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Positive Regulation of Pyoluteorin Biosynthesis in *Pseudomonas* sp. M18 by Quorum-Sensing Regulator VqsR

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The biocontrol rhizobacterium *Pseudomonas* sp. M18 can produce two kinds of antibiotics, namely pyoluteorin (Plt) and phenazine-1-carboxylic acid (PCA), and is antagonistic against a number of soilborne phytopathogens. In this study, a *luxR*-type quorum-sensing regulatory gene, *vqsR*, was identified and characterized immediately downstream of the Plt gene cluster in strain M18. A *vqsR*-inactivated mutant led to a significant decrease in the production of Plt and its biosynthetic gene expression. However, this was restored when introducing the *vqsR* gene by cloning into the plasmid pME6032 *in trans*. The *vqsR* mutation did not exert any obvious influence on the production of PCA and its biosynthetic gene expression and the production of *N*-acylhomoserine lactones (C4 and C8-HSLs) and their biosynthetic gene *rhlI* expression. Accordingly, these results introduce VqsR as a regulator of Plt production in *Pseudomonas* spp., and suggest that the regulatory mechanism of *vqsR* in strain M18 is distinct from that in *P. aeruginosa*. In addition, it was demonstrated that *vqsR* mutation did not have any obvious impact on the expression of Plt-specific ABC transporters and other secondary metabolic global regulators, including GacA, RpoS, and RsmA.

Keywords: Quorum-sensing, VqsR, antifungal compounds, pyoluteorin, phenazine-1-carboxylic acid

The plant growth-promoting rhizobacterium (PGPR) *Pseudomonas* sp. M18 produces two different types of antifungal metabolites, namely pyoluteorin (Plt) and phenazine-1-carboxylic acid (PCA), both of which can suppress soilborne phytopathogenic fungi in the rhizosphere. Until now, this is the first example of the production of these two antibiotics in one single strain [14]. Plt can effectively suppress *Pythium spp.*, especially *Pythium ultimum*, which causes seedling damping-off and root rot in host plants, including many economically important crops [2]. Plt is an aromatic polyketide antibiotic produced by several *Pseudomonas* species, including *P. fluorescens* Pf-5, CHA0, and *Pseudomonas* sp. M18 [2, 14]. The Plt biosynthetic, regulatory, and ABC (ATP-binding cassette) transport gene cluster has already been cloned and characterized in both *P. fluorescens* Pf-5 [4, 20, 2] and *Pseudomonas* sp. M18 [15, 16]. Phenazine and its biosynthetic mechanisms have also been studied in detail in the strains *P. fluorescens* 2-79 [24], *P. aureofaciens* 30-84 [28], and *P. aeruginosa* PAO1 [24]. The biosynthesis of secondary metabolites, including these antibiotics, is subject to the regulation of a complicated network consisting of various regulatory genes and environmental factors [11]. The regulatory genes involved in regulating the biosynthesis of Plt and PCA include the two-component global response regulatory system GacS/GacA, the principal sigma factors RpoD and RpoS, the secondary metabolic repressors RsmA and RsmB, the small RNAs (RsmX, RsmY, and RsmZ) sequestering the repressors RsmA and RsmB [11], the pathway-specific regulators PltR and PltZ [16], and a cell density-dependent quorum-sensing (QS) system [11].

QS functions on the basis of a two-component system, consisting of a signal molecule (autoinducer) synthetic enzyme LuxI (PhzI, RhlI, and LasI) and response transcriptional regulator LuxR (phzR/RhlR, and LasR) [35]. Typically, QS regulates the expression of a target gene by binding a LuxR-autoinducer complex to an upstream *lux* box in that gene [36]. Previous work by the current authors suggested that the RhlI/RhlR QS system negatively regulates Plt production, which is the first example of a QS regulatory function for Plt production [37]. In addition to these LuxI/LuxR pairs, there are also several orphan LuxR family regulators, such as QscR [21] and VqsR [17]. VqsR was first characterized as a virulence and quorum-sensing regulator (designated as *vqsR*) in *P. aeruginosa* [17]. As such, the above-mentioned QS regulators or systems constitute a complex hierarchical network that globally regulates the transcription of certain genes in response to

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increasing cell densities. It has already been reported that QscR negatively regulates phenazine biosynthesis [21], whereas the inactivation of VqsR abrogates the production of *N*-acyl-homoserine lactone (AHL) and decreases the production of phenazine antibiotics in *P. aeruginosa* [17].

Surprisingly, a *vqsR* homologous gene (namely *orfX*) and two other open reading frames (*orfV* and *orfW*) have been identified immediately downstream of the Plt biosynthetic and ABC transport gene cluster (about 30 kb in length) in *Pseudomonas* sp. M18 [15, 16]. However, Plt is not produced by *P. aeruginosa*, and the *plt* gene cluster has not been found in the complete genomic sequence of *P. aeruginosa* PAO1. Therefore, this gene context prompted further study of the regulatory mechanism of *vqsR* on the biosynthesis of Plt and PCA in *Pseudomonas* sp. M18.

Accordingly, this study examined the identification and characterization of *vqsR* in *Pseudomonas* sp. M18, and found that *vqsR* is involved in the positive regulation of Plt biosynthesis. In addition, the influence of *vqsR* on PCA biosynthesis and the *rhl* quorum-sensing system was also investigated. Finally, since it has already been reported that the biosynthesis of Plt and PCA is subject to regulation by several important global regulators, including GacA [7], RpoS [9], and RsmA [38], the relation of *vqsR* to these three regulators was explored to improve the current understanding of the complicated hierarchical regulatory pathway or network of antibiotic biosynthesis in *Pseudomonas* sp. M18.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* was grown at 37°C in a Luria-Bertani (LB) broth [30], whereas the *Pseudomonas* sp. M18 and its derivatives were routinely grown at 28°C in an LB and King's medium B (KMB) broth [19] for Plt production or pigment production medium (PPM) for PCA production [22]. As the indicator strain for AHL production [25, 32], *Chromobacterium violaceum* CV026 was grown at 28°C in an LB broth, and 5% sucrose was supplemented to counter-select the suicide plasmid pEX18Gm. When required, X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) was used for blue/white screening, 4 mg/ml ONPG (ortho-nitrophenyl-β-D-galactopyranoside) for β-galactosidase assaying, and 1 mM IPTG

Table 1. Strains and plasmids used in this study.

