

Comparative Systems Biology Analysis To Study the Mode of Action of the Isothiocyanate Compound Iberin on *Pseudomonas aeruginosa*

Sean Yang-Yi Tan,^{a,b} Yang Liu,^a Song Lin Chua,^{a,c} Rebecca Munk Vejborg,^d Tim Holm Jakobsen,^d Su Chuen Chew,^{a,e} Yingying Li,^{a,b} Thomas E. Nielsen,^f Tim Tolker-Nielsen,^d Liang Yang,^{a,b} Michael Givskov^{a,d}

Singapore Centre on Environmental Life Sciences Engineering (SCELSE), Nanyang Technological University, Singapore^a; School of Biological Sciences, Nanyang Technological University, Singapore^b; NUS Graduate School of Integrative Sciences and Engineering, National University of Singapore, Singapore^c; Costerton Biofilm Center, Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen N, Denmark^d; Interdisciplinary Graduate School, Nanyang Technological University, Singapore^e; Department of Chemistry, Technical University of Denmark, Kongens Lyngby, Denmark^f

Food is now recognized as a natural resource of novel antimicrobial agents, including those that target the virulence mechanisms of bacterial pathogens. Iberin, an isothiocyanate compound from horseradish, was recently identified as a quorum-sensing inhibitor (QSI) of the bacterial pathogen *Pseudomonas aeruginosa*. In this study, we used a comparative systems biology approach to unravel the molecular mechanisms of the effects of iberin on QS and virulence factor expression of *P. aeruginosa*. Our study shows that the two systems biology methods used (i.e., RNA sequencing and proteomics) complement each other and provide a thorough overview of the impact of iberin on *P. aeruginosa*. RNA sequencing-based transcriptomics showed that iberin inhibits the expression of the GacA-dependent small regulatory RNAs RsmY and RsmZ; this was verified by using *gfp*-based transcriptional reporter fusions with the *rsmY* or *rsmZ* promoter regions. Isobaric tags for relative and absolute quantitation (iTRAQ) proteomics showed that iberin reduces the abundance of the LadS protein, an activator of GacS. Taken together, the findings suggest that the mode of QS inhibition in iberin is through downregulation of the Gac/Rsm QS network, which in turn leads to the repression of QS-regulated virulence factors, such as pyoverdine, chitinase, and protease IV. Lastly, as expected from the observed repression of small regulatory RNA synthesis, we also show that iberin effectively reduces biofilm formation. This suggests that small regulatory RNAs might serve as potential targets in the future development of therapies against pathogens that use QS for controlling virulence factor expression and assume the biofilm mode of growth in the process of causing disease.

Quorum sensing (QS) is a cell-to-cell communication system widely distributed among bacteria in which small diffusible signal molecules are employed to regulate gene expression in a dose-dependent manner (1). After reaching a threshold concentration, the QS signal molecules will bind to and activate their receptors, which results in a coordinated population expression of QS-regulated genes. These genes include those that upregulate the synthesis of QS signal molecules (autoinduction) but, more importantly, they also include genes that encode virulence factors required for bacterial infections (2). Thus, QS inhibitors (QSIs) have been proposed as antipathogenic agents and have been shown to attenuate the capability of pathogens to cause infections (3, 4). QSIs possess different modes of action, including interfering with the synthesis of quorum-sensing signaling molecules (5) or competitively binding to the QS signal receptors (6). The regulation of the bacterial QS systems is complex, and this further expands the targets for the design of novel QSIs (7, 8).

Isothiocyanates (ITCs) are biologically active compounds found in cruciferous vegetables and have gained research interest as cancer chemopreventive agents (9). Sulforaphane (SFN) (10), allyl isothiocyanate (AITC) (11), and phenethyl isothiocyanate (PEITC) (12) are examples of ITCs with such cancer-preventing activities. In addition to their cancer-preventing activities, ITCs are also known for their antimicrobial activity (13, 14).

Recently, Jakobsen et al. (15) described several ITC-containing compounds (iberin, cheirolin, iberiverin, and alyssin) found in various foods; a few of the tested food extracts were found to actively inhibit QS in *Pseudomonas aeruginosa*, particularly the *las* and *rhl* system. Among these ITCs, iberin (Fig. 1), which the authors identified as the QSI component of horseradish, had the

greatest QS-inhibiting effect. Using a DNA microarray approach, iberin was found to inhibit 49 QS-controlled genes, including *lasB*, *rhlAB*, *chiC*, *lecA*, *pivA*, and *phz*. This study also suggested that iberin inhibits the *rhl* QS system (but not the *las* QS system) by blocking the interaction of *N*-butanoyl homoserine lactone (C_4 -HSL) with its cognate receptor RhlR. The mode of inhibition in iberin was speculated to occur through competition with acyl-homoserine lactone (AHL) molecules for their receptor; however, iberin bears weak structural similarity to these molecules.

Therefore, we decided to elucidate the underlying mechanisms of the inhibition of QS by iberin in *P. aeruginosa*. With cRNA sequencing (RNA sequencing [RNA-Seq])-based transcriptomics and isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomics approaches, we investigated the various genes and proteins affected by iberin treatment. We also show that iberin inhibits the expression of the small regulatory RNAs *rsmY* and *rsmZ*, which in turn regulate the levels of pyoverdine production (16). Hence, we propose that the mode of inhibition by iberin is through targeting the Gac/Rsm network in *P. aeruginosa*. Lastly,

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Address correspondence to Liang Yang, yangliang@ntu.edu.sg.

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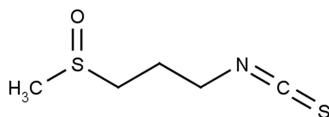


FIG 1 Chemical structure of the isothiocyanate compound 1-isothiocyanato-3-(methyl-sulfinyl) propane, otherwise known as iberin.

we show that iberin effectively inhibits *P. aeruginosa* biofilm formation. As such, iberin and ITCs therefore are an interesting class of QSIs with a novel mode of action, and the use of systems biology analyses provides insight for the development of dual functioning antivirulence and antibiofilm drugs.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* strain PAO1 (17) was used for all experiments. For marker selection in *P. aeruginosa*, 30 μg gentamicin (GEN) ml^{-1} , 50 μg tetracycline (TET) ml^{-1} , and 200 μg carbenicillin (CAR) ml^{-1} were used, as appropriate. Batch cultivation of *P. aeruginosa* was carried out at 37°C in ABT minimal medium (18) supplemented with 0.25% (wt/vol) glucose and 0.25% (wt/vol) Casamino Acids (ABTGC medium). *P. aeruginosa* cells were harvested at late-log phase for both the RNA-Seq and iTRAQ proteomic analyses.

A PAO1 suspension (optical density at 600 nm [OD₆₀₀], 0.01) was added to a 24-well plate with or without 500 μM iberin. The ABTGC medium was used for culturing bacteria, and each well had a final volume of 1 ml. The plate was incubated at 200 rpm and 37°C. After reaching an OD₆₀₀ of 0.5 (measured using an Infinite 200 Pro Series plate reader [Tecan], approximately after 3.5 h of incubation), the cultures were mixed immediately with 2 volumes of RNAprotect bacterial reagent (Qiagen). After 5 min of incubation at room temperature, the samples were centrifuged at 7,000 $\times g$ for 5 min at 4°C, the supernatant was removed, and the pellets were stored at -80°C .

RNA preparation. Total RNA was extracted with the RNeasy Protect bacterial minikit with on-column DNase digestion, according to the manufacturer's instructions (Qiagen). The integrity of total RNA and DNA contamination was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies) and Qubit 2.0 fluorometer (Invitrogen). The 16S, 23S, and 5S rRNAs were removed using the Ribo-Zero magnetic kit (bacteria) (Epicentre).

