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Molecules and Cells

Growth Phase-Differential Quorum Sensing Regulation of Anthranilate Metabolism in *Pseudomonas aeruginosa*

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***Pseudomonas* quinolone signal (PQS) plays a role in the regulation of virulence genes and it is intertwined in the *las*/*rhl* quorum sensing (QS) circuits of *Pseudomonas aeruginosa*. PQS is synthesized from anthranilate by *pqsA-D* and *pqsH* whose expression is influenced by the *las*/*rhl* systems. Since anthranilate can be degraded by functions of *antABC* and *catBCA*, PQS synthesis might be regulated by the balance between the expression of the *pqsA-D/phnAB, pqsH*, *antABC*, and *catBCA* gene loci. *antA* and *catA* are repressed by LasR during log phase and activated by RhlR in late stationary phase, whereas *pqsA-E/phnAB* is activated by LasR in log phase and repressed by RhlR. QscR represses both but each repression occurs in a different growth phase. This growth phase-differential regulation appears to be accomplished by the antagonistic interplay of LasR, RhlR, and QscR, mediated by two intermediate regulators, AntR and PqsR, and their cofactors, anthranilate and PQS, where the expressions of *antR* and *pqsR* and the production of anthranilate and PQS are growth phase-differentially regulated by QS systems. Especially, the anthranilate level increases in an RhlR-dependent manner at late stationary phase. From these results, we suggest that RhlR and LasR regulate the anthranilate metabolism in a mutually antagonistic and growth phase-differential manner by affecting both the expressions and activities of AntR and PqsR, and that QscR also phase-differentially represses both LasR and RhlR functions in this regulation.**

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that causes serious infections in plants, animals, and humans. This versatile and ubiquitous organism is a major cause of nosocomial infections and is responsible for chronic lung infections in immunocompromised individuals and those suffering from cystic fibrosis (Van Delden and Iglewski, 1998). As in many bacteria, the expression of numerous virulence factors in this bacterium is controlled by small diffusible signaling molecules in a cell density-dependent manner known as 'quorum sensing' (QS) (Fuqua et al., 2001; Kim et al., 2009). In *P. aeruginosa*, QS relies on the activation of specific transcriptional regulators (LasR and RhlR) by corresponding signals synthesized by their cognate acyl HSL synthases (LasI and RhlI). The signals produced by LasI and RhlI are *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12) and *N*-butanoyl-L-homoserine lactone (C4), respectively. *P. aeruginosa* has a third LasR-RhlR homolog, QscR which was known as an orphan receptor (Chugani et al., 2001), but it was recently reported that QscR shares 3OC12 as its cognate signal with LasR (Lee et al., 2006). These three QS regulators and two signal synthases comprise a hierarchical cascade, where *las* regulates *rhl* and *qscR*, which co-ordinate the expression of numerous genes (more than 6% of the *P. aeruginosa* genome), many of which encode virulence and survival factors (Lequette et al., 2006; Schuster and Greenberg, 2006; Schuster et al., 2003; Venturi, 2006).

In addition to acyl-HSLs, another type of signal molecule, *Pseudomonas* quinolone signal (PQS; 2-heptyl-3-hydroxy-4-quinolone), is produced by *P. aeruginosa* and plays a role in the regulation of virulence genes (Pesci et al., 1999). PQS signaling seems intricately intertwined in the QS circuits and is required for the expression of some RhlR-dependent genes at the onset of the stationary phase (Diggle et al., 2003; McKnight et al., 2000; Pesci et al., 1999). While the PQS functions are not fully understood yet, the metabolic pathway and genes for PQS biosynthesis have been partly elucidated. The precursor is anthranilate (Calfee et al., 2001), from which PQS is synthesized by the products of the *pqsABCD* and *phnAB* gene cluster and *pqsH* (Deziel et al., 2004; Gallagher et al., 2002) (Fig. 1). Because anthranilate is also a precursor of tryptophan biosynthesis (Calfee et al., 2001; Essar et al., 1990; Oglesby et al., 2008) and can be degraded into TCA cycle intermediates by the functions of *antABC* and *catA* (encoding anthranilate 1,2-dioxygenase complex and catechol 1,2-dioxygenase, respectively) (Oglesby et al., 2008), anthranilate is an important intermediate at the metabolic branch point during the PQS biosynthesis (Fig. 1).

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Previous transcriptome analysis of QS signaling showed that *antABC* in anthranilate degradation is uniquely regulated by the QS system in a very complicated manner; its expression was induced at late stationary phase in signal mutant (*lasI*⁻, *rhlI*⁻), repressed by the addition of 3OC12 and restored again by the co-addition of C4 with 3OC12 (Schuster et al., 2003). A recent real-time PCR analysis showed RhlR-dependent activation and LasR-dependent repression of *antA* (Oglesby et al., 2008). These results indicated that the anthranilate-degrading function of the *antABC* operon might be antagonistically regulated by the QS regulators. Also, the expression of the *pqsABCDE/phnAB* operon for the PQS synthesis is also regulated by QS systems where they are activated by *las* system and repressed by *rhl* system (McGrath et al., 2004; Wade et al., 2005). These results further imply that PQS biosynthesis could be finely tuned by the QS systems at the metabolic branch point, anthranilate. Interestingly, these complicated regulations appear to occur with different timing. While RhlR-activation of *antA* expression occurs in late stationary phase, the LasR-repression of *antA* occurs at the entry of stationary phase (Oglesby et al., 2008).

