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A genetically engineered whole-cell pigment-based bacterial biosensing system for quantification of *N*-butyryl homoserine lactone quorum sensing signal

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ABSTRACT

N-Acyl homoserine lactone (AHL) is a widely conserved quorum sensing (QS) signal of Gram-negative bacteria and has received attention in fighting against human diseases and environmental pollution. However, a method for quantifying AHL is lacking although it is urgently required for diagnosis and bioprocess manipulation. This work screened out an aromatics degrader *Pseudomonas aeruginosa* for biosensing system development, which produced a blue–green pigment regulated by the RhlI–RhlR QS system. By taking advantage of the recognition of *N*-butyryl homoserine lactone (BHL, the signal molecule of RhlI–RhlR QS system and an AHL) by the product of *rhlR*, a new whole-cell biosensor *P. aeruginosa* Δ*rhlIR*/pYC-rhlR (*rhlI*$^{-}$ *rhlR*$^{++}$) was developed. It was constructed through abolishing its BHL production by in-frame deletion of *rhlIR* and over-expressing *rhlR* by introducing a multi-copy plasmid pYC-rhlR into Δ*rhlIR*. By using the pigment production which responded to exogenous BHL as biosensor output, BHL quantification in samples was simply done spectrophotometrically. Under optimum conditions, the calibration curve had the limit of detection (LOD), the 50% activation/effect concentration, the limit of quantification (LOQ), and the quantitative detection range of 1.3 nM, 2.77 ± 0.45 μM, 5.7 nM and 0.11–49.7 μM, respectively. The biosensor output was stable, culture samples could be stored 10 days under −20 °C, and this sensing system was resistant to interferences by toxic aromatic pollutants. It was successfully applied to environmental samples even without extraction. The new whole-cell biosensing system provided a simple, stable, toxic pollutants-tolerant, and cost-effective tool for quantitative investigation of the QS signals' role in environmental processes.

1. Introduction

N-Acyl homoserine lactone (AHL) is a widespread signal molecule for cell-to-cell communication through quorum sensing (QS) in Gram-negative bacteria. This AHL-mediated QS plays an important role in many biological processes including bacteria-related human diseases (Bassler and Losick, 2006; Cooley et al., 2008). For example, for the opportunistic pathogen *Pseudomonas aeruginosa*, its QS systems including the *las* system (transcriptional activator LasR plus AHL synthase LasI) and the *rhl* system (RhlR and *N*-butyryl homoserine lactone (BHL) synthase RhlI) control the expression of a wide variety of virulence genes. Inhibition or mutation of these QS systems could result in less production of its virulence factor and less virulent to various animal models (Ishida et al., 2007; Juhas et al., 2005). In fact, AHLs were demonstrated to be potential biomarkers for the diagnosis and management of some bacteria-related human diseases (Kumari et al., 2008).

In environmental bioprocesses, AHL producers such as *Pseudomonas*, *Agrobacterium*, and *Aeromonas* were isolated from activated sludges and water reclamation systems (Hu et al., 2003; Morgan-Sagastume et al., 2005). Addition of AHLs changed the bacterial community composition and improved phenol biodegradation efficiency in industrial activated sludge (Valle et al., 2004). Moreover, AHL production by bacteria in membrane bioreactors was observed, and it accelerated the membrane biofouling in advanced wastewater treatment (Yeon et al., 2009). These findings implied that AHLs- or AHL-mediated QS might play a role in pollutant treatment processes, and AHL/QS manipulation was presumed to be a promising strategy to regulate these complex processes at the population/community level (Manefield and Whiteley, 2007). Definite determination of the AHL level and the AHL kinetic profile are greatly required in view of bioprocess regulation and optimization. Nevertheless, they were not reported due to the lack of suitable quantification methods.