RNA sequencing and data analysis. Gene expression analysis (two biological replicates) was conducted by Illumina RNA sequencing (RNA-Seq technology). The rRNA-depleted RNA was fragmented to 200- to 300-bp fragments, and then first- and second-strand cDNA were synthesized, followed by end repair and adapter ligation. After 12 cycles of PCR enrichment, the quality of the libraries was assessed using the Bioanalyzer (Agilent Technologies, USA). The libraries were sequenced using the Illumina HiSeq 2000 platform with a paired-end protocol and read lengths of 100 nucleotides.

The sequence reads were assembled and analyzed in the RNA-Seq and expression analysis application of CLC Genomics Workbench 6.0 (CLC bio, Aarhus, Denmark). The PAO1 genome (<http://www.ncbi.nlm.nih.gov/nuccore/110645304>) was utilized as the reference genome for the assembly. The following criteria were used to filter the unique sequence reads: minimum length fraction of 0.9, minimum similarity fraction of 0.8, and maximum number of two mismatches. The data were normalized by calculating the reads per kb per million mapped reads (total reads/mapped reads in millions \times gene length in kb) for each gene and annotated with PseudoCAP (see <http://www.geneontology.org/GO.current.annotations.shtml>). A *t* test was performed on the transformed data (0.5 was added to each number to deal with zero counts) to identify the genes with significant changes in expression ($P < 0.05$; fold change, >2.0 or less than -2.0) between the control and iberin-treated samples.

iTRAQ proteomics analyses. The iTRAQ proteomics experiment was performed at the Proteomic Core Facility of the Biological Research Center, School of Biological Sciences, Nanyang Technological University, Singapore, and carried out as per our previous study (19).

Protein preparation and digestion. *P. aeruginosa* PAO1 was grown in ABTG medium (AB minimal medium with 2.5 $\mu\text{g}/\text{ml}$ thiamine and 0.5% glucose) with or without 500 μM iberin to late-log phase at 37°C, with shaking, after which the cells were harvested. After harvesting, the cell pellet was washed with 1 \times phosphate-buffered saline (PBS) and resuspended in 2 ml of lysis buffer containing 0.5 M triethyl ammonium bicarbonate (TEAB), 0.1 M SDS, and protease inhibitor cocktails (Sigma-Aldrich). The cells were ruptured by sonication, and the cell debris was removed by centrifugation at 16,000 $\times g$ and 4°C for 15 min. Three biological replicates for each growth condition were pooled, and 200 μg of proteins from each growth condition were dissolved in an equal volume of sample buffer (Invitrogen) supplemented with 0.5% 2-mercaptoethanol and denatured by boiling at 95°C for 5 min. One-dimensional (1D) gel electrophoresis was carried out using 10% SDS-PAGE for in-gel digestion.

The proteins were first reduced in 5 mM Tris-(2-carboxyethyl)phosphine (TCEP) for 1 h at 60°C, followed by blocking cysteine residues in 10 mM methyl methanethiosulfate (MMTS) for 30 min at room temperature in the dark. Trypsin was added at a ratio of 1:50 (trypsin to sample). It was then incubated at 37°C overnight. The tryptic peptides were extracted by 50% acetonitrile (ACN)–5% acetic acid from gel three times and were desalted using Sep-Pak C₁₈ cartridges (Waters, Milford, MA) and dried in a SpeedVac (Thermo Electron, Waltham, MA). All chemicals were purchased from Sigma-Aldrich, unless stated otherwise.

iTRAQ labeling. The iTRAQ labeling of the tryptic peptides was performed using a 4-plex iTRAQ reagent kit (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. Two hundred micrograms of peptides from each condition were individually labeled with respective isobaric tags (control sample, 114; iberin-treated sample, 115), followed by 2 h of incubation, quenching by water, desalting using C₁₈ solid-phase extraction cartridge, and then vacuum centrifugation to dryness. The iTRAQ-labeled peptides were reconstituted in buffer A (10 mM ammonium acetate, 85% acetonitrile, 0.1% formic acid) and fractionated using an electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) column (200 by 4.6 mm, 5- μm particle size, 200-Å pore size) by a high-performance liquid chromatography (HPLC) system (Shimadzu, Japan) at flow rate of 1.0 ml/min, using our previously optimized protocol (20). The HP liquid chromatograms were recorded at 280 nm, and fractions were collected online using an automated fraction collector. Twenty fractions were collected and concentrated using a vacuum centrifuge and reconstituted in 3% ACN with 0.1% formic acid for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS. The peptides were separated and analyzed on a home-packed nanopore C₁₈ column (15 cm by 75 μm ; RepronSil-Pur C₁₈-AQ, 3 μm ; Dr. Maisch, Germany) with a PicoFrit nanospray tip (New Objective, Woburn, MA, USA) on a Tempo nano-multidimensional LC (MDLC) system coupled with a QSTAR Elite hybrid LC-MS/MS system (Applied Biosystems). Peptides from each fraction were analyzed in triplicate by

TABLE 1 Characteristics of the bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference
PAO1	Prototypic wild-type strain	17
$\Delta pvdA$	Isogenic <i>pvdA</i> mutant of PAO1	33
PAO1 miniTn7- <i>gfp</i>	PAO1 carrying the miniTn7- <i>gfp</i> that constitutively expresses <i>gfp</i>	33
PAO1 <i>lasB-gfp</i>	PAO1 carrying the <i>lasB-gfp</i> fusion reporter	39
PAO1 <i>rsmZ-gfp</i>	PAO1 carrying the <i>rsmZ-gfp</i> fusion reporter	30
PAO1 <i>rsmY-gfp</i>	PAO1 carrying the <i>rsmY-gfp</i> fusion reporter	30
PAO1 <i>pvdA-gfp</i>	PAO1 carrying the <i>pvdA-gfp</i> fusion reporter	33

LC-MS/MS over a gradient of 90 min. The flow rate of the LC system was set to a constant 300 nl/min. Data acquisition in QSTAR Elite was set to positive ion mode using the Analyst QS 2.0 software (Applied Biosystems). MS data were acquired in positive ion mode with a mass range of 300 to 1,600 *m/z*. Peptides with +2 to +4 charge states were selected for MS/MS. For each MS spectrum, the three most abundant peptides above a five-count threshold were selected for MS/MS and dynamically excluded for 30 s, with a mass tolerance of 0.03 Da. Smart information-dependent acquisition was activated with automatic collision energy and automatic MS/MS accumulation. The fragment intensity multiplier was set to 20, and the maximum accumulation time was 2 s.

Data analysis. The spectra acquired from the three technical replicates were submitted to ProteinPilot (version 3.0.0.0; Applied Biosystems) for peak-list generation, protein identification, and quantification. The user-defined parameters of the Paragon algorithm in the ProteinPilot software were configured as follows: (i) sample type, iTRAQ 2-plex (peptide labeled); (ii) cysteine alkylation, MMTS; (iii) digestion, trypsin; (iv) instrument, QSTAR Elite electrospray ionization (ESI); (v) special factors, urea denaturation; (vi) species, none; (vii) specify processing, quantitation and bias correction; (viii) identification (ID) focus, biological modifications and amino acid substitutions; (ix) database, *P. aeruginosa* PAO1 PAO1-UW; (x) search effort, thorough ID; and (xi) result quality, unused ProtScore (conf) of >0.05 (10.0%). The default precursor and MS/MS tolerance for the QSTAR ESI MS instrument were adopted automatically by the software. For iTRAQ analysis, the peptide for quantification was automatically selected by the Pro Group algorithm to calculate the reporter peak area, error factor (EF), and *P* value. The resulting data were auto-bias corrected by a built-in ProteinPilot algorithm to eliminate any variations imparted due to the unequal mixing during the combination of different labeled samples. During bias correction, the software identifies the median average protein ratio and corrects it to unity and then applies this factor to all quantitation results.