In this study, to better understand the growth phase-dependent QS-regulation of anthranilate in detail, we comparatively examined the QS-regulation pattern of two metabolic pathways, from anthranilate to the PQS synthesis or to TCA cycle through the growth of *P. aeruginosa*. Our data show that the two pathways are intricately regulated by the antagonistic interplay of three QS receptors at different times in growth phase mediated by two intermediate regulators, AntR and PqsR, and their co-factors.

MATERIALS AND METHODS

Plasmids, bacterial strains and growth conditions

The *P. aeruginosa* strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth containing 50 mM MOPS [3-(*N*-morpholino) propanesulphonic acid, pH 7.0] at 37°C with vigorous shaking. Growth was monitored at OD_{600}. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 100 μg/ml; gentamicin, 12.5 μg/ml (for *E. coli*) or 100 μg/ml (for *P. aeruginosa*); $HgCl_2$, 7.5 μg/ml.

RNA isolation and real-time PCR analysis

For the real-time PCR analysis, cells from seed culture were inoculated into fresh medium (initial OD_{600} = 0.01) and cultivated; samples were taken at the indicated growth points for RNA extraction. The sampled cells (about 2×10^9) were directly mixed with RNA Protect Bacteria reagent (Qiagen) to stabilize RNA and lysed by lysozyme and brief sonication. RNA was purified by RNeasy mini columns (Qiagen) according to the manufacturer's protocol. Contaminated DNA was removed by on-column DNase I (Qiagen) digestion and additional RQ1 DNase I (Promega) digestion for 1 h at 37°C. DNase I was removed by RNeasy column purification. We confirmed RNA integrity by agarose gel electrophoresis and ruled out genomic DNA contamination by PCR amplification with primers to a ribosomal protein gene, *rplU* (no amplification was detected). For the cDNA synthesis, we annealed 12 μg of purified RNA with 750 ng of semi-random decamer primers of 75% G+C content (5′-$(NS)_5$-3′). In 30-μl reaction volume, 750U of Superscript II reverse transcriptase (Life Technologies), 1× 1st strand buffer, 10 mM DTT, 0.5 mM dNTPs, and 1U RNaseIN (Ambion) were mixed and incubated at 25°C for 10 min, 37°C for 1 h, 42°C for 1 h, and then 70°C for 10 min (for enzyme inactivation). After RNA removal by alkaline hydrolysis, final cDNA products were purified by Qiaquick PCR purification kit (Qiagen) and qualified on agarose gel. For real-time PCR, primers to the genes of interest were designed using primer express software (Taqman). The 1 ng of cDNA and 300 nM of the specific primers were mixed in 25-μl reaction volume of SYBR Green PCR amplification Master Mix (Applied Biosystems) in 96-well optical plate, and applied to real-time PCR machine (Applied Biosystems Model 7000). Reaction conditions were as follows: first stage, 2 min at 50°C; second stage, 10 min at 95°C; and third stage, 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing and extension).

Primer extension analysis

Primer extension of *pqsA* was performed using the Primer Extension System (PES) AMV Reverse Transcriptase kit (Promega). The primer (5′-gatcgaaatcgaggcggaacagaa-3′) was end-labeled by phosphorylation with [γP^{32}]-ATP, annealed with 20 μg of total RNA, and extended according to the manufacturer's protocol. The same primer was used in a sequencing reaction with Sequenase version 2.0 kit (USB). The products from the primer extension and sequencing reactions were resolved on a 6% polyacrylamide gel and visualized on X-ray film or phosphor imager (Typhoon).

Construction of dual plasmid system

To assess the activity of the regulators, we used a dual plasmid system as previously reported (Lee et al., 2006); this system is composed of the promoter-reporter fusion plasmid and the regulator-expressing plasmid. These two plasmids are compatible and replicable in both *P. aeruginosa* and *E. coli*. The transcriptional fusions, *antA-lacZ* (pJL201) and *pqsA-lacZ* (pJL301), were constructed by directional cloning of 856-bp and 631-bp PCR products of the upstream region of *antA* and *pqsA* genes into the *Sma*I site of pQF50. To express the regulators, the *antR* and *pqsR* orfs were amplified using primers containing an *Xba*I site and cloned into the *Xba*I site of pJN105 (pJN105A and pJN105P, respectively). The correct size, orientation, and sequence of all constructs were confirmed by sequencing.

β-galactosidase activity assay

For the *Pseudomonas* dual plasmids assay, PAO1 was transformed with two plasmids and fresh medium was inoculated with seed culture to the initial OD_{600} = 0.04 with 0.4% L-arabinose for the protein induction. Cells were collected at the indicated OD, and β-galactosidase activity was assayed by Galacto-Light Plus™ kit as recommended (Tropix). For the *E. coli* assay, DH5α cells harboring two plasmids were inoculated into fresh medium to the initial OD_{600} = 0.04 and grown to $OD_{600} \approx 0.3$. Then, 0.4% L-arabinose was added together with anthranilate or the *Pseudomonas* spent medium at the indicated concentration for 2 h, and β-galactosidase activity was then assayed. Results are given in units of β-galactosidase activity per OD_{600}. All measurements were performed at least three times and the most representative results were presented.

