A novel two-component system BqsS-BqsR modulates quorum sensing-dependent biofilm decay in *Pseudomonas aeruginosa*

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**Introduction**

*Pseudomonas aeruginosa* can grow either as planktonic- or biofilm-form in response to environmental changes. Recent studies show that switching from biofilm to planktonic lifestyle requires rhamnolipids. Here we report the identification of a novel two-component system BqsS-BqsR that regulates biofilm decay in *P. aeruginosa*. BqsS is a multidomain sensor kinase and BqsR is an OmpR-like response regulator. Deletion of either bqsS or bqsR in *P. aeruginosa* mPAO1 resulted in a significant increase in biofilm formation. Time course analysis showed that the bqsS-bqsR mutants were defective in biofilm dispersal and in rhamnolipid production. Mutation of the BqsS-BqsR two-component system did not affect the biosynthesis of long chain quorum sensing (QS) signal N-3-oxo-dodecanoyl-homoserine lactone (3OC12HSL) but resulted in reduced production of the short chain QS signal N-butyryl-L-homoserine lactone (C4HSL) and the *Pseudomonas* quinolone signal (PQS). Exogenous addition of C4HSL, PQS or -butyryl-L-homoserine lactone (C4HSL) and the *Pseudomonas* lasI gene that encodes the biosynthesis of the long-chain QS signal leads to formation of flat, undifferentiated biofilms. While in another experiment, the lasI mutant biofilms were found indistinguishable from the wild-type biofilms at all time points with respect to both average thickness and roughness. In chronic infection, *P. aeruginosa* seems to adopt a surface-attached life style—biofilms. Formation of biofilms, in many cases, drastically increases the bacterial resistance to antibiotics treatment and host immune responses. As such, understanding the genetic basis and molecular regulatory mechanisms of *P. aeruginosa* involved in biofilm formation and development would be essential for control and prevention of biofilm formation, and for treatment of *P. aeruginosa* infections.

In response to environmental changes, *P. aeruginosa* can switch between planktonic growth and biofilm lifestyle. Biofilm formation is a dynamic process that involves several stages, including initial attachment, microcolony formation, biofilm maturation and ultimately biofilm dispersion. Dispersion is an important, but less understood stage of biofilm development, in which a subpopulation of biofilm cells detach and swim away, reverting to a planktonic lifestyle. Detachment/dispersion of cells from biofilms is essential for the maintenance and continuation of biofilms. In addition, dispersion also plays an important role in pathogenesis as the process creates mobile bacteria (single cells or aggregates) that can cause infection and promote dissemination from an initial infection point to other sites. Dispersion is a complicated process that involves multiple steps, including degradation of biofilm matrix, activation of motility and physiological changes, which prepare cells for the conditions outside biofilms. Several environmental and biochemical factors have been demonstrated to influence biofilm dispersion, such as availability of nutrients that promotes bacterial cells to move out of biofilms, extracellular hydrolytic enzymes that degrade biofilm matrix, activation of motility and physiological changes, which prepare cells for the conditions outside biofilms. Several environmental and biochemical factors have been demonstrated to influence biofilm dispersion, such as availability of nutrients that promotes bacterial cells to move out of biofilms, extracellular hydrolytic enzymes that degrade biofilm matrix, activation of motility and physiological changes, which prepare cells for the conditions outside biofilms.
in regulation of biofilm decay in *P. aeruginosa*. We showed that the null mutants of the two-component system were defective in biofilm decay and in rhamnolipid production. Our data also suggest that BqsS-BqsR may control biofilm decay by modulating biosynthesis of QS signals C4HSL and PQS (Pseudomonas quinolone signal).

**Results**

Deletion of *bqsS* and *bqsR* results in enhanced biofilm formation. Transposon mutagenesis was conducted to identify the genes of *P. aeruginosa* implicated in biofilm production. Screening of about 20,000 transposon mutants led to identification of fifteen mutants with altered biofilm phenotypes. While most of the biofilm mutants were owing to mutation of the previously identified genes (data not shown), a few mutants were found to contain transposon insertion in unreported genes. One mutant, in which *PA2656* was disrupted by Marinar transposon insertion at the position 386 bp downstream the start codon ATG, was found to produce a significantly higher amount of biofilm than the wild-type strain mPAO1. *PA2656* encodes a putative two-component sensor, containing a HAMP (Histidine kinases, Adenylyl cyclases, Methyl binding proteins, Phosphatases) domain, a phospho-acceptor domain, a histidine kinase-like ATPase domain, and a type I export signal peptide at N-terminal ([http://v2.pseudomonas.com/](http://v2.pseudomonas.com/)) (Fig. 1A). At the upstream of *PA2656*, the gene *PA2657* was predicted to encode a response regulator that contains a CheY-type REC signal receiver domain at the *N*-terminal and an effector domain (reg_C) associated with DNA- and RNA-polymerase binding at the C-terminal (Fig. 1A). Given their roles in biofilm development and QS as discussed below, these two genes were named as *bqsS* and *bqsR*, respectively. A BLAST search and gene context analysis showed that *bqsS* and *bqsR*, are highly conserved among Pseudomonas species, including *P. mendocina, P. fluorescens, P. syringae, P. putida* and *P. entomophila*, with an identity about 60%–80% at the amino acid level. In addition, other bacterial species, such as *Nitrospumucens oceani, Alcanivorax borkumensis, Escherichia coli* and *Limnobacter* sp. also contain homologues of BqsS and BqsR. None of these homologues has been previously characterized except the QseB of *E. coli*, which is a response regulator modulating bacterial cell motility and flagella biosynthesis.19