The following cutoffs were used for protein identification: an unused protein score of ≥ 2 (i.e., 99% confidence of identification) and having >1 peptide identified; in this way, a total of 1,948 proteins were identified. Using a *P* value cutoff of 0.05 and a fold change of at least +2.0 (for upregulation) and -2.0 (for downregulation), the abundances of 119 proteins were found to be significantly affected by iberin; the abundance of 27 proteins was upregulated, while the abundances of 92 proteins were downregulated (shown in Tables 2 and 3, respectively).

Biosensor assay. The growth and *gfp* expression of the *P. aeruginosa* strains containing the *lasB-gfp*, *rsmY-gfp*, or *rsmZ-gfp* biosensors in the presence of iberin were monitored using a Victor X multilabel plate reader (PerkinElmer). These strains were cultivated in 96-well microtiter plates with ABTGC medium with different concentrations of iberin at 34°C without shaking. The OD₄₅₀ (or OD₆₀₀, in the case of the strain with *lasB-gfp*) and green fluorescent protein (GFP) fluorescence (in relative fluorescence units [RFU], with excitation and emission wavelengths of 485 and 535 nm, respectively) were measured every 30 min until the culture reached stationary growth phase.

The growth, *gfp* expression, and pyoverdine production of the *P. aeruginosa* strain containing the *pvdA-gfp* biosensor in the presence of iberin were measured using an Infinite 200 Pro Series plate reader (Tecan). These strains were cultivated in 96-well microtiter plates with ABTGC medium with different concentrations of iberin at 37°C without shaking. OD₆₀₀ readings, GFP fluorescence (in relative fluorescence units [RFU], with excitation and emission wavelengths of 485 and 535 nm, respectively) and pyoverdine fluorescence (in RFU, with excitation and emission wavelengths of 398 and 460 nm, respectively [21]) were measured every 30 min until the culture reached stationary growth phase.

Biofilm assay. Overnight cultures of PAO1 were diluted 100-fold in 50-ml BD Falcon tubes containing 10 ml of ABTGC medium with 500 μ M iberin. No iberin was added in the controls. Sterilized coverslips were placed into the medium and incubated at 37°C under static conditions for the cultivation of surface-attached biofilm at the air-liquid interface. Im-

ages of 1-day-old biofilms were captured using confocal microscopy at $\times 63$ magnification (Zeiss LSM 780 confocal system) and analyzed using the IMARIS software package (Bitplane, Zurich, Switzerland).

Nucleotide sequence accession numbers. The RNA-Seq data sets are available in the NCBI GEO Sequence Read Archives under accession no. SRP028308 and SRR950389.

RESULTS AND DISCUSSION

Comparative analysis of the mode of QS inhibition in iberin. In order to study the mechanisms behind the inhibition of QS in iberin, we first wanted to find out the changes in gene expression and protein abundance of *P. aeruginosa* PAO1 in the presence or absence of medium containing 500 μ M iberin. RNA-Seq and iTRAQ proteomics were used to examine the changes in gene expression and protein abundance, respectively.

Prior to the extraction of samples for either proteomics or RNA-Seq analysis, we optimized the appropriate time point for harvesting the cells by using *P. aeruginosa* PAO1 containing the *lasB-gfp* reporter, which can monitor the onset of QS induction of gene expression (Fig. 2). Based on this assay, we chose the sampling time point to be in the late-log phase of growth. Hence, for the actual RNA-Seq and iTRAQ proteomics experiments, we cultured *P. aeruginosa* PAO1 in the presence or absence of 500 μ M iberin and harvested the samples for subsequent analyses when the cultures reached late-log phase.

The genes and proteins whose expression or abundance was significantly increased or decreased in the presence of 500 μ M iberin are summarized in Table 2 (upregulated genes/proteins) and Table 3 (downregulated genes/proteins), respectively. For the RNA-Seq results, 62 genes had increased expression (>5-fold) and 79 genes had decreased expression (>5-fold) in the presence of iberin. In the iTRAQ proteomics results, 27 proteins had increased abundance (>2-fold) and 92 proteins had decreased abundance (>2-fold). For these results in Tables 2 and 3, we had determined an arbitrary cutoff of ± 5 -fold change for upregulation and downregulation of gene expression, respectively, in the RNA-Seq results. Nevertheless, we have included in the supplemental material all the genes in the RNA-Seq results with more than a ± 2.0 -fold change. The complete lists of the 145 upregulated and 258 downregulated genes in the RNA-Seq results are presented in Tables S1 and S2 in the supplemental material, respectively.

Iberin was able to affect the expression of genes regulated by QS, with reference to the list of QS-regulated genes in Hentzer et al. (4); these are marked with an "X" in Tables 2 and 3. Among the genes/proteins that had significantly increased expression/abundance in the presence of iberin, only 3% of the upregulated genes (2 of 62) in the RNA-Seq results were QS regulated, which supported the previous view of iberin acting as a QSI and not a QS activator. None of the 27 upregulated proteins in the iTRAQ proteomics results was related to QS control. Among the genes/proteins that had significantly decreased expression/abundance in the presence of iberin, 49% of the downregulated genes (39 of 79) in the RNA-Seq results and 27% of the downregulated proteins (25 of 92) in the iTRAQ proteomics results were QS regulated.

Surprisingly, the groups of genes and proteins up- or downregulated by iberin, as identified by either RNA-Seq or iTRAQ proteomics analysis, do not share much in common. Only 9 of the 81 unique significantly upregulated genes/proteins (11%) and 16 of the 157 unique significantly downregulated genes/proteins

TABLE 2 Genes/proteins found to be significantly upregulated upon iberin treatment^d