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The crucial roles of AHLs both in bacteria-related diseases and in pollutant treatment processes point out the importance of quantification methods for AHL signals. Due to the lack of a significant chromophore in AHL molecules, the application of conventional physical–chemical analytical methods for its quantification is usually limited to a procedure coupled with expensive mass spectrometry (MS), such as LC–MS and GC–MS as reported (Cataldi et al., 2007; Morin et al., 2003). In addition, physical–chemical analysis cannot provide information about the bioavailability of analytes (Gu and Chang, 2001). Biosensor assays provide an alternative method for sensitive, fast, and cost-effective quantification, and they may also give bioavailability information (D'Souza, 2001; Kumari et al., 2006; Steindler and Venturi, 2007). Until now, only a few biosensors have been developed for AHL quantification (Boettcher and Ruby, 1995; Le Berre et al., 2008; Pearson et al., 1997; Ravn et al., 2001; Séveno et al., 2001; Shaw et al., 1997; Yan et al., 2007). These biosensors usually involve *Escherchia coli* or AHL synthase mutants of *Agrobacterium*, which carries a recombinant plasmid containing *lacZ*, *luxCDABE,* or *gfp* fused with the promoter of target genes to be activated by QS (Kumari et al., 2006; Steindler and Venturi, 2007). Apparently these methods each have one or more significant drawbacks. The β-galactosidase enzyme assay (*lacZ*) is time and cost consuming due to its in vitro enzyme assay (Blosser and Gray, 2000). For luminescence (*luxCDABE*) assays, the AHLs-induced luminescence is not very stable due to durative changes of cell metabolic activity and the impossible storage of culture samples for later assays (Kohlmeier et al., 2007; Neilson et al., 1999). And the fluorescence bioassay (*gfp*) requires an elaborate fluorescence detector (Leskinen et al., 2008). Using natural pigment as the biosensor output may overcome those problems (Blosser and Gray, 2000). As reported, until now *Chromobacterium violaceum* CV026 was the sole natural pigment biosensor for AHL quantification. However, the attempt to detect/quantify AHLs in activated sludge by using *C. violaceum* CV026 was not successful (Valle et al., 2004), which may be partially due to violacein inhibition by co-existing long-chain AHLs (C_{10}–C_{14} in acyl chain length) (McClean et al., 1997) and the loss of biosensor viability in toxic samples. Therefore, a new biosensor that can be applied in toxic ecosystems is required in order to obtain quantitative insight into the role of AHLs in polluted environments (Manefield and Whiteley, 2007).

To that end, an aromatic pollutants degrader *P. aeruginosa* CGMCC 1.860, which could produce a blue–green pigment under the control of BHL (one of AHLs), was screened out. By genetic modification, a pigment-based biosensor that could effectively respond to low levels and a wide concentration range of BHL was constructed. Furthermore, we have developed a biosensing system with spectrophotometric detection, based on this biosensor, that provides rapid, sensitive, and quantitative detection of BHL. This biosensing system was further validated in wastewater samples.

2. Materials and methods

2.1. Bacterial strains and culture conditions

P. aeruginosa CGMCC 1.860 (Sun et al., 2006), which was eventually selected as the parent strain for biosensor construction, was originally isolated from trash rubber (Table S1 and S2). Bacteria were routinely grown with shaking in LB broth at 30 °C. For the biosensor assay, *Pseudomonas* broth (PB) (Ishida et al., 2007) was used. Where required, antibiotics were used at the following concentrations: ampicillin (100 μg/ml) or tetracycline (20 μg/ml) for *E. coli*; ampicillin (100 μg/ml) and tetracycline (250 μg/ml) for *P. aeruginosa*.

2.2. Chemicals and reagents

N-Butyryl-L-homoserine lactone (>98%, BHL) and *N*-(3-oxo)-dodecanoyl-L-homoserine lactone (>98%, OdDHL) were purchased from Cayman Chemical Co. (Michigan, USA). Phenanthrene (>97%) was purchased from Sigma–Aldrich Co. (Steinheim, Germany). All antibiotics used were purchased from Ameresco Inc. (Ohio, USA). HPLC-grade acetonitrile was purchased from Fisher Scientific Co. (Pennsylvania, USA). All other chemical reagents were analytical grade and supplied by Sinopharm Chemical Reagent Co. (Shanghai, China). Stock solutions of 1 mg/l BHL or OdDHL were prepared in HPLC-grade acetonitrile and stored at −70 °C. Enzymes for DNA manipulation were obtained from Fermentas Co. (Vilnius, Lithuania) and Takara Biotech. (Dalian, China).

Industrial wastewater was taken from the wastewater treatment center of Shanghai Dye Chemical Industry Co. (Minhang, Shanghai, China). Municipal wastewater was obtained from Shanghai Minhang Water Clarification Co. (Minhang, Shanghai, China).

