needs further research to ascertain whether these sRNAs along with Hfq control antibiotic biosynthesis in the M18 strain. In addition, the presence of multiple promoters in the longer intergenic region will prompt us to further assess whether there exist any *cis*-encoded sRNAs in the opposite direction of *phzM*, *qscR*, and *pltR* genes.

We also questioned whether two important sRNAs, RsmY and RsmZ, are involved in Hfq-mediated regulation of antibiotic biosynthesis in *P. aeruginosa* M18. These two sRNAs have been extensively studied for their involvement in the control of secondary metabolism and quorum sensing in *P. fluorescens* CHAO and *P. aeruginosa* (24, 40). They can sequester the translational repressor RsmA, which in turn blocks ribosome binding at the RBS of target mRNAs (18, 47). Although Hfq has been reported to be able to bind to and stabilize RsmY (44), there appears to be no direct evidence that Hfq regulates the expression of other target genes in collaboration with these two sRNAs. In *P. aeruginosa* M18, the *rsmY* mutation led to an almost complete inhibition of Plt biosynthesis and a mild increase in PCA biosynthesis, while the *rsmZ* mutation did not show a significant influence on Plt and PCA biosynthesis (data not shown). However, Hfq was shown to exert both strong negative regulation on Plt biosynthesis and significant positive regulation on PCA biosynthesis in M18. Therefore, it can be reasonably speculated that Hfq and RsmY/RsmZ control antibiotic biosynthesis through two distinct regulatory pathways and mechanisms in *P. aeruginosa* M18. In addition, in *P. aeruginosa*, two Hfq-dependent sRNAs, PrrF1 and PrrF2, have been characterized that are involved in iron homeostasis (52), and two other Hfq-dependent sRNAs, PhrS and PhrD, in PqsR-mediated quorum sensing (40). PhrS, expression of which is induced under low-oxygen conditions, activates PqsR expression and thus elevates PYO and PQS levels (40). However, PYO production was significantly enhanced in the *hfq* mutant M18hfq. Therefore, whether the *P. aeruginosa* M18 Hfq protein regulated PYO biosynthesis in cooperation with the PhrS sRNA remains unknown.

Hfq and/or Hfq-dependent sRNA has often been reported to perform two opposing functions in a target mRNA-specific way. For example, the Hfq-dependent sRNA, DsrA, not only activates the translation of RpoS but also represses the translation of H-NS (4). It can be proposed that whether one target mRNA is positively or negatively regulated by Hfq likely depends on the secondary structure of the target mRNA leader. As shown in this study, a similar secondary structure with a short single-stranded AU-rich spacer (a potential Hfq-binding motif) linking two stem-loops occurs in the mRNA leaders of three negative Hfq regulons, *pltR*, *phzM*, and *qscR*. Therefore, this typical RNA secondary structure could be a common characteristic of mRNAs subject to negative regulation by Hfq. The similar RNA structure has also been reported in other Hfq negative regulons, such as the *rpoS* mRNA repressed by the Hfq-dependent OxyS sRNA (55) and the autorepressed *hfq* mRNA (48) in *E. coli*. Interestingly, similar RNA struc-

FIG 9 A schematic model outlining the regulation of antibiotic biosynthesis by Hfq in the rhizobacterium *P. aeruginosa* M18. Thick arrows, positive regulation; thick lines with flattened ends, negative regulation; thin arrows, biogenesis and transport. Three regulatory pathways of Hfq are (i) the negative regulatory pathway of Hfq on Plt production and its biosynthetic gene expression exclusively mediated by PltR, (ii) the positive regulatory pathway of Hfq on PCA production and the *phz2* operon expression, and (iii) the negative regulatory pathway of Hfq on PYO production and the *phzM* expression. Ellipses indicate the Hfq-dependent mediators of transcriptional regulation. In addition, the *plt* operon is transcriptionally activated by the LysR-type regulator PltR and autoinduced by Plt itself. The ABC (ATP-binding cassette) transport system PltHIJKNO is probably responsible for exporting Plt out of the cell (20). Three important QS systems, Las, Rhl, and PQS, strongly repressed Plt biosynthesis in *P. aeruginosa* M18. The PCA biosynthesis was almost entirely inhibited by the Rhl or PQS inactivated mutant and was strongly repressed by the Las system (8, 30, 54). Furthermore, *phzM* and its regulatory genes *lasI* and *ptsP* were expressed in a temperature-dependent manner (19).

tures were also found in other Hfq-dependent sRNAs such as OxyS, which was shown to repress *fhlA* and *rpoS* translation (56). In collaboration with an unidentified sRNA, Hfq may repress its target mRNAs through several possible mechanisms, including stabilization of the inhibitory stem-loop structures, blocking of ribosome binding, and antisense degradation.

The ubiquitously expressed protein Hfq has been increasingly reported to function as a central regulator for adjusting global gene expression profiles to be responsible for the fitness, virulence, and stress or host response in most bacteria (6, 7). Transcriptomic results have indicated that Hfq directly or indirectly regulates almost 20% of all *Salmonella* genes and 15% of all *P. aeruginosa* genes (7). Multiple transcriptional or post-transcriptional regulators mediate bacterial transcriptomic regulation by Hfq. Such a global cascade regulatory network might be thought of as an economical and energy-saving regulatory strategy in bacteria. *P. aeruginosa* M18, an effective bio-control strain isolated from the rhizosphere of sweet melon, probably has evolved a unique profile of secondary metabolites, including antibiotics, during long-term inhabitation of

rhizosphere soil. This can be partly illustrated by our previous results that the temperature-dependent and strain-specific characteristics of the M18 strain contributed to the production of the strong antifungal compound PCA rather than the virulence factor PYO (19). Hfq may provide a new pathway by which *P. aeruginosa* M18 can inhibit PYO biosynthesis and concomitantly promote PCA biosynthesis.

In summary, our results demonstrate that the Hfq chaperone protein is a critical regulator of *P. aeruginosa* M18 secondary metabolism at both the posttranscriptional and transcriptional levels (the latter mediated by PltR and QscR). Further studies will focus on identifying the potential sRNAs involved in the Hfq-dependent regulation of antibiotic biosynthesis by high-throughput methods, such as deep-sequencing, tilting array, and Hfq-RNA coimmunoprecipitation (co-IP). These studies might help unravel the Hfq-dependent genomic expression profile; reconstruct the Hfq-dependent global regulatory network of secondary metabolism, including antibiotic biosynthesis; and determine the molecular mechanism by which Hfq controls the growth of cells and the utilization of carbon in *P. aeruginosa* M18.

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