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Molecular mechanisms of master regulator VqsM mediating quorum-sensing and antibiotic resistance in Pseudomonas aeruginosa

Haihua Liang¹,²,†, Xin Deng²,†, Xuefeng Li³, Yan Ye³ and Min Wu³

¹Key Laboratory of Resources Biology and Biotechnology in Western China, Ministry of Education, College of Life Science, Northwest University, Xi’an, ShaanXi 710069, China, ²Department of Chemistry and Institute for Biophysical Dynamics, The University of Chicago, Chicago, IL 60637, USA and ³Department of Basic Science, School of Medicine and Health Science, University of North Dakota, 501 North Columbia Rd, EJRF Building, Room 2726, ND 58203, USA

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ABSTRACT

Pseudomonas aeruginosa is a Gram-negative bacterium that causes a large number of opportunistic infections, especially in hospitals and in patients with cystic fibrosis, which leads to chronic infection of the lungs and is the main cause of early mortality (1). These infections are made possible through the production of an arsenal of virulence factors such as toxins, proteases, hemolysins and phenazines, many of which are regulated by quorum-sensing (QS) signals (2,3).

Bacteria are highly social organisms capable of sophisticated co-operative behaviors mediated via QS (4). It is a form of bacterial cell–cell communication utilized by many species to sense population density and coordinate cognate gene expression (5,6). The QS signaling network of P. aeruginosa is one of the most complicated QS systems in all bacterial species (2). P. aeruginosa possesses at least three well-defined QS systems including two N-acyl-homoserine lactone (AHL) based signaling systems (las and rhl) and one 2-alkyl-4-quinolones (AQs) based signaling system. The las system produces and responds to N-3-oxo-dodecanoyl homoserine lactone (3-oxo-C12-HSL), which is produced by the LasI synthase and recognized by the transcriptional regulator LasR (7,8). The rhl system produces N-butanoyl homoserine lactone (C₄-HSL), which is produced by the Rhl synthase and sensed by the transcriptional regulator RhlR. The las and rhl systems regulate over 10% of the P. aeruginosa genome (9). Besides 3-oxo-C₁₂-HSL and C₄-HSL, P. aeruginosa produces diverse AQs as the third group of QS signal molecules (10). The major AQ signals include 2-heptyl-3-hydroxy-4-quinolone (the Pseudomonas quinolone signal [PQS]) and 2-heptyl-4-quinolone (10,11). The AQ system is also involved in the regulation of virulence factor production, biofilm maturation and motility phenotypes

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium that contributes to bacterial homeostasis and pathogenicity. Although the AraC-family transcription factor VqsM has been characterized to control the production of virulence factors and QS signaling molecules, its detailed regulatory mechanisms still remain elusive. Here, we report that VqsM directly binds to the lasI promoter region, and thus regulates its expression. To identify additional targets of VqsM in P. aeruginosa PAO1, we performed chromatin immunoprecipitation (ChIP) followed by high-throughput DNA sequencing (ChIP-seq) and detected 48 enriched loci harboring VqsM-binding peaks in the P. aeruginosa genome. The direct regulation of these genes by VqsM has been confirmed by electrophoretic mobility shift assays and quantitative real-time polymerase chain reactions. A VqsM-binding motif was identified by using the MEME suite and verified by footprint assays in vitro. In addition, VqsM directly bound to the promoter regions of the antibiotic resistance regulator NfxB and the master type III secretion system (T3SS) regulator ExsA. Notably, the vqsM mutant displayed more resistance to two types of antibiotics and promoted bacterial survival in a mouse model, compared to wild-type PAO1. Collectively, this work provides new cues to better understand the detailed regulatory networks of QS systems, T3SS, and antibiotic resistance.

†To whom correspondence should be addressed. Tel: +86 029 88302132; Fax: +86 029 88302132; Email: lianghh@nwu.edu.cn
Correspondence may also be addressed to Min Wu. Tel: +1 701 777 4875; Fax: +1 701 777 2382; Email: min.wu@med.und.edu

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Recently, a new QS signal IQS (2-[2-hydroxyphenyl]thiazole-4-carbuldehyde) has been identified in *P. aeruginosa*. The disruption of IQS biosynthesis paralyses the *pqs* and *rhl* systems and attenuates bacterial virulence (13).

These QS systems are arranged hierarchically with the *las* system positively regulating both the *rhl* (14, 15) and *AQ* (16) systems. The *rhl* system negatively regulates the *AQ* system (17). Each of these systems is modulated by an array of additional regulators at both transcriptional and post-transcriptional levels. For example, the regulator RsaL acts as a major transcriptional repressor of the *las* system by directly binding to the *lasI* promoter, which controls the maximal levels of AHLs and thus virulence factor production (18, 19). Several factors involved in regulating the activation threshold of quorum-regulated genes have been identified, such as QscR (20) and QteE (21). Recently, we also have reported that VqsR directly binds to the *qscR* promoter to control *P. aeruginosa* QS-regulated phenotypes (22).

VqsM has been characterized as a global regulator of QS and virulence in *P. aeruginosa*. Transcriptional analysis revealed that approximately 300 genes were influenced by a *vqsM* mutation, which is highlighted by a group of virulence factors and QS-regulators (i.e. RsaL, LasI, LasR, VqsR) (23). Here, we attempt to explore the underlying regulatory mechanism of VqsM by searching for its direct targets via electrophoretic mobility shift assays (EMSA) and ChiP-seq experiments. The VqsM consensus sequence was identified by using the Multiple EM for Motif Elicitation (MEME) suite and verified by footprint assays *in vitro*, which led us to uncover several unprecedented regulatory pathways, such as VqsM/LasI, VqsM/ExsA and VqsM/NfxB. In sum, this work not only pinpoints the binding motif and direct *in vivo* targets of VqsM, but also demonstrates that VqsM plays important roles in tuning T3SS and antibiotic resistance.