Strain no.	Gene	QS ^b	Description of product ^c	RNA-seq (500 μM)		iTRAQ proteomics (500 μM)	
				Fold change ^d	P value	Fold change ^d	P value
PA0140	<i>ahpF</i>		Alkyl hydroperoxide reductase subunit F			4.85	<0.01
PA0182			Probable short-chain dehydrogenase	7.6	0.04		
PA0185			Probable permease of ABC transporter	8.9	0.04		
PA0201			Hypothetical protein	25.9	0.01		
PA0202			Probable amidase	20.7	0.03		
PA0283	<i>sbp</i>		Sulfate-binding protein precursor	26.0	0.01		
PA0284			Hypothetical protein	32.9	0.04		
PA0565			Conserved hypothetical protein			2.91	<0.01
PA0849	<i>trxB2</i>		Thioredoxin reductase 2			2.94	0.01
PA0865	<i>hpd</i>		4-Hydroxyphenylpyruvate dioxygenase	42.2	0.04	2.75	<0.01
PA0878			Hypothetical protein	7.9	0.04		
PA1240			Probable enoyl-CoA hydratase/isomerase			3.45	<0.01
PA1260			Amino acid ABC transporter periplasmic-binding protein			4.49	<0.01
PA1285			Probable transcriptional regulator	5.2	0.05		
PA1310	<i>phnW</i>		2-Aminoethylphosphonate-pyruvate aminotransferase	19.3	0.05		
PA1332			Hypothetical protein	17.7	0.05		
PA1334			Probable oxidoreductase	74.7	0.04	5.74	<0.01
PA1493	<i>cysP</i>		Sulfate-binding protein of ABC transporter	8.2	<0.01		
PA1999	<i>dhcA</i>		DhcA, dehydrocarnitine CoA transferase, subunit A	9.1	0.01		
PA2000	<i>dhcB</i>		DhcB, dehydrocarnitine CoA transferase, subunit B	9.5	<0.01		
PA2008	<i>fahA</i>		Fumarylacetoacetase	54.8	0.03		
PA2009	<i>hmgA</i>		Homogentisate 1,2-dioxygenase	62.0	0.02		
PA2197			Conserved hypothetical protein			2.07	<0.01
PA2247	<i>bkdA1</i>		2-Oxoisovalerate dehydrogenase (alpha subunit)			2.71	<0.01
PA2248	<i>bkdA2</i>		2-Oxoisovalerate dehydrogenase (beta subunit)			2.98	<0.01
PA2249	<i>bkdB</i>		Branched-chain alpha-keto acid dehydrogenase (lipoamide component)			2.64	<0.01
PA2277	<i>arsR</i>		ArsR protein	15.3	0.05		
PA2278	<i>arsB</i>		ArsB protein	9.6	0.02		
PA2310			Hypothetical protein	20.5	0.03		
PA2311			Hypothetical protein	51.8	0.05		
PA2312			Probable transcriptional regulator	29.6	<0.01		
PA2327		X	Probable permease of ABC transporter	6.6	0.05		
PA2359			Probable transcriptional regulator	34.5	0.03		
PA2444	<i>glyA2</i>		Serine hydroxymethyltransferase	37.4	0.05		
PA2445	<i>gcvP2</i>		Glycine cleavage system protein P2			3.67	<0.01
PA2483			Conserved hypothetical protein			3.30	<0.01
PA2490			Conserved hypothetical protein	16.4	0.05		
PA2491	<i>mexS</i>		MexS	34.1	0.03	5.08	0.01
PA2493	<i>mexE</i>		RND multidrug efflux membrane fusion protein MexE precursor			5.69	<0.01
PA2494	<i>mexF</i>		RND multidrug efflux transporter MexF	129.4	0.04	5.69	<0.01
PA2495	<i>oprN</i>		Multidrug efflux outer membrane protein OprN precursor	102.3	0.03	5.34	<0.01
PA2535			Probable oxidoreductase			3.02	<0.01
PA2575			Hypothetical protein	28.5	0.02	4.01	<0.01
PA2579	<i>kynA</i>		L-Tryptophan:oxygen 2,3-oxidoreductase (decyclizing) KynA	5.3	0.05		
PA2580			Conserved hypothetical protein	26.1	0.01	4.58	0.01
PA2594		X	Conserved hypothetical protein	9.0	0.02		
PA2599			Conserved hypothetical protein	12.1	0.05		
PA2600			Hypothetical protein	21.0	<0.01		
PA2610			Conserved hypothetical protein	5.2	0.03		
PA2758			Probable transcriptional regulator	63.7	0.02		
PA2759			Hypothetical protein	179.3	0.01		
PA2767			Probable enoyl-CoA hydratase/isomerase	5.8	0.03		
PA2786			Hypothetical protein	8.6	0.05		
PA2812			Probable ATP-binding component of ABC transporter	20.4	0.03		
PA2813			Probable glutathione S-transferase	32.7	0.04		
PA2844			Conserved hypothetical protein	8.3	0.04		

(Continued on following page)

TABLE 2 (Continued)

Strain no.	Gene	QS ^b	Description of product ^c	RNA-seq (500 μM)		iTRAQ proteomics (500 μM)	
				Fold change ^d	P value	Fold change ^d	P value
PA2845			Hypothetical protein	91.2	0.04		
PA2931	<i>cifR</i>		CifR	10.4	0.03		
PA2932	<i>morB</i>		Morphinone reductase	45.7	0.03		
PA3035			Probable glutathione S-transferase	8.0	<0.01		
PA3126	<i>ibpA</i>		Heat shock protein IbpA			4.37	<0.01
PA3222			Hypothetical protein	5.4	0.04		
PA3229			Hypothetical protein	311.5	0.04		
PA3230			Conserved hypothetical protein	69.5	0.01		
PA3938			Probable periplasmic taurine-binding protein precursor	24.5	0.05		
PA3446			Conserved hypothetical protein	63.5	0.05		
PA3450			Probable antioxidant protein	39.4	0.01		
PA3931			Conserved hypothetical protein	44.7	0.05		
PA4166			Probable acetyltransferase	77.6	0.02		
PA4167	<i>dkgB</i>		Probable oxidoreductase	112.0	<0.01	4.93	<0.01
PA4173			Conserved hypothetical protein	12.4	0.01		
PA4236	<i>katA</i>		Catalase			4.01	<0.01
PA4354			Conserved hypothetical protein				
PA4355	<i>pyeM</i>		PyeM	37.3	<0.01		
PA4356	<i>xenB</i>		Xenobiotic reductase			4.28	<0.01
PA4385	<i>groEL</i>		GroEL protein	6.4	0.04	2.48	<0.01
PA4386	<i>groES</i>		GroES protein	6.4	0.03		
PA4387			Conserved hypothetical protein	8.0	0.05		
PA4613	<i>katB</i>		Catalase			5.10	<0.01
PA4623			Hypothetical protein	727.4	0.01		
PA4973	<i>thiC</i>		Thiamine biosynthesis protein ThiC			2.58	<0.01

^a Upregulation was defined as a >5-fold increase in the RNA-Seq result and a >2-fold increase in the iTRAQ proteomics result. Only genes/proteins showing a significant difference in expression from the control (i.e., *P* value < 0.05) were selected.

^b An “X” indicates that the gene/protein is regulated by quorum sensing (4), in reference to the QS-regulated genes/proteins, as determined previously.

^c CoA, coenzyme A; RND, resistance-nodulation-cell division.

^d Empty lines indicate no significant change in expression levels.

(10%) were detected by both methods. Among the 16 downregulated genes/proteins, 14 (i.e., 88%) were subject to QS control, with important examples being chitinase (*chiC*), protease IV (*piv*), and rhamnosyltransferase chain B (*rhlB*). This finding suggests the strong inhibition of these particular QS-regulated virulence factors by iberin on both the transcriptional and posttranscriptional/translational levels.

The suppression of many known QS-regulated genes (4) was observed only by the RNA-Seq and not the iTRAQ proteomics analysis, and vice versa (Table 3). For example, the decreased expression of genes encoding hydrogen cyanide synthase (*hcnA* [PA2193], *hcnB* [PA2194], and *hcnC* [PA2195]), lectin (*lecA* and *lecB* [PA2570/PA3361]), rhamnosyltransferase chain A (*rhlA* [PA3479]), and elastase (*lasB* [PA3724]) was mainly reflected in the RNA-Seq but not the proteomics results (Table 3). This indicates that the expression of these virulence factors was strongly inhibited by iberin on the transcriptional level, which would also be expected to manifest as a reduction in the protein content. However, the missing correlation to the proteomic analysis suggests that these proteins were below the detection limit of the iTRAQ method. In fact, our iTRAQ protocol allowed for the detection (with 99% confidence) of only a total of 1,948 proteins. Elastase (LasB) was detected in only the RNA-Seq (−124.4-fold decrease) and not the proteomics analysis, which agrees with our previous paper in which iTRAQ proteomics was used to study the effects of the QSI 5-imino-4,6-dihydro-3H-

1,2,3-triazolo[5,4-*d*]pyrimidin-7-one on *P. aeruginosa* (22). In that study, elastase was also not detected through proteomics. Since only the cells and not the surrounding medium were subjected to iTRAQ analysis, this extracellular enzyme was likely not to be present intracellularly in large enough amounts to be detected. We put forward that this explanation is also valid for lectin (*LecA* and *LecB*).

Our results also show that iberin was able to inhibit the expression of multiple components of the type III secretion system (T3SS) of *P. aeruginosa*, which is an important system in pathogenesis (23). Gene expression of the effector protein ExoT and ExoS toxins known to impair phagocytosis by host immune cells (24, 25) was reduced by −2.0- and −3.0-fold in the RNA-Seq results, respectively (see Table S2 in the supplemental material). Also, the expression levels of the genes for the T3SS regulatory proteins ExsB, ExsD, ExsC, and ExsE (26) were reduced by −3.5-, −6.6-, −3.2-, and −4.4-fold, respectively. The expression levels of the genes for the needle components PscC and PscG were reduced −2.1- and −2.6-fold, respectively. Lastly, gene expression of the translocator protein PopB (27) was decreased −6.7-fold in the RNA-Seq results.