To understand the role of BqsS-BqsR two-component system in biofilm development, we generated the *bqsS* and *bqsR* deletion mutants Δ*bqsS* and Δ*bqsR*, respectively, using *P. aeruginosa* mPAO1 as the parental strain. After grown in LB medium for 16 h at 37°C, biofilm mass was detected by crystal violet staining. Both Δ*bqsS* and Δ*bqsR* mutants showed enhanced biofilm formation compared with their parental strain (Fig. 1B and C). To further confirm the role of the BqsS-BqsR two-component system, the wild-type *bqsS* and *bqsR* genes were placed under the control of the lac promoter and introduced into the Δ*bqsS* and Δ*bqsR* mutants, respectively. The complemented strains were cultured under the same conditions as mPAO1 and its deletion mutants before determination of biofilm formation. The results showed the biofilm production by the complemented strain Δ*bqsS*Δ*bqsS* and Δ*bqsR*Δ*bqsR* was substantially decreased to a level even lower than that of the parental wild-type strain. Consistent with the notion that the response regulator BqsR acts at the downstream of the sensor BqsS, the data also showed that the strain Δ*bqsS*Δ*bqsR*, in which the response regulator gene *bqsR* was expressed in the sensor mutant Δ*bqsS*, produced much less biofilm (Fig. 1B and C). In contrast, expression of the sensor gene *bqsS* in the deletion mutant Δ*bqsR* was unable to rescue the biofilm phenotype of the response regulator mutant (Fig. 1B and C).

The BqsS-BqsR two-component system is involved in regulation of biofilm decay. To understand how the two-component system could influence biofilm formation, a time course analysis of biofilm formation was performed on these strains over a period of 32 h. The results showed that the major differences between the wild-type mPAO1 and the mutants were in biofilm accumulation and in extent of biofilm decay (Fig. 2A). mPAO1 displayed a pattern of time-dependent accumulation and decline of biomass; in the first 16 h of incubation, the biomass of biofilms increased and reached a maximum amount at the time point of 16 h followed by a progressive decrease. In contrast, both Δ*bqsS* and Δ*bqsR* mutants, particularly the Δ*bqsS* mutant, produced abundant biofilms and displayed reduced biofilm decay over the course of the experiment. As expected, the...

**Figure 1.** Null mutation of the BqsS-BqsR two-component system results in enhanced biofilm formation in *P. aeruginosa* strain mPAO1. (A) Genetic organization and domain structures of the sensor kinase BqsS and the response regulator BqsR. Gene orientation is indicated by arrow. Domain structure prediction was done using the SMART program ([http://smart.embl-heidelberg.de/](http://smart.embl-heidelberg.de/)). Symbol: REC, cheY-homologous receiver domain; reg_C: response regulator receiver domain; SP, signal peptide; HAMP, histidine kinases, adenylyl cyclases, methyl binding proteins, phosphatases domain; HisKA, phospho-acceptor domain; HATPase, histidine kinase-like ATPase domain. (B) Visualization of biofilm formation on the walls of the polystyrene tubes by crystal violet staining. (C) Quantification of biofilm formation. The data shown are the means of triplicates and the standard deviation (SD) is shown by error bar. The following bacterial strains were used in this experiment: mPAO1 (mP), the bqsS deletion mutant Δ*bqsS*Δ*bqsS* (ΔS), the bqsR mutant Δ*bqsR*Δ*bqsR* (ΔR), and the complemented strains Δ*bqsS*Δ*bqsS* [ΔS(S)], Δ*bqsS*Δ*bqsR* [ΔS(R)], Δ*bqsR*Δ*bqsR* [ΔR(S)] and Δ*bqsR*Δ*bqsS* [ΔR(S)].
BqsS-BqsR controls QS-dependent biofilm decay

Co-cultured in 2:1 and 1:1 ratio. After 24 h incubation in polystyrene tubes at 37°C, biofilm formation was quantified by crystal violet staining. The results showed that the amount of biofilms in the ΔbqsS-mPAO1 mix culture was significantly decreased in comparison with the ΔbqsS monoculture (Fig. 3A).