Strains and plasmids	Genotype and/or relevant characteristics*	Reference
E. coli		
DH5α	*supE*44 Δ*lac*U169(Φ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Sambrook and Russell [30]
SM10	*thi*-1 *thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r	Sambrook and Russell [30]
Pseudomonas sp. M18		
Wild-type	Rhizosphere isolate, Plt^+ PCA^+ Ap^r Sp^r	Huang *et al.* [16]
M18X	*vqsR*::Km^r, Sp^r Km^r	This study
C. violaceum CV026	Signal molecular reporter, Km^r	McClean *et al.* [25]
Plasmids		
pLAP1, 2	Cosmids containing parts of *plt* gene cluster screened from M18 genomic library, Km^r	Huang *et al.* [16]
pDSK519	Source of Km^r cassette, Km^r	Keen *et al.* [18]
pEX18Gm	Gene replacement vector with MCS from pUC18, $oriT^+$ $sacB^+$, Gm^r	Hoang *et al.* [13]
pME6032	pVS1-p15A *E. coli-Pseudomonas* shuttle vector, $lacI^q$-*Ptac* expression vector, Tc^r	Heeb *et al.* [12]
pOER	858 bp DNA fragment containing *vqsR* and its SD sequence cloned into pME6032, Tc^r	This study
pME6015	pVS1-p15A *E. coli-Pseudomonas* shuttle vector for translational *lacZ* fusions and promoter probing, Tc^r	Schnider-Keel *et al.* [31]
pME6522	pVS1-p15A *E. coli-Pseudomonas* shuttle vector for transcriptional *lacZ* fusions and promoter probing, Tc^r	Blumer *et al.* [3]
pEXXK	1.7 kb Km^r cassette inserted into *vqsR* at blunted-BamHI site in pEX18Gm, Gm^r Km^r	This study
pMEAZ	*pltLA'-'lacZ* translational fusion in pME6015, Tc^r	Huang *et al.* [16]
pMEZA	*phzA'-'lacZ* translational fusion in pME6015, Tc^r	Ge *et al.* [7]
pHZLF	*pltH'-'lacZ* translational fusion in pME6015, Tc^r	Huang *et al.* [15]
pHZCF	*pltH'-'lacZ* transcriptional fusion in pME6522, Tc^r	Huang *et al.* [15]
pMEGA	*gacA'-'lacZ* translational fusion in pME6015, Tc^r	Ge *et al.* [8]
pMEIZ	*rhlI'-'lacZ* translational fusion in pME6015, Tc^r	Tang *et al.* [34]
pMERS	*rpoS'-'lacZ* translational fusion in pME6015, Tc^r	Ge *et al.* [10]
pMERA	*rsmA'-'lacZ* translational fusion pME6015, Tc^r	Ge *et al.* [10]

*Plt, pyoluteorin; PCA, phenazine-1-carboxyl acid; r, antibiotic-resistant; Km, kanamycin; Tc, tetracycline; Gm, gentamicin; Ap, ampicillin; Sp, spectinomycin; Rif, rifampicin.

(isopropyl-beta-D-thiogalactopyranoside) for blue/white screening and promoter induction. Antibiotics were used in the medium at the following concentrations (μg/ml): for pseudomonad, gentamicin (Gm) 40, kanamycin (Km) 50, spectinomycin (Sp) 100, and tetracycline (Tc) 125; for *E. coli*, Km 50, Gm 15, ampicillin (Ap) 100, and Tc 25; and for *C. violaceum* CV026, Km 50.

General Molecular Biology Techniques and Sequence Analysis

The molecular biology methods not described in detail were performed using standard methods [30]. The restriction endonucleases, DNA-modifying enzymes, *Taq* DNA polymerase, DNA molecular weight markers, DNA-purifying kits, and other associated products were all used as recommended by the manufacturers (MBI Fermentas or TaKaRa).

ORFs were initially predicted using Gene Mark HMM [23], and a database search for similar protein sequences was carried out using BLASTX of NCBI. Putative promoters were predicted using NNPP (Promoter Prediction by Neural Network) [29], and a probable domain homology search was performed using Pfam [1] and PROSITE [33]. In addition, multiple sequence alignments were performed using CLUSTAL W [35].

Construction of Chromosomal *vqsR* Inactivated Mutant Strain

Plasmid pUCX is a *vqsR* gene-containing derivative of pUC18 obtained from an M18 chromosomal genomic shotgun library of a 14 kb EcoRI fragment of the cosmid pLAP2. As such, a 1.3 kb EcoRI-XbaI fragment containing *vqsR* and its flanking sequence from pUCX was inserted into the suicide plasmid pEX18Gm carrying the *sacB* gene as a counter-selectable marker to construct pEXX. A 1.7 kb kanamycin resistance cassette from pDSK519 was inserted into pEXX at the blunt-ended BamHI site to construct plasmid pEXXK containing a *vqsR*::km^R fragment. The BamHI site is located at nucleotide 88 of the *vqsR* gene. The resulting plasmid pEXXK was mobilized from the *E. coli* SM10 donor into strain M18 by biparental mating. Transconjugants were selected on LB plates containing Sp to counter-select *E. coli* SM10 and Km, and after a second crossing-over, Km^{R}Gm^{S}Sac^R recombinants were then obtained. The resultant marker-exchanged mutant, designated M18X, was further confirmed by a PCR analysis and Southern analysis (data not shown).