**MATERIALS AND METHODS**

**Bacterial stains and culture conditions**

The bacterial strains and plasmids used in this study are listed in Table S1. *P. aeruginosa* PAO1 and derivatives were grown at 37°C on Luria-Bertani (LB) agar plates or in broth with shaking at 200 revolutions per minute (rpm). Antibiotics were used at the following concentrations: for *Escherichia coli*, gentamicin (Gm) at 10 μg/ml and ampicillin at 100 μg/ml; for *P. aeruginosa*, gentamicin (Gm) at 50 μg/ml in LB or 150 μg/ml in PIA (*Pseudomonas* Isolate Agar); Tetracycline at 150 μg/ml in LB or 300 μg/ml in PIA.

**Expression and purification of truncated, RsaL, and ExsA proteins**

The truncated gene encoding and full-length *rsaL* gene were polymerase chain reaction (PCR) amplified from *P. aeruginosa* chromosomal DNA by using the primers *vqs*Mpf/*vqs*Mpr and *rsa*Lpf/*rsa*Lpr (Supplementary Table S2). The PCR product was introduced into pMCSG7 (24) by ligation independent cloning to generate pMCSG7-*vqsM*. The resulting plasmid was transformed into BL21star (DE3) and pMCSG7-*rsaL*. 10 ml of overnight pre-cultures grown from a single colony were inoculated into 1 l of autoclaved LB medium containing 100 μg/ml ampicillin. The cells were grown at 37°C, 250 rpm to OD_600~0.6 and then the temperature was reduced to 16°C. Protein expression was induced with 1 mM IPTG (Iso-propyl β-D-1-Thiogalactopyranoside). The overnight culture was harvested at 4°C by centrifugation at 6300 g for 8 min. All subsequent steps were performed at 4°C. The pellet was suspended in 20 ml buffer A (10 mM Tris-Cl [pH 7.4], 500 mM NaCl, 1 mM DTT) and 10 mM PMSF (Phenylmethylsulfonyl fluoride). The cells were lysed by sonication and centrifuged at 12,000 rpm for 25 min. The supernatant was filtered through a 0.45 μm filter and applied to a Ni-NTA column. The column was washed with 5% buffer B (10 mM Tris-Cl [pH 7.4], 500 mM imidazole, 500 mM NaCl, 1 mM DTT) and eluted with a linear gradient from 5% to 100% buffer B over 40 ml. Peak fractions were pooled and kept at 4°C. The purity was verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Supplementary Figure S2A). The full-length exsA gene was PCR amplified from *P. aeruginosa* genomic DNA by using the primers ExsApf/ExsApr, and then cloned into pMCSG19. The resulting pMCSG19-exsA was transformed into a BL21star strain containing a plasmid pAK1037. The following purification procedures for ExsA were identical to those for VqsMt and RsaL.

**Electrophoretic mobility shift assays**

Various amounts of VqsM1 truncated proteins were incubated with different DNA probe (Supplementary Table S2) in 25 μl of the gel shift-loading buffer (20 mM Tris-Cl, pH 7.4, 50 mM KCl, 5 mM MgCl₂, 10% Glycerol and 3 μg/ml sheared salmon sperm DNA). After incubation at room temperature for 20 min, the samples were analyzed by 6% polyacrylamide gel electrophoresis in 0.5X TBE (Tris/Boric Acid/EDTA) buffer at 90 V for 90 min. The gels were stained by SYBR GOLD dye and subjected to screen on a phosphor screen (BAS-IP, Fuji).

**Dye primer based DNase I footprint assay**

The DNA footprint assay was followed as previously described (25). Briefly, a 301-bp promoter fragment of the *lasI* promoter region that encompasses bases from −246 to +55 was generated by PCR with primers *lasI*Pr (carrying 6-FAM at the 5’ end) and *lasI*Sr. 40 nM of one end 6-FAM-labeled *lasI* promoter probe was incubated with varying amounts of His6-VqsM protein ranging from 0 to 4 μM in gel shift-loading buffer (20 mM Tris-Cl, pH 7.4, 50 mM KCl, 5 mM MgCl₂, 10% Glycerol and 3 μg/ml sheared salmon sperm DNA). After several optimization experiments, the nuclease digestion was found to work best with 0.05 units of DNase I (New England Biolabs, NEB) per 20 μl reaction for 5 min at 25°C. The reaction was stopped with 0.25 M EDTA and extracted with phenol–chloroform–isoamylalcohol (25:24:1). Control digestions with the *lasI* promoter probe were done with 10 μM of bovine serum albumin (BSA) instead of His-VqsM. The DNA fragments were purified with the QIAquick PCR Purification kit (Qiagen) and eluted in 15 μL distilled water. About 5 μl of digested DNA was added to 4.9 μl HiDi formamide (Applied
Table 1. The Minimum Inhibitory Concentrations (MICs) of antibiotics against wild-type PAO1, ΔvqsM and ΔvqsM complemented (ΔvqsM/p-vqsM) strains in LB broth were determined.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>MIC (µg/ml)a</th>
<th>ΔvqsM</th>
<th>ΔvqsM/p-vqsM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>6.0 ± 1.0</td>
<td>192 ± 6.0</td>
<td>6.0 ± 1.0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>16.0 ± 2</td>
<td>256 ± 8.0</td>
<td>32.0 ± 2.0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>6.0 ± 1.0</td>
<td>6.0 ± 1.0</td>
<td>6.0 ± 1.0</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>80 ± 4.0</td>
<td>80 ± 4.0</td>
<td>80 ± 4.0</td>
</tr>
</tbody>
</table>

aMICs (Minimum Inhibitory Concentrations) were determined by serial 2-fold dilutions in LB medium. The MIC presents the concentrations at which no obvious growth was observed after 24 h of incubation at 37°C. The values are the modes of three independent experiments.