The above data suggest that wild-type P. aeruginosa might produce an extracellular factor(s) that promotes biofilm decay. To test this possibility, ΔbqsS was inoculated in conditioned LB broth that contained 12.5% or 25% of filter-sterilized 24 h culture supernatants of the wild-type strain mPAO1. The bacteria were then grown at 37°C for 24 h before measurement of OD600 and biofilm formation. The results showed that inclusion of 12.5–25% of culture supernatants in conditioned medium had no obvious effect on growth rate of the mutant ΔbqsS (data not shown), but reduced mutant biofilm mass by up to 3-fold (Fig. 3B). The results imply the presence of extracellular factor(s) in supernatants of P. aeruginosa that reduces biofilm biomass by either inhibiting biofilm formation or promoting biofilm decay.

The BqsS-BqsR two-component system controls rhamnolipid production. Several recent publications showed that rhamnolipids play a role in biofilm dispersion, detachment and biofilm structural development. Therefore, rhamnolipid production was investigated in mPAO1 and its derivatives by using thin-layer chromatography (TLC). The extracted rhamnolipids were separated on TLC plates. Purified rhamnolipids (JBR599; Jeneil Biosurfactant Co.,) were used as a control. TLC results showed that wild-type mPAO1 and the two complemented strains produced two rhamnolipids, whereas the mutants ΔbqsS and ΔbqsR yielded little or no rhamnolipids (Fig. 4A).
We also found that deletion of \textit{bqsS} and \textit{bqsR} in wild-type strain \textit{mPAO1} decreased bacterial swarming motility by 27% and 19%, respectively, and the mutant phenotype was restored to the wild-type level by in trans expression of the corresponding wild-type gene. In contrast, deletion of the two-component system merely caused less than 5% change in swimming and twitching motility. These results appear to agree with the phenotype of decreased rhamnolipid production, since rhamnolipids facilitate biofilm formation of the \textit{mPAO1} and its derivatives (Fig. 4B). Similar to the pattern of PQS production, complemented strains produced higher levels of pyocyanin than wild-type, in particular the strain \textit{mPAO1ΔbqsR}, which produced over 7-fold less amount of C4HSL than its wild-type and complemented strain (Fig. 5A).

The above results suggest that the \textit{BqsS-BqsR} two-component system may influence expression of the genes encoding rhamnolipid biosynthesis and hence modulate production of rhamnolipids in \textit{P. aeruginosa}.

The \textit{BqsS-BqsR} two-component system influences production of C4HSL and PQS quorum sensing signals. Quorum sensing (QS) regulates expression of \textit{rhlAB} and \textit{rhlC} genes and production of rhamnolipids.\textsuperscript{22-24} It is intriguing to determine whether the \textit{BqsS-BqsR} two-component system influences rhamnolipid production directly or via modulation of QS signaling. We therefore examined production of \textit{N-acyl-L-homoserine lactones} (AHLs) QS signals in strain \textit{mPAO1} and its derivatives. AHLs were extracted from the overnight cultures grown at 37°C. The bioassay showed that strain \textit{mPAO1} and its mutants \textit{ΔbqsS} and \textit{ΔbqsR} produced similar levels of the long chain AHL signal 3OC12HSL (data not shown). However, decreased production of C4HSL was observed in the mutants \textit{ΔbqsS} and \textit{ΔbqsR}, especially the sensor mutant \textit{ΔbqsS}, which produced over 7-fold less amount of C4HSL than its wild-type and complemented strain (Fig. 5A).

Biosynthesis of the short chain C4HSL signal is not only regulated by the long chain signal 3OC12HSL but is also under positive control by the \textit{Pseudomonas} quinolone signal (PQS), which is a QS signal linking the \textit{las} and \textit{rhl} QS systems.\textsuperscript{25,26} To determine whether \textit{BqsS-BqsR} could influence PQS signaling, the signal extracts from strain \textit{mPAO1} and its derivatives were determined by TLC analysis following an established method.\textsuperscript{27,30} The results showed that the mutants \textit{ΔbqsS} and \textit{ΔbqsR} produced fewer PQS signals than strain \textit{mPAO1}, whereas their complemented strains produced much more PQS than the wild-type (Fig. 5C, left). Consistently, production of cytotoxin pyocyanin, which is positively regulated by PQS, was also reduced in \textit{ΔbqsS} and \textit{ΔbqsR} (Fig. 5B). Similar to the pattern of PQS production, complemented strains produced higher levels of pyocyanin than wild-type, in particular the strain \textit{ΔbqsS(ΔbqsS)} (Fig. 5B). The TLC analysis also detected anthranilate, which is a precursor of PQS,\textsuperscript{28} in the wild-type and its deletion mutants; however, the precursor was hardly detectable in both complemented strains (Fig. 5C). One plausible explanation is that overexpression of \textit{BqsS} or \textit{BqsR} in the complemented strains may facilitate conversion of anthranilate to PQS.