Construction of *vqsR* Gene Overexpression Vector pOER

A 858-bp fragment containing *vqsR* with its Shine-Dalgarno (SD) sequence was PCR-amplified from a partial *plt* gene cluster containing the cosmid pLAP2 using the primers Phr1 (5'-CGC GGA ATT CAG AGG AGT AGT TGT GTG GAT-3') and Phr2 (5'-AAT TAG ATC TGC GGC GCA CTA GCG TGC GTC-3'). The restriction sites for EcoRI and BglII are underlined. The PCR product recovered from the gel was digested with EcoRI and BglII and cloned into the *E. coli-pseudomonad* shuttle expressional vector pME6032 (medium copy number), thereby creating the *vqsR* overexpression plasmid pOER. The *vqsR* gene was transcribed under the control of the *tac* promoter in pME6032.

Quantitative Assay of Plt and PCA by HPLC

The amounts of Plt and PCA produced by *Pseudomonas* sp. M18 and its derivatives were assayed by high-performance liquid chromatography (HPLC). The Plt and PCA were extracted from the culture suspensions of the M18 strain and its derivatives using ethyl acetate and chloroform, respectively, and subjected to HPLC (Shimadzu LC-8A) using a C18 reverse-phase column eluted with methanol-water (70:30) at a flow rate of 2.0 ml/min. The Plt and PCA were then detected by UV spectroscopy at λ=308 nm and 248 nm and a retention time of 3.1 min and 1.78 min, respectively. The Plt and PCA were both quantified using authentic samples as the standard. The authentic Plt and PCA were purified from culture supernatants of M18, and their structure and purity confirmed by an elemental analysis, their ultraviolet absorbance spectrum, mass spectrum with electrospray ionization, and ^{1}H and ^{13}H nuclear magnetic resonance (NMR) [14].

β-Galactosidase Assays

The *Pseudomonas* sp. M18 and its derivatives were grown at 28°C with shaking at 220 rpm in 500-ml flasks containing 100 ml of KMB or LB media supplemented with 0.05% Triton X-100. Appropriate antibiotics were added when required. The media were then inoculated with cell cultures grown overnight in a KMB broth based on a proportion of 1 to 100 (v/v). The cultures were sampled after a certain period of time and assayed for β-galactosidase activities according to the method of Miller [26] and as described by Huang *et al.* [16].

TLC Analysis of AHLs

For the extraction of AHL, the *Pseudomonas* sp. M18 and its derivatives were grown at 28°C with shaking at 220 rpm in 500-ml flasks containing 100 ml of KMB or LB media, and then 1 ml of the culture at an OD_{600} of 5.0 to 6.0 was subsequently harvested. The culture supernatants were extracted three times with 1 ml of ethyl acetate, and the dried extracts containing AHL were resuspended in 20 μl of ethyl acetate. The *N*-butanoyl-L-homoserine lactone (BHL) and *N*-hexanoyl-L-homoserine lactone (HHL) were analyzed using a thin layer chromatography (TLC) bioassay, as described previously [25, 32]. The AHL samples and standards (Fluka) were spotted (2 μl) onto TLC silica gel RP-C18 plates (Merck) for migration and *C. violaceum* CV026 was used as the reporter strain [25, 32].

Nucleotide Sequence Accession Number

The nucleotide sequences of the *vqsR* gene reported in this paper and the *plt* biosynthetic and ABC transport gene cluster have been deposited in the GenBank under the accession number AY394844.

RESULTS

Identification of *vqsR* Gene in *Pseudomonas* sp. M18

The Plt biosynthetic, regulatory, and ABC transport gene cluster in *Pseudomonas* sp. M18 (Fig. 1) was cloned, sequenced, and identified from five *plt*-positive cosmids, pLAP1 to pLAP5 [15, 16]. An open reading frame of 807 bp, *orfX* [15], which was highly homologous to the *vqsR* gene (PA2591) of *P. aeruginosa* PAO1 (99% nucleotide sequence identity), was identified downstream of the *plt* gene cluster and two hypothetical proteins encoding the genes of *orfV* and *orfW* (Fig. 1). Thus, *orfX* was also named *vqsR* in this study. A putative promoter region and

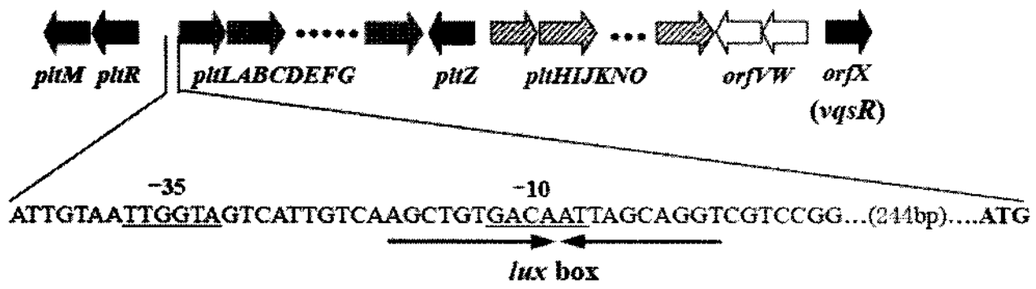

Fig. 1. Genetic organization of the *vqsR* gene, pyoluteorin biosynthetic structural genes (*pltLABCDEFG* and *pltM*), ABC (ATP-binding cassette) transporter genes (*pltHIJKNO*), two regulatory genes (*pltR* and *pltZ*), and two hypothetic genes (*orfV* and *orfW*). The sequences of the putative promoter elements (−35 and −10 regions) are underlined.
The position of the putative *lux* box is indicated by the convergent arrows.