Preparation of the *Pseudomonas* spent media and anthranilate assay

A single fresh colony of each *Pseudomonas* strain of interest was inoculated and grown to OD_{600} = 3.5. Cells were removed by two successive centrifugations (16,000× *g* at 4°C for 10 min) and the supernatant was taken and kept on ice for use. The spent media were always prepared freshly. To measure the anthranilate levels in the media, the spent media from the indicated OD were used in the *E. coli* dual plasmids assay with

Table 1. Strains and plasmids used in this study

Names	Genotype	References
P. aeruginosa		
PAO1	Wild type prototroph	Pearson et al. (1997)
PAO-R3	*qscR* mutant of PAO1, Gm^R	Chugani et al. (2001)
PAO-R1	*lasR* mutant of PAO1, Tc^R	Pearson et al. (1997)
PDO111	*rhlR*::Tn*501*, PAO1, Hg^R	Brint and Ohman (1995)
PAO lasR rhlR	*lasR::Tc*^{R}*ΔrhlR::Gm*^{R}, Tc^R , Gm^R	Schuster et al. (2003)
PAO1 *phnA*	*phnA*::IS*lacZ*/hah (PTL 7254), Tc^R	Jacobs et al. (2003)
PAO1 *phzE1*	*phzE1*::IS*phoA*/hah (PTL 37013), Tc^R	Jacobs et al. (2003)
E. coli		
DH5α	*supE44ϕΔlac*U169 (*ϕ80 lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Sambrook et al. (1989)
Plasmids		
pQF50	Broad-host-range *lacZ* fusion vector, Ap^R	Farinha and Kropinski (1990)
pJL201	*antA-lacZ* fusion in pQF50, Ap^R	This work
pJL301	*pqsA-lacZ* fusion in pQF50, Ap^R	This work
pJN105	*araC*-pBAD cassette cloned in pBBR1MCS-5	Newman and Fuqua (1999)
pJN105L	*lasR* orf in pJN105, Gm^R	Lee et al. (2006)
pJN105R	*rhlR* orf in pJN105, Gm^R	Schuster and Greenberg (2007)
pJN105A	*antR* orf in pJN105, Gm^R	This work
pJN105P	*pqsR* orf in pJN105, Gm^R	This work

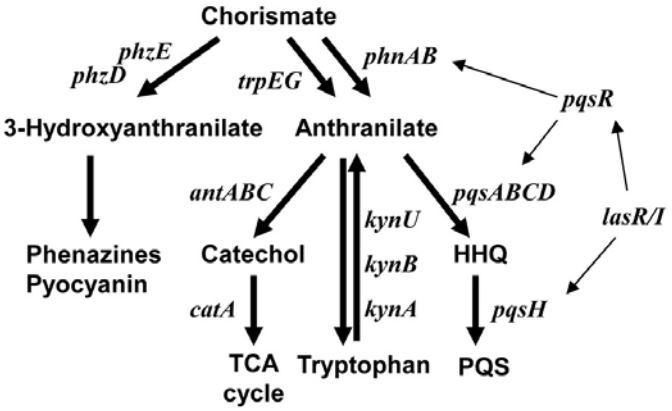

Fig. 1. Anthranilate metabolism and related genes. Arrows indicate the transcriptional activation. HHQ is 4-hydroxy-2-heptylquinoline and PQS is *Pseudomonas* quinolone signal.

pJN105A and pJL201, and β-galactosidase activity was assayed as described above.

RESULTS

Metabolic pathways from anthranilate are growth phase-differentially regulated by the antagonistic interplay of three QS regulators

To address details of the timing and antagonistic regulation of anthranilate metabolism by QS systems, we investigated whether the expression of *catA*, a gene at the next step in the anthranilate degradation pathway, is synchronously regulated with *antA*. The real-time PCR analysis showed that *catA* is regulated in a pattern similar to *antA*, where the *catA* expression was induced in late stationary phase, which disappeared in *rhlR*$^-$ mutant, and dramatically derepressed in *qscR*$^-$ mutant (Fig. 2). Transcriptome analysis also showed RhlR-dependent activation and LasR-dependent repression of c*atA* (Schuster et al., 2003). Together with the *antA* regulation pattern, this indicated that LasR could repress the expression of anthranilate degradation function during exponential and early stationary phases, but the C4-activated RhlR could overcome this repression in the late stationary phase to activate anthranilate degradation.

For better comparison, we investigated the expression timing and patterns of the *pqsABCDE/phnAB* gene cluster in another pathway toward the PQS synthesis by real-time PCR analysis under the same conditions. Because *pqsABCDE* and *phnAB* are synchronously regulated by the QS system (Deziel et al., 2004; Schuster et al., 2003), we probed the *phnA* expression. Our results showed that the *pqsA-E/phnAB* gene cluster was derepressed in the *rhlR*$^-$ mutant throughout growth, and while it was activated at late log phase in wild type, its activation was delayed to the late stationary phase in the *lasR*$^-$ mutant (Fig. 2). This indicated that LasR affected the timing of *pqsA-E/phnAB* gene cluster induction but not the expression level. Instead, the expression level of this gene cluster was more strongly regulated by RhlR, as demonstrated by the much higher level of expression in *rhlR*$^-$ mutant (Fig. 2). Like the *antA* and *catA* expressions, the most dramatic change in the expression of the *pqsA-E/phnAB* gene cluster was observed in the *qscR*$^-$ mutant, where both the expression level and timing were particularly altered. The expression of *pqsA-E/phnAB* gene cluster was much advanced toward early exponential phase and also derepressed to a high level (Fig. 2).