The reverse transcription polymerase chain reaction (RT-PCR) analysis confirmed the role of the \textit{BqsS-BqsR} two-component system in modulation of transcriptional expression of \textit{pqsA} and \textit{phnA}, which are involved in PQS biosynthesis.\textsuperscript{29} In both mutants \textit{ΔbqsS} and \textit{ΔbqsR}, the transcripts level of \textit{pqsA} and \textit{phnA} were decreased in comparison with \textit{mPAO1} and corresponding complemented strains (Fig. 5D).

Exogenous addition of rhamnolipids, C4HSL or PQS reduces biofilm formation of the \textit{bqsS} mutant to wild-type level. The above results suggest that the \textit{BqsS-BqsR} two-component system may modulate QS-dependent rhamnolipid production and hence influence biofilm decay. We therefore tested whether exogenous addition of rhamnolipids and QS signal molecules to the \textit{ΔbqsS} mutant could restore biofilm production to a wild-type level. Each test molecule was added to a final concentration of 50 and 100 μM, respectively, to LB liquid medium before inoculation of \textit{AbaqS}. Quantification of biofilm 24 h after inoculation showed that addition of either rhamnolipids or C4HSL or PQS could reduce biofilm accumulation to a level similar to the wild-type \textit{mPAO1} (Suppl. Fig. S1A–C). To further assess the effect of these molecules on biofilm development at various growth stages, we monitored bacterial growth rate and biofilm mass at four time points, i.e., 6 h, 16 h, 24 h and 32 h after...

Figure 4. Effect of \textit{bqsS-bqsR} mutation on rhamnolipid production and \textit{rhlA} expression. (A) TLC plate assay of rhamnolipids production. \textit{P. aeruginosa} strains described in Figure 1 were grown at 37°C for 24 h, and rhamnolipids were extracted from the supernatants for TLC analysis using standard rhamnolipids (Rh) as a positive control and the extracts from \textit{las} and \textit{bqsS} double mutant (IS) was used as negative control. Two predominant rhamno-

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which bacteria sense and respond to environmental changes and coordinate lifestyle transition are still poorly understood. In this study, we showed that null mutation of a novel multidomain sensor kinase BqsS and its cognate response regulator BqsR, in particular BqsS, resulted in enhanced biofilm formation and significantly reduced biofilm dispersion/detachment. Expression of the wild-type bqsS and bqsR in corresponding mutants restored the normal pattern of wild-type biofilm dispersion/detachment (Figs. 1B and C; 2A). In addition, overexpression of the response regulator BqsR in the sensor mutant ΔbqsS could restore its biofilm dispersion/detachment to the wild-type level (Fig. 1B and C). Cumulatively, these results have established an important role of the BqsS-BqsR two-component system in modulation of P. aeruginosa biofilm dispersion/detachment.

One of the key phenotypes regulated by the BqsS-BqsR two-component system is biosynthesis of rhamnolipids. Mutation of BqsS-BqsR resulted in significantly reduced production of rhamnolipids (Fig. 4A), and expression of rhlAB (Fig. 4B), whose products are involved in production of rhamnolipids.18,19 Rhamnolipids appear to have multiple roles in P. aeruginosa biofilm development, including promoting microcolony formation in the early growth phase and facilitating migration-dependent structural development in the late growth phase. It has been shown recently that P. aeruginosa rhlA mutants, deficient in synthesis of rhamnoslipids, are not capable of forming microcolonies in the initial phase of biofilm development.19 In contrast, mutation of bqsS or bqsR appeared to significantly affect the biofilm development in the late growth phase but had little effect on the biofilm formation during early phase (Fig. 2A). One plausible explanation is that BqsS-BqsR mutants were still able to produce a basal level of rhamnoslipids as evident from TLC analysis of rhamnoslipids and rhlAB expression analysis (Fig. 4A and B), which is sufficient to support initial biofilm formation.