Biosystems) and 0.1 µl GeneScan-500 LIZ size standards (Applied Biosystems). The samples were analyzed with the 3730 DNA Analyzer, with G5 dye set, running an altered default genotyping module that increased the injection time to 30 s and the injection voltage to 3 kV, in the sequencing facility at the University of Chicago. Results were analyzed with Peak Scanner (Applied Biosystems).

ChIP-seq analysis

Chromatin immunoprecipitation (ChIP) was performed as previously described (26) with minor changes. Wild type P. aeruginosa MPAO1 containing empty pAK1900 or pAK1900-VqsM-VSV was cultured in LB medium supplemented with ampicillin until the mid-log phase (Optical Density; OD = 0.6), before it was treated with 1% formaldehyde for 10 min at 37°C. Cross-linking was stopped by addition of 125 mM glycine. Bacterial pellets were washed twice with a Tris buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl), and then re-suspended in 500 µl IP buffer (50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, mini-protease inhibitor cocktail (Roche) and the DNA was sonicated to 100–300 bp. Insoluble cellular debris was removed by centrifugation and the supernatant used as input sample in IP experiments. Both control and IP samples were washed by protein A beads (General Electric), and then incubated with 50 µl agarose-conjugated anti-VSV (vesicular stomatitis virus) antibodies (Sigma) in IP buffer. Washing, crosslink reversal, and purification of the ChIP DNA were conducted by following previously published protocols (26). DNA fragments (150–250 bp) were selected for library construction and sequencing libraries prepared using the NEXTflexTM ChIP-Seq Kit (Bioo Scientific). The libraries were sequenced using the HiSeq 2000 system (Illumina). ChIP-seq reads were mapped to the P. aeruginosa genomes, using TopHat (version 2.0.0) with two mismatches allowed (27). Only the uniquely mapped reads were kept for the subsequent analyses. The enriched peaks were identified using MACS software (version 2.0.0) (28), which was followed by MEME analyses to generate the VqsM-binding motif (29). The ChIP-Seq data files have been deposited in NCBI’s (National Center of Biotechnology Information) Gene Expression Omnibus (GEO) and can be accessed through GEO Series accession number GSE57284.

Construction of P. aeruginosa vqsM gene deletion mutant

For vqsM gene replacement, a sacB-based strategy was employed (30). To construct the VqsM null mutant (ΔvqsM), PCRs were performed to amplify sequences upstream (1500 bp) and downstream (1503 bp) of the intended deletion. The upstream fragment was amplified from PAO1 genomic DNA using primers VqsMmfl (with EcoRI site) and VqsMmmr1 (with XbaI site), while the downstream fragment was amplified with primers, VqsMmmf2 (with XbaI site) and VqsMmmr2 (with HindIII site). These primers are listed in Supplementary Table S2. The two PCR products were digested with EcoRI–XbaI or XbaI–HindIII, respectively, and then cloned into EcoRI/HindIII digested gene replacement vector pEX18aap via a three-piece ligation, which yielded pEX18aap-VqsM. A gentamicin resistance cassette was digested from pPS858 (31) with XbaI. The fragment was cloned into XbaI digested pEX18aap-VqsM. The resulting plasmid, pEX18aap-VqsMGm, was electroporated into wild-type PAO1 with selection for gentamicin resistance. Colonies were screened for gentamicin resistance, carbenicillin sensitivity and loss of sucrose (5%) sensitivity, which typically indicates a double crossover event and thus the occurrence of gene replacement. The ΔvqsM strain was further confirmed by PCR and Southern blot analysis.

Construction of the promoter-reporter plasmids

The plasmid pMS402 carrying a promoterless luxCDABE reporter gene cluster was used to construct promoter-lux fusions of exsA or mftA as reported previously (32,33). These promoter regions were amplified by PCR using the primers shown in Supplementary Table S2 and cloned into the BamHI-Xhol site upstream of the lux genes in pMS402 and cloned into the BamHI-Xhol site upstream of the lux genes in pMS402. The construct was transformed into PAO1 strains by electroporation. Cloned promoter sequences were confirmed by DNA sequencing.

Luminescence screening assays

Expression of lux-based reporters from cells grown in liquid culture was measured as counts per second of light production in a Victor3 Multilabel Plate Reader (Perkin-Elmer, USA) or Synergy 2 (Biotek) as previously described by our group (33). Overnight cultures of the reporter strains were diluted to an A600 of 0.2 and cultivated for an additional
2 h before use. The cultures were inoculated into parallel wells of a black 96-well plate with a transparent bottom. A 5-μl volume of the fresh cultures was inoculated into the wells containing a total volume of 95 μl medium plus other components, and the A_{600} value in the wells was adjusted to around 0.07. A 60-μl volume of filter-sterilized mineral oil was added to prevent evaporation during the assay. Promoter activities were measured every 30 min for 24 h. Bacterial growth was monitored at the same time by measuring the OD at 595 nm in a Victor3 Multilabel Plate Reader.

**Western blot hybridization**

Overnight cultures of the *P. aeruginosa* strains containing ExsA-FLAG in CTX1 plasmid were transformed into the same fresh LB medium to an A_{600} of 0.02 and cultivated for additional 3 h. 100-μl cultures were centrifuged and the pellets were resuspended in 10 μl phosphate buffered saline (PBS) before mixed with an equal volume of 2×SDS loading buffer. After boiling for 5 min, the samples were subjected to SDS-PAGE and then transferred to nitrocellulose membrane (Life technologies). The membrane was blocked with 5% BSA and incubated with anti-FLAG antibody (1:8000) from mouse (Sigma) at 4°C overnight; then the blot was incubated with horseradish-peroxidase-conjugated secondary antibody (goat anti-mouse IgG). Signals were detected by a luminal enhancer solution detection kit (Thermo Scientific).