While *pqsA-E/phnAB* genes in a cluster have been reported to be synchronously regulated by QS (Schuster et al., 2003), an

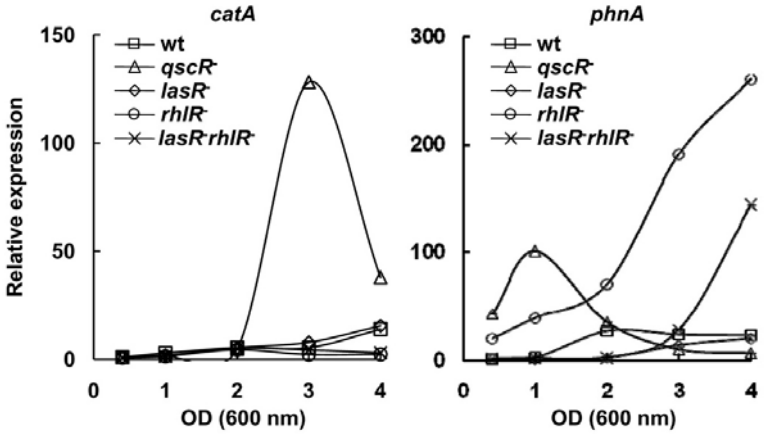

Fig. 2. The expression patterns of *catA* and *phnA*. The left panel shows the expression patterns of *catA*, and the right panel, that of *phnA* in various QS mutants throughout growth, as measured by real-time PCR analysis. The expression levels are indicated as relative expression units when the expression level of wild-type PAO1 at OD_{600} = 0.4 is 1.

independent study claimed that *pqsA-E* and *phnAB* genes are separately transcribed as two distinct polycistronic operons (McGrath et al., 2004). To exclude any possibility that the expression pattern of *phnA* might be different from that of *pqsA*, we confirmed the *pqsA* expression by primer extension analysis, finding that the *pqsA* expression pattern was same as that of *phnA* in Fig. 2 (Fig. 3). Therefore, these particular expression patterns of the *pqsA-E/phnAB* gene cluster in *rhlR*⁻ and *qscR*⁻ mutants are most likely achieved by transcriptional regulation on the *pqsA* promoter.

Interestingly, the RhlR repression affected the expression of the *pqsA-E/phnAB* gene cluster even in the early exponential phase when RhlR is not fully active. Because the *rhl* system is dependent on the *las* system in the QS hierarchy (Latifi et al., 1996; Pesci et al., 1997), this early repression of the LasR-activated gene by RhlR was not expected. Another interesting regulation was found in the *lasR*⁻*rhlR*⁻ double mutant where the derepression of the *pqsA-E/phnAB* gene cluster was detected only in late stationary phase (Fig. 2). This implied that other factor(s) may be involved in the activation of this operon in late stationary phase, which is repressed by RhlR in wild type.

These results showed the growth phase-differential regulation of anthranilate metabolism by the antagonistic interplay of three QS regulators, LasR, RhlR, and QscR, where the destination of anthranilate is finely tuned by QS systems depending on the growth phase.

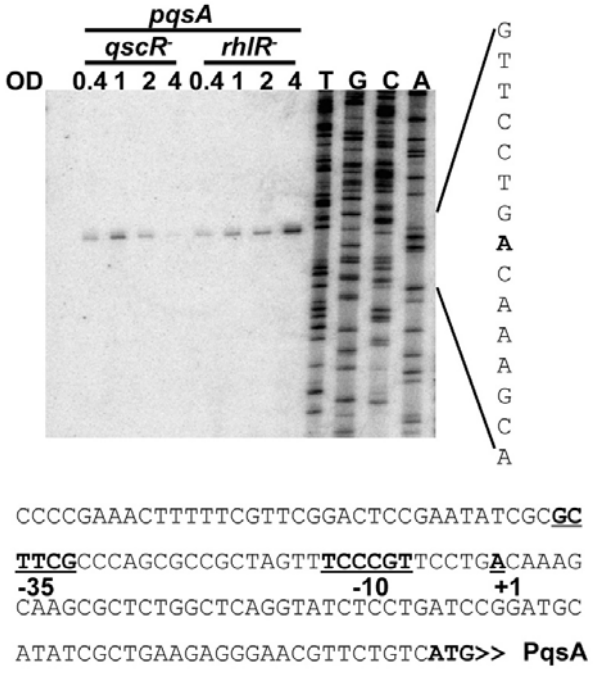

Fig. 3. Primer extension analysis on *pqsA* promoter. Primer extension experiments were quantitatively carried out to measure the transcript level of the *pqsA* gene. The information about the *pqsA* promoter such as +1 sites, -35/-10 boxes, and the start codon was indicated by underlines and bold letters. RNAs were extracted from the indicated growth points (OD_{600}) of each mutant strain.

Two direct regulators, AntR and PqsR, mediate this well-timed QS regulation, partly through the QS-regulated expression of *antR* and *pqsR* genes

For PQS synthesis, PqsR has been reported as a direct activator of *pqsA* promoter (McGrath et al., 2004; Wade et al., 2005). For the *antABC* operon, AntR (encoded by PA2511), which is divergently transcribed from *antA*, has recently been suggested as a direct activator (Oglesby et al., 2008). Therefore, the QS regulation of anthranilate degradation and PQS synthesis could be indirectly achieved through the regulation of *antR* and *pqsR* expression. However, the recently reported expression patterns and timings of *antR* and *pqsR* were different from those of *antABC/catA* and *pqsA-E/phnAB* operons. As shown in Fig. 2, the *pqsA-E/phnAB* gene cluster was differently expressed in *rhlR* and *qscR* mutants (Fig. 2), whereas *pqsR* was similarly expressed in both mutants (Oglesby et al., 2008). This means that the *pqsR* expression pattern does not fully reflect the *pqsA* expression pattern. Moreover, when we modified the expression level and timing of LasR and RhlR by arabinose-induced overexpression in PAO1 cells, the well-timed regulation was not significantly altered (Fig. 4). So, it was apparent that the complex regulation of anthranilate metabolism requires something other than the QS regulation of the *antR* and *pqsR* gene expressions.