Previous studies showed that rhamnolipids production is dependent on the short chain signal C4HSL-mediated QS system,22,23 and to a much lesser extent on the long chain signal 3OC12HSL-dependent inoculation. At a final concentration of 50 mg/L (rhamnolipids) or 50 μM (C4HSL, PQS), the molecules neither affect the growth rate of ΔbqsS (Suppl. Fig. S1D), nor the initial biofilm formation (6 h) in comparison with the wild-type control, but significantly increased subsequent biofilm decay (Fig. 6).

Discussion

P. aeruginosa is able to grow either as planktonic- or biofilm-form and undergoes transition between these two lifestyles in response to environmental changes. However, the molecular mechanisms by which bacteria sense and respond to environmental changes and coordinate lifestyle transition are still poorly understood. In this study, we showed that null mutation of a novel multidomain sensor kinase BqsS and its cognate response regulator BqsR, in particular BqsS, resulted in enhanced biofilm formation and significantly reduced biofilm dispersion/detachment. Expression of the wild-type bqsS and bqsR in corresponding mutants restored the normal pattern of wild-type biofilm dispersion/detachment (Figs. 1B and C; 2A). In addition, overexpression of the response regulator BqsR in the sensor mutant ΔbqsS could restore its biofilm dispersion/detachment to the wild-type level (Fig. 1B and C). Cumulatively, these results have established an important role of the BqsS-BqsR two-component system in modulation of P. aeruginosa biofilm dispersion/detachment.

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LadS35 were found to affect biofilm formation by controlling the QS system.23 Our bioassay results showed that null mutation of either BqsS or BqsR did not affect 3OC12HSL accumulation in bacterial culture, but drastically reduced the production of C4HSL signals (Fig. 5A). Chemical and genetic analysis also showed that the BqsS-BqsR two-component mutants produced substantially reduced PQS signals (Fig. 5C). In light of our current results and the previous findings that C4HSL production is regulated positively by PQS signal,30 we propose that the BqsS-BqsR two-component system may regulate P. aeruginosa biofilm decay through modulation of PQS, C4HSL and rhamnolipids production. The notion is strengthened by the findings that exogenous addition of either PQS, C4HSL or rhamnolipids at a physiological relevant concentration to the BqsS mutant was able to reduce the biofilm formation to a level similar to the wild-type strain mPAO1 (Fig. 6). Nevertheless, at this stage we have not yet tested the possibility that BqsS-BqsR could also regulate C4HSL biosynthesis in a PQS-independent manner. Neither would we rule out that other QS-dependent factors (apart from rhamnolipids) may also be implicated in the process of biofilm decay.

It is worthy noting that deletion of the sensor kinase gene bqsS resulted in much more significant phenotype changes, including biofilm decay (Figs. 1B and C; 2A), rhamnolipids production (Fig. 4A), and C4HSL and PQS signal biosynthesis than the response regulator mutant ΔbqsR (Fig. 5A and C). One likely explanation is that the sensor BqsS may be able to communicate with more than one response regulators. Signal transduction cross-talk has been found between about 3% of non-cognate sensor/response regulator pairs out of 27 two-component systems in Escherichia coli.31 The possibility that the sensor BqsS communicates with other response regulators in modulation of biofilm decay warrants further investigation.

It remains highly intriguing to determine the environmental signal(s) that activates the BqsS-BqsR two-component system. P. aeruginosa has more than 60 known or predicted two-component systems.32 Among them, three sensor kinases, GacS,33 RetS34 and LadS35 were found to affect biofilm formation by controlling the intracellular level of small regulatory RNAs. RetS and LadS are modular proteins sharing a similar domain architecture; both contain a N-terminal 7TM-DISMED2 (7-transmembrane-receptor with diverse intracellular signaling modules extracellular domain 2), followed by a 7TM-DISM_7TM domain (seven transmembrane segments found adjacent to 7TM-DISM domain), and then C-terminal histidine kinase and response regulator receiver domains. The sensor RetS is larger than LadS because the former contains an additional response regulator domain at its C-terminal. Of particular note is that these two closely related sensors appear to play opposite roles on biofilm formation. The retS mutant showed a hyperadhesive phenotype and overproduction of biofilms,34 whereas deletion of ladS resulted in loss of adherence and decreased biofilm formation.34 Sequence alignment and domain analysis show that these two hybrid sensor proteins do not seem to share significant similarity with BqsS except the histidine kinase domain. The GacS-GacA two-component system has been reported to play a role in regulation of C4HSL production and biofilm formation in response to an unknown environmental signal.35,34,36 The domain analysis and sequence alignment show that BqsS and GacS share the conserved HAMP-HisKA-HATPase domains with about 44% similarity but have little homology at the first 200 amino acids at the N-terminal, which may be implicated in sensing a signal ligand. Similar to the mutant ΔbqsR, the gacA mutant of P. aeruginosa strain PAO1 generates substantially less C4HSL signal,33,37 but the effect of the gacA mutation on biofilm formation in this strain has not been tested. In P. aeruginosa strain PA14, however, mutation of gacA does not seem to affect the C4HSL signal production but results in substantially decreased biofilm formation.38 Despite this apparent inconsistency in modulating QS signal production, the role of GacS-GacA in positive regulation of biofilm formation appears conserved in various bacterial species, including P. aeruginosa PA14,38 P. aeruginosa PAK,34 Pseudomonas sp. KL28,39 and Erwinia chrysanthemi.40 In all the cases, mutation of either GacS or GacA resulted in decreased biofilm formation, which is a sharp contrast to the negative regulatory role of the BqsS-BqsR system on biofilm development. Collectively, the available evidence seems to suggest that the BqsS-BqsR and GacS-GacA two-component systems may respond to different signals and use different mechanisms to influence biofilm formation.