**RNA isolation**

To isolate the RNA for quantitative real-time PCR (RT-qPCR) analysis, all *P. aeruginosa* strains were grown at 37°C overnight in brain--heart infusion broth (BHI), diluted 100-fold in fresh 10 ml BHI in a 50-ml conical tube (BD Biosciences), and incubated at 37°C with shaking at 250 rpm for 6 h. The bacteria were harvested and disrupted mechanically (Fast Prep FP120 instrument; Qbio-gene). The RNaseasy Mini Kit (Qiagen) was used for the subsequent RNA purification. RNA concentration and purity were determined by UV absorption at 260 and 280 nm.

**Quantitative RT-PCR (RT-qPCR)**

RT-qPCR analysis was performed with SuperScript III Platinum SYBR Green One-Step qPCR Kit w/ROX (Invitrogen) and the ABI 7300 Real-Time PCR System. The 96-well RT-qPCR plate was prepared by following the manufacturer’s recommendation. Five nanograms of each sample were added into each well. Each reaction was performed in triplicate in 25-μl reaction volumes, with 16S rRNA as a control. For each reaction, 200 nM primers (Supplementary Table S2) were used for RT-qPCR.

**Biofilm formation assay**

Biofilm formation was measured in a static system as previously described (34) with minor modifications. Cells from overnight cultures were inoculated at 1:100 dilutions into LB medium in polystyrene tubes (Costar) and grown at 30°C for 10 h. A 250 μl volume of 1% crystal violet was added to each tube and stained for 15 min prior to removal by aspiration. Wells were rinsed three times by submerging the tubes in distilled water, and the remaining crystal violet was dissolved in 1 ml of 95% ethanol. A 1 ml portion of this solution was transferred to a new polystyrene tube, and the absorbance was measured at 600 nm.

**Animal infection and bacteria burden assay**

Bacteria were grown overnight in Luria-Bertani (10) broth at 37°C with gentle shaking. The next day, the bacteria were pelleted by centrifugation at 5000 × g and resuspended in 10 ml of fresh LB broth and allowed to grow until the mid-logarithmic phase. OD_{600} nm was measured, density was adjusted to ~0.25 OD (0.1 OD = 1 × 10^6 cells/ml). C57BL6 mice, a strain sensitive to *P. aeruginosa* infection, were purchased from the Harlan Laboratory (Indianapolis, IN). The animal experiments have been approved by the University of North Dakota institutional animal care and use committee. Mice were randomly assigned to different group (six each group), and were lightly anesthetized with 20 mg/kg ketamine plus 5 mg/kg diazepam. Then we intranasally instilled 5 × 10^6 colony-forming units (CFUs) of *P. aeruginosa* and monitored the animals with infection for up to 72 h (35,36). Intranasal instillation of equal amount of PBS was performed as controls. Moribund mice were euthanized to obtain the lung for analysis. After bronchoalveolar lavage (BAL), the trachea and lung were excised for homogenization. In selected experiments to check the efficiency of instillation, we used intratracheal instillation and ventilation procedures to confirm evenness of distribution in the lung. Alveolar macrophages (AM) from BAL fluid and ground lung were homogenized with PBS containing 0.1% Triton X-100, and were spread on LB agar plates to enumerate bacterial levels. Fifty microliter of the homogenates or AM cells was inoculated to plain agar plate and grown in 37°C incubator overnight and colonies were counted. Bacterial clearance was calculated by comparing with the control (37). Triplicates were done for each sample and control.

**Nitroblue tetrazolium assay**

This assay is widely used to measure the superoxide release of cells. AM cells isolated from lavage fluid were cultured in 96-well plates and incubated at 37°C with 5% CO_2 overnight. One microgram/milliliter nitroblue tetrazolium (NBT) dye (Sigma, St Louis, MO) was added to each well of the plate following the manufacturer’s instructions. The yellow color NBT can change to blue upon reduction by released superoxide (38). The reaction was terminated by adding 10 μl of stop solution as above. The plate was left at room temperature overnight for complete dissolution of dye product, and a multiscan plate reader to quantify the dye conversion read the absorbance at 560 nm. Each experiment was conducted in triplicate (37).

**Histological analysis**

After BAL procedures and serum collection, lung and other tissues were fixed in 10% formalin or Optical Cutting Temperature compound (Sakura Finetek USA, Torrance, CA)
using a routine histologic procedure (39). Ten microliter of BAL and serum were applied on a microscope slide. After staining with a HEMA kit (ThermoFisher), the numbers of polymorphonuclear leukocyte were counted using a light microscope. Homogenizations of lung and other tissues were done using liquid nitrogen and then dissolved in radioimmunoprecipitation assay buffer and sonicated for three times at 10 s with 10 s intervals for next analysis. The formalin-fixed tissues were used for hematoxylin and eosin (H&E) staining to examine tissue damage post-infection (40).

**Growth tests of *P. aeruginosa* in the presence or absence of antibiotics**

Effects of antibiotics on the growth of strains including wild-type PAO1, $\Delta vqsM$ and $\Delta vqsM$ complementary ($\Delta vqsM/p-vqsM$) were tested by measuring growth with Bioscreen C and EZExperiment software (Growth Curves USA). In brief, $\sim 2 \times 10^5$ cfu of bacteria was inoculated into 200 $\mu$l of LB broth containing a designated amount of antibiotics and incubated for 24 h with interval shaking at 37°C. For data stability, the bacterial growth was measured by OD$_{600}$.