the SD sequence of the *vqsR* gene were found to be located within the 152 bp intergenic region between the two oppositely transcripted genes *vqsR* and *orfV*. The *vqsR* gene encoded a putative protein containing 268 amino acid residues with a molecular mass of 30,591 Da in *Pseudomonas* sp. M18. A protein homology search disclosed an extensive similarity between VqsR and other bacterial regulators belonging to the LuxR family, including LuxR (*Vibrio fischeri*), PhzR (*P. chlororaphis*), RhlR (*P. aeruginosa*), LasR (*P. aeruginosa*), and RhiR (*Rhizobium leguminosarum* bv. *viciae*). The signature pattern for the LuxR family (Prosite Accession No. PDOC00542, Pfam Accession No. PF00196) was located near the C-terminus of these proteins: G-[LIVMFYS]-x(2,3)-[TS]-[LIVMT]-x(2)-[LIVM]-x(5)-[LIVQS]-[STAGENQH]-x-[GPAR]-x-[LIVMF]-[FYST]-x-[HFY]-[FV]-x-[DNST]-K-x(2)-[LIVM]. For VqsR, a conserved signature containing a helix-turn-helix motif was located in the region from amino acid residues 199 to 256. Moreover, a *lux* box (5'-ACCTACCAGAACTGGTAGTT-3'), which shared homology with the DNA sequence for the *lux* box identified in *V. fischeri* [5], was found at −95 to −114 upstream of the start codon (GTG) of *vqsR* in *Pseudomonas* sp. M18. These *lux* boxes usually function as binding sites for LuxR-autoinducer complexes.

Inhibited Biosynthesis of Plt by Inactivating the *vqsR* Gene in M18X Strain

To determine the effect of the *vqsR* mutation on Plt and PCA production, a *vqsR*::km^R chromosomal inactivation mutant, M18X, of strain M18 was constructed and verified, as described in Materials and Methods. The dynamic curves of the Plt production by strain M18X and its parental strain M18 were determined in KMB and PPM, respectively (Figs. 2A and 2B). Owing to the inactivation of *vqsR*, the Plt production kinetics were significantly altered in both media, where strain M18X produced less than 40% of the Plt production by the parental M18 strain. The Plt production by strain M18 reached a maximum of 83.5 μg/ml in the KMB after 96 h, whereas the parallel cultures of strain M18X only produced 30 μg/ml (Fig. 2A). The influence of the *vqsR* inactivation on PCA production in