In summary, we have demonstrated that BqsS-BqsR modulates biofilm decay by regulating biosynthesis of two QS signals, i.e., PQS and C4HSL. As these QS signal molecules are implicated in regulation of diverse biological functions,18,25,26,41 the BqsS-BqsR two-component system could also be an excellent model for further investigation on how bacteria may respond to environmental cues to adjust the QS-dependent social behaviors.

**Materials and Methods**

**Bacterial strains, media, growth and motility assay.** The P. aeruginosa strains and other bacteria used in this study are listed in Table 1. Unless otherwise indicated, bacteria were routinely grown at 37°C in Luria-Bertani broth (LB). Antibiotics were used when necessary at the following concentrations: carbenicillin, 300 μg/ml for P. aeruginosa, 200 μg/ml for Escherichia coli; tetracycline, 100 μg/ml for P. aeruginosa and 10 μg/ml for E. coli. For bacterial growth assay, overnight culture was diluted in LB broth and incubated in the Bioscreen C apparatus (Labsystems, Helsinki, Finland) at 37°C with moderate and continuous shaking. OD660 was measured every 30 min or otherwise indicated and each culture had 5 duplicates. Conditioned LB broth was obtained by addition of 12.5% to 25% of filter-sterilized overnight supernatants from strain mPAO1. Cell motility assays were performed as previously described.42

**DNA manipulation and deletion mutagenesis.** The plasmids used in this study are listed in Table 1. To generate the bqsS and bqsR deletion mutants of P. aeruginosa, plasmids pEX-S and pEX-R were constructed as following: two PCR fragments flanking bqsS or bqsR were amplified from the genomic DNA of wild-type strain mPAO1. The 5’- and 3’-flanking regions of bqsS were amplified using the primer pair bqsS5f/bqsS5r (5’-AGCAGATCCTGACGAGGATGCCTGCCGCT3’), 5’-GAGAGACTCTGTGTAAGCCCTGCTGCATGATTGCTCAG-3’), and bqsS3f/bqsS3r (5’-GATGAGATCCTGACGAGGATGCCTGCCGCTGTAAGCCCTGCTGATGATTGCTCAG-3’), respectively. Similarly, the two flanking regions of bqsR were amplified using bqsR5f/bqsR5r (5’-AGCAGATCCTGACGAGGATGCCTGCCGCTGTAAGCCCTGCTGATGATTGCTCAG-3’), 5’-GCTATAGAATCGGCCCCGTTAGTCAG3’), respectively. The two PCR fragments of each gene were fused by overlap extension.
Table 1   Bacterial strains and plasmids used in this study