**RESULTS**

**VqsM directly binds to *lasI* promoter region**

Alignment of VqsM with other AraC proteins including AraC (*E. coli*) and ExsA shows that VqsM carries a predicted helix-turn-helix DNA binding domain at the C terminal (aa 240–325), which is conserved among them (Supplementary Figure S1). In order to perform EMSA assays that would reveal direct targets of VqsM, we sought to express and purify the full-length VqsM in *E. coli*, which unfortunately largely formed insoluble inclusion bodies. We then tested 10 different VqsM truncated versions (Supplementary Figure S2A), and obtained a soluble protein that contains the predicted DNA-binding domain (C185–325, designated as VqsM$^t$) (Supplementary Figure S2B). VqsM$^t$ was used in the following biochemical assays throughout the study. Given that VqsM tunes expression of many QS-related genes including *rhlR*, *lasI*, *rhlI*, *vqsR* and *rsaL*, we speculated that VqsM might directly control these QS genes via interaction with their promoter regions. In order to test this hypothesis, the DNA-binding properties of VqsM$^t$ were investigated by performing EMSA using different DNA probes encompassing the promoter regions of these QS-related genes. Interestingly, the VqsM$^t$ protein can directly bind to the *lasI-rsaL* intergenic region (Figure 1A), but not to other tested promoter regions (Supplementary Figure S2C). This result indicates that VqsM directly regulates *lasI*, and thus controls other QS-genes downstream of the *lasR*/I cascade. To verify the activity of truncated VqsM and the full-length VqsM-VSV (For next ChIP-seq assay) *in vivo*, the expression of *lasI* was measured in the wild-type PAO1, the $\Delta vqsM$ and the $\Delta vqsM$ complemented ($\Delta vqsM$ carrying p-VqsM, p-VqsM$^t$ and p-VqsM-VSV, respectively) strains. The activity of *lasI-lux* in vqs$M$ mutant with complemented plasmids could be restored to wild-type levels (Supplementary Figure S3), indicating that VqsM$^t$ and VqsM-VSV are as functional as the full length VqsM.

To further characterize the specific DNA sequence that VqsM recognizes in the intergenic region between *rsaL* and *lasI*, a dye-primer-based DNase I footprint assay was performed on both strands of a DNA fragment encompassing the entire intergenic region (Supplementary Table S2). Using Peak Scanner Software (Applied Biosystems), we compared the electropherograms with and without VqsM$^t$ (Figure 1B), which uncovered a specific VqsM$^t$-protected region containing a putative 15-bp motif (5′-TGATCTTTTCGGACG-3′, -102 to -88 from the *lasI* transcriptional starting site) (Figure 1C). The lack of dyad symmetry in this motif suggests that VqsM binds DNA as a dimer. Subsequently, we repeated the EMAS using a truncated *lasI* probe without the 15-bp motif, which abolished the binding of VqsM$^t$ (Figure 1D). This result confirmed that the motif is crucial to the DNA-binding ability of VqsM. We further performed the EMAS using a group of *lasI*-p probes containing the VqsM motif with difference point mutations that were introduced by PCR (mutated in each pair of specific nucleotides in the binding motif, Supplementary Table S2). VqsM$^t$ displayed much lower affinities with mutated probes 99G/T, 98A/G, 97T/G, 95T/G, 94T/C, 90G/T, 89G/A and 87C/A (Supplementary Figure S4), indicating that these nucleotides are essential for the interaction between VqsM and its own cognate motif.

**Genome-wide analysis of the VqsM-binding regions by ChIP-seq**

The identification of the VqsM motif in the *lasI* promoter led us to globally characterize all VqsM-binding loci on the chromosome of *P. aeruginosa* using the ChIP-seq (ChIP followed by high throughput DNA sequencing) experiments (26). VSV-tagged full length VqsM was overexpressed from plasmid pAK1900 and then transformed into a wild-type strain. Sequence reads were obtained from two independent ChIP-seq assays using VSV specific antibody and mapped to the *P. aeruginosa* genome. Using MACS software (28), we identified 48 enriched loci (*P*-value $< 0.5$) harboring VqsM-binding peaks (Supplementary Table S3), which were enriched by >1.5-fold but were absent in control samples using wild type PAO1 without any VSV tags. These 48 loci are located across the genome and were sited both in intergenic regions (50%) and within coding regions (50%), suggesting that VqsM is a global transcriptional regulator in *P. aeruginosa* (Figure 2A).

Diverse functions are encoded by VqsM-bound genes include QS, virulence, regulatory proteins, transporters, metabolism and hypothetical proteins (Figure 2B). Notably, VqsM directly binds to several QS and transcription regulator genes, such as its own promoter (Figure 2C) and *pvdQ* that encodes 3-oxo-C$_7$-homoserine lactone acylase (41,42). VqsM also directly binds to a group of conserved regulatory proteins, including PA2005, PA2588, PA3565 and PA5324. It has been recently reported that PA5324 (SphR) is a sphingolipid-responsive transcription factor in *P. aeruginosa* (43). In addition, more than one-third of the VqsM-binding sites are various metabolic genes involved in several pathways including TCA metabolism (*aacA*), DNA repair
The VqsM protein directly binds to the lasI promoter region. (A) EMSA experiment showed that vqsM directly binds to rsaL-lasI intergenic region but cannot bind to the lasR promoter. PCR products containing the rsaL-lasI or lasR promoter regions were added to the reaction mixtures at 50 nM each. VqsMt protein was added to reaction buffer in lanes with 0.1, 0.2, 0.5, 1.0 μM, respectively. No protein was added in Lane 1. (B) VqsMt directly binds to the motif (TGATCTTTTCGGACG) in the lasI promoter region. Electropherograms showing the protection pattern of the lasI promoter region after digestion with DNase I following incubation in the absence or presence of 400 nM VqsMt. The region of interest identifies the area that shows significant reduction in the peak pattern compared with the control. (C) Sequence of the lasI promoter region. The lasI ATG starting codon is in boldface and underlined, and the nucleotides complementary to the starting codon of rsaL (CAT) are in boldface and double underlined, and the asterisk represents the transcriptional starting site. The sequence protected by VqsMt in the DNase I protection assay is boxed by a rectangle. The sequence protected by LasR is boldface, and RsaL-binding sequence is underlined. (D) Mutation in the binding motif affects the DNA-binding affinity of VqsMt. EMSA experiment showed that VqsM could not bind to the region without the binding motif. PCR products containing the rsaL-lasI intergenic region (lasI-p), lasI-p1 (start from the binding motif) and lasI-p2 (without the binding motif) regions were added to the reaction mixtures at 40 nM each.