In the previous study by Jakobsen et al. (15), it was shown that iberin was able to compete effectively with C₄-HSL for binding to RhlR, with the expression levels of *rhlR*, *rhlB*, and *rhlA* reduced by −5.3-, −42.1-, and −59.0-fold, respectively, upon the addition of 64 μg/ml iberin to PAO1, as determined by DNA microarray.

TABLE 3 Genes/proteins found to be significantly downregulated upon iberin treatment^a

Strain no.	Gene	QS ^b	Description of product ^c	RNA-seq (500 μM)		iTRAQ proteomics (500 μM)	
				Fold change ^d	P value	Fold change ^d	P value
PA0059	<i>osmC</i>	X	Osmotically inducible protein OsmC			-2.91	<0.01
PA0081	<i>fha1</i>		Fha1			-2.25	<0.01
PA0084	<i>tssC1</i>		TssC1			-2.01	<0.01
PA0088	<i>tssF1</i>		TssF1			-2.13	<0.01
PA0122	<i>rahU</i>	X	RahU	-32.8	0.05	-5.50	<0.01
PA0176	<i>aer2</i>		Aerotaxis transducer Aer2			-3.67	<0.01
PA0180	<i>cttP</i>		Chemotactic transducer for trichloroethylene (positive chemotaxis), CttP			-2.10	<0.01
PA0523	<i>norC</i>		Nitric-oxide reductase subunit C	-10.9	0.04		
PA0575			Conserved hypothetical protein			-2.18	<0.01
PA0586			Conserved hypothetical protein			-2.17	<0.01
PA0587			Conserved hypothetical protein			-2.38	<0.01
PA0704			Probable amidase			-5.21	<0.01
PA0707	<i>toxR</i>		Transcriptional regulator ToxR	-5.4	0.02		
PA0852	<i>cbpD</i>	X	Chitin-binding protein CbpD precursor	-22.0	0.04	-4.92	<0.01
PA1001	<i>phnA</i>	X	Anthranilate synthase component I	-7.2	0.01		
PA1027			Probable aldehyde dehydrogenase			-3.22	<0.01
PA1127			Probable oxidoreductase			-2.09	<0.01
PA1130	<i>rhlC</i>	X	Rhamnosyltransferase 2	-11.7	0.04		
PA1131		X	Probable MFS transporter	-8.9	0.05		
PA1134			Hypothetical protein	-6.0	0.01		
PA1214			Hypothetical protein	-8.2	0.03		
PA1215			Hypothetical protein	-7.7	0.02		
PA1216			Hypothetical protein	-9.0	0.04		
PA1220			Hypothetical protein	-5.4	0.03		
PA1221			Hypothetical protein	-6.8	0.04		
PA1245			Hypothetical protein PA1245			-5.46	<0.01
PA1246	<i>aprD</i>	X	Alkaline protease secretion protein AprD			-2.43	<0.01
PA1247	<i>aprE</i>	X	Alkaline protease secretion protein AprE			-2.99	<0.01
PA1248	<i>aprF</i>	X	Alkaline protease secretion outer membrane protein AprF precursor			-3.84	<0.01
PA1249	<i>aprA</i>	X	Alkaline metalloproteinase precursor			-3.99	<0.01
PA1300			Probable sigma-70 factor, ECF subfamily	-5.5	0.03		
PA1318	<i>cyoB</i>		Cytochrome <i>o</i> ubiquinol oxidase subunit I			-2.15	<0.01
PA1323		X	Hypothetical protein	-6.9	0.03		
PA1324		X	Hypothetical protein	-7.7	0.05	-3.96	<0.01
PA1327			Probable protease			-3.80	<0.01
PA1408			Hypothetical protein PA1408			-2.31	<0.01
PA1529	<i>ligA</i>		DNA ligase			-2.03	<0.01
PA1559			Hypothetical protein	-7.3	0.03		
PA1560			Hypothetical protein	-8.9	0.02		
PA1705	<i>pcrG</i>		Regulator in type III secretion	-7.3	0.03		
PA1706	<i>pcrV</i>		Type III secretion protein PcrV	-6.1	0.03		
PA1708	<i>popB</i>		Translocator protein PopB	-6.7	0.03		
PA1710	<i>exsC</i>		ExsC, exoenzyme S synthesis protein C precursor.	-6.6	0.01		
PA1730			Conserved hypothetical protein			-3.06	<0.01
PA1871	<i>lasA</i>	X	LasA protease precursor	-25.5	0.02		
PA1875		X	Probable outer membrane protein precursor	-5.1	0.04		
PA1906			Hypothetical protein	-20.4	0.01		
PA1912	<i>femI</i>		ECF sigma factor, FemI	-5.1	0.01		
PA1914			Conserved hypothetical protein	-14.0	0.02		
PA1927	<i>metE</i>		5-Methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase			-3.19	<0.01
PA1930			Probable chemotaxis transducer			-2.66	<0.01
PA2067			Probable hydrolase	-16.4	0.04		
PA2068		X	Probable MFS transporter	-37.5	0.02		
PA2069		X	Probable carbamoyl transferase	-107.8	0.02	-5.97	<0.01
PA2072			Conserved hypothetical protein			-2.48	<0.01

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TABLE 3 (Continued)

Strain no.	Gene	QS ^b	Description of product ^c	RNA-seq (500 μM)		iTRAQ proteomics (500 μM)	
				Fold change ^d	P value	Fold change ^d	P value
PA2144	<i>glgP</i>		Glycogen phosphorylase			-2.55	<0.01
PA2151			Conserved hypothetical protein			-3.18	<0.01
PA2152			Probable trehalose synthase			-4.32	<0.01
PA2153	<i>glgB</i>		1,4-α-Glucan branching enzyme			-3.44	<0.01
PA2159			Conserved hypothetical protein	-5.4	0.04		
PA2160			Probable glycosyl hydrolase			-4.85	<0.01
PA2162			Probable glycosyl hydrolase			-3.89	<0.01
PA2163			Hypothetical protein PA2163			-3.32	0.03
PA2164			Probable glycosyl hydrolase			-2.15	0.01
PA2167			Hypothetical protein	-5.3	0.02		
PA2169			Hypothetical protein	-6.7	0.03		
PA2170			Hypothetical protein	-9.1	0.04		
PA2177			Probable sensor/response regulator hybrid			-2.35	<0.01
PA2193	<i>hcnA</i>	X	Hydrogen cyanide synthase HcnA	-27.7	0.05		
PA2194	<i>hcnB</i>	X	Hydrogen cyanide synthase HcnB	-15.9	0.01		
PA2195	<i>hcnC</i>	X	Hydrogen cyanide synthase HcnC	-12.6	<0.01		
PA2261			Probable 2-ketogluconate kinase			-3.08	0.04
PA2300	<i>chiC</i>	X	Chitinase	-29.9	0.03	-5.71	<0.01
PA2305	<i>ambB</i>	X	AmbB			-2.30	<0.01
PA2322			Gluconate permease	-11.4	0.02		
PA2345			Conserved hypothetical protein			-2.60	<0.01
PA2363			Hypothetical protein PA2363			-2.41	0.01
PA2371			Probable ClpA/B-type protease			-2.78	<0.01
PA2377			Hypothetical protein	-8.5	0.05		
PA2384			Hypothetical protein	-7.4	<0.01		
PA2414		X	L-Sorbose dehydrogenase	-10.5	0.02		
PA2415		X	Hypothetical protein	-8.6	0.02		
PA2416	<i>treA</i>		Periplasmic trehalase precursor			-2.79	0.04
PA2427			Hypothetical protein	-6.9	0.01		
PA2448			Hypothetical protein PA2448			-3.16	<0.01
PA2566		X	Conserved hypothetical protein	-5.9	0.04		
PA2570	<i>lecA</i>	X	LecA	-38.9	0.03		
PA2573			Probable chemotaxis transducer			-2.94	<0.01
PA2587	<i>pqsH</i>	X	Probable FAD-dependent monooxygenase			-2.19	<0.01
PA2588		X	Probable transcriptional regulator	-8.9	0.02		
PA2717	<i>cpo</i>		Chloroperoxidase precursor			-2.88	0.01
PA2788			Probable chemotaxis transducer	-5.8	0.03	-2.64	<0.01
PA2815	<i>fadE</i>		Probable acyl-CoA dehydrogenase			-2.51	<0.01
PA2920			Probable chemotaxis transducer			-2.03	<0.01
PA3040			Conserved hypothetical protein			-2.15	0.03
PA3118	<i>leuB</i>		3-Isopropylmalate dehydrogenase			-2.15	<0.01
PA3155	<i>wbpE</i>		UDP-2-acetamido-2-dideoxy-D-ribo-hex-3-uluronic acid transaminase, WbpE			-2.03	<0.01
PA3305.1	<i>phrS</i>		PhrS	-6.5	0.05		
PA3326	<i>clpP2</i>	X	ClpP2	-12.7	0.04	-4.97	0.01
PA3327	<i>fabH2</i>	X	Probable nonribosomal peptide synthetase	-8.1	0.02	-4.69	<0.01
PA3328	<i>fabH2</i>	X	Probable FAD-dependent monooxygenase	-27.0	0.01	-4.93	<0.01
PA3329	<i>fabH2</i>	X	Hypothetical protein	-11.4	0.04	-5.28	<0.01
PA3330	<i>fabH2</i>	X	Probable short-chain dehydrogenase	-31.5	0.03		
PA3331	<i>fabH2</i>	X	Cytochrome P450	-15.9	0.03	-4.61	<0.01
PA3332	<i>fabH2</i>	X	Conserved hypothetical protein	-19.5	0.03	-3.07	<0.01
PA3333	<i>fabH2</i>	X	3-Oxoacyl-[acyl-carrier-protein] synthase III	-19.0	0.03		
PA3334		X	Probable acyl carrier protein	-14.0	0.02		
PA3346			Probable two-component response regulator			-2.35	<0.01
PA3361	<i>lecB</i>	X	Fucose-binding lectin PA-III	-35.3	0.02		
PA3408	<i>hasR</i>		Heme uptake outer membrane receptor HasR precursor			-2.71	<0.01
PA3418	<i>ldh</i>		Leucine dehydrogenase			-2.42	<0.01
PA3431			Conserved hypothetical protein	-6.1	0.05		