Notably, we found PqsR repressed *antA* expression (Fig. 4) as recently reported (Oglesby et al., 2008). This indicates that PqsR can drive PQS production both by activating the PQS synthetic genes and by repressing the anthranilate degrading genes at the same time.

Cofactors of AntR and PqsR are QS-dependently produced

Since AntR and PqsR require cofactors, anthranilate and PQS

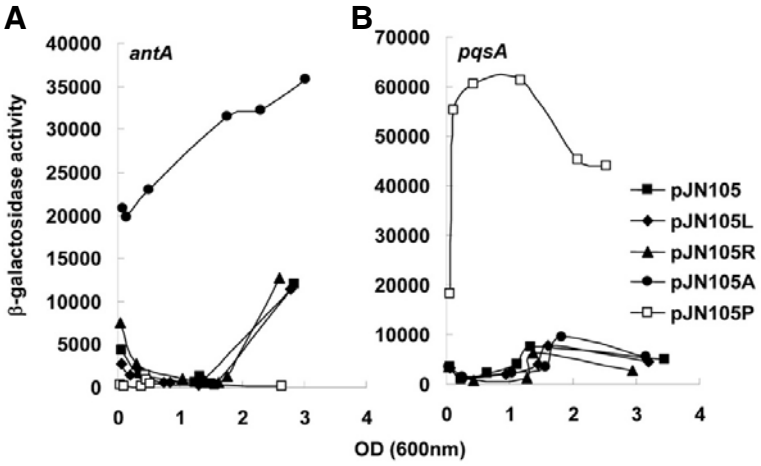

Fig. 4. Effect of the overexpression of AntR, PqsR, and the QS regulators on the expression of *antA* (A) and *pqsA* (B) in *P. aeruginosa* PAO1. The regulator-expressing plasmid (pJN105P, pJN105A, pJN105L, or pJN105R) was transformed into PAO1 cells that carried reporter fusion plasmid (pJL201 or pJL301). Cells containing two plasmids were grown with 0.4% L-arabinose, and β-galactosi-dase activity was measured at the indicated growth points throughout growth.

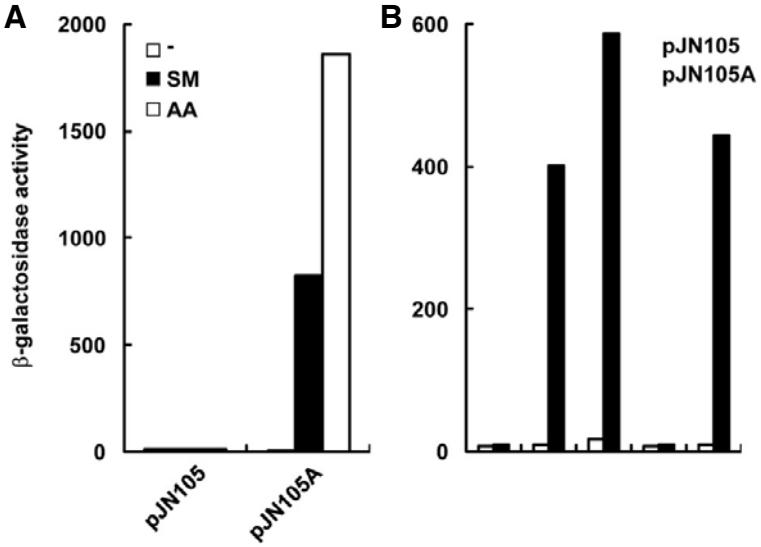

Fig. 5. Anthranilate-dependent activation of *antA* (A) and RhlR-dependent secretion of anthranilate. (A) the secreted anthranilate in the *Pseudomonas* spent medium was detected using the *E. coli* dual plasmid assay, which has two compatible plasmids, pJN105A and pJL201 (*antA-lacZ*). Cells were treated with 0.1 mM anthranilate (AA) or 20% PAO1 spent medium (SM) for 3 h. (B) treatment with the *Pseudomonas* spent medium from various R mutants at 20% for 3 h and β-gal activity was measured.

(or HHQ), respectively (Oglesby et al., 2008; Wade et al., 2005; Xiao et al., 2006), cofactor production could affect the QS regulation of anthranilate metabolism. The PQS and HHQ production is known to be activated by the *las* system (McGrath et al., 2004). However, which QS system influences the anthranilate production responsible for AntR activation was not clear. *phnAB* encodes the anthranilate synthase that is responsible for PQS production, but as shown in Fig. 2, the *phnAB* expression is facilitated by LasR and repressed by RhlR, which is opposite to the RhlR-dependent expression patterns of *antA* and *catA*. Another important anthranilate source for PQS synthesis is the kynurenine pathway, in which anthranilate is supplied through tryptophan degradation (Farrow and Pesci, 2007; Oglesby et al., 2008). However, the genes involved in this pathway, *kynB* and *kynU*, were positively regulated by *lasR* function (Schuster et al., 2003). This implied that the anthranilate produced by *phnAB* or kynurenine pathways might be insufficient or not responsible for the activation of the AntR function. So, we investigated the excretion of anthranilate in the spent media from various QS mutants, using an *E. coli* dual plasmid reporter system. This system carries AntR-expressing and *antA-lacZ* fusion plasmids that sensitively respond to anthranilate in the spent medium (Fig. 5A). Of the spent media from various QS mutants, only one from the *rhlR*⁻ mutant failed to activate AntR (Fig. 5B). Since anthranilate is considered permeable and the exogenous supplement of anthranilate can complement intracellular function (Farrow and Pesci, 2007), this result indicated that the anthranilate accumulation is RhlR-dependent. As the *antA* and *catA* expressions were turned on in late stationary phase (Fig. 2) (Oglesby et al., 2008), the anthranilate excretion also occurred in late stationary phase (Fig. 6). These results suggested that anthranilate might accumulate at late stationary phase to a sufficient level to activate AntR and this accumulation is RhlR-dependent. Consistently, the exogenous addition of anthranilate to a level high enough to activate AntR advanced the *antA* expression (Fig. 7).