Validation of ChIP-seq results in vitro and in vivo

As aforementioned, we have identified 48 enriched loci harboring VqsM-binding peaks in the P. aeruginosa genome (Supplementary Table S3). To validate the ChIP-seq data, five promoter regions (PA2227/PA2228, PA2588, PA5324, PA3106 and PA3342) of VqsM target genes were tested in EMSA with VqsM in vitro, and the rhlR promoter was used as a negative control. As shown in Figure 3, VqsMt efficiently bound to all five probes in a concentration-dependent manner, while the negative control rhlR promoter still remained unbound even at the highest protein concentration.

To further confirm VqsM-binding peaks in vivo, we next sought to investigate the gene expression of several newly identified VqsM-dependent targets by performing quantitative RT PCR in the wild type, the ΔvqsM, and the complemented (ΔvqsM/p-vqsM) strains. A non-peak region (ahlF) was used as a negative control. The expression of PA2227/PA2228, PA2588, PA5324, PA3106 and PA3342, but not ahlF, was clearly affected by deletion of vqsM (Supplementary Figure S5). The decreased expression of PA2588 in ΔvqsM strain is consistent with a previous study (23). Together, these results strongly confirm the high accuracy of the ChIP-seq results.
Figure 2. ChIP-seq reveals in vivo binding sites of VqsM on the PAO1 chromosome. (A) Pie chart of 48 VqsM-binding peaks. (B) Pie chart displaying the percentage of VqsM targets with functional categories defined in the *Pseudomonas* database (http://pseudomonas.com). (C) VqsM binds to its own promoter region from the ChIP-seq experiment. (D) Most significant motif derived from ChIP-seq binding sequence returned by the MEME tool (29).

VqsM directly binds to *exsA* and controls the type III secretion system

Given that the *lasI* promoter was missing from the ChIP-seq peaks, we next sought to comprehensively search for targets of VqsM by using the VqsM motif (*GG[A/T]T/C/G][C/G][T/A][G][C/A][G][C/T/A][T/C]CGGCCA*) that we have identified. The *P. aeruginosa* genome was searched for the motif by using the Regulatory Sequence Analysis Tools (RSAT, http://rsat.ulb.ac.be/rsat/). Among these potential VqsM-bound promoters, we selected some interesting ones that were not included in the ChIP-seq data. For example, we found that the promoter region of *exsA* and *nfxB* shares a conserved sequence including the VqsM motif (Figure 4A).

Type III secretion system (T3SS) is a specialized secretion system that facilitates the delivery of bacterial effector proteins into eukaryotic host cells (44). Previous studies show that the expression of T3SS genes is tightly regulated and under the direct transcriptional control of the master regulator ExsA, a member of the AraC family of transcriptional activators (45). The *P. aeruginosa* T3SS translocates four effectors (ExoS, ExoT, ExoU and ExoY) with anti-host properties (46). *exsA* has been previously shown to be co-transcribed with *exsC*, *exsE* and *exsB* in the same operon, which is driven by the promoter upstream of *exsC* (47). In addition, the intergenic region upstream *exsA* displayed a relative weak promoter activity, suggesting that it contains a separate promoter. This is supported by the identification of a transcription start site in front of *exsA* (48) identification of a potential VqsM motif in the upstream region of *exsA* led us to determine if there is a direct interaction between VqsM and the *exsA* promoter. As expected, VqsM indeed directly bound to the *exsA* promoter region (Figure 4B).

Given this direct interaction, we next attempted to determine whether the expression of *exsA* is regulated by *vqsM* in vivo. To this end, we constructed an *exsA* promoter-*lux* fusion (*exsA-*lux, Supplementary Table S1) and then measured its activity in the wild-type PAO1, the Δ*vqsM* strain and its complemented strain (Δ*vqsM*/p-*vqsM*). As expected, the relative activity of *exsA-*lux in the *vqsM* mutant was about 6-fold lower compared to that of wild-type PAO1. Introduction of the plasmid p-*vqsM* into the *vqsM* mutant restored the *exsA-*lux activity to the wild-type level (Figure 4C). These results were also confirmed by immuno-
VqsM deletion mutant is more resistant to an array of antibiotics

Besides exsA, nfxB also carries a putative VqsM binding motif in its promoter (Figure 4A), which led us to test whether VqsM also directly bind to the nfxB promoter region. To this end, a fragment of nfxB promoter harboring the putative VqsM motif was amplified by PCR using corresponding primers (Supplementary Table S2). Expectedly, VqsM was able to retard the mobility of the nfxB promoter as identified by the EMSA (Figure 5A). To further test whether VqsM regulates the expression of nfxB in vivo, we constructed an nfxB promoter-lux (nfxB-lux, Supplementary Table S1) and then measured its activity in the wild type, the ΔvqsM strain and its complemented strain (ΔvqsM/p-vqsM). As shown in Figure 5B, the activity of nfxB-lux was 8-fold lower in the vqsM mutant compared to the wild type.
Figure 4. VqsM directly binds to the exsA promoter region and controls its activity. (A) Alignment of the genes (exsA, nfxB, lasI, PA2227/PA2228 and PA2588) with the consensus sequence by MEME suite. (B) EMSA experiment showed that VqsM directly binds to the exsA promoter region. PCR product containing the exsA promoter region was added to the reaction mixtures at 50 nM. VqsM protein was added to reaction buffer in lanes with 0.1, 0.2, 0.5, 1.0 μM, respectively. No protein was added in Lane 1. (C) The transcription level of exsA was elevated in wild-type PAO1, ΔvqsM and ΔvqsM complemented (ΔvqsM/p-vqsM) strains, respectively. The asterisks indicate that the expression of exsA in wild-type PAO1 and ΔvqsM complemented strains is statistically different from ΔvqsM strain as determined by a student’s t-test (P < 0.005). (D) Western blot confirms that the expression of exsA was drastically decreased in the ΔvqsM strain compared to the wild-type PAO1. The wild-type PAO1, the ΔvqsM, and the ΔvqsM complemented strains containing the integrated single-copy plasmid CTX-exsA-flag (Supplementary Table S1) were cultured at OD600 = 0.6 and OD600 = 1.0. The whole-cell extracts from the designated strains were subjected to SDS/PAGE separation and subsequent immuno-blotting.

