Bacteria with multiple drug resistance (MDR) have become a global issue worldwide, and hundreds of thousands of people’s lives are threatened every year. The emergence of novel MDR strains and insufficient development of new antimicrobial agents are the major reasons that limit the choice of antibiotics for the treatment of bacterial infection. Thus, preserving the clinical value of current antibiotics could be one of the effective approaches to resolve this problem. Here we identified numerous novel small RNAs that were downregulated in the MDR clinical isolates of Pseudomonas aeruginosa (P. aeru), and we demonstrated that overexpression of one of these small RNAs (sRNAs), AS1974, was able to transform the MDR clinical strain to drug hypersusceptibility. AS1974 is the master regulator to moderate the expression of several drug resistance pathways, including membrane transporters and biofilm-associated antibiotic-resistant genes, and its expression is regulated by the methylation sites located at the 5’ UTR of the gene. Our findings unravel the sRNA that regulates the MDR pathways in clinical isolates of P. aeru. Moreover, transforming bacterial drug resistance to hypersusceptibility using sRNA could be the potential approach for tackling MDR bacteria in the future.

INTRODUCTION

Pseudomonas aeruginosa (P. aeru) is a Gram-negative bacterium of the family Pseudomonadaceae, and it can switch from being an environmental isolate (soil and water) to a human pathogen.1 P. aeru causes nosocomial pneumonia, catheter and urinary tract infections, sepsis in burn wound and immunocompromised patients, as well as chronic pulmonary inflammation in cystic fibrosis patients. One of the major characteristics of this bacterium is its resistance to antibiotics, which is due to the highly coordinated and complex transcriptional regulatory networks it possesses, resulting in the assimilation of signals originating from a multitude of different environments, such as the expression of different sets of genes to facilitate growth in drug-induced stress environments.2–4 In recent years, a steady increase in the multiple drug resistance (MDR) of P. aeru has been reported.5 Moreover, the emerging MDR strains were resistant to fluoroquinolones, cephalosporins, carbapenems, and aminoglycosides. Thus, the choice for clinical treatment of P. aeru infection is very limited.16

Variations of the genomes are one of the hallmarks of bacterial survival in adaptation to environmental change, and studies of the transcriptomes always provide us a snapshot of the bacterial response to variations of the external environments. To date, several complete genomes1,2,8,9 and numerous DNA microarray-derived transcriptomes have been reported.3 Although these transcriptomes were sequenced and available online, the understanding of gene regulation in this bacterium in responses to environmental variations is still restricted to the limiting resolutions and the lack of quantification information of the microarrays.3 In particular, the genomic variations of MDR P. aeru have not been defined yet.

In the last decade, an increasing number of small regulatory RNAs has been described in different pathogenic bacteria, such as Escherichia coli, Staphylococcus aureus, Vibrio cholerae, and P. aeru (PAO1).10–15 The majority of small RNA (sRNA)-induced post-transcriptional events commonly required the bacterial Sm-like protein called Hfq, which is one of the most abundant RNA-binding proteins in bacteria. Hfq was first identified as a host factor required for phase QB RNA replication in Escherichia coli,16 and it was recently demonstrated to have important physiological roles, such as quorum sensing, stress response, and virulence factor regulation, in numerous model bacteria.17 Hfq interacts with both regulatory sRNAs and mRNAs, and it facilitates the interaction between the short, imperfect antisense sRNAs and their corresponding target mRNAs post-transcriptionally.17,18 Nevertheless, Hfq can also act alone as a translational repressor of mRNA as well as modulate mRNA decay by stimulating polyadenylation.19,20 In P. aeru, numerous sRNAs have been found to regulate different physiological processes, such as the quorum sensing network, iron homeostasis,21,22 biofilm formation,23–27 survival under stresses, and modulation of virulence factors and pathogenicity.28 However, little is known about the regulatory mechanism of sRNAs in controlling MDR pathways.
In this study, we compared the sRNA repertoires of three MDR clinical isolates and three drug susceptibility strains, and we identified three novel sRNAs that were downregulated in MDR strains. We showed that one of the sRNAs, AS1974, played significant roles in regulating various drug resistance pathways in *P. aeruginosa* and was able to transform the bacteria from drug resistance to drug susceptibility. This knowledge of sRNA regulation could be utilized for tackling the MDR bacteria in the future.