(Continued on following page)

TABLE 3 (Continued)

Strain no.	Gene	QS ^b	Description of product ^c	RNA-seq (500 μ M)		iTRAQ proteomics (500 μ M)	
				Fold change ^d	P value	Fold change ^d	P value
PA3460			Probable acetyltransferase			-3.61	<0.01
PA3461			Conserved hypothetical protein			-3.91	<0.01
PA3477	<i>rhlR</i>	X	Transcriptional regulator RhlR	-5.7	<0.01		
PA3478	<i>rhlB</i>	X	Rhamnosyltransferase chain B	-27.6	<0.01	-5.47	<0.01
PA3479	<i>rhlA</i>	X	Rhamnosyltransferase chain A	-110.4	<0.01		
PA3520		X	Hypothetical protein				
PA3554	<i>arnA</i>		ArnA			-2.56	<0.01
PA3613			Hypothetical protein PA3613			-2.14	<0.01
PA3621.1	<i>rsmZ</i>		Regulatory RNA RsmZ	-4.0	0.04		
PA3691		X	Hypothetical protein PA3691			-3.43	<0.01
PA3692	<i>lptF</i>	X	Lipotoxon F, LptF			-2.23	<0.01
PA3724	<i>lasB</i>	X	Elastase LasB	-124.4	0.02		
PA3734			Hypothetical protein	-5.6	0.05		
PA3923			Hypothetical protein			-2.07	<0.01
PA3974	<i>ladS</i>		Lost adherence sensor, LadS			-2.03	0.01
PA4078			Probable nonribosomal peptide synthetase			-3.60	<0.01
PA4112			Probable sensor/response regulator hybrid			-2.82	<0.01
PA4141		X	Hypothetical protein	-144.7	0.02		
PA4142		X	Probable secretion protein	-43.3	0.01	-2.15	0.03
PA4143			Probable toxin transporter	-20.8	0.02	-5.39	<0.01
PA4144			Probable outer membrane protein precursor	-16.2	<0.01		
PA4156			Probable TonB-dependent receptor	-11.5	<0.01		
PA4159	<i>fepB</i>		Ferrienterobactin-binding periplasmic protein precursor FepB	-9.7	0.02		
PA4160	<i>fepD</i>		Ferric enterobactin transport protein FepD	-5.0	0.03		
PA4175	<i>piv</i>	X	Protease IV	-24.3	<0.01	-2.56	<0.01
PA4207	<i>mexI</i>		Probable RND efflux transporter			-2.94	<0.01
PA4209	<i>phzM</i>	X	Probable phenazine-specific methyltransferase	-25.1	0.02		
PA4213	<i>phzD1</i>		Phenazine biosynthesis protein PhzD	-71.8	0.05		
PA4217	<i>phzS</i>	X	Flavin-containing monooxygenase			-4.69	0.02
PA4228	<i>pchD</i>		Pyochelin biosynthesis protein PchD			-2.92	<0.01
PA4229	<i>pchC</i>		Pyochelin biosynthetic protein PchC				
PA4230	<i>pchB</i>		Salicylate biosynthesis protein PchB			-3.15	<0.01
PA4362			Hypothetical protein PA4362			-2.37	<0.01
PA4471			Hypothetical protein	-16.0	0.04		
PA4570			Hypothetical protein	-5.7	0.01		
PA4624			Hypothetical protein PA4624			-2.62	<0.01
PA4785			Probable acyl-CoA thiolase			-2.21	<0.01
PA4835			Hypothetical protein PA4835			-2.58	<0.01
PA4836			Hypothetical protein PA4836			-2.33	<0.01
PA5058	<i>phaC2</i>	X	Poly(3-hydroxyalkanoic acid) synthase 2			-4.42	<0.01
PA5061			Conserved hypothetical protein			-4.40	0.01
PA5113			Hypothetical protein			-4.40	0.01
PA5171	<i>arcA</i>		Arginine deiminase			-2.23	<0.01
PA5213	<i>gcvP1</i>		Glycine cleavage system protein P1			-3.27	<0.01
PA5220		X	Hypothetical protein	-8.5	0.01		

^a Downregulation was defined as a >5-fold decrease in the RNA-Seq result and a >2-fold decrease in the iTRAQ proteomics result. Only genes/proteins showing a significant difference in expression from the control (i.e., P value < 0.05) were selected. Empty lines within fold change columns indicate no significant change in expression levels.

^b An "X" indicates that the gene/protein is regulated by quorum sensing (4), in reference to the QS-regulated genes/proteins, as determined previously.

^c MFS, major facilitator superfamily; ECF, extracytoplasmic function; FAD, flavin adenine dinucleotide; CoA, coenzyme A; RND, resistance-nodulation-cell division.

^d Empty lines indicate no significant change in expression levels.

Also, iberin inhibited rhamnolipid production by PAO1 in a concentration-dependent manner, with complete inhibition occurring at a concentration of 200 μ M iberin. Similarly, our results showed that the expression levels of the *rhlR*, *rhlB*, and *rhlA* genes were significantly decreased in the RNA-Seq results by -5.7-, -27.6-, and -110.4-fold, respectively. Our iTRAQ result showed only RhlB being significantly downregulated (-5.47-fold).