2.3. DNA manipulation and mutant construction

The DNA fragment containing the *rhlIR* gene (GenBank Accession No. FJ213455) was amplified by PCR using *P. aeruginosa* CGMCC 1.860 genomic DNA and specifically designed primers rhlIRUF 5′-GCATCGCTCACGAGAAGTAC-3′ and rhlIRDR 5′-GCCGCCCCTGTATTACTTG-3′ (Fig. S1). The nucleotide sequence showed 99% identity to that of *P. aeruginosa* PAO1. The DNA fragment (FJ213455) was cloned into pUC19, resulting in pUC-rhlIR. The chromosomal deletion mutants included Δ*rhlIR* and Δ*rhlI*, Δ*rhlIR* (1003 bp in-frame deletion in *rhlIR*, *rhlI*$^-$*rhlR*$^-$) was obtained by conjugating a suicide plasmid pYC2000Δ*rhlIR* into *P. aeruginosa* CGMCC 1.860, and Δ*rhlI* (246 bp in-frame deletion in *rhlI*, *rhlI*$^-$*rhlR*$^+$) was obtained by pYC2000Δ*rhlI* conjugation (Fig. S2). Allelic exchange selection was conducted as reported (Philippe et al., 2004). Plasmids pYC2000Δ*rhlIR* and pYC2000Δ*rhlI* were constructed as follows. A 1003 bp *Kpn*I–*Bam*HI fragment deletion of *rhlIR* gene in pUC-rhlIR was introduced by endonuclease excision, end polishing and ligation, resulting in pUC-ΔrhlIR. The ΔrhlIR fragment was excised from the vector using *Eco*RI and *Hind*III, and inserted into the *Pst*I site of pYC2000, resulting in pYC2000Δ*rhlIR*. An approximate 1.9 kb *Bam*HI fragment of FJ213455 was excised with *Sty*I and ligated with T4 DNA ligase; thus a 246 bp *Sty*I fragment was deleted in the *rhlI* gene. This ΔrhlI fragment was inserted into the *Pst*I site of pYC2000, resulting in pYC2000Δ*rhlI*. Plasmid pYC2000 was constructed by inserting the tetracycline resistance cartridge from pBR322 at the *Ssp*I site of pDS132 (Philippe et al., 2004). Mutants were confirmed by PCR analysis, DNA sequencing, and complementation. A broad host range multi-copy plasmid pYC1000 derived from pME6000 (Reimmann et al., 2002), by replacing its *tetAR-lacZ* fragment with *tet* from pBR322, was used to construct a series of expression plasmids. pYC-rhlI and pYC-rhlIR were obtained by inserting the DNA fragments containing an intact *rhlI* gene (about 2.0 kb *Bam*HI fragment of FJ213455) or *rhlIR* gene (FJ213455) into the *Aat*II site of pYC1000, respectively. pYC-rhlR was constructed by digesting pYC-rhlIR with *Kpn*I and re-ligating. Plasmid transformation for *P. aeruginosa* was performed by conjugation using *E. coli* S17-1 as the donor strain (Reimmann et al., 2002).

2.4. RT-PCR

Total RNA was isolated using Trizol Reagent (Invitrogen Co., CA, USA), treated with DNase I (Promega Co., Madison, USA) at 37 °C for 30 min and heat-treated in the presence of EDTA at 65 °C for 10 min. The cDNA was synthesized from the total RNA and then amplified by PCR with the following primers: for *rhlR* the primers were 5′-CCGATGCTGATGTCCAACC-3′ and

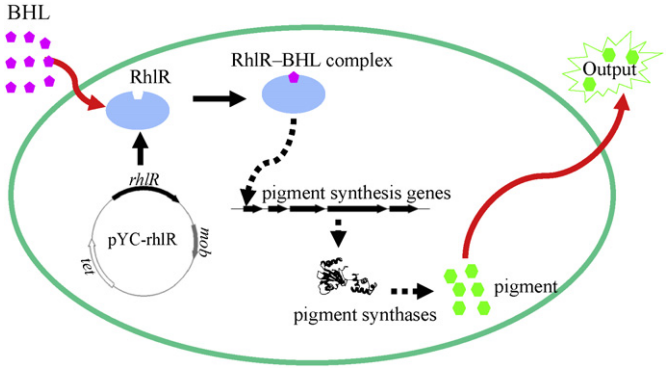

Fig. 1. Schematic of the genetically engineered pigment-based whole-cell sensing system.

5′-GTGGCAGCCAGCGTCTTG-3′; for *rhlI* the primers were 5′-CAGCGATCCGTCGGTATGG-3′ and 5′-CGTCTCGCCCTTGACCTTC-3′; for *16S rDNA* the primers were 5′-GGCTGCTGGCACGAAGTTAG-3′ and 5′-TTGGACAATGGGCGAAAGC-3′. The temperature cycle used for PCR was as follows: 28 cycles at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s.

2.5. Sample preparation

Aliquots of 5 ml bacteria cultures or wastewater samples were centrifuged at 12,000 × *g* for 4 min; then the supernatants were extracted with 5 ml of dichloromethane three times. The organic phase was pooled, dried over anhydrous magnesium sulfate, filtrated, and evaporated to dryness at 30 °C (Morin et al., 2003). The residue (sample extracts) was stored at −70 °C for later biosensor assay. For direct quantification, the wastewater or wastewater culture supernatant was only filtrated with a 0.22 μm filter.

