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Building Quantitative Gene Regulatory Mechanism in Quorum Sensing in *Pseudomonas aeruginosa* Using Transcriptomic Data

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ABSTRACT

A large amount of transcriptomic data provides opportunities 1) to verify the gene regulatory mechanism, which is usually obtained from a single experiment, at population level; 2) to uncover the gene regulatory mechanism at population level; and 3) to build a quantitatively gene regulatory mechanism. One of the best studied regulatory mechanisms in bacteria is the quorum sensing (QS), which plays an important role in regulation of bacteria population behaviors such as antibiotic production, biofilm formation, bioluminescence, competence, conjugation, motility and sporulation. *Pseudomonas aeruginosa* is a Gram-negative bacterium causing diseases in plants, animals, humans, and its biofilm and drug-resistance become great concerns in clinics. *P. aeruginosa* has three QS systems including a specific one for *Pseudomonas*. In this study, the transcriptomic data of *P. aeruginosa* were combined from 104 publications and QS gene expressions were analyzed under different experimental conditions. The results demonstrate the quantitatively regulatory mechanisms of QS genes at population level including 1) to rank and group QS-related genes according to their activity; 2) to quantitatively define the role of a single global regulator; 3) to find out the probability that a global regulator impacts QS genes and the probability that a QS gene responds to global regulators; and 4) to search for overlapped genes under four types of experimental conditions. These results provide integrative information on understanding the regulation of QS genes at population level.

1. INTRODUCTION

From big data to knowledge is the landmark for big data era [1] because the exponential growth of data provides an opportunity to use big data to generate new knowledge. For gene regulatory mechanisms, the large amount of transcriptomic data provides opportunities 1) to verify the gene regulatory mechanism, which is usually obtained from a single experiment, at population level ; 2) to uncover the gene regulatory mechanism at population level; and 3) to build a quantitatively gene regulatory mechanism.

Quorum sensing (QS) in bacteria is a cell-to-cell communication through production and detection of autoinducers, and response to autoinducers. Thus, a bacterium can coordinate its own behavior with a population behavior upon the concentration of autoinducers, which is subject to cell density [2]. At genetic level, QS regulatory mechanism has been well studied over years, *i.e.* bacteria regulate their gene expression according to their population size [2]. This regulation results in antibiotic production, biofilm formation, bioluminescence, competence, conjugation, motility and sporulation.

For bacteria, QS is generally composed of two components: 1) an autoinducer synthase, which is a member of LuxI family (accession no. pfam00765) and produces an N-acylhomoserine lactone (AHL); and 2) a transcriptional regulator, which is a member of LuxR family (accession no. pfam03472) and activates or represses the transcription of targeted genes after binding of AHL [3-5].

Pseudomonas aeruginosa is a Gram-negative bacterium causing diseases in plants [6], animals [7], and humans, where the cystic fibrosis is a major concern [8-10]. *P. aeruginosa* has three QS systems. The first is LasI-LasR, which is related to synthesis and use of N-(3-oxo-dodecanoyl)-L-homoserine lactone ($3OC_{12}$-HSL) [8]. The second is RhlI-RhlR, which is related to synthesis and use of N-(butanoyl)-L-homoserine lactone (C_4-HSL) [11]. These two QS systems essentially are N-acylated homoserine lactone (AHL)-based QS systems [12] in many bacteria. The third is the *Pseudomonas* quinolone signal (PQS)-based system, PqsABCDH-PqsR, which is related to synthesis and use of 2-heptyl-3-hydroxy-4-quinolone (HHQ) [13, 14].

For these three QS systems, their gene regulatory mechanism is the objective of many studies and reviews [15-17]. Basically, this regulatory mechanism indicates that QS system is a positive feedback system at QS level, *i.e.* a close loop exists for each pair, LasI-LasR through 3OC12-HSL, RhlI-RhlR through C4-HSL, and PqsABCDH-PqsR through HHQ.

The regulatory mechanism among three QS systems in *P. aeruginosa* includes both positive and negative feedbacks: 1) LasR regulates both RhlR and PQS positively through the complex 3OC12-HSL–LasR [12, 18-20]; and 2) PQS positively regulates *rhlI* expression [21]. The only negative regulation among QS systems is that RhlR negatively regulates PqsR in *P. aeruginosa* [12, 22]. But this negative feedback seems not to be sufficient to stop QS because QS response is not reversed for small decreases in population density in *P. aeruginosa* [16].

Usually, QS-controlled genes are not active unless the population density reaches the threshold [23]. Therefore there is a regulatory mechanism that preventing QS genes from expression at "prequorum" period [23, 24]. The genes controlling QS genes are termed differently such as super-regulators of QS [17], QS regulators [23], global regulators [25-27].

Negative global regulators for both LasI-LasR and RhlI-RhlR systems are 1) QteE inhibits QS system by reducing LasR protein stability and reducing RhlR levels [23]; 2) MvaT (PA4315) works as a repressor for QS [25]; 3) AlgR2 (AlgQ, PA5255) negatively modulates *lasR* and *rhlR* [28]; and 4) RpoN (σ54, PA4462) inhibits *lasR* and *lasI* genes at a low cell density [26] and inhibits *rhlR* and *rhlI* genes throughout growth because a potential RpoN recognition sequence exists in the *rhlI* promoter [26, 29].

Negative global regulators for LasI-LasR system include 1) QscR (PA1898) represses LasI [18, 30]; 2) QslA (PA1244) inhibits LasR by preventing its binding to DNA [31, 32]; and 3) RsaL (PA1431) inhibits *lasI* by binding to *rsaL-lasI* promoters [33, 34], then negatively feedbacks itself [35], and controls over 130 genes [36]; and 4) RsmA (PA0905) inhibits *lasI* [37].

Negative global regulators for RhlI-RhlR system are 1) DksA (PA4723) inhibits *rhlI* and further inhibits both *rhlAB* (rhamnosyltransferase for virulence factor rhamnolipid) and *lasB* (elastase) [38-40] be-

cause the expression of *lasB* needs the complex C4-HSL–RhlR [41]; 2) Lon (PA1803) negatively regulates RhlR/RhlI by degrading LasI [42]; 3) RpoS (PA3622) inhibits *rhlI* [18, 43-45], although an opposite result was observed [18].

The negative global regulator for PQS system is QslA (PA1244), which negatively regulates PqsR protein [31].

Positive global regulators for LasI-LasR system include 1) GacA (PA2586)/GacS (PA0928) promoting *lasR* in *P. aeruginosa* [46, 47] and *P. putida* [48]; 2) Vfr (PA0652) promoting *lasR* in *P. aeruginosa* through the cyclic AMP receptor protein-binding and *rhlR* [49]; 3) VqsR (PA2591) promoting *lasI* [27].

Additionally, VqsM (PA2227) is sometimes positioned beyond global regulators because it positively regulates VqsR (PA2591), RpoS (PA3622) and PprB (PA4296) [50].

Collectively, there are eleven negative global regulators (AlgR2, DksA, Lon, MvaT, QscR, QslA, QteE, RpoN, RpoS, RsaL and RsmA), and four positive global regulators (GacA, GacS, Vfr and VqsR). Moreover, there is interaction among global regulators, for example, VqsR (PA2591) directly inhibits QscR (PA1898) through binding to *qscR* promoter region [51].