As mentioned above, this increase of anthranilate is not likely dependent on either *phnAB* or kynurenine pathways, and is *phnA*-independent (Fig. 6). This means that anthranilate should accumulate due to another factor that is activated by RhlR. There are multiple anthranilate synthase homologues in addition to PhnAB in *P. aeruginosa* (Essar et al., 1990; Farrow and Pesci, 2007; Gallagher et al., 2002; Stover et al., 2000). Two of them, PhzE1 and PhzE2 (responsible for phenazine synthesis), are activated in an RhlR-dependent manner, whereas others such as PabB (responsible for para-aminobenzoate synthesis), PchA (responsible for salicylate synthesis) and TrpEG (responsible for tryptophan synthesis) are not QS-dependent (Schuster

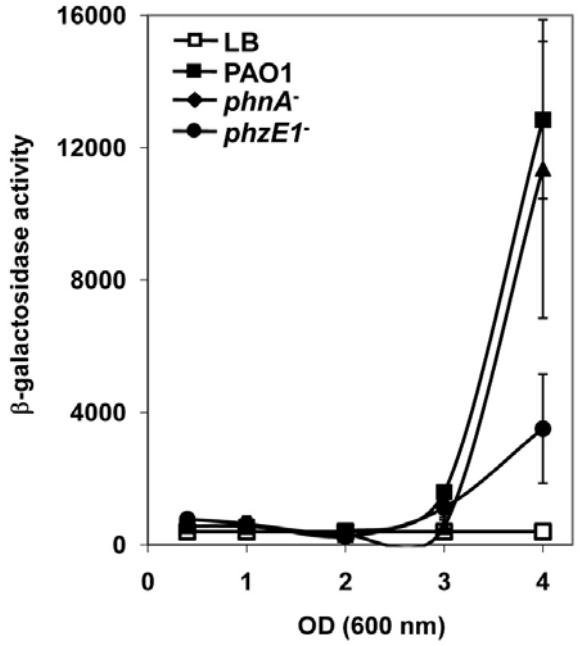

Fig. 6. The *phnAB*-independent secretion of anthranilate in late stationary phase. The anthranilate in the spent media from *phnA*⁻ and *phzE1*⁻ mutant strains were detected throughout growth by using the *E. coli* dual plasmid assay used in Fig. 5. Since the β-galactosidase activity of this bioassay has fairly good linear correlation with the concentration of exogenously added synthetic anthranilate, this method was used to estimate the anthranilate level. The anthranilate level in the culture media of wild-type PAO1 at late stationary phase (OD_{600} = 4) was estimated at 49.3 μM and that of *phzE1*⁻ mutant was at 13.5 μM.

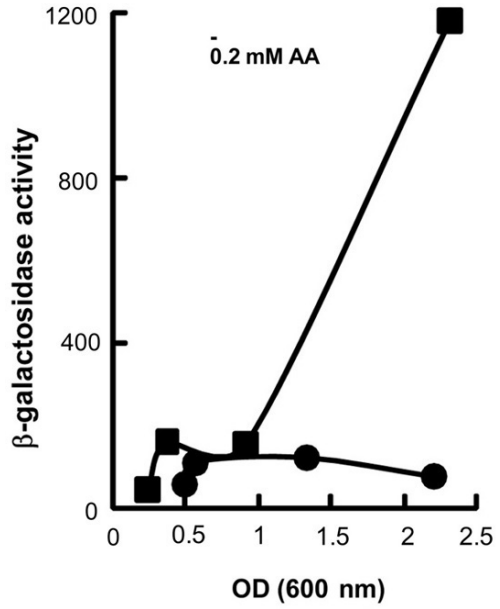

Fig. 7. Advanced activation of *antA* by the exogenously added anthranilate in *P. aeruginosa* PAO1. The *antA-lacZ* fusion plasmid (pJL201) was transformed into *P. aeruginosa* PAO1 and cultivated in LB broth containing 0.2 mM anthranilic acid (AA). Aliquots of culture were taken and β-galactosidase activity was assayed.

et al., 2003).

PhzE1 and PhzE2 have almost identical amino acid sequences, and the PhzEs have been suggested as an anthranilate synthase lacking the lyase activity of TrpE subunits, which cleaves pyruvate from 2-amino-deoxychorismic acid (ADC) (Mavrodi et al., 2006). So, the product of the PhzE-catalyzed reaction has been known to be ADC, an anthranilate analogue that has a pyruvate moiety. Together with PhzD, PhzE has been suggested to generate 3-hydroxyanthranilate instead of anthranilate (Fig. 1).