2.6. Biosensor assay

The schematic of the biosensing system is shown in Fig. 1. The BHL receptor RhlR was overexpressed by a multi-copy plasmid pYC-rhlR. RhlR could recognize the BHL, which was freely diffused into cells and to form an RhlR–BHL complex. Under the activation of this complex, the green–blue pigment that reflected the BHL concentration could be produced and then monitored using a spectrophotometer. For the biosensor assay, AHL stock solution or sample extracts were diluted to appropriate concentrations as required using HPLC-grade acetonitrile. The acetonitrile solution of AHL standard or sample extract of 10 μl was pipetted into the bottom of a test tube (100 mm length × 12 mm i.d.) (10 μl HPLC-grade acetonitrile was used as blank sample), and the acetonitrile solvent was removed by evaporation under a gentle stream of nitrogen gas. Then 1 ml of diluted overnight biosensor culture (diluted to an OD_{600} of about 0.01 with fresh PB medium) was added to the test tube, mixed thoroughly, and incubated with shaking at 30 °C. After 24 h incubation, this culture sample was centrifuged at 12,000 × *g* for 5 min. The supernatant was extracted with 0.5 ml chloroform twice, and the absorbance of the pooled chloroform extracts was monitored at 299 nm with a UV–vis spectrophotometer. For quantification, the biosensor output was proportional to the concentration of BHL in the solution based on the calibration curve. For quantification of water samples with the AHL extraction procedure, the results were corrected with the varied extraction efficiency in different water samples (Morin et al., 2003).

2.7. Spiked water sample analysis

Water samples that were not expected to contain BHL were fortified with this analyte to evaluate the reliability of the developed biosensor assay and potential matrix effect. These 5 ml samples were spiked with BHL standard at 0.86 (5 nmol), 4.3, 8.6, 17.2 and 21.5 μg. These spiked water samples were then extracted with dichloromethane as described in "Sample preparation". The extracts were dissolved in 50 μl acetonitrile, and 10 μl of the acetonitrile solution was used for BHL quantification following the procedure of the "Biosensor assay".

2.8. Data analysis

Calibration curve was fitted with a four-parameter logistic model using the Origin 7.0 program (OriginLab Co., MA, USA):

$$y = \frac{A1 - A2}{1 + ([x]/[x_0])^p} + A2$$

where $[x]$ is the analyte concentration, $[x_0]$ is the analytic concentration at inflection, A_1 and A_2 are the lower and upper biosensor output to the titration curve, and p is the slope at the inflection point (Long et al., 2008).

3. Results and discussion

3.1. Strain screening and BHL regulation on pigment production

The BHL(RhlI)–RhlR QS system in *P. aeruginosa* was reported to control a large set of bacterial phenotypes such as rhamnolipid production, biofilm formation, and virulence factor production (Juhas et al., 2005). Also, BHL producers such as *Aeromonas* were isolated from activated sludge (Morgan-Sagastume et al., 2005). However, the quantification method for BHL (which has the shortest acyl-chain among these AHLs) was especially lacking as it had low signal response in MS detection (Cataldi et al., 2007; Morin et al., 2003) and was not included in some previously reported biosensors (e.g., Shaw et al., 1997). To develop a pigment-based biosensor for definite quantification of BHL in environmental samples, nine environment isolates belonging to *Pseudomonas* and *Burkholderia* genera were chosen for strain screening (Table S2). The screening was carried out based on their pollutant biodegradation ability and pigment production ability. Eventually, *P. aeruginosa* CGMCC 1.860, which could degrade a broad spectrum of aromatics and produce blue–green pigment with 1 mM BHL addition, was selected for biosensor construction (Table S2). Further analysis showed that this strain had an intact BHL(RhlI)–RhlR QS system (Fig. 2A and GenBank Accession No. FJ213455). Its BHL synthesis ability was determined by AHLs plate and TLC assay (McClean et al., 1997, data not shown).

To study the regulation of the BHL(RhlI)–RhlR QS system on pigment production, the *rhlIR* gene cluster was abolished using gene in-frame-deletion technology (Fig. S2). Suicide plasmid pYC2000Δ*rhlIR* was constructed as described in Section 2. By conjugation of pYC2000Δ*rhlIR* with *P. aeruginosa* CGMCC 1.860, 27 positive clones which had about a 1 kb in-frame deletion in the *rhlIR* cluster were obtained after 2 cycles of selection (Table S3). This *rhlIR* abolished mutant designated as Δ*rhlIR* was confirmed by PCR, RT-PCR (Fig. 2A), and DNA sequencing. The pigment production was then examined in wild type and Δ*rhlIR*. As shown in Fig. 2B, pigment production could be observed in the wild type strain with addition of 1 mM BHL, and it was completely abolished by *rhlIR* deletion in strain Δ*rhlIR*. However, by complementation with pYC-rhlIR in strain Δ*rhlIR*, pigment production could be restored and even overproduced without BHL addition. These results indicate the production of this pigment was under the control of the