### RESULTS

#### Characterization of MDR Strains of Clinical Isolates

Six representative strains from clinical isolates, including 3 drug susceptible and 3 MDR of *P. aeruginosa*, were selected and cultured for further characterization. To define the susceptibility and resistance groups to different antibiotics, minimal inhibition concentration (MIC) assay was performed. ATCC 27853 was used as a reference to test the antimicrobial activity and susceptibility, with well-defined MIC values representative of susceptible, intermediate, and resistant phenotypes,

#### Identification of MDR-Specific sRNAs in *P. aeruginosa*

Hfq is the RNA chaperone that can be used as a tool to enrich the sRNA repertoire of MDR strains. For each sample, more than 400,000 reads were mapped to the reference transcripts according to the protocol supplied by the company (Life Technologies). 203,349 reads were mapped to the reference transcripts. The corresponding sRNA sequences were identified as sRNA with a 15% abundance in the MDR strains and 0% abundance in the susceptible strains. The sRNA sequences were reverse complemented and used to design the sRNA sequences.

The total of 17 sRNAs were identified, including 15 sRNAs targeted against the fluoroquinolone, 1 sRNA targeted against the beta-lactam, and 1 sRNA targeted against the aminoglycoside.

### Table 1. MIC of Six Clinical Isolates and the sRNA-Transformed Strains

| Class             | Wild-Type Strains | Transformed-Strains | Quality Control Range for ATCC 27853 |
|-------------------|------------------|---------------------|-------------------------------------|
| **Aminoglycoside**|                  |                     |                                     |
| AMIKACIN**        | 1                | 4                   | > 128                               |
| GENTAMICIN**      | 0.5              | 2                   | > 128                               |
| TOBRAMICIN**      | 0.5              | 0.5                 | > 128                               |
| CEFTRIAXONE**     | 32               | 64                  | > 128                               |
| CEPFODOXIME**     | 128              | 128                 | > 128                               |
| CEFOTAXIME/ CLAVULANIC ACID | 32 | 64        | > 128                               |
| CEFOTAXIME**      | > 128            | > 128               | > 128                               |
| CEFAMANDOLE**     | 2                | 2                   | > 128                               |
| CEFAZIDIME**      | 2                | 2                   | > 128                               |
| CEPFIRAXONE**     | 32               | 64                  | > 128                               |
| CEPFOXAN**        | 8                | 8                   | > 128                               |
| CPIFUXAMOZINE**   | 8                | 8                   | > 128                               |
| CEPFOXAN**        | 8                | 8                   | > 128                               |
| CEPFOXAN**        | 8                | 8                   | > 128                               |
| IMPENEM**         | 16               | 16                  | > 128                               |
| SULTAMICILLIN**   | > 128            | > 128               | > 128                               |
| AMICILLIN**       | > 128            | > 128               | > 128                               |
| MINOFLOXIN**      | 4                | 4                   | > 128                               |
| PENOFLOXIN**      | 2                | 2                   | > 128                               |
| PEPFLOXIN**       | 2                | 2                   | > 128                               |
| NIFLOXIN**        | 0.5              | 0.5                 | > 128                               |
| SPARFLOXIN**      | 0.5              | 0.5                 | > 128                               |
| LEVOFLOXIN**      | 0.5              | 0.5                 | > 128                               |
| CEFLOXACIN**      | 0.12             | 0.12                | > 128                               |
| MOXIFLOXIN**      | 1                | 1                   | > 128                               |
| PIPACILLIN/TAZOBACTAM** | 4 | 4       | > 128                               |
| PIPACILLIN**      | 4                | 4                   | > 128                               |
| MOXIFLOXIN**      | 4                | 4                   | > 128                               |
| PIPACILLIN**      | 4                | 4                   | > 128                               |
| MOXIFLOXIN**      | 1                | 1                   | > 128                               |
| PIPACILLIN**      | 4                | 4                   | > 128                               |
| MOXIFLOXIN**      | 1                | 1                   | > 128                               |
| PIPACILLIN**      | 4                | 4                   | > 128                               |
| MOXIFLOXIN**      | 1                | 1                   | > 128                               |
| PIPACILLIN**      | 4                | 4                   | > 128                               |
| CHLORAMPHENICOL**| 64               | 128                 | > 128                               |

The MIC of six clinical isolates and the sRNA-transformed strains were measured using microbroth method. Different classes of antibiotics including aminoglycoside, cephalosporin, beta-lactam, extended-spectrum penicillin beta-lactam, fluoroquinolone were used. Antibiotics susceptibility and resistance strains are highlighted as pale yellow and blue colors respectively. R3-AS1974, R3-AS2779 and R3-IGR2780 represent R3 strains transformed with the corresponding sRNA while R3-AS1974-Reverse represents R3 strains transformed the sRNA which sequence was reverse and complementary to AS1974 (negative control). R3-empty vector was used as vector control. The strain ATCC27853 was used for quality control.
the reference genome PAO1, and most of the reads were located at either coding regions or intergenic regions (Figure S1).