In the 1990s, transcription of *aprA* (PA1249), *lasA* (PA1871) and *toxA* (PA1148) was found to require LasR [52]. Since then, the number of genes under QS control in *P. aeruginosa* varies from experiment to experiment, ranging from 1% - 4% genes [43], to 10% genes [15, 16], and to 11% genes [53]. Recently, the number of QS-controlled genes by *las* and *rhl* systems reached 616 genes [53].

Also, two AHL-acylases, PvdQ (PA2385) and QuiP (PA1032), play a role in controlling AHL homeostasis by adjusting its level [54, 55]. Still, the import-export pumps, *i.e. mexE* (PA2493), *mexF* (PA2494), *oprN* (PA2495) [56], *mexG* (PA4205), *mexH* (PA4206), *mexI* (PA4207) and *opmD* (PA4208) [57] are important for QS. Moreover, *pfm* (PA2950), enoyl-CoA reductase, has its influence on QS [58].

At this point, the QS regulatory mechanism is clear, but its quantification is needed because each individual gene responds differently. For example, some genes well respond to *lasA,* some genes respond well to *rhlAB*, and some genes equally respond to both [15, 59]. Moreover, QS regulatory mechanism was usually obtained from an individual experiment. However, the performance of each component at population level has yet to fully understand.

A way to deal with the abovementioned issues is to assemble and analyze the transcriptomic data together because they dynamically and globally record the gene response to various experimental conditions. In this study, the transcriptomic data from 104 journal publications on *P. aeruginosa* were combined and QS gene expressions under different conditions were analyzed in order at population level 1) to rank QS genes according to their activity, 2) to verify the regular pathways, and 3) to uncover QS regulatory mechanism, and 4) to build a quantitative gene regulatory mechanism.

2. MATERIALS AND METHODS

Affymetrix *P. aeruginosa* array is widely used in transcriptomic studies. Platform GPL84 is specially designed for *P. aeruginosa* PAO1 and contains 5549 *P. aeruginosa* genes. Gene Expression Omnibus (GEO) requires archiving all the transcriptomic data [60, 61]. The GPL84 datasets from 104 journal publications (Supplementary Info) were analyzed for this study. Yet, these 104 studies were all the studies we could find at the beginning of our study.

Basically, these data come from different experiments with different measurement standards and cut-off values. Therefore, we reformatted them according to the measurement standard of signal intensity with cut-off value of 2, which is the statistical significance used in these studies (Supplementary Datasets 1 and 2).

For an individual transcriptomic study, the focuses are usually placed on how many genes are up/down-regulated and which gene is up/down-regulated under experimental conditions. As a result, we can estimate a gene's activity at population level through counting its up/down-regulated frequency in all available transcriptomic studies. Indeed, this activity represents a gene's function under all the experimental conditions, so it is an activity at population level. In this manner, we have the activity of all up-regulated and down-regulated QS-related genes at population level (**Figure 1** and **Figure 2**, and Supplementary

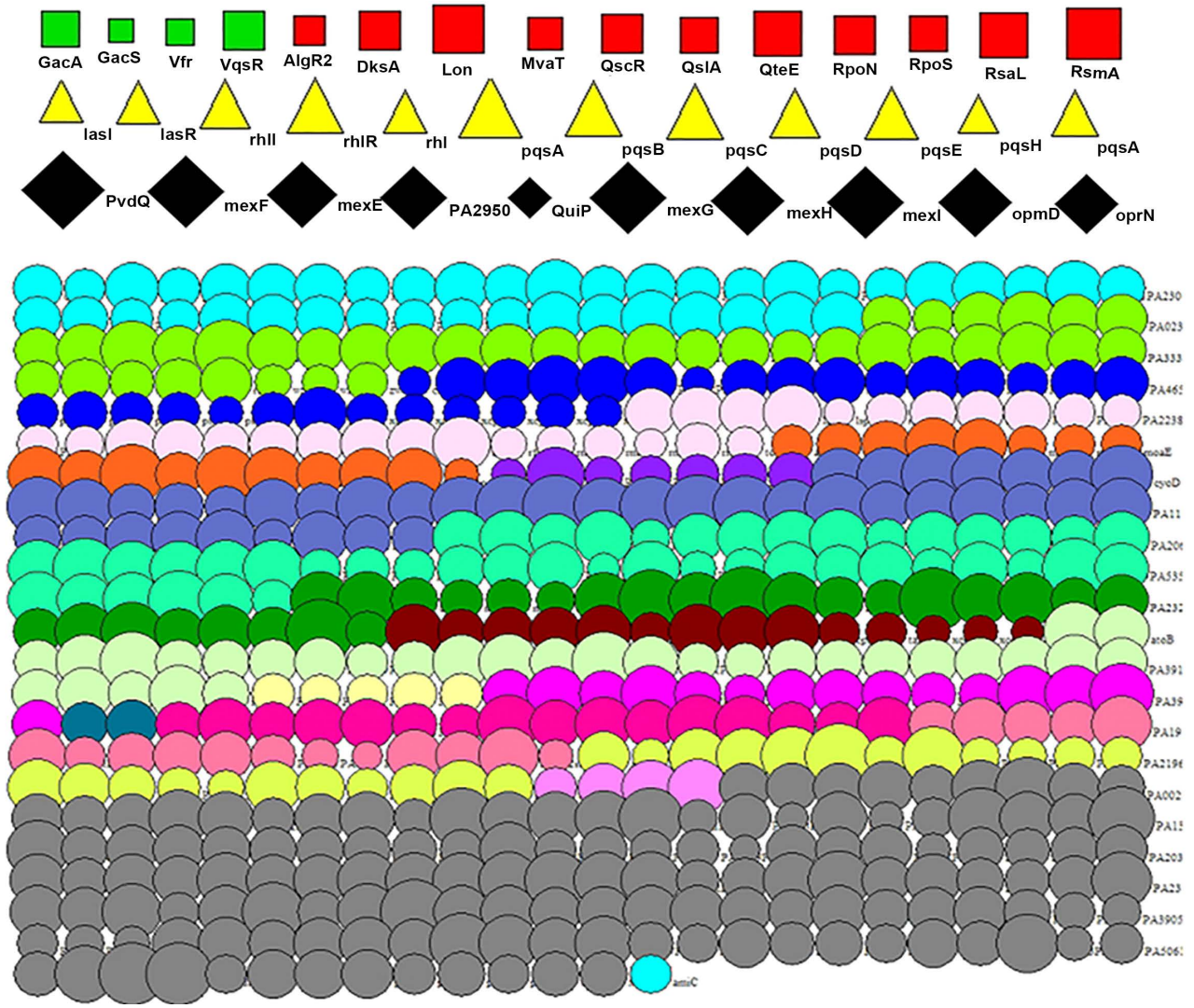

Figure 1. Quantitative activity of QS-related genes under up-regulation. Each symbol represents a gene and its size is proportional to its up-regulation frequency at population level. From the top row down, 1) green and red squares are positive and negative global regulators, 2) yellow triangles are QS genes, 3) black diamonds are the genes affecting AHL levels, and 4) circles are QS-controlled genes, whose encoding proteins are colored according to *Pseudomonas* classification [63, 64] as follows: 1) cyan—amino acid transport and metabolism; 2) lime green—carbohydrate transport and metabolism; 3) blue—cell motility; 4) pink—cell wall/membrane/envelope biogenesis; 5) orange—coenzyme transport and metabolism; 6) purple—defense mechanisms; 7) cadet blue—energy production and conversion; 8) teal blue—general function prediction only; 9) olive green—inorganic ion transport and metabolism; 10) maroon—intracellular trafficking, secretion and vesicular transport; 11) light green—lipid transport and metabolism; 12) light yellow—nucleotide transport and metabolism; 13) magenta—posttranslational modification, protein turnover and chaperones; 14) midnight blue—replication, recombination and repair; 15) wild strawberry—secondary metabolites biosynthesis, transport and catabolism; 16) salmon—signal transduction mechanisms; 17) green yellow— transcription; 18) lavender—translation, ribosomal structure and biogenesis; and 19) gray—function unknown or unclassified. For data details, see Supplementary Data Fig-1-2.xlsx.