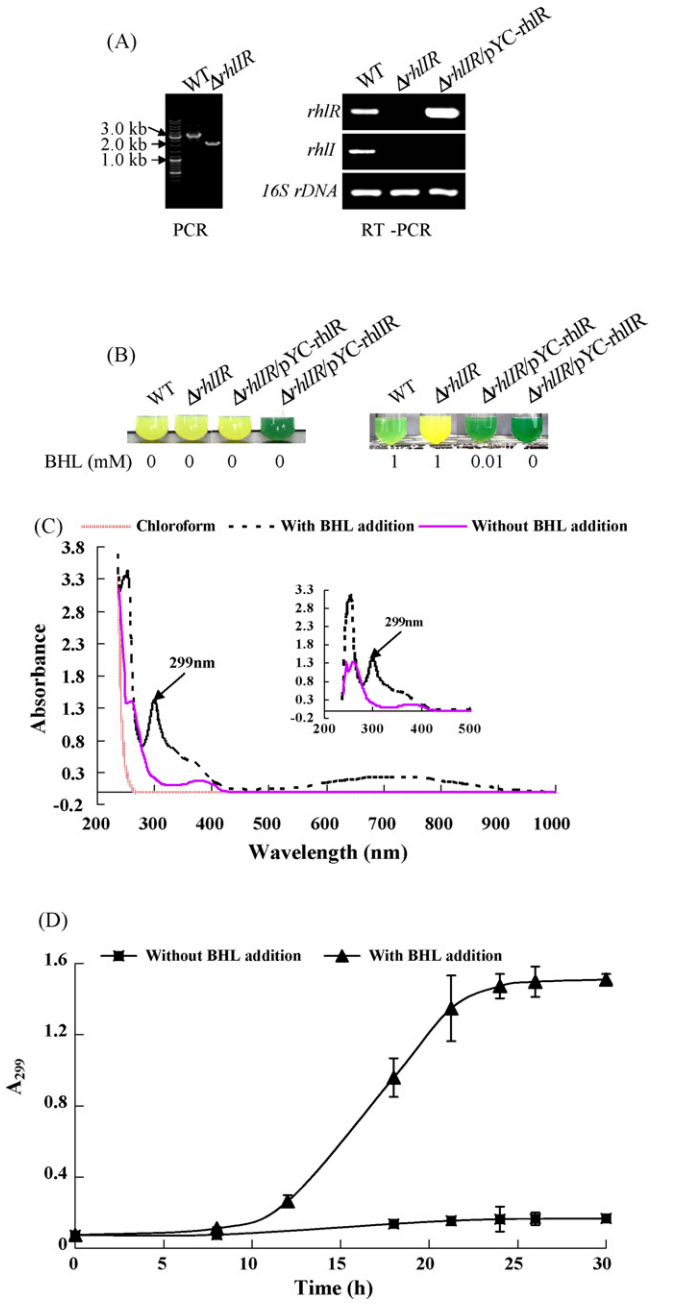

Fig. 2. Biosensor construction and its response to BHL. (A) PCR of *rhlIR* gene fragment from the genome DNA using primers of rhlIRUF and rhlIRDR, and RT-PCR analysis of the expression of *rhlI* and *rhlR*. (B) Pigment production in related mutants with or without BHL addition. (C) Full wavelength scanning of chloroform, chloroform extracts of biosensor culture without BHL addition (blank), and chloroform extracts of biosensor culture with 1 μM BHL addition; inset is the absorption curve of chloroform extracts extracted with the absorption of chloroform. (D) Typical time-course of pigment production by the biosensor in response to 1 μM BHL.

BHL(RhlI)–RhlR QS system and imply its potential for biosensor development.

3.2. Biosensor construction and biosensing system development

For a whole-cell biosensor, production of the analyte by the biosensor strain should be avoided, and a functional receptor for recognition of the target analyte is needed. RhlI has been shown to be responsible for BHL synthesis, and RhlR was characterized as the BHL receptor in *P. aeruginosa* (Juhas et al., 2005). So, two strains Δ*rhlI* (*rhlI*$^{-}$*rhlR*$^{+}$) and Δ*rhlIR*/pYC-rhlR (*rhlI*$^{-}$*rhlR*$^{++}$) without functional RhlI but with active RhlR were expected to be constructed. The strain Δ*rhlI* was obtained according to the procedure of Δ*rhlIR* construction described above using suicide plasmid pYC2000Δ*rhlI* (Table S3). Strain Δ*rhlIR*/pYC-rhlR was obtained by transforming pYC-rhlR into Δ*rhlIR*.