To identify the sRNAs that are specific to MDR, sRNAs that expressed with the fold change in log2 scale > 1 and q value ≤ 0.05 between drug-susceptible or drug-resistant strains were selected. We finally identified three sRNAs, including IGR2780, AS1974, and AS2779 (Figure 1). IGR2780 is located at the intergenic region between PA2770 and PA2771. AS1974 and AS2779 are located at the antisense region of mexR and PA2769, respectively. As shown in the northern blot analysis of Figure 1, three sRNAs were downregulated in all drug-resistant strains compared with all drug-susceptible strains.

To further characterize these sRNAs, we identified the transcription start site (TSS) and the promoters of each sRNA using 5’ rapid amplification of cDNA ends (RACE) analysis. Intriguingly, both AS1974 and AS2779 possessed two TSSs and thus different isoforms, whereas IGR2780 had only one. Moreover, all of them were under the control of Sigma70 promoter. We also predicted the secondary structures of the sRNAs using RNAfold30 and CentroidFold.31 IGR2780 contained a typical terminator, which is a stem-loop with a stretch of poly(U) sequence, whereas AS1974 and AS2779 contained only a typical I-shaped terminator. Notably, there was a canonical terminator in the middle of AS2779, suggesting that the RNA polymerase may stop the transcription at that position and generate a shorter form of AS2779.

Interaction between sRNAs and Hfq

To understand the sRNA-Hfq interaction, in vitro RNA binding and electrophoretic mobility shift assay (EMSA) were performed using in vitro-transcribed 32P-32P-labeled RNA and purified recombinant P. aeruginosa Hfq protein. RsmY sRNA, which was previously shown to bind Hfq, was used as the positive control,32 whereas RsmY-reverse (the RNA with the reverse and complement sequence of RsmY) was the negative control in this experiment.

As shown in Figure S2, all sRNAs directly bound to Hfq protein with high specificity, except the rsmY-reverse sRNA (negative control). Moreover, the apparent dissociation constants (Kd) calculated from the binding isotherms of EMSA were less than 150 nM, indicating their high affinity to Hfq protein (Figure 1). Noteworthy, we found that IGR2780 and AS2779 sRNAs possessed two cooperative binding sites to Hfq, which was similar to DsrA sRNA, the translational regulator of two global transcription regulators, H-NS and RpoS, in bacteria.33 These results suggested the versatility of these sRNAs to pair effectively with various target(s) at different extents in an Hfq-dependent manner.

Figure 1. Characterization of MDR-Specific sRNAs

(A–C) Northern blot analysis, 5’ RACE, secondary structure prediction, and electrophoretic mobility shift assay (EMSA) of different sRNAs were used to characterize the MDR-specific sRNAs: (A) AS1974, (B) AS2779, and (C) IGR 2780 sRNAs. For the northern blot analysis, the expressions of sRNAs were detected by specific probes designed for each sRNA and are shown. The bands of the sRNAs are indicated and SS was used as the loading control. The relative intensities of the bands were quantitated and analyzed by Image Processing and Analysis in Java software (ImageJ). The intensities of three drug-resistant strains (R) were compared with three drug-susceptible strains (S) using boxplot. The transcription start sites (TSSs) of each sRNA were determined using 5’ RACE and the PCR results are shown. The band found in TAP and adaptor-treated RNA was cloned and sequenced to determine the TSSs. The secondary structure of each sRNA was predicted using the TSSs found in 5’ RACE, and its orientation in the genome is depicted. For EMSA, increasing concentrations of Hfq protein were titrated with labeled sRNAs. The binding isotherms of each sRNA to Hfq are plotted, and the dissociation constants were calculated by fitting the curve with the Hill equation. All P. aeruginosa strains were grown in nutrient broth at 250 rpm and 37°C and harvested at stationary phase (optical density at 600 [OD600] 1.5–1.7).
Overexpression of AS1974 sRNA Induces Hypersusceptibility of the Resistance Strain

As shown in Figure 1, three sRNAs were downregulated in drug-resistant strains compared with drug-susceptible strains. To find out if the sRNAs directly play a role in the intrinsic drug resistance of bacteria, we overexpressed each of the sRNAs in the resistance strains of P. aeruginosa, and we performed MIC measurements. Individual sequences of sRNAs were inserted into the modified pMMB66EH vector that was engineered for RNA expression and transformed into the resistance strains by electroporation. Empty vector was used as a control, and the strain of ATCC27853 was used as a reference for MIC measurement.

As shown in Table 1, overexpression of AS1974 in the transformed resistance strains displayed hypersusceptibility to aminoglycoside, cephalosporin, meropenem, and ertapenem, whereas IGR2780 and AS2779 had no effect on MIC, suggesting the functional roles of AS1974 sRNA in regulating the gene expression of these particular drug resistance pathways. To rule out the possibility of the non-specific effect from the sRNAs produced from the vector, we included AS1974-reverse as a negative control, which produced the same length but reverse and complement sequence of AS1974 in bacterial cell. As expected, the sRNA-reverse negative control showed no effect on the MIC (Table 1), indicating the specificity of AS1974 in regulating the drug resistance of P. aeruginosa.