In Jakobsen et al. (15), the expression of *lasB* was found to be decreased by 89.8-fold upon the addition of 64 μ g/ml iberin to PAO1, as determined by DNA microarray. However, they also showed that iberin was unable to competitively inhibit *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo- C_{12} -HSL)-driven expression of the *lasB-gfp* fusion when present in an *Escherichia coli* background (15). In the present study, neither the transcriptomic nor proteomic analysis showed iberin to inhibit the expression of

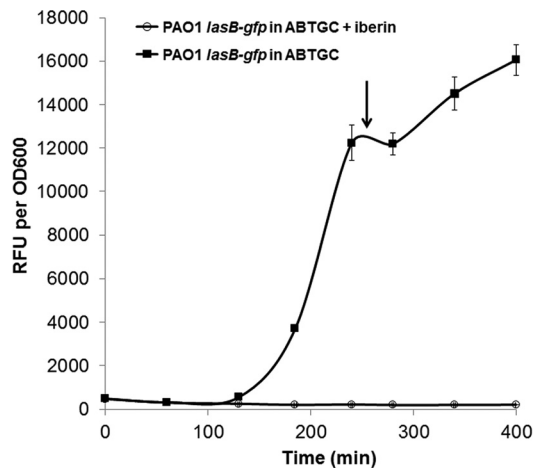


FIG 2 Growth and normalized *gfp* gene expression (RFU/OD₆₀₀) of the *P. aeruginosa* PAO1 biosensor strains carrying *lasB-gfp* in ABTGC medium with 500 μ M iberin. Every 60 min, growth and *gfp* fluorescence were measured using OD₆₀₀ values and relative fluorescence units (RFU), respectively. The experiments were performed in triplicate; only representative ones are shown. The arrow indicates the sampling time point for RNA sequencing, while error bars are the averages of triplicate readings.

the major QS regulator, LasR. These findings taken together strongly suggest that iberin does not target LasR, and it does not compete with 3-oxo-C₁₂-HSL for binding to LasR. Despite this, the *lasB* gene, the expression of which is positively regulated by

LasR, was found to be reduced by 124.4-fold in the RNA-Seq results. Thus, a comparison of the mRNA and corresponding protein content does not reveal the mechanisms by which iberin inhibits the *las* QS-controlled gene expression.

Analysis of the inhibition of small regulatory RNAs by iberin. One valuable advantage of the RNA-Seq method over the DNA microarray method used previously (15) is that RNA-Seq is able to monitor the expression of small regulatory RNAs. Our RNA-Seq results showed that the expression of two small regulatory RNAs, encoded by the *phrS* and *rsmZ* genes (PA3305.1 and PA3621.1, respectively), was repressed by iberin treatment by 6.5-fold and 4.0-fold, respectively, compared to the untreated control (Table 3). PhrS and RsmZ have been reported to positively regulate alkyl quinolone-based and AHL-based QS systems, respectively (28, 29). A Δ *rsmY* Δ *rsmZ* mutant was shown to have deficient synthesis of the exoproducts hydrogen cyanide, pyocyanin, elastase, chitinase, and chitin-binding protein (29). This phenotype agrees with our RNA-Seq results showing that iberin inhibited the transcription of genes involved in the production of those exoproducts. Hence, we wanted to confirm whether iberin inhibited *rsmY/rsmZ* expression.

In order to test the inhibitory effects of iberin on *rsmY* and *rsmZ* expression, PAO1 strains containing *rsmY-gfp* or *rsmZ-gfp* plasmids (30) were cultured in the presence of various concentrations of iberin. Our results show that iberin inhibited the expression of *rsmY* and *rsmZ* in a dose-dependent manner (Fig. 3); how-

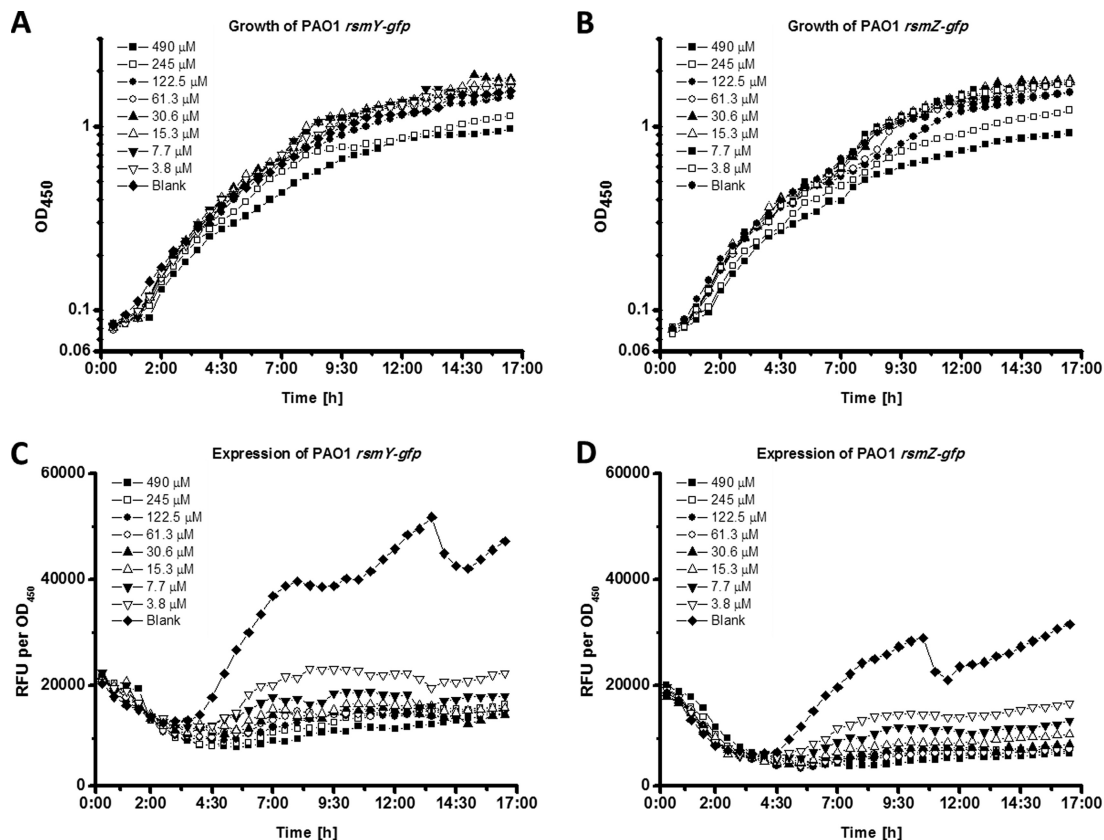


FIG 3 Growth and normalized *gfp* gene expression (RFU/OD₄₅₀) of the *P. aeruginosa* PAO1 biosensor strains carrying *rsmY-gfp* (A and C) and *rsmZ-gfp* (B and D) in ABTGC medium containing various concentrations of iberin. Every 30 min, growth and *gfp* fluorescence were measured using OD₄₅₀ values and relative fluorescence units (RFU), respectively. The experiments were performed in triplicate; only representative ones are shown.

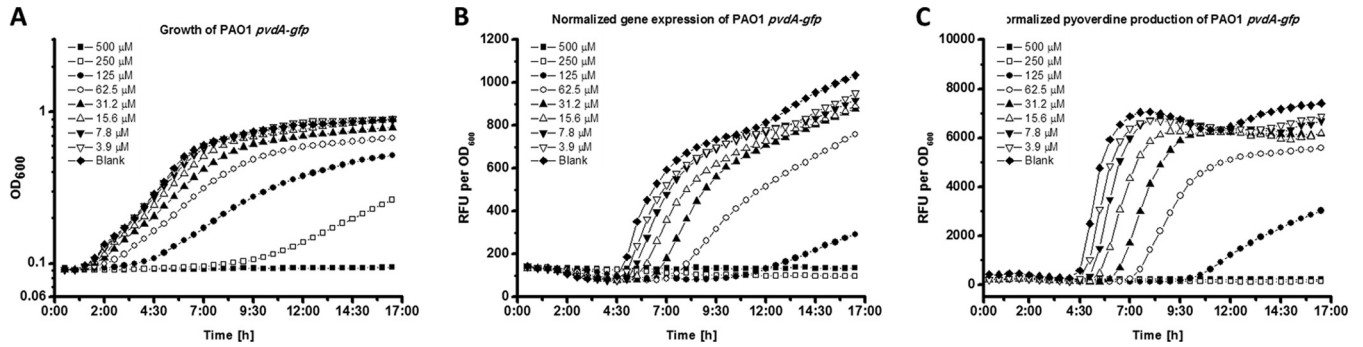


FIG 4 Growth (A), normalized *gfp* expression (B), and normalized pyoverdine production (C) of the *P. aeruginosa* PAO1 biosensor strain carrying *pvdA-gfp* in ABTGC medium containing various concentrations of iberin. Every 30 min, growth, *gfp* fluorescence, and pyoverdine production were measured using OD₆₀₀ values and relative fluorescence units (RFU), respectively. Normalization was calculated as RFU divided by OD₆₀₀. The experiments were performed in triplicate; only representative ones are shown.

ever, high levels of iberin were growth inhibiting.