with that in the wild-type PAO1, which could be fully restored by p-vqsM. Collectively, these data demonstrate that VqsM directly controls the nfxB expression at the transcriptional level by binding to its promoter region.

Given that a nfxB mutant is more resistant to norfloxacin (49), but more susceptible to β-lactam and aminoglycoside antibiotics (50), we envisioned that the vqsM mutant might display altered susceptibility to various antibiotics. To this end, we compared the growth of various P. aeruginosa strains in LB medium when supplemented with seven antibiotics including tetracycline, kanamycin, ciprovofloxacin, tobramycin, ceftazidime, polymixin B, and ampicillin, respectively. Interestingly, the ΔvqsM strain had a clear growth advantage in the presence of 100 μg/ml tetracycline or 60 μg/ml kanamycin than wild-type PAO1 and ΔvqsM complemented (ΔvqsM/p-vqsM) strains (Figure 5C and D). We further monitored the MICs of the wild-type PAO1, the ΔvqsM and the complemented (ΔvqsM/p-vqsM) strains to an array of antibiotics (Table 1). The ΔvqsM strain was 32-fold more resistant to tetracycline and 16-fold more resistant to kanamycin than the wild-type PAO1. Notably, the nfxB mutant and the vqsM mutant displayed different resistance to other antibiotics, such as ciprofloxacin and aminoglycosides, which suggests that other pathways rather than nfxB contribute to the response of VqsM to antibiotics. For example, mexR was shown to be upregulated in a vqsM mutant (23). A group of genes encoding transcription factors and transporters are directly targets of VqsM in our current study, which may play roles in the complicated regulation of antibiotic resistance by vqsM.
VqsM affects *P. aeruginosa* biofilm formation

Since VqsM directly regulates lasI and its product contributes significantly to the virulence-related phenotypes of *P. aeruginosa*, we sought to test biofilm production in the wild-type PAO1, the ΔvqsM and the complemented strain (ΔvqsM/p-vqsM) in polystyrene tubes. Biofilm production was determined by crystal-violet staining of the tubes where the bacteria were cultured. At 24 h after incubation at 37°C, the ΔvqsM strain produced significantly more biofilm than that of both the wild-type strain and the complemented strain. Moreover, constitutive expression of lasI in the ΔvqsM strain decreased its biofilm production (Supplementary Figure S7), which indicates that VqsM negatively regulated biofilm via LasI.

Lack of vqsM alters *P. aeruginosa* virulence and promotes its survival in a mouse model

The drastically changed QS-regulated genes and phenotypes in the ΔvqsM strain led us to test if vqsM also contributes to *P. aeruginosa* virulence in a mouse model of acute pneumonia. Therefore, C57BL/6 mice were infected intranasally with approximately 1 × 10^7* wild-type PAO1, ΔvqsM strain or complemented strain (ΔvqsM/p-vqsM). The Kaplan–Meier survival analysis showed that deletion of vqsM significantly improved mouse survival versus wild-type PAO1. We showed that ΔvqsM strain infection exhibited significantly decreased mortality with no death until 38 h and more than 70% of mice survived at 48 h, whereas wild-type PAO1 infection caused mouse death at 15 h post-infection with 50% and 83.3% mortality at 24 and 48 h, re-
The vqsM deletion mutant decreases the virulence of P. aeruginosa. C57BL6 mice were intranasally challenged with ΔvqsM, ΔvqsM complemented (ΔvqsM/p-vqsM) strains and wild-type PAO1 at 10⁷ cfu in 50 μL PBS and moribund mice were killed to obtain survival data (Kaplan–Meier Curve with Log-Rank test, \( P = 0.034, n = 6 \)). The vqsM deletion mutant (ΔvqsM) decreased lung injury and inflammatory response in mice (indicated by arrows). C57BL6 mice were intranasally challenged with vqsM deletion mutant (ΔvqsM), vqsM deletion mutant complemented (ΔvqsM/p-vqsM) and wild-type PAO1 at 10⁷ cfu in 50 μL PBS and mice were killed for histological analysis 48 h after infection (H&E stain, original mag ×400). Data were representative of six mice. Bacterial burdens of AM of BAL were detected by CFU assay. 5000 AM of each mouse incubated in a LB-agar dish at 37°C overnight (\( * P < 0.05 \)). Superoxide levels were determined by an NBT assay (One-Way ANOVA and Tukey Test, \( ** P < 0.01, n = 6 \)). Data were mean ± SEM and representative of three experiments.

DISCUSSION

The P. aeruginosa QS systems are sophisticatedly regulated and integrated with the global regulatory networks within the bacterium. However, the detailed impacts and molecular mechanisms of a group of important QS regulators on acyl-HSL productions and their interactions with las, rhl and PQS systems remain unknown. Although a previous study showed that a vqsM mutation has dramatic effects on acyl-HSL production and QS-regulated virulence factors (23), the detailed regulatory mechanism has yet to be elucidated. By testing an array of truncated VqsM constructs, we obtained a highly soluble protein that was used in all biochemical assays in this study (Supplementary Figure S2A). EMSA analysis showed that VqsM directly binds to the lasI promoter region (Figure 1A), which explains that the lasI transcription level was compromised in the vqsM mutant. Two other QS regulators LasR and RsaL have been identified to directly bind to the lasI promoter region (19,51). Furthermore, we also demonstrated that VqsM binds to a conserved motif (TGATCTTTTCG-AACG), which is located between -102 and -88 from the lasI transcriptional starting site. By blasting the VqsM
Figure 7. Schematic diagram of VqsM regulation in the QS regulatory cascade of *P. aeruginosa*. The potential regulatory pathways and interplays of VqsM are proposed according to our observations and previous studies. The typical QS systems including the *las*, *rhl* and PQS systems and their interactions were summarized based on previous reports. In the present study, we showed that VqsM directly binds to the *lasI* promoter region and regulates the expression of *lasI*, while indirectly regulating the Rhl system. In addition, the VqsM directly binds to the *exsA* and *nfxB* promoter regions. Solid arrows indicate positive regulation and solid T-bars present negative regulation.

amino acid sequence using the BLASTP, we did not find significant homology with functionally characterized proteins in *P. aeruginosa*. VqsM belongs to the AraC-family protein was shown to directly control Las-QS in this bacterium. Unlike some identified QS regulators, such as QscR and LasR, which need special signals to bind to their cognate DNA, the DNA binding of VqsM was independent of the known signals such as 3OC12-HSL, C4-HSL or PQS (Supplementary Figure S8). A possible signal domain may be removed from the truncated VqsM that may need an uncharacterized signal. Given that LasR and RsaL also bind to the *lasI* promoter, we tested whether they compete with VqsM. Only addition of RsaL, not LasR, changed the DNA-binding pattern of VqsM by forming a supershift, suggesting that RsaL can compete with VqsM in DNA binding (Supplementary Figure S9A and B). We also performed EMSA by mixing VqsM with ExsA, another AraC-family protein. Addition of ExsA generated a weak supershift, which suggests that a protein–protein interaction between VqsM and ExsA is probably not a major regulation of DNA binding of VqsM. The evolvement of other protein–protein interactions or transcriptional regulation of VqsM may play bigger roles in tuning its DNA binding (Supplementary Figure S9C).

In this study, we have developed a ChIP-seq method to identify all potential *in vivo* binding sites of VqsM in *P. aeruginosa*. Through analysis of the chromosome-wide DNA-binding profiles, 48 targets were detected. The majority of the genes (37%) are involved in metabolism, indicating the profound roles of VqsM in a variety of metabolic pathways. Among other major VqsM targets are several transcription factors, such as *vqsM* itself, and PA2588 whose functions have not yet been reported. Recently, it has been reported that PA5324 (SphR) is a sphingosine-responsive transcription factor in *P. aeruginosa*, suggesting the regulatory role of VqsM in the sphingosine pathway (29). Our results provide the new cues to study the interaction between *P. aeruginosa* and its host. In addition, we also found that the PA5324 protein regulates the pyocyanin production (data not shown), which is strictly controlled by QS. We plan to study the function and regulation of PA5324 and PA2588 proteins in the future. Confirmation of VqsM binding to these sites was verified *in vitro* by using VqsM (Figure 3), demonstrating the high accuracy of ChIP-seq data. We also defined a consensus VqsM-binding sequence (GG[A/T][T/A][A/T]/
assays (Figure 2B).

Although missing in the ChIP-seq data, lasI, exsA and nfxB were identified as novel direct targets of VqsM via biochemical and bioinformatic analyses, which have been experimentally verified in vivo and in vitro. At least two possibilities might explain the omission of these genes in the ChIP-seq results. First, we observed high unspecific peaks corresponding to these three genes in the control sample without VSV antibody, suggesting the possible limitation in application of this antibody in our ChIP-seq procedure. Second, the interaction between VqsM and these missing targets might not be strong enough in the culture conditions used in the ChIP-seq protocol (in pAK1900 vector, VSV tagged, mid-log phase). Although the ChIP-seq needs optimization in the future, the combinations of the ChIP-seq analyses and biochemical assays are powerful tools to comprehensively pinpoint the targets of VqsM in vivo.

Moreover, our data also provide insights into the importance of the VqsM in the pathogenicity and surface-associated behaviors of P. aeruginosa. We examined the ability of the mutated vqsM to maintain pulmonary infections in a mouse model. When infected within 48 h, mice inoculated with the vqsM mutant showed a significantly lowered bacterial load compared to those infected with wild-type PAO1 (Figure 6A). We also demonstrated that the vqsM mutant infection exhibited decreased lung injury in mice and decreased superoxide production in host macrophage cells compared to wild-type PAO1 (Figure 6B–D). The observed pathogenesis loss is consistent with the decreased activity of Type III secretion genes (Supplementary Figure S6), which may be a potential mechanism to evade the host defense in an acute infection model. Thus, these critical in vivo results indicate that VqsM is physiologically relevant to the infection of mammals and may prove to be clinically significant. On the other hand, the different regulation of VqsM in biofilm (Supplementary Figure S7) is similar to observations of a sadB mutation that utilizes a chemotaxis-like regulatory pathway to irregularly regulate two key surface behaviors (52), suggesting multifaceted regulatory mechanisms connecting both biofilm formation and virulence.

The results presented in this work provide an improved understanding of the complex regulatory mechanisms involved in lasI transcription and provide an integrative picture of the P. aeruginosa QS networks (Figure 7). By directly binding to lasI, exsA, nfxB, and at least 48 other loci in the genome, VqsM sits on a center position mediating a large group of cellular pathways ranging from QS to antibiotic resistance, and P. aeruginosa pathogenicity. Our ChIP-seq results provide a useful database that would allow us to characterize more functions and targets such as PA2588 and PA5324 of VqsM in the future. We envision that tuning its expression and developing an inhibitor are of importance for the development of strategies to control P. aeruginosa acute infections.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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