As a result, Δ*rhlI* and Δ*rhlIR*/pYC-rhlR could neither produce any BHL themselves nor synthesize the blue–green pigment without exogenous BHL (Fig. 2B and data not shown). In this way, the production of pigment was under the control of exogenously added BHL, which could diffuse into the cell and be recognized by the RhlR regulator (synthesized by genome *rhlR* in Δ*rhlI* or by plasmid pYC-rhlR in Δ*rhlIR*/pYC-rhlR) to form a RhlR–BHL binding complex. Upon binding, this complex could activate the expression of pigment synthases, which then synthesize pigment to generate a color change that can be detected as output by spectrophotometry (Fig. 1). Thus, these genetically modified strains were tested as biosensor candidates regarding their response to exogenously added BHL. It was found that Δ*rhlI* showed only low levels of pigment production when 1 mM BHL was added. However, Δ*rhlIR*/pYC-rhlR showed relatively high pigment production with addition of only 10 μM BHL (Fig. 2B). This enhancement of sensitivity was obviously due to the overexpression of *rhlR* (Fig. 2A) and implies that *rhlR* overexpression might be a promising approach to improve biosensor sensitivity. Hence, *P. aeruginosa* CGMCC 1.860 Δ*rhlIR*/pYC-rhlR was chosen as the new BHL biosensor by using its pigment production as output.

To develop a quantitative biosensing system, the method for pigment quantification, culture medium, and length for cell incubation were all examined. This pigment could be effectively extracted with chloroform and showed strong absorbance peaks at 251 and 299 nm (Fig. 2C). The absorbance of 299 nm was chosen for pigment quantification because there was almost no interference from chloroform solvent and the blank (chloroform extract of biosensor culture without BHL addition). To ensure maximum pigment production, the PB medium was selected after testing M9, LB, PYO and PB media (Fig. S3). As shown in Fig. 2D, by using PB medium, the pigment production in response to BHL could achieve its maximum level at 24 h and be stable for at least another 6 h. So, the optimum culture conditions were incubation of the cells for 24 h at 30 °C in PB medium, after which the pigment produced was extracted into chloroform and quantified by A_{299} measurement.

Once the culture conditions and pigment quantification methods for this biosensing system were optimized, the biosensor was exposed to varying concentrations of BHL. As shown in Fig. 3, pigment production (A_{299}) responded to BHL in a dose-dependent

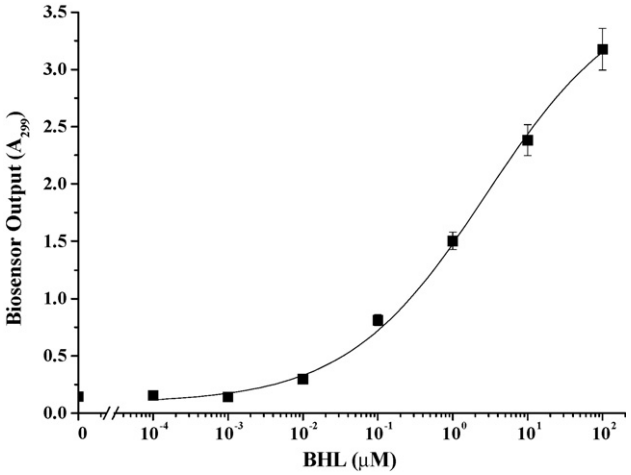

Fig. 3. Biosensor dose–response to BHL under optimum conditions and its logarithmic calibration plot.