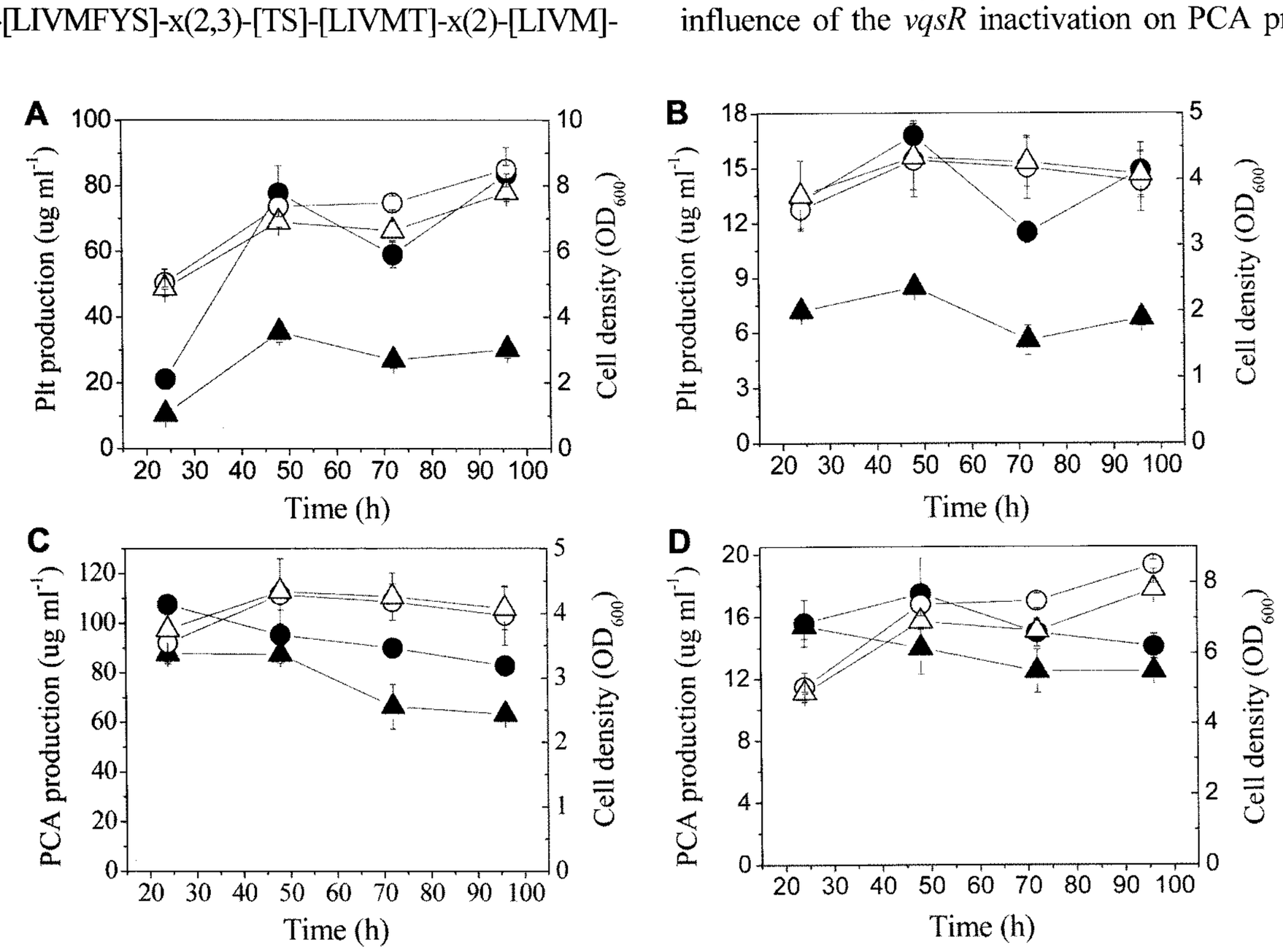

Fig. 2. Plt production (**A**, **B**), PCA production (**C**, **D**), and cell growth (OD_{600}) by wild-type M18 strain (●, ○) and *vqsR* mutant M18X strain (▲, △) in KMB broth (**A**, **D**) and PPM (**B**, **C**).
Solid symbols, Plt and PCA production; open symbols, OD_{600}. Each value is the mean±SD of four cultures.

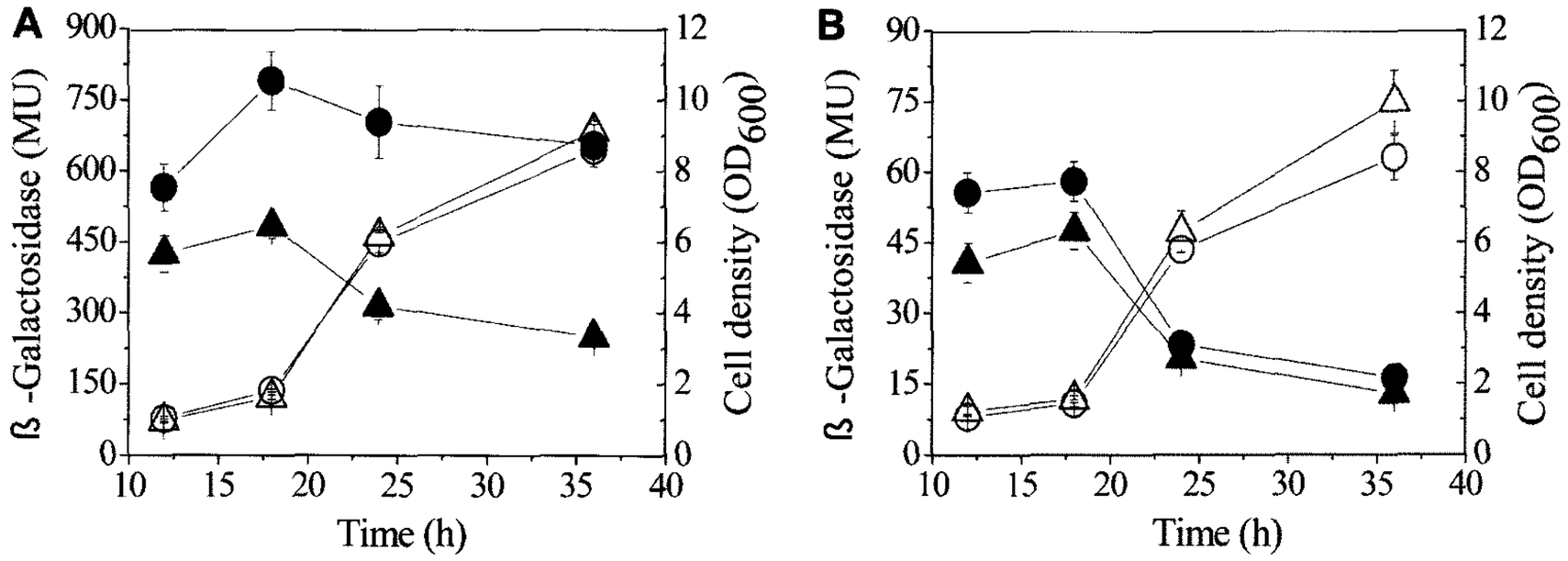

Fig. 3. Influence of *vqsR* on Plt (**A**) and PCA (**B**) biosynthetic gene expression.
The β-galactosidase activities resulting from *pltA'-'lacZ* translational fusion in pMEAZ (**A**) and *phzA'-'lacZ* translational fusion in pMEZA (**B**) were determined in the wild-type M18 strain (●, ○) and *vqsR* mutant M18X strain (▲, △) in a KMB broth. Solid symbols, β-galactosidase activities; open symbols, OD_{600}. Each value is the mean±SD of four cultures.

the PPM and KMB media is shown in Figs. 2C and 2D. Although the *vqsR* mutant strain M18X produced slightly less PCA than the wild-type M18 strain in both media, the differences were not statistically significant (data not shown). Additionally, the cell growth of the M18 and M18X strains did not display obvious differences in the KMB and PPM (Fig. 2), indicating that *vqsR* positively regulated Plt biosynthesis, yet did not exert an obvious influence on PCA biosynthesis.

Positive Regulation of Expression of Plt Biosynthetic Genes by *vqsR*

β-Galactosidase activity analyses were also performed to quantify the regulatory effect of *vqsR* on the expression of