Direct Control of MDR Gene Expression by AS1974 sRNA

One of the major functions of sRNAs is to suppress the target gene expression under particular conditions or in response to external stimuli. To find out the regulatory roles of AS1974 sRNAs in various drug resistance pathways, we sequenced the transcriptomes of six strains, and we used our newly developed program to correlate sRNA repertoires with transcriptomes (unpublished data). Since sRNAs often hybridize with their corresponding target mRNAs and activate the RNA degradation mechanism, those sRNA-regulated genes were supposed to be downregulated in the strains when the sRNA was highly expressed. As a result, we identified numerous target genes involved in drug resistance pathways, which were upregulated in all drug resistance strains, such as genes of membrane proteins (mexD, chtA, and prc), transporters (major facilitator superfamily), flagella (pilD), and antibiotic resistance (ndvB). To validate the transcriptome result, we performed real-time qPCR experiments to measure and compare the transcript levels of these genes in both drug-susceptible and drug-resistant strains. As shown in Figure 2A, all the target genes were upregulated in the drug-resistant strains compared with the drug-susceptible strains, suggesting

Figure 2. Analysis of AS1974 Target Gene Expression

(A) Comparison of AS1974 target gene expression among antibiotic-resistant and -susceptible strains. The relative mRNA expressions of AS1974 target genes were measured using real-time PCR. The genes of three drug-resistant strains (R) were compared with three drug-susceptible strains (S) using boxplot, and the results were expressed as the means of at least 3 independent experiments. The bacteria were cultured in nutrient broth at 250 rpm and 37°C. The cells were harvested at stationary phase (OD600 = 1.5–1.7). Data are presented as mean ± SD, and comparisons were analyzed using unpaired t test (two tailed: *p < 0.05, **p < 0.01, and ***p < 0.001). (B) Suppression of drug-resistant genes in AS1974-overexpressed transconjugants. The relative expressions of AS1974 target genes were measured in transconjugants that overexpressed AS1974 (R3-AS1974) and compared with control (transconjugants with empty vector [R3-EV]) using real-time PCR. The bacteria were cultured in nutrient broth including 128 mg/mL tetracycline at 250 rpm and 37°C. The cells were harvested at stationary phase (OD600 = 1.5–1.7). The results were expressed as the means of at least 3 independent experiments. Data are presented as mean ± SD, and comparisons were analyzed using unpaired t test (two tailed: *p < 0.05, **p < 0.01, and ***p < 0.001).
the role of AS1974 sRNA in post-transcriptional regulation of these transcripts.

To further investigate if these drug-resistant genes were directly controlled by this sRNA, we overexpressed AS1974 in the resistance strain (Figure 5A), and we measured their transcript levels by qPCR. Empty vector was used as a negative control. As shown in Figure 2B, all the target gene expressions were downregulated upon the overexpression of sRNA, indicating the functional roles of AS1974 in controlling the drug resistance of bacterial strains. Notably, ndvB is the biofilm-specific antibiotic-resistant gene. Overexpression of ndvB protein secretes the glucans that bind to the aminoglycoside, which prevents the molecules from entering the cell, and, therefore, the bacteria become resistant to aminoglycoside. These phenotypes were observed in our MIC study of transconjugant that overexpressed AS1974 sRNA (Table 1). We also compared transcriptome profiles of AS1974-transformed and control strains, and we found that less than 1% of the total transcripts was affected, indicating the off-target effect of AS1974 was minimal (Figure S5).

**DISCUSSION**

P. aeruginosa is a Gram-negative, opportunistic pathogen that is commonly found in nosocomical infection of patients with chronic obstructive pulmonary disease and cystic fibrosis as well as immunocompromised patients such as AIDS patients. The emergence of multidrug resistance of P. aeruginosa, which has caused a very high mortality rate, reinforces its infection as a serious threat to the public health, according to the antibiotic resistance threat reports in the United States published by the Centers for Disease Control and Prevention in 2013. In the last decade, extensive studies of numerous major drug resistance mechanisms have been performed, such as derepression of chromosomal AmpC cephalosporinase, diminished outer membrane permeability, overexpression of active efflux system with wide substrate profiles, and synthesis of aminoglycoside-modifying enzymes. Nevertheless, the occurrence of drug resistances from multifactorial regulatory systems in bacteria remains unclear. Small regulatory RNAs have been recently shown to play significant roles in controlling multiple pathways involved in bacterial antibiotic resistance, such as regulating RNA synthesis to make bacteria highly tolerant to different antibiotics, controlling cell membrane integrity, and membrane transporters to prevent the attack from molecules. Therefore, sRNAs indeed represent a group of potential antimicrobial molecules for future development to combat the multidrug-resistant pathogen.