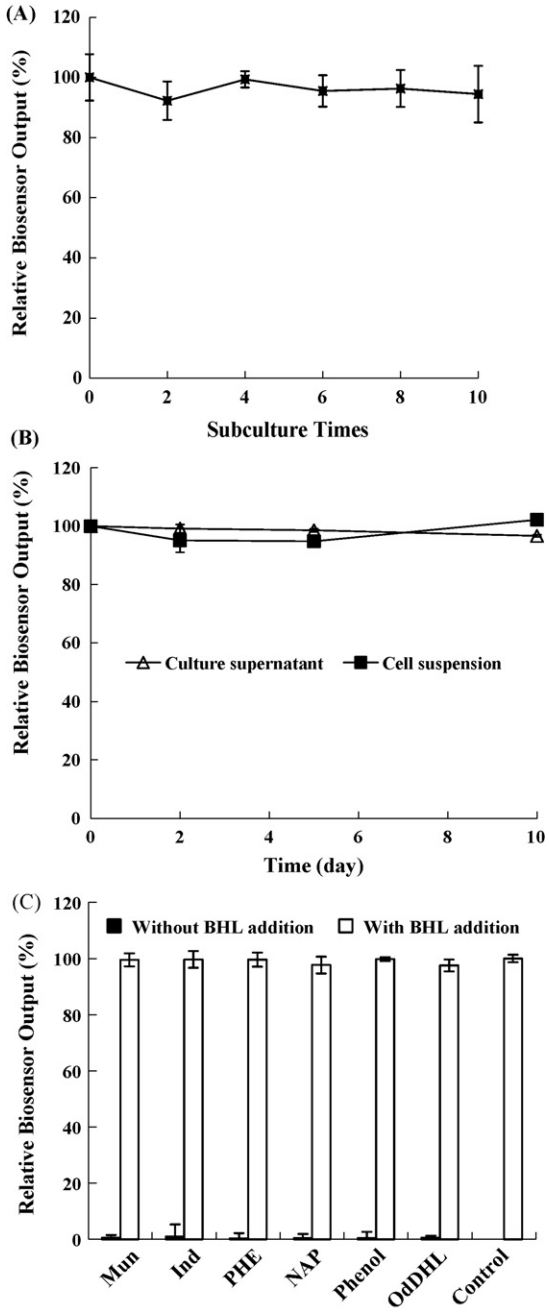

Fig. 4. (A) Stability of the biosensor strain, subculture was carried out on PB slant and the subculture period was 12 h; (B) stability of culture sample stored at −20 °C as cell suspension or culture supernatant; (C) effects of interferences on biosensor response, and the output was normalized with blank control. Mun, municipal wastewater; Ind, industrial wastewater; PHE, phenanthrene; NAP, naphthalene. Phenanthrene, naphthalene and phenol were added to a final concentration of 100 μM. OdDHL was added to a final concentration of 5 μM. For wastewater interferences, extract of 1 ml wastewater was used. BHL was added at a concentration of 5 μM.

manner ranging from 1 nM to 100 μM. So, A_{299} was defined as the biosensor output. A calibration curve (R^2 = 0.996) was obtained based on a four-parameter logistic model as described in data analysis (Fig. 3). The limit of detection (LOD) determined using the mean blank values plus three times standard deviation was 1.3 nM (S/N = 3), and the limit of quantification (LOQ) determined using the mean blank values plus 10 times standard deviation was 5.7 nM (S/N = 10). The 50% activation/effect concentration of this biosensor assay for BHL was 2.77 ± 0.45 μM. The dynamic determination

Table 1
Quantification results of water samples spiked with synthetic BHL.

Origin	BHL (μg)		CV[a] (%)	Recovery (%)
	Amount spiked	Determined by biosensor (mean)		
Distilled water	0.86	0.90	5.2	105
	4.3	4.26	2.5	99.1
	8.6	8.31	3.8	96.6
	17.2	16.1	4.7	93.6
	21.5	20.5	6.0	95.3
Wastewater[b]	0.86	0.78	8.4	90.7
	4.3	4.24	6.5	98.6
	8.6	8.18	3.9	95.1
	17.2	17.1	4.2	99.4
	21.5	20.4	8.5	94.9

[a] CV, coefficient of variation (n = 3).
[b] Wastewater was the industrial wastewater as described in Section 2.

range of BHL corresponding to 20–80% maximum biosensor output was 0.11–49.7 μM. The sensitivity of this developed sensing system for BHL quantification was comparable to the currently available β-galactosidase biosensor assay (Pearson et al., 1997), but with a wider quantitative detection range. Also, this system showed about four orders of magnitude lower LOD than the reported sole pigment biosensor *C. violaceum* CV026 (McClean et al., 1997). In addition, the LOD was much lower than the reported physical–chemical analytical methods, i.e., approximately 300 times lower than LC–MS (Morin et al., 2003) and about 4000 times lower than GC–MS (Cataldi et al., 2007).

The stability of this biosensing system was also examined. The biosensor strain was relatively stable, as the relative biosensor output was steady between 92% and 99% within 10 times subculture on PB slant (Fig. 4A). Moreover, it could be stored in 15% glycerol at −70 °C for at least 3 months without significant effect on its response to BHL. The stability of culture samples was also tested. As shown in Fig. 4B, whether the culture sample was stored as the culture supernatant or as a cell suspension at −20 °C, the biosensor output was relatively stable for 10 days. As a result, this culture sample could be stored simply as a cell suspension or the culture supernatant for later assay.

The next step in our research was to evaluate the effect of various interferences on this biosensing system. *N*-3-Oxo-dodecanoyl-homoserine lactone (OdDHL) is another main AHL synthesized by LasI of *P. aeruginosa*, and it usually coexists with BHL in samples (Pearson et al., 1997). Hence, the interference from OdDHL should be taken into account. In the case of violacein biosensor *C. violaceum* CV026, as the pigment production induced by short chain AHLs (such as BHL) could be inhibited by the co-existing long-chain AHLs (such as OdDHL) (McClean et al., 1997), its application to complex samples containing long-chain AHLs would be difficult. As shown in Fig. 4C, OdDHL alone could not produce obvious biosensor output. It also had no significant effect on the biosensor output after co-addition with BHL. In order to apply this biosensor to environmental samples, the interferences from aromatic pollutants were also tested. Due to its pollutants degradation ability, aromatics such as phenol, naphthalene and phenanthrene did not affect the response to BHL (Fig. 4C). These results suggest that the biosensing system is applicable in complicated environmental samples.