The expression patterns of phzE1 and 2 are similar, but the expression level of *phzE*1 has been known to be much higher than that of *phzE*2 (Ledgham et al., 2003; Schuster et al., 2003). We suspected that the *phzE* might be responsible for the RhlR-dependent anthranilate accumulation, because both *phz* operons are RhlR-dependently expressed in stationary phase (Chugani et al., 2001; Ledgham et al., 2003; Schuster et al., 2003), and their expressions are derepressed and advanced in *qscR*⁻ mutant (Chugani et al., 2001; Ledgham et al., 2003; Lequette et al., 2006), which is similar with the *antA* and *catA* expression patterns. As we expected, the anthranilate level in the culture medium of the *phzE1* mutant was lower than those in wild type (Fig. 6). To exclude the possibility that our AntR reporter system nonspecifically detected 3-hydroxyanthranilate, we tested the synthetic 3-hydroxyanthranilate for AntR activation and found that 3-hydroxyanthranilate failed to activate AntR significantly (Fig. 8). This suggests that anthranilate is accumulated in late stationary phase by *rhl* function and the anthrani-

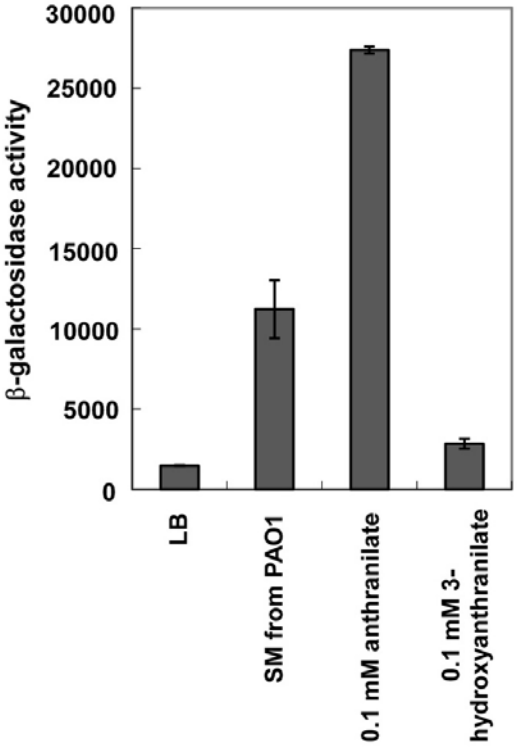

Fig. 8. Specific response of AntR to anthranilate. *E. coli* dual plasmid assay used in Fig. 5 was treated with anthranilate and 3-hydroxyanthranilate, and β-galactosidase activity was assayed. Spent medium (SM) from PAO1 overnight culture was added to 20% and fresh LB medium was used as control.

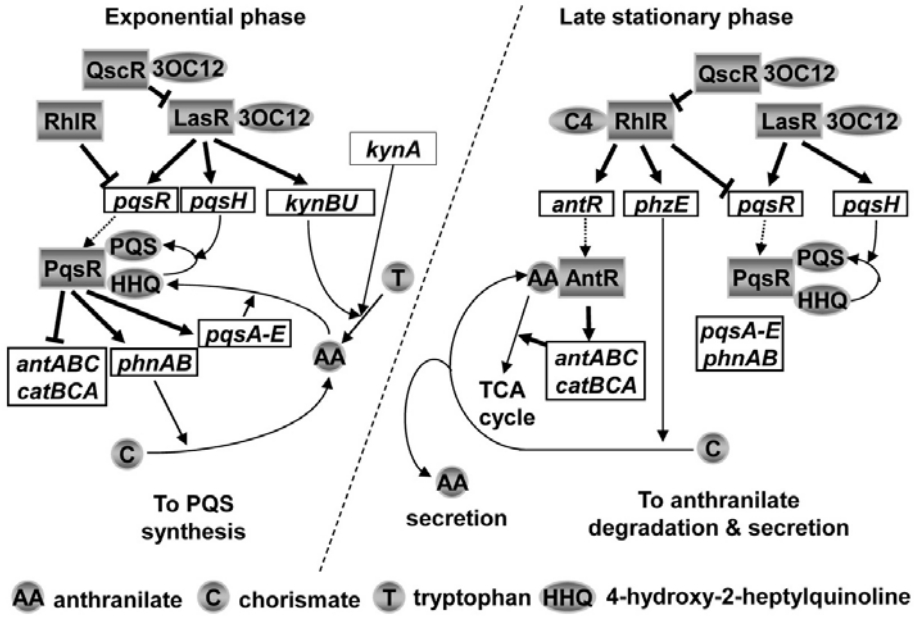

Fig. 9. Summary of the proposed regulatory circuit. The anthranilate metabolism is differentially regulated along with the growth phase. Until onset of stationary phase (left), 3OC12-activated LasR activates the PQS synthesis by increasing the PqsR level (Deziel et al., 2004; Oglesby et al., 2008; Schuster et al., 2003; Wade et al., 2005). PqsR also represses anthranilate degradation functions (Fig. 4). During this period, anthranilate as PQS precursor can be supplied from chrismate and tryptophan by the functions of *phnAB* and *kynA/BU* (Calfee et al., 2001; Farrow and Pesci, 2007; Gallagher et al., 2002), which are activated by LasR (Fig. 2) (Schuster et al., 2003), and RhlR represses the PQS synthesis (Figs. 2 and 3). Even though RhlR is supposed to be inactive at early exponential phase because of the premature C4-production, RhlR is likely to repress the *pqsR* function (Figs. 2 and 3) (Oglesby et al., 2008). In stationary phase (right), the C4-activated RhlR function inhibits PqsR (Fig. 2), which may remove the repression on the *antA* expression and interrupt the conversion of anthranilate to PQS. The RhlR-activated *antR* expression and anthranilate accumulation turn on the anthranilate degradation (Figs. 2, 5, and 6) (Oglesby et al., 2008), and the RhlR-activated *phzE* might influence the anthranilate excretion and accumulation (Fig. 6). QscR strongly represses both *lasR* and *rhlR* functions, but in a growth phase-differential manner (Figs. 2 and 3). See the text for details. The genes are represented in white boxes, proteins in gray boxes, small molecules in gray circles.

late accumulation may be significantly influenced by the *rhl*-dependent *phzE* function, although it has been previously suggested to be responsible for phenazine synthesis.