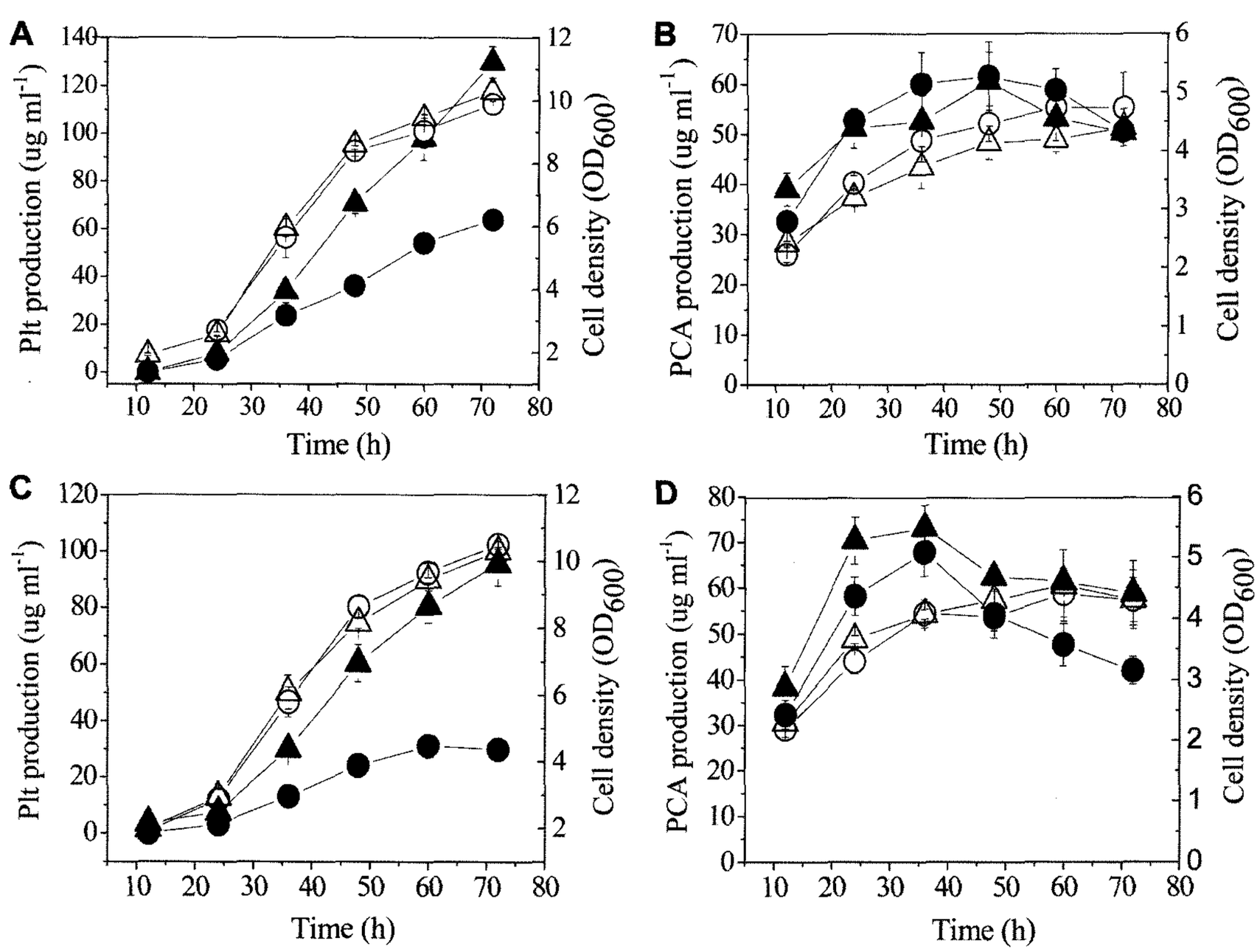

Fig. 4. Plt (**A**, **C**) and PCA (**B**, **D**) production by wild-type M18 strain (**A**, **B**) or *vqsR* mutant M18X strain (**C**, **D**), harboring pOER (▲, △) in which the *vqsR* gene was overexpressed and the wild-type M18 strain harboring empty vector pME6032 alone (●, ○), growing in KMB (for Plt production) and PPM (for PCA production), respectively.
Solid symbols, Plt or PCA production; open symbols, OD_{600}. Each value is the mean±SD of four cultures.

the Plt and PCA biosynthetic genes in *Pseudomonas* sp. M18 (Fig. 3). The vectors pMEAZ [16] and pMEZA [7], which carried a *pltA'-'lacZ* and *phzA'-'lacZ* translational fusion, respectively, were both introduced into the *vqsR* mutant M18X strain and wild-type M18 strain, respectively. The β-galactosidase activity was then assayed in KMB. The expression of the *pltA'-'lacZ* translational fusion in the M18X strain was severely reduced to about 40–60% of that in the wild-type M18 strain throughout the growth cycle. However, the expression of the *phzA'-'lacZ* translational fusion in the *vqsR* mutant M18X was not obviously altered compared with that in the M18 strain (Fig. 3). Thus, the results from the β-galactosidase activity analyses were consistent with those from the Plt and PCA quantitative assays in the M18 and M18X strains, thereby extending and corroborating the positive regulation by *vqsR* on Plt biosynthesis and no impact on PCA biosynthesis.

Enhancement of Plt Biosynthesis Resulting from Overexpression of *vqsR* Gene

To analyze the influence of *vqsR* overexpression on Plt and PCA biosynthesis, the *vqsR* overexpression plasmid pOER and empty plasmid pME6032 (as the control) were introduced into the wild-type M18 strain and *vqsR* mutant M18X strain, respectively. The Plt production was then assayed in KMB and the PCA production in PPM (Fig. 4). The overexpression of *vqsR* resulting from pOER in the wild-type M18 strain led to a one-fold increase of Plt production (129.9 μg/ml at 72 h) when compared with the production by the M18 strain carrying the empty plasmid pME6032 (63.8 μg/ml after 72 h) (Fig. 4A). Yet, more significantly, the *vqsR* overexpression resulted in a more than 2-fold increase of Plt production, from 29.8 to 95.5 μg/ml after 72 h, by the *vqsR* mutant M18X strain (Fig. 4C). Nonetheless, multiple copies of *vqsR* did not cause a significant alteration in PCA production by either the wild-type M18 strain or the *vqsR* mutant M18X strain (Figs. 4B and 4D). In addition, the *vqsR* overexpression had no significant impact on the growth of strains M18 and M18X (Fig. 4).

