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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

A novel whole-cell biosensor of *Pseudomonas aeruginosa* to monitor the expression of quorum sensing genes

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Abstract

A novel whole-cell biosensor was developed to noninvasively and simultaneously monitor the in situ genetic activities of the four quorum sensing (QS) networks in *Pseudomonas aeruginosa* PAO1, including the *las*, *rhl*, *pqs*, and *iqs* systems. *P. aeruginosa* PAO1 is a model bacterium for studies of biofilm and pathogenesis while both processes are closely controlled by the QS systems. This biosensor worked well by selectively monitoring the expression of one representative gene from each network. In the biosensor, the promoter regions of *lasI*, *rhlI*, *pqsA*, and *ambB* (QS genes) controlled the fluorescent reporter genes of Turbo YFP, mTag BFP2, mNEON Green, and E2-Orange, respectively. The biosensor was successful in monitoring the impact of an important environmental factor, salt stress, on the genetic regulation of QS networks. High salt concentrations (≥ 20 g·L^{-1}) significantly downregulated *rhlI*, *pqsA*, and *ambB* after the biosensor was incubated for 17 h to 18 h at 37 °C, resulting in slow bacterial growth.

Keywords Whole-cell biosensor · Gene expression · Fluorescence · Quorum sensing · *Pseudomonas aeruginosa* PAO1 · Salt stress

Introduction

Numerous assays are available to analyze gene expression, such as reverse transcription quantitative polymerase chain reaction (RT-qPCR) (VanGuilder et al. 2008) and RNA-Seq (Ramsköld et al. 2012). These methods have their advantages but require cell lysis prior to measuring gene expression. A whole-cell biosensing assay, on the other hand, elicits real-time and noninvasive in situ information about gene expression in living cells with high selectivity and sensitivity (Brutesco et al. 2017; Daunert et al. 2000; Idil et al. 2017; Vasilescu et al. 2017). This approach fuses the promoter of a gene of interest to a heterologous reporter gene and quantifies gene expression through examining the activities of the reporter (Avison 2008; Elad and Belkin 2013; Hakkila et al. 2002; Whangsuk et al. 2016). Fluorescent genes have unique merits among available reporters. Fluorescent genes work as reporters through spontaneous fluorophore synthesis and do not require a cofactor to function (Damron et al. 2013; Teng et al. 2015). In addition, high temporal resolution and noninvasive monitoring of genetic activities in living cells is straightforward using fluorescent reporters (Zaslaver et al. 2006). Moreover, the emerging development of fast-maturing fluorescent proteins (FPs) with high photostability/brightness but low cytotoxicity provides a promising opportunity to simultaneously measure activities of multiple target genes (e.g., multicolor labeling of living cells) (Chudakov et al. 2010; Dedecker et al. 2013; Shaner et al. 2005; Ueno and Nagano 2011).

A traditional fluorescent whole-cell biosensor often contains only one promoter-reporter fusion. For instance, one study monitored how iron concentration affected the expression of two iron-dependent genes *bfrB* and *pvdA* in *Pseudomonas aeruginosa* PAO1 (Parrello et al. 2015) using two whole-cell biosensors. Promoters of *bfrB* and *pvdA* were

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in separated artificial plasmids, which, in turn, were in different biosensors. However, the use of multiple biosensors increases the complexity of the gene expression monitoring process since handling of several biosensors is needed. Moreover, different biosensors tend to have different growth rates due in part to additives such as antibiotics necessary to maintain plasmids. The differences in fluorescent intensity could result from the differences in bacterial growth rate. Consequently, monitoring the regulation of multiple genes by tracking the changes in fluorescence intensity with separate plasmids or biosensors may introduce inaccuracies.

Therefore, it is useful to construct a single biosensor with multiple promoter-reporter fusions that can easily track expression of several genes simultaneously. Most of the work related to dual or multilabeling of bacterial strains with different fluorescent reporters is done in *Escherichia coli*. An *E. coli* biosensor containing three distinguishable fluorescent reporters fused to three different responsive promoters was successful in simultaneous screening the activities of three transcription factors (AraC, LacI, and TetR) (Cox III et al. 2010). Another *E. coli* whole-cell biosensor utilized two separate fluorescent reporters in the same cells to monitor changes in gene expression in response to both zinc and copper (Ravikumar et al. 2012).

Surprisingly, the application of a dual or multilabeling approach to monitor gene regulation has not been broadly disseminated among bacteria exhibiting quorum sensing (QS) behavior such as *P. aeruginosa*. For example, a dual-labeled biosensor of *P. aeruginosa* PAO1 used green fluorescent protein (GFP) to track QS signaling in a strain which constitutively expressed red fluorescent protein (RFP) (Hentzer et al. 2002; Hentzer et al. 2003); however, this approach did not simultaneously probe multiple QS pathways. Therefore, application of the dual or multilabeling method in *P. aeruginosa* PAO1 will enable investigations of its genetic regulation of QS systems.

P. aeruginosa PAO1 is a model microorganism for biofilm and pathogenesis studies while its QS systems govern both processes. Biofilm formation of *P. aeruginosa* requires coordination of numerous genes in its QS signaling networks (de Kievit et al. 2001; Li et al. 2007; Rasamiravaka et al. 2015; Shrout et al. 2011; Solano et al. 2014; Wagner et al. 2003). In addition, QS systems in *P. aeruginosa* are closely related to its virulence or pathogenesis (Lesic et al. 2009; McGrath et al. 2004; Smith and Iglewski 2003; Vandeputte et al. 2010; Whiteley et al. 1999; Winzer and Williams 2001). Furthermore, the QS systems in *P. aeruginosa* are tightly interconnected with other regulatory networks and are viewed as "a network of networks" (Schuster and Greenberg 2006). Thus, understanding the genetic activities of the QS networks in *P. aeruginosa* provides a wealth of information to develop efficient biofilm and pathogen control strategies. Consequently, this study focused on developing a whole-cell biosensor of *P. aeruginosa* PAO1 to simultaneously monitor the expression of multiple QS genes.

To date, *las* system, *rhl* system, *pqs* system, and *iqs* system are the four hierarchical QS networks that have been discovered and investigated in *P. aeruginosa* PAO1 (Deng et al. 2013; Lee et al. 2013; Lee and Zhang 2015; Li et al. 2007). We selected one representative QS gene from each network to construct the whole-cell biosensor.

In the *las* system, *lasI* (ordered locus name PA1432) encodes the synthetase of LasI, an enzyme directing the synthesis of an extracellular diffusible *N*-Acyl homoserine lactone (AHL) autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL or OdDHL) (Li et al. 2007; Pearson et al. 1994; Pesci et al. 1997; Wargo and Hogan 2007). OdDHL recognizes and actives the cognate response regulator LasR protein to regulate many downstream genes (Kim et al. 2015; O'Loughlin et al. 2013; Schuster and Greenberg 2007; Steindler et al. 2009). In addition, LasR as a transcriptional activator from the *las* system activates *lasI* or stimulate its expression (Frederix and Downie 2011; Kiratisin et al. 2002; Seed et al. 1995), suggesting that *lasI* is induced upon autoactivation of its own QS system (i.e., the *las* network).

rhlI (ordered locus name PA3476) holds a similar role in the *rhl* system where it drives the generation of RhlI, which, in turn, produces another AHL autoinducer *N*-butanoyl-L-homoserine lactone (C4-HSL or BHL) (Pearson et al. 1995; Pesci et al. 1997; Rasamiravaka et al. 2015; Steindler et al. 2009). BHL binds to its cognate transcriptional regulator RhlR and regulates a large regulon of genes (Lee and Zhang 2015; O'Loughlin et al. 2013; Steindler et al. 2009). However, experimental data indicate that RhlR/C4-HSL might not be able to activate the transcription of *rhlI* (Morales et al. 2017) even though such an activation was proposed before (Latifi et al. 1996).

pqsA (ordered locus name PA0996) is an important gene in the *pqs* network. This network generates a key QS signal molecule 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) (Diggle et al. 2007). PQS regulates numerous genes (such as virulence genes) (Diggle et al. 2007; Heeb et al. 2011; Mooij et al. 2011; Williams and Cámara 2009) and bridges the *las* and *rhl* systems (McKnight et al. 2000). *pqsA* encodes PqsA, an anthranilate-coenzyme A ligase, which initiates the biosynthesis of PQS (Coleman et al. 2008; Gallagher et al. 2002; Lee and Zhang 2015).

In the *iqs* QS system, *ambBCDE* encodes proteins responsible for the generation of 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) (Lee et al. 2013). IQS is a newly discovered intercellular communication signal substance with a great impact on gene expression in *P. aeruginosa*. *ambB* (ordered locus name PA2305) is also associated with the production of PQS and BHL.

Therefore, this study selected *lasI*, *rhlI*, *pqsA*, and *ambB* as the target genes when designing the biosensor. Hereinafter, the "biosensor" refers to the *P. aeruginosa* PAO1 strain

containing a plasmid with four promoter-reporter fusions unless otherwise specified. We named this plasmid pSEVA-QSGEF, where the "SEVA" and the "QSGEF" stand for Standard European Vector Architecture and Quorum Sensing Gene Expression Factor, respectively. The pSEVA-QSGEF was created through inserting a synthesized DNA fragment with the four fusions (i.e., the "QSGEF") into a vector backbone (i.e., pSEVA-P_{lac}-M Cherry). Relying on this biosensor, we simultaneously determined the impact of salt stress on the expression of multiple QS genes in this strain.

Materials and methods

Bacterial strains, plasmids, and general DNA manipulation

Table 1 shows bacterial strains and plasmids used in this study. Bacteria were stored at −80 °C in glycerol and routinely incubated in lysogeny broth-Miller (LB-Miller) (Fisher Scientific) with shaking (200 rpm) or on LB-Miller agar plate (Fisher Scientific) at 37 °C (aerobic growth). Kanamycin (Sigma-Aldrich) at the final concentrations of 350 mg·L^{-1} (for *E. coli*) or approximately 500 mg·L^{-1} (for *P. aeruginosa* PAO1) was used in a culture medium when incubating a bacteria strain harboring a plasmid DNA except for the cell culture incubated immediately before fluorescence spectrum acquisition (the final culture). All plasmids shared a common structure, the SEVA (Martínez-García et al. 2015; Silva-Rocha et al. 2013). Tables 1 and S1 show plasmid DNA construction procedure and their sequences, respectively. Table S2 indicates primer sequences and PCR conditions for plasmid DNA construction.

General DNA manipulations (e.g., restriction enzyme digestion and ligation) and plasmid construction were performed following standard molecular biology techniques (Davis et al. 1986; Green and Sambrook 2012; Innis et al. 1990). Restriction enzymes and T4 DNA ligase enzyme for DNA restriction digest and ligation, respectively, were provided by New England BioLabs® Inc. Traditional PCRs were conducted in a MJ Mini™ Gradient Thermal Cycler (BIO-RAD), using GoTaq® DNA Polymerase from Promega. The oligonucleotide primers were designed using Primer3 (Koressaar and Remm 2007; Rozen and Skaletsky 2000; Untergasser et al. 2012) and synthesized by Integrated DNA Technologies Inc. except for the standard primer M13 Reverse. Table S2 shows the sequences of oligonucleotide primers and the PCR conditions for amplifying promoter regions from the genomic DNA of *P. aeruginosa* PAO1. DNA Sanger sequencing was performed using a 3730*xl* 96-capillary Applied Biosystems™ DNA Analyzer with an Applied Biosystems™ BigDye® Terminator Cycle Sequencing kit (both from Thermo Fisher Scientific). Recombinant plasmids were first introduced into *E. coli* TOP10 competent cells via chemical transformation (Chung et al. 1989; Hanahan 1983; Inoue et al. 1990), verified by DNA Sanger sequencing (Table S2), and then delivered into *P. aeruginosa* PAO1 cells through electropermeabilization using a MicroPulser™ Electroporator (BIO-RAD).

Construction of the multicolor whole-cell biosensor

A 5531-bp DNA fragment (i.e., QSGEF) (Fig. S1) was synthesized by GenScript (GenPlus™ Economy Service). Briefly, several sub-fragments of this DNA fragment were generated via de novo synthesis and then assembled to form QSGEF. The synthesized QSGEF was subsequently inserted into the *Pac*I/*Spe*I cloning site of vector pSEVA-P_{lac}-mCherry (4074 bp, Table S1) by T4 ligation. This subcloning generated the recombinant plasmid pSEVA-QSGEF (8498 bp, Table S1). Figure 1 shows the map of pSEVA-QSGEF. In pSEVA-QSGEF, promoter regions of *lasI* (P_{lasI}), *rhlI* (P_{rhlI}), *pqsA* (P_{pqsA}), and *ambB* (P_{ambB}) controlled Turbo YFP, codon-optimized mTag BFP2, mNEON Green, and E2-Orange genes, respectively. Table S3 shows the amino acid sequences of these four FPs as well as mCherry. The multicolor whole-cell biosensor was constructed by introducing pSEVA-QSGEF into *P. aeruginosa* PAO1 via electropermeabilization. This biosensor was stored at −80 °C in glycerol, and its stock cell culture was recovered on LB-Miller agar plates with 500 mg·L^{-1} of kanamycin to obtain single colonies.

Codon optimization of the mTag BFP2 gene

mTag BFP2 had a relatively weak fluorescence signal compared with the other three selected FPs (i.e., Turbo YFP, mNEON Green, and E2-Orange) under the control of P_{lac} in *P. aeruginosa* PAO1 (Fig. S2). Therefore, we optimized the codons of the mTag BFP2 gene using an OptimumGene™-Codon Optimization service (GenScript) to enhance its expression in *P. aeruginosa* PAO1 (amino acid sequence not altered). Table S4 compares the nucleotide sequences of mTag BFP2 before and after the codon optimization. We used the codon-optimized mTag BFP2 gene in QSGEF (Fig. S1).

Fluorescence spectrum acquisition

To determine fluorescence signals of the multicolor whole-cell biosensor (i.e., *P. aeruginosa* PAO1 harboring the pSEVA-QSGEF), a single colony of this strain was incubated overnight in 20 mL of LB-Miller with 500 mg·L^{-1} of kanamycin (37 °C, 200 rpm) (the pre-culture). This high concentration of kanamycin was used when incubating *P. aeruginosa* PAO1 harboring a plasmid DNA especially pSEVA-QSGEF in the pre-culture because *P. aeruginosa* is naturally or intrinsically resistant to kanamycin (Aires et al. 1999; Hächler et al. 1996; Kwon and

Table 1 Bacterial stains and plasmids

Name	Description	Source or reference
Strains		
E. coli TOP10	From TOPO® TA Cloning® Kit for Sequencing (Catalog number K4575J10, Thermo Fisher Scientific)	Invitrogen™
P. aeruginosa PAO1	*P. aeruginosa* (Schroeter) Migula (strain designation: PAO-PR1) from American Type Culture Collection (ATCC® 39018™). A wild-type opportunistic pathogen	Le Berre et al. 2008; Stover et al. 2000; Zhang and Hu 2013
Plasmids[a]		
pSEVA237R[b]	Broad-host-range (BHR) plasmid; the reporter gene was promoterless; Km^r. Plasmid DNA length 3,816 bp	Bonnichsen et al. 2015; Silva-Rocha et al. 2013
pSEVA-P_{lac}-M Cherry	Derived from pSEVA237R by replacing the *Kpn*I-*Bam*HI-*Xba*I fragment with a *Kpn*I-P_{lac}-*Xba*I fragment; Km^r. The P_{lac} (i.e., $P_{A1/04/03}$) is an isopropyl-thio-β-D-galactoside-inducible *lac* promoter (Andersen et al. 1992; Choi and Schweizer 2006; Ind et al. 2009). Plasmid DNA length 4,074 bp	This study
pSEVA-P_{lac}-Turbo YFP	Derived from pSEVA-P_{lac}-M Cherry by replacing the reporter gene with a Turbo YFP gene (Norris et al. 2010); Km^r. Plasmid DNA length 4,095 bp	This study
pSEVA-P_{lac}-mNEON Green	Derived from pSEVA-P_{lac}-M Cherry by replacing the reporter gene with an mNEON Green gene (Shaner et al. 2013); Km^r. Plasmid DNA length 4,074 bp	This study
pSEVA-P_{lac}-E2-Orange	Derived from pSEVA-P_{lac}-M Cherry by replacing the reporter gene with an E2-Orange gene (Strack et al. 2009); Km^r. Plasmid DNA length 4,041 bp	This study
pSEVA-P_{lac}-mTag BFP2	Derived from pSEVA-P_{lac}-M Cherry by replacing the reporter gene with an mTag BFP2 gene (Wickersham et al. 2013); Km^r. Plasmid DNA length 4,062 bp	This study
pSEVA-P_{lasI}-mNEON Green	Derived from pSEVA-P_{lac}-mNEON Green by replacing the *Kpn*I-P_{lac}-*Xba*I fragment with a *Kpn*I-P_{lasI}-*Xba*I fragment; Km^r. The *Kpn*I-P_{lasI}-*Xba*I fragment was PCR amplified using the primer set of P_{lasI}-Forward and P_{lasI}-Reverse (Table S2). Plasmid DNA length 4,026 bp	This study
pSEVA-P_{rhlI}-mNEON Green	Derived from pSEVA-P_{lac}-mNEON Green by replacing the *Kpn*I-P_{lac}-*Xba*I fragment with a *Kpn*I-P_{rhlI}-*Xba*I fragment; Km^r. The *Kpn*I-P_{rhlI}-*Xba*I fragment was PCR amplified using the primer set of P_{rhlI}-Forward and P_{rhlI}-Reverse (Table S2). Plasmid DNA length 4,106 bp	This study
pSEVA-P_{pqsA}-mNEON Green	Derived from pSEVA-P_{lac}-mNEON Green by replacing the *Kpn*I-P_{lac}-*Xba*I fragment with a *Kpn*I-P_{pqsA}-*Xba*I fragment; Km^r. The *Kpn*I-P_{pqsA}-*Xba*I fragment was PCR amplified using the primer set of P_{pqsA}-Forward and P_{pqsA}-Reverse (Table S2). Plasmid DNA length 4,442 bp	This study
pSEVA-P_{ambB}-mNEON Green	Derived from pSEVA-P_{lac}-mNEON Green by replacing the *Kpn*I-P_{lac}-*Xba*I fragment with a *Kpn*I-P_{ambB}-*Xba*I fragment; Km^r. The *Kpn*I-P_{ambB}-*Xba*I fragment was PCR amplified using the primer set of P_{ambB}-Forward and P_{ambB}-Reverse (Table S2). Plasmid DNA length 4,007 bp	This study
pSEVA-QSGEF[c]	Derived from pSEVA-P_{lac}-M Cherry; Km^r. pSEVA-P_{lac}-M Cherry was linearized by double enzyme digestion with the restriction enzymes of *Pac*I and *Spe*I. QSGEF was digested using the same restriction enzyme couple. The *Spe*I-T0-*neo*-*oriT*-*rep*-T1-*Pac*I fragment from pSEVA-P_{lac}-M Cherry and the restriction enzyme digested QSGEF were connected to generate pSEVA-QSGEF (Table S1) (T4 ligation). Plasmid DNA length 8,498 bp	This study

[a] Table S1 shows the DNA sequences of all plasmids

[b] Sequence of pSEVA237R is also available at http://beta.labgeni.us/registries/SEVA/pSEVA237R/

[c] Figure 1 shows the map of pSEVA-QSGEF

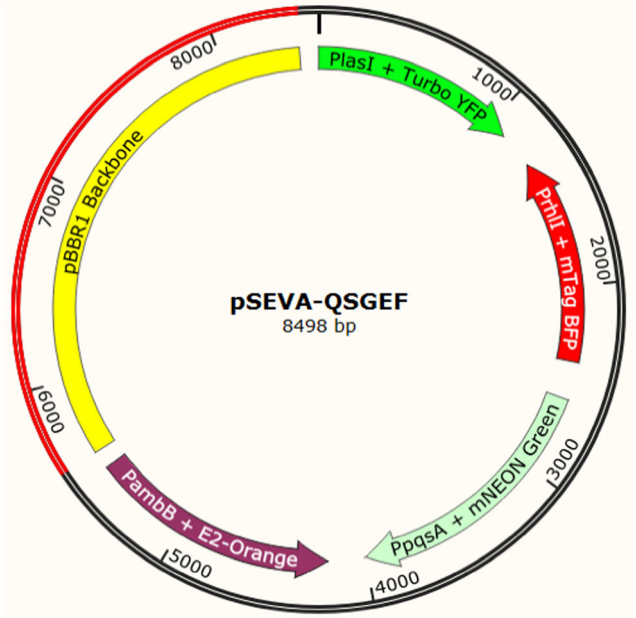

Fig. 1 Map of pSEVA-QSGEF. The map was drawn using SnapGene® Viewer Version 3.0.3 (http://www.snapgene.com). pSEVA-QSGEF is an SEVA plasmid with a stable, medium-copy-number, and BHR origin of replication in the backbone. The backbone also contains a kanamycin resistance gene

Lu 2006; Morita et al. 2014). An aliquot (0.6 mL) of the cell culture was washed using 0.6 mL of phosphate-buffered saline (PBS, pH 7.4, containing 8.00 g·L^{-1} of sodium chloride, 0.20 g·L^{-1} of potassium chloride, 1.44 g·L^{-1} of sodium phosphate dibasic, and 0.24 g·L^{-1} of potassium phosphate monobasic) and resuspended in 0.6 mL of PBS. An aliquot (60 μL) of this cell suspension (with or without dilution) was added into 60 mL of fresh antibiotic-free culture media (the final culture) with varying sodium chloride concentrations (from 0.1 g·L^{-1} to 30 g·L^{-1}, Table 2) and incubated (37 °C, 200 rpm) for 16 h, 17 h, to 18 h, or 26 h to 28 h. No antibiotics were added into the final culture so that the monitoring of gene expression would not be affected. The incubated cell culture was washed twice with and finally resuspended in a sodium chloride solution (8.5 g·L^{-1}) to reach an optical density at 600 nm (O.D.$_{600}$) (measured using a model 4001/4 Thermo Scientific™ GENESYS™ 20 Visible Spectrophotometer and standard plastic cuvette cells with a path length of 1 cm) in the range of 0.990 to 1.000. Synchronous fluorescence spectra of this final cell suspension were measured using an F-4500 Fluorescence Spectrophotometer (Model 250-0551, Hitachi High-Technologies Corporation) and standard quartz cuvette cells with a path length of 1 cm. As a negative control, synchronous fluorescence spectra of *P. aeruginosa* PAO1 without any plasmid were checked following the same procedure. In addition, synchronous fluorescence spectra of *P. aeruginosa* PAO1 strains with a single promoter-reporter fusion (plasmid-encoded) were assayed in a similar manner.

Impacts of salt stress on the growth of *P. aeruginosa* PAO1

A single colony of *P. aeruginosa* PAO1 without any plasmid was incubated overnight in 20 mL of antibiotic-free LB-Miller (37 °C, 200 rpm) to reach the stationary phase, according to the growth curve shown in Fig. S3. An aliquot (0.6 mL) of the cell culture was washed with and resuspended in 0.6 mL of PBS. Subsequently, this cell suspension was diluted to 10^{-3} in PBS. An aliquot (60 μL) of the 10^{-3} cell suspension was mixed with 240 μL of fresh culture media (antibiotic-free) with varying sodium chloride concentrations (0.1 g·L^{-1}, 5 g·L^{-1}, 10 g·L^{-1}, 20 g·L^{-1}, and 30 g·L^{-1}) in a 96-well plate. According to the growth curve of *P. aeruginosa* PAO1 (Fig. S3), the initial cell density for each well was approximately 1.2×10^5 colony forming units (CFU) mL^{-1}. The 96-well plate was incubated at room temperature for up to 60 h, and the O.D.$_{600}$ of the cell culture in each well was measured hourly using a VICTOR^3$_{TM}$ 1420 Multilabel Counter (PerkinElmer®) (the plate was shaken each hour for mixing immediately before the O.D.$_{600}$ measurement). To obtain the specific growth rates of *P. aeruginosa* PAO1 at different sodium chloride concentrations, the O.D.$_{600}$ was plotted on a base-2 logarithmic scale against incubation time on a liner scale (semilogarithmic display). The slopes of such plots for the early logarithmic (exponential) phase were used to calculate the specific growth rates (Herbert et al. 1956).

Statistical analysis

Statistical analysis was performed with MedCalc Version 17.9.7 (MedCalc Software, Belgium). The significance level was set to be 0.05.

Accession numbers of plasmid DNA

The sequences of the plasmid DNA used in this study were deposited in GenBank. The accession numbers of pSEVA-P_{lac}-mCherry, pSEVA-P_{lac}-Turbo YFP, pSEVA-P_{lac}-mNEON Green, pSEVA-P_{lac}-E2-Orange, pSEVA-P_{lac}-mTag BFP2, pSEVA-P_{lasI}-mNEON Green, pSEVA-P_{rhlI}-mNEON Green, pSEVA-P_{pqsA}-mNEON Green, pSEVA-P_{ambB}-mNEON Green, and pSEVA-QSGEF are MH013362, MH013363, MH013364, MH013365, MH013366, MH013367, MH013368, MH013369, MH013370, and MH013371, respectively. In addition, the GenBank access numbers of the codon-optimized mTag BFP2 gene and the QSGEF are MH013372 and MH013373, respectively.

Results

We simultaneously determined the impact of salt stress on the expression from the four selected QS genes (i.e., *lasI*,

Table 2 Culture media used to test the impact of salt stress on the expression of QS genes using the multicolor whole-cell biosensor

Medium	Tryptone ($g \cdot L^{-1}$)	Yeast extract ($g \cdot L^{-1}$)	Sodium chloride ($g \cdot L^{-1}$)	Note
Medium 1	10	5	0.1	Low salt stress; generates a salinity close to that of drinking water and freshwater (Bervoets et al. 1996; Cotruvo 2015; McLusky and Elliott 2004; McPherson and Hammett 1991); the United States Environmental Protection Agency (USEPA) sets the secondary maximum contaminant level (SMCL) of total dissolved solids (TDS) in drinking water to be 500 mg L^{-1} (USEPA Accessed 2017)
Medium 2	10	5	5	LB-Lennox (Lennox 1955)
Medium 3	10	5	10	Common recipe of LB (Cold Spring Harbor Laboratory Press 2006; MacWilliams and Liao 2006); LB-Miller (Miller 1972); generates a salinity close to that of human blood (Sims et al. 1983; Strahl et al. 2002)
Medium 4	10	5	20	Medium-high salinity level
Medium 5	10	5	30	High salt stress; generates a salinity close to that of seawater (Feng et al. 2008; Ji et al. 2007; Taylor et al. 2000)

rhlI, *pqsA*, and *ambB*) using the newly developed multicolor whole-cell biosensor. Salt stress was selected as an important environmental parameter to validate the biosensor because it has a significant impact on biofilm formation and virulence of *P. aeruginosa*. For example, sodium chloride at 0.5 M (29.2 g L^{-1}) reduces the thickness and biomass amount of *P. aeruginosa* PAO1 biofilm by approximately 50% and approximately 67%, respectively (Bazire et al. 2007). Compared with the control, *P. aeruginosa* PAO1 grown in M9 minimal medium amended with 0.4 M (23.4 $g \cdot L^{-1}$) of sodium chloride produces roughly 2.5 times more neuraminidase (Tang et al. 1996), a virulence factor for many pathogens including *P. aeruginosa* PAO1 (Cacalano et al. 1992; Engibarov et al. 2015; Ghazaei et al. 2010). *P. aeruginosa* is ubiquitous in various environments (Stover et al. 2000; Teitzel and Parsek 2003; Wolfgang et al. 2003), and it would be of importance to study how salt stress affects its QS systems. Therefore, the current study screened the responsese of the selected four QS genes to salt stress using the whole-cell biosensor.

First, we individually monitored the responses of the selected QS genes to salt stress using *P. aeruginosa* PAO1 strains containing pSEVA-P_{lasI}-mNEON Green, pSEVA-P_{rhlI}-mNEON Green, pSEVA-P_{pqsA}-mNEON Green, or pSEVA-P_{ambB}-mNEON Green. The four selected QS genes, especially *lasI* and *rhlI*, were all downregulated when the sodium chloride level in the culture media increased from 1.25 $g \cdot L^{-1}$ to 12.85 $g \cdot L^{-1}$ (Figs. S4 and S5).

We also exposed the multicolor whole-cell biosensor to culture media with varying sodium chloride concentrations (Table 2). We chose sodium chloride concentrations of 0.1 $g \cdot L^{-1}$, 10 $g \cdot L^{-1}$, and 30 $g \cdot L^{-1}$ to represent the salinities of drinking water, human blood, and seawater, respectively. In addition, the concentration range of sodium chloride in this study well covered the salinity of municipal sewage, which typically is less than 1% or 10 parts per thousands (ppt) (Ho et al. 2013; Wu et al. 2008; Ye and Zhang 2011). Salt stress had an obvious impact on the expression of *rhlI*, *pqsA*, and *ambB*. At the incubation time of 17 h to 18 h at 37 °C, sodium chloride statistically significantly impacted the expression of *rhlI*, *pqsA*, and *ambB* as determined by one-way ANOVA ($F(4, 15) = 32.1$, p value < 0.001), the Kruskal-Wallis test (p value = 0.006), and one-way ANOVA ($F(4, 15) = 7.5$, p value = 0.002), respectively (Fig. 2). For all those three QS genes, the post hoc test further indicated that the expression level at 30 $g \cdot L^{-1}$ of sodium chloride was statistically significantly lower than those at all other four tested sodium chloride concentrations (the SNK procedure, p value < 0.05). For the incubation time of 16 h at 37 °C, a similar expression pattern was found for *rhlI*, *pqsA*, and *ambB*, but the fluorescence signals for *pqsA* and *ambB* were too low to detect at the sodium chloride concentration of 30 $g \cdot L^{-1}$ (Fig. S6). In contrast, when the incubation time reached 26 h to 28 h at 37 °C, increase in sodium chloride concentration showed only a slight impact on the expression from *rhlI*, *pqsA*, and *ambB* as determined by one-way ANOVA ($F(4, 15) = 0.267$, p value = 0.895), one-way ANOVA ($F(4, 15) = 0.363$, p value = 0.831), and the Kruskal-Wallis test (p value = 0.699), respectively (Fig. 3). Together, the results suggest that the impact of salt stress on the expression of *rhlI*, *pqsA*, and *ambB* varied over time as the bacterium adapted to the new environments.

Our results are consistent with a previous study which used a *luxCDABE*-based reporter system to individually measure the regulation of important QS genes such as *lasI* and *rhlI* in

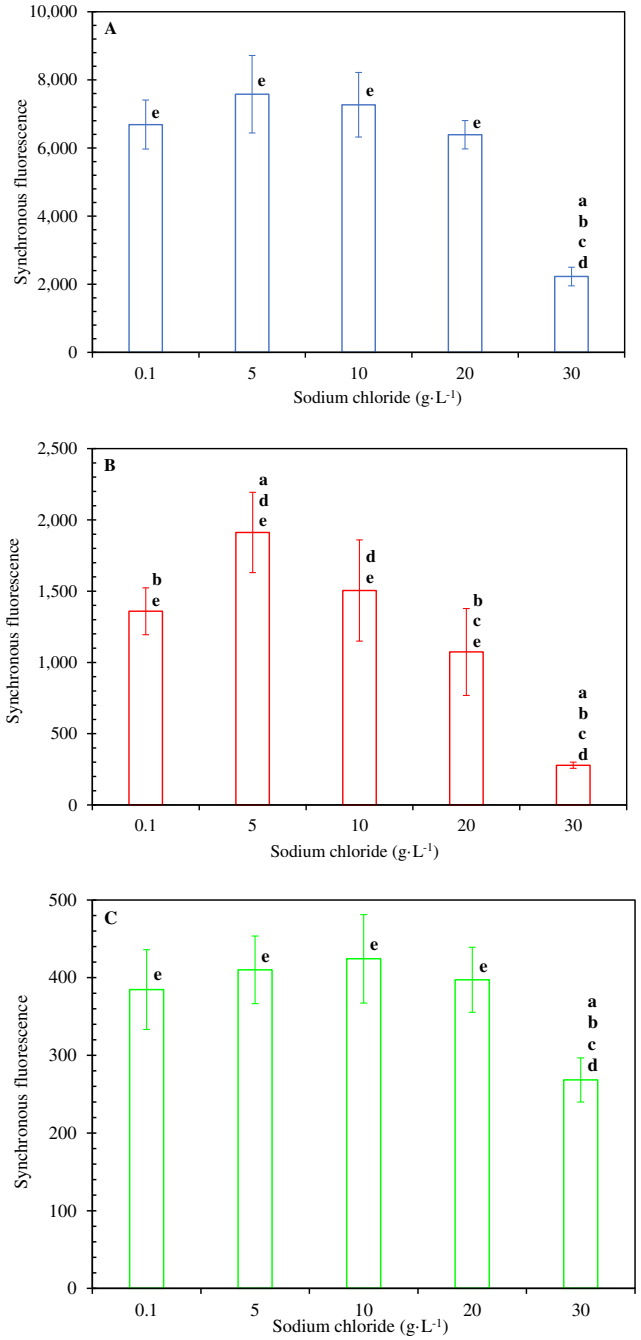

Fig. 2 Expression of (**A**) *rhlI*, (**B**) *pqsA*, and (**C**) *ambB* (measured in fluorescence units) at different sodium chloride concentrations using the multicolor whole-cell biosensor after 17 h to 18 h of incubation at 37 °C. The fluorescence signals were generated from cell cultures with an $O.D._{600}$ in the range from 0.990 to 1.000. The error bars indicate standard deviations of four independent experiments. The gene expression levels at sodium chloride concentrations of 0.1 $g{\cdot}L^{-1}$, 5 $g{\cdot}L^{-1}$, 10 $g{\cdot}L^{-1}$, 20 $g{\cdot}L^{-1}$, and 30 $g{\cdot}L^{-1}$ are denoted as "a," "b," "c," "d," and "e," respectively. Each letter above a column indicates statistically significant difference (p value < 0.05) between expression levels

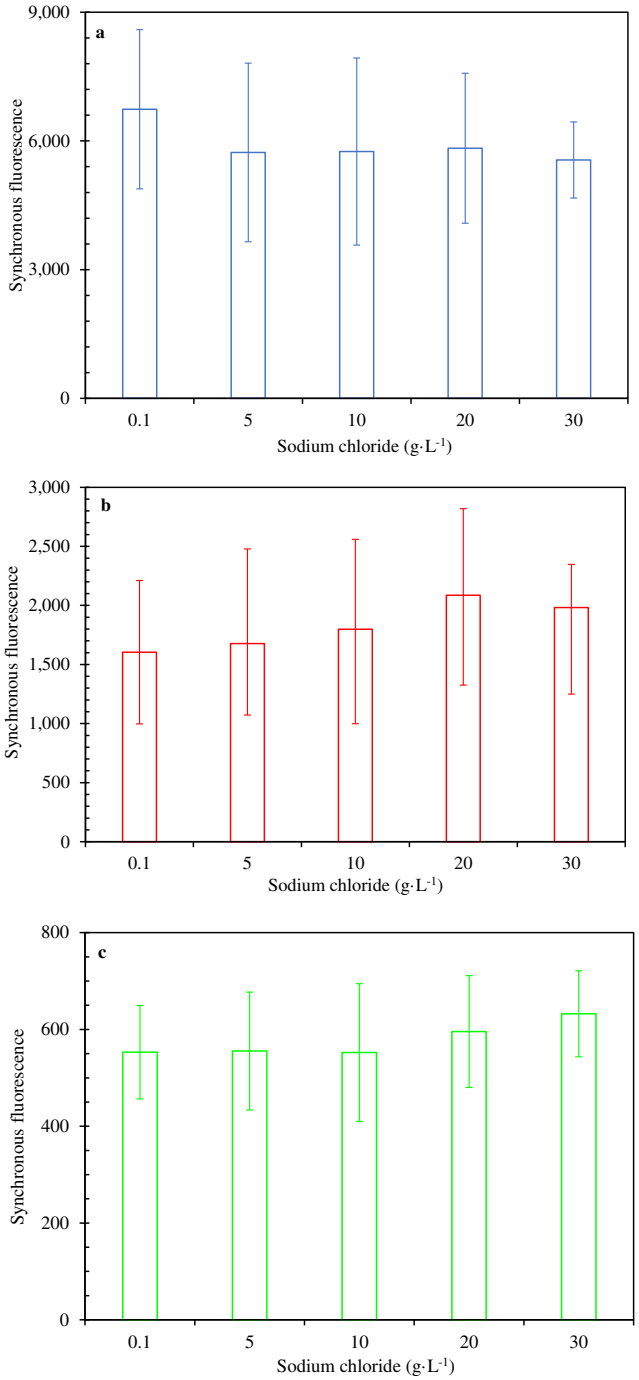

Fig. 3 Expression of (**a**) *rhlI*, (**b**) *pqsA*, and (**c**) *ambB* (measured in fluorescence units) at different sodium chloride concentrations using the multicolor whole-cell biosensor after incubation for 26 h to 28 h at 37 °C. The fluorescence signals shown in Fig. 3(**a**) were generated from two times diluted cell culture, while the original cell culture had an $O.D._{600}$ in the range from 0.990 to 1.000. The fluorescence signals shown in Fig. 3**b**, **c** were generated from cell cultures with an $O.D._{600}$ in the range 0.990 to 1.000. The error bars indicate standard deviations of four independent experiments

P. aeruginosa PAO1 (Duan and Surette 2007). Similarly, an RT-qPCR assay revealed that sodium chloride at 29.22 $g{\cdot}L^{-1}$ could reduce the relative expression levels of *lasI* and *rhlI* by approximately 90% and approximately 70% to 92%, respectively, in *P. aeruginosa* PAO1 (Bazire et al. 2005; Bazire et al. 2009). The results of the bioluminescent and the RT-qPCR assays as orthogonal methods suggest that our

newly developed whole-cell biosensor is reliable to detect the expression of QS genes (at least for *rhlI*). In addition, this new whole-cell biosensing approach is superior to the bioluminescent and the RT-qPCR assays in that it is capable to simultaneously and noninvasively monitor the expression of multiple genes in a single set of experiment. However, further transcriptomic analysis of the impact of salt stress on native QS genes in the chromosome is required to fully validate the feasibility of using this biosensor to screen the expression of *rhlI*, *pqsA*, and *ambB*.

The activities of QS systems are closely associated with cell population density (Swift et al. 2001; Waters and Bassler 2005; Whitehead et al. 2001). Figure 4 shows the representative growth curves of *P. aeruginosa* PAO1 without any plasmid DNA at varying sodium chloride levels. Table 3 indicates the specific growth rate of this strain for each salinity level. Sodium chloride concentration had a statistically significant impact on the specific growth rate as determined by one-way ANOVA ($F(4, 10) = 20.3$, p value < 0.001). Moreover, the specific growth rates at sodium chloride concentrations of 20 g·L^{-1} and 30 g·L^{-1} were statistically significantly lower than those at sodium chloride concentrations of 5 g·L^{-1} and 10 g L^{-1} (the Student-Newman-Keuls (SNK) procedure, p value < 0.05), indicating that a high salinity adversely affected the growth of *P. aeruginosa* PAO1. Those findings are in line with previous research studies where a high salt stress repressed the growth of *P. aeruginosa* PAO1 (Aspedon et al. 2006; Bazire et al. 2009; D'Souza-Ault et al. 1993; Havasi et al. 2008; Nostro et al. 2005).

Table 3 Impact of sodium chloride concentration on the growth of *P. aeruginosa* PAO1

Sodium chloride concentration in the culture medium (gL^{-1})	Specific growth rate (h^{-1})	
	Average (n = 3)	Standard deviation (n = 3)
0.1	0.142 b/c/d	0.007
5	0.180 a/d/e	0.005
10	0.174 a/d/e	0.010
20	0.155 a/b/c	0.008
30	0.140 b/c	0.003

Data are generated from three independent experiments. The specific growth rates at sodium chloride concentrations of 0.1 g·L^{-1}, 5 g·L^{-1}, 10 g·L^{-1}, 20 g·L^{-1}, and 30 g·L^{-1} are denoted as "a," "b," "c," "d," and "e," respectively. The letters following a specific growth rate indicate statistically significant difference (SNK procedure, p value < 0.05) between specific growth rates

Discussion

Selection of target QS genes

It is essential to select appropriate target genes so that monitoring of the regulation of QS systems with the new biosensing approach would be meaningful. As discussed above, *lasI*, *rhlI*, *pqsA*, and *ambB* are responsible for the biosynthesis of important QS signal molecules OdDHL, BHL, PQS, and IQS, respectively. Characterization of the expression patterns of these QS genes promotes a better understanding of QS network-regulated processes such as biofilm formation (Erken et al. 2013; Nadell et al. 2008; Parsek and Greenberg 2005). Indeed, *lasI*, *rhlI*, *pqsA*, and *ambB* impact biofilm formation in *P. aeruginosa* (Davies et al. 1998; de Kievit et al. 2001; Sakuragi and Kolter 2007; Shih and Huang 2002; Yang et al. 2007) and are also essential for its virulence (Déziel et al. 2005; Kim et al. 2010; Lee et al. 2013; Rumbaugh et al. 1999; Wu et al. 2001).

This study designed the whole-cell biosensor to selectively monitor the activities of *lasI*, *rhlI*, *pqsA*, and *ambB* as representative QS genes due to their unique roles in the QS networks as well as their essential functions in biofilm process and pathogenesis in *P. aeruginosa* PAO1. We first

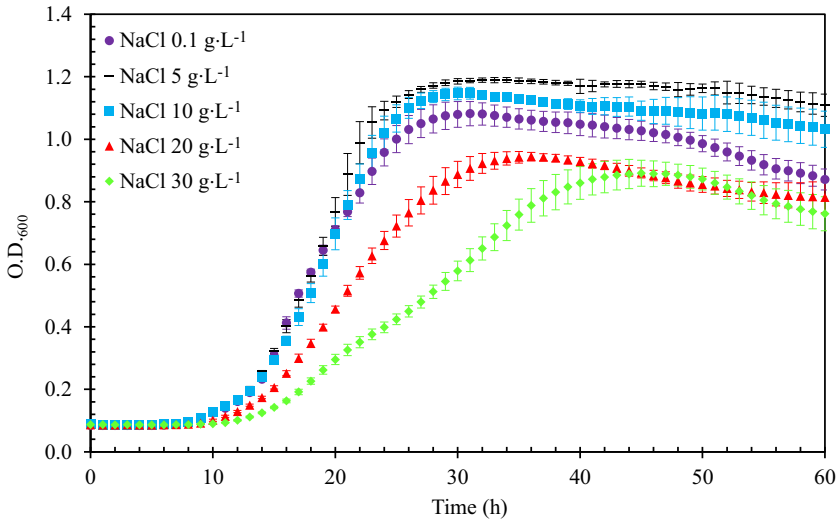

Fig. 4 Growth curves of *P. aeruginosa* PAO1 (change in optical density O.D.$_{600}$ with time) in culture media with different sodium chloride concentrations. Table 2 shows the recipes of those media. The error bars indicate the standard deviation of eight biological replicates

individually fused P_{lasI}, P_{rhlI}, P_{pqsA}, or P_{ambB} upstream of the mNEON Green gene in separate plasmids (Table S1) and checked the synchronous fluorescence signals of *P. aeruginosa* PAO1 strains with those plasmids. Figure S7 reveals that the mNEON Green gene yielded strong fluorescence signals when fused downstream of each promoter. These four promoter regions were, therefore, suitable candidates to monitor the regulation of the QS networks in *P. aeruginosa* PAO1. However, different promoters clearly showed different genetic activities (Fig. S7). Specifically, the activities of P_{lasI} and P_{rhlI} were much more intense than those of P_{pqsA} and P_{ambB}, indicating the different expression levels among the four QS networks. It is also worth noting that when designing the multicolor biosensor, we incorporated the entire intergenic region upstream of a QS gene with an extension of 90 bp into each (i.e., upstream and downstream) flanking coding region as the "promoter region" to surely include the promoter (Zaslaver et al. 2006).

Selection of fluorescent reporter genes

We tested the feasibility of ten fluorescent genes as potential reporters in *P. aeruginosa* PAO1, namely, Clover, DsRed-Express2, E2-Crimson, E2-Orange, mCandinal2, M Cherry, mNEON Green, mTag BFP2, SFGFP, and Turbo YFP genes. We constructed ten plasmids where each plasmid contained one fluorescent gene under the control of P_{lac} and individually delivered those plasmids into *P. aeruginosa* PAO1 to form ten new strains. Fluorescence signal strengths of these ten strains were individually recorded. In addition, since certain FPs exhibit toxicity to host cells and limit their application as whole-cell tracers (Liu et al. 1999; Shemiakina et al. 2012; Strack et al. 2008), we checked the toxicities of these ten FPs to *P. aeruginosa* PAO1. Among them, E2-Crimson, mCandinal2, and SFGFP exhibited toxicity to *P. aeruginosa* PAO1 and/or yielded low fluorescence intensities (data not shown).

The remaining seven FPs showed no obvious toxicity to the host cell and generated strong fluorescence signals with an order of strengths of Clover > mNEON Green > E2-Orange > Turbo YFP > DsRed-Express2 ≈ mCherry ≈ mTag BFP2 (Fig. S8). The fluorescence signal of Clover was considerably more intense than and might override or interrupt the signals of other FPs. Thus, Clover gene was excluded from the biosensor design. However, we included the mNEON Green, E2-Orange, and Turbo YFP genes in the biosensor since they produced strong and comparable fluorescence without obvious cytotoxicity or impact on cell growth (data not shown).

Next, we selected mTag BFP2 based on its clear spectral separation from mNEON Green, E2-Orange, and Turbo YFP. mTag BFP2 was also not toxic to the host cell. Nonetheless, the fluorescence signal strength of mTag BFP2 was only 17% to 31% of these three FPs (Figs. S2 and S8). To ensure that the four selected FPs have comparable fluorescence signal strengths, we optimized the codon usage for the mTag BFP2 gene to improve its expression in *P. aeruginosa* PAO1 (Table S4). The codon optimization upgraded the codon adaptation index (CAI) and the frequency of optimal codons (FOP) of the mTag BFP2 gene for *P. aeruginosa* PAO1 from 0.55 to 1.00 and from 71% to 99%, respectively, without alerting the fluorescence signal peak location. Consequently, the codon optimization significantly improved the fluorescence signal strength of mTag BFP2 (Fig. S9). In addition, Fig. S9 shows that even though after codon optimization, mTag BFP2 had a very strong fluorescence signal, it did not affect the fluorescence signals of other selected FPs such as mNEON Green and E2-Orange.

Fluorescence signals of the four chosen FPs (i.e., Turbo YFP, mTag BFP2, mNEON Green, and E2-Orange) must be completely separated so that we could simultaneously detect the expression of the four QS genes (i.e., *lasI*, *rhlI*, *pqsA*, and *ambB*). The synchronous fluorescence peak locations of mTag BFP2, mNEON Green, Turbo YFP, and E2-Orange in *P. aeruginosa* PAO1 (425 nm, 501 nm, 522 nm, and 541 nm, respectively) were at least 19 nm apart from one another (Figs. S2 and S8). Therefore, a clean separation of the synchronous fluorescence signals from all four FPs was achieved.

Coupling of promoters and reporters

Promoters of the four target QS genes had considerably different strengths (Fig. S7). For instance, the synchronous fluorescence was larger than 5000 for P_{rhlI} but less than 130 for P_{pqsA} when measured using the mNEON Green gene as the reporter. The selected reporter genes also had varying fluorescence signal strengths (Fig. S2). In a multicolor whole-cell biosensor, strong fluorescence signals might interrupt or mask the weak ones, negatively affecting the accuracy of gene expression monitoring. Detection of gene expression by monitoring the changes in multiple fluorescent genes/proteins is more feasible if the fluorescence signals have comparable strengths. Therefore, we coupled strong promoters with weak fluorescent genes ans also weak promoters with strong fluorescent genes in the biosensor. Based on the relative fluorescence signal strengths of the promoters (Fig. S7) and reporters (Fig. S2), they were coupled as follows: the mTag BFP2 gene under the control of P_{rhlI}, the Turbo YFP gene under the control of P_{lasI}, the E2-Orange gene under the control of P_{ambB}, and the mNEON Green gene under the control of P_{pqsA} (Figs. 1 and S1). Specifically, after codon optimization, mTag BFP2 showed the highest fluorescence signal strength among the selected reporter proteins (Fig. S9). P_{rhlI} also showed the highest fluorescence signal strength among the selected promoters (Fig. S7). However, the P_{rhlI}-mTag BFP2 gene fusion would not affect other fusions because the fluorescence signal peak of mTag BFP2 was far away from other reporters.

Genetic independence in pSEVA-QSGEF

It is important to avoid potential crosstalk and minimize the interactions among the four open reading frames (ORFs) (i.e., fusions of promoters and fluorescent reporter genes) in pSEVA-QSGEF. Thus, we put two different transcriptional terminators immediately upstream and downstream of each ORF (Fig. S1). Experiments have confirmed that those 16 different terminators in pSEVA-QSGEF have high terminator strengths (i.e., being able to reduce more than 60-fold downstream expression) in *E. coli* (Chen et al. 2013). The other two terminators [i.e., T0 from phage lambda (Martínez-García et al. 2015; Wright et al. 2014; Zobel et al. 2015) and T1 from the *rrnB* operon of *E. coli* (Chan et al. 2016; Farzadfard and Lu 2014; Martínez-García et al. 2015)] in the backbone of pSEVA-QSGEF are also efficient in stopping transcription. Table S5 summarizes the basic properties for the 18 Rho-independent terminators in pSEVA-QSGEF. These terminators function intrinsically through generating self-annealing hairpin (i.e., stem-loop) structures and 3′-end U-tracts in the transcribed mRNA without the need of any other proteins (Craig et al. 2014; Ermolaeva et al. 2000; Farnham and Platt 1981; Peters et al. 2011). We put two different terminators immediately upstream of each ORF to ensure that the read-through transcription was blocked even if one of the two terminators had a low terminator strength (Mairhofer et al. 2014). In addition, we arranged the four ORFs in QSGEF in alternating orientations, where (1) the Turbo YFP gene and the mTag BFP2 gene were converging, (2) the mNEON Green gene and the E2-Orange gene were converging, and (3) the mTag BFP2 gene and the mNEON Green gene were diverging (Figs. 1 and S1). This special arrangement further hindered crosstalk between two consecutive ORFs. Finally, we employed double stop codons (SCs) at the end of each fluorescent gene (i.e., 5′-TAG TAA-3′ for the E2-Orange gene and 5′-TAA TGA-3′ for other three fluorescent reporter genes) to appropriately terminate gene translation. Those design strategies potentially made the four ORFs in QSGEF genetically independent.

Biocompatibility of pSEVA-QSGEF

To successfully detect gene expression with an engineered plasmid, the plasmid must be biocompatible (e.g., nontoxic) to the host cells (Cox III et al. 2010; Shaner et al. 2005; Tao et al. 2007). As mentioned above, the fluorescent genes in QSGEF did not show obvious toxicity to *P. aeruginosa* PAO1. In addition, the four promoter regions in QSGEF originally exist in the natural chromosome of *P. aeruginosa* PAO1. However, pSEVA-QSGEF significantly impacted the growth of *P. aeruginosa* PAO1 (data not shown). This might be caused by the large size of this plasmid DNA (8498 bp).

Furthermore, the backbone of pSEVA-QSGEF has a stable, medium-copy-number (moderate-copy-number), and BHR origin of replication (Silva-Rocha et al. 2013; Szpirer et al. 2001) from a small BHR plasmid pBBR1 (Antoine and Locht 1992). Previous studies showed that plasmids with the pBBR1 replicon can be stably maintained without antibiotic selection for more than 30 generations in several bacterial taxa (Miller et al. 2000) and have a medium-copy-number in multiple bacterial species (Elzer et al. 1995; Jittawuttipoka et al. 2009; Khan et al. 2008; Kovach et al. 1995). In general, a medium-copy-number plasmid has 100 copies or less in each host cell. For example, each *E. coli* MM294 cell contains 30 to 40 copies of pBBRl-derived plasmids (Antoine and Locht 1992). Since pSEVA-QSGEF is a large plasmid (8498 bp), we speculated that it behaved like a medium-copy-number plasmid in *P. aeruginosa* PAO1. This medium-copy-number characteristic of pSEVA-QSGEF prevented undesired nonspecific transgene expression (which might occur from high-copy-number plasmids), minimized background levels, reduced stress or burden caused by harboring this plasmid, and helped to maintain a tight regulation of expression in the biosensor. However, the large size of this plasmid could affect its stability, especially after many generations. Further studies are needed to verify its long-term stability in the biosensor without antibiotic pressure.

Prevention for genetic instability in QSGEF

Genetic instability often causes engineered circuits (e.g., plasmids) to fail to possess their designed functions (Canton et al. 2008; Cox III et al. 2010). Genetic instability is a complicated multiparameter phenomenon, but homologous recombination is a main reason (Jack et al. 2015). Repeated use of terminators in an engineered plasmid (Krom et al. 2015; Miller et al. 2000) induces serious homologous recombination and greatly reduces the evolutionary half-life of the circuit (Sleight et al. 2010). In this study, we avoided the repeated use of terminators. Moreover, homologous recombination rate is high when different terminators in an engineered plasmid share a contiguous sequence or a mismatch-free sequence longer than the minimal efficient processing segment (MEPS) (Haber 2014; Kahl 2015). Different bacterial strains have different MEPSs. The estimated MEPSs of *E. coli*, *Agrobacterium*, *Bacillus subtilis*, and *Streptococcus pneumoniae* are 23 bp (Shen and Huang 1986; Smith 1988), 27 bp (Costechareyre et al. 2009), 21 bp (Majewski and Cohan 1999), and 27 bp (Majewski et al. 2000), respectively. Therefore, it is reasonable to assume that the MEPS of *P. aeruginosa* is approximately 23 bp based on the phylogenetic relationship between these strains and *P. aeruginosa* (Brown and Volker 2004; Casciotti and Ward 2005; Gupta 2000; Jackson and Dugas 2003; Maggi et al. 2008; O'Neill et al. 1992; Willems and Collins 1993; Williams et al. 2010). When designing QSGEF, we used a

stricter criterion and made sure that no terminator pairs shared a contiguous stretch of sequence of or longer than 16 bp. The four fluorescent genes in QSGEF might be another source of homologous recombination because they shared the same ribosome-binding site (RBS) (i.e., 5′-AGG AGG-3′) (Borrero-de Acuña et al. 2014; Silva-Rocha et al. 2013), AT-rich spacer region (i.e., 5′-AAA AAC AT-3′) (Cheng and Patterson 1992; Miller and Lindow 1997), and start codon (i.e., 5′-ATG-3′) (Hoseini and Sauer 2015). However, the EFM Calculator (barricklab.org/django/efm/) (Jack et al. 2015) predicted that the rates of repeat-mediated deletions (RMDs) in QSGEF caused by these long repeated regions were less than 5×10^{-9} mutation events per cell per generation (MECG), suggesting that QSGEF was highly resistant to homologous recombination.

In addition to homologous recombination, short insertions and/or deletions induced by simple sequence repeats (SSRs) destabilizes an engineered plasmid. In QSGEF, P_{rhlI} has the highest SSR rate (i.e., 3.18×10^{-5} MECG) due to the presence of 3′-CAC ACA CA-5′ (EFM Calculator prediction) (Jack et al. 2015). Multiple studies have fused this promoter region to a reporter gene in a plasmid and successfully detected the expression of *rhlI* (de Kievit et al. 2002; Duan and Surette 2007; Whiteley et al. 2000). Indeed, we did not find any obvious SSR-driven mutations in P_{rhlI} when using pSEVA-P_{rhlI}-mNEON Green to study the expression pattern of *rhlI* (data not shown). Other regions in QSGEF had lower SSR rates according to the EFM Calculator (Jack et al. 2015). Overall, QSGEF was a robust and stable DNA fragment with extremely low mutation rates and high genetic stability.

Modularity of QSGEF

QSGEF contains three essential groups of elements: (1) promoter regions, (2) fluorescent reporter genes (including the RBSs, spacers, and stop codons), and (3) terminators. We put unique restriction sites upstream and downstream of each of those individual components in QSGEF (Fig. S1) so that replacement of any of them is feasible. For instance, we may monitor the expression of other *P. aeruginosa* PAO1 genes by simply replacing the promoter regions in QSGEF. It is also possible to use other fluorescent genes as reporters when necessary. Furthermore, the QSGEF scaffold can be moved into different vectors (Cox III et al. 2010) to examine gene expression in a broad range of bacterial species using this noninvasive approach.

Applications of the novel whole-cell biosensor

We designed this biosensor to study how salt stress would affect the expression of important QS genes in *P. aeruginosa* PAO1. Since QS networks are closely related to biofilm formation and pathogenesis, such information generated by the biosensor could help to develop more efficient biofilm and pathogen control approaches. A recent studies has developed a MATLAB™ program using tensor rank decomposition or PARAllel FACtor analysis (PARAFAC) to separate the impact of multiple factors on the fluorescence signals (Parrello et al. 2015). This program successfully decomposed the raw synchronous spectra of a mixed culture of two *P. aeruginosa* PAO1 bioreporters to a fluorescence profile as the function of iron concentration and a fluorescence profile as the function of the ratio of the two strains. Further work is needed to investigate and separate the effects of multiple environmental stress factors on the expression of the selected QS genes using the novel biosensor and the MATLAB™ program.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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