DISCUSSION

Together with published data and suggestions, our results can be incorporated into the proposed regulatory circuit as summarized in Fig. 9. In this study, we showed that the induction and repression of genes in anthranilate metabolism are tuned along with growth by the antagonistic interplay among three QS regulators, LasR, RhlR, and QscR, although there is no direct regulation by these regulators. Even QscR, which showed the most dramatic repression of the expressions of *antA* and *pqsA in vivo*, did not bind to any promoter of these genes *in vitro* (Lee et al., 2006). Apparently QS systems affect all regulations through the direct regulators, AntR and PqsR. As it has been suggested that an unknown 'super-regulation' influences QS and the induction of most target genes is not simply affected by levels of signals or receptor proteins in *P. aeruginosa* (Schuster and Greenberg, 2007; Schuster et al., 2003), our results also show the complicated QS regulation around anthranilate metabolism, such that the overexpression of LasR or RhlR failed to disrupt this well-timed regulation (Fig. 3). This means that the activities of AntR and PqsR could not be immediately altered by changing the level of QS regulators, and some other factors should be involved in the 'super-regulation'.

One reason for this complicated regulation could be the fine QS-regulation in the production of cofactors for AntR and PqsR. This means that QS systems regulate anthranilate metabolism both through the expressions of *antR* and *pqsR* genes and through cofactor production. While PhnAB has been suggested as the anthranilate synthase responsible for PQS synthesis (Calfee et al., 2001; Gallagher et al., 2002), many publications claimed the presence of multiple synthases and sources of anthranilate (Aendekerk et al., 2005; Essar et al., 1990; Farrow and Pesci, 2007; Gallagher et al., 2002). Our result also suggested that another possible factor influenced anthranilate accumulation in late stationary phase. While the other anthranilate sources, such as the *phnAB* function, kynurenine pathway, or trpEG are LasR-dependent or QS-independent (Schuster et al., 2003), the anthranilate excretion was RhlR-dependent (Fig. 5). This apparent inconsistency led us to investigate another anthranilate synthase, PhzE, which is *rhlR*-dependently induced in stationary phase (Schuster et al., 2003) and strongly derepressed and advanced in *qscR*⁻ mutant (Chugani et al., 2001; Ledgham et al., 2003; Lequette et al., 2006). Our result showed reduced excretion of anthranilate in *phzE1*⁻ mutant (Fig. 6). We assume that the anthranilate produced by PhnAB or tryptophan degradation until stationary phase might be rapidly consumed in PQS synthesis by PqsA-D and thus does not accumulate, but once RhlR is activated, the RhlR-induced PhzE could influence the anthranilate accumulation in stationary phase. However, although PhzE is homologous to other anthranilate synthases, its function has been postulated to produce 3-hydroxyanthranilate instead of anthranilate (Mavrodi et al., 2001). But since 3-hydroxyanthranilate did not activate AntR significantly (Fig. 8), it is still unknown how PhzE affects anthranilate accumulation.

As shown in Fig. 2, *pqsA* was constantly up-regulated throughout the growth in the *rhlR*⁻ mutant, whereas its expression in *qscR*⁻ mutant was activated in a much earlier phase and rapidly decreased in stationary phase. Interestingly, the *pqsR* expression pattern was similar in both mutants but the level was higher in the mutants than in wild type at early log phase (Oglesby et al., 2008). Although the repressions of *pqsR* by

RhlR and QscR can explain the higher expression of *pqsA* in *qscR*⁻ and *rhlR*⁻ mutants at early log phase, it is hard to explain why the *pqsA* expression in late stationary phase was decreased in the *qscR*⁻ mutant but continuously increased in the *rhlR*⁻ mutant. This result strongly suggests an inhibitory effect on PqsR activity at the post-transcriptional level, which may be activated by RhlR but repressed by QscR in stationary phase.

Interestingly, QscR acted as a repressor of both *antA/catA* and *pqsA-E/phnAB* operons in a growth phase-differential manner, where the *qscR*⁻ mutation strongly derepressed *pqsA-E/phnAB* during log phase, but repressed it in stationary phase; in contrast, the same *qscR*⁻ mutation strongly derepressed *antA/catA* only in late stationary phase (Fig. 2). The log phase-derepression of *pqsA-E/phnAB* by the *qscR*⁻ mutation may be due to the higher expression level of *pqsR* in the *qscR*⁻ mutant and self-boosting effect by higher expression of *pqsA-E/phnAB* in the *qscR*⁻ mutant. One clue for the stationary phase-repression of *pqsA-E/phnAB* by the *qscR*⁻ mutation may be the anthranilate accumulation, in that the anthranilate accumulation can be facilitated by the *qscR*⁻ mutation because QscR strongly represses both *phzE1* and *2* (Chugani et al., 2001; Ledgham et al., 2003; Lequette et al., 2006). The opposite attitude toward *phzE* expression may be a reason why RhlR and QscR show a similar repressive effect on *pqsA-E/phnAB* during log phase, but a dramatically different effect in stationary phase.

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