In this work, we identified several novel sRNAs in clinical isolates of P. aeruginosa using Hfq immunoprecipitation followed by sRNA sequencing, and we characterized their RNA-binding capabilities as well as functional roles in the context of drug resistance. We
showed that the expressions of these sRNAs were significantly downregulated in the multidrug-resistant strains compared with those in the drug-susceptible strains. Moreover, overexpression of AS1974 sRNA transformed the bacteria from resistant strains of aminoglycoside, meropenem, and some of the board-spectrum cephalosporin to drug-sensitive strains. This is of significant importance because sRNAs have been considered as the potential antimicrobial molecules for multidrug resistance pathogens with the shortage of efficient antibiotics in clinical sectors, and we proved the concept here that the sRNA is capable of eliminating the drug resistance of clinical isolates of *P. aeruginosa*. In other words, sRNA treatment in combination with old non-functional antibiotics (those that have been shown to be resistant and cannot be used for treatment) may potentially be the strategic approach to target the widely spreading multidrug resistance of bacterial infection in the world.

Although the delivery of the sRNA to the pathogen in the human body is still an unsolved problem, this approach indeed opens up a new direction for the potential strategy of treatment as well as future development of antimicrobial agents.

One of the functional features of sRNA is to act as a master regulator to control multiple cellular pathways simultaneously.
to provide an immediate response to the environmental changes such as stress. Here we found that regulation of AS1974 was capable of modulating the gene expression of various pathways, including efflux pumps, cell wall turnover, biofilm formation, motility, and iron acquisition. Moreover, most of them are related to the multidrug resistance. For example, MexD is the component of the multidrug efflux system (MexC-MexD-OprJ) in P. aeru, which can pump the antibiotics out of the cell. PA2055 is the major facilitator superfamily (MFS) transporter located at the inner membrane of bacteria, which responds and transports small molecules outside the bacteria in order to reduce the effective concentration of drugs in the cells. The periplasmic tail-specific protease (prc) is an enzyme involved in C-terminal processing of penicillin-binding protein 3 (PBPs), and inactivation of the prc gene in E. coli results in multiple antibiotic susceptibility.\(^4\) \(^2\) \(^4\) \(^4\) ndvB is the biofilm-specific antibiotic resistance gene from which the translated protein can secrete glucans to sequester the aminoglycoside and prevent it from entering the bacterial cells.\(^4\) In summary, the sRNA-regulated gene expression of multiple pathways simultaneously dictated the highly regulated and coupled molecular mechanism of multidrug resistance in P. aeru.

In our qPCR results, AS1974 sRNA downregulated the component of the multidrug efflux system, MexC-MexD-OprJ, which was the commonly found efflux pump in P. aeru, and it provided resistance to aminoglycosides, chloramphenicol, and most β-lactams antibiotics except sulbenicillin and carbenicillin to the bacteria.\(^4\) \(^6\) In our MIC result (Table 1), however, the AS1974-overexpressed strain still showed resistance to some antibiotics such as chloramphenicol. As the antibiotic resistance of bacteria is often attributed to multiple mechanisms, the observed resistance was indeed due to the contribution of other mechanisms. For example, the bacteria could secrete chloramphenicol acetyltransferase (CAT) to detoxify the chloramphenicol\(^4\) \(^6\) and become resistant to this particular antibiotic, which explains why AS1974-expressing strains were resistant to chloramphenicol and even the efflux system was affected.

Apart from the genes described above that target different types of molecules to protect the bacteria from antibiotics attack, other functional genes regulated by AS1974 were also found in other pathways, such as PA1940, pilD, and chtA. PA1940 is the putative catalase involved in oxidative stress, pilD functions in pilus biogenesis, and chtA is the TonB-dependent siderophore receptor that anchors on the outer membrane of the cell and controls iron acquisition. These genes have been shown to play roles in bacterial virulence, motility, and iron homeostasis. Intriguingly, chtA and prc are the major components of the cell surface signaling (CSS) regulatory system that controls extracytoplasmic function (ECF) sigma factors to regulate vital functions in the bacterial response to the environment in Gram-negative bacteria, and they were shown to play significant roles in the activation of the aerobactin-mediated CSS system in P. putida. Therefore, we speculate that AS1974 is capable of mediating the physiology of bacteria, in particular to the external stimuli, since sRNA provide intermediate responses to the bacterial host to adapt to environmental changes for survival.

**MATERIALS AND METHODS**

**Media and Growth Conditions**

P. aeru and Escherichia coli (E. coli) strains were grown aerobically at 37°C in 100 mL Difco Nutrient Broth (Becton Dickinson) and Luria-Bertani (Affymetrix) medium separately with shaking at 250 rpm or on Difco Nutrient agar (Becton Dickinson) and Luria-Bertani (LB) agar (Affymetrix) plates. Ampicillin was used at the concentration of 100 μg/mL.