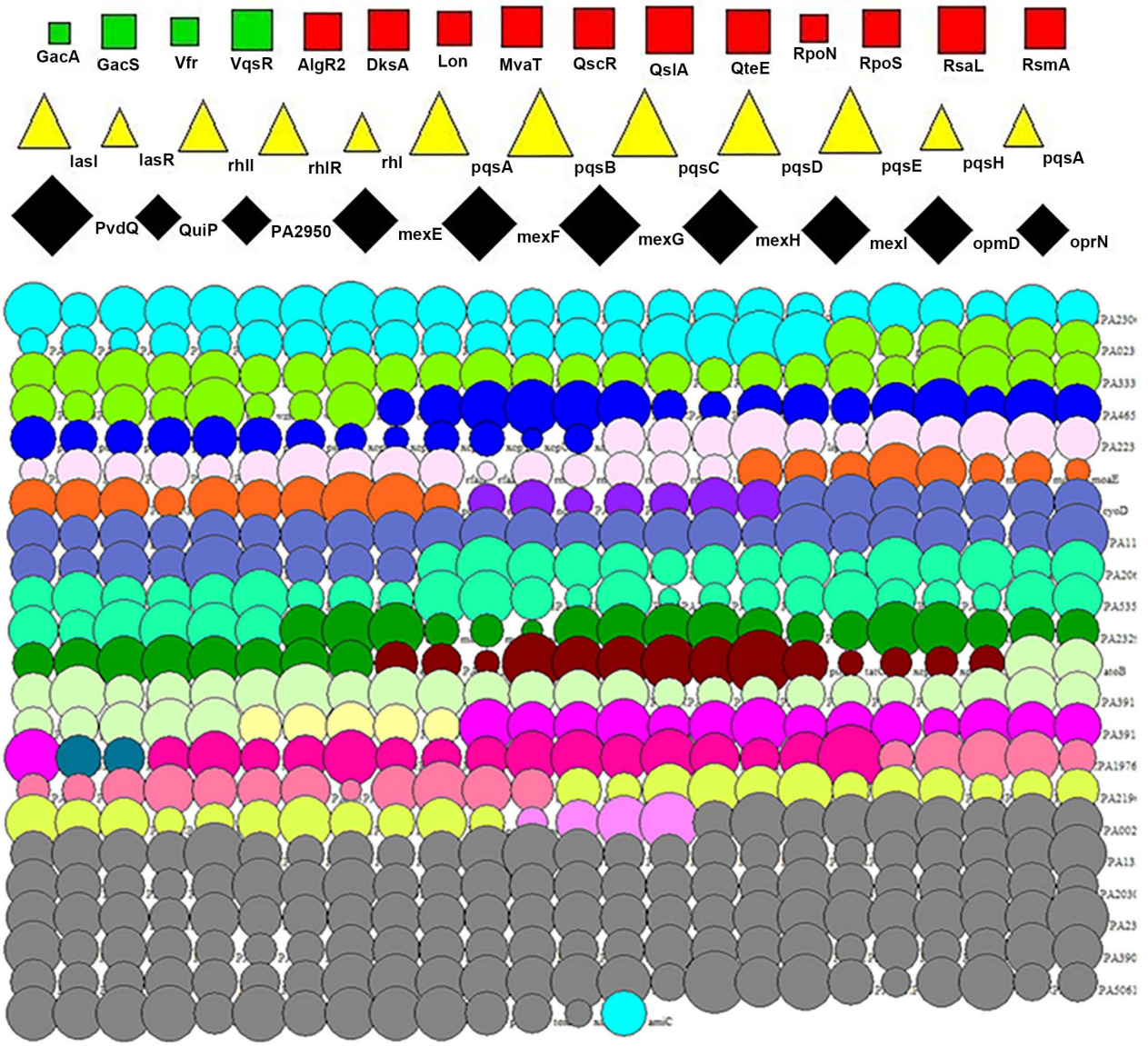

Figure 2. Quantitative activity of QS-related genes under down-regulation at population level (for the information on symbols and colors, refer to legend of Figure 1).

Data Fig-1-2). Subsequently, we can rank QS-related genes according to their activity at population level, and group QS-related genes (Tables 1-3).

Because QS regulatory pathways are usually intertwined together, it is hard to quantitatively define the role of a single global regulator. Therefore, the four scenarios were examined according to QS regulatory hierarchy: 1) which QS gene positively reacted to the up-regulation of a single global regulator with the rest global regulators unchanged (red number in Figure 3); 2) which QS gene negatively reacted to the up-regulation of a single global regulator with the rest global regulators unchanged (blue number in Figure 3); 3) which QS gene positively reacted to the down-regulation of a single global regulator with the rest global regulators unchanged (red number in Figure 4); and 4) which QS gene negatively reacted to the down-regulation of a single global regulator with the rest global regulators unchanged (blue number in Figure 4). These analyses can be done through looking at the abovementioned four scenarios in each transcriptomic dataset, and then summing them up. In this way, the relationship between a global regulator and each individual QS gene was established.

Table 1. Grouping of global regulators in up/down-regulations according to their activity.

Global Regulators		Up-Regulations	Down-Regulations	Group
Negative Regulators	AlgR2	8	12	III
	DksA	14	13	I
	Lon	20	9	II
	MvaT	10	13	III
	QscR	14	14	I
	QslA	12	17	III
	QteE	17	15	I
	RpoN	13	6	III
	RpoS	11	12	I
	RsaL	17	18	I
	RsmA	24	13	II
Positive Regulators	GacA	11	4	II
	GacS	5	9	III
	Vfr	7	6	I
	VqsR	14	14	I

Table 2. Grouping of up/down-regulations of QS genes according to their activity.

QS Genes	Up-Regulations	Down-Regulations	Group
lasI	12	19	III
lasR	12	9	II
rhlI	16	17	I
rhlR	21	16	II
rhl	12	9	II
pqsA	25	24	I
pqsB	21	28	III
pqsC	20	28	III
pqsD	16	25	III
pqsE	19	27	III
pqsH	10	12	I
pqsR/mvfR	14	11	II

In reality, a global regulator has effects not only on a single QS gene but also on other QS genes through either direct or indirect pathway. For this consideration, we also have four scenarios: 1) both a global regulator and QS genes up-regulated (red number in **Figure 5**); 2) a global regulator up-regulated but QS genes down-regulated (blue number in **Figure 5**); 3) both a global regulator and QS genes down-regulated (blue number in **Figure 6**); and 4) a global regulator down-regulated but QS genes up-regulated (red number in **Figure 6**). These analyses can be done through counting the relevant gene

Table 3. Grouping of up/down-regulations of genes that control AHL homeostasis according to their activity.

Gene Name	Up-Regulations	Down-Regulations	Group
mexE	18	16	I
mexF	21	21	I
mexG	21	26	III
mexH	20	21	I
mexI	21	18	I
opmD	19	18	I
oprN	15	11	II
pfm	16	9	II
pvdQ	26	26	I
quiP	7	8	I

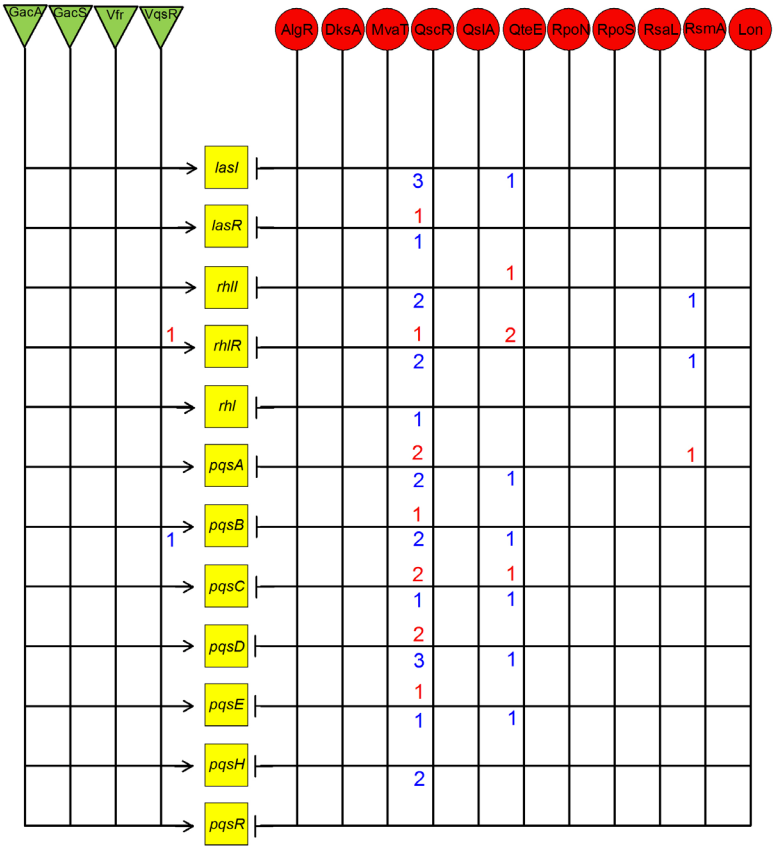

Figure 3. Frequency of up/down-regulation of QS genes in response to the up-regulation of a single global regulator with the rest global regulators unchanged. The red number above the horizontal line is the frequency of up-regulation of QS genes. The blue number below the horizontal line is the frequency of down-regulation of QS genes. Green triangles and red circles represent stimulating (arrow) and inhibiting (vertical-line end) global regulators, and yellow squares represent QS genes.

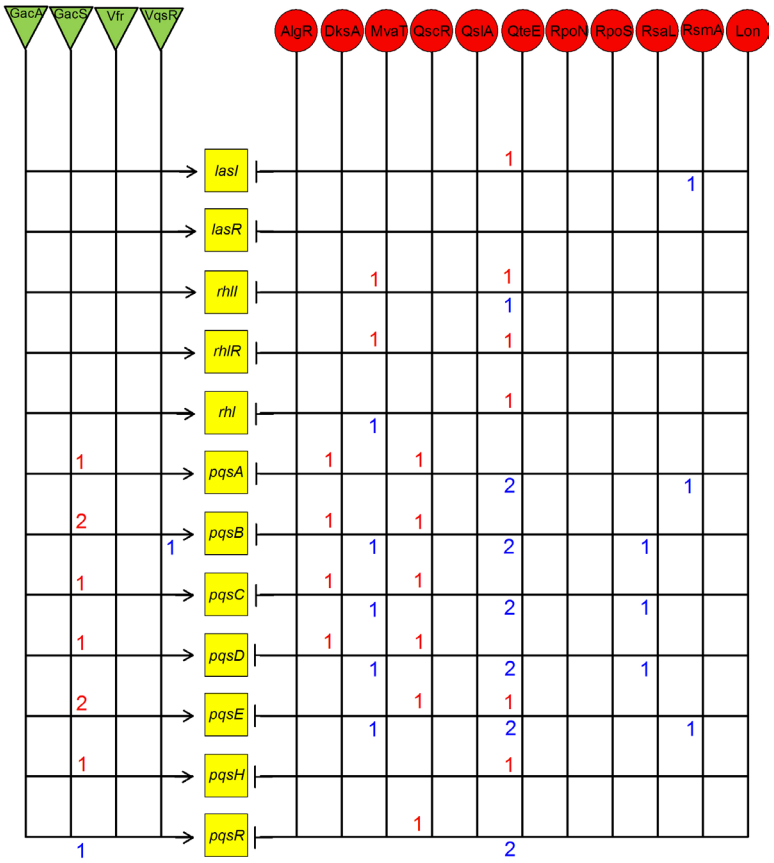

Figure 4. Frequency of up/down-regulation of QS genes in response to the down-regulation of a single global regulator with the rest global regulators unchanged (for the information on symbols and colors, refer to legend of Figure 3).

up/down-regulations in the four scenarios throughout all transcriptomic data, and then computing their probability. This probability is the chance that a global regulator impacts on QS genes.

Oppositely, a QS gene responds to global regulators differently. Similarly, we again have four scenarios: 1) both a QS gene and global regulators up-regulated (pink number in **Figure 7**), 2) a QS gene up-regulated but global regulators down-regulated (green number in **Figure 7**), 3) both a QS gene and global regulators down-regulated (green number in **Figure 8**), and 4) a QS gene down-regulated but global regulators up-regulated (pink number in **Figure 8**). The analysis procedure is exactly similar to the method for **Figure 5** and **Figure 6**.

To conduct our study at population level with data from various sources, it is important to know the overlapped genes across different experimental conditions. If there are no overlapped genes, then we cannot combine all the datasets together for analysis. The experimental conditions in transcriptomic data from the 104 journal publications can be grouped into four types: 27 nutrition (starvation) related experiments, 38 stress related experiments, 14 habitat related experiments and 36 mutant related experiments. This analysis can be done through counting QS-related genes in each experiment and then using Venn diagram [62] to present the overlapped QS-related genes in **Figure 9** and **Figure 10**.

3. RESULTS AND DISCUSSION

In this study, we combined the transcriptomic data from 104 journal publications in order to understand how QS-related genes function at population level in a quantitative manner.

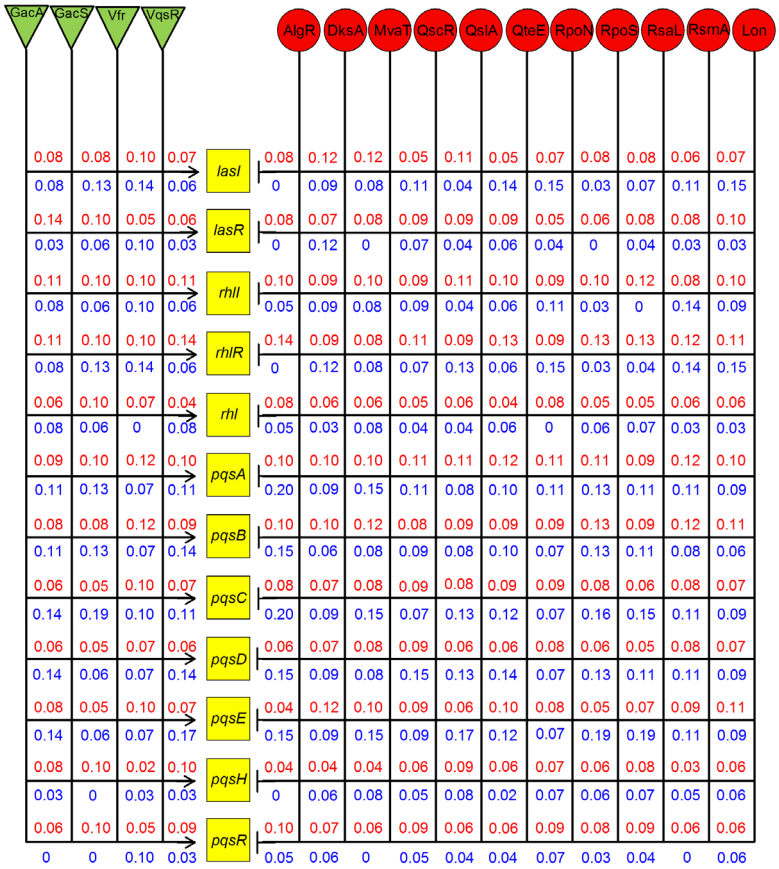

Figure 5. Probability that an up-regulated global regulator impacted on QS genes. Red number above horizontal line is the probability that a global regulator up-regulated QS genes. Blue number below horizontal line is the probability that a global regulator down-regulated QS genes. Green triangles and red circles represent stimulating (arrow) and inhibiting (vertical-line end) global regulators.

Figure 1 and Figure 2 show the activity of QS-related genes at population level in terms of symbol size, *i.e.* the larger the symbol size is, the stronger the gene's activity is. In Figure 1 and Figure 2, genes are arranged according to the cascade of QS gene regulatory mechanism, namely, global regulators (square symbols) regulate QS genes (triangle symbols), which then regulate QS-controlled genes (circle symbols), while AHL-acylases, enoyl-CoA reductase and import-export pumps (diamond symbols) adjust AHL homeostasis. Because most of the 104 studies were not specifically designed to investigate QS, so Figure 1 and 2 summarize the quantitative QS-related gene's activity under various experimental conditions.

The first striking feature in Figure 1 and Figure 2 is that the size of symbols varies differently, that is, the frequency of up/down-regulations is different. This is understandable because each gene has a different activity. Basically, we can determine a gene's role by comparing its frequency with others. For global regulators in up-regulation (Figure 1), RsmA with frequency of 24 is the most active, followed by Lon (with frequency of 20), RsaL and QteE (frequency of 17 for both). For global regulators in down-regulation (Figure 2), RsaL with frequency of 18 is the most active, followed by QslA with frequency of 17 and QteE with frequency of 15.

Interestingly, VqsR plays an equal role in both up-regulation (Figure 1) and down-regulation (Figure 2). This is possible because LasR directly binds to the promoter region of VqsR [65], and LasR is more

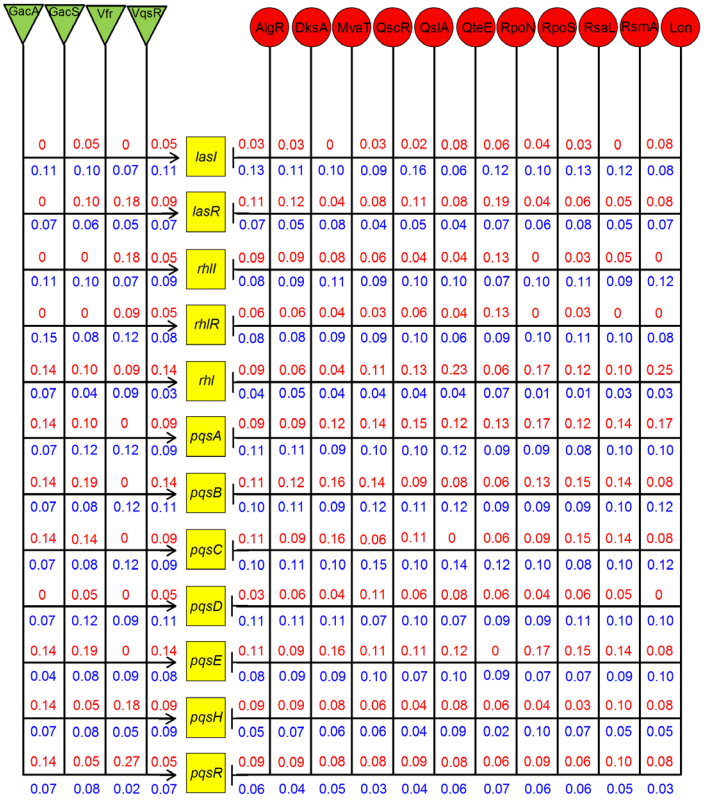

Figure 6. Probability that a down-regulated global regulator impacted on QS genes. Red number above horizontal line is the probability that a global regulator down-regulated but QS genes was up-regulated. Blue number below horizontal line is the probability that both global regulator and QS genes down-regulated. Green triangles and red circles represent stimulating (arrow) and inhibiting (vertical-line end) global regulators.

active in **Figure 1**, so more active VqsR in up-regulation would be due to that LasR promotes VqsR [65]. Similarly, QscR acted as the same as VqsR did in **Figure 1** and **Figure 2**, which could be attributed to the fact that VqsR directly inhibits QscR [51].

Taking both up-regulation and down-regulation together into account, the global regulators can be classified into 3 groups according to their activity (**Table 1**). Group I includes DksA, QscR, QteE, RpoS, RsaL, Vfr and VqsR, which have an equal or somewhat equal activity in both up-regulation and down-regulation. Group II includes Lon, RsmA and GacA, which have a stronger activity in up-regulation than in down-regulation. Group III includes AlgR2, MvaT, QslA, RpoN and GacS, which have a smaller activity in up-regulation than in down-regulation. This grouping provides useful information on access the activity of each global regulator because all global regulators were studied individually, so it is hard to know which one is more active than others.

For QS genes in up-regulation, *pqsA* with frequency of 25 is the most active, followed by *rhlR* and *pqsB* (frequency of 21 for both), and *pqsC* with frequency of 20. For QS genes in downregulation, *pqsC*, *pqsB*, *pqsE*, *pqsD* and *pqsA* are more active because their frequency ranged from 28 to 24. The QS genes can also be classified into 3 groups (column 4 in **Table 2**). Group I comprises *rhlI*, *pqsA* and *pqsH*, which have an equal or somewhat equal activity in both up-regulation and down-regulation. Group II comprises *lasR*, *rhlR*, *rhl* and *pqsR/mvfR*, which activate strongly in up-regulation. Group III comprises *lasI*, *pqsB*, *pqsC*, *pqsD* and *pqsE*, which activate strongly in down-regulation.

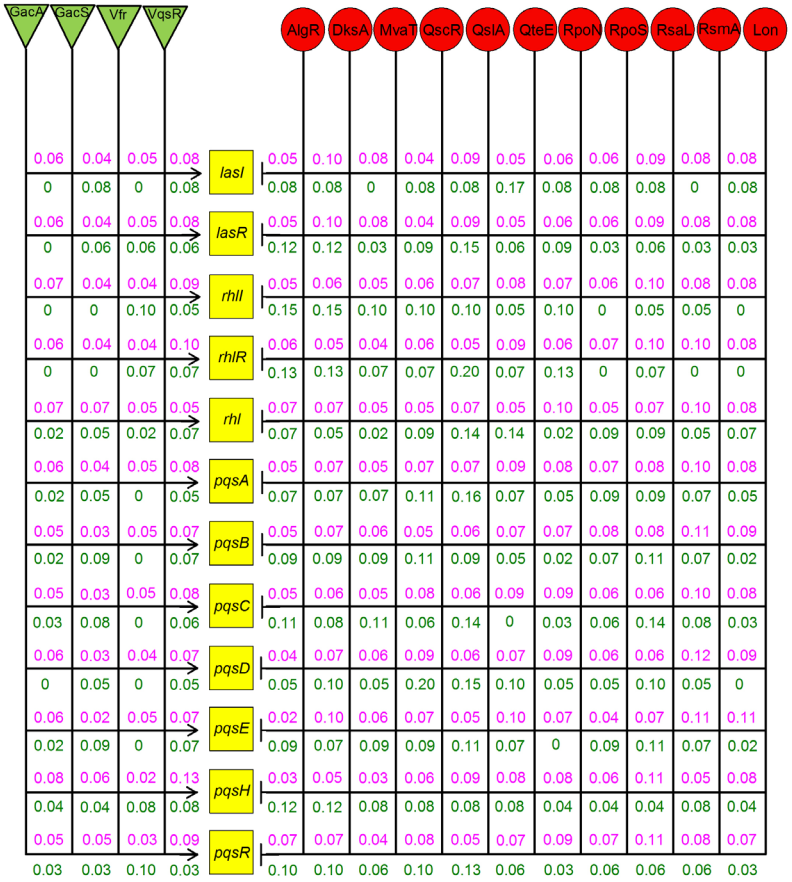

Figure 7. Probability that an up-regulated QS gene responded to global regulators. Pink number above horizontal line is the probability that both a QS gene and global regulators up-regulated. Green number below horizontal line is the probability that a QS gene up-regulated but global regulators down-regulated. Green triangles and red circles represent stimulating (arrow) and inhibiting (vertical-line end) global regulators.

For the genes that control AHL homeostasis, *pvdQ* is the most active with frequency of 26 in up-regulation, followed by *mexF*, *mexG* and *mexI* (frequency of 21 for each), and *mexH* with frequency of 20. For these genes in down-regulation, *mexG* and *pvdQ* are the most active (frequency of 26 for both), followed by *mexF* and *mexH* (frequency of 21 for both). Similarly, these genes can be classified into 3 groups (column 4 in **Table 3**). Seven genes (*mexE*, *mexF*, *mexH*, *mexI*, *opmD*, *pvdQ* and *quiP*) belong to Group I with an equal or somewhat equal activity in both up-regulation and down-regulation. Two genes (*oprN* and *pfm*) belong to Group II being more active in up-regulation than in down-regulation, which is plausible because enoyl-CoA reductase (*pfm*) leads to an increase in autoinducer level rather than a decrease in autoinducer level [66]. Only one gene (*mexG*) belongs to Group III being less active in up-regulation than in down-regulation.

Collectively, **Figure 1** and **Figure 2** together with **Tables 1-3** indicate which gene is more active at three different population levels. When QS-controlled genes are up-regulated, very active genes are as follows (*norB*, PA0515, *norC*, PA0525, PA4880, PA3235, *narG*, *nirC*, PA5481, PA5482, *narH*, PA1323, *nirM*, *gcdH*, PA2151, PA0510, *coxB*, PA2156, PA4739, *narK1*, PA2142, PA3913, *nirF*, PA0567, PA0714, PA1168, PA4139). When QS-controlled genes are down-regulated, very active genes are as follows (PA4306, *phzD2*, *phzC2*, *phzE2*, PA4131, *hcnA*, PA1869, PA4648, *lasA*, *norC*, PA1177, *phzA1*, *hvn*, PA0122, PA2372).

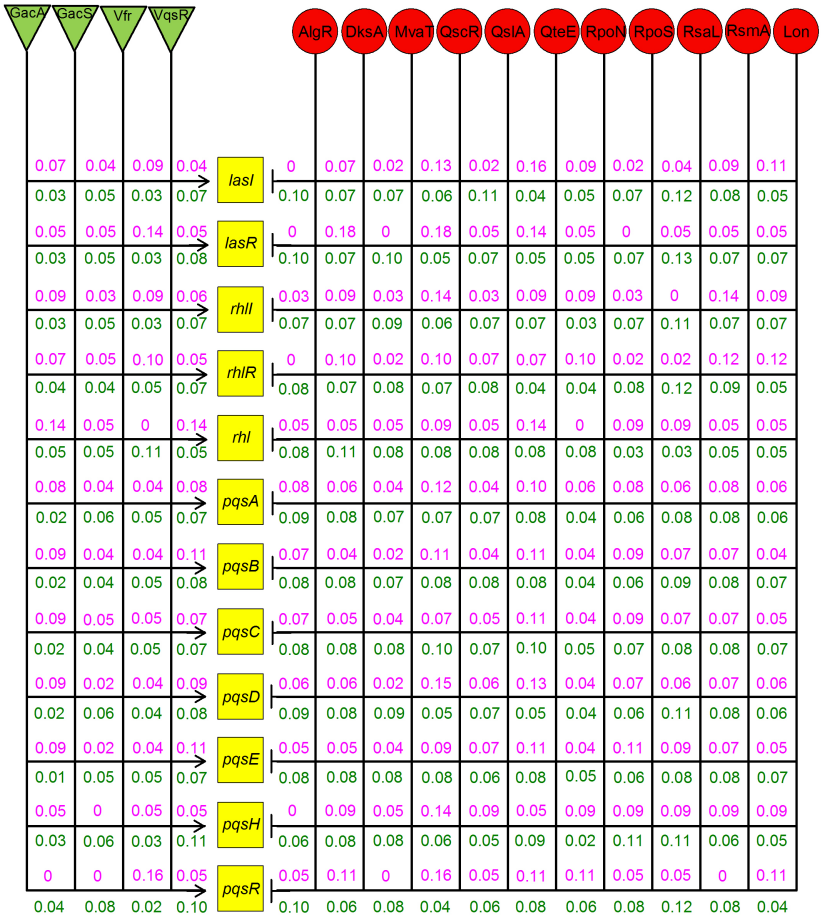

Figure 8. Probability that a down-regulated QS gene responded to global regulators. Pink number above horizontal line is the probability that a QS gene down-regulated but global regulators up-regulated. Green number below horizontal line is the probability that both a QS gene and global regulators down-regulated. Green triangles and red circles represent stimulating (arrow) and inhibiting (vertical-line end) global regulator.

The quantitatively hierarchical description of QS-related genes is the key point to reveal the underlining mechanism [67]. Individual studies often concentrate on pinpointing and finding out individual gene's regulatory mechanism. With numerous transcriptomic data, it is possible to build a hierarchical regulation of QS-related genes in a quantitative and precise way.

Figure 3 and **Figure 4** show the frequencies of how QS genes reacted to the up/down-regulation of a single global regulator with the rest global regulators unchanged. These figures can be read as follows with respect to the colored numbers. For example, when VqsR up-regulated alone in **Figure 3**, it had a positive impact on *rhlR* with frequency of 1 and a negative impact on *pqsB* with frequency of 1. At first glance, there are not many numbers in both **Figure 3** and **Figure 4**, *i.e.* a single global regulator does not play a very big role in up/down-regulating QS genes when it acts alone. Of 15 global regulators, eleven in **Figure 3** and seven in **Figure 4** had no impact on QS genes when they acted alone. This once again demonstrates that global regulators are more likely to work together rather than alone. Comparing these two figures, we can find that there are more colored numbers in **Figure 4** than in **Figure 3**, *i.e.* the down-regulation of a single global regulator impacted more on QS genes than its up-regulation did. As can be seen in **Figure 4**, DksA and QscR activated the expression of PQS-induced QS genes. On the contrary, VqsR repressed the

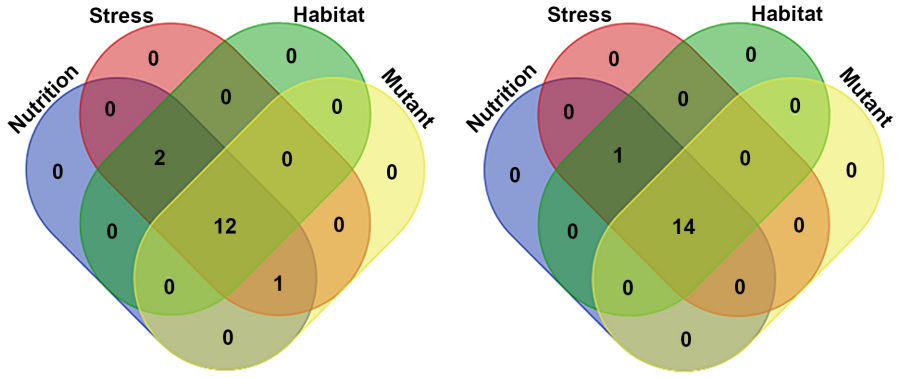

Figure 9. **Overlap of global regulators in up-regulation (left panel) and down-regulation (right panel) under four types of experimental conditions.**

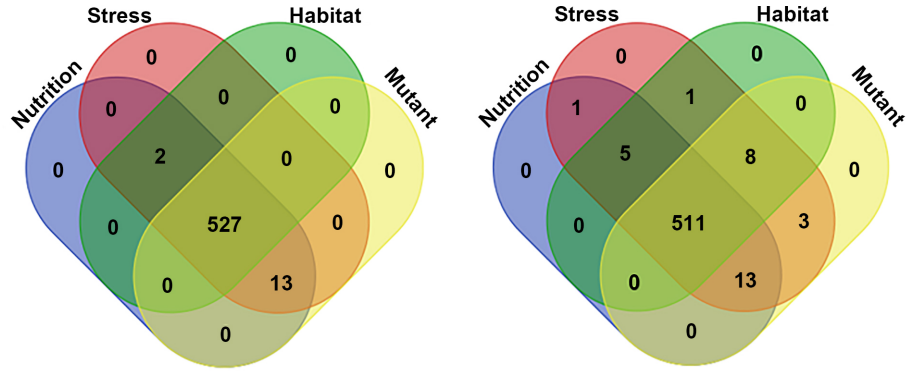

Figure 10. **Overlap of QS-controlled genes in up-regulation (left panel) and down-regulation (right panel) under four types of experimental conditions.**

expression of *pqsB*; RsaL repressed the expression of *pqsB*, *pqsC* and *pqsD*; and RsmA repressed the expression of *lasI*, *pqsA* and *pqsE*.

Furthermore, some global regulators reveal different effects on different QS genes. For instance, when VqsR up-regulated, *rhlR* was up-regulated but *pqsB* down-regulated (**Figure 3**); when RsmA up-regulated, *rhlI* and *rhlR* were down-regulated but *pqsA* was up-regulated (**Figure 3**); when GacS down-regulated, *pqsA* to *pqsH* were up-regulated but *pqsR* was down-regulated (**Figure 4**); when MvaT down-regulated, *rhlI* and *rhlR* were up-regulated but *rhl*, *pqsB*, *pqsC*, *pqsD* and *pqsE* down-regulated (**Figure 4**). More complicated influence of single global regulator on QS genes can be found in the up-regulation of QscR and QteE (**Figure 3**) as well as in the down-regulation of QteE (**Figure 4**). Interestingly, a global regulator can play opposite roles in experiments because both red and blue numbers appeared for the same QS gene, for example, QscR impacted on *lasR*, *rhlR*, *pqsA*, *pqsB*, *pqsC*, *pqsD* and *pqsE* in **Figure 3**, and QteE impacted on *rhll* and *pqsE* in **Figure 4**.

Previous studies demonstrated that QscR (PA1898) was a negative global regulator repressing LasI [18, 30]. Recent works emphasize its role in the regulation of QS by affecting several AHLs [68], which highlights new strategies to treat bacterial infections [69, 70]. More recently, a chromatin immunoprecipitation analysis demonstrates the mechanism of QscR to regulate QS, that is, QscR bounds to the promoter of a single operon for three genes PA1895 to PA1897, which link to *qscR* [71]. Here, our analysis shows that QscR alone has broad effects on QS genes. It is a real global regulator because it can repress not only on

LasI-LasR system but also on RhlI-RhlR and PQS systems (blue numbers in QscR column in **Figure 3**). Meanwhile, it can activate three QS systems as indicated by the red numbers in the same column. Recently, experiments conformed that there is a regulatory link between *vqsR* and PQS, where RpoS plays an important role [72].

QteE, quorum threshold expression protein, displays active influence on QS in *P. aeruginosa* in both its alone up- and down-regulation. In general, this global regulator activates RhlI-RhlR systen but represses PQS system although dual effects can be found in some cases, including *rhlI*, *pqsC* and *pqsE* (**Figure 3** and **Figure 4**). For LasI-LasR system, QteE negatively influences the activity of *lasI* but has no effect on *lasR*. Of course, there are overlaps in gene functions; for example, there is an overlap between QslA and QteE regulons, which affect the induction of 999 genes and the repression of 798 genes [73].

Figure 5 and **Figure 6** display the probability that a global regulator impacted on QS genes. For example, considering the probability that GacA up-regulated QS genes (red numbers in the first column in **Figure 5**), GacA had the strongest impact on lasR with a probability of 0.14, strong impact on *rhlI* and *rhlR* with a probability of 0.11 for each, good impact on *pqsA* with a probability of 0.9, fair impact on *lasI*, *pqsB*, *pqsE* and *pqsH* with a probability of 0.08 for each, and weak impact on *rhl*, *pqsC*, *pqsD* and *pqsE* with a probability of 0.06 for each. In terms of the probability that GacA down-regulated QS genes (blue numbers), GacA has the strongest impact on *pqsC*, *pqsD* and *pqsE* with a probability of 0.14 for each, strong impact on *pqsA* and *pqsB* with a probability of 0.11 for each, fair impact on *lasI*, *rhlI*, *rhlR* and *rhl* with a probability of 0.08 for each, and weak impact on *lasR* and *pqsH* with a probability of 0.03 but no impact on *pqsR*.

Similarly, let us look at how GacA impacts on QS genes during its down-regulation (see the first column in **Figure 6**). The blue numbers indicate similar effects of GacA on QS genes, that is, GacA down-regulated while QS genes are also down-regulated. GacA has strong impact on *rhlR* with a probability of 0.15, good impact on *lasI* and *rhlI* with a probability of 0.11 for each, fair impact on *lasR*, *rhl*, *pqsA*, *pqsB*, *pqsC*, *pqsD* and *pqsH* with a probability of 0.08 for each, and weak impact on *pqsE* with a probability of 0.04. On the contrary, red numbers show opposite effects of GacA on QS genes, that is, GacA down-regulated whereas QS genes are up-regulated. GacA affects strangely on *rhl*, *pqsA*, *pqsB*, *pqsC*, *pqsE*, *pqsH* and *pqsR* with a probability of 0.14 for each. However, it has no influence on *lasI*, *lasR*, *rhlI*, *rhlR* and *pqsD*. In this manner, we have a probabilistic view of how a global regulator affects QS genes.

Figure 7 and **Figure 8** display the probability that a QS gene responded to global regulators. For example, the probability that up-regulated *lasI* responded to up-regulated global regulators (pink numbers in the first row in **Figure 7**) ranges as follows: 0.10 for DksA, 0.09 for QslA and RsaL, 0.08 for MvaT, RsmA, Lon and VqsR, 0.06 for RpoN, RpoS and GacA, 0.05 for AlgR2, QteE and Vfr, 0.04 for QscR and GacS. The probability that up-regulated *lasI* responded to down-regulated global regulators (green numbers in the second row in **Figure 7**) ranges as follows: the strongest response to QteE had a probability of 0.17, and equal response to 8 global regulators (AlgR2, DksA, QscR, QslA, RpoN, RpoS, RsaL, Lon, GacS and VqsR) had a probability of 0.08, but there was no response to 4 global regulators (MvaT, RsmA, GacA and Vfr).

Again taking *lasI* as an example, the probability of down-regulated *lasI* responding to up-regulated global regulators (pink numbers in the first row in **Figure 8**) ranges as follows: 0.16 for QteE, 0.13 for QscR, 0.10 for Lon, 0.09 for RpoN, RsmA and Vfr, 0.07 for DksA and GacA, 0.04 for RsaL, GacS and VqsR, 0.02 for MvaT, QslA and RpoS, and 0 for AlgR2. The probability of down-regulated *lasI* responding to down-regulated global regulators (green numbers in the second row in **Figure 8**) ranges as follows: 0.12 for RsaL, 0.11 for QslA, 0.10 for AlgR2, 0.08 for RsmA, 0.07 for 4 global regulators (DksA, MvaT, RpoS and VqsR), 0.06 for QscR, 0.05 for 3 global regulators (RpoN, Lon and GacS), 0.04 for QteE, and 0.03 for GacA and Vfr. In this manner, we get a probabilistic view of response of QS genes to global regulators.

Of those QS-related genes, some interesting points can be observed on global regulators, which in turn regulate QS genes. For example, LasR induces the expression of RsaL [35], which in turn inhibits QS system [33, 34], so this is a negative feedback loop. In addition, the LasR/OdDHL complex regulates VqsR

[65], which in turn stimulates QS genes [27], so this is a positive feedback loop.

Collectively, **Figures 5-8** demonstrate the interaction between global regulators and QS genes at population level. This interaction is balanced and weighed by different probabilities.

However, the quantitative regulation relationship between QS genes and QS-controlled genes cannot be presented in such a graphic manner due to the difficulties in drawing so many genes and lines. Grouping and stratifying of QS-controlled genes are needed for their analyses in future.

Figure 9 and **Figure 10** illustrate the overlap of genes from global regulators and QS-controlled genes in up-regulation and down-regulation under four types of experimental conditions. In **Figure 9**, 12 (DksA, VqsR, QslA, MvaT, GacA, RpoS, RsaL, Lon, RsmA, QteE, QscR, RpoN) and 14 (DksA, VqsR, QslA, MvaT, RpoS, RsaL, Lon, RsmA, Vfr, QteE, QscR, GacS, AlgR2, RpoN) global regulator genes overlapped under four types of experimental conditions for up-regulation and down-regulation. This suggests that the activity of global regulators is rather independent of experimental conditions, and provides the evidence to combine all the transcriptomic data together. Yet, 2 (Vfr, AlgR2) and 1 (GacA) global regulators overlapped under three experimental conditions (nutrition, stress and habitat) for up-regulation and down-regulation, indicating that these three global regulators are less affected by mutation. For up-regulation, GacS appears under three experimental conditions (nutrition, stress and mutant), suggesting that it is less sensitive to habitat conditions.

Similarly, a vast majority of QS-controlled genes are independent of experimental conditions as demonstrated in **Figure 10**, where 527 and 511 QS-controlled genes overlapped under four types of experimental conditions for up-regulation and down-regulation. In addition, 2 and 5 QS-controlled genes overlapped under three types of experimental conditions (nutrition, stress and habitat), indicating that they are less sensitive to mutation; 13 QS-controlled genes overlapped under three types of experimental conditions (nutrition, stress and mutant) for both up-regulation and down-regulation, indicating that they are less affected by habitat conditions. Finally, 8 (stress, habitat and mutant), 3 (stress and mutant), 1 (nutrition and stress) and 1 (stress and habitat) QS-controlled genes overlapped in down-regulation (right panel in **Figure 10**), from which we can trace the common characteristics of these genes. For instance, *moaE* was down-regulated by nutrition and stress conditions whereas PA3242 was down-regulated by habitat and stress conditions. *rfaE*, PA0158 and PA3678 can be affected by mutants and stresses. Among 8 genes were down-regulated by stress, habitat and mutant, 3 genes, PA0567, PA1323 and PA3662, encode hypothetical proteins, which are free from the influence of nutrition.

In other words, what is the quantitatively regulatory mechanism of QS systems in *P. aeruginosa.* Clearly, the answer to this question is important because the first two QS systems in *P. aeruginosa* have close relationships with virulence [74], including following notorious virulence genes: *lasB* (PA3724) [75, 76], *lasA* (PA1871) [77, 78], *toxA* (PA1148) [79, 80], *aprA* (PA1249) [81], *rhlA* (PA3479), *rhlB* (PA3478) [82, 83], *lecA* (PA2570) [84], *hcnA* (PA2193), *hcnB* (PA2194) and *hcnC* (PA2195) [85], *phzA* (PA4210), *phzB* (PA4211), *phzC* (PA1901), *phzD* (PA1902), *phzE* (PA1903), *phzF* (PA1904), *phzG* (PA1905) and *phzM* (PA4209) [86-88]. The third QS system also has a relationship with virulence factors, e.g. pyocyanin [88], elastase, PA-IL lectin and rhamnolipids [7, 67, 89]. Usually, QS plays an important role in biofilm [90], and the signal molecules synthesized by QS systems affect eukaryote [91, 92]. Consequently, QS systems are considered as a target in therapy [93], although there are different viewpoints [94-97]. Indeed, the molecules with similar structures to AHL were intensively examined for their potential as antagonists to QS [98-100], and the development of inhibitors for QS is in therapeutic agendas [69, 70, 101].

In conclusion, it is well recognized that QS systems have very complicated regulatory mechanism at different levels in response to different stresses and stimuli. In this study, we concentrate our efforts on the general trend at population level rather than individual cases, which are heavily influenced and shadowed by various controllable and uncontrollable factors, by means of integrative analysis of transcriptomic datasets from 104 journal publications. The results provide not only quantitative information on how QS-related genes as whole behave in response to various stresses and stimuli at population level, but also the base to reveal some negative pathway which may reverse the QS process and thus reverse some clinical

outcomes [102].

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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