| Strain or plasmid       | Relevant genotype or phenotype                        | Source or reference                      |
|-------------------------|-------------------------------------------------------|-----------------------------------------|
| P. aeruginosa           |                                                       |                                         |
| mPAO1                   | Prototrophic laboratory strain                        | University of Washington Genome Centre  |
| ΔbqsS::Tn                | mPAO1 mutant with transposon inserted in the coding region of bqsS | This study                              |
| ΔbqsS                   | The bqsS deletion mutant of mPAO1 with a 1309-nt internal coding region being deleted | This study                              |
| ΔbqsR                   | The bqsR deletion mutant of mPAO1 with a 639-nt internal coding region being deleted | This study                              |
| ΔbqsS(bqsS)             | ΔbqsS containing pUCP-bqsS                             | This study                              |
| ΔbqsS(bqsR)             | ΔbqsS containing pUCP-bqsR                             | This study                              |
| ΔbqsR(bqsR)             | ΔbqsR containing pUCP-bqsR                             | This study                              |
| ΔbqsR(bqsS)             | ΔbqsR containing pUCP-bqsS                             | This study                              |
| E. coli                 |                                                       |                                         |
| DH5x                    | F\sup{\text{\textregistered}}80dlacZΔM15 endA1hsdR17 [r\text{\textregistered}6 m\text{\textregistered}1] supE44 thi-1 gyrA96 Δ[::lacZYA-argF] recA pro (RP4-2Tet::Mu Kan::Tn7) | Gibco                                   |
| S17-1                   |                                                       |                                         |
| Chromobacterium violaceum |                                                       |                                         |
| CV026                   | Indicator strain for detecting C4HSL                  | McClean et al.49                        |
| Plasmid                 |                                                       |                                         |
| pUCP19                  | E. coli—P. aeruginosa shuttle vector with the lac promoter [plac], Amp\textsuperscript{\textregistered}/Cb\textsuperscript{\textregistered} | ATCC 87110                             |
| pEX18Ap                 | P. aeruginosa gene replacement vector; sacB, Amp\textsuperscript{\textregistered}/Cb\textsuperscript{\textregistered} | Hoang et al.43                          |
| pME6010                 | pVS1-p15A shuttle vectors                             | Heeb et al.44                           |
| pUCP-bqsS               | pUCP19 containing bqsS under the control of plac     | This study                              |
| pUCP-bqsR               | pUCP19 containing bqsR under the control of plac     | This study                              |
| pEX-S                   | pEX18Ap carrying the bqsS flanking region with the gene being deleted | This study                              |
| pEX-R                   | pEX18Ap carrying the bqsR flanking region with the gene being deleted | This study                              |
| pME2-lacZ               | pME6010 carrying a full-length lac gene               | This study                              |
| prhlA-lacZ              | pME2-lacZ carrying rhlA promoter in front of lac gene | This study                              |

a Amp\textsuperscript{\textregistered}, ampicillin resistant; Cb\textsuperscript{\textregistered}, carbenicillin resistant.

PCR. The resulted fusion PCR fragments contain the truncated bqsS and bqsR, in which a 1290-nt coding region (from 18–1308 bp) of bqsS and a 638-nt coding region (from 26–664 bp) of bqsR were deleted, respectively. After purification with QIAquick PCR purification kit (QIAGEN), the fusion fragments were digested with SacI and XbaI (the site is underlined in primer sequence) and separately cloned into the corresponding site of pME2-lacZ, which was then digested with HindIII and EcoRI (underlined in the primer sequence). The resultant constructs were introduced into E. coli S17-1 by electroporation and then mPAO1 by biparental mating. The generated bqsS and bqsR deletion mutants were confirmed by PCR.

For mutant complementation, the coding regions of the wild-type bqsS and bqsR genes were amplified from mPAO1 genomic DNA using primer pairs Sf/Sr (5'-CACGGATCCCTGTGGATGCGCGC-3', 5'-AGCGATCGACCTTGAGCATG-3'), respectively. The PCR products were cloned into the downstream of lac promoter in the shuttle vector pUCP19 (ATCC 87110) digested by XbaI/BamHI (for bqsS) or HindIII/BamHI (for bqsR). The resultant constructs were mobilized into E. coli separately and sequenced before introducing to corresponding mutants as described in the previous section.

To construct the prhlA-lacZ reporter plasmid, a 433 bp fragment corresponding to -377 to +56 bp relative to the translational start site of the rhlA gene was amplified from the genomic DNA of P. aeruginosa by PCR using the primer pair prhlAr 5'-TGACACAGAGTTCCATGGTTTCGCCGCAAC-3' and prhlAr 5'-CGGAAATTTCGATGTACCGCGAGCC-3'. The fragment was then digested with HindIII and EcoRI (underlined in the primer sequence) and then cloned in the same sites of pME2-lacZ sequence, which was derived from pME6010.44 The resultant constructs were used to transform P. aeruginosa by electroporation, and transformants were selected on LB agar plates containing relevant antibiotics.