**Bacterial Strains, Plasmids, and Primers**

Bacterial strains, plasmids, and primers used in this study are listed in Table S1.

**The MIC Measurement**

The MICs of 11 antibiotics, including penicillin G, cefotaxime, chloramphenicol, tetracycline, erythromycin, lincomycin, ciprofloxacin (Sigma, St. Louis, MO, USA), levofloxacin (Daichi Sankyo, Tokyo, Japan), gatifloxacin (Bristol-Myers Squibb, Moreton, UK), moxifloxacin (Bayer Healthcare Pharmaceuticals, Berlin, Germany), and linezolid (Pfizer, N, USA), were determined using the microbroth dilution method, according to the Clinical and Laboratory Standards Institute (CLSI).

**Total RNA Preparation**

Six P. aeru strains, PA51530 (S1), PA83365 (S2), PA85710 (S3), PA191712 (R1), PA194803 (R2), and PA185388 (R3), were grown (100-mL culture) and harvested at OD600 1.7 (stationary phase). The cell pellet was lysed in 0.5 mg/mL lysozyme (Sigma–Aldrich) and extracted using 15 mL Trizol (Invitrogen). The total RNA was then treated with DNase I (New England Biolabs), and the rRNA was depleted using MICROBExpress Bacteria mRNA Enrichment Kit (Ambion), followed by Ribo-Zero rRNA Removal Kits (Gram-Negative Bacteria, Epicentre), according to the manufacturer’s instructions. The quality and integrity of the extracted RNA was determined by Bioanalyzer (Agilent Technologies) and Qubit 2.0 Fluorometer (Life Technologies).

**Purification of Recombinant Hfq Protein**

The gene of hfq from P. aeru was cloned into pET28a vector and transformed into BL21(DE3) pLysS E. coli. Overexpression of the recombinant protein was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when OD600 reached 0.6 at 37°C. After 4 h induction, cells were harvested and lysed in lysis buffer (20 mM Tris, 150 mM NaCl, 0.5% Triton X-100, 0.4 mM phenylmethylsulfonyl fluoride (PMFS), and 2 mM 2-mercaptoethanol [β-ME] [pH 7.5]), followed by 5-min sonication on ice. The lystate was then centrifuged at 13,000 rpm at 4°C for 45 min, and the supernatant was loaded onto column with TALON affinity resins (Clontech Laboratories). After extensive wash of the column, the protein was eluted and dialyzed overnight. The sample was then concentrated and the purity was analyzed by SDS-PAGE.
RNA Immunoprecipitation with Recombinant Hfq Protein and sRNA Sequencing

100 ng purified recombinant Hfq protein was immobilized on the 50 μL Gammabind G Sepharose beads (GE Healthcare) with anti-His6 antibody, as described previously.49 Beads with antibody were incubated with 2 min. DNA oligonucleotide probe specific for sRNA was radioactively labeled with [γ-32P] ATP using T4 polynucleotide kinase (New England Biolabs) and further purified by ethanol precipitation. The purified RNA then underwent rRNA depletion using Ribo-Zero rRNA Removal Kits (Gram-Negative Bacteria, Epicentre), according to the manufacturer’s instructions, and the sequencing libraries were constructed and sequenced using Ion Torrent PGM sequencer, according to the protocol supplied by the company (Life Technologies).

Northern Blot Analysis

Six *P. aeru* strains, S1, S2, S3, R1, R2, and R3, were grown in 100 mL Nutrient broth with 250 rpm at 37°C and harvested at OD600 1.5–1.7 (stationary phase). The cell pellet was lysed as described previously. Total RNA (10 μg) was denatured at 70°C for 5 min in Gel Loading Buffer II (Ambion) and loaded onto 6% urea denaturing polyacrylamide gels. RNA was then transferred to Hybond-XL membrane (Amersham) and cross-linked under a UV light of 120 ml/cm² for 2 min. DNA oligonucleotide probe specific for each sRNA was radioactively labeled with [γ-32P] ATP using T4 polynucleotide kinase (New England Biolabs) and further purified by Centri Spin column-20 (Princeton Separations). The membranes were incubated with probes at 42°C overnight after prehybridization with UltraHyb buffer (Ambion), and then washed twice with 20 mL 0.2 × sodium-sodium citrate (SSC) and 0.1% SDS for 10 min. The membranes were exposed to a phosphor screen overnight and visualized using a PhosphorImager (Typhoon TRIO, Amersham Biosciences).