The expression of the GacA-dependent small RNAs, *rsmY* and *rsmZ*, is essential for AHL-mediated QS and virulence in *P. aeruginosa* (29). Previous works aiming to elucidate QS inhibitory mechanisms have reported that the QS inhibitor azithromycin inhibits the expression of *rsmY* and *rsmZ* transcription through the PA0588-PA0584 gene cluster (31). However, our transcriptomic data did not show that iberin affected the expression of the PA0588-PA0584 gene cluster, which suggests that iberin uses a different mechanism from that of azithromycin to inhibit the expression of *rsmY* and *rsmZ*.

Our proteomics result shows that iberin led to a 2-fold decrease in the abundance of the lost adherence sensor protein LadS

(PA3974). LadS is a sensor kinase that phosphorylates GacS, which in turn is responsible for activating GacA and the expression of *rsmY* and *rsmZ* (32). This result suggests that iberin post-transcriptionally inhibits the expression of LadS, which in turn leads to reduced activation of GacS, thus reducing RsmY and RsmZ levels.

A study by Frangipani et al. (16) showed that RsmY and RsmZ sequester RsmA, a situation that allows for the production of the siderophores pyochelin and pyoverdine. Pyoverdine is important for development of the characteristic mushroom-like structures of *P. aeruginosa* biofilms (33), and it is also believed to play a role in its virulence, for example, in the production of the virulence factor pyocyanin (34). Hence, we were interested to find out

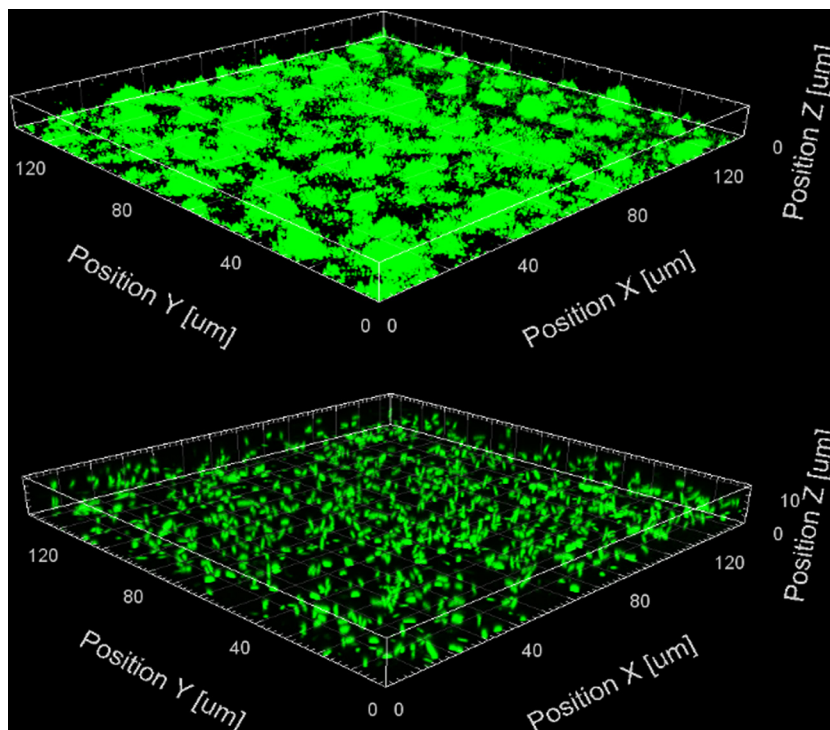


FIG 5 Three-dimensional confocal images of 1-day-old miniTn7-*gfp*-tagged *P. aeruginosa* PAO1 slide biofilms with either ABTGC medium (control, top panel) or 500 μM iberin (bottom panel). Images were obtained using confocal microscopy at $\times 63$ magnification (Zeiss LSM 780 confocal system), and analyzed using the Imaris software package (Bitplane AG). Only a representative image of three replicates is shown.

whether iberin might affect pyoverdine production. A *P. aeruginosa* PAO1 wild-type strain containing the *pvdA-gfp* reporter was cultured in various concentrations of iberin. Iberin inhibited the expression of the *pvdA-gfp* genes and the production of pyoverdine (Fig. 4B and C), indicating that the inhibition of pyoverdine synthesis occurs on a transcriptional level, mediated through released RsmA protein, which is usually sequestered by the small regulatory RNA molecules. This is supported by our RNA-Seq results, in which the expression levels of the pyoverdine biosynthesis genes *pvdA*, *pvdP*, *pvdG*, and *pvdS* were reduced by -3.2 -, -3.6 -, -3.9 -, and -3.6 -fold, respectively (see Table S2 in the supplemental material).

The exopolysaccharides Pel and Psl, along with alginate, are important components of the extracellular matrix of *P. aeruginosa* biofilms (35). It has been shown that both the *pel* and *psl* polysaccharide genes are posttranscriptionally regulated by RsmY and RsmZ (29, 36). As such, we performed a slide biofilm assay in order to determine if iberin was able to reduce biofilm formation by *P. aeruginosa* through the downregulation of *pel* and *psl*. Figure 5 shows that the sample treated with 500 μ M iberin had significantly less biofilm formation than the control sample in ABTGC. This suggests that iberin may be used not only as an antipathogenic agent but also as an antibiofilm agent.

In addition to RsmY and RsmZ, PhrS was also reported to be an important small regulatory RNA that can stimulate the synthesis of the *Pseudomonas* quinolone signal (PQS) via activating PqsR (28). PQS quorum sensing is well known to regulate the synthesis of the virulence factor pyocyanin and rhamnolipid and the release of extracellular DNA in *P. aeruginosa* (37, 38). In our RNA-Seq results, we see that iberin reduced the expression of *phrS* by -6.5 -fold. Also, the expression of the *pqs* operon (PA0996-PA1001) was reduced by 3- to 4-fold and the phenazine biosynthesis genes (*phnA* and *phnB*) by 7.2- and 4.8-fold, respectively (see Table S2 in the supplemental material). As such, iberin has an inhibitory effect on the expression of the *pqs* QS system and phenazine biosynthesis genes.

In summary, our study sheds light on the QS inhibitory mechanisms of a natural food-derived QSI, iberin, by using complementary transcriptomic and proteomic approaches. Iberin is an efficient QSI, not from its interaction with the central transcriptional QS regulators LasR and RhIR but rather through its effect on the expression of the small regulatory RNAs RsmY and RsmZ, as well as PhrS. From our analysis of gene expression and protein content, we believe that iberin modulates the Gac/Rsm network of *P. aeruginosa* through posttranscriptional inhibition of LadS. Lastly, we also show that iberin effectively reduces biofilm formation, highlighting its potential use as both an antipathogenic and antibiofilm agent. Further studies will be carried out to investigate the possible interaction of iberin with LadS through biochemical and phosphoproteomics methods.

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