RNA purification and RT-PCR analysis. Overnight culture of P. aeruginosa wild-type mPAO1 and its derivatives were diluted in LB broth and incubated at 37°C until the OD\textsubscript{600} reached about 1.5. Total RNA samples were then isolated using the RNeasy miniprep kit (QIAGEN). Reverse transcription polymerase chain reaction (RT-PCR) was performed using QIAGEN OneStep RT-PCR Kit according to the manufacturer’s protocol. The primer pairs used for pqsA and phnA are: pqsAF/pqsAR, (5'-TGATGCAACGCTGGCGCAAC-3', 5'-CTTCTGAGGTGCGGTGCGTCTGTCGCTTGGTA-3'; 5'-CGGAAATTTCGATGTACCGAGCC-3'; HindIII and BamHI site underlined); Rf/Rr (5'-TGACACAGAGTTCCATGGTTTCGCCGCAAC-3', 5'-AGCGATCGACCTTGAGCATG-3'). The PCR products were cloned into the downstream of lac promoter in the shuttle vector pUCP19 (ATCC 87110) digested by XbaI/BamHI (for bqsS) or HindIII/BamHI (for bqsR). The resultant constructs were mobilized into E. coli separately and sequenced before introducing to corresponding mutants as described in the previous section.

β-galactosidase assay. For measurement of β-galactosidase activity, P. aeruginosa strains containing the prhlA-lacZ construct
were grown overnight in LB with shaking at 37°C. The starter cells were inoculated in LB at a ratio of 1:100, and then grown at the same temperature to an OD_{600} of 1.5. β-galactosidase activity was determined using a standard protocol. Results are given as Miller units (MU) of β-galactosidase activity per OD_{600}.

**Biofilm formation assay and quantification.** Biofilm formation assay was performed according to O’Toole and Kolter with minor modifications. Briefly, overnight bacterial cultures were diluted to the same concentration of OD_{600} = 0.002 with fresh LB broth. The diluted cultures (1 ml) were transferred to a 14-ml polystyrene tube (17 x 100 mm; FALCON, 352057) and incubated at 37°C with shaking at 210 rpm for a period as stated. Bacterial cultures were carefully removed for measurement of OD_{600}. The bacterial cells bound to the wall of the tubes (biofilms) were stained with 0.1% crystal violet (Sigma) for 15 min at room temperature and the tubes were then rinsed several times with water. The tubes were air-dried at room temperature and then photographed. For quantification, the attached cells (biofilms) were suspended in 2 ml of 75% ethanol. The absorbance at 595 nm was measured with a spectrophotometer. Each experiment was repeated at least three times.

**Preparation of rhamnolipids and TLC assay.** Bacterial cells were removed from the cultures grown at 37°C for 24 h by centrifugation, and the supernatant was adjusted to pH 2 with concentrated HCl. Rhamnolipids were then extracted from the acidified supernatants (1 ml) twice with an equal volume of chloroform-ethanol (2:1, v/v) mixture. The pooled organic phases were evaporated to dryness, and the remaining residues were dissolved in 0.5 ml of methanol. After air dry, each sample was resuspended in 20 μl of methanol. An aliquot of each sample (5 μl) was spotted and analyzed by thin-layer chromatography (TLC; silica gel 60 plates, F_{254}; Merck, Darmstadt, Germany) with a mobile phase consisting of chloroform-methanol-water (65:15:2 by volume). Commercial rhamnolipids (JBR599; Jeneil Biosurfactant Co., LCC) were used as a standard control in TLC analysis.

**Pyocyanin, AHL and PQS extraction and assay.** _P. aeruginosa_ strains were grown in LB or _Pseudomonas_ medium A^47 (for pyocyanin) broth for 24 h at 37°C with shaking. Pyocyanin was extracted from 5 ml of culture supernatant with a 3-ml volume of chloroform and the chloroform phase was extracted with 0.2 N HCl. The OD_{420} of the aqueous phase was measured. The AHL signals were extracted from 10 ml of supernatants with an equal volume of acidified ethyl acetate by vigorous vortexing, followed by centrifugation. The organic phase was transferred to a fresh tube and dried to completion. The extracted compounds were dissolved in 100 μl methanol for bioassay. Long chain AHL signal 3OC12HSL were assayed by plate diffusion assay as described. For detection of C4HSL, _Chromobacterium violaceum_ CV026 was used as an indicator strain and LB agar medium was used for bioassay. Bioassay plates were incubated at 28°C for 40 h. The relative amounts of C4HSL were quantified as described. PQS extraction and assay were conducted as described by Diggle et al. For each sample, 5-μl extracts were spotted onto a Silica 60 F 254 plate (10 x 20 cm; Merck), along with synthetic PQS standard. TLC plates were soaked for 30 min in 5% KH₂PO₄ and activated at 100°C for 1 h before use. Chromatography was performed with a solvent mixture of dichloromethane-methanol (95:5). Upon completion, the plates were air-dried and visualized under UV light and photographed using Alphalmager (Alpha Innotech). For determination of anthranilate, TLC plates were left for about 24 h at room temperature until yellow spots became visible.

**Note**

Supplementary materials can be found at: www.landesbioscience.com/supplement/DongCIB1-1-Sup.pdf

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