EMSA

The RNAs were *in vitro* transcribed and labeled with [α-32P] UTP using a MEGAScript kit (Ambion). The labeled sRNAs were further purified by 8M urea denaturing gel electrophoresis and precipitated with ethanol for subsequent experiments. To set up the EMSA reaction, 10,000 cpm labeled RNA was incubated with increasing concentrations (0–400 nM) of purified recombinant Hfq protein in binding buffer (50 mM Tris, 250 mM NH₄Cl [pH 7.5], 1 × RNasin, and 10 μM tRNA) at 37°C for 30 min. The samples were then mixed with 2 μL loading buffer (75% glycerol, 0.01% xylencyanol, and 0.01% bromphenol blue) and separated on 8% 1 × Tris/Borate/EDTA (TBE) polyacrylamide gel. The image was analyzed by autoradiography. Signals of free and binding RNAs were quantified with ImageJ (NIH), and the binding percentages, which is equal to the intensity of all binding complex over the total intensity (including the free RNA and bound RNA), were calculated. The curves were fitted with the Hill equation using the Igor Pro program (WaveMetrics), using default settings to estimate the K₅ values of each binding isotherm.

Construction of pMMB66EH-rrnBter-tet

Plasmid pMMB66EH (ATCC 37620) was purchased from ATCC. To construct pMMB66EH-rrnBter-tet, the transcription unit (TU) of tetracycline resistance gene was cloned from the pACYC184 vector, using the primers of Tet-PstI-F (5’-CGACTGCAAGATTTCAG TGCAATTATCTC-3’) and Tet-PstI-R (5’-GATCTGAGGTTC ACGTTCTCCGCAGAATTG-3’), and inserted into the PstI site. The clone carrying pMMB66EH-rrnB-tet with the desired direction was selected and validated by Sanger sequencing (Beijing Genomics Institute [BGI]).

Construction of pMMB66EH-rrnBter-tet-sRNA

The sRNA sequences were amplified from *P. aeru* genomic DNA by PCR and cloned into the Xhol and KpnI sites of pMMB66EH-rrnBter-tet to generate pMMB66EH-rrnBter-tet-sRNA constructs. The clone carrying pMMB66EH-rrnB-tet-sRNAs was selected and validated by Sanger sequencing (BGI). The recombinant plasmids were then transformed into *P. aeru* by electroporation for further studies.

Electroporation

The sRNAs were amplified from genomic DNA and cloned into our in-house RNA expression vector (pMMB66EH-rrnBter-tet). The vector carrying sRNA was then transformed into *P. aeru* MDR strains by electroporation under the conditions of 25 microfarad (μF), 2.5 kV, and 200Ω.50 The transformed cells were plated on the nutrient agar plates with tetracycline at the concentrations of 16, 32, 64, and 128 μg/mL and incubated at 37°C overnight.

Real-Time qPCR

Six *P. aeru* strains, S1, S2, S3, R1, R2, and R3, were grown at 37°C overnight in nutrient broth with shaking at 250 rpm and harvested at stationary phase (OD600 = 1.5–1.7). The growth conditions for R3-EV and R3-AS1974 transformed strains were the same as described above, except for using tetracycline at 128 mg/mL. Total RNA extracted from *P. aeru* was reverse transcribed using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). Real-time qPCR was performed on an ABI Fast 7500 real-time PCR machine (Applied Biosystems). All real-time PCR assays were run in a total reaction of 10 mL consisting of 1× SYBR Green Master Mix (Applied Biosystems) and 200 nM primers. Cycling conditions were 2 min at 50°C, then 10 min at 95°C, followed by 40 cycles of amplification (15 s at 95°C and 1 min at 60°C). Melting curves were determined by an auto-dissociation program. The housekeeping gene rpsL was used for normalization. The comparative CT method (2⁻ΔΔCT) was used to calculate the relative expression level of the target genes. The results were expressed as the means of at least 3 independent experiments. Data are presented as mean ± SD, and comparisons were analyzed using unpaired t test (two tailed: *p < 0.05, **p < 0.01, and ***p < 0.001).

RACE

5′ RACE was carried out using FirstChoice RML-RACE kit (Ambion) with a modified protocol. Total RNA was treated with Tobacco acid
pyrophosphatase (TAP). 5' RACE RNA adaptor was ligated to the TAP-treated RNA, followed by reverse transcription using random primers. Controls without TAP treatment and/or without adding adaptor were included. PCR was performed using a forward primer specific to the 5' RACE adaptor and reverse primers specific to target sRNAs. The amplified PCR fragment was subsequently cloned into pGEM-T easy vector (Promega) and then sequenced. TSSs of sRNAs were identified by sequencing the junction between the adaptor and the sRNAs.

**Methylation Analysis by Bisulfite Sequencing**

The bisulfite sequencing of genomic DNA (gDNA) was performed using EpiTect Plus DNA Bisulfite Kit (QIAGEN), according to the manufacturer’s protocol. Briefly, 0.5 μg gDNA of six clinical strains was added individually to the PCR tubes containing DNA Protect Buffer and Bisulfite Mix and placed into the thermal cycler for bisulfite conversion. The converted DNAs were then purified and subjected to PCR amplification with the primers located at ~150 and 50 nt of AS1974 sRNA for Sanger sequencing. The methylated cytosine residues that could not be converted to uracil showed the cytosine in sequencing results.

**ChIP-qPCR**

ChIP was performed as previously described with minor changes.51,52 Briefly, *P. aeruginosa* strains PA51530 (S1) and PA185388 (R3) were grown in 50-mL cultures to OD600 = 1.7 (stationary phase) and treated with 100 μL of formaldehyde (final concentration of 1%) for 20 min at room temperature. The cross-linking reactions were quenched by cacodylate at the final concentration of 250 mM. The cells were harvested by centrifugation, washed twice with ice-cold Tris-buffered saline (20 mM Tris-HCl [pH 7.5], 150 mM NaCl), and stored at −80°C. The pellets were resuspended in immunoprecipitation buffer (50 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, and Roche complete protease inhibitor cocktail) and sonicated by bioruptor (Bioruptor Plus, Diagenode) to shear DNA to an average size of 0.25–1 kb. Cell debris was then removed by centrifugation, and the supernatant with an equal amount of protein concentration was used for the immunoprecipitation experiment. Gammabind G Sepharose (GE Healthcare), was incubated with the supernatant overnight at 4°C on a rotating wheel. The beads were then collected and washed twice with immunoprecipitation (IP) buffer, once with IP buffer plus 500 mM NaCl, once with wash buffer III (10 mM Tris-HCl [pH 8.0], 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet-P40, and 0.5% sodium deoxycholate), and once with Tris-EDTA buffer (pH 7.5). Immunoprecipitated complexes were eluted from the beads by treatment with 100 μL elution buffer (50 mM Tris-HCl [pH 7.5], 10 mM EDTA, and 1% SDS) at 65°C for 30 min. Samples were then treated with RNase A for 2 h at 37°C, and the cross-links were reversed by overnight incubation at 65°C in 0.5× elution buffer with proteinase K. The DNA after proteinase digestion was further purified using a DNA purification Kit (GE Healthcare) and used for qRT-PCR experiments, as described above.

**PAS1974-GFP Reporter Construction**

PAS1974-GFP reporter plasmids were constructed by fusing the promoter region of sRNAs AS1974, including 130 bp (AS1974-S)/112 bp (AS1974-L) upstream of the start codon, to GFP and cloned into pMMB66EH vector. Antibiotic marker tet-R from pACYC184 was also cloned into pMMB66EH in order to perform selection after transformation to the bacterial strains. pMMB66EH with the *gfp* gene but without a promoter was used as a negative control plasmid, whereas pMMB66EH with the *gfp* gene controlled by the Ptac promoter was used as a positive control plasmid. Three methylation sites (M1, M2, and M3) were individually mutated from C to T in the PAS1974-GFP plasmid. For promoter region, position −10 of the promoter region was mutated from TATCCT to CCCGGG. The plasmids were subsequently transformed into the *P. aeruginosa* drug-resistant strain R3 (PA185388) and drug-sensitive strain S2 (PA83365).

**PAS1974-GFP Reporter Assays**

Reporter strains were cultured in nutrient broth (NB) supplemented with 32 μg/mL (for S2) and 128 μg/mL (for R3) tetracycline at 37°C. Cultures were diluted 3-fold in NB and added to 96-well clear-bottom assay plates for measurement. NB only was used as blanks. After overnight growth, fluorescence signal was measured on a multi-plate reader with the excitation wavelength at 405 nm and the emission wavelength at 510 nm. Relative GFP fluorescence intensity was calculated as follows: the fluorescence intensity/OD600 nm at the test time point.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found with this article online at https://doi.org/10.1016/j.omtn.2019.02.011.

**AUTHOR CONTRIBUTIONS**

T.C.K.L. conceived the idea, designed and supervised the project, and wrote the manuscript. C.O.K.L., C.H., Q.P., J.L., N.W.S.L., and I.L.A. performed experiments. Q.H. and T.F.C. analyzed data. M.I. provided all *P. aeruginosa* strains. T.F.C., A.K., M.I., and E.C. provided advice.

**ACKNOWLEDGMENTS**

We sincerely thank the Food and Health Bureau, Hong Kong (11100462 and 11101518) from the HKSAR Government; Shenzhen Science and Technology Program from Shenzhen Government, China; and SRG-Fd (CityU 7004404) from City University of Hong Kong, Hong Kong for the financial support on this study. We would like to thank Dr. Ming Chan for his advice in ChIP-qPCR experiments.

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