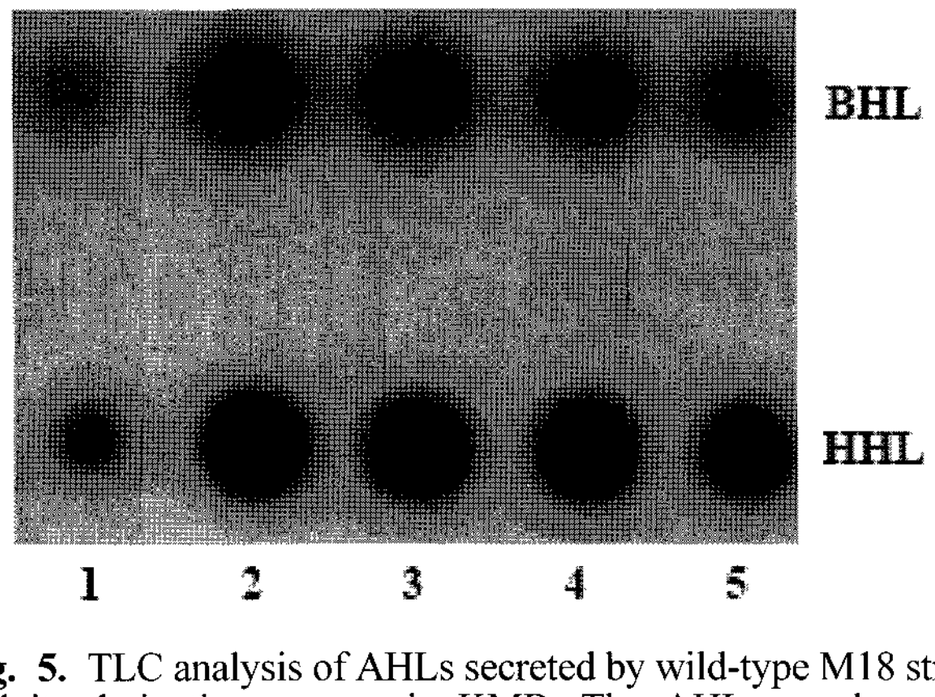

Fig. 5. TLC analysis of AHLs secreted by wild-type M18 strain and its derivatives grown in KMB. The AHL samples were visualized using the *C. violaceum* CV026 reporter strain.
1, BHL and HHL markers; 2, wild-type M18 strain; 3, *vqsR* mutant M18X strain; 4, M18 strain harboring empty vector pME6032; and 5, M18 strain harboring pOER in which the *vqsR* gene was overexpressed.

Influence of *vqsR* on Quorum Sensing

The influence of the *vqsR* mutation on *N*-acyl-homoserine lactone production and its biosynthetic gene *rhlI* expression in *Pseudomonas* sp. M18 was assessed in KMB. The results from the TLC assays showed no significant differences between the AHL levels produced by strains M18 and M18X in the KMB (Fig. 5). Similarly, the *vqsR* gene overexpression resulting from the vector pOER did not significantly alter the AHL production by the wild-type M18 strain when compared with that by the M18 strain carrying the empty control plasmid pME6032 (Fig. 5).

The plasmid pMEIZ, harboring a translational fusion of the AHL biosynthetic gene *rhlI* and reporter gene *lacZ*, allowed the *rhlI* expression to be monitored in the wild-type M18 strain and its derivative strains. The plasmid pMEIZ was introduced into the M18 strain and its *vqsR* mutant strain, respectively, and the β-galactosidase activities then assayed in KMB. The results showed that the *vqsR* mutation did not cause a significant difference between the M18X strain and its parental M18 strain (Table 2).

Table 2. Influence of *vqsR* on *rhlI*, *gacA*, *rpoS*, *rsmA*, and pyoluteorin ABC transport gene expression.

Time	Strain	*rhlI'-'lacZ* (pMEIZ)[a]	*gacA'-'lacZ* (pMEGA)[a]	*rpoS'-'lacZ* (pMERS)[b]	*rsmA'-'lacZ* (pMERA)[b]	*pltH'-'lacZ* (pHZLF)[a]	*pltH'-'lacZ* (pHZCF)[a]
18 h	M18	209.1±17.5	228.7±14.6	1024.8±79.5	79.2±7.5	11.5±1.5	40.1±3.0
	M18X	197.3±11.7	212.7±11.3	1041.7±19.8	70.2±6.3	12.5±0.8	38.9±3.5
24 h	M18	234.6±5.9	252.2±13.9	1328.5±82.8	67.0±2.7	12.9±1.2	25.2±1.3
	M18X	233.0±8.3	232.2±11.4	1340.9±47.1	66.6±2.8	11.0±1.1	22.9±1.7

The β-galactosidase activities (Miller units) resulting from the target genes and *lacZ* translational or transcriptional fusion reporter vectors were determined in the wild-type strain M18 and *vqsR* mutant strain M18X.
[a]β-Galactosidase activities were determined in KMB.
[b]β-Galactosidase activities were determined in LB media.
Plasmids pHZLF and pHZCF carried the *pltH'-'lacZ* translational and transcriptional fusions, respectively.
Each value is the mean±SD of four cultures.

Influence of *vqsR* on *gacA*, *rpoS*, *rsmA*, and Plt ABC Transport Gene Expression

The biosynthesis of antibiotics, including Plt and PCA, in *Pseudomonas* is under the control of a complex regulatory network, which is composed of a number of pathway-specific regulators and global regulators, such as the two-component response regulator GacA, stationary phase sigma factor RpoS, and small RNA-binding protein RsmA [11]. Translational fusions of the *lacZ* reporter gene with *gacA* (in the plasmid pMEGA), *rpoS* (pMERS), and *rsmA* (pMERA) allowed the expression of these genes to be monitored in the *vqsR* mutant M18X strain and its parental M18 strain, thereby enabling the hierarchical relation of VqsR to GacA, RpoS, and RsmA to be determined in the complex regulatory network of Plt and PCA. The β-galactosidase activity assays showed that the *vqsR* mutation did not lead to a significant difference in the expression of these three genes between the *vqsR* mutant M18X strain and its parental M18 strain (Table 2).

The *pltHIJKNO* ABC transport gene cluster, which is required for Plt production and involved in the export and resistance of Plt, is located immediately downstream of the Plt biosynthetic structural and regulatory gene cluster (*pltLABCDEFG*, *pltRM*, and *pltZ*). The *pltH'-'lacZ* translational fusion plasmid pHZLF and transcriptional fusion plasmid pHZCF were introduced into the *vqsR* mutant M18X strain and its wild-type M18 strain, respectively. The β-galactosidase activities were then measured in KMB. However, the expression of the *pltH'-'lacZ* translational and transcriptional fusions in the *vqsR* mutant M18X strain was not altered when compared with that in the wild-type M18 strain (Table 2), suggesting that *vqsR* did not influence the expression of the Plt ABC transport system.

Discussion

LuxIR-type quorum-sensing (QS) systems are widespread among Gram-negative bacteria and involved in the regulation of diverse and important biological processes, including antimicrobial compound biosynthesis. Most known cases are involved in the regulation of phenazine production, for example, PhzIR, LasIR, QscR, and VqsR in *Pseudomonas* spp. [36]. In previous research, the current authors were the first to report on an *rhl*-like QS system regulating the production of the polyketide antibiotic pyoluteorin in *Pseudomonas* sp. M18. Therefore, this study identified the orphan *luxR*-type regulatory gene, *vqsR*, downstream of the *plt* gene cluster, thereby facilitating a study of the regulatory mechanism of an AHL-dependent QS on two different types of antibiotics, pyoluteorin and phenazine, in the unique M18 strain.

The *vqsR* mutant of M18 was shown to produce significantly lower amounts of Plt when compared with the wild-type M18. However, the ability to produce Plt was restored when introducing the plasmid pOER, in which *vqsR* had been overexpressed under the control of the *tac* promoter, into the *vqsR* mutant. Concomitant with the upregulation of Plt production by VqsR, the expression of Plt biosynthetic genes was also positively regulated by VqsR. A similar complementation of the *vqsR* mutant with the cloned *vqsR* gene in the plasmid pOER also restored the decreased expression of Plt biosynthetic genes. As such, this study provides the first example to date of the involvement of VqsR in the regulation of Plt production and its biosynthetic genes expression. However, the molecular mechanism by which VqsR positively regulates the production of Plt and expression of Plt biosynthetic genes directly or indirectly through other intermediates is currently unknown. The presence of a typical *lux* box upstream of the *pltLABCDEFG* operon (Fig. 1), which is characteristic of LuxR-regulated target genes [6], suggests that the LuxR-type regulator VqsR likely regulates the expression of Plt biosynthetic genes *via* binding at the *lux* box.

VqsR was first identified as the fourth LuxR-type global regulator of quorum sensing and virulence in *P. aeruginosa* [17], and subsequent transcriptome analyses have suggested that VqsR modulates the expression of a wide variety of QS-regulated genes and virulence factors in *P. aeruginosa* [17], as *vqsR* mutation in *P. aeruginosa* abrogates the production of AHLs and dramatically decreases protease and phenazine production [17]. In contrast to *P. aeruginosa*, at least under the current experimental condition, *vqsR* mutation in *Pseudomonas* sp. M18 did not lead to obvious changes in the production of PCA and its biosynthetic gene expression or the production of AHLs (BHL and HHL) and their synthase gene *rhlI* expression, indicating a distinct regulatory activity of *vqsR* in *Pseudomonas* sp. M18. Thus, in strain M18, VqsR may control Plt biosynthesis through other regulatory pathways distinct from the BHL- and HHL-mediated QS pathways. Similar to other global regulatory systems, including GacA [7], RhlIR [37], and RsmA [38], VqsR had a different influence on PCA and Plt production, respectively, also revealing a more sophisticated regulatory network involved in the control of antibiotic production in strain M18, in contrast to other *Pseudomonas* spp., including *P. fluorescens* Pf-5 and *P. aeruginosa* PAO1 [11, 36].

In addition, the *lacZ* reporter gene expression assays in this study showed that VqsR did not exert a significant influence on the expression of the Plt-specific ABC transporter (PltHIJKNO) [15] and other global regulators, including the two-component response regulator GacA [7], secondary metabolism repressor RsmA [38], and stationary phase sigma factor RpoS [9], suggesting that VqsR was not located upstream of these three global regulators in the hierarchical regulatory network of Plt and PCA, and did not control the biosynthesis of Plt indirectly through these

regulators or transporters in *Pseudomonas* sp. M18. In terms of the biological significance, the regulation of QS regulators or systems, such as VqsR and RhlIR [37], in relation to Plt biosynthesis allows the fine-tuning of a balanced production of the two different types of antibiotics, Plt and PCA, in *Pseudomonas* sp. M18. Nonetheless, since multiple QS pathways occur in bacteria, further experiments are needed to determine the QS pathway by which VqsR regulates Plt biosynthesis and the relations between VqsR and other QS response regulators. Additional studies are also needed to determine the detailed regulatory mechanism of VqsR on Plt biosynthesis and other cell activities, along with an extensive search for other regulators and pathways involved in secondary mechanisms, including antibiotic biosynthesis in *Pseudomonas* sp. M18. Consequently, understanding the regulation of Plt and PCA production, both positive and negative, will provide the basis for the metabolic engineering of strain M18 to improve its antibiotic production and biocontrol performance.

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