To examine the influence of the matrix effect and establish the credibility of this new biosensing system, water samples spiked with different amounts of BHL were tested. As shown in Table 1, the amounts of BHL quantified by the sensing system corresponded well to the real amounts spiked. The recovery of all measured samples was between 90% and 105%. The results indicate that this

Table 2
Determination of BHL concentration in wastewater by the developed biosensor.[a]

Sample No.	Medium	Sterile	Inocula[b]	OD_{600}	BHL concentration (μM)	
					A[c]	*B*
1	Ind.[d]	No	DIR-IR	1.13	NT[e]	23.3 ± 1.42
2	Ind.	No	DIR-IR	2.34	NT	35.7 ± 3.68
3	Ind.	No	DIR-I	2.19	NT	12.6 ± 2.52
4	Mun.	No	DIR-I	2.50	12.7 ± 2.50	12.0 ± 1.23
5	MM9	Yes	DIR-I	2.04	9.63 ± 1.22	10.7 ± 0.31
6	MM9 + Phena.	Yes	DIR-I	2.19	10.3 ± 1.63	10.9 ± 0.77
7	MM9 + Phena.	Yes	WT	1.95	NT	0.91 ± 0.16

[a] Samples were the culture supernatant of *P. aeruginosa* strains cultivated in wastewater.
[b] Inocula were *P. aeruginosa* CGMCC 1.860 and related mutants, i.e., DIR-IR, Δ*rhlIR*/pYC-rhlIR; DIR-I, Δ*rhlIR*/pYC-rhlI; WT, wild type.
[c] A, direct quantification, samples filtrated with 0.22 μm filter and without extraction; B, extracted with dichloromethane.
[d] Culture medium, Ind., industrial wastewater with 8 g/l glucose and 2 g/l NH_4Cl; Mun., municipal wastewater with 8 g/l glucose and 2 g/l NH_4Cl; MM9 + Phena., MM9 medium with 0.1 mM phenanthrene. MM9 medium was M9 medium with 8 g/l glucose and 2 g/l NH_4Cl.
[e] Not tested.

biosensor assay shows good reliability and that possible interference from wastewater was negligible.

3.3. Application to wastewater samples

To date, there is no report on quantification of AHLs in environmental samples containing toxic compounds. Attempts to use reported biosensors for AHLs detection in such environmental samples failed (Manefield and Whiteley, 2007; Valle et al., 2004). To further apply this biosensing system to complex/toxic environmental samples, a set of wastewater samples from different wastewater treatment plants and man-made wastewater with polycyclic aromatic hydrocarbons (PAH) was analyzed by this sensing system. There was no significant detectable biosensor output for these wastewater samples without inoculation. When the industrial wastewater was inoculated with Δ*rhlIR*/pYC-rhlIR, a high concentration of BHL was favorably reached, i.e., about 23 μM at OD_{600} = 1.1 and about 36 μM at OD_{600} = 2.3 (Table 2). When Δ*rhlIR*/pYC-rhlI, which had no functional *rhlR*, was used as inoculum, BHL concentration was only about one third of that inoculated with *rhlIR*/pYC-rhlIR, which was in accordance with previous reports (Chen et al., 2005) (Table 2). The BHL concentration was also determined in municipal and man-made wastewater inoculated with Δ*rhlIR*/pYC-rhlI (Table 2). The possibility of quantifying BHL in environment samples without concentration or extraction was also tested. The filtrated samples (100 μl) were directly mixed with 900 μl diluted biosensor culture for the biosensor assay. The results (Table 2) obtained by direct quantification without sample extraction were in agreement with that of quantification after extraction.

4. Conclusions

A new pigment-based whole-cell bacterial biosensing system for quorum sensing signal quantification was developed and its application to wastewater samples was also demonstrated. Although chloroform could effectively extract the blue–green pigment from the medium, a solvent with less volatility may be further explored. The developed sensing system for BHL quantification had much higher sensitivity than either a violacein pigment biosensor or reported physical–chemical methods. It also had a comparable sensitivity to the β-galactosidase biosensor, while it possessed merits such as its simple spectrophotometric detection procedure, output stability, and interference resistance. Further increase of the biosensor sensitivity may be reached by using an even higher expression system to overproduce the transcription activator RhlR. Moreover, the high tolerance of the biosensor strain to environmental pollutants allowed its applicability to BHL quantification in environmental samples with toxic pollutants even without extraction. Therefore, this biosensing system could be employed to interpret the role of AHLs in environmental biological processes. It is also expected to be applicable for the diagnosis of bacteria-related human diseases. The idea for construction of biosensors, using an environmental isolate with tolerance to environmental interference and natural pigment as the biosensor output, may be also useful for developing other biosensors for the analysis of environmental samